Mucus Layer Properties and Dynamics in Reef Corals

By

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A Thesis submitted to Newcastle University
For the degree of
Doctor of Philosophy

School of Biology
Newcastle University

October 2008

Abstract

Mucus functions as the first line of defence against infections, amongst other roles. The protective role of mucus depends upon gel-forming properties of mucin molecules that are encoded by the MUC genes. Failure of this protective barrier has been associated with changes in structure, function and physical properties of mucins in human diseases (cancer, IBD and cystic fibrosis) and has been proposed as a prognostic tool for early diagnosis of these diseases. The study of coral mucus is in its infancy and early investigations on coral mucus gave incomplete and variable chemical composition data. The dynamic nature of the coral surface mucus layer (SML) in limiting pathogens remains unexplored. The present study attempts to detect coral muc genes by tracing the evolution of muc genes from cnidarians to mammals using bioinformatics tools, examines the coral mucin molecule and investigates the response of epidermal and surface mucus to the changing environmental conditions.

The presence of a continuous SML at least 145 μm thick on the coral epithelium has been demonstrated for the first time. Rheological studies confirmed the presence of high molecular weight, polymeric glycoprotein similar to mammalian mucin. The study has also developed molecular tools (primers) based on bioinformatics information and has detected tandem repeats rich in serine, threonine and proline and C-terminal Cysteine Knot regions homologous to those of human gel forming mucins. A variation in the epidermal mucus content as well as SML was noticed in between the species as a response to bleaching (loss of zooxanthellae and complete white appearance of coral tissue) and changing environmental conditions (tidal cycle). Thus, study of mucus indicates the health of corals and plays an important role in survival of coral during diseases and environmental stressful conditions.

Acknowledgements

It is a pleasure to thank the many people who made this thesis possible. I want to express my sincere gratitude to my Ph.D. supervisor, Dr. J. C. Bythell for his inspiration, and his great efforts to explain things clearly and simply. Throughout the research, he provided valuable feedback and sound advice. I wish to acknowledge my indebtedness to Dr. J. Pearson for all the help in rheology and for providing a stimulating and fun environment which made learning an easy process. My sincere thanks are due to Dr. Barbara Brown for practical information on intertidal corals of Thailand. I also wish to express my warm and sincere thanks to Dr N. J. Morris and Dr. H. Magalon for their help in Chapter 2 dealing with bioinformatics.

I am also grateful to the Leverhulme Trust for funding this project (F/00 1125/S). This project would have been a distant dream without their support.

I am eternally grateful to my parents for their constant support and encouragement without which this work would not have happened. I wish to thank my best friend and critic Mr. Savan Mayekar for all the entertainment and fun during the long stressful times. Finally I would like to acknowledge Mr. M. Prabandham for helping me take the very first step for this research.

Statement of Sole Authorship

All the data presented here results from my own research and that no part of this thesis contains data produced by other authors, with the exception that the Bioinformatics Support Unit of Newcastle University conducted the required searches to generate the raw dataset of coral mucin homologs in *Nematostella vectensis* genome and *Acropora millepora* EST database. No part of this thesis may be copied or reproduced in any other form without the prior consent of the author.

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Abbrevations

ABBREVATIONS	DESCRIPTION
AMOP	adhesion-associated domain in MUC4 and other proteins
BSM	bovine submaxillary mucin
CK	cysteine knot
CT	cytoplasmic tail
DDDG	different domains in different MUC genes
EGF	epidermal growth factor
EST	expressed sequenced tags
FIM-A.1	frog integumentary mucin A
FIM-B.1	frog integumentry mucin B
FIM-C.1	frog integumentary mucin C
G’	storage/elastic moduli
G’’	loss/viscous smoduli
GM	*Goniopora djiboutiensis* mucin
GPI	glycosylphosphatidylinositol
GX1M	*Goniopora stoksii* – Extract 1 mucin
HSPG2	heparan sulphate proteoglycan 2
*hu*MUC	human mucin gene
IIM	invertebrate intestinal mucin
MD	million dalton
MM	*Montastrea faveolata* mucus
NIDO	nidogen

PSM	porcine salivary mucin
PTS	proline, threonine and serine amino acid residue
RGM	reduced *Goniopora stoksii* mucin
SEA	sea urchin enterokinase
SML	surface mucus layer
SP	signal peptide
SST	sea surface temperature
Susd2	sushi domain containing protein 2
SXC	six cysteine domain
TcMUC	*Trypanosoma cruzi* mucin gene family
TcSMUC	*Trypanosoma cruzi* small mucin gene family
TIL	trypsin like inhibitor
TM	trans-membrane domain
Vf	volume fraction
VNTR	variable number of tandem repeat
vWD	von Wilibrand factor type D domain
vWF	von Willebrand factor

CHAPTER 1

General Introduction

1.1 IMMUNE SYSTEM IN ANIMALS

In vertebrates, defences against microbes are mediated by the initial reaction of innate immunity, followed by the responses of adaptive immunity. Innate/natural immunity acts as a first line of defence and acts before the establishment of infection (Abbas et al. 2007). It consists of four major components:

1. Physical and chemical barriers such as epithelia, mucus and antimicrobial substances produced at the epithelial surfaces.
2. Phagocytic cells (neutrophils, macrophages) and Natural Killer cells (NK)
3. Blood proteins including members of the complement system and mediators of inflammation, and;
4. Cytokines which regulate and coordinate activities of innate immunity.

The innate immune system recognises the structures that are common to microbes and may not necessarily distinguish the fine differences between specific microbes. In contrast, adaptive immunity shows extraordinary ability to distinguish closely-related molecules and microbes. It also possesses an ability to remember and respond quickly when exposed to the same pathogen repeatedly. Adaptive immunity consists of lymphocytes and antibodies which are generated against the antigens (Abbas et al. 2007).

Immune responses across animal groups reveal innate immunity as phylogenetically the oldest form of host defence and adaptive immunity has evolved later. The majority of invertebrates immobilise foreign particles by surrounding them with the cells that destroy the foreign particle. These cells resemble phagocytes of vertebrates and are called phagocytic ameobocytes in acoelomates, haemocytes in molluscs and arthropods, coelomocytes in annelids and blood leucocytes in tunicates. Adaptive immunity was first seen in the jawed vertebrates and became more specialized with evolution (Abbas et al. 2007) (Table 1).

Group	Innate Immunity		Adaptive Immunity	
	Phagocytes	NK cells	Antibodies	T and B lymphocytes
Protozoa	+	-	-	-
Porifera	+	-	-	-
Annelida	+	+	-	-
Arthropoda	+	-	-	-
Elasmobranch (Shark, rays)	+	+	+ (IgM)	+
Teleosts (eel, tuna, catfish)	+	+	+ (IgM, others?)	+
Amphibia	+	+	+ (2 or 3 classes)	+
Reptilia	+	+	+ (3 classes)	+
Aves	+	+	+(3 classes)	+
Mammalia	+	+	+ (7 classes)	+

Table 1 Evolution of immune systems in animal. Table showing the presence and absence of various components of innate and adaptive immunity in various group of animal (+ present, - absent) (Abbas et al. 2007) IgM – immunoglobulin M molecule.

It is clear from Table 1 that the invertebrate immune system consists of innate immunity only. Corals represent the earliest metazoan phylum which evolved tissue-level organisation. An acquired immune system like vertebrates is not known in cnidarians to date but cnidarians show the ability to distinguish between self and non-self, indicating the expression of cell surface molecules (Krupp 1985). Recently during a genome-wide comparison of immune related proteins, cell-associated pattern recognition receptors (Toll-like receptors) and the C3 pathway of the complement system which are involved in innate immunity have found to be present in the lower metazoans including the coral *Acropora millepora* and sea anemone *Nemtostella vectensis* (Hemmrich et al. 2007). Mucus along with its underlying epithelium and stinging cells or nematocysts forms the first line of defence in corals. Mucus acts as a physical barrier that limits the entry of microbes and protects the integrity of epithelium. Coral mucus layer exhibits the following structural similarities with the human colonic mucus layer:

1. In corals, mucus is stored in the mucocytes which consists of a number of sac-like structures similar in appearance to those of the goblet cells of human (Figure 2).
2. A distinct microbial community is associated with healthy coral. This microbial community structure present on the coral surface mucus changes during diseases (Pantos et al. 2003, Pantos & Bythell 2006) and in response to changed environmental conditions (Ritchie 2006, Bourne et al. 2007). Similar changes in beneficial (non pathogenic) bacterial community to a pathogen-dominated one has been demonstrated during infection in human mucosal systems (Corfield et al. 2001, Pearson & Brownlee 2005).

3. In humans, evidence suggests that mucus provides receptors, which are similar to the mucosal receptors, for the pathogens to bind (Moncada et al. 2003). These pathogens are then sloughed off eventually thereby preventing the pathogens from reaching the mucosal surface for example – ameobiasis (Variyam 2007). Although such a processes have not been seen in corals, in an isolated study done on the Mediterranean coral *Oculina pentagonica*, the bleaching agent *vibrio shiloi* has been proposed to bind with the beta-D-galactopyranoside-containing receptor present in coral mucus (Banin et al. 2001).

4. Staining of the coral and human tissue using Periodic Acid Schiff's reagent /Alcian blue (PAS/AB) demonstrates acidic nature of mucus. The PAS/AB stains mucus in a blue/purple color showing that the carbohydrate component of both the mucins contain sulphate and other anionic residues (Figure 1 and Figure 2) (Krupp 1985, Strugala et al. 2003)

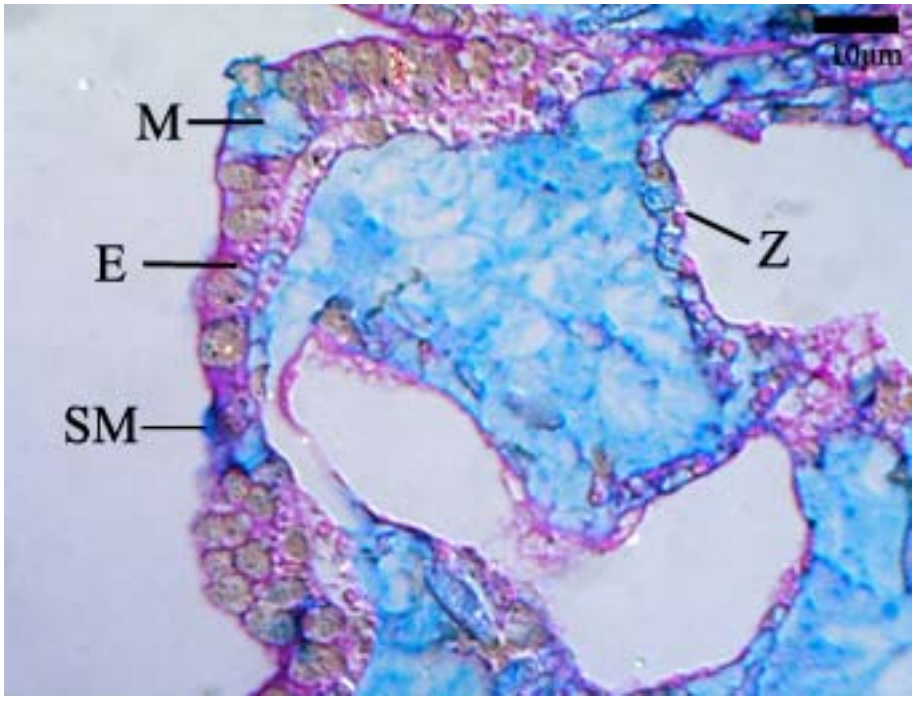


Figure 1 *Porites cylindrica* tissue showing epidermis (E), mucocytes (M), zooxanthellae (Z) and surface mucus (SM).Tissue stained with Periodic acid Schiff's reagent /Alcian Blue (PAS/AB)

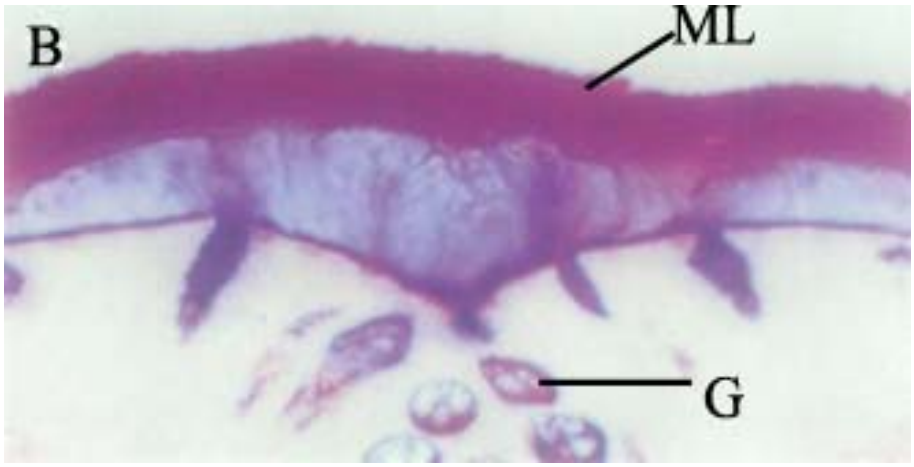


Figure 2 Human colon tissue with mucus layer (ML) stained in blue purple colour, G – goblet cells (Stain – Periodic Acid Schiff's reagent/Alcian Blue (PAS/AB). Magnification x613.Figure taken from histochemistry of surface mucus gel layer of the human colon (Matsuo et al. 1997).

1.2 THE FUNCTIONAL ROLE OF MUCUS IN CORALS

Mucus in vertebrates mainly acts as a protective and lubricating layer. Amongst invertebrates, other functions such as locomotion, mating, homing and food capture have been known along with the fundamental function of protection (Denny 1989). Thus, the mucus layer in invertebrates performs far more diverse functions compared to the mucus layer of vertebrates. Coral mucus performs the following functions which help the coral to resist effects of the changing surrounding conditions:

Defence

The surface mucus layer (SML) protects the coral surface from a variety of physical-chemical and microbial invasions. The SML performs a barrier function, a mucociliary function in which the exuded mucus is swallowed along with food and associated microbes are digested and shows presence of antimicrobial substances in mucus (Brown & Bythell 2005). Ducklow and Mitchell (1989) and Vacelet and Thomassin (1991) have counted more bacteria in the mucus compared to the surrounding water column. In Mediterranean coral *Oculina pentagonica* mucus has been demonstrated to provide receptors to the pathogen which then targets the symbionts resulting in bleaching of the coral (Banin et al. 2001). A decrease in antibacterial properties of the mucus in response to changing environmental conditions has been recently demonstrated (Ritchie 2006; Bourne et al. 2007). Thus, these studies highlight the fact that corals possess not only an ability to respond to microbial attack but are also affecting and limiting the growth of the harmful microbes on the epithelial surface.

Protection from UV radiation

Many marine animals have been shown to secrete mycosporine like amino acid (MAA's) which have a capacity to absorb the ultra violet A (320-400 μm) and UV B (280-320 μm) radiations (Norris 1999). Although the mechanism of protection adopted by corals is still largely unknown, it has been identified that the mucus of corals contains mycosporine like amino acid (MAAs) which are considered as UV absorbing molecules in coral mucus. It has been shown that MAA's concentration in mucus of the corals varies according to the species and/ location (Teai et al. 1998). Thus, coral mucus probably acts as a sunscreen and protects the surface from detrimental effects of solar radiations.

Feeding

Corals possess cilia on their oral ectoderm surface with the help of which they direct a mucus web entangled with suspended food particles towards the polyp mouth, then into the pharynx and then coelenteron where it gets digested (Figure 3). Duerden (1906) discussed the significance of mucus in food collection and described membrane-like mucus for polyp surface cleaning and watery mucus for carrying food towards the mouth. This observation is interesting since similar bilayer mucus structure has been demonstrated in the gastrointestinal tract of mammals (Strugala et al. 2003). Lewis and Price (1975) and Lewis (1977) showed that the mucus net substantially increases the chances of availability and capturing food and widens the potential food resource by entangling zooplankton and suspended bacterial aggregates (Sorokin 1973, Bak et al. 1998) with other particulates such as silt and sand. A large quantity of energy has been demonstrated to be spent by coral polyp on mucus formation. In *Acropora acuminat* and

A. variabilis nearly 40% and 8% of total photosynthate carbon goes to form the mucus respectively, whereas in *Porites porites* 45% of the energy was utilised in synthesizing mucus (Edmunds & Davies 1986). The ingestion of the mucus web along with entrapped food particles by the polyp is proposed to be beneficial since polyp regains a part of energy which has been spent on synthesis of mucus.

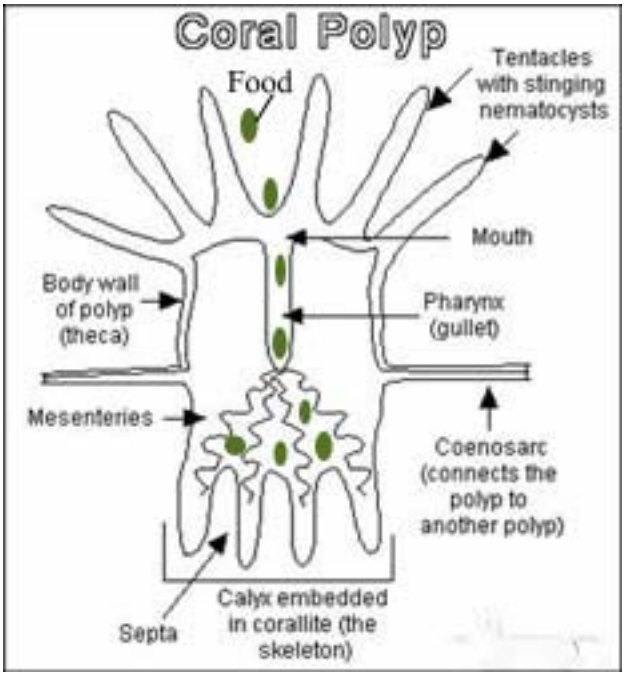


Figure 3 Feeding behaviour in coral. The figure shows the mesenteries, pharynx, tentacles and body wall of coral. The food (shown green in color) is captured by coral with the help of tentacles, nematocysts and exuded mucus. The food gets entangled in mucus and is finally engulfed.

Protection from sedimentation

Daily and seasonal variations along with the storm and land runoff results in increased of sedimentation and turbidity in sea water leading to accumulation of sediments on the reef surface. It is reported that coral forms mucus strings which help in the removal of non nutritive particles away from the coral surface (Duerden 1906). During increased sedimentation, it is observed that more energy is used by corals in keeping the surface clean. Edmunds and Davies (1989) and Coffroth (1983) suggested that secretion of

mucus string to keep the coral surface clear off the sediments (Edmunds & Davies 1989) is a secondary function performed by the corals in absence of sufficient water currents in *Porites furcata* (Coffroth 1983). Thus one of the important roles of mucus is to keep the coral head clean by driving the sediments away from the surface.

As a source of primary productivity

It is believed that mucus increases primary productivity of the reef ecosystem as 40-50% of the net carbon fixed by the corals is released as mucus (Crossland et al. 1980). Johannes (1967) studied the organic aggregates in the corals reef of Eniwetok Atoll and concluded that these suspended organic aggregates in the mucus of corals act as an important source of food to filter feeders, zooplanktons, and copepods (Johannes 1967). Benson and Muscatine (1974) recognised wax ester and triglycerides as major lipid components in the mucus that act as an energy resource for small fishes. Coles and Strathmann (1973) reported that fishes ingest the mucus in the form of the tiny particles and do not feed on the mucus aggregates. Thus, coral mucus traps energy and helps in energy transfer and recycling of nutrients in coral reefs (Wild et al. 2004a, Wild et al. 2004b).

Bioaccumulation of pollutants

Oil spillage is one of the major pollutant threats which coral reefs face more frequently apart from the addition of sewage in the vicinity. Exposure of corals to oil/aromatic hydrocarbons is well studied (Mitchell & Chet 1975, Neff & Anderson 1981, Peters 1981). Neff and Anderson (1981) suggested that mucus has an ability to absorb the hydrocarbons thereby protecting the underlying tissue from damage. Peters et al.

(1981), found highly increased mucus secretion activity in the epidermis and mesenteric area. Many cells were swollen to the extent that their cell walls were broken and large vacuoles were visible. These changes were observed after 2, 4 and 6 weeks of exposure but during the 8-12 weeks of exposure, the mucus secretory cells atrophied and disappeared completely from many places. In case of chronic exposure even in small concentration, *Manicina areolata* showed cell degeneration and atrophy with a reduced ability to reproduce. Thus it seems that mucus secreting cells are profoundly affected by the presence of oil pollution in the surroundings.

1.3 SYNTHESIS AND COMPOSITION OF CORAL MUCUS

The release of coral mucus on the epithelial surface has been observed to attract particulate matter. Mucus entangled with particulate matter forms a web-like structure which has been visualised using scanning microscopy by Ducklow and Mitchell (1979b). The released mucus is found to be rich in organic content compared to surrounding water (Coles & Strathman 1973). A part of this mucus may be utilised as food (Johannes 1967, Bensen & Muscatine 1974) while the remaining mucus entangled with particulate matter is reported to settle down in sediments where benthic and bacterial decomposition of the mucus occurs (Wild et al. 2004b). Thus, coral mucus recycles the minerals and energy in the reef ecosystem (Wild et al. 2004a).

In the earlier part of last century, major work was carried out to understand the biochemical composition of coral mucus and to study the functional roles of mucus (as discussed in section 1.2). However, without better understanding of mucus layer

properties and its dynamics, it is difficult to explain all the functional roles of coral mucus. Corals share a symbiotic relation with the zooxanthellae which occupy endodermal tissue of the host. It is demonstrated that zooxanthellaes participate in photosynthesis and store the fixed carbon in the form of lipids in its cytoplasm which finally gets incorporated into the host mucus (Crossland et al. 1980). Thus zooxanthellae were thought to be the carbon source for the host mucus. A recent study has demonstrated that *Montipora capitata* can fulfil all its metabolic demands by hetrotrophic feeding under stressful conditions (bleaching) while species like *Porites lobata* and *P. compressa* could not do so (Grottoli et al. 2006). This indicates that corals can switch their carbon source under stressful conditions and further poses a question about the exact source of carbon for host mucus synthesis.

Biochemical analysis of various species of coral mucus revealed that newly secreted mucus consists of varying proportions of the protein, lipid and polysaccharide (Meikle et al. 1988, Brown & Bythell 2005). No common dominant component could be established in the coral mucus. In fact, investigators have described differences in the mucus components within the same species. For example; in the reef coral *Fungia fungities* mucus was reported to consist of 5% protein, 2.5% polysaccharides and 4% lipids respectively (Ducklow & Mitchell 1979b), whereas Meikle et al. (1988) claimed 72.7%-50.2% protein, 4.5%-5.1% polysaccharides and 4.4% lipids for the same species of coral. These inconsistencies in biochemical composition of coral mucus could have been anticipated due to adoption of widely different sample collection, methods and analytical techniques (Brown & Bythell 2005). Since the method of collection and analysis of coral mucus is not standardised, definition of coral mucus is also not clear. It

has been proposed that different types of mucus with different properties may be produced at different sites within the coral colony (Brown and Bythell 2005). An isolated study done on *Acropora formosa* has shown a glycoprotein with oligosaccharide side chains similar to those found in humans, responsible for viscoelastic properties of mucus (Meikle et al. 1987), but little information is available on its structure or its effects on mucus function. Thus, neither the definition, content nor the sources along with the mucus layer properties are clearly understood in corals.

1.4 THE DIFFERENCE BETWEEN MUCUS AND MUCINS

Mucus is the slimy secretion consisting of mucins and a combination of other substances such as inorganic salts, immunoglobulin and lipids suspended in water giving it characteristic lubricating properties (Pearson & Brownlee 2005). The functional properties of mucus depend on its capacity to form a gel on the epithelial surface. This gel-forming property is controlled by the amount, size and the degree of cross-linking present between the mucus glycoprotein (mucin) (Smith 2002, Pearson & Brownlee 2005). Mucins are filamentous, high molecular weight glyco-proteins (0.5-20 MD). There are two major forms of mucin known in humans – monomeric and oligomeric. The monomeric form is generally present on the cell surface whereas the oligomeric mucin has been held responsible for the gel like properties of secreted mucin (Thornton & Sheehan 2004).

1.5 STRUCTURE OF A HUMAN GEL FORMING MUCIN

A typical gel forming mucin is a highly glycosylated molecule in which the carbohydrate side chains are attached to a protein core. The mucin molecule consists of the following two major regions based on their susceptibility to proteolytic digestion (Allen & Pearson 1993):

1. The glycosylated or variable number of tandem repeat (VNTR) region
2. Non-glycosylated region

1.5.1 THE GLYCOSYLATED OR VARIABLE NUMBER OF TANDEM REPEAT (VNTR) REGION

The central highly glycosylated tandem repeat region of mucins is rich in proline (P), theronine (T) and/or serine (S) amino acid residues, hence also referred as the PTS region (Figure 4). The VNTR region varies in number as well as in length amongst different mucin molecules; however the three amino acids (PTS) together form more than 40% of the total amino acids in the VNTR region in all mucin molecules (Lang et al. 2004). The hydroxyl side chains of serine and threonine form O-glycosidic bonds with the carbohydrate side chains giving the mucin molecule its characteristic “bottle brush” shape. The sequence of the VNTR region is not conserved amongst the different mucin molecules. This region regulates the saccharide side chain formation and also determines the structure of saccharide at that site (Bhavanandan et al. 1999). Carbohydrate forms nearly 80% of mucin by weight and shows great structural diversity (Corfield et al. 2001, Thornton & Sheehan 2004). It is believed that this saccharide diversity provides binding sites for invading viruses and bacteria. The pathogens bind to

these sugar chains and get removed with the sloughing of the mucus layer and are thus prevented from reaching the epithelial surface. The exact number of oligosaccharides per mucin molecule is difficult to determine as glycosylation is not a high fidelity process, which results in variation in the amount and length of saccharide chains around the protein core. This is termed as polydispersity of mucins (Bhavanandan et al. 1999).

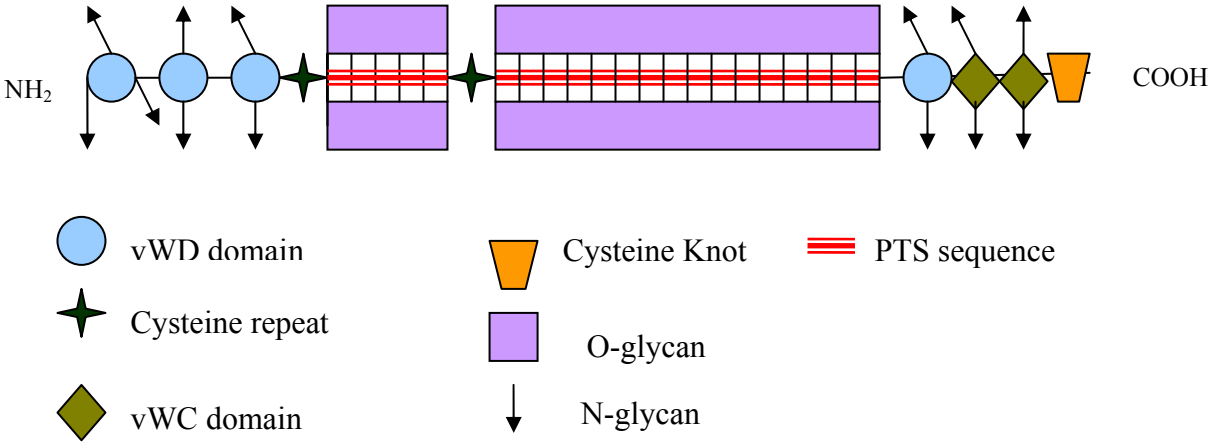


Figure 4 Structure of gel forming mucin molecule encoded by the human MUC2 gene, showing different domains and pattern of glycosylation.vWD, vWC and CK domain are the non glycosylated regions whereas the central PTS domain (also called the VNTR region) represents the glycosylated region of the mucin molecule.

1.5.2 NON GLYCOSYLATED REGION

On both sides of the VNTR region, towards the N and C terminal regions, non-glycosylated regions are present. The C and N terminal region bear potential N-glycosylation sites (Figure 4). Von Willebrand Factor type D (includes vWD1, vWD2, VWD' and VWD3) domains and other vWF domains (Von Willebrand Factor type B (vWB) and Von Willebrand Factor type C (vWC)) are found on the N-terminus while the other vWD4 is present towards the C terminal. These domains share sequence homology with the D-domains (D1, D2, and D'and D4) of the von Willebrand Factor protein, hence are named after it. The D domains of the mucin molecules form disulphide bridges similar to those which are seen with the von Willebrand Factor (vWF) protein. Thus, structurally and functionally the vWF and mucin are closely related (Furlan 1996; Desseyn et al. 2000). A Cysteine Knot region (CK domain) having highly conserved cysteine amino acids is found at the end of the C terminal. The CK and the various vWD regions are believed to be involved in polymerization of mature mucin molecules. Disulphide bridges result in formation of dimers and finally multimers which results in mucin polymeric structure. This polymeric structure controls the gel forming properties of the mucins (Pearson et al. 2000, Pearson & Brownlee 2005).

1.6 THE STRUCTURE OF MUCIN-ENCODING MUC GENES

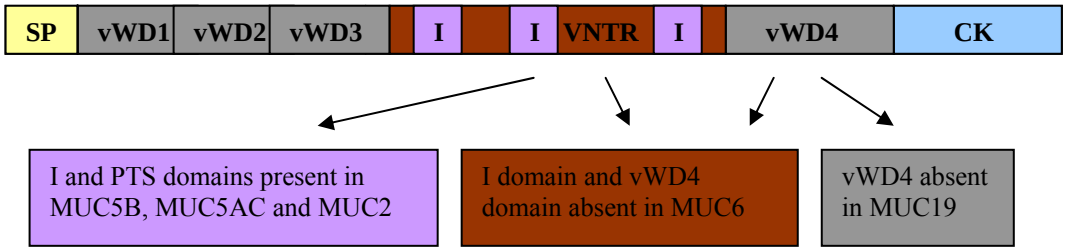
With the availability of the complete human genome dataset, a lot more information has been acquired from the mucin cDNA clones. Mucins are encoded by the MUC genes

and currently there are more than 19 MUC genes reported in humans. These are categorized into 2 subclasses, gel forming MUC genes and membrane bound MUC genes. The gel forming MUC genes consist of MUC2, MUC5B, MUC5AC, MUC6 and the recently added MUC19. All the remaining MUC genes (13 or more in number) come under membrane bound MUC genes. Some MUC genes could not be categorised in these 2 subclasses for example MUC7. The gel forming MUC genes and the membrane bound MUC genes do not share sequence homology or common ancestral origin. But still they are both kept in the same family in view of their protective role and the biophysical properties (Dekker et al. 2002). Hence, structurally the gel forming and membrane bound MUC gene differ considerably. In contrast, the analysis of the gene sequence in the gel forming MUC gene subclass reveals a high degree homology and seems to show a common ancestry (Desseyn et al. 1997, Keates et al. 1997, Buisine et al. 1998).

1.6.1 STRUCTURE OF GEL FORMING MUC GENES

Gel forming MUC genes consist of a signal peptide (SP) followed by three vWD domains and a central VNTR domain. The C terminal ends with a CK domain which can be preceded by one or more vWF domains (vWD, vWB and vWC domains) in some gel forming mucins (MUC2, MUC5B and MUC5AC). These vWD, B and C domains play an important role in polymeric structure formation of mature mucin molecules via disulphide bridge formation. The VNTR domain varies in number as well as length and can be interrupted by the unique cysteine rich super-repeats in some gel forming MUC genes (MUC5B, MUC5AC and MUC2). The cysteine rich super repeats are highly conserved amongst MUC5B, MUC5AC and MUC2, but their exact role is

still unknown (Verma et al. 1994). Thus, the SP, three vWD, VNTR and CK domains are common for all the gel forming MUC genes (Figure 5).

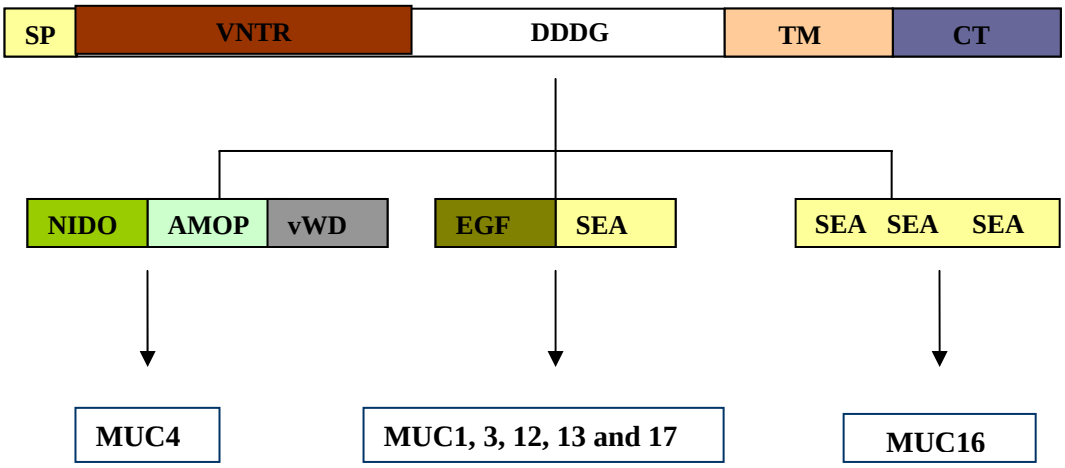


SP – Signal Peptide
vWD1, vWD2, vWD3 and vWD4 – von Willibrand Factor type D domain
I – Cysteine rich super repeats
VNTR – Tandem repeats
CK – Cysteine Knot

Figure 5 General structural organization of gel forming human MUC genes depicting absence and presence of various domains in the different gel forming MUC genes.

1.6.2 STRUCTURE OF MEMBRANE BOUND MUC GENES

The membrane bound MUC genes consist of a signal peptide (SP) followed by the VNTR region. The C terminal has a Trans-membrane domain (TM) which ends in Cytoplasmic tail region (CT). The TM domain is believed to help in the attachment of the mucin molecule to the epithelium. The region between the VNTR and TM domains has been found to vary amongst the membrane bound MUC genes in domain structure (Figure 6). This region can be occupied by one or multiple Sea Urchin Enterokinase (SEA) and Epidermal Growth Factor (EGF) domains. The EGF domain is commonly found in a variety extracellular proteins and in mucins it helps in mucosal restitution (Desseyn et al. 2008). The SEA domain is absent in MUC4 and in addition to EGF, adhesion-associated domain in MUC4 and other proteins (AMOP), Nidogen (NIDO) and vWD domains have been detected (Figure 6). The function of NIDO is unknown while it is speculated that AMOP is somehow involved in the adhesion process (Ciccarelli et al. 2002, Desseyn et al. 2008). Thus, the SP, VNTR, TM and CT regions are common to all the membrane bound MUC genes (Figure 6).



SP – Signal Peptide
VNTR – Tandem Repeat
DDDG- Different domains in different MUC genes
vWD – von Wilibrand factor type D domain
TM – Trans-membrane Domain
CT – Cytoplasmic tail
NIDO – Nidogen
AMOP – adhesion-associated domain in MUC4 and other proteins
EGF – Epidermal growth Factor
SEA – Sea Urchin Enterokinase

Figure 6 General structural organization of human membrane bound MUC genes depicting the domain similarities and differences between each member of membrane bound MUC gene.

1.7 THE FUNCTIONAL ROLE OF MUCIN

MUC genes are multi-domain structures and the domain architecture of gel forming and membrane bound mucins markedly differ from each other. The majority of membrane bound mucins (MUC1, MUC4 and MUC16) have been reported to be over-expressed in diverse human carcinomas (Duraisamy et al. 2006, Duraisamy et al. 2007), and changes in expression of gel forming mucins have been observed during respiratory and gastrointestinal tract disorders (Corfield & Warren 1996, Corfield et al. 2001, Thornton & Sheehan 2004). Investigations into gel forming mucins during diseases (respiratory tract diseases such as cystic fibrosis (CF), chronic obstructive pulmonary disease (COPD) and asthama) have revealed an overproduction of mucus with altered rheological properties.

The rheological properties of mucus gel depend on the amount, molecular size and morphology of oligomeric mucins which can undergo alteration during these disease processes. Thus, production of mucus with "correct properties" is significant for maintaining good health (Thornton & Sheehan 2004, Taylor et al. 2005). Rheologically, mucus possesses two important properties which play a big role in the proper functioning of the gastrointestinal and the respiratory tracts at the physiological level in humans. Mucus behaves as a viscoelastic gel at low levels of applied stress or strain and becomes viscous fluid with large increases in stress or strain. Secondly, mucus shows the property of rheological reversibility, wherein it recovers its optimum gel properties after mechanical disruption such as that induced by coughing. These two properties of

the mucus gel allow it to act as a reusable gel, as a lubricant and to assist locomotion under different physiological conditions (Pearson et al. 2000). Similar behaviour of mucus would be expected in case of corals where the surface mucus layer (SML) is subjected to a variety of environmental insults such as physical damage, microbial invasion, aerial exposure, sediment overload and pollutants. However, little information is available regarding structure, molecular size, physical/rheological properties of the coral mucus/mucin, since the majority of work studying coral mucus was carried out two decades ago and with little further advances in this area, the existence of the coral mucin molecule has not been unequivocally demonstrated.

1.8 AIM OF THIS STUDY

With the available resources from different fields, efforts have been made to investigate the mucus layer properties and dynamics in reef building corals. A vast amount of genomic information is becoming available about the mucin encoding MUC genes which has been exploited in the present study to better understand the genetic basis of mucus in corals. Various bioinformatic tools have been used to search the relevant mucin encoding sequences in the EST database of *Acropora millepora*. This sequence information has been used to generate the molecular tools to further enhance the mucus related gene expression studies in corals (Chapter 2).

In absence of a known consistent mucus composition across the reef corals, this study focuses on finding the mucin component of mucus using standard techniques used to

isolate and detect mucins in mammals. It further investigates the gel forming properties of isolated mucin using standard rheological methods (Chapter 3).

A method to measure the thickness of mucus layer developed for use in the human gastrointestinal tract was adopted and used successfully under submerged conditions for measuring coral SML. The SML thickness changes in four species of corals were measured under different environmental conditions (bleaching and tidal cycle) to understand the impact of these changed conditions on mucus dynamics (Chapter 4).

Volume fraction (volume of mucus present in the total volume of coral epidermis) of coral tissue of the epidermal mucus was determined in histological fixed sections during a 1991 bleaching event in order to better understand the response of mucus to bleaching stress. Similar studies were carried on two other species of corals undergoing thermal stress in a tank experiment (Chapter 4 and 5).

Within-colony changes in the mucus volume fraction and SML thickness were carried out on two species of corals experiencing bleaching. The epidermal mucus content of each colony was compared with the SML thickness to understand the relation between the two (Chapter 6).

CHAPTER 2

A bioinformatics approach to detect Cnidarian mucin gene sequences and the development of molecular tools for mucin gene expression

2.1 INTRODUCTION

Better understanding of human genome function in the post genomic era has become possible due to the availability of bioinformatics and molecular tools. The accessibility of the other vertebrate and invertebrate genome information has further increased the possibility of exploring the functional comparisons and evolutionary relationship between animal groups. In order to explore the evolution of gel forming mucin encoding muc gene or genes, information regarding various muc genes and or domains in different animal groups known to date is necessary. The human gel-forming MUC genes share sequence homology at the C and the N terminals between themselves (Desseyn et al. 1997, Keates et al. 1997, Buisine et al. 1998, Escande et al. 2004). Based on this structural homology, evolutionary relationship of the gel-forming MUC genes in humans has been proposed (Desseyn et al. 2000) (section 2.2.1). In other vertebrates such as rat, mice, pig and frog substanitial genetic information is available which show evidence of Muc genes or domains being present in these groups. Amongst the invertebrates few mucin like proteins and domains have been reported. The extensive information available on the human MUC genes (section 2.2) combined with

fragmentary evidence of the mucin like proteins or genes in other vertebrate (section 2.3) and invertebrates (section 2.4) can be utilized to detect and characterise any mucin gene homologues in corals. In this chapter, the muc gene domains or homologues of different animal proteins or genes were then utilised to explore the evolutionary relationship of coral muc genes with other MUC genes reported in different animal groups.

2.2 EVOLUTION OF THE MUC GENE FAMILY IN HUMANS

Mucus has been observed in all groups of animals, but the fundamental understanding of mucus gels and mucin proteins has been acquired from the invaluable work done on mammalian mucosal systems (Forstner 1995, Vanklinken et al. 1995, Corfield & Warren 1996, Perez-Vilar & Hill 1999, Offiner & Troxler 2000, Pearson et al. 2000, Corfield et al. 2001, Dekker et al. 2002, Escande et al. 2004, Duraisamy et al. 2006, Paulsen et al. 2006, Perez-Vilar & Mabolo 2007, Desseyn et al. 2008). The human MUC (*hu*MUC) family is still expanding and can be broadly grouped into gel-forming and membrane-bound mucins (section 1.6). The human MUC gene family has been comparatively well studied compared to other animal groups. The available MUC gene structure and sequence details can be utilised to trace the evolutionary relationships of various MUC genes with each other and with other vertebrate and invertebrate core proteins.

2.2.1 GEL FORMING MUC GENES IN HUMANS

There are more than 20 MUC genes known and only five have been categorised as gel-forming MUC genes (section 1.6.1). MUC2, MUC5B, MUC5AC and MUC6 genes form a cluster on the 11p15.5 chromosome. Recently, MUC19 has been added to the gel forming family. It is found on chromosome 12q12 and has been claimed to have gel-forming features similar to that of porcine salivary mucin (PSM) (Chen et al. 2004), but to date the mucin protein encoded by MUC19 has not been isolated and characterised.

MUC5B is considered as a model gene for evolutionary studies of *hu*MUC gel-forming genes since the complete cDNA and genomic sequences of MUC5B have been available for several years (Desseyn et al. 2000). The MUC5B gene possesses a central variable number of tandem repeat (VNTR) region, the sequence of which differs amongst the MUC genes and cannot be used for evolutionary studies (Desseyn et al. 2000; Lang et al. 2004). The regions upstream of the VNTR domain have four von Willebrand factor D domain (vWD) (D1, D2, D' and D3) domains that are involved in polymerization of the mucin molecule. The region downstream of VNTR domain has vWD4, B, C and CK domains. All gel-forming MUC genes possess Cysteine Knot (CK) domains which are important for the mucin polymer formation (structure of gel forming MUC gene described in details in section 1.6.1). The upstream and downstream cysteine-rich vWD domain (D1, D2, D', D3 and D4) architecture of gel-forming MUC genes has been found to be closely related to the human blood glycoprotein, vWF (Desseyn et al. 2000). Thus, vWF and human gel-forming MUC genes share various vWD domains while the VNTR region is the additional domain in MUC genes.

It has been suggested by Desseyn et. al (2000) that the vWF gene evolved from a common ancestor of the gel-forming mucin and vWF. The vWF gene evolved first from the common ancestor and appeared on chromosome 12, by duplication of the C domain and triplication of B domains (Figure 7). After the separation of vWF, the ancestral MUC gene had D1-D2-D'-D3-D4-B-C-CK domains with it (grey box in Figure 7). MUC6 separated from the rest of the MUC2-MUC5B and MUC5AC group by the loss of exons encoding the D4, B and C domains (Figure 7). In due course of time MUC2, MUC5AC and then MUC5B were formed from a common ancestorMUC2-5ACB (Figure 7). Since the VNTR domains are poorly conserved amongst and between the different species, it has been speculated that they must have been evolved independently (Desseyn et al. 2000). The VNTR regions must have been added after the separation and before the evolution of MUC6 as they are important for glycosylation of mucin molecules and are found in all the MUC genes. The VNTR region is interrupted by a number of cysteine rich domains called cysteine super repeats. These cysteine repeats of the central VNTR domain have been suggested to have evolved by successive gene duplication events in MUC2, 5B and 5AC (Desseyn et al. 2000).

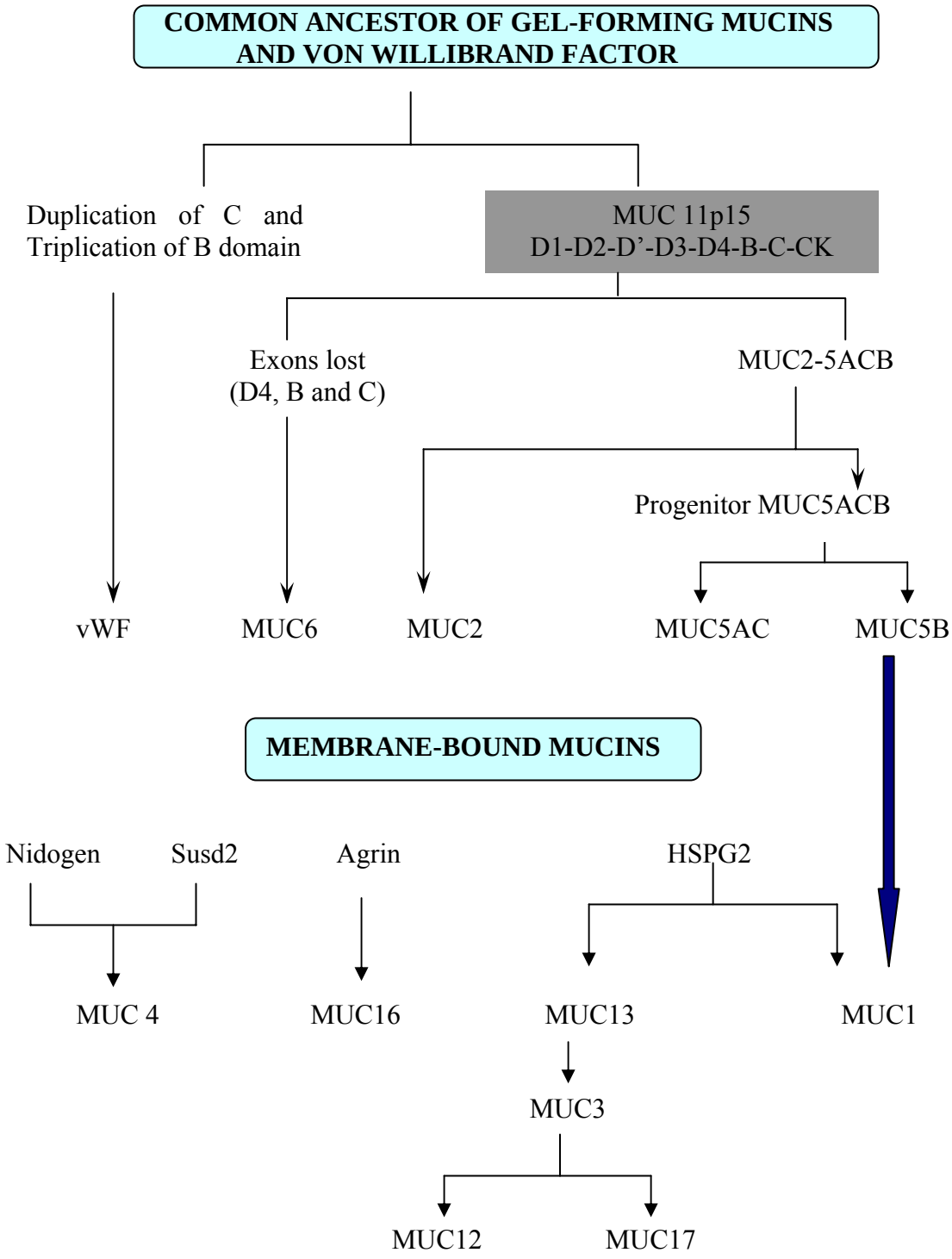


Figure 7 Evolution of gel-forming and membrane-bound mucins in humans. Duraisamy et. al has shown that CT & N terminal sequence between SEA & TR domains in MUC1 have evolved from MUC5B. This possible evolutionary relationship between the 2 subgroups of MUC gene has been represented by blue arrow. (vWF-von Willebrand Factor, Sus2 – Sushi containing 2 protein, Agarin-proteoglycan, HSPG2-heparan sulfate proteoglycan 2) Grey box represents the ancestors of all the gel forming MUC genes (Desseyn et al. 2000, Duraisamy et al. 2006, Duraisamy et al. 2007).

2.2.2 EVOLUTION OF MEMBRANE-BOUND MUC GENE

The membrane-bound mucins are involved in the cell-cell interactions, adhesion and signalling via their ecto-domains that extend from the apical cell surface (Duraisamy et al. 2006). A common domain of the membrane-bound MUC genes except MUC4 is Sea urchin Enterokinase (SEA). The SEA domain sequence analysis of membrane-bound MUC genes suggests three different ancestors (Duraisamy et al. 2006, Duraisamy et al. 2007, Desseyn et al. 2008). Accordingly, the three sub-groups of the membrane-bound MUC genes are:

A) MUC1 (one SEA domain)

B) MUC4 (SEA domain absent)

C) MUC16 (19 SEA domains)

A genome wide search revealed that the MUC1, 3, 12, 13 and 17 homologues were absent in chicken, fish and worm genomes. Thus, it appears that these are restricted to mammals only (Duraisamy et al. 2006). A phylogenetic tree was built using the SEA domain sequence alignment of all membrane-bound mucins and all proteins containing SEA domains (Duraisamy et al. 2006) indicated that MUC1, MUC13, MUC3, MUC12 and MUC17 originated from heparan sulphate proteoglycan 2 (HSPG2). It was also suggested that MUC3 evolved from MUC13, which gave rise to MUC 12 and MUC 17 (Figure 7). HSPG2 is a large single-chain polypeptide found in mammals (human, cow, rat and mouse) that has homologues in the frog (*Xenopus laevis*), fish (*Danio rerio* and *Tetraodon nigroviridis*), insect (*Drosophila melanogaster*), nematode (*Caenorhabitis elegans*) and sea urchin *(Strongylocentrotus purpuratus*) (Iozzo 2005).

The second subgroup of membrane-bound mucin i.e. MUC4 lacks a SEA domain, but possesses nidogen (NIDO), AMOP (adhesion associated domain in MUC4) and vWD domains. MUC 4 has been proposed to have evolved by two evolutionary events. The first event involved incorporation of NIDO and an EGF-like domain from an ancestor common to Nidogen and the second would have involved incorporation of AMOP and a vWD domain common to Sushi domain containing 2 protein (Susd2) (Figure 7). NIDO is an extracellular domain in nidogen, a sulphated glycoprotein widely distributed in basement membranes that binds to laminin and collagen IV, and thereby contributes to cell adhesion and cell extracellular matrix interactions (Ciccarelli et al. 2002, Desseyn et al. 2008).

The third subgroup of membrane-bound mucin, MUC16 contains 19 SEA domains. The large number of SEA domains have been speculated to be added by duplication events and evolved from agrin (Duraisamy et al. 2006). Agrin is a heparan sulphate proteoglycan, a component in glomerular basement membranes and may play a role in cell-matrix interactions.

The human gel-forming and membrane-bound MUC genes do not share structural homology and share similar biophysical properties (Dekker et al. 2002). A recent study using Blast analysis of MUC1 has shown that MUC1-CT (cytoplasmic tail) and the region between the SEA domain and TR domain on the MUC1 N-terminal has evolved from the gel-forming mucin MUC5B (represented by a blue arrow in Figure 7). Thus, evidence suggests that membrane-bound mucins or at least the MUC1 sub group have evolved from the gel-forming mucins (Duraisamy et al. 2007) (Figure 7).

2.3 AVAILABLE INFORMATION ON NON-HUMAN MUCIN GENES

Characterisation of various proteins from different groups of animal have revealed the presence of highly glycosylated proteins having tandem repeats rich in proline, thronine and serine (PTS) amino acid residues (Gowda & Davidson 1994), Wang et al. 1997, Theodoropoulos et al. 2001, Neuhaus et al. 2007). These have been referred to as mucin or mucin-like proteins. A vast amount of information about the gel-forming mucin genes is known in higher vertebrates especially in humans while corals are lower metazoan animals, thus there exists a substantial evolutionary gap between the two groups. It would be impossible to detect the evolutionary relationships unless the information about mucins in "intermediate animal groups" i.e. in other invertebrates and vertebrates is known. This section deals with the reported glycoprotein and the mucin domains known in vertebrates other than mammals.

A high molecular weight glycosylated protein containing predominantly O-linked and a small proportion of N-linked oligosaccharides has been described in cobra venom. This protein has been named "cobra venom mucin". The purified venom mucin has been reported to contain about 85% carbohydrate and 15% protein but it does not form a viscous aqueous solution unlike other mucins. It is suggested that venom mucin interacts non-covalently with a number of small venom proteins and glycoproteins and help these to remain in soluble form in the venom (Gowda & Davidson 1994). Thus, this cobra venom protein appears to possess structural similarity with mucin molecule but further functional evidence is needed to confirm it as mucin.

In frog *Xenopus laevis*, 3 integumentary mucins (FIM-A.1, FIM-B.1 and FIM-C.1) have been reported with O-glycosylated, threonine-rich domains and cysteine-rich modules (Hoffmann & Hauser 1993, Joba & Hoffmann 1997). The cysteine-rich modules in FIM-A.1 and FIM-C.1 consist of six highly conserved cysteine residues with a potential to form 3 intermolecular disulphide bridges like the cysteine knot region of the human mucins. Although the function of this domain is still unknown, it is speculated to play a role in the gastro-intestinal epithelium reconstitution process (mucosal healing). FIM-B.1 has 2 cysteine-rich modules called short consensus repeats (SRC) which are also refered by other names such as Complement control protein motif (CP) or "Sushi structure". The other cysteine-rich module is CC29 found at the C terminus and shows homology (29 out of 30 cysteine residues are conserved) with vWF, BSM, PSM and MUC2 (Hoffmann & Hauser 1993). CC29 function is not known but corresponding region in vWF helps in dimerization by disulphide bridges. The amino terminal of FIM-B.1 has been described to share homology with the pro-vWFD domains (Probst et al. 1990, Joba & Hoffmann 1997). Thus, it seems that the various integumentary mucins of *X. laevis* seem to form a polymeric glycoprotein via di-sulphide bridge formation similar to those found in vWF and human mucins.

Six mucin genes have been predicted using bioinformatics searches from the puffer fish genome (*Fugu rubripes*), out of which 3 have been proposed to be gel forming having general structure homologous to *hu*MUC2 (Lang et al. 2004). The vWD domain structure in these gel forming mucin sequences has been shown to be highly conserved using the multiple alignments but little information is available about the characterization of these mucins. Recently 2000 kD and 70-700 kD molecular weight

glycoproteins were categorised as “mucin like” based on the gel filtration and histological studies (Neuhaus et al. 2007). These glycoproteins were isolated from the common carp *Cyrinus carpio* intestine and needs further investigation to confirm their structure and gel forming properties. Thus, neither the structure nor the properties of mucin gene/protein have been completely elucidated to date in vertebrates other than mammals.

2.4 MUCIN GENE/PROTEINS IN INVERTEBRATES

The structural information for mucin encoding MUC genes amongst the invertebrates is fragmentary although a large number of invertebrates having glycosylated molecules with functional and biochemical similarity to vertebrate mucins (henceforth the invertebrate mucin proteins/domains have been referred as mucin analogues) have been reported. The mucin analogues have been reported to consists of mucin like domains with either conserved cysteine amino acid repeats (Loukas et al. 2000b, Sarauer et al. 2003) or the tandem repeats rich in amino acids serine, threonine and proline (Kramerov et al. 1996). These reported mucin analogues have been best studied in host–parasite relationships in diseases such as amoebiasis, trypanosomiasis and toxocarosis or for some commercial reason such as pest control (Wang & Granados 1997, Hicks et al. 2000, Acosta-Serrano et al. 2001, Theodoropoulos et al. 2001).

Amongst the various protozoan parasites such as *Trypanosoma* species, *Leishmania* species, *Trichomonas* species and *Entamobeba histolytica*, comparatively well studied is *T. cruzi*.

T. cruzi secrete mucin like surface molecules that can be categorised in two distinct mucin gene families (TcMUC – *T. cruzi* mucin gene family and TcSMUC - *T. cruzi* small mucin like gene family) based on life stage of the host in which they are secreted (Hicks et al. 2000, Buscaglia et al. 2006). In mammal host stage, the mucin analogues consist of tandem repeats rich in threonine and proline amino acid residues along with a glycosylphosphatidylinositol (GPI) at the C terminal end and a hyper-variable N terminal region. The molecular weight of this protein has been found to range from 60-200 KD. The second group of mucins (TcSMUC) which is expressed in insect host stage lacks the tandem repeat but has a central region rich in threonine, proline and serine. These protein molecules have been found with a molecular weight in range of 30-50 KD. Although the exact function of these mucin molecules is still under investigation, it has been suggested by Hicks et al. 2000, Acosta-Serrano et al. 2001 that these mucin analogues help in evading the host immune response in mammalian host stages. Thus, these mucin gene families seem to be highly specialised in functions that are essential for leading a successful parasitic life which is not required in free living animals.

In the nematode parasite *Toxocara canis* mucin like genes named nmuc-1, muc-2, muc-3 and muc-4 have been characterised and their products are collectively referred as the TES-120 glycoprotein family (Gems & Maizels 1996, Loukas et al. 2000a, Theodoropoulos et al. 2001). These genes encode for mucin like glycoproteins in the infective larval stages and are believed to help the larva in overcoming the host immune system. TES-120 peptide consists of central a region rich in serine and threonine and a conserved 36-amino acid six-cysteine domain (SXC). This six-cysteine domain is

suggested to fulfil the cross-linking function of cysteine-rich flanking domains in vertebrate mucins (Doedens et al. 2001). Another *T. caris* mucin gene, Tc muc-5 has been described to encode a product that is both larger and more divergent than the other *T. canis* mucins (Doedens et al. 2001). Tc muc-5 contains a putative signal peptide followed by two six-cysteine (SXC) domains, an extended threonine-rich central mucin core domain and two C-terminal SXC domains. It is belived that Tc muc-5 do not form a part of TES family since 6.7% of Tc muc-5 protein show lysine amino acid residue which has been found to be absent in the TES family glycoprotein (Doedens et al. 2001).

Wang et al. (1997) have reported the characterisation of the first invertebrate intestinal mucin (IIM) isolated from larval stage of moth *Trichoplusia ni.* The larval stage of *T. ni* is one of the most important pest of crop plants in the crucifer family. The cDNA sequence of IIM shows two tandem repeat-rich in proline, threonine and alanine interrupted by three cysteine-rich domains similar to those of *hu*MUC 2 gene. The cDNA was isolated from the peritrophic membrane specific to insects and the cysteine-rich domains were shown to be binding with chitin. The core protein of MUC2 has been demonstrated to contain more than 5100 amino acid residues whereas mature IIM contains only 763-782 amino acids (Wang & Granados 1997). Thus, there appears to be overall structural homology between IIM and the human intestinal MUC2 but it cannot be considered true representation of a mucin gene in invertebrates since *Trichoplusia ni* lacks a true mucosal epithelium and the gene product appears to be too small to form a polymeric mucin molecule.

In *Drosophila melanogaster*, a surface protein called haemomucin has been reported to be involved in immune responses and is found to be deposited in the peritrophic membrane, but the size of protein is small, ranging from 100-220 kD (Theopold et al. 1996). Similarly a 90 kD secreted glycoprotein obtained from the embryonic cells of *D. mealnogaster* has been proposed as first invertebrate mucin type glycoprotein with serine, threonine and proline amino acid residues constituting up to 30% of the protein while 40% of the glycoprotein was carbohydrate (Kramerov et al. 1996). Another protein has been characterised and named AgMuc1 in *Anopheles gambiae*. AgMuc1 appears to have a structural architecture similar to the human membrane-bound mucins with putative glycosylphosphatidylinositol (GPI) anchor sequence, central core of seven repeating TTTTVAP motifs flanked by hydrophobic N- and C-terminal domains (Shen et al. 1999). Thus amongsts arthopodes, characterization of insect intestinal mucins has been carried out essentially to develop new strategies for disease and pest control.

Recently, a jellyfish glycoprotein named Qniumucin (Masuda et al. 2007) has been reported to consist of tandem repeats of 8 amino acid residues. The sugar side chain analysis of qniumucin showed high percentage of N-acetylgalactosamine (GalNac), arabinose and galactose (Masuda et al. 2007). The presence of arabinose is unusual as it is not natural product in animals and is generally found in the plants. It is interesting to note that Qniumucin tandem repeats start at the N terminus of the molecule directly without any other domain unlike the vertebrate mucin genes. Further investigations are required to confirm the polymeric structure of the protein.

The gel forming human MUC genes exhibit a multi-domain structure. Although the exact function of each domain has remained unclear in some cases, the domain origin can be traced in various animal groups. Lang et al. (2007) conducted a genome-wide search amongst the completely sequenced animal groups. The result of this search revealed mucin related domains in most animal groups (Table 2.)

Animal group	Example	N terminal 3 vWD domains	Central TR rich in STP	C terminal vWD and or CK domain
Mammalia	*Homo/Mus/Rattus*	P	P	vWD and CK
Aves	*Gallus gallus*	P	P	vWD and CK
Amphibia	*Xenopus tropicalis*	P	P	vWD and CK
Pisces	*Tetraodon nigroviridis*	P	P	vWD and CK
Cephalochordata	*Brachiostoma floridae*	P	P	vWD and CK
Urochordata	*Cliona intestinalis*	P	P	X
Echinodermata	*Stronglyocentrotus purpuratus*	P	P	X
Arthropoda	*Drosophila melanogaster*	P	X	vWD and CK
Nematoda	*Caenorhabditis elegans*	X	X	X
Cnidaria	*Nematostella vectensis*	P	P	X*

P = present, X = not known

Table 2 Presence of known mucin-associated domains in different animal groups (Lang et al. 2007) * denotes that the CK domain has been detected in the *N. vectensis* in the present study (see section 2.4.4. and Figure 8)

A comparison of *Acropora millepora* Expressed Sequence Tags (ESTs) with complete genome of humans, *D. melanogaster* and *C. elegans* has revealed a stronger match with humans than with *D. melanogaster* and *C. elegans* genomes (Kortschak et al. 2003). This indicates that arthropoda and nematoda genomes are highly modified and there is considerable loss of metazoan genes. In arthropods *(D. melanogaster)* and nematodes *(C. elegans)* the mucin encoding domains are unknown to date (Table 2) but mucin analogues have been characterised (discussed in above section). *D. melanogaster* and *C. elagans* are notable exceptions (Table 2) in lacking important central VNTR domains but it is unclear whether these genes are absent or they are too strongly altered to be identified as mucin genes.

The evolutionary relationships between the gel-forming MUC genes in humans have been reasonably well elucidated while in other vertebrates, mucin domain homologs have been reported. Although the information about mucin genes and or proteins is very fragmentary amongst invertebrates, glycoprotein with limited homology to mucins have been reported in many animal groups. Amongst invertebrates many functions of mucus have been recognised such as locomotion in gastropods and slugs (Alexander 2006, Davies & Blackwell 2007, Ewoldt et al. 2007), mating in echinoderms and nemerteans (Thiel & Junoy 2006, Von Byern & Klepal 2006), and feeding (Lewis & Price 1975, Lewis 1977, Goldberg 2002, Ribak et al. 2005). These biologically important functions have been known in invertebrates along with the fundamental function of protection (Denny 1989). Thus, the mucus in invertebrates performs far more diverse functions compared to the mucus of vertebrates. Hence it is not possible to detect those mucin domains or genes which are highly specialised for carrying out particular function

restricted to that animal group using the vertebrate mucin gene information. For example the glycoprotein families in *T. caris* and *T. cruzi* have been suggested to help the parasite in evading the host immune mechanisms and successful establishment of the infection and disease in the host. This function of mucin seems to be limited to the parasite species and would not be useful in detecting mucin homologs in corals.

2.5 AIMS

Human MUC19 (Chen et al. 2004) and mucin genes in *Fugu rubripes* (Lang et al. 2004) have been detected using a variety of bioinformatics tools on completely annotated genomic databases. Here, the extensive information available on the human MUC genes has been utilized to:

1. Determine whether there are any gel-forming mucins gene homologs in corals.
2. Determine the complete strucutre and domain architecture of coral mucin homologs.
3. Understand evolutionary relationship of coral muc genes with other reported MUC genes

2.6 METHODS AND RESULTS

Corals and sea anemones come under the class Anthozoa of phylum Cnidaria. Anthozoan datasets are limited but a relatively large number of anthozoan Expressed Sequence Tags (ESTs) have been sequenced by Dr. David Miller and colleagues (Kortschak et al. 2003). Many of these sequences are available in Genbank or at http://cbis.anu.edu.au/coral/. *Nematostella vectensis* is a sea anemone and has been

completely sequenced and annotated by 2 different groups, StellaBase (Sullivan et al. 2006) and the Joint Genome Institute (http://genome.jgi-psf.org/Nemve1/Nemve1.home.html). Apart from these resources, the raw dataset of *Acropora millepora* (coral) has been made available on the trace archive at NCBI. All these resources were used as starting points to investigate the coral mucin genes.

In order to explore the possibility of mucin genes in cnidarians, a general search using NCBI blastp was performed. The outcome revealed homology of a part of all human gel-forming MUC genes with Fibrillin, Nematocyst outer wall antigen precursor [*Hydra vulgaris*], Integrin subunit betaCn1 [*Acropora millepora*], EGF-like protein [*Podocoryne carnea*], Head-activator binding protein precursor, [*Hydra viridis*] Dickkopf-3 related protein [*Hydra magnipapillata*] and Galaxin [*Galaxea fascicularis*]. This confirmed that the various domains of mucin genes are widespread in the different animal genomes/proteins and therefore the search requires specific bioinformatics tools to detect the cnidarian mucin gene. The Bioinformatics Support Unit of Newcastle University conducted the required searches using PTSFind, Hidden Markov Model and Rapid Automatic Detection and Alignment of Repeats to find the complete or a part of mucin gene in *Acropora millepora* and sea anemone genome. Details of PTSFind, Hidden Markov Model and Rapid Automatic Detection and Alignment of Repeats are mentioned in 2.4.1, 2.4.2 and 2.4.3 sections as provided by the Bioinformatic Unit of Newcastle University.

2.6.1 PTSFIND

This was a reimplementation of the PTSPRED algorithm described by Lang et al. (2004). A sliding window of 100 amino acids was applied to a protein sequence, and the

serine/threonine (S/T) and proline (P) abundance within that window was assessed at each step. If the S/T abundance was greater than 40% and the P abundance was greater than 5% (the most stringent criteria applied by Lang et al. (2004), then the region within the window was considered a PTS domain. If more than one domain overlaped then they were merged. The 100 aa window was moved along a sequence in 10 aa steps.

2.6.2 PFAM SEARCHES

Sequences were searched for the presence of the 5 Pfam (a database of protein families http://pfam.sanger.ac.uk/) domains (vWD, TIL, CK, SEA and EGF) commonly found in human mucins. The Hidden Markov Model for each of the domains was searched for in each of the three datasets using the hmmsearch algorithm of the hmmer program (Eddy 1998). All domains found with an E-value below the standard cut-off value of 10.0 were considered.

2.6.3 REPEATS

It was recognized that the PTS domains of mucins often contain tandem repeats. It was decided, therefore, to examine the repeating sequences found within mucin candidates by analyzing them using the RADAR (Rapid Automatic Detection and Alignment of Repeats) algorithm (Heger & Holm 2000). Thus RADAR helped in finding out the tandem repeat rich in PTS amino acid residues in cnidarian genome dataset.

The above mentioned PTSFind, Pfam searches and Repeats programmes were delevoped and used by the Bioinformatic Unit of Newcastle University to find the coral mucin gene and or domains.

2.6.4 METHOD FOR PHYLOGENETIC TREE CONSTRUCTION

Cysteine Knot sequences for various animals were obtained from National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov) using the blastp tool with default setting. Various proteins showing homology with human mucin CK domains were acquired using the blast tool with default setting from Ensembl (http://www.ensembl.org/index.html). The CK domains were found in mammals, birds, amphibians, fishes, urochordates and arthropods. The cnidarian sequences were obtained from the searches conducted by the Bioinformatic Unit of Newcastle University using PTSfind and Pfam methods. Two *A. millepora* ESTs showed all mucin specific conserved cysteine residues and motifs, but DY587151 EST was truncated and had half of the CK domain signature (CK signature in section 2.5). Hence it was not considered in the phylogenetic tree construction. There were six sequences found in *N. vectensis* which had CK domain architecture but the mucin specific cysteine knot signature was found in two ESTs only (Figure 8 and Figure 12). Details of the final sequences are mentioned in Table 1. The sequences were further analysed manually for the conserved domain architecture of CK domain. The final thirty sequences were aligned by ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html) using default parameters and phylogenetic analysis was done using TREECON version 3.1 (http://bioinformatics.psb.ugent.be/software/details/3). The ClustalW2 alignment was used in constructing the phylogenetic tree and method for distance calculation was Poisson's correction. Neighbour joining was used to generate the phylogenetic tree.

Animal group	Species	Sequence details
Mammals	*Homo sapiens* *Mus musculus* *Porsine submaxillary mucin* *Bovine submaxillary mucin*	MUC2,MUC5B,MUC5AC MUC19 and MUC6 Muc2, Muc5B, Muc19 and Muc6 AAC62527 P98091
Aves	*Gallus gallus*	XP_001234582, XP_421033, NP_989992 ENSGALP00000010855.
Amphibia	*Xenopus tropicalis*	ENSXETP00000040991,ENSXETP00000054681ENSXETP00000042206,ENSXETP00000042205ENSXETP00000041142,ENSXETP00000022821ENSXETP00000042198
Pisces	*Tetradon nigroviridis*	ENSTNIP00000007660, CAF93238
Urochordata	*Cliona intestinalis*	ENSCINP00000026267
Arthropoda	*Drosophila melanogaster*	AAF55915 (gi7300771)
Cnidaria	*Nematostella vectensis* *Acropora millepora*	DV090713, DV082209 DY584564, DY577643

Table 3 Details of cysteine knot sequences and proteins with accession numbers. The sequences were obtained from the NCBI and Ensembl database as mentioned in methods section 2.4.4.

2.6.5 CNIDARIAN MUC GENE SEQUENCES IN EST DATABASES

The bioinformatics tools used for searching coral EST database and *N. vectensis* genome did not produced a full sequence for a Cnidarian mucin gene; however it provided information about the possibility of mucin gene domain orthologues in *A. millepora* ESTs. The search produced 725 JGI (Joint Genome Institute), 922 StellaBase and 158 *A. millepora* ESTs, which were homologous to the various vWD, PTS and CK domains of human gel-forming mucins. Some of them were homologous to the

membrane-bound mucin domains such as Epidermal Growth Factor (EGF) and Trypsin inhibitor-like cysteine rich domain (TIL). The *A. millepora* ESTs sequences (158) were manually scrutinised to detect tandem repeats, conserved motifs, position and number of cysteine amino acid residues in the ESTs. In three EST sequences, presence of a mucin-specific Cysteine Knot domain could be clearly established, although a sequence was truncated. The multiple sequence alignment of the CK region of human gel-forming MUC gene and *A.millepora* ESTs confirmed the CK signature in the ESTs as shown in Figure 8.

```
CLUSTAL 2.0.8 multiple sequence alignment

MUC19_Human             ------------------------CCYTCKNNCRSSLVNVTVIYSG---CKKR--VQMA 30
MUC2_Human              -------------------------RNETRVPCSTVPVTTEVSYAG---CTKT--VLMN 29
MUC5AC_Human            -------------------------PYQNQSTCAVYHRSLIIQQQG---CSSSEPVRLA 31
MUC5B_Human             --------------------KTGCCYSCEEDSCQVRINTTILWHQG---CET--EVNIT 34
MUC6_Human              -------------------------TPTSPGVCSVREQQEEITFKG---CMAN--VTVT 29
DY587151_Acropora       ------------------------------------------------------------
DY584564_Acropora       LSFAERRLGAMTFLSLIIIVVLLLGQMTFPHASGKEICERKPVSMNV---CGGR-EIRAY 56
DY577643_Acropora       -----QIMTNCNRLVATWILILAFQLSLSSTFSCRFKKYREVIKASDGYKCKQT-RVKLG 54

MUC19_Human             KCTGECEK-TAKYNHDI---LLLEHSCLCCREENYELRDIVLDCPD----GSTIPYQYKH 82
MUC2_Human              HCSGSCGT-FVMYSAKA---QALDHSCSCCKEEKTSQREVVLSCPN----GGSLTHTYTH 81
MUC5AC_Human            YCRGNCGDSSSMYSLEG---NTVEHRCQCCQELRTSLRNVTLHCTD----GSSRAFSYTE 84
MUC5B_Human             FCEGSCPG-ASKYSAEA---QAMQHQCTCCQERRVHEETVPLHCPN----GSAILHTYTH 86
MUC6_Human              RCEGACIS-AASFNIIT---QQVDARCSCCRPLHSYEQQLELPCPDPSTPGRRLVLTLQV 85
DY587151_Acropora       ---GDVGY--------------VSTCTCCQPYLTQVENVFLQCPS----GESRSTKFAI 38
DY584564_Acropora       KCQGACES-ESKILMGD---PWFRAECRCCKSIRTETK--SVPCPG----GDEE--KIRF 104
DY577643_Acropora       RCVGMCDSHAIPIPIGDDGVPKFQTECKCCAPKEIRERTFRFSGEG-----CDKSIVVSQ 109
                           *                         * **        .   .   .

MUC19_Human             ITTCSCLD-ICQLYTTFMYS---------------------- 101
MUC2_Human              IESCQCQDTVCGLPTGTSRRARRSPRHL-------------- 109
MUC5AC_Human            VEECGCMGRRCPAPGDTQHSEEAEPEPS-------------- 112
MUC5B_Human             VDECGCTPFCVPAPMAPPHTRGFPAQEATAV----------- 117
MUC6_Human              FSHCVCSSVAC------------------------------- 96
DY587151_Acropora       IKTCRCRQFKCVSEPDKSGMKEVDAQGKVFESNPNNIEKRSL 80
DY584564_Acropora       IHACGCGNCNGA------------------------------ 116
DY577643_Acropora       IRSCECKNCSRR----------------------------- 121
                        .  * *
```

Figure 8 Multiple sequence alignment of *Acropora millepora* ESTs with that of human CK domains found in MUC2, MUC5B, MUC6, MUC19 and MUC5AC. Conserved cysteine residues are in yellow color. Cysteine in pink colour is the characteristic of mucin CK domain. DY is the accession numbers for the sequences in the National Center for Biotechnology Information (NCBI) database * represents the identical amino acid residues.

There were six sequences containing the cysteine knot domain signature in *Nematostella vectensis* annotated genome database. However the mucin specific cysteine knot with full signature (details in section 2.5) was found only in DV082209 and DV090713. The multiple sequence alignment of all these sequences is shown in Figure 9.

```
CLUSTAL 2.0.8 multiple sequence alignment


DV082277_Nematostella      ----------------------------HIAYRSTIMSLKLKRNDLRG-- 20
SB_33643_Nematostella      -----------------------------XGGSTIMSLKLKRNDLRG-- 18
SB_57158_Nematostella      ----------------MNTVIVCAVVVVLCATAQSRPSKLEKEGLSGTN 33
DV082209_Nematostella      --------------------FIDRRDKSVSHVKRSEKHRIKMGGVSPIY 29
DV090713_Nematostella      IAGVLKAILCPFATSSIYKFIHKSRYFLRCASSKRCTSARWFASQTHSLA 50
DV083228_Nematostella      --------------------------------GPATIPPKCERQ------ 12
                                                          :

DV082277_Nematostella      --------------------DWCKLR---------PVLQKLHHPGCNSS 40
SB_33643_Nematostella      --------------------DWCKLR---------PVLQKLHHPGCNSS 38
SB_57158_Nematostella      --------EEVFIMDNSLLKRGWCKTK---------PVKQIIRIEGCEPA 66
DV082209_Nematostella      GTLVVALLVIISEMSAEANYKEVCQPR---------QGSVDVRLTGCPEG 70
DV090713_Nematostella      PQFGMIVRVVLAVVAVSFIQASLGQFEGMQIQFDRVSVTRRVSYPGCETK 100
DV083228_Nematostella      --------------------EFG--------------AKQIKIGACYSN 27
                                                       :   .*

DV082277_Nematostella      FIMNNM-CYGQCMSFFIPRH------FTSCAFCTPVSKNVVSVHLKCAG- 82
SB_33643_Nematostella      FIMNNM-CYGQCMSFFIPRH------FTSCAFCTPVSKNVVSVHLKCAG- 80
SB_57158_Nematostella      EIVNNF-CYGQCNSLYIPHYNRQTPAFESCATCIPVRVHRRSVYLNCPN- 114
DV082209_Nematostella      KAFLHL-CVGTCYTEDNVVR-----DEASCTCCKPTKFGSVQVDVECRHN 114
DV090713_Nematostella      IVKVHA-CKGTCRSLTEILSAHP-WTNTICECCKMTDVKKEKVTLTCSN- 147
DV083228_Nematostella      QTYLHTGCGGYCDSSATATHGVSLTLDHKCTCCSAAKVTRFNVYMVCPD- 76
                               :  * * * :               *  *   .     .* : *

DV082277_Nematostella      -DLKVVK--KVSIIQSCSCRPCGNQYI---- 106
SB_33643_Nematostella      -DLKVVK--KVSIIQSCSCRPCGNQYI---- 104
SB_57158_Nematostella      -AKQKRKRHRYTYVKRCRCVTFRLRAAGVAK 144
DV082209_Nematostella      KAWGIVK-HVMREHEHCACVPCLG------- 137
DV090713_Nematostella      ---GDLHTRILSSAVGCKCQLCQD------- 168
DV083228_Nematostella      --KSKNFETMFPVIDSCTMSFYLVLQRHHR- 104
                                         *
```

Figure 9 CK domain signature in *Nematostella vectensis*, cyteines in yellow coloured are conserved in all the sequences, cysteine in pink colour is the characteristic conserved cysteine in mucins. Thus DV082209 and DV090713 are the potential mucin CK domain sequences in *N. vectensis*.

Similarly, three different ESTs were selected representing the VNTR region showing tandem repeats on the basis of proline, threonine and serine amino acid composition. The details of these sequences are summarized in Table 4.

Contig Number	No. of repeat	Type of repeat	Repeat sequence
DY583662	Two repeats of 38aa residue	Identical	TLSFNSTVLPSGSVTTSPSQNRTVMPIYNTTVMPT
DY587256	Three repeats of 25aa residue	Degenerate	PSSNASGKPAQKSIPSRNPTSTKSS PSSSLQKLPNLSSALPLSTSAPSTS STTAVLKVTSHVSAPPRSSGK
DY578567	Nine repeats of 7aa residues	Identical	TSSTSAS

Table 4 Details of ESTs with possible tandem repeats, rich in proline, threonine and serine amino acid residues in the database of *A. millepora* obtained by the various bioinformatic tools.

2.6.6 CONFIRMATION OF MUC GENE DOMAINS IN *A. MILLEPORA* GENOMIC DNA

The above searches resulted in selection of six ESTs showing significant homology to CK and TR domains of human mucins (Figure 8 and Table 4). These ESTs were used in developing primers to sequence the CK and TR domains in *A. millepora* genomic DNA (gDNA). For this, *A. millepora* tissue was collected in the field (PMBC Phuket, Thailand) using an airgun and frozen (-80°C) until further use. The frozen samples were sent to the laboratory (Newcastle University, UK). DNA was extracted from the tissue slurry of *A. millepora* preserved at -80°C using the DNeasy tissue kit (Qiagen) following the manufacturers protocol. In order to increase the specificity of the PCR reaction external and internal primers were designed and nested PCR protocol was followed. External and internal primers specific to each domain were designed manually and then subsequently tested with Primer3 (Rozen & Skaletsky 2000). For external primers (AJ1, AJ2, AJ3, AJ4, AJ5 and AJ6), PCR were carried out in a Px2 Thermo® thermocycler in a total volume of 10µl using 1 µl DNA template, 400 nM each primer, 50 µM each dNTP, 1 µl Q-Biogen *T.Pol* incubation mix (10 mM TrisHCl pH 9, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1 % Triton 100x, BSA or gelatin 0.2 mg/mL), 0.25 U of *Taq* DNA polymerase (Q-Biogen). The cycling protocol was: 1 × 94°C (5 min), 5× (30 s at 94°C, 30 s at 40°C, 30s at 72°C), 35× (30 s at 94°C, 30 s at 50°C, 30s at 72°C), and 72°C (5 min). For all internal primers (AM1-AM6; see Table 5), PCRs were carried out in a total volume of 30 µl using the same proportions as above and using 1.5-3µl of DNA or PCR products for the nested PCR. The cycling protocol was: 1 × 94°C (3 min), 35× (30 s at 94°C, 30 s at 50°C, 30s at 72°C), and 1 × 72°C (5 min). Products from the

PCR reactions were electrophoresed on 1.4% agarose gels to check product size. Successfully amplified products were then purified using QIAquick PCR purification kit (Qiagen) following the manufacturer's instructions for subsequent direct sequencing. To check primer specificity, fragments were sequenced in both directions using 3.2 pmol of both primers. Reagents and reaction conditions were as specified in the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction kit (PE Applied Biosystems, Foster City, California).

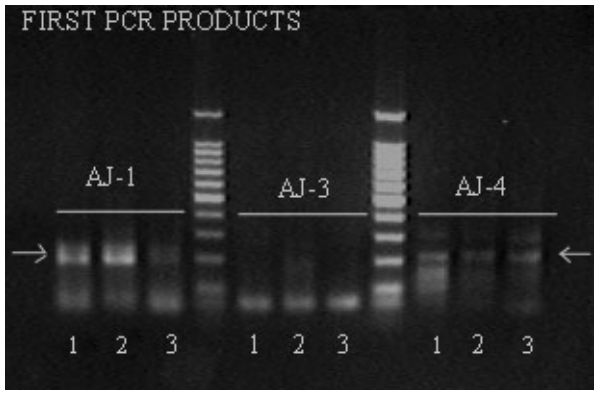


Figure 10 PCR product confirmation for primers AJ1, AJ3 and AJ4. CK domain specific primers AJ1, AJ3 and TR domain primer AJ4 showing product with the three different extracts represented as 1, 2 and 3 of *A. millepora* DNA. The product (represented by arrows in the diagram) size for CK is nearly 200 bp whereas multiple products ranging from 200-300 bp seen for TR domain.

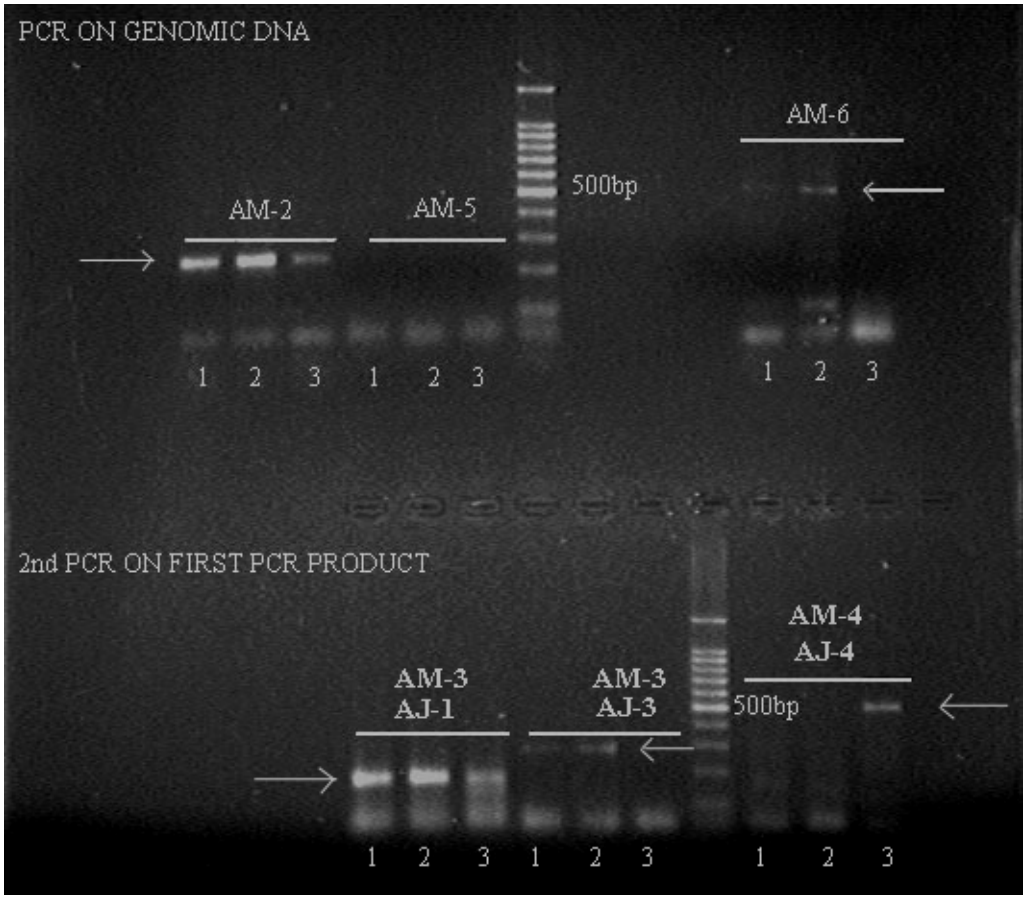


Figure 11 PCR products obtained from different primers.TR specific primers AM5 and AM6 used on three different genomic DNA extracts. Product size nearly 500 bp obtained with AM6, no product with AM5 while CK domain specific AM2 revealed product of size 200 bp.Products are indicated by arrows. Second PCR done by following nested protocol using AM1, AM3 and AM4 was done on products of AJ1, AJ3 and AJ4. Product of size 150-200 bp obtained from AM1 and AM3 while TR specific AM4 showed product of size 500 bp.

Chromatograms were checked using Chromas2 software (http://www.technelysium.com.au), and sequences aligned using ClustalW (Larkin et al. 2007). Sequences were aligned to other related sequences obtained from GenBank (through Blast search, http://www.ncbi.nlm.nih.gov/BLAST/). This analysis showed several primers were successful in amplifying the cysteine knot (CK) signature and mucin domain rich in PTS residues from *A. millepora* genomic DNA (Table 5).

Contig. Number	Primer	Domain	PCR done on	PCR Protocol
DY587151	AJ1 5′TGGAGATGTCGGTTACGTGT3′ 5′AGCATCGACCTCTTTCATGC3′ AM1 5′CGGTTACGTGTCCACTTGC3′ 5′TCTTTCATGCCGCTCTTGT3′	Cysteine Knot	Genomic DNA First PCR Product	Nested
DY577643	AM2 5′AATTGTCACGGCAATAACGAG3′ 5′GAAAGTGGACGACAGCGATAG3′	Cysteine Knot	Genomic DNA	PCR
DY584564	AJ3 5′CATAGTTAAGCTTTGCGGAGAGAC3′ 5′TTGATTCAATGACAAGGCAGA3′ AM3 5′TAATCGTCGTGTTGCTGCTC3′ 5′AACCGCAGGCATGTATGAAG3′	Cysteine Knot	Genomic DNA First PCR Product	Nested
DY587256	AJ4 5′AGCAGCAGATCCGAGGAA3′ 5′GCGGGAATTCGATTGAGA3′ AM4 5′TCCGAGGAAGAAAATATCCAGA3′ 5′TGCTAGTAGGGTTTCTCGAAGG3′	Mucin domain/Tandem Repeat (Degenerate 25 amino acid repeat)	Genomic DNA First PCR Product	Nested
DY578567	AM6 5′CAGTTTCGTCGGAAACGAAT3′ 5′TGGAAGTTGACACTGATGACG3′	Mucin domain/Tandem Repeat (Identical 7 amino acid repeat)	Genomic DNA	PCR

Table 5 Details of the successful primer sets which amplified sequences for CK and mucin domains of *A. millepora* DNA using PCR.

Sequences obtained from genomic DNA (gDNA) were 97%, 98% and 96% similar to the CK sequences, (DY 587151, DY577643 and DY584564 respectively) from the EST database used to design the primers. The specific MUC gene cysteine signature of CK domain was preserved in the coral gDNA sequences (Figure 12, Figure 13 and Figure 14)

DY587151

A Amino acid sequence of CK domain and conserved cysteine knot signature represented by cysteine amino acid residues

```
A.m     GDVGYVSTCTCCQPYLTQVENVFLQCPSGESRSTKFAIIKTCRCRQFKCVSEPDKSGMKEVDAQGKVFE
CK S            CXCC             C               CRC    C
```

B Multiple alignment of EST DY587151 and CK signature sequence retrieved from gDNA of *A. millepora*

```
CLUSTAL W (1.81) multiple sequence alignment

DY587151   CGGTTACGTGTCCACTTGCACCTGTTGTCAACCATATCTGACACAAGTTGAAAATGTGTT
Acro1-AM1  CGGTTACGTGTCCACTTGCACCTGTTGTCAACCATATTTGACACAAGACGAAAATGTGTT
           ************************************ *********  ***********

DY587151   TTTGCAATGCC-CCAGCGGAGAAAGTCGTTCGACCAAGTTTGCGATCATCAAGACCTGTC
Acro1-AM1  TTTGCAATGCCACCAGCGGAGAAAGTCGTTCGACCAAGTTTGCGATAATCAAGACCTGCC
           *********** ********************************** ********** *

DY587151   GTTGTCGACAGTTCAAATGTGTTTCTGAACCAGACAAGAGCGGCATGAAAGA
Acro1-AM1  GTTGTCGACAGTTCAAATGTGTTTCTGAACCAGACAAGAGCGGCATGAAAGA
           ************************************************************
```

Figure 12 A- CK domain signature in *Acropora millepora* database and B - the multiple alignments of the same with sequence obtained by primer AJ3 and AM3 using nested PCR protocol (Table 5 and Figure 10).Conserved cysteine amino acid residues in yellow shade and the extra cysteine residue before cysteine no. 4 is in pink colour.* represents the identical amino acid amongst the sequences

DY577643

A Amino acid sequence of CK domain and conserved cysteine knot signature represented by cysteine amino acid residues

```
YKCKQTRVKLGRCVGMCDSHAIPIPIGDDGVPKFQTECKCCAPKEIRERTFRFSGEGCDKSIVVSQIRSCECKNCS
  C         CXGXC                    CXCC               C           CEC  C
```

B Multiple alignment of EST DY577643 and CK signature sequence retrived from gDNA of *A. millepora*

```
CLUSTAL W (1.81) multiple sequence alignment

DY577643        TTTTCCTTTGGGATCAAGTGCTCCGAACTCTCTGTGACTTAATTGTCACGGCAATAACGA
Acro2-AM2       -------------------------------------TTTATTGTCACGGCAATAACGA
Acro1-AM2       -------------------------------------TTTATTGTCACGGCAATAACGA
PHU52-AM2       -------------------------------------TTAATGTCACGGCAATAACGA
                                                     * * *****************

DY577643        GTCATCACGAGATCCATCTTAGAAGTGTTCATAGAAAGCACAACTTTGACAAGTACTGTC
Acro2-AM2       GTCATCACGAGATCCATCTTAGAAGTGTTCATAGAAAGCACAACTTTGACAAGTACTGTC
Acro1-AM2       GTCATCACGAGATCCATCTTAGAAGTGTTCATAGAAAGCACAACTTTGACAAGTACTGTC
PHU52-AM2       GTCATCACGAGATCCATCTTAGAAGTGTTCATAGAAAGCACAACTTTGACAAGTACTGTC
                ************************************************************

DY577643        AGATTTGACGTACCCACCGCCAAATAGCGCTTGTCTTGCGGATAAAACATGAAATATTC-
Acro2-AM2       AGATTTGACGTACTCACCGCCAAATAGCGCTTGTCTTGCGGATAAAACATGAAATATTC-
Acro1-AM2       AGATTTGACGTACTCACCGCCAAATAGCGCTTGTCTTGCGGATAAAACATGAAATATTC-
PHU52-AM2       AGATTTGACGTACTCACGGCCAAATAGCGCTTGTCTTGCGGATAAAACATGAAATATTCC
                ************* *** ****************************************

DY577643        ATTGTTTTGTTAACAGATAATGACGAACTGCAATCGCCTGGTGGCAACGTGGATACTCAT
Acro2-AM2       ATTGTTTTCTTAACAGATAATGACGAACTGCAATCGCCTGGTGGCAACGTGGATACTCAT
Acro1-AM2       ATTGTTTTCTTAACAGATAATGACGAACTGCAATCGCCTGGGGGCAACGTGGATACTCAT
PHU52-AM2       ATTGTTTTCTTAACAGATAATGACGAACTGCAATCGCCTGGGGGCAACGGGGATACTCTT
                ******** ******************************** ******* ******** *

DY577643        CCTCGCTTTTCAGCTATCGCTGTCGTCCACTTTCTCGTGCCGCTTCAAGAAGTACAGAGA
Acro2-AM2       CCTCGCTTTTCAGCTATCGCTGTCGTCCACTTTC--------------------------
Acro1-AM2       CCTCGCTTTTCAGCTATCGCTGTCGTCCACTTTCAA-------------------------
PHU52-AM2       CCTCGCTTTTCAGCTATCGCTGTCGTCCACTTTC--------------------------
                *********************************

DY577643        AGTGATCAAAGCAAGCGATGGTTATAAATGTAAGCAGACCAGAGTAAAACTAGGCAGATG
Acro2-AM2       ------------------------------------------------------------
Acro1-AM2       ------------------------------------------------------------
PHU52-AM2       ------------------------------------------------------------

DY577643        CGTTGGTATGTGTGATTCGCATGCTATTCCGATACCCATTGGCGACGATGGGGTGCCCAA
Acro2-AM2       ------------------------------------------------------------
Acro1-AM2       ------------------------------------------------------------
PHU52-AM2       ------------------------------------------------------------

DY577643        GTTTCAAACAGAATGCAAATGCTGTGCTCCGAAGGAAATACGAGAGAGAACCTTTCGATT
Acro2-AM2       ------------------------------------------------------------
Acro1-AM2       ------------------------------------------------------------
PHU52-AM2       ------------------------------------------------------------

DY577643        CAGCGGTGAAGGGTGCGATAAGTCTATCGTCGTCTCCCAAATTCGGTCCTGTGAATGC
Acro2-AM2       ------------------------------------------------------------
Acro1-AM2       ------------------------------------------------------------
PHU52-AM2       ------------------------------------------------------------
```

Figure 13 A- CK domain signature in *Acropora millepora* database and B – alignment of sequence upstream of the CK domain has been retrieved from the lab work, using primer AM2 used directly on the genomic DNA.

DY584564

A Amino acid sequence of CK domain and conserved cysteine knot signature represented by cysteine amino acid residues

```
EIRAYKCQGACESESKILMGDPWFRAECRCCKSIRTETKSVPCPGGDEEKIRFIHACGC
      CXGXC                CXCC           C             C.C
```

B Multiple alignment of EST DY54564 and CK signature sequence retried from gDNA of *A. millepora*

```
CLUSTAL W (1.81) multiple sequence alignment

Acro2-AM3  TTAATCGTCGTGTTGCTGCTCGGCCAAATGACCTTTCCCCACGCTTCTGGCAAGGAGATA
DY584564   ------------------------------------------------------------


Acro2-AM3  TGCGAGAGAAAACCGATATCGATGAATGTTTGCGGTGGACAGCAAATCAAGGCATACAAG
DY584564   ------------------------------------- GAAATCAGGGCATACAAG
                                                  ****************

Acro2-AM3  TGTCAGGGAGCTTGCGAATCCGAGAGTAAAATACTAATGGGCGATCCCTGGTTTCGAGCT
DY584564   TGTCAGGGAGCTTGCGAATCCGAGAGTAAAATACTAATGGGCGATCCCTGGTTTCGAGCT
           ************************************************************

Acro2-AM3  GAATGCAGGTGCTGCAAAAGCATCCGAACAGAAATAAAGTCTGTGCCATGCCCAGGTGGA
DY584564   GAATGCAGGTGCTGCAAAAGCATCCGTACAGAAACAAAGTCTGTGCCATGCCCAGGAGGA
           ************************************************************

Acro2-AM3  GATGGGGAAATAATTCGCTTCATACATGCCTGCGGT------
DY584564   GATGAGGAAAAAATTCGCTTCATACATGCCTGCGGTTGC
           ****************************************
```

Figure 14 A- CK domain signature in *Acropora millepora* database and B - the multiple alignments of the same with sequence obtained by primer AM3 using nested PCR protocol. Conserved cysteine amino acid residues in yellow shade and the extra cysteine residue before cysteine no. 4 is in pink color. Acro2 is the DNA

Similarly, the TR domain specific sequence retrived from gDNA of *A. millepora* showed 94% similarity with EST DY578567. A 21bp tandem repeat (shown in grey colour in Figure 16) was conserved in EST DY587256 as follows

DY587256

```
CLUSTAL W (1.81) multiple sequence alignment


DY587256        -GCACGAGTTCTAATTGTCATGGAGGAAAAAGATAAAGAAGATCTTTCCGGTGCTAACAA
PHU52-AM4       TTCCGAGGAAGAAAATATCCAGATG--AGTAGTCGAAGTAGTTTTG-GAGTTATTGAAGA
                  *. ..*:: :**:*.**.:*.:*  *.:**: .***:**:* *   .* *. *.*..*

DY587256        AGCAGCAGATCCGAGGAAGAAAATATCCAGAGAAGACAAGAGCAAGAAAGCGGTTAGTTC
PHU52-AM4       GACTTCAGATAGACAATCGAAGAAGCTTGAAGAATTTTGGCACAATTCGCCAGAACCTTC
                ..*: *****. ....:.***.*:.   ..**** : :.*..*** :.. *.*::. ***

DY587256        CGTTAAAGGAAAAAAAACGG------ACAGTCATGTAGCAGTCAAACGGACTTCGTCAAA
PHU52-AM4       AGACGACG--AACAAATCGGGGACCCGCAACCATGAAATGGATTCCCTTCCTTGAGCCAT
                .*: .*.*  **.***:***      .**. ****:*. .*: :..*  .*** . *.*:

DY587256        CCCGAGTTCAAATGCAAGCGGTAAACCAGCT-CAGAAATCGATTCC-TTCGAGAAACCCT
PHU52-AM4       TACAGCTCCTCGTGCTCGC--ACTACTTGCAACAGGATTATTTGCCGTACGAAAATTCCT
                 .*.. * *:..***:.**  :.:** :**: ***.*:*. :* ** *:***.**: ***

DY587256        ACTAGCACCAAGAGCTCTCCATCGTCCAGTCTCCAGAAGCTTCCCAATCTGTCCTCGGCT
PHU52-AM4       TCAATTTTCTGTAGTACTCTA----CGGGAAACGCGTATCGGAGCGAAAGGTCAAGGGCG
                :*:*  : *:. ** :*** *    * .*:.:* .*:* *  . *.*:. ***.: ***

DY587256        CTCCCTCTATCGACATCAG---CTCCCAGCACAAGCGCC----TCTACTACTGCTGTCTT
PHU52-AM4       AAACGGTAATAGAATACAAGTTCGCATTACACAA-CGCCGAAGACGTAGAGCTCAAAACC
                .:.*   :**.**.::**.   * *. :.***** ****    :* :. *   *:.:.

DY587256        GAAGGTTACCTCTCATGTCAGCGCCCCTCCGCGAAGCTCTGGAAAGACAACCGGAAAAAC
PHU52-AM4       AAAGGTAAACTTTCA------CTATCCATAGACATTTTGGATAAGTACGGTCGCTTTGCC
                .*****:*.** ***      * . **: .*. *:  *  . **. **.. ** ::::.*

DY587256        AGCCAAGGACAAAACGTCTGATAAGACCACAGCAGGGAAAAGTAAGAAGTCGATACCTGT
PHU52-AM4       GAGGAAGACCCCGTCGTCTACGCAGGCC-CTACAG----------ATATCGATCTCATA
                ..  ***..*...:*****.. .**.** *:.***           *:.*****. *: :

DY587256        TAGTCCTCCAAATTCTTAGTACCTGCAGGTAATATCTCAATCGAATTCCCGCGGCCACCT
PHU52-AM4       CGG--CACG----ACAAAAAACCTTCG---AAAGACTTGAACAGGCCTTCGAGAAACCCT
                 .*  *:*     :*::*.:**** *.   **:.:** .*:*...    **.*....***

DY587256        GATGGCG-
PHU52-AM4       ACTAGCAA
                ..*.**.
```

Figure 15 Multiple sequence alignment of EST DY587256 and retrieved sequence from lab done on PHU52 DNA of *A. millepora*. * represents identical amino acid residues in two sequences.

DY578567

```
CLUSTAL W (1.81) multiple sequence alignment


Acro1-AM6      TAGTTTCGTCGGAAACGAATTCGTCAATGTCAGCCACGCCAACAATAATGTCACCCATG
Acro2-AM6      TTAGTTTCGTCGGAAACGAATTCGTCAATGTCAGCCACGCCAACAATAATGTCACCCATG
DY578567       -CAGTTTCGTCGGAAACGAATTCGTCAATGTCAGCCACGCCAACAATAATGTCACCCATG
                 ************************************************************

Acro1-AM6      TCGGCATCAATGTCATCCATGTCGGCATCAACGTCATCAATGTCATCCATGTCGACATCA
Acro2-AM6      TCGGCATCAATGTCATCCATGTCGGCATCAACGTCATCAATGTCATCCATGTCGACATCA
DY578567       TCGGCATCAATGTCATCCATGTCGGCATCAACGTCATCAATGTCATCCATGTCGACATCA
               ************************************************************

Acro1-AM6      ACGTCATCCATGTCGGCATCAACGTCATCCATGTCGGCATCAACGTCATCCACGTCGGCA
Acro2-AM6      ACGTCATCCATGTCGGCATCAACGTCATCCATGTCGGCATCAACGTCATCCACGTCGGCA
DY578567       ACGTCATCCATGTCGGCACCAACGTCATCCATGTCGGCATCAACGTCATCCACGTCGGCA
               ****************** ****************************************

Acro1-AM6      TCAACGTCATCCACGTCGGCATCAACGCCATCCACGTCGGCATCAACGTCATCCACGTCG
Acro2-AM6      TCAACGTCATCCACGTCGGCATCAACGCCATCCACGTCGGCATCAACGTCATCCACGTCG
DY578567       TCAACGTCATCCACGTCGGCATCAACGCCATCCACGTCGGCATCAACGTCATCCACGTCG
               ************************************************************

Acro1-AM6      GCATCAACGTCATCCACGTCGGCATCAACGTCATCCACGTCGGCATCAACGCCATCCACG
Acro2-AM6      GCATCAACGTCATCCACGTCGGCATCAACGTCATCCACGTCGGCATCAACGCCATCCACG
DY578567       GCATCAACGTCATCCACGTCGGCATCAACGCCATCCACGTCGGCATCAACGTCATCCACG
               ***************************** ******************* ********

Acro1-AM6      TCGGCATCAACGCCATCCACGTCGGCATCAACGTCATCCACGTCGGCATCAACGTCATCC
Acro2-AM6      TCGGCATCAACGCCATCCACGTCGGCATCAACGTCATCCACGTCGGCATCAACGTCATCC
DY578567       TCGGCATCAACGTCATCCACGTCGGCATCAACGTCATCCACGTCGGCATCAACGCCATCC
               ************ ****************************************** *****

Acro1-AM6      ACGTCGGCATCAACGTCATCCACGTCGGCATCAACGCCATCCACGTCGGCATCAACGTCA
Acro2-AM6      ACGTCGGCATCAACGTCATCCACGTCGGCATCAACGCCATCCACGTCGGCATCAACGTCA
DY578567       ACGTCGGCAACAACGTCATCCACGTCGGCATCAACGCCATCCACGTCGGCATCAGCGCCA
               ********* ********************************************* ** **

Acro1-AM6      TCCACGTCGGCATCAACGTCATCCACGTCGGAATCAACCTCATCCATGTCGACATCAACG
Acro2-AM6      TCCACGTCGGCATCAACGTCATCCACGTCGGAATCAACCTCATCCATGTCGACATCAACG
DY578567       TCCACGTCGGCATCAGCGCCATCCACGTCGGCATCAGCGCCATCCACGTCGGCATCAACG
               *************** ** ************ **** *  ****** **** ********

Acro1-AM6      TCATCCATGTCGGTATCAACGTCATCCATGTCGTCATCAGTGTCAACTAC--CAA-----
Acro2-AM6      TCATCCATGTCGGTATCAACGTCATCCATGTCGTCATCAGTGTCAACTAC--CAA-----
DY578567       TCATCCACGTCGGCATCAACGCCATCCACGTCGGCATCAACGCCATCCACGTCGACATCC
               ******* ***** ******* ****** **** *****  * ** * **  * *

Acro1-AM6      ---------------------------------------------
Acro2-AM6      ---------------------------------------------
DY578567       ATGTCGGTATCAACGTCATCCATGTCGTCATCAGTGTCAACTTCCA
```

Figure 16 **M**ultiple sequence alignment of EST DY578567 and retrieved sequence from lab done on 2 different extracts of (Acro1 and Acro2) DNA of *A. millepora*. The base pairs in grey color represent the tandem repeats of 21 basepairs. The primers used were AM6 (Table 5 and Figure 9). * represents the identical amino acid residues in two sequences.

2.7 DISCUSSION

Identification of mucin genes in genomic datasets has always been a difficult task given the multi-domain structure, occurrence of the different domains in more than one protein and the lack of sequence conservation in the signature VNTR region. In the present study, this was further complicated by the availability of Cnidarian genomic information in EST form (150-1000 bp) but not of the complete annotated genome. The present results indicate a strong possibility of mucin genes being present in the *A. millepora* genome. Mucin-like sequences sharing sequence homology with the various domains found in the human mucin genes were detected in the *A. millepora* EST database using bioinformatic tools and by using the generated primers on genomic DNA (gDNA). The molecular tools (primers) will allow further work to obtain more complete sequences of the mucin genes.

Mucins are high molecular weight glycoprotein multimers (2-40 MD) consisting of high levels of carbohydrate (nearly 80%) and protein (nearly 20%). The characteristic central VNTR region to which the carbohydrates are attached via O-glycosidic bonds with serine (S) and or thereoine (T) residues and could not be used for coral mucin gene detection since composition of the VNTR domain varies amongst the MUC genes. Hence human gel-forming MUC genes characterized by non PTS cysteine rich regions such as vWD, and CK domains were used for finding coral mucin genes. The number and position of cysteine amino acid residues in these non PTS domains on the N and C terminal have been observed to be highly conserved amongst the mammalian gel-

forming mucins (Desseyn et al. 1997, Keates et al. 1997, Buisine et al. 1998, Escande et al. 2004). Human gel-forming mucins have been placed together in one group since each gel-forming MUC gene possess Cysteine Knot domain at their C terminal end (Desseyn et al. 1997). The CK domain along with various vWD domain present at the ends of the MUC gene help in polymerisation of the mucin monomer. The functional effectiveness of the mucin barrier is related to this polymeric structure of mucins (Pearson & Brownlee 2005). Thus, CK has been predicted to be one of the significant domains for gel-forming mucins.

The Cysteine knot structure is found at the C terminus of all the mammalian gel forming MUC genes. The CK domain encodes for dimerization and multimerization amongst mucin monomers by forming disulphide bridges (Offiner & Troxler 2000, Bell et al. 2001). A pair of cysteines is necessary for disulphide bond formation. If one half of the disulphide bond pair is lost then protein folding is affected. This can result in loss of vital function of the protein (mucin) (Vitt et al. 2001). Experimental replacement of cysteine amino residues by alanine in rat mucin has resulted in production of impaired dimers and affected the function of the mucin barrier (Bell et al. 2001). Thus, the correct positioning of cysteine residues is important in mucins. Considering the importance of CK domain, the presence of cysteine residues at particular positions and their number formed one of major criteria in searching the available lower metazoan database.

Cysteine knot sequences have been reported in the other extracellular proteins which form dimers such as transforming growth factor (TGB-β), glycoprotein hormones (GPHs) and platelet derived factors (PDGFs). These proteins are referred as members of

CK superfamily. The Cysteine Knot consists of a ring structure of amino acids joined by disulphide bonds between the cysteine residues Cys2 and Cys5, Cys3 and Cys6 (numbered from the N terminal of the knot) (Bell et al. 2001) (Figure 17). The third disulphide bond is formed between the Cys1 and Cys4 that penetrates the ring thus resulting in a knot like structure. The three dimensional structure of cysteine knot can be described as a “hand” containing two fingers and a heel (Figure 17). This arrangement exposes the hydrophobic residues to the aqueous environment leading to formation of homo or hetero dimers (Bell et al. 2001; Vitt et al. 2001). Members of all the CK superfamily contain the important C-X-G-X-C and C-X-C motifs that participate in ring formation (Vitt et al. 2001).

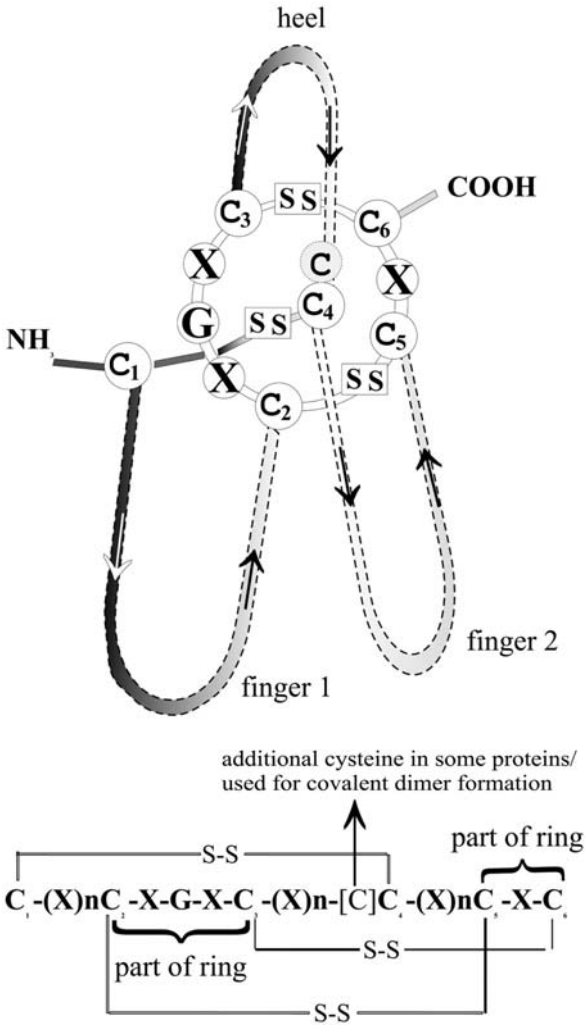


Figure 17 Schematic Drawing of the 10-Membered Cysteine Knot Structure Arrows indicates the direction (N to C terminal) of the amino acid chain. SS indicates disulfide bonds. The six cysteines involved in knot formation are numbered consecutively and their spacing is given in the *lower panel*. Cysteines 2 and 3 form disulfide bonds with cysteines 5 and 6, respectively, thus forming a ring. The ring is penetrated by the third disulfide bond formed between cysteines 1 and 4. The amino acid chains between cysteines 1 and 2 and between 4 and 5 typically form finger-like projections, whereas the segment between cysteines 3 and 4 forms an α-helical structure and is designated as a heel. In some of the known cystine knot proteins an additional cysteine is located in front of cysteine 4, which was found to be essential for covalent dimer formation (Vitt et al. 2001).

The mucin CK structure differs from the members of all other CK super family by:

(1) Presence of additional cysteine residue just before the 4th cysteine residue. This 4th cysteine is a characteristic feature of all the gel-forming mucins and vWF CK domain (Bell et al. 2001; Vitt et al. 2001). (2) Cysteine amino acid residue C^a, C^b, C^c and C^d (Figure 18) are conserved in mucins and vWF but are found to be absent in many other CK containing proteins.

Thus, the mucin specific Cysteine knot signature can be represented as:

$$C^1(X)_nC^a\text{-}(X)_n\mathbf{C^2\text{-}X\text{-}G\text{-}X\text{-}C^3}\text{-}(X)C^b\text{-}(X)_n\text{-}C^*\text{-}C^4\text{-}(X)_nC^c\text{-}(X)_n\mathbf{C^5\text{-}X\text{-}C^6}\text{-}(X)_nC^d$$

Figure 18 The signature of mucin specific cysteine knot domain. The numbered cysteine amino acid residues ($C^{1\text{-}6}$) participate in the internal cysteine knot ring formation by forming three di-sulphide bonds. The four cysteine amino acid residues labeled as $C^{a\ldots d}$ are specific to mucins and vWF while they are found to be absent in other members of CK superfamily. C* is the extra cysteine residue conserved just before the 4th cysteine amino acid. X represents any amino acid. The motif CXGXC and CXC which are common for all CK domain proteins are shown in bold.

The bioinformatics searches identified three mucin-specific CK domain sequences in the *A. millepora* EST database (Figure 8). Although DY587151 was missing the important first part and motif CXGXC, presence of CXCC i.e. extra C* infront of C^4 and conserved CXC motif along with C^d indicates the strong possibility of third mucin specific CK sequence in *A. millepora* genome (Figure 8 and Figure 12). In the *N. vectensis* genome, six sequences representing CK domains were identified. All the sequences had conserved CXGXC and CXXC motif but only three sequences showed extra cysteine amino acid (C*) residue conservation in CXXC motif (Figure 9). DV083228 did not show the conservation of the CXC motif which participates in CK ring formation. The presence of CK signature of mucin in the *A. millepora* and *N.*

vectensis sequences clearly established the strong possibility of mucin genes in cnidaraians.

The cysteine residues retrieved in the sequences obtained from the *A. millepora* genomic DNA in the laboratory has demonstrated the known CK motif such as CXGXC, CXCC and CXC (Figure 12, Figure 13 and Figure 14). EST DY587151 obtained from the *A. millepora* EST database was truncated; hence it showed an incomplete CK domain signature starting with CXGXC motif (Figure 7). These motifs form an important part of the 3 dimensional structure of the cysteine knot (Figure 17), participating in the internal disulphide bridge formation. Dimerization process has been reported to be impaired by the mutation of cysteine (C^b) in $C^bXC^*C^4$ motif while mutation of cysteine amino acid residue (C^2) in C^2XGXC^3 motif has shown poor secretion of mucin dimers (Parez-villar et al. 1999).The additional cysteine residue (C*) just before the 4th cysteine amino acid in $C^bXC^*C^4$ was also conserved in all the three retrieved CK sequences of *A. millepora*. The presence of the above mentioned motifs and the additional cysteine before the 4th cysteine amino acid residue along with four conserved cysteine amino acid residues in two CK domain sequences indicates two different CK Knots and possibility of the third CK domain specific to gel-forming mucin in *A. millepora*. These retrieved sequences do not seem to form part of same gene, as the amino acid residues in between conserved motifs varies substantially (Figure 12, Figure 13 and Figure 14) and the presence of more than one CK domain is unknown in the same mucin gene in other organisms. Thus, presence of three CK sequences in *A. millepora* and two in *N. vectensis* suggests that these sequences represent more than one gel forming MUC gene in cnidarians.

A phylogenetic tree was constructed on the basis of the cysteine knot domain sequences specific to mucins to understand the divergence and evolutionary relationship of the lower metazoan mucin CK sequences with that of the well documented vertebrate mucin and other animal mucin CK proteins. As shown in Figure 19 *A. millepora* (DY584564) and *N. vectensis* (DV090713) share a close relationship with each other and appear as a distinct group along with other cnidarian sequences. The lower metazoan muc genes seems to posses more divergent sequences from the other animal mucin gene CKs. Human gel forming MUC genes group into distinct clusters (MUC6, MUC19 and MUC2) alongside closely related muc genes from other vertebrate mucin CKs. *D. melanogaster* belongs to the phylum Arthropoda and is an invertebrate animal, hence it would be expected to have a CK domain close to the cnidarian sequence but it is shown here to be more closely related to the human MUC19 group and related sequences from other vertebrates rather than lower metazoan CKs. The constructed phylogenetic tree is unrooted and hence it is not possible to determine the evolutionary path and the common ancestor for different mucin CKs. Other members of the CK super family (such as transforming growth factor (TGB-β), glycoprotein hormones (GPHs) and platelet derived factors (PDGFs) or other proteins (such as vWF or split like protein) could be possibly used to root the present tree but the evolutionary relation between the mucin CKs and these protein is unclear and members of the CK superfamily have been well studied in higher vertebrates (humans mainly) which makes it difficult to use any one of these as a reliable root. Hence an unrooted tree was constructed since it was difficult to find a protein or domain which would have diverged long ago and symbolizes the first common ancestor of CK sequences.

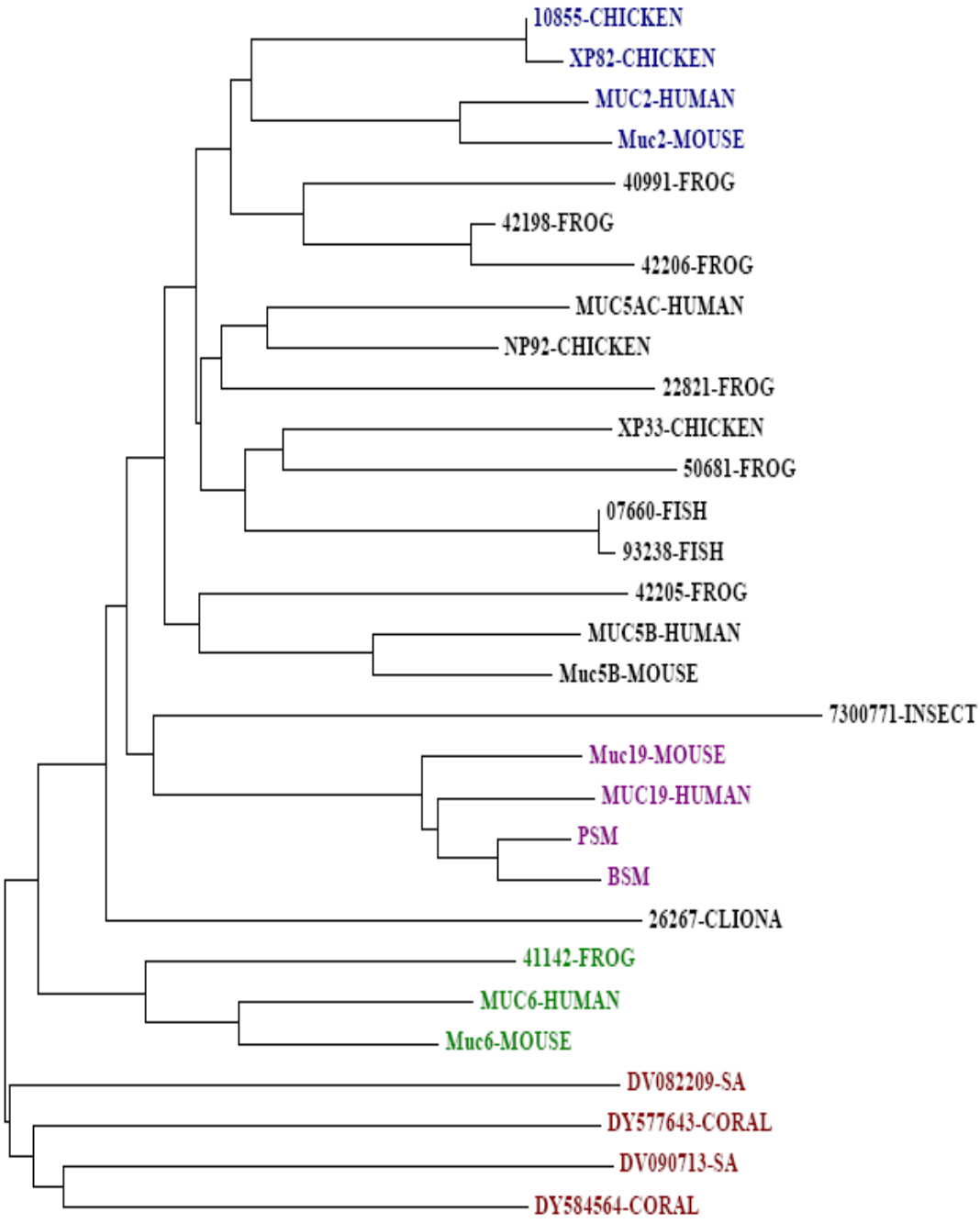


Figure 19 Unrooted phylogenetic tree depicting the evolution of mucin specific cysteine knot domain in known mucin genes and proteins. The cnidarian sequences cluster with each other shown in red colours while the MUC6, MUC19 MUC2 groups have been represented in green, purple, and blue colours respectively.The details of the name and accession numbers of the sequences are mentioned in Table 3.

There were three EST sequences selected from the database to design the primers specific to VNTR region, out of which two pairs of primers worked and amplified the sequences from gDNA of *A. millepora* but the third one could not give the product. Out of two retrieved sequences DY578567 tandem repeat is similar to human MUC 5AC. However, the other tandem repeat does not show any similarity with TR domain of any other mucins. DY578567 had seven amino acid repeat consisting of (Thr – Ser – Ser – Thr – Ser –Ala – Ser) whereas MUC 5AC consists of a repeat of eight amino acid (Thr – Thr – Ser – Thr – Thr – Ser – Ala – Pro). The position of three amino acids is identical in both the repeats. The percentage of serine and threonine is more than 80% in both the repeats. There can be maximum six glycan bonds possible in both the repeats and three sites of glycosylation are common in both is shown in Figure 20

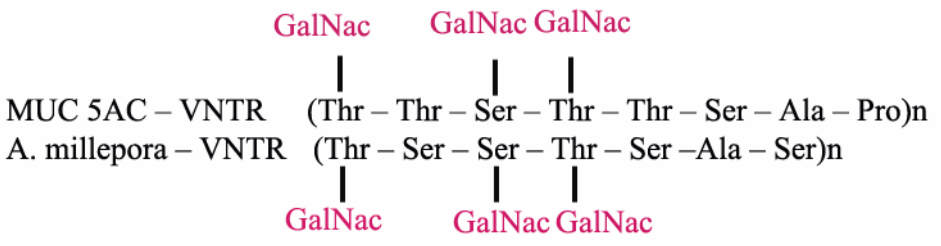


Figure 20 Demonstration of the sites of O glycosylation in *A. millepora* tandem repeat similar to that found in the human MUC5AC gene

Thus, there seems to be a similarity in the pattern of glycosylation of the tandem repeat sequence with that of the human MUC5AC VNTR region. The presence of more than one CK domain has been established in cnidarians. The developed primers specific to the CK and tandem repeat have successfully amplified these domains in *A. millepora*.The CK and the tandem repeat strongly indicates polymeric high molecular weight mucin in *A. millepora*. Although the cnidarian muc genes CKs seem to posses

more divergent sequences than the other animal mucin gene CKs, the process of mucin synthesis involving disulphide bridges in dimer formation has been indicated to be similar to that found in mucins and vWF.Thus it is highly likely that coral mucin may have overall structure and properties similar to human gel forming MUC genes.

2.8 CONCLUSIONS

Clear evidence has been demonstrated to support the first aim of the chapter, to determine whether there are any gel-forming mucin gene homologs in coral. The presence of more than one gel-forming mucin gene has been indicated in cnidarians. The second aim of finding the complete structure of gel-forming coral muc genes was partially achieved by the characterisation of two different VNTR regions along with three Cysteine Knot domain sequences. The evolutionary relationship of muc genes and proteins based on CK domain architecture confirmed that the muc genes in cnidarians show strong divergence to all other mucin genes. The cnidarian mucin-specific CK sequences might represent are ancestral form of the vertebrate and invertebrate muc genes, but this needs further investigation. Although a link between the VNTR and CK domain has not yet been established, the VNTR and CK domain together indicate a strong potential for cnidarian gel-forming mucin genes.

CHAPTER 3

Investigation of rheological properties of coral mucus

3.1 INTRODUCTION

Rheology is the study of deformation and flow of matter. The behaviour (elastic or plastic) and the response of a fluid to shear forces (stress or strain) determines the rheological properties of the fluid. Values of shear stress, shear rate, and shear strain are primary parameters for quantitative specification of both the flow conditions and the liquid response to applied shear stress. Mucus possesses elastic behaviour as well as fluid behaviour in response to applied shear force. The rheological properties of mucus are responsible for the protection and lubrication of the underlying surfaces.

The human mucosal system is well studied and defines mucus as a secretion containing mainly mucin along with immunoglobulins, lipids, enzymes, proteins and nucleic acid (Pearson and Brownlee 2005). Studies done on gastrointestinal and respiratory system diseases reveal that the protective property of the mucus lies in its ability to form a gel and this gel-forming ability is imparted by the mucin. Mucin is a high molecular weight (2-10 MD) polymeric glycoprotein consisting of glycosylated subunits joined by disulphide bridges. This polymeric structure as well as length of the mucin strands is responsible for the gel formation and the rheological properties of the mucus (Pearson et al. 2000, Perez-Vilar & Mabolo 2007). Being a heterogenous molecule consisting of subunits, it is practically very difficult to protect the polymeric mucin molecule from

degradation after collection, but use of a range of protein inhibitors during isolation helps in preventing degradation. Thus, due to inherent heterogeneity and polydispersity of the mucin molecule (details in 1.5.1) a range of molecular weights is reported, but they are characterised by their large size in millions of Daltons. In order to characterise the coral mucin molecule, it is necessary to establish information regarding its size, molecular weight and the degree of polymerization that will contribute to its rheological properties.

The previous biochemical compositional studies demonstrate that coral mucus consists of protein, polysaccharide and lipids along with the coral tissues, zooxanthellae, bacteria, nematocysts, planktons, filamentous algae and sediments in varying quantities (Brown & Bythell 2005). Although the studies done on the composition of coral mucus propose the presence of probable glycoprotein, virtually nothing is known about its structure, molecular size or its composition (Meikle et al. 1987, 1988, Brown & Bythell 2005). Uncertainty surrounds coral mucus as well as coral mucin because neither the definition of nor the composition of mucus has been clearly worked out. Besides this, the majority of coral mucus compositional studies were carried out before the 1990's, when sparse information was available on the molecular and genetic basis of mucus production and secretion.

Recent studies on the coral surface microbial communities have renewed interest in the surface mucus layer and its inherent protective (antibacterial) properties. A loss of antibiotic property of mucus during the summer bleaching event and a shift in the microbial community with an increase in the number of bleaching- associated *Vibrio*

species has been demonstrated prior to bleaching (Ritchie 2006, Bourne et al. 2007). 2007). However, these studies focus on determining the changes in the antimicrobial properties of mucus and shaping of microbial community structure in response to different environmental stressors rather than understanding their interactions and the components of mucus that could lead to this change.

Rheological properties of mucin can be determined by solution viscosity measurements, whereas the gel strength and the degree of polymerisation are obtained by gel rheology measurements and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) respectively. The solution viscosity of purified mucin solution rises asymptotically with increased concentration of mucin until a gel is formed (Allen & Pearson 1993). This behaviour of mucin is unlike a Newtonian fluid's behaviour where viscosity follows a linear relationship with the increased solute concentration. The solution viscosity measurement not only provides information regarding the size and shape of mucin but also indicates the molecular interaction occurring within and between the mucin molecules before gel formation (Allen & Pearson 2000).

In gel rheology measurement, the gel is exposed to sinusoidal oscillating stress or strain and resulting stress or strain is measured. In other words, energy is put into the gel system, the elastic component of the system stores the energy and is termed as storage/elastic modulus (G') while a part of the energy dissipates which is measured and is called the loss/viscous modulus (G''). If the gel is entirely elastic then stress and strain would be in phase and if purely viscous then they would be 90° out of phase. Any lag between the input and output is the phase angle. The smaller the phase angle, the

more elastic the system is and larger the angle the more fluid and viscous the system is. Thus, the value of phase angle and G' provides information about the gel strength.

Since mucin is a high molecular weight glycoprotein, SDS PAGE (Sodium dodecyl sulphate polyacrylamide gel electrophoresis) of purified mucin helps in finding the approximate molecular weight range and also the degree of polymerization. Highly polymeric mucin species remain near the point of application in the stacking gel, while the rest migrate up to and into the running gel after the completion of the electrophoresis. Thus, a combination of all the above described techniques has been employed to understand the behaviour of coral mucin molecule/s. The present study focuses on coral mucin molecule/s and their rheological properties, thus making a distinction of coral mucus from its major component mucin which has not been studied previously.

3.2 METHODS AND MATERIAL

3.2.1 SAMPLE COLLECTION AND SOLUBILISATION:

The corals *Goniopora stoksii, Acropora millepora, Fungia fungitis* and *Montastrea faveolata* were aerially exposed and inverted on a beaker to promote mucus secretion and the exuded mucus was immediately frozen at -20°C and then stored at -80°C until further use. This method of mucus collection is referred as "milking of corals" (Ducklow & Mitchell 1979b, Brown & Bythell 2005). Other samples of *Goniopora stoksii* were obtained by snap-freezing the coral directly in liquid nitrogen in the laboratory and preserved at -80°C until further use.

Coral mucus samples (volume) were measured and homogenised briefly, if needed (1 minute, speed 4 on Silverson homogeniser) on ice. The sample was mixed with the inhibitor buffer (10 mmol.l^{-1} EDTA, 100 mmol.l^{-1} α aminocaproic acid, 10 mmol.l^{-1} N-ethyl melimide, 1mmol.l^{-1} iodoacetamide, 5 mmol.l^{-1} benzamide HCl, 1mmol.l^{-1} PMSF and 2 ml propanol mixed in Phosphate Buffer Saline (PBS) (pH 7.3) in order to inhibit any proteolytic activity. Coral mucus samples and the proteolytic inhibitors solutions were mixed in a 1:9 ratio. PMSF and benzamide HCl were added to the rest of the buffer just before adding the sample to the buffer. A further 25 ml of PBS (pH 7.3) was added to this mixture and solution was stirred at 4°C for 24 h. The samples were then centrifuged at 10,000 rpm for 1 h at 4°C and supernatants were extensively dialysed against distilled water. Different methods of solubilisation were tried to extract the mucin from the collected samples. Based on method of collection and solubilisation, the following samples were obtained from various corals

:

Species	Collection method	Solubilised in	Named as
Goniopora dubitiensis	Aerially exposed/milking	PBS and inhibitors	*Goniopora* mucin (GM)
Goniopora stoksii	Freeze dried in liquid nitrogen and made into slurry	PBS and inhibitors	*Goniopora* – Extract 1 mucin (GX1M)
Goniopora stoksii	undissolved *Goniopora* extract 1	Guanidinium chloride	*Goniopora* – Extract 2 mucin (GX2M)
Acropora millepora	Aerially exposed/milking	PBS and inhibitors	*Acropora* mucin (AM)
Montastrea faveolata	Aerially exposed/milking	PBS and inhibitors	*Montastrea* mucin (MM)

Table 6 Method of sampling and solubilisation of the collected mucus from various species of coral. First *Goniopora stoksii* mucin (GX1M) was extracted from the whole tissue in PBS and inhibitor solution. Then the undissolved material was then solubilised in guanidinium chloride solution for 24 hours and mucin was extracted (GX2M). This procedure was done to achieve complete solubilisation of the mucin.

3.2.2 CAESIUM CHLORIDE EQUILIBRIUM DENSITY GRADIENT ULTRACENTRIFUGATION

In order to obtain purified mucin, CsCl equilibrium density gradient centrifugation was performed. The dialysed samples were weighed and CsCl was added equivalent to approximately 60% of the total volume of sample. Samples were adjusted to the recommended starting density for the mucin isolation of 1.42g.ml^{-1} (Davies & Carlstedt 2000). The samples were then centrifuged for 48 hours at 40,000 rpm at 4°C in Centricon T-1170 centrifuge. After centrifugation, 9 fractions were recovered from the gradient by piercing the bottom of the tubes and collected fractions in separate tubes. The fractions were analysed for density (by weighing a known volume) and fractions were dialysed extensively against distilled water to remove the caesium salt. The

dialysed samples were then subjected to periodic acid Schiff's assay (PAS) at 555 nm to determine glycoprotein distribution and concentration. Presence of protein in the samples was also determined by calculating 260/280 nm ratio.

3.2.3 PERIODIC ACID SCHIFF'S (PAS) ASSAY

Quantification of glycoprotein was carried out using the PAS Assay (Mantle & Allen 1978). In this assay the 1, 2 glycol groups present in the glycoprotein are oxidised to form di-aldehyde, which then binds the Schiff's reagent producing a magenta colour. Standard curves were prepared using glycoprotein concentrations ranging from zero to 100 μg of papain-digested pig gastric mucin and total volume was made up to 1 ml using distilled water. 1ml of fractions obtained from CsCl gradient were used as samples. To this sample, 100 μl of 0.1% periodic acid in 7% acetic acid was added (sample solution). A 0.017 $g.ml^{-1}$ sodium metabisulphite solution was made in Sigma Schiff's reagent (solution 2). The two solutions (sample solution and solution 2) were incubated at 37^0C for an hour in water bath. 100 μl of activated Schiff's reagent (solution 2) was added to each of the sample tubes and was left half an hour for colour development at room temperature. Absorbance was measured at 555 nm against the reagent blank. The amount of glycoprotein in the sample was obtained from the standard curve.

3.2.4 REDUCTION OF NATIVE *GONIOPORA STOKSII* MUCIN (RGM)

A mucin solution was reconstituted by dissolving 5 mg of lyophilised material in 10 ml of distilled water. The solution was dialysed against 0.2 $mol.l^{-1}$ NaCl, 0.03 $mol.l^{-1}$ sodium azide with 0.2 $mol.l^{-1}$ βmercaptoethanol added at 4°C and incubated for 24 hours. The reduced material was then dialysed for 24 h against 0.2 $mol.l^{-1}$ sodium chloride containing 0.03 $mol.l^{-1}$ sodium azide and 0.22 $mol.l^{-1}$ iodoacetamide (3 changes). This reduced and blocked glycoprotein preparation was finally dialysed against distilled water at 4°C for 24 h and then freeze dried.

3.2.5 SDS PAGE

SDS PAGE was conducted using the Pharmacia gel system (Pharmacia Ltd.). The isolated freeze dried coral mucin samples and standards were solubilised in 0.0625 $mol.l^{-1}$ Tris buffer, pH 6.8, containing 2% (w/v) SDS, 10% (v/v) glycerol and 0.001% bromophenol blue. Samples were heated to 100°C for two minutes in a water bath before being applied to the gels (Phastgel™ Gradient 4-15%). Gels were run at a constant current of 10 mA for 260Vh.

3.2.6 PERIODIC ACID SCHIFF'S STAINING (PAS) FOR 4-15% SDS PAGE GEL

The gels were fixed overnight in 7% acetic acid, followed by 1 h oxidation step using periodic acid (0.2% periodic acid in 7% acetic acid). 0.4 g Sodium metabisulphite was added to 24 ml of Schiff's reagent (Sigma) and was incubated at 37°C for 1 h in a

waterbath. This activated Schiff's solution was then added to the oxidised gel and again incubated for half an hour at 37°C. The gels were then scanned using a Biorad GS-800 densitometer. The amount of polymeric material present in a sample was calculated by determining the amount of material that remained at the point of application compared to the amount that had moved from the point of application to the interface between the stacking and running gel and into the running gel. This SDS PAGE method has previously been validated (Rankin et al. 1995, Newton et al. 1998).

3.2.7 SOLUTION VISCOSITY MEASUREMENTS

Solution viscosity measurements were carried out using a Contraves low shear 30 (Contraves A.G). The equipment consists of a Couette rotating cup and a bob. The viscometer is controlled by an electronic speed programmer (Rheoscan 20) which allowed the speed (shear rate) to be increased or decreased under controlled conditions. Approximately 2 ml of sample was placed in the cup and the bob was lowered into the sample avoiding trapping of any air bubbles. For solution viscosity measurements 20 mg of the *Goniopora* species mucin was dissolved in 2 ml of the PBS solution. Shear force was applied to the sample and the signal from it was converted into chart recorder output. The recorder produces a plot of percentage deflection (amount of torque transferred from the rotating cup via the viscous solution to the bob) on the *y*-axis and shear rate (speed the cup turns) on *x*-axis. The gradient of the deflection vs shear plot is directly proportional to viscosity; the relative viscosity (η_{rel}) can be determined from

$$\eta_{rel} = \frac{\text{Gradient of sample solution plot}}{\text{Gradient of solvent (PBS) plot}}$$

Specific viscosity (η_{sp}) was calculated from relative viscosity as follows;

$$\eta_{sp} = \eta_{rel} - 1$$

Using specific viscosity, reduced viscosity (η_{red}) was obtained as follows;

$$\eta_{red} = \eta_{sp}/C \qquad (C = \text{concentration in } mg.ml^{-1})$$

To obtain intrinsic viscosity ln η_{rel}/C was plotted against glycoprotein concentration (C) and extrapolated to zero concentration (Kramer plot). Alternatively, a value for intrinsic viscosity can also be obtained by plotting η_{red} versus C extrapolated to zero concentration (Huggins plot). Both Kramer and Huggins plots can be used together or independently to obtain the intrinsic viscosity. The molecular weight (MD) of the mucin was calculated using the intrinsic viscosity [η] from the Mark-Houwink equation which is:

$$[\eta] = K M^{\alpha}$$

where, K and α are Mark-Houwink constants that depend upon the type of mucin polymer, solvent, and the temperature of the viscosity determinations, the exponent 'α' is a function of polymer geometry, and varies from 0.5 to 2.0. These constants were determined experimentally by measuring the intrinsic viscosities of several pig gastric mucin polymer samples for which the molecular weight has been determined by light scattering and sedimentation methods. For pig gastric mucin, K and α were 3.4865×10^{-5} and 0.56 respectively.

3.2.8 GEL RHEOLOGY MEASUREMENTS

Gel rheology measurements were carried on Bohlin rheometer, in which the native mucus gel samples were placed between the serrated plates, which were closely set up

to a gap of 0.150 mm. Samples were covered by a Perspex cover to prevent desiccation. Then the samples were subjected to electronically control sinusoidal deformations. A viscoelastic region i.e. "the shear independent plateau" is determined first where the moduli $G^{'}$ (elastic moduli) and $G^{''}$ (viscous moduli) remain constant over a range of shear stress. This is described as an amplitude sweep and is carried out at a frequency of 1 Hz at 25°C. The moduli change constantly before the shear independent pleatue (Figure 26). With increase of shear stress after the shear independent pleateau, a point is reached where $G^{'}$ will collapse in value and $G^{''}$ goes on increasing; this is the nonviscoelastic region where the gel is being disrupted. Once the viscoelastic region is determined, a frequency sweep was performed at 25°C between 0.002-30 Hz with the rheometer operating in auto stress mode. The initial stress is set in the middle of the viscoelastic region with the target strain also set in this region.

3.2.9 CENTRIFUGAL CONCENTRATION OF THE *M. FAVEOLATA* SAMPLE

The *M. faveolata* milked mucus sample (120 ml) as collected from the field was thoroughly mixed and then 2 ml of sample was used to obtain the initial solution viscosity measurement on the cup and bob viscometer (Contraves low shear 30). The sample was subjected to centrifuge using a Centricon filter device. The cut off limit of the filter was 50,000 D. Samples were concentrated at 2000g at 10°C in stepwise manner until 120 ml of mucus sample was concentrated to 2.7ml. After every step, solution viscosity measurements were recorded using the cup and bob viscometer. A gel rheology measurement was carried on this concentrated sample followed by the

isolation of mucin using the equilibrium CsCl purification method as described earlier. The CsCl purified fractions were extensively dialysed against distilled water at 4°C. The quantification of mucus glycoprotein in the fractions was done using the PAS assay. The fractions were then freeze dried and weighed.

3.3 RESULTS

3.3.1 SOLUTION VISCOSITY

Molecular weight was determined for purified *Goniopora* species mucin (GM, GX1M and GX2M) by calculating various parameters such as relative viscosity, specific viscosity. *Goniopora* mucin was used for this study as mucin yield from other species was insufficient. Huggins and Kramer plots were produced and extrapolated to infinite dilution to obtain the intrinsic viscosity and hence molecular weight (Figure 21). Ideally, both Kramer and Huggins plots should provide the same intrinsic viscosity reading but either of the two plots can also be used independently to obtain the intrinsic viscosity (Figure 21). Here, the values obtained from Kramer plot were used as intrinsic viscosity although the values of Kramer and Huggins plot for the samples were similar (Table 7).

Mucin sample type	Huggins plot value $\eta_{red} = \eta_{sp} / C$ (ml.mg^{-1})	Kramer plot value $\frac{\ln(\eta_{rel})}{C}$ (ml.mg^{-1})	Molecular weight $M^{\alpha} = \frac{[\eta]}{K}$ Dalton
Goniopora djiboutiensis Mucin (GM)	0.148	0.132	2.45 x 10^6
Goniopora stoksii – Extract 1 mucin (GX1M)	0.095	0.109	1.46 x 10^6
Goniopora stoksii – Extract 2 mucin (GX2M)*	0.07	0.074	8.73 x 10^5
Reduced *Goniopora stoksii* Mucin (RGM)*	0.046	0.038	530,000

Table 7 Molecular weight of *Goniopora* species.showing the various parameters and calculated molecular weight of the *Goniopora* species mucin. * means results are not very reliable due to the limited amount of mucin available (mucin concentration range 3 mg.ml^{-1} – 0.5 mg.ml^{-1})

The *Goniopora* species mucus extracts showed the presence of high molecular weight (1.46 x 10^6 - 2.45 x 10^6 dalton) native mucin molecule. This native mucin molecule consisted of subunits (530,000 dalton) presumably joined by the disulphide bridges. The use of PBS first on GX1M may have solubilised all the available mucins and dissolving the same tissue extract in guanidinium chloride may have resulted in solubilisation of previously un-dissolved mucin such as incompletely biosynthesised mucin. This may be the reason for lower molecular weight of *G. stoksii* extract 2 having 8.7 x 10^5 dalton than PBS solubilised mucin (1.46 x 10^6 dalton). Thus, the above molecular weights indicate a polymeric structure of *Goniopora* mucin consisting of an average three polymers of size 5 x 10^5 dalton.

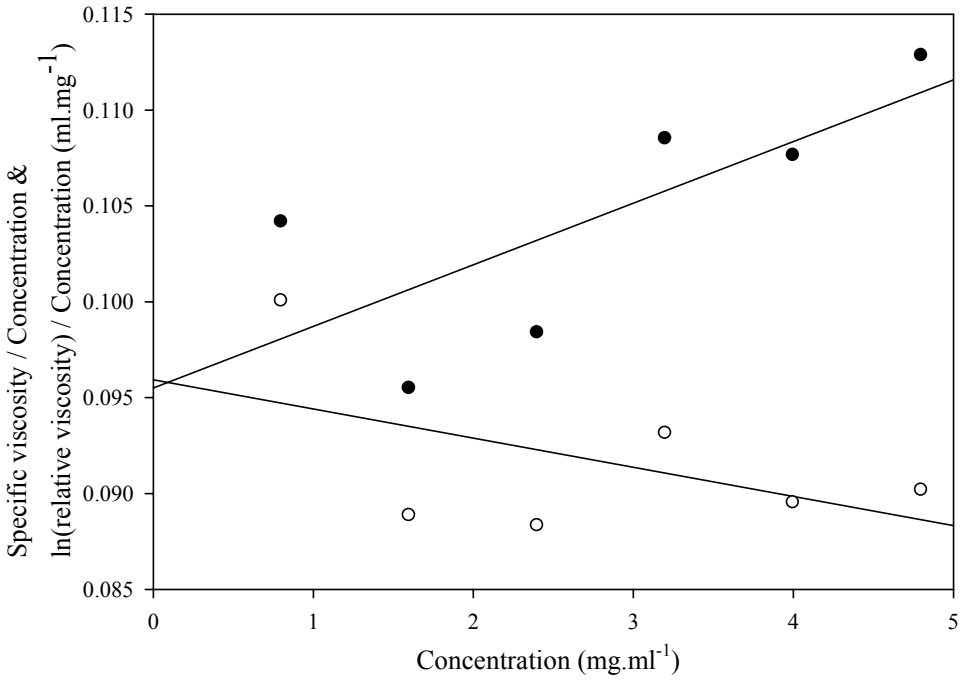


Figure 21 Huggin's ● and Kramer ○ plot for the native *Goniopora stoksii* mucin from which intrinsic viscosity was obtained.

A *M. faveolata* mucus (MM) sample of unknown mucin concentration was subjected to solution viscosity measurements in a stepwise manner while concentrating the 120 ml of sample to 2.7 ml. The concentrations of the solution were back calculated from the amount of lyophilised material obtained from the CsCl equilibrium density gradient purification of 2.7 ml sample. Molecular weight was determined from the solution viscosity measurements based on three different assumptions (Table 8):

1. Mucin was distributed across all the CsCl gradient fractions. This would be unlikely but considered (total lyophilised material)
2. Mucin was only present in fractions numbered 3-7 of the CsCl gradient as shown in the mammalian mucins (Davies & Carlstedt 2000).
3. Mucin was only present in fractions numbered 3-7 of the CsCl gradient, but the values were corrected for the glycoprotein content determined by PAS assay.

Sample	Criteria	Lyophilised material in mg	Concentration of original milked sample ($mg.ml^{-1}$)	Concentration of final (2.7 ml) sample ($mg.ml^{-1}$)	Kramer plot value	Molecular weight $M^{\alpha} = \frac{[\eta]}{K}$
MM – All fractions	Total lyophilised material	23.83	0.198	3.27	0.31	11.26×10^6
MM – 3-7 fractions	Total lyophilised mucin fraction 3-7	13.41	0.1127	1.84	0.54	30.34×10^6
MM – 3-7 PAS positive	Total mucin fractions (3-7) calculated using PAS positivity of fractions	1.8	0.015	0.25	4.37	12.69×10^8

Table 8 Molecular weight of *M. faveolata* mucin. Table showing molecular weight of *M. faveolata* (MM) milked sample along with various criteria adopted to obtain it. Note Kramer plot value has been used to calculate the molecular weights.

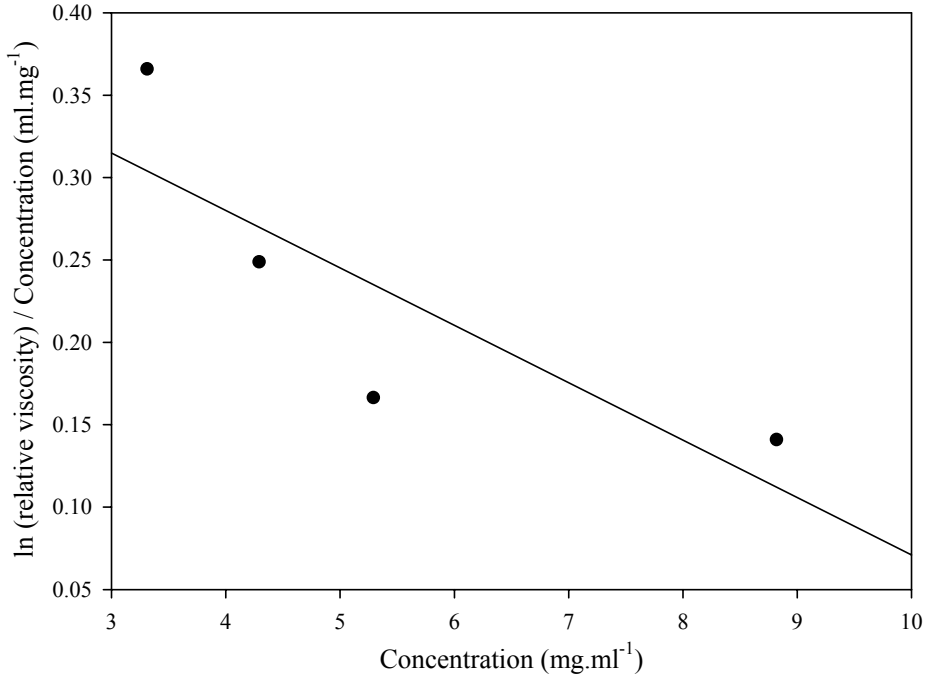


Figure 22 Kramer plot for *M. faveolata* mucin (MM) considering the total lyophilised material as mucin. The intrinsic viscosity obtained is 0.31

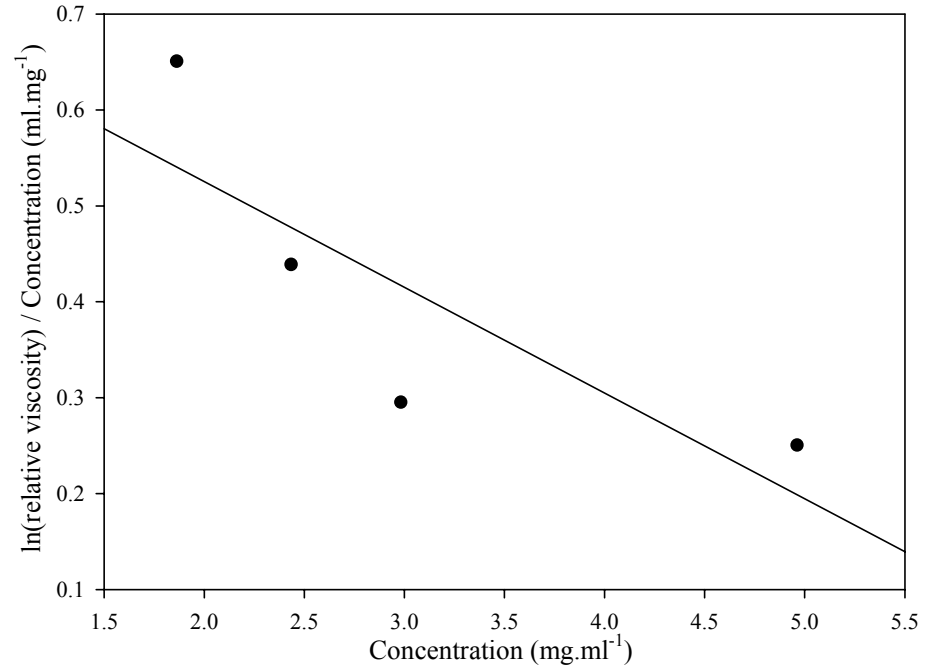


Figure 23 Kramer plot for pooled CsCl purified fractions 3-7 of *M. faveolata* mucin (MM) having the expected density for mucins.

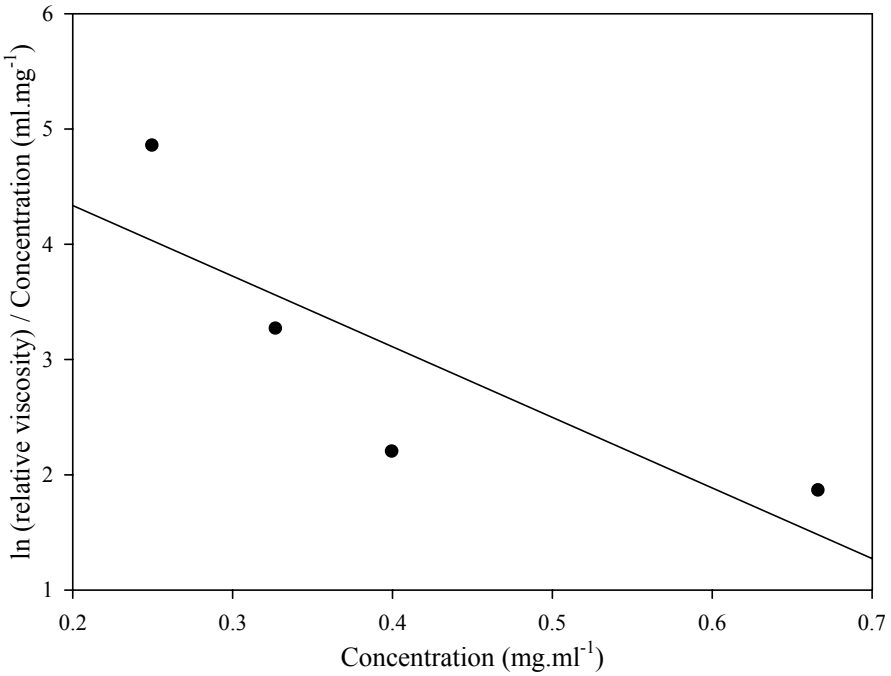


Figure 24 Kramer plot for PAS positive glycoprotein obtained by CsCl purified fractions 3-7 of *M. faveolata* mucin (MM) having the correct density for mucins.

Nearly 80% of the weight of pig gastric mucin is found to be PAS positive. The adjacent hydroxyl groups present on the sugar backbone in pig mucin are oxidised by the periodic acid to form aldehyde group. This aldehyde group forms magenta colour on reaction with the Schiff's reagent, thus carbohydrates present in pig gastric mucin show PAS staining. Based on this criterion, it is possible to estimate the amount of mucin present in the pig mucin. In case of *M. faveolata*, only 7.5% of total lyophilised material has been found to be PAS positive (Table 8). Thus, the result shows that *M. faveolata* mucin is either staining less with Schiff's reagent as compared to the pig gastric mucin which has been used as a standard or has sugar groups which do not have adjacent hydroxyl group attached to them. However, the coral mucin molecule appears to be big compared to pig gastric mucin (Table 8).

3.3.2 SDS PAGE

The amount of polymeric material present in *Acropora millepora* that remained at the point of application of the SDS PAGE gel was 64% of total, compared to the amount that had moved from the point of application to the interface between the stacking and running gel and into the running gel (36%) (Figure 25). The material which remained at the point of application in the stacking gel is polymeric high molecular weight mucin, whereas the material in the running gel represents the degraded or the smaller subunits of the mucin. The standard used was native pig gastric mucin. The standards also showed the same kind of the trend (62% polymeric and 38% degraded) confirming that the degree of polymerisation in the pig mucin and *A. millepora* mucin are similar.

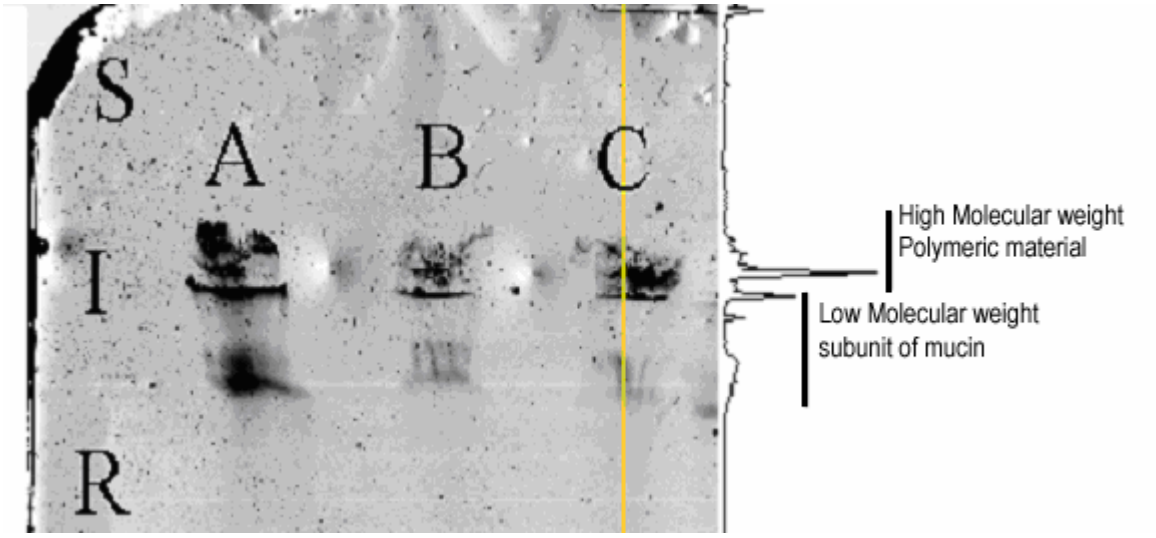


Figure 25 SDS Page of *Acropora millepora* mucin compared with the native pig gastic mucin. A – Pig mucin B & C - *A. mllepora* mucin, I – interface between stacking and running gel, S – stacking gel and R–running gel. Staining method used is periodic acid Schiff's stain.

3.3.3 GEL RHEOLOGY MEASUREMENTS

The gel rheology measurements carried directly on the milked mucus samples showed absence of gel properties but when the gel rheology measurements were done on the concentrated *M. faveolata* mucus samples, data revealed that concentrated mucus samples were starting to behave like a gel. The amplitude sweep was carried out at shear stress of 0.3 – 100 Pa. At shear stress (1.2 – 100 Pa), the value of elastic moduli (G') is greater than viscous moduli (G'') (Figure 26). G' and G'' appear to be nearly constant between 22-270 Pa. This is the shear-independent region. Shear stress of 62.931 Pa was selected (G' = 0.5 Pa and G''= 0.2 Pa) and the sample was subjected to frequency sweep at 0.1-10 Pa at 25°C (Figure 27). The target strain 3.65 was achieved at G' = 0.6 Pa and G''= 0.1Pa. Figure 27 shows that the moduli are frequency dependent increasing with increasing frequency. This result suggests that there is evidence of shear independent region; however the moduli are extremely low. Pig gastric mucin shows a strong gel forming at a concentration above 20 $mg.ml^{-1}$ with a G' and G'' values around 100 Pa. In *M. faveolata* a very weak gel would be expected based on the values of G' and G'' moduli.

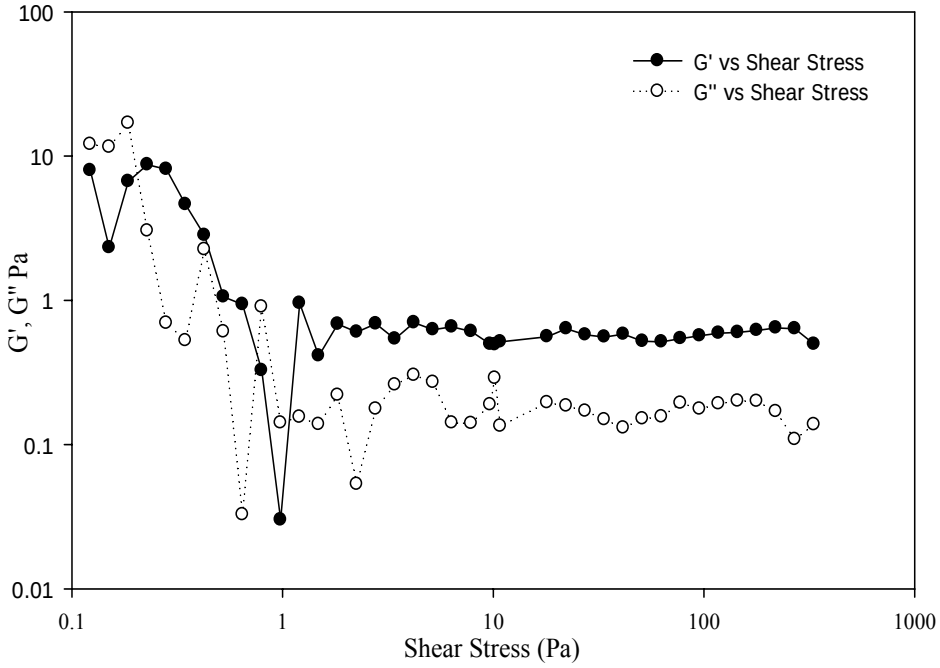


Figure 26 Concentrated sample of *M. faveolata* subjected to amplitude sweep with shear stress ranging from 0.1 – 410 Pa. G' and G'' appears to be nearly constant between 22-270 Pa showing shear independent region.

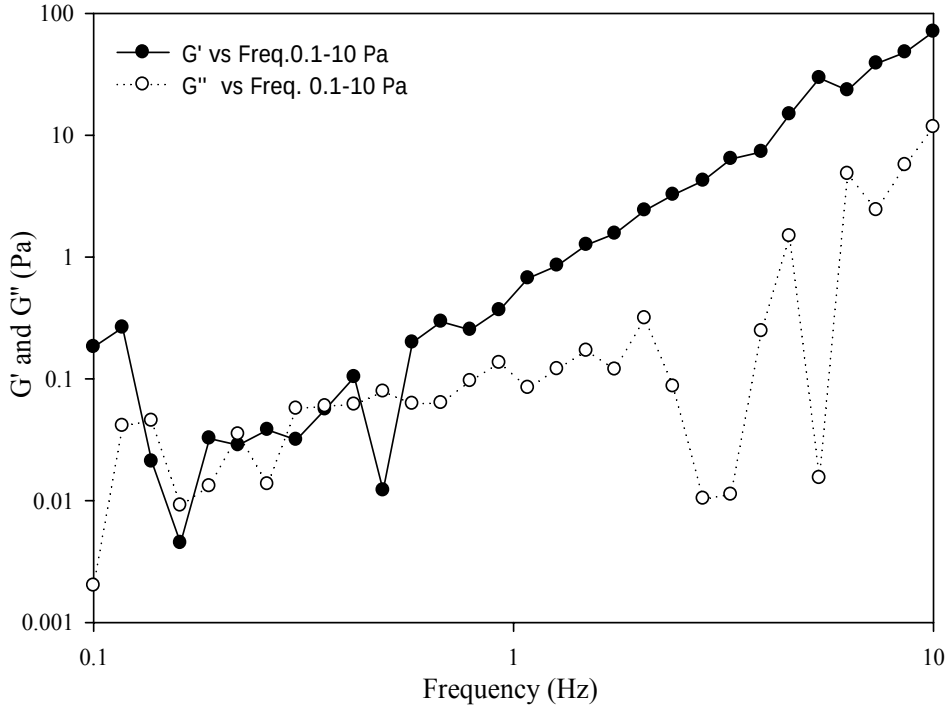


Figure 27 shows a very weak gel which is frequency dependent with the moduli increasing as the frequency increases. G' is almost 100 Pa at 10 Hz and G'' is around 10 Pa at 10 Hz.

3.4 DISCUSSION

Mammalian rheological studies reveal that mucus gel properties are influenced and to a large extent controlled by high molecular weight polymeric glycoprotein mucin (Pearson et al. 2000, Taylor et al. 2005). One of the initial difficulties in studying vertebrate mucin molecule was the extreme large size, its solubilisation and preservation apart from the inherent heterogeneity and polydispersity of the molecule (Allen & Pearson 1993, Corfield 2000). Although the term mucin has been used in coral literature, its structure and role of mucin in affecting the properties of coral surface mucus layer dynamics is not presently understood (Brown and Bythell 2005). Isolation of a glycoprotein responsible for gel forming properties of mucus was explored in a study done on *Acropora formosa* (Meikle et al. 1987). The collected mucus samples were subjected to centrifugation, dialysis and finally reduced using sodium borohydride. The reducing agent does help in solubilisation of mucin but does not help in purification of mucin molecule. During the present study, a better method of preservation and standard method of CsCl gradient ultracentrifugation which concentrates the mucin in a particular density range during isolation was followed (Van Klinken et al. 1998, Davies & Carlstedt 2000). It is documented that solubilisation of freshly collected mucus without degradation is difficult hence the use of chaotropic solvents such as guanidine has been common practice in vertebrate mucin studies (Van Klinken et al. 1998). Long term observation revealed that guanidine results in disappearance of important structural information making the preparation less valid for functional studies (Perez-Vilar & Mabolo 2007). Instead solubilisation can be acheieved by proteolytic inhibitors along with controlled homogenisation at low temperature. Hence in the present study the coral

mucin solubilisation was achieved by proteolytic inhibitors in PBS first and then the undissolved mucin was subjected to guanidine solubilisation. Thus, both the methods were followed to obtain maximum yield.

The molecular weight of native *Goniopora* species mucin (1.5-2 x 10^6 Dalton) in present study was found to be much lower than the reported *A. formosa* mucin (40 x 10^6 Dalton) (Meikle et al. 1988). The reported molecular weight 40 x 10^6 dalton of *A. formosa* by Meikle et al. (1987) seems to be unrealistic using the described standards having molecular weight equivalent to the reduced subunit size of known native mucin molecules. Standard used in the study were thyroglobulin (Mr = 669000 Dalton) and ferritin (Mr = 440,000 Dalton) and aldolase (Mr = 158,000 Dalton) on CL-4B and CL-2B column (Meikle et al. 1987; Meikle et al. 1988). The native vertebrate mucin molecules have been described to possess a molecular weight range of 0.2–40 million Daltons (Corfield & Warren 1996, Offiner & Troxler 2000, Pearson et al. 2000). *Goniopora* species demonstrated molecular weight 1.5-2 x 10^6 daltons which agrees with the molecular weight of known mucin molecules.

The most important parameter for gel formation is appropriate amounts of dissolved mucin having a polymeric structure (Sellers et al. 1988, Pearson et al. 2000, Thornton & Sheehan 2004). The pig gastric mucin which was used as a standard forms a gel only after it reaches a concentration higher than 20mg.ml^{-1} (Pearson et al. 2000). The amount of lyophilized coral mucin and initial values of viscous and elastic moduli suggests a very low concentration of coral mucin in the samples. This low concentration of mucin is not sufficient to form a gel on the coral surface but with increase in concentration of

mucin, increase in solution viscosity measurement and gel rheology measurement was observed. Pig mucus gel rheology studies suggest intermolecular interactions and arrangement of molecules within the gel system (G'>G'') during gel formation (Taylor et al. 2005). It is observed that during excess shear stress or strain, the value of viscous moduli (G'') becomes greater than elastic moduli (G') resulting in gel becoming viscous fluid and recovers its optimum gel properties after removal of mechanical disruption (Taylor et al. 2003). Although the elastic moduli for *M. faveolata* mucus gel was very low compared to pig mucus, the gel rheology of concentrated *M. faveolata* mucus showed a begining of frequency dependent increased intermolecular interaction (G'>G'') with the increasing stress (Figure 27). Similarly, solution viscosity plot started to show a tendency towards asymptotic rise when plotted against the concentration. This suggests that *M. faveolata* mucin is capable of forming gel provided appropriate quantity of mucin is dissolved in the mucus.

The lower mucin concentration in milked coral samples can be explained based on the sampling method. The milked mucus sampling technique was used to reduce the possibility of contaminants which may act as a gel inhibitor (Sellers et al. 1991) and to obtain the clear sticky mucus from the surface of the coral. It is now ascertained that probably the majority of milked mucus is released from the mouth of the coral polyp (John Bythell per comm.) consisting of mainly the coelenteron (gut) sea water, tissue debris with dissolved mucin. This viscous fluid like mucus released from coral mouth seems to explain the functional role of ciliary feeding in which corals produce mucus which covers the mouth and surrounding area. The food gets entangled in this mucus and corals have been reported to ingest the food entangled mucus strings (Yonge 1930,

Lewis & Price 1975, Lewis 1977, Brown & Bythell 2005). Thus for feeding purposes the mucus required by coral should be more fluid for an easy flow and should be sticky enough to entangle the prey but do not necessarily needs the gel like behaviour of mucus.

Mucin was detected in CsCl purified fractions by PAS assay. This assay has been designed to detect the sugar with adjacent hydroxyl group in the mammalian mucin (Mantle and Allen 1978). During isolation of vertebrate mucin it is observed that nearly 80% total material present in 3 till 7 CsCl purified fractions show PAS positive result. Hence quantification of mucin is possible in vertebrate mucus. Coral mucus demonstrated PAS positivity but the quantification based on PAS assay for *M faveolata* mucin could not match with the lyophilised material. The molecular weight of 13 x 10^8 for *M faveolata* mucin based on PAS assay seems be too big and incorrect. *M faveolata* result suggests that the PAS positivity of coral mucin is different than pig mucin which needs to be considerd in the study of gel forming properties of coral mucin. The possibility of coral mucus possessing some sugar chains those are totally different from the mammalian mucin is expected as large quantity of arabinose which is a plant associated sugar has been reported in corals (Meikle et al. 1987, 1988, Wild et al. 2005). Although the sugar groups present in coral mucus are known to a large extent from biochemical (Ducklow & Mitchell 1979b, Meikle et al. 1987), exact chemical linkages between the sugar molecules have not been fully elucidated.

When a mucin molecule is released onto the epithelium, it undergoes hydration and mucin gene products anneal to form the human mucus gels (Piludu et al. 2003). Based

on bioinformatic and retrieved CK sequences from the coral genome from Chapter 2, the potential for more than one gel forming muc gene has been demonstrated in corals. Thus, mucus obtained by the milking technique could be a combination of the exuded surface mucus layer and mucus released from the oral cavity. There is possibility of different kinds of mucin molecule/s encoded by different mucin genes in the collected samples similar to those of humans (for example the MUC5AC and MUC6 are found in human stomach).

Thus the rheological measurements indicate that coral mucus consists of high molecular weight glycoproteins with similar degree of polymerisation and capable of gel formation similar to pig gastric mucin.

CHAPTER 4

Impact of stress on thickness of surface mucus layer in reef corals

4.1 INTRODUCTION

Mucus gels are secreted by virtually all animals studied to date. In reef corals, it forms an important part (approximately 20-45%) of the daily energetic demands of the organism (Crossland et al. 1980). It was initially thought to perform the functions of sediment removal (Hubbard & Pocock 1972) and ciliary feeding (Duerden 1906, Yonge 1930), but a variety of roles have emerged, some of which are still under investigation. One such major role of the surface mucus layer (SML) is protection against a range of hostile conditions including desiccation, microbial invasion, sedimentation and pollution (Brown and Bythell 2005). An immediate increase in surface mucus secretion has been reported as a response to the environmental impacts in the surroundings such as presence of crude oil (Mitchell and Chet 1975; Neff and Anderson 1981), copper sulphate exposure (Mitchell and Chet 1975), decreased salinity and sediment overload. Histology of healthy coral tissue reveals the presence of widely distributed and abundant mucus glands cells in the epidermis and gastrodermis. An increased abundance and size of epidermal mucus-producing cells was seen in *Manicina areolata* on long term exposure to crude oil (Peters 1981). Similar increased abundance of epidermal mucus producing cells was reported in *Goniastrea aspera* during a natural

bleaching event elicited by elevated sea surface temperature experiment (Brown et al. 1995). Since environmental stresses appear to increase the rate of surface mucus secretion, these observations of increased mucus cells within the tissues indicate both increased mucus synthesis and secretion must occur in response to stress.

In humans, the gastrointestinal tract, especially the colon, is densely colonised by anaerobic bacteria (10^{11} to 10^{12} cells.ml^{-1} of intestinal content), which practically occupy every available niche in the outer mucus layer (Laux et al. 2005). This natural gut micro-flora, together with the biophysical properties of the mucus layer itself, provide protection from the infective stages of pathogens and several mechanisms have been elucidated for this protective role . For example, the beneficial bacteria of the gut microflora have been shown to produce glycosidases and proteases that degrade the resistant lectin covering of the *Entameoba* traphozoites, which renders them less capable of binding to epithelial cell surfaces (Variyam 2007). In addition to direct competitive roles, pro-biotic bacteria such as *Lactobacillus plantarum* and *Lactobacillus rhamnosus* have been shown to inhibit the in vitro adherence of *Escherichia coli* to the intestinal lining of human epithelial cells by stimulating the host cells to up-regulate the expression of MUC2 and MUC3 mucins (Mack et al. 1999). In addition to the physical barrier of the SML, mucus provides other beneficial properties. For example, the surface of epithelial cells and the mucus gel in the human intestine have been found to contain similar ligands recognised by amoebic adherence glycoprotein. The adherence of amoebic traphozoites to the mucus gel, which eventually sloughs off, thereby protects the epithelium from infection (Variyam 1995, 1996, Belley et al. 1999, Moncada et al. 2005, Variyam 2007). Thus, the mucus gel

layer and its microflora play a significant role in limiting the activities of the pathogens. While relatively little is known of these processes in reef corals, a loss of antibacterial properties in the mucus and a shift in the microbial community to domination by potentially harmful Vibrio species under unfavourable conditions have recently been reported (Ritchie 2006; Bourne et al. 2007). Thus, there is growing evidence suggesting that the SML promotes beneficial microbes by creating a favourable micro-environment inside the SML under healthy conditions.

The efficiency of the SML as a protective physical barrier depends on its thickness, composition, gel strength (Sellers et al. 1991) and the duration for which the mucus layer remains on surface. Out of these four factors, mucus composition has been addressed previously, whereas the other three factors have been barely addressed to date. Chemical composition of mucus in corals has remained questionable since various authors have used widely different analysis techniques and sample collection methods, resulting in wide variance in reported composition (Brown and Bythell 2005). Varying mucus compositions have been reported for different coral species (Meikle et al. 1988) and have been shown to be influenced by the depth, ageing and level of contamination by surrounding water and particulate material (Ducklow & Mitchell 1979a, b, Daumas et al. 1981, Coffroth 1984, 1990). Despite these differences, coral mucus has been found to contain polysaccharide, protein, lipid, zooxanthellae and surrounding sea water contaminants in varying quantity. The mucus layer thickness and changes over time and over the surface of the coral colony have not been previously described.

Here we have adapted a methodology that has been successfully used to measure the thickness of the outer sloppy and inner adherent mucus gel in the gastrointestinal tract of the mammalian system (rat)(Atuma et al. 2001, Strugala et al. 2003). This method has been adapted to an aquatic environment. The structureless, gel-like, nearly transparent and dynamic nature of mucus under aquatic conditions has posed difficulties for *in vivo* mucus studies to date. This is a first attempt to measure the thickness of SML in corals and to examine its role under different environmental conditions.

4.2 MATERIALS AND PROCEDURES

4.2.1 EXPERIMENTAL SETUP

Coral SML thickness measurements were carried out at the Phuket Marine Biological Centre, Thailand in February 2007. Similar sized colonies (approximately 8 x 5 cm) of different coral species were collected from the same vicinity (except bleached colonies) using hammer and chisel, without touching the live tissue as far as possible. Detached colonies were collected under water and prevented from exposure to air. They were acclimatized in a running sea-water aquarium for at least 1 hour. A single colony was then transferred on to an inclined base plate in a measuring tank under water (Figure 29). The base plate was perpendicular to the tank wall and had an adjustment lever. The base plate was inclined so that the coral surface became perpendicular to the measuring micropipette (determined visually).

4.2.2 MUCUS GEL MEASURING SYSTEM

SML thickness measurements were carried on using a glass micropipette held by a micromanipulator (Leitz, Wetzlar, Germany). The micromanipulator was connected with a digital micrometer which recorded the distance covered by the micropipette. Tip diameter of the glass micropipette was formed at 1-2 μm by pulling the glass tubing (1.2 mm OD and 0.6mm ID; Rederick Haer, Brunswick, ME) using a pipette puller (pp-83; Narishinge Scientific Instrument Laboratories, Tokyo, Japan) (Figure 28).

Under a binocular microscope, a tranlucent mucus gel layer around the coral surface was clear in comparison to the overlaying sea water, but the interface of the mucus layer and sea water was indistinguishable. A drop of seawater containing carbon particles (activated charcoal-10μm in size, extra pure, Kebo Lab) was introduced using a dropper. Immediately the carbon particles started sinking into the water and reached the water-mucus gel interface. Carbon particles appeared to settle down on the interface and thus helped in visualisation of outer layer of the mucus gel. The micropipette was brought to this point perpendicular to the coral surface (estimated visually) and carbon particles were used as an indicator that the outer surface of the mucus layer had been reached. The micrometer was switched on and started to record the distance as the micropippette was advanced deforming the gel layer towards the polyp. The polyp structure, due to its natural pigmentation was easily visible under microscope. Measurements were carried out from the region falling between septa A and septa B as illustrated in the Figure 30. The advancement of micropipette was stopped as soon as the retraction of tentacle or tissue was observed. The thickness reading was measured from micrometer in microns. The SML thickness measurement technique was used on

the reef corals belonging to family Faviidae. *Goniastrea aspera, Favites abdita, Goniastrea retiformis* and *Platygyra daedalea* SML were recorded under different environmental conditions to test the reliability of the technique. 15 random readings were recorded and samples were collected in replicates of three.

Figure 28 Gel measurement assembly with M- micromanipulator, P-micropipette, MT-measuring tank, C-coral, B-base plate, D-digital indicator, DM-dissecting microscope

Figure 29 Coral (C) is present in the tank (MT) under water before the transfer of the coral into the gel measuring assembly for measuring the surface mucus thickness measurement. MT-measuring tank, C-coral, AL – adjustment lever

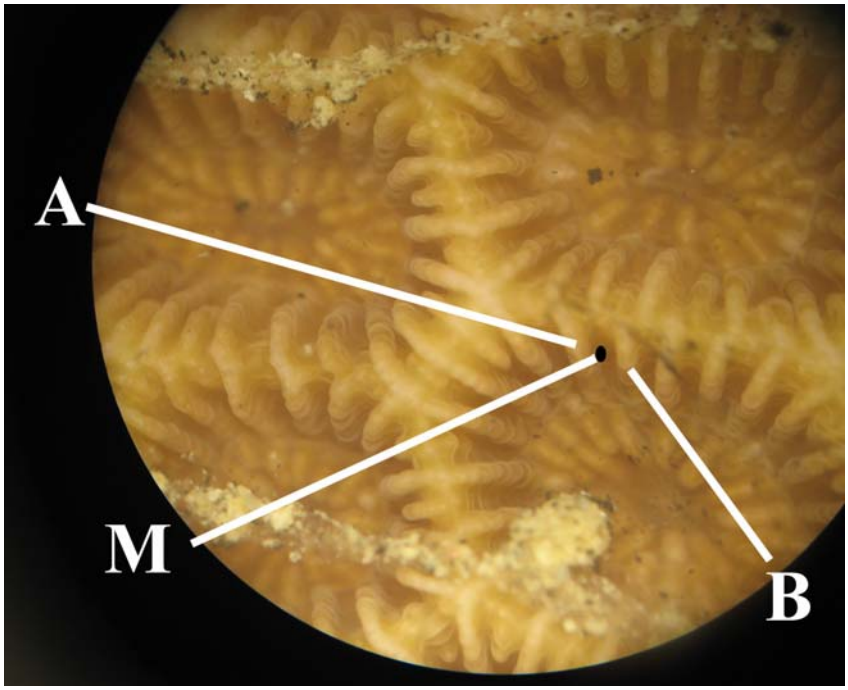


Figure 30 Measurement site on the polyp. A and B are the septa and M represents the measurement site where micropepitte was held to record the measurement on the micromanipulator on the surface of *G. aspera*

4.2.3 ASSESSMENT

Corals possess a variety of shapes ranging from smooth rounded to highly uneven branched forms. Mucus gel flows and therefore acquires the shape of the underlying coral surface. Measurement of such an asymmetric and variable shape was bound to introduce error due to the visual estimation of the perpendicular angle to the coral surface. Measurements at random locations across the coral surface were obtained on uneven coral surface using an inclined base-plate and adjustable micromanipulator. A micropipette was inserted perpendicular to the coral surface (estimated visually) and angular deviation from perpendicular was assessed using a protractor (Figure 31). Mean deviation (n = 32) was found to be 1.9 ± 2.04º (± 95% confidence interval), which equates to an overestimation of mucus thickness of 0.24%.

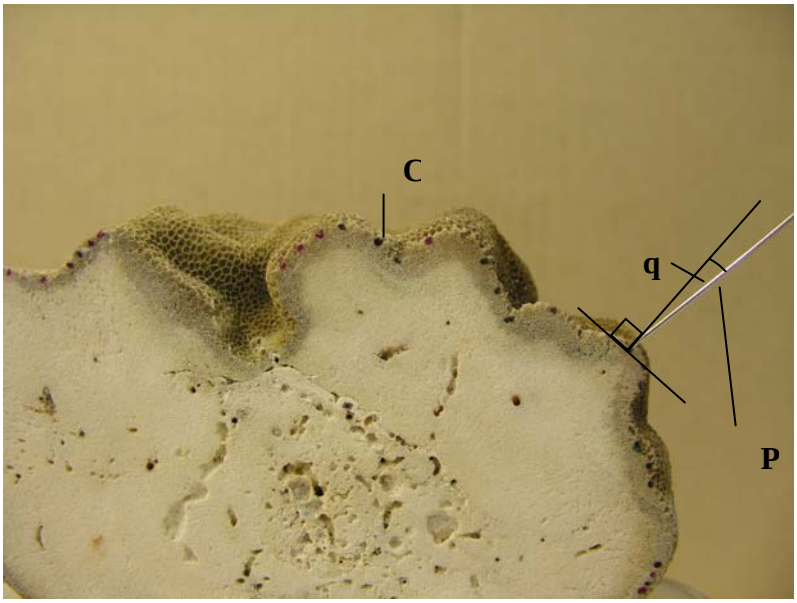


Figure 31 shows the standardization of the SML thickness measurement process using a sectioned massive Porites species colony. The micromanipulator needle P represents the perpendicular angle estimated by eye and q is the angle of deviation of P from the actual perpendicular. The angle of q has been estimated using a protractor independently and C is the coral surface.

4.3 RESULTS

Visibly healthy colonies of *Goniastrea aspera* and *Favites abdita* were collected on same day to compare the SML thickness in different species on a given day. In *G. aspera* and *F. abdita,* the mean SML thickness (n = 3 colonies) was found to be 490 ± 0.58 [95%CL] µm and 496 ± 0.59 [95%CL] µm respectively (Figure 32). A nested ANOVA showed no significant difference in SML thickness between coral colonies ($F_{(1, 4)} = 0.06$, $p = 0.5$) or between the species ($F_{(1, 4)}, = 0.06$, $p = 0.8$).

To assess the effects of aerial exposure of corals during the natural tidal cycle, visibly healthy colonies of *Goniastrea aspera* were collected on 4 different days at the end of spring tidal sequence (Figure 33). The colonies collected on the first day had experienced the most extreme tidal conditions and had been repeatedly aerially exposed at low tide (in the early morning and mid afternoon) over a period of 5 days. The colonies collected over the subsequent 3 days from the same location had been continuously submerged throughout this period. There were significant differences noted in SML thickness between collection days (nested ANOVA; $F_{(3, 8)} = 64.04$, $p < 0.001$). The SML was 1.8 fold thicker on colonies collected on the first day compared to the colonies collected subsequently.

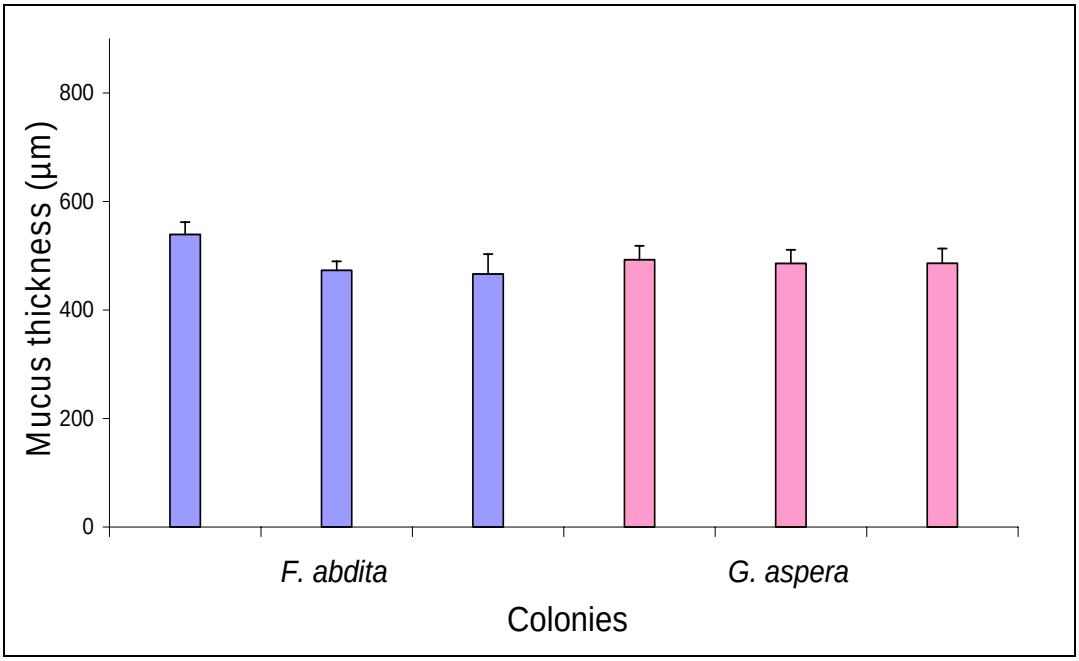


Figure 32 SML thickness measurements for three apparently healthy colonies of *G.aspera* and *F. abdita* (error bars – 95% confidence interval)

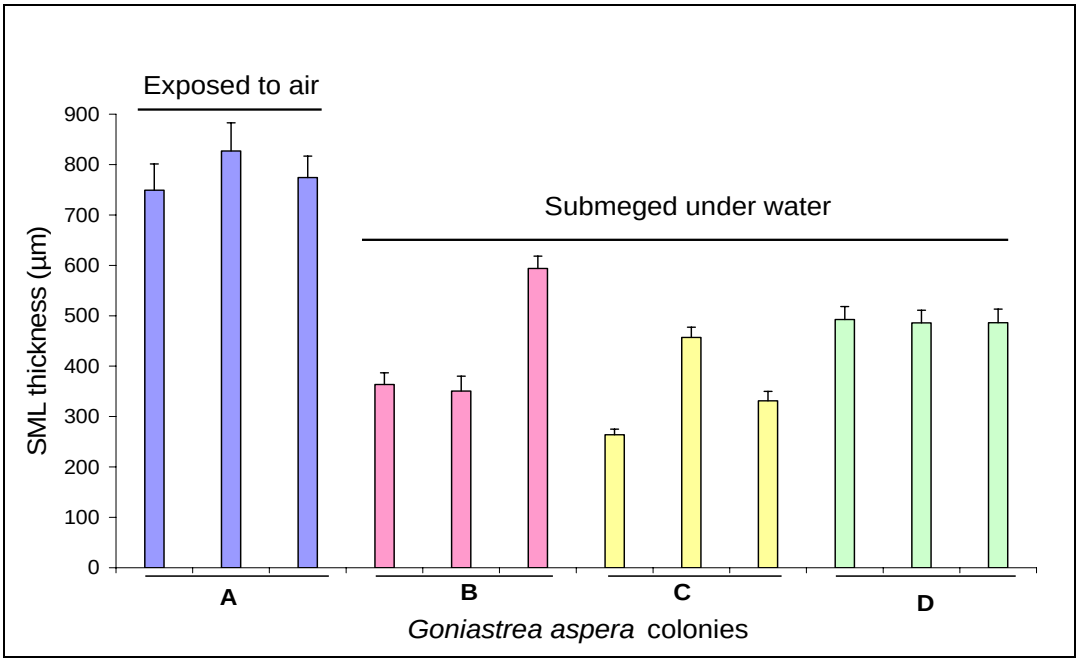


Figure 33 SML thickness in *G. aspera* on four (22nd, 24th, 26th and 28th of Feb 2007) different days in a tidal cycle (error bars – 95% confidence interval). A,B,C and D are the colonies collected on different days of tidal cycle.

Colonies of *Goniastrea retiformis* and *Platygyra daedalea* were selected to compare between areas of solar bleaching (visibly white tissues) that had developed on the west sides of colonies, with the rest of the colony which was normally coloured (Brown et al. 1994). In *P. daedalea*, the SML thickness on bleached tissue (315 μm) was approximately half that of visibly coloured tissue (700 μm; Figure 34; nested ANOVA; $F(2, 3) = 21.72$, $p < 0.001$). There was no significant difference in SML thickness between coral colonies in either the normally pigmented or bleached tissues (nested ANOVA; $F(2, 3) = 1.12$, $p = 0.333$). In contrast, *G. retiformis* revealed no consistent pattern (Figure 35). Mucus thickness in different colonies varied significantly (nested ANOVA; $F(2, 3) = 8.41$, $p < 0.001$) and there was no significant effect of bleaching (nested ANOVA; $F(2, 3) = 2.25$, $p = 0.088$).

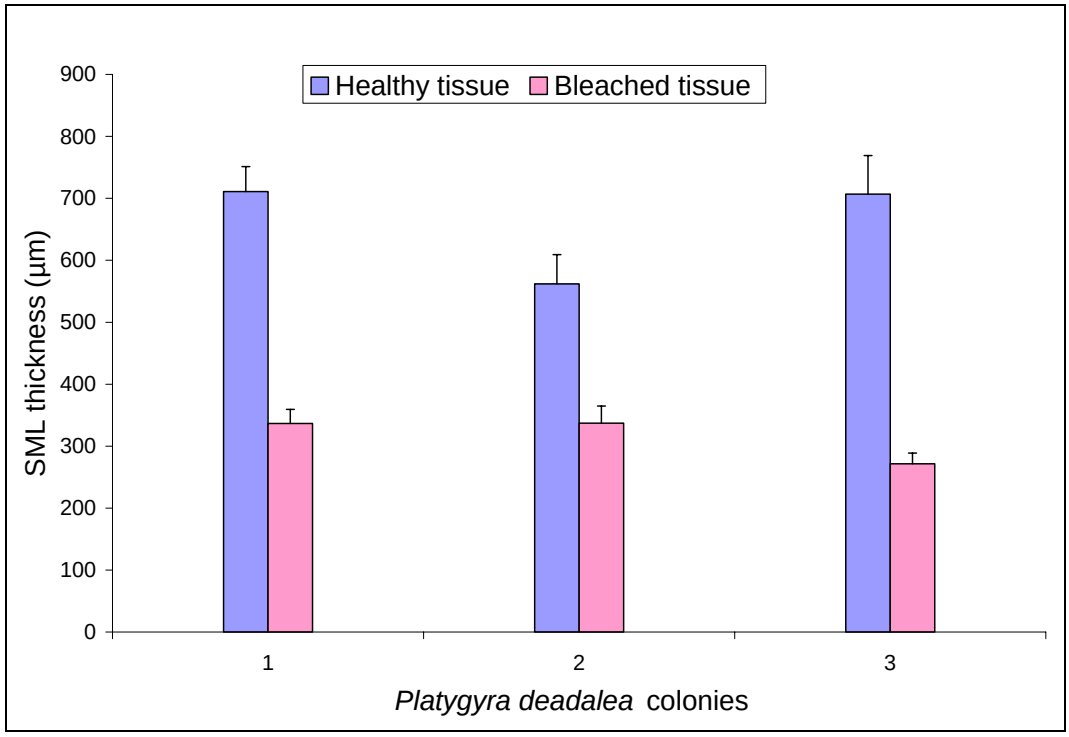


Figure 34 SML thickness in bleached and healthy part in 3 colonies *Platygyra daedalea* colony (error bars- 95% confidence interval)

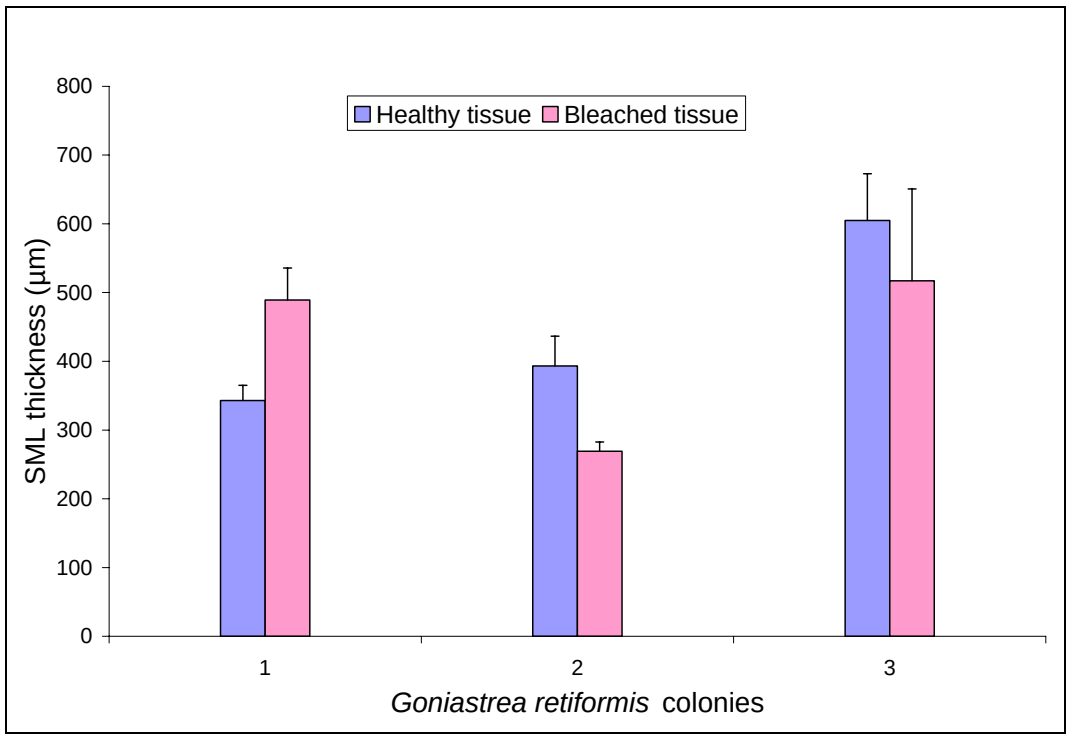


Figure 35 SML thickness in bleached and healthy part of the *Goniastrea retiformis* colony (error bars – 95% confidence interval)

4.3.1 OBSERVATIONS DURING THE MEASUREMENT

A key observation was the presence of a layer of mucus over the apparently pigmented, pale and completely white polyps in all the experiments. The mucus layer was observed under the microscope to be clear and slightly more viscous than water. Where sediments were present on the coral surface, mucus strings trapped with sediments were clearly visible in addition to the clear mucus. It was difficult to distinguish the outer layer of mucus but the settlement of charcoal particles on the gel-water interface made the distinction quite clear under the microscope. Over time (approx. 5 minutes), some of the charcoal particles were entangled in mucus and were observed to move over the colony (Figure 36 and Figure 37). Near the edge of the colony, mucus strings were observed to move away into the surrounding water. Interestingly, the charcoal particles showed circular movements near the polyp's mouth in apparently healthy portions of the colony. These polyps were fully expanded and appeared to push the charcoal particles towards the mouth in form of mucus-particle aggregates (Figure 36). In bleached colonies, the dead area was covered with sediments and was found to be devoid of mucus. The completely white (bleached but tissue-covered) areas of tissue consistently showed the presence of a mucus layer on them.

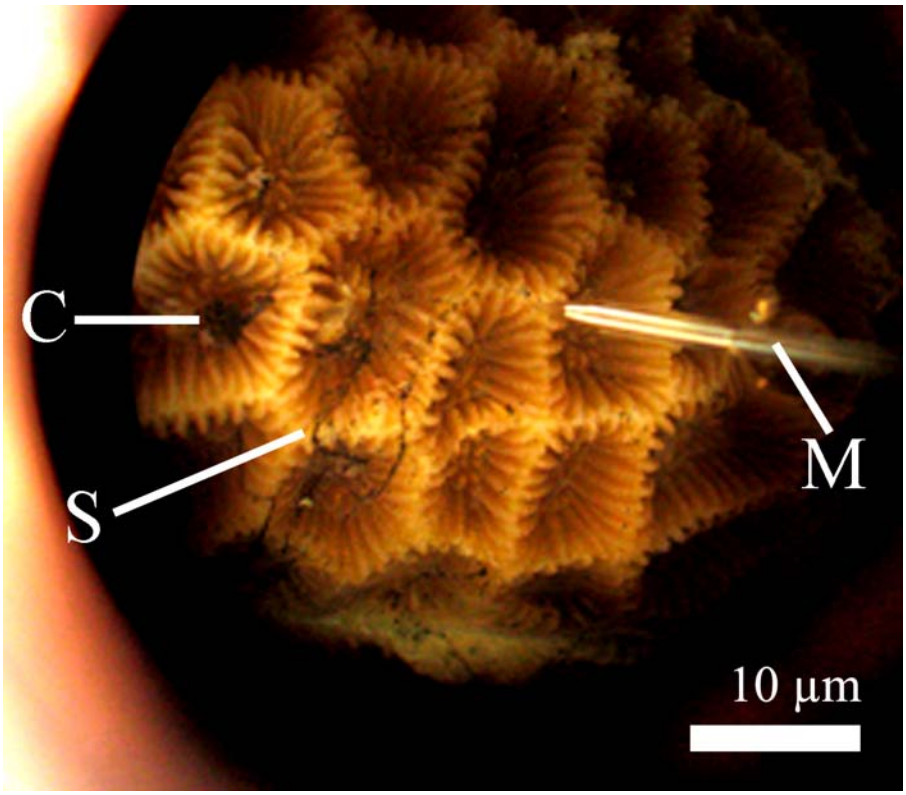


Figure 36 *Goniastrea aspera* healthy colony with mucus entangled carbon string C- carbon aggregates and S – mucus string entangled with carbon particles M- micromanipulator

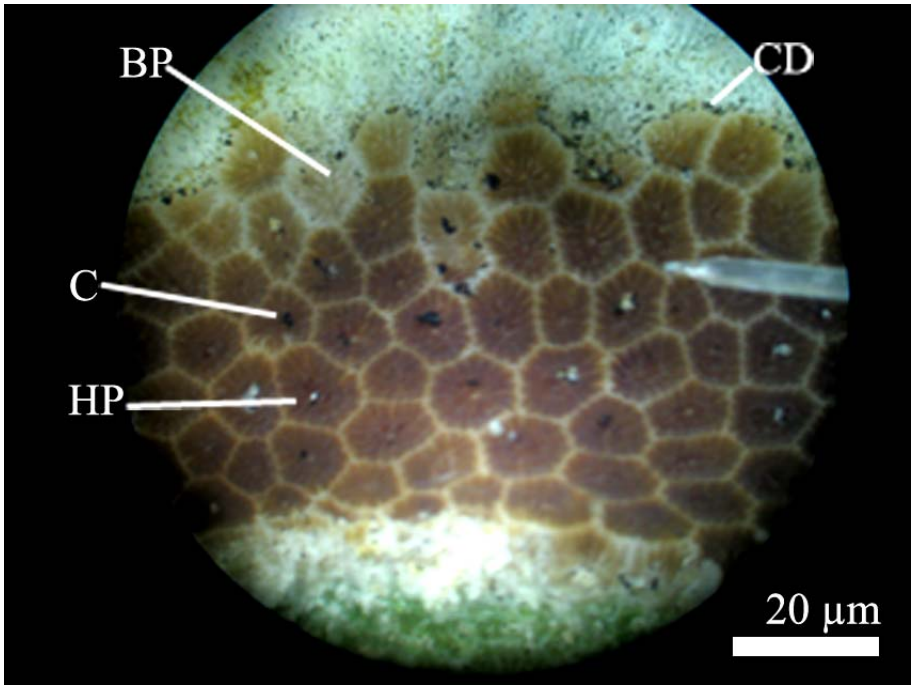


Figure 37- *Goniastrea retiformis* colony with bleached and healthy polyps Bleached/Healthy colony (HP – healthy polyp, BP-bleached polyp, CD – carbon deposition at the interface of dead and living tissue

4.4 DISCUSSION

Described herein is a novel method to study the dynamic nature of the coral SML in semi-natural conditions underwater. Obtaining realistic estimates of thickness of the delicate mucus gel layer *in situ* is difficult and the presence of the surrounding seawater makes it even more complicated in corals. While the mucus layer can sometimes be preserved using histological methods (Ducklow & Mitchell 1979b, Johnston & Rohwer 2007), fixation, dehydration and embedding will likely lead to shrinkage and deformation. Conventional techniques often result in complete dissolution and loss of the surface mucus gel layer (Allen and Pearson 2000).

A rapid extrusion of mucus from mucus producing cells and a continuous mucus layer has been visualised under the light microscope as a response to low calcium/calcium free water in frozen sections of *Galaxia fasicularis* (Marshall & Clode 2004). However, it is unlikely that freeze sectioning will be generally useful for mucus layer studies in corals since the freezing process is time consuming and may itself, alter the mucus secretion rates. Thus, it is challenging to study and measure the SML in corals, without disturbing the natural rate of secretion and turnover of the SML. This chapter describes a novel method to measure the thickness of coral surface mucus and provides an opportunity to examine the dynamics of the layer over time and under different conditions. The technique is non-destructive and non-invasive.

The method showed a measurable layer of mucus over the coral surface in all of the 450 readings undertaken during the study, which was at least 145 μm. A similar SML

thickness was detected in visibly healthy colonies of 2 different species (*G. aspera* and *F. abdita*) and the method was successful in detecting changes in the mucus layer as a result of stressful conditions. The thickness of the SML of *G. aspera* was increased (1.8 fold) due to periods of tidal aerial exposure compared with periods of continuous submergence. This increase in mucus thickness upon aerial exposure is presumably a response to avoid the deleterious effect of desiccation of coral surface. A clear effect of solar bleaching was observed in *P. daedalea* in which SML was reduced by half in bleached polyps as compared to visibly healthy polyps. In contrast, *G. retiformis* SML thickness was very variable in both healthy and bleached parts of the selected colonies and showed no significant differences due to bleaching. This result may, however, reflect different levels of severity of the solar bleaching in the two species rather than a difference in the response of mucus layer reduction due to bleaching. This is the first time any method has revealed a decrease in surface mucus secretion due to bleaching, which has been long speculated and which is likely related to the decreasing densities of zooxanthellae and lower photosynthetic carbon production (Fitt et al. 2001; Douglas 2003; Brown and Bythell 2005).

Microscopic observations during the study also re-emphasized the functional role of mucus in ciliary feeding (Figure 36) which has been previously reported from early studies of coral physiology (Yonge 1930; Lewis 1977). The continuous release of carbon particle-entangled mucus strings from the colony edges into the surrounding seawater and continuous maintenance of a measurable mucus layer (145 μm) on coral surface highlights the dynamic nature of the SML, and supports the conclusion that the SML is continuously and rapidly renewed.

The described method is non-invasive and non-destructive in contrast to the known conventional methods of visualising the mucus layer using histological studies. This method also allows the study of the mucus layer in semi-natural conditions underwater. The present study shows that the method can be effectively used on small sized colonies freshly collected from the field where it provides an opportunity to study the SML dynamics over time and under different environmental conditions.

CHAPTER 5

Significance of stored mucus content in coral epidermis

5.1 INTRODUCTION

The existence of the reef ecosystem over the past 200 million years, points to the fact that corals have evolved an efficient defense mechanisms to protect them from the frequent changing surrounding conditions and from a variety of invaders (Sutherland et al. 2004, Mydlarz et al. 2006). Acquired immune systems like higher vertebrates have not been shown in invertebrates. The identified defence mechanisms in invertebrate include physico-chemical barriers such as surface mucus layer (SML), melanin deposition, primitive immune memory, antioxidants and amebocytes showing phagocytosis (Mullen et al. 2004, Mydlarz et al. 2006).

Mucosal systems have been comparatively well studied in humans especially under disease conditions. The majority of infections enter the human body after crossing a surface mucus layer barrier (Moncada et al. 2003, Lidell et al. 2006). Studies focused on diseases of the human gastro-intestinal system, pulmonary tract and tumors have described the SML as an essential physical barrier that lubricates as well as protects the underlaying epithelia (Corfield & Warren 1996, Corfield et al. 2001, Perez-Vilar et al. 2003). Over-production of mucus has been recognized as an important disease associated factor in human asthma, chronic bronchitis, cystic fibrosis, tumors and malignant growths (Ogata et al. 1992, Jeffery 1997, Packer et al. 2004, Thornton & Sheehan 2004). With increased understanding of human mucin gene expression and

mucus layer properties, modification of the quantity of mucus is considered as a first detectable indication of changed health status. Based on these findings, mucus glycoprotein has been recommended as a prognostic tool for early diagnosis of fatal diseases like carcinomas, tumor outgrowths and stomach ulcers (Hube et al. 2004, Kocer et al. 2004, Paulsen et al. 2006).

An increasing trend of coral mortality in recent years (Harvell 1999, Ritchie et al. 2001, Walther et al. 2002, Wilkinson 2004), due to occurrences of temperature-associated bleaching and subsequent disease outbreaks, calls for the better understanding of mucus associated processes (Brown and Bythell 2005; Ritchie 2006). Mucus is a detectable, immediate response known in corals to the changed environmental conditions. The surface mucus layer represents an interface between the external surrounding conditions and the surface epithelium of coral. As a general response to increase external perturbations, elevated levels of mucus have been observed during natural tidal and experimental aerial exposure (Daumas et al. 1981; Wild et al. 2004a; Wild et al. 2004b), sedimentation (Loya & Rinkevich 1980, Daumas et al. 1981) and pollution (Peters 1981). This change in exuded mucus is either due to increased synthesis or release of the stored mucus from the tissues or may be a combination of both. The increase in abundance and size of the mucocytes in tissues represent the increased synthesis and storage of the mucus. Mucus is released on the surface by mucocytes present in the epidermis. The rate of release of mucus on surface and its subsequent rate of loss into the surrounding water determines the thickness of the surface mucus layer. Thus study of change in the volume fraction (V_f) of mucus in the epidermis under different environmental conditions could reflect alteration in surface mucus layer (SML)

thickness which is mainly responsible for the protection of coral surface from infections. Thus, change in V_f may be viewed as an adjustment of coral system to face the stress. The measurement of volume fractions of mucocyte (V_f) in fixed histological sections, represents the maximum amount of stored mucus that can be exuded by the polyp at a given time. In this study, variation in mucus volume fraction (V_f) in coral epidermis has been investigated using histological sections to determine how epidermal mucus responds to:

1. Increased sea surface temperature during a natural bleaching event.
2. An experimental temperature treatment study focused on the continual exposure to thermal stress of bleached corals and its impact on mucus quantity in epidermis.

5.2 METHODS

5.2.1 SAMPLES COLLECTED DURING 1991 NATURAL BLEACHING EVENT

Samples were provided from a collection made five weeks after the actual report of bleaching in May 1991, from the southeast part of Phuket Island, Thailand (provided by B.E. Brown). An elevated temperature of 1-2°C higher than normal summer maximum sea surface temperature (SST) was monitored and there was 0.8°C mean temperature elevation for the whole year over the normal mean temperature. Samples of *Goniastrea retiformis*, *G.aspera, Favites abdita* and *Coeloceris mayeri* were collected from parts of coral colony that were either completely white (bleached), and completely pigmented (unbleached) from a single colony. Further samples were collected in March 1993,

when the sea surface temperature (SST) was normal and no bleaching was seen. These were used as control to compare with bleached samples and unbleached samples of 1991.

Samples collected for histology were anaesthetised in 1:1 solution of 0.36 M magnesium chloride and sea water for 30 minutes and fixed in 10% formalin in sea water for 48 h. All samples were stored in 2% formalin in sea water and returned to UK. Samples were decalcified in a 1:1 solution of 5% formic acid and 5 % formalin which was changed after every 12 hours until the entire skeleton had dissolved. Small (1-2 polyps) pieces of tissue were dissected out, dehydrated in a graded series of acetone and embedded in TAAB resin supplied by the TAAB Laboratories Equipment Ltd Berkshire, UK.

5.2.2 EXPERIMENTAL TEMPERATURE STRESS TREATMENTS

Samples were provided from a heat stress experiment conducted at the Heron Island Research Station, GBR, Australia, during summer (February-March) 2002 for 12 days. Coral nubbins were acclimatised for one week in the tanks before the start of the experiment. *Stylophora pistilata* and *Porites cylindrica* were collected from 0-2 m depth and were already undergoing partial bleaching with visible discoloration at the time of collection due to elevated natural sea surface temperatures. Nubbins (small piece of coral colony is called nubbin) were maintained in tanks at raised temperature of 30 to 32°C for 5 days before returning to ambient temperature for the rest of the experiment. Control tanks were maintained at ambient environment temperature of 28°C throughout the experiment.

Samples were collected every day for the first five days and then on the 10th and 12th day of the experiment from both the tanks. Electron microscope samples were fixed in glutaraldehyde, post fixed in osmium tetraoxide and embedded in TAAB resin. Light microscope samples were fixed in 4% paraformaldehyde and embedded using Technovit resin following the protocol as supplied by the manufacturer (Heraeus Kulzer, Germany).

5.2.3 HISTOLOGY

For light microscopic (LM) studies, 0.5µm sections were made using a glass knife on a Riechejung ultramicrotome. Sections were mounted on coated microscope slides dipped in 2% 3, aminopropyltriethoxysilane / acetone solution for one minute then changed to 100% acetone solution for 1 minute. Slides were washed in double distilled water with agitation and air dried at 60°C overnight before use. Sections were stained using the toluidine blue or periodic acid Schiff's/alcian blue (PAS/AB) stain. Mucus in cnidarians has been defined as glycoprotein-lipid complex having terminal oligosaccharide side chains similar to human mucus (Ducklow and Mitchell 1979b; Brown and Bythell 2005). These oligosaccharide side chains impart negative charge to the molecule and can be detected easily by cationic dyes. Toluidine Blue is a basic dye and is particularly useful because of its metachromatic properties that imparts purple or red colour to the mucin in the sections (Flint 1992). Further confirmation of the mucin was done by the using Periodic Acid Sciff's /Alcian Blue stain (PAS/AB). PAS/AB is to date the most accurate, easy reliable technique available to detect the acid mucin in tissue, although it more time consuming as compared to Toluidine blue (Corfield 2000).

Toluidine blue stain (pH 2-2.5) was applied for 2-3 minutes on a hot plate. Excess stain was removed in running distilled water and sections were mounted using a mounting media, DPX. The PAS/AB sections were subjected to acetone for 5 minutes to remove resin, then were hydrated and subjected to Alcian blue staining (pH-2.5) for 10 minutes. The excess stain was removed from tissue by distilled water and then oxidised with 1% aqueous periodic acid for 7 minutes. Tissues were rinsed in distilled water and stained with Schiff's reagent for 25 minutes. Sections were washed in running tap water for 5-10 minutes and mounted using histomount.

5.2.4 VOLUME FRACTION MEASUREMENT

Digital photographs of the sections were obtained by focusing on the outer epidermis of the polyp, avoiding the stomodaeum and tentacle regions. The sections were subjected to grid analysis and the number of grid points covering the whole epidermis and epidermal mucocytes were counted using a software Volumeasure downloaded from http://imaging.sbes.vt.edu/laboratory/CTL/Software/VoluMeasure/index.html (Figure 38).The grid points were utilized to obtain the Volume fraction (V_f) of mucocytes in epidermis:

$$V_f = \frac{\text{Number of points overlaying mucocytes}}{\text{Total number of points on epidermis}}$$

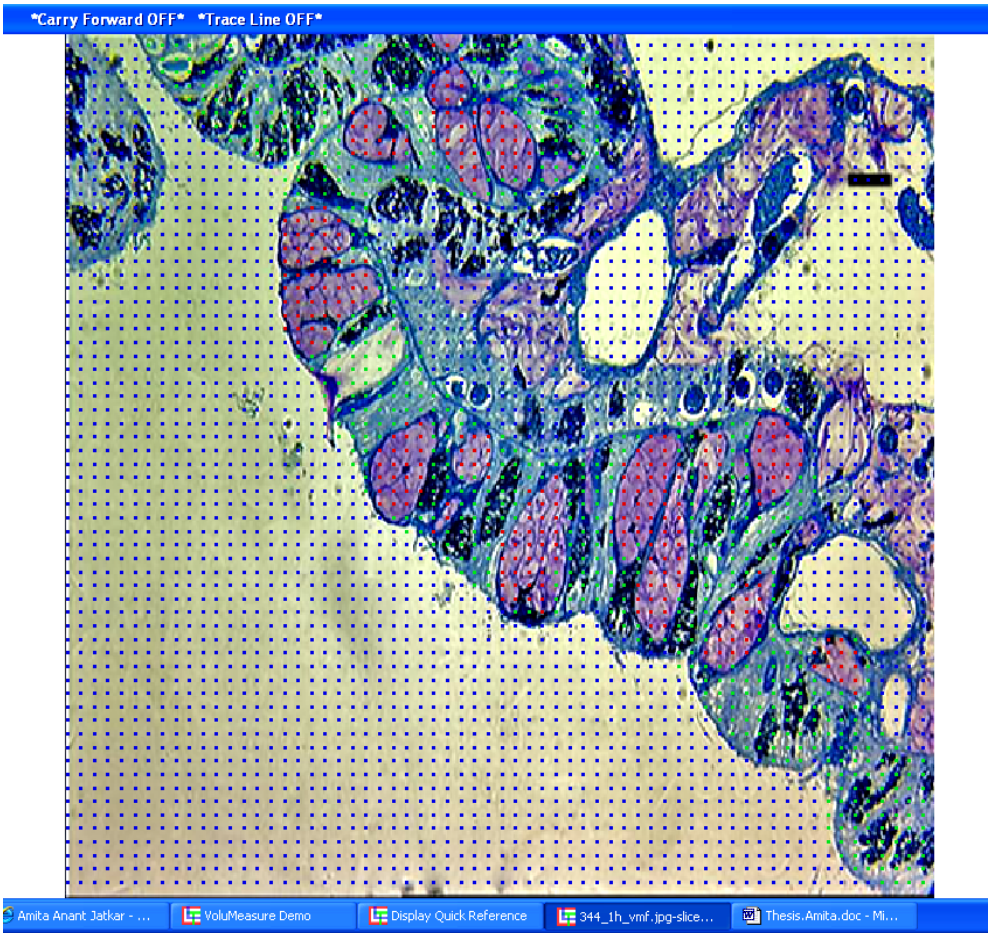


Figure 38 Digital micrograph showing grid points counting method to measure the volume fraction (V_f) of mucocytes in the epidermis. The software Volumeasure counts the grid points covering the epidermis represented by the light blue colour. This provides the total number of grid points covering the epidermis. The software then counts the grid points which cover the mucocytes represented by the red points which gives the number of points overlaying mucocytes.V_f is calculated by the formula mentioned above.

5.3 RESULTS

5.3.1 THE 1991 BLEACHING EVENT IN THAILAND

Under normal environmental conditions (controls), a large variation was seen in epidermal mucus content (V_f) in different species. V_f ranged from 0.9 in *G. aspera* to 0.06 in *G. retiformis* (Figure 39). There was no consistent response in V_f of corals to bleaching between the species. In *G. aspera* there was a 1.3 fold decrease of V_f in bleached tissue as compared to controls whereas, in *F. abdita*, a 9 fold increase of epidermis mucus content (Vf) was measured (Figure 39). In other species, (*Coeloceris mayeri* and *Goniastrea retiformis*) there was little difference between control and bleached tissues leading to a strong statistical interaction between the condition (bleached, healthy) and species (2 way ANOVA; $F_{(6, 71)} = 23.89$, $p < 0.001$).

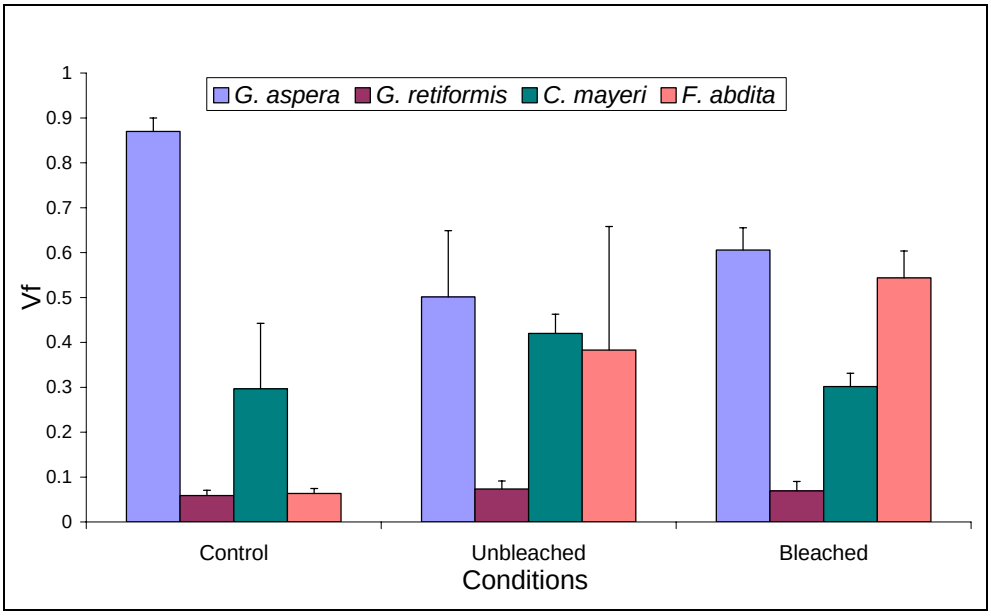


Figure 39 Quantitative mucus content in epidermis of *G. aspera*, (GA) *G. retiformis*, (GR) *Coeloceris mayeri* (CM) and *Favites abdita* (FA) during a natural bleaching event of 1991 in Phuket Island, Thailand. (Bleach – completely white or bleached samples, Unbleach – pigmented or healthy samples collected during bleaching event, Control – samples collected at normal sea surface temperature) (error bars – 95% confidence interval)

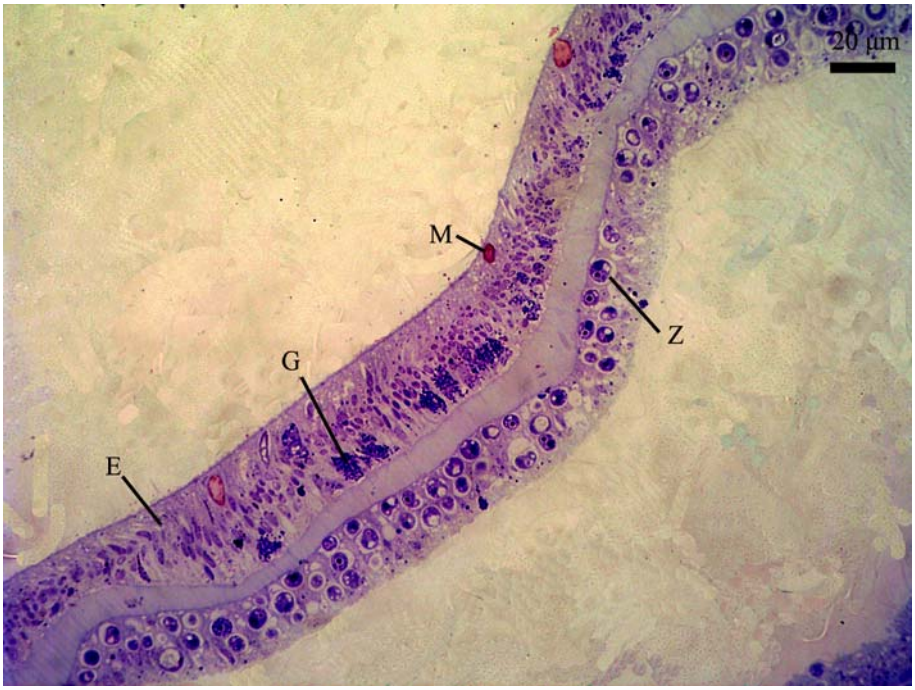


Figure 40 *Favites abdita* healthy tissue showing mucocytes (M) and epidermis (E). the dark granules are visible accumulated towards the mesoglea and zooxanthellae (Z) are present in the endodermis layer.

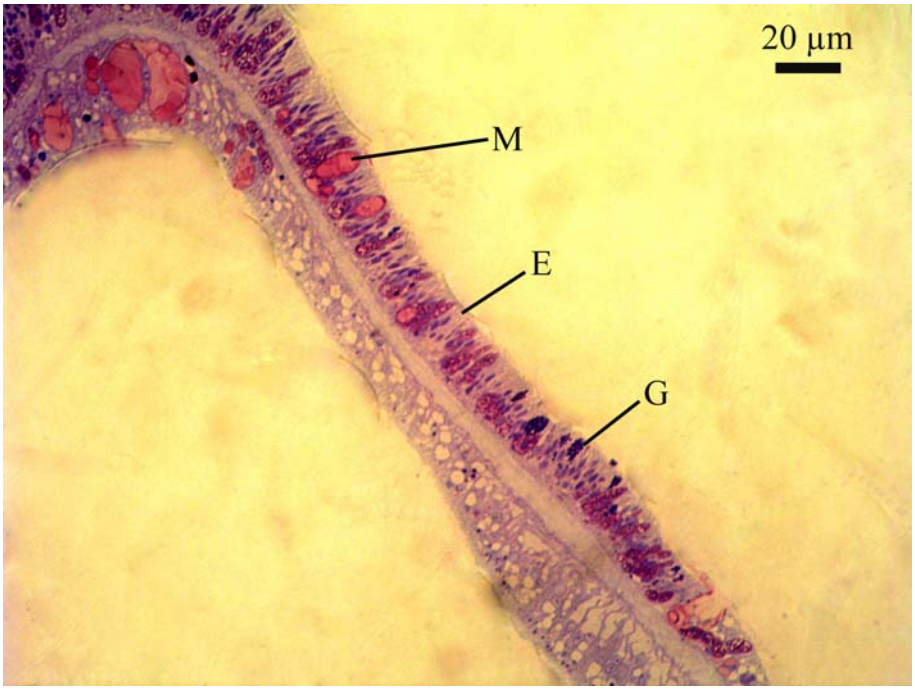


Figure 41 *Favites abdita* bleached tissue revealing dark granules towards the outer side of the epidermis (E) Mucocytes (M), and dark granules (G)

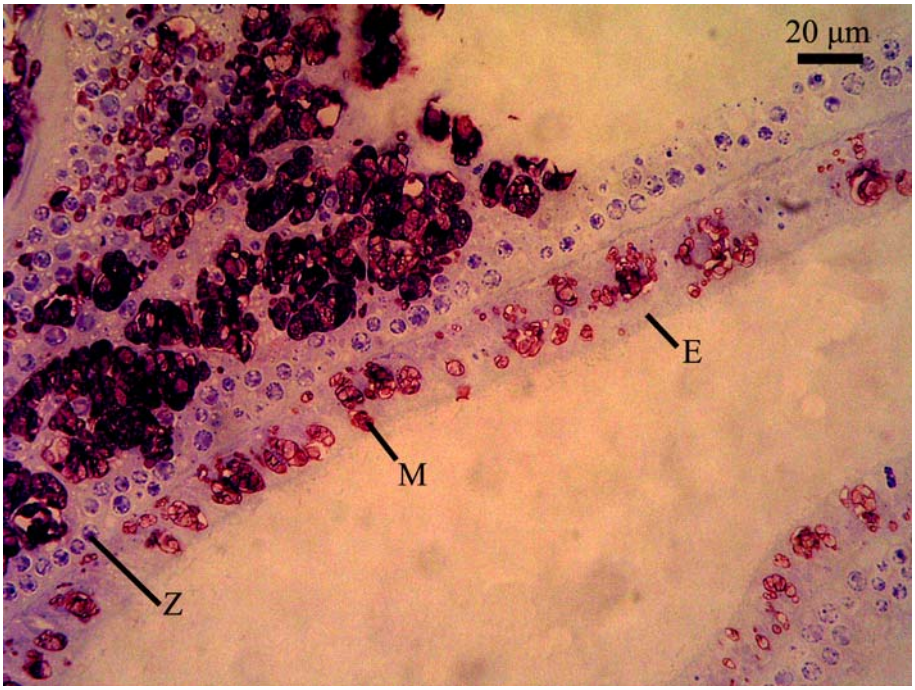


Figure 42 *Coeloceris mayeri* healthy tissue showing mucocytes (M), epidermis (E) and zooxanthellae (Z). Note that there are no the dark granules in the epidermis

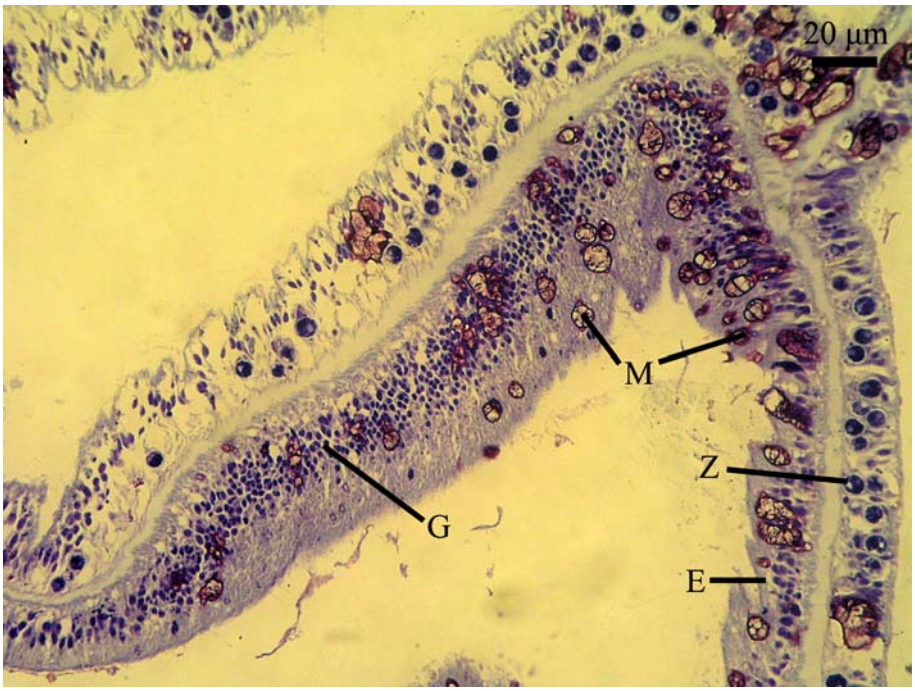


Figure 43 *Coeloceris mayeri* bleached tissue displaying mucocytes (M) in epidermis (E) and zooxanthellae (Z) in the endodermis. The dark granules (G) are plenty in epidermis which appeared as a reposnse to bleaching.

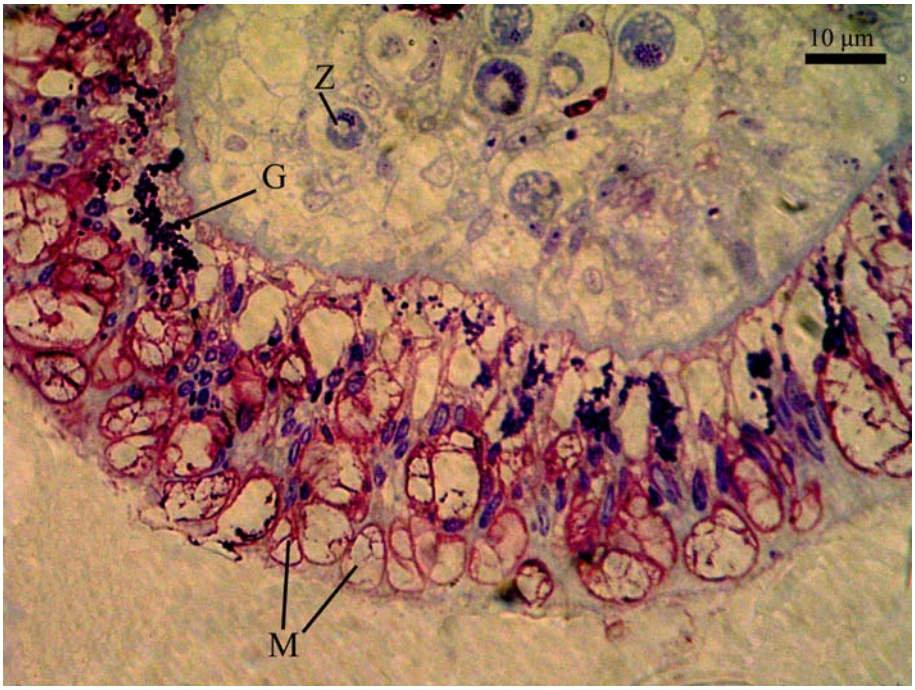


Figure 44 *Gonastrea aspera* healthy tissue with plenty of mucocytes (M) in epidermis with dark granules (G) and zooxanthellae (Z) in endodermis.

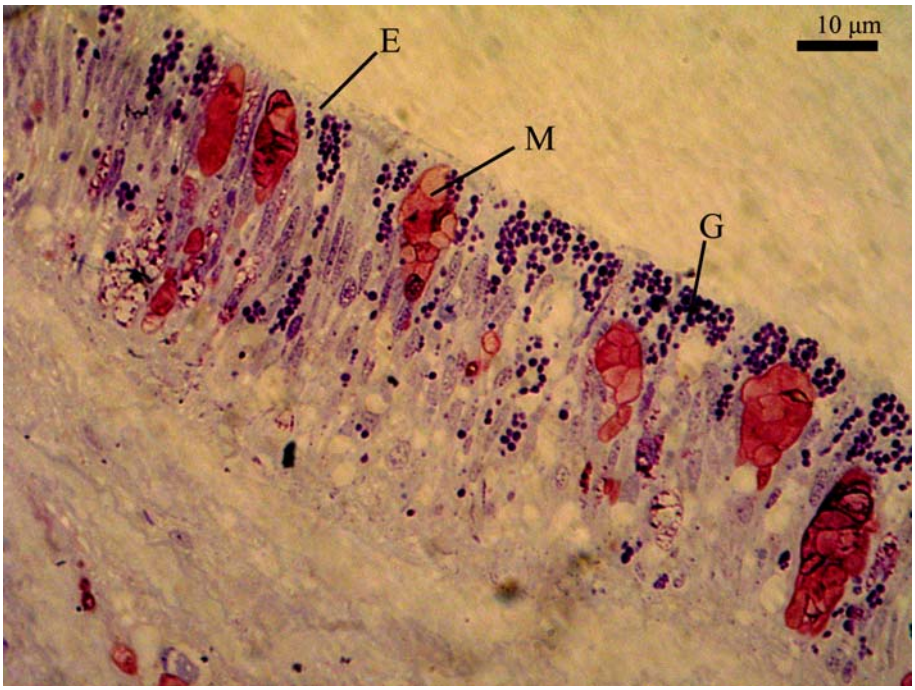


Figure 45 *Gonastrea aspera* bleached tissue with mucocytes (M) in epidermis. Note the position of dark granules (G) which has shifted from inner epidermis in Figure 43 G to be situated near the outer epidermis

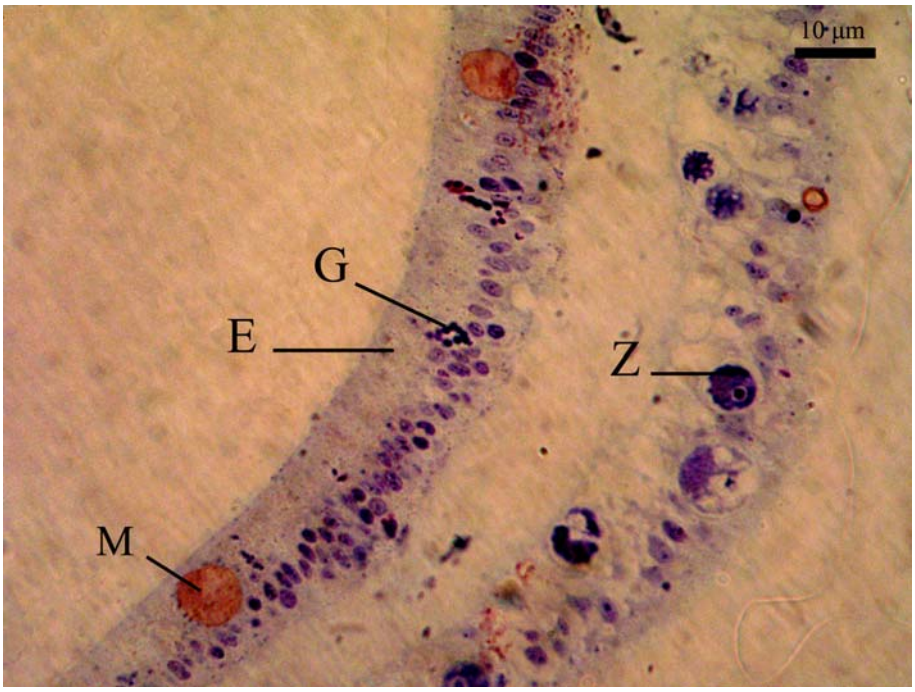


Figure 46 *Goniastrea. retiformis* healthy tissue with mucocytes (M), epidermis (E), Zooxanthellae (Z) and dark granules (G)

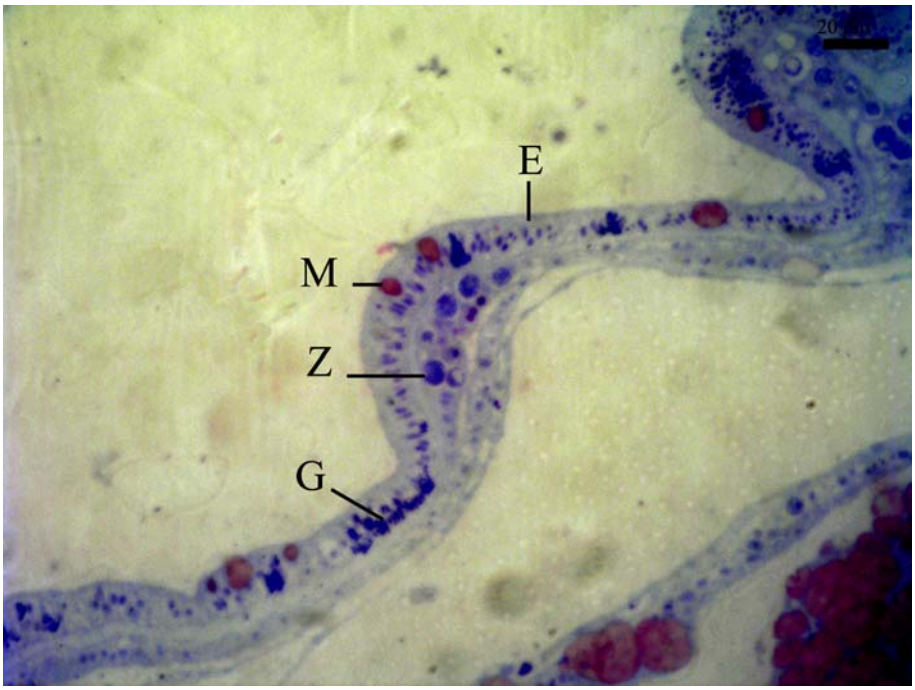


Figure 47 *Goniastrea. retiformis* bleached tissue with Mucocytes (M), epidermis (E), Zooxanthellae (Z) and dark granules (G)

The spatial arrangement of the cellular component in the epidermis has been observed to change as a response to bleaching. In epidermal tissue, mucocytes and dark granules are clearly seen. In healthy tissue of *G. aspera* and *F. abdita* these dark granules are situated more towards the mesoglea in the epidermis (Figure 40 and Figure 44). Function of these granules is unknown but they are highly refractive and may be photo protective pigments or melanin deposits (Caroline Palmer, pers. comm.). In healthy as well as the bleached tissue of *F. abdita* and *G aspera*, few mucocytes were completely filled with stored mucus while others appeared empty or clear revealing numerous sacs (Figure 41 and Figure 44). In other species the epidermal mucocytes were completely filled.

In control healthy tissue, the dark granules were present in the form of clusters situated more towards the mesoglea except in *C.mayeri* (Figure 42) in which they are completely absent in healthy tissue and became prominently visible in bleached sections (Figure 43). In bleached sections, these granules were seen more spread out in epidermis and sometimes close towards the periphery (Figure 41, Figure 43 and Figure 45).

5.3.2 THE TEMPERATURE STRESS EXPERIMENT

In the temperature stress experiment, the majority of the temperature stressed *Stylophora pistilata* nubbins were dead by the end of the 10th day of the experiment while *Porites cylindrica* nubbins survived to the end of the experiment and remained pigmented throughout. Histology revealed that the *S. pistilata* epidermis was without

mucocytes (Figure 48 - B, D and F) whereas *P. cylindrica* had ample reserves of mucocytes in the epidermis (Figure 48 - A, C and E).

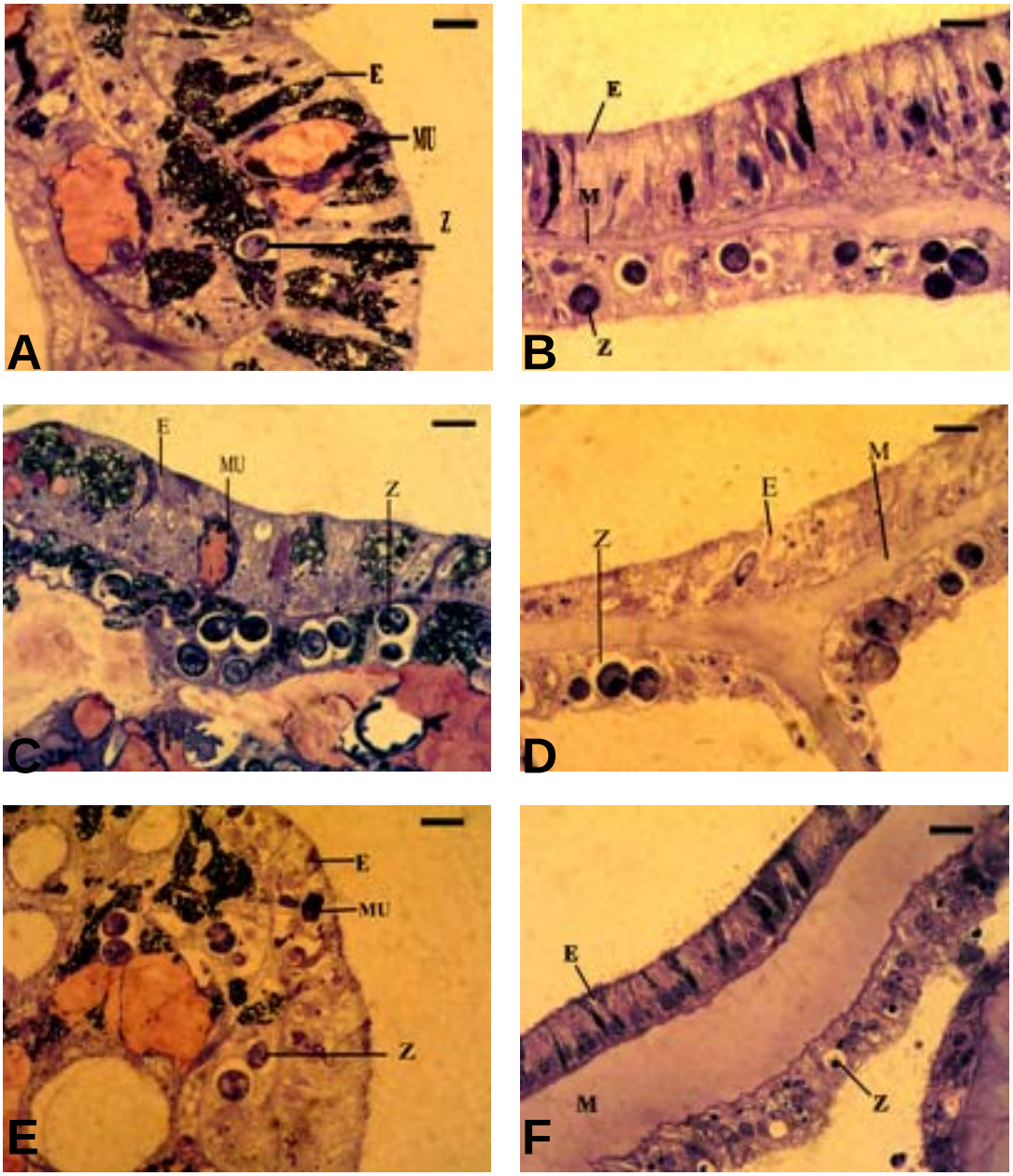


Figure 48 *P. cylindrica & S. pistilata* tissue stained with toluidine blue revealing mucus (pink) in epidermis and endodermis. A & B – control sections of day zero of *P. cylindrica* and *S. pistillata* respectively. C & D- temperature stressed sections of day five of *P. cylindrica & S. pistillata* respectively. E & F - temperature stressed sections of day twelve of *P. cylindrica & S. pistillata* respectively. Scale – 10 µm (MU- mucopolysaccharide, Z- zooxanthellae, E – epidermis and M- mesoglea)

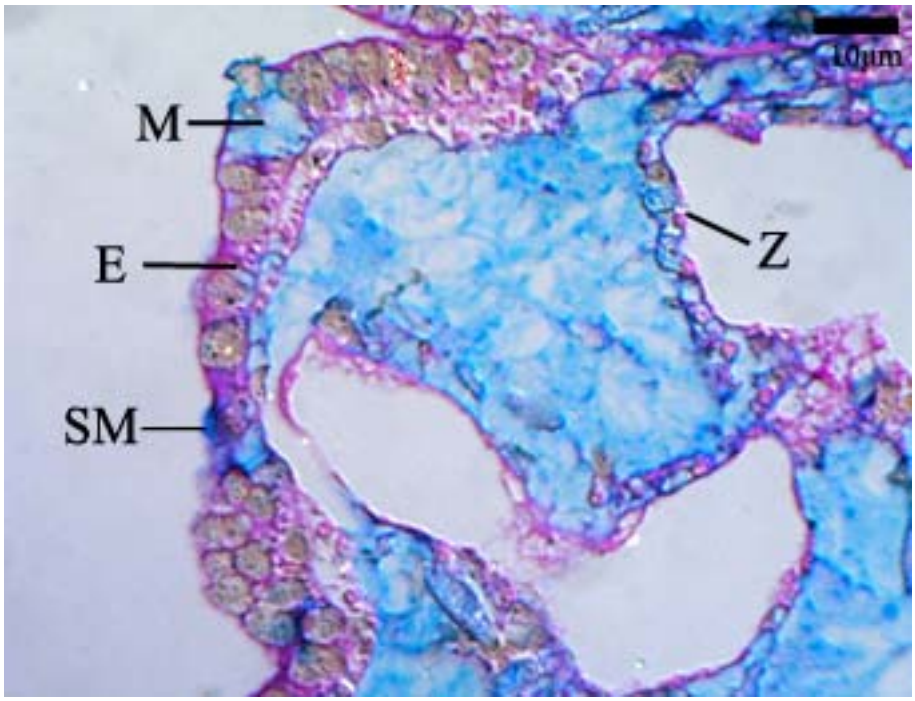


Figure 49 *Porities cylindrica* tissue stained with mucus specific PAS/AB stain. (M-mucocytes, SM-mucus on surface, E-epidermis and Z - zooxanthellae) The mucus is stained blue colour and indicates the acidic nature of mucus.

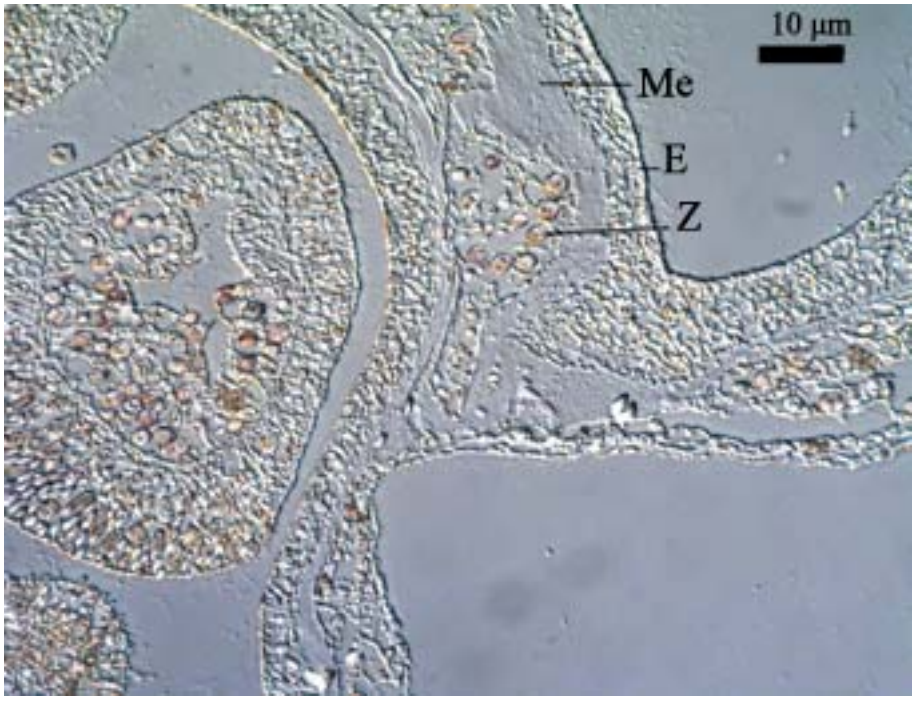


Figure 50 *Stylophora pistilata* tissue stained with mucus specific PAS/AB stain. (M-mucocytes, E-epidermis and ME- mesoglea) note the tissue is without mucus.

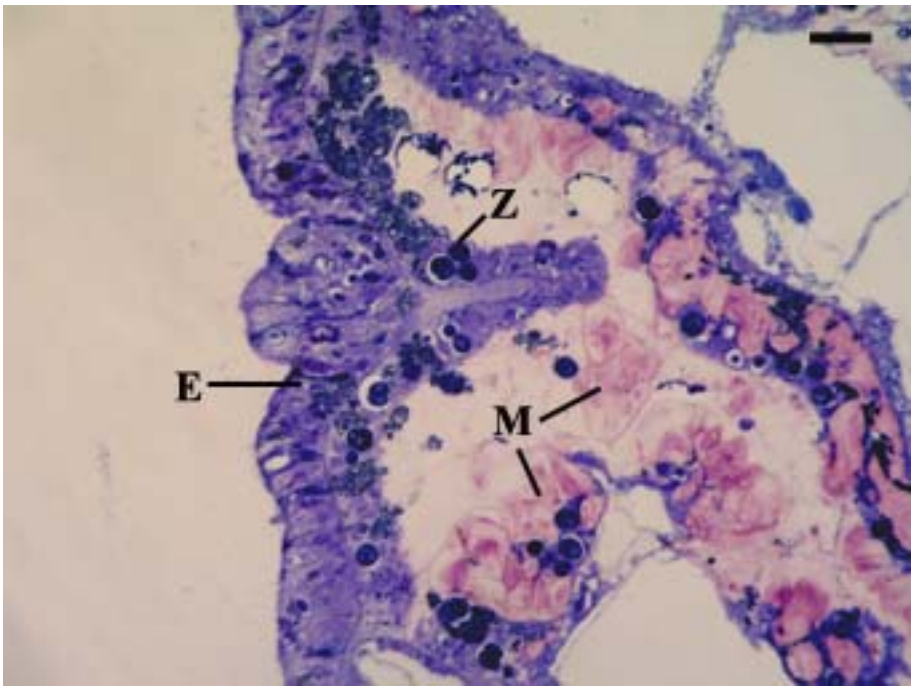


Figure 51 Treated *P. cylindrica* tissue stained with methylene blue revealing mucus (M) mainly in the endodermis and gastrodermis while epidermis comparatively (E) has little amount of mucus. Z – zooxanthellae undergoing mitotic division and zooxanthellae seen to be released in gastrodermis.

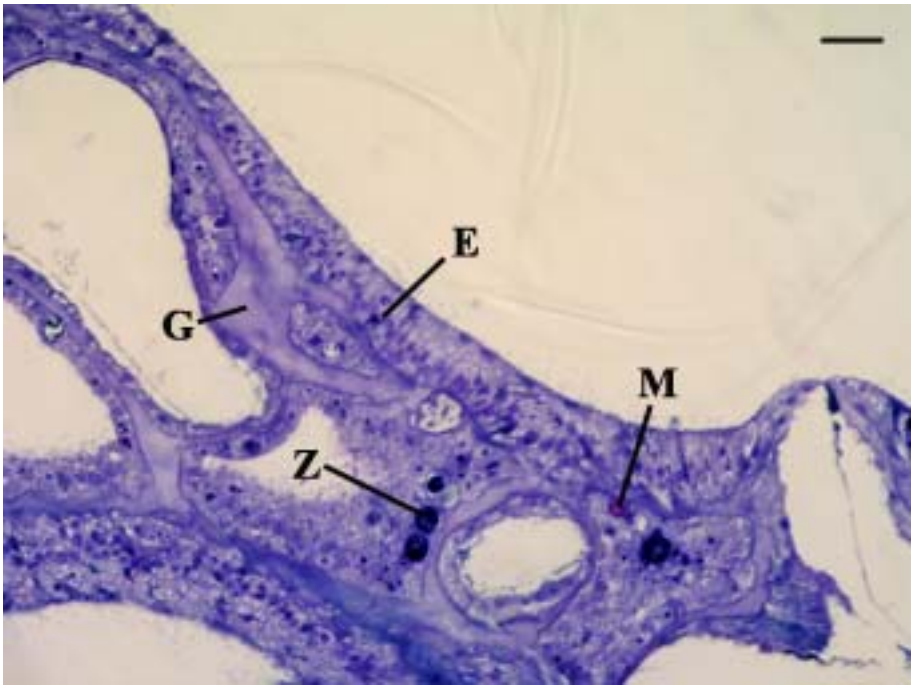


Figure 52 *S. pistillata* tissue showing degrading zooxanthellae (Z), mesoglea (G). Note the epidermis has no mucus while evidence of very little mucus (M) is present in the gastrodermal tissue

In all samples, both treatment and control bleaching signs were clearly evident. Zooxanthellae in the endodermis were in state of active mitotic division (Figure 48 – B, E, and Figure 51), increased vacuolation (Figure 48 - C), and were undergoing in-situ degeneration (Figure 48 – D, F). Release of zooxanthellae into the coelenteron was also observed (Figure 51). This clearly shows that control (untreated) corals were already undergoing bleaching prior to the experiment. The mucus in the treated *P. cylindrica* tissue was seen to be more restricted to endodermis as compared to the epidermis with the progresses of the experiment and at the end of experiment on twelfth day the tissue had demonstrated small mucocytes in its epidermis (Figure 48 - E). *S. pistillata* tissue had very little mucus in its endodermis (Figure 52)

Mucus-specific PAS/AB staining confirmed that the epidermis of *S. pistilata* was devoid of mucus and mucocytes (Figure 50) while *P. cylindrica* epidermis had mucus reserves in the form of mucocytes. (Figure 49) In *S. pistilata* although the epidermis had no mucocytes in surface tissue, the deeper gastrodermal tissue had some mucus present. Quantitative comprision of mucus content V_f in the epidermis of *S. pistilata* and *P cylindrica* could not be carried out as mucus V_f was virtually absent in *S. pistilata*.

5.4 DISCUSSION

5.4.1 THE 1991 BLEACHING EVENT IN THAILAND

The present comparative study of coral tissue under bleached (visibly completely white tissue), unbleached (pigmented tissue collected during bleaching event) and control (pigmented tissue at normal sea surface temperature) conditions, not only revealed

variation in V_f between the species but also highlighted the profound differences in response of each species to the same temperature exposure during a natural bleaching event.

The studied intertidal corals on reef flat are exposed aerially during every spring tide and bleaching is observed following the severe summer season (Brown et al. 1994, Brown et al. 2000). During aerial exposure the main function of protective mucus blanket would be to prevent desiccation and shield the epidermis from solar radiations. Obviously the coral colonies which are exposed for longer duration will have more mucus releasing capacity to promote lubrication and prevent desiccation. Four species used in the study occupy different positions on the reef flat ranging from outer to mid and inner reef flat (Ditlev 1978, Brown et al. 1994, Brown et al. 2002b). *G. aspera* inhabits near shore areas as compared to rest of the species which occupy relative deeper water away from shore. *G. aspera* must have been exposed to temperature stress quite early and for longer duration as compared to other species. Thus, a higher relative exposure and increased need for preventing desiccation and any other invasion may be the reason for relative higher quantity of mucus volume fraction in healthy tissues of *G. aspera*.

In all the studied species, bleaching pattern and response of *G. aspera* have been well documented. Bleaching response of *G. aspera* species have been established as highly directional depending upon the solar irradiation and duration of exposure (Brown et al. 1994, Brown et al. 2000, Brown et al. 2002a). Within colony susceptibility to bleaching is a characteristic of *G. aspera* and investigations have shown that it is the previous

experience of the colony to exposed solar radiation that shapes the bleaching response in *G. aspera* (Brown et al. 2002a, Brown et al. 2002b). The present study has been carried out in intertidal corals of Thailand in which solar irradiation has been demonstrated as an important factor along with elevated temperature in shaping bleaching. Thus, the presence and changing position of dark granules in bleaching tissue could be a response to shield the solar radiations. The dark granules were present in control healthy tissues of three species and appeared in the fourth (*C. mayeri)* species upon bleaching.

5.4.2 THE TEMPERATURE STRESS EXPERIMENT

Bleaching of corals was observed in the field when the samples for present experiment were collected. The healthy colonies of *S. pistilata* and *P. cylindrica* revealed ample quantities of mucus in their tissues (Richman et al. 1975, Crossland 1987, Harland et al. 1993). Thus, absence of mucus in the epidermis of control *S. pistilata* tissues seems to be a response to the field bleaching event.

Although the exact relation between the symbiotic component and coral host remains unclear, carbon source of mucus for the host has been proposed to be the zooxanthellae (Patton & Burries. 1983, Brown & Bythell 2005). The degrading and decreasing status of zooxanthellae during bleaching has been related to the decreased photosynthetic ability of the zooxanthellae (Anthony et al. 2007). This suggests that mucus synthesis in the host will be affected in the severely bleached conditions. The decreased density of zooxanthellae has also been associated with decreased in tissue biomass (Fitt et al. 2000) presumably suggesting the use of stored energy and to meet the metabolic

demands of bleaching coral. The temperature stress experiment can be considered an extension of natural bleaching event exposing *S. pistilata* and *P. cylindrica* to the extreme bleaching conditions. Thus, absence of mucus in control and death of majority of treated *S. pistillata* probably reflects complete utilization of its carbon reserve. The absence of mucus also points to the increased susceptibility of *S. pistilata* presumably due to absence of the surface mucus layer. Thus, failure of primary defence system along with the inability to fulfil the metabolic demand may have caused the death of *S. pistilata*.

5.5 CONCLUSION

Bleaching is a complex phenomenon triggered by many factors and it is difficult to pinpoint one factor responsible for a particular response of the host. In 1991 study number of individually or together factors may have played a role in altering mucus volume fraction in the epidermis. These factors may include relative positioning of the species on reef flat, duration of exposure to the temperature and / solar stress, individual resistance of colony and the genotype of the zooxanthellae

Clearly, variability in the epidermal mucus content V_f has been demonstrated between and within the species during a 1991 bleaching event. Although no clear explanation can be accounted for the V_f variability, the potential of V_f to affect the surface mucus layer dynamics remains significant. The suggestive absence of SML in *S. pistilata* upon severe bleaching also needs to be further investigated. Hence, it is essential to know

more about mucus content V_f of epidermis and the SML during healthy and bleached conditions. This has been explored in chapter 6.

CHAPTER 6

Within-colony epidermal mucus content and surface mucus thickness in two species of corals

6.1 INTRODUCTION

The coral surface is constantly exposed to a variety of physical, chemical and microbial insults and therefore needs an extensive protective system for survival. Copious mucus release has been reported on exposure to air (low tide) (Krupp 1984), heavy sedimentation (Hubbard & Pocock 1972) and pollutants (Mitchell and Chet 1975; Neff and Anderson 1981; Peters 1981). The physical barrier function of the surface mucus layer (SML) depends on the thickness of the SML (Chapter 4). The SML thickness is maintained by the amount of mucus released to the surface and the amount of mucus lost to the surrounding sea-water (described in Chapter 4 and 5). These two important processes affect the thickness of the SML and virtually no information is available about these processes, although it is estimated that 40% of total fixed carbon in *A. acuminata* is lost to surrounding sea water (Crossland et al. 1980).

Mucus is a carbon rich compound believed to be constituted indirectly from the carbon which is fixed by the zooxanthellae found in the endodermal tissue of the host (Brown and Bythell 2005). Evidence of dynamic nature of coral mucus was noticed during the

measurement of the SML thickness when charcoal particles entangled with mucus were seen to be lost in form of strings in surrounding water (Chapter 4). Such a loss of the top mucus layer is a normal phenomenon commonly observed in the gastrointestinal tract of vertebrates, where it has been demonstrated that mucus layer sloughing helps in limiting microbes from penetrating the epithelium. It is well documented that the coral SML harbours a microbial community which changes during diseases (Frias-Lopez et al. 2002, Pantos et al. 2003, Frias-Lopez et al. 2004, Pantos & Bythell 2006). Thus, it can be assumed that the loss of SML may be an important factor in maintaining sterile condition around the epithelium similar to higher vertebrates.

Mucin synthesis and secretion is comparatively well documented in mammals. Mucin secretory granules are released from the golgi complex and stored underneath the plasma membrane as mucin granule masses. Mucin synthesis within the cell follows either:

1) a steady vesicular constitutive/unregulated pathway, with no storage and no receptor mediated secretory regulation or
2) as a regulated/stimulatory pathway which involves packaging, storage and requires stimuli for release.

A single granule released constantly from the peripheral zone of the mucin granule mass is believed to follow the constitutive pathway. This is believed to be the continuous process responsible for maintaining thickness of mucus layer on the epithelium (Forstner & Forstner 1994, Forstner 1995, Corfield et al. 2001, Brownlee et al. 2003). Mucin is released to the cell surface by exocytosis and increased mucus demand results in fusion of many neighbouring pores with the plasma membrane called compound

exocytosis. A prolonged stimulus resulting in accelerated exocytosis with emptying of stored mucin and cavitation of central storage area is seen during regulatory pathway (Figure 53).

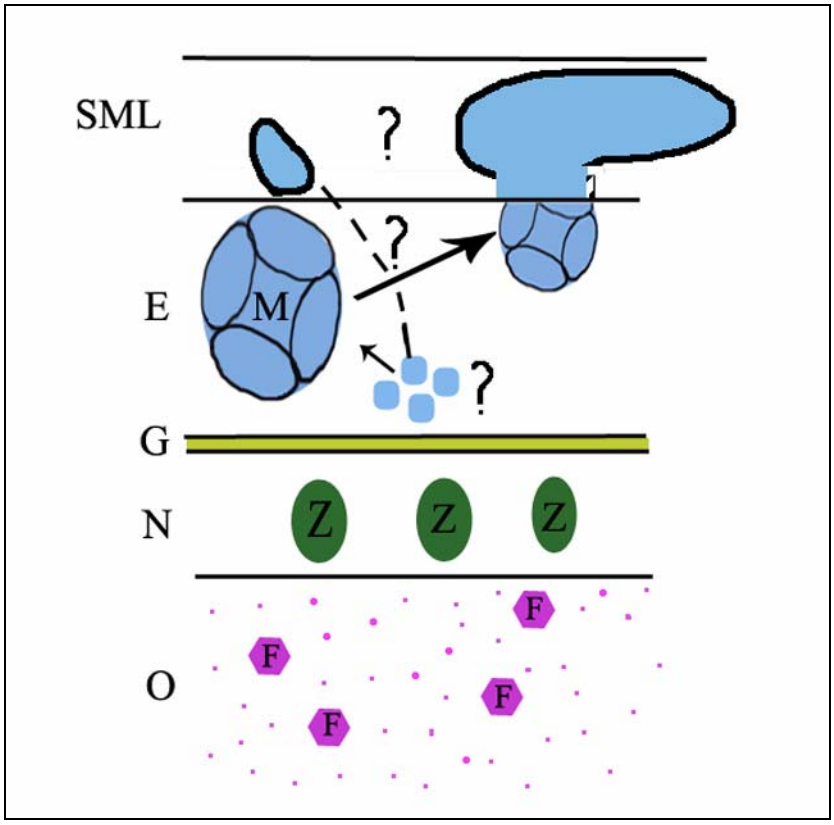


Figure 53 Mucin secretion model depicting the release of mucus to the epithelial surface by either constitutive pathway in dotted line and possible compound exocytosis shown by arrows M – mucin granule, SML – surface mucus layer, E-epidermis, G- mesoglea, N- endodermis, O-coelenteron, F – food particle and Z-zooxanthellae

Although a sharp distinction in constitutive and stimulatory processes of release is not always possible but a considerable amount of cytoplasm loss has suggested that compound exocytosis is an extreme process reserved for all emergencies (Forstner & Forstner 1994, Forstner 1995). Thus a general response of mucus release to the surface is quite different from a stimulatory response.

There is a possibility that the above process may also be occurring in corals but no information is available about these processes. The present study focuses on finding how the volume fraction of mucus in the epidermis changes within a colony in response to bleaching. The epidermal mucus content (V_f) has been determined after measuring the SML thickness on the same colony of corals. The V_f and SML thickness were recorded as a response of mucus to the bleaching within the same colony.

6.2 METHOD

A single colony of each of *Goniastrea retiformis* and *Platygyra daedalae* undergoing bleaching were collected from Phuket Marine Biological Centre (PMBC) site. Similar sized (8 x 5 cm) colonies were selected and SML thickness was measured as described in methods section of Chapter 4 at the PMBC laboratory. Three colonies of each species were used for SML measurement as well as for the histological studies. After SML measurement, healthy and bleached tissues of the colony were fixed in 4% paraformaldehyde separately for histological studies (method described in Chapter 5). 0.5 μm sections were stained with toluidine blue and volume fraction was calculated using Volumeasure software as described in methods section of Chapter 5.

6.3 RESULTS

In healthy part of the colony of *P. daedalae* more mucus was present in its tissue as well as a thicker layer on the surface as compared to the healthy part of *G. retiformis* sample. The V_f in *P. daedalae* (0.075) was three times more than V_f in the healthy polyps of *G.retiformis* (0.025). The SML on the healthy polyps of *P.daedalea* (700 nm) was twice as thick compared to the SML thickness (350 nm) present on the surface of healthy *G. retiformis* polyps (Figure 55 and Figure 57).

G. retiformis and *P. daedalae* displayed a completely different pattern of behaviour in the epidermal mucus content and in the SML thickness recorded as a response to bleaching. The SML thickness and the volume fraction measurement (V_f) in *G. retiformis* showed a positive relationship (Figure 54 and Figure 55) while there was a decrease in V_f in *P. daedalea* with an increase in SML thickness (Figure 56 and Figure 57). A significant difference was found between the mucus volume fractions (2 way ANOVA; $F_{(1, 1)} = 154.51$, $p<0.001$) as well as in the SML thickness (2 way ANOVA; $F_{(1, 1)} = 7.59$, $p < 0.01$) of *G. retiformis* and *P. daedalea*.

Note: Dataset used in this Chapter is same as the data from Chapter 4.

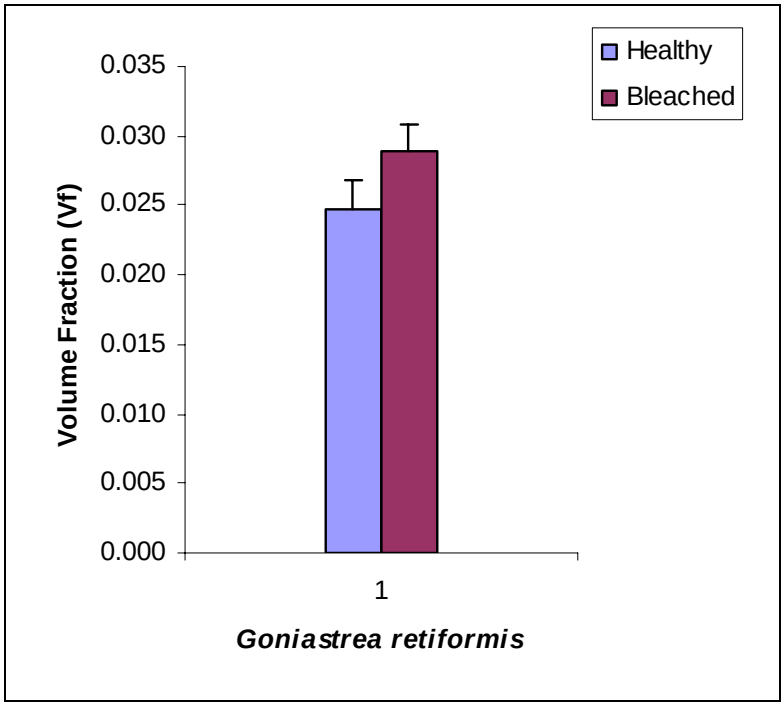


Figure 54 *G. retiformis* showing the Volume fraction (V_f) of mucus in the epidermal tissue of healthy and bleached portion of the same colony (Error bars – 95% confidence interval).Data same as in Figure 35.

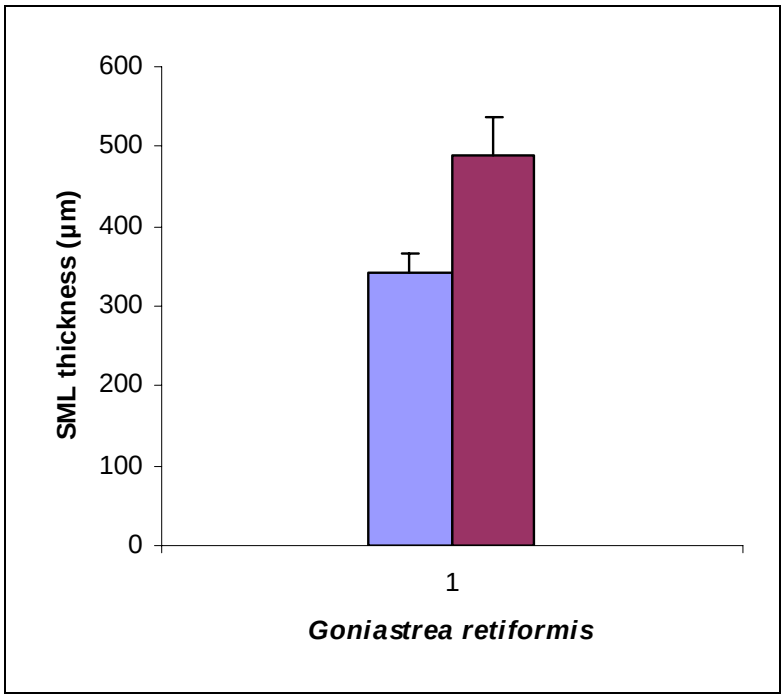


Figure 55 The surface mucus layer thickness in healthy and bleached polyps within the same colony of *G. retiformis* (Error bars – 95% confidence interval). Data same as in Figure 35

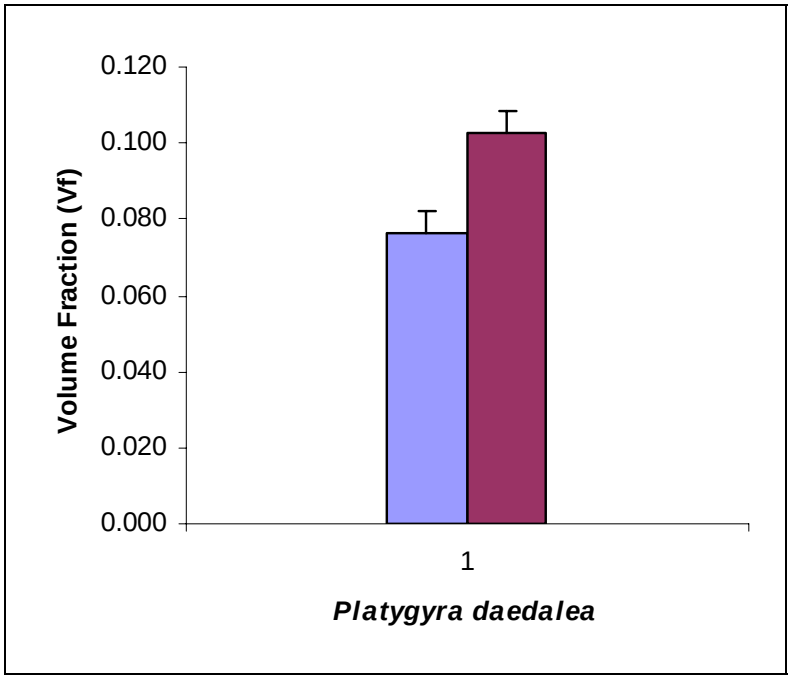


Figure 56 *P. dedalea* showing the Volume fraction of mucus in the tissue of bleached and healthy portion of the same colony (Error bars – 95% confidence interval). Data same as in Figure 34

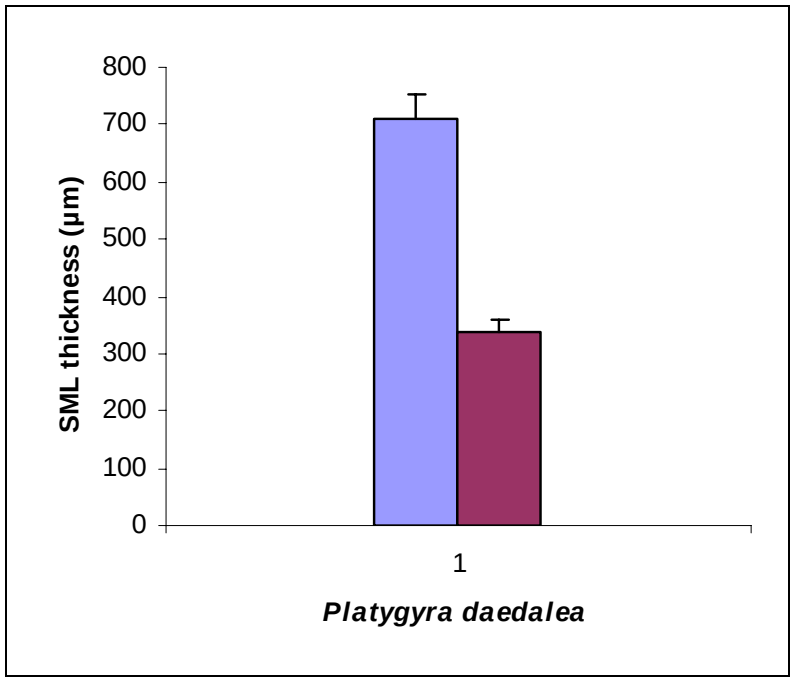


Figure 57 *P. daedalea* showing the surface mucus layer thickness in the tissue of bleached and healthy portion of the same colony (Error bars – 95% confidence interval) Data same as in Figure 34

The volume fraction dataset was transformed to natural logarithm using a Box Cox transformation in Minitab. A two way ANOVA was carried out on the transformed dataset which revealed a significant difference in the mucus content (V_f) between the bleached and healthy tissue within the colony (2 way ANOVA; $F_{(1, 1)} = 10.75$, $p<0.001$). The mucus volume fraction of the two species was affected differently under healthy and bleached conditions since a strong interaction of species and condition was seen (2 way ANOVA; $F_{(1, 1)} = 6.83$, $p < 0.01$).

The SML thickness of the bleached and the healthy tissue of the 2 species also varied significantly within the colony (2 way ANOVA; $F_{(1, 1)} = 6.07$, $p < 0.001$) and the SML thickness was also influenced by the condition as well as by the species involved (2 way ANOVA interaction; $F_{(1, 1)} = 38.23$, $p<0.001$).

In *P. daedalea* healthy tissue sections, mucocytes were observed actively releasing mucus to the epidermal surface (Figure 58) while in bleached tissue sections the mucocytes were mainly seen within the epidermal layer and number of small mucus granules were apparent in epidermis (Figure 59). The number of mucocytes in the healthy tissue of *G retiformis* tissue was less in number compared with *P daedalae* tissue (Figure 60 and Figure 61)

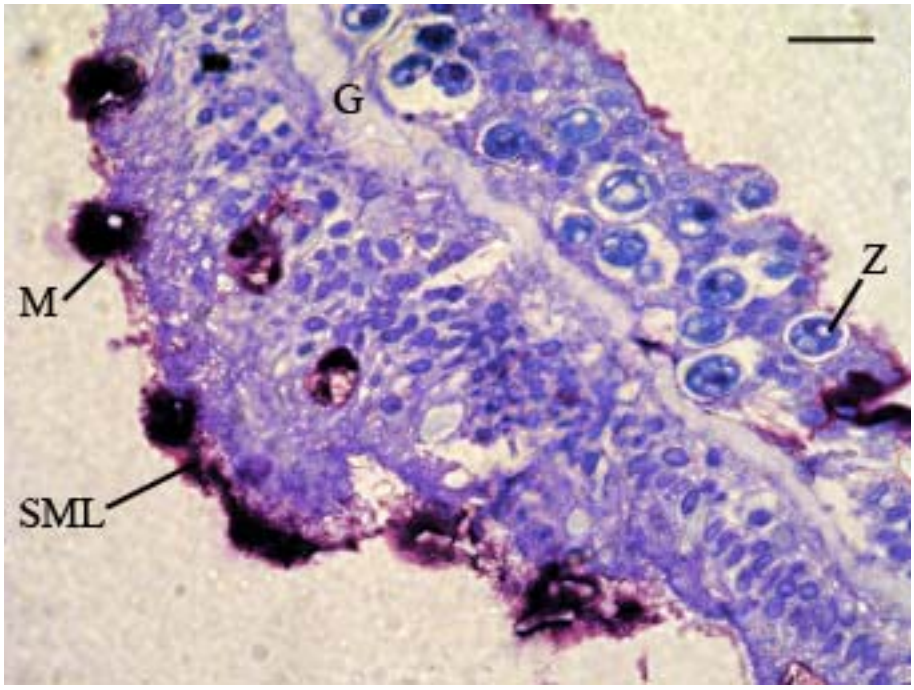


Figure 58 Healthy *P. daedalae* tissue showing mucocytes releasing mucus on the surface forming surface mucus layer (SML), M-mucocytes and Z-zooxanthellae

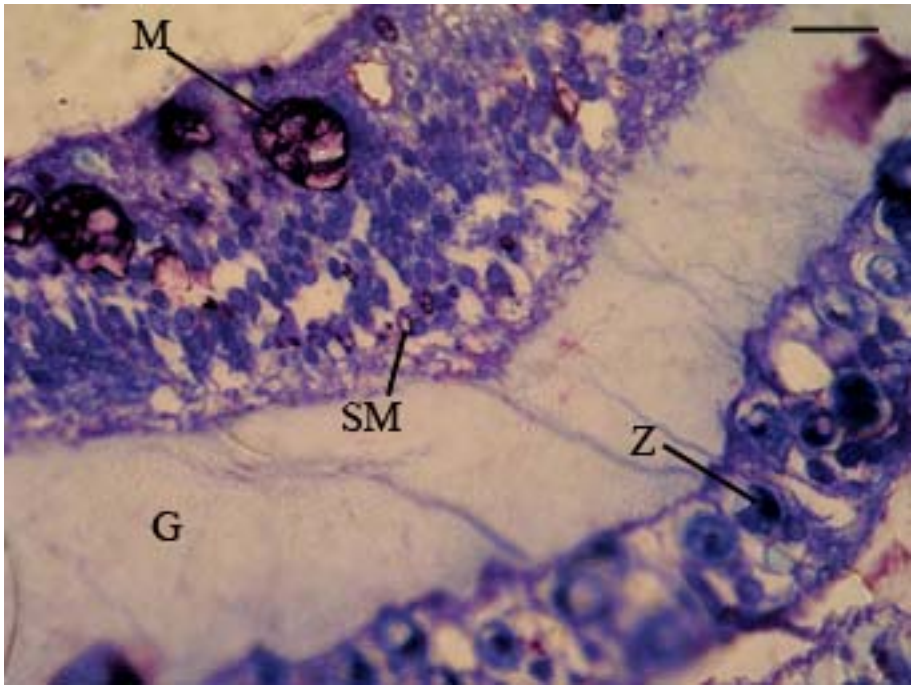


Figure 59 Bleached tissue of *P. daedalae* showing mucocytes in the epidermis, zooxanthellae (Z) and mesoglea (G). Note the appearance of small mucocytes (SM) in the epidermis.

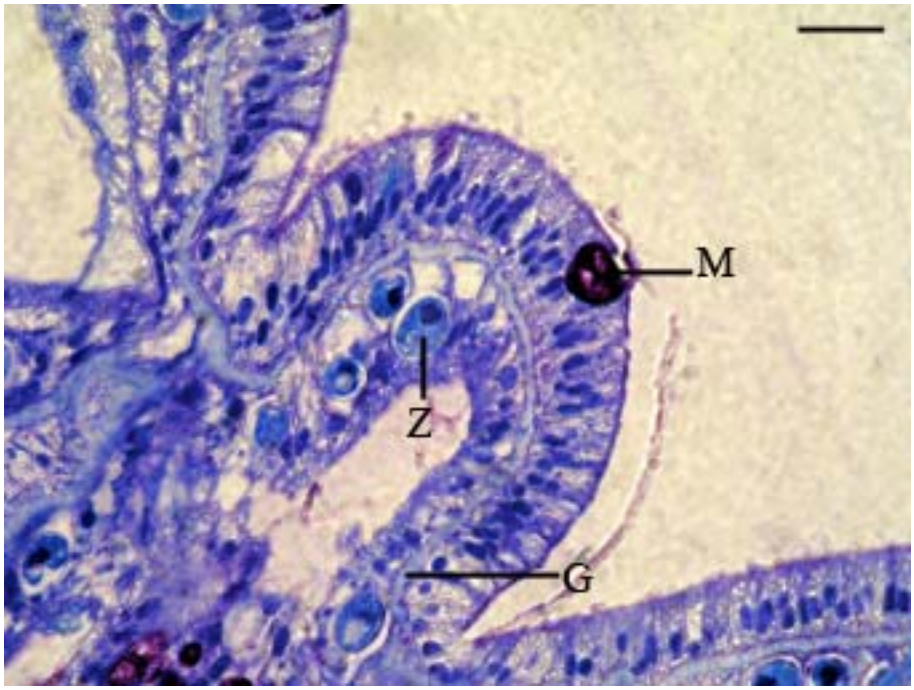


Figure 60 Healthy tissue of *Goniastrea retiformis* revealing mucocyte (M), mesoglea (G) and healthy zooxanthellae (Z)

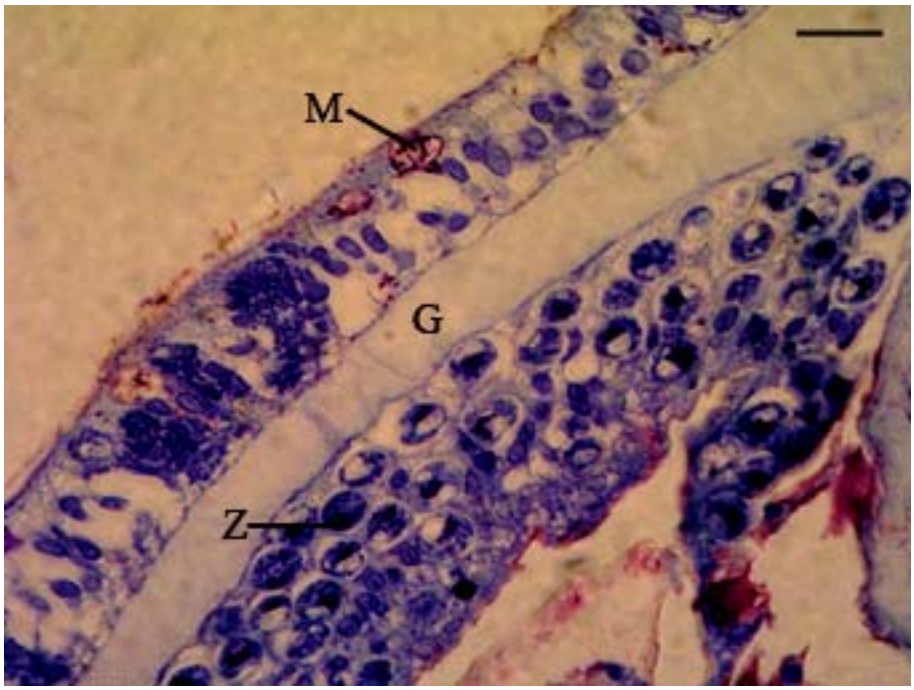


Figure 61 Bleached tissue of *Goniastrea retiformis* revealing mucocyte (M), mesoglea (G). The zooxanthellae (Z) are in degrading state revealing increased cavitation and insitu disintegration.

6.4 DISCUSSION

A balance of mucus synthesis, release and dynamic SML is crucial in maintaining sterile conditions around epithelium (Chapter 4 and 5). A mucus blanket is the first obstacle for the pathogens and disruption of it is a prerequisite to make host susceptible to infections (Moncada et al. 2003). Pathogens adopt a variety of mechanisms in order to achieve this. They may secrete toxins that triggers release of large quantity of mucus; (example *vibrio cholerae* (Lencer et al. 1990) and bacterial infection by *Yersinia enterocolitica* (Mantle et al. 1989) or inhibit biosynthesis of mucins (Byrd 2000, Moncada et al. 2003, Moncada et al. 2005). Recently, progressive decrease and discontinuity in the SML thickness in the mammalian gastrointestinal tract has been found to be closely related to the severity of ulcerative colitis and Crohn's diseases (Corfield et al. 2000, Shirazi et al. 2000, Strugala et al. 2008). Thus, SML thickness indicates the health status of the associated organ/animal. In corals, bleaching can be described as a complex physiological phenomenon which may or may not result in serious disorder depending upon the duration of the stress. Corals are being reported to recover from mild versions of bleaching with time but extension of thermal stress beyond a threshold results in large scale mortality (Brown 1997, Coles & Brown 2003, Douglas 2003, Anthony et al. 2007). In present study, the SML thckness response of the two species to bleaching has been totally different, but the notable observation was a change in the SML thickness in both species over the bleached polyps as compared with the healthy polyps. Although the reason for the change in SML thickness cannot be ascertained, its change can be an important indicator of changed health status of coral as is demonstrated in mammalian gastrointestinal tract.

Although the present study was carried out within one colony, the mucus output in the healthy tissue of *P. dedalea* was much greater than *G. retiformis*. It is difficult to allocate one particular reason for this greater mucus output but the position of coral species on the reef and the exposure of coral to air and radiation might be a factors in coral mucus synthesis and relase as discussed in chapter 4. An increase in the epidermal mucus volume fraction has been observed in the bleached tissues of both the species as compared to healthy tissues. This increased V_f may indicate an anticipation of increased need for the mucus by the bleached polyps but it is very difficult to determine from the present study whether this increase in V_f results in actual increased release of mucus to the surface, however quantitative presence of mucus granule mass in the epithelium in general could represent increased possibility of mucus secretion to the surface. For example, mucus secreting goblet cells form 10% of the duodenal tract in humans while in distal colon it increases to 24%, where digested food remains for a long time and the barrier protective function of mucus becomes crucial to limit the entry of pathogens into the epithelium (Forstner and Forstner 1994).

Although the source of carbon for the coral mucus is debatable, it is believed that majority of coral carbon need is satisfied by symbiont algae present in coral tissue (Patton & Burries. 1983, Crossland 1987, Brown & Bythell 2005). With the advancement of bleaching, decrease and in-situ degradation of zooxanthellae leading to complete loss of zooxanthellae in severe bleaching has been widely reported (Brown et al. 1995, Brown 1997, Douglas 2003, Anthony et al. 2007). It is highly likely that the two species of coral used in present study may have just started experiencing mild form of bleaching which may have resulted in transfer of stored inner tissue mucus content to

the epidermis. This could be the reason for the detection of increased V_f in the two species.

Thus, from the present study it is clear that there was an increase in V_f in the bleached tissue of two species in response to bleaching. It is clear that the SML of two species is affected during the bleaching event. Various factors responsible for the bleaching of the studied intertidal corals have been outlined in Chapter 5 and it is difficult to allocate a particular reason and relation of the change in V_f and SML thickness for the corals.

CHAPTER 7

General discussion

7.1 INTRODUCTION

The coral reef ecosystem is undergoing dramatic changes induced by global climate change. (Fourth Inter-governmental Panel on Climate Change report IPCC; http://www.ipcc.ch/). Frequent episodes of bleaching and emergence of novel diseases amongst reef building corals in the past 2-3 decades has pressed for a need to study the immune system and related stress responses of corals (Sutherland et al. 2004, Mydlarz et al. 2006). Coral immune system study is still in its infancy and the known defence responses consist of surface mucus, melanin deposition and nematocysts (Harvell 1999, Mydlarz et al. 2006). Investigations into the microbial community associated with coral surface mucus layer, under different environmental conditions, have started to unravel the protective nature of surface mucus. Changes in the mucus microbial community structure during diseases have been demonstrated, highlighting the possible role of mucus in protection similar to human gastro-intestinal mucus (Pearson and Brownlee 2005). Recently, a decrease in the antibiotic activity of coral mucus has been reported during stressful summer months (Ritchie 2006; Bourne et al. 2007). The biochemical constituents of the coral mucus have been studied earlier demonstrating a highly variable mucus composition which has been suggested because of different sampling and analysis methods (Brown & Bythell 2005). The protective nature of SML depends on its constituents, gel forming property and the thickness of the mucus layer (Allen &

Pearson 2000, Pearson et al. 2000, Atuma et al. 2001, Strugala et al. 2003). The study addresses coral mucus encoding muc gene, mucin properties and the dynamic nature of the SML which have remained unexplored to date. There were three overall main aims of this work

1. Development of molecular tools to detect coral gel forming mucin gene/genes
2. To address the question does coral possesses mucus glycoprotein – mucin responsible for the gel forming properties of mucus
3. To investigate the dynamic nature of SML

1. Development of molecular tools to detect coral gel forming mucin gene/genes

The muc gene encoding mucin molecule has not been detected in cnidarians. The present study is first of its kind and a step towards finding the domains or complete coral muc gene/genes. Two sequences representing characteristic mucin domains (VNTR) responsible for the heavy glycosylation of the mucin were obtained from EST databases as well as retrieved from the genomic DNA of *A. millepora* (Chapter 2). Three mucin-specific CK domains have been retrieved from *A. millepora*. The presence of more than one CK domain is unknown in the same mucin gene in other organisms. This indicates that there is likely to be more than one gel forming mucin gene in corals. The coral CK domains share a high degree of homology with the human gel forming MUC genes (Chapter 2). The CK domain in human gel forming MUC genes are believed form the disulphide bridge responsible for polymeric structure of mucins (Bell et al. 2001, Vitt et al. 2001). A similar process of polymerisation

resulting in formation of a polymer which is essential for the gel forming properties of mucin is anticipated in coral mucin and has been preliminary demonstrated in Chapter 3.

Primers were developed specific to CK and VNTR regions of the *Acropora millepora* to further study the mucin gene expression under different environmental conditions. Oligonucleotide probes can be generated based on the obtained tandem repeat sequences from genomic DNA. The probes will be a useful molecular tool to detect the changes in expression levels of mucin during stressful conditions such as bleaching.

Thus, from this study it can be implied that coral mucus contains a glycosylated molecule (mucin) showing polymerization processes similar to human mucins (Chapter 3).

2. To address the question - does coral possesses mucus glycoprotein – mucin responsible for the gel forming properties of mucus

The definition and distinction between coral mucus and mucin is not clearly defined in the coral literature (Brown and Bythell 2005; Chapter1). This study demonstrates the presence of a high molecular weight glycoprotein in coral mucus which showed similar degree of polymerisation to polymeric pig gastric mucin (Chapter 3). Mucin is the major component of mucus responsible for the gel forming properties of the mucus (Pearson et al. 2000) but its presence, concentration, molecular size and properties were not known in cnidarians.

Although mucin concentrations in the studied coral mucus (milked) were very low and the collected mucus was not having a gel structure, a tendency to form a gel at higher concentrations of mucin in the solution was demonstrated (Chapter 3). This result points towards the possibility of coral's ability to secrete gels with different strengths, varying in mucin concentration.

The coral polyp has a mouth surrounded by tentacles.The mouth leads into a sac like body cavity (coelenteron). The body wall is diploblastic consisting of ectoderm, endoderm and acellular mesoglea is found in between them (Figure 63). The structure of the coral SML has not been completely understood to date. Durden (1906) had mentioned the presence of two layers of mucus while observing the feeding behaviour of corals (Duerden 1906). However after this, no other study has described the structure of the coral SML. Similar SML structure having number of layers was observed during the thermal stress experiment in *Porites cylindrica* tissue in the present study (Figure 64) (Chapter 5). In a recent study where the SML was preserved (Figure 64) it was revealed that more than one layer of mucus was present on the coral surface (Bythell et al. 2008). All these studies indicate the possible existence of a double layer structure of coral surface mucus layer. Preservation of this bilayer structure is quite difficult in corals where the SML remains in contact with sea water unlike mammalian gastrointestinal tract where the mucus layer is found on inner side and comparatively easy to preserve. In addition to this it is very tricky to work with coral SML as any kind of environmental stress or manipulation is anticipated to result in change in the rate of mucus relase to the surface. Mucus

bilayer has been well demonstrated in human gastrointestinal tract with a sloppy top layer constantly eroded and remaining in contact with food and microbiota, whereas a more tightly held adherent layer protects the epithelial surface from strong ionic (acidic/alkaline) conditions and infections.

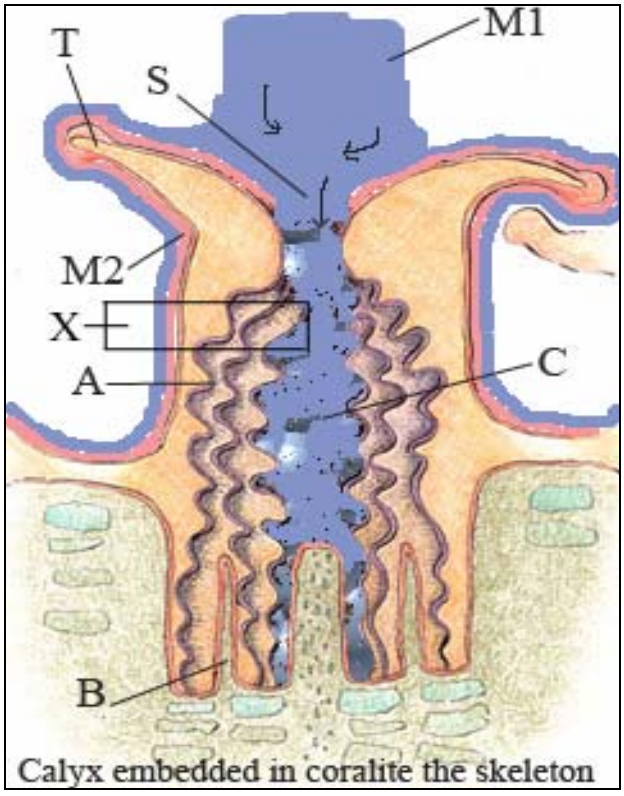


Figure 62 Coral polyp mucus model displaying two different mucus layers, sloppy M1 mucus layer, low in concentration of mucin and more adherent M2 mucus gel layer; arrows represent ciliary feeding C- coelenteron (gut), A – mesentaries, B- septa, S- mouth, T – tentacles. The box X represents diploblastic tissue layer shown in Figure 63

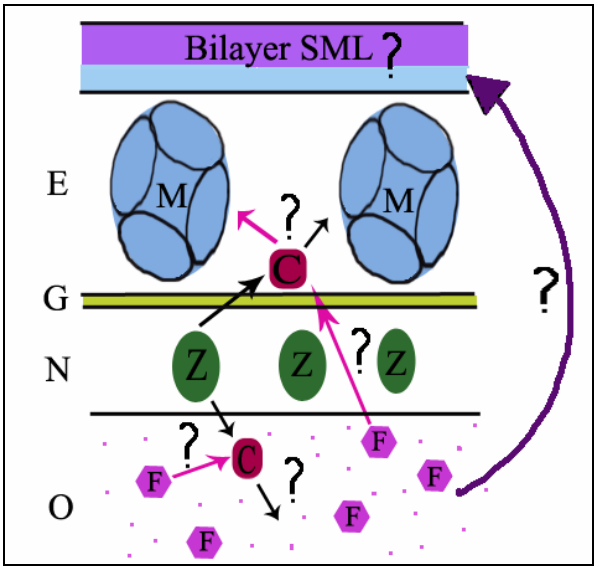


Figure 63 Diploblastic bodywall of coral (from figure 61 above) with epidermis (E), endoderm (N) with mesoglea in between (G). Coelenteron (O) consists of food particle. Mucus is exuded and can form the top layer of SML represented by dark purple colour. Source of carbon for mucus producing mucocytes (M) can be the zooxanthellae (Z) represented by black arrows or the food (F) from coelenterons represented by red arrows.

It is well documented that the exuded mucus entangles the food particle from surrounding water and pushes it towards the mouth and finally it is swallowed by coral (Figure 62). During milking it is belived that large quantity of mucus is exuded from mouth (Figure 62 and Figure 65). Similar process was noticed during the SML thickness measurement when charcoal particles were driven towards the mouth of the polyps in all species of corals (Figure 66) (Chapter 4). Thus, it is logical to assume that for the purpose of food capture, coral requires mucus to have more flowing property than gel forming. This means that the mucus produced from mouth (coelenterons) should have very low concentration of the mucin similar to human saliva which does not form a gel at its native concentration (per comm.. Pearson J) and prepares the food for swallowing.

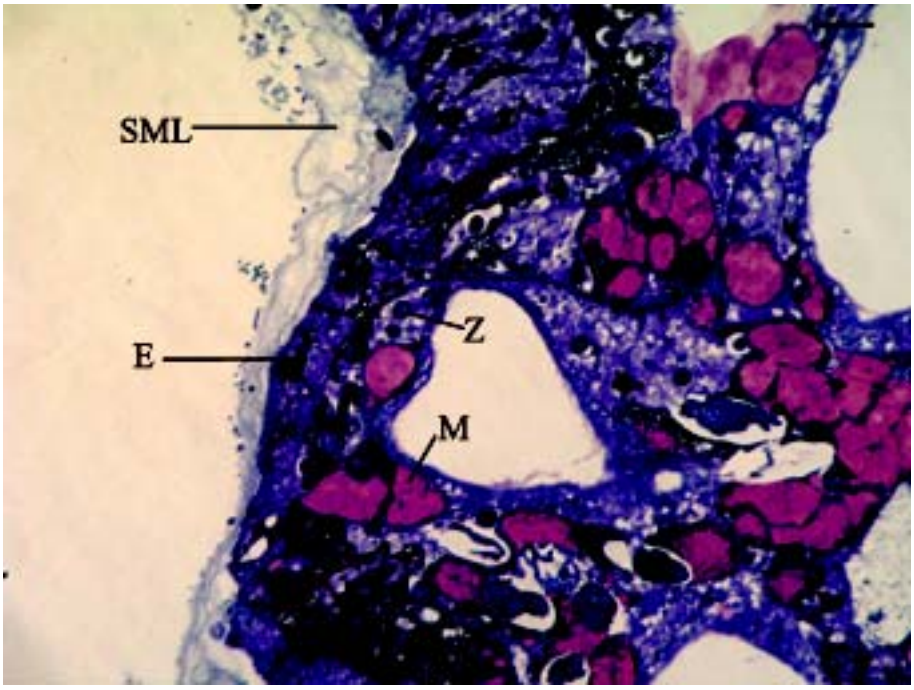


Figure 64 *Porities cylindrica* demonstrating the many layers of the SML and large quantity of mucus deposit in the inner gastrodermal tissue; E – epidermis, M-mucocyte, Z-zooxanthellae and SML – surface mucus layer

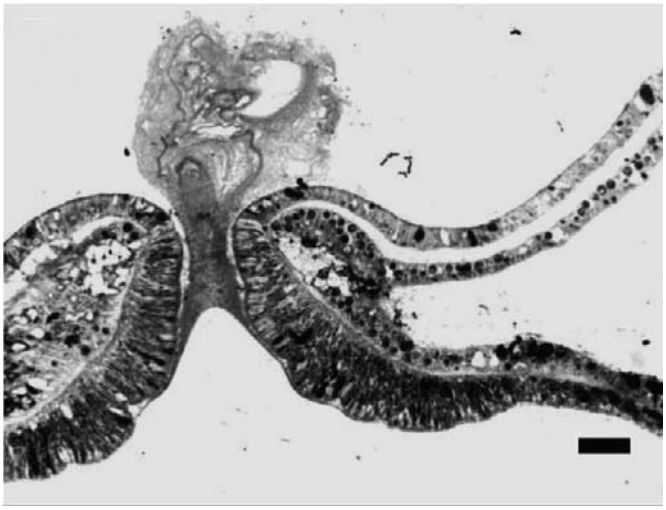


Figure 65 *Goniastrea aspera* demonstrating mucus release from the mouth (Figure published in mucus review (Brown & Bythell 2005)

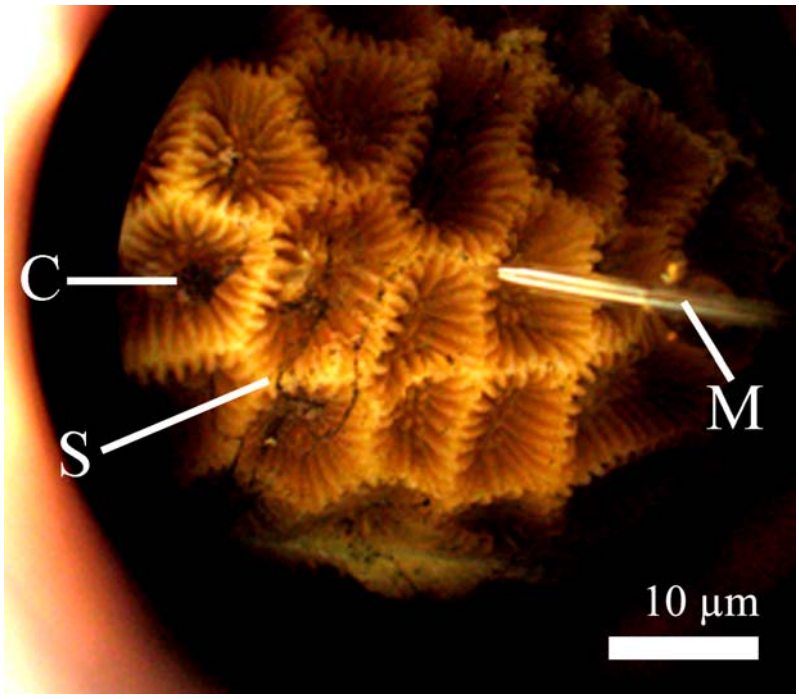


Figure 66 *Goniastrea aspera* showing the movement of mucus entangled charcoal particle into the mouth (C). S is the sting of mucus with charcoal in it and M is the micromanipulator probe.

The gel rheology of *M. faveolata* demonstrated the property of coral mucus to approach gel formation (Chapter 3). The bioinformatic and laboratory result from the *A. millipora* tissue does indicate a strong possibility of different kinds of gel forming muc genes in corals (Chapter 2). Thus, mucus released on the coral epithelial surface other than mouth may have gel forming properties. The large quantity of mucus released by the mouth with relatively greater flowing property would probably cover the surface of the coral polyp. A mucus bilayer structure as reported originally by Durden (1906) is possible in which epithelial gel layer secreted onto the surface by underlaying epidermal mucocytes is probably covered by the (low concentration mucin) mucus layer exuded from the mouth. Similar bilayer structure with adherent gel layer and sloppy (low concentration mucin) layer model is well documented in human gastrointestinal tract (Atuma et al. 2001; Strugala et al. 2003).

During milking of mucus an initial secretion of large quantity of mucus has been reported on aerial exposure and with the passage of time depletion in quantity of mucus is observed. When such a coral is allowed to recover for some time, coral head again starts to ooze out the mucus. Thus, milking presumably results in emptying of gut content which mainly consists of sea water, mucin and tissue debris. This mucus could be a mixture of more than one muc gene product similar to the saliva of humans where MG1 and MG2 have been reported to be products and MUC5B and MUC7 (Offiner & Troxler 2000, Piludu et al. 2003). Thus the sampling process explains the detection of low concentration of mucin and absence of gel structure in the collected mucus samples used in chapter 3.

3. To investigate the dynamic nature of SML

It is believed that coral surface always remains covered with mucus and has a dynamic nature. The dynamic nature of SML depends on the turn-over rate of release of mucus on coral surface and rate of loss of SML from the epithelial surface. A lot of confusion remains in literature about the rate of release and loss of mucus. Ecologists have used the term "rate of production of mucus" to describe the term rate of loss of the SML into the surrounding sea water. Accordingly they have quantified mucus found in the sea water although it has been reported that mucus lost from coral surface undergoes degradation with the passage of time (Coles & Strathman 1973, Ducklow & Mitchell 1979a). The SML thickness has been reported by combining above data with the estimated surface area of coral head (Wild et al. 2005, Koren & Rosenberg 2006). In another approach, cell biologists have used rate of production term to mean either the rate of synthesis of mucus inside cell or the rate of production of mucus on the surface (Crossland et al. 1980, Krupp 1984). Thus there remains a need to define these terms clearly.

Mucus is continuously released to the epithelial surface and mucus sloughing is an important protective mechanism known in the gastrointestinal tract in humans (Belley et al. 1999, Laux et al. 2005). During healthy conditions a delicate balance is maintained between the rate of loss of SML and its replenishment on the surface. Thus, a change in mucus thickness has been related to disease conditions (e.g. ulcerative colitis) in mammals. A thinning of a mucus layer

along with decrease in number of goblet cells in tissue with the progression of ulcerative colitis and Crohn's has been reported (Corfield et al. 2000, Strugala et al. 2008). Similar studies were not known in corals but the present study measured the coral SML thickness using the non-invasive and non-destructive method for the first time (Chapter 4). There was presence of continuous SML of at least 145 μm in four species of corals studied. A comparatively thinner layer of SML was observed over the bleached area in *P. daedalae* colony as compared to the healthy tissue. Although this is a species- specific response but it may be a significant one considering the barrier and protective function of SML.

Chapter 5 and 6 demonstrated a variation in the mucus content (V_f) within and in between the different species in response to natural bleaching and thermal stress. Although, the effect of variability of epidermal mucus content on surface mucus layer is not completely understood, the absence of mucus in tissue of *S. pistilata* and its subsequent death in response to thermal stress indicates the important role of mucus in survival of corals (Chapter 5).

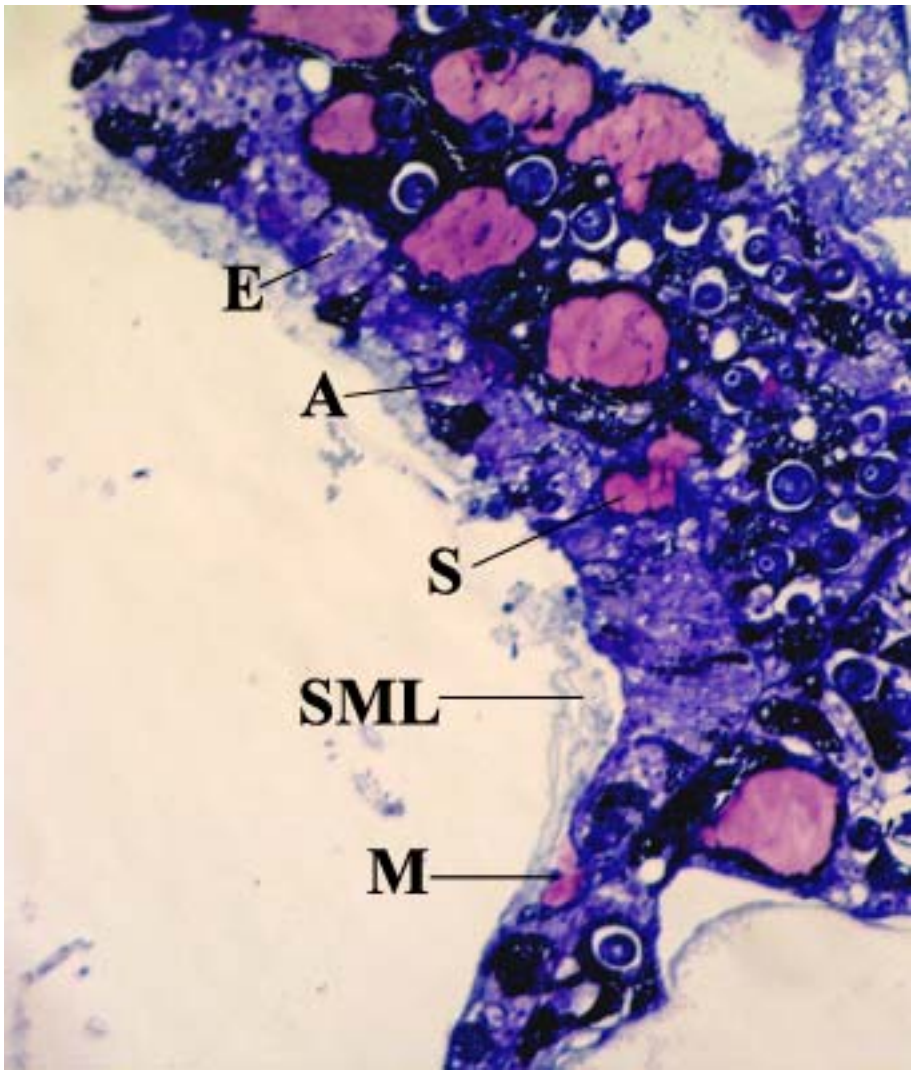


Figure 67 *Porities cylindrica* tissue showing mucocyte (M) releasing mucus on the epithelial surface SML- surface mucus layer, S-mucocyte transferred from endoderm to epidermis layer A- continous release of mucus onto the epidermal surface.

The process of synthesis of mucin inside the cell involves regulatory or unregulatory pathway in higher vertebrates (described in detail- Chapter 6 introduction section). These pathways subsequently result in steady release of mucin or stimulatory release of mucin via compound exocytosis on the surface. Figure 67 of *P. cylindrica* section suggests that these pathways may be present in the coral where the mucocyte may release its mucus by exocytosis (M). A steady continous release of mucus onto the epithelial surface can be seen in form of small mucin droplets (A). Transfer of mucin storage from inner endodermal tissue to the epidermis has been depicted and labelled by S in the Figure 67. Thus the regulatory or unregulatory pathways are possible to be present in corals but it needs further study for confirmation.These processes are important to understand the mucus synthesis inside cell, its release on surface, its affect on the SML thickness and its consequent influence on the health of coral under stressful conditions.

Coral shares a symbiotic relationship with zooxanthellae which occupy endodermal tissue of the host. It is demonstrated that zooxanthellae participate in the photosynthesis and stores the fixed carbon in the form of lipids in its cytoplasm which finally gets translocated to the host (Crossland et al. 1980) and probably gets incorporated into the mucus. Thus zooxanthellae were thought to be the carbon source for the host mucus. However a recent study has demonstrated that *Montipora capitata* can fulfil all its metabolic demands by hetrotrophic feeding under the stressful conditions (bleaching) while the species like *Porites lobata* and *P. compressa* could not do so (Grottoli et al. 2006). This

study has raised the possibility that few species of coral are more adaptable than others and can switch their carbon source under stressful conditions (Figure 66).

Thus, there still remain many aspects of coral mucus biology which need further investigation however the present study enhances the understanding of the dynamics of coral mucus layer and mucin genes. The presence of a high molecular weight glycoprotein with polymeric structure similar to mammalian mucin.was also demonstrated for the first time.

7.2 FUTURE WORK

The possibility of at least three coral gel-forming muc genes was established in this study, however the structure of coral mucin encoding muc gene and properties of coral mucin has not been complete elucidated. A link between the VNTR and CK domain in cnidarians needs to be established. The origin of coral mucus, synthesis, storage and mechanism of its release on surface remains elusive and future work should involve study of these physiological processes. The rheological properties of mucus play an important role in effective functioning of the mucus barrier (Pearson et al. 2000). The composition of oligosaccharide side chains, degree of glycosylation and the amount of dissolved native mucin molecule in different coral mucus should be further investigated.

The structure, properties of human mucin along with the involved microbes has been reported to change during diseases. Similar changes in microbial community structure have been reported in bleaching and diseased corals (Frias-Lopez et al. 2002, Pantos & Bythell 2006). Thus, it is important to consider not only the response of the Fhost in limiting the invading organism but also the mechanism of invasion of the microbes in order to control and manipulate the situation. Mucin structural and functional changes have been proposed as a prognostic tool to detect tumour, cancer and other gastrointestinal diseases. Similar studies can be conducted in diseased corals and health status of coral can be evaluated if proper molecular tools are available. These evaluations may play a deciding role in initiating the early conservation action.

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