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An ancient and variable mannose-binding lectin from the coral *Acropora millepora* binds both pathogens and symbionts

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Summary
Corals form the framework of the world's coral reefs and are under threat from increases in disease and bleaching (symbiotic dysfunction), yet the mechanisms of pathogen and symbiont recognition remain largely unknown. Here we describe the isolation and characterisation of an ancient mannose-binding lectin in the coral *Acropora millepora*, which is likely to be involved in both processes. The lectin ('Millectin') was isolated by affinity chromatography and was shown to bind to bacterial pathogens as well as coral symbionts, dinoflagellates of the genus *Symbiodinium*. cDNA analysis of Millectin indicate extensive sequence variation in the binding region, reflecting its ability to recognise various mannose-like carbohydrate structures on non-self cells, including symbionts and pathogens. This is the first mannose-binding lectin to show extensive sequence variability as observed for pattern recognition proteins in other invertebrate immune systems and, given that invertebrates rely on non-adaptive immunity, is a potential keystone component of coral defence mechanisms.

Introduction

Scleractinian corals are sessile marine invertebrates that form the framework of coral reefs and sustain an enormous biodiversity. However, coral reefs are experiencing increased levels of stress from a range of factors including diseases and bleaching—loss of their algal symbionts

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(*Symbiodinium*) [1–3]. There has been a rapid increase in investigation of coral diseases and stress responses over the last decade, yet study of the scleractinian coral immune system remains in its infancy. Little is known about the molecular mechanisms underlying pathogen defence and symbiont uptake in corals despite their inherent association with coral disease and bleaching. A fundamental feature of the innate immune system of all metazoans is the ability to identify non-self utilising pattern recognition receptors (PRRs) to recognise and bind surface structures on external foreign entities [4]. A PRR binding event can initiate signalling cascades that induce a downstream immune response or immediate reactions such as agglutination of invading cells and opsonisation for phagocytosis [4,5]. In addition, agglutination and phagocytosis assist in symbiont uptake by animals [6,7]. We therefore hypothesised that keystone elements of non-self recognition in both coral immune responses and symbiotic processes may be found amongst the pattern recognition proteins. The interest in invertebrate PRRs has also increased over the last few years as it has become evident that invertebrates may utilise an alternative adaptive immune system involving highly variable PRRs [8–13]. An important group of PRRs in vertebrates and invertebrates are the lectins, proteins that can recognise carbohydrate structures on non-self cells [14–16]. Mannans, consisting of multiple densely packed terminal mannose residues, are abundant carbohydrate entities on the surface of non-metazoan cells and function as key targets for several lectins involved in immunity, including that of the well-studied vertebrate mannose-binding lectin (MBL) [17]. Bioinformatics studies on expressed genes have found that corals appear to have high ancestral complexity and have many genes in common with vertebrates [18]. Some of these genes do not appear in many other invertebrates, suggesting extensive gene loss in these taxa [18]. Hence, studies into coral immunity may provide novel information on the evolution of the early innate immune system of animals. One example reflecting the presence of unexpected immune-related genes in corals is the discovery of complement C3 [19,20]. This molecule interacts with MBL in a complex system in vertebrates and a more simplified system in higher invertebrates, further fuelling the interest to investigate coral lectins with preference for mannose. We used mannose affinity chromatography to isolate a protein from the coral *Acropora millepora* that we have named Millectin, which is the first functional PRR to be described in a scleractinian coral. The protein binds both pathogens and algal symbionts, suggesting lectins may have been co-opted from an ancient innate immune system into a role in selecting and maintaining the photosynthetic endosymbionts in reef-building corals. Millectin is an ancient relative of the collectins MBL and SP-A and has extensive sequence variation in the binding region, providing insight into the function and evolution of the innate immune system.

Material and methods

Materials

A. millepora used in this study was collected from the reef flat on Heron Island, Great Barrier Reef, Australia (23°27′12″S, 151°55′47″E). Fragments of 10×10 cm were transported live to the laboratory where protein extractions were performed. Coral fragments destined for RNA extraction and subsequent cDNA library construction were snap frozen in liquid nitrogen immediately after collection and stored at −80 °C. Bacterial isolates used in this study all came from the Aquatic Animal Health Laboratory strain collection at the University of Queensland (UQ) with the exception of *Vibrio coralliilyticus*, which was isolated from mucus of *A. millepora* from Heron Island. Maintained *Symbiodinium* cultures were provided by Eugenia Sampayo, Centre for Marines Studies, UQ. All chemicals used in this study came from Sigma (Castle Hill, Australia) unless stated otherwise.

Isolation of *V. coralliilyticus* from *A. millepora*

A coral piece was collected using gloves from the reef flat at Heron Island, rinsed in filter-sterilised (0.22 μm) seawater to remove loosely associated microbes and allowed to produce mucus (from air exposure) for 30 s. A mucus drop was spread onto a marine agar plate (BD Bioscience, USA) and grown at 25 °C overnight. Individual colonies of interest were picked and re-streaked. Ribosomal 16s DNA (rDNA) was amplified from pure isolates in a PCR reaction according to Lane 1991 [21] utilising primers 27f (5′-AGA GTT TGA TCM TGG CTC AG-3′) and 1492r (5′-TAC GGY TAC CTT GTT ACG ACT T-3′) [21]. Purified PCR products were sequenced in both directions by the Australian Genome Research Facility (AGRF, Brisbane, Australia).

Isolation of lectin (Millectin) from *A. millepora*

The lectin was isolated and purified using mannose affinity chromatography essentially as described for teleost lectins by Ewart et al. [22] with some modifications. Briefly, coral tissue and mucus were removed by airbrushing coral pieces into ice-cold sterile Tris-buffered calcium saline (TCS; 10 mM Tris–HCl, 150 mM NaCl, 10 mM $CaCl_2$, pH 7.4) with additions of 0.5 mM phenylmethyl sulfonyl fluoride and 1 μg ml^{-1} each of pepstatin and leupeptin. The resultant slurry was homogenised and coral algal symbionts removed by centrifugation at 1500*g* for 10 min at 4 °C. Coral tissues were again homogenised and centrifuged at 5600*g* for 1 h at 4 °C to remove membranes and debris. Non-specific agarose-binding proteins were removed from the supernatant using agarose beads (Ultragel-A4, Sigma, Castle Hill, Australia), prior to affinity chromatography. Mannose-binding proteins were recovered by affinity by incubation of the supernatant with mannan-conjugated agarose beads (Sigma, Castle Hill, Australia), for 10 h at 4 °C. The beads and bound proteins were then packed into 5 ml Econo-Pac columns (BioRad, Gladesville, Australia) for washing and elution. The columns were washed with 3 column volumes of TCS, and protein was eluted in sterile Tris-buffered saline (TBS; 10 mm Tris–HCl, 150 mM NaCl, pH 7.4) containing D- (+)-mannose (200 mM). The eluate was collected in 50 μl aliquots.

SDS-PAGE

All sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed in a Hoeffer SE260