IV Bacterial interactions with the immune system

CHAPTER 14

Host responses to bacteria: Innate immunity in invertebrates

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14.1. INTRODUCTION

In Chapter 10, a brief introduction was provided of our current understanding of the bacterial recognition and effector systems involved in insect immunity. This chapter continues this theme but introduces the reader to a less well-known, but equally important, arena of innate immunity, namely that of marine invertebrates. Enormous advances have been made over the last decade in deciphering how marine organisms, such as the sea urchin, which live within ecosystems containing numerous bacteria, have evolved to maintain an equilibrium with such microbes.

14.2. THE MARINE ENVIRONMENT

14.2.1. Plankton

The marine environment is home to all ranges of organisms. A major subdivision of the marine habitat is the water column versus the sediment (see also Chapters 1 and 2). At the surface of the water column is the complex ecosystem of the plankton. The planktonic organisms are a complex assemblage of single-celled creatures including both eubacteria and archaebacteria, and eukaryotes – both single-celled and multicellular phytoplankton and zooplankton. Many of the single-celled organisms are described as picoplankton, based on their small size, 0.2 to 2.0 μm, and are too small to collect or concentrate by filtering, and if collected, are typically impossible to culture. Because of the difficulties in analyzing these populations, other approaches to characterize the plankton have employed more classic methods to estimate biomass and productivity of plankton in ocean waters

(Pedros-Alio et al. 1999), but correct or accurate estimates have not been previously obtained. Overall, it has been difficult to ascertain the actual population sizes and the true biomass of marine plankton. However, rRNA isolation from marine waters, followed by reverse-transcriptase and polymerase chain reaction (RT-PCR) and fragment sequencing, have indicated that the surface waters between the California coastline and Santa Barbara Island of the Channel Islands have 2×10^6 bacteria/ml and 5×10^5 archaea/ml (Massana et al. 2002). At a depth of 100 meters, these methods have demonstrated that there are 4×10^5 bacteria/ml and 5×10^5 archaea/ml, with the archaea being different from those at the surface. These results have led to more detailed analysis of the organisms present by preparing bacterial artificial chromosome (BAC) libraries from DNA collected from marine environments (DeLong 2001). The BAC plasmid allows the ligation of large DNA inserts (50 to 150 kb) so that very large amounts of chromosomal DNA can be incorporated into a genomic library. The generation of such libraries is described in Chapter 8. The production of BAC libraries has also been applied to an acidophilic biofilm surviving on drainage from a mine (Tyson et al. 2004) and to the prokaryotic populations in the oligotrophic water of the Sargasso Sea (Venter et al. 2004). Both communities included nonculturable organisms and were assumed to be of low complexity, which aided in disentangling the mixed genomic samples during data acquisition and evaluation. The assembly of whole and partial genomes from these environmental systems identified many new species and large numbers of previously unidentified genes, and resulted in the characterization of metabolic and cooperative strategies used for survival in habitats where the major resource was either geochemical, in the absence of light, or low levels of marine nutrients combined with high levels of light. These methods appear to be an excellent initial approach for characterizing a complex microbial community that can be used to direct and optimize future analyses of environmental microbial samples.

In addition to the prokaryote fraction of the plankton, there are large numbers of phyto- and zooplankton present in the surface waters. Analysis of phytoplankton plastid DNA has been used to characterize that fraction of the plankton (Rappe et al. 2000). The zooplankton includes many invertebrate larval forms that remain in the plankton for days or weeks and employ this part of their life cycle as an effective dispersal mechanism for species that have sedentary adult forms. There are two general types of larvae found in the zooplankton, based on whether or not they feed. Lecithotrophic larvae derive all their required nutrients from the large nutrient-rich egg, which enables them to develop and metamorphose into a juvenile adult without feeding (Villinski et al. 2002). Planktotrophic larvae do not have nutrient

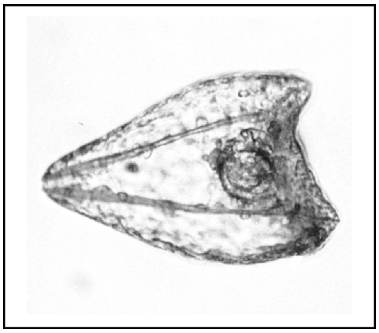

Figure 14.1. Early pluteus of the purple sea urchin, *Strongylocentrotus purpuratus*, also called the prism prior to the development of arms. The oral ectoderm to the right and the stomach is located just below. The image was provided by Ken Brown, George Washington University.

stores and feed on other members of the community to grow during their planktonic phase. Sea urchins exhibit both lecithotrophic and planktotrophic life histories, depending on species.

The sea urchin begins life as an egg that is fertilized in the water column and proceeds to undergo embryogenesis. After a few days, depending on water temperature and species, indirect development culminates in the production of a feeding pluteus (Fig. 14.1), which matures into a larva (Fig. 14.2). The pluteus and larvae are bilateral, with an internal skeleton constructed of calcium and protein that supports the organism, including several "arms" that extend out from the central body. The pluteus and larva are covered with a ciliated ectodermal layer and have a functional digestive tract with coelomic spaces between the ectoderm and the gut (Hyman 1955). The spaces are thinly populated with multipolar or stellate blastocoelar cells that reach long processes across the blastocoelar spaces, around the gut, and out into the arms (Tamboline and Burke 1992) (Fig. 14.3). The ciliated ectodermal cells have regions where the cells are columnar and are more tightly packed, and are located at the intersection between the oral and aboral regions. These cells and their high concentration of cilia are known as the ciliary bands or epaulettes (Strathmann 1975) and enable the larval sea urchin to swim and remain near the surface within the plankton and to simultaneously function to sweep food into the mouth (Strathmann 1971; Strathmann et al. 1972). Echinoderm larvae are omnivorous and will eat almost any particle that will fit down their esophagus, including all types of single-celled plankton and all types of multicellular organisms, including other members of the zooplankton.

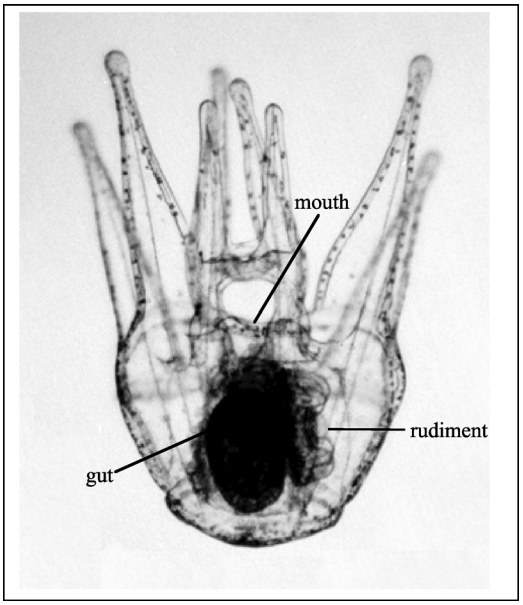

Figure 14.2. Two-week-old larva of the purple sea urchin, *S. purpuratus*. Laboratory-reared larva with eight arms and fully formed and functional gut, which is full of phytoplankton. The adult rudiment is located to the right of the gut. Pigment cells can be seen in the ectoderm. The image was generously contributed by R. Andrew Cameron, Caltech.

As the larva grows during its time in the plankton, it serves as a nutrient supplier and a protector for the adult rudiment (Fig. 14.2) (Hinegardner 1975). The rudiment initially appears on the left side of the gut and essentially forms the ventral half of the pentamerous adult sea urchin having five spines and five tube feet (Hinegardner 1975). Important cues to initiate metamorphosis of the larva into the juvenile of many sedentary invertebrates, including sea urchins, are low–molecular weight organic compounds derived from bacteria (Hinegardner 1969; Cameron and Hinegardner 1974). At the time of metamorphosis, the larv, leaves the plankton and sinks to the bottom. Given a receptive substrate and the proper cues, the rudiment is everted from the side of the larva with the remnant larval tissues remaining as a "turban" on the dorsal side (Hyman 1955). After metamorphosis, the reorganization of the larval turban tissues generates the remaining organ systems of the juvenile, including the dorsal half of the sea urchin, a new digestive tract, and other internal organs, including the gonads. Once the new gut becomes functional, the juvenile begins to feed on biofilms that form on rocks in the marine environment (Leahy et al. 1978), including surface-adhering diatoms

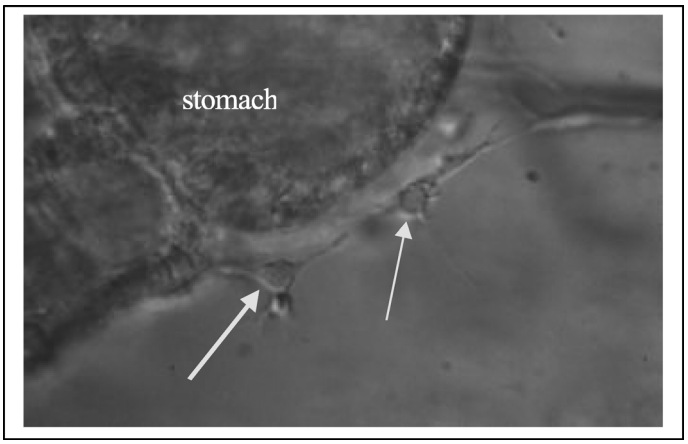

Figure 14.3. Blastocoelar cells. Cell bodies from two blastocoelar cells (arrows) are shown within the blastocoelar space near the wall of the stomach. Each cell is probably positioned near the basement membrane that lines the blastocoelar space, and each has long processes extending from the sides of the cell body. For more information, see Tamboline and Burke (1992). The image was generously contributed by Robert Burke, University of Victoria.

(Hinegardner 1975). As the juvenile sea urchin grows larger, it graduates to eating macroalgae such as giant kelp (Fig. 14.4).

14.3. MICROBIAL CONTACT WITH INVERTEBRATE SURFACES

14.3.1. Larvae

Marine invertebrates such as sea urchins live in environments rich in microbial assemblages. The hatched embryo and larvae contact multitudes of microbes in the water column. Adhesion and colonization of the surfaces of such invertebrates by microbes can lead to infection of the developing embryos, larvae, or adults unless microbial contact and proliferation are controlled by the surface layer of the host cells. Pigment cells located in the body wall of many sea urchin plutei and larvae, including *Strongylocentrotus purpuratus*, may be involved in surface protection. These cells arise from the secondary mesenchyme cells (Gustafson and Wolpert 1967; Gibson and Burke 1985; Kominami et al. 2001), which migrate across the basement membrane that lines the blastocoel, and enter the ectoderm. They extend two or three long processes in a stellate morphology, and can move about within the epithelium (Fig. 14.5) (Gibson and Burke 1985, 1987). These cells

Figure 14.4. Adult purple sea urchins. The image was taken near the coastline of Southern California by Dr. Susan Fuhs while on scuba.

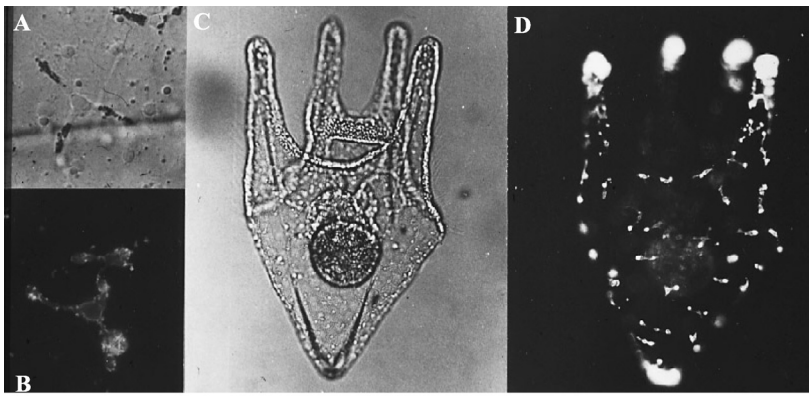

Figure 14.5. Pigment cells in a pluteus of *S. purpuratus.* **(A, B)** Close-ups of tripolar pigment cells showing granules containing echinochrome. **(C)** Pluteus with a gut containing food. The oral ectoderm and mouth are located at the base of the arms at the top of the body. The spherical structure within the body is the stomach. This bright field image is the matched pair to that shown in **D. (D)** The pluteus is incubated with a monoclonal antibody specific for pigment cells (Gibson and Burke 1985). The multipolar pigment cells are spread throughout the ectoderm, with concentrations at the tips of the arms and the tip of the aboral ectoderm at the bottom of the image. The plate was generously provided by Robert Burke, University of Victoria.

are conspicuously red because of the production of a naphthaquinone called echinochrome A, which is contained within spherical granules (Gibson and Burke 1987). Echinochrome is produced by a set of enzymes encoded by genes that are specifically expressed in pigment cells in the late embryo and pluteus (Calestani et al. 2003). Gene knockouts showed that echinochrome production could be blocked in the pigment cells, resulting in uncharacteristically clear embryos and plutei. Echinochrome A is a small double-ring compound with six hydroxyl groups and two carbonyl groups (Kuhn and Wallenfells 1940). Isolated echinochrome A functions as an effective antibacterial compound, particularly against vibrios, which are known to adversely affect sea urchins (Service and Wardlaw 1984, 1985). Although little is known of the functions of the pigment cells in the pluteus and larva, it is not too speculative to suggest that they have defense capabilities to protect the larval sea urchin from attachment and colonization of the ectoderm by marine microbes. This might include migration of the pigment cells to regions of microbial contact followed by degranulation of the pigment granules and release of echinochrome A.

14.3.2. Adults

Surface contact between microbes and the ectoderm of the adult sea urchin presents problems similar to those of the larva in the plankton. A number of marine bacterial species have been isolated and characterized from swabs of the peristomial membrane that surrounds the mouth of the eastern Atlantic sea urchin, *Echinus esculentus*. However, the bacterial composition resembled that of the sand and seawater in which the animal was living (Unkles 1977), suggesting that the microbes were not in permanent association with the host skin. The presence of large numbers of microbes in the sea urchin habitat may not be generally pathogenic, but may at least be opportunistic. Injuries or wear to the tips of the spines in addition to injuries to the skin from puncture wounds from other sea urchins sometimes results in the colonization at these sites by a variety of microbes (Johnson and Chapman 1970; Heatfield and Travis 1975a,b). Skin infections elicit responses by the red spherule cells (see description of coelomocytes below) that form red or black rings (depending on sea urchin species) around infected areas (Hinegardner 1975; Höbaus 1979). Commonly, skin infections result in loss of tissue and spines, but recovery has been observed with a regrowth of skin including new spines. Artificial injuries from surgical implants of allografted tissues also show an infiltration of red spherulous cells to the edges of the graft bed, which dissipate after a few days

(Hinegardner 1975; Coffaro and Hinegardner 1977). The red spherule cells of the adult may have functions similar to those described for the pigment cells in the larva. They can extend short pseudopodia, are slightly mobile, and are filled with granules containing echinochrome A (Johnson 1969).

In addition to the activities of the red spherule cells and echinochrome A, extracts from coelomocytes and the body wall (plates, tube feet, spines, pedicellaria) from the Atlantic green sea urchin, *Strongylocentrotus droebachiensis*, were found to be active against *Vibrio anguilarum* and *Corynebacterium glutamicum* but not against *Escherichia coli* and *Staphylococcus aureus* (Haug et al. 2002). Extracts from cell-free coelomic fluid, gut, and eggs were not active against any of the bacteria. Extracts from other echinoderms including a sea star, *Asterias rubens*, and a sea cucumber, *Cucumaria frondosa*, showed antibacterial activities. The type of compounds responsible for these activities was suggested to be a complex mixture, some sensitive to heat or protease treatment, some not sensitive.

14.4. MICROBIAL CONTACT WITH THE GUT

Because the diet of the larva, juvenile, and adult sea urchin includes prokaryotes, either because they are small enough for the larva to eat or because they are present in or on the food ingested by the juvenile and adult, it can be assumed that when in the gut, microbes may become detrimental if not kept under control while passing through the gut. There is no information regarding microbes that have been isolated from the gut of the larva or the juvenile, but bacteria have been isolated and characterized from gut of the adult. All were Gram-negative bacteria, including vibrios, pseudomonads, aeromonads, and flavobacteria, with 2×10^7 culturable bacteria from three-centimeter gut sections (Unkles 1977). However, only fourteen percent of the sea urchins tested had culturable bacteria in their gut. It is not known whether the gut bacteria identified in these studies were just "tourists," microbes that were passing through and had originally been associated with the food eaten by the sea urchin, or if they were intestinal commensals or "residents." Because only a few of the sea urchins tested had culturable microbes, the conclusion is that what was found were tourists. On the other hand, the status of tourist versus resident may be based on the contributions to the host from the microbe. For example, several nitrogen-fixing *Vibrio* species were isolated from the gut of two sea urchin species (Guerinot and Patriquin, 1981).

14.5. DEFENSES IN BODY SPACES

14.5.1. Phagocytic Cells in Embryos, Plutei, and Larvae

In general, immune defense in embryonic and larval sea urchins have received very little attention. Silva (2000) showed that when yeast were injected into the blastocoel of the mid gastrula, they were readily phagocytosed by the secondary mesenchyme cells present in the blastocoel at that time of development. Similarly, when bacteria were injected into the blastocoel of four-day plutei, they were also phagocytosed, probably by blastocoelar cells (J. P. Rast, personal communication). It seems feasible that phagocytic activity in embryos is mediated by secondary mesenchyme cells and by the blastocoelar cells in plutei and larvae (Tamboline and Burke 1992). Both of these cell types may be of significant importance in host protection against microbes and other foreign contact in the internal body spaces of the embryo and larva.

14.5.2. Adult Coelomocytes

There are four morphologically distinct types of coelomocytes in the fluid of the coelomic cavity of the adult sea urchin (Johnson 1969; Gross et al. 2000), and are present in large numbers (1 to 7×10^6/ml) (Smith et al. 1992; Pancer et al. 1999). The most common type (sixty-six to seventy-five percent of all cells) is the phagocyte, which is potentially a complex set of several subtypes based on differences in both morphology and function (Edds 1993; Gross et al. 2000). These cells have an extensive cytoskeleton; are highly amoeboid, mobile, and phagocytic; and are thought to be the primary immune defense cells. The red spherule cells discussed above with respect to skin infections are usually found in the coelomic fluid (zero to twenty percent) and are considered to have important roles in immune functions based on their echinochrome A content. The spherical-shaped vibratile cells (approximately fifteen percent) are packed with granules and are not amoeboid, but have a single flagellum that is used for active swimming through the coelomic fluid. The colorless spherule cells (zero to five percent) are similar in morphology and mobility to the red spherule cells, but they do not produce echinochrome. Their functions and activities are unknown. The numbers of each type of coelomocyte in the coelomic fluid are estimates and may be quite variable. Significant changes in numbers of all coelomocytes (1.5-fold increase) have been noted in sea urchins after immune challenge (Clow et al. 2000). This result may be mostly based on

the sevenfold increase in phagocytes containing the sea urchin complement homologue (see below). Coelomocytes have been known for some time to maintain sterility in the coelomic cavity of adult sea urchins. Injected bacteria and other foreign particles are quickly removed from the coelomic cavity by the coelomocytes (Unkles 1977; Yui and Bayne 1983; Plytycz and Seljelid 1993; for review, see Smith and Davidson 1994). Antibacterial activity is not only present in the red spherule cells based on the echinochrome activity (Messer and Wardlaw 1979), but coelomocytes also produce lysozyme, which is active against Gram-negative marine bacteria (Messer and Wardlaw 1979; Gerardi et al. 1990; Haug et al. 2002). In association with their active phagocytic activity, phagocytes have been shown to produce increased amounts of hydrogen peroxide with repeated foreign challenge (Ito et al. 1992).

14.5.3. Molecular Analysis of Immune Responses in Coelomocytes

The molecular analyses of echinoderm immunology initially employed genomics that capitalized on a previous characterization of profilin expression in coelomocytes. The gene encoding profilin, *SpCoel1*, was induced after injury or exposure to lipopolysaccharide (LPS) (Smith et al. 1992, 1995). It was assumed that profilin was involved in modulations of the cytoskeleton (dos Remedios et al. 2003) in the coelomocytes during immune activation based on the extensive cytoskeleton in the amoeboid phagocytes (Smith et al. 1992). Consequently, the *SpCoel1* expression pattern was used as a marker to identify sea urchins with activated coelomocytes to enable the identification of other genes activated by LPS. Activated coelomocytes were used in an expressed sequence tag (EST) study that resulted in the identification of a number of interesting immune response genes, including one encoding a C-type lectin and two others encoding complement components (Smith et al. 1996).

14.5.4. Lectins

Innate immunity is composed of numerous detection systems and effector responses directed toward classes or subclasses of microbes based on the macromolecular components or signatures of those microbes. Lectins compose a major type of effector molecule in multicellular organisms and function either as small, single-domain proteins or as one of many domains in a mosaic protein. They bind carbohydrates through their carbohydrate recognition domain (CRD) and are a complex set of proteins with

at least seven different structural CRDs that bind a wide range of carbohydrates (Drickamer and Fadden 2002). One domain type that has received a significant amount of attention is the C-type lectin, which is stabilized through calcium binding (Drickamer 1988, 1993, 1999). The functions of many lectins have been identified and characterized with regard to immune responses in eukaryotes including both vertebrates and invertebrates, and a number of them show enhanced expression in response to immune challenge (reviewed by Vasta 1992; Weis et al. 1998; Vasta et al. 1999; Feizi 2000; Kogelberg and Feizi 2001; Natori 2001; Vasta et al. 2001; Bianchet et al. 2002).

A C-type lectin identified originally as an EST in LPS-activated coelomocytes (Smith et al. 1996) showed significant similarities to echinoidin, a lectin from a different species of sea urchin, *Anthocidaris crassispina* (Giga et al. 1987). Echinoidin from *S. purpuratus* (called SpEchinoidin) is a single-domain C-type lectin with a CRD with a binding motif of glutamine, proline, and asparagine, which is predictive for binding galactose and/or galactose derivatives in a Ca^{++}-dependent manner (Drickamer 1993). The expression pattern of SpEchinoidin was of particular interest with respect to immune responsiveness in the sea urchin because it was found exclusively in the phagocyte class of coelomocytes and only after activation by LPS (Smith et al., unpublished). Immune challenge of sea urchins with either LPS or heat-killed bacteria resulted in the appearance of a significant number of small lectins in the coelomic fluid between 12 and 48 hours after challenge. Subsets of the lectins isolated by differential affinity chromatography using galactose, mannose, N-acetyl-glucosamine, and fucose indicated that SpEchinoidin and the diverse array of inducible lectins is a dynamic response that may be essential for recognizing a variety of sugars on the surface of potential pathogens present in inappropriate places in the host body, such as the coelomic cavity.

14.5.5. Complement

Two other ESTs matched to homologues of complement components. *Sp064* encoded a homologue of vertebrate C3, called SpC3, and was the first complement component identified in an invertebrate (Smith et al. 1996; Al-Sharif et al. 1998). The deduced protein was 210 kd, with a conserved internal cleavage site that yields α and β chains that are disulfide linked. A conserved thioester site and an associated functional histidine were located in the α chain. Phylogenetic analysis of SpC3 compared with other members of the thioester protein family, which includes C3, C4, C5, and alpha 2 macroglobulin (α2M), indicated that SpC3 was the first divergent complement protein

in the deuterostome lineage, falling at the base of the complement protein clade. Transcripts from *Sp064* are present in two subsets of phagocytes (Gross et al. 2000). Investigations of the SpC3 content in the coelomic fluid showed changes in response to both LPS and to injury, with faster increases in response to LPS (Clow et al. 2000). Expression of *Sp064* in coelomocytes also revealed slight increases with respect to immune challenge, and the number of coelomocytes containing SpC3 increased in response to challenge from LPS. These results indicated that responses to a perceived bacterial challenge by the sea urchin are similar in many ways to acute-phase reactants in higher vertebrates. More detailed analysis of the conserved thioester site of SpC3 indicated that it could probably mediate all the basic functions of C3 proteins that have been characterized in detail from mammals (Smith 2002). Analyses of opsonization functions showed that SpC3 may be a major opsonin in the coelomic fluid and significantly increased phagocytosis of SpC3-opsonized yeast (Smith 2001; Clow et al., submitted).

Almost no molecular investigations of immune function in embryos and larvae of marine invertebrates have been conducted; however, the presence of transcripts from *Sp064* throughout embryogenesis has been reported (Shah et al. 2003). An increase in expression was noted prior to and throughout gastrulation during normal embryogenesis, and exposure of early embryos to heat-killed marine bacteria significantly increased *Sp064* message content in late embryos and plutei. Given that SpC3 functions as a powerful opsonin (Smith 2001; Clow et al., unpublished) and that the secondary mesenchyme and perhaps the blastocoelar cells are phagocytic (Silva 2000), embryos and larvae may employ a complement-mediated opsonization in their coelomic spaces to augment phagocytosis of foreign particles prior to their destruction.

The second sea urchin complement component was initially thought to be a complement receptor because of the short consensus repeats (SCRs) that were identified in the EST (Smith et al. 1996). However, sequence analysis of the full-length cDNA showed that it was a homologue of factor B (Bf) called SpBf (Smith et al. 1998). SpBf is a mosaic protein composed of five SCRs (vertebrate Bf proteins typically have three), a von Willebrand factor A domain, and a serine protease domain. Phylogenetic analysis of SpBf indicated that it was the most ancient member of the vertebrate Bf/C2 family. Additional phylogenetic analysis of the SCRs indicates that five SCRs in SpBf may be ancestral to three SCRs, which is the typical pattern in the vertebrate Bf/C2 proteins. The gene encoding SpBf, *Sp152*, is constitutively expressed in phagocyte coelomocytes and is not induced by LPS (Terwilliger et al., submitted). *Sp152* has three alternatively spliced messages in which either SCR1, SCR4, or both exons were deleted. Deletions of SCR1 introduced

a frame shift. However, mRNAs with SCR4 deletions (SpBfΔ4) encoded a putatively functional protein with four SCRs rather than five. Comparisons among full-length SpBf, SpBfΔ4, and Bf/C2 proteins from other species, including carp (Nakao et al. 1998, 2002), suggested that the early evolution of this gene family may have involved gene duplications in addition to deletions of exons encoding SCRs.

14.5.6. Functions of the Complement System in Invertebrate Deuterostomes

Since the identification of a simpler complement system in the sea urchin (reviewed in Gross et al. 1999), an increasing number of complement homologues have been found in other invertebrates. Initially, only deuterostome invertebrates appeared to have complement homologues, including tunicates (Ji et al. 1997; Nonaka et al. 1999; Nair et al. 2000; Raftos et al. 2001; Sekine et al. 2001; Fujita 2002; Raftos et al. 2002; Marino et al. 2002; Endo et al. 2003; Azumi et al. 2003) and the cephalochordate *Amphioxus* (Suzuki et al. 2002), suggesting that the system was specific to the deuterostome lineage. However, complement homologues have been identified in nondeuterostomes, including a gorgonian coral, *Swiftia exerta* (accession no. AAN86548), and ESTs that match to complement components have been found in the Hawaiian bobtail squid, *Euprymna scolopes*, a protostome and member of the Mollusca (M. McFall-Ngai, personal communication). Furthermore, complement-like proteins with conserved thioester sites and opsonin functions have been identified in insects (Lagueux et al. 2000; Levashina et al. 2001; Blandin and Levashina 2004). Consequently, it appears that complement and complement-like components are present throughout the animal kingdom, and therefore this system appears to be significantly more ancient than previously thought.

A simpler complement system composed of homologues of C3, factor B, and mannose binding–associated serine protease (MASP) was proposed to function based on mannose-binding lectin (MBL) recognition activity in association with the amplification feedback loop of the alternative pathway (Smith et al. 1999). Careful analysis of the deduced amino acid sequences of SpC3 and SpBf from the sea urchin revealed conserved cleavage sites for factor I and C3-convertase in SpC3 (Al-Sharif et al. 1998) and conserved sites in SpBf that were involved in both Mg^{++} binding and the activity of the serine protease domain as controlled by putative factor D protease cleavage at a conserved site (Smith et al. 1998). This was interpreted to predict the formation of a C3-convertase complex and feedback loop activation of the alternative

pathway (Smith et al. 1999; Smith et al. 2001). The proposed selective advantage of an amplification feedback loop is that it would coat pathogens with complement proteins more quickly and more efficiently than a simple opsonin that binds upon contact as a result of simple diffusion (Smith et al. 2001). However, this prediction of invertebrate complement function may be too simple. Homologues of most of the components involved in the vertebrate complement pathways have been identified in the genome of the tunicate *Ciona intestinalis* (Azumi et al. 2003). This strongly suggests that at least the protochordates have a mostly complete complement system, which lacks only the ability to be activated by antibodies. It is currently not clear how many additional complement components may be found in other members of the animal kingdom.

Because complement proteins C3 and C4 in the higher vertebrates are known to form covalent thioester bonds with amines and hydroxyls on any molecule, they have the ability to bind to any surface, including self cells. The consequence of this chemical reactivity is the activation of opsonic functions in addition to the lytic functions of the terminal complement pathway that leads to cell lysis and inflammation. To control this activity, a complement regulatory system acts to protect self cells against autologous complement attack and thereby to direct the attack toward foreign pathogens, and it blocks depletion of complement components from uncontrolled activation (Liszewski et al. 1996). Because the sea urchin complement system resembles the alternative pathway and the tunicate system resembles most of the higher vertebrate system, both may require significant regulation. Two additional cDNAs from the sea urchin encode mosaic proteins with domains homologous to known complement regulatory proteins such as factor H and factor I (Multerer and Smith, in press). These domains include multiple SCRs, a fucolectin domain, ser/thr/pro-rich regions, a cys-rich region, and a factor I membrane attack complex (FIMAC) domain, which is also found on C6 and C7 of the terminal complement pathway. The genes appear to be members of a small gene family, are constitutively expressed in all tissues of the sea urchin, and are not induced in response to immune challenge. Although these are the first possible examples of invertebrate complement regulatory proteins, other proteins with similar function are expected to be identified in other invertebrates that employ thioester proteins in opsonization functions
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14.6. IMMUNE DIVERSITY IN INVERTEBRATES

Genes involved in invertebrate immunity are generally understood to be set in the genome as a result of evolutionary selection for protection

against the pathogens with which the host comes into contact in its environment. The proteins encoded by these genes are designed to recognize or interact with microbial molecules that are essential for the viability of the microbes and are associated with broad classes of organisms. They have been described as pathogen-associated molecular patterns (PAMPs) by the late Charles Janeway (Janeway 1989) and include LPS (Gram-negative bacteria), flagellin (bacterial), double-stranded RNA (viral), unmethylated CpG motifs (bacterial), peptidoglycan (Gram-positive bacteria), techioic acid (Gram-positive), mannans (bacteria), and β-glucans (fungi). The major class of receptors that recognize the PAMPs, called pattern-recognition receptors (PRRs), are members of the Toll family that have been identified throughout both the animal and plant kingdoms (reviewed in Imler and Zheng 2004). These and other host–bacterial recognition receptors are reviewed in detail in Chapter 15. The best characterized set of Toll receptors are those in *Drosophila*, the organism in which Toll was originally identified as a gene involved in early dorsoventral polarity during embryogenesis (Lemaitre et al. 1997 – see Chapter 10). The PRRs in *Drosophila* initiate two signaling pathways, Toll and "immune deficiency" (*imd*), which result in the transcription of multiple genes encoding antimicrobial peptides (reviewed in Hoffmann 2003; Hetru et al. 2003 – also see Chapter 10). In general, the antimicrobial peptides are directed toward certain classes of microbes (Lemaitre et al. 1997; Hultmark 2003), including defensins, which act on Gram-positive bacteria; drosomycin, which is antifungal; and attacins, which are anti–Gram-negative. The immune response in *Drosophila* is very effective in protecting the host from all types of microbial attack and is designed for broad recognition and broad effector specificity.

Although the diversity of innate immune responsiveness in invertebrates has appeared to be limited, recent advances seem to suggest that the capabilities of generating significant immune diversity in invertebrates may be greater than previously known (Warr et al. 2003; Du Pasquier and Smith 2003). One example is the family of scavenger receptors with cysteine-rich (SRCR) domains that have been implicated in the development and regulation of the vertebrate immune system (Resnick et al. 1994; O'Keeffe et al. 1999). A family of perhaps 150 polymorphic SRCR genes that are constitutively expressed have been characterized in coelomocytes (Pancer et al. 1999; Pancer 2000, 2001). Comparisons among the encoded proteins showed that they are mosaic proteins with multiple SRCR domains plus a variety of other domains, including von Willebrand repeats, SCRs, transmembrane regions, extracellular matrix-like domains, RGD motifs, and epidermal growth factor repeats. Of note was that some SRCR genes were differentially expressed in

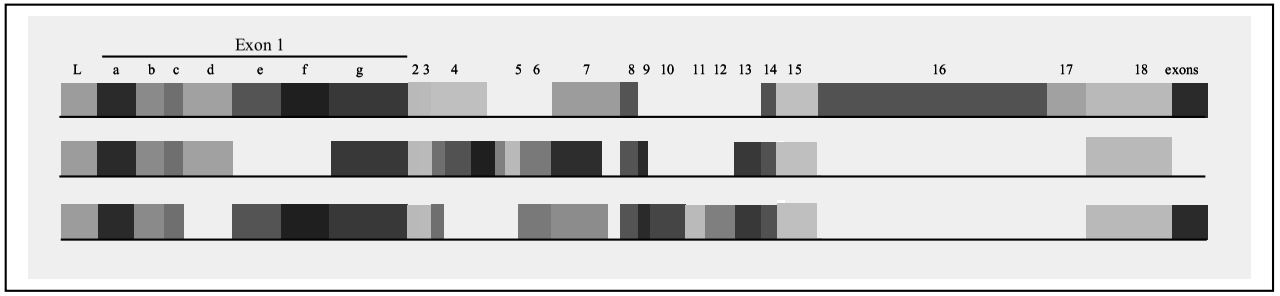

Figure 14.6. Alignment of three full-length *185/333* cDNA sequences. The amino acids deduced from the cDNA sequences were used to generate the alignment with Bioedit. Colored blocks represent exons, and missing blocks represent regions of deleted sequence due to alternative splicing. A leader and eighteen exons were identified from 81 full-length sequences. Exon 1 is shown as a set of subexons, "a" through "g," based on alternative splicing from within the exon as identified by alignments and by cryptic splice sites (see Fig. 14.7). Exons 4 and 7 are shown in multiple colors to indicate significant sequence variability. Exon 18 is shown in two colors of blue to denote a nucleotide variation that introduces an early stop codon.

coelomocytes from different sea urchins, whereas other SRCR genes were expressed with little variation among animals. Furthermore, the expression patterns of several SRCR genes indicated that they changed over time in response to immune challenge and injury, and revealed different expression patterns among the animals analyzed. The sea urchin SRCR gene family and its expression patterns are complex and dynamic and appear to mediate a diverse response to microbial challenge.

An unexpectedly higher level of immune diversity has recently been identified in another set of transcripts expressed in sea urchin coelomocytes in response to LPS or bacteria that are represented by two GenBank submissions, DD185 (accession no. AF228877: Rast et al. 2000) and EST333 (accession no. R62081; Smith et al. 1996), and hereafter called *185/333*. Preliminary analysis of *185/333* expression patterns showed that it was strikingly up-regulated in response to bacterial challenge and was not expressed in unchallenged coelomocytes (Rast et al. 2000), a result that was confirmed from screens of cDNA libraries constructed from bacterially activated versus nonactivated coelomocytes (Nair et al., unpublished). Hundreds of variants of these transcripts were identified by EST matches from an activated coelomocyte library using a subtracted probe specific for transcripts up-regulated by LPS (Nair et al., unpublished). Comparisons among the EST sequences revealed two very surprising results. First, there was a significant level of alternative splicing of eighteen exons, which was confirmed from full-length cDNA sequences (Fig. 14.6). Complexity in some of the exons from different cDNA sequences was also identified. This is illustrated in exons 4 and 7, which are shaded with different colors and sizes (Fig. 14.6). These regions are shown in this way because the sequences did not align well for these exons and they may actually represent nonoverlapping regions rather than variations of the same exon. In addition, exon 1 is shown as subexons "a" through "g" rather than as seven different exons. This was based on alignments that showed the alternative splicing and then based on cryptic splice sites that were identified in appropriate positions relative to the alignment (Fig. 14.7).

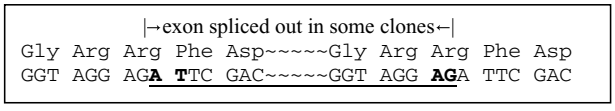

Figure 14.7. Cryptic splice sites within exon 1 of *185/333*. Splicing from within exon 1 was first identified from alignments and then from cryptic splice sites positioned at the edges of the subexons. An example of cryptic splice sites in exon 1 are indicated in bold and are located within the codons for arginine and phenylalanine. ~~, sequence omitted.

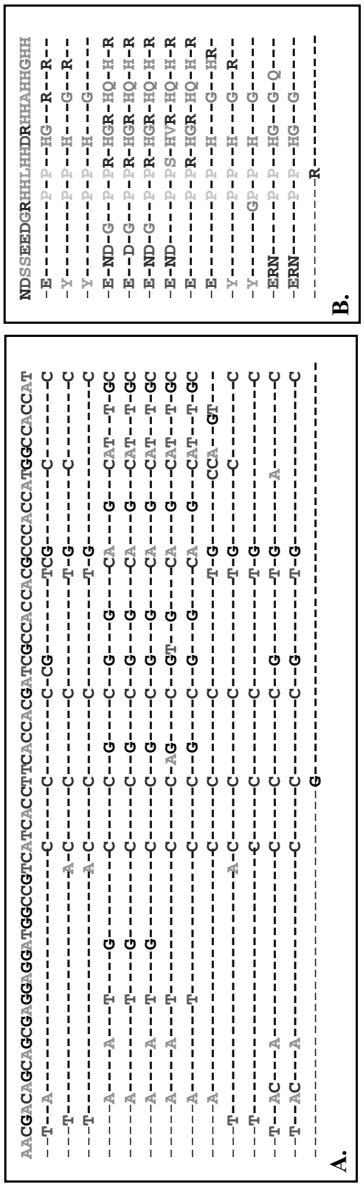

Figure 14.8. Sequence variation in *185/333* cDNAs. **(A)** Sequences from exon 7 are shown to illustrate the variability in nucleotide sequences among fourteen cDNAs. **(B)** The same region is shown translated into protein sequence. Variations in nucleotide sequence are reflected in variations in amino acid sequences.

The deduced 185/333 proteins were composed of an N-terminal glycine-rich region and a C-terminal histidine-rich region. All have a leader, a variety of repeats, acidic regions, histidine patches, and N-linked glycosylation sites only in the histidine-rich region, and most have an RGD motif. Proteins range in size from 26 kd to 55 kd, depending on splicing; are composed mostly of random coils, and do not contain cysteines.

The second unexpected result from the analysis of the *185/333* cDNAs was a significant level of nucleotide variations among the cDNA sequences. Part of exon 7 is shown and illustrates the nucleotide variation for fourteen sequences (Fig. 14.8A). This variation is reflected in the diversity of the corresponding amino acid sequences (Fig. 14.8B). The nucleotide variability was not random, was located throughout the cDNAs at specific positions, and was not clustered at exon boundaries. Based on a combination of nucleotide sequence variability plus alternative splicing, we identified sixty-six unique cDNAs of eighty-one full-length sequences. Estimates of the number of genes in the genome is presently unknown; however, preliminary analysis of the ESTs suggests that there may be hundreds. Overall, the *185/333* set of cDNAs from LPS-activated coelomocytes show an unexpectedly high diversity, perhaps because of the expression of many genes, a signficant amount of alternative splicing, and nucleotide variations within the same exon. If these transcripts are expressed, it is expected that they would generate significant diversity of a set of similar proteins. Based on the expression patterns in response to challenge from injected bacteria or LPS (Rast et al. 2000; Nair et al., unpublished), we hypothesize that the *185/333* genes encode antimicrobial proteins that are important in host defense.

14.7. CONCLUSIONS

Outside of insects, including *Drosophila* and mosquitoes, whose immune systems have received significant attention, little is known of the relevant genes, proteins, and immune mechanisms that function in most of the other invertebrates. Of interest is whether invertebrates, as a broad group, harbor resident gut-associated microbes and if there are residents present on the skin. Of course, the answer to this question may depend on the species under investigation. For example, Caribbean gorgonians have a resident microbial community found in association with the mucus covering on their surfaces (K. Kim, personal communication). In contrast, sea urchins may not (Unkles 1977). At present, little is known about this question and few investigators are pursuing it.

The rate of progress for understanding tidbits of invertebrate immunology has been slow. However, an exception is expected for investigations of the immune system of *Caenorhabditis elegans*. With the availability of a sequenced and assembled genome, a complete characterization of the developmental program, and multitudes of powerful genomic, proteomic, cellular, and molecular technologies that can be applied to this species, studies of the immune system is under way, and is expected to yield interesting and important information on how this primitive innate system functions (Millet and Ewbank 2004). Overall, the application of modern approaches to questions of immune responses to pathogens and interactions with bacterial assemblages that share habitats with invertebrates is expected to yield speedy advances in the future.

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