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Review

Antiviral immunity in marine molluscs

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Marine molluscs, like all living organisms, are constantly exposed to viruses and have evolved efficient antiviral defences. We review here recent developments in molluscan antiviral immunity against viruses belonging to the order *Herpesvirales*. Emerging results suggest an interferon-like response and autophagy are involved in the antiviral defence of bivalves to viral infection. Multifunctional plasma proteins from gastropods and bivalves have been identified to have broad-spectrum antiviral activity against mammalian viruses. The antiviral defences present in molluscs can be enhanced by genetic selection, as shown by the presence of oyster strains specifically resistant to ostreid herpesvirus type 1. Whether varying amounts or different isoforms of these antiviral plasma proteins contributes to genetic resistance is worthy of further research. Other evolutionarily conserved antiviral mechanisms, such as RNA interference and apoptosis, still need further characterization.

Introduction

Molluscs do not encode a classical acquired immune system (Bachère *et al.*, 1995; Loker *et al.*, 2004) yet thrive in the ocean, which is rich in viruses (Suttle, 2007). Molluscs occupy a wide variety of ecological niches in the ocean and some species are colonial, filter-feeders and can live for up to 400 years (Philipp & Abele, 2010). Molluscs could not be ecologically successful without effective innate responses to protect themselves from fast-evolving pathogens, such as viruses (Loker *et al.*, 2004).

Viruses infecting marine molluscs have been interpreted as members of the families *Herpesviridae*, *Iridoviridae*, *Papovaviridae*, *Togaviridae*, *Reoviridae*, *Birnaviridae* and *Picornaviridae* (reviewed by Renault & Novoa, 2004). Some of these molluscan viruses threaten the commercial viability of aquaculture enterprises (Segarra *et al.*, 2014a) and others have had a detrimental impact on wild fisheries (Crane *et al.*, 2013). Very few studies have investigated the antiviral responses of molluscs (reviewed by Loker *et al.*, 2004). Routine techniques used in virology and immunobiology are complicated by the absence of continuous cell lines for marine molluscs (Yoshino *et al.*, 2013) and the fact that marine viruses cannot be propagated in the freshwater pond snail (*Biomphalaria glabrata*) embryonic cell line (Bge) or in molluscan primary cell cultures (further details in Garcia *et al.*, 2011). Therefore, knowledge gaps remain regarding the antiviral responses of molluscs. Previously, researchers had a tendency to fill knowledge gaps in molluscan antiviral immunity by using what is known about other invertebrate phyla, such as arthropods (*Drosophila* and *Penaeus*). Emerging research now suggests that the antiviral response of molluscs is different to model invertebrate species (Loker *et al.*, 2004).

Here, we review the antiviral responses of molluscs against viruses belonging to *Herpesvirales*. We focus on the *Herpesvirales* order of viruses because few other studies exist regarding mollusc responses to other diseases of potential viral aetiology (Martín-Gómez *et al.*, 2014a, b). We therefore start by describing viruses belonging to the family *Malacoherpesviridae* within the order *Herpesvirales*. We discuss recent studies conducted on bivalves and gastropods and highlight similarities in their antiviral responses against viruses. The picture that emerges from these studies is that an interferon-like response and autophagy appear to be important antiviral responses of molluscs, but other evolutionarily conserved or novel antiviral responses for inhibiting viral infection and replication should not be overlooked.

Herpesvirus infections of marine molluscs

Viruses belonging to the family *Malacoherpesviridae* from the order *Herpesvirales* are known to cause disease in marine molluscs (Davison *et al.*, 2005, 2009; Savin *et al.*, 2010). The intentional translocation of marine molluscs around the world for aquaculture is considered to be the main reason for the geographical expansion of the family *Malacoherpesviridae* (Breener *et al.*, 2014; Mineur *et al.*, 2015). Marine molluscs farmed outside their natural

distribution range are also naïve to viruses endemic to that region. Not surprisingly, viruses belonging to the *Malacoherpesviridae* have caused disastrous economic consequences for farmed and wild fisheries (Hooper *et al.*, 2007; Mineur *et al.*, 2015; Segarra *et al.*, 2010). Up to now, the family *Malacoherpesviridae* incorporates two groups of viruses that are described in further detail below.

Ostreid herpesvirus 1 (OsHV-1)

OsHV-1 belongs to the genus *Ostreavirus* from the family *Malacoherpesviridae* (Davison *et al.*, 2009). The first description of herpes-like virus associated with mollusc mortality was reported in 1972 in the Eastern oyster (*Crassostrea virginica*) from the east coast of the USA (Farley *et al.*, 1972). Since this time, herpes-like viruses have been described in other species of oyster (Burge *et al.*, 2006; Hine *et al.*, 1992; Renault *et al.*, 1994), scallops (Arzul *et al.*, 2001a; Ren *et al.*, 2013) and clams (Renault *et al.*, 2001). The virus has been purified from naturally infected *Crassostrea gigas* larvae (Le Deuff & Renault, 1999) and its genome entirely sequenced (Davison *et al.*, 2005). Several genotypes of OsHV-1 have been detected by conventional PCR, targeting a specific area of the genome (Arzul *et al.*, 2001b; Martenot *et al.*, 2011; Segarra *et al.*, 2010). The reference (OsHV-1 ref) and variant (OsHV-1 var.) genotypes were associated with sporadic mortality events of *Crassostrea gigas* larvae and spat (oysters less than 1 year old) (Garcia *et al.*, 2011). From 2008, *Crassostrea gigas* mortalities greatly increased on the French coast and spread to other European countries (Renault *et al.*, 2012). These high mortalities were linked to the emergence of a newly described OsHV-1 genotype labelled μVar (Segarra *et al.*, 2010). In addition, mortality events of *Crassostrea gigas* were reported in 2010 from New Zealand and Australia in association with a virus closely related to μVar (Jenkins *et al.*, 2013; Keeling *et al.*, 2014). Numerous genomic and proteomic studies investigating the host–pathogen interactions of *Crassostrea gigas* and OsHV-1 have been undertaken (Corporeau *et al.*, 2014; Du *et al.*, 2013; Fleury & Huvet, 2012; Green & Montagnani, 2013; Jouaux *et al.*, 2013; Normand *et al.*, 2014; Renault *et al.*, 2011; Segarra *et al.*, 2014a, b, c; Tamayo *et al.*, 2014).

Acute viral necrosis virus (AVNV) is the causative agent of a serious disease of Chinese scallops, *Chlamys farreri* (Ren *et al.*, 2013). The complete genome sequence of AVNV indicates it's a variant of OsHV-1 (Ren *et al.*, 2013). Since the 1980s, AVNV has caused disease events in summer of farmed *Chlamys farreri* in China, with mortality reaching >90 % within 5–8 days after first appearance (Fu *et al.*, 2005). Studies investigating the physiological and immunological responses of *Chlamys farreri* infected with AVNV have been undertaken (Chen *et al.*, 2011, 2013, 2014; Tang *et al.*, 2010; Xing *et al.*, 2008).

Abalone herpesvirus (AbHV)

The first description of AbHV, associated with high mortality of abalone, was reported in 2005 in farmed abalone *Haliotis diversicolor supertexta* from Taiwan (Chang *et al.*, 2005). Histopathology of moribund abalone indicated the nervous system was the target tissue and electron microscopic examination demonstrated herpes-like viral particles within the degenerated cerebral ganglion cells (Chang *et al.*, 2005). AbHV was likely to occur outside of Taiwan with pre-existing reports of amyotrophia and mortality of Japanese black abalone (*Haliotis discus discus*) associated with a virus-like particle (Nakatsugawa *et al.*, 1999; Otsu & Sasaki, 1997). In late 2005 there was the emergence of AbHV in farmed and wild abalone populations in Victoria, Australia (Hooper *et al.*, 2007). This outbreak of AbHV was linked to the collection of abalone to be used as broodstock and the translocation of abalone between farms to exchange genetics for breeding programmes or for production purposes (Hooper *et al.*, 2007). Purification of herpesvirus-like particles and partial genome sequencing confirmed AbHV forms part of an ancient clade with its nearest relatives being herpesvirus belonging to OsHV-1 (Savin *et al.*, 2010). A single study investigating the immunological response of hybrid abalone (*Haliotis laevigata* × *Haliotis rubra*) to AbHV has been undertaken (Dang *et al.*, 2013).

Innate antiviral responses of marine molluscs

Antiviral immunity in molluscs is poorly understood compared with that of other invertebrate phyla, with only a few studies conducted in bivalves (mostly focused on *Crassostrea gigas* and *Chlamys farreri*), limited work in gastropods (*Haliotis* spp.) and no studies of cephalopods. Studies investigating the antiviral response of molluscs have mainly focused on identifying antiviral compounds, measuring immune enzyme activity or characterizing the transcriptional response to OsHV-1 infection (Chen *et al.*, 2013; Dang *et al.*, 2011, 2013; Fleury & Huvet, 2012; Green & Montagnani, 2013; Green *et al.*, 2014c; Jouaux *et al.*, 2013; Moreau *et al.*, 2015; Normand *et al.*, 2014; Olicard *et al.*, 2005a, b; Renault *et al.*, 2011; Rosani *et al.*, 2014; Segarra *et al.*, 2014a, c). These studies suggest that the molluscan antiviral response has similarities to the vertebrate interferon pathway (Green & Montagnani, 2013). However, this interpretation may be biased by the reliance on transcriptional studies to characterize antiviral responses. Bioinformatic analysis of the oyster genome reveals molluscs also have the potential to control viral infections using RNA interference (RNAi) and programmed cell death (PCD) responses (Fig. 1). We therefore make comparisons with other phyla to highlight potential knowledge gaps in our understanding of mollusc antiviral immunity.

Interferon-like response

The interferon system is crucial for resistance of mammals and other vertebrates to viral infection (Randall & Goodbourn, 2008). Vertebrate cells produce interferons upon recognition of virus-derived nucleic acids, such as dsRNA (Randall & Goodbourn, 2008). Interferons are secreted

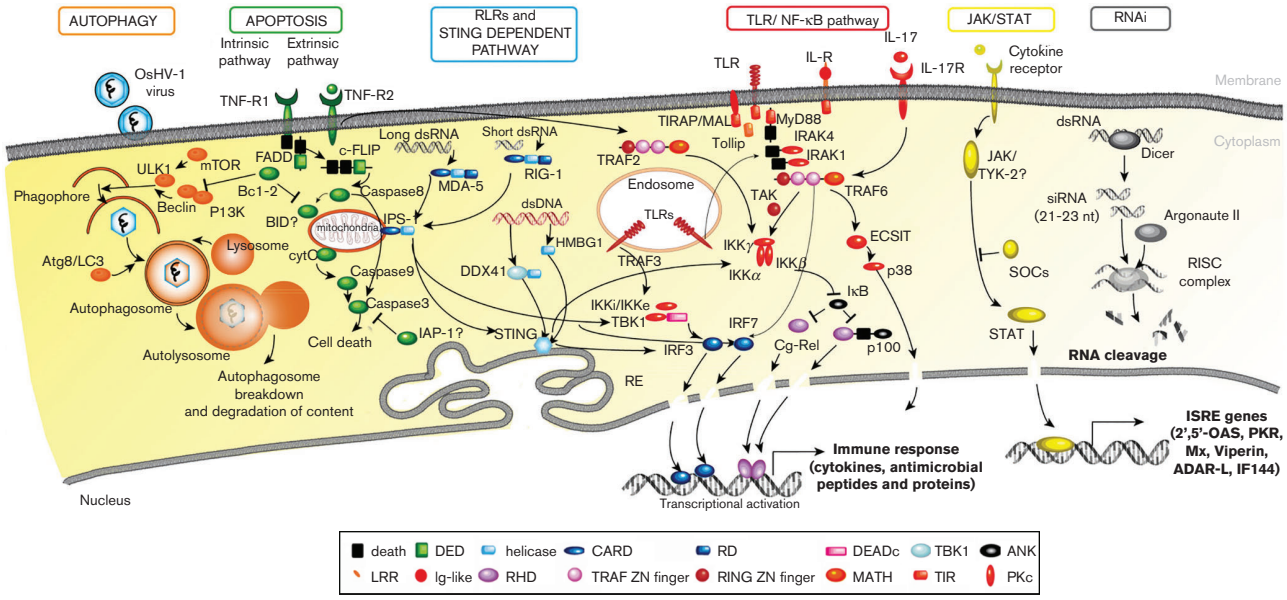


Fig. 1. Conserved antiviral signalling pathways in the oyster. This conceptual diagram was built from the identification of conserved sequences identified in the genome of *Crassostrea gigas* (Zhang *et al.*, 2012). Most of the inferences between the components of these pathways were inferred from vertebrate and insect literature and remain to be elucidated in molluscs. Characteristic domains of the proteins are indicated: TIR, Toll/IL1 receptor; Ig, immunoglobulin; DD, death domain; LRR, leucine rich repeat; TM, transmembrane; PKc, protein kinase; RHD, rel homology domain; IPT, immunoglobulin-like fold, plexins, transcription factors; RING, RING-finger (really interesting new gene) domain; zf-TRAF, TRAF type zinc finger; MAT, meprin and TRAF-C homology domain; UBL-TBK1-like, ubiquitin-like domain of human Tbk1 and similar proteins.

proteins that induce an antiviral state by binding to distinct receptors present on all nucleated cells (Biron & Sen, 2001). Receptor engagement activates signal transduction via the Jak-STAT pathway, leading to the transcription of hundreds of interferon stimulated genes (ISGs) (Robertsen, 2006; 2008). The products of these ISGs exert numerous antiviral effector functions (Schoggins & Rice, 2011). The interferon response has traditionally been thought to be a vertebrate innovation because the genomes of model invertebrates (i.e. *Drosophila* and mosquitoes) do not encode interferon or its major effectors (Loker *et al.*, 2004; Robalino *et al.*, 2004). Subsequently, it has been demonstrated that arthropods have a transcriptional response to viral infection that signals through a secreted peptide, vago, that induces the expression of antiviral effectors via the Jak-STAT pathway (Deddouche *et al.*, 2008; Paradkar *et al.*, 2012, 2014). Several important contrasts exist between the vertebrate interferon response and the arthropod transcriptional response to viral infection. *Drosophila* and mosquitoes cannot distinguish non-specific nucleic acids to activate vago expression, but instead rely on a component of the RNAi-mediated antiviral response, dicer-2, to recognize replicating viruses and activate vago via a signalling pathway involving TRAF and Rel2 (Deddouche *et al.*, 2008; Paradkar *et al.*, 2014). Secondly, antiviral effectors that are induced by vago (i.e. CG12780, vir1 and CG9080) have no resemblance to vertebrate ISGs (Dostert *et al.*, 2005).

Although no interferon–cytokine homologue has yet been identified in molluscs, oysters (*Crassostrea gigas*) have a transcriptional response to viral infection (OsHV-1) that closely resembles the vertebrate interferon response. Firstly, the immune system of *Crassostrea gigas* can recognize non-specific nucleic acids (i.e. poly I : C) to induce an antiviral response that inhibits subsequent infection with OsHV-1 (Green & Montagnani, 2013). This response was antiviral because inducing an anti-pathogen response by injecting *Crassostrea gigas* with heat-killed *Vibrio* bacteria provided no protection against subsequent OsHV-1 infection (Green & Montagnani, 2013). Several evolutionary conserved nucleic acid sensors and their downstream signalling molecules, including Toll-like receptors (TLRs), RIG-like receptors (RLRs), interferon regulatory factors (IRFs) and stimulator of interferon (STING), have been identified in the *Crassostrea gigas* genome (Figs 2 and 3, Table 1). Secondly, the *Crassostrea gigas* genome encodes many classic ISGs (Table 1), such as 2′-5-oligoadenylate synthetase (OAS) (Fig. 4, GenBank accession nos EKC21335 & EKC26578), Mx protein (GenBank no. KC28205), viperin (GenBank no. EKC28205) and ADAR-L (GenBank no. EKC20855) (Zhang *et al.*, 2012). Finally, OsHV-1 infection of *C. gigas* coincides with elevated mRNA levels of many genes involved in the interferon response, such as virus recognition receptors (i.e. TLRs and RLRs), signal transducers (i.e. MyD88, STING, SOC), transcription factors (IRFs, NF-κB) and antiviral effectors (i.e. viperin, IFI44,

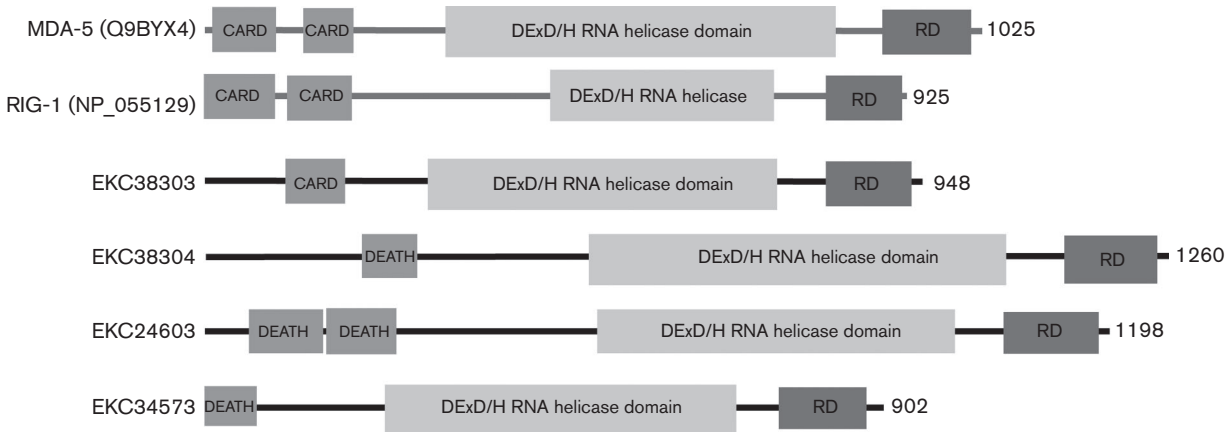


Fig. 2. Schematic representation of the primary structural and functional domains of RIG-like receptors (RLRs) from human (MDA-5 and RIG-1) and oyster (EKC38303, EKC38304, EKC24603 and EKC34573). CARD, Caspase activating recruitment domain; DEATH, death domain superfamily, which contains the death domain, pyrin, CARD and death effector domain families; RD, regulatory domain.

ADAR-L, TRIM, etc.) (see Table 1 and references within). Many of these genes are also induced in the gill, mantle and hemocyte tissue of *Crassostrea gigas* injected with poly I : C (Green *et al.*, 2014a, b; Green & Montagnani, 2013). Collectively, these studies have demonstrated the oyster can recognize virus-associated molecular patterns to induce a systemic transcriptional response that is capable of controlling OsHV-1 infection and replication. Future research should attempt to identify an oyster interferon cytokine because there is evidence that vertebrate interferons can elicit an antiviral response in pearl oysters (*Pinctada fucata*) (Miyazaki *et al.*, 2000) and can phosphorylate STAT-like proteins in the mussel, *Mytilus galloprovincialis* (Canesi *et al.*, 2003).

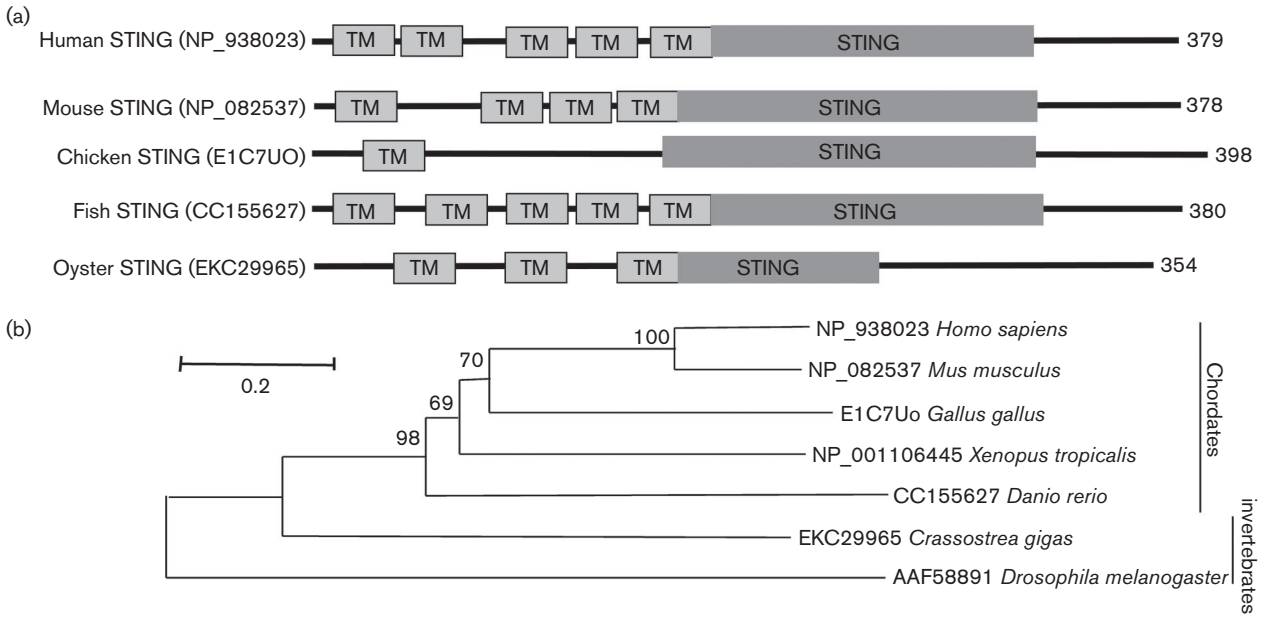


Fig. 3. Bioinformatic analysis of stimulator of interferon (STING). (a) Schematic representation of the primary structural and functional domains of STING from human (NP_938023), mouse (NP_082537), chicken (E1C7U0), fish (CCl55627) and oyster (EKC29965). (b) Unrooted phylogenetic tree of vertebrate and invertebrate STING amino acid sequences. The tree was reconstructed using the neighbour-joining algorithm in the MEGA v5.1 program (Tamura *et al.*, 2011). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances that were used to infer the phylogenetic tree (scale bar, 0.2 amino acid substitutions per site). Bootstrap values (shown at nodes) are based on 1000 resamplings of the data.

Table 1. Key antiviral genes identified in the *Crassostrea gigas* genome involved in virus recognition receptors, antiviral signalling molecules, antiviral effectors and key components in PCD and RNAi

The percentage amino acid identity and E-value of these *Crassostrea gigas* genes to antiviral genes from *Homo sapiens* is provided. References refer to previous studies that have shown these *Crassostrea gigas* genes are differentially expressed in response to OsHV-1 infection.

Gene	*C. gigas* GenBank no.	*H. sapiens* GenBank no.	E-value	aa identity (%)	OsHV-1 refs
Virus recognition					
TLR-3	EKC35956	NP_003256	$3.00e^{-46}$	26	Fleury & Huvet (2012)
RLH	EKC38303	O95786	$3.00e^{-108}$	34	He *et al.* (2015)
RLH	EKC38304	O95786	$7.00e^{-69}$	35	He *et al.* (2015)
HMGB	EKC40290	AAI41845	$4.00e^{-53}$	54	
cGAS	EKC29902	NP_612450	$1.00e^{-18}$	29	
Antiviral signalling					
IRF-8	EKC26205	NP_002154	$1.00e^{-47}$	31	Rosani *et al.* (2014)
IRF-2	EKC43155	NP_002190	$5.00e^{-32}$	50	Green & Montagnani (2013)
STING	EKC29965	NP_938023	$2.00e^{-40}$	31	
SOC-1	EKC24772	NP_003868	$3.00e^{-31}$	41	Rosani *et al.* (2014)
JAK	EKC41693	NP_004422	$3.00e^{-47}$	37	He *et al.* (2015)
STAT	EKC39332	NP_001171551	$2.00e^{-10}$	36	He *et al.* (2015)
Caveolin-1	EKC31086	NP_001744	$2.00e^{-29}$	40	He *et al.* (2015)
Antiviral effectors					
OAS	EKC21335	BAB18647	$1.00e^{-37}$	29	
OAS	EKC26578	BAB18647	$1.00e^{-19}$	34	
Mx	EKC33820	NP_001138397	$1.00e^{-54}$	33	
Viperin	EKC28205	AAL50053	$3.00e^{-163}$	63	Rosani *et al.* (2014)
ADAR-L	EKC20855	NP_056655	$1.00e^{-145}$	47	Rosani *et al.* (2014)
IFI44	FJ440108	NP_006811	$5.00e^{-39}$	47	Renault *et al.* (2011)
RNAi					
Dicer-2	EKC26346	NP_085124	0	43	He *et al.* (2015)
TRBP	XP_011456094	NP_004169	$3.00e^{-49}$	36	
AGO-2	EKC19600	NP_036286	0	73	
AGO-2	EKC35067	NP_036286	0	64	
Apoptosis					
TNF	EKC35160	NP_003801	$5.00e^{-13}$	32	He *et al.* (2015)
TNF	EKC39243	NP_003801	$2.00e^{-14}$	27	He *et al.* (2015)
TNF	ADX31292	NP_003801	$1.00e^{-13}$	23	He *et al.* (2015)
TNFR1	EKC38398	NP_001241	$5.00e^{-15}$	31	
SODD	EKC42633	NP_004865	$2.00e^{-15}$	39	
ADAMS-17	EKC21816	AAB51514	$1.00e^{-78}$	29	
Autophagy					
Beclin	EKC28450	NP_003757	0	62	Moreau *et al.* (2015)
P13K	EKC39750	NP_002636	0	45	
Akt	EKC33169	AAH20479	$1.00e^{-115}$	45	
mTOR	EKC29347	NP_004949	0	63	
ATG1 (ULK1)	EKC18065	NP_055498	$4.00e^{-117}$	47	Moreau *et al.* (2015)
ATG8 (LC3)	EKC40439	NP_115903	$2.00e^{-61}$	75	Moreau *et al.* (2015)
ATG18 (WIPI1)	EKC39143	NP_057087	0	69	

RNA interference (RNAi)

dsRNA is an important regulator of gene expression in animals (Meister & Tuschl, 2004; Randall & Goodbourn, 2008). It can induce a transcriptional response (i.e. interferon-pathway) and it can also regulate different types of post-transcriptional gene processes that are collectively referred to as RNAi (Meister & Tuschl, 2004). RNAi is highly evolutionarily conserved process triggered by dsRNA precursors that vary in length and origin (Jeang, 2012; Kemp & Imler, 2009; Wang *et al.*, 2010). According to their origin or function, three types of naturally occurring small RNA have been characterized: (i) short interfering RNAs (siRNAs) are generated from dsRNA either derived from exogenous sources such as viruses or encoded by the cell genome, (ii) microRNAs (miRNAs) are generated from cell-encoded transcripts and ultimately function to regulate gene expression at the level of translation, and (iii) PIWI-interacting RNAs (piRNAs) are cell-encoded

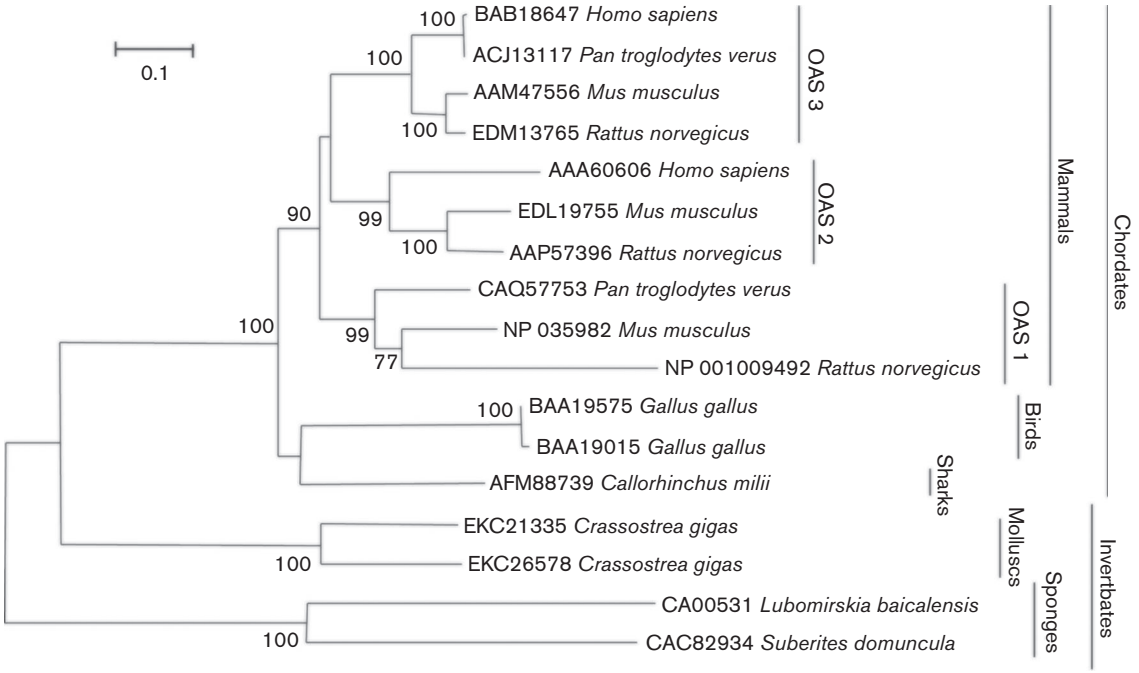


Fig. 4. Unrooted phylogenetic tree based on representative vertebrate and invertebrate 2′5′-oligoadenylate synthetase (OAS) amino acid sequences. Biological activity for invertebrate OAS has been demonstrated (Kuusksalu *et al.*, 1998). See Fig. 3 for details on reconstruction of phylogenetic tree. Scale bar indicates an evolutionary distance of 0.1 amino acids per position in the sequence. The statistical significance of interior nodes was determine by performing bootstrap analysis based on 1000 resamplings of the data.

and do not require processing to function in the epigenetic control of genomic elements in the germ-line (reviewed by Kingsolver *et al.*, 2013). We focus on reviewing what is known about the siRNA and miRNA pathways in molluscs because these two pathways are well characterized in the antiviral response of arthropods and mammals. The siRNA-pathway is recognized as an important defence mechanism in arthropods against RNA and DNA viruses (Blair, 2011; Kemp & Imler, 2009; Kemp *et al.*, 2013), whereas, the rarity of siRNAi-pathways in the antiviral immunity of vertebrate cells against herpesviruses is now thought to be because this group of vertebrate viruses have been tightly selected not to maintain siRNA producing sequences (Jeang, 2012). The miRNA-pathway is involved in regulating many cellular processes, including innate immune responses of mammalian cells (Pauley & Chan, 2008). The miRNA-pathway mediates host gene expression by blocking translation through incomplete binding with 3′-UTR of a target gene or by directing degradation of a target mRNA (Chen *et al.*, 2014). Analysis of small expression profiles of virus infected mammalian cells has also revealed several herpesviruses express viral-miRNAs to inhibit the host's antiviral innate immune response (Cullen, 2011). The role of miRNAs in viral pathogenesis of arthropod cells is not clear (Kingsolver *et al.*, 2013).

The RNAi system is clearly functional in molluscs, with several researchers utilizing RNAi to investigate gene expression on defence proteins, gonad maturation and shell formation (reviewed by Owens & Malham, 2015). However, the importance of the siRNA- and miRNA-pathways in the antiviral defence of mollusc cells is unknown. Differences exist between the number of RNAi-related enzymes encoded by genomes of molluscs and other animals. The oyster genome encodes a single orthologue of both dicer and the RISC loading complex subunit (TRBP), but only two argonaute proteins (Table 1). Insects have two dicer proteins; dicer-1 is required for the production of miRNA, whereas dicer-2 is required for processing dsRNA to generate siRNA (Kingsolver *et al.*, 2013). In contrast, mammalian cells have only one dicer enzyme that is utilized by both the siRNA and miRNA pathways (de Jong *et al.*, 2009) but they have four argonaute proteins (AGO1–4), with only AGO2 known to function in the siRNA and miRNA pathways (Pauley & Chan, 2008). The only study covering an aspect of molluscan RNAi-mediated antiviral immunity was recently performed by Chen *et al.* (2014), where *Chlamys farreri* miRNAs and miRNA expression profiles in response to AVNV infection was analysed by deep sequencing technology. Sequence homology searches of *Chlamys farreri* miRNAs against the *Chlamys farreri* expressed sequence tag (EST) database revealed scallop miRNAs may target immune- and stress-related genes for post-transcriptional regulation (Chen *et al.*, 2014). Further research is now required to determine if these scallop miRNAs suppress translation of immune- and stress- related

genes. In their study, Chen *et al.* (2014) did not state whether they observed AVNV-derived siRNAs (vsiRNAs) in their dataset. This is despite the fact that the entire AVNV genome is sequenced (Ren *et al.*, 2013) and may have provided evidence that the RNAi-mediated antiviral response is functional in molluscs.

Programmed cell death (PCD)

Apoptosis and autophagy are forms of PCD. PCD is a fundamental cellular response to prevent viral replication and protein synthesis in virus-infected cells (Quinlan, 1999). The processes of apoptosis and autophagy are extensively described for mammalian and arthropod cells (Degterev & Yuan, 2008; Lamiable & Imler, 2014). Genes encoding the apoptotic machinery are highly conserved in molluscs (Zhang *et al.*, 2011), but the role of the apoptosis system in molluscan immunity has received little attention (Zhang *et al.*, 2011). The large number of apoptosis inhibitors encoded in the genomes of OsHV-1, AVNV and AbHV (Davison *et al.*, 2005; Ren *et al.*, 2013; Savin *et al.*, 2010) implies apoptosis may represent an important antiviral response of molluscs and warrants further investigation.

Autophagy is another highly evolutionally conserved process of PCD (He & Klionsky, 2009). Autophagy has a role in cellular homeostasis and is also an innate immune mechanism that can selectively target intracellular pathogens and cytosolic proteins for enzymatic degradation (He & Klionsky, 2009; Richetta & Faure, 2013; Sumpter & Levine, 2010). Protein kinase R (PKR) is a vertebrate interferon stimulated gene that can trigger autophagy upon dsRNA binding through a pathway involving eIF2α (reviewed by Richetta & Faure, 2013). Vertebrate cells can utilize this PKR and eIF2α-dependent autophagy pathway to degrade both herpes simplex virus type 1 (HSV-1) virions and HSV-1 proteins (Tallóczy *et al.*, 2006). The importance of autophagy is cell-type dependent, with neuron cells, but not mitotic cells, relying on autophagy in HSV-1 defence (Yordy & Iwasaki, 2013). An intracellular DNA sensor must also be able to provoke autophagy in response to herpesvirus infection (McFarlane *et al.*, 2011). Human cytomegalovirus (HCMV) and HSV-1 induced autophagy in human fibroblasts and this response was retained when cells were infected with UV-irradiated HCMV (McFarlane *et al.*, 2011). Other pathogen sensors (such as TLR3, TLR4, TLR7, TLR8 and NOD2) are also known to induce autophagy upon binding with their specific ligand, via a MyD88 independent pathway (reviewed by Richetta & Faure, 2013). Autophagy is also an antiviral mechanism utilized by arthropods (Nakamoto *et al.*, 2012). In *Drosophila*, Toll-7 recognizes vesicular stomatitis virus (VSV) at the plasma membrane and induces antiviral autophagy via an NF-κB-independent pathway (Nakamoto *et al.*, 2012).

Autophagy appears to be an important antiviral response of oysters (Moreau *et al.*, 2015). OsHV-1 induces autophagy in mantle tissue of *Crassostrea gigas* and survival assays using a known inhibitor (NH_4Cl) of autophagy demonstrated this antiviral response had a protective role in *Crassostrea gigas* against OsHV-1 (Moreau *et al.*, 2015). Furthermore, microarray-based gene expression studies have observed elevated expression of genes associated with autophagy in *Crassostrea gigas* undergoing a mass mortality event in California, USA (Moreau *et al.*, 2015). Chaney & Gracey (2011) did not investigate the cause of mortality, but OsHV-1 is often associated with oyster mortality in California (Burge *et al.*, 2006). Future research should identify which OsHV-1 ligand induces autophagy and whether autophagy targets the OsHV-1 virion or an essential OsHV-1 protein.

Antiviral compounds

The occurrence and function of antimicrobial peptides (AMPs) in marine bivalves has been well studied in relation to anti-bacterial and anti-fungal immunity (reviewed by Bachère *et al.*, 2015; Schmitt *et al.*, 2010). Less is known regarding the activity of AMPs against molluscan viruses. Investigations into the antiviral activity of molluscan AMPs and tissue homogenates is hampered by the lack of continuous cell lines from marine molluscs (Yoshino *et al.*, 2013) and the fact that OsHV-1 cannot be cultured in primary cell cultures from bivalves (details in Garcia *et al.*, 2011). Numerous studies have therefore utilized a heterologous model involving HSV-1 and African green monkey kidney (Vero) cells to identify antiviral compounds in tissue homogenates from commercially important bivalves (Carriel-Gomes *et al.*, 2006; Defer *et al.*, 2009; Green *et al.*, 2014c; Olicard *et al.*, 2005a, b; Segarra *et al.*, 2014a; Zeng *et al.*, 2008) and gastropods (Dang *et al.*, 2011; Zanjani *et al.*, 2014).

Haemocyanins and haemocyanin-derived peptides from marine and terrestrial gastropods have potent anti-herpesvirus activity (Nesterova *et al.*, 2011; Zagorodnya *et al.*, 2011; Zanjani *et al.*, 2014). The primary function of haemocyanins is the transport of molecular oxygen to respiring tissues (Coates & Nairn, 2014; Zanjani *et al.*, 2014), but their contribution to innate immunity is often overlooked (Coates & Nairn, 2014). Haemocyanins are copper containing glycoproteins and a major haemolymph component, approximately 50 % to >90 %, of some mollusc species (Coates & Nairn, 2014). Abalone haemocyanin inhibits HSV-1 infection of Vero cells, presumably by blocking viral entry (Dang *et al.*, 2011; Zanjani *et al.*, 2014). The anti-HSV-1 activity of abalone plasma does not increase above baseline levels in response to experimental infection with AbHV (Dang *et al.*, 2013). However, the anti-HSV-1 activity of abalone plasma appears to be influenced by temperature with higher anti-HSV-1 activity occurring in summer than in winter (Dang *et al.*, 2012).

The genomes of pteriomorph bivalve genera, such as *Crassostrea*, *Mytilus* and *Argopecten*, do not encode a haemocyanin gene (Lieb & Todt, 2008). Yet, their plasma also has anti-HSV-1 activity (Carriel-Gomes *et al.*, 2006; Defer *et al.*, 2009; Olicard *et al.*, 2005b). Anti-HSV-1 activity of *Crassostrea gigas* haemolymph corresponds to a copper containing glycoprotein, termed cavortin (GenBank no.

AY551094) that exerts its antiviral activity by interfering with virus replication (Green *et al.*, 2014c). Cavortin is the major plasma protein in the oyster and the protein has an extracellular superoxide dismutase domain (Gonzalez *et al.*, 2005; Itoh *et al.*, 2011). The anti-HSV-1 activity of oyster haemolymph from juveniles and adults is similar (Green *et al.*, 2014b) and varies throughout the year, with adult oysters having higher activity during the summer/autumn period compared with winter (Olicard *et al.*, 2005a). The anti-HSV-1 activity of oyster hemolymph could not induced by injecting oysters with poly I : C (Green *et al.*, 2014b).

Genetics and physiology

Genetics, host physiology and the environment are all important determinants of mollusc survival to viral infection. Survival of *Crassostrea gigas* to OsHV-1 is positively correlated with oyster age and size (Dégremont, 2013; Paul-Pont *et al.*, 2014; Peeler *et al.*, 2012; Pernet *et al.*, 2012). Water temperature is also a factor in the expression of disease caused by OsHV-1 infection. The water temperatures must exceed 16 °C for OsHV-1 to cause mortality of juvenile *Crassostrea gigas* (Petton *et al.*, 2013) and mortality of *Chlamys farreri* to AVNV occurs in late summer (Fu *et al.*, 2005). It is unknown how host physiology and the environment influences disease expression of abalone to AbHV.

Oyster age and water temperature is also known to influence antiviral gene expression in *Crassostrea gigas* stimulated with poly I : C (Green *et al.*, 2014b). At 22 °C, juvenile oysters express antiviral genes earlier and to a higher magnitude compared with adult oysters. In contrast, water temperature of 12 °C delayed antiviral gene expression in adult oysters and inhibited the antiviral response of juvenile oysters (Green *et al.*, 2014b). Many of these antiviral genes are involved in processes that prevent cell transcription and translation and it should be determined whether the vigorous antiviral response of juvenile oysters at 22 °C is contributing to an immune-mediated disorder leading to higher mortality. Intervention studies on commercial oyster farms have demonstrated farm husbandry, such as raising the intertidal growing height, can reduce mortality of *Crassostrea gigas* by primarily reducing exposure risk to OsHV-1 (Paul-Pont *et al.*, 2013). These farm husbandry practices may also provide additional protection by reducing the available feeding time and thereby, limiting energy allocation to immunity.

The antiviral defences present in molluscs can be enhanced by genetic selection (Dégremont, 2011, 2013; Sauvage *et al.*, 2010). Segarra *et al.* (2014c) compared the susceptibility of bi-parental *C. gigas* families to OsHV-1 and confirmed that susceptibility to OsHV-1 infection had a significant genetic component. Viral DNA was detected earlier and the overall amount of viral DNA was higher for a low surviving oyster family compared with a high surviving family (Segarra *et al.*, 2014c). The high surviving family presumably controls OsHV-1 replication from exceeding the viral DNA threshold [8.8×10^3 copies (mg of tissue)$^{-1}$] for mortality to occur (Oden *et al.*, 2011). Similar observations are reported for abalone with a proportion of the population testing PCR-positive for AbHV (low amounts of viral DNA), but displaying no clinical signs of disease (Crane *et al.*, 2013; Dang *et al.*, 2013). Selective breeding programmes would benefit from identifying the genetic mechanism(s) utilized by resistant molluscs for maintaining viral loads below the mortality threshold. A good starting point would be the antiviral plasma proteins (haemocyanin and cavortin) that are known to interfere with mammalian virus replication (Dang *et al.*, 2011; Green *et al.*, 2014c; Olicard *et al.*, 2005b). Normand *et al.* (2014) concluded expression levels of superoxide dismutase metalloenzymes (i.e. cavortin) may partly determine resistance of *Crassostrea gigas* to OsHV-1 associated mortality. Plasma antiviral activity is a trait under genetic control in populations of *Crassostrea gigas* (Green *et al.*, 2014c) and abalone (unpublished data).

Conclusions

Compared with only a few years ago, remarkable progress has been made on characterizing the antiviral mechanisms in molluscs. Recent findings indicate that many features of the inducible antiviral response of molluscs are shared with the mammalian interferon pathway. Laboratory and field studies have also highlighted the importance of autophagy in the oyster's antiviral response. The contribution of other evolutionarily conserved antiviral mechanisms, such as RNAi and apoptosis, will no doubt be evaluated once appropriate methodologies and tools become available to study them. Of note, genetic selection of *Crassostrea gigas* to OsHV-1 has led to the development of susceptible and resistant family lines. In consequence, genetic screens between families with contrasted survival to OsHV-1 infection hold great promise in identifying the major antiviral pathways in molluscs.

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