**Introduction**

The fundamental role of lipids as structural molecules is well established; however, their role as signaling molecules is relatively new territory, opening up new avenues of scientific investigation into the basis of physiological processes. Ceramide is an important component of sphingolipids and has been implicated as a signaling molecule in cell proliferation and differentiation, inflammation, and programmed cell death, or apoptosis (Ballou et al. 1996, review; Hannun 1994, review). The ceramide synthesis and sphingolipid metabolism pathway has been characterized in mammals (Hannun 1994). It has become increasingly apparent that these recently unveiled roles of ceramide underlie some well-understood processes (inflammation) and others that have remained mostly elusive (apoptosis). The presence and role of ceramide in invertebrates remains to be characterized. Oysters express genes that are homologous to many of those found in the vertebrate ceramide pathway, but their function in specific physiological responses remains a mystery. In this study, we characterize some genes in the ceramide pathway in invertebrates through the model species *Crassostrea gigas* (the Pacific oyster). The wealth of genomic resources available for *C. gigas* and its ease of use in controlled laboratory experiments make it an ideal organism for this endeavor.

Ceramide synthesis can occur via a variety of different pathways and can affect a number of different physiological processes. Ceramide itself can be synthesized *de novo* (Hannun 1994) or through catabolism of sphingolipids by sphingomyelinases or ceramide synthases (Ballou et al. 1996). Agonists that stimulate sphingomyelin hydrolysis leading to an accumulation of intracellular ceramide include tumor necrosis factor α (TNFa), interleukin-1β, γ-interferon, vitamin D3, nerve growth factor, complement complexes, and others (Ballou et al. 1996). Many of these genes have already been proven to have roles in the immune response, leading to the proven conclusion that ceramide itself is instrumental in the stress and immune responses in vertebrates. Ceramide activates the NfkB pathway (Ballou et al. 1996) a key component of the immune response and a pathway that has been characterized in invertebrates. C2-ceramide also has antiproliferative effects on leukemia cells and enhances the secretion of interleukin-2 in lymphocytes (Hannun 1994).

 There are links between ceramide signaling and the stress response beyond innate immunity. In leukemia cells exposed to a variety of environmental stressors, all cells increased levels of ceramide and decreased amount of sphingomyelin (Verheij et al. 1996). In aquatic organisms, changes in intracellular ceramide in sea bass gills are associated with abrupt shifts in environmental salinity (El Babili et al. 1996). The exact role and mechanisms of ceramide in this response seems to be that it signals stress-induced apoptosis by way of regulation of sphingolipid metabolism (Verheij et al. 1996; Hannun & Luberto 2000). In fact, TNFa works through ceramide in both apoptosis and inflammation (Verheij et al. 1996). Ceramide’s roles are many and varied, but can be broadly categorized as instrumental in cellular response to stressors. Given the evidence that oysters (and most likely all invertebrates) have genes homologous to those known to be in the vertebrate ceramide pathways, it is likely that ceramide plays the same crucial roles in the invertebrate system.

 Oysters are ideal model organisms for many studies in genomics and transcriptomics due to the wealth of genomic resources available, the simplicity of their physiology, and their sensitivity to changes in the environment. A number of stress-response genes have been characterized for *C. gigas* (e.g. *hsp70*, Gourdon et al. 2000; *superoxide dismutase*, Gonzalez et al. 2005), and many more have been sequenced but not yet identified. Molecular analysis can be specific enough to pinpoint discrete physiological pathways that are affected by environmental contaminants (Svendsen et al. 2008). Concurrently, molecular tools allow insight into disturbances in fundamental biological processes such as reproduction, immune response, and growth. Through comparisons of gene expression patterns at different life stages and under different nutritional and environmental conditions, we will also be able to better predict environmental effects realized at the population level.

 The objectives of this study are to characterize four genes involved in the pathway of ceramide synthesis and catabolism: *serine palmitoyltransferase*, *3-ketodihydrosphingosine reductase*, *acid ceramidase*, and *ceramide glucosyltransferase*. Secondly, we investigate the role of the ceramide pathway in the invertebrate immune response through measuring the expression levels of these four genes after *C. gigas* was exposed to the bacterium *Vibrio vulnificus*.

**Methods**

*Animals, tissue collection, and bacterial challenges*

 Adult Pacific oysters (*C. gigas)* were acquired from Taylor Shellfish (Quilcene, WA). Tissue was dissected using sterile techniques from gill, mantle, muscle, and visceral mass and stored in RNAlater (Ambion, Carlsbad, CA) until analysis. *V. vulnificus* were grown from plated cultures in 400 mL O/N culture (1x LB, 1% NaCL) at 37C on a shaker set at 150 rpm. The culture was pelleted at 4300 rpm at 25C and the supernatant was removed and pelleted bacteria was resuspended in 50 mL of sea water. Sixteen adult oysters in 8 L of sea water were subjected to *Vibrio vulnificus* ( 4.56x1019 cfu/L ) via a 3 hour immersion bath. Control oysters (n=16) were likewise placed in 8L of sea water. Following exposure, gill tissue was dissected from 8 oysters from each treatment and immediately frozen on dry ice to be stored at -80C.

 RNA isolation was carried out using Tri-Reagent (Molecular Resources Center, Cincinnati, OH) as per manufacturer’s protocol. Following RNA isolation, samples were treated with Turbo DNAase (Ambion) according to manufacturer’s protocol in order remove any potential genomic DNA carry over.  All samples were evaluated to insure genomic DNA was absent by performing PCR on RNA samples alongside controls. Total RNA was stored at -80℃.

*Gene discovery and sequencing*

 In order to identify genes involved in ceramide metabolism in the Pacific oyster, publically available pyrosequencing data were mined for sequences based on sequence similarity. Roche 454 sequencing data from a 6-day old *C. gigas* larvae transcriptome library were downloaded from NCBI’s Short Read Archive (accession SRX032364). Reads were quality trimmed and de novo assembled using CLC Genomics Workbench v3.7. Using NCBI’s BLAST, these contigs were compared to published homologous proteins in the SwissProt database (http://expasy.org/sprot/). As the ESTs added more base pairs onto the acquired sequences, we continued to re-compare with homologous full-length sequences to make sure our contiguous sequences were full-length.

Primers were then designed to encompass the full-length coding sequence using the assembled ESTs. Primers for sequencing and quantitative PCR (qPCR) were designed using Primer3 in Geneious Pro v.4.5.8 (Table 1). This search returned a number of genes probably involved in the ceramide pathway that were not further characterized in this study. These genes are listed in Table 2 along with NCBI accession numbers corresponding top hit putative homologs. Full sequences assembled from available ESTs in GenBank were found for all four genes *sptlc1*, *3KDSR*, *AC,* and *GlcCer*.

Full length cDNA products were amplified and cloned to achieve the sequences of the four target genes in their entirety. Equal concentrations of cDNA from the control and *Vibrio*-exposed groups were pooled and amplified (n=8 in each pool). The PCRs were done in 25 uL reactions with 12.5 uL Apex TaqRED master mix (Genesee Scientific, San Diego, CA), 8.5 uL water, 0.5 uL of each 10 uM primer (forward and reverse, Invitrogen, San Diego, CA), and 3 uL cDNA template. The thermalcycler protocol was: 1. 95C for 10 minutes; 2. 40 cycles of 95C for 30 seconds, 55C for 30 seconds, and 72C for 30 seconds; 3. 72C for 10 minutes. PCR products were run on a 1.5% agarose gel and bands were excised and purified using Ultrafree ®-DA columns (Millipore, Billerica, MA).

 Cloning was accomplished using a TOPO TA cloning kit (Invitrogen) and following manufacturer’s protocol. LB plates with 50 mg/uL of Kanamyacin were used to grow the colonies. After about 24 hours, white colonies were restreaked on a new plate and left to incubate 72 hours at room temperature. After incubation, restreaked colonies were touched with a sterile toothpick, which was then dropped into 5 mL of 1xLB with 50 mg/mL Kanamyacin and left to grow overnight at 37C and shaking at 250 rpm. A Qiagen miniprep kit (Qiagen, Valencia, CA) was then used to isolate the plasmids, following the manufacturer’s protocol. Plasmid DNA was resuspended in 30 uL of EB buffer (Qiagen). Sequencing using both forward and reverse M13 primers (Invitrogen) was done on an ABI 3700. ???

 Sequences were trimmed and translated in Geneious Pro v. 4.8.5. Alignments were done in ClustalX v. 2.1. The Transmembrane Prediction Tool plugin (Suchard) in Geneious was used to predict transmembrane domains.

*Pathway phylogeny*

Translated protein sequences for the four genes were aligned with homologous sequences in other model organisms to determine the evolutionary relationship of these key enzymes.

 Using NCBI’s HomoloGene, protein sequences for the desired genes in *Homo sapiens*, *Mus musculus*, *Danio rerio*, *Xenopus tropicalis* and *Caenorhabditis elegans* were acquired where available. Sequences were aligned in ClustalX 2.1 and the alignment was exported to Geneious. Using the PhyML plugin in Geneious, maximum likelihood phylogenetic trees of the protein sequences were constructed based on the James-Taylor-Thornton (JTT) model and bootstrapped 100 times (Guindon & Gascuel 2003; Jones et al. 1992). PhyML builds phylogenetic trees based on a hill-climbing algorithm in which tree topology and branch lengths of a unique tree are modified to that the tree likelihood increases until an optimum is reached (Guindon & Gascuel 2003). The JTT model builds protein relationships based on a mutation data matrix, which clusters homologous sequences, tallies the observed mutations (ignoring gaps), and related the number of mutations to those expected by chance (Jones et al. 1992).

*qPCR amplification*

DNA-free RNA was reverse transcribed to complementary DNA (cDNA) using M-MLV reagents (Promega, Madison, WI). RNA concentrations were determined on a NanoDrop1000 (Thermo-Fisher, Waltham, MA). For reverse transcription, 2 ug of RNA were diluted in sterile nanopure water so that the total volume was 17.75 uL. Oligo dT primers (0.5 uL; Promega) were added to the RNA and water and heated at 70C for 5 minutes. The samples were then put immediately on ice for <10 minutes. To each reaction, 5 uL M-MLV 5x buffer, 1.25 uL 10 mM dNTPs and 0.5 uL M-MLV reverse transcriptase were added. The reactions were incubated at 42C for 1 hour with a 3 minute deactivation at 95C.

 Quantitative PCR (qPCR) was performed on all cDNA samples to determine the tissue-specific function of the ceramide pathway genes and their role in response to environmental stressors. qPCR reactions totaled 25 uL and included 12.5 uL of 2x Immomix (Bioline, London, UK), 9.5 uL water, 1.0 uL 50 uM SYTO-13 (Invitrogen), and 0.5 uL of each primer, forward and reverse (Invitrogen). qPCRs were done on a Bio-Rad CFX96 (Hercules, CA) following the thermalcycler protocol: 1. 95C for 10 min; 2. 40 times 95C for 15s, 55C for 15s, 72C for 30s, and a plate read; 3. 95C for 10s; 4. melt curve from 65C to 95C at 0.5C increments for 5s; 5. plate read. Average Ct (fluorescence-based cycle threshold) values across replicates and average gene efficiencies were calculated in PCR Miner (Zhao & Fernald 2005, http://www.miner.ewindup.info/version2). Gene expression (R0) was calculated based on the equation R0 = 1/(1+E)Ct, where E is the average gene efficiency and Ct. All expression values were normalized to expression in the housekeeping gene *elongation factor 1α*. All qPCRs were run in duplicate and significant differences in expression were determined via pairwise t-tests in R.

**Results**

*Gene discovery*

Complete full-length mRNA sequences, including start and stop codons, for AC, 3KDSR, and sptlc1 were assembled from the sequenced cDNA (Figure 1). The GlcCer sequence is missing some of the 3’ end and the publically available ESTs do not provide the missing sequence (Figure 1). Putative transmembrane domains were identified in the protein translations for all sequences (Figure 2). *C. gigas* sptlc1 shares 12 of the 21 amino acids in the *H. sapiens* transmembrane domain that begins at residue 21. All sequences aligned for 3KDSR share a putative hydrophobic domain from residues 3-23 (residues 5-25 for *C. gigas*). Only the *H. sapiens* protein sequence for AC has a putative transmembrane domain, but *C. gigas* has 3 putative domains in its GlcCer sequence compared to one in all the other comparative sequences. The GlcCer putative domains are located at residues 48-89, 348-368, and 377-397. *C. gigas* 3KDSR also possesses a conserved catalytic site and a NADH/NADPH binding site (Kihara & Igarashi 2004).

>*C. gigas* *sptlc1*

ATGTATGATATGTTTCAAGCCCTGTTGCAGGCTCCCTCATATCATCTTTTCTTCGAAGCATTGCTTATCATATGGATTTTCAAGCTGTTGTTTTTCTCTAAAGCCTACGCCCCAGAATCCGTCCTAACCGAAAAGGAAAAGGAGGAGTTGATTGCAGAATGGCAGCCGGAACCTCTAGCTCCCGAAATTCCAGACGACCACCCTGTTTTAATGGCGATGGAAAATAACATTATTACAGGGAAACCAGGAAAATATGTAACCATTAATGGAAAATCTTGTGTTAACATGGCTACATTAAACTTTCTTGGTATGGCAGGGAACCCATCTGTAGAGGCAGAGGCCATCAAAACTCTGAAAAAGTATGGAGTGGGATCGTGTGGACCAAGAGGTTTCTATGGCACAATGGACGTCCATTTAGAACTTGAAGACAAAATAGCAAAGTTCATGAATTGTGAGGAAGCTATATTATATGCTTTTGGCTTTGCGACCATAGCGAGTGCTATCCCAGCTTACTCGAAACGTGGAGATGTAATATTTGCTGATGAGGGAGTATGCTTTGCTATACAGAAAGGACTCGTTGCCTCAAGAAGCAAAATAAAGTGGTTCAAACACAACGATATGGAGGATCTGGAGCGTCTACTTATTGAACAAGCAAAGGAGGACAAGAAAAACCCTAAGAAAGCCAAAGTGACCAGAAGATTTCTTGTTGTGGAAGGACTCTACATTAACTATGGTGACTTATGTCCTCTTCCAAAATTAGTTGAACTCAAGTGGAAGTATAAAGTCCGCCTTTTTCTGGAAGAAAGTTTGTCATTTGGCATTCTTGGTAGCAATGGGAAAGGTGTAACAGAACACTACAATATCTCTCCAGATGATATTGACTTGATTGCGGCCTCATTAGAAAATGCTATTGGATCAACAGGAGGCTTTTGCTGTGGGAAGAAATATATTGTGGACCATCAACGTTTGTCAGGACTTGGATATTGCTTCTCAGCATCTTTACCTCCCATGTTAGCAACTGCAGCTATCGAGTCCCTGCGTTTGATTGATGAAAAACCAGGAATGCTTGTTGAATTGCGAGAAAATTGTGAAAAAATTCACAGCAAGCTGAGCGATATAAATGGAACCGTCATTGTAGGGGAACCTATTTCCCCAGTCAAGCACATTAGACTTGCAGAGCCAAGTACTGACAGGGACTTTGATGTGCAGACTCTGCAGAAGATAGCCGATCTCTCGAGAGACAACAAAGTTGCTGTGACGTTGGCTCGCTACTTAGAAGAGGAGGAACATAAACTTCCATTGCCAAGCATCCGGATATCTGTGAACAACCAGCTTTCAGATGAAGAAATTGACACTGTCTTCACTACACTAAGTGAAGCTTTTCAGAAAATCATCACTCATTAA

>*C. gigas* *3KDSR*

ATGTTGATTTATTGTGGAATTTTGTTGATTTTAGTAATTTTATTTGCATTATCAAAACTTCTATCGGCAAAGAAAATAAAGCTAAAAGGAGCTCATGTATTGATAACAGGTGGATCTAGTGGTATTGGTAAAGCCCTTGCTATTGAGGCGGTTAAAAATGGAGCTAATGTCACTATCATGGCCAGGAATGAAAAAAAACTAGAAGATGCAAAAGAAGAAATAGAAAGTCACATAAAAGATAAAGATGCTCAGAAGGTTTTTTCAACATCTGTGGACATAACAAAAAGTGCAGAAATGGTTAAAGAAGCTGTTAAAAAGGCTGAAAACAATCTGGGACCAGTTACAATTCTAATTAACAATGCAGGCAGTGCAGTGGCTGGAAATTTTGAAGACACTTCATCTGAGCAATTTCAGCGCATGATGGATTTGAATTTTCTCGGAGGTGTCAATGTCACCAAGGCTGCCTTAAAAGGGATGAAGGACAACAATGGAGGAAGAATAGTTTTCATCTCATCCCAGGCGGGACAAGTGGGCGTGTTTGGCTACACCGCCTACTCAGCCTCAAAATTCGCTCTCAGGGGATTCGCAGAGTCCCTTCAAATGGAGGTAAAACCATACAATATTTATGTTACCATGGCATTTCCACCTGACACAGACACTCCTGGTCTAGCAGAGGAGAATAAATCTAAGCCCAGGGAAACACTGCTCATATCAGATACAGTAGGACTGTTTTCTCCCAGTGATGTGGCCAAGTCAGTTTTCAAGGATGCTGTGAATGGGAAATTTTTGAGTTACGTTGGTATGGATGGCTGGTTGCTTTGCAACTTATCAAGCGGGATGTCCCCTGCAACGTCCGTTTTGGATTTACTTCAGCAGATATTCACTATGGGACTGTTCCGCTTTGTTTGTCAGTTTTATTTGTTACATTTTGACCGGATTATAAAAAAGTGTAAAGAAGAGAAGCATGCAAAACCCAAGACATCATAAGATTTCATACGTATTTAATGGTTTTTTTTAAAACAATTTAAATATATTTATGAACAAATTATACATTTATCAGTTGTATTTGAAATATCGGTATATTAATTTGCTTTTACATTCAATGAATTCACACTTGCAAAAAATATTT

>*C. gigas AC*

ATGTGGATACCGGTGGTCTTGTCGCTTTCTGTTGTATTTTGGGTGGGGAAYTGTCAATTTCCACCGTTTAATGAGACCTGTGTTTCCAATGCATATCCGCCAAATAAAACAAATGCAGTTCCTTGGTATACTATTAACCTAGACTTACCACCAAAGCAAAGATGGACTCAAGTTGGCCAGGAGAAGAAAGCAGGGATAAAGAAACTGCTGGTTTCCTTCATTGATTTTGCCAAAGCATGGAGTGAGAATGCCACAGGGAAAGTGGTCGACTTCCTTATTAATGAAGGGGGCAAAATAGAYGAWACTCTCCCCCAGCCTTATGCAGATGAAATGAGGGGGTTGGCTGAAGCGACAGGGATACCACTTGGAGAAATAGTGCTGTACAACATTTTCTATGAACTTTTCACTGTGTGTACCTCCATCGTAGCAGAAGATCCATCTGGAAAACTTTTCCATGTACGAAATCTGGATTTTGGTCTATTTTTGGGATGGGATGTTAAAACTAAGACATGGGAAATAACGGATGCCCTGCGTCCTACTGTGGTTAACTTGGATTTCCAGAAGGGAGGAAAAACTGTCTTCAAATCTGTGAACTTTGCAGGATACATTGGTATCCTTACAGCTGTGAAACCAGGAATGTTCACACTAACAATGAATGAAAGATTCAACATGGATGGTGGATACATAGGTGTACTTGAGTGGATTCTGGGGATCAGGACAGGGAAGTGGATGGGTTTCCTGACCAGAGAGACAATGGAAAAAGCTGGCTCCTACCAAGAGGCTGTAGCTATGTTGTCCCAGACAGAAATGCTGGCTCCTGCATATTTCATTGTTGGAGGCAATAAGTCAGGAGAGGGCTGTGTGATCACCAGGTCTCGTGAAAAAGCTATTGATGTGTGGAGGATGGATCAAGCAAATAATTGGTACATCCTGGAGACAAATTACGACCATTGGGAAAATCCCCTCTTCTTGGATGACCGCAGAACTCCAGCTCACAAGTGTATGCAGAAAACGACCTCCAAGAATGTGTCAATCAAAGGACTGTTTAATGTATTGTCCTCAAAACCTGTTTTAAATAAGTTGACGACTTACTCCGCACTGATGCAGGTGAACTCGGGACATCTGGAGAGCTGGTTGCAGTACTGTCCAGATCCATGTCAGCCATTTTAA

>*C. gigas GlcCer*

ATGAGCGTAGCTCACTATTTTGCTTTCTCTCTTGCTATTTTAATTTTAGGAGGATGGTGKTTCGTTTGGTTTATGCACATTTTAGCCTTAATCTATGGAAAATACCGTCTACACCATCCTATTCCGCCTCCCTCCCSGGAAGACCTCCAGGGGGTGTCCATCATTAAACCCCTGGTCGGGGTGGATCCAAACCTGTATTTCAATTTAGAGTCTTTTTTCACCACTGTCTATCCATCCTTTGAACTCCTCTTCTGTTTACAAGATGAAAGTGATCCTGCTCTTATGGTTGTTAAAGCTCTCATGGAAAAATACCCAAAAGTAGATGCTAAAATTTTTATAGGTGTAAAATATGTTGGCCCAAACGGGAAAGTTAACAACATGTGCAAAGCATATGAGGCAGCCAAATATGATTTAATAGTCATATCTGATAGTAGTATATTAATGAAACCAGACTCACTCATGGACATGATGTCCTTCATGAAGCCAGATGTCGGTCTAGTGTTGCAAATGCCATATTGTTGTACTCGCAAGGGGTTCGCTGCTGTTTACGAAAAGGTTTATTTTGGAACGTTTCAGTCCAGAAACTGTCTTAGTGCTAATTCTGTAGGAATCAATTGTTCAACGGGCATGTCCTGCCTTTTCCGAAAAGACATTTTAGAAAAATCAGGGGGCTTGGCTCCTTTAGGAAAATATTTAGCAGAAGATTATTTTATTTCTGAAAACATTAGAAAAGAGGGATATAAAACAGTATTATGTAGTCAACCAGCACAGCAAAATTCTGGACAGTACAACATAGGCCATTTTCATCAACGACTCATCAGGTGGTCCCAGCTGCGCATCTCTCTGTTACCTCATCTCATCCTGTTTGAGCCCCTGTCGGAATGTATGCTGATGGGGGTGATTGCCTCGTGGGCTGCCGAGTACATCTTTGGCATCAGCTCAATGGGCTTCTTCCTCATGCATGTTCTAGTCTGGTTCCTGTTCGATTACGCTCTCCTTACATGTGTTGAGAATGGGCCACTTCCTTTTTCTAAGTTTGAATTCCTGGTTGCCTGGATTCTACGAGAAGTGTTATCCATATGGAAGGGCTTCTCGTGTACTTTCGTGTGTTCGCCTCTAAGGGC

*Pathway phylogeny*

 All four genes show similar phylogenetic topologies (Figure 3 a-d). When the *C. elegans* sequence was available, it clustered with the *C. gigas* sequence with a bootstrap value of 100%. Similarly, *H. sapiens* and *M. musculus* sequences always clustered together with a bootstrap of 100%. This identical pattern across all four genes suggests separate vertebrate and invertebrate lineages of the ceramide pathway components.

*Gene expression*

 All four genes showed similar patterns of tissue distribution. All were expressed the most highly in gill tissue. The overall trend for hierarchical expression (greatest to least) was: gill > digestive gland > mantle > adductor muscle (Figure 4 a-d; Table 4). The only exception to this trend is in 3KDSR (Figure 4b), where the mRNA expression levels between the mantle and digestive gland are approximately equivalent. Fold over minimum sets the minimum expression level to be in the muscle (Table 4). The fold-over-minimum expression differences in the gill are 47.7-fold for Sptlc1, 4.9-fold for 3KDSR, 27.9-fold for GlcCer, and 2.5-fold for AC.

 In general, production of ceramide seems to be up-regulated during a *C. gigas* immune challenge with the pathogen *Vibrio vulnificus* (Figure 5; Table 4). Both sptlc1 and 3KDSR show higher levels of gene expression in the *Vibrio*-exposed oysters compared to controls, although the difference is not significant for either gene (p=0.068 and p=0.079, respectively). AC is also expressed more highly during the immune challenge (p=0.045), whereas there is no statistical difference in GlcCer expression (p=0.47). Sptlc1 is expressed 1.3-fold higher in the *Vibrio*-exposed versus the control oysters; 3KDSR is expressed 1.6-fold higher; GlcCer expression in bacteria-exposed oysters is 0.7 of the control expression; and AC is expressed 1.4-fold higher in the bacteria challenge (Table 4). All fold differences are based on mean expression values for exposed versus control.

**Discussion**

We have characterized four major components of the ceramide biosynthesis pathway in *Crassostrea gigas*, using laboratory and *in silico* analysis: *serine palmitoyltransferase* (*sptlc*), *acid ceramidase* (*AC*), *3-ketodihydrosphingosine reductase* (*3KDSR*), and *ceramide glucosyltransferase* (*GlcCer*).  The discovery of these genes in *C. gigas* adds to a growing group of apoptosis-related genes in molluscs (Lee et al. 2010; Zhang et al. 2011; Romero et al. in press).  Unlike the previously described genes in molluscs, however, the genes described here are directly responsible for generation of ceramide in *de novo* (*sptlc, 3KDSR*) and catabolic synthesis (*AC*) and generation of sphingolipids from ceramide (*GlcCer*).  Further *in silico* analysis of the *C. gigas* transcriptome also shows that there are a number of other genes in this same ceramide biosynthesis pathway that have not yet been fully characterized.  Full characterization of the ceramide pathway will increase our understanding of how this commercially and environmentally important species interacts with its environment and the stressors it encounters therein.

*Gene Structure and Function*

*C. gigas* has ESTs that correspond with high homology to a majority of the elements in the ceramide pathway (Figure 5).  The genes not identified in this study or in Zhang et al. (2011) were discovered through *in silico* analysis of *C. gigas* 454 contigs of larval sequences and through BLAST searches based on homology.  We identified almost all the enzymes necessary for de novo ceramide synthesis, assuring the conservation of this segment of the pathway in Pacific oysters.  We also identified a number of enzymes responsible for transformation of ceramide into other lipid products: ceramide kinase (leads to ceramide 1-P), ceramide synthase (sphingosine), and sphingomyelin synthase (sphingomyelin).  Also identified were a variety of caspases, TNF superfamily receptors, RIP and FADD subunits of the TNFa receptor.  Even though a few components of the overall ceramide pathway were not found in our searches, they most likely exist within *C. gigas*, but have either not been sequenced or the inherent poor quality and incomplete sequences of ESTs make them unidentifiable via homology searches.  The discovery of the enzymes that regulate generation and transformation of ceramide are of particular importance in understanding *C. gigas* response to its environment since these are the genes that will respond and adjust to changes.  Since some of these genes come from a larval transcriptome, we now have evidence of the presence of this pathway in early stages of development of *C. gigas*.  Apoptosis is an instrumental process during development as cells and tissues rearrange and differentiate.  Genes in the ceramide pathway are essential to development in a number of vertebrates (e.g. Eliyahu et al. 2007; Yabu et al. 2001) and the presence of these genes in the oyster provide evidence of their importance in invertebrate development as well.  Below we discuss in detail the four genes characterized in this study and their potential roles in oyster physiology.

Serine palmitoyltransferase catalyzes the first step of ceramide *de novo* biosynthesis: the condensation of serine and palmitoyl-CoA to 3-ketosphinganine (reviewed in Hannun 1994; Fig. 5). Sptlc mediates the rate-limiting step in the pathway of *de novo* ceramide biosynthesis (Perry 2002; Merrill et al. 1985).  There are two forms of human Sptlc – LCB1 and LCB2. The human LCB1, shown in the alignment (Figure 2a), has a putative transmembrane domain starting at residue 21 and extending 21 amino acids (Hanada 2003). This domain is predicted in by the Transmembrane Predictor (Suchard in Geneious) and the *C. gigas* protein shares 11 of those 21 amino acids, suggesting that it may have a similar topology. The mammalian transmembrane domain spans the endoplasmic reticulum, with the N terminus within the ER (Yasuda et al. 2002; Hanada 2003).  LCB2 has a conserved motif that binds pyridoxal phosphate (PLP) (Hanada 2003), but *C. gigas* has an asparagine in that position (residue 315 on our alignment), which is the same as the *H. sapiens* LCB1 form of the enzyme.  The basic function of Sptlc is conserved across taxa and is instrumental in determining levels of ceramide and other sphingolipid intermediates (Hanada 2003).  The expression of Sptlc mRNA and protein in vertebrates is sensitive to a number of biological processes, including stage of development, external stress stimuli, and apoptotic stimuli that activate the enzyme post-transcriptionally (Hanada 2003). Sptlc1 is responsible for accumulation of intracellular ceramide during cellular stress (Perry 2002; Perry et al. 2000).   These enzymatic functions are probably conserved in *C. gigas* considering the high degree of homology between the oyster and human amino acid sequences: 59.8% pairwise identity (over 479 residues).   The *C. gigas* sequence shares 51.0% pairwise identity over 475 amino acid residues with the *C. elegans* Sptlc1, indicative of a very high degree of homology between these taxa.

The product of the reaction catalyzed by Sptlc is 3-ketosphingasine. 3-ketosphingasine is reduced by a NADPH-dependent reductase to dihydrosphingosine. The enzyme that catalyzes this reaction is 3-ketodihydrosphingonsine reductase (3KDSR). This enzyme is much less studied than Sptlc and was not characterized in humans until 2004 (Kihara & Igarashi 2004). Human 3KDSR has 332 amino acids and shares 23.5% pairwise identity with its yeast homolog (Kihara & Igarashi 2004). Over their entire sequence, the protein sequences for *H. sapiens* and *C. gigas* 3KDSR share 50.8% identity. The *C. gigas* sequence shares a predicted transmembrane domain with the other aligned sequences at the N-terminus spanning residues 3-23 (on the *H. sapiens* sequence). The *C. gigas* protein sequence also shares a putative active site motif and a NADH or NADPH binding site with *H. sapiens, M. musculus*, and *D. rerio*. The active site motif, first identified as being conserved across humans, mice, and yeast, follows the pattern *Tyr-X-X-X-Lys* (Kihara & Igarashi 2004). All four amino acid sequences in Figure 2b share even more of this motif: *Tyr-Ser-X-Ser-Lys*, beginning at residue 187 on the alignment. The possible NADH or NADPH binding site is characterized by the motif *Gly-Gly-X-X-X-Gly-X-Gly* (Kihara & Igarashi 2004) and begins at residue 39. This motif is actually identical in its entirety – *Gly-Gly-Ser-Ser-Gly-Ile-Gly* – across all four sequences/taxa in Figure 2b. The high degree of conservation for both of these motifs is suggestive of their importance in mechanistic function of 3KDSR.

Ceramidases are responsible for the catabolic generation of ceramide from sphingosine. “Acid” refers to the pH optimum of this particular form the enzyme, with the maximum activity of the *H. sapiens* enzyme occurring at pH 3.8-4.3 (Bernardo et al. 1995). *In vitro*, AC can accomplish its reverse reaction as well – the generation of sphingosine from ceramide (Okino et al. 2003). AC is made up of two subunits: an unglycosylated α-subunit of approximately 13 kDa and a N-glycosylated β-subunit of about 40 kDa (Koch et al. 1996; Bernardo et al. 1995). Only *H. sapiens* shows a predicted N-terminal transmembrane domain from residues 5-25 (Figure 2c). Four mutations that cause cessation of enzymatic activity have been identified in *H. sapiens* AC, which all cause Farber’s Disease resulting in death by a few years of age (Koch et al. 1996; Li et al. 1999). Increased expression of AC has been found to upregulate metabolism of intracellular ceramide in mammals and this turnover of ceramide to sphingosine removes long-chain ceramides that inhibit insulin signaling (Strelow et al. 2000; Chavez et al. 2005). Similarly to human tissues (Li et al. 1999), AC is also ubiquitously expressed in different oyster tissues, implying that both *de novo* and catabolic generation of ceramide are important in *C. gigas*. The *C. gigas* amino acid sequence shares 46.6% pairwise identity over 402 residues with *C. elegans* and 49.4% identity with the *H. sapiens* sequence over 398 residues.

Ceramide glucosyltransferase, or glucosylceramide synthase, catalyzes the reaction that generates glycospingolipids (GSLs) from ceramide (reviewed in Ichikawa & Hirabayashi 1998). GSLs are important constituents of plasma membranes. The ubiquitous expression of GclCer in both *C. gigas* and *H. sapiens*, coupled with a putative C+G-rich regulatory region in *H. sapiens*, demonstrates its probable role in cell viability (Ichikawa et al. 1996). The structure of the *C. gigas* GlcCer is different from *H. sapiens* in that it does not share the latter’s N-terminal transmembrane domain that spans residues 70-90 in Figure 2d (Ichikawa et al. 1996). Three transmembrane domains are predicted for the *C. gigas* amino acid sequence: a N-terminal segment from residue 48-90 and two closer to the C-terminal end from 348-368, and 377-397. *D. rerio*, *C. elegans*, and *M. musculus* also only have one putative transmembrane domain at the N-terminal, implying that the *C. gigas* structure is an evolutionarily derived form of the enzyme. Despite these differences in topology, *C. gigas* and *H. sapiens* share a 45.9% pairwise amino acid identity over 396 residues. Compared to 40.9% shared between *C. elegans* and *C. gigas* over 468 residues. GlcCer is present in all animals and many plants, underlining the universal use of GSLs in plasma membranes (Ichikawa & Hirabayashi 1998). GlcCer also serves as an efficient reaction to ceramide accumulation (Ichikawa & Hirabayashi 1998), which can occur during cellular stress.

 *Gene Expression and Role in the Immune Response*

 The expression patterns of the four genes described in this paper support that ceramide synthesis - both *de novo* and catabolic - is important in the *C. gigas* immune response. Genes important in the oyster immune response are frequently expressed in gill tissue (Seo et al. 2010; Yu et al. 2011; Romero et al. 2011), although this expression pattern could be an artifact of the expression profiles of hemocytes that have infiltrated the gills. The oyster encounters its environment via its gills, which constitute a large portion of *C. gigas*’s surface area and are constantly filtering water. Thus, it follows logically that cells in the gills would activate the pathways necessary to respond to environmental stress. Given the extensive studies of the role of the ceramide pathway in vertebrates and the high degree of identity between oyster and vertebrate genes in this pathway, it is probable that changes in ceramide metabolism are important in the Pacific oyster response to environmental stressors.

 In support of the hypothesis that ceramide generation and metabolism change in response to environmental stressors in *C. gigas*, three genes characterized in this study show signs of upregulation in an immune challenge. Adult oysters were exposed to the marine bacterium *Vibrio vulnificus* for three hours and gene expression of Sptlc1, 3KDSR, AC, and GlcCer was assessed in gill tissue. Three of these genes – Sptlc1, 3KDSR, and AC – were upregulated compared to oysters that were not exposed to *V. vulnificus* (although only the expression difference for AC was significant at p<0.05). Sptlc1 and 3KDSR catalyze the first two steps in the *de novo* synthesis of ceramide and AC catalyzes the catabolic generation of ceramide from sphingosine. The lack of up-regulation of GlcCer, which catalyzes the generation of glycosphingolipids from ceramide further implies that the *Vibrio* exposure is stimulating an accumulation of intracellular ceramide and potentially an apoptotic response. Further research that investigates changes in cellular apoptosis during an immune challenge in *C. gigas* will help to support or refute this role for ceramide generation.

The taxonomic universality and importance of the ceramide pathway and apoptosis in regulating an organism’s response to environmental changes is becoming increasingly acknowledged.  Ceramide is an important component of the cyclooxygenase and prostanglandin synthase pathway, modulating secretion of prostaglandin E2 and activating transcription of cyclooxygenase (Hannun 1994).  TNFa works through ceramide in both apoptosis and inflammation  (Verheij et al. 1996).  Stress-induced apoptosis is signaled by ceramide upon exposure to ionizing radiation, hydrogen peroxide, UV radiation, and heat shock (Verheij et al. 1996).  Catabolic generation of ceramide is an important immediate and efficient response to radiation (Haimovitz-Friedman 1994).  Abrupt changes in salinity can trigger shifts in ceramide metabolism in sea bass, most likely is an effort to mediate cellular rearrangement after osmotic shock (El Babili et al. 1996).

Recent studies have found that apoptosis regulation is prevalent in invertebrates and probably instrumental in response to environmental stress.  Zhang et al. (2010) found 52 apoptosis-related genes in *Bombyx mori*, including 5 members of the caspase family and 2 in the TNF superfamily.  The disk abalone, *Haliotis discus discus*, constitutively expresses caspase-8, the highest levels being in the gill (Lee et al. 2010).  There is immediate upregulation of this gene in gill tissue and hemocytes following bacterial and viral challenge (Lee et al. 2010).  In addition to the two genes discussed in this study, four more apoptotic genes were recently described in *C. gigas*: Fas-associated protein with death domain (FADD), inhibitor of apoptosis (IAP), and initiator and effector caspases (Zhang et al. 2011).  Similarly to the disk abalone and the expression patterns seen for *C. gigas* *sptlc*, both caspases showed the highest transcript levels in gill and mantle tissue and the lowest in the gonad and digestive gland (Zhang et al. 2011).  FADD and IAP had the highest mRNA transcript levels in the hemolymph (Zhang et al. 2011).  Overall, Zhang et al. found that there was a relatively high level of complexity in the *C. gigas* apoptosis components that they described, between those of ecdysozoa and deuterostomes, indicating gene loss in the former or possible expansion in the latter.  In accordance with our findings and with Lee et al.’s (2010), FADD, IAP, and the caspases showed increased expression immediately following challenge with a *Vibrio* bacteria until expression peaked at 12 hours post-challenge (Zhang et al. 2011).  Since vertebrate apoptosis and ceramide synthesis are sensitive to a wide range of environmental stressors beyond disease, the significant role in immunity played by the ceramide pathway is probably a more universal response to perceived environmental stress.