Putative role for bacterial symbionts in the biosynthetic origin of Tyrian purple in (Dicathais orbita), a Muricidae mollusc

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Putative role for bacterial symbionts in the biosynthetic origin of Tyrian purple in *Dicathais orbita*, a Muricidae mollusc

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Declaration

I certify that the work presented in this thesis is, to the best of my knowledge and belief, original, except as acknowledged in the text, and that the material has not been submitted, either in whole or in part, for a degree at this or any other university.

I acknowledge that I have read and understood the University's rules, requirements, procedures and policy relating to my higher degree research award and to my thesis. I certify that I have complied with the rules, requirements, procedures and policy of the University (as they may be from time to time).

Ajit Kumar Ngangbam Date- 03/08/2016

Abstract

The neogastropod molluscs of the family Muricidae are well known for the production of the historically important dye Tyrian purple. 6, 6' Dibromoindigo, the major pigment in Tyrian purple dye was the first marine natural product to be structurally defined, however even a century later, the biosynthetic origin of Tyrian purple and the role of microbial symbionts in its production is not known. 6, 6' Dibromoindigo is the brominated derivative of the blue dye indigo which is usually derived from a range of bacteria and plants. The broad objective of this thesis was to establish the biosynthetic origin and identify genes involved in the biosynthesis of bromoindole derivatives in muricid molluscs. The first aim of the thesis was to generate the transcriptome of hypobranchial gland, prostate gland, albumen gland, capsule gland, mantle and foot tissues of *Dicathais orbita* using the Illumina HiSeq 2000 platform. This investigation generated over 201 million high quality reads and was *de novo* assembled into 219,437 contigs. In total, 76,152 contigs that contained candidate-coding regions were annotated with reference to the Nr, Swiss-Prot and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases. This study revealed that *D. orbita* expresses a number of genes associated with indole, sulfur and histidine metabolism pathways that are relevant to Tyrian purple precursor biosynthesis. However, there were no matches to known bromoperoxidase enzymes within the *D. orbita* transcripts.

The second aim of the thesis was to establish whether distinct bacterial communities occur in the biosynthetic organs of *D. orbita* and to identify any indole producing bacteria using 16S rRNA sequencing. Biochemical activity profiles and the bacterial communities cultured from *D. orbita* were not significantly different between males and females, but there were significant differences according to the source tissue. Bacterial communities from foot tissue were similar to seawater, but the Tyrian purple producing hypobranchial glands and reproductive organs had a low diversity of culturable bacteria with limited biochemical activity.

Three indole producing bacteria were also identified from these biosynthetic organs. Biochemical and molecular analysis revealed that these indole-producing isolates matched closely to *Vibrio* spp.

The third aim of the thesis was to assess the diversity and identity of bacteria associated with the Tyrian purple producing hypobranchial gland, in comparison with foot tissue using a high-throughput sequencing bacterial profiling approach. This investigation revealed a highly diverse bacterial assemblage associated with the hypobranchial gland and foot tissues of *D. orbita*. The dominant bacterial phylum in the 16S rRNA bacterial profiling data set was *Proteobacteria* followed by *Bacteroidetes*, *Tenericutes* and *Spirochaetes*. In comparison to the hypobranchial gland, the foot had significantly higher bacterial diversity and a different community composition, based on taxonomic assignment at the genus level. Indole producing *Vibrio* spp. and bacteria with brominating capabilities were also observed in the hypobranchial gland of *D. orbita*.

The final aim of the thesis was to culture bacterial symbionts from the Tyrian purple producing hypobranchial gland and screen the isolates for indole production and bromoperoxidase genes using molecular methods. The ability of bromoperoxidase positive isolates to produce the brominated indole precursor to Tyrian purple was also established by extraction of the culture and analysis using liquid chromatography mass spectrometry (LCMS). In total, 32 bacterial isolates were cultured from *D. orbita* hypobranchial glands using marine agar, marine agar with hypobranchial gland extracts, blood agar, thiosulfate citrate bile salts sucrose agar and cetrimide agar at pH 7.2. These bacterial isolates included 26 *Vibrio* sp., two *Bacillus* sp., one *Phaeobacter* sp., one *Shewanella* sp., one *Halobacillus* sp., and one *Pseudoalteromonas* sp. The two *Bacillus* species were the only isolates found to have coding sequences for bromoperoxidase enzymes. Bromoperoxidase producing *Bacillus* sp. cultured from the hypobranchial glands in tryptone broth supplemented with KBr, were able to produce

the brominated compound, tyrindoxyl sulphate which is the ultimate precursor of Tyrian purple in *D. orbita*.

In summary, this thesis used a range of culture and culture independent approaches, modern gene sequencing approaches and chemical profiling techniques, such as liquid chromatography mass spectrometry, to understand the role of microbial symbionts and biosynthetic origin of Tyrian purple in *D. orbita*. This thesis offers new opportunities for sustainable production of Tyrian purple and has greatly increased the molecular understanding of Tyrian purple producing muricid molluscs.

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List of Abbreviations

Acetonitrile	ACN
Amygdalin	AMY
Analytical profile index	API
Arabinose	ARA
Arginine dihydrolase	ADH
Australian Genome Research Facility	AGRF
Basic Local Alignment Search Tool	BLAST
Blood agar	BA
Bromine	Br
Calcium carbonate	$CaCO_3$
Capsule gland	CG
Celsius	C
Centimeter	cm
Cetrimide agar	CA
Citrate utilization	CIT
Colony forming units	CFUs
Daltons	Da
Deoxynucleotide triphosphates	dNTPs
Deoxyribonucleic acid	DNA
Diode array detection	DAD
East	E
Ecteinascidin 743	ET-743
Egg capsules	EC
Electrospray ionisation	ESI
European Nucleotide Archive	ENA
Expressed sequence tag	EST
Female	F
Female foot	F2F, F3F
Female hypobranchial gland	F1H, F2H
Food and Drug Administration	FDA
Forward primer	FP

Gelatinase	GEL
Gene ontology	GO
Glucose	GLU
Glycerol asparagine agar	GA
Gram	g
High Performance Liquid Chromatography	HPLC
Hours	hrs
Hydrochloric acid	HCl
Hydrogen sulfide	H_2S
Hypobranchial gland	HBG
Hypobranchial gland dorsal swabs	HGDS
Hypobranchial gland homogenates	HGH
Hypobranchial gland ventral swabs	HGVS
Indole production	IND
Inositol	INO
Kyoto Encyclopedia of Genes and Genomes	KEGG
Liquid chromatography mass spectrometry	LCMS
Lysine decarboxylase	LDC
Magnesium chloride	MgCl
Male	m
Male foot	M2F, M3F
Male hypobranchial gland	M1H, M2H
Mannitol	MAN
Marine agar with hypobranchial gland extracts	MAH
Mass spectrometry	MS
Melibiose	MEL
messenger RNA	mRNA
Metagenome Analyzer	MEGAN
Microgram	µg
Microlitre	µl
Micrometer	µm
Micromolar	µM
Milligram	mg

Millilitre	ml
Millimetre	mm
Millimolar	mM
Minutes	min
Molar	M
Molecular mass	m/z
Nanogram	ng
National Center for Biotechnology Information	NCBI
New South Wales	NSW
Non metric multidimensional scaling	nMDS
Non redundant	Nr
Nuclear magnetic resonance spectroscopy	NMR
Nucleotide BLAST	BLASTN
Open reading frame	ORF
Operational taxonomic unit	OTU
Ornithine decarboxylase	ODC
Ortho NitroPhenyl-βD Galactopyranosidase	ONPG
Penis	P
Permutational Multivariate Analysis of Variance	PERMANOVA
Phosphate buffer saline	PBS
Plymouth routines in multivariate ecological research	PRIMER
Polymerase chain reaction	PCR
Potassium bromide	KBr
Potential hydrogen	pH
Principal coordinates ordination	PCO
Prostate gland	PG
Rectal gland	RG
Reverse primer	RP
Revolutions per minute	rpm
Rhamnose	RHA
Ribonuclease Ribose nucleic acid	RNase
Ribose nucleic acid	RNA

ribosomal RNA	rRNA
Saccharose	SAC
Seawater	SW
Seconds	s
Similarity percentages	SIMPER
Sodium chloride	NaCl
Sorbitol	SOR
South	S
Species	sp
Standard error	SE
Subspecies	spp
Temperatures	temp
Thiosulfate citrate bile salts sucrose	TCBS
Total ion current-mass spectrum	TIC-MS
Trifluoroacetic acid	TFA
Tryptophan deaminase	TDA
Tyrian purple	TP
Ultraviolet	UV
United States of America	USA
Urease	URE
Volt	V

Chapter 1: General Introduction

1.1 Project summary

Marine invertebrates are an important source of biologically active compounds that provide leads for drug development, however, in most cases the biosynthetic origin of these compounds is unknown. *Dicathais orbita* is a predatory muricid snail distributed along the southern coast of Australia and it is an ideal model species for investigating the production of Tyrian purple which is produced in the hypobranchial gland, reproductive organs and egg capsules of muricid molluscs including *D. orbita*. Tyrian purple and its brominated indole dye precursors have anticancer and antimicrobial properties. This project aimed to establish the biosynthetic origin and identify genes involved in the biosynthesis of bromoindole derivatives in muricid molluscs. The objectives of this project include: i) Generation of the full transcriptome of *D. orbita* to identify expressed biosynthetic genes potentially involved in Tyrian purple precursor production; ii) comparison of symbiotic culturable heterotrophic bacterial communities associated with different tissues of *D. orbita* and the identification of indole producing bacteria from the biosynthetic organs which could contribute to Tyrian purple production; iii) metagenomic characterization of bacterial communities associated with the Tyrian purple producing gland in *D. orbita* to quantify bacterial diversity and; iv) to specifically culture bacteria potentially involved in Tyrian purple precursor synthesis, and then screen the bacterial isolates for indole production and bromoperoxidase genes. This project employed a combination of transcriptomics, traditional bacterial cultured techniques, targeted PCR screening and metagenomic approaches to identify biosynthetic genes and bacterial symbionts potentially associated with Tyrian purple production in *D. orbita*. The outcomes of this project have implications for the sustainable production of Tyrian purple and anticancer precursor compounds, and will help to determine the potential role of symbiotic bacteria in the biosynthesis of Tyrian purple precursors.

1. 2 Marine invertebrates derived secondary metabolites

Oceans occupy 70% of the Earth's surface and provide habitat for 34 of the 36 animal phyla (Wright 2000, Levinton 2001, Sima & Vetvicka 2011b). There is high species diversity in the marine environment with up to 1000 different species per square meter in tropical reef zones (Pomponi 1999, Jimeno 2002). Nearly 60% of all the marine animal species are invertebrates and most of the invertebrates belong to the phyla Arthropoda, Mollusca, Annelida, Echinodermata, Bryozoa, Cnidaria, Porifera, Platyhelminthes and sub-phylum Tunicata (Ausubel et al. 2010, Leal et al. 2012b). Most marine invertebrates are sessile and soft bodied and depend on chemical defences. These chemical defences have been developed through their evolutionary history to perform ecological functions of paralysing prey or preventing predation, pathogens, competition (Faulkner 2000, Haefner 2003). Secondary metabolites are small organic molecules that are less than 3000 Da in size and can act as a chemical defence (Kinghorn et al. 2009). Beyond the ecological role of secondary metabolites, these novel chemicals also provide opportunities that aid human society in the form of bio-products. A large number of marine natural products have been isolated and characterized in association with the high diversity of marine invertebrates (Molinski et al. 2009, Senthilkumar & Kim 2013).

Research efforts on natural products from marine organisms involve identifying biochemical properties and mechanisms of action, with less focus on their ecological functions (Paul et al. 2007). To date 16,617 marine compounds have been reported from different marine organisms (Hu et al. 2015). The percentage of new marine natural products from different Phyla of marine invertebrates discovered since 1990 to 2009 (Fig. 1.1) are dominated by Porifera (48.8%) and Cnidaria (28.6%), with between 5-10% also coming from Echinodermata, Chordata and Mollusca, whilst the Hemichordata, Bryozoa, Annelida, Platyhelmintes,

Arthropoda and Brachiopoda contribute only 1.7% (Leal et al. 2012b) (Fig. 1.1). The Phylum Porifera, with around 4852 compounds, is considered the largest source of new marine natural products (Mehbub et al. 2014). This pattern indicates that marine invertebrates that branched off earlier in animal evolution, mainly the sponges and cnidarians, are a particularly good source of natural products. However, it is not clear whether an equal amount of natural product research has been invested in species from the other phyla. Therefore, the pattern observed in Figure 1.1 could actually be driven by research bias towards certain phyla (Brahmachari 2015) that have previously been found to produce interesting natural products. This problem in research bias is supported by research on molluscan natural products, where less than 1% of species have been studied and most of these are soft bodied opisthobranchs (Benkendorff 2014). Despite a higher diversity of species, and the discovery of many pharmaceutically important compounds, relatively few shelled molluscs have been studied due to early assumptions based on a greater need for chemical defence in soft bodied species. Thus marine invertebrate phyla that evolved later, from bilaterally symmetrical ancestors, may be equally good sources of novel marine compounds, but require further strategic research to investigate these properly.

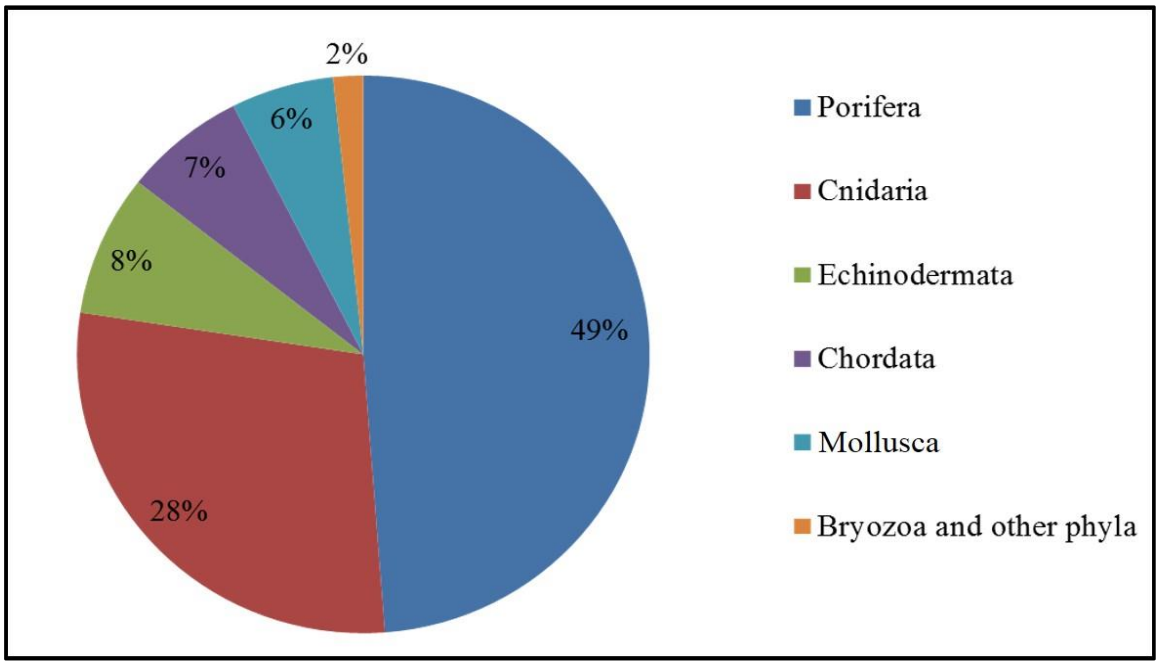

Fig. 1.1. Percentage of new marine natural products from different marine invertebrate phyla (data sourced from Leal et al., 2012b).

The diversity of natural products spans a range of chemical groups including terpenoids, alkaloids and other aliphatic compounds (Leal et al. 2012a, Mehbub et al. 2014). These chemical groups are represented in a number of phyla including, Porifera, Cnidaria, Mollusca and Echinodermata (Fig. 1.2). A review of new natural products from marine invertebrates between 2000-2010 shows that not all chemical classes are dominated by the Porifera and Cnidaria (Leal et al. 2012a, Mehbub et al. 2014) (Fig. 1.2). Marine natural products reported from Cnidaria and Mollusca are mainly terpenoids, whereas alkaloids are a dominant group in Chordata (Leal et al. 2012a), terpenoids and alkaloids from Porifera (Mehbub et al. 2014), while aliphatic compounds dominate Echinodermata (Leal et al. 2012a). Within the Mollusca, a dominance of terpenes was reported in the Gastropoda, whereas bivalves are dominated by sterols and aliphatic nitrogenous compounds (Benkendorff 2010). Rosegheni et al. (1996) has reported a number of biogenic amines specifically in Neogastropods. Also within the

neogastropods, Muricidae contain alkaloids (Benkendorff et al. 2015) and the *Conus* sp., (Conidae) produce neurotoxic peptides such as Ziconotide and contulakin G (Newman & Cragg 2004). The large diversity of conotoxins explains the domination of amino acid and peptides reported from molluscs (Fig. 1.2).

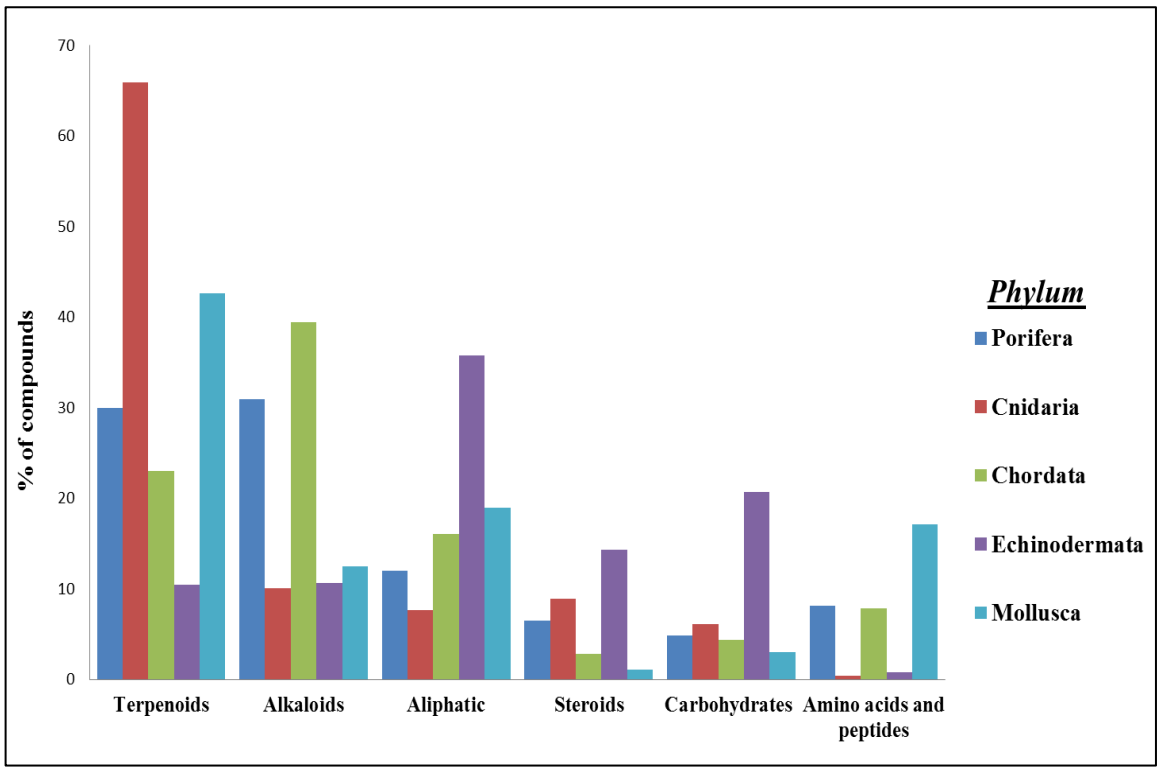

Fig. 1.2. Percentage of new marine natural products in different chemical classes from different marine invertebrate phyla (data sourced from Leal et al., 2012a).

Marine organisms have been of much research focus during the last forty years for their ability to produce natural products with potential for therapeutic application (Sima & Vetvicka 2011a). Importantly, marine organisms are routinely linked to the production of bioactive compounds with antimicrobial, antifungal, antiviral, antiprotozoal, anthelminthic and anticancer properties (Donia & Hamann 2003, Nagle & Zhou 2009, Malaker & Ahmad 2013). Of 16,617 marine compounds, at least 4,196 compounds are reported to have bioactive

properties (Hu et al. 2015). Among these, 2225 (56%) compounds have anticancer activity, 521 (13%) have antibacterial properties, 14 % of the compounds have antivirus, antifungal, heart and vascular disease prevention, neuron protection or neurotoxicity, pesticidal activities and the activities of the remaining 16 % bioactive compounds could not be identified (Hu et al. 2015). Many secondary metabolites produced by many marine invertebrates have antitumor properties (Sima & Vetvicka 2011a) and the antitumor properties of marine natural products have driven the investigation into useful leads for the development of new chemotherapeutic agents.

Sourcing natural products in quantities required for drug development is the main impediment to the development of marine natural products (Molinski et al. 2009, Berrue et al. 2011, Martins et al. 2014) and so sustainable production of marine natural products, beginning with an understanding of their biosynthetic origin, requires investigation. Currently, one of the biggest challenges faced by researchers, or pharmaceutical companies, in the production and development of successful marine natural products is the supply, (Berrue et al. 2011, Martins et al. 2014) because many of these molecules are not only novel, but complex and difficult to synthesize (Berrue et al. 2011). Aquaculture of marine invertebrates along with genetic modification or microbial fermentation are alternative options for supplying sufficient amounts of pharmaceutical compounds for clinical testing and, commercialization (Sipkema et al. 2005, Benkendorff 2009). The commercialization of new marine drugs is challenging and needs to consider economic viability for sustainable production during the stages of development and drug approval.

1. 3 Role of microbial symbionts in producing marine invertebrate secondary metabolites

The involvement of marine microbes, associated with the host invertebrate, in the biosynthesis of the secondary metabolites is of growing research interest (Berrue et al. 2011). Marine microbial symbionts provide an alternative for identifying new marine natural products and producing them on a larger scale for drug development. A large number of marine natural products reported from marine invertebrates, including sponges, molluscs, tunicates and bryozoans, are non-ribosomal peptides, polyketides and hybrid molecules consistent with bacterial metabolites (Faulkner 2000). The structural similarities between bacterial and marine invertebrate natural products provide some indication that certain marine invertebrate natural products could actually be derived from microbial symbionts, rather than being synthesized directly by the host marine invertebrates (Lane & Moore 2011). Secondary metabolites, such as the polyketide onnamides with antitumor properties, have been confirmed to arise from bacterial symbionts of the host sponge *Theonella swinhoei* (Piel et al. 2004). Several other secondary metabolites have also been reported from microbial symbionts associated with marine sponges (Unson et al. 1994, Sacristan-Soriano et al. 2011, Freeman et al. 2015, Santos et al. 2015).

Microbial populations associated with other marine invertebrates are less-well studied compared to sponges. Nevertheless, bryozoans are also a source of bioactive metabolites such as the alkaloid bryostatins and phidolopins, which have been reported from microbial symbionts (Anthoni et al. 1990). Pyrrole pigments, such as tambjamines from bryozoans, were found to be related to bacterial products and could be of symbiotic origin or from a dietary source (Anthoni et al. 1990). Another antitumor metabolite, macrocyctic lactone bryostatin, which was first isolated from the bryozoan species *Bugula neritina* (Pettit et al. 1982, Sima & Vetvicka 2011b), requires a microbial symbiont for synthesis (Davidson et al. 2001, Lopanik

et al. 2004, Sudek et al. 2007). It is now confirmed that *Candidatus* Endobugula sertula, a microbial symbiont is the true producer of bryostatins and not the bryozoan, *Bugula neritina* (Lopanik 2015). Ecteinascidin 743 (ET-743), an anticancer agent is a tetrahydroisoquinoline marine natural product previously discovered from another ascidian *Ecteinascidia turbinata* (Rinehart et al. 1990). Rath et al. (2011) hypothesized that ET-743 is the natural product of a marine bacterial symbiont based on the structural similarities observed to three other bacterial secondary metabolites; that is saframycin Mx1 from *Myxococcus xanthus* Gram negative bacteria, saframycin A from *Streptomyces lavendulae* Gram positive bacteria, and safracin B from *Pseudomonas fluorescens*, a Gram negative bacterium (Arai et al. 1980, Ikeda et al. 1983, Irschik et al. 1988, Rath et al. 2011). Very recently, *Candidatus* Endoecteinascidia frumentensis, abacterial symbiont of *E. turbinata* has been identified as the true source of ET-743 (Schofield et al. 2015).

Recently, studies have also begun to investigate the microbial origin of some secondary metabolites found in molluscs. *Streptomyces* sp., a bacterial symbiont in the turrid snail *Lienardia totopotens*, was reported to be the source of peptide compounds namely totopotensamide A and totopotensamide B (Lin et al. 2012). It was also demonstrated that nocapyrones, a polyketide pyrone in the cone snail, *Conus rolani* is synthesized by bacteria, through the application of various chromatographic and molecular techniques such as genome sequencing (Lin et al. 2013). This shows that shelled gastropods can also be a potential source of microbially derived secondary metabolites. These examples suggest a microbial origin for a diverse range of marine invertebrate natural products, but more research is required to confirm biosynthesis by the bacterial symbionts in many cases.

Many marine bacteria cannot be easily cultured (Amann et al. 1995, Joint et al. 2010), however, the potential to culture using traditional or specially modified techniques for marine

bacteria, does provide an advantage for large scale production of natural products. Marine microbial symbionts associated with the host invertebrate having biomedical importance may provide an alternative for identifying new marine natural products and producing them on a larger scale for drug development (Gulder & Moore 2009). These microbial symbionts can possibly solve the sustainable supply issue for many marine invertebrates that produce bioactive compounds.

1. 3.1 Culture approaches used for isolating marine bacterial symbionts

A range of different culture media have been used to culture bacteria from marine invertebrates (Table 1.1). The majority of studies available on isolating marine bacteria used traditional nutrient media such as marine agar, nutrient agar, Luria-Bertani Medium, Bacto Marine Broth, Tryptic Soya Agar and Zobell Agar (Table 1.1). Overall, marine agar is the most commonly used bacterial media for isolating bacteria from marine invertebrates and a range of different types of bacteria have been recovered using this media. For example, Kurahashi and Yokota (2002) used marine agar for isolating bacteria from molluscs by incubating at 20°C for one week (Kurahashi & Yokota 2002). *Pseudoalteromonas, Vibrio*, *Photobacterium* and *Listonella* were also isolated by using marine agar plates from marine invertebrate samples incubated at 25 °C for 18 hours (Wilson et al. 2010). However, other media under specific conditions can target specific bacterial symbionts. For example, *Bacillus* sp. was isolated using Zobell agar from the egg capsules of *Concholepas concholepas* incubated at 20°C for 5 days (Leyton & Riquelme 2010). *Pseudomonas fulva* was isolated from the gills of the bivalve mollusc, *Geukensia demissa*, using Luria-Bertani Medium and Bacto Marine Broth incubated at 25 °C overnight in a rotary shaker (Loomis & Zinser 2001). Many studies have trialled a range of different culture media and conditions to maximise the diversity of bacteria recovered.

Romanenko et al. (2008) used Marine 2216 Agar and Tryptic Soya Agar for isolating marine bacteria, such as *Pseudoalteromonas, Pseudomonas and Sphingomonas* from *Anadara broughtoni*, an ark shell, by incubating at a range of temperatures with different incubation periods. Many epibiotic bacterial colonies were isolated from molluscan egg masses by using Zobell marine agar and Actinomycetes agar when incubated for 7 days at room temperature (Vijayalakshmi et al. 2008). The M1 agar, the Gause mineral agar and the glycerol asparagine agar (GA) were used for isolating *Marinobacterium, Pseudoalteromonas* and *Vibrio* from soft corals incubated at 25°C for 5 days (Chen et al. 2012). The incubation temperatures (15-28°C) used are generally selected to match the temperature range in the waters from which the samples were collected. Some studies used 37°C for isolating pathogenic bacteria (Bauwens et al. 2006), which is the internal body temperature of terrestrial vertebrates, including humans, but this is not ecologically realistic for marine microbial culture and more susceptible to contamination from human skin bacteria.

Table 1. 1. Examples of traditional methods for isolating marine bacteria from the tissues of marine invertebrates

Host	Media	Temp.	Duration	Bacterial sp.	Locations	References
Geukensia demissa,	Luria-Bertani Medium and Bacto Marine Broth	25°C	24 hrs	*Pseudomonas fulva*	Connecticut, U.S.A	Loomis and Zinser, 2001
Anadara broughtoni	Marine 2216 Agar and Tryptic Soya Agar	15, 28 and 37 °C	3,5,14,21days	*Gammaproteobacteria, Alphaproteobacteria,Bacillus and Saccharothrix*	Sea of Japan, Russia	Romanenko et al., 2008
Sea urchins, oysters, sponges	Marine agar	25 °C	18 hr	*Proteobacteria*	Sydney Harbour, Australia	Wilson et al., 2010
Mollusc	Marine Agar	20°C	1 week	*Vibrio, Rhodobacter, Alteromonas*	Kanto area, Japan	Kurahashi and Yokota, 2002
Seaweeds, Barnacles, Molluscan egg	Zobell Marine agar and Actinomycetes agar	37°C	7 days	Epibiotic bacteria	Tuticorin and Kovalam, India	Vijayalakshmi et al., 2008
Concholepas concholepas	Zobell agar	20°C	5 days	*Bacillus pumilus, Bacillus licheniform,* or *Bacillus* sp.	San Jorge Bay, Chile	Leyton and Riquelme, 2010
Soft corals	M1 agar, Gause mineral agar and glycerol asparagine agar	20°C	5 days	*Vibrio,Pseudovibrio, Pseudoalteromonas, Enterovibrio*	Nanwan Bay, Southern Taiwan	Chen et al., 2012
Stellaster equestris and soft coral	Marine agar	22–25 °C	5–7–10 days	*Erythrobacter*	South China Sea	Ivanova et al., 2004

Many attempts have been made to “culture the unculturable” bacteria from different environments. The majority of the media used to culture bacteria is nutrient rich, which mainly supports the growth of faster growing bacteria and creates unfavourable conditions for slow growing species (Connon & Giovannoni 2002, Vartoukian et al. 2010). The optimization of inoculum size, nutrient media, pH and incubation times also increases the recovery of previously uncultured microbes (Davis et al. 2005, Vartoukian et al. 2010, Pham & Kim 2012). *Bacteroides forsythus*, a fastidious anaerobic bacteria, can be easily cultured by supplementing synthetic tissue culture media with N-acetylmuramic acid (Wyss 1989). Increasing the incubation time to 24 weeks, coupled with lower temperature, results in the successful isolation of strains from the SAR11 clade (Alphaproteobacteria) (Song et al. 2009). Marine invertebrate extracts in artificial sea water can also mimic the natural environment for culturing previously uncultured bacteria (Li & Liu 2006, Montalvo et al. 2014, Xing et al. 2014). When compared with traditional culture methods, a combination of dilute media and longer incubation periods should be considered for isolating or culturing marine bacterial symbionts from marine molluscs.

Many bacteria are difficult to culture and only an estimated 0.001–0.1% of marine microbes can be successfully cultured (Amann et al. 1995). Therefore, molecular methods provide an alternative to culture dependent practices for identifying marine microbial symbionts and the biosynthetic origin of marine metabolites. Culture independent methods such as metagenomics have been used to study the unculturable microbial communities in environmental samples and provides a mechanism for estimating the total diversity of the microbial community (Handelsman 2004, Riesenfeld et al. 2004, Tringe et al. 2005). These results could then be used to direct the culture methods according to the types of microbes present in a sample. However, methods for culturing recalcitrant microbes could be key in many cases to achieve the ultimate goal of sustainable production of secondary metabolites of

microbial origin. In cases where this cannot be achieved, heterologous expression of the biosynthetic genes encoding the compound of interest may provide an alternative.

1. 4 Molluscan diversity and derived secondary metabolites

Molluscs, which include approximately 7% of living animals, occupy the second largest animal phylum on Earth, after the arthropods. There is an estimated 200,000 species and currently 52,525 species names are accepted for marine molluscs (Bouchet 2006, Benkendorff 2010, Benkendorff 2014). Molluscs are diverse and have adapted to a broad range of habitats including the marine environment, brackish water, freshwater and terrestrial regions resulting in a range of different lifestyles and feeding niches (Lindberg et al. 2004). Molluscs can be classified into seven classes namely Gastropoda (snails and slugs), Bivalvia (clam, mussels, scallops, oysters etc), Cephalopoda (octopus, squids, cuttlefish etc), Scaphopoda (tusk shells), Monoplacophora (monoplacophorans- one shell), Polyplacophora (chitons), and Aplacophora (aplacophorans-lack shells) (Lindberg et al. 2004, Saxena 2005). The class Gastropoda alone constitutes around 90% of the molluscan diversity (Benkendorff 2010) and is found across all marine and freshwater habitats (Lindberg et al. 2004). The second largest group of molluscs, and most diverse class after the gastropods, is Bivalvia with 10,000–20,000 species (Lindberg et al. 2004, Benkendorff 2010). The large adaptive capability within molluscan biology suggests their chemistry or secondary metabolism may also be diverse (Benkendorff 2010).

Molluscs are an important source of bioactive compounds and dyes. The diverse nature of molluscs in the marine environment has driven many chemical investigations towards isolating and identifying novel bioactive compounds. Among the marine molluscs, gastropods and bivalves have been of major interest to chemists, thereby yielding a wide range of chemical classes, some of which are commercially available and many others in clinical trials. For example, conus extract CGX-1160 (Newman & Cragg 2004), dolastatins 10 (Madden et al.

2000, Perez et al. 2005), kahalalide F and ES285 (Faircloth & Cuevas 2006). The isolation and identification of new bioactive compounds from marine molluscs is important mainly because of their potential application in therapeutics and pharmaceutical development (Benkendorff 2010, Sarumathi et al. 2012). The sole marketed pharmaceutical product derived from marine molluscs is the pain relief medication Ziconotide, a neurotoxic conotoxin isolated from the venom of the marine cone snail, *Conus magus* (McIntosh et al. 1982). It was approved by the United States Food and Drug Administration in 2004 under the commercial name PRIALT(R) (Skov et al. 2007, Joseph et al. 2011)**.** Besides therapeutic and pharmaceutical products, there are also important nutraceuticals or natural health products from molluscs. These include the patented and clinically tested mussel extract, Lyprinol. It is a commercially available lipid extract obtained from *Perna canaliculus*, the New Zealand green lipped mussel, and is used for treating osteoarthritis, asthma and cancer (Gibson & Gibson 1998, Sukumaran et al. 2010, Abdulazim et al. 2012). Another patented product from molluscs is Cadalmin TM GMe, which was developed from extracts of the Indian Green mussel, *Perna viridis* (Rao et al. 2011). These examples demonstrate the potential value of strategic research into the development of molluscan bioactive compounds and extracts.

1. 4. 1 Muricids as a source of Tyrian purple and its bioactive precursors

The muricids, also known as murex snails and rock snails, are a family of neogastropods, which display unique shells and occur globally (Radwin & D'Attilio 1976, Taylor et al. 1980, Carpenter & Niem 1998). Muricids are predatory marine gastropods that prey on other marine invertebrates such as bivalves, gastropods, barnacles, tube worms and ascidians (Taylor et al. 1980, Carpenter & Niem 1998). Marine gastropods of the family Muricidae are well known for the production of the dye Tyrian purple (Cooksey 2001, Westley

& Benkendorff 2008). Tyrian purple, also known as royal purple, is a dye of historical importance since it was the first true purple dye (Westley & Benkendorff 2008). Although Tyrian purple was discovered from Mediterranean muricids and named after the Phoenician city of Tyre (Cooksey 2001), it was referred to as *Purpura* in ancient times and was regarded as a luxury good (Melo 2009).

The hypobranchial glands of Muricidae are the only natural source of Tyrian purple (Cooksey 2001). The hypobranchial gland of live muricids do not contain Tyrian purple, but it is formed through a series of reactions on smears of the gland taken from live specimens (Baker & Sutherland 1968). Consequently, although biosynthesis of the precursors of Tyrian purple occurs in the hypobranchial gland, the deep purple colour is an artefact generated from exposure to sunlight and oxygen (Cooksey 2001). The generation of Tyrian purple from the main precursor, tyrindoxyl sulphate stored in the hypobranchial gland is well characterized (Baker & Sutherland 1968, Baker & Duke 1973, Cooksey 2001, Westley & Benkendorff 2009). However, the entire biosynthetic pathway for the production of Tyrian purple precursors is yet to be elucidated.

Friedlander first identified the chemical structure of Tyrian purple as 6,6'-dibromoindigo from *Murex brandaris* (Freidlander 1909) and this finding has given a strong basis for the investigation into the generation of Tyrian purple dye in *D. orbita*. Another major achievement was from the work of Baker and Sutherland in 1968, which identified colorless tyrindoxyl sulphate (Fig. 1. 3) as the ultimate dye precursor in the hypobranchial gland of *D. orbita* and they also identified an enzyme with sulphatase activity that can hydrolyse tyrindoxyl sulphate (Baker & Sutherland 1968). The ethanol extracts of *D. orbita* hypobranchial gland also revealed the presence of choline and showed that tyrindoxyl sulphate is stored as choline ester salt (Baker & Duke 1976). The hydroxylation of this salt by the arylsulphatase enzyme in

the hypobranchial gland results in the generation of intermediate Tyrian purple precursors (Baker & Sutherland 1968, Benkendorff 2013) (Fig. 1. 3). The other intermediate precursors of Tyrian purple were isolated and identified as tyrindoxyl, tyrindolinone, tyrindoleninone (Baker & Duke 1973, Baker & Duke 1976, Cooksey 2001) and tyriverdin (Baker & Sutherland 1968, Christophersen et al. 1978). Minor components of the Tyrian purple dye include 6-bromoisatin, which is a yellow oxidation byproduct and the precursor of the red Tyrian purple isomer 6,6'-dibromoindirubin (Baker & Sutherland 1968, Cooksey 2001).

The biochemical pathway for Tyrian purple production from *D. orbita* is outlined in Figure 1. 3. It is hypothesized that dietary derived tryptophan **(1)**, which is an essential amino acid, may be the source for indole production, in which tryptophanase enzymes act on tryptophan to produce indoles **(2)** (Verhecken 1989, Naegel & Alvarez 2005, Westley & Benkendorff 2009, Laffy 2011). Indoxyl may be formed through hydroxylation of indoles, then sulphation of indoxyl results in indoxyl sulphate **(3).** As molluscs, like all animals, cannot biosynthesize tryptophan (Westley 2008, Westley & Benkendorff 2009), this essential amino acid might be diet or bacteria derived. Westley and Benkendorff, (2009) have identified the presence of tryptophan in the hypobranchial and rectal gland of *D. orbita* from histochemical analysis.

The role of bromoperoxidase in the addition of bromine to the 6-position of tyrindoxyl sulphate **(4)** has also been suggested (Benkendorff 2013, Benkendorff et al. 2015). This is supported by bromoperoxidase activity in homogenates of *Murex trunculus* hypobranchial gland (Jannun & Coe 1987) and also by histochemical analysis in the gland of *D. orbita* (Westley 2008, Westley & Benkendorff 2009). Bromoperoxidase enzyme activity occurs in the hypobranchial and rectal gland, as well as female reproductive organs (capsule and albumen glands) of *D. orbita* (Westley 2008). There appears to be no other reports of bromoperoxidase

enzymes in the phylum Mollusca. However, bromoperoxidase enzymes have been previously identified from marine bacteria such as *Streptomyces* sp. (Butler & Walker 1998) and *Synechococcus* sp. (Johnson et al. 2011). This suggests the possibility of a role for symbiotic bacteria in the bromination of Tyrian purple precursors.

Tyrindoxyl sulfate, the ultimate Tyrian purple precursor in *D. orbita* is oxidized by arylsulphatase to produce tyrindoxyl **(5)** (Baker & Sutherland 1968). This enzyme has been detected histochemically in the hypobranchial and rectal glands, and in the vascular sinus of *D. orbita* (Westley 2008). The arylsulphatase coding gene is the only gene involved in Tyrian purple production that has so far been identified from a transcriptome of the hypobranchial gland of *D. orbita* (Laffy *et al*., 2013). Laffy et al. (2013) reported expression of arylsulphatase in the hypobranchial gland, but no gene expression from mantle of *D. orbita*, confirming this enzyme is not acquired from bacterial symbionts or dietary sources, but is a *D. orbita* gene expressed in the gland. The full length arylsulphatase gene sequence from *D. orbita* is now available in NCBI Genbank with accession number HM246144 (Laffy 2011), along with another putative arylsulphatase gene sequence with accession number GD253910 (Laffy et al. 2013). All of remaining biosynthetic genes involved in Tyrian purple production are yet to be identified.

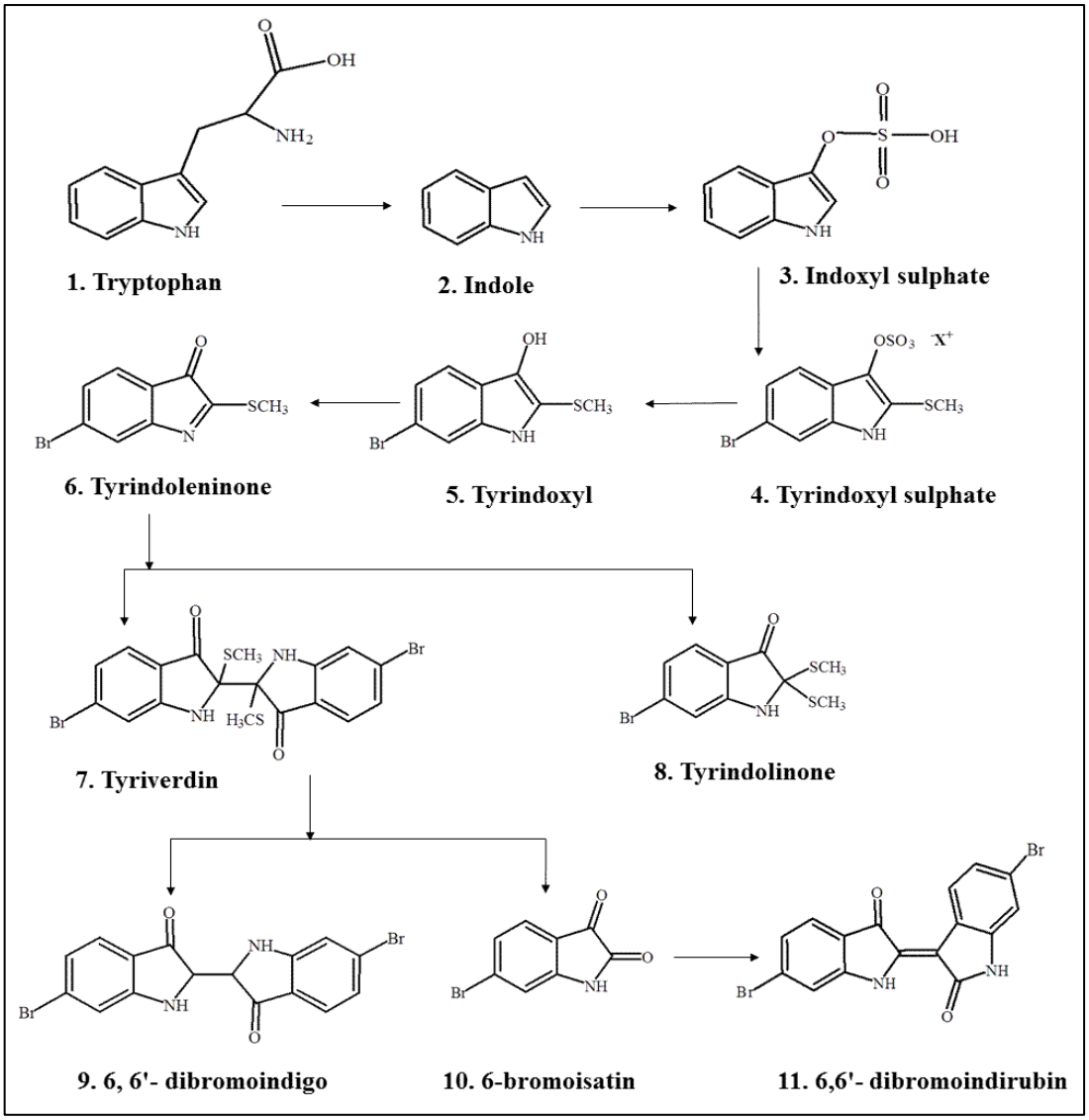

Fig. 1.3. The proposed generation of Tyrian purple and derivatives from tryptophan in *Dicathais orbita*.

1. 4. 2 Bioactivity of *Dicathais orbita* secondary metabolites

Extracts from the hypobranchial glands and egg masses of muricids have been an area of interest for their bioactive properties (Benkendorff et al. 2015). The extracts and Tyrian purple precursors from *D. orbita* have anticancer and antimicrobial properties (Table 1.2) (Benkendorff 2010, Westley et al. 2010b, Edwards et al. 2012, Benkendorff 2013, Esmaeelian et al. 2013, Esmaeelian et al. 2014, Benkendorff et al. 2015). The anti-cancer properties include

inhibition of the proliferation of a range of cancer cell lines by the brominated indoles, mainly tyrindoleninone, tyrindolinone, 6-bromoisatin and 6,6'-bromoindirubin and also crude extracts from the egg masses and hypobranchial glands (Meijer et al. 2003, Vine et al. 2007, Westley et al. 2010b, Benkendorff et al. 2011, Benkendorff et al. 2015). *In vitro* studies of semi purified extracts from *D. orbita* indicates that tyrindoleninone has cytotoxic activity against U937 and Jurkat lymphoma cell (Benkendorff et al. 2011). Edwards et al. (2012) also reported the specific anticancer properties of brominated indoles isolated from *D. orbita* in treating female reproductive cancer cell lines including uterine, granulosa and endometrial derived cancer cells, with significantly lower activity against freshly isolated healthy granulosa cells. Crude extracts of *D. orbita* containing tyrindoleninone and 6-bromoisatin produce a dose-dependent increase in apoptotic response of DNA damaged cells in the distal colon of mice, using a prevention model for colon cancer (Westley et al. 2010b). An increase in apoptosis was reported after two weeks of daily administration of crude extracts of *D. orbita* at a dose of 1.0 mg/g (Westley et al. 2010b). A more recent study on the purified compounds reported 6-bromoisatin as the principle active compound *in vivo*, and the most active compound against colon cancer cells *in vitro* (Esmaeelian et al. 2013). Synthetic 6-bromoisatin inhibits colon cancer cell proliferation and prevents early tumour formation in rodent colons (Esmaeelian et al. 2014). These *D. orbita* extracts also show no major toxic side effects in mice after 4 weeks of daily administration (Westley et al. 2013) and did not cause inflammation when administered alone in a rat model for mucositis (Yazbeck et al. 2015). 6, 6' Dibromoindirubin was found to be an inhibitor of glycogen synthase kinase-3(GSK-3), thereby also showing potential as an anticancer agent (Meijer et al. 2003). *D. orbita* is an edible snail and has been included as part of the traditional human diet. Therefore a purified extract concentrating the active factors could serve as a potential novel medicinal and functional food, to prevent colorectal cancer in humans (Benkendorff 2013, Benkendorff et al. 2015, Yazbeck et al. 2015).

Muricid extracts also show antimicrobial activity (Table 1.2). The brominated indole precursors of Tyrian purple show strong inhibitory activity against a range of pathogenic bacteria (Benkendorff et al. 2000, Benkendorff et al. 2001a). The bacteriolytic properties of extracts from *D. orbita* egg masses are mainly due to the purple precursor tyrindoleninone, whereas tyriverdin is a bacteriostatic compound and 6-bromoisatin is mildly cytotoxic (Benkendorff et al. 2000). Antimicrobial activities have also been reported from other muricids species such as *Chicoreus virgineus, C. ramosus, Rapana rapiformis, Cronia margariticola, Murex tribulus, Thais biserialis, T. bufo* and *T. tissoti* (Table 1.2) (Benkendorff et al. 2001a, Murugan & Ramasamy 2005). Lim et al. (2007) reported the antifouling properties of *D. orbita* egg capsules and antibacterial properties were demonstrated against biofilm forming microbes, such as *Pseudoalteromonas* sp. S91 (Lim et al. 2007). The egg masses of *D. orbita* have high concentrations of tyrindoleninone, tyriverdin and 6-bromoisatin with strong antimicrobial properties (Benkendorff et al. 2001a, Westley 2008) and also shown antibacterial activities against some marine bacteria (Benkendorff et al. 2001b) (Table 1.2). These finding highlight the importance of indole derivatives in muricids, which are thought to be maternally transferred from the hypobranchial glands to the egg masses, as a chemical defence against pathogenic organisms (Rudd et al. 2015). Overall, the Australian mollusc, *D. orbita,* is a highly valuable species as a model for Tyrian purple biosynthesis and its potential in future medicinal research.

Table 1. 2. Bioactivity of crude extracts, egg mass and isolated compounds from muricid species.

Muricidae sp.	Source	Compound	Bioactivity	Target tissue or cells	References
Dicathais orbita	Egg mass extract	Tyrindoleninone and 6-bromoisatin	Anticancer	Reproductive cancer cell lines, colorectal cancer cell lines	(Edwards et al. 2012, Esmaeelian et al. 2013, Benkendorff et al. 2015)
	Hypobranchial glands, egg masses extract	Brominated indoles	Anticancer	Colon cancer prevention (mouse model), small intestine and liver toxicity	(Westley et al. 2010b, Westley et al. 2013, Benkendorff et al. 2015)
	Egg mass extracts, murex remedy	Tyrindoleninone and 6-bromoisatin	Anticancer	Human carcinoma and lymphoma cells	(Benkendorff et al. 2011)
	Hypobranchial gland	Choline esters	Muscle relaxant	-	(Baker & Sutherland 1968, Roseghini et al. 1996, Benkendorff 2013, Rudd et al. 2015)
	Hypobranchial gland	Mixture of brominated indoles mainly tyrindoleninone	Anticancer	Colorectal cancer and novel functional food	(Yazbeck et al. 2015)
	Hypobranchial gland	Tyrindoleninone, tyrindolinone and 6-bromoisatin	Anticancer	Female reproductive cells	(Edwards et al. 2014)
	Egg mass	Tyriverdin, 6-bromoisatin	Bacteriostatic, Antimicrobial	*P. aeruginosa, E. sericolicida, V. angillarium, S. aureus and E. coli*	(Benkendorff et al. 2000)
	Egg mass	-	Antibacterial	*V. anguillarum, V. alginolyticus, V. harveyi* and *E. sericolicida*	(Benkendorff et al. 2001b)
	Egg capsules	-	Antifouling	*Pseudoalteromonas* sp.	(Lim et al. 2007)
Hexaplex trunculus	Tyrian purple dye	6,6'-dibromoindirubin	Anticancer	Glycogen synthase kinase-3 inhibitor	(Meijer et al. 2003)
Chicoreus virgineus, C. ramosus	Whole body and egg methanol extracts	Crude extracts	Antimicrobial	Biofilm forming bacteria	(Murugan & Ramasamy 2005)

Thais sp.	Whole body methanol extracts	Crude extracts	Antimicrobial	Biofilm forming bacteria	(Murugan & Ramasamy 2005)
Rapana rapiformis	Whole body and egg acetone extracts	Crude extracts	Antimicrobial	Biofilm forming bacteria	(Murugan & Ramasamy 2005)
Cronia margariticola	Whole body methanol dichloromethane and egg methanol extracts	Crude extracts	Antimicrobial	Biofilm forming bacteria	(Murugan & Ramasamy 2005)
Murex trapa *Murex tribulus* *Muricanthus fulvescens* *Ocenebra erinacea Urosalpinx cinerea Urosalpinx heptagonalis* *Bolinus brandaris* *Chicoreus brunneus* *Chicoreus florifer* *Chicoreus ramosus* *Chicoreus torrefactus* *Concholepas concholepas* *Hexaplex truculus*	Hypobranchial gland	murexine dihydromurexine, *n*-methylmurexine , senecioylcholine and tigloylcholine	Muscle relaxant	-	(Roseghini et al. 1996)
Not Applicable	Synthetic	Isatin derivatives, 6-bromoisatin	Anticancer	Lymphoma cells	(Vine et al. 2007)
Not Applicable	Synthetic	6-bromoisatin	Anticancer	Colorectal cells	(Esmaeelian et al. 2014)
Not Applicable	Synthetic	Indirubin derivative-6-Bromoindirubin-3'-oxime (6BIO)	Anticancer	Lungs	(Braig et al. 2013)

1. 4. 3 Hypobranchial gland of muricid molluscs

The Tyrian purple precursor, tyrindoxyl sulphate, occurs in the hypobranchial glands, as well as the egg masses and reproductive glands of both male and female *D. orbita* (Westley & Benkendorff 2008). The dye pigment is also associated with mucus secreted by *Plicoplura pansa* (Naegel 2004). Tyrian purple can also be obtained from the mucus of *D. orbita* after anesthetisation in 7% ethanol or using benzocaine (Noble et al 2009). The function and anatomical position of the hypobranchial gland may explain these various sources of Tyrian purple precursors in the mollusc.

The hypobranchial gland is uniquely found in gastropod molluscs with a primary function to produce mucus (Westley et al. 2010b). The hypobranchial gland lies within the mantle cavity where it is thought to filter foreign particles, like bacteria, in the sticky mucus (Westley et al. 2010a). The hypobranchial gland lies adjacent to the rectal gland and the reproductive organs in muricids (Benkendorff et al. 2004a, Westley & Benkendorff 2009). In *D. orbita*, the rectal gland is embedded in the medial hypobranchial gland, making it difficult to separate these organs, but the rectal gland is visible as a pigmented elongated structure (Westley & Benkendorff 2009). The hypobranchial gland and the gonoduct (capsule and albumen glands) have the same biochemical secretions and biosynthetic enzyme activities, which provides evidence that Tyrian purple precursor production may also occur in the gonoduct (Benkendorff et al. 2004a, Westley 2008, Westley & Benkendorff 2008, Westley & Benkendorff 2009) and mass spectrometry imaging has demonstrated that these compounds are transferred into the egg masses during the reproductive phase (Rudd et al. 2015).

Within the Muricidae, the hypobranchial gland plays an important role in the biosynthesis of Tyrian purple and its precursors, however, its detailed ecological function is

not fully understood. Amongst the gastropods, the Muricoidea superfamily of neogastropods have particularly well developed hypobranchial glands (Roseghini et al. 1996) and a detailed study on histomorphology and histochemistry of the hypobranchial gland of *D. orbita* was carried out by Westley et al. (2009, 2010). In *D. orbita*, the hypobranchial gland is made up of ciliated and non-ciliated pseudostratified epithelium and is divided into three areas; medial, branchial and rectal area. The medial area lies in a lower depression between the branchial and rectal area and is the main pigmentation area where colour changes occur when exposed to sunlight and oxygen, accompanied by the release of sulfurous odour. The bromoperoxidase activity occurs in the rectal area of the gland and tryptophan is stored in the branchial area before being brominated or converted to tyrindoxyl sulfate for Tyrian purple production (Westley et al. 2010b).

A preliminary gene expression study of *D. orbita* hypobranchial gland undertaken by Laffy et al. (2011) revealed a high proportion of unknown genes. A total of 110 *D. orbita* genes were identified and 24 of these coded for rRNA genes. The other *D. orbita* genes contribute to protein synthesis, choline ester regulation, electron transport, neurotransmitter and cellular processing. In addition to the *D. orbita* genes, a further 61 genes were identified that were consistent with ciliate protozoans (Laffy et al. 2009, Laffy 2011, Laffy et al. 2013). Laffy, (2011) confirmed the presence of symbiotic ciliates in the hypobranchial gland of *D. orbita* by histology, however, the role of the ciliates is not clear. Considering cilliates are typically bacterivorous, it is likely that the hypobranchial gland also supports populations of bacteria, as their abundance in the hypobranchial gland indicates that there is likely to be a bacterial source of food to support them (Benkendorff 2013). The ciliates do not appear to be the source of Tyrian purple, however, as there was no evidence of tyrindoxyl sulfate secretion or storage associated with the ciliates form histochemical evidence (Laffy *et al.* unpublished

data). Thus further research is required on both the transcriptome of *D. orbita* and the microbial symbionts to help identify the biosynthetic origin of Tyrian purple precursors.

1. 5 Marine bioinformatics- an overview of omics approach

Species specific gene expression may provide insights into the biosynthetic processes and origin of marine secondary metabolites. Comparisons of gene expression within different tissues and under different environmental conditions could be useful if the biosynthetic organs and conditions for secondary metabolite production are known. A bioinformatics approach could ultimately help identify the structure, function and evolution of proteins involved in the biosynthesis of small molecules (Luscombe et al. 2001, Vinithkumar 2006), including secondary metabolites produced by marine invertebrates or their associated microbes.

More generally, the use of bioinformatics is required to analyse and interpret complex molecular data from marine organisms. Bioinformatics is a term that encompasses the computing tools that underpin the new "omics" era of biological research, with applications in genomics, transcriptomics, proteomics and metabolomics (Luscombe et al. 2001). Marine bioinformatics uses computer and networking technologies to collect, store, analyse, interpret and disseminate data on marine organisms, including taxonomic classification, phylogenetic and biomolecular structure, as well as gene sequence data (Vinithkumar 2006).

1. 5. 1 Genomics

Genomics is based on DNA sequencing and is now a well-established discipline. According to the Genomes OnLine Database, as of 3rd January 2016, 2215 genomes (Eukaryota, bacteria, archaea) have been completed and 7131 permanent draft genomes are currently reported in the database (Reddy et al. 2015). The nucleotide sequence data of different marine invertebrate phyla presently available (as of 18th November 2015) in GenBank NCBI are presented in Table 1.3. Most of these nucleotide sequences are based on taxonomic or phylogenetic markers such as 16 and 28s rRNA, cytochrome oxidase etc. The Phylum Mollusca has the most nucleotide sequences compared to other marine invertebrates (Table 1.3). Among the Mollusca, the class Gastropoda has the highest number of nucleotide sequences (Table 1.3) thus reflecting gastropods as the most diverse group currently under investigation within the Mollusca.

Table 1.3. Number of nucleotide sequences available in GenBank NCBI database for different marine invertebrate phyla.

Phyla/ subphyla	Number of GenBank nucleotide sequence
Annelida	1112785
Brachiopoda	60426
Cnidaria	1880123
Echinodermata	2723901
Tunicata	1860537
Porifera	159276
Bryozoa	13024
Crustacea	3667387
Mollusca	4234890
Class	
Gastropoda	2917900
Bivalvia	1128021
Cephalopoda	182646
Polyplacophora	4978
Scaphopoda	101
Monoplacophora	53

Full genome sequences are extremely useful in the study of developmental biology and evolution. However, they can also provide a valuable insight into the potential biosynthetic genes in species known to *de novo* synthesised secondary metabolites. At present, the completed genome sequences from molluscs are limited to three species: *Crassostrea gigas*, a pacific oyster (Zhang et al. 2012), *Octopus bimaculoides* (California two-spot octopus) (Albertin et al. 2015) and *Lottia gigantea*, a giant owl limpet (Simakov et al. 2013). Among the molluscs, the smallest genome at 420.54 Mb (megabases) is from the Eogastropoda gastropod *Lottia gigantea* (Gregory 2005), while the largest genome, of 7677.3 Mb, is predicted to be from the Caenogastropoda gastropod snail, *Diplommatina kiiensis kiiensis* (Gregory 2005). The most highly evolved Neogastropoda, including the Muricidae, have relatively large genomes ranging from 1956 Mb (*Olivella biplicata*) to 5750.64 Mb (*Neobuccinum eatoni*) (Gregory 2005).

The publication of the first complete mollusc genomes has provided a useful scaffold for identifying coding gene regions in other molluscs. *Lottia*, a gastropod is from a lineage of Eogastropoda that are known to have diverged early from all other orthogastropods (including the caeno and neogastropods) (Lindberg et al. 2004). Bivalves, cephalopods and gastropods diverged several million years ago and consequently more genome research on the diverse gastropods is required. A preliminary genome sequencing indicates that the *D. orbita* genome is very large, with many repetitive sequences (Benkendorff, Pers. Comm.) providing challenges in compiling the full genome. Consequently, there are advantages in first focusing on the transcriptome to identify the genes that are coded and expressed in the genome. Hence a transcriptome approach was undertaken for the identification of expressed genes in this study.

1. 5. 2 Transcriptomics

The "post-genomics era" has recently seen an expansion in the application of bioinformatics in the fields of transcriptomics, proteomics and metabolomics (Zhang et al. 2010). Transcriptomics describes genome wide mRNA analysis and thus provides information on the genes expressed by an organism under certain circumstances or stage of development in a particular tissue or cell type (Brown 2002, Adams 2008) and thus provides opportunities for comparisons of gene expression within different tissues and under different environmental conditions. If enough different tissues are sequenced with sufficient depth, it can also provide detailed information on the organisms coding genes and a good scaffold for compiling the species genome.

Transcriptomics is commonly applied to the identification of genes involved in the biosynthesis of secondary metabolites (Sun et al. 2010) and can also establish cellular and molecular interactions. Transcriptomic studies in molluscs have been used for establishing the developmental processes (Heyland et al. 2011, Huang et al. 2012), cellular and physiological mechanisms (Zhang et al. 2012, Meng et al. 2013), immunology (Manubens et al. 2010, Franchini et al. 2011, Romero et al. 2012, Pauletto et al. 2014, He et al. 2015), shell formation (Jackson et al. 2006, Gaume et al. 2014, Jia et al. 2015, Liang et al. 2015), host parasite interactions (Knight et al. 2002, Lockyer et al. 2008, Coustau et al. 2015) and functioning of nervous systems (Moroz et al. 2006, Feng et al. 2009, Wollesen et al. 2014, Adamson et al. 2015).

Transcriptome profiling studies on predatory marine neogastropods are very limited. These include gonadal tissues of *Reishia* (Thais) *clavigera* with 104,207,350 reads, 197,324 transcripts (Ho et al. 2014), the mantle, foot, gills and gonadal tissues of *Concholepas*

concholepas with 140,756 reads (Cardenas et al. 2011), the alimentary canal and salivary glands of *Colubraia reticulata* with 220,305,266 reads, 144,380 transcripts (Modica et al. 2015), the venom glands of *Conus consors*, 213,561 reads (Terrat et al. 2012) and the venom ducts of *C. tribblei* with 829,718,44 reads (pooled from three *C. tribblei*) and 327,700 transcripts and *C. lenavati* with 940,013,72 reads (pooled from three *C. lenavati*) and 298,481 transcripts (Barghi et al. 2015).

In cases where the biosynthetic organ is known within a species, it is possible to compare the gene expression in this tissue to a tissue that does not produce the secondary metabolites. A suppressive subtraction hybridisation clone library was created to target the genes that were upregulated in the hypobranchial gland, a biosynthetic organ known as the source of Tyrian purple in *D. orbita* (Baker & Sutherland 1968), relative to the mantle tissue that does not produce Tyrian purple (Laffy, 2011, Laffy et al., 2013). Using this approach, an arylsulfatase enzyme was identified and then confirmed to be upregulated in the hypobranchial gland by real-time PCR (Laffy et al., 2013). However, other biosynthetic enzymes such as tryptophanase and bromoperoxidase (Laffy 2011), which are also thought to play an important role in Tyrian purple precursor biosynthesis (Westley et al. 2006, Benkendorff 2014), were not identified in a *D. orbita* EST hypobranchial gland library, using the primers designed from sequences of marine algae and bacteria. This may be due to the limitations in the technique used, or it may imply that the ultimate precursor of Tyrian purple is supplied to the host by a microbial symbiont. These transcriptome studies on *D. orbita* were limited by the short sequence reads generated by suppressive subtractive hybridisation, which may explain why many of the gene sequences were not identified. More detailed transcriptomics using longer sequence reads are required to resolve the likely biosynthetic origin of the Tyrian purple precursor *D. orbita* secondary metabolites.

1. 5. 3 Metagenomics- Culture independent approach for profiling bacterial symbionts

Metagenomics captures the genomes of micro-organism communities rather than individual genomes and facilitates assessments linked to physiology and genetics of uncultured microbes (Handelsman 2004). Marine microbe metagenomics databases are currently one of the richest databases in the field of marine ecology and oceanography (Gilbert & Dupont 2011). Metagenomics can be used to identify the diversity and interaction of endo-symbiotic bacteria within marine invertebrates.

Metagenome analysis involves direct isolation of total genomic DNA from an environmental sample (Fig. 1.4). Previously, the bacterial DNA would be amplified then used to create a clone library by ligating into a suitable vector and transformation of *Escherichia coli* (Li & Qin 2005). However, this approach has been largely replaced by approaches that allow rapid bacterial profiling and searching for bacterial genes within eukaryotic genomes (Thomas et al. 2010). High-throughput sequencing based on 16S rRNA gene is commonly used for detailed studies of the microbial communities by isolating the total DNA from the environment without the need for traditional culture techniques of the microbial communities (Roesch et al. 2007, Petrosino et al. 2009). For example, 16S rRNA universal bacterial primers can amplify the majority of bacterial target sequences, which are then submitted for direct sequencing by the Illumina MiSeq or Hi Seq, followed by bioinformatics analysis to establish the number and type of distinct species (Webster et al. 2010, Alex & Antunes 2015, He & Zhang 2015). This approach can be applied to gain an overview of the types of microbial symbionts within host marine invertebrates for comparison with the diversity of culturable micro-organisms.

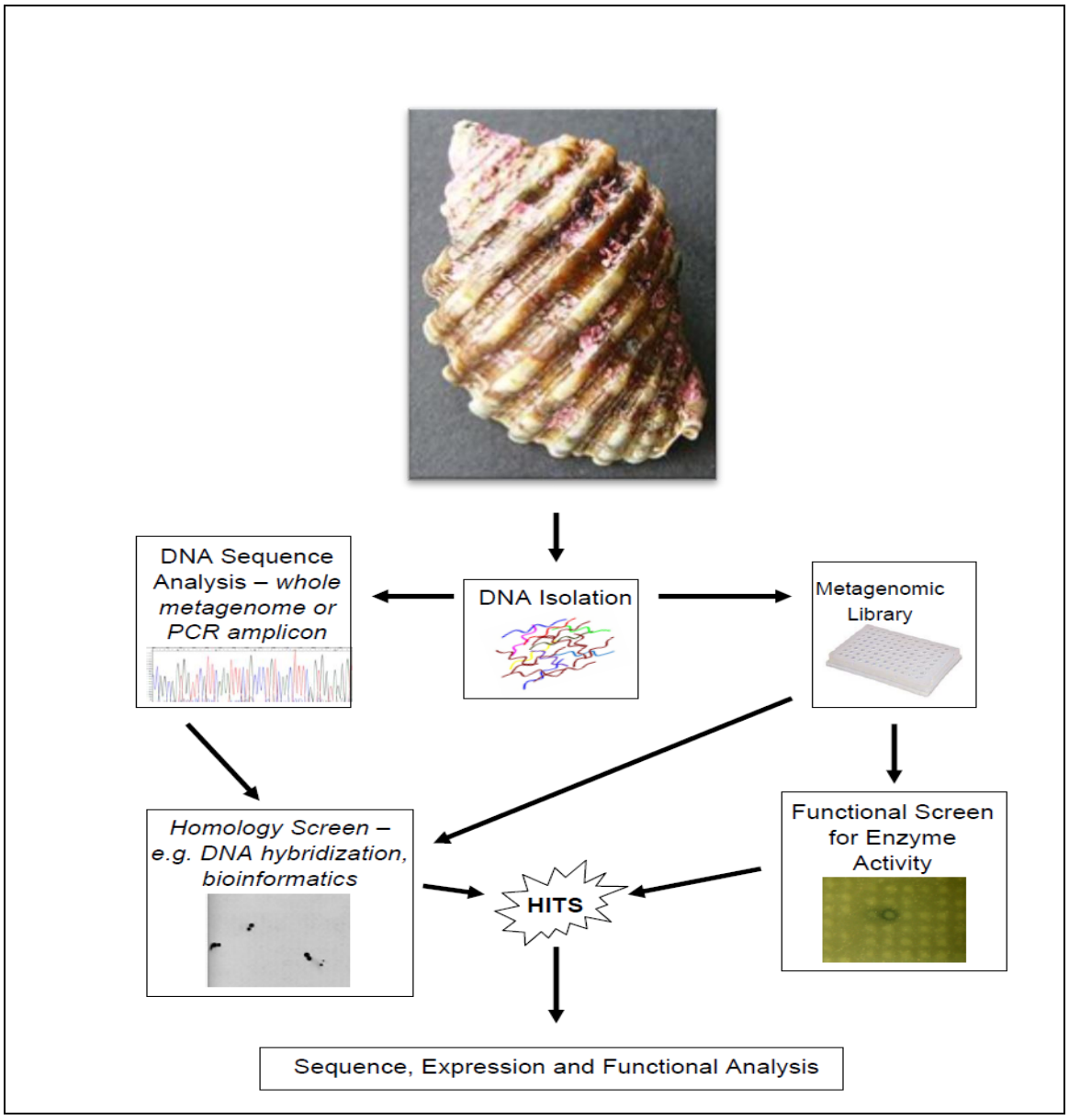

Fig. 1. 4. Construction of metagenomic library and its application, adapted from (Kennedy et al. 2010).

1. 6 Thesis aims, significance, structure and objectives

With the advances being made to explore the biosynthetic origin of marine secondary metabolites, and the underlying mechanisms at a molecular level, there is promise in advancing our knowledge and understanding of the biosynthetic pathway of Tyrian purple

and its precursors in *D. orbita* by using a range of culture and culture independent approaches on the hypobranchial gland. This thesis aims to establish the biosynthetic origin and identify genes involved in the biosynthesis of 6-bromoindole derivatives in Muricidae molluscs. This study involves a combination of traditional bacterial culturing techniques, targeted isolation of bacteria involved in Tyrian purple precursor synthesis and screening of the isolates for indole production and bromoperoxidase genes, high-throughput bacterial profiling using 16S rRNA bacterial genes and transcriptomic approaches for identifying biosynthetic genes associated with Tyrian purple precursor synthesis in *D. orbita.*

In this thesis, samples from both male and female *D. orbita* were taken to allow more comprehensive coverage of the transcriptome (Chapter two) and bacterial communities (Chapter three) from the gender specific reproductive organs. Furthermore, in Chapter three and Chapter four, the bacterial communities were compared between male and female hypobranchial glands, as previous studies have demonstrated different compositions of the extracts. The female glands when compared to male have higher amounts of reduced compounds, such as tyrindoleninone and tyriverdin, whereas males glands have more oxidised compounds namely, 6-bromoisatin and 6,6'dibromoindirubin (Westley & Benkendorff 2008).

1. 6. 1 Thesis structure

This thesis is presented in manuscript format. The underlying principal concept of the thesis is to present a progressive body of research, although each chapter is intended for independent publication, or in the case of chapter two, three and four have already been published in peer reviewed international journals. All the chapters have been formatted in a consistent manner and have adopted the referencing format outlined in Journal of Shellfish

Research in order to maintain the uniformity and continuity of presentation. The literature cited in each manuscript has been complied as a single reference list at the rear of the thesis to avoid repetition. All experiments in subsequent chapters have been designed in collaboration with my supervisors, who are also co-authors on the publications. I was solely responsible for conducting the experiments and obtaining the data. Supervisors and other co-authors assisted with some of the data annotation and analysis (Appendix I). I prepared the first draft of all manuscripts. The following outlines the objectives of each chapter and indicates the publication status.

1. 6. 2 Chapter objectives

Chapter two. Transcriptome of the Australian mollusc *Dicathais orbita* provides insights into the biosynthesis of indoles and choline esters.

Objective: Chapter two aims to generate transcriptome libraries from the hypobranchial gland, prostate gland, capsule gland, albumen gland, and mantle and foot tissues of *D. orbita* using Illumina HiSeq to profile the gene expression and identify expressed genes potentially involved in the biosynthesis of Tyrian purple dye precursors.

Chapter three. Indole producing bacteria from the biosynthetic organs of a muricid mollusc could contribute to Tyrian purple production.

Objective: Chapter three aims to compare the culturable bacteria and biochemical profiles of microbial communities derived from different tissues of *D. orbita,* in order to establish whether any unique bacteria or distinct bacterial communities are associated with the biosynthetic organs compared to tissues that are known not to be involved in Tyrian purple

production. A secondary aim was to culture aerobic heterotrophic bacteria from these tissues and then identify any indole producing bacteria using 16S rRNA sequencing.

Chapter four. Characterization of bacterial communities associated with the Tyrian purple producing gland in a marine gastropod.

Objective: Chapter four aims to quantify the diversity of bacteria associated with the Tyrian purple producing hypobranchial gland using high-throughput sequencing (454 GS FLX Titanium) of the variable region V1-V3 16S rRNA bacterial gene. Comparison of these sequences with equivalent sequence data from the foot tissue contributed to the identification of bacteria specifically associated with, or more abundant in, the Tyrian purple producing hypobranchial gland. A further aim was to identify bacteria with potential to produce indoles and brominated compounds based on published information.

Chapter five. Evidence for a bacterial origin for Tyrian purple precursors in muricid molluscs.

Objective: Chapter five aimed to expand the culture conditions used in Chapter three to target additional bacteria identified in Chapter four that are potentially involved in Tyrian purple precursor synthesis, and then screen the isolates for indole production and bromoperoxidase genes using molecular methods. The bacteria were also screened for their ability to produce tyrindoxyl sulfate in potassium bromide supplemented media using liquid chromatography mass spectrometry.

1. 6. 3 Publications which are incorporated into thesis and contribution statement

The statement of authorships to a joint publication are provided in Appendix I.

Chapter 2–Ngangbam, A. K., Baten, A., Waters, D. L. E. and Benkendorff, K. 2016. Transcriptome of the Australian mollusc *Dicathais orbita* provides insights in the biosynthesis of indoles and choline esters. *Marine Drugs 2016, 14, 135*; *doi:10.3390/md14070135*

http://www.mdpi.com/1660-3397/14/7/135

My principal supervisor, K.B. initiated the study and all authors contributed to the experimental design. I did the sampling, dissections, RNA extraction and RNA quality check for transcriptome sequencing, A.B. assembled and annotated the transcriptome. All authors contributed to the data analysis and interpretation. A.B. and I wrote the first draft of the paper together with input from K.B on biosynthetic pathways and editorial feedback from D.W.

Chapter 3– Ngangbam, A. K., Waters, D. L. E., Whalan. S., Baten. A. and Benkendorff, K. 2015. Indole producing bacteria from the biosynthetic organs of Muricid mollusc could contribute to Tyrian purple production. *J Shellfish Res*. 2015; 34: 443-454.

http://www.bioone.org/doi/abs/10.2983/035.034.0228

I conceived and designed the study with input from all supervisors who are coauthors on the paper. All sampling, dissections, culturing and biochemical analyses were undertaken by myself under initial supervision from K.B. I was also responsible for the DNA extraction, DNA quality check, PCR and submission of samples for genetic sequencing. Sequence alignment, interpretation and gene bank submission was undertaken with the assistance of D.W. Statistical analyses were undertaken with assistance from K.B. I prepared the first draft of the paper with editorial input from all supervisors.

Chapter 4– Ngangbam, A. K., Baten, A. Waters, D. L. E., Whalan, S. and Benkendorff, K. 2015. Characterization of bacterial communities associated with the Tyrian purple producing gland in a marine gastropod. *PloS one,* 10(10), e0140725.

http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0140725

All authors conceived and designed the experiments. I did the sampling, dissections, total DNA extraction, DNA quality check and submission of samples for high-throughput sequencing (454 GS FLX Titanium). A.B. provided the data assemblage and annotation. Statistical analyses were undertaken with assistance from K.B. I prepared the first draft of the paper with editorial input from all supervisors.

Chapter 5- Ngangbam, A. K., Smith, J., Mouatt, P. Waters, D. L. E., Whalan, S. and Benkendorff, K. Evidence of a bacterial origin for Tyrian purple in muricid molluscs.

This chapter in confidential as it has commercial potential and is currently under assessment for potential provisional patent application.

I conceived and designed the experiment with input from all supervisors who are coauthors on the chapter. All sampling, dissections, culturing, DNA extraction and quality check, PCR and submission of samples for genetic sequencing performed by myself, under initial supervision from K. B and J. S. who helped in design the various bacterial culture conditions. D. W. provided assistance for sequence alignment, interpretation and gene bank submission. P. M. performed the LCMS analyses on extracts from the bacterial cultures. I prepared the first draft of the chapter with editorial input from all supervisors.

Chapter 2. Transcriptome of the Australian mollusc *Dicathais orbita* provides insights in the biosynthesis of indoles and choline esters

Ngangbam, A.K., Baten, A., Waters, D.L.E. and Benkendorff, K. Transcriptome of the Australian Mollusc *Dicathais orbita* provides insights into the biosynthesis of indoles and choline esters. *Mar. Drugs* 2016, 14, 135.

2. 1 Abstract

Dicathais orbita is a mollusc of the Muricidae family and is well known for the production of the expensive dye Tyrian purple and its brominated precursors that have anticancer properties, in addition to choline esters with muscle-relaxing properties. However, the biosynthetic pathways that produce these secondary metabolites in *D. orbita* are not known. Illumina HiSeq 2000 transcriptome sequencing of hypobranchial glands, prostate glands, albumen glands, capsule glands, and mantle and foot tissues of *D. orbita* generated over 201 million high quality reads that were de novo assembled into 219,437 contigs. Annotation with reference to the Nr, Swiss-Prot and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases identified candidate-coding regions in 76,152 of these contigs, with transcripts for many enzymes in various metabolic pathways associated with secondary metabolite biosynthesis represented. This study revealed that *D. orbita* expresses a number of genes associated with indole, sulfur and histidine metabolism pathways that are relevant to Tyrian purple precursor biosynthesis, and many of which were not found in the fully annotated genomes of three other molluscs in the KEGG database. However, there were no matches to known bromoperoxidase enzymes within the *D. orbita* transcripts. These transcriptome data provide a significant molecular resource for gastropod research in general and Tyrian purple producing Muricidae in particular.

2. 2 Introduction

Dicathais orbita is well known for the production of the dye Tyrian purple, which is a historically important colourant exclusively produced by the Muricidae family of marine molluscs (Westley & Benkendorff 2008, Benkendorff et al. 2015). Tyrian purple is not produced within the mollusc but is formed from oxidative and photolytic reactions from a

precursor tyrindoxyl sulfate (Fig. 2.1A), which is stored as a salt of the choline ester murexine (Fig. 2.1B) (Baker & Duke 1976, Benkendorff 2013). Once the salt is liberated by an aryl sulfatase enzyme, a range of intermediate brominated indole precursors are produced, which have anticancer and antimicrobial properties (Benkendorff et al. 2000, Westley et al. 2010b, Edwards et al. 2012, Esmaeelian et al. 2013, Esmaeelian et al. 2014). The major dye component of Tyrian Purple, 6, 6' dibromoindigo, was the first marine natural product to be structurally elucidated (Freidlander 1909), however, a century later, limited information is available on the biosynthesis or gene regulation of this secondary metabolite.

The "post-genomics era" has seen an expansion in the application of bioinformatics to the fields of transcriptomics, proteomics and metabolomics (Zhang et al. 2010). Transcriptomics provides information on the genes expressed by an organism under certain circumstances or stage of development, in a particular tissue or cell type (Brown 2002, Adams 2008). Mollusc transcriptome studies have been instrumental in establishing the gene expression events associated with shell formation (Jackson et al. 2006), host parasite interactions (Lockyer et al. 2008, Knight et al. 2014), nervous system function (Moroz et al. 2006, Feng et al. 2009, Sadamoto et al. 2012), immune defence (Romero et al. 2012, Chavez et al. 2013), developmental processes (Ho et al. 2014, Powell et al. 2015) and cellular and physiological mechanisms (Zhang et al. 2012, Meng et al. 2013). Only a few transcriptome profiling studies have been undertaken on predatory marine neogastropods including the gonadal tissues of *Reishia* (Thais) *clavigera* (Ho et al. 2014), the mantle, foot, gills and gonadal tissues of *Concholepas concholepas* (Cardenas et al. 2011), the alimentary canal and salivary glands of *Colubraia reticulata* (Modica et al. 2015), the venom glands of *Conus consors* (Terrat et al. 2012) and the venom ducts of *C. tribblei, C. lenavati* (Barghi et al. 2015) and *Lophiotoma olangoensis*, a Turrid snail (Watkins et al. 2006).

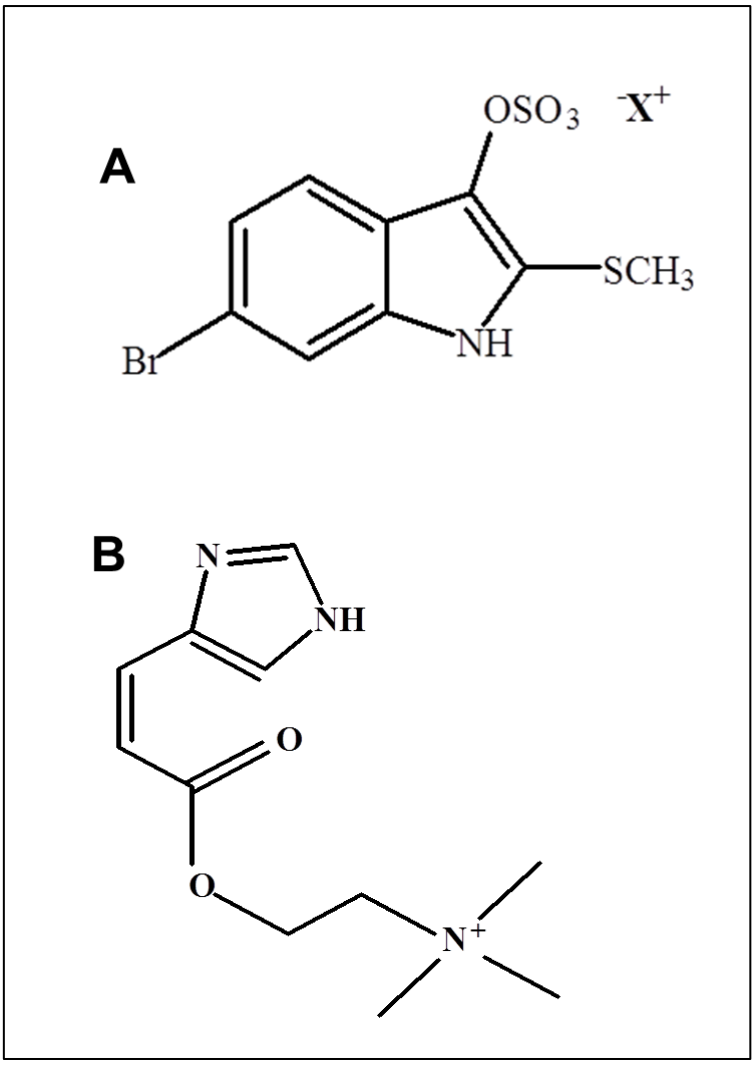

Fig. 2.1. Tyrindoxyl sulfate (**A**), the ultimate Tyrian purple precursor in *Dictahais orbita*, is held as a salt of the choline ester murexine (**B**).

Transcriptomics can identify genes involved in the biosynthesis of secondary metabolites (Urbarova et al. 2012, Zhang et al. 2014). The Australasian mollusc *D. orbita* is an ideal model species for gene expression studies of biosynthetic pathways that may be involved in the synthesis of biologically active secondary metabolites (Benkendorff 2013). A preliminary transcriptome study of *D. orbita* used suppressive subtractive hybridisation to identify genes that were upregulated in the hypobranchial gland, the biosynthetic organ

where Tyrian purple is produced (Laffy et al. 2013). This study confirmed the hypobranchial gland is a significant site for protein synthesis and regulation, but the only enzyme associated with Tyrian purple production identified was arylsulphatase (Laffy et al. 2013). However, that study was limited by short read lengths and a low total number of reads. Therefore, the aim of this study was to generate a more comprehensive transcriptome from the hypobranchial glands, prostate gland, capsule gland, albumen gland, and mantle and foot tissues of *D. orbita* (Muricidae Neogastropoda) and then search these transcriptomes for potential metabolic pathways that could contribute to indole and choline ester biosynthesis using the Kyoto Encyclopedia of Genes and Genomes (KEGG) PATHWAYS database.

2. 3 Materials and Methods

2. 3. 1 Specimens collection

Eighteen adult specimens of *D. orbita* (Table 2.1) were collected during low tide from the sub-tidal and intertidal rocky reefs of Flat rock, Ballina (28°84′ S and 153°60′ E), NSW, Australia. Six spawning females were collected during the breeding season August 2014 and a further six females and six males were collected after breeding season in January 2015, under the permit number F89/1171-6.0 issued by Department of Primary industries, NSW Government, Australia. Total RNA was extracted from the hypobranchial glands of the females collected in August 2014 and from five different tissues from female and three tissues from male *D. orbita* collected (Fig. 2.2) in January 2015 (Table 2.1).

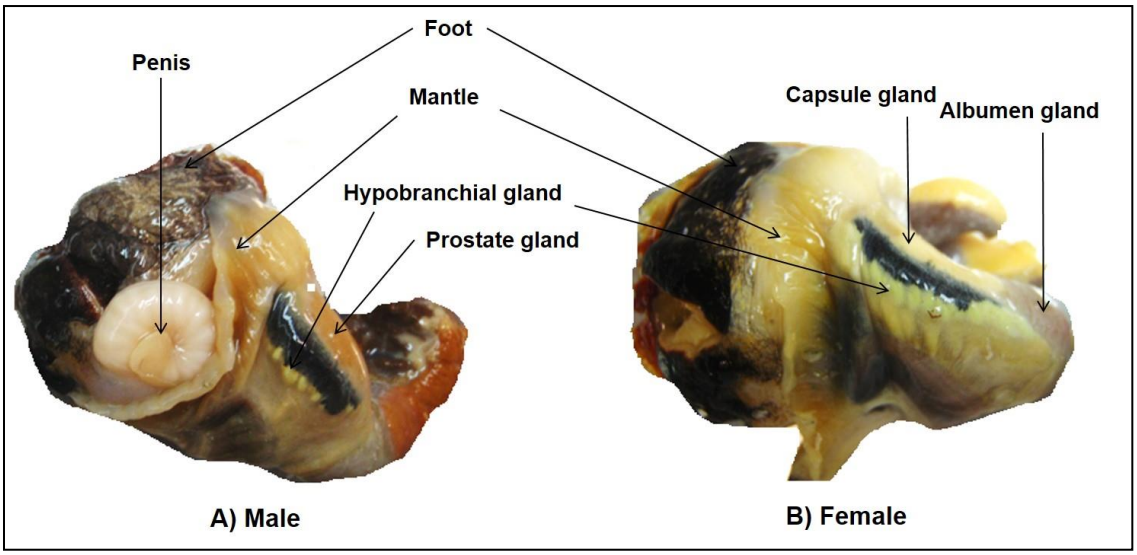

Fig. 2.2. *Dicathais orbita* male (**A**) and female (**B**) tissues used for RNA extraction to generate the transcriptome.

Table 2.1. Summary of the number of raw sequencing reads and the percent remaining after quality control from 14 tissue samples of *Dicathais orbita*

Snails (S)	Description	Raw Reads	High Quality Reads	
			Number	Percent
S1 + S2 + S3	Female hypobranchial gland 1, August, Breeding season, 2014	15,531,322	15,100,466	97.23
S4 + S5 + S6	Female hypobranchial gland 2, August, Breeding season, 2014	15,693,385	15,258,671	97.23
S7 + S8 + S9	Female hypobranchial gland 1, January, 2015	15,835,271	15,425,533	97.41
S10 + S11 + S12	Female hypobranchial gland 2, January, 2015	16,457,635	15,990,724	97.16
S13 + S14 + S15	Male hypobranchial gland 1, January, 2015	16,142,317	15,684,926	97.17
S16 + S17 + S18	Male hypobranchial gland 2, January, 2015	17,461,007	16,997,497	97.35
S7 + S8 + S9	Female foot 1, January, 2015	16,015,535	15,595,463	97.38
S10 + S11 + S12	Female foot 2, January, 2015	17,057,433	16,653,222	91.40
S13 + S14 + S15	Male foot 1, January, 2015	14,241,690	13,885,327	97.50
S16 + S17 + S18	Male foot 2, January, 2015	15,813,363	15,406,030	97.42
S7 + S8 + S9	Capsule gland, January, 2015	15,805,867	15,291,498	96.75
S7 + S8 + S9	Albumen gland, January, 2015	14,442,864	14,011,099	97.01
S13 + S14 + S15	Prostate gland, January, 2015	15,600,688	15,113,842	96.88
S10 + S11 + S12	Mantle 1, January, 2015	16,273,556	15,804,247	97.12
-	Total	222,371,933	216,218,545	-

2. 3. 2 Transcriptome sequencing

Three independent replicate snails were used for each tissue sample summarised in Table 2.1. The tissues were stabilized prior to RNA extraction in RNase free 2 ml Eppendorf tube using 600 µl of RNA*later* RNA stabilization reagent (Qiagen, Chadstone, Victoria, Australia). The stabilized tissue was incubated at 4 °C overnight and stored at -80 ^{o}C, prior to extracting the total RNA. The total RNA was extracted from the RNA*later* stabilized tissue using the E.Z.N.A. Mollusc RNA Kit (Omega bio-tek, Norcross, GA, U.S.A.) following the manufacturer's instructions. The concentration and quality (purity and integrity) of total RNA was assessed by NanoDrop and the Agilent Bioanalyzer 2100 System (Agilent Technologies, Santa Clara, CA, U.S.A). The total RNA extracted from three biological replicates of each tissue type (hypobranchial gland, prostate gland, albumen gland, capsule gland, mantle and foot) was pooled within the same tube for each tissue in equal masses. The pooled extracted RNA was stored at -80 ^{o}C until further used. The RNA samples were shipped to Macrogen Inc. (Seoul, South Korea) for high throughput sequencing. Prior to shipping, each RNA sample was precipitated in a mixture of 2 X ethanol (96 %) and 0.1 X sodium acetate (3 M). mRNA isolation and library construction were performed by Macrogen (Seoul, South Korea). The libraries were sequenced using the Illumina HiSeq 2000 platform (HCS2.2.38 version, Illumina, Seoul, South Korea).

2. 3. 3 De novo transcriptome assembly and annotation

FASTQ format raw sequencing reads were checked for quality using FASTQC (version 0.10.4, http://www.bioinformatics.babraham.ac.uk/projects/fastqc) (Andrews). The adapter sequences, poly-N stretches and low quality reads (Phred score <20) were removed using the BBDuck module of the BBMap software package (version 34_90,

http://sourceforge.net/projects/bbmap) using command "bbduk.sh in1=in1.fastq in2=in2.fastq out1=out1.fastq out2=out2.fastq ref=adapters.fa qtrim=rl trimq=20 ktrim=rl k=28 hdist=1 minlength=20". BLAST search of over-represented k-mers against NCBI confirmed these were mostly mitochondrial and ribosomal *Dicathais obita* genes and no evidence of bacterial contamination was found. CLC Genomics Workbench, version 4.9 (CLC Bio, Aarhus, Denmark; www.clcbio.com) with the option to map reads back to contigs, automatic word size and automatic bubble size was used to *de novo* assemble the high quality reads. All the contigs were clustered using CD-hit-est (version v4.6.1, http://weizhongli-lab.org/cd-hit) (Li & Godzik 2006) and Transdecoder (version 2.0.3, http://transdecoder.github.io/) was used to identify candidate coding regions within transcript sequences.

BLAST analysis was done against non-redundant protein database Nr (Mollusc specific proteins), Swiss-Prot and KEGG protein databases. Gene ontology analysis was performed with Interproscan (version 5.10.50, https://code.google.com/archive/p/interproscan) (Zdobnov & Apweiler 2001) using command "interproscan.sh -appl ProDom,PfamA,PANTHER -i longest_orfs.pep.fa -o out.txt -f TSV -goterms -iprlookup -pa" and plotted using WEGO (http://wego.genomics.org.cn/cgi-bin/wego/index.p) (Ye et al. 2006). *D. orbita* transcripts were searched for bromoperoxidase gene by aligning against known bromoperoxidase genes available in NCBI GenBank using BLAST (e value 1×10^{-3}) (Table 2.2).

Table 2.2. List of known bromoperoxidsae genes available in NCBI GenBank used for BLAST against *D. orbita* transcripts.

Accession numbers	Source species
AJ491786.1	*Laminaria digitata*
JX128092.1	*Gracilaria changii*
AF218810.1	*Corallina officinalis*
XM_001822243.2	*Aspergillus oryzae*
XM_003234475.1	*Trichophyton rubrum*
XM_002679630.1	*Naegleria gruberi*
NM_001157259.1	*Zea mays*
KLO55315.1	*Mycobacterium haemophilum*
AKJ56790.1	*Bacillus thuringiensis*
KLI98717.1	*Luteimonas* sp. FCS-9
YP_029199.1	*Bacillus anthracis* str. Sterne
KKM37545.1	*Bacillus anthracis*
KKI91829.1	*Bacillus* sp. SA1-12
KXH89531	*Bacillus* sp. JH7
KXI43580	*Bacillus cereus*
ALQ29400.1	*Arthrobacter* sp. YC-RL1
KND40451.1	*Streptomyces stelliscabiei*
WP_046733028	*Streptomyces* sp. MUSC119T

2. 3. 4 Nucleotide sequence accession number

All raw sequence data were deposited in the European nucleotide archive (ENA) with the accession numbers PRJEB12262. Assembled contigs are available from the authors upon request.

2. 4 Results and Discussion

2. 4. 1 De novo transcriptome assembly

Transcriptome sequencing across the six different tissue types (hypobranchial glands, prostate gland, capsule gland, albumen gland, mantle and foot tissues) resulted in approximately 222 million sequencing reads (Table 2.1). Raw sequencing reads in FASTQ format were first checked for quality using FASTQC (Andrews) followed by removal of adapter sequences, poly-N stretches and low quality (Phred score < 20) reads using the BBDuck module of the BBMap software package (version 34_90, http://sourceforge.net/projects/bbmap), which resulted in 216 million high quality reads.

High quality reads were de novo assembled into 219,437 contigs using CLC Genomics server, version 4.9 (CLC Bio, Aarhus, Denmark; www.clcbio.com) (Table 2.3). Transdecoder (version 2.0.3, http://transdecoder.github.io/) identified 76,152 contigs that contained candidate-coding regions that were used for annotation and further downstream analysis.

Table 2.3. Summary statistics of the assembled contigs using CLC *de novo* assembler.

Contig summary statistics	**bp (Base pair)**
Number of contigs	219,437
Total assembly length	117,767,308
N50	608
Mean contig length	537
Largest contig length	12,897
Number of contigs larger than 500bp	59,144
Number of contigs larger than 1000bp	22,818

2. 4. 2 Transcriptome annotation

Basic Local Alignment Search Tool (BLAST) analysis was performed using 76,152 contigs with Open Reading Frames (ORF)s against non-redundant protein database National Center for Biotechnology Information (NCBI) Nr (Mollusc specific proteins), Swiss-Prot and KEGG protein databases. Overall 28,364 contigs (~37%) had significant BLAST hits (e value 1×10^{-5}). The *D. orbita* contig BLAST hit rate was in a similar range to other whole mollusc genome/ transcriptome studies with hits ranging from 25-40% (Prentis & Pavasovic 2014). A total of 24996 contigs were assigned to various cellular components, molecular function and biological process gene ontology (GO) categories, as shown in Figure 2.3. General cell and cell parts were the most frequent subcategories of the cellular components ontology category, while binding and cellular process was most represented subcategory of molecular function and biological process. Analysis of KEGG pathway showed the largest number of contigs were involved in metabolic pathways (914 contigs), followed by biosynthesis of secondary metabolites (304 contigs) and microbial metabolism in diverse environment (173 contigs) (Table 2.4). Previous studies have revealed a diversity of bacterial symbionts in *D. orbita* tissues (Ngangbam et al. 2015a, Ngangbam et al. 2015b). However, we checked the overrepresented k-mers generated in the quality control phase of RNAseq reads and confirmed these are mostly mitochondria RNA rather than bacterial (<0.1%).

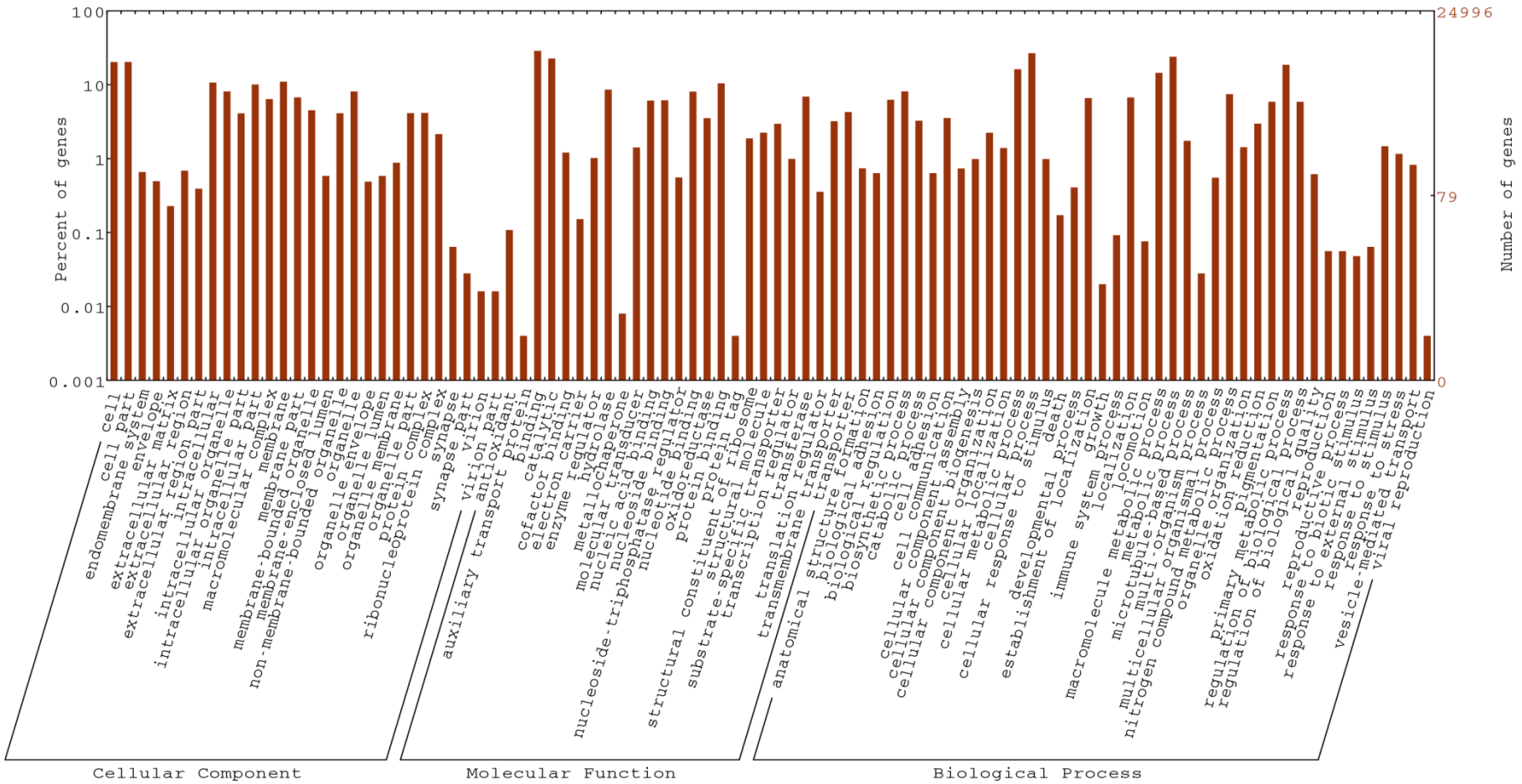

Fig. 2.3. The proportion and number of *Dicathais orbita* contigs assigned to gene ontology (GO) terms from biological process, cellular component and molecular function. Biological process was the most highly represented GO category followed by cellular component and molecular function.

Table 2.4. Analysis of KEGG pathway showing the top 20 metabolic pathway involving the largest number of contigs in *D. orbita* trancriptome.

Sl. No.	Top 20 metabolic pathway
1.	ko01100 Metabolic pathways (914)
2.	ko01110 Biosynthesis of secondary metabolites (304)
3.	ko01130 Biosynthesis of antibiotics (211)
4.	ko01120 Microbial metabolism in diverse environments (173)
5.	ko00230 Purine metabolism (120)
6.	ko03013 RNA transport (118)
7.	ko04144 Endocytosis (1118)
8.	ko03010 Ribosome (117)
9.	ko01200 Carbon metabolism (113)
10.	ko05016 Huntington's disease (112)
11.	ko03040 Spliceosome (106)
12.	ko00190 Oxidative phosphorylation (105)
13.	ko04141 Protein processing in endoplasmic reticulum (103)
14.	ko00240 Pyrimidine metabolism (98)
15.	ko05010 Alzheimer's disease (92)
16.	ko01230 Biosynthesis of amino acids (91)
17.	ko04120 Ubiquitin mediated proteolysis (88)
18.	ko05169 Epstein-Barr virus infection (88)
19.	ko05166 HTLV-I infection (86)
20.	ko04142 Lysosome (80)

2. 4. 3 Tryptophan metabolism and phenylalanine, tyrosine, tryptophan biosynthetic pathways

Specific searches in the KEGG PATHWAY database (Kanehisa & Goto 2000) were undertaken to identify genes potentially involved in the biosynthesis of Tyrian purple precursors and choline esters. Indoles like tyrindoxyl sulfate are thought to be derived from

tryptophan metabolism and we identified 28 enzyme contigs mapped to 35 reactions in the tryptophan metabolism pathway (Fig. 2.4; note that there are multiple KEGG enzyme (EC) numbers for some enzymes, and they can occur at different positions in the pathways, generating more matches to reactions that the number of matching contigs; this applies to all other pathways below). The list of 28 mapped contigs with the KEGG orthology assignment is provided in Table 2.5.

Table 2.5. List of the 28 mapped contigs and with the KEGG orthology assignment in *D. orbita* trancriptome for tryptophan metabolism.

Sl. No.	List of the enzymes in *D. orbita* trancriptome for tryptophan metabolism
1.	ko:K00022 HADH; 3-hydroxyacyl-CoA dehydrogenase [EC:1.1.1.35]
2.	ko:K00128 E1.2.1.3; aldehyde dehydrogenase (NAD+) [EC:1.2.1.3]
3.	ko:K00149 ALDH9A1; aldehyde dehydrogenase family 9 member A1 [EC:1.2.1.47 1.2.1.3]
4.	ko:K00164 OGDH; 2-oxoglutarate dehydrogenase E1 component [EC:1.2.4.2]
5.	ko:K00252 GCDH; glutaryl-CoA dehydrogenase [EC:1.3.8.6]
6.	ko:K00274 MAO; monoamine oxidase [EC:1.4.3.4]
7.	ko:K00452 HAAO; 3-hydroxyanthranilate 3,4-dioxygenase [EC:1.13.11.6]
8.	ko:K00453 E1.13.11.11; tryptophan 2,3-dioxygenase [EC:1.13.11.11]
9.	ko:K00463 INDO; indoleamine 2,3-dioxygenase [EC:1.13.11.52]
10.	ko:K00486 KMO; kynurenine 3-monooxygenase [EC:1.14.13.9]
11.	ko:K00626 E2.3.1.9; acetyl-CoA C-acetyltransferase [EC:2.3.1.9]
12.	ko:K00816 CCBL; kynurenine---oxoglutarate transaminase / cysteine-S-conjugate beta-lyase / glutamine---phenylpyruvate transaminase [EC:2.6.1.7 4.4.1.13 2.6.1.64]
13.	ko:K00825 AADAT; kynurenine/2-aminoadipate aminotransferase [EC:2.6.1.7 2.6.1.39]
14.	ko:K01426 E3.5.1.4; amidase [EC:3.5.1.4]
15.	ko:K01432 AFMID; arylformamidase [EC:3.5.1.9]
16.	ko:K01556 KYNU; kynureninase [EC:3.7.1.3]
17.	ko:K01593 DDC; aromatic-L-amino-acid decarboxylase [EC:4.1.1.28]
18.	ko:K01667 tnaA; tryptophanase [EC:4.1.99.1]
19.	ko:K01692 paaF; enoyl-CoA hydratase [EC:4.2.1.17]
20.	ko:K01782 fadJ; 3-hydroxyacyl-CoA dehydrogenase / enoyl-CoA hydratase / 3-hydroxybutyryl-CoA epimerase [EC:1.1.1.35 4.2.1.17 5.1.2.3]
21.	ko:K03781 katE; catalase [EC:1.11.1.6]
22.	ko:K07408 CYP1A1; cytochrome P450, family 1, subfamily A, polypeptide 1 [EC:1.14.14.1]
23.	ko:K07511 ECHS1; enoyl-CoA hydratase [EC:4.2.1.17]
24.	ko:K07514 EHHADH; enoyl-CoA hydratase / 3-hydroxyacyl-CoA dehydrogenase / 3,2-trans-enoyl-CoA isomerase [EC:4.2.1.17 1.1.1.35 5.3.3.8]
25.	ko:K07515 HADHA; enoyl-CoA hydratase / long-chain 3-hydroxyacyl-CoA dehydrogenase [EC:4.2.1.17 1.1.1.211]
26.	ko:K11182 AOC1; diamine oxidase [EC:1.4.3.22]
27.	ko:K11818 CYP83B1; cytochrome P450, family 83, subfamily B, polypeptide 1 [EC:1.14.-.-]
28.	ko:K14085 ALDH7A1; aldehyde dehydrogenase family 7 member A1 [EC:1.2.1.31 1.2.1.8 1.2.1.3]

The annotated genomes of only three other molluscs are available for comparison in the KEGG PATHWAYS database, the gastropod *Lottia gigantea*, cephalopod *Octopus bimaculoides* and the bivalve *Crassostrea gigas*. Nearly all the genes involved in tryptophan metabolism found in *D. orbita* (Fig. 2.4) were identical to those found in the other three molluscs (Fig. 2.5). However, an important point of difference is that unlike these other molluscs, the *D. orbita* transcriptome contained tryptophanase (4.1.99.1; Fig. 2.4), which converts tryptophan to indole. Consistent with the other molluscs such as *L. gigantea* and *C. gigas*, *D. orbita* expresses aromatic-L-amino-acid decarboxylase (4.1.1.28; Fig. 2.4) that converts tryptophan to tryptamine. However, we did not detect a transcript for tryptophan 5-monooxygenase (1.14.16.4), which converts tryptophan to 5-hydroxy-L-tryptophan, despite the presence of aromatic-L-amino-acid decarboxylase (4.1.1.28) involved in the production of serotonin. As the other three molluscs all contain matches to tryptophan 5-monoxygenase in their genomes (Fig. 2.5), it is possible this gene is also present in *D. orbita*, but was not detected in our transcriptome due to low expression. Nevertheless, it appears likely that *D. orbita* diverts the conversion of tryptophan away from 5-hydroxy-L-tryptophan in favour of indoles, to facilitate Tyrian purple precursor production by higher expression of the tryptophanase gene

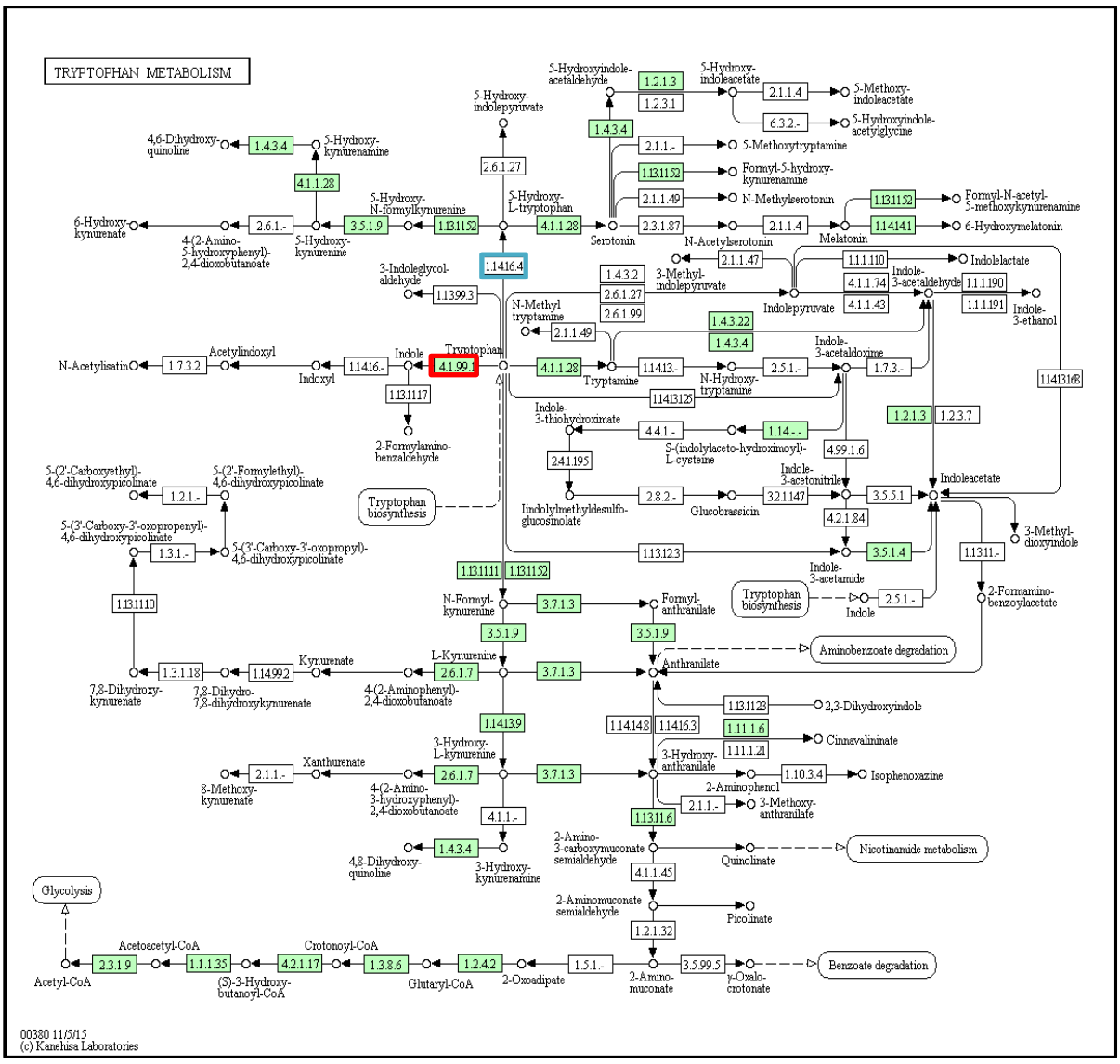

Fig. 2.4. Tryptophan metabolism pathway with matches to *Dicathais orbita* contigs filled in green. The match to a tryptophanase relevant to indole biosynthesis is highlighted by the red box, whereas the tryptophan 5-monoxygenase that was not detected in our transcriptome is highlighted in a blue box.

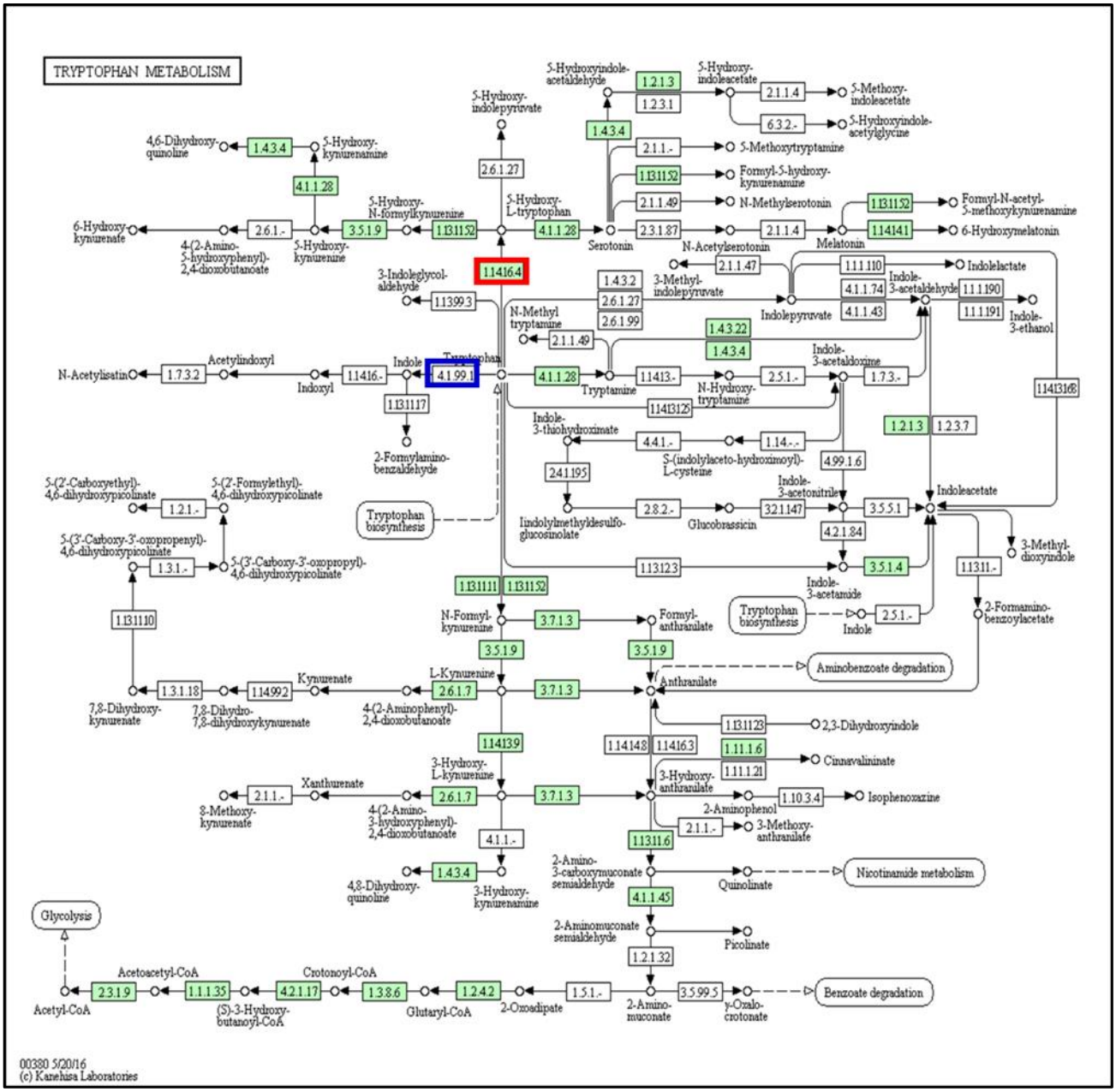

Fig. 2.5.A. Tryptophan metabolism pathways for *Crassostrea gigas* showing enzyme matches in green including tryptophan 5-monoxygenase (red box), which was missing form *Dicathais orbita*, but no match to tryptophanase (blue box).

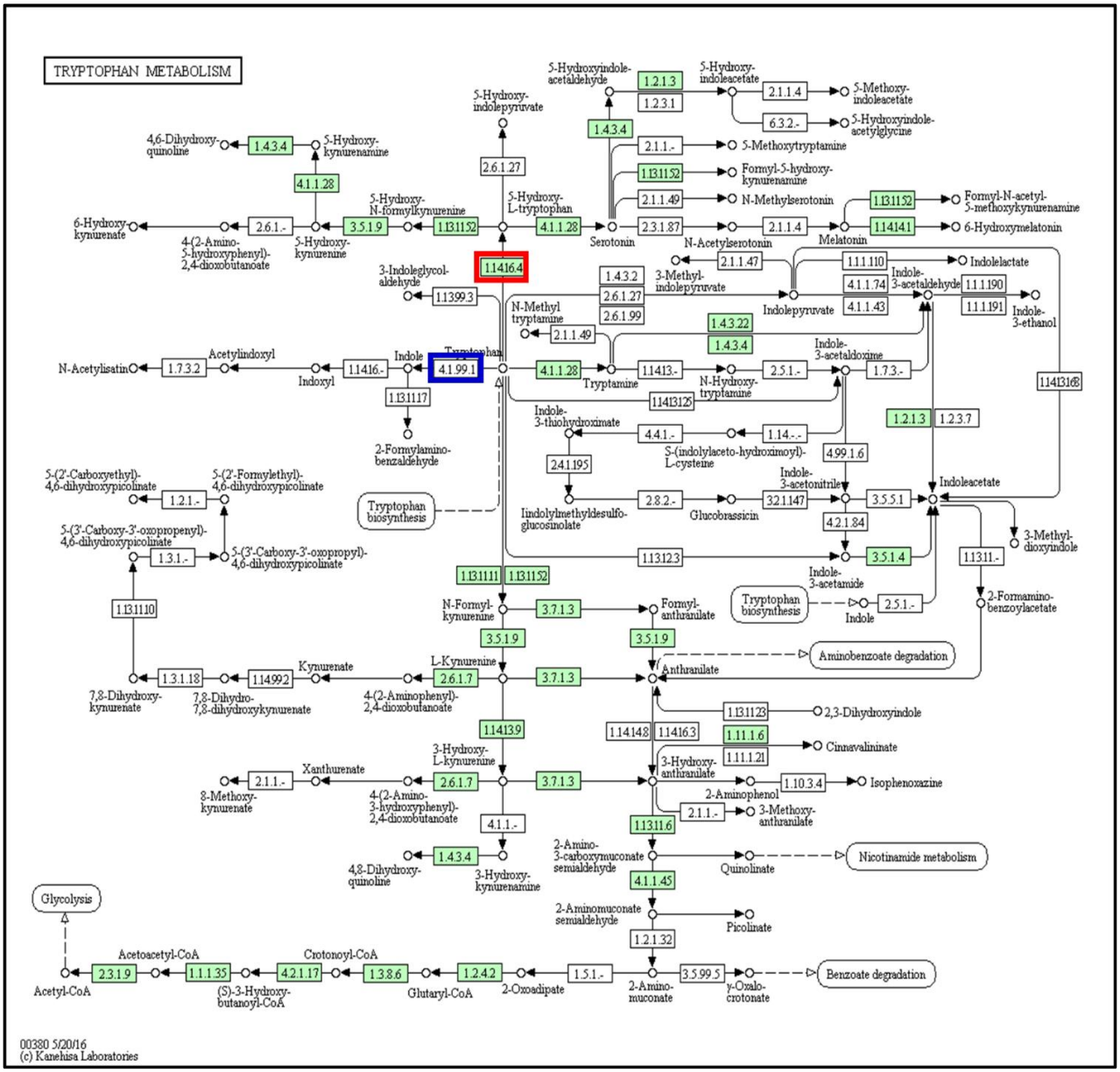

Fig. 2.5.B. Tryptophan metabolism pathways for *Lottia gigantea* showing enzyme matches in green including tryptophan 5-monoxygenase (red box), which was missing form *Dicathais orbita*, but no match to tryptophanase (blue box).

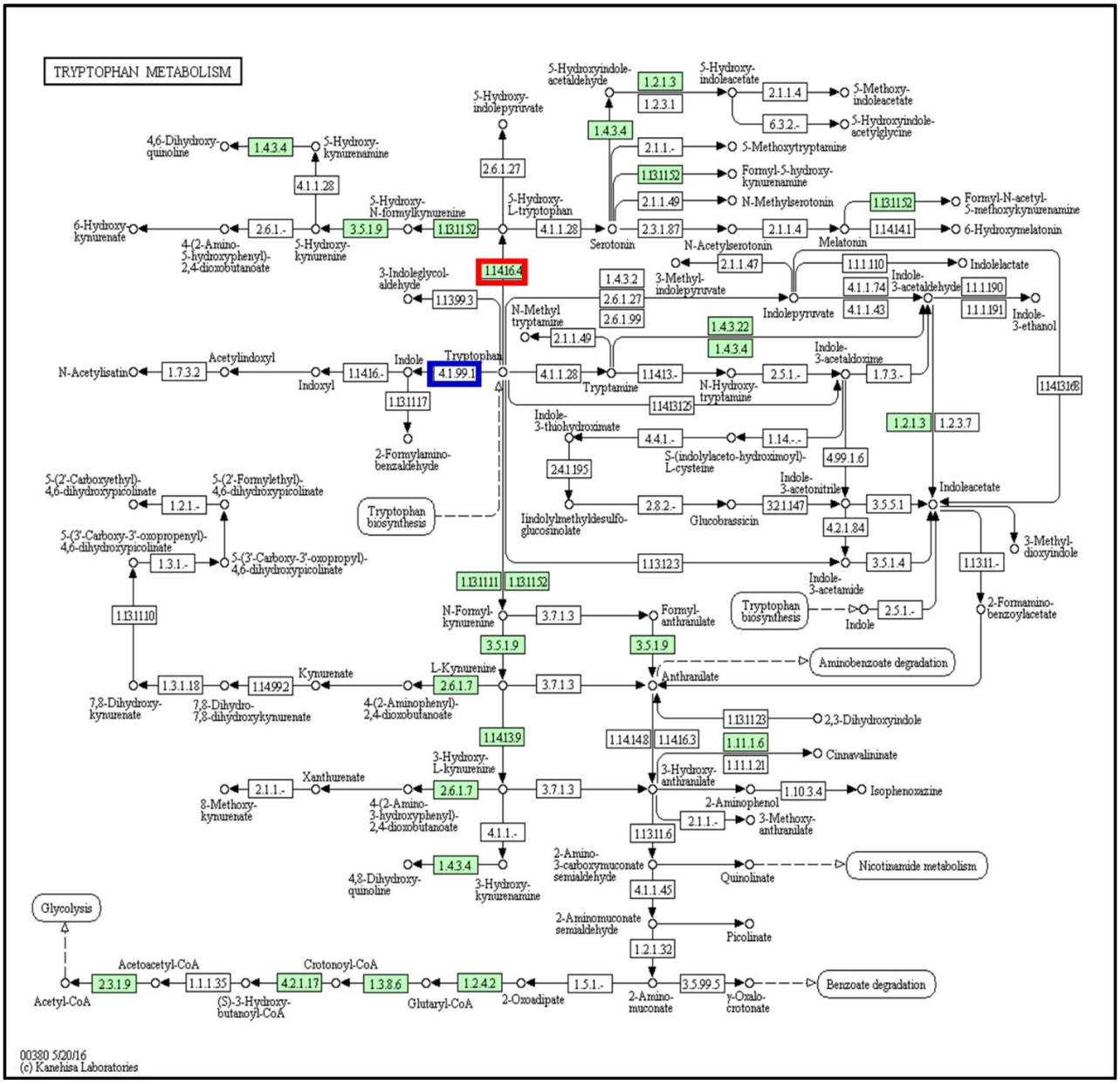

Fig. 2.5.C. Tryptophan metabolism pathways for *Octopus bimaculoides* showing enzyme matches in green including tryptophan 5-monoxygenase (red box), which was missing form *Dicathais orbita*, but no match to tryptophanase (bluebox).

In the *D. orbita* transcriptome, we found no match on the reaction pathway for the conversion of indole to indoxyl, a precursor to indoxyl sulphate (Fig. 2.4, 1.14.16). However, in bacteria cytochrome P450 enzymes (Banoglu et al. 2001, Arora et al. 2015), and/or mono- or dioxygenases (O'connor & Hartmans 1998, Han et al. 2008) are involved in the formation of indoxyl sulfate and indigo. We found matches to two cytochrome P450 enzymes, as well as a monoxidase and several dioxygenases in the *D. orbita* transcriptome (Table 2.5). Our recent studies have also revealed numerous *Vibrio* sp. that have the ability to synthesize indoles in the Tyrian purple producing tissues of *D. orbita* (Ngangbam et al. 2015a, Ngangbam et al. 2015b) and these may provide a supplementary source of indoles for Tyrian purple production.

In the phenylalanine, tyrosine and tryptophan biosynthetic pathway (Fig. 2.6), there was a match in the *D. orbita* transcriptome to tryptophan synthase alpha chain (4.2.1.20; Fig. 2.6), which converts tryptophan to indoles and vice versa, the initial precursors for Tyrian purple biosynthesis (Verhecken 1989, Westley et al. 2006, Benkendorff 2013). This tryptophan synthase was only found in the *D. orbita* transcriptome and not found in the annotated genomes of *L. gigantea, O. bimaculoides* and *C. gigas* (Fig. 2.7). Most of the genes involved in phenylalanine, tyrosine and tryptophan biosynthesis in *D. orbita* were found to be different to the other three molluscs and this Neogastropoda had more matches to these aromatic amino acid biosynthesis reactions (14 genes, Fig. 2.6) compared to *L. gigantea* (5 genes), *O. bimaculoides* (5 genes) and *C. gigas* (5 genes) (Fig. 2.7).

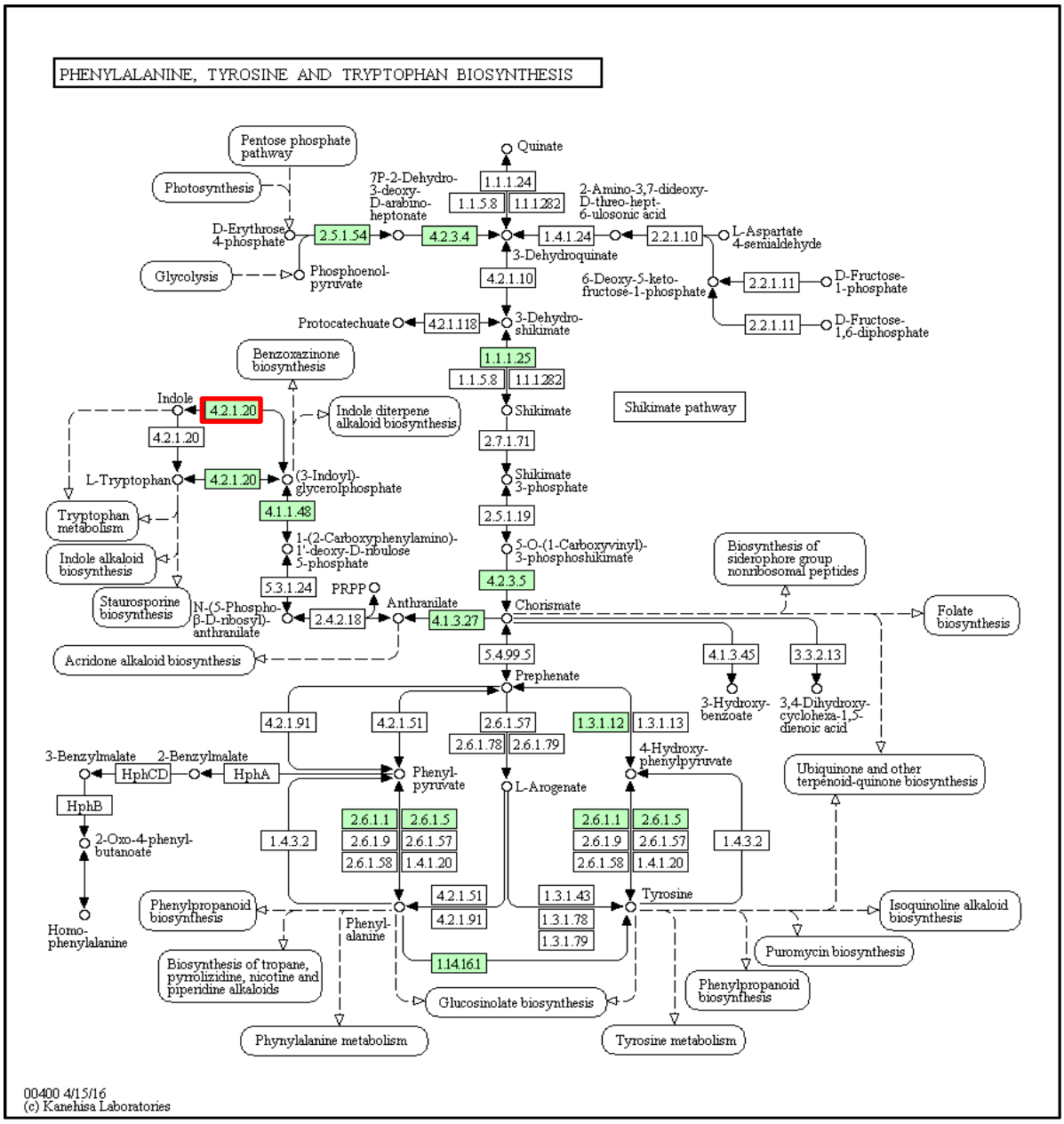

Fig. 2.6. Phenylalanine, tyrosine and tryptophan biosynthetic pathway showing matches to *Dicathais orbita* contigs highlighted in green, with tryptophan synthase highlighted in the red box.

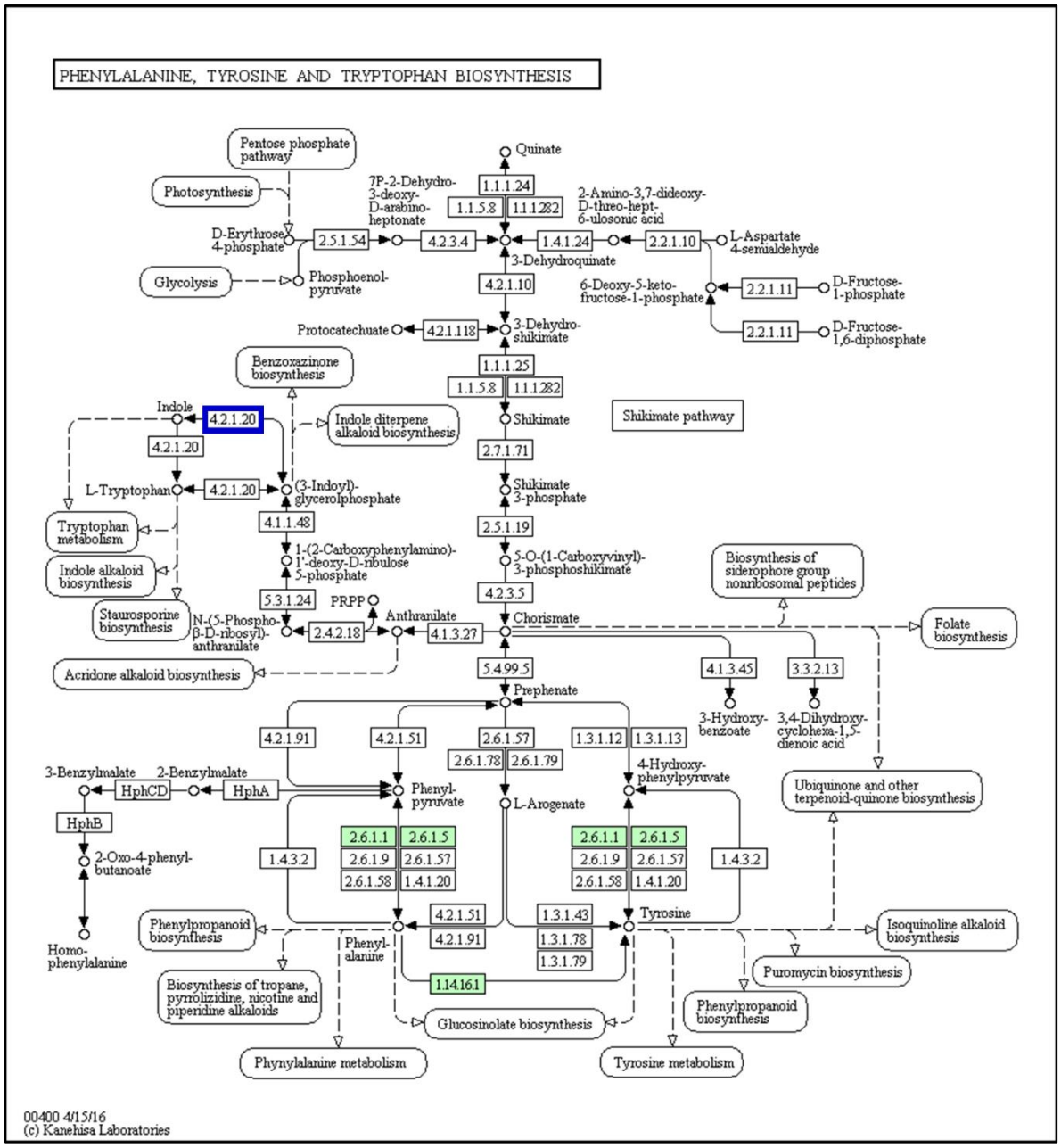

Fig. 2.7.A. Phenylalanine, tyrosine and tryptophan biosynthetic pathways for *Crassostrea gigas* with enzyme matches in green, but with no match to tryptophan synthase highlighted in the blue box.

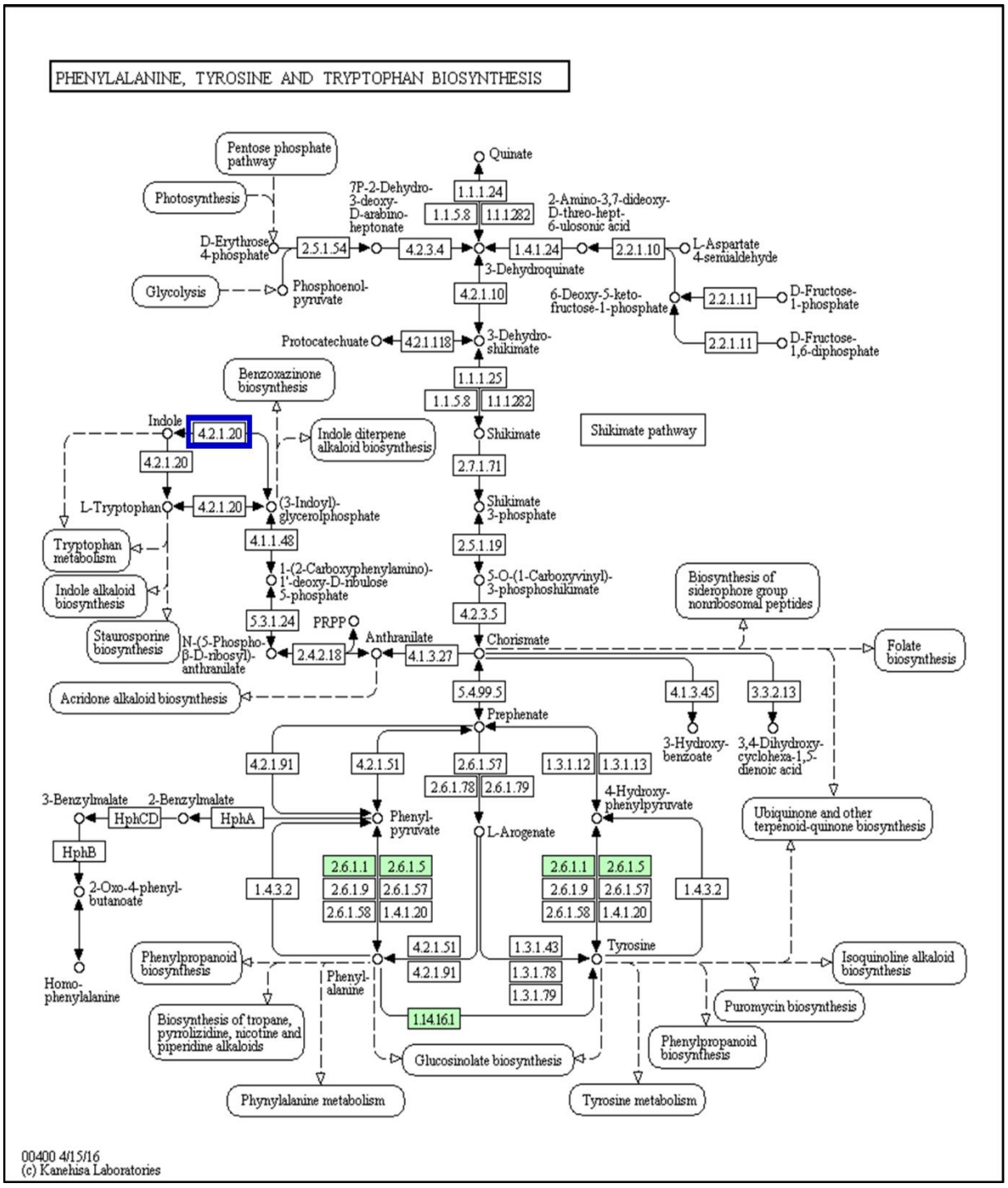

Fig. 2.7.B. Phenylalanine, tyrosine and tryptophan biosynthetic pathways for *Lottia gigantea* with enzyme matches in green, but with no match to tryptophan synthase highlighted in the blue box.

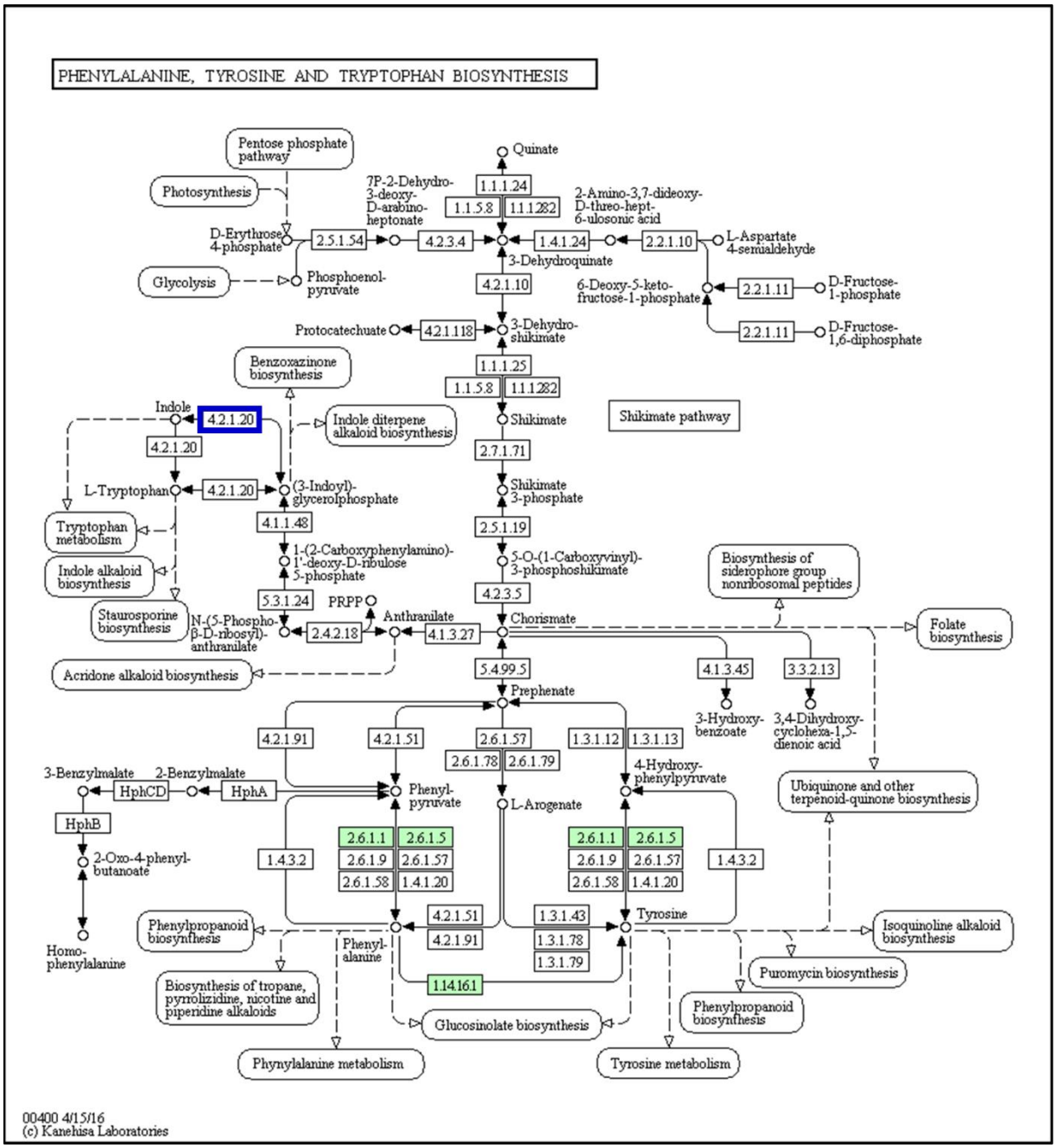

Fig. 2.7.C. Phenylalanine, tyrosine and tryptophan biosynthetic pathways for *Octopus bimaculoides* with enzyme matches in green, but with no match to tryptophan synthase highlighted in the blue box.

2. 4. 4 Sulfur, cysteine and methionine metabolisms pathway in *Dicathais orbita*

Tyrindoxyl sulphate contains a methane thiol group at the 2′ position of the indole ring (Fig. 2.1A). Examination of the *D. orbita* transcripts with reference to the sulfur metabolism pathway revealed that most of the genes involved in *D. orbita* sulfur metabolism were found to be similar to other three molluscs. Enzymes like dimethyl-sulfide monooxygenase (1.14.13.131; Fig. 2.8) that produces methyl mercaptan from dimethyl disulfide were not detected in any of the molluscs, including *D. orbita*. Overall, the number of genes detected in the *D. orbita* sulfur metabolism pathway (15 genes, Fig. 2.8) was only slightly higher than the number identified in the genome of *L. gigantea* (12 genes), *O. bimaculoides* (12 genes) and *C. gigas* (14 genes) (Fig. 2.9).

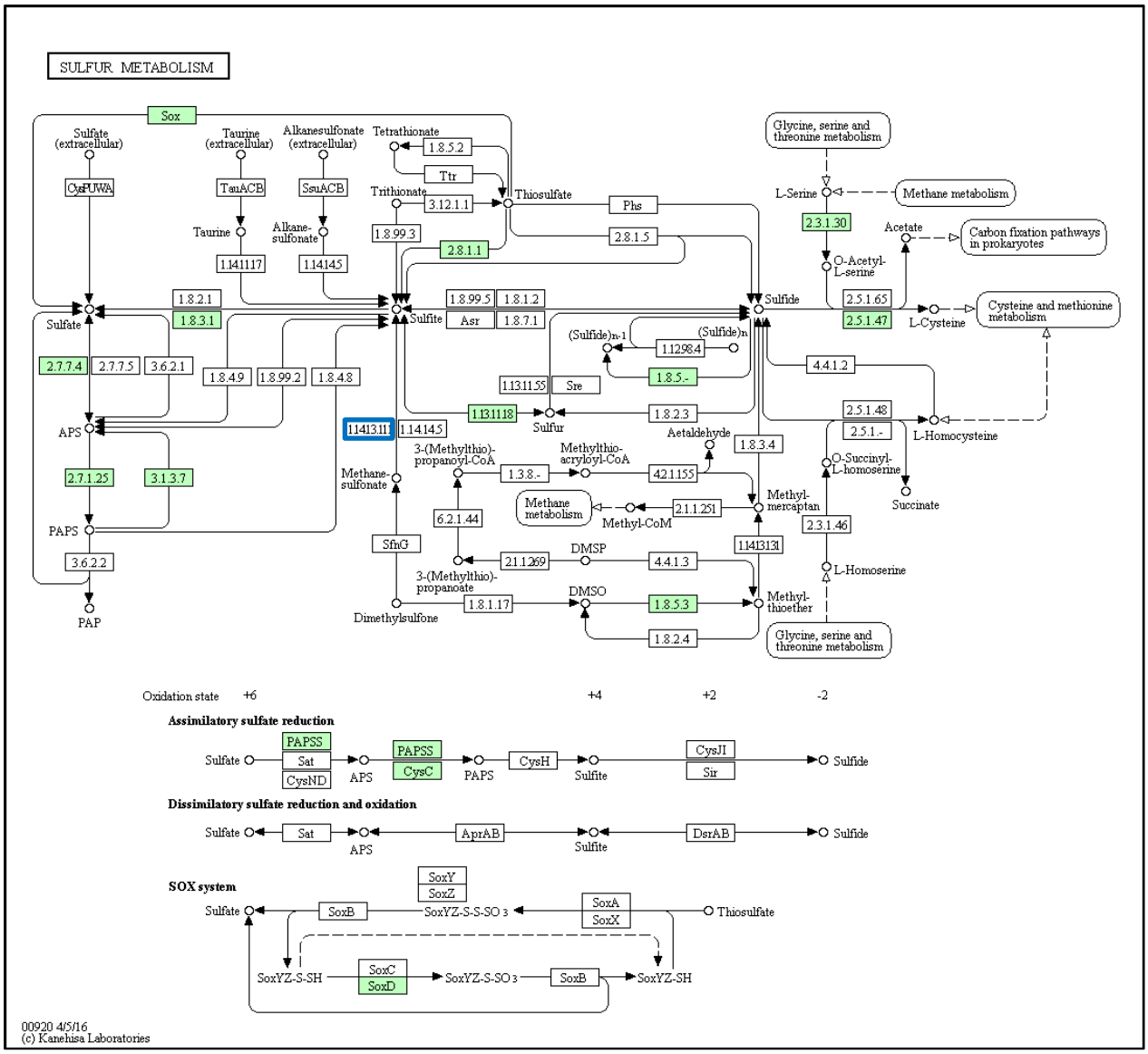

Fig. 2.8. Sulfur metabolism pathway with matches to *Dicathais orbita* contigs highlighted in green; there was no match to dimethyl-sulfide monooxygenase in the *D. orbita* transcriptome (bluebox).

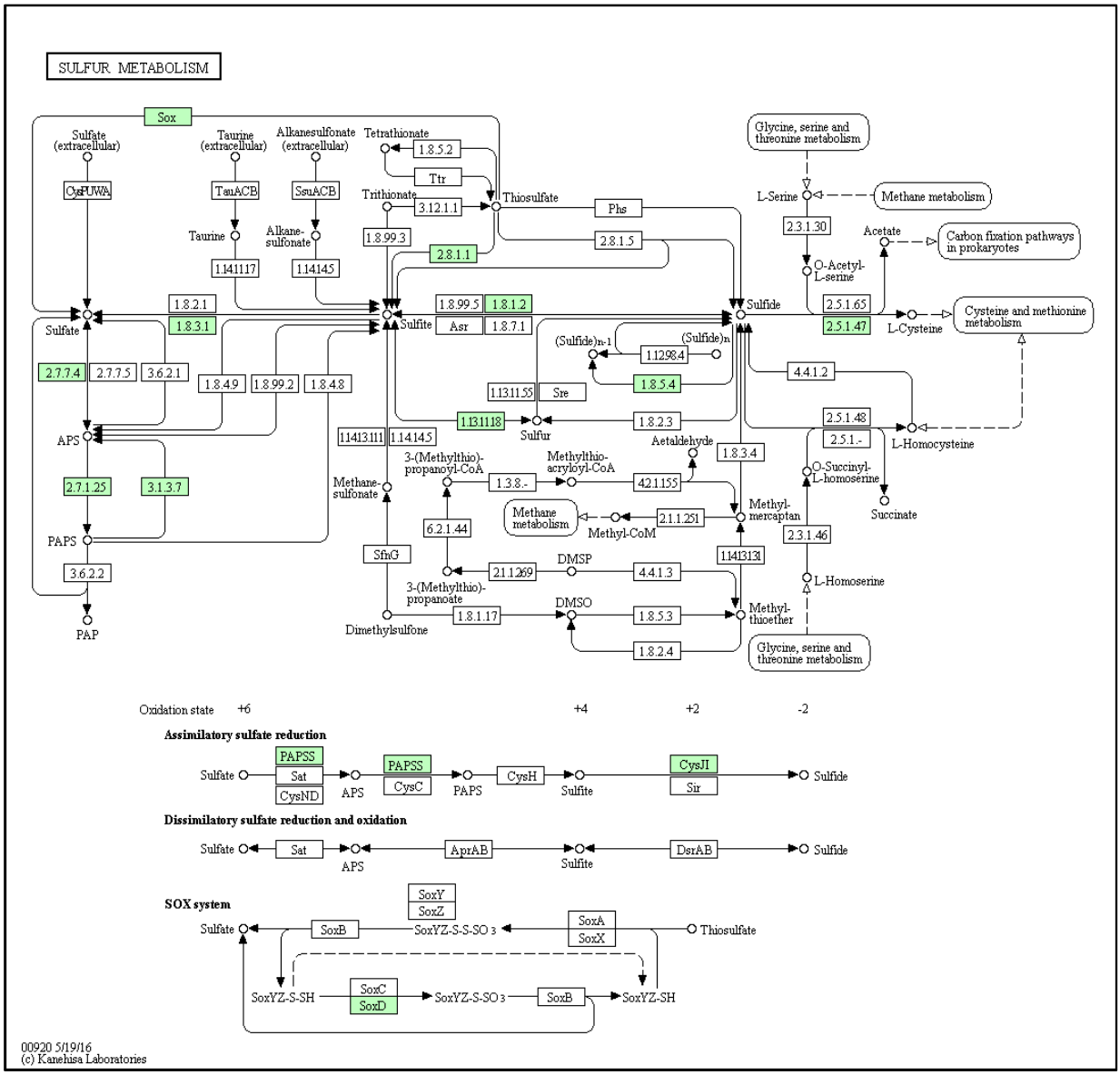

Fig. 2.9.A. Sulfur metabolism pathways for *Crassostrea gigas*.

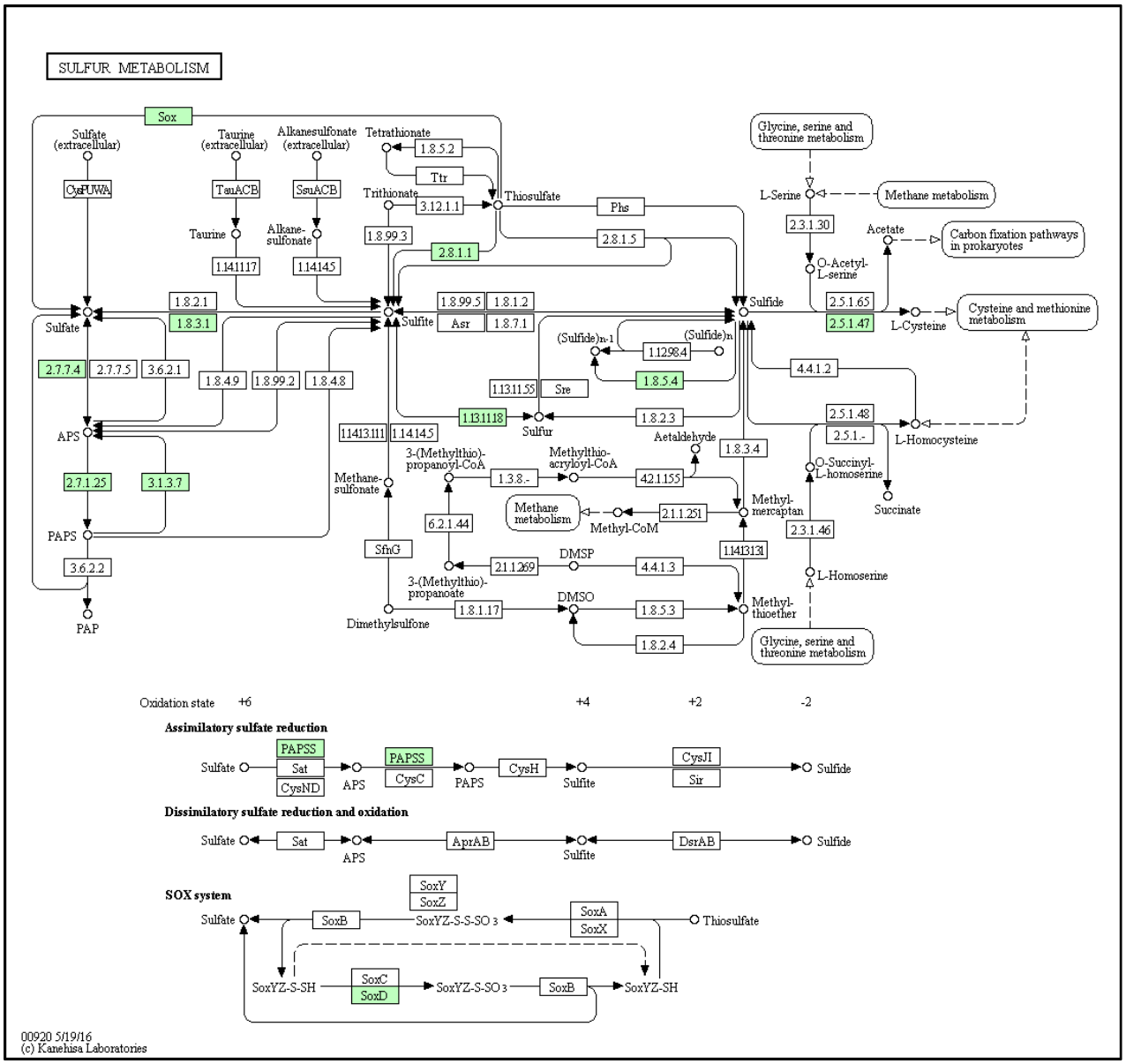

Fig. 2.9.B. Sulfur metabolism pathways for *Lottia gigantea.*

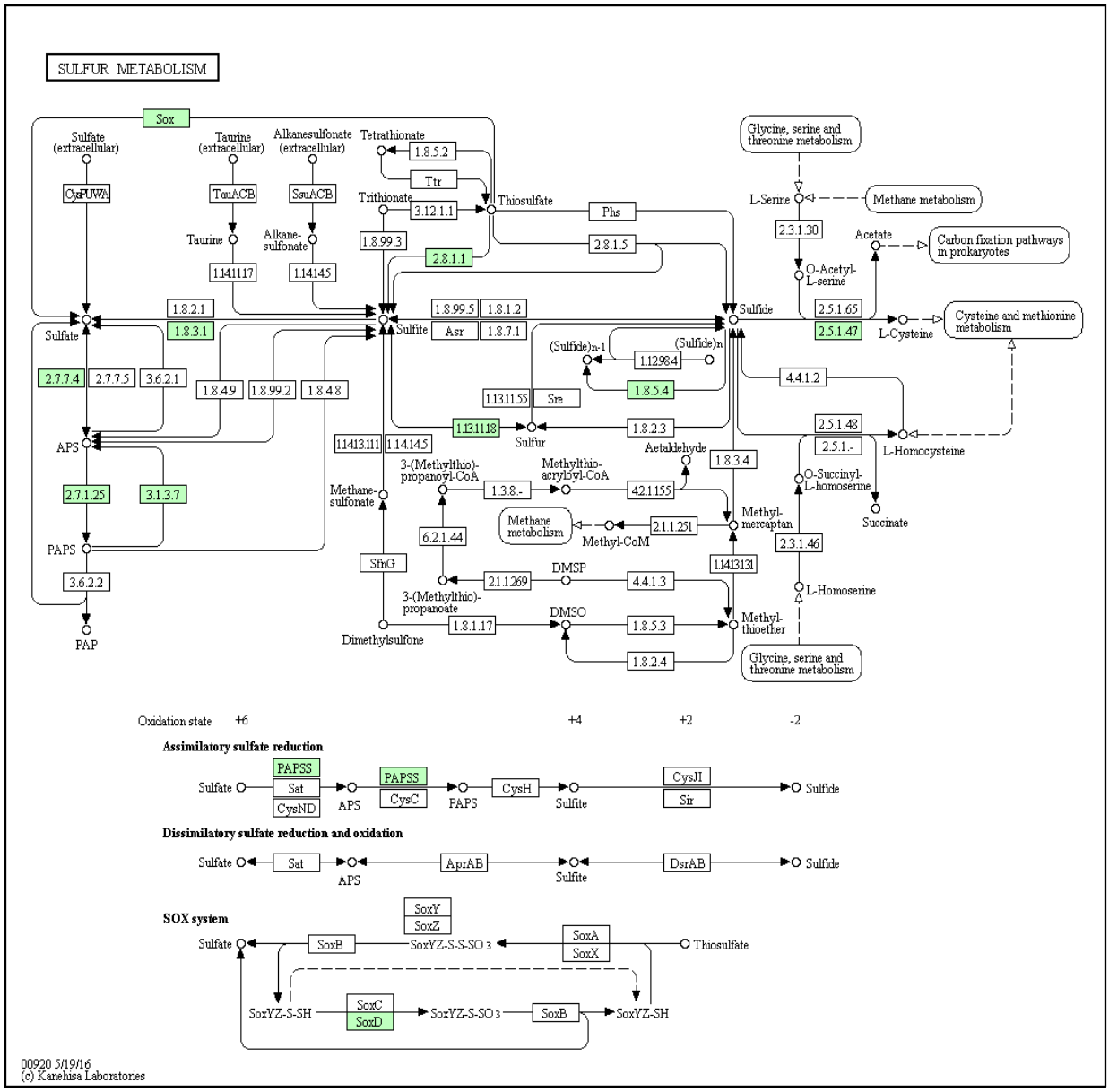

Fig. 2.9.C. Sulfur metabolism pathways for *Octopus bimaculoides.*

The metabolism of sulfur containing amino acids provides another possible source of the methanethiol group in tyrindoxyl sulfate. Enzyme matches in the cysteine and methionine metabolism pathway (Fig. 2.10) indicate that *D. orbita* has the ability to produce 3-methylthioproprionate, 3-mercaptopyruvate and thiosulfate, but no match was found to methionine-gamma-lyase (4.4.1.11; Fig. 2.10), which converts the amino acid L-Methionine directly into methanethiol. Nevertheless, we did detect a transcript for tyrosine aminotransferase (2.6.1.5; Fig. 2.10), which may play a role in placing the methane thiol onto the aromatic indole ring. Tyrosine aminotransferase genes were also found in the *Lottia*, octopus and oyster genomes (Fig. 2.11). Overall, the *D. orbita* transcriptome had more matches to enzymes in the cysteine and methionine metabolism pathway (41 genes, Fig. 2.10) compared to *L. gigantea* (32 genes), *O. bimaculoides* (30 genes) and *C. gigas* (33 genes) (Fig. 2.11). This ability to metabolise sulfur from various sources is consistent with the biosynthesis of indole mercaptans in Muricidae.

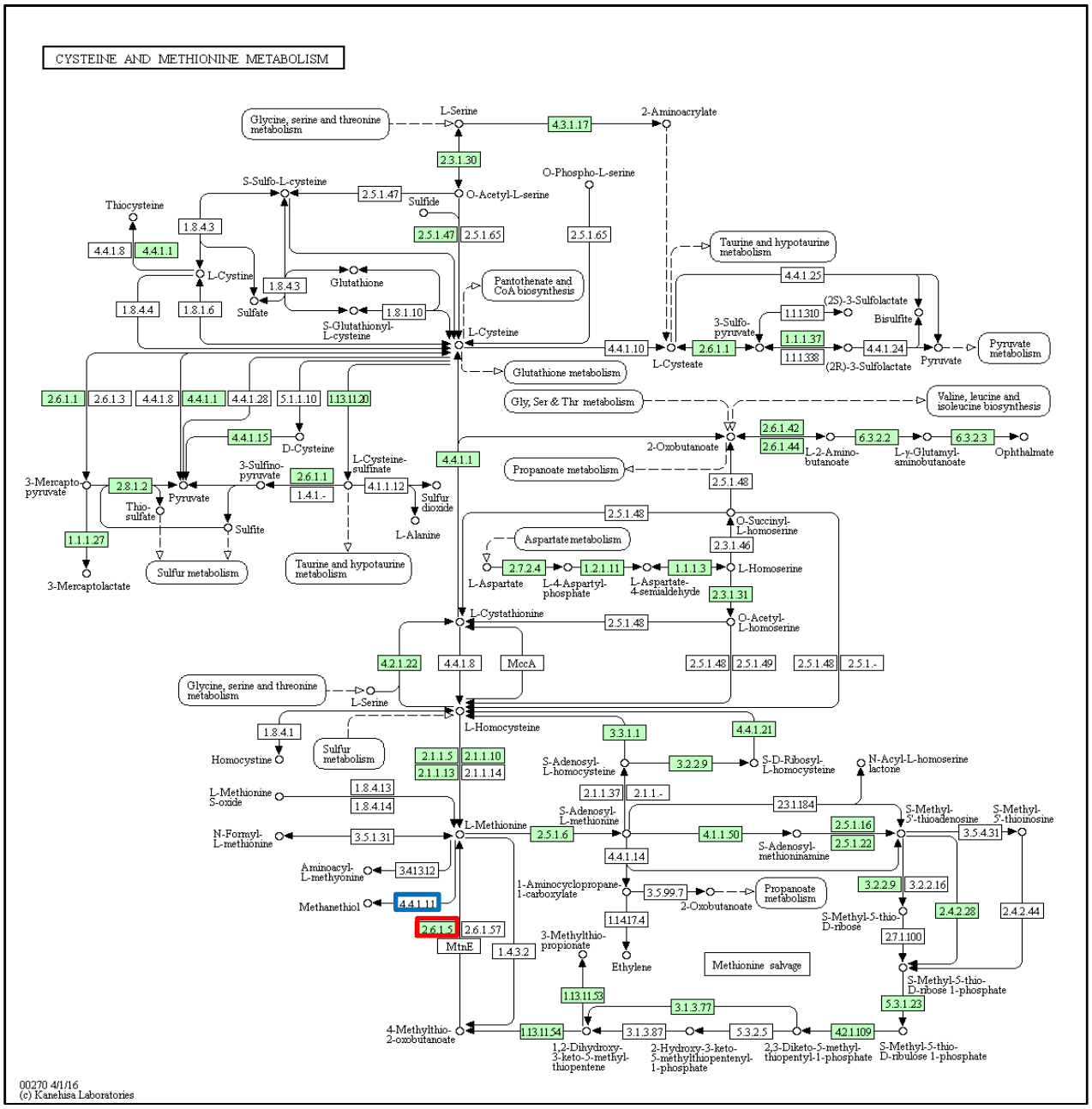

Fig. 2.10. Cysteine and methionine metabolism pathway showing matches to *Dicathais orbita* contigs highlighted in green, including tyrosine aminotransferase (red box), but no match was found to methionine-gamma-lyase (blue box).

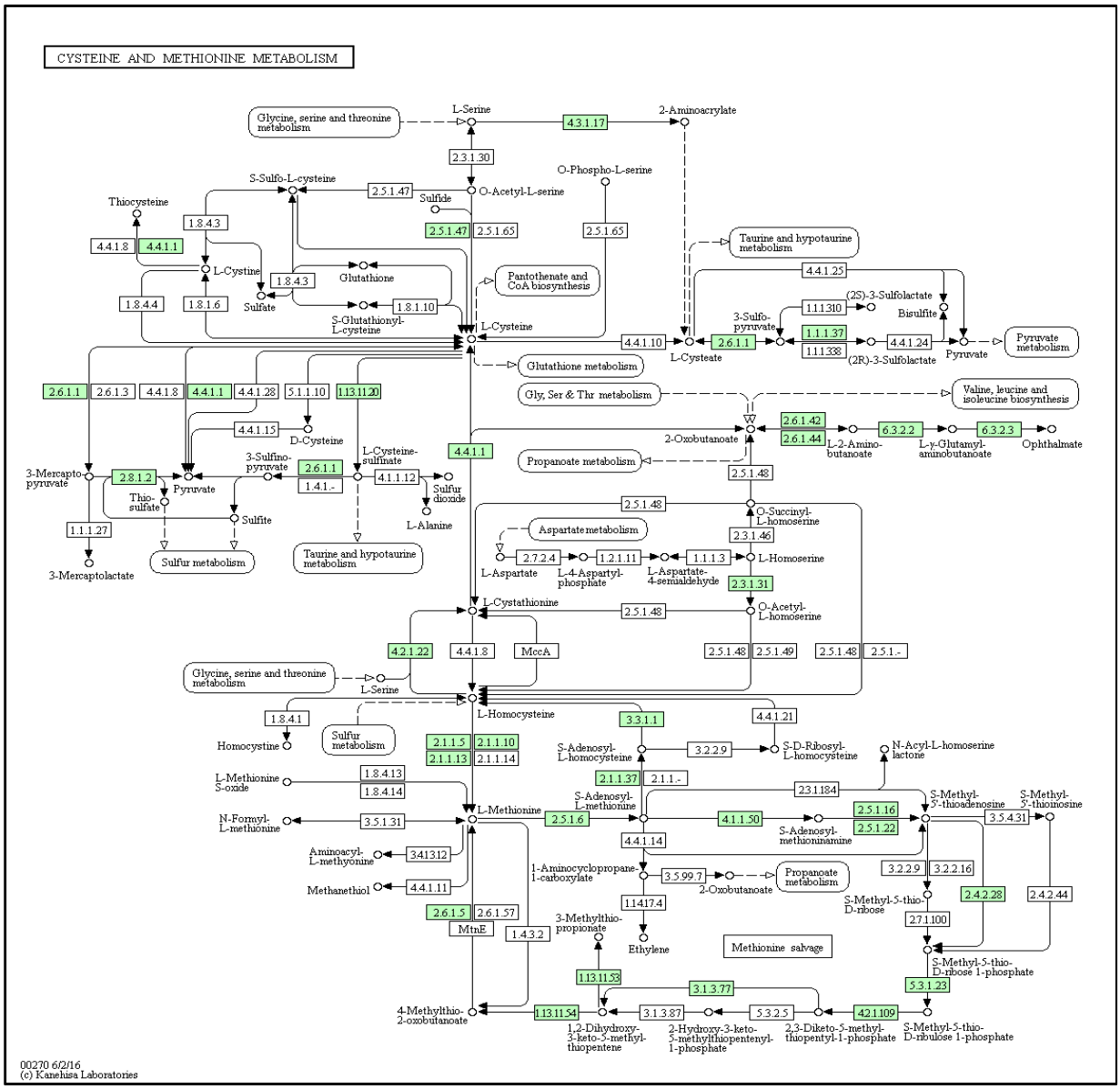

Fig. 2.11.A. Cysteine and methionine metabolism pathway of *Crassostrea gigas*.

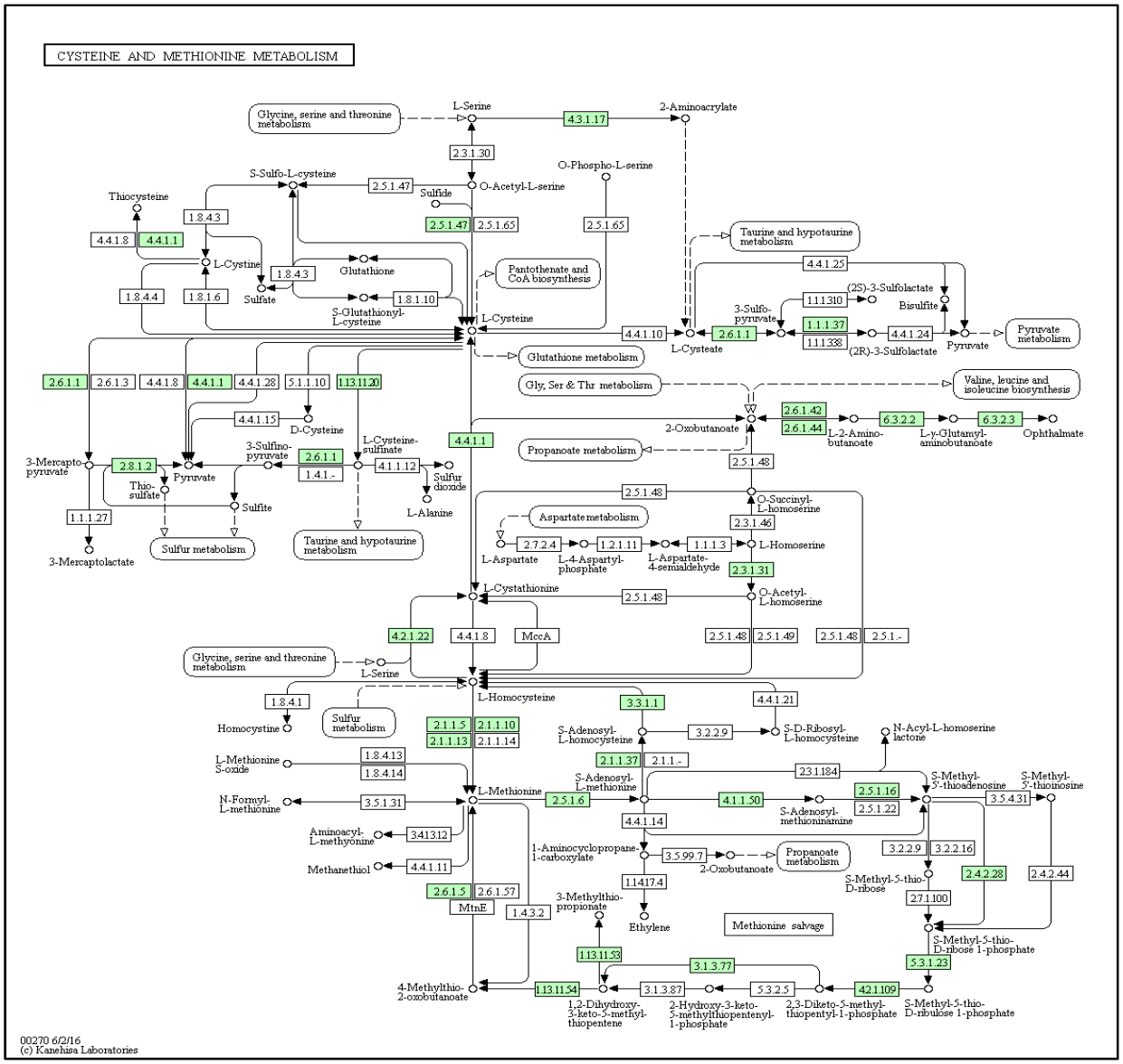

Fig. 2.11.B. Cysteine and methionine metabolism pathway of *Lottia gigantea*.

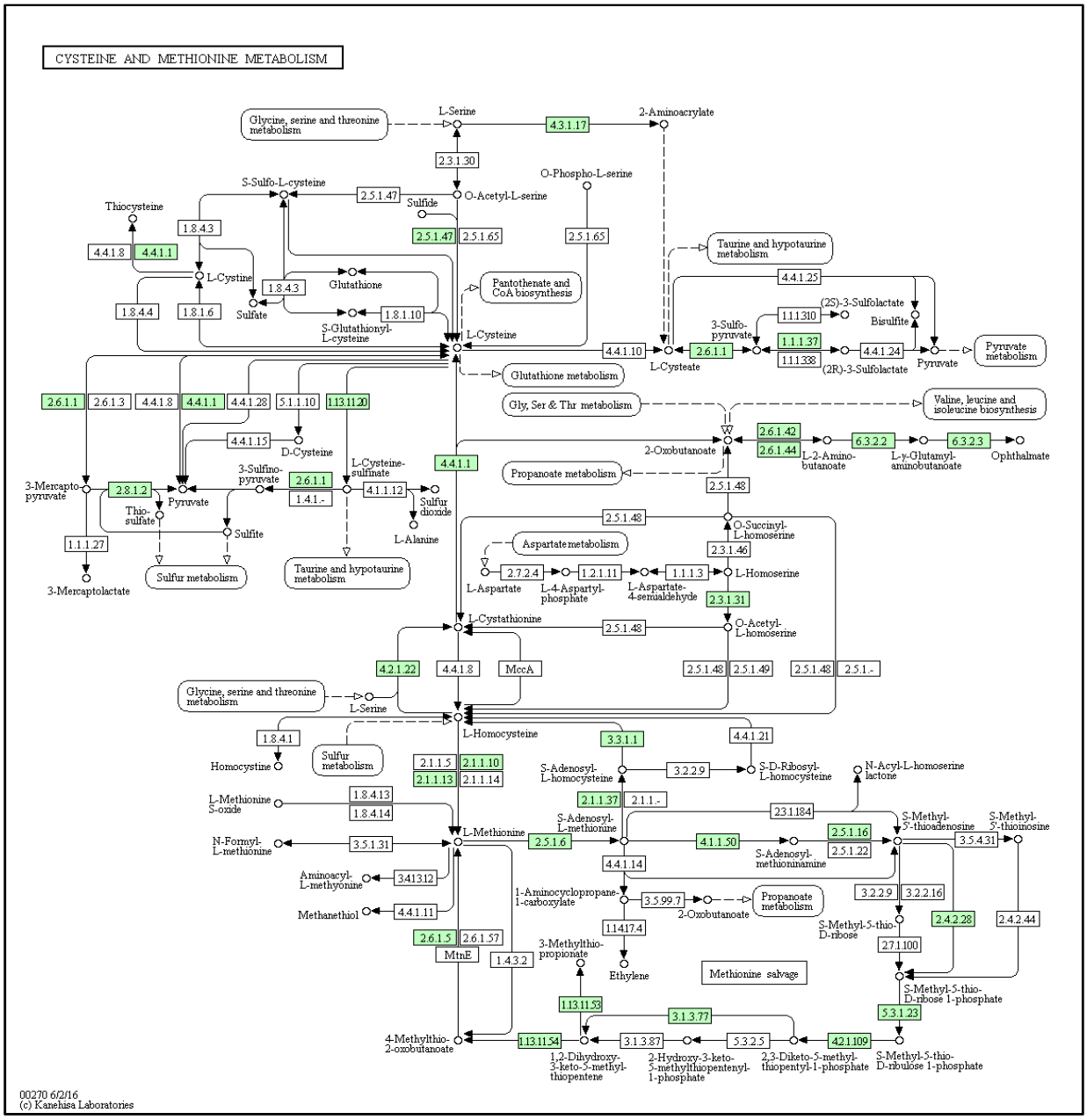

Fig. 2.11.C. Cysteine and methionine metabolism pathway of *Octopus bimaculoides.*

2. 4. 5 Bromoperoxidase enzymes

Tyridoxyl sulfate is a 6-brominated indole derivative (Figure 2.1A) and bromoperoxidase activity has been detected in the hypobranchial glands of *D. orbita* (Westley & Benkendorff 2009) and other Muricidae species (Jannun & Coe 1987). Consequently, a search was undertaken for bromoperoxidase genes by aligning *D. orbita* transcripts against known bromoperoxidase genes using BLAST (e value 1×10^{-3}). However, no evidence of matches to bromoperoxidase genes was found using these sequences. This is consistent with a previous study that examined the transcripts that were up-regulated in the *D. orbita* hypobranchial glands relative to other *D. orbita* tissues, which also found no matches to bromoperoxidase genes (Laffy et al. 2013). There appears to be no previous reports of bromoperoxidase coding genes or transcripts in any gastropods or other molluscs. However, recent metagenomic analyses of *D. orbita* hypobranchial glands revealed the presence of bacterial symbionts known to produce bromoperoxidase enzymes (Ngangbam et al. 2015a). Our *D. orbita* transcriptome data therefore supports the suggestion that symbiotic bacteria play a role in the biosynthesis of Tyrian purple.

2. 4. 6 *Dicathais orbita* glycerophospholipid and histidine metabolism pathway

Tyrindoxyl sulfate is stored as a choline ester of murexine, which contains a choline ester group and imidazole moiety (Figure 2.1B). The glycerophospholipid metabolism pathway has a role in the biosynthesis of choline esters (Quastel et al. 1936) and more matches to reactions in the glycerophospholipid metabolism pathway (53 genes), were found in the *D. orbita* transcriptome (Fig. 2.12) compared to the annotated genomes of other

molluscs (*L. gigantea* = 48, *O. bimaculoides* = 40 and *C. gigas* = 44 genes) (Fig. 2.13). The *D. orbita* transcriptome included matches to choline/ethanol amine kinase (2.7.1.32; Fig. 2.12), which produces choline from phosphocholine, as well as choline O-acetyltransferase (2.3.1.6; Fig. 2.12) and acetylcholinesterase (3.1.1.7; Fig. 2.12), which produce acetylcholine. Laffy *et al.* (Laffy et al. 2013) found that acetylcholinesterase (3.1.1.7) transcripts were upregulated in the hypobranchial gland of *D. orbita* relative to foot tissue. The octopus *O. bimaculoides* and gastropod *L. gigantea* genomes were also found to contain matches to these enzymes for acetylcholine biosynthesis, whereas the oyster *C. gigas* lacks phosphocholine phosphatase (Fig. 2.13). In comparison to the gastropods and the bivalve, the octopus *O. bimaculoides* was found to lack any matches to genes in the phosphothanolamine N-methyltransferase pathway for the production of phosphocholine from phosphoethanolamine (Fig. 2.13C).

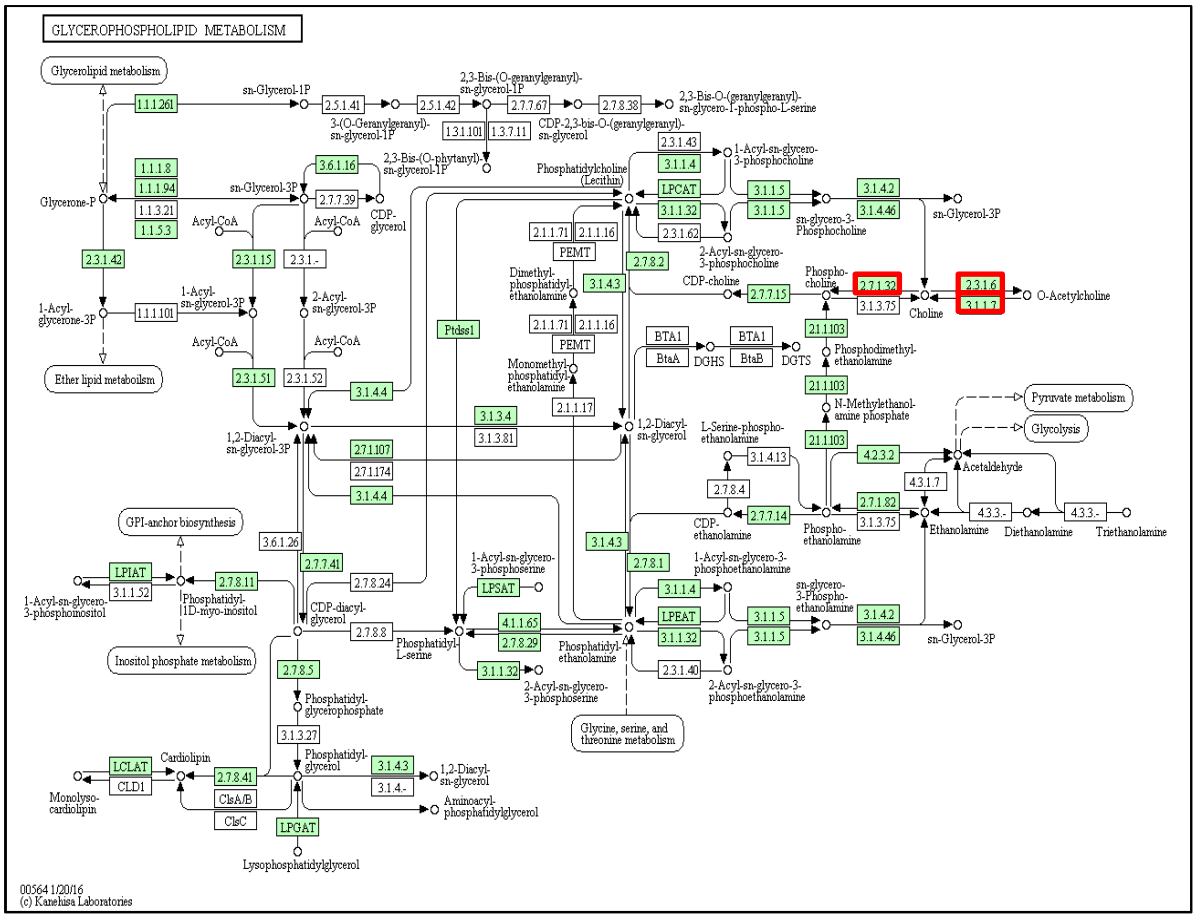

Fig. 2.12. Glycerophospholipid metabolism pathway with matches to *D. orbita* contigs highlighted in green including choline kinase, choline O-acetyltransferase and acetylcholinesterase (red boxes) used to generate the acetyl choline moiety found in murexine.

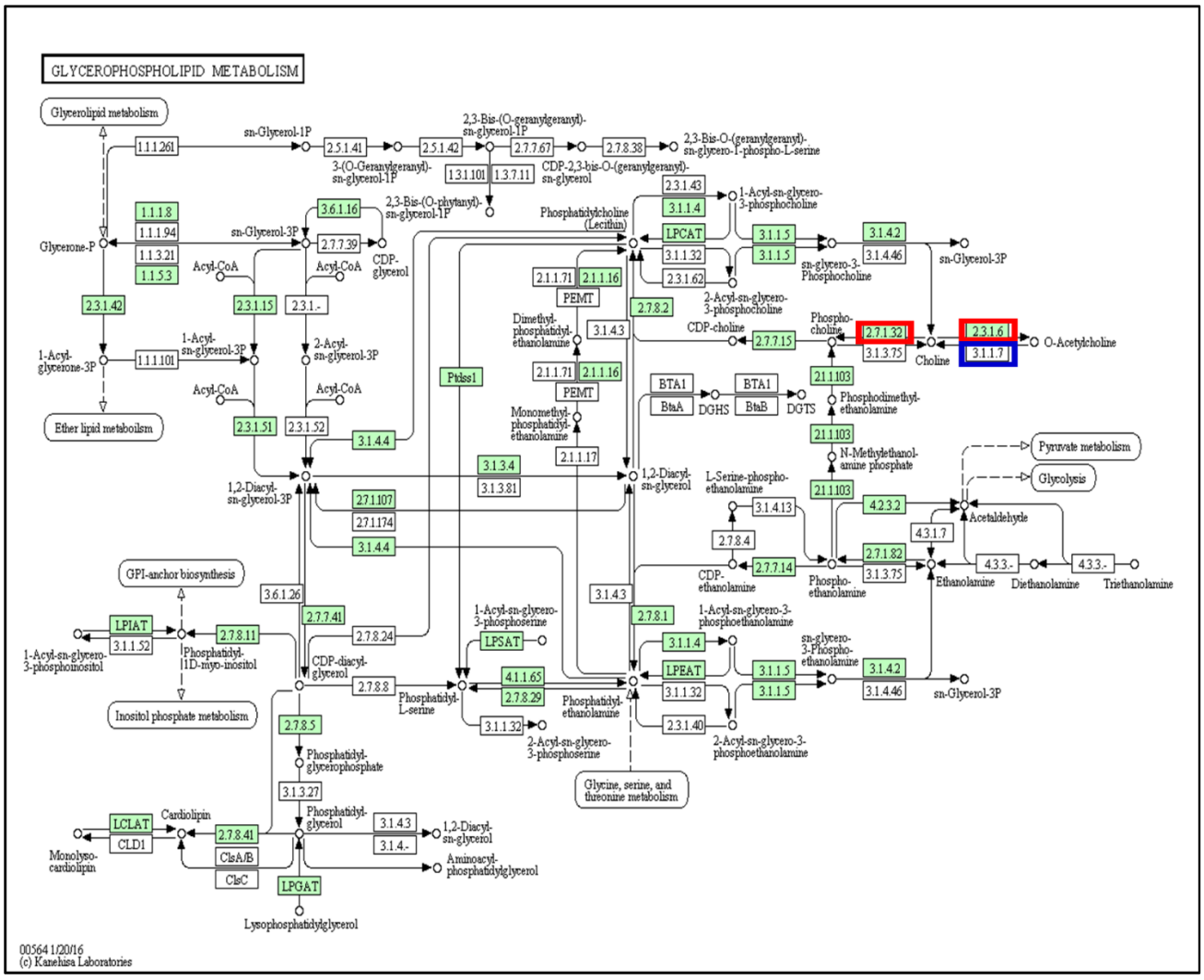

Fig.2.13.A. Glycerophospholipid metabolism pathway of *Crassostrea gigas* showing enzyme matches in green with those relevant to choline ester synthesis highlighted in red (present) and blue (absent).

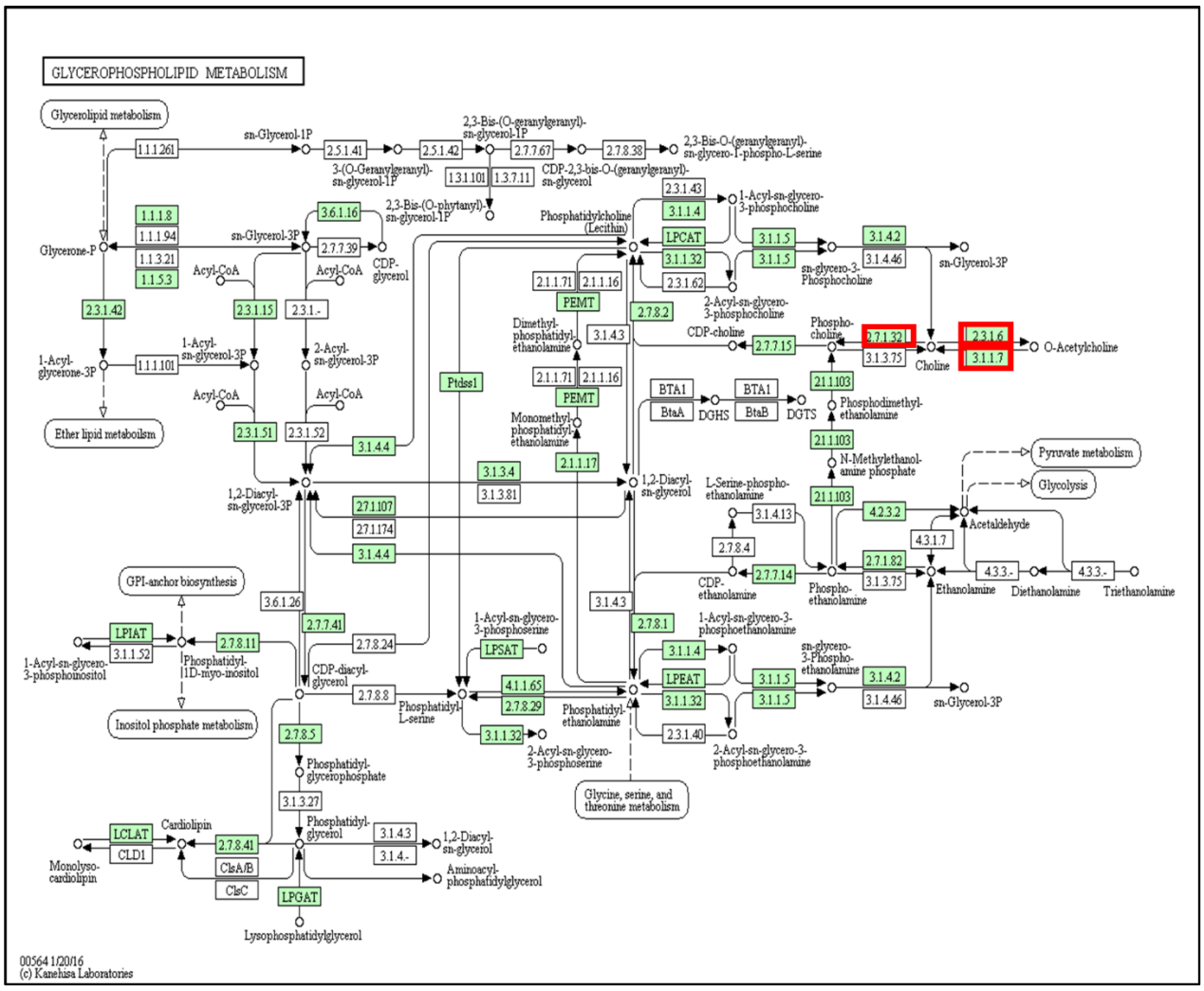

Fig.2.13.B. Glycerophospholipid metabolism pathway of *Lottia gigantea* showing enzyme matches in green with those relevant to choline ester synthesis highlighted in red (present) and blue (absent).

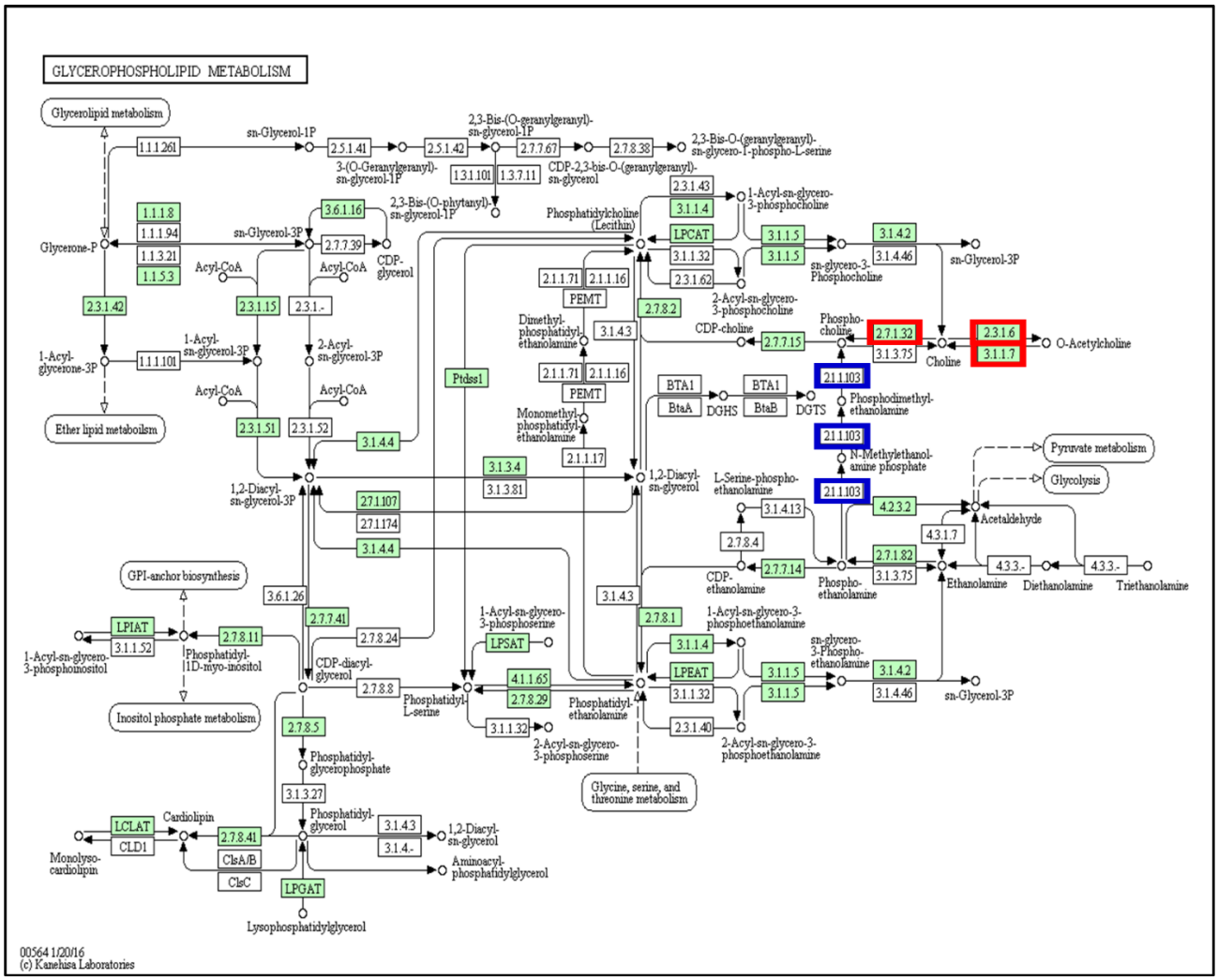

Fig.2.13.C. Glycerophospholipid metabolism pathway *Octopus bimaculoides* showing enzyme matches in green with those relevant to choline ester synthesis highlighted in red (present) and blue (absent).

Choline or acetyl choline combines with imidazole to produce the muscle relaxant murexine (Roseghini et al. 1996) (Figure 2.1B). Imidazole is a derivative of the amino acid histidine (Sundberg & Martin 1974) and there were several matches to the histidine metabolism pathway in the *D. orbita* transcriptome (Fig. 2.14). These include, diamine oxidase (1.4.3.22; Fig. 2.14), aldehyde dehydrogenase (NAD+) (1.2.1.3; Fig. 2.14) and monoamine oxidase (1.4.3.4; Fig. 2.14), which convert histamine into imidazole. There was also a match to histidine ammonia-lyase (4.3.1.3; Fig. 2.14), which converts L-histidine to urocanate, which could feasibly combine with choline ester to form murexine. All these biosynthetic enzymes are also found in the *L. gigantea, O. bimaculoides* and *C. gigas* genomes (Fig. 2.15).

Previous studies of choline esters in molluscs have focused on the predatory neogastropods and there is no record of murexine or similar derivatives being isolated from limpets or oysters. Roseghini et al. (Roseghini et al. 1996) found no evidence for imidazole or acryl choline esters in 27 species from 8 families of herbivorous and scavenging gastropods, including three Patellidae limpets, while at least one of these compounds was found in 53 of 55 species of the predatory Muricoidae superfamily. This implies the Neogastropoda have evolved a specific murexine biosynthesis pathway and, consistent with this, the *D. orbita* transcriptome had more matches to enzymes in the histidine metabolism pathway (19 genes) when compared to *L. gigantea* (10 genes), *O. bimaculoides* (11 genes) and *C. gigas* (13 genes) (Fig. 2.15). Specifically, the enzyme involved in imidazole biosynthesis imidazoleglycerol-phosphate dehydratase (4.2.1.19; Fig. 2.14) was only found in the *D. orbita* transcriptome.

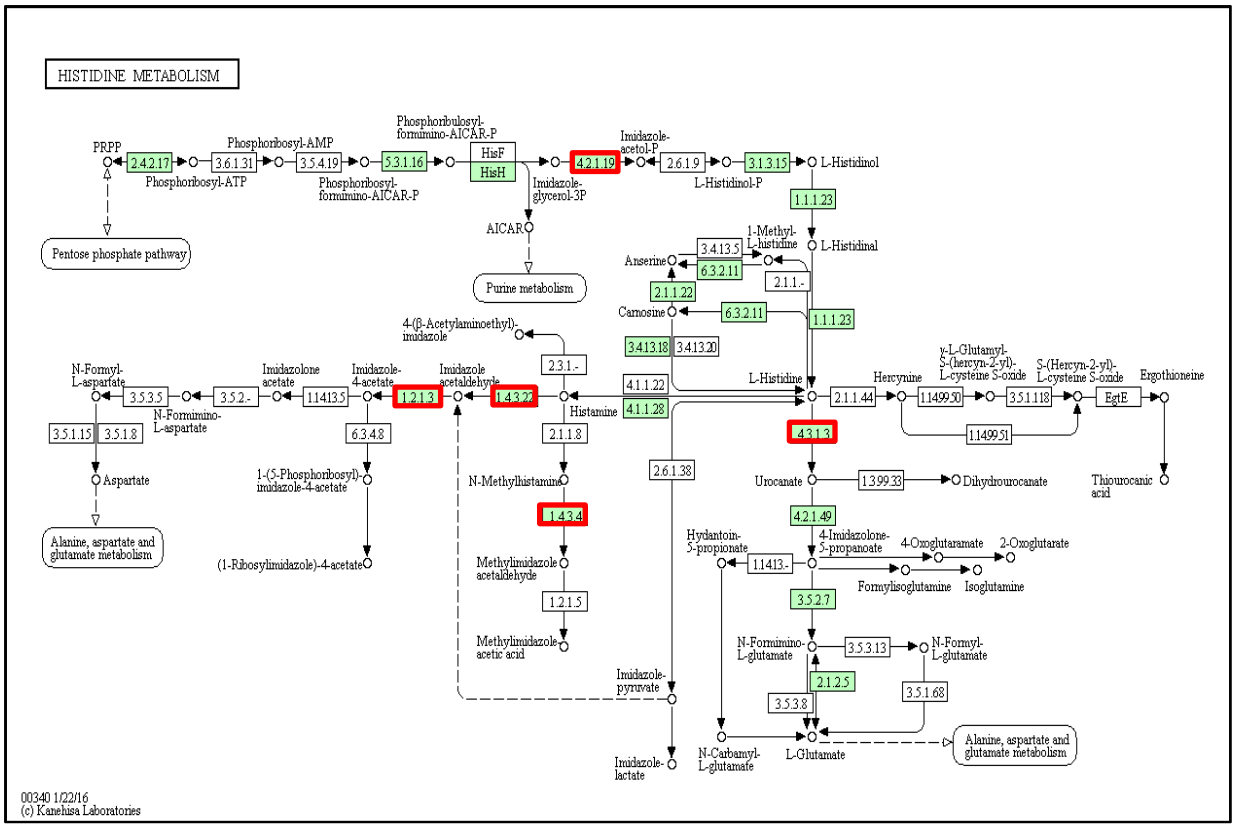

Fig. 2.14. Histidine metabolism pathway showing matches to *Dicathais orbita* contigs highlighted in green, including several enzymes that convert histidine into imidazole (red boxes) and imidazoleglycerol-phosphate dehydratase (red box top pathway).

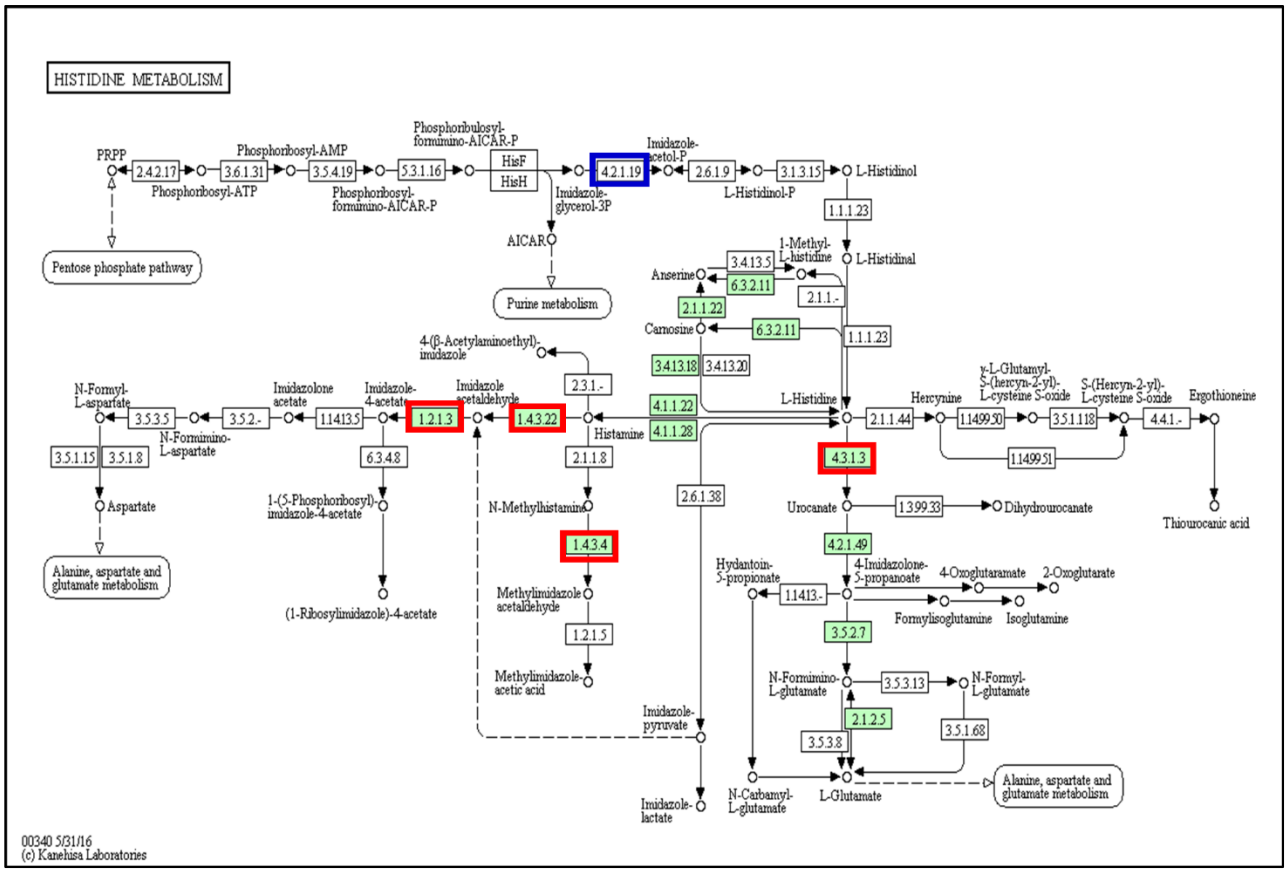

Fig. 2.15.A. Histidine metabolism pathway of *Crassostrea gigas* showing matching enzymes in green, including several enzymes that convert histidine into imidazole (red boxes) but not imidazoleglycerol-phosphate dehydratase (bluebox).

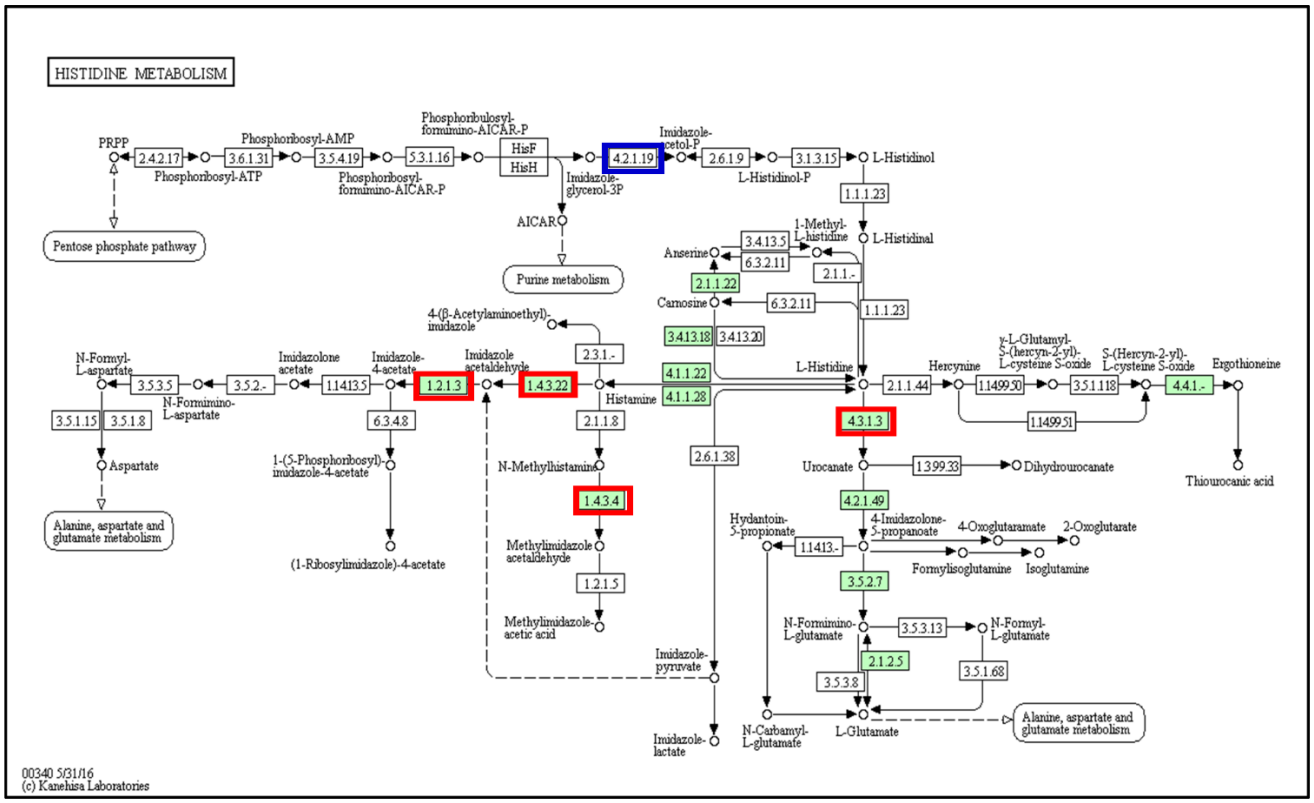

Fig. 2.15.B. Histidine metabolism pathway of *Lottia gigantea* showing matching enzymes in green, including several enzymes that convert histidine into imidazole (red boxes) but not imidazoleglycerol-phosphate dehydratase (bluebox).

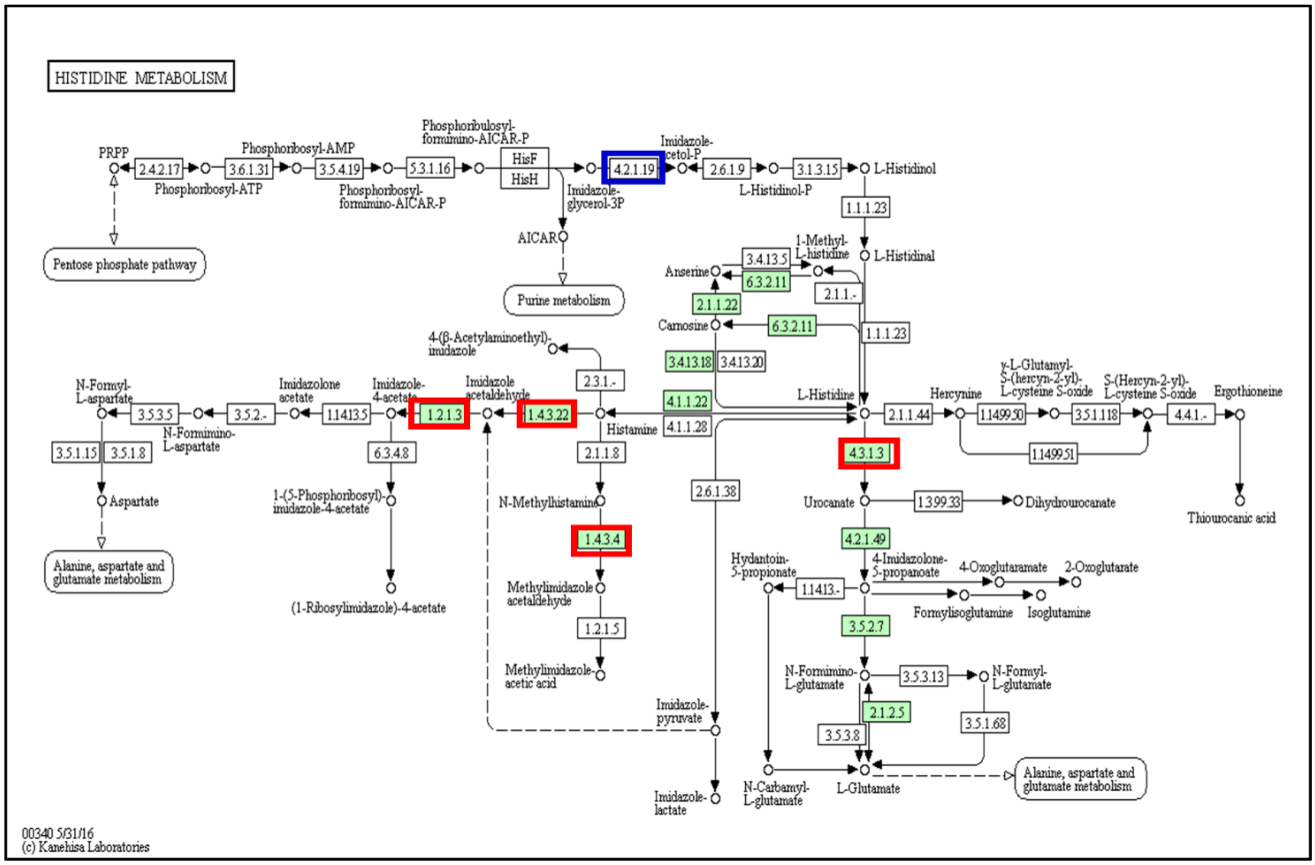

Fig. 2.15.C. Histidine metabolism pathway of *Octopus bimaculoides* showing matching enzymes in green, including several enzymes that convert histidine into imidazole (red boxes) but not imidazoleglycerol-phosphate dehydratase (bluebox).

2.5 Conclusions

This transcriptome study of *D. orbita* generated over 216 million high quality reads that were *de novo* assembled into 219,437 contigs, of which 76,152 contigs contained candidate-coding regions that were annotated with Nr, Swiss-Prot and KEGG databases. This provides a significant new molecular resource for neogastropod molluscs, and adds to pool of genomic data for molluscs in general. Several genes were also identified in *D. orbita* that are potentially associated with indole, sulfur and histidine metabolism pathways relevant to Tyrian purple precursor biosynthesis. However, most of these genes were not found in the fully annotated genomes of *L. gigantea*, *O. bimaculoides* and *C. gigas* available in the KEGG database. It appears the neogastropod *D. orbita* has evolved a complex suite of metabolic capabilities that are not represented in the more primitive orthogastropods or bivalves, for which complete genome sequences are available.

2. 6 Acknowledgments

This work was supported by Southern Cross University International Postgraduate Research Scholarship and School of Environment, Science and Engineering Postgraduate support for A.K.N. and a philanthropic grant to K.B.

Chapter 3. Indole producing bacteria from the biosynthetic organs of muricid mollusc could contribute to Tyrian purple production.

Ngangbam AK, Waters DLE, Whalan S, Baten A, Benkendorff K. Indole producing bacteria from the biosynthetic organs of Muricid mollusc could contribute to Tyrian purple production. J Shellfish Res. 2015; 34: 443-454.

3. 1 Abstract

The Muricid mollusc, *Dicathais orbita*, produces Tyrian purple which is a brominated derivative of the blue dye indigo. This study aimed to establish whether distinct bacterial communities occur in the organs of *D. orbita* associated with Tyrian purple production and to identify indole producing bacteria using 16S rRNA gene sequencing. Biochemical profiles of microbial communities from different *D. orbita* tissues were investigated and the composition of aerobic heterotrophic bacterial populations from homogenates and swabs assessed. There were significant differences in biochemical activity profiles and bacterial communities cultured from different *D. orbita* tissues, but no significant differences between males and females. Bacterial communities derived from foot tissue and sea water samples were similar. The biochemical and molecular evidence from swabs and tissue homogenates suggest indole-producing isolates are *Vibrio* spp. This study suggests Tyrian purple indole precursors could be obtained from opportunistic ubiquitous bacteria.

3. 2 Introduction

Molluscs are an important source of potentially useful secondary metabolites, including biologically active compounds and dyes (Benkendorff 2010). Marine gastropods of the family Muricidae produce Tyrian purple (6, 6'dibromoindigo) (Cooksey 2001), a dye of significant historical importance that can be obtained from no other natural source (Westley & Benkendorff 2008). The precursors of Tyrian purple have interesting pharmacological properties (Benkendorff 2013), including the specific inhibition of a range of cancer cell lines (Benkendorff et al. 2011, Edwards et al. 2012, Esmaeelian et al. 2013) and prevention of early stage colon cancer formation in rodent models (Westley et al. 2010b,

Esmaeelian et al. 2014). These brominated indole precursors are stored as choline ester salts in the hypobranchial glands of Muricidae (Baker & Sutherland 1968, Benkendorff 2013). The main choline ester is murexine which has a muscle relaxing activity and is an effective pain killer (Roseghini et al. 1996). These pharmacological properties of Muricidae extracts and secondary metabolites have raised interest in their potential for development as new nutraceutical products (Benkendorff 2009, Benkendorff 2013).

Sustainable supply is a major impediment to the development of new marine nutraceuticals and pharmaceuticals (Benkendorff 2009, Molinski et al. 2009, Berrue et al. 2011). Many marine natural products are complex and difficult or expensive to chemically synthesize (Sipkema et al. 2005, Berrue et al. 2011), as is the case for some of the bioactive brominated indole precursors of Tyrian purple (Benkendorff 2013). In such cases, further insight into the biosynthetic origin, including potential microbial symbionts and, the specific genes involved in the biosynthesis of these secondary metabolites may provide options for supplying sufficient amounts of marine compounds for clinical testing and commercialization (Sipkema et al. 2005, Berrue et al. 2011, Lane & Moore 2011).

Secondary metabolites associated with marine invertebrates are often produced by symbiotic bacteria (Berrue et al. 2011) and comparative genetic studies of 16S rRNA highlight the co-evolution between symbiotic microbes and their invertebrate hosts (Radjasa et al. 2011), particularly in the Porifera (sponges) (Thacker & Starnes 2003). Secondary metabolites, such as the polyketide onnamides with antitumor properties, arise from bacterial symbionts of the host sponge *Theonella swinhoei* (Piel et al. 2004), whereas bryostatins, anticancer agents first isolated from the bryozoan *Bugula neritina,* require the microbial symbiont *Endobugula sertula* for their synthesis (Davidson et al. 2001, Lopanik et al. 2004, Sudek et al. 2007). Several other secondary metabolites are due to microbial

symbionts associated with marine sponges (e.g. (Unson et al. 1994, Sacristan-Soriano et al. 2011)), although microbial populations associated with other marine invertebrates are less-well studied.

Nevertheless, studies in the last decade have begun to investigate the microbial origin of some secondary metabolites found in molluscs. For example, dolastatin 10, an antitumor agent (Kindler et al. 2005) originally isolated from the marine gastropod *Dolabella auricularia*, (Pettit et al. 1987) was recently found to be associated with the marine cyanobacterium *Symploca* sp. (Luesch et al. 2001). Another antitumor depsipeptide, kahalalide F (Cruz et al. 2009), was originally isolated from *Elysia rufescens*, but is now thought to be of bacterial (Davis et al. 2013) or algal (*Bryopsis pennata*) origin (Henrıquez et al. 2005). Recently, *Streptomyces* sp. cultivated from the gastropod *Lienardia totopotens*, was reported to be the source of the peptide compound totopotensamide A and totopotensamide B (Lin et al. 2012). Lin et al. (2013), also reported that pyrone polyketides found in the cone snail *Conus rolani*, are synthesized by bacteria. These examples highlight the potential for shelled molluscs to be a source of microbially derived secondary metabolites.

Structural similarity between marine invertebrate secondary metabolites and microbial compounds has been used to infer a microbial origin. For example, Rath et al. (2011), hypothesized that Trabectedin (ET-743) (D'Incalci & Galmarini 2010) is the product of a marine bacterial symbiont *Ecteinascidia turbinata* because of its structural similarities to secondary metabolites of the bacterial species *Myxococcus xanthus*, *Streptomyces lavendulae* and *Pseudomonas fluorescens* (Arai et al. 1980, Ikeda et al. 1983, Irschik et al. 1988, Rath et al. 2011). A large number of natural products from marine invertebrates, including non-ribosomal peptides, polyketides and hybrid molecules are consistent with

bacterial metabolites (Faulkner 2000). These structural similarities suggest many marine invertebrate natural products could be partially or wholly derived from microbial symbionts rather than being synthesized by the host alone (Lane & Moore 2011).

The biosynthetic origin of Tyrian purple which has been exclusively isolated from Muricidae molluscs is not currently known. Indigo (which has the same structure as Tyrian purple but without the two attached bromines), is produced by a diverse range of bacterial species residing in soil (Lim et al. 2005), marine (Mercadal et al. 2010) and sludge environments (Qu et al. 2010, Qu et al. 2012). The structural similarities between indigo and 6, 6'dibromoindigo suggest the possible involvement of symbiotic bacteria in the biosynthesis of this compound in muricid molluscs.

Tyrian purple is generated from indoxyl sulphate precursors in the hypobranchial glands of Muricidae (Baker & Sutherland 1968). The essential amino acid tryptophan, has been hypothesized to be the ultimate precursor for indoles production, in which tryptophanase enzymes act on tryptophan to produce indoles (Verhecken 1989, Naegel & Alvarez 2005, Westley et al. 2006, Benkendorff 2013, Laffy et al. 2013). Indeed, tryptophan appears to be stored in the hypobranchial glands of a range of muricids (Srilakshmi 1991, Naegel & Aguilar-Cruz 2006). Westley and Benkendorff (2009), have identified the presence of tryptophan in the hypobranchial gland, and female reproductive organs, of the Australian muricid *Dicathais orbita* based on histochemical analysis. This is consistent with reports of Tyrian purple and indole precursors occurring in the hypobranchial glands, reproductive organs (Westley & Benkendorff 2008) and egg capsules of Muricidae (Palma et al. 1991, Benkendorff et al. 2000, Benkendorff et al. 2001a). Like all animals, molluscs cannot biosynthesize tryptophan, (Verhecken 1989, Rouhbakhsh et al. 1997, Westley et al. 2006) so this essential amino acid must be derived from either the diet or symbiotic bacteria.

Previous researchers have assumed a dietary origin of tryptophan (Verhecken 1989, Westley et al. 2006, Westley et al. 2010a), but here we propose a bacterial role in Tyrian purple precursor synthesis.

Indole biosynthesis can be detected in microbial communities using biochemical tests such as the indole (tryptophan degradation) test and tryptophanase activity (Holding & Collee 1971, Lammert 2007). More generally, biochemical kits can be used to identify bacteria and elucidate differences in bacterial communities based on the substrates that they can metabolise. Biochemical kits, such as API 20E, are typically used for identification of Gram negative rods and Enterobactericeae, but have been successfully applied to the preliminary characterisation of microbial communities in marine samples (Alcaide et al. 1999, Xie et al. 2007, Peters et al. 2012) and this approach enables functional profiling of heterotrophic bacterial communities in different tissues.

The ability to culture bacteria using traditional techniques is an advantage for subsequent large scale production of marine natural products and so it is useful to investigate the diversity of bacteria that can be cultured from biosynthetic organs using traditional culture techniques. The main aim of this study was to compare the biochemical profiles of microbial communities derived from different tissues of *D. orbita,* in order to establish whether any unique bacteria or distinct bacterial communities are associated with the biosynthetic organs compared to tissues that are known not to be involved in Tyrian purple production. A secondary aim was to culture aerobic heterotrophic bacteria from these tissues and then identify any indole producing bacteria using 16S rRNA gene sequencing. Using this approach, three indole producing bacteria were isolated and cultured from the biosynthetic organs of *D. orbita*, thus confirming symbiotic bacteria could contribute to Tyrian purple production.

3. 3 Materials and Methods

3. 3. 1 Sample collection and preparation

The muricid mollusc *Dicathais orbita* was collected under permit number P10/0069-1.0 issued by Primary Industries, NSW Government, Australia. The snails were collected from the sub-tidal and intertidal rocky reefs near Ballina (28°84′ S and 153°60′ E) on the north coast of NSW, Australia during low tides in August and November 2013. Snails were transported live to Southern Cross University in Lismore (~40 min) and held in aerated seawater tanks until processing (within 24 hrs). Twelve snails were collected in August for standardised comparison of the predominant culturable heterotrophic bacteria from different tissues; three male and three female *D. orbita* were used for biochemical characterisation and a further three independent males and three females were used for culturing the predominant heterotrophic symbiotic bacteria. From each sample, swabs were taken from the outer surface of the tissues before dissection and homogenates were subsequently prepared from the inner tissue surfaces which were untouched during swabbing.

Supplementary sampling during November 2013 involved seven snails (swabs and homogenates) and egg capsules of *D. orbita* (Fig. 3.1A). These were used to obtain additional cultures and further characterise the bacterial colonies by Gram staining, motility, oxidase and indole tests (see below).

The shell of *D. orbita* was removed according to Westley and Benkendorff (2008), using a bench vice with pressure applied at the point between the primary body whorl and spire. Tissues from the foot, hypobranchial, rectal, prostate (males, Fig. 3.1B) and capsule glands (females, Fig. 3.1C) were dissected under sterile conditions (Westley & Benkendorff 2008). An incision was made into each tissue and swabbed across a 0.3 cm^2 surface area with a sterile cotton bud, then placed in 2 ml microcentrifuge tubes containing 0.5 ml sterile

sea water. Swabs were taken from three male and three female snails to provide independent replicates for each tissue type (Fig. 3.1). Additionally, tissue homogenates weighing 0.1 g were taken from a separate region of each sample of foot muscle, hypobranchial, rectal, prostate and capsule glands. These were prepared in 0.5 ml sterile seawater by homogenising the tissues with a UV treated mortar and pestle.

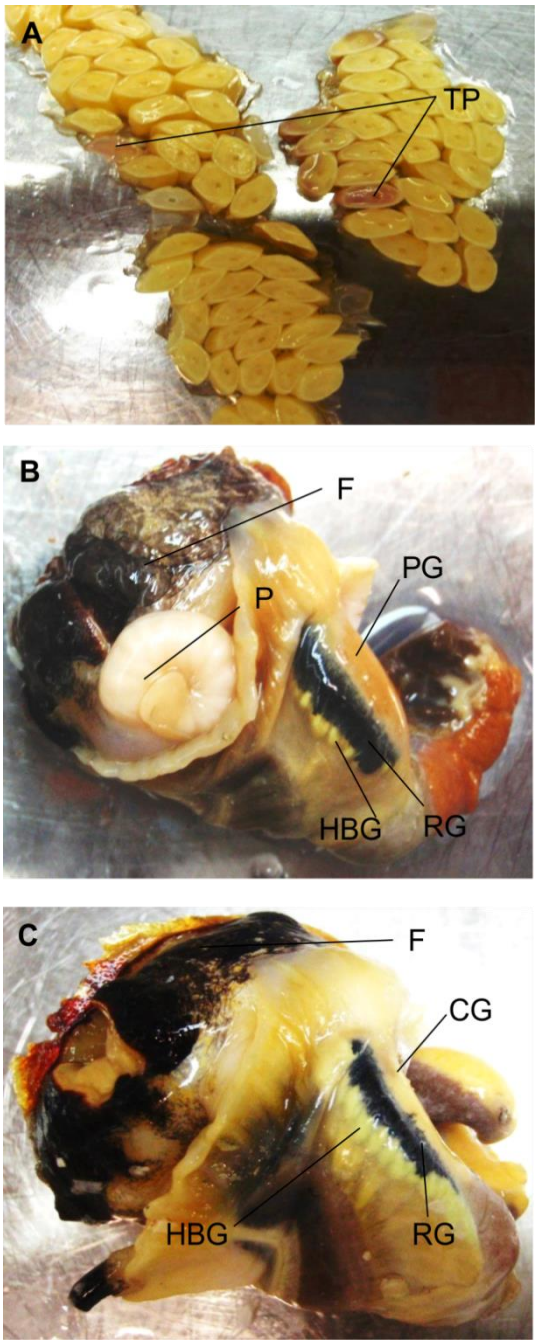

Fig. 3. 1. The *Dicathais orbita* tissues used for sampling bacterial communities. (A) Egg capsules of *D. orbita* showing some Tyrian purple = TP staining within a couple of oxidised capsules. The intermediate precursors of TP appear yellow in the HBG (hypobranchial gland) of adults and freshly laid capsules. (B) Male *D. orbita* dissection showing PG, prostate gland; P, penis; as well as the HBG, RG, rectal gland; F, foot muscle. (C) Female *D. orbita* dissection showing CG, capsule gland; RG, HBG, and F.

3. 3. 2 Biochemical characterisation

Biochemical characterisation of the bacterial communities from each of the triplicate tissue swabs and homogenate samples was undertaken using a biochemical characterization kit, (API 20E, BioMerieux, Marcy I'Etoile, France) according to the manufacturers's instructions. Triplicate samples of fresh seawater from the same location where the snails were collected were used as background controls. The swab and homogenate suspensions were diluted in 5 ml sterile seawater. After vortexing, 200 µl of the suspension was pipetted into each well of the biochemical test strip. Anaerobic conditions were maintained by overlaying the wells with mineral oil for the tests of arginine dihydrolase (ADH), ornithine decarboxylase (ODC), lysine decarboxylase (LDC), hydrogen sulfide and urease. The mineral oil does not create an anoxic environment itself but effectively provides a barrier against the continual diffusion of oxygen into the media and thus prevents potential false positives. Tests for citrate (CIT), acetoin production (VP) and gelatinase utilization (GEL) were performed by filling both the well and cupule of the test strips. All other tests were performed with only the well filled, but not the cupule. Test strips were incubated at 25 °C for 48 hrs.

3. 3. 3 Heterotrophic bacterial culture and biochemical identification of indole producing bacteria

Two types of agar, marine agar (Difco Marine Broth 2216 and Bacto Agar) and nutrient agar (Peptone- 5 g, Yeast extract- 3 g, NaCl- 5 g, Agar- 15 g, Milli-Q water- 1000 ml) were used for the recovery of aerobic bacteria. A threefold dilution series was prepared from each of the triplicate swab and homogenate samples and then aliquots (100 µl) were plated in triplicate and spread evenly onto agar and incubated at 25 ^{o}C for 48 to 72 hrs.

Seawater controls were prepared in triplicate. Morphologically distinct colonies were described and counted at the lowest dilution possible then converted to colony forming units per ml.

Supplementary swabs and homogenates from the tissues of seven additional snails were undertaken by streaking onto fresh marine agar to obtain pure cultures. Gram staining and oxidase tests were performed for each of the pure cultures and each isolate was analysed for indole production using API 20E test strips. The indole test (BioMerieux, Marcy I'Etoile, France) was performed using 5 ml of sterile sea water containing a loop of an individual colony from each pure culture. The test strips were inoculated with the bacterial suspension and incubated for 24 hrs at 25 °C. After 24 hrs of incubation, one drop of JAMES reagent (BioMerieux, Marcy I'Etoile, France) was added to the test strips. The formation of a pink colour in the whole cupule was scored as a positive reaction. The indole positive cultures were preserved by diluting 1:1 in sterile marine broth containing 30 % glycerol and stored at -80 ^{o}C pending further analysis.

3. 3. 4 16S rRNA analysis

Indole positive colonies isolated from biosynthetic tissues were identified by 16S rRNA gene sequencing. DNA of the indole positive isolates was extracted (QIAamp DNA Mini Kit, Qiagen) and 16S rRNA amplified using primer pair 27F-5′-GAGAGTTTGATCCTGGCTCAG-3′ and 1492R 5′-CTACGGCTACCTTGTTACGA-3′ (Li & Liu 2006, Chen et al. 2012). The PCR mix included 2.5 µl of 10x PCR buffer; 2.5 µl of dNTPs (2mM), 1.25 µl of 50 mM $MgCl_2$; 1 µl genomic DNA (35-80 ng); 0.4 µl *Taq* polymerase and 1 µl forward primer (FP) (10 µM), 1 µl reverse primer (RP) (10 µM), 15.35 µl Milli-Q water in a final volume of 25 µl. PCR cycle conditions comprised an initial

denaturation at 94 °C for 5 min followed by 30 cycles of 45 s at 95 °C, 1 min at 58 °C and 1 min at 72 °C. The PCR amplicons were separated by agarose gel electrophoresis (1.5%) followed by visualisation with GelRed staining under UV irradiation, purified in accordance with the manufacturer's instructions (QIAquick PCR Purification Kit, Qiagen) and sequenced by the Australian Genome Research Facility (AGRF), Brisbane, using Applied Biosystems 3730 and 3730xl capillary sequencers. DNA sequences were analysed using Sequence scanner software v1.0 and compared with sequences in the NCBI GenBank database by BLASTN. All nucleotide sequences were submitted to GenBank with the following GenBank accession nos. KM242644, KM242645, KM242646, KM242647, KM242648 and KM242649.

3. 3. 5 Statistical analyses

Multivariate analyses of the biochemical activity (presence/absence) were analysed using PRIMER 6 and PERMANOVA. Owing to a lack of independence in the source snails, separate analyses were run for the swab and homogenate samples. Similarity matrices were generated using Euclidean distance for the biochemical profiles. All analyses were run using 9999 permutations of the data. For the biochemical data, two factors nested PERMANOVAs were run with "gender" (male, female) and "tissue" (foot, hypobranchial gland, rectal gland, prostate, capsule gland) nested in gender. Pairwise tests were then undertaken on the significant factors. Non metric multi-dimensional scaling (nMDS) plots were used to graphically represent the data. Seawater was included as an additional variable in the plots for homogenates.

To assess the richness (number of morphologically distinct colony types), total abundance (CFU/ml from 0.3 cm^2 swabs or 0.1 g homogenates) and diversity (Shannon's H

index) from the standardised sampling of seawater and each *D. orbita* tissue, the DIVERSE function in PRIMER 6 was used. Three factor nested univariate analyses were then performed on the Bray-Curtis similarity matrices, with a dummy variable of 1. Separate analyses were run on the swabs and homogenates. The factors used in each analysis were "gender", "tissue" (nested in gender) and "agar" (marine or nutrient) with pairwise tests undertaken to establish significant differences between tissues, split for agar type in cases where the interaction term was significant. Multivariate analyses were not undertaken on the cultured bacterial communities due to uncertainty regarding whether morphologically similar bacteria were actually the same across all the different tissue samples.

3. 4 Results

3. 4 .1 Biochemical comparison of the bacterial communities associated with different tissues of *Dicathais orbita*

API 20E biochemical analysis of swabs and homogenates revealed the same biochemical substrates were utilised by bacteria in the foot (non-Tyrian purple producing tissue) of males and females and sea water (Table 3.1). There was urea metabolism in foot tissue swabs which was absent in foot homogenates and all other *D. orbita* tissues (Table 3.1). No biochemical reactions were observed in homogenates of the hypobranchial and rectal gland from male and female snails (Table 3.1). Positive biochemical reactions were recorded in swabs taken from the hypobranchial and rectal glands, although there was some inconsistency between replicate samples for many of the tests, with only one or two out of three positive reactions for many of the biochemical tests (Table 3.1). All other tissues showed a high degree of consistency in the biochemical profiles between replicates (Table 3.1). Indole positive reactions were observed in swab samples from all tissues, as well the seawater and homogenates from all tissues except the hypobranchial and rectal gland. ADH

(L-arginine) metabolism was absent in swabs from the hypobranchial gland of both sexes, but was present in some samples from all other tissues. The metabolism of ODC (L-ornithine) and LDC (L-lysine) were less frequent in swabs from the hypobranchial gland, but these amino acids were consistently metabolised in all other tissues. The metabolism of sodium thiosulfate, which leads to hydrogen sulfide (H_2S) production, was less apparent in swabs from male tissues compared with females (Table 3.1). The metabolism of sugars such as RHA (L-rhamnose) and MEL (D-melibiose) was only detected in swabs from one replicate of the female biosynthetic organs (hypobranchial and rectal glands) and generally not in these tissues from males, except one replicate of the male prostate gland (Table 3.1). ARA (L-arabinose) was only associated with the female internal organs, but was not detected in homogenates or swabs from the male tissues except for the foot (Table 3.1).

Table 3.1. API 20E biochemical test of *Dicathais orbita* tissue homogenates and swabs.

Test	Homogenates									Swabs							
	CG	PG	RG		HBG		Foot		SW	CG	PG	RG		HBG		Foot	
	F	M	F	M	F	M	F	M	SW	F	M	F	M	F	M	F	M
ONPG	+++	+++	-	-	-	-	+++	+++	+++	+++	+++	+++	+++	+++	++	+++	+++
ADH	+++	+++	-	-	-	-	+++	+++	+++	+++	+++	+++	+	-	-	+++	+++
LDC	+++	+++	-	-	-	-	+++	+++	+++	+++	+++	++	+++	+	-	+++	+++
ODC	+++	+++	-	-	-	-	+++	+++	+++	+++	+++	+++	+++	+	+	+++	+++
CIT	+++	+++	-	-	-	-	+++	+++	+++	+++	+++	+++	+++	+++	+	+++	+++
H_2S	+++	+++	-	-	-	-	+++	+++	+++	++	-	+	-	+	-	+++	+++
URE	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	++	+++
TDA	+++	+++	-	-	-	-	+++	+++	+++	+++	+++	+++	+++	+++	++	+++	+++
IND	+++	+++	-	-	-	-	+++	+++	+++	+++	+++	+++	+++	+++	++	+++	+++
VP	+++	+++	-	-	-	-	+++	+++	+++	+++	+++	+++	+++	+++	++	+++	+++
GEL	+++	+++	-	-	-	-	+++	+++	+++	+++	+++	+++	+++	++	++	+++	+++
GLU	+++	+++	-	-	-	-	+++	+++	+++	+++	+++	+++	+++	+++	++	+++	+++
MAN	+++	+++	-	-	-	-	+++	+++	+++	+++	+++	+++	+++	+++	++	+++	+++
INO	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
SOR	+++	+++	-	-	-	-	+++	+++	+++	+++	++	++	+++	+++	-	+++	+++
RHA	+++	++	-	-	-	-	+++	+++	+++	++	+	+	-	+	-	+++	+++
SAC	+++	+++	-	-	-	-	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
MEL	+++	++	-	-	-	-	+++	+++	+++	-	+	+	+	+	-	+++	+++
AMY	+++	+++	-	-	-	-	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
ARA	+++	-	-	-	-	-	+++	+++	+++	+++	-	++	-	+	-	+++	+++

"+", positive biochemical test in a single replicate; "++" and "+++" indicate positive tests in 2 or 3 replicate samples respectively; "-" = negative biochemical test in all three replicates.CG, capsule gland; PG, prostate gland; RG, rectal gland; HBG, hypobranchial gland; SW, sea water; F, female; M, male; Biochemical test: ONPG, Ortho NitroPhenyl-βD Galactopyranosidase; ADH, Arginine dihydrolase; LDC, Lysine decarboxylase; ODC, Ornithine decarboxylase; CIT, Citrate utilization; H_2S, H_2S production; URE, Urease; TDA, Tryptophan deaminase; IND, Indole production; VP, Acetoin production; GEL, Gelatinase; GLU, Glucose; MAN, Mannitol; INO, Inositol; SOR, Sorbitol; RHA, Rhamnose; SAC, Saccharose; MEL, Melibiose; AMY, Amygdalin; ARA, Arabinose.

Multivariate analyses for biochemical activity profiles from bacterial communities associated with swabs from *D. orbita* showed that there was no significant difference between males and females (Pseudo F = 0.702, p = 0.626). However, there was a significant difference between tissue samples within the sexes (Pseudo F = 3.781, p < 0.01). Multivariate analyses for the biochemical activity profiles of bacterial communities from the homogenates from *D. orbita* showed similar results to the swabs (Fig. 3.2). There was no significant difference between the homogenates sampled from males and females (Pseudo F = 0.377, p = 0.945), but there was a significant difference between the tissue samples within the sexes (Pseudo F = 157.5, p < 0.01).

Non-metric Multi-Dimensional Scaling of swab samples revealed some variation among samples, with the ordination showing separation of hypobranchial and prostate gland samples from foot samples (Fig. 3.2A). The nMDS analysis and pairwise tests between tissues from swab samples of male and female showed that the hypobranchial gland was significantly different to the foot in female (p = 0.026) and males (p = 0.009). The female capsule glands and male prostate and rectal glands were also significantly different to the foot (p < 0.05). The rectal gland was not significantly different from the hypobranchial gland (p = 0.302), capsule (p = 0.489) or prostate gland (p = 0.358).

The nMDS analyses of homogenates produced an ordination where tight clustering of hypobranchial and rectal gland samples occurred and both showed clear separation from all the other tissues (Fig. 3.2B). In addition, the ordination showed foot and female capsule gland samples grouping close to seawater samples (Fig. 3.2B). Pairwise tests between tissues for the homogenates in PERMANOVA confirmed that the hypobranchial and rectal glands were different to all other tissues (p < 0.05). Furthermore, the rectal gland was different to the prostate gland (p < 0.01). The prostate gland was also different from the hypobranchial gland (p < 0.01), but not to the foot (p = 0.054).

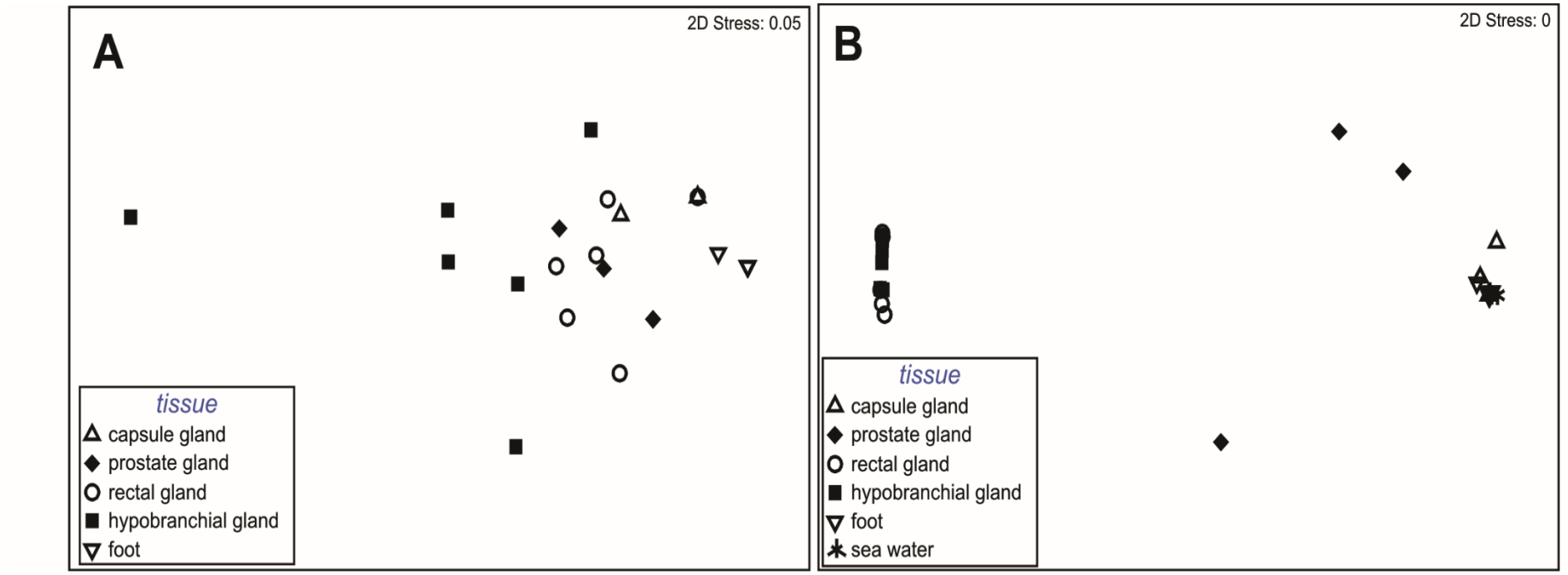

Fig. 3. 2. Differences in the multivariate bacterial biochemical profiles from various *Dicathais orbita* tissues. Non-parametric multidimensional scaling from Euclidean Distance similarity matrices based on 20 biochemical reactions from API 20E substrates in (A) swabs and (B) homogenates of seawater and different *D. orbita* tissues.

3. 4. 2 Bacterial diversity cultured from different tissues of *Dicathais orbita* and identification of indole producing bacteria

There was substantial variation in the types of bacterial colonies isolated from the different tissue and seawater samples, with on average less than four colony types per sample (Table 3.2), compared with a collective total of 16 distinct colonies (Table 3.3). The maximum number of morphologically distinct bacteria cultured from individual samples from seawater and some prostate samples was five (data not shown). The highest abundance and diversity of bacterial colonies was found in the seawater and the foot samples, whereas the lowest bacterial richness, abundance and diversity was recovered from the hypobranchial and rectal glands of *D. orbita* (Table 3.2). The reduced bacterial diversity in these biosynthetic tissues is likely to account for the fewer biochemical reactions (Table 3.1) and consequently the separate clustering based on biochemical profiles (Figure 3.2), in comparison to other tissues.

Univariate PERMANOVA for the richness of bacterial colony types from the homogenate samples revealed that on average, significantly more morphological distinct types of bacteria were cultured on marine agar (mean 1.9±1.3) compared to nutrient agar (mean 1.1±1.1) (Pseudo $F = 6.791$, $p = 0.026$). Similarly for the swab samples, there was a greater richness of bacterial species cultured on marine (mean 2.3±1.3) than nutrient agar (mean 1.4±1) (Pseudo $F = 11.448$, $p = 0.008$). For both homogenate and swab samples, there was no significant interaction between agar and gender or tissue ($p > 0.05$) and no significant difference between males and females ($p > 0.4$).There was, however, a significant difference in the richness of bacterial species cultured from the different tissues (Homogenates: Pseudo $F = 4.072$, $p = 0.001$; Swabs: Pseudo $F = 2.967$, $p = 0.012$). Pairwise tests on the homogenates revealed fewer types of distinct bacteria in the hypobranchial gland of females

compared to all other tissues (Table 3.2). In males, the hypobranchial glands and the rectal glands had fewer types of bacteria than the foot, but the rectal glands were not different from any other tissue (Table 3.2). Very similar results were found for bacterial richness in the swabs, with significantly fewer morphological types isolated from the hypobranchial glands in comparison to the foot tissue, as well as the capsule gland in females (Table 3.2).

Analysis of the total abundance of bacteria from homogenate samples revealed no significant differences or interactions between agar and gender ($p > 0.05$). There was, however, significantly higher abundances of CFUs on marine agar (mean 41.5±43) than nutrient agar (15.5±19) (Pseudo $F = 4.787$, $p = 0.033$) from swab samples. For both the homogenate and swab samples, there was a significant difference between tissues (homogenates Pseudo $F = 9.95$, $p < 0.01$; swabs Pseudo $F = 3.689$, $p < 0.01$). The hypobranchial gland homogenates had significantly fewer CFUs than all other tissue homogenates, except the male rectal glands (Table 3.2). There were also fewer bacteria cultured from female rectal glands compared to female foot tissues (Table 3.2). Similarly in female swab samples, there were significantly fewer bacteria cultured from the hypobranchial glands than all other tissues, whereas in the males, significantly fewer CFUs were cultured form both the rectal and hypobranchial gland compared to the foot (Table 3.2). Very similar statistical outcomes were found using the Shannon's diversity index (Table 3.2), with tissue type being the main significant factor in both homogenates (Pseudo $F = 3.957$, $p = 0.003$) and swabs (Pseudo $F = 4.152$, $p = 0.002$).

Table 3. 2. Diversity of bacteria cultured from different tissues of *Dicathais orbita*.

Preparation	Agar	Sex	Tissue	Richness (Mean±SD)	Total abundance (Mean±SD)	H index (Mean±SD)
Homogenates	Marine agar	NA	Sea water	3.33 ± 2.08	18.66 ± 10.40	0.92 ± 0.80
		Female	Foot	3.0 ± 0.0^{a}	14.0 ± 5.29^{a}	0.95 ± 0.07^{a}
			Hypobranchial gland	0.33 ± 0.57^{b}	0.33 ± 0.57^{c}	0.0 ± 0.0^{b}
			Rectal gland	1.66 ± 0.57^{a}	2.33 ± 0.57^{b}	0.45 ± 0.39^{ab}
			Capsule gland	2.33 ± 0.57^{a}	7.33 ± 6.11^{ab}	0.68 ± 0.21^{a}
		Male	Foot	3.0 ± 1.0^{a}	14.66 ± 4.50^{a}	0.86 ± 0.31^{a}
			Hypobranchial gland	1.0 ± 0.0^{b}	1.0 ± 0.0^{b}	0.0 ± 0.0^{b}
			Prostate gland	1.33 ± 0.57^{ab}	9.66 ± 2.51^{a}	0.15 ± 0.27^{b}
			Rectal gland	1.0 ± 0.0^{b}	1.33 ± 0.57^{b}	0.0 ± 0.0^{b}
	Nutrient agar	NA	Sea water	3.0 ± 2.0	13.0 ± 6.24	0.83 ± 0.75
		Female	Foot	1.0 ± 1.73^{a}	3.66 ± 6.35^{a}	0.27 ± 0.48^{a}
			Hypobranchial gland	0.0 ± 0.0^{b}	0.0 ± 0.0^{c}	0.0 ± 0.0^{b}
			Rectal gland	0.33 ± 0.57^{a}	0.33 ± 0.57^{b}	0.0 ± 0.0^{b}
			Capsule gland	1.0 ± 1.0^{a}	5.33 ± 6.80^{bc}	0.23 ± 0.40^{a}
		Male	Foot	2.0 ± 1.0^{a}	7.66 ± 4.04^{a}	0.51 ± 0.52^{a}
			Hypobranchial gland	1.0 ± 1.0^{b}	1.66 ± 2.08^{b}	0.22 ± 0.38^{b}
			Prostate gland	1.33 ± 0.57^{ab}	8.66 ± 2.51^{a}	0.16 ± 0.28^{b}
			Rectal gland	0.66 ± 0.57^{b}	0.66 ± 0.57^{b}	0.0 ± 0.0^{b}
Swabs	Marine agar	Female	Foot	3.0 ± 0.0^{a}	55.33 ± 32.47^{a}	0.78 ± 0.27^{a}
			Hypobranchial gland	1.0 ± 0.0^{b}	2.66 ± 1.52^{b}	0.0 ± 0.0^{b}
			Rectal gland	2.0 ± 1.0^{ab}	24.66 ± 10.40^{a}	0.49 ± 0.43^{a}
			Capsule gland	2.0 ± 0.0^{a}	46.66 ± 51.38^{a}	0.50 ± 0.16^{a}
		Male	Foot	3.0 ± 0.0^{a}	78.67 ± 28.59^{a}	0.63± 0.29^{a}
			Hypobranchial gland	2.0 ± 1.0^{b}	11.0 ± 5.29^{b}	0.51 ± 0.45^{b}
			Prostate gland	3.0 ± 2.64^{ab}	90.33 ± 78.42^{ab}	0.64 ± 0.56^{ab}
			Rectal gland	1.33 ± 0.57^{b}	22.66 ± 29.14^{b}	0.15 ± 0.27^{b}
	Nutrient agar	Female	Foot	1.0 ± 1.0^{ab}	22.33 ± 32.80^{a}	0.22 ± 0.39^{a}
			Hypobranchial gland	0.66 ± 0.57^{a}	0.66 ± 0.57^{b}	0.0 ± 0.0^{b}
			Rectal gland	1.66 ± 1.52^{ab}	20.66 ± 21.54^{a}	0.43 ± 0.39^{a}
			Capsule gland	1.33 ± 0.57^{b}	22.0 ± 33.80^{a}	0.13 ± 0.24^{a}
		Male	Foot	1.0 ± 1.0^{a}	25.67 ± 9.02^{a}	0.79 ± 0.23^{a}
			Hypobranchial gland	0.66 ± 0.57^{b}	1.33 ± 1.52^{b}	0.0 ± 0.0^{b}
			Prostate gland	1.66 ± 1.52^{ab}	27.66 ± 24.37^{ab}	0.44 ± 0.41^{ab}
			Rectal gland	1.0 ± 0.0^{ab}	3.33 ± 1.15^{b}	0.0 ± 0.0^{b}

Richness, number of morphologically distinct bacteria; Total abundance, number of colony forming units per ml from a 0.3 cm2 swab or 0.1 g tissue homogenate; H, Shannons diversity index. Different small letters indicate statistically difference between tissues nested in sex in PERMANOVA pair-wise tests ($p < 0.05$).

In total, sixteen morphologically distinct bacterial colonies were isolated from the supplementary sampling of *D. orbita* adults and egg capsules, (Table 3.3). Only two of the most abundant colony types (LC1 and SC2) were ubiquitous across all tissues and seawater (Table 3.3). Gram staining, however, revealed at least two distinct bacteria with similar colony morphologies for LC1, as well as LC6. Most of the bacterial isolates were Gram negative, oxidase positive and motile, except isolates LC1-a, SB4 and LC61-a which were Gram positive (Table 3.3). Three Gram negative isolates, namely LC1-b isolated from rectal gland, LR9 from prostate gland and SC2 from hypobranchial gland homogenates and egg capsules, were found to be indole positive based on API 20E biochemical tests (Table 3.3). These isolates were also oxidase positive and motile.

Table 3.3. Summary of the morphologically distinct bacteria isolated from seawater and different tissues of *Dicathais orbita*.

Bacterial isolates	Description	Gram stain	Motility test	Oxidase test	Indole test	SW	F	HBG	RG	PG	CG	EC
LC1	Large colony, circular, creamy colour					X	X	X	X	X	X	
	LC1-a (Gram +)	Gram +	Motile	+	-		**X**					
	LC1-b (Gram -)	Gram-	Motile	+	+				**X**			
SC2	Small colony, circular, creamy colour	Gram-	Motile	+	+	X	X	**X**	X	X	X	**X**
ST3	Small colony, circular, transparent	Gram-	Motile	+	-	X	X			X		**X**
SB4	Small colony, circular, dark brown in colour	Gram +	Motile	+	-		X		**X**			
ST5	Small colony, watery appearance, transparent	Gram-	Motile	+	-				X	**X**		
LC6	Large colony, irregular edge, creamy colour						X	X		X		
	LC6-a (Gram +)	Gram +	Motile	+	-				**X**	**X**	**X**	**X**
	LC6-b (Gram -)	Gram-	Motile	+	-				**X**	**X**		**X**
SG7	Small colony, circular, light green colour	NT	NT	NT	NT						X	
LY8	Large colony, circular, yellow colour	NT	NT	NT	NT	X				X		
LR9	Large colony, irregular, rhizoid	Gram-	Motile	+	+					**X**		
LT10	Large colony, circular, transparent	Gram-	Motile	+	-		**X**					
SP11	Small colony, circular, pink colour	Gram-	Motile	+	-	X	X		**X**		**X**	
LP12	Large colony, circular, pink colour	Gram-	Motile	+	-			**X**		X		**X**
SCY13	Small colony, circular, yellow colour	Gram-	Motile	+	-		**X**		**X**			
RC14	Round colony, dull creamy colour	Gram-	Motile	+	-						**X**	
LI515	Large colony, irregular shape, transparent	Gram-	Motile	+	-				**X**			
STS16	Small colony, transparent, shiny appearance	Gram-	Motile	+	-			**X**				

SW, seawater; F, foot; HBG, hypobranchial gland; RG, rectal gland; PG, prostate gland; CG, capsule gland; EC, egg capsules; X indicates presence in the respective tissue and those in bold were isolated and cultured for Gram staining, motility, oxidase and indole testing. NT = not tested due to insufficient growth in culture.

3. 4. 3 Molecular identification of indole producing bacteria

Analysis of 16S rRNA gene sequences using BLASTN revealed isolate LC1-b had 100% (FP) sequence similarity to *Vibrio* sp. P1S6, (GenBank accession no. JX477117.1) and 98% (RP) similarity to *Vibrio pomeroyi* strain VSG520, (GenBank accession no. KC534198.1) (Table 3.4). Isolate SC2 had 97% (FP) and 99% (RP) sequence similarity to *Vibrio* sp. (GenBank accession no. KF577048.1) and *Vibrio* sp. V140 (GenBank accession no. DQ146978.1), respectively. Similarly, isolate LR9 showed 99% (FP and RP) sequence similarity to *Vibrio gigantis* strain PJ-21, (GenBank accession no. KC261280.1) and *Vibrio sp*. V140, (GenBank accession no. DQ146978.1). Overall, partial sequencing of 16S rRNA revealed that all three indole producing bacteria from *D. orbita* are likely to be *Vibrio* spp.

Table 3.4. Results of BLASTN analysis showing the closest match to other 16S ribosomal RNA gene, partial sequences in GenBank for each indole producing bacterial isolate cultured from *Dicathais orbita* tissues.

Bacterial isolates	**GenBank accession nos.**	**Length (base pair)**	**Identity (%)**	**Closest match and accession number**
LC1-b				
FP	KM242647	427	100	*Vibrio* sp. P1S6, (JX477117.1)
RP	KM242644	904	98	*Vibrio pomeroyi* strain VSG520, (KC534198.1)
SC2				
FP	KM242648	648	97	*Vibrio* sp. (KF577048.1)
RP	KM242645	1115	99	*Vibrio* sp. V140, (DQ146978.1)
LR9				
FP	KM242646	435	99	*Vibrio gigantis* strain PJ-21, (KC261280.1)
RP	KM242649	605	99	*Vibrio* sp. V140, (DQ146978.1)

FP, forward primer; RP, reverse primer.

3. 5 Discussion

This study establishes the potential for ubiquitous indole producing marine bacteria to contribute to Tyrian purple precursor synthesis in Muricidae molluscs. Biochemical analysis revealed microbial communities have the capacity to produce indole in all tissues sampled from *D. orbita*, as well as in seawater controls. Three indole positive bacterial species were isolated from various *D. orbita* tissues and these all closely matched to *Vibrio* sp. The abundance, diversity and richness of morphologically distinct bacteria from foot tissue were similar to seawater, but significantly fewer bacteria were isolated from the hypobranchial and rectal glands. No bacteria were identified that were unique to the hypobranchial and rectal glands, which are organs that store tryptophan (Westley & Benkendorff 2009) and are the main site for Tyrian purple production (Westley & Benkendorff 2008). The reduced microbial biochemical substrate utilisation in these purple-producing glands appears to be due to a relatively low diversity and abundance of culturable bacteria.

No biochemical reactions were observed in the homogenates of hypobranchial and rectal glands, indicating that something within these glands may be inhibiting the habitation, growth, viability, or metabolism of heterotrophic aerobic bacteria. This is likely to be due to the presence of brominated indole precursors of Tyrian purple in homogenates from these glands, which show strong inhibitory activity against a range of Gram positive and Gram negative bacteria (Benkendorff et al. 2000, Benkendorff et al. 2001a). It could be assumed that any specialised endosymbionts involved in Tyrian purple precursor synthesis would be naturally resistant to the bioactive properties. Indeed in previous studies on sponges, chemical extracts from the sponges have been used to effectively "simulate" the sponge environment. Using this approach, Li and Liu (2006), successfully isolated bacteria belonging to

Actinobacterium and *Bacteroidetes* from the sponge *Craniella austrialiensis*. However, this approach is not likely to be successful if the microbial symbionts produce inactive precursors that are stored by the host invertebrate with a controlled release mechanism. The hypobranchial gland of *D. orbita* has an highly compartmentalised structure with nine distinct cell types storing a range of vesicles with different staining reactions (Westley et al. 2010a). Two distinct types of secretory cells are believed to be associated with the separate storage of tyrindoxyl sulphate and an aryl sulphatase enzyme (Westley et al. 2010a). Homogenisation of the hypobranchial gland would break down the cell structure, thus initiating the hydrolysis of tyrindoxyl sulphate by aryl sulphatase (Westley & Benkendorff 2008, Benkendorff 2013). Any bacteria associated with the gland would then be exposed to the antimicrobial brominated indole precursors of Tyrian purple, resulting in potential cell death or the inhibition of normal metabolic activity. This would then lead to no or few viable bacteria remaining in hypobranchial gland homogenates, thus explaining the lack of biochemical reactions in these samples.

Swabs taken from incisions of hypobranchial and rectal glands also resulted in relatively few biochemical reactions and significantly fewer bacteria were cultured from these glands in comparison with other tissues. Low bacterial diversity and abundance in the biosynthetic glands might be due to the presence of "unculturable bacteria". Many bacterial symbionts are difficult to culture, with only an estimated 0.001–0.1% of marine microbes being successfully cultured (Ferguson et al. 1984, Amann et al. 1995). Furthermore, tissues associated with particular secondary metabolites, such as Tyrian purple precursors, are likely to support distinct chemical environments. This may lead to selection of well-adapted highly specialised symbiotic bacteria that are particularly difficult to culture using standard culture conditions. For example, preliminary data indicates that the hypobranchical glands of *D. orbita*

have a pH of less than 5 and a different oxidation-reduction potential in comparison to other tissues, such as the foot (unpublished data). Recent studies on the effects of low pH on marine bacterial communities have demonstrated significant effects on community composition and metabolism (Krause et al. 2012, Siu et al. 2014). Similarly, multivariate analyses of the biochemical profiles of swabs from *D. orbita* confirmed that the hypobranchial and rectal glands were statistically different to other tissue samples. This implies that the bacteria associated with these glands have distinct metabolic requirements, likely to result from adaptations to the specific chemical environment within these glands.

The hypobranchial gland is a reducing environment with high production of mercaptans, including dimethyl disulfide (Benkendorff et al. 2001a). Many bacterial species can metabolise compounds such as thiosulfate to produce H_2S (Clarke 1953). Biochemical tests revealed thiosulphate metabolism, leading to hydrogen sulphide production was more common in bacterial swabs from female hypobranchial glands and reproductive organs than the equivalent male tissues. This is consistent with previous findings of higher concentrations of methane thiol containing intermediate precursors of Tyrian purple in female glands compared to oxidised products in the males (Westley & Benkendorff 2008). Consequently, bacteria involved in thiosulfate metabolism may facilitate the synthesis of reduced Tyrian purple precursors in females, which are then transferred to the egg capsules for maximal antimicrobial protection of the encapsulated embryos (Benkendorff *et al.* 2000).

Biochemical tests can provide a simple culture independent method for assessing some properties of the resident bacteria. The absence of urea metabolism in most of the *D. orbita* tissue samples, with the exception of swabs from the foot, suggest that urea is not an important source of nitrogen for these symbiotic bacteria and the source of nitrogen for the microbial communities might be amino acids (Table 3.1). The preferred source of nitrogen for most

bacteria is ammonium (Warner 1956, Masepohl et al. 2001), amino acids, purines and polyamines (Masepohl et al. 2001). The absence of L-arginine metabolism and reduced levels of L-ornithine and L-lysine metabolism from the hypobranchial gland swabs may indicate the presence of uncommon enteric bacterial communities that are not easily cultured in nutrient rich broth, as is often the case with marine bacteria. The use of dilute nutrient media can facilitate the culturing of previously unculturable bacteria from aquatic environments (Connon & Giovannoni 2002, Rappe et al. 2002). Enzyme activity associated with the metabolism of sugar substrates, such as L-rhamnose, D-melibiose and L-arabinose was also less common in the Tyrian purple producing biosynthetic organs, particularly in male *D. orbita*. This biochemical profile is in common with *Roseivirga echinicomitans* sp. nov., a novel marine bacterium isolated from sea urchins (*Strongylocentrotus intermedius*) which also does not utilize these sugars (Nedashkovskaya et al. 2005). These biochemical profiles further support the presence of specific bacterial communities that are using alternative carbon sources in the biosynthetic tissues of *D. orbita*.

Ubiquitous bacteria were present in all the non-Tyrian purple associated organs of *D. orbita*. The similar biochemical profiles and bacterial communities present in the seawater and foot of *D. orbita* suggests the foot is mostly occupied by opportunistic bacteria from the external environment. The foot of marine gastropods is directly exposed to sea water, whereas the internal organs may be exposed to reduced bacterial loads within the mantle cavity. Anatomical studies, have suggested that the production of mucus from the hypobranchial gland in gastropods facilitates the binding and removal of particulate matter introduced in the respiratory current (Fretter & Graham 1994, Fretter et al. 1998). The hypobranchial glands of *D. orbita* secrete highly sulphated mucopolysaccharides (Westley et al. 2010a, Laffy et al. 2013), which are commonly associated with antimicrobial defence (Westley et al. 2010a) and could therefore effectively trap and kill bacteria as seawater passes into the mantle cavity.

Although some seawater associated bacteria were found in all of the reproductive organs, particularly in the male prostate, mucus secretions may reduce the number of opportunistic bacteria reaching other internal organs.

Only three indole producing bacterial species were cultured from *D. orbita* tissues and these were all found to have greater than 97% 16S rRNA gene sequence similarity to *Vibrio* sp. Greater than 97% 16S rRNA gene sequence similarity is the benchmark for bacterial species differentiation (Stackebrandt & Goebel 1994). Species of the highly diverse Vibrionaceae family are commonly associated with marine invertebrates (Cheng et al. 1995, Sawabe et al. 2003, Thompson et al. 2004, Chimetto et al. 2011, Lasa et al. 2013) and marine sediments (Baross & Liston 1970, Williams & Larock 1985, Urakawa et al. 2000). Furthermore, many marine Vibrios produce indole, including *V. gigantis, V. pomeroyi* (Beleneva & Kukhlevskii 2010), and *V. parahaemolyticus* (Alcaide et al. 1999) which were most similar to the indole producing bacteria from *D. orbita*. Consequently, these *Vibrio* spp. could be providing some of the basic building blocks for Tyrian purple precursor synthesis, which may be acquired opportunistically and stored within the biosynthetic organs. Furthermore, some *Vibrio* sp. produce tryptophanase (Klug & DeMoss 1971) and may also produce haloperoxidase (Small & McFall-Ngai 1999, Nishiguchi et al. 2004). Bromination of tryptophan or indole precursors is essential for Tyrian purple biosynthesis and bromoperoxidase activity has been reported in the hypobranchial glands of Muricidae (Jannun et al. 1981), including *D. orbita* (Westley & Benkendorff 2009). More broadly, marine Vibrionaceae have thiol peroxidase (Cha et al. 2004) and produce a range of bioactive compounds including the indole derivative indazole-3-carbaldehyde (Indazole) (Fotso Fondja Yao et al. 2010). Consequently, it is plausible that endosymbiotic *Vibrio* spp. are involved in the biosynthesis of Tyrian purple precursors. However further studies, similar to those used for identifying halogenases in sponge microbiota (Ozturk et al. 2013), will be required to confirm this.

In conclusion, this study is the first report on the diversity of bacterial communities associated with *D. orbita*. The presence of indole producing bacteria in the tissues of *D. orbita* that are associated with Tyrian purple synthesis suggest a possible role for ubiquitous symbiotic bacteria in the production of early precursors to Tyrian purple in Muricidae molluscs. Nevertheless, to further investigate any unique symbionts and their capacity to brominate indole precursors, other bacteria associated with the hypobranchial gland may need to be identified through culture independent methods. Our preliminary metagenome data reveals higher diversity of bacterial taxa in the foot than the hypobranchial gland of *D. orbita* and also indicates higher proportion of *Vibrio* spp. in the hypobranchial glands (unpublished data), thus supporting the conclusions drawn from heterotrophic cultural bacterial in this study.

3. 6 Acknowledgments

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Chapter 4. Characterization of bacterial communities associated with the Tyrian purple producing gland in a marine gastropod.

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4. 1 Abstract

Dicathais orbita is a marine mollusc recognised for the production of anticancer compounds that are precursors to Tyrian purple. This study aimed to assess the diversity and identity of bacteria associated with the Tyrian purple producing hypobranchial gland, in comparison with foot tissue, using a high-throughput sequencing approach. Taxonomic and phylogenetic analysis of variable region V1-V3 of 16S rRNA bacterial gene amplicons in QIIME and MEGAN were carried out. This analysis revealed a highly diverse bacterial assemblage associated with the hypobranchial gland and foot tissues of *D. orbita*. The dominant bacterial phylum in the 16S rRNA bacterial profiling data set was *Proteobacteria* followed by *Bacteroidetes*, *Tenericutes* and *Spirochaetes*. In comparison to the foot, the hypobranchial gland had significantly lower bacterial diversity and a different community composition, based on taxonomic assignment at the genus level. A higher abundance of indole producing *Vibrio* spp. and the presence of bacteria with brominating capabilities in the hypobranchial gland suggest bacteria have a potential role in biosynthesis of Tyrian purple in *D. orbita*.

4. 2 Introduction

Tyrian purple is a dye of historical and religious importance (Cooksey 2001, Westley & Benkendorff 2008) and its indole precursors are reported to have potential anticancer and antimicrobial properties (Benkendorff et al. 2000, Westley et al. 2010b, Edwards et al. 2012, Benkendorff 2013, Esmaeelian et al. 2013, Esmaeelian et al. 2014). Muricid molluscs are the only natural source of Tyrian purple (Cooksey 2001), which is formed as a secondary metabolite from indoxyl sulfate precursors stored in the hypobranchial gland (Baker &

Sutherland 1968, Benkendorff 2013). The main pigment in Tyrian purple (6, 6' dibromoindigo) was the first marine natural product to be structurally elucidated (Freidlander 1909), however, even a century later, little information is available on its biosynthesis or the potential role of endosymbiotic bacteria in its production (Benkendorff 2013). 6, 6' Dibromoindigo is the brominated derivative of the blue dye indigo, produced by plants (Maugard et al. 2001, Bechtold et al. 2002) and a range of bacteria (Lim et al. 2005, Qu et al. 2010, Qu et al. 2012). The formation of halogenated marine natural products (mostly containing chlorine and bromine), requires enzymes, such as halogenases and haloperoxidases (Gribble 2004). Bromoperoxidases are believed to be involved in the bromination of indoxyl sulfate precursors, resulting in Tyrian purple biosynthesis in muricid molluscs (Jannun & Coe 1987, Westley & Benkendorff 2009). Several marine bacteria such as *Psychrobacter* sp., *Vibrio* sp., *Pseudomonas* sp. and *Streptomyces* sp. produce halogenases (Small & McFall-Ngai 1999, Nishiguchi et al. 2004, Wynands & Pee 2004, Zeng & Zhan 2011, Ozturk et al. 2013), while *Pseudomonas* sp. (van Pee & Lingens 1985, Wiesner et al. 1985, Itoh et al. 1994), *Streptomyces* sp. (Lingens 1985, van Pee et al. 1987, Zeiner et al. 1988, Knoch et al. 1989) and *Synechococcus* sp. (Johnson et al. 2011) are known to produce bromoperoxidases.

Bacterial profiling using pyrosequencing is an efficient approach for identifying the diversity of endo-symbiotic bacteria and their interactions within marine invertebrates. For instance, metagenome analysis has revealed the remarkable diversity of bacterial symbionts in sponges (Wang 2006, Webster et al. 2010) and the presence of biosynthetic genes in sponge microbial symbionts (Schirmer et al. 2005, Pimentel-Elardo et al. 2012). Other studies have highlighted the host–symbiont biochemical interactions between *Proteobacterium* sp. and the deep sea tube worm, *Riftia* sp. (Laue & Nelson 1994, Felbeck & Jarchow 1998, Handelsman

2004). Thus, targeted metagenomic studies can elucidate a range of species associations and functional relationships.

The Australian muricid, *Dicathais orbita,* provides a useful model for studying Tyrian purple, and the biosynthesis of anticancer brominated indoles more generally (Benkendorff 2013). In preliminary studies, three indole producing bacteria were cultured from the Tyrian purple producing hypobranchial glands of *D. orbita*. However, these studies relied on traditional culture methods and yielded a relatively low number of bacteria, with just 16 distinct strains isolated from all tissues, only three of which were from the Tyrian purple producing gland (Ngangbam et al. 2015b). Culturing most marine bacteria is difficult, with only an estimated 0.001–0.1% of marine microbes being successfully cultured (Amann et al. 1995). Therefore, the aim of this study was to assess the diversity of bacteria associated with the Tyrian purple producing hypobranchial gland using high-throughput sequencing (454 GS FLX Titanium) of the variable region V1-V3 of the 16S rRNA bacterial gene. Comparison of these sequences with equivalent sequences isolated from foot tissue will contribute to the identification of bacteria specifically associated with, or more abundant in, the Tyrian purple producing hypobranchial gland. A further aim was to identify bacteria with potential to produce indoles and brominated compounds, based on their taxonomic affiliation.

4. 3 Materials and Methods

4. 3. 1 Sample collection and maintenance

Adult specimens of *D. orbita* were collected under permit number F89/1171-6.0 and P10/0069-1.0 issued by Department of Primary Industries, (NSW) Australia. Six live snails were collected from the intertidal rocky reefs of Flat Rock, Ballina (28°84′ S and 153°60′ E),

NSW, Australia, during low tides in April and July 2014. Snails were held in aerated seawater tanks for a maximum of 24 hours before processing.

4. 3. 2 Snail dissection and total DNA extraction

The hard shell of *D. orbita* was removed by applying pressure between the primary body whorl and spire using a bench vice (Westley et al. 2010a). The hypobranchial glands and the foot were carefully rinsed by pipetting with sterile sea water to remove any sediment before dissecting. Total genomic DNA from triplicate female and male hypobranchial glands, as well as the female and male foot, was extracted using the QIAmp DNA mini kit (Qiagen) following the manufacturer's instructions. DNA quality and concentrations were determined with agarose gel electrophoresis and a NanoDrop 2000 Spectrophotometer (Thermo Scientific) and then stored at –20 °C pending analysis. Only those samples that passed quality control checks were used in the 16s rRNA bacterial profiling libraries, so in total only duplicate samples were obtained for each gender and tissue combination.

4. 3. 3 Roche GS- FLX amplicon sequencing

Bacterial diversity of the biosynthetic organ (hypobranchial glands) and non-biosynthetic tissues (foot) of *D. orbita* were characterised by high-throughput sequencing (454 GS FLX amplicon sequencing) (Binladen et al. 2007) using the primer pair of 27F/519R that targeted the variable region V1-V3 of 16S rRNA bacterial gene (Kumar et al. 2011, Fagervold et al. 2014). DNA samples were shipped to Macrogen Inc, South Korea (http://dna.macrogen.com/eng) for high-throughput sequencing. GS FLX data processing was performed using Roche GS FLX software (v 2.9) in two stages, image processing and signal

processing. Image processing involves normalization of raw images and generation of raw signals. In the signal processing stage, correction, filtering, and raw signal trimming were done prior to base calling with corresponding quality score of reads. Sequence reads from each sample were segregated with in-house script (Macrogen) using the tag (Barcode) sequences, and by matching the initial and final bases of the reads to the known tag sequences used in the preparation of the libraries.

4. 3. 4 Bioinformatics analysis

Sequences were filtered for low quality bases and chimeric sequences. Only sequences of 100 bp., or more, were selected for final analysis. All sequence analyses were performed using QIIME version 1.8.0 (Caporaso et al. 2010) and open-reference operational taxonomic units (OTUs) picking strategy was employed. OTUs were picked based on 97% sequence similarity using UCLUST algorithm (Edgar 2010) and taxonomies were assigned against the well curated Silva_119 database (Pruesse et al. 2007). The parameters used for OTU picking and taxonomic assignments are as follows: pick_otus.py -i all.merged.min100bp.fasta - -threads =8 and assign_taxonomy.py -i rep_set.fna –r /Silva119_for_Qiime/rep_set/97/Silva _119_ rep _set 97.fna -t/Silva119_for_Qiime/taxonomy /97/ taxonomy_97_all_levels.txt -o taxonomy _results/ -e 0.01 --uclust_similarity=0.85. Sample specific OTUs were retrieved from all the OTUs and aligned against the same database by BLAST (Altschul et al. 1997). Finally, the taxonomic classification were plotted using metagenome analyser (MEGAN5) (Huson et al. 2007).

All 16S rRNA gene sequences were deposited in the European Nucleotide Archive (ENA- http://www.ebi.ac.uk/ena) under accession number PRJEB9174.

4. 3. 5 Statistical analyses

A full model two factor permutational analysis of variance was run using Primer v. 6 with PERMANOVA add-on, to compare the bacterial communities between the hypobranchial gland and foot tissue of male and female *D. orbita*. Bray Curtis similarity matrices with a dummy value of 1 were generated from the untransformed OTU data at the genus level. Initial analyses were performed using the number of reads as a covariate to establish whether the unequal number of reads between samples influenced the outcomes. However, as the covariate was not significant (Pseudo $F = 9.83$, $p = 0.96$), the covariate was removed and the results are presented from the reduced two factor model. Additional analyses were also performed using a reduced data set excluding the low read samples (i.e. F1H, F2F and M2H) and these produced comparable results to the full data set (Table 4.1). All PERMANOVA analyses were performed using 9999 permutations. Principal Coordinates Ordination (PCO) was undertaken to represent the data graphically. Similarity of Percentages (SIMPER) was run to establish which bacterial taxa contributed to the dissimilarity between the hypobranchial gland and foot tissue.

The DIVERSE function in PRIMER 6 was used to analyse the genus richness and diversity (Shannon's H index), which was calculated from the relative abundance (% of reads) for each distinct OTU in each sample, but excluding the unassigned taxa. Univariate PERMANOVAs of genus richness and diversity were performed using Euclidean distance similarity matrices.

Table 4.1. Summary of statistical analyses for genus level using a reduced data set (F2H, M1H, F3F, M2F and M3F) excluding samples with low number of reads (F1H, M2H and F2F). Univariate PERMANOVA was performed on Euclidean distance similarity matrices for species richness and diversity, whereas multivariate PERMANOVA was performed using Bray-Curtis similarity matrices for community composition based on the number of reads.

Analysis	**Tissues**		**Gender**		**Tissue and Gender interaction**	
	Pseudo F value	**P (perm) value**	**Pseudo F value**	**P (perm) value**	**Pseudo F value**	**P (perm) value**
Community composition	29.99	0.03	11.66	0.09	21.58	0.07
H diversity	1907.40	0.03	226.63	0.10	582.74	0.07
Richness	66.31	0.06	467.11	0.03	9.56	0.84

4. 4 Results

4. 4. 1 Bacterial profiling of the hypobranchial gland and foot of *Dicathais orbita*

A total of 149,804 reads, with an average length of 436.301 base pairs, were obtained from the eight samples (four hypobranchial gland and four foot) of *D. orbita* (Table 4.2). Total acceptable reads, for operational taxonomic unit (OTU) assignment for the eight samples, ranged from 637 to 36,728 in the hypobranchial gland and foot (Table 4.2). At least one replicate from each sample type had > 15,000 reads. The total number of shared (non-overlapping) operational taxonomic units (OTUs) resulting from the bacterial profiling data set was 3585. The foot samples had a higher number of OTUs than the hypobranchial gland, across all taxonomic levels (Table 4.2).

Rarefaction curves indicated the richness of bacterial taxa had not peaked at the maximum number of sequences read, with the exception of female hypobranchial gland 2, which reached an asymptote of < 70 bacterial genera after ~ 10,000 sequences (Table 4.2). The number of OTUs is likely to be highly under-represented in the other female hypobranchial gland sample (F1H = 17) and male hypobranchial gland 2 (M2H =71, Table 4.2), due to the low number of sequence reads (Fig. 4.1). The alpha diversity rarefaction plots also showed that the female hypobranchial gland had lower bacterial diversity than the male hypobranchial gland and foot samples of *D. orbita* (Fig 4.1).

Table 4.2. Summary of *Dicathais orbita* hypobranchial gland and foot tissue 16S rRNA bacterial profiling.

Tissue	**Hypobranchial gland**				**Foot**				
Gender	Female	Female	Male	Male	Female	Female	Male	Male	Total
Sample[1]	F1H	F2H	M1H	M2H	F2F	F3F	M2F	M3F	-
Reads	637	36,728	35,548	3,601	1,326	15,305	28,611	28,048	149,804
Total bases	253,914	15,930,139	15,597,012	1,544,017	597,192	6,681,905	12,563,818	12,191,707	65,359,704
Average read length	398.6	433.7	438.8	428.8	450.4	436.6	439.1	434.7	-
Operational taxonomic units[2]	50	526	1672	250	290	1055	1440	1496	3585
Number of Phyla	5	9	18	11	17	16	25	24	28
Number of Classes	11	21	36	21	30	32	55	53	65
Number of Orders	13	35	80	35	48	70	103	98	143
Number of Families	15	48	127	48	69	108	148	152	243
Number of Genera	17	66	221	71	124	204	277	288	443

[1] The samples are labelled such that the first letter refers to the gender, the number to different replicate snails within each gender and the second letter to the tissue type.

[2] OTUs are shared among multiple samples and are based on 97% sequence similarity criteria in the Silva_119 database.

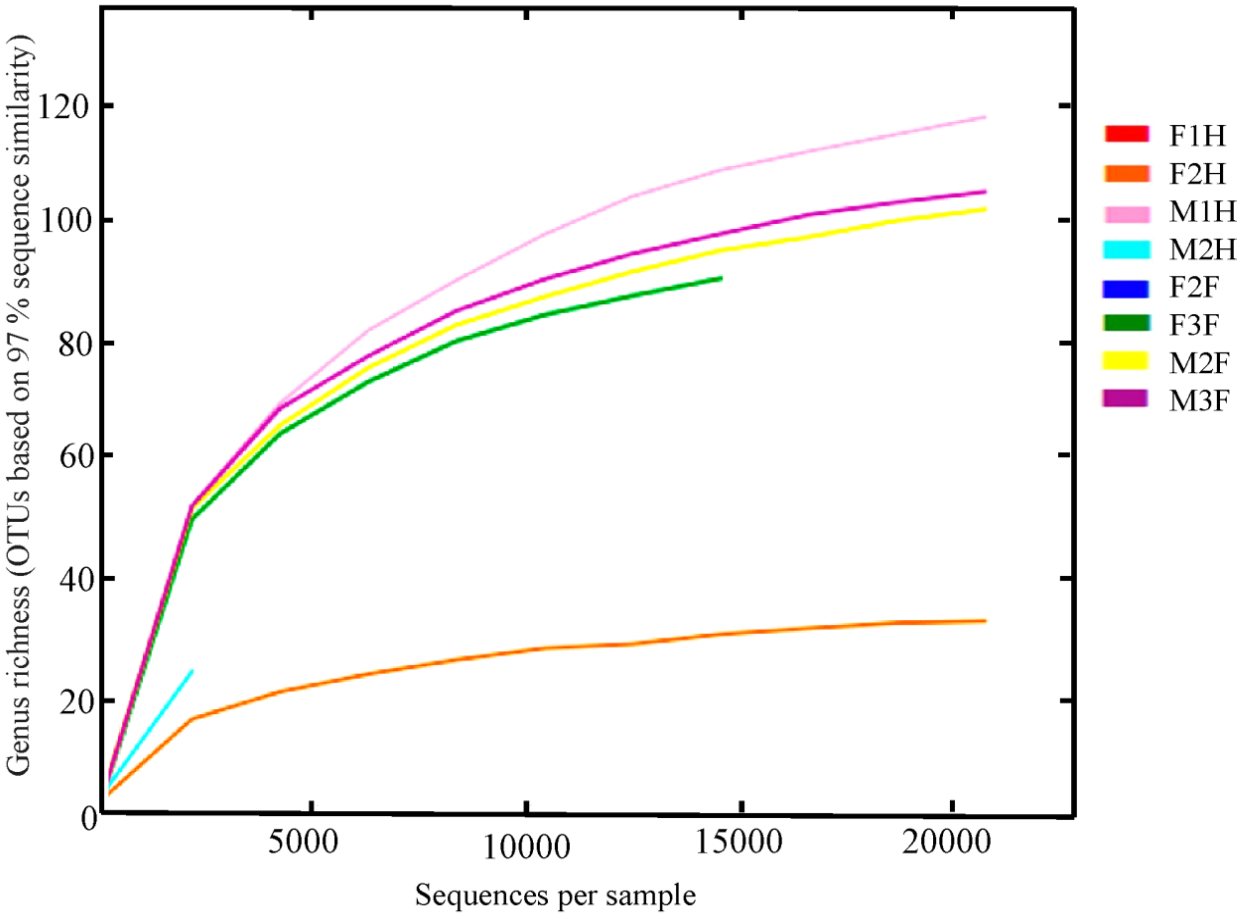

Fig. 4.1. Alpha diversity showing the richness of bacterial community diversity within *Dicathais orbita* foot (F2F, F3F, M2F and M3F) and hypobranchial gland samples (F1H, F2H, M1H and M2H) (F, female; M, male). The phylogenetic diversity metric consists of genus richness based on 3585 observed OTUs at the 97% sequence similarity level and 443 possible observed genus. Sample with reads of more than 3000 are visible.

4. 4. 2 Bacterial taxonomic diversity of the hypobranchial gland and foot of *Dicathais orbita*

Altogether, 28 different bacterial phyla were observed in the bacterial profiling data set; however, only dominant phyla are presented (Fig.4.2). Bacterial groups that could

not be assigned to any phyla equated to 6.8%. The dominant phylum was *Proteobacteria*, representing 32.2% of the bacterial abundance in all *D. orbita* samples (Fig. 4.2). Bacteria from the phylum *Tenericutes* were more abundant in the hypobranchial gland compared to the foot (Fig. 4.2). *Bacteriodetes* were more abundant in foot tissues than female hypobranchial glands (Fig. 4.2). Bacteria from the phylum *Spirochaetes* were also more abundant in the foot than the hypobranchial gland samples (Fig. 4.2).

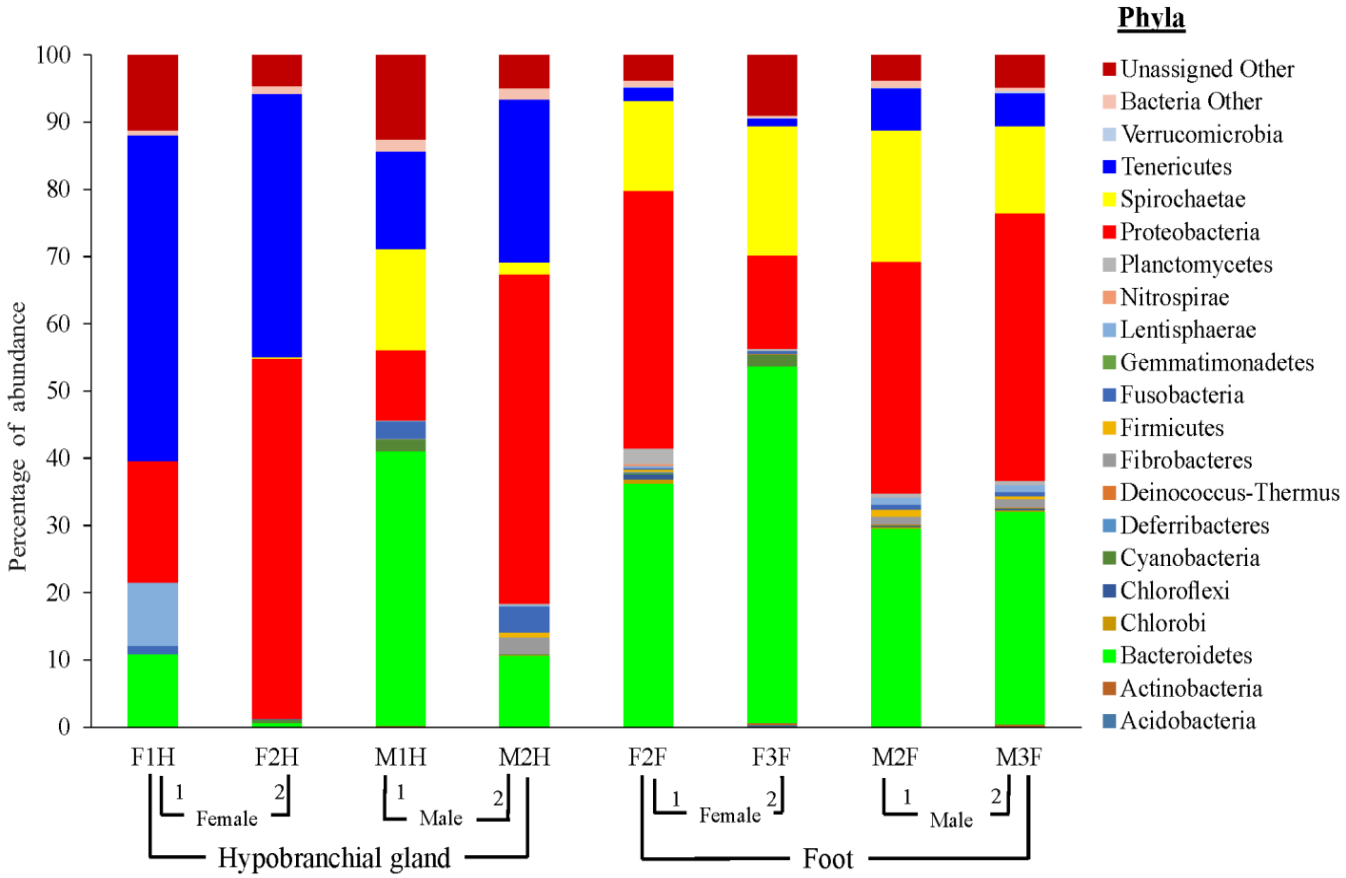

Fig. 4.2. Phylum-level taxonomic diversity associated with the female (F) and male (M) hypobranchial gland (F1H, F2H, M1H and M2H) and foot (F2F, F3F, M2F and M3F) of *Dicathais orbita* bacterial profiling. All the minor phyla and unnamed, but previously identified bacterial phyla (such as BD1-5, CKC4, candidate division BRC1, OD1, OP8, SR1, TM7, SHA-109, and TM6) are grouped into "Bacteria Other".

Phylogenetic analysis revealed male foot tissue (M3F) had greater taxonomic diversity than the hypobranchial gland (Fig. 4.3). *Flavobacteriales*, *Sphingobacteriales* and *Rhodobacterales* were more common in the foot, while *Vibrionales* was more dominant in hypobranchial gland (Fig. 4.3). *Vibrionales* was the dominant order in the female hypobranchial gland (Fig. 4.3A) and representatives from this order were observed in all samples of the foot and hypobranchial gland (Fig. 4.3). *Mycoplasma*, in the phyla *Tenericutes*, was found to be more dominant in the hypobranchial gland when compared to *D. orbita* foot samples (Fig. 4.3).

Fig. 4.3. Phylogenetic tree of *Dicathais orbita* samples generated from 16S rRNA sequences by MEGAN. A= Female hypobranchial gland (F2H); B= Male hypobranchial gland (M1H); C= Female foot (F3F); D= Male foot (M3F). All these sample types have more than 15,000 reads.

Altogether, 443 known bacterial genera were identified in the foot and hypobranchial gland of the *D. orbita* bacterial profiling dataset, based on >97% sequence similarity. In total there were 169 distinct bacterial genera present in the foot, 52 in the hypobranchial gland and 222 common bacterial genera between the foot and hypobranchial gland of *D. orbita*. On average, a higher number of distinct bacterial genera were recorded in the foot compared to hypobranchial gland samples (Fig. 4.4A). Univariate PERMANOVA analysis confirmed there was significantly different genus richness between tissue types (Pseudo $F = 8.54$, $p = 0.04$). However, genus richness was not significantly different between genders (Pseudo $F = 6.33$, $p = 0.06$), and there was no interaction between gender and tissue type (Pseudo $F = 2.49$, $p = 0.86$).

Using Shannon's diversity index to assess richness and evenness of the bacterial communities, higher diversity was consistently detected in the foot compared to the hypobranchial gland of *D. orbita* (Fig. 4.4B). Univariate PERMANOVA analysis revealed genus diversity (H′) was significantly higher in the foot than the hypobranchial gland (Pseudo $F = 18.44$, $p = 0.01$). There was no significant difference according to gender (Pseudo $F = 3.71$, $p = 0.13$), and no interaction between gender and tissue (Pseudo $F = 1.79$, $p = 0.25$).

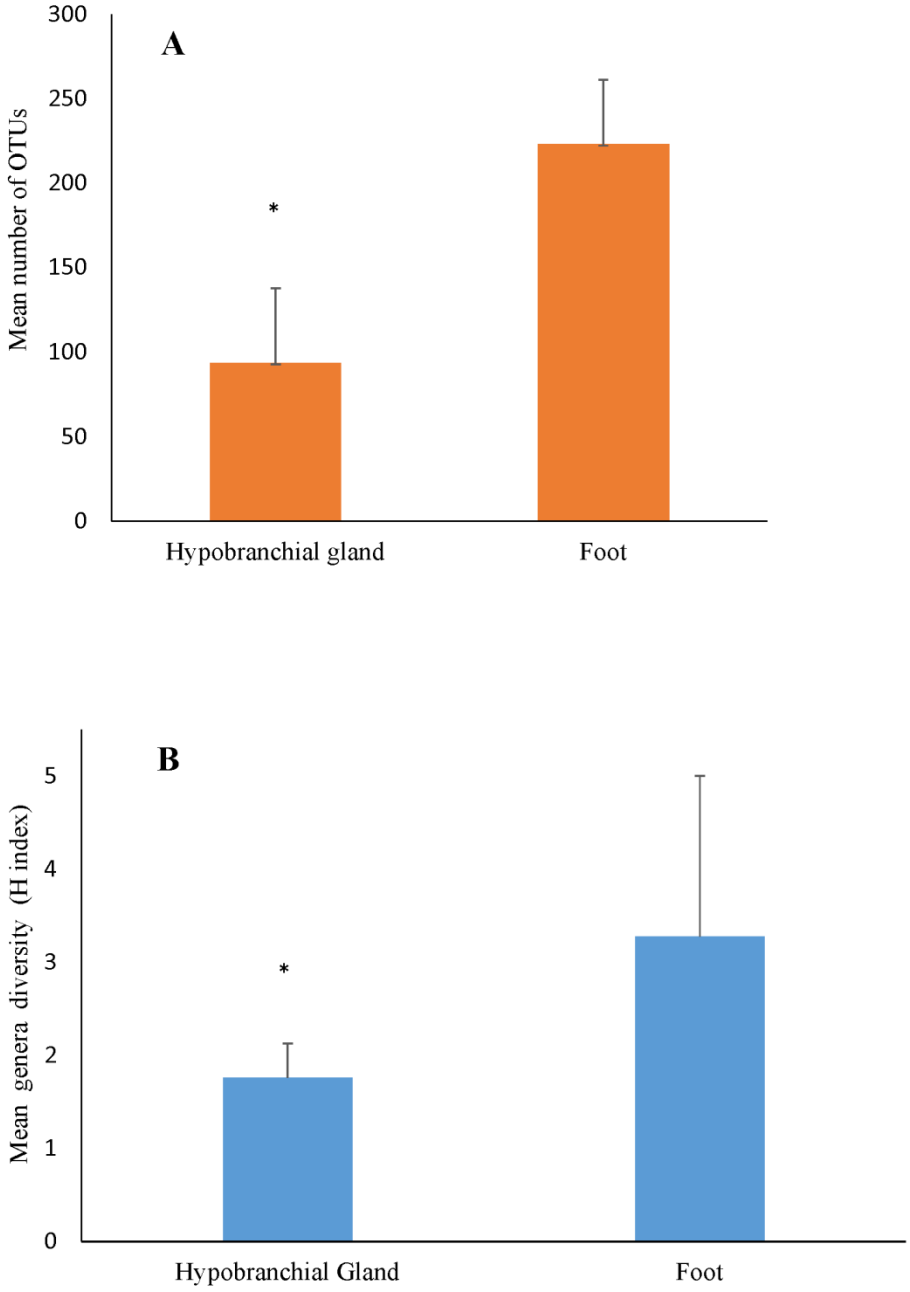

Fig. 4.4. Mean (+S.E.) number of OTUs in the hypobranchial gland and foot tissue of *Dicathais orbita*, showing the mean proportion unique to individuals samples of foot and hypobranchial gland tissue. (A) = OTUs richness, (B) = H index/diversity.

4. 4. 3 Bacterial community structure in the hypobranchial gland and foot of *Dicathais orbita*

Principal Coordinates Ordination (PCO) revealed separation of bacterial communities, based on genera level OTUs, between the hypobranchial gland and foot samples (Fig. 4.5). The bacterial communities of hypobranchial gland samples were more variable and also showed separation between male and females, whereas foot samples clustered together on the left hand side of the plot (Fig. 4.5). Multivariate analyses of genera OTUs associated with the hypobranchial gland and foot of *D. orbita*, revealed a significant difference between these tissues (Pseudo $F = 5.46$, $p = 0.02$). However, there was no significant difference according to gender (Pseudo $F = 0.58$, $p = 0.67$) and no interaction between gender and tissue (Pseudo $F = 2.01$, $p = 0.17$). Similar results were found when just the presence and absence of bacteria in the samples are considered (rather than relative abundance). Here, the PCO plot also revealed a general pattern of foot samples clustering separately and hypobranchial gland samples being more variable between the individual snails than foot samples (Fig. 4.6).

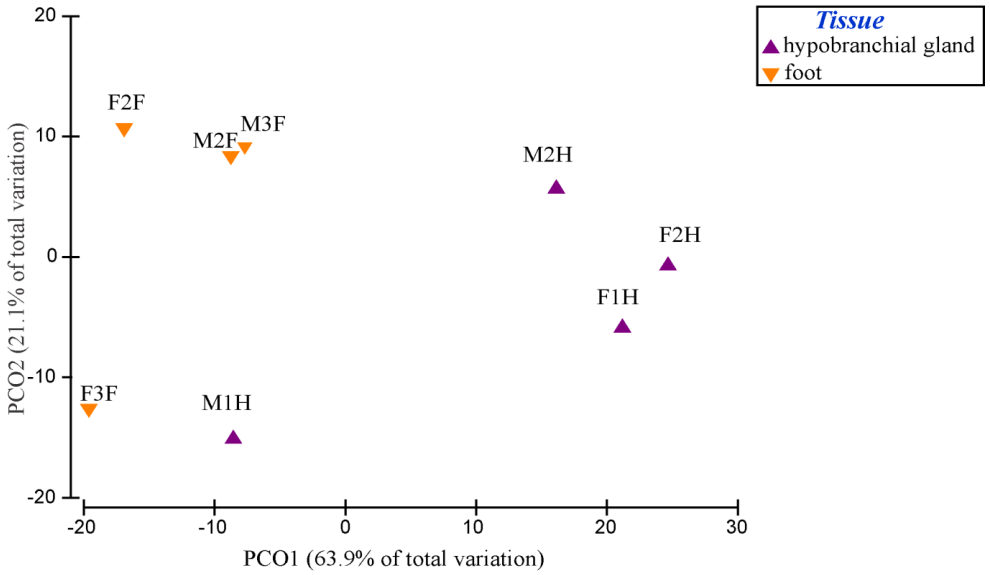

Fig. 4.5. Principal Coordinates Ordination (PCO) of bacterial genus composition, based on a Bray Curtis similarity matrix of the relative abundance of OTUs at 97% sequence similarity level for the hypobranchial gland (purple) and foot (orange) of female (F) and male (M) *Dicathais orbita*.

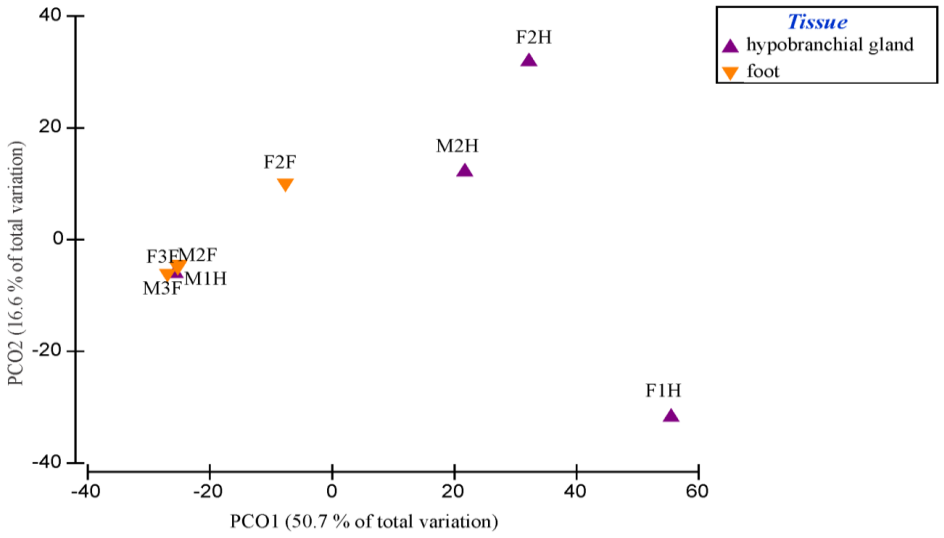

Fig. 4.6. Principal Coordinates Ordination (PCO) of bacterial genus associated with hypobranchial gland (purple) and foot (orange) of female (F) and male (M) *Dicathais orbita* after presence/ absence transformation.

SIMPER (Similarity of percentages) analysis revealed high dissimilarity between the bacterial communities of the hypobranchial gland and foot of *D. orbita* (Table 4.3, Average dissimilarity = 68.51 %). Of the 443 bacterial genera, four contributed approximately 50% of the dissimilarity between the tissues. *Vibrio* and *Mycoplasma* were more abundant in the hypobranchial gland, whereas *Chitinophagaceae* and *Spirochaeta* were more abundant in the foot (Table 4.3). A relatively small number of genera (e.g. *Mycoplasma*, *Vibrio*), along with an average of approximately 8% unassigned bacteria contributed to the similarity between hypobranchial gland samples of *D. orbita* (Table 4.4A). However, 30 diverse bacteria contributed to 90% of the similarity between the foot samples (Table 4.4B).

Table 4.3. Similarity of percentages (SIMPER) analysis showing the bacterial genus that contribute most to the differences between hypobranchial gland and foot of *Dicathais orbita* (Average dissimilarity = 68.51).

Genus	Group Hypobranchial gland Av. Abundance[1]	Group Foot Av. Abundance[2]	Av. Diss.[3]	Diss./ SD[4]	Contrib. %[5]	Cum. %[6]
Mycoplasma	0.32	0.04	13.99	2.03	20.42	20.42
Vibrio	0.27	0.09	10.83	1.27	15.81	36.24
Chitinophagaceae; Other	0.08	0.15	8.07	1.29	11.78	48.02
Spirochaeta	0.04	0.16	6.20	1.96	9.05	57.07
Owenweeksia	0.01	0.07	3.29	2.16	4.80	61.87
Unassigned; Other	0.08	0.05	2.03	1.26	2.97	64.84
Rhodobacteraceae; Other	0.01	0.05	1.81	1.98	2.65	67.49
Oligosphaeria; uncultured bacterium	0.02	0.01	1.29	0.68	1.89	69.38
Marinilabiaceae; uncultured	0.02	0.01	1.08	1.32	1.57	70.95
Rhodobacteraceae; uncultured	0.01	0.03	1.05	1.94	1.54	72.48
Flavobacteriaceae; uncultured	0.01	0.03	0.98	1.55	1.43	73.92
Propionigenium	0.02	0	0.72	1.49	1.06	74.97
Roseovarius	0	0.01	0.72	1.70	1.05	76.02
Flavobacteriaceae; Other	0.01	0.02	0.64	1.66	0.93	76.95
Marinifilum	0.01	0	0.59	1.03	0.87	77.82
Saprospiraceae; uncultured	0	0.01	0.52	2.46	0.77	78.59
Bacteria; Other	0.01	0	0.50	2.50	0.72	79.31
Bacteroidetes; Other	0.01	0	0.49	0.75	0.71	80.02
Fibrobacteria Incertae Sedis; possible genus03	0.01	0.01	0.47	1.13	0.69	80.71
Flammeovirgaceae; Other	0	0.01	0.37	1.06	0.54	81.25
Aureispira	0	0.01	0.35	1.69	0.51	81.75

[1,2]Average abundance; [3]Average dissimilarity; [4]Ratio of dissimilarity/standard deviation; [5]Percent contribution; [6]Cumulative percent contribution.

Table 4.4A. Percentage of similarity (SIMPER) analysis showing genus that contribute to similarity in replicate *D. orbita* hypobranchial gland samples (Average similarity: 45.47).

Genus	**Av. Abund**	**Av. Sim[1]**	**Sim/ SD[2]**	**Contrib. %**	**Cum. %**
Mycoplasma	0.32	21.81	2.24	47.97	47.97
Vibrio	0.27	13.09	0.99	28.79	76.76
Unassigned;Other;Other;Other;Other;Other	0.08	5.81	2.19	12.77	89.54
Bacteria;Other;Other;Other;Other;Other	0.01	0.93	4.18	2.04	91.57

[1]Average similarity; [2]Similarity/standard deviation ratio.

Table 4.4B. Percentage of similarity (SIMPER) analysis showing genus that contribute to similarity in replicate *D. orbita* foot samples (Average similarity: 60.14).

Genus	Av. Abund	Av. Sim	Sim/ SD	Contrib. %	Cum. %
Spirochaeta	0.16	13.99	5.75	23.26	23.26
*Chitinophagaceae;*Other	0.15	7.2	6.53	11.97	35.23
Owenweeksia	0.07	5.51	1.59	9.16	44.39
Unassigned;Other;Other;Other;Other;Other	0.05	3.96	8.86	6.58	50.97
Vibrio	0.09	3.84	0.74	6.39	57.36
Rhodobacteraceae;Other	0.05	3.72	3.04	6.18	63.54
Rhodobacteraceae;uncultured	0.03	2.82	18.13	4.69	68.24
Mycoplasma	0.04	2.05	1.44	3.41	71.65
Flavobacteriaceae;uncultured	0.03	1.73	2.68	2.88	74.53
Flavobacteriaceae;Other	0.02	1.71	17.95	2.84	77.36
Saprospiraceae;uncultured	0.01	0.97	8.18	1.61	78.98
Roseovarius	0.01	0.93	0.96	1.54	80.52
Roseobacter clade NAC11-7 lineage	0.01	0.61	6.53	1.02	81.54
Polaribacter	0.01	0.45	2.72	0.75	82.29
Aureispira	0.01	0.42	1.22	0.69	82.98
Colwellia	0.01	0.4	1.15	0.67	83.65
Marinilabiaceae; uncultured	0.01	0.4	0.78	0.66	84.31
Aquimarina	0.01	0.38	4.2	0.63	84.94
Lutibacter	0.01	0.35	1.16	0.58	85.52
Propionigenium	0	0.32	3.03	0.52	86.04
Tenacibaculum	0	0.31	3.16	0.52	86.57
Cohaesibacter	0.01	0.3	0.93	0.5	87.07
Arcobacter	0	0.29	2.42	0.48	87.54
Maribacter	0	0.28	0.91	0.47	88.01
Psychroserpens	0	0.26	1.15	0.44	88.45
Sulfitobacter	0	0.26	3.05	0.43	88.88
Fibrobacteria Incertae Sedis; possible genus 03	0.01	0.22	0.47	0.37	89.26
Portibacter	0	0.22	9.03	0.37	89.62
Flavobacteriales;Other	0	0.21	2.89	0.36	89.98
*Oligosphaeria;*uncultured bacterium	0.01	0.21	0.57	0.35	90.33

4. 4. 4 Biosynthetic capabilities of the bacterial symbionts

Of the possible 443 bacterial genera identified from the tissues of *D. orbita*, only 22 bacterial species are known to have biosynthetic capabilities directly relevant to Tyrian purple precursor biosynthesis (Fig. 4.7, Table 4.5). A greater proportion of the bacteria found only in the hypobranchial glands (9.6%) were found to have indole and/ or brominating capabilities compared to those only found in the foot (0.6% , Fig. 4.7). There were 21 indole producing species detected across 9 genera (Fig. 4.7) and the majority of these were *Vibrio* spp. common to both the foot and hypobranchial gland samples (Fig. 4.7). Three species were detected that are known to produce both indoles and brominated secondary metabolites and a further three species produce bromoperoxidase enzymes (Fig.4.7, Table 4.5). More specifically, bacteria from three genera that were detected more frequently in the hypobranchial gland, namely *Bacillus*, *Pseudomonas* and *Synechococcus*, are known to produce bromoperoxidase (Table 4.5). *Pseudomonas* spp., and several other bacteria found in the hypobranchial gland, are also known to produce oxidised sulphur metabolites, whereas three sulphur reducing bacteria were found exclusively in the foot tissue (Table 4.5).

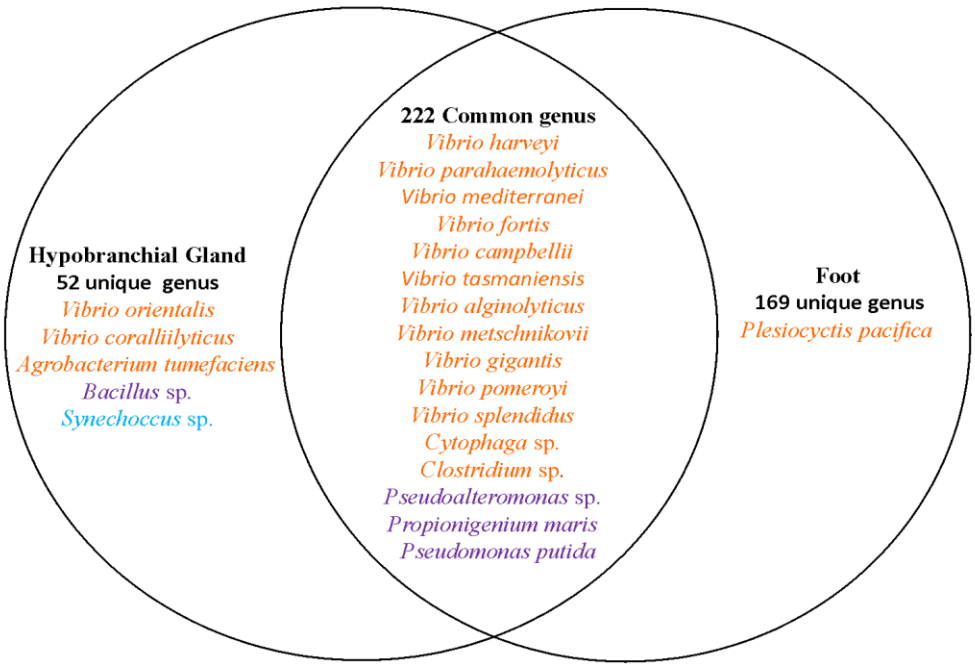

Fig. 4.7. Venn diagram showing shared and non-shared bacterial species between the hypobranchial gland and foot of *Dicathais orbita*. The number of species that have biosynthetic capabilities relevant to Tyrian purple production are highlighted in different colours (Orange = indole producers; Blue = brominating enzymes; Purple = indole producers and brominating capabilities).

Table 4.5. *Dicathais orbita* associated bacteria that have been previously shown to produce indoles, brominated secondary metabolites or enzymes associated with their biosynthesis or sulphur metabolizing bacteria.

Bacteria	Foot[1]	Hypobranchial gland[1]	Indole	Bromoperoxidase	Brominated compounds	Sulphur metabolism
Vibrio parahaemolyticus	3/4	3/4	Yes (Pandey et al. 2010)	-	-	-
Vibrio orientalis	0/4	1/4	Yes (Lambert et al. 1998)	-	-	Yes, cleave dimethylsulfoniopropionate (DMSP) (Curson et al. 2012)
Vibrio mediterranei	4/4	3/4	Yes (Pujalte & Garay 1986)	-	-	-
Vibrio fortis	2/4	2/4	Yes (Thompson et al. 2003)	-	-	-
Vibrio campbellii	3/4	1/4	Yes (Haldar et al. 2011)	-	-	-
Vibrio coralliilyticus	0/4	2/4	Yes (Ben-Haim et al. 2003)	-	-	Yes, used dimethylsulfoniopropionate (Garren et al. 2014)
Vibrio tasmaniensis	2/4	4/4	Yes (Noguerola & Blanch 2008)	-	-	-
Vibrio alginolyticus	3/4	2/4	Yes(Noguerola & Blanch 2008)	-	-	-
Vibrio metschnikovii	3/4	2/4	Yes (Lee et al. 1978)			
Vibrio gigantis	1/4	3/4	Yes (Beleneva & Kukhlevskii 2010)	-	-	-
Vibrio harveyi	3/4	2/4	Yes (Hashem & El-Barbary 2013)	-	-	-
Vibrio pomeroyi	1/4	1/4	Yes (Gomez-Gil et al. 2003)	-	-	-
Vibrio splendidus	1/4	2/4	Yes (Lambert et al. 1998)	-	-	-
Desulfotalea arctica	2/4	1/4	-	-	-	Yes, sulphur reducing bacteria (Knoblauch et al. 1999)
Cytophaga sp.	3/4	3/4	Yes (Shaaban et al. 2002)	-	-	-
Propionigenium maris	4/4	4/4	Yes (Janssen & Liesack 1995)		Yes, 2,4,6-tribromophenol (TBP) to monobromophenols (Watson et al. 2000)	Yes, succinate decarboxylation (Janssen & Liesack 1995)
Clostridium sp.	1/4	1/4	Yes (Kohda et al. 1997)	-	-	Yes, produces elemental sulphur from thiosulphate(Schink & Zeikus 1983)
Bacillus sp.	0/4	1/4	Yes, indole 3 acetic (Idris et al. 2007)	Yes (Read et al. 2003)	-	-
Desulfobulbus mediterraneus	1/4	0/4	-	-	-	Yes, sulphur reducing bacteria (Sass et al. 2002)
Desulfoluna spongiiphila	1/4	0/4	-	-	-	Yes, dehalogenating bacterium (Ahn et al. 2009)
Desulfoluna butyratoxydans	1/4	0/4	-	-	-	Yes, sulphur reducing bacteria (Suzuki et al. 2008)
Rhodovulum sulfidophilum	3/4	0/4	-	-	-	Yes, oxidation of thiosulfate and sulfide (Appia-Ayme et al. 2001)
Sulfitobacter mediterraneus	4/4	3/4	-	-	-	Yes, sulfite-oxidizing (Pukall et al. 1999)
Ruegeria pomeroyi	1/4	1/4	-	-	-	Yes, dimethylsulphoniopropionate (DMSP)(Reisch et al. 2013)
Synechococcus sp.	0/4	1/4		Yes (Johnson et al. 2011)	-	-
Desulfobulbus mediterraneus	1/4	0/4	-	-	-	Yes, sulphur reducing bacteria (Sass et al. 2002)
Agrobacterium tumefaciens	0/4	1/4	Yes, indole acetic acid (Inze et al. 1984)	-	-	-
Plesiocystis pacifica	3/4	0/4	Yes (Iizuka et al. 2003)	-	-	-

Pseudomonas putida	1/4	2/4	Yes, indole-3-acetic acid (Patten & Glick 2002)	Yes (Itoh et al. 1994)	Yes, dibromoethenes (Hur et al. 1994)	Yes, oxidized to methanesulfonate (Vermeij & Kertesz 1999)
Pseudoalteromonas sp.	4/4	2/4	Yes (Yang et al. 2007)	-	Yes, 2,4-dibromo-6-chlorophenol (Jiang et al. 2000)	-
Leucothrix mucor	2/4	1/4	-	-	-	Yes, thiosulfate oxidation (Grabovich et al. 1999)

[1]This indicates the proportion of samples of each tissue types in which each bacterial species was detected.

4. 5 Discussion

This study determined differences in bacterial community composition in the Tyrian purple producing hypobranchial gland and the non-biosynthetic foot tissues of the muricid mollusc, *D. orbita*. Bacterial taxa representing 3585 OTUs from 28 different phyla, 243 families and 443 genera (Table 4.2) were observed in the bacterial profiling data set of *D. orbita*. Phylogenetic analysis highlighted the presence of more complex bacterial communities in the foot compared to the hypobranchial gland, and this was supported by significantly lower OTU richness and diversity in the gland than the foot. PCO and multivariate analysis revealed significantly different bacterial community structure between the two tissues, and dissimilarity analysis revealed a higher abundance of *Vibrio, Mycoplasma* and unassigned bacteria in the biosynthetic hypobranchial gland. Consistent with previous culture studies (Ngangbam et al. 2015b), *Vibrios* were the dominant indole producing bacteria detected. However, 16S rRNA bacterial profiling also revealed the presence of bromoperoxidase producing bacteria such as *Bacillus*, *Pseudomonas* and *Synechococcus,* and bacteria known to produce brominated secondary metabolites, such as *Pseudoalteromonas* and *Propionigenium,* in the Tyrian purple producing gland.

The taxonomic diversity of symbiotic bacteria, with a dominance of *Proteobacteria* in *D. orbita*, is comparable with previous 16S rRNA analyses of marine mollusc associated bacteria. Metagenomic (16S rRNA) bacterial diversity studies of the molluscan sea slug, *Elysia chlorotica,* from a total number of reads among samples ranging from 4601 to 11374, produced 199 to 889 OTUs derived from 5 to 9 distinct phyla (Devine et al. 2012). Another metagenomic study of the 16S rRNA gene of a bivalve mollusc (internal body parts without the shell) resulted in the discovery of 3553 OTUs from 44 phyla, in which *Proteobacteria* was found to be the

most abundant phylum (Cleary et al. 2015). The metagenome of the digestive tract of a marine limpet also revealed diverse microbial communities with the most dominant phylum being *Proteobacteria* (Dudek et al. 2014). Indeed, *Proteobacteria* accounts for more than 40% of all known prokaryotic genera (Kersters et al. 2006) and is the dominant bacterial phyla reported from other marine invertebrate taxa, including the sponge *Halichondria* sp. (Naim et al. 2014), the coral *Porites astreoides* (Wegley et al. 2007) and the oyster *Crassostrea* sp.(Trabal Fernandez et al. 2014).

Significantly different bacterial community structure, with a higher richness and diversity of OTUs, was observed in the foot relative to the hypobranchial gland samples of *D. orbita* (Figs 4.3- 4.5). This is consistent with a previous study on heterotrophic culturable bacteria, where no bacterial biochemical activity was detected in the homogenised glands and significantly fewer species were isolated from swabs of the hypobranchial gland in comparison to the foot tissue of *D. orbita* (Ngangbam et al. 2015b). The lower Shannon's diversity index from the hypobranchial gland coupled with the SIMPER analyses imply the bacterial community in this gland is dominated by two abundant symbiotic genera (i.e. *Mycoplasma*, *Vibrio*), some unassigned, possibly novel bacteria and a larger number of genera detected in low abundance, many of which may be rare opportunists or contaminants. This pattern of bacterial diversity is consistent with a highly specialised internal environment; indeed the hypobranchial gland has a low pH (Ngangbam et al. 2015b) and produces secretions containing antibacterial sulphated mucopolysaccharides and brominated indoles that would be expected to kill the majority of opportunistic bacteria (Westley et al. 2010a, Laffy et al. 2013). *Mycoplasma*, the best known genus in the class *Mollicutes*, are common parasites in marine organisms and can persist in extreme environments including low pH and low oxygen (Giebel et al. 1991, Maniloff et al. 1992). They lack a cell wall and are unaffected by many antibiotics (Razin et al. 1998). They are also common laboratory contaminants (Nikfarjam & Farzaneh

2012), but may exist as parasites or commensals within the hypobranchial gland, which provides a rich source of carbohydrates. To establish the potential for vertical or horizontal transmission of the bacterial symbionts in *D. orbita,* future bacterial profiling studies could include samples of the egg capsules, water and benthic substrate for comparison. Interestingly, a preliminary study on the culturable heterotrophic bacteria from *D. orbita* tissues identified at least one indole producing bacteria common to the hypobranchial gland and egg capsules (Ngangbam et al. 2015b). The precursors of Tyrian purple are found in the egg capsules (Benkendorff et al. 2000), as well as the female capsule gland, which lies adjacent to the hypobranchical gland in *D. orbita* (Westley & Benkendorff 2008, Rudd et al. 2015) suggesting a potential role of vertical transmission of biosynthetic symbiotic bacteria.

The dominance of *Vibrionaceae* in the hypobranchial glands of *D. orbita* implies these bacteria are selectively retained or are able to multiply within this unusual mucus producing organ. *Vibrios* are commonly associated with marine organisms (Cheng et al. 1995, Thompson et al. 2004, Chimetto et al. 2011) and species such as *V. parahaemolyticus, V. orientalis* and *V. campbellii* are pathogens of marine invertebrates (Sutton & Garrick 1993, Wang et al. 2015, Zhang et al. 2015) with *V. orientalis, V. harveyi, V. coralliilyticus* and *V. splendidus* being specific mollusc pathogens (Sutton & Garrick 1993, Sugumar et al. 1998, Ben-Haim et al. 2003, Chen et al. 2015). However, *Vibrio* species can also be endosymbionts such as those found in the viscera of the muricid, *Nucella lapillus,* and mucus of the sea slug, *Elysia rufescens* (Davis et al. 2013, Pratheepa et al. 2014), as well as *V. fischeri,* which is found in squid bioluminescent organs (Small & McFall-Ngai 1999). *Vibrio* species are known, not only for their symbiotic relationships with marine molluscs, but also for the production of important secondary metabolites (Ruby 1996, Kimbell & McFall-Ngai 2003, Mansson et al. 2011). A previous study successfully cultured three indole producing *Vibrio* species from the biosynthetic organs of *D. orbita* (Ngangbam et al. 2015b). Many additional indole producing

Vibrio species from the hypobranchial gland of *D. orbita* were identified in this study (Table 4.5). The relatively high concentration of these *Vibrios* in the hypobranchial gland, and their capacity for indole synthesis, suggest they may contribute to Tyrian purple precursor synthesis in the hypobranchial glands of Muricidae. Indole producing *Vibrio* species, including *V. orientalis* and *V. coralliilyticus* were found exclusively in the hypobranchial gland, but not in the foot. A range of other bacterial genera that produce indoles were also detected in the hypobranchial gland. These include *Bacillus,* (Idris et al. 2007) *Propionigenium,* (Janssen & Liesack 1995) and *Pseudomonas,* which produce indoles such as indole-3-acetic acid (Patten & Glick 2002). Hence, indole precursors may be opportunistically acquired from more than one bacterial species for Tyrian purple production in muricids.

It has been suggested bromoperoxidase plays a role in Tyrian purple biosynthesis through the addition of bromine to the 6-position of tyrindoxyl sulphate (Westley et al. 2006, Benkendorff 2014). This is supported by evidence of bromoperoxidase activity in *Trunculariopsis (Murex) trunculus* hypobranchial gland homogenates (Jannun & Coe 1987) and histochemical sections from *D. orbita* hypobranchial and rectal glands (Westley 2008, Westley & Benkendorff 2009). Several bacterial genera were detected in our bacterial profiling studies that are known to produce bromoperoxidase (Table 4.5), including *Pseudomonas* (Itoh et al. 1994) and bacteria of the *Bacillaceae* family (Read et al. 2003). The cyanobacterium, *Synechococcus* produce vanadium dependent bromoperoxidase (Johnson et al. 2011); this enzyme is implicated in the biosynthesis of marine halogenated natural products of pharmacological importance (Butler & Carter-Franklin 2004) and can also react with indole to produce region specific brominated indole products (Martinez et al. 2001, Butler & Carter-Franklin 2004). *Bacillus, Pseudomonas* and *Synechococcus* were all present in the hypobranchial gland of *D. orbita* and these bromoperoxidase producing bacterial genera are

the priorities for future targeted culture work to further investigate their role in Tyrian purple production in *D. orbita*. Future studies could also apply functional metagenomics approaches to uncover the brominating enzymology associated with the hypobranchial glands of Muricidae by screening specifically for bromoperoxidase and brominase genes (Agarwal et al. 2014).

Several other marine bacteria found in the hypobranchial glands are known to produce halogenases and could provide an alternative path for brominating indole precursors of Tyrian purple. Halogenating enzymes such as brominases, responsible for the synthesis of polybrominated metabolites, including phenol and imidazole structures, have been identified in marine bacteria (Agarwal et al. 2014). Tribromoimidazole, a brominated secondary metabolite found within the eggs of muricid molluscs, may be produced by brominase activity (Benkendorff et al. 2004a). *Pseudomonas* sp. also produce halogenase enzymes (Wynands & Pee 2004) and we detected *Pseudomonas* spp. in the hypobranchical glands of *D. orbita*. Other marine studies have isolated a tryptophan 6-halogenase with brominating activity from *Streptomyces* sp. (Zeng & Zhan 2011) and a novel halogenase gene from *Psychrobacter* sp. (associated with the marine sponge *Crambe crambe*) (Ozturk et al. 2013), a genus that was also detected in our *D. orbita* study. Other bacteria detected in the hypobranchial gland of *D. orbita*, including *Vibrio,* and *Pseudoalteromonas,* have previously been found to produce brominated secondary metabolites (Table 4.5). For example, *Vibrio* sp. (strain KMM-81-1) associated with the marine sponge (*Dysidea* sp.) produces brominated secondary metabolites (Elyakov et al. 1991), and several species of *Pseudomonas* produce brominated nitrophenyl pyrrole compounds (Ajisaka et al. 1969, van Pee et al. 1983), while *Pseudoalteromonas* sp. can produce pentabromopseudilin and bromophene (Feher et al. 2010). However, these bacteria were not specifically associated with the hypobranchial glands of *D. orbita* and thus appear less likely candidates for providing tissue localised precursors to Tyrian purple.

It is possible bacteria may be responsible for several steps which occur early in the Tyrian purple biosynthetic pathway. Enzymes such as sulphur transferase and sulphur reductase may be involved in contributing the methane thiol group on the indole ring of tyrindoxyl sulfate. High concentrations of mercaptan and dimethyl disulfide are present in muricid hypobranchial glands that produce Tyrian purple (Benkendorff et al. 2001a, Westley & Benkendorff 2008, Benkendorff 2013). *Pseudomonas* found in the hypobranchial gland is known to utilize dimethyl disulfide (Ito et al. 2007). Several bacteria that metabolise sulphur, such as *V. orientalis* and *V. coralliilyticus* are exclusively found in the hypobranchial gland and also utilize dimethylsulfoniopropionate, an organosulfur compound that produces dimethyl sulfide and methanethiol as a breakdown product (Curson et al. 2012, Garren et al. 2014). *Sulfitobacter mediterraneus* was detected in the foot and hypobranchial gland and is a sulfite-oxidizing bacteria (Pukall et al. 1999) that may catalyse the production of indoxyl sulfate. Thus, it is possible the various sulphur metabolizing bacteria found in the hypobranchial gland play important roles in Tyrian purple precursor production.

A difference in the 16S rRNA bacterial profiles of male and female hypobranchial glands was expected on the basis that previous chemical studies have suggested a difference in the oxidation and reduction state of indole dye precursors in male and female hypobranchial glands. Specifically, the female glands were found to contain higher amounts of reduced methanethiol derivatised indoles, such as tyrindoleninone and tyriverdin, whereas males contained more oxidised end-products 6-bromoisatin and 6,6'dibromoindirubin (Westley & Benkendorff 2008). This could imply the presence of sulfur-reducing bacteria in the female hypobranchial glands, although we actually found more evidence for known sulfur-reducing bacteria in the foot tissue (Table 4.5) and none were unique to the female hypobranchial gland. Nevertheless, the reducing environment of the female gland could explain why the bacterial

community structure was noticeably more distinct from the foot communities than the male glands (Fig. 4.5) and the tendency towards lower phylogenetic complexity in the female compared to male hypobranchial glands (Table 4.2, Fig. 4.3). However, consistent with a previous culture based study (Ngangbam et al. 2015b), there were no significant difference in the bacterial communities isolated from male and female samples and no interaction between tissue and gender. In both studies the lack of a significant gender effect could be influenced by a consistent bacterial community structure within the foot tissues and low power to detect a gender difference, specifically in the hypobranchial glands, due to relatively high variability and low replication of male and female samples within this tissue type (e.g. Fig. 4.5 PCO). Consequently, future functional metagenomics studies aimed specifically towards examining the sulphur metabolising bacteria in male and female hypobranchicial glands of Muricidae are warranted.

Overall, a larger number of bacterial taxa were found in the foot compared with the hypobranchial gland of *D. orbita*, however, a higher abundance of *Vibrio* and some unique microbial symbionts were observed in the hypobranchial gland. Some of the bacteria identified in the hypobranchial gland are known to produce indole and bromoperoxidase or other enzymes which may contribute to Tyrian purple precursor synthesis. Future studies will aim to culture these microbial symbionts associated with the hypobranchial gland and further analysis will be undertaken to identify genes that may be associated with Tyrian purple precursor production.

4. 6 Acknowledgments

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Chapter 5. Evidence of a bacterial origin for Tyrian purple precursors in muricid molluscs

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5. 1 Abstract

The secondary metabolite Tyrian purple, also known as royal purple, is a dye with historical importance for humans. The biosynthetic origin of Tyrian purple in Muricidae molluscs is not currently known. A possible role for symbiotic bacteria in the production of early precursors to Tyrian purple in the Australian species, *Dicathais orbita,* which is an ideal model species for muricid molluscs, has been proposed. This study aimed to culture bacterial symbionts from the Tyrian purple producing hypobranchial gland and screened the isolates for indole production and bromoperoxidase genes using molecular methods. The ability of bromoperoxidase positive isolates to produce the brominated indole precursor to Tyrian purple was then established by extraction of the culture and analysis using liquid chromatography mass spectrometry (LCMS). In total, 32 bacterial isolates were cultured from *D. orbita* hypobranchial glands using marine agar, marine agar with hypobranchial gland extracts, blood agar, thiosulfate citrate bile salts sucrose agar and cetrimide agar at pH 7.2. These included 26 *Vibrio* sp., two *Bacillus* sp., one *Phaeobacter* sp., one *Shewanella* sp., one *Halobacillus* sp., and one *Pseudoalteromonas* sp. The two *Bacillus* species were the only isolates found to have coding sequences for bromoperoxidase enzymes. Bromoperoxidase producing *Bacillus* sp. cultured from the hypobranchial glands in tryptone broth supplemented with KBr, produced the brominated precursor to Tyrian purple, tyrindoxyl sulphate. This study provides evidence that symbiotic *Bacillus* sp. could be the ultimate source of Tyrian purple.

5. 2 Introduction

Many marine invertebrates produce secondary metabolites that contribute to a suite of ecological roles, including paralysing prey, and preventing predation (Pawlik 1993), pathogens

(Engel et al. 2002), surface fouling (Boltovskoy & Cataldo 1999) and competitors (Haefner 2003). Beyond the ecological roles of secondary metabolites, these chemicals also provide opportunities that aid human society in the form of novel bio-products. A large number of marine natural products have been isolated and characterized from marine invertebrates (Molinski et al. 2009), including antimicrobial, antifungal, antiviral, antiprotozoal, anthelminthic and anticancer compounds (Donia & Hamann 2003, Nagle & Zhou 2009, Malaker & Ahmad 2013), as well as dyes and pigments (Benkendorff et al. 2004b, Westley & Benkendorff 2008, Prota 2012, Pereira et al. 2014).

The secondary metabolite Tyrian purple, also known as royal purple, is a dye with historical importance for humans (Westley & Benkendorff 2008). The indole precursors of Tyrian purple are known to have antimicrobial and anticancer properties (Benkendorff et al. 2000, Westley et al. 2010b, Edwards et al. 2012, Benkendorff 2013, Esmaeelian et al. 2013, Esmaeelian et al. 2014). Tyrian purple, a brominated derivative of indigo which has the same structure as Tyrian purple but without the bromines, is produced by bacteria (Lim et al. 2005, Qu et al. 2010, Qu et al. 2012). The synthesis of tyrindoxyl sulfate, which is the ultimate Tyrian purple precursor in the hypobranchial gland of muricids, requires bromoperoxidase to brominate the indole precursors (Jannun & Coe 1987, Westley & Benkendorff 2009, Westley et al. 2010a). Bacterial species such as *Bacillus* sp., *Synechococcus* sp., and *Pseudomonas putida*, which were identified in *D. orbita* tissue (Ngangbam et al. 2015a), have been previously found to produce bromoperoxidase enzymes (Read et al. 2003, Johnson et al. 2011, Itoh et al. 1994). Several other bacterial species also produce bromoperoxidase enzymes (Lingens 1985, Wiesner et al. 1985, van Pee et al. 1987, Zeiner et al. 1988, Knoch et al. 1989) and indoles (Alcaide et al. 1999, Patten & Glick 2002, Beleneva & Kukhlevskii 2010).

There is growing research interest in the involvement of bacteria-host invertebrate associations in the biosynthesis of marine secondary metabolites (Berrue et al. 2011). Structural homology between marine invertebrate natural products and microbial metabolites can provide an indication that these natural products are obtained from the microbes rather than being synthesized by the host (Lane & Moore 2011). Several marine natural products such as non-ribosomal peptides, polyketides and hybrid molecules reported from marine invertebrates are consistent with bacterial metabolites (Faulkner 2000). For example, the rod shaped bacteria *Candidatus* Endobugula sertula associated with the bryozoan *Bugula neritina,* are considered to be the original producer of bryostatin, an anticancer agent which was initially thought to be produced by the bryozoans themselves (Lopanik et al. 2004, Choi & Oh 2015). The structural similarity between Tyrian purple and indigo, coupled with the presence of indole and bromoperoxidase producing bacteria in the *D. orbita* hypobranchial gland (Ngangbam et al. 2015a, Ngangbam et al. 2015b) suggests a potential role of bacterial symbionts in Tyrian purple precursor's synthesis.

The major issue in the development of any new marine pharmaceutical product is a sustainable supply (Benkendorff 2009, Molinski et al. 2009, Berrue et al. 2011). Identification of bacteria involved in the biosynthesis of marine secondary metabolites can provide options for supplying sufficient quantities of marine compounds for clinical testing and commercialization (Sipkema et al. 2005, Benkendorff 2009). Many marine bacteria cannot be easily cultured (Amann et al. 1995, Joint et al. 2010), however, the ability to culture bacteria using traditional techniques provides an advantage for large scale production of natural products. Marine microbial symbionts associated with the host invertebrate may provide an alternative for producing compounds of biomedical importance on a larger scale for drug

development (Gulder & Moore 2009) and possibly solve the sustainable supply issue faced with the marine invertebrates.

Many approaches have been used to "culture the unculturable" bacteria from marine invertebrates. As an example, the optimization of growth media, incubating temperature and pH increases the recovery of microbes that are unculturable under standard conditions (Davis et al. 2005, Vartoukian et al. 2010, Pham & Kim 2012). Sterile marine water can be used to mimick the natural environment for culturing previously uncultured bacteria (Vartoukian et al. 2010) while marine invertebrate extracts can also mimic the chemical environment within hosts (Montalvo et al. 2014, Xing et al. 2014). For example, Li and Liu (2006) used sponge extracts in artificial seawater to "simulate" the sponge natural environment and resulted in the isolation of bacteria belonging to *Actinobacterium* and *Bacteroidetes.*

The muricid mollusc, *Dicathais orbita*, is an ideal model species for studying the biosynthesis of anticancer brominated indoles (Benkendorff 2013). Some indole producing bacteria have been previously isolated from the hypobranchial gland of *D. orbita* (Ngangbam et al. 2015b), but their ability to produce brominated metabolites was not determined. A recent culture independent metagenomic study on the hypobranchial gland, reported a large number of microbial symbionts, some of which have the capacity to produce both indoles and brominated compounds (Ngangbam et al. 2015a). This study aimed to culture bacteria potentially involved in Tyrian purple precursor synthesis, and then screen the isolates for indole production and bromoperoxidase genes using molecular methods. The bacteria were also screened for their ability to produce tyrindoxyl sulfate in potassium bromide supplemented media using liquid chromatography mass spectrometry (LCMS).

5. 3 Materials and Methods

5. 3. 1 Sample collection, preparation and culturing

D. orbita (n = 15 snails) were collected from subtidal and intertidal rocky reefs near Ballina (28°84′ S and 153°60′ E), northern NSW, Australia. Samples were collected during low tide on 11th December 2014 under permit number F89/1171-6.0 issued by Primary Industries, NSW Government, Australia. Snails were transferred live to Southern Cross University and processed immediately. The hard shells were removed and the snails dissected according to Westley & Benkendorff (2008). Hypobranchial glands (Fig. 5.1) were removed aseptically under laminar flow and rinsed three times with sterile seawater to remove any external bacteria loosely associated with the gland. An aqueous extract of hypobranchial gland was prepared separately for incorporation into bacterial culture media by homogenising 2 g hypobranchial gland (15 snails) with 35 ml of phosphate buffer saline (PBS) solution in a blender. The extract solution was filter sterilized through a 0.25 μm syringe filter (Minisart, Sartorius) before adding to autoclaved marine agar. The pH of the hypobranchial gland was measured using a pH microprobe (Orion, pH Micro Electrode, Thermo Scientific) and found to be mean 4.5 (± 0.08 st. dev, n = 3).

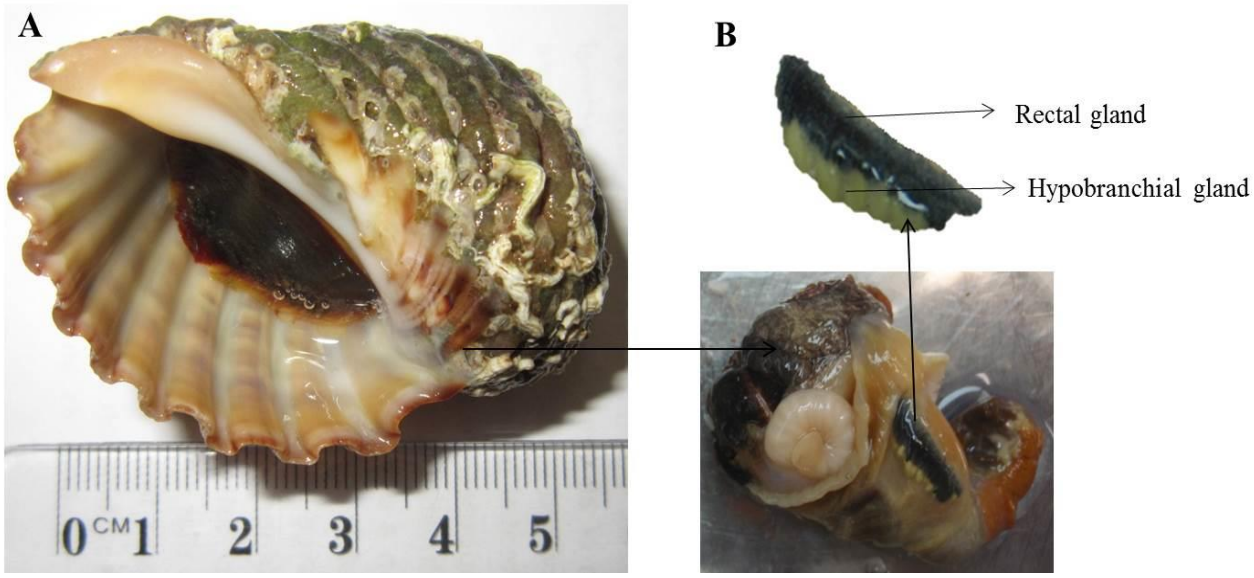

Fig. 5.1. *Dicathais orbita* hypobranchial gland used for isolating and culturing bromoperoxidase and indole producing bacteria. A) *Dicathais orbita*; B) Hypobranchial and rectal gland.

Culturing potential *D. orbita* hypobranchial gland microbial symbionts was undertaken using five different growth media: marine agar (pH 7.2), marine agar (pH 4.5), marine agar and hypobranchial gland extract (pH 7.2), marine agar and hypobranchial gland extract (pH 4.5), blood agar, TCBS (thiosulfate citrate bile salts sucrose) agar and cetrimide agar. These media were chosen based on their potential to provide favourable conditions which may not be provided by standard growth media. Marine agar with hypobranchial gland extract was used to mimic the natural environment of the *D. orbita* hypobranchial gland. TCBS and cetrimide agar was used as selective media for isolating *Vibrio* sp. and *Pseudomonas* sp. respectively (Gomez-Gil et al. 1998, Jaksic et al. 2002, Phatarpekar et al. 2002). Blood agar was used as enriched media to isolate fastidious bacterial symbionts (Parija 2014). Marine agar and marine agar supplemented with 10% aqueous gland extract plates were used at pH 7.2 and adjusted to pH 4.5 using small amounts of HCl in order to match the pH of the hypobranchial gland lumen.

Three approaches were used to isolate and culture bacteria from hypobranchial glands. The sampling approaches included: (1) sampling homogenates of whole hypobranchial glands; (2) taking dorsal swabs of glands and (3) taking ventral swabs of glands. In all cases, three hypobranchial glands, with an approximate total weight of 0.25 g, were used. Homogenates were prepared using a sterile mortar and pestle whereas swab samples were taken using sterile cotton swabs. Each of the samples was diluted in 9 ml of sterile sea water, mixed thoroughly by vortexing, and three fold dilutions were prepared with sterile seawater. Additional concentrated homogenates were also prepared from six individual hypobranchial glands. Following Marinho et al. (2009), these were homogenised separately in marine broth at a concentration of 1g/ ml then directly plated onto marine agar and cetrimide agar for maximum recovery of bacterial symbionts, including *Pseudomonas* sp.

A 100 µl aliquot from each homogenate and swab sample was spread onto duplicate agar plates. The agar plates were incubated for 14 days at 25 °C. Agar plates were observed daily for bacterial colonies and the colony size and morphology were recorded. Morphologically distinct colonies were selected for further indole testing and molecular identification of the isolates. All genetically distinct isolates were screened for bromoperoxidase genes and a subset of these were analysed for brominated indole production.

5. 3. 2 16S rRNA sequencing and indole producing bacteria

Morphologically distinct colonies were subjected to 16S rRNA sequence analysis. DNA was extracted using Qiagen DNA extraction kits (QIAmp DNA mini kit, Qiagen). PCR reactions comprised 2.5 µl of 10x PCR buffer; 2.5 µl of dNTPs (2mM), 1.25 µl of 50 mM $MgCl_2$; 1 µl genomic DNA (35-80 ng); 0.4 µl *Taq* polymerase and 1 µl forward primer (27F)

(10 µM), 1 µl reverse primer (1492R) (10 µM), 15.35 µl Milli-Q water in a final volume of 25 µl. PCR cycle conditions consisted of an initial denaturation at 94 °C for 5 min, followed by 30 cycles of 45 s at 95 °C, 1 min at 58 °C and 1 min at 72 °C. PCR amplicons were separated by agarose gel electrophoresis and visualised by GelRed staining under UV irradiation. Positive PCR products were purified using QIAquick PCR Purification Kit (Qiagen) in accordance with the manufacturer's instructions and sequenced by Macrogen Inc, South Korea (http://dna.macrogen.com/eng). DNA sequences were analysed using sequence scanner software v1.0 and compared with sequences in the NCBI GenBank database by BLASTN. All 16S rRNA gene sequences from the 32 bacterial isolates were lodged with GenBank under accession number KR855712, KR338844 - KR338874. The isolates were preserved by diluting 1:1 in sterile marine broth containing 30 % glycerol and then stored in -80^{o}C for further analysis.

Gram staining (100x magnification, OL) was performed on the pure cultures and distinct colonies were analyzed for indole production in Tryptone broth (Casein enzymatic hydrolysate 10.000 g, NaCl- 5 g, pH-7.5). The indole test was performed by using a loopful of colony from pure culture inoculated into tryptone broth and incubated for 24 hours at 25°C. The formation of a red ring on addition of 1 ml Kovac's reagent (Fluka) indicated a positive reaction.

5. 3. 3 Bromoperoxidase gene screening

Genomic DNA of the 32 bacterial isolates were screened for bromoperoxidase genes using primer pair BBFp (CCCATG TGG ACC ACC CTT TAT) and BBRp (TAA GTG GTC GAT CTT GGGAAT). These primers were designed based on the bromoperoxidase consensus sequence derived from five *Bacillus* strains (Lilles 2011). The PCR reaction was composed of

2.5 µl of 10x PCR buffer; 4 µl of dNTPs (2mM), 1 µl of 50 mM $MgCl_2$, 2 µl genomic DNA, 0.5 µl *Taq* polymerase, 1.5 µl forward primer (FP) (10 µM), 1.5 µl reverse primer (RP) (10 µM), 12 µl Milli-Q water in a final volume of 25 µl. PCR cycle conditions comprised an initial denaturation at 94 °C for 5 min followed by 30 cycles of 1 min at 94 °C, 1.30 min at 58 °C and 1 min at 72 °C. PCR amplicons were separated and visualised by agarose gel electrophoresis and GelRed staining under UV irradiation. Positive bromoperoxidase fragments, approximately 700 bp in size, were purified and sequenced by Applied Biosystems 3730 and 3730xl capillary sequencers (Australian Genome Research Facility, Brisbane and further analysed using sequence scanner software v1.0 and compared with sequences in the NCBI GenBank database by BLASTN. Nucleotide sequences for putative bromoperoxidase genes were submitted to NCBI GenBank under accession number KT180165 and KT180166.

5. 3. 4 Liquid chromatography mass spectrometry (LCMS) analysis of bacterial extracts

Bromoperoxidase containing *Bacillus* sp. M1 and F1 and a subset of bromoperoxidase negative bacterial species, including *Vibrio* sp. B1, *Pseudoalteromonas* sp. T2 and *Phaeobacter* sp. C3 were analysed for the possible production of any brominated compounds. Bacteria were grown in sterilized Schott bottles containing 60 ml marine broth and tryptone broth, since these contain tryptophan which is suspected to be the ultimate precursor for Tyrian purple (Benkendorff 2013), and supplemented with 0.2 g potassium bromide (KBr). The bacterial growth was maintained for 24 hrs at 25 °C. A control of tryptone broth without bacterial innoculation was also maintained with KBr for comparison. Exponentially growing cultures were centrifuged at 6000 rpm for 10 min to separate the cells and supernatant. Extraction of the supernatant was performed by ion exchange chromatography by passing the supernatant through a diaion resin (Diaion HP 20, Supelco, Bellefonte PA, USA), then washing

the column with methanol, before drying the methanol extract using a stream of high purity nitrogen gas. The cell pellet was also extracted in chloroform: methanol (1:1) and dried under nitrogen gas. These extracts were analysed using LCMS.

LCMS analysis was undertaken using an Agilent 1260 infinity High Performance Liquid Chromatography (HPLC) system coupled with a 6120 Quad mass spectrometer (MS). The HPLC utilized a Phenomenex luna C18 reversed phase column (100 x 4.6 mm) using a solvent gradient from 10 to 95% acetonitrile (ACN) with 0.005% trifluoroacetic acid (TFA) over 18 minutes at a flow rate of 0.75 mL/min. Peak absorption was monitored using parallel UV/Vis diode-array detection (DAD). Electrospray ionisation (ESI) mass spectrometry was used in the positive and negative ion modes. Agilent ChemStation was used to analyze the LCMS data. Characteristic ion cluster patterns from Br^{79} and Br^{81} in the mass spectrum were used for identifying the presence of any brominated compounds (Westley & Benkendorff 2008).

5. 4 Results

5. 4. 1 Bacterial isolation

Thirty two distinct bacteria were isolated in total (Table 5.1). Six, 10 and 16 bacterial morphotypes were cultured from the samples of hypobranchial gland homogenates, hypobranchial gland dorsal swabs and hypobranchial gland ventral swabs respectively (Table 5.1). All of the distinct types of bacteria were recovered on marine agar at 7.2 pH and no additional bacteria were cultured by incorporating hypobranchial gland extract into the media. No bacterial colonies were observed at pH 4.5 on marine agar with or without 10% aqueous gland extract when incubated at 25° C. A subset of the bacteria was recorded on the other

growth media: 25 % in cetrimide agar, 72 % in blood agar, 78 % marine agar with hypobranchial gland extracts and 81% in thiosulfate citrate bile salts sucrose agar (Table 5.1).

5. 4 .2 Molecular identification of cultivated bacteria and indole producing bacteria

Analysis of 16S rRNA gene sequences, of the 32 isolates cultured revealed 26 *Vibrio* spp., two *Bacillus* sp., one *Phaeobacter* sp., one *Shewanella* sp., one *Halobacillus* sp., and one *Pseudoalteromonas* sp (Table 5.1).

Four *Vibrio* sp. (KR338857, KR338858, KR338859, KR338870) two *Bacillus* sp. (KR338869, KR855712) from hypobranchial gland homogenates (Table 5.1); seven *Vibrios* (KR338845, KR338846, KR338851, KR338853, KR338854, KR338856, KR338871) one *Phaeobacter* sp. (KR338852) one *Shewanella* sp. (KR338855), and one *Pseudoalteromonas* sp. (KR338872) from hypobranchial gland dorsal swabs (Table 5.1); 15 *Vibrio* sp. (KR338844, KR338847- KR338850, KR338860- KR338863, KR338865- KR338868, KR338873, KR338874), and one *Halobacillus* sp. (KR338864) were cultured from hypobranchial gland ventral swabs sample (Table 5.1).

Sequence similarity to partial 16S rRNA gene sequence available in NCBI GenBank of all the isolates ranged from 97 % to 100 % (Table 5.1). Twenty five of the isolates were indole positive (Table 5.1). Partial 16S rRNA sequence analysis revealed that all 25 indole producing bacteria from the hypobranchial gland of *D. orbita* were Vibrionaceae (Table 5.1). All 32 bacterial isolates were motile with 29 being Gram negative and three being Gram positive (*Bacillus* sp., *Bacillus thuringiensis* and *Halobacillus* sp.)

Table 5.1. BLASTN analysis of partial 16S rRNA gene sequence derived from bacteria isolated from *D. orbita* hypobranchial gland cultured on different agar media with closest match to NCBI GenBank data.

Closest match and accession number	Agar media					GenBank accession nos.	Length (base pair)	Identity (%)	Indole	Gram stain	Tissue source
	MA	TCBS	CA	MAH	BA						
Vibrio sp. (KM369853.1)	+*	+	-	-	+	KR338844	1149	100	+	Gram-	HGVS
Vibrio chagasii (NR117891.1)	+*	+	+	+	+	KR338845	1083	99	+	Gram-	HGDS
Vibrio alginolyticus (KF886646.1)	+*	+	+	+	+	KR338846	1115	99	+	Gram-	HGDS
Vibrio sp. (KP126921.1)	+*	+	-	-	+	KR338847	510	100	+	Gram-	HGVS
Aliivibrio sp. (FR744854.1)	+*	+	-	-	+	KR338848	1087	99	+	Gram-	HGVS
Vibrio azureus (JF412237.1)	+*	+	-	-	+	KR338849	1119	99	+	Gram-	HGVS
Vibrio pomeroyi (KM014017.1)	+*	+	-	-	+	KR338850	1090	99	+	Gram-	HGVS
Vibrio sp. (KM369851.1)	+*	+	+	+	-	KR338851	1004	100	+	Gram-	HGDS
Phaeobacter sp. (GQ906799.1)	+*	+	-	+	-	KR338852	1044	100	-	Gram-	HGDS
Vibrio sp. (GQ406789.1)	+*	+	+	+	-	KR338853	1046	99	-	Gram-	HGDS
Vibrio sp. NB0059, (KP770076.1)	+*	+	+	+	+	KR338854	1096	100	+	Gram-	HGDS
Shewanella sp. (JF825445.1)	+*	+	+	+	+	KR338855	1058	98	-	Gram-	HGDS
Vibrio mediterranei, (HF541948.1)	+*	+	-	+	+	KR338856	1116	99	+	Gram-	HGDS
Vibrio sp. (KF188532.1)	+*	-	-	+	-	KR338857	1096	100	+	Gram-	HGH
Vibrio sp. (KF188531.1)	+*	-	-	+	-	KR338858	1066	99	+	Gram-	HGH

Vibrio sp. (HG942391.1)	+*	-	-	+	-	KR338859	626	99	+	Gram-	HGH
Vibrio sp. (KP126921.1)	+*	+	-	+	+	KR338860	951	97	+	Gram-	HGVS
Vibrio sp. (KM369860.1)	+*	+	-	+	+	KR338861	796	99	+	Gram-	HGVS
Vibrio sp. (KM369853.1)	+*	+	-	+	+	KR338862	1091	100	+	Gram-	HGVS
Vibrio sp. (GQ406789.1)	+*	+	-	+	+	KR338863	1079	100	+	Gram-	HGVS
Halobacillus sp. (FM992846.1)	+*	+	-	+	+	KR338864	1109	100	-	Gram +	HGVS
Vibrio sp. (FJ457587.1)	+*	+	-	+	+	KR338865	1004	100	+	Gram-	HGVS
Vibrio sp. (KM369853.1)	+*	+	-	+	+	KR338866	1038	99	+	Gram-	HGVS
Vibrio splendidus (AB038030.1)	+*	+	-	+	+	KR338867	1083	100	+	Gram-	HGVS
Vibrio harveyi (KR003734.1)	+*	+	-	+	+	KR338868	973	100	+	Gram-	HGVS
Bacillus sp. (KJ756140.1)	+*	-	-	+	-	KR338869	1034	100	-	Gram +	HGH
Bacillus thuringiensis (KC355253.1)	+*	-	-	+	-	KR855712	1180	99	-	Gram +	HGH
Vibrio sp. (HF937138.1)	+*	-	-	+	-	KR338870	866	99	+	Gram-	HGH
Vibrio sp. (EU340847.1)	+*	+	+	+	+	KR338871	1110	99	+	Gram-	HGDS
Pseudoalteromonas sp. (KP301110.1)	+*	+	+	+	+	KR338872	973	99	-	Gram-	HGDS
Vibrio sp. (FJ457361.1)	+*	+	-	-	+	KR338873	1056	99	+	Gram-	HGVS
Vibrio jasicida (AB562594.1)	+*	+	-	-	+	KR338874	907	99	+	Gram-	HGVS

MA, marine agar; TCBS, thiosulfate citrate bile salts sucrose; CA, cetrimide agar; MAH, marine agar with hypobranchial gland extracts; BA, blood agar; +, bacterial isolates; -, no bacterial isolates; +*, bacterial isolates used for further cultivation for screening bromoperoxidase enzyme; HGVS, Hypobranchial gland ventral swabs; HGDS, Hypobranchial gland dorsal swabs and; HGH, Hypobranchial gland homogenates.

5. 4. 3 Putative bromoperoxidase gene screening by PCR

PCR of DNA derived from the 32 distinct bacterial isolates using primer pairs BBFp and BBRp amplified putative bromoperoxidase coding gene sequences from two *Bacillus* sp., but failed to amplify any DNA from the remaining 30 bacterial isolates. BLASTN comparison of gene sequence amplified from the two *Bacillus* sp. against the NCBI database revealed 97% sequence similarity with *Bacillus thuringiensis* MC28- bromoperoxidase (CP003687.1) (Table 5.2).

Table 5.2. *Bacillus* sp. putative bromoperoxidase gene BLASTN analysis showing the closest match in NCBI GenBank.

Bacterial isolates	**GenBank accession nos.**	**Length (base pair)**	**Identity (%)**	**Closest match, accession number, position and protein id**
Bacillus sp.	KT180165	628	97	*Bacillus thuringiensis* MC28-bromoperoxidase (CP003687.1); 2239980-2240816 and AFU13721.1
Bacillus thuringiensis	KT180166	634	97	*Bacillus thuringiensis* MC28-bromoperoxidase (CP003687.1); 2239980-2240816 and AFU13721.1

5. 4. 4 Bacterial extract analysis for brominated compounds by liquid chromatography mass spectrometry (LCMS)

LCMS analysis revealed the presence of an HPLC peak with a retention time of around 15 min in diaion resin supernatant extracts from *Bacillus* sp. (KR338869) and *Bacillus thuringiensis* (KR855712) cultures, which were not present in the tryptone broth control supplemented with KBr (Fig. 5.2). Major ions in total ion current-mass spectrum (TIC-MS), obtained at the apex of this peak, were m/z 336, 338, which corresponds to the molecular ion of tyrindoxyl sulfate (i; Br^{79}, Br^{81} (Fig. 5.3). The diode array revealed UV absorption maxima at 228 and 302 nm (Fig. 5.3). The UV trace, HPLC retention time and mass spectrum are all consistent with tyrindoxyl sulfate standard isolated from the hypobranchial gland and confirmed by nuclear magnetic resonance H^1NMR (600 MHz, CD_sCN, 25^oC aromatic protons δ 7.65 (1H, d), 7.55 (1H, d), 7.20, 1H, dd), methyl protons δ 2.5 (3H, s).

The peak corresponding to tyrindoxyl sulphate was also observed in chloroform/ methanol extracts from the cell pellets of *Bacillus* sp. (KR338869) and *Bacillus thuringiensis* (KR855712) (data not shown). However, no peaks corresponding to tyrindoxyl sulfate, and no other brominated compounds were detected in extracts from cell pellets or supernatant of *Pseudoalteromonas* sp. (KR338872), *Phaeobacte*r sp. (KR338852) or *Vibrio* sp. (KR338845) cultures (Fig. 5.2).

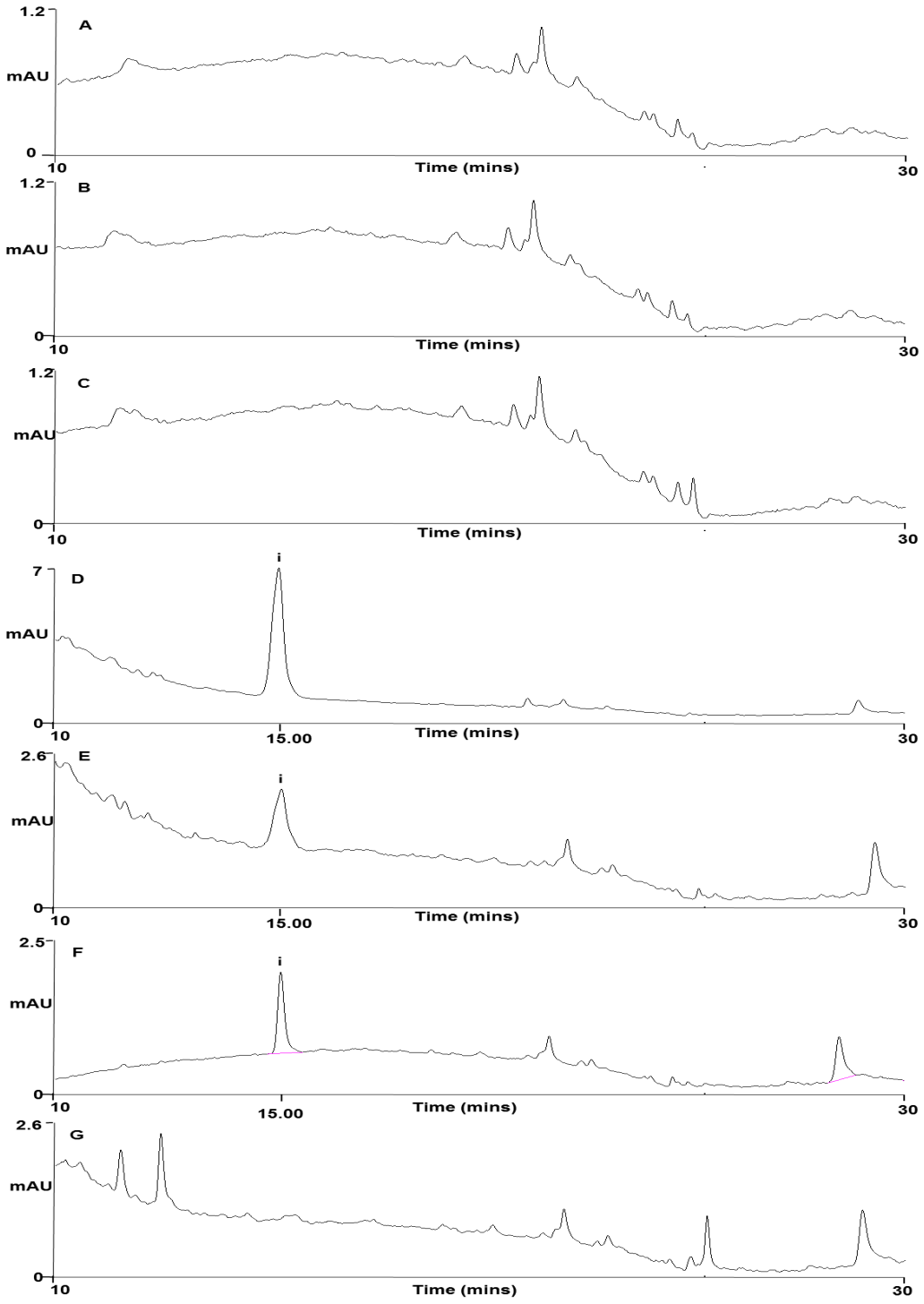

Fig. 5.2. Liquid chromatography-mass spectrometry analysis of diaion resin chromatography extracts of A) *Pseudoalteromonas* sp. T2 (KR338872); B) *Phaeobacte*r sp. C3 (KR338852); C) *Vibrio* sp. B1 (KR338845); D) *Bacillus* sp. M1 (KR338869); E) *Bacillus* sp. F1 (KR855712) and; F) tyrindoxyl sulfate standard isolated from *D. orbita* hypobranchial gland G) Tryptone broth control. The chromatogram obtained from the diode array at 280 nm shows the presence of tyrindoxyl sulfate (i).

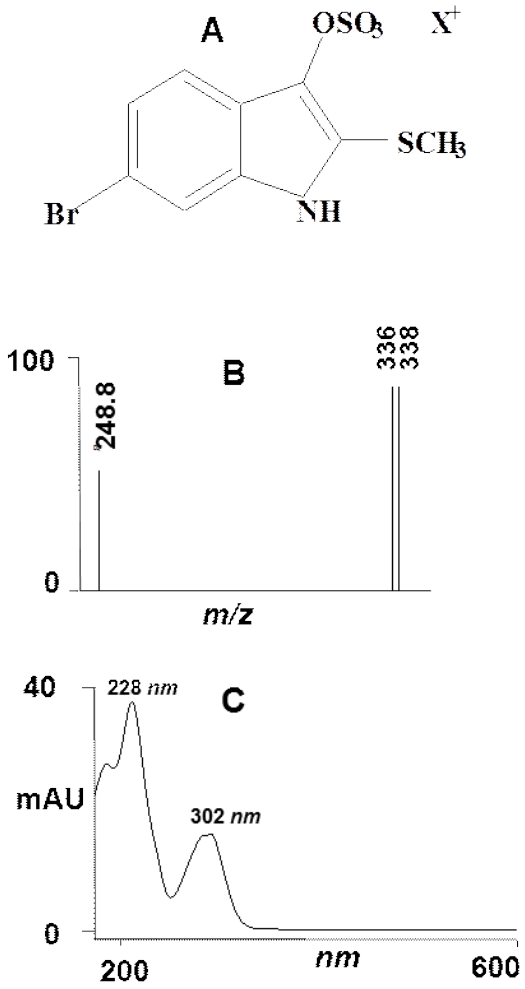

Fig. 5.3. Tyrindoxyl sulfate A) chemical structure; B) TIC-MS (Total ion current-mass spectrum), obtained from the apex of the major chromatographic peak obtained at 15.00 min showing signals with the molecular mass (m/z 336, 338) and; C) UV- Vis spectra of tyrindoxyl sulfate.

5. 5 Discussion

This study provides the first evidence of a bromoperoxidase producing bacteria that is capable of biosynthesizing the brominated precursor of Tyrian purple in the hypobranchial gland of a muricid mollusc. Tyrian purple is a dye of historical importance that traditionally could only be obtained by extraction from the Muricidae. However, only 1g of dye is obtained

from approximately 12000 snails (Cooksey 2001), highlighting the need for sustainable methods if Tyrian purple dye is to be to supplied on a large industrial scale. Although Tyrian purple can be chemically synthesized (Imming et al. 2001, Schatz 2001, Wolk & Frimer 2010) there is still a demand for the natural product. Targeting organisms to supply biomaterials can place populations at risk, as demonstrated by the decline of the central American Muricidae *Plicopurpura pansa* populations due to overharvesting for purple dye (Flores-Garza et al. 2012). Presently, *P. pansa* is considered a threatened species and is under special protection by the Mexican government (Flores-Garza et al. 2012). The discovery that muricid bacterial symbionts contribute to Tyrian purple precursor biosynthesis provides an alternative and more sustainable method to source this natural dye.

The low microbial diversity observed in hypobranchial gland homogenates and the identification of 25 indole positive bacteria that were all *Vibrio* sp. is consistent with previous studies (Ngangbam et al. 2015a, Ngangbam et al. 2015b). However, this study also identified two *Bacillus* species which have coding sequences for bromoperoxidase enzymes. Bacterial species belonging to the *Bacillaceae* family are known to produce bromoperoxidase (Read et al. 2003) along with several other bacteria (van Pee & Lingens 1985, Wiesner et al. 1985, Knoch et al. 1989, Itoh et al. 1994). Bromoperoxidases produced by marine bacteria are often involved in the biosynthesis of halogenated natural products of pharmacological importance (Butler & Carter-Franklin 2004) and this enzyme has the capability to react with indole specifically in the 6' position for producing 6-brominated indoles (Martinez et al. 2001, Butler & Carter-Franklin 2004). Bromoperoxidase activity has been reported in the hypobranchial glands of *D. orbita* (Westley & Benkendorff 2009) and other muricid (Jannun & Coe 1987). This study confirms that a bromoperoxidase associated with bacteria in the hypobranchial

gland of a muricidae is capable of brominating indoxyl sulfate in the 6’ position on the aromatic ring to form tyrindoxyl sulfate.

Bromoperoxidase producing *Bacillus* sp., isolated from the hypobranchial glands of *D. orbita,* were able to produce the brominated Tyrian purple precursor, tyrindoxyl sulphate. Importantly, hypobranchial gland bacteria that did not contain a bromoperoxidase gene, such as *Pseudoalteromonas* sp. T2, *Phaeobacte*r sp. C3 and *Vibrio* sp. B1, failed to produce brominated Tyrian purple precursors. Marine *Pseudoaltermonas* have previously been found to contain halogenase enzymes and produce small polyaromatic brominated secondary metabolites (Jiang et al. 2000, Feher et al. 2010). Given that no brominated compounds were detected from the *Pseudoaltermonas* in this study, this bacterium is less likely to play a role in Tyrian purple precursor synthesis. On the other hand, *Bacillus* sp. have been isolated from the egg masses of *Concholepas concholepas* (Leyton & Riquelme 2010), another Muricidae species that produces Tyrian purple in its hypobranchial glands and egg masses (Benkendorff et al. 2004b). The fact that this bacteria is associated with the eggs also indicates possible maternal transmissiom of the bacterial symbionts. Overall, this study identifies the potential for an association between Muricidae and *Bacillus* for Tyrian purple precursor production. This could be further tested by screening the other Muricidae species for bromoperoxidase containing *Bacillus* species.

Despite having the capacity to produce tyrindoxyl sulfate from tryptophan containing broth, the *Bacillus* species isolated in this study were not positive for indole production. However, 25 indole positive bacteria were isolated and all were identified as *Vibrionaceae*. The finding that *Vibrio* spp. are the dominant bacterial species in the hypobranchial glands of *D. orbita* was consistent with our previous metagenomic study on *D. orbita* hypobranchial glands (Ngangbam et al. 2015a). Several marine *Vibrio* spp. are known to produce indoles

(Pujalte & Garay 1986, Alcaide et al. 1999, Thompson et al. 2003, Beleneva & Kukhlevskii 2010, Pandey et al. 2010), but there are no reports of bromoperoxidase genes being isolated from marine *Vibrio* spp.. Consistent with this, none of the *Vibrio* spp. isolated in this study contained coding sequences for putative bromoperoxidases. The dominance of *Vibrio* spp. in the hypobranchial gland of *D. orbita* suggests *Vibrio* spp. may contribute indoles, which are then brominated by *Bacillus* sp. for Tyrian purple precursor synthesis. This implies a novel interaction between distinct endosymbiotic bacteria in the hypobranchial glands of Muricidae.

Overall, this study provides evidence that *Bacillus* spp. containing bromoperoxidase enzymes that occur in the hypobranchial gland of *D. orbita* are capable of producing brominated precursors for Tyrian purple biosynthesis. However, there remains a possible role for marine *Vibrio* spp. in contributing non-brominated indoles, which provide the scaffold for bromination and generation of the ultimate precursor to Tyrian purple. Hence, the role of symbiotic bacteria in the biosynthesis of Tyrian purple precursors is highlighted and provides scope for future studies on potential sustainable production of Tyrian purple through the application of bacterial culture and/or genetic engineering.

5. 6 Acknowledgments

I would like to thank Roselyn Regino-Valles for helping P. M in isolating tyridoxyl sulfate from *D. orbita* and Wendy Loa-Kum-Cheung from the Eskitis Institute, Griffith University for the NMR data on this compound and David Rudd for assisting with the NMR interpretation. I also appreciate feedback on the manuscript from Bijayalakshmi Devi Nongmaithem. This study was supported by the SESE, SCU PG support for A.K.N. and philanthropic grant to K.B.

Chapter 6. General discussion and future directions

6. 1 General discussion and synthesis

D. orbita, a mollusc of the Muricidae family, is well known for the production of the indole dye Tyrian purple and murexine, a choline ester. Accordingly, this thesis investigated the transcriptome of the Australian mollusc *D. orbita* (Chapter 2) and identified various genes associated with indole, histidine and sulfur metabolism pathways that could be associated with Tyrian purple biosynthesis. Transcriptome sequencing of several *D. orbita* tissues generated over 200 million good quality reads that were *de novo* assembled into 219,437 contigs and identified many genes expressed in this neogastropod that were not found in the annotated genomes of other molluscs available in GenBank and KEGG databases (Chapter 2). This transcriptome study provides a good resource for exploring further differential gene expression studies to identify genes upregulated in the biosynthetic tissues which could help confirm the biosynthetic enzymes required for indole and choline ester synthesis. Furthermore, based on Rudd et al. (2015), differential gene expression during egg deposition could help identify regulatory genes for choline esters in the reproductive tract of *D. orbita*. However, we did not find any brominating enzymes in the *D. orbita* transcripts responsible for producing brominated indole precursors in Tyrian purple synthesis. This investigation therefore lead us to study the possible role of microbial symbionts associated with *D. orbita* Tyrian purple production.

Bioactive secondary metabolites produced in marine invertebrates are often associated with symbiotic bacteria (Konig et al. 2006). Investigation of the microbial communities associated with different *D. orbita* tissues using traditional culture techniques (Chapter 3) initially identified three indole producing *Vibrio* spp. in the Tyrian purple biosynthetic hypobranchial glands. Culture and biochemical characterisation of the bacterial communities also revealed a lower diversity of bacteria and distinct communities in *D. orbita* biosynthetic glands compared to foot tissue and seawater. However, the majority of marine microorganisms

are not easily culturable, it is estimated only about 0.001–0.1% of marine microbes have been successfully cultured (Amann et al. 1995), and the majority of the bacteria specifically associated with hypobranchial glands appear to be unculturable. Hence, the culture independent method of metagenomics was used to further investigate any unique symbionts and their capacity to brominate the indole precursors.

High-throughput genetic profiling was used to investigate the unculturable bacteria in *D. orbita* tissues in Chapter 4. Highly diverse bacterial communities associated with both the hypobranchial gland and foot tissues of *D. orbita* were observed in the metagenome dataset, with phylum *Proteobacteria* as the dominant bacteria. Nevertheless, consistent with the preliminary culture work (Chapter 3), the Tyrian purple biosynthetic hypobranchial gland was found to have significantly lower bacterial diversity and a different bacterial community composition when compared to the foot tissue (Chapter 4). A higher abundance of indole producing *Vibrio* spp. were again detected, and genetic bacterial profiling also revealed bacteria with brominating capabilities in the hypobranchial gland (Chapter 4). This finding, along with the absence of brominating genes in the *D. orbita* transcriptome (Chapter 2), suggested bacteria likely have a role in Tyrian purple biosynthesis. This thesis further investigated the evidence of a bacterial origin for Tyrian purple in muricid molluscs using additional culture work to target the bacteria with brominating capabilities. Two *Bacillus* species, isolated from hypobranchial gland homogenates using marine agar at 7.2 pH, were confirmed to contain bromoperoxidase genes by targeted PCR amplification and sequencing. These bromoperoxidase producing *Bacillus* sp. cultured from the hypobranchial glands in tryptone broth with KBr were then shown by LCMS analysis to produce tyrindoxyl sulphate, a brominated precursor to Tyrian purple. This provides the first evidence confirming the role of bacteria in Tyrian purple synthesis.

6. 2 Future directions

The ability to culture symbiotic bacteria that play a role in secondary metabolite production can provide options for sustainable production. Large scale sustainable supply of marine secondary metabolites is still a major challenge for clinical testing and commercial production for pharmaceutical companies (Benkendorff 2009, Molinski et al. 2009, Berrue et al. 2011, Martins et al. 2014, Gomes et al. 2016). Microbial fermentation technology is a suitable approach for large scale bacterial culture for the sustainable supply of marine secondary metabolites (Salomon et al. 2004, Radjasa & Sabdono 2009, Penesyan et al. 2010, Waters et al. 2010). Pfefferle et al. (2000) demonstrated that by optimizing the fermentation conditions of bacterial genus *Streptosporangium*, such as increasing the speed of the stirrer or agitation speed from 500 rpm to 750 rpm, increasing oxygen supply from 2 v v^{-1} min^{-1} to 4 v v^{-1} min^{-1}, pH 7.3, complex media (comprising of glucose, soluble starch, glycerol, cornsteep powder, peptone, yeast extract NaCl and $CaCO_3$) and also the agitation system using marine impeller system, could all increase the secondary metabolite production significantly. *Bacillus* sp. with bromoperoxidase enzymes identified in this thesis, provides future possibilities for the sustainable production of Tyrian purple and further research could focus on optimizing *Bacillus* sp. culture conditions for Tyrian purple production.

Recombinant technology is another strategy that could be used to facilitate the large scale sustainable production of marine secondary metabolites for drug development (Wagner-Dobler et al. 2002, Long et al. 2005, Pickens et al. 2011, Gomes et al. 2016). Li et al. (2009) demonstrated that heterologous gene expression in *Streptomyces cinnamonensis* increased the production of polyketide products. Furthermore, the recombinant bacteria *S. lividans* shows increased production of the secondary metabolite daptomycin through the elimination of genes coding for actinorhodin (Penn et al. 2006). The application of recombinant technology can be

used for expression of targeted genes for brominated indole synthesis in a heterologous host. For example, it is possible inserting the *Bacillus* bromoperoxidase gene specifically into a *Vibrio* spp. that produces indole could create a bacterium with the full suite of biosynthetic capabilities and increase the scale of Tyrian purple precursor production, an approach that could be used as an alternative to wild harvest of Muricidae molluscs.

Co-culturing techniques can increase the yield of secondary metabolites over a single microbial culture, enhance the chemical diversity generated by the microbial cultures and influence the growth of recalcitrant microbes (Conway et al. 2012, Dashti et al. 2014, Marmann et al. 2014). For example, Conway et al. (2012) revealed that co-culturing *Pseudomonas aeruginosa* with *Roseobacter denitrificans* altered gene expression in seconday metabolite biosynthetic pathways in both species compared to single cultures. An example of enhanced chemical diversity resulting from co-culturing is the co-culturing of *Actinokineospora* sp. and *Nocardiopsis* sp. which resulted in the production of new secondary metabolites which were not detected in a single microbial culture (Dashti et al. 2014). Hence, co-culturing techniques using indole producing *Vibrio* spp. and brominating *Bacillus* sp. could be done as future work for exploring novel brominated indole biosynthesis having pharmaceutical importance.

To further investigate whether a symbiotic relationship between *Vibrios* and *Bacillus* sp. leads to the production of tyrindoxyl sulfate in *D. orbita,* controlled culture experiments could be undertaking using the following model. The brominating *Bacillus* sp. could be co-cultured with indole producing *Vibrio* sp. in minimal marine media that doesn't contain any indole and/or tryptophan, but supplemented with KBr as a source of bromine ions. Control cultures of *Bacillus* sp. could be prepared without the *Vibrio* in the same minimal media (negative control) and in media supplemented with indole (positive control). Control culture of *Vibrio* sp. without the *Bacillus* would also be required in the minimal media. The expected

outcomes from this model would be production of indoxyl sulfate by *Vibrio* spp. alone and tyrindoxyl sulfate by co-culturing of *Vibrio* spp. with *Bacillus* sp.

Tyrian purple is completely insoluble and traditional methods of dying (with both indigo and Tyrian purple) therefore require the precursors to get the compound into the cloth, where it is subsequently converted into the final pigment by chemical reactions (Baker & Sutherland 1968, Baker & Duke 1976, Cooksey 2001). Of all the intermediate precursors, tyrindoxyl sulfate is the most stable so it can be stored in this form until the first reaction is initiated. The first reaction is the hydrolysis of the indoxyl sulfate into the alcohol tyrindoxyl. This is performed by an aryl sulfatase enzyme in the mollusc, but it can also be artificially induced using acid, such as HCl (Baker & Duke 1976, Westley & Benkendorff 2008). Trindoxyl is unstable and rapidly converts to the ketone tyrindoleninone, which spontaneously dimerizes with tyrindoxyl to form tyriverdin. Tyriverdin is light sensitive and on exposure to sunlight undergoes photolytic cleavage to produce Tyrian purple. Thus, the careful application of HCl to initiate the acid hydrolysis of tyrindoxyl sulfate is all that is required to produce Tyrian purple.

Overall, this thesis highlights the importance of using a combination of traditional and non-traditional bacterial culture techniques and modern genomic approaches to investigate symbiotic bacterial communities in marine invertebrates. The contributions of this research to the proposed model of Tyrian purple biosynthesis are represented in figure 6.1 (highlighted in red). Gene profiling can identify candidate bacteria with biosynthetic genes whereas culture work, along with chemical profiling using techniques such as liquid chromatography mass spectrometry analysis is required to identify bacterial symbionts with the capacity to biosynthesise marine secondary metabolites.

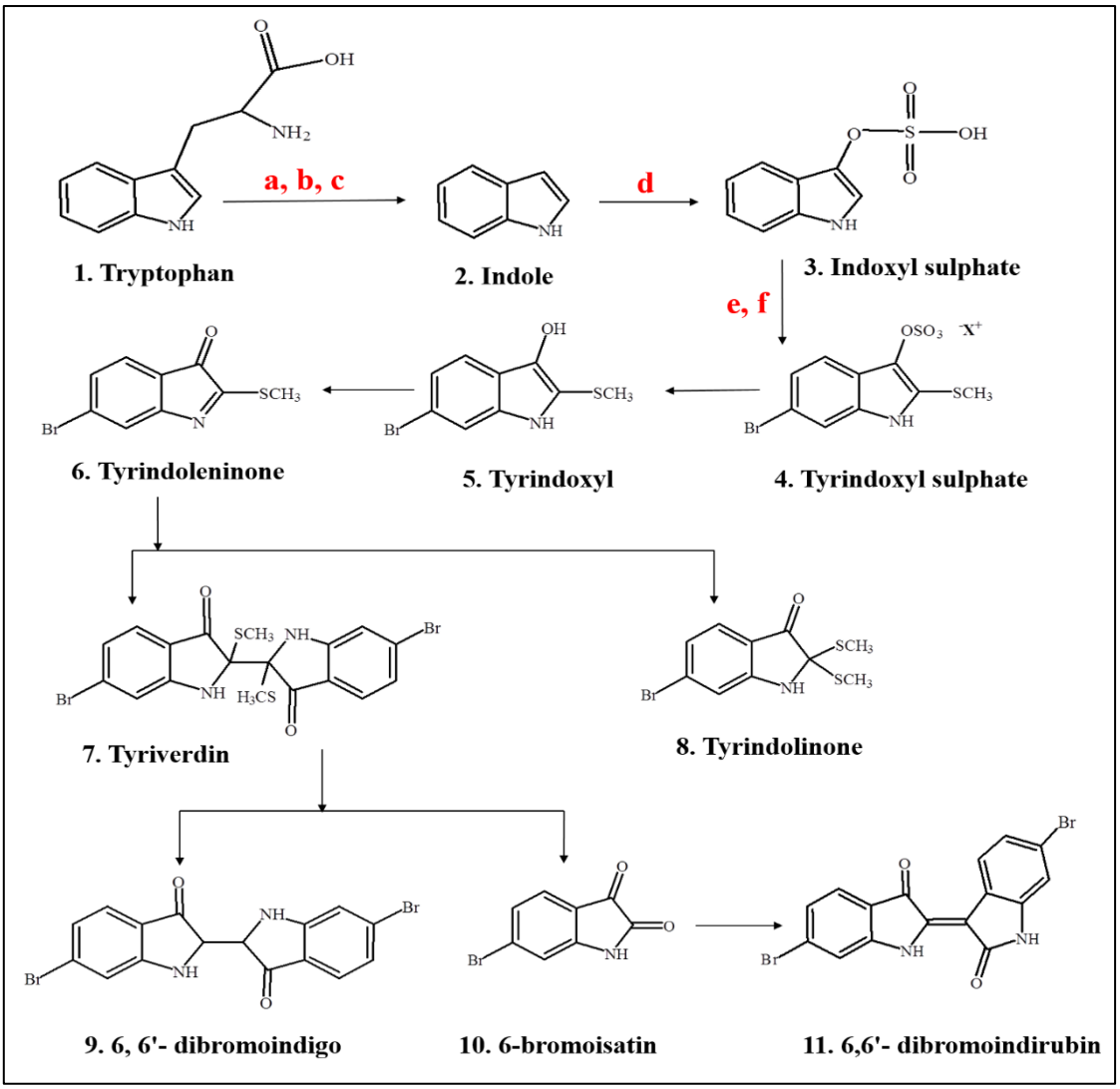

Fig. 6. 1. Proposed model of Tyrian purple biosynthesis after including the relevant enzymes/ genes and bacterial species associated with Tyrian purple biosynthesis identified in this thesis. **a**, *D. orbita* tryptophanase (tryptophan to indole); **b**, Tryptophan synthase *D. orbita* (tryptophan to indoles); **c**, *Vibrio parahaemolyticus, V. orientalis, V. mediterranei, V. fortis, V. campbellii, V. coralliilyticus, V. tasmaniensis, V. alginolyticus, V. metschnikovii, V. gigantis, V. harveyi, V. pomeroyi, V. splendidus* (source of indoles and indoxyl sulfate); **d**, *D. orbita* cytochrome P450 enzymes (indoxyl sulfate formation); **e,** *Bacillus thuringiensis* bromoperoxidase (bromination of indoxyl sulfate to produce brominated tyrindoxyl sulfate) and; **f**, *D. orbita* tyrosine aminotransferase (addition of methane thiol in the indole ring).

7. Appendix

Statement of Authorship Chapter 2 Publication

Title of Paper	Transcriptome of the Australian mollusc *Dicathais orbita* provides insights into the biosynthesis of indoles and choline esters
Publication Status	☑ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Baten*, A., Ngangbam*, A. K., Waters, D.L.E. and Benkendorff, K. Transcriptome of the Australian Mollusc *Dicathais orbita* provides insights into the biosynthesis of indoles and choline esters. *Mar. Drugs* 2016, 14, 135. * equal contributions

Principal Author

Name of Principal Author (Candidate)	Ajit Kumar Ngangbam		
Contribution to the Paper	Experimental design, sampling, dissections, RNA extraction and RNA quality check for transcriptome sequencing, data analysis and interpretation Prepared the first draft of the introduction, materials and methods sections 3.1, 3.2 and 3.4, and results and discussion KEGG pathway analysis sections 2.3, 2.4, 2.5 and 2.6 and Figures 1, 3 to 9 Overall contributions were equal to the primary author listed on the manuscript (Dr Abdul Baten)		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature. I am the primary author of this paper.		
Signature		Date	21/07/2016

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and

Name of Co-Author	Abdul Baten
Contribution to the Paper	Experimental design, assembled and annotated the transcriptome, data quality control and interpretation and editorial input

Signature		Date	27-07-2016

Name of Co-Author	Daniel L. E. Waters		
Contribution to the Paper	Experimental design and editorial feedback		
Signature		Date	27/07/2016

Name of Co-Author	Kirsten Benkendorff		
Contribution to the Paper	Initiated the study, experimental design, assistance with data analysis for KEGG biosynthetic pathways and feedback on draft manuscripts and responses to reviewers		
Signature		Date	26/07/2016

Statement of Authorship Chapter 3 Publication

Title of Paper	Indole producing bacteria from the biosynthetic organs of Muricid mollusc could contribute to Tyrian purple production
Publication Status	☑ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted w ork w ritten in manuscript style
Publication Details	Ngangbam, A. K., Waters, D. L. E., Whalan. S., Baten. A. and Benkendorff, K. 2015. Indole producing bacteria from the biosynthetic organs of Muricid mollusc could contribute to Tyrian purple production. *J Shellfish Res*. 2015; 34: 443-454.

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Statement of Authorship Chapter 4 Publication

Title of Paper	Characterization of bacterial communities associated with the Tyrian purple producing gland in a marine gastropod
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Statement of Authorship Chapter 5 Manuscript

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