**Bacterial communities associated with the Southern Ocean vent gastropod, *Gigantopelta chessoia*: indication of horizontal symbiont transfer**

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**Abstract**

Recently discovered hydrothermal vents of the East Scotia Ridge (ESR) in the Southern Ocean host unique faunal communities that depend on microbial chemosynthetic primary production. These highly abundant invertebrates gain energy from either grazing on free-living microbes or via hosting symbiotic chemoautotrophic microorganisms. T. The main objective of this study was to characterise microbes associated with a newly discovered species of hydrothermal vent gastropod and therefore increase knowledge of ecosystem functioning in this largely unknown Antarctic hydrothermal vent system. We investigated the phylogenetic composition of bacteria associated with the gills and oesophageal gland of the ESR peltospirid gastropod, Gigantopelta *chessoia* by molecular cloning and terminal restriction fragment length polymorphism (T-RFLP). 16S rRNA gene clone libraries revealed host tissue specific combinations of bacteria. The oesophageal gland contained one Gammaproteobacteria OTU whereas a more diverse community of Gamma, Epsilon and Deltaproteobacteria was isolated from the gills. T-RFLP analysis revealed that juvenile bacterial communities were more closely related to adult gillassociated bacterial communitiesthan oesophageal gland bacteria. Oespohageal gland Gammaproteobacteria exhibited a higher sequence similarity with sulphur oxidising bacteria isolated from cold seep sediments and with thioautotrophic endosymbionts than with bacteria found in the surrounding water column, suggesting that these endosymbionts were not acquired directly from the water column. Juvenile G. chessoia were located within the mantle cavity of adults and we speculate that Gammaproteobacterial endosymbionts in the oesophageal gland could be transmitted horizontally from adults to juveniles via the gills due to the close contact of juveniles with adults’ gills.

**Keywords:** Symbiont; Hydrothermal vent; gastropod; Antarctic; chemosynthesis; microbial diversity

**Introduction**

Deep-sea hydrothermal vent ecosystems are hotspots of biological productivity, hosting unique animal communities dependent on energy derivation from the oxidation of reduced compounds (e.g. sulphide, hydrogen and methane) by chemoautotrophic microbes, often in symbiosis (Cavanaugh et al. 1981, Dubilier et al. 2008). Recently discovered East Scotia Ridge (ESR) hydrothermal vents represent an independent vent biogeographic province (Rogers et al. 2012) that is dominated by endemic species like the yeti crab *Kiwa* t*yleri* Thatje 2015 (Thatje et al. 2015), the stalked barnacle *Vulcanolepas scotiaensis* Buckeridge & Linse 2013 (Buckeridge et al. 2013), seaspiders of the *Sericosura* spp. (Arango and Linse 2015), a lepetodrillid limpet and the peltospirid gastropod *Gigantopelta chessoia* Chen, Linse, Roterman, Copley and Rogers 2015 (Chen et al. 2015a; Marsh et al. 2012).

Endobionts in hydrothermal vent gastropods were first discovered in 1988 in the Western Pacific with abundant sulphur oxidation enzymes co-located with bacteria in specialized gill cells suggesting chemoautotrophy and symbiosis (Stein et al. 1988). Since this early study, a number of hydrothermal vent gastropod species have been found to contain bacterial endobionts in their gills, with some species containing only Gamma or Epsilonproteobacteria (Suzuki et al. 2005) whilst others contain both (Suzuki et al. 2006).

Research into gastropod endobionts has focused on phylogenetic analyses of bacteria located within the gills, however, one study identified an abundance of Gammaproteobacteria from one OTU contained within the oesophageal gland of the large-sized peltospirid ‘scaly-foot gastropod’ Chrysomallon squamiferum (Chen et al. 2015a) from the Central Indian Ridge (Chen et al. 2015b; Goffredi et al. 2004). In *C. squamiferum* the oesophageal gland is extremely enlarged (10-1000 times larger than other gastropods; Warén et al. 2003) and highly vascularised. The animal also has a hypertrophied circulatory system that is a possible adaptation to supply the host and its symbiotic bacteria with oxygen and sulphur (Chen et al. 2015b; Chen et al. 2015c). The genome of this Gammaproteobacterial endosymbiont has since been sequenced revealing very low genetic diversity, likely driven by high host selectivity during horizontal transmission (Nakagawa et al. 2014). Recently, a new genus of large-sized peltospirid gastropod, *Gigantopelta*, has been discovered from vents on the ESR and the South West Indian Ridge. They also have enlarged oesophageal glands, a characteristic hinting that these species may also host associated symbiotic bacteria (Chen et al. 2015a).

This study investigated the presence and phylogenetic composition of bacteria found in the gills and oesophageal gland of the ESR peltospirid gastropod, *G. chessoia* (Chen et al. 2015a), by molecular cloning and T-RFLP of 16S rRNA genes.

**Materials and Methods**

**Sample collection**

Samples were collected from active sites in the E2 hydrothermal vent field on the ESR in the Southern Ocean (Fig. 1) during the ChEsSO cruise JC80 in December 2012. *Gigantopelta chessoia* (Chen et al. 2015a) were collected using the remotely operated vehicle (ROV) Isis deployed from RRS James Cook. Whole adult gastropods were fixed in 96 % ethanol within 4 hours of collection and subsequently photographed. Gills and oesophageal glands were dissected from 12 adult *G. chessoia* (> 40 mm shell length) using sterile techniques and frozen at -80 °C within 12 hours of collection. Three juvenile gastropods (5-6 mm shell length) were also collected from within the mantle cavity of adult snails from the same site and frozen whole at -80 °C.

**DNA isolation and amplification for cloning**

DNA was isolated from between 10 and 20 mg of tissue from the gills and oesophageal gland of one host snail using the DNeasy blood and tissue kit (Qiagen) according to the manufacturer’s recommendations. Duplicate extractions were performed and the concentration and quality of extracted DNA was measured using a NanoDrop 2000 spectrophotometer, with yields of 131 to 451 ng.µL-1. 16S rRNA genes were amplified by PCR using the forward primer 27F (5’-AGA GTT TGA TCC TGG CTC AG-3’; Lane 1991) and the reverse primer 1492R (5’-GGY TAC CTT GTT ACG ACT T-3’; Lane 1991). Final reaction volumes of 25 µL contained 0.3 µM of each primer, 100-200 ng template DNA, 1 x Maxima buffer, 1.25 U Maxima Hot Start Taq DNA polymerase, 2 mM MgCl2 and 0.2 mM of each dNTP (all reagents from Thermo Fisher Scientific Inc.). PCR conditions were an initial denature of 4 min at 95 °C followed by 30 cycles of 30 s at 95 °C, 1 min 30 s at 55 °C and 1 min at 72 °C with a final extension period of 7 min at 72 °C.

**16S rRNA gene cloning**

16S rRNA genes isolated and amplified from the gills and oesophageal gland were cloned and transformed into TOP10 competent *Escherichia coli* cells using the TOPO TA cloning kit (Thermo Fisher Scientific Inc.) according to the manufacturer’s recommendations. Clones were grown on LB agar plates containing 50 µg.mL-1 kanamycin and 60 µg.mL-1 X-Gal for 18 hours at 37 °C. Colonies were picked and grown on in liquid LB medium containing kanamycin for 18 hours at 37 °C. Transformed *E. coli* were centrifugally separated and washed from media before being lysed by incubation for 10 min at 95 °C. Cloned inserts were amplified using M13f and M13r primers and the Thermo Maxima Hot Start kit as described above (Thermo Fisher Scientific Inc.). PCR conditions were an initial denature for 4 min at 95 °C followed by 25 cycles of 30 s at 95 °C, 30 s at 55 °C and 1 min 30 s at 72 °C followed by a final extension of 7 min at 72 °C.

Inserted 16S rRNA genes from 29 clones from the oesophageal gland and 46 clones from the gills were sequenced bidirectionally using Sanger sequencing (LGC Genomics, Berlin, Germany), curated and manually edited using MEGA version 6 (Tamura et al. 2013). The 16S rRNA gene sequence data reported here are available in the DDBJ/EMBL/GenBank databases under the accession numbers KU942528-KU942604. The mean final contig length was 970 base pairs. Maximum likelihood phylogenetic trees were constructed and tested with 1000 bootstrap replications in MEGA version 6 (Tamura et al. 2013). Shannon and Simpson diversity indices were calculated along with rarefaction analyses and Good’s coverage estimator using mothur version 1.38.1 (Schloss et al. 2009).

**Terminal Restriction Fragment Length Polymorphism (T-RFLP)**

Variability in community composition of bacteria in the gills and oesophageal glands of the 15 individuals was assessed using T-RFLP. Total DNA from individual gill and oesophageal gland tissues of 12 adults and from the total tissues of three 3 juveniles was extracted and purified using the Qiagen DNeasy kit as above. Due to low DNA concentrations obtained from the 3 juveniles, these DNA extracts were pooled into 1 sample for subsequent amplification. 16S rRNA genes were amplified from all 24 adult tissue DNA extracts and the 3 whole juvenile DNA extracts using a FAM labelled 27F forward primer and an unlabelled 907R reverse primer (5’-CCG TCA ATT CMT TTR AGT TT-3’; Lane 1991). Reaction volumes and composition were as described above with duplicate amplifications performed. PCR conditions were an initial denature of 4 min at 95 °C followed by 30 cycles of 30 s at 95 °C, 1 min at 55 °C and 30 s at 72 °C with a final extension period of 7 min at 72 °C.

A restriction endonuclease was selected using REPK (Collins and Rocap 2007) and cloned 16S rRNA gene sequences from this study along with 16S rRNA gene sequences obtained from water samples and associated with *Kiwa tyleri* from the same hydrothermal vent site (Zwirglmaier et al. 2015). Amplified 16S rDNA was digested using the CfoI restriction enzyme (Promega, Madison, WI, USA) for 3 h at 37 °C followed by a final denaturation period of 15 min at 65 °C according to the manufacturer’s recommendations. Fragment length analysis by capillary electrophoresis was performed by Eurofins MWG Operon, Ebersberg, Germany) and fragments were sized using the ROX1000 size standard and peak scanner software (Thermo Fisher Scientific Inc.). Fragment lengths were binned into 1 bp size groups and fragments constituting less than 1% of the total fluorescence in all samples were removed. Shannon diversity indices were calculated using the proportion of different fragments in each sample and the mean of the diversity indices were compared for fragments obtained from oesophageal gland and gill-associated bacteria. Correspondence analysis was also performed to compare all samples, gill bacteria and oesophageal gland bacteria, using R (R Core Team 2013). **Results**

Adult *G. chessoia* (Chen et al. 2015a) were found to reach up to 50 mm in shell diameter (Fig. 2). Juvenile *G. chessoia* between 5 and 6 mm in shell diameter were found within the mantle cavity of some adult snails, close to the adults’ gills, during dissection.

The majority of 16S rRNA clone sequences obtained from the gills and oesophageal gland were Gammaproteobacteria. Bacterial 16S rRNA gene clones from oesophageal gland DNA formed a single OTU which clustered with bacteria isolated from sediments at a methane seep (98 % sequence similarity in BLASTn) and thioautotrophic symbionts isolated from bivalve gills, tubeworms and the ‘scaly-foot 'gastropod *C. squamiferum* (96 %, 94 % and 93 % similarity respectively; Fig. 3a)*.* 16S rRNA gene clone sequences from this Gammaproteobacterial OTU exhibited only a maximum of 92 % similarity to clones from water samples obtained at the same E2 vent site. The same OTU dominated the gill DNA clone library (73 % of clones), however a greater bacterial diversity was associated with gill tissue, including Epsilonproteobacteria (25 % of clones) that cluster with epibionts of the ESR yeti crab *Kiwa tyleri* and bacteria cloned from water samples, both collected from the E2 vent field (> 95 % sequence similarity; Fig. 3b). A Deltaproteobacterial clone which clustered with symbionts of the gutless worm *Olavius algarvensis* was also isolated from the gills (Fig. 3c).

Shannon (H) and Simpson (D) diversity indices were 0 and 1 respectively for the oesophageal gland clone library at all sequence similarities calculated from 80 to 99 %. This lack of diversity is as expected due to only 1 OTU present. In contrast, a higher diversity was found in the gill clone library with Shannon diversity indices of between 0.9 and 1.0 for sequence similarities between 90 and 99 % and Simpson diversity indices of 0.5 for sequence similarities over 90 % (Fig. 4). As only one OTU was sequenced from the oesophageal gland clone library, rarefaction analysis indicated that all diversity was captured in the 29 clones sequenced (Fig. 5). In contrast the gradient of a rarefaction curve for the gill clone library decreased with increasing number of clones sampled, although the curve remained non-linear at the sampling of 48 clones, indicating that this clone library did not capture the full diversity of 16S rRNA sequences in the gill. The Good’s coverage estimator for gill-associated bacterial OTUs is 0.95 for sequence similarities of 97 % and 0.94 for sequence similarities of 99 %, indicating that the gill clone library captured 94 or 95 % of the OTUs defined at 97 or 99 % similarity. As shown in the rarefaction analysis, Good’s coverage of the oesophageal gland clone library showed that all of the diversity was captured by the clone library with a value of 1.0 for OTUs defined at a 99 % sequence similarity.

Bacterial community composition varied more between individual host snail gills than between host oesophageal glands as shown by correspondence analysis of T-RFLP data (Fig. 6a). The bacterial community of juvenile host snails was more closely related to the community associated with adult *G. chessoia* gills than with adult oesophageal glands (Fig. 6a) and was more closely related to Gammaproteobacteria clones than Epsilonproteobacteria clones isolated from adult gills (Fig. 6b). Higher diversities of terminal restriction fragments (TRFs) were obtained from gill-associated bacteria (mean H = 1.9, standard error of the mean = 0.1) compared to oesophageal gland-associated bacteria (mean H = 1.5, standard error of the mean = 0.1). Diversity of bacterial TRFs obtained from the pooled sample of juvenile snails is higher (H = 2.3) than diversities calculated from both of the adult tissue-associated bacterial TRFs.

**Discussion**

Since the discovery of chemoautotrophy in hydrothermal vent bacteria, a variety of metabolic pathways have been identified to convert inorganic carbon (C1 compounds) into organic carbon (C3 compounds) (Cavanaugh et al. 2006; Campbell and Cary 2004). Campbell et al. (2003) discovered citrate lyase genes in epibionts of polychaete worms (Alvinellidae), indicating that carbon is chemoautotrophically fixed via the reductive tricarboxylic acid (rTCA) cycle in these Epsilon-proteobacteria (Campbell and Cary 2004) and changing the previous theory that all carbon fixation by chemoautotrophs occurs via the Calvin Benson cycle (Felbeck et al. 1981).

Gammaproteobacteria and Epsilonproteobacteria endosymbionts have been found to co-occur in hydrothermal vent gastropods (*Alviniconcha*) which fix carbon via the Calvin-Benson cycle and rTCA cycle respectively (Stein et al. 1988). However, studies of stable isotope ratios in foot tissue from *G. chessoia* indicate that carbon is fixed primarily via the Calvin-Benson cycle using RuBisCO Form 1 (Reid et al. 2013). This is consistent with the dominance of Gammaproteobacteria in both the gills and the oesophageal gland clone library results presented here. These data indicate a symbiotic relationship between the gastropod and its hosted bacteria with the dominant chemoautotrophic Gammaproteobacteria providing energy for their host.

Juvenile *G. chessoia*-associated bacterial community composition exhibited greater similarity to communities associated with adult host gills and was more similar to gill Gammaproteobacteria than adult oesophageal gland bacterial communities and adult gill Epsilonproteobacteria, however it is important to note that only 3 juvenile snails were obtained and these were pooled into one sample. These initial data suggest that Gammaproteobacteria associated with the gills are transmitted vertically or acquired in early life whereas Epsilon and Deltaproteobacteria in the gills and Gammaproteobacteria in the oesophageal gland are acquired horizontally from the environment in later life although further analyses with a larger data set would be required to confirm this. Due to the location of juveniles within the mantle cavity and proximity to the gills of the adults, it is possible that juveniles acquire the Gammaproteobacteria from the adult gills in early life and transfer them via their gills to the oesophageal gland.

The gills are in direct contact with the surrounding hydrothermal fluid and free living bacteria whereas the oesophageal gland has no direct contact with the external environment and Gammaproteobacterial endosymbionts located there must be fully supported by the host. The presence of only one Gammaproteobacterial OTU in the oesophageal gland highlights the selectivity of this specialised, isolated environment over its associated bacteria. As the gill and oesophageal gland share a single dominant OTU, there could be transfer of bacteria between the two tissues, although this would require further life-history evidence to confirm. The Gammaproteobacteria may live as ectobionts on the gill (before being internalised in the oesophageal gland), enabling them to be ingested by juveniles. The low similarity of oesophageal gland Gammaproteobacteria endosymbionts to free living bacteria in the surrounding hydrothermal water suggests that these symbionts may not be acquired directly from the environment every host generation. However, it cannot be ruled out that these Gammaproteobacteria exist in very low concentrations in the water column, and were therefore not detected previously (Rogers et al. 2012). If this was the case, there would need to be a high specificity for selection in the gill tissue.

This study is the first characterisation of bacterial communities in the gills and oesophageal gland of the recently discovered G. chessoia and provides suggestions into the inter-generational transmission of symbionts between host individuals.

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**References**

Arango CP, Linse K (2015) New *Sericosura* (Pycnogonida: Ammotheidae) from deep-sea hydrothermal vents in the Southern Ocean. Zootaxa 3995:037-050. doi: 10.11646/zootaxa.3995.1.5

Buckeridge JS, Linse K, Jackson JA (2013) *Vulcanolepas scotiaensis* sp. Nov., a new deep-sea scalpelliform barnacle (Eolepadidae: Neolepadinae) from hydrothermal vents in the Scotia Sea, Antarctica. Zootaxa 3745:551-568. doi: 10.11646/zootaxa.3745.5.4

Campbell BJ, Cary SC (2004) Abundance of reverse tricarboxylic acid cycle genes in free-living microorganisms at deep sea hydrothermal vents. Appl Environ Microbiol 70(10):6282-6289.doi: 10.1128/AEM.70.10.6282-6289.2004

Campbell BJ, Stein JL, Cary SC (2003) Evidence of chemolithoautotrophy in the bacterial community associated with *Alvinella pompejana*, a hydrothermal vent polychaete. Appl Environ Microbiol 69(9):5070-5080.doi: 10.1128/AEM.69.9.5070-5078.2003

# Cavanaugh CM, Gardiner SL, Jones ML, Jannasch JW, Waterbury JB (1981) Prokaryotic cells in the hydrothermal vent tube worm *Riftia pachyptila* Jones: possible chemoautotrophic symbionts. Science 213:340–342. doi: 10.1126/science.213.4505.340

# Cavanaugh CM, McKiness ZP, Newton ILG, Stewart FJ (2006) Marine chemosynthetic symbioses. In Dworkin M et al (ed) The prokaryotes. A handbook on the biology of Bacteria. Volume 1: Symbiotic associations, biotechnology and applied microbiology, 3rd Ed. Springer, New York, USA, pp 475-507. doi: 10.1007/0-387-30741-9\_18

Chen C, Linse K, Roterman CN, Copley JT, Rogers AD (2015a) A new genus of large hydrothermal vent-endemic gastropod (Neomphalina: Peltospiridae). Zool J Linn Soc Lond 175:319-335. doi: 10.1111/zoj.12279

Chen C, Linse K, Copley JT, Rogers AD (2015b) The ‘scaly-foot gastropod’: a new genus and species of hydrothermal vent-endemic gastropod (Neomphalina: Peltospiridae) from the Indian Ocean. J Mollus Stud 81:322-334. doi: 10.1093/mollus/eyv013

Chen C, Copley JT, Linse K, Rogers AD, Sigwart JD (2015c**)** The heart of a dragon: 3D anatomical reconstruction of the ‘scaly-foot gastropod’ (Mollusca: Gastropoda: Neomphalina) reveals its extraordinary circulatory system. Front Zool 12:13. doi: 10.1186/s12983-015-0105-1

Collins RE, Rocap G (2007) REPK: an analytical web server to select restriction endonucleases for terminal restriction fragment length polymorphism analysis. Nucleic Acids Res. 35:W58-W62. doi: 10.1093/nar/gkm384

# Dubilier N, Bergin C, Lott C (2008) Symbiotic diversity in marine animals: the art of harnessing chemosynthesis. Nat Rev Microbiol 6:725-740. doi: 10.1038/nrmicro1992

# Felbeck H, Childress JJ, Somero GN (1981) Calvin-Benson cycle and sulphide oxidation enzymes in animals from sulphide-rich habitats. Nature 293:291-293. doi: 10.1038/293291a0

Goffredi SK, Waren A, Orphan VJ, Van Dover CL, Vrijenhoek RC (2004) Novel forms of structural integration between microbes and a hydrothermal vent gastropod from the Indian Ocean. Appl Environ Microbiol 70(5):3082-3090. doi: 10.1128/AEM.70.5.3082-3090.2004

Lane DJ (1991) 16S/23S rRNA sequencing. In: Stackebrandt E and Goodfellow M (eds) Nucleic acid techniques in bacterial systematics. John Wiley and Sons, New York, pp 115-175

Marsh L, Copley JT, Huvenne VAI, Linse K, Reid WDK, Rogers AD, Sweeting CJ, Tyler PA (2012) Microdistribution of faunal assemblages at deep-sea hydrothermal vents in the Southern Ocean. PLoSOne 7(10):e48348. doi:10.1371.journal.pone.0048348

Nakagawa S, Shimamura S, Takaki Y, Suzuki Y, Murakami S, Watanabe T, Fujiyoshi S, Mino S, Sawabe T, Maeda T, Makita H, Nemoto S, Nishimura S, Watanabe H, Watsuji T, Takai K (2014) Allying with armored snails: the complete genome of gammaproteobacterial endosymbiont. ISME J 8:40-51. doi: 10.1038/ismej.2013.131

R Core Team (2013). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0

Reid WDK, Sweeting CJ, Wigham BD, Zwirglmaier K, Hawkes, JA, McGill RAR, Linse K, Polunin NVC (2013) Spatial differences in East Scotia Ridge hydrothermal vent food webs: influences of chemistry, microbiology and predation on trophodynamics. PLoS One 9(6):e65553. doi: 10.1371.journal.pone.006553

# Rogers AD, Tyler PA, Connelly DP, Copley JT, James R, Later RD, Linse K, Mills RA, Garabato AN, Pancost RD, Pearce DA, Polunin NV, German CR, Shank T, Boersch-Supan PH, Alker BJ, Aquilina A, Bennett SA, Clarke A, Dinley RJ, Graham AG, Green DR, Hawkes JA, Hepburn L, Hilario A, Huvenne VA, Marsh L, Ramirez-Llodra E, Reid WD, Roterman CN, Sweeting CJ, Thatje S, Zwirglmaier K (2012) The discovery of new deep-sea hydrothermal vent communities in the Southern Ocean and implications for biogeography. PLOS Biol 10(1):e1001234. doi: 10.1371/journal.pbio.1001234

# Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, Lesniewski RA, Oakley BB, Parks DH, Robinson CJ, Sahl JW, Stres B, Thallinger GG, Van Horn DJ, Weber CF (2009) Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl Environ Microbiol 75(23):7537-7541. doi: 10.1128/AEM.01541-09

Stein JL, Cary C, Hessler RR, Ohta S, Vetter RD, Childress JJ, Felbeck H (1988) Chemoautotrophic symbiosis in a hydrothermal vent gastropod. Biol Bull 174:373-378. doi:10.2307/1541963

Suzuki Y, Sasaki T, Susuki M, Nogi Y, Miwa T, Takai K, Nealson KH, Horikoshi K (2005) Novel chemoautotrophic endosymbiosis between a member of the Epsilonproteobacteria and the hydrothermal-vent gastropod *Alviniconcha* aff. *hessleri* (Gastropoda: Provannidae) from the Indian Ocean. Appl Environ Microbiol 71(9):5440–5450. doi: 10.1128/AEM.71.9.5440-5450.2005

# Suzuki Y, Kojima S, Sasaki T, Suzuki M, Utsumi T, Watanabe H, Urakawa H, Tsuchida S, Nunoura T, Hirayama H, Takai K, Nealson KH, Horikoshi K (2006) Host-symbiont relationships in hydrothermal vent gastropods of the genus Alviniconcha from the Southwest Pacific. Appl Environ Microbiol 72(2):1388–1393. doi: 10.1128/ AEM.72.2.1388-1393.2006

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013) MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol Biol Evol 30:2725-2729. doi: 10.1093/molbev/mst197

Thatje S, Marsh L, Roterman CN et al (2015) Adaptations to hydrothermal vent life in *Kiwa tyleri*, a new species of yeti crab from the East Scotia Ridge, Antarctica. PLoS ONE 10(6):e0127621. doi: 10.1371/journal.pone.0127621

Warén A, Bengtson S, Goffredi, SK, Van Dover CL (2003) A hot-vent gastropod with iron sulfide dermal sclerites. Science 302:1007. doi: 10.1126/science.1087696

# Zwirglmaier K, Reid W, Heywood J, Sweeting CJ, Wigham BD, Polunin NV, Hawkes JA, Connelly DP, Pearce D, Linse K (2015) Linking regional variation of epibiotic bacterial diversity and trophic ecology in a new species of Kiwaidae (Decapoda, Anomura) from East Scotia Ridge (Antarctica) hydrothermal vents. Microbiol Open 4(1):136-150. doi: 10.1002/mbo3.227

**Fig. 1 Map showing location of E2 hydrothermal vent field**

**Fig. 2** Photograph of an adult *Gigantopelta chessoia*. The operculum (op), foot (fo) and mantle (ma) are labelled

**Fig. 3** Maximum Likelihood phylogenetic tree of cloned 16S rRNA gene partial sequences of Gamma (**a),** Epsilon (**b**) and Deltaproteobacteria (**c**) isolated from the gills (clones A19-1 to A19-48) and oesophageal gland (clones A19-49 to A19-77) of Gigantopelta chessoia. Clone sequences isolated from samples in the water column at E2 (Rogers et al. 2012) and from Kiwa tyleri (Thatje et al. 2015) also at the E2 vent site (Zwirglmaier et al. 2015) are included in addition to similar sequences from GenBank identified by BLASTn. Accession numbers are shown for all sequences. Scale bars represent the average number of nucleotide changes per site. Numbers represent bootstrap values based on 1000 permutations. Classification to the Order level is shown where possible.

**Fig. 4** Shannon and Simpson diversity indices for the gill clone library as a function of the sequence similarity threshold to define an operational taxonomic unit (OTU)

**Fig. 5** Rarefaction curves for gill and gland clone libraries calculated for OTUs delineated at 99 % sequence similarity

**Fig. 6** Correspondence analysis of 16S rDNA fragment data from T-RFLP of (**a**) all snail gills and glands in addition to a pooled sample of 3 whole juvenile snails and (**b**) selected Gamma and Epsilonproteobacteria clones from snail gills compared to the T-RFLP signature of 3 combined juvenile snails

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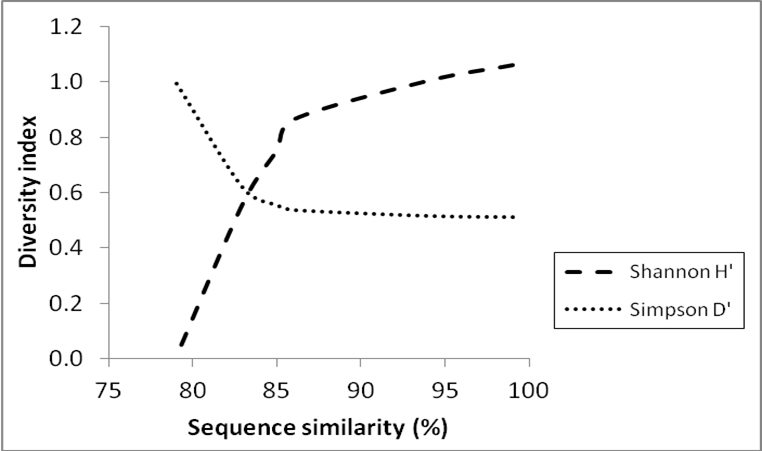
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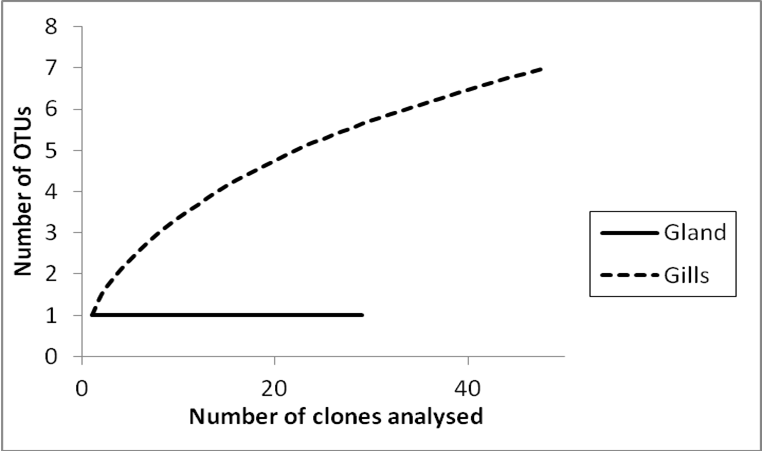
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**C:\7 - Journals & Paper\4 - Publications in review and press\Heywood et al Gigantopelta microbiology\Heywood symbionts revision\Fig3a_1.tifFig. 6** Correspondence analysis of 16S rDNA fragment data from T-RFLP of (**a**) all snail gills and glands in addition to a pooled sample of 3 whole juvenile snails and (**b**) selected Gamma and Epsilonproteobacteria clones from snail gills compared to the T-RFLP signature of 3 combined juvenile snails