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ORIGINAL PAPER

Microbial communities of the carapace, gut, and hemolymph of the Atlantic blue crab, *Callinectes sapidus*

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Abstract The Atlantic blue crab *Callinectes sapidus* is an important fisheries resource that is subject to mortality and morbidity from hemolymph infections. We used culture-independent methods based on the analysis of 16S rRNA genes to characterize and quantify the microflora from the carapace, gut, and hemolymph of *C. sapidus* with the goals of (1) characterizing the *C. sapidus* microbial assemblage and (2) identifying the reservoirs of potential pathogens associated with the crab. We found that the carapace, gut and hemolymph microflora have a core Proteobacteria community with contributions from other phyla including Bacteroidetes, Firmicutes, Spirochetes, and Tenericutes. Within this Proteobacteria core, γ-Proteobacteria, including the members of the Vibrionaceae that are closely related to potential pathogens, dominate. Bacteria closely related to hemolymph pathogens were found on the carapace, supporting the hypothesis that punctures, molting damage, or broken dactyls may be routes for hemolymph infections. These results provide some of the first data on the blue crab microbial assemblage obtained with culture-independent techniques and offer insights into the routes of infection and potential bacterial pathogens associated with blue crabs.

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Introduction

The Atlantic blue crab *Callinectes sapidus* is an important marine resource (Phillips and Peeler 1972). As such, diseases of blue crabs are of commercial importance and factors that affect either crab health or the risk to humans of handling and consuming blue crabs are of interest. Previous culture-based studies have identified potential pathogens including *Vibrio cholerae*, *Vibrio parahaemolyticus*, and *Vibrio vulnificus* (Sizemore et al. 1975; Davis and Sizemore 1982; Welsh and Sizemore 1985) associated with *C. sapidus* (Krantz et al. 1969). These bacteria have been found within gills, in viscera, in processed meat taken from healthy crabs, and within the hemolymph of diseased crabs held in commercial tanks (Tubiash et al. 1975).

Many *Vibrio* spp. are commensals, but some species can be opportunistic pathogens that impact host physiology and health (Austin and Zhang 2006). Similar bacteria species have been isolated from the carapace of healthy crabs and from those with erosive lesions and shell disease (Noga et al. 2000). The gut microflora community directly affects digestion, growth, and nutrition (Harris 1993). Furthermore, sublethal levels of bacterial infection have been shown to alter the metabolism and resistance to fatigue of shrimp and crabs (Burnett et al. 2006; Scholnick et al. 2006; Thibodeaux et al. 2009; Roman et al. unpublished data), which consequently may impact their reproduction and survival.

The presence of these pathogens is also of concern for human health as pathogenic *Vibrio* spp. in crab meat have been implicated in incidents of foodborne illness (CDC

1971, 1976, 1999; Molenda et al. 1972), and *V. vulnificus* has been linked to septicemia cases in humans handling and ingesting crabs (Blake et al. 1979).

Based on studies of vertebrates, it has been assumed that hemolymphs of healthy invertebrates are sterile. However, culture-based studies have indicated that healthy *C. sapidus* naturally harbor low populations of bacteria in their hemolymphs that may be capable of causing infections (Davis and Sizemore 1982; Welsh and Sizemore 1985). Counts of bacteria in the hemolymph are higher in crabs that are missing appendages or that have been injured or stressed during capture and holding (Tubiash et al. 1975; Welsh and Sizemore 1985). The abundance of bacterial cells in samples of hemolymph fluids from infected crabs varies widely, from 1.8×10^3 to 6.7×10^5 CFU mL^{-1} (Welsh and Sizemore 1985; Davis and Sizemore 1982). Davis and Sizemore (1982) evaluated 81 crabs and divided the crab population into four categories based on the level of bacterial infection. Ten percent had light hemolymph infections ($<10^3$ CFU mL^{-1}), 52 % had moderate infections (10^3–10^5 CFU mL^{-1}), 25 % had heavy infections ($>10^5$ CFU mL^{-1}), and only 12 % were found to have sterile hemolymphs. A study of bacteria associated with freshly captured *Cancer magister* (Dungeness crabs) also reported low levels of bacteria in hemolymph fluid ($<10^2$ CFU mL^{-1}; Faghri et al. 1984).

The relative abundance of *Vibrio* spp. in crab hemolymph bacterial populations is also highly variable. Monthly mean relative abundance of *Vibrio* spp. in *C. sapidus* hemolymph fluids ranged from 6 to 64 % of total CFU (Welsh and Sizemore 1985). Incidence of *Vibrio* spp. within the hemolymph was higher in crabs subjected to commercial handling, in crabs from warmer water, and in those that already had hemolymph infections (defined as $>10^2$ CFU mL^{-1}; Welsh and Sizemore 1985). Sizemore et al. (1975) reported an average of 21 % *V. parahaemolyticus* within the hemolymph bacterial community of crabs in Chesapeake Bay during the months of May, June, and July when the concentrations of this bacterium increased in the water. Another study using crabs from Galveston Bay, Texas, reported *V. parahaemolyticus* in 23 % of hemolymph samples. *V. cholerae* and *V. vulnificus* were detected at lower incidences: 2 and 7 %, respectively (Davis and Sizemore 1982). In addition to *Vibrio* spp., these studies documented the presence of *Pseudomonas* sp., *Acinetobacter* sp., *Aeromonas* sp., *Bacillus* sp., *Flavobacterium* sp. (Sizemore et al. 1975), and *Photobacterium* sp. (Gomez-Gil et al. 2010) in crab hemolymphs. Thus, the presence of bacteria in the hemolymph of crustaceans may be a natural occurrence, or colonization, and not an infection (Gomez-Gil et al. 1998).

These culture-based studies have provided valuable insights into the composition of crab-associated microbial communities and have yielded isolates for detailed physiological investigation. However, culture-based methods are known to provide biased assessments of the microbial community, as typically <1 % of the cells known to be present by direct microscopic enumeration produces colonies on solid media (Ferguson et al. 1984; Head et al. 1998). This bias also applies to *Vibrio* species (Thompson et al. 2004), for example, leading to questions of the role of these "viable but non-culturable" bacteria in the epidemiology of cholera outbreaks (Huq et al. 1990). One goal of the present study was to characterize and quantify the microbial assemblage associated with *C. sapidus* using culture-independent techniques. A second goal was to use this information to evaluate the potential for bacteria associated with the carapace or the gut to serve as inocula for hemolymph infections.

Methods

Sample collection and DNA extraction

Male *C. sapidus* ($n = 7$; wet weight range = 78.5–207 g) were caught in a crab pot (Crabs 1; 4–7) and by trawl (Crabs 2 and 3) in Charleston Harbor, South Carolina, during June 2010. The crabs were examined and injuries were noted and categorized as old versus new based on their appearance. Crabs were banded, weighed, measured, and placed in individual holding tanks of well-aerated, static seawater (30 psu; 24–26 °C), and then sampled after being held in quarantine for 24 h. Hemolymph samples were taken by sterilizing the carapace around the pericardial sinus with betadine (povidone-iodine solution USP, 10 %) and isopropanol and then inserting a 23-gauge needle attached to a 1-mL syringe through the carapace into the pericardial sinus to collect 500 µL of hemolymph. Hemolymph samples were placed in PowerBead tubes (MoBio Laboratories), immediately vortexed, and placed on ice. Sterile swabs of ~ 6 cm^2 of the carapace and 10 mm^2 clips of the carapace were collected, put in PowerBead tubes, and processed as above. The same region was swabbed and clipped for all samples. Once the carapace was removed, the gut (mid to hindgut) was excised aseptically and placed in PowerBead tubes and processed as above. DNA extractions were completed using the MoBio PowerSoil DNA Extraction Kit as per kit instructions.

Sequencing and sequence analysis

DNA was amplified using Illustra puReTaq Ready-To-Go PCR Beads (GE Healthcare) with the bacteria-specific 16S rRNA primers 27F/1492R (Table 1; Lane 1991) with the

Table 1 Primers used in this study

Primers	Gene	Use	Primer sequences (5′–3′)	Reference
27F	16S rRNA	Sequencing	AGAGTTTGATCMTGGCTCAG	(Lane 1991)
1492R	16S rRNA	Sequencing	GGTTACCTTGTTACGACTT	(Lane 1991)
BACT1369F	16S rRNA	qPCR	CGGTGAATACGTTCYCGG	(Suzuki et al. 2000)
PROK1492R	16S rRNA	qPCR	GGWTACCTTGTTACGACTT	(Suzuki et al. 2000)

following PCR conditions: initial denaturation at 95 °C for 5 min; 35 cycles of denaturation at 95 °C for 45 s, annealing at 62 °C for 30 s, and extension at 72 °C for 1 min; and finishing with a final extension at 72 °C for 45 min. Amplified DNA was electrophoresed on a 1 % agarose gel, bands of the expected product size were excised, then the DNA in them was extracted and purified using QIAGEN QIAquick gel extraction kits. DNA extracted from the gel was cloned with TOPO TA cloning kits (Invitrogen) using the pCR 4.0-TOPO TA vector and chemically competent *E. coli* TOP10 cells. Clones were selected randomly and sequenced using the 27F primer by Georgia Genomics (Athens, Georgia) or Genewiz (South Plainfield, New Jersey). We used Sanger sequencing to obtain longer sequences and thus increased phylogenetic resolution. All sequences were checked for chimeras using the Bellerophon server (Huber et al. 2004). Taxonomic identities were assigned to each sequence using both RDP SeqMatch (Cole et al. 2007, 2009) and BLAST searches against the nonredundant nucleotide database (NCBI GenBank) and then grouped phylogenetically. Sequences were assigned to a genus if there was >95 % similarity (Tindall et al. 2010) and to a species if there was >97 % similarity to the reference sequence (Stackebrandt and Goebel 1994; Tindall et al. 2010).

Of a total of 846 sequences (combined libraries for gut, carapace clip, carapace swab, and hemolymph samples), 26 sequences (~3 %) were discarded because they were poor quality or chimeric. A total of 239 sequences (415–1,118 bp; median = 992) was retrieved from gut libraries; 201 sequences (366–1,242 bp; median = 827) from the carapace clip libraries; 189 sequences (693–1,129 bp; median = 973) from the carapace swab libraries; and 193 sequences (362–1,267 bp; median = 927) from the hemolymph libraries. Sequences identified as cyanobacteria or chloroplasts contributed 27, 41, 0.84, and 4.2 % to the 16S rRNA clone libraries from the carapace clip, carapace swab, gut, and hemolymph communities, respectively. These sequences were excluded from further analysis. Sequences are available from NCBI GenBank under accession numbers KC917584–KC918339.

Quantitative PCR

Quantitative PCR (qPCR) used a BioRad iCycler and the primers given in Table 1. qPCR cycling conditions followed those published in Buchan et al. (2009). qPCRs were run in 25 μL with 1× iQ SYBR Green Supermix (BioRad Laboratories), forward and reverse primers, nuclease free water, and 3 μL of template DNA. All reactions were run in triplicate with standards ranging from 10^1 to 10^7 copies per μL^{-1} (Kalanetra et al. 2009). Because there was no robust way to normalize across the different sample types used in this study, we took care to be consistent from crab to crab in our sampling and extraction protocols and qPCR data are reported as copies of 16S rRNA genes mL^{-1} of final, purified template DNA extract.

Statistical analysis

The software package PRIMER (v.6; Clarke and Gorley 2006) was used for nonmetric multidimensional scaling analysis (NMDS) of ribotype distributions and to compare the composition of clone libraries from crab samples at both phylum and genus levels of phylogenetic discrimination. Multiresponse permutation procedures (MRPP) were performed in R (R Core Team 2009) using the vegan statistical package (Oksanen et al. 2009) to test whether there was a significant difference between clustered groups. MRPP was run with the Bray–Curtis distance matrix with 999 permutations.

Results

Crab condition

During pre-quarantine physical inspection, Crab 1 (C1) was found to be the missing part of the tip of a cheliped, Crab 2 (C2) and Crab 4 (C4) were both missing an entire cheliped, and Crab 4 (C4) also had extensive algal growth on its carapace. All injuries appeared to have occurred prior to capture and had healed externally. At the time of the initial examination, Crab 3 (C3) appeared physically healthy with no injuries; however, after 24 h in quarantine, C3 became extremely lethargic and moribund. All appendages were intact on the rest of the specimens and they were outwardly healthy in appearance in both pre- and post-quarantine.

Gut community

As evident in Fig. 1a, the composition of the gut microflora varied among crabs. We detected a total of 8 different phyla in the bacteria sequences retrieved from gut samples. Forty-seven percent of these ribotypes was assigned to the phylum Proteobacteria, which was the most frequently encountered taxon. Ribotypes assigned to the phyla Spirochetes, Bacteroidetes, Fusobacteria, and Firmicutes were found in most gut samples with relative abundances (all samples) of 10–12 %. The Fusobacteria sequences retrieved from specimens C1, C2, C5, and C7 were >97 % similar to *Propionigenum maris* (Y16800). C1, C3, and C6 all contained sequences most closely related to the phylum Tenericutes, which were 90–93 % similar to uncultured Mycoplasmataceae (EU646198).

γ-Proteobacteria were the most abundant class of Proteobacteria, accounting for 71 % of all Proteobacteria sequences retrieved from gut samples (Fig. 2a). Within the γ-Proteobacteria, 46 % were most closely related to *Photobacterium* spp., 26 % to *Marinobacter* sp., 23 % to *Vibrio* spp., 2.5 % to *Escherichia* spp., and 2.5 % to *Thalassomonas* sp. (Fig. 2b). The *Photobacterium* spp. clones could be further assigned at >97 % sequence similarity to either *Photobacterium damselae* subsp. *damselae* (AB032014, DQ005198) or *P. damselae* subsp. *piscida* (AY147859). Some of the *Vibrio* spp. clones could be further assigned at >97 % sequence similarity to *Vibrio harveyi* (JN183120, HQ439528) and *Vibrio xuii* (JN183156). ε-Proteobacteria were also important, contributing 27 % of the Proteobacteria community. All of the ε-Proteobacteria sequences were >97 % similar to *Arcobacter* sp. (FJ573217, HE565357).

Carapace community

Proteobacteria dominated the microbial assemblage found in carapace clip samples, accounting for 59 % of all bacteria sequences retrieved (Fig. 1b). Carapace clip samples included bacteria from the exterior of the carapace as well as those living within the carapace matrix. As with the gut samples, carapace clip libraries from crabs C3 and C6 both contained sequences representative of the Tenericutes (Mycoplasmataceae). The Proteobacteria were represented by γ-(54 %) and α-Proteobacteria (43 %) (Fig. 2a). Most of the α-Proteobacteria sequences were >97 % similar either to *Erythrobacter* sp. or to members of the family Rhodobacteraceae (*Oceanicola* sp., *Roseobacter* sp.,

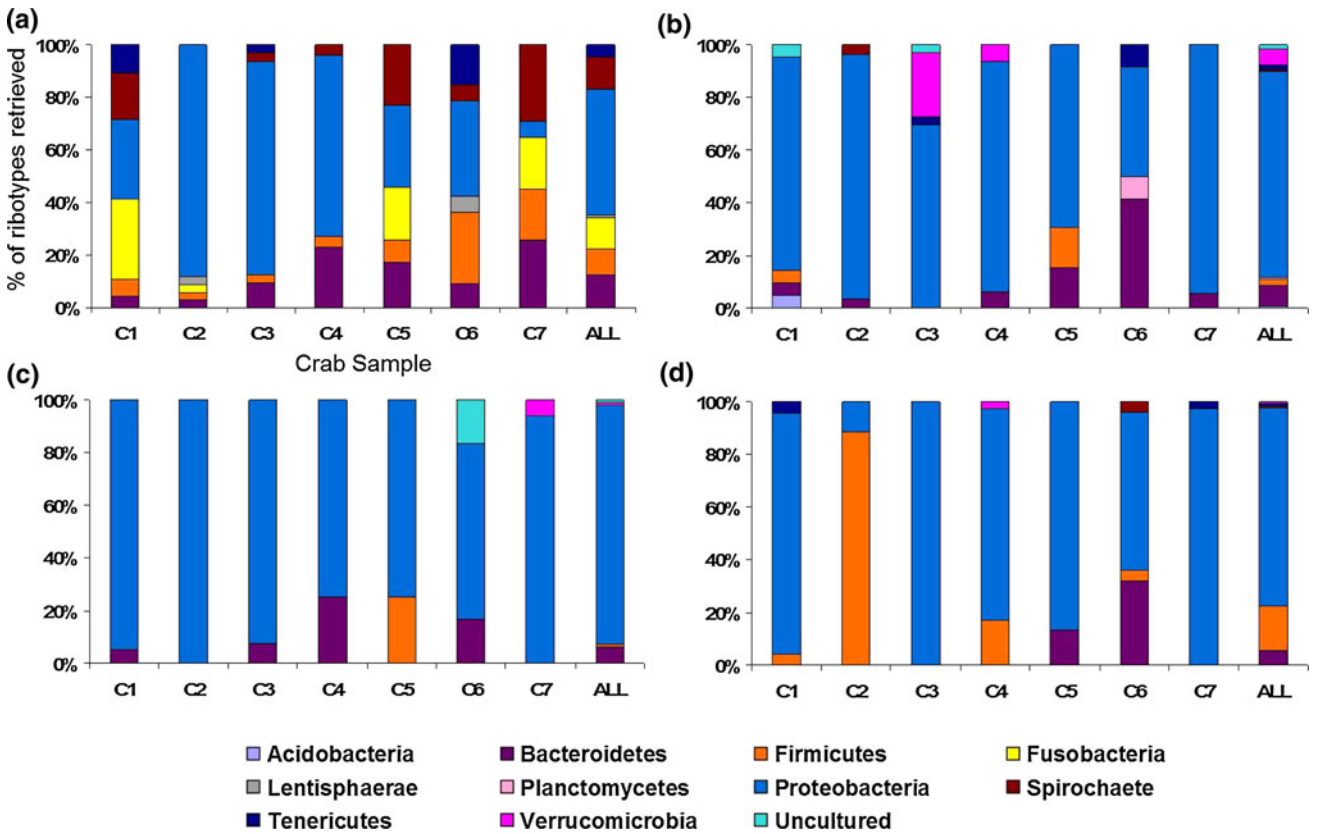

Fig. 1 Phyla (% of bacterial ribotypes) detected in **a** gut; **b** carapace clip; **c** carapace swab; and **d** hemolymph libraries. *C1–C7* refer to the seven different crabs that were sampled; *ALL* corresponds to combined data from all crabs sampled

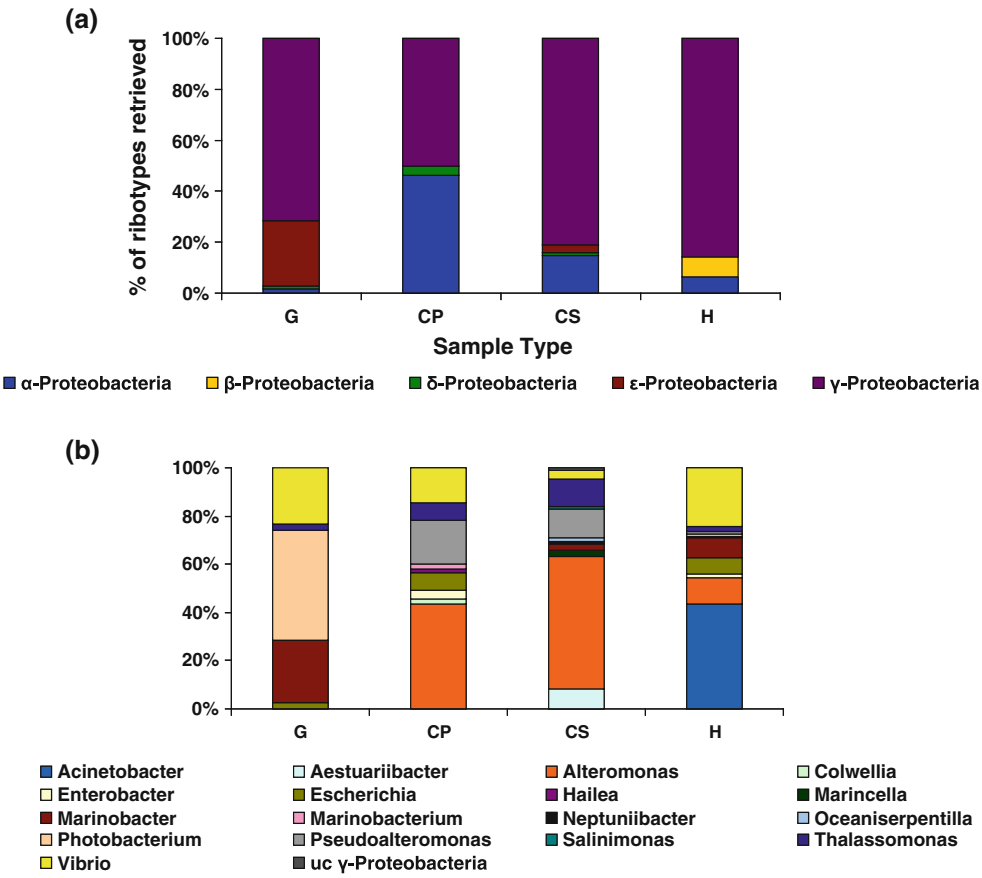

Fig. 2 Contribution of **a** all Proteobacteria; and **b** γ-Proteobacteria ribotypes to bacteria sequences retrieved from gut (*G*), carapace clip (*CP*), carapace swab (*CS*), and hemolymph (*H*) samples. Data from all seven crabs are combined for each sample type

Roseovarius sp., and *Ruegeria* sp.). Thirty-seven percent of the γ-Proteobacteria sequences were most similar to *Alteromonas* sp., 18 % to *Pseudoalteromonas* sp., and 14 % to *Vibrio* spp. Of the *Vibrio* spp. sequences, 25 % were most similar to *V. harveyi* (EU251517) (Fig. 2b). The bacterial ribotypes found in the carapace swab samples (Fig. 1c) were similar to those reported for the carapace clip, with 55 % of the ribotypes identified as Proteobacteria. The Proteobacteria in these samples were comprised of 81 % γ-Proteobacteria and 15 % α-Proteobacteria (Fig. 2a). Fifty-four percent of the γ-Proteobacteria ribotypes were most similar to *Alteromonas* sp. with additional smaller contributions from *Pseudoalteromonas* sp. (12 %), *Thalassomonas* sp. (10 %), and *Vibrio* spp. (3 %) (Fig. 2b).

Hemolymph community

Seventy-two percent of the bacteria sequences retrieved from hemolymph samples belonged to the phylum Proteobacteria (Fig. 1d). Ribotypes associated with the Proteobacteria almost completely dominated the hemolymph assemblages of all crabs except C2 and C6. The library from crab C2 contained primarily Firmicutes (85 % *Bacillus* sp.) with only a small contribution from Proteobacteria. In contrast, ribotypes retrieved from the hemolymph of crab C6 were a combination of Proteobacteria (60 %) and Bacteroidetes (32 %). A few (∼3 %) Tenericutes (Mycoplasmataceae) were found in hemolymph samples from crabs C1 and C7, and Mycoplasmataceae ribotypes accounted for 10 % of the ribotypes retrieved from the gut of crab C1. However, no Tenericutes were found in the hemolymph samples of crabs C3 or C6, despite the presence of Tenericutes ribotypes in both gut and carapace clip samples from these crabs. The Proteobacteria assemblage in these hemolymph samples was comprised of 86 % γ-Proteobacteria, 7.8 % β-Proteobacteria, and 6.5 % α-Proteobacteria (Fig. 2a). Most of the γ-Proteobacteria were *Acinetobacter* sp. (43 %), *Vibrio* spp. (24 %), and *Alteromonas* sp. (10 %) (Fig. 2b). The *Acinetobacter* sp. sequences were >97 % similar to *A. junii* (FJ447529, JX490076). Most of the *Vibrio* spp. ribotypes, including all of those from crab C3, were >97 % similar to *V. harveyi* (EU251517).

Statistical analysis

We compared the composition of libraries from our samples using NMDS. When we compared composition at the level of bacterial phylum (Fig. 3), all gut samples clustered

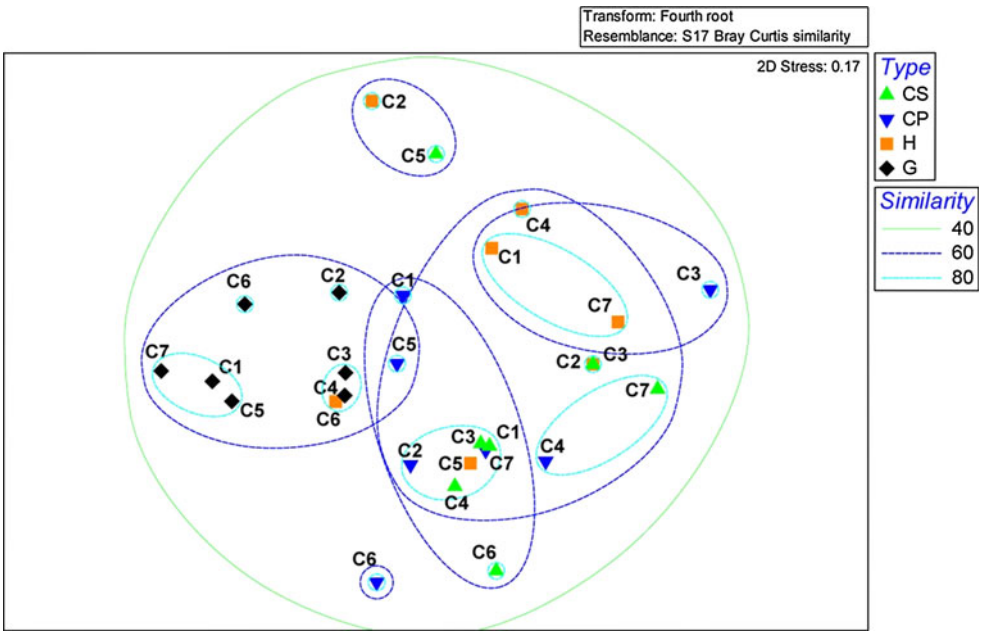

Fig. 3 Nonmetric multidimensional scaling analysis of the distribution of bacterial phyla found in carapace clip (*CP*), carapace swab (*CS*), gut (*G*), and hemolymph (*H*) samples. Samples from Crabs 1–7 (*C1–C7*) are displayed in a two-dimensional space and clustered according to the similarity of the Bacterial assemblages they contain. Note that in many instances, the 80 % similarity cutoff only included one sample. The *C2*/*CS* point overlaps that of the *C3*/*H* sample. Samples not present in the plot were below the 40 % similarity cutoff. MRPP analysis indicates that clusters defined at 60 and 80 % similarity are significantly different ($p = 0.002$ and $p = 0.001$, respectively)

together and were at least 60 % similar to each other. Most of the carapace clip and carapace swab samples also clustered together with at least 60 % similarity. The libraries from C5 hemolymph, C2 and C7 carapace clip, and C1, C3, and C4 carapace swab were dominated (>75 %) by Proteobacteria with contributions from Bacteroidetes (<25 %) and clustered together with 80 % similarity at the phylum level. The carapace clip from C6 was the only sample with similar contributions (~42 %) of Bacteroidetes and Proteobacteria and thus did not cluster with any of the other samples. MRPP indicates that clusters defined at 60 and 80 % similarity are significantly different from each other ($p = 0.002$ and $p = 0.001$, respectively).

We also compared the composition of γ-Proteobacteria ribotypes in the libraries from our samples (Fig. 4). This analysis showed that libraries from hemolymph samples are distinct from those obtained from carapace and gut samples of the same crab. Hemolymph samples of crabs C3 and C4 clustered with either gut or carapace samples of other crabs, but not with the gut or carapace samples from C3 or C4. Carapace clip and carapace swab samples from crabs C1, C2, and C3 clustered together with at least 60 % similarity. These samples all contained elevated abundances of *Alteromonas* sp. ribotypes. Gut samples from crabs C1, C4, C5, and C6 all had higher incidence of both *Photobacterium* sp. and *Vibrio* spp. than other crabs and clustered together with 60 % similarity. The hemolymph sample from crab C3 was dominated by *Vibrio* spp. related to *V. harveyi* and clustered with the gut samples from C1, C4, C5, and C6 with 40 % similarity. MRPP indicates that clusters separated at the 20, 40, 60, and 80 % similarity level were all significantly different from each other at $p = 0.001$.

qPCR analysis

All gut samples had similar bacterial abundances, ranging between 2.1×10^8 and 4.3×10^9 copies of 16S rRNA genes mL^{-1} of template (Fig. 5). Abundances in carapace swab samples were between 3.8×10^6 and 2.1×10^8 copies of 16S rRNA genes mL^{-1} of template. Carapace clip samples had bacterial abundances between 2.4×10^6 and 6.5×10^7 copies of 16S rRNA genes mL^{-1} of template. Hemolymph samples ranged from 5.8×10^4 to 1.5×10^9 copies of 16S rRNA genes mL^{-1} of template. These same hemolymph samples plated on marine agar yielded counts ranging from 0 to 1.5×10^4 CFU mL^{-1} of hemolymph fluid (K. and L. Burnett, unpublished data). The hemolymph sample from crab C3 had the highest bacterial abundance detected by both qPCR (1.5×10^9 copies of 16S rRNA genes mL^{-1} of template) and CFU counts (1.5×10^4 CFU mL^{-1} of hemolymph fluid). Bacterial abundances (qPCR) in carapace clip, carapace swab, and gut samples from crab C3 were similar to those reported in the other crabs.

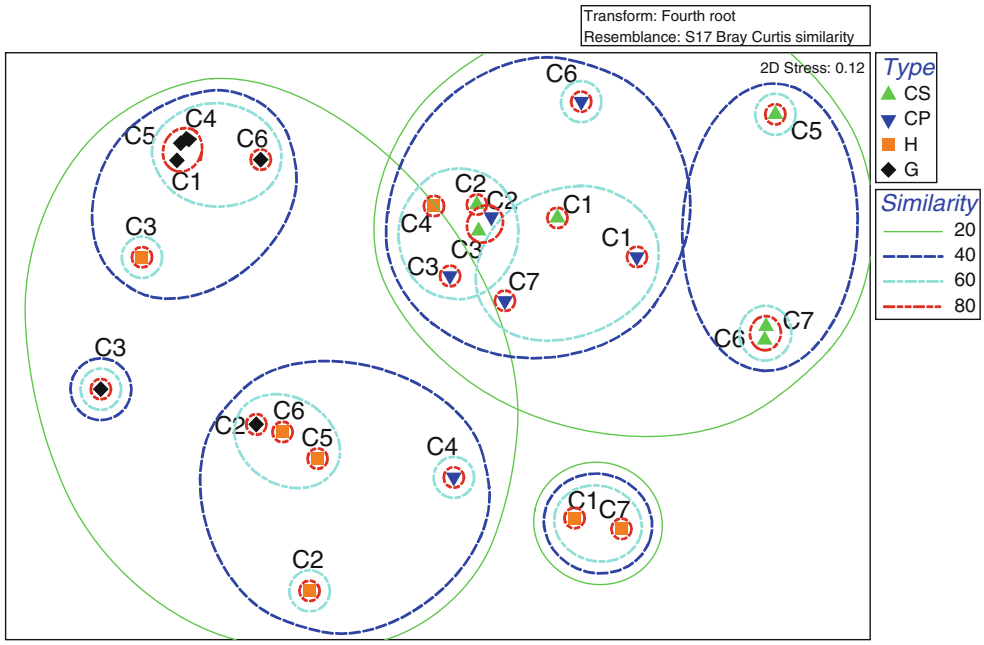

Fig. 4 Nonmetric multidimensional scaling analysis of γ-Proteobacteria ribotypes retrieved from carapace clip (*CP*), carapace swab (*CS*), gut (*G*), and hemolymph (*H*) samples. Samples are displayed in a two-dimensional space and clustered according to similarity. Note that in many instances, the similarity cutoff only included one sample. Samples not shown in the plot were below the 20 % similarity cutoff. MRPP analysis indicates that clusters separated at the 20, 40, 60, and 80 % similarity level were all significantly different at $p = 0.001$

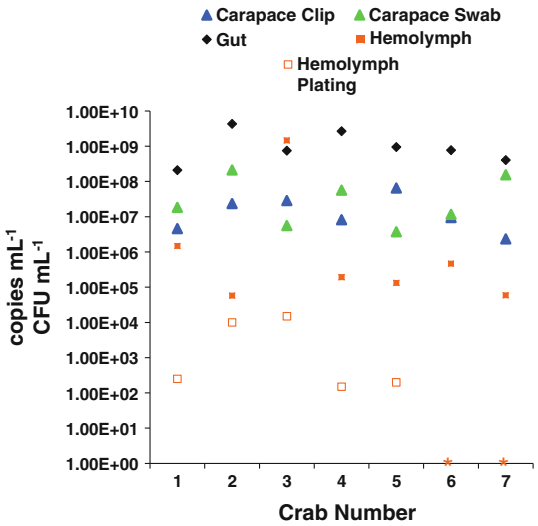

Fig. 5 qPCR analysis of the abundance of bacteria in carapace clip, carapace swab, gut, and hemolymph samples. Abundance is reported as copies of 16S rRNA genes mL^{-1} of DNA extract from each sample and thus is comparable across crabs but not between sample types. Hemolymph plating refers to hemolymph samples plated on marine agar (CFU mL^{-1} of hemolymph fluid) (K. and L. Burnett, unpublished data). *Asterisk* indicates that hemolymph plates for C6 and C7 had no colony growth

Discussion

Data from this study show that the microflora of *C. sapidus* is more diverse than previously reported (Table 2). The carapace, gut, and hemolymph all have a core Proteobacteria community (47–72 % of the ribotypes detected) that is dominated by γ-Proteobacteria (54–86 %). However, other phyla including Acidobacteria, Bacteroidetes, Firmicutes, Fusobacteria, Lentisphaerae, Planctomycetes, Spirochetes, Tenericutes, and Verrucomicrobia all contribute to the blue crab microbial assemblage.

Of the four sample types, the gut microflora was most diverse (Fig. 1a). A previous study of banana prawns (*Penaeus merguiensis*) found that healthy prawns had a more diverse gut microflora than diseased prawns (Oxley et al. 2002). This may suggest that the reported diverse gut microflora is indicative of healthy *C. sapidus*. We found the gut microflora community of the blue crab to be similar to previous studies that repeatedly documented Proteobacteria (Harris 1993; Oxley et al. 2002), Bacteroidetes (Li et al. 2007), Firmicutes, and Tenericutes (Li et al. 2012) in crustacean gut microflora (Harris 1993; Oxley et al. 2002; Li et al. 2012). The gut microbial assemblage often includes bacteria with protease, lipase, and chitinase enzymes that aid in digestion and nutrient availability (Harris 1993).

Some of these bacteria, such as the Mycoplasmataceae, are notoriously difficult to culture, hence their absence in previous studies using solely culture-based methods. Although some Mycoplasmataceae have been classified as pathogenic, others have been observed to be commensals and natural components of bacterial communities (Giebel et al. 1990). *Mycoplasma* sp. have been associated with

Table 2 Phylogenetic affiliation and abundance of bacteria in the blue crab microbial assemblage determined using culture-based versus culture-independent analyses

Sample type and assemblage composition	Concentration	Reference
Carapace clip		
Alteromonas sp., *Pseudoalteromonas* sp., *Erythrobacter* sp., Verrucomicrobia, *Vibrio* spp. (*V. harveyi*), Rhodobacteraceae (*Oceanicola* sp., *Roseobacter* sp., *Roseovarius* sp., and *Ruegeria* sp.), Bacteroidetes, Mycoplasmataceae	2.4×10^6–6.5×10^7 copies mL^{-1}	This study
Carapace swab		
Alteromonas sp., *Pseudoalteromonas* sp., *Thalassomonas* sp., *Aestuariibacter* sp., Rhodobacteraceae (*Roseobacter* sp., *Roseovarius* sp., *Loktanella* sp.), Bacteriodetes, *Vibrio* spp.	3.7×10^6–2.1×10^8 copies mL^{-1}	This study
Vibrio sp., *Pseudomonas* sp.	NR	(Cook and Lofton 1973)
Vibrio spp.; *V. cholerae*, *V. parahaemolyticus*, *V. vulnificus*	NR	(Davis and Sizemore 1982)
Vibrio sp., *Pseudomonas* ssp., *Aeromonas* sp., *Plesiomonas* sp.	NR	(Noga et al. 1994)
Achromobacter spp., *Acinetobacter* spp., *Aeromonas* spp., *Plesiomonas* spp., *Pseudomonas* spp., *E. coli*, *Serratia* sp., *Vibrio* spp. including *V. alginolyticus*, *V. mimicus*, *V. parahaemolyticus*, *V. vulnificus*	NR	(Noga et al. 2000)
Gut		
P. damselae (subsp. *damselae*, *piscida*), *Arcobacter* sp., *Spirochaeta* sp., Bacteroidetes, *P. maris*, Firmicutes (*Bacillus* sp., *Paenibacillus* sp.) Marinobacter sp., *Vibrio* spp. (*V. gallicus*, *V. harveyi*, *V. tubiashii*, *V. xuii*), Mycoplasmataceae, *Escherichia* sp., *Thalassomonas* sp.	2.1×10^8–4.3×10^9 copies mL^{-1}	This study
Hemolymph		
A. junii, *Bacillus* sp., *Vibrio* spp. (*V. harveyi*), *Alteromonas* sp., Marinobacter sp., *Escherichia* sp., Bacteroidetes, *Methylobacterium* sp., *Comamonas* sp., *Diaphrobacter* sp., *Paenibacillus* sp., *Thalassomonas* sp., Mycoplasmataceae	5.8×10^4–1.5×10^9 copies mL^{-1}	This study
Clostridium botulinum type F	NR	(Williams-Walls 1968)
V. parahaemolyticus	NR	(Krantz et al. 1969)
Vibrio spp., *V. parahaemolyticus*	NR	(Colwell et al. 1975)
Vibrio spp. (especially *V. parahaemolyticus*), *Pseudomonas* sp., *Acinetobacter* sp., *Aeromonas* sp., *Bacillus* sp., *Flavobacterium* sp., coliforms	NR	(Sizemore et al. 1975)
Vibrio spp.; *V. cholerae*, *V. parahaemolyticus*, *V. vulnificus*	8.6×10^1–3.0×10^7 bacteria mL^{-1}	(Davis and Sizemore 1982)
Vibrio spp.	0–9.5×10^4 CFU mL^{-1}	(Welsh and Sizemore 1985)

NR not reported

the gut microflora of a variety of terrestrial and marine hosts including rats (Giebel et al. 1990), termites (Wong et al. 2000; Hongoh et al. 2003), fish (Holben et al. 2002; Bano et al. 2007; Ward et al. 2009), abalone (Tanaka et al. 2004; Huang et al. 2010), lobsters (Meziti et al. 2010), and the marsh fiddler crab (Gulmann 2004). None of the Mycoplasmataceae sequences we retrieved clustered with the *Mycoplasma* spp. found in termite (Hongoh et al. 2003) or fish gut studies (Holben et al. 2002; Bano et al. 2007; Ward et al. 2009). Our sequences did, however, cluster with uncultured Mycoplasmataceae from guts of mud crabs *Scylla paramamosain* (Li et al. 2012) and with symbionts from isopod midguts (Fraune and Zimmer 2008).

We did not retrieve *V. cholerae*, *V. parahaemolyticus*, or *V. vulnificus* from any of the crabs we sampled; however, we retrieved many sequences that were similar to other potential pathogens. *A. junii*, *Alteromonas* sp., *Bacillus* sp., *E. coli*, *P. damselae subsp. damselae*, *P. damselae subsp. piscida*, *Pseudoalteromonas* sp., and *V. harveyi* are all potentially pathogenic, and sequences assigned (>97 % similarity) to these species were associated with blue crabs in our study. *V. harveyi* and both subspecies of *P. damselae* are known to be opportunistic pathogens of both finfish and

shellfish (Thyssen et al. 1998; Fouz et al. 2000; Austin and Zhang 2006). *P. damselae* subsp. *damselae* is also documented as a human pathogen with 3 cases reported in 2010 and an incidence of 0.01 per 100,000 persons (CDC 2011).

Previous studies (Tubiash et al. 1975; Welsh and Sizemore 1985) reported that crabs with physical injuries had increased levels of hemolymph bacterial colonization. Of the crabs sampled in this study, C1, C2, and C4 had sustained injuries prior to capture that resulted in partial (dactyl) or complete (chelipeds) loss of appendages. The hemolymph sample from crab C1 had the second highest abundance of bacteria in these samples, with 1.5×10^6 copies of 16S rRNA genes mL^{-1} of template. Crabs C2 and C4 had much lower concentrations, in the range of 10^4–10^5 copies of 16S rRNA genes mL^{-1} of template. Crab C3 had no injuries, but at the time of dissection had the highest concentration of bacteria in its hemolymph (10^9 copies of 16S rRNA genes mL^{-1} of template), with all sequences retrieved having >97 % similarity to the opportunistic pathogen *V. harveyi*. When we assessed the abundance of bacteria in crab hemolymph samples using published classifications based on plating (Davis and Sizemore 1982), 29 % had sterile hemolymphs, 42 % had light level colonization, and 29 % had moderate-level colonization. No crabs had high-level (>10^5 CFU mL^{-1} of hemolymph fluid) colonization. In contrast, if we convert our qPCR data (copies of 16S rRNA genes mL^{-1} of template) to estimates of genome (cell) abundance by dividing by an average copy number of 3.3 16S rRNA genes/genome (the average of the ribosomal gene copy numbers for genera present in this study's clone libraries; refs (Klappenbach et al. 2001; Lee et al. 2009), we can estimate abundance as genomes (cells) mL^{-1} of template. We then use the categories proposed by (Davis and Sizemore 1982) to classify crab hemolymph samples by qPCR assay: 86 % had moderate-level colonization and 14 % had high-level colonization. Although the previously injured crabs C1, C2, and C4 only had light levels of bacterial colonization and were apparently healthy, their hemolymph communities were dominated by potential pathogens: *A. junii* (C1); *Bacillus* sp. (C2); and *Alteromonas* sp., *Bacillus* sp., *P. damselae*, and *Vibrio* spp. (C4). Bacteria thus occur naturally in the hemolymph; however, the origin and the actual function (whether beneficial or antagonistic) of these bacteria remains unclear.

Conclusions

1. A more diverse (and abundant) microbial community is associated wild blue crabs than previously reported. Many of these microbes are closely related to overt or opportunistic pathogens of shellfish with implications for blue crab health. Some of these same pathogens may also be of public health concern.
2. The gut microflora community is similar among sampled crabs but different from that found in either carapace or hemolymph bacterial communities. While overall composition of the hemolymph microflora community is not the same as that of the carapace or the gut, sequences representing many of the same phyla (Bacteroidetes, Firmicutes, and Proteobacteria) and even ribotypes (i.e., *Alteromonas* sp., *Escherichia* sp., and *Vibrio* sp.) that were found in carapace and gut samples were also found in hemolymph samples.
3. Given the adverse consequences of sublethal levels of hemolymph bacteria on crustacean metabolism and performance (Burnett et al. 2006; Scholnick et al. 2006; Thibodeaux et al. 2009; Roman et al. unpublished data), the high incidence of bacterial infection in the blue crab population documented in the present study points to larger concerns regarding the long-term health and fitness of these wild populations.

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