

ADDITIONAL FILE for

Gill-associated bacteria are homogeneously selected in amphibious mangrove crabs to sustain host intertidal adaptation

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Supplementary Method

Method S1. Quantification of ammonia concentration in crab gills. Male individuals of *Cranuca inversa* and *Thalamita crenata* were collected from the Ibn Sina Field Research Station mangrove at KAUST (KSA) and kept in dedicated aquaria with fresh sediment for *C. inversa* and filtered fresh seawater flushed with air to maintain an oxygen saturation of 98%, at 21°C, 1 atm (assessed through a Fibox4 logger, Presence, Regensburg, Germany). After 12 h of acclimation, 10 individuals of each species were sacrificed, and the left gills were extracted. Gills were weighed and soaked with 300 µL of sterile ultrapure water (Invitrogen, Waltham, USA). Samples were then manually homogenised with plastic pestles for 1.5 µL tubes and centrifuged for 5 min, 13000g. After centrifugation, the supernatants were collected to be centrifuged again for another 10 min at 13000g. The final supernatant was used to quantify ammonia concentration in the crab gills using the ammonia assay kit MAK310 (Merck, Darmstadt, Germany) following the manufacturer's instructions. Fluorescence readings were performed with a TECAN infinite 200 pro spectrophotometer (TECAN, Grödig, Austria) in 96-well clear bottom black polystyrene microplates (Corning, NY, USA). Results were calculated following manufacturers' indications and normalised on the fresh weight of initial gill tissue.

Supplementary Tables

Table S1. Pairwise comparison of the bacterial beta-diversity among Sites and Species (including sediments).

Pairs	F-model	r-square	adjusted-<i>p</i>
KSA_albimana vs KSA_inversa	2.765	0.217	0.043
KSA_albimana vs KSA_urvillei	3.104	0.154	0.003
KSA_albimana vs KSA_sediment	12.800	0.561	0.004
KSA_albimana vs KY_sediment	10.787	0.519	0.002
KSA_albimana vs KY_urvillei	7.444	0.383	0.001
KSA_albimana vs ZA_sediment	12.437	0.580	0.003
KSA_albimana vs ZA_occidentalis	2.585	0.190	0.017
KSA_albimana vs ZA_chlorophthalmus	1.800	0.141	0.087
KSA_albimana vs ZA_urvillei	3.597	0.217	0.002
KSA_inversa vs KSA_urvillei	5.882	0.257	0.001
KSA_inversa vs KSA_sediment	21.168	0.679	0.004
KSA_inversa vs KY_sediment	16.879	0.628	0.003
KSA_inversa vs KY_urvillei	12.065	0.501	0.001
KSA_inversa vs ZA_sediment	21.919	0.709	0.006
KSA_inversa vs ZA_occidentalis	8.811	0.445	0.001
KSA_inversa vs ZA_chlorophthalmus	3.402	0.236	0.003
KSA_inversa vs ZA_urvillei	5.110	0.282	0.001
KSA_urvillei vs KSA_sediment	12.760	0.429	0.001
KSA_urvillei vs KY_sediment	11.027	0.393	0.001
KSA_urvillei vs KY_urvillei	5.333	0.219	0.001
KSA_urvillei vs ZA_sediment	11.354	0.415	0.001
KSA_urvillei vs ZA_occidentalis	2.820	0.135	0.002
KSA_urvillei vs ZA_chlorophthalmus	4.413	0.197	0.001
KSA_urvillei vs ZA_urvillei	3.751	0.158	0.001
KSA_sediment vs KY_sediment	12.165	0.549	0.003
KSA_sediment vs KY_urvillei	38.467	0.762	0.002
KSA_sediment vs ZA_sediment	14.351	0.615	0.002
KSA_sediment vs ZA_occidentalis	18.688	0.629	0.001
KSA_sediment vs ZA_chlorophthalmus	12.602	0.534	0.001
KSA_sediment vs ZA_urvillei	16.965	0.566	0.001
KY_sediment vs KY_urvillei	30.006	0.714	0.001
KY_sediment vs ZA_sediment	1.077	0.107	0.35
KY_sediment vs ZA_occidentalis	15.683	0.588	0.003
KY_sediment vs ZA_chlorophthalmus	10.705	0.493	0.001
KY_sediment vs ZA_urvillei	14.452	0.526	0.001
KY_urvillei vs ZA_sediment	42.899	0.796	0.002
KY_urvillei vs ZA_occidentalis	11.060	0.460	0.001
KY_urvillei vs ZA_chlorophthalmus	7.001	0.350	0.001
KY_urvillei vs ZA_urvillei	4.142	0.216	0.006
ZA_sediment vs ZA_occidentalis	18.649	0.651	0.003
ZA_sediment vs ZA_chlorophthalmus	11.903	0.543	0.001
ZA_sediment vs ZA_urvillei	15.972	0.571	0.001
ZA_occidentalis vs ZA_chlorophthalmus	4.825	0.287	0.001
ZA_occidentalis vs ZA_urvillei	5.588	0.285	0.001
ZA_chlorophthalmus vs ZA_urvillei	4.116	0.227	0.001

Table S2. List of FISH probes used in this study.

Name	Sequence (5'→3')	Fluorescent	Target group	% FA (40°C)	Reference
EUB338*	GCT GCC TCC CGT AGG AGT	Cy3	Most bacteria	10	Amann R. I., Binder B. J., Olson R. J., Chisholm S. W., Devereux R. and Stahl D. A. (1990). Appl. Environ. Microbiol. 56: 1919–1925.
EUB338II*	GCA GCC ACC CGT AGG TGT	Cy3	<i>Planctomycetales</i>	10	Daims H., Brühl A., Amann R., Schleifer K.-H. and Wagner M. (1999). Syst. Appl. Microbiol. 22: 434–444.
EUB338III*	GCT GCC ACC CGT AGG TGT	Cy3	<i>Verrucomicrobiales</i>	10	Daims H., Brühl A., Amann R., Schleifer K.-H. and Wagner M. (1999). Syst. Appl. Microbiol. 22: 434–444.
ALF968	GGT AAG GTT CTG CGC GTT	Cy5	<i>Alphaproteobacteria</i>	35	Neef A. (1997). Anwendung der in situ Einzelzell-Identifizierung von Bakterien zur Populationsanalyse in komplexen mikrobiellen Biozönosen. Doctoral thesis (Technische Universität München).
BET42a	GCC TTC CCA CTT CGT TT	FITC	<i>Betaproteobacteria</i>	35	Manz W., Amann R., Ludwig W., Wagner M. and Schleifer K.-H. (1992). Syst. Appl. Microbiol. 15: 593 - 600.
GAM42a	GCC TTC CCA CAT CGT TT	Competitor probe for BET42a	<i>Gammaproteobacteria</i>	35	Manz W., Amann R., Ludwig W., Wagner M. and Schleifer K.-H. (1992). Syst. Appl. Microbiol. 15: 593 - 600.
HGC236	AAC AAG CTG ATA GGC CGC	Cy5	<i>Actinobacteria</i>	10 - 20	Erhart, R., D. Bradford, R. J. Seviour, R. Amann, and L. L. Blackall (1997). Syst. Appl. Microbiol. 20:310-318:
ILU470	GTAGTTGGCCGCACCTTCTT	FITC	<i>Acidimicrobiales</i>	10 – 20	This work
NONEUB-Cy5	ACT CCT ACG GGA GGC AGC	Cy5	/	***	Wallner G., Amann R. and Beisker W. (1993). Cytometry. 14: 136–143.
NONEUB-Cy3	ACT CCT ACG GGA GGC AGC	Cy3	/	***	Wallner G., Amann R. and Beisker W. (1993). Cytometry. 14: 136–143.
NONEUB-FITC	ACT CCT ACG GGA GGC AGC	FITC	/	***	Wallner G., Amann R. and Beisker W. (1993). Cytometry. 14: 136-143.

* Applied as an equimolar mix; *** the same stringency conditions of the FISH probe with the same fluorescent

Table S3. General statistics for metagenomes and assemblies of fiddler gill and burrow sediments microbiomes.

Table S4. Summary of 16S rRNA gene sequences retrieved from individual metagenomes under study.

Table S5. List of KEGG orthology (KO) further investigated in this study related to carbon, sulfur, and nitrogen metabolism as well as the detoxification of sulfur compounds and xenobiotics.

Table S6. General information and statistics for metatranscriptomes and assemblies from the gill tissues of the fiddler crab *C. inversa* collected in the Red Sea, KAUST coastline mangroves.

Supplementary Figures

Figure S1. Rarefaction (A) and Goods' coverage index (B) of the bacterial 16S rRNA gene amplicon sequencing dataset.

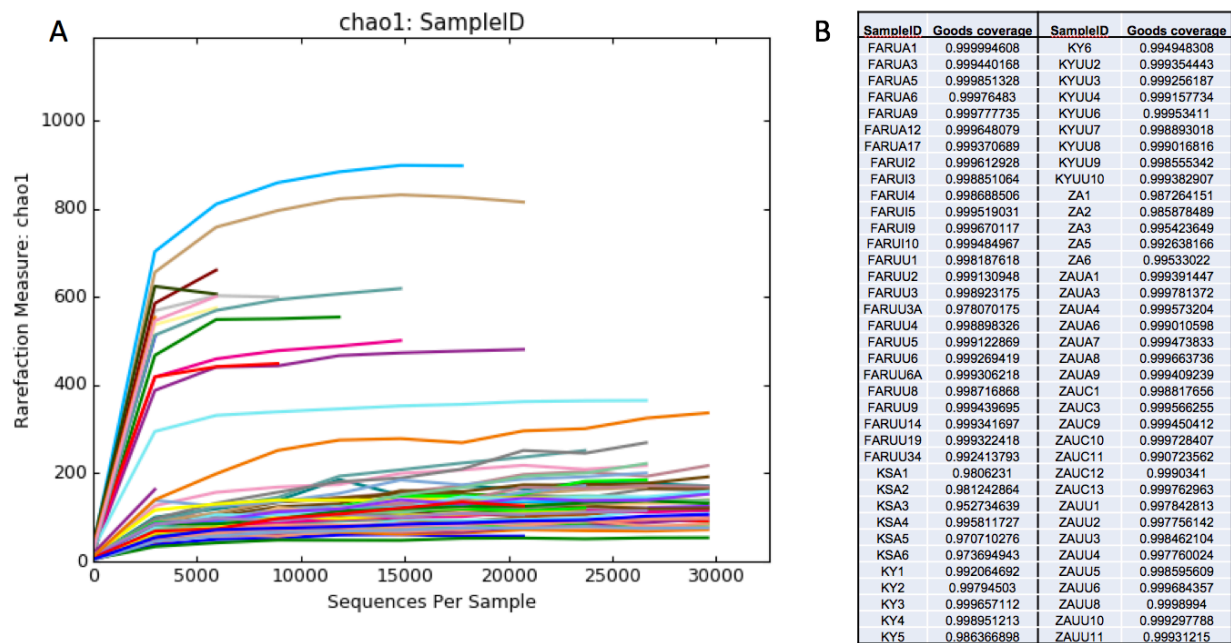
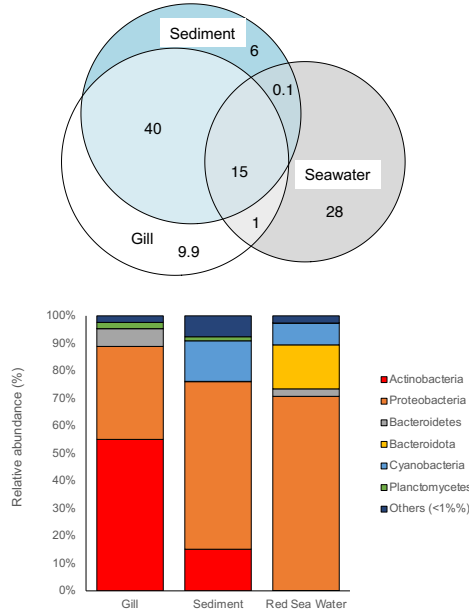


Figure S2. Eulero-Venn Diagram that shows the shared OTUs (numbers represent the percentage weighted for the OTUs relative abundance) among sediment, seawater and crab gills, and Taxonomy of the overall samples (A) and considering only the samples from Red Sea (B).

A - Overall samples



B - KSA samples

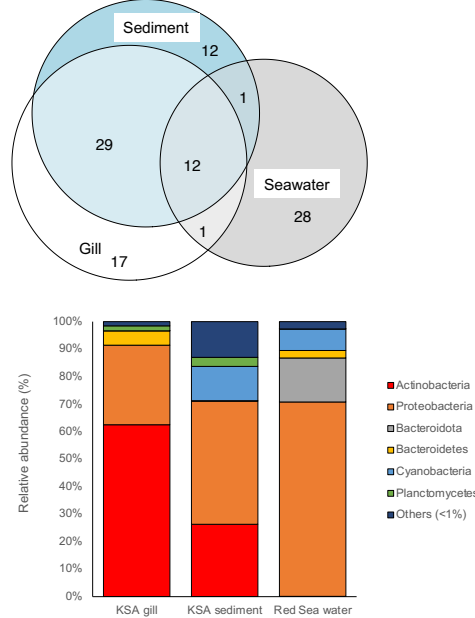
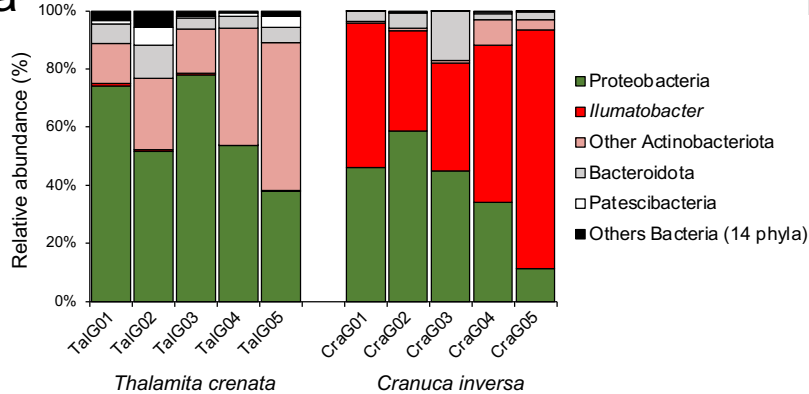


Figure S3. (A) Taxonomical composition of bacterial communities in *T. crenata* and *C. inversa* highlights the large presence of *Ilumatobacter* sp. in the semiterrestrial fiddler crab gills and its paucity in the aquatic crabs. Notably, the “other Actinobacteria” detected in *T. crenata* mainly belonged to *Propionibacteriaceae* and *Microtrichaceae*. (B) Quantification of ammonia concentration in the crab gills. Significantly different ammonia concentrations on the gill of the swimming crabs *Thalamita crenata* and the fiddler crabs *Cranuca inversa* (Mann-Whitney test, $U=14$, $p<0.0052$, $n=10$).

a



b

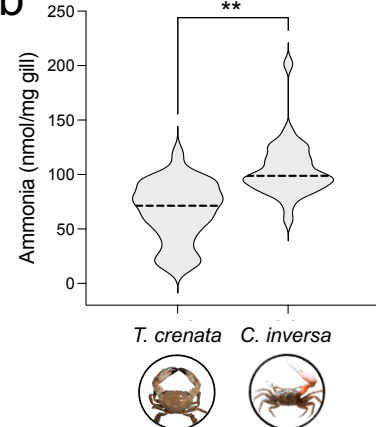


Figure S4. Constrained analysis of principal coordinates of the phylogenetic distances (alpha PD) among the bacterial microbiomes across (A) sites and (B) crab species.

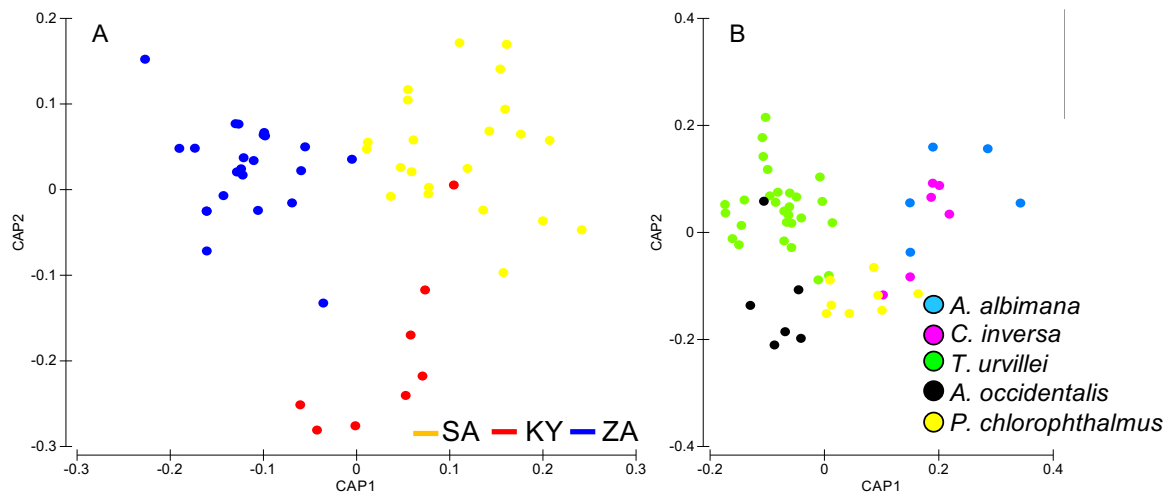


Figure S5. Scanning electron microscope imaging of fiddler crabs gill studied where we can see the constant coverage of the bacterial layer in all the species investigated.

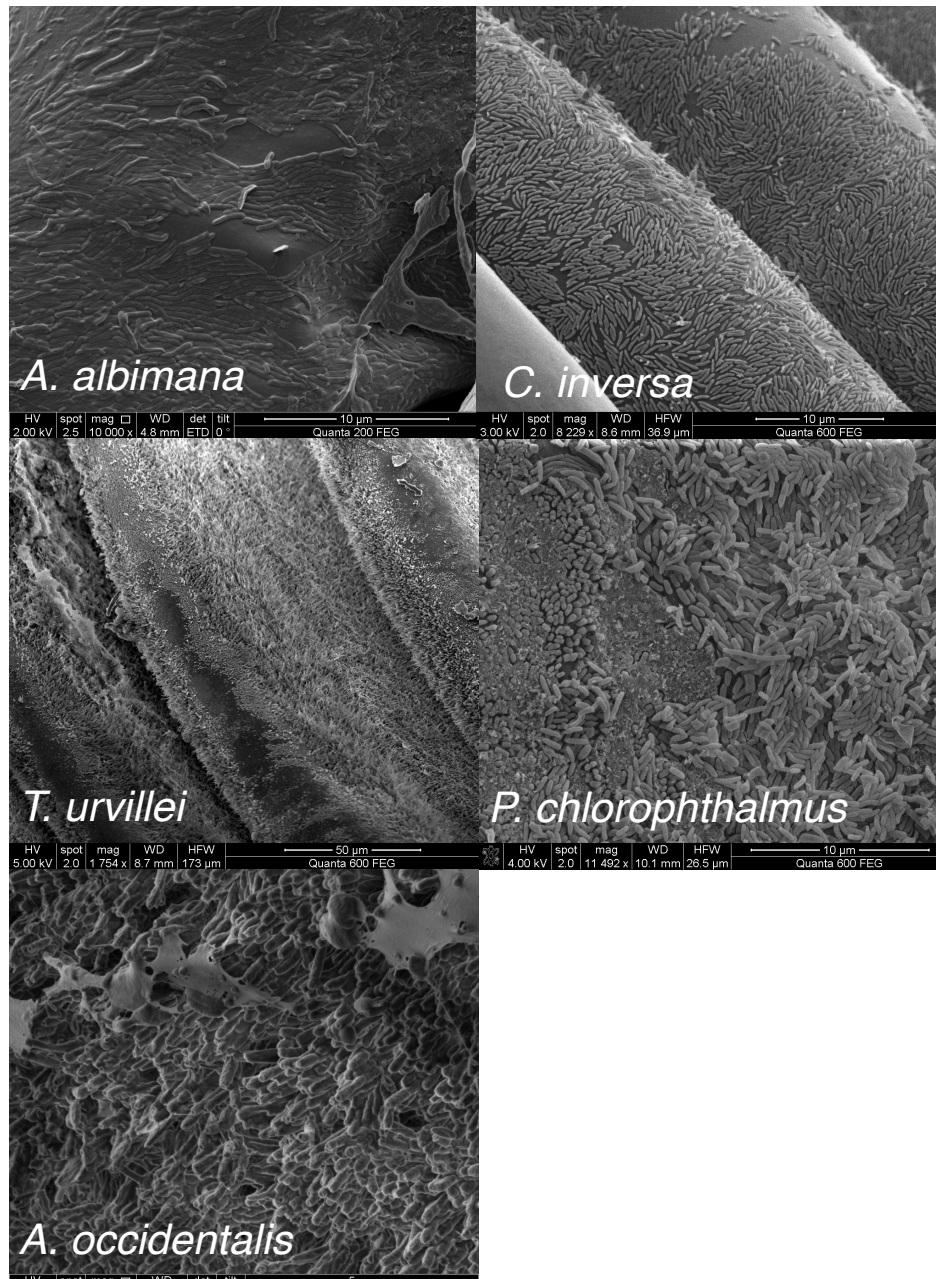


Figure S6. Bright-field (a) and FISH negative controls (b) of fiddler crabs gill lamellae.

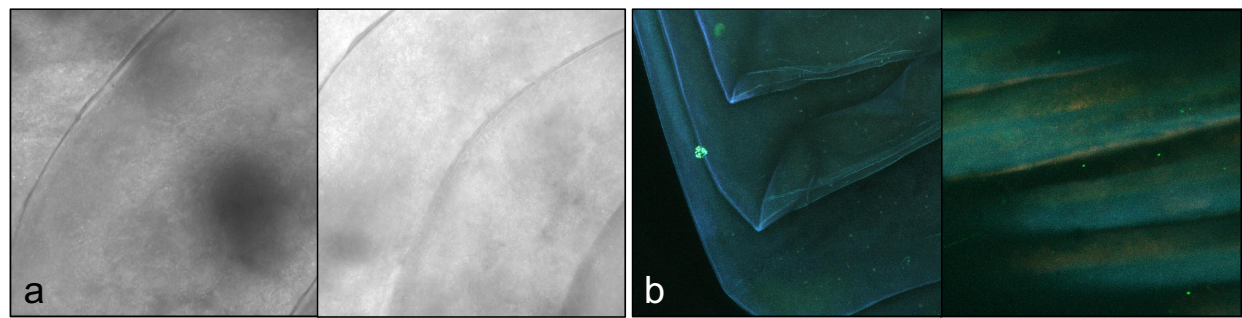
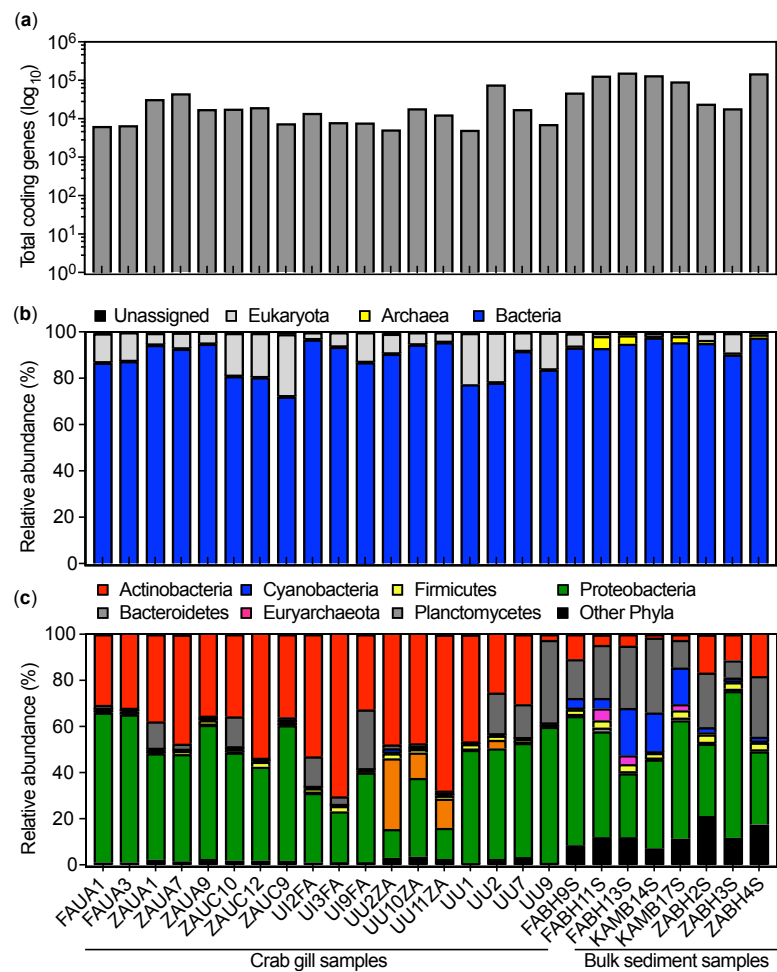


Figure S7. Metagenomic protein-coding gene sequence space of crab and bulk sediment microbiomes. (a) Total counts of predicted protein-coding genes in individual samples. (b) Relative abundance of prokaryotic (bacteria and archaea) relative to eukaryotic genes in sequenced metagenomes. (c) Phylum-level taxonomic breakdown of bacterial genes indicates Actinobacteria's prevalence in crab samples.



(a) Bar chart showing the total 16S reads retrieved (log₁₀) for 24 samples. The y-axis ranges from 10⁰ to 10⁵. The x-axis labels are FAUA1, FAUA3, ZUA1, ZUA7, ZUA9, ZUA10, ZUA12, ZUA13, ZUA14, ZUA15, ZUA16, ZUA17, ZUA18, ZUA19, ZUA20, ZUA21, ZUA22, ZUA23, ZUA24, ZUA25, ZUA26, ZUA27, ZUA28, ZUA29.

(b) Stacked bar chart showing the relative abundance (%) of 16S rRNA reads for five categories: Unassigned (black), Unknown Archaea (orange), Unknown Bacteria (light blue), Archaea (yellow), and Bacteria (dark blue). The y-axis ranges from 0 to 100%.

(c) Stacked bar chart showing the relative abundance (%) of 16S rRNA reads for ten bacterial phyla: Actinobacteria (red), Cyanobacteria (blue), Firmicutes (yellow), Proteobacteria (green), Bacteroidetes (grey), Euryarchaeota (pink), Planctomycetes (orange), and Other Phyla (black). The y-axis ranges from 0 to 100%.

Crab gill samples: FAUA1, FAUA3, ZUA1, ZUA7, ZUA9, ZUA10, ZUA12, ZUA13, ZUA14, ZUA15, ZUA16, ZUA17, ZUA18, ZUA19, ZUA20, ZUA21, ZUA22, ZUA23, ZUA24, ZUA25, ZUA26, ZUA27, ZUA28, ZUA29.

Bulk sediment samples: FABH9S, FABH11S, FABH13S, KAMB4S, KAMB17S, ZABH2S, ZABH3S, ZABH4S.

Figure S9. Krona graph showing the taxonomic breakdown of the representative protein-coding gene catalogue predicted from all metagenomes.

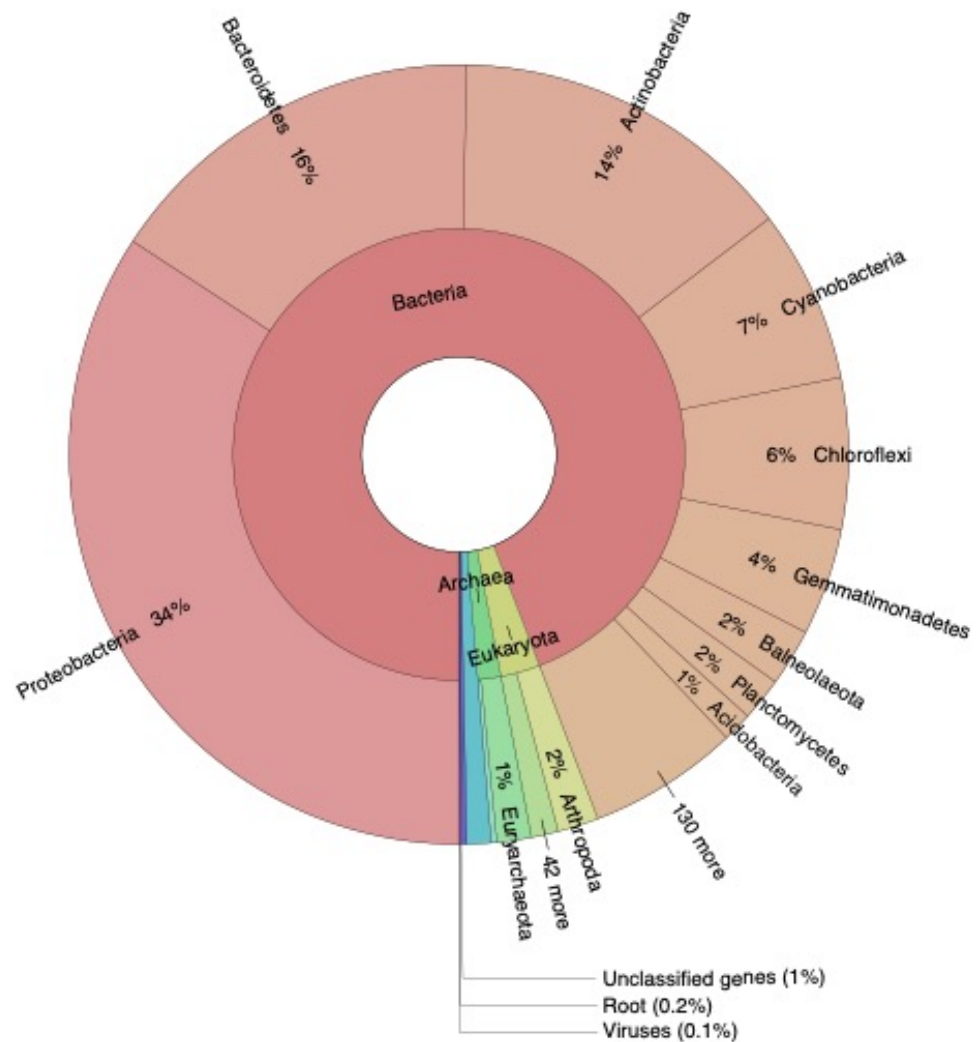


Figure S10. Total counts of predicted protein-coding genes and the corresponding domain. Additional information is provided in Table S5.

