The Gills of Reef Fish Support a Distinct Microbiome Influenced by Host-Specific Factors

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ABSTRACT Teleost fish represent the most diverse of the vertebrate groups and play important roles in food webs, as ecosystem engineers, and as vectors for microorganisms. However, the microbial ecology of fishes remains underexplored for most host taxa and for certain niches on the fish body. This is particularly true for the gills, the key sites of respiration and waste exchange in fishes. Here we provide a comprehensive analysis of the gill microbiome. We focus on ecologically diverse taxa from coral reefs around Moorea, sampling the gills and intestines of adults and juveniles representing 15 families. The gill microbiome composition differed significantly from that of the gut for both adults and juveniles, with fish-associated niches having lower alpha diversity values and higher beta diversity values than those for seawater, sediment, and alga-associated microbiomes. Of ~45,000 operational taxonomic units (OTUs) detected across all samples, 11% and 13% were detected only in the gill and the intestine, respectively. OTUs most enriched in the gill included members of the gammaproteobacterial genus *Shewanella* and the family *Endozoicimonaceae*. In adult fish, both gill and intestinal microbiomes varied significantly among host species grouped by diet category. Gill and intestinal microbiomes from the same individual were more similar to one another than to gill and intestinal microbiomes from different individuals. These results demonstrate that distinct body sites are jointly influenced by host-specific organizing factors operating at the level of the host individual. The results also identify taxonomic signatures unique to the gill and the intestine, confirming fish-associated niches as distinct reservoirs of marine microbial diversity.

IMPORTANCE Fish breathe and excrete waste through their gills. The gills are also potential sites of pathogen invasion and colonization by other microbes. However, we know little about the microbial communities that live on the gill and the factors shaping their diversity. Focusing on ecologically distinct types of coral reef fish, we provide a comprehensive analysis of the fish gill microbiome. By comparison to microbiomes of the gut and the surrounding environment, we identify microbes unique to the gill niche. These microbes may be targets for further studies to determine the contribution of the microbiome to waste exchange or host immunity. We also show that despite exhibiting a unique taxonomic signature, the gill microbiome is influenced by factors that also influence the gut microbiome. These factors include the specific identity of the host individual. These results suggest basic principles describing how association with fishes structures the composition of microbial communities.

KEYWORDS intestine, gut, bacteria, teleost, coral reef, Moorea

Teleost fish are among the most biodiverse of coral reef inhabitants, representing an estimated 6,000 to 8,000 total reef species (1). Reef fishes engage in complex energy and material transfer processes involving a range of trophic and symbiotic relationships. Understanding the microbiota of the gills, which play a critical role in respiration and waste exchange, provides insights into the unique environmental pressures shaping these microbial communities.

Received 8 January 2018 Accepted 12 February 2018 Accepted manuscript posted online 16 February 2018 Citation Pratte ZA, Besson M, Hollman RD, Stewart FJ. 2018. The gills of reef fish support a distinct microbiome influenced by host-specific factors. Appl Environ Microbiol 84:e00063-18. https://doi.org/10.1128/AEM.00063-18.

Editor Andrew J. McBain, University of Manchester
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interactions, while also serving as reservoirs and vectors of microorganisms (2, 3). Such diversity makes reef fish communities useful for exploring the factors shaping animal-associated microbiomes (4). For fishes in general, it remains unclear how microbiome diversity is partitioned among niches, notably between the fish intestine and other body sites. In particular, our understanding of the mucosal microbiome of the fish gill remains limited. Given the role of the gills as a primary site for gas and waste exchange in fishes, as well as for mucosal immune interactions, osmoregulation, and detoxification (5), gill-associated microbiomes may play critical roles in overall fish health and physiology. In the present study, using reefs as a model system for sampling broadly across host diversity, we explore patterns of organization in the fish gill microbiome.

Animal-associated microbiomes often contain hundreds of bacterial or archaeal species, many of which are hypothesized to contribute to host health, behavior, and development (6, 7). These contributions are principally mediated by microbial chemical transformations, including the breakdown of host food and waste products and the synthesis of molecules that interact directly with host cells (8). These chemical transformations and the genetic diversity underlying these processes are becoming better understood for the microbiome of the vertebrate gastrointestinal (GI) tract, primarily the large intestine, where the vast majority of microbiome biomass is located (9). However, microbes exist in diverse niches throughout the animal body, including in association with non-GI mucosal surfaces that also function in chemical or gas exchange and as barriers or entry points for pathogens. Characterizing non-GI mucosal microbiomes—including those of the gills, lungs, and skin—is therefore critical for identifying novel functions of animal-associated microbes and for understanding the connectivity between environmental microbial communities and those of the host.

In fish, the mucosal surface of the gills may be a relatively unique habitat for host-associated microbes. The fish gill is potentially influenced by high local concentrations of oxygen and nutrients (e.g., ammonia and urea [10]), in contrast to the GI tract, where oxygen may be more limiting and for which the genomic potential for anaerobic fermentative metabolism has been demonstrated (3); however, variation in intestinal oxygen availability across fishes with differing dietary ecologies and GI structures is not well understood. Furthermore, the gills may undergo high levels of mucosal discharge, especially during times of environmental stress (e.g., sediment loading [11]), may exhibit high connectivity with the surrounding water microbiome and between the water and bloodstream (5), and may be important access points for pathogens (12). The extent to which the gill community is more or less influenced by colonization by external microbes than are microbiomes of other body site niches, such as the GI tract, remains uncertain; indeed, the fish intestinal microbiome is presumably also highly connected to the environmental microbiome via the intake of food as well as seawater, which saltwater fish regularly “drink” to help maintain water balance. Together these factors suggest a need for further work to explore the gills as sites for unique assembly processes or novel microbe-microbe or microbe-host interactions, potentially involving symbioses. For example, the gill microbiome of rainbow trout was shown to have antifungal properties (13), and a recent study of carp gills identified intracellular bacterial symbionts that oxidize host-excreted ammonia as an energy source (14), thereby removing a potential toxin that can cause tissue damage at high concentrations (15). However, while the gill microbiome is likely distinct from that of the surrounding environment (e.g., see reference 16), the full diversity of the gill microbiome, the factors structuring this diversity, and the extent to which this diversity is specific to the gill or a host individual remain unknown.

Focusing on a single reef system around the Island of Moorea in French Polynesia, thereby minimizing confounding effects of geography, we present a comprehensive analysis of gill microbiome diversity across diverse reef fish taxa. We sampled fishes representing 15 families, diverse diet ecologies, and both juveniles and adults to test for a shared gill microbiome structure and the extent to which this structure varies from those of the intestinal microbiome and the microbiome of the surrounding environ-
ment. These analyses identify key microbial taxa specific to the gill niche and also provide support for the hypothesis that the gill and intestinal microbiomes are influenced by similar host-specific organizing factors, whose strengths vary with life stage.

RESULTS

Microbiome samples. We analyzed 552 microbiomes from Moorea, French Polynesia (see Fig. S1 in the supplemental material), including 399 fish microbiomes representing the gills of 141 adults and 46 juvenile recruits and the intestines of 176 adults and 36 recruits. An additional 50 gill and 97 intestine samples were collected but failed to yield visible PCR products. Fish microbiome samples spanned 53 species within 15 families in the order Perciformes (Table 1; Fig. 1), with each family represented by 1 to 36 individuals. Based on the literature, the sampled taxa are roughly equally distributed among the three diet categories of carnivore, herbivore, and omnivore (Fig. 1; Table S1). However, some taxa likely do not adhere strictly to a single diet category; a comprehensive assessment of fish microbiome structuring based on diet should therefore involve a coupled assessment of diet contents.

The nature of the field collections did not allow recovery of the same set of host taxa between spearfished (adult) and crest net-collected (recruit) sample sets. While adult individuals were obtained for all 15 families, recruits were obtained only for the Chaetodontidae, Holocentridae, Lutjanidae, and Pomacanthidae families (Table 1). Therefore, comparisons of microbiomes between adults and recruits should be interpreted with caution, as differences may be due to changes in host factors, such as fish age, or to differences in host taxonomic composition.

We therefore emphasize comparisons between the gill and intestinal niches within a life stage, as the taxonomic representation of host individuals was relatively uniform between these niches within a life stage (Table 1). These results are presented below and are contextualized by comparison to microbiomes of seawater, sediment, algae, and coral from around Moorea (“environmental” samples in Table 1 [n = 153]).

Niche-specific structuring of 16S rRNA gene sequence diversity. Microbiome diversity (alpha and beta) varied significantly between animal-associated and environmental samples. In total, we recovered ~45,000 microbial operational taxonomic units (OTUs; 97% similarity clusters) across all samples after rarefaction to a common se-
Of these, 11% and 13% were detected only in fish gills and intestines (recruit or adult), respectively. On average, of the OTUs detected in fish-associated niches, 75 to 85% were also detected in environmental samples (Table S2). Of the environmental samples, sediment and alga samples contributed the highest percentages of unique OTUs (22% and 20%, respectively), despite being represented by considerably fewer samples. These results are consistent with fish gill and intestinal microbiomes having significantly lower Chao1-estimated OTU richness values than those of microbiomes of seawater, algae, and sediment (P < 0.01; Tukey post hoc test) (Table S3), which were, on average, 3 to 11 times higher (Fig. 2). Rather, fish microbiome richness was comparable to that of the coral (Porites) microbiome. On average, Chao1 estimates for intestinal samples were comparable to those reported previously (2, 17, 18). Despite their high level of richness, environmental microbiomes of algae, water, and sediment clustered according to sample type based on a nonmetric multidimensional scaling (NMDS) analysis of Bray-Curtis dissimilarity (OTU level) (Fig. 3). In contrast, fish-associated microbiomes exhibited high intersample variation (Fig. 2 and 3; Fig. S2), notably with those of adult fish (both gill and intestine) having significantly higher values for dispersion (distance from individual samples to the centroid of the sample type) than those of all other microbiome types, excluding those of the recruit intestine (P < 0.01; Tukey post hoc test) (Table S4).

Focusing on the fish-associated samples, we observed significant differences in microbiome structure based on body site. While microbiomes of the four fish sample types exhibited comparable levels of alpha diversity (Fig. 2; Table S3), with the exception of those of the recruit intestine, analysis of similarity (ANOSIM) based on Bray-Curtis matrices revealed that the taxonomic composition differed significantly between the gill and the intestine for both recruit and adult data sets (P < 0.01; ANOSIM) (Table 2; see Fig. 5). The magnitude of dissimilarity between gill and intestinal microbiomes was greater for recruits (ANOSIM global R = 0.47) than for adults (R = 0.11) (Table 2), although we caution that comparison across life stages is confounded by differences in host taxonomic composition.
Gill and intestinal microbiomes also differed in the extent to which they were structured based on presumed host diet. For both recruit and adult fish, the intestinal microbiome composition varied significantly among carnivores, herbivores, and omnivores ($P < 0.05$; ANOSIM) (Table S5). However, diet type-specific microbiome clustering was most pronounced for the adult intestine (Fig. 4). Among the intestinal samples, the microbiomes of adult carnivorous fish showed the tightest clustering, with ANOSIM $R$ values of 0.52 and 0.32 for comparisons against herbivore and omnivore microbiomes.

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respectively \( (P < 0.01) \) (Table S5), whereas the latter microbiomes were more similar to one another \( (R = 0.13) \). Interestingly, adult gill microbiomes were also significantly partitioned according to diet type \( (P < 0.01) \), although the extent of this structuring was less than that for the adult intestine samples (global \( R \) values of 0.22 and 0.35 for gill and intestine comparisons, respectively). Significant structuring based on diet type was not observed for the gill microbiomes of recruits (Table S5).

Analyses of paired data sets (gill and intestinal microbiomes from the same individual) provided evidence that gill and intestinal microbiomes were influenced jointly by the same host-specific assembly factors. Using data from all adult individuals for which paired data sets were available \( (n = 110) \), the average Bray-Curtis similarity between gill and intestinal microbiomes within an individual was significantly greater \( (>2\text{-fold}) \) than that between gill and intestinal microbiomes from different individuals \( (P < 0.001) \) (Table 3), with gill and intestine data sets from the same individual sharing,

### TABLE 2 One-way ANOSIM results for comparisons of gill and intestinal microbiomes of recruit and adult fish

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Global ( R ) value</th>
<th>( P ) value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adult gill vs adult intestine</td>
<td>0.109</td>
<td>0.001</td>
</tr>
<tr>
<td>Adult gill vs recruit gill</td>
<td>0.362</td>
<td>0.001</td>
</tr>
<tr>
<td>Adult intestine vs recruit intestine</td>
<td>0.240</td>
<td>0.001</td>
</tr>
<tr>
<td>Recruit gill vs adult intestine</td>
<td>0.154</td>
<td>0.001</td>
</tr>
<tr>
<td>Recruit gill vs recruit intestine</td>
<td>0.465</td>
<td>0.001</td>
</tr>
<tr>
<td>Recruit intestine vs adult gill</td>
<td>0.579</td>
<td>0.001</td>
</tr>
</tbody>
</table>

![FIG 4](http://aem.asm.org/) Similarities in composition among Moorea microbiomes grouped according to presumed host diet category. Clustering is based on nonmetric multidimensional scaling analysis of all microbiome samples according to Bray-Curtis distances evaluated at the OTU level (97% sequence similarity). Host diet category designations are based on the data in Table S1 in the supplemental material.
on average, ~24% of OTUs (Table S2). Significantly higher similarity within versus between individuals was also observed for adults of the Chaetodontidae. Consistently higher similarity within versus between individuals was observed for all other comparisons, including those involving recruits; however, these differences were not significant, potentially due to smaller sample sizes (Table 3).

**Gill-associated taxa.** The taxonomic signature of the gill microbiome was distinct from that of the intestinal microbiome. Analysis of microbial taxa at the class/subclass level or above showed that gill and intestinal microbiomes share many of the same broad taxonomic groups, with both niches dominated by members of the Gammaproteobacteria (Fig. 5), and that there is a high level of variation among gill and intestinal microbiomes (Fig. S2). However, in both adults and recruits, gill microbiomes were enriched (with higher proportional abundances) in members of the Betaproteobacteria (5.9- to 72.0-fold), Flavobacteria (2.0- to 5.9-fold), and Saprospirae (2.8- to 11.9-fold) compared to intestinal microbiomes. In contrast, in both life stages, intestinal microbiomes were enriched (5.8- to 185.9-fold) in Brevinematae, members of the Spirochaetes reported for microbiomes of both aquatic and terrestrial animals (17, 19).

These broad patterns are supported by analysis at finer taxonomic levels. DESeq2 tests revealed 177 microbial OTUs significantly enriched in the gill microbiome (recruit and adult data combined) compared to all other data sets (Table 4; Table S6). The most enriched taxa, based on changes in mean proportional abundance, included an un-identified betaproteobacterial OTU with highest similarity to unclassified environmental clones from diverse aquatic habitats (data not shown) and multiple OTUs of the gammaproteobacterial genus *Shewanella* and the family Endozoicimonaceae (Table 4). Several of these groups were highly prevalent. Notably, one of the Endozoicimonaceae OTUs was detected in 81% of all adult gill samples, at an average abundance of 4%, but in only 14% of adult intestine samples, at an average abundance just above the detection limit (Table S7). In contrast, the OTUs most enriched in the intestine compared to the gill included multiple unclassified members of the Brevinematae and the family *Pirellulaceae* of the phylum Planctomycetes (Tables S8 and S9). The most intestine-enriched OTUs included a member of the genus *Epulupiscium* (Firmicutes), a potential fish symbiont linked to algal polysaccharide degradation in reef surgeonfish (3), as well as many diverse phototrophic cyanobacterial lineages, presumably reflecting cells consumed as or in association with food.

**DISCUSSION**

The gills of reef fish host a unique and complex microbiome. Analysis of 53 fish species common to South Pacific reefs confirmed that the microbiomes of the gill and intestine are distinct from one another and from those of surrounding environmental reservoirs. Like the fish intestine and corals, the gill contains significantly fewer OTUs than those in the surrounding seawater and nearby sediment and algae. This is

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**TABLE 3** Similarities between gill and intestinal microbiomes

<table>
<thead>
<tr>
<th>Fish group (n)</th>
<th>Mean Bray-Curtis similarity value(a)</th>
<th>Within individual</th>
<th>Between individuals</th>
<th>(P) value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adults</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All taxa (110)</td>
<td>19.6</td>
<td>9.6</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Chaetodontidae (27)</td>
<td>22.2</td>
<td>12.3</td>
<td>0.003</td>
<td></td>
</tr>
<tr>
<td>Pomacanthidae (5)</td>
<td>21.9</td>
<td>21.5</td>
<td>0.97</td>
<td></td>
</tr>
<tr>
<td>Holocentridae (10)</td>
<td>19.5</td>
<td>17.2</td>
<td>0.44</td>
<td></td>
</tr>
<tr>
<td>Recruits</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All taxa (24)</td>
<td>21.2</td>
<td>17.3</td>
<td>0.08</td>
<td></td>
</tr>
<tr>
<td>Chaetodontidae (6)</td>
<td>16.2</td>
<td>14.1</td>
<td>0.67</td>
<td></td>
</tr>
<tr>
<td>Pomacanthidae (3)</td>
<td>33.3</td>
<td>23.0</td>
<td>0.31</td>
<td></td>
</tr>
<tr>
<td>Holocentridae (15)</td>
<td>20.7</td>
<td>16.9</td>
<td>0.07</td>
<td></td>
</tr>
</tbody>
</table>

\(a\)Potential values range from 0 to 100, with higher values indicating higher levels of similarity.
consistent with recent comparisons showing decreased diversity in animal-associated microbiomes compared to that in free-living microbiomes (20). Indeed, the vast majority of gill and intestine OTUs were also detected in environmental samples (Table 1; see Table S5 in the supplemental material), suggesting that both body niches select for a nested subset of the external community.

However, the gill also includes potentially niche-specific members. Of the >45,000 OTUs recovered across all data sets, ~10% were detected only in the gill samples. Of these, 177 OTUs were significantly enriched in the gill compared to all other sample types. The fish gill is particularly enriched in certain members of the gammaproteobacterial genus *Shewanella* and the family *Endozoicimonaceae*. *Shewanella* species are metabolically diverse and common members of free-living microbial communities in diverse aquatic habitats and of fish microbiomes, including those in the intestine and in association with disease (2, 18, 21, 22). The most enriched *Shewanella* OTU (814252) (Table 4; Table S6) was 100% identical to an environmental sequence recovered from Pacific herring (GenBank accession number JQ191540.1) and 99% identical to the common fish associate *Shewanella putrefaciens* and the potential human pathogen *Shewanella alga*. Endozoicomonads have been recovered ubiquitously from mucosal surfaces of marine invertebrates, particularly corals (23, 24), but have been reported less commonly for fish (23). In the present study, the three most enriched endozoicomonad OTUs share 99 to 100% identity with sequences recovered from diverse marine hosts, with the most enriched OTU (370251) (Table 4; Table S6) being 100% identical to a putative symbiont isolated from the coral *Montipora* (25). Although studies of aquacultured fishes implicate endozoicomonads in diseases of the fish gill (26, 27), our results suggest that this group is a stable and enriched component of the fish gill niche.

**FIG 5** Average microbiome taxonomic compositions of all sample types. Taxa were grouped at the class/subclass level by using a rarefied OTU table (3,000 sequences per sample). All 239 detected classes were present in either gill or intestine samples and at least one environmental sample (coral, algae, sediment, or water). The bar plot shows the log fold change in mean proportional abundance between gill and intestine data sets. The microbiome taxonomic compositions of individual fish are shown in Fig. S2 in the supplemental material.
TABLE 4 Most enriched OTUs in gill microbiomes

<table>
<thead>
<tr>
<th>Phylum</th>
<th>Class</th>
<th>Order</th>
<th>Family</th>
<th>Genus</th>
<th>Species</th>
<th>Change</th>
<th>Relative abundance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proteobacteria</td>
<td>Betaproteobacteria</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>9.4</td>
<td>1.21 ± 7.6</td>
</tr>
<tr>
<td>Gammaproteobacteria</td>
<td>Legionellales</td>
<td>Francisellaceae</td>
<td>NA</td>
<td>NA</td>
<td>7.5</td>
<td>0.59 ± 2.7</td>
<td></td>
</tr>
<tr>
<td>Gammaproteobacteria</td>
<td>Oceanospirillales</td>
<td>Endozoicimonaceae</td>
<td>NA</td>
<td>6.3</td>
<td>5.66 ± 13.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gammaproteobacteria</td>
<td>Alteromonadales</td>
<td>Shewanellaceae</td>
<td>Shewanella</td>
<td>NA</td>
<td>5.8</td>
<td>2.24 ± 6.7</td>
<td></td>
</tr>
<tr>
<td>Gammaproteobacteria</td>
<td>Oceanospirillales</td>
<td>Endozoicimonaceae</td>
<td>NA</td>
<td>5.7</td>
<td>0.08 ± 0.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gammaproteobacteria</td>
<td>Alteromonadales</td>
<td>Shewanellaceae</td>
<td>Shewanella</td>
<td>NA</td>
<td>5.1</td>
<td>0.29 ± 1.4</td>
<td></td>
</tr>
<tr>
<td>Gammaproteobacteria</td>
<td>Alteromonadales</td>
<td>Shewanellaceae</td>
<td>Shewanella</td>
<td>NA</td>
<td>4.8</td>
<td>0.19 ± 0.9</td>
<td></td>
</tr>
<tr>
<td>Alphaproteobacteria</td>
<td>Rickettsiales</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>4.8</td>
<td>0.22 ± 2.5</td>
<td></td>
</tr>
<tr>
<td>Gammaproteobacteria</td>
<td>Oceanospirillales</td>
<td>Endozoicimonaceae</td>
<td>NA</td>
<td>4.8</td>
<td>1.44 ± 6.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gammaproteobacteria</td>
<td>Enterobacteriales</td>
<td>Entero bacteriaceae</td>
<td>NA</td>
<td>4.5</td>
<td>1.36 ± 2.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alphaproteobacteria</td>
<td>Rickettsiales</td>
<td>Anaplasmataceae</td>
<td>Neorickettsia</td>
<td>NA</td>
<td>4.5</td>
<td>0.95 ± 3.3</td>
<td></td>
</tr>
<tr>
<td>Alphaproteobacteria</td>
<td>Rhizobiales</td>
<td>Methylobacteriaceae</td>
<td>Methylobacterium</td>
<td>NA</td>
<td>4.4</td>
<td>0.05 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>Alphaproteobacteria</td>
<td>Rhizobiales</td>
<td>Bartonellaceae</td>
<td>NA</td>
<td>NA</td>
<td>4.3</td>
<td>0.06 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>Bacteroidetes</td>
<td>Cytophagia</td>
<td>Cytophagales</td>
<td>“Amoebophilaceae”</td>
<td>NA</td>
<td>4.2</td>
<td>0.05 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>Proteobacteria</td>
<td>Gammaproteobacteria</td>
<td>Enterobacteriales</td>
<td>Enterobacteriaceae</td>
<td>NA</td>
<td>4.2</td>
<td>1.69 ± 6.8</td>
<td></td>
</tr>
<tr>
<td>Firmicutes</td>
<td>Clostridium</td>
<td>Clostridiaceae</td>
<td>Clostridium</td>
<td>NA</td>
<td>4.1</td>
<td>0.03 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>Fusobacteria</td>
<td>Fusobacteria</td>
<td>Fusobacteriaceae</td>
<td>Cetobacterium</td>
<td>NA</td>
<td>4.1</td>
<td>1.07 ± 2.9</td>
<td></td>
</tr>
<tr>
<td>Proteobacteria</td>
<td>Alphaproteobacteria</td>
<td>Rickettsiales</td>
<td>Rickettsiaceae</td>
<td>Wolbachia</td>
<td>NA</td>
<td>4.1</td>
<td>0.70 ± 1.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Sphingomonadales</td>
<td>Sphingomonas</td>
<td>4.0</td>
<td>0.05 ± 0.1</td>
</tr>
</tbody>
</table>

aThe OTUs listed are 16S rRNA gene 97% similarity clusters identified as significantly differentially abundant by analysis of all fish gill data sets versus all other data sets (adult and recruit fish intestine and environmental samples) by use of DESeq2. OTUs are ranked by fold change. Complete lists of all gill-enriched and intestine-enriched OTUs can be found in Tables S6, S8, and S9 in the supplemental material. NA, not available.
bLog2 fold change in mean proportional abundance.
cMean proportional abundance (%) across all fish gill samples (± standard deviation (SD)), based upon the rarefied (3,000 sequences) OTU table.
dWolbachia endosymbiont of Chorthippus parallelus.

Other gill-enriched OTUs include groups previously associated with external surfaces of fish, including the skin and fins. These include Shewanella, Cetobacterium, and Clostridium (28, 29) as well as representatives of the obligately intracytoplasmic genus Neorickettsia (30), which has been implicated in fish-transmitted disease in mammals (31), and the methylotrophic genus Methylobacterium, which has been suggested to be a fish commensal with a role in beneficial antibacterial activity (32). This suggests that the gill microbiome likely resembles those of other exterior habitats of the fish body. Like that of the gills, the skin and fin microbiomes are also associated with mucus and presumably shaped by high connectivity to the external environment. Indeed, the microbiome composition of the skin mucus has been used as a marker of harvest location for commercial food fish (33, 34), and sampling date and location have been shown to play a significant role in structuring the microbiome of the dorsal fin (30). In the present study, the high percentage of gill OTUs shared with the environmental samples (>75%) (Table S2) indicates that the gill microbiome may be influenced similarly by variation in the surrounding free-living microbial community.

It is also possible that the gill-specific microbiome is influenced by the unique functions of the gills in waste excretion, gas exchange, and body site-specific immune activity (35). For example, the degree to which the microbiome interacts with fish immune factors can differ between the skin and gills (36). Also, ammonia excretion in fish occurs primarily through the gills (37) and may select for ammonia-utilizing microbes (14). In the present study, the set of OTUs enriched in the gill niche did not include taxonomic groups canonically associated with ammonia oxidation (Table S6). However, analysis involving only the gill and intestine data sets (no environmental data sets) identified two OTUs of the ammonia-oxidizing Thaumarchaeota among the taxa most enriched in the gill compared to those most enriched in the intestine (Table S8). We caution, however, that the metabolic functions of the gill microbiome remain essentially unknown. Exploring these functions directly through metagenomics, physiological assays of gill tissue, or culture of the dominant gill-enriched microbes identified here is necessary to identify the chemical and ecological processes unique to the gill niche.
**Shared assembly processes.** Despite clear taxonomic differences, gill and intestinal microbiomes show similarities suggesting that these niches are influenced partly by the same organizing factors. Both niches appear to be structured jointly by processes operating at the level of the host individual. In recruits and adults and across different fish families, the gill microbiome of an individual, on average, shares 20 to 25% of its OTUs with the microbiome of the intestine (Table S2) and is more similar to the intestinal microbiome of the same individual than to that of a different fish (Table 3). This pattern can be explained if the gill microbiome exchanges members with the microbiomes of other body sites, potentially via the bloodstream. Indeed, a study of wild-caught snapper showed broad similarity between gill and blood microbiomes (38). Gill and intestinal microbiomes of the same fish may also be similar because they were colonized at the same time from the same free-living pool (in contrast to other individuals) or are jointly influenced by systemic host factors operating across body sites, potentially including the chemical or immune cell composition of host mucus (39–41). Indeed, microbes interact with the immune system in both the gills and the intestine, and the coating of microorganisms by certain immunoglobulins has been shown to be higher in these niches than at other microbiome sites (36, 42). This suggests the occurrence of similar immune interactions at these sites and raises the possibility of similar selections for specific microbes. However, it should be noted that our study could not distinguish between microbes that maintain longer-term residence in the host and more transient (allochthonous) members of the microbiome. It is possible that if they were distinguished, these two communities would exhibit differential patterns of relatedness across body niches (gill and intestine); this topic deserves further study.

For adult individuals, fish microbiome composition varied among host species grouped by diet category. This effect was greatest in the adult intestine, consistent with a recent analysis of freshwater fishes showing gut microbiome structuring based on trophic level (22). Interestingly, the gill microbiome of adults was also significantly, though much more subtly, influenced by diet category. This is consistent with the high beta diversity observed for both gill and intestine data sets from adult fish (Fig. 2 and 3). We caution, however, that diet is confounded by host phylogenetic identity (Fig. 1), and the high level of microbiome structuring in adult fish is potentially due to sampling across a wide range of diverse host types, each of which covaries with a range of variables (e.g., habitat use and host physiology) in addition to diet. Indeed, a prior study examining Atlantic salmon reared on various diets revealed no effect of diet variation on gill microbiome structure (43). In our study, microbiome composition also varied significantly among adult microbiomes partitioned according to host family (data not shown). Thus, although the data confirm a general effect of host specificity on gill microbiome assembly, we cannot definitively link this effect to variation in diet.

Although comparisons between recruit and adult samples should be interpreted with caution, as these two categories were not represented by exactly the same host taxa, the overall trends in this study suggest that processes of gill microbiome structuring may vary with life stage. In comparison to those of adult fish, the gill and intestinal microbiomes of juvenile recruits were less likely to vary significantly among hosts grouped by diet type (Fig. 4; Table S5). This is consistent with a stronger role for stochastic colonization of the microbiome in young fish, followed by enhanced selection for host-specific assemblages as fish age. A study of captive zebrafish showed that nonneutral processes, such as selection for the host environment, play a more dominant role in microbiome assembly in adults than in juveniles (44). This is perhaps not surprising, as the microbiome is presumably responsive to many of the processes that occur as fish age, including immune system development, increased specialization of the host diet, and exposure to different external environments (e.g., off reef versus on reef). Indeed, a prior study of wild-caught damselfish and cardinalfish indicated a significant shift in intestinal microbiome composition over the transition from pelagic larvae before reef settlement to postsettlement juveniles and adults (2). This transition...
may involve shifts in diet (45). Indeed, we know less about the diets of young fish, and our diet category designations were inferred from studies focused predominantly on adults. If microbiome assembly pressures change markedly over the host life span, then microbiome compositional variation between body sites may be less than the variation in the same body site sampled at different host life stages. In the present study, gill microbiomes of adults were more similar to intestinal microbiomes of adults (R = 0.11 by ANOSIM) than to recruit gill microbiomes (R = 0.36). This is intriguing given the presumably large differences in the chemistries of the gill and intestinal niches, notably with the latter influenced by incoming food. Together the data confirm that host factors influence the gill microbiome but likely vary over time, with a potentially greater overall effect on microbiome assembly in adult individuals.

Conclusions. The reef fish gill microbiome appears to be organized by a combination of stochastic and deterministic processes, including connectivity with the surrounding environment and host factors that vary with fish species and life stage, potentially including diet. Our results for paired gill and intestine data sets from the same individual provide the strongest evidence for microbiome organizing processes shared across body sites and also suggest a significant role for interindividual effects on microbiome assembly. Nonetheless, several microbial OTUs appear to be enriched consistently in the gill compared to all other studied niches. The results suggest these taxa as targets to identify microbial functions potentially specific to the gill niche. Although beyond the scope of this study, mining of these data sets, which span a broad spectrum of fish types, may also identify microbial taxa significantly associated with specific host types or ecological strategies. Thousands of microbial OTUs were detectable only in the fish gill or intestine, confirming these fish-associated niches as distinct reservoirs of marine microbial diversity. Understanding this diversity will inform our understanding of fish health and ecology as well as basic principles describing how host association structures microbiome assembly.

MATERIALS AND METHODS

Collection. Between 23 January and 15 February 2016, 207 adult fish and 55 juvenile recruits spanning 15 families and 53 species (Table 1; Fig. 1) were collected from 6 sites (see Fig. S1 in the supplemental material) around the island of Moorea, French Polynesia, via spear gun and hand nets (adults) or via crest net (recruits and some adults) (see references 46 and 47 for full descriptions of crest net methods). The exact capture locations of individual fish were not recorded. We therefore cannot discount the possibility of local variation in the water column microbiome as a determinant of gill/intestinal microbiome diversity; however, the relatively low beta diversity of water column microbiome samples from the different collection sites (Fig. 2) argues against a strong intersite effect.

All fish specimens were immediately euthanized either by pithing or by use of a lethal dose of tricaine mesylate (MS222) and placed directly on ice in coolers until arrival at the CRIOBE Research Station (typically 2 h later, and no longer than 6 h later). Each fish was measured (fork length by height) and photographed for identification (described below). Specimens were either dissected within ~12 h of capture (kept at 4°C or below until dissection), with most dissections performed within 2 to 5 h, or frozen at −20°C until dissection. All specimens were dissected in trays wiped with 100% ethanol. Due to their small size, which limited sample processing in the field, recruits were sorted according to species and date and frozen whole in 15-ml conical tubes filled with RNA/DNA stabilizing buffer (25 mM sodium citrate, 10 mM EDTA, 5.3 M ammonium sulfate, pH 5.2) until dissection at Georgia Tech. For both recruits and adults, either the entire gill or a gill fragment was dissected and placed in a cryovial, which was then filled with RNA/DNA stabilizing buffer. For each adult individual, we also dissected the entire intestine, which in teleost fish consists of the small intestine, where the majority of food digestion takes place, followed by the rectum; teleost fish lack the true large intestine present in other vertebrates. Adult fish intestines were strung out and cut in half, and the proximal half of each was preserved in a cryovial as described above. Specimens were either dissected within 10% ethanol in dry ice until dissection. Samples of seawater, sediment, coral, and dominant reef alga species (Chaetomorpha sp., Sargassum sp., filamentous turf) were also collected from each sampling site to characterize the microbiome of the surrounding environment. These samples are collectively termed “environmental samples” throughout this study. Three to 10 samples were collected per environmental sample type per site. For analysis of seawater microbiomes, ~0.5 liter of water was collected by bottle during dives (scuba or free). Bottles were placed on ice until return to the research station, where the water was filtered through a 0.2-μm isopore membrane filter (Millipore). Filters were
immersed in RNA/DNA stabilizing buffer in cryovials and frozen. Samples of sediment were scooped (without touching by hand) into sterile 50-ml Falcon tubes or Whirl-Pak bags and placed on ice until preservation. For preservation, residual seawater was decanted and replaced with RNA/DNA stabilizing buffer, and the sample was then frozen. To sample the coral microbiome, small (~1 cm²) fragments of massive Porites sp. coral (including mucus, tissue, and skeleton) were removed from the coral head by use of a hammer and chisel. Coral fragments were placed in a Whirl-Pak bag underwater and immediately placed on ice. The tubes were vortexed for 15 s to dispel intestinal content, the intestinal lining was subsequently removed, and extraction was performed following the manufacturer’s instructions. For algae, the entire original sample (filter plus algal fragment, coral fragment, and filter, respectively) was placed directly into a PowerBead tube, and extraction was performed following the manufacturer’s instructions. For algae, coral, and seawater samples, the entire original sample (filter plus algal fragment, coral fragment, and filter, respectively) was placed directly into a PowerBead tube, and extraction was performed following the manufacturer’s instructions.

**Host identification and diet type determination.** Where possible, host taxonomic identification was determined by comparing specimens to a standard reference book (48). For each identified taxon, the nucleotide sequence of the cytochrome c oxidase 1 (COI) gene was obtained from Fishbase (www.fishbase.org). Any fish that could not be identified was genotyped according to previously described methods (49). Briefly, the COI gene was amplified by PCR, using primers C_FishF1t1 and C_FishR1t1 in 25-μl reaction mixtures with gill DNA as the template (Table 5). PCR products were verified in a 0.8% agarose gel, visualized with GelRed (Biotium), and purified using an ExSap-IT PCR product cleanup kit (Affymetrix). Cleaned PCR products were then sequenced bidirectionally using Sanger technology. All generated and Fishbase-obtained sequences were then aligned using ClustalW (50) and used to estimate the host phylogeny by use of maximum likelihood (ML) with the Kimura two-parameter model of substitution in MEGA (51), with clade support assessed via bootstrapping (1,000 replicates). The ML topology was visualized using the Interactive Tree of Life (52). The broad diet category (omnivore, herbivore, or carnivore) for each host species was estimated based on a comprehensive literature review, the results of which are shown in Table S1.

**Microbiome community composition.** Microbial community composition was assessed by Illumina sequencing of the V3-V4 region of the 16S rRNA gene. The V3-V4 region was PCR amplified using primers F515 and R806 (53, 54), each appended with barcodes and Illumina-specific adapters as described previously (54) (Table 5). Reaction mixtures included 1 to 2 μl DNA template, 22 μl Platinum PCR SuperMix (Life Technologies), 0.5 μl (each) forward and reverse primers (total concentration, 0.4 μM), and 1 to 2 μl bovine serum albumin (BSA) (20 mg/ml; New England BioLabs Inc.). PCR conditions were an initial 3-min denaturation at 94°C, followed by 30 cycles of denaturation at 94°C (45 s), primer annealing at 55°C (45 s), and primer extension at 72°C (90 s) and then a final extension at 72°C for 10 min. Amplicon libraries were purified by use of Difinity RapidTip PCR purification tips (Difinity Genomics, NY), quantified on a Qubit machine (Life Technologies), and pooled at equimolar concentrations. Amplicons were sequenced on an Illumina MiSeq machine across six different runs, using a V2 500-cycle kit (250 × 250 bp) with 10% PhiX to increase read diversity.

Sequences were trimmed using Trim Galore, with the criteria of a minimum length of 100 bp and a Phred score of >25. Paired reads were then merged using FLASH (55), with the criteria of a minimum length of 250 bp per input read, a minimum length of 300 bp for merged fragments, and a maximum

### Table 5. Primers and associated sequences

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5'–3')</th>
<th>Reference(s)</th>
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</tr>
<tr>
<td>R806</td>
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</tr>
<tr>
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<td>FR1d_t1 ACCTCAGGGTGACCGAAGAATCAGAA&lt;sup&gt;b&lt;/sup&gt;</td>
<td>49</td>
</tr>
</tbody>
</table>

<sup>a</sup>Index and adapter sequences are not shown.

<sup>b</sup>M13 tails are not shown.
within-individual similarity was compared by use of the Bray-Curtis similarity, obtained by calculating the mean Bray-Curtis similarity for all possible pairwise gill versus intestine comparisons involving datasets from different individuals. We also conducted this analysis after reformatting in Phyloseq (58), and the program DESeq2 (59) was used to detect differentially abundant taxa. Sequences sharing 97% nucleotide similarity were clustered into operational taxonomic units (OTUs) by open-reference picking with the uclust tool (57), with taxonomy assigned to OTUs by comparison to the Greengenes database (56). Singletons were excluded from the analysis; all OTUs reported here are represented by at least two sequences. Sequences classified as “chloroplast” or not classified as “bacteria” or “archaea” were removed. QIIME OTU tables were used for further analyses as described below.

Statistical analyses. We assessed alpha and beta diversity values by using rarefied OTU data based on a uniform depth of 3,000 sequences (sequence counts per sample ranged from 3,073 to 234,562). Chao1 estimates of OTU richness (alpha diversity) were obtained through QIIME. Bray-Curtis dissimilarity matrices were calculated based on the rarefied OTU data and used to measure beta diversity as the distance from individual samples to the centroid of the sample type (i.e., dispersion), using PERMDISP in the program PRIMER 7 (Primer-E Ltd.). We tested for significant variation in both alpha (Chao1) and beta (dispersion) diversity among samples by using analysis of variance (ANOVA) followed by Tukey post hoc tests. The Bray-Curtis matrices were also used for nonmetric multidimensional scaling (NMDS) and an analysis of similarity (ANOSIM) to test for variation in community composition among sample groups, also in PRIMER 7. The latter involved a one-way ANOSIM using life stage (recruit and adult) and sample type (gill and intestine) as factors. Four separate one-way analyses were conducted for each sample type (recruit or adult × gill or intestine), using presumed diet category (herbivore, carnivore, or omnivore) (Table S1) as a factor.

To assess the potential of host-specific factors to influence microbiome structuring across body sites, we tested whether similarity between gill and intestinal microbiomes varies within versus between individuals. For this analysis, we used data only for individuals for which we had paired gill and intestine data sets (n = 24 for recruits; n = 110 for adults), using a uniform number of sequences (n = 3,000) per data set. The Bray-Curtis similarity between gill and intestinal microbiomes (evaluated at the OTU level) was calculated for each individual and then averaged across individuals (within individual). Mean within-individual similarity was compared by use of the t test (two-tailed) to between-individual similarity, obtained by calculating the mean Bray-Curtis similarity for all possible pairwise gill versus intestine comparisons involving data sets from different individuals. We also conducted this analysis after partitioning samples based on host taxon, focusing on the following three fish families represented by multiple individuals in both adult and recruit sample sets: Chaetodontidae, Pomacanthidae, and Holocentridae.

Nonrarefied count data were used to detect OTUs that differed significantly in proportional abundance among sample groups. For this analysis, the OTU table containing absolute sequence counts was reformatted in Phylloseq (58), and the program DESeq2 (59) was used to detect differentially abundant taxa. Nonrarefied data were used because the use of rarefied data for differential abundance testing has been shown to lead to false-negative results (lower power) due to the exclusion of data (60). DESeq2 performs an internal variance stabilization procedure that adjusts nonrarefied counts via a log-like transformation to account for variation in sequencing depth.

Accession number(s). All sequence data generated in this study have been deposited in the NCBI Sequence Read Archive under BioProject ID numbers PRJNA401167 and PRJNA401168.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.00063-18.

SUPPLEMENTAL FILE 1, PDF file, 1.2 MB.

ACKNOWLEDGMENTS

We thank A. Caughman for assistance with data management, P. Ranjan for the jackknife perl script, and R. Brooker, W. Feeney, L. Johnston, D. Dixon, and V. Liao for help with sample collection.

This work was supported by Simons Foundation award 346253 (to F.J.S.) and by the Teasley Endowment to the Georgia Institute of Technology.

We declare that we have no competing commercial or financial interests.

Z.A.P. and F.J.S. designed the study. Z.A.P. and M.B. designed the sampling strategy and collected samples. Z.A.P. and R.D.H. processed samples. Z.A.P. and F.J.S. analyzed and interpreted data and prepared the manuscript. All authors read and approved the final manuscript.

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