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Article

Long-Read Sequencing Unlocks New Insights into the *Amphidinium carterae* Microbiome

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Abstract: Dinoflagellates are one of the largest groups of marine microalgae and exhibit diverse trophic strategies. Within these niches, dinoflagellates can produce secondary metabolites that are known to be toxic, which can lead to ecologically harmful blooms. *Amphidinium carterae* is one species of dinoflagellate that produces toxic compounds and is used as a model for dinoflagellate studies. The extent to which the microbiome surrounding *A. carterae* affects its growth and secondary metabolite synthesis is still being assessed, as well as the impact of bacterial data on sequencing and assembly. An antibiotic cocktail was previously shown to eliminate 16S amplification from the dinoflagellate culture. Even with drastically reduced bacterial numbers, bacterial populations are likely to be present. In this experiment we used novel Nanopore long-read sequencing techniques on *A. carterae* cultures to assemble 15 full bacterial genomes ranging from 2.9–6.0 Mb and found that the use of antibiotics decreased the percentage of reads mapping back to bacteria. We also identified shifts in the microbiome composition and identified a potentially deleterious bacterial species arising in the absence of the antibiotic-treatment. Multiple antibiotic-resistance genes were identified, as well as evidence that the bacterial population do not contribute to toxic secondary metabolite synthesis.

Keywords: dinoflagellate; microbiome; long-read sequencing

1. Introduction

Dinoflagellates are unicellular organisms, which occupy multiple trophic levels in marine ecosystems, ranging from photo-autotrophy, predation, mixotrophy, to even parasitism [1–3]. They are a significant component of phytoplankton communities, responsible for producing a large portion of the world's oxygen and serve as the primary food source for a wide array of marine organisms, including zooplankton, small fish, and filter feeders [4–6]. Their ability to photosynthesize and sometimes feed enables them to thrive in various environmental conditions, making them vital for maintaining and disturbing ecological balance and supporting biodiversity in aquatic habitats.

Some species of dinoflagellates are also known to cause harmful algal blooms (HABs), which can have devastating ecological and economic impacts [7–9]. These blooms occur when dinoflagellate populations grow explosively under favorable conditions, producing toxins that can kill fish, shellfish, and other marine life, and pose serious health risks to humans through seafood consumption or direct exposure. The toxins can also lead to significant economic losses in fisheries, tourism, and public health sectors. On a broader level the single-species dominance of a bloom influences the microbial community both directly and indirectly.

Many dinoflagellate cultures contain abundant bacterial populations, likely originating from the original sample isolated from the environment [10–12]. The role of bacterial populations on dinoflagellate toxin production is one that remains hotly debated, with prior research showing evidence of dinoflagellate dependence on bacteria for toxin-production and others showing the opposite [13–18]. Some of these studies involve the filtering of bacteria from dinoflagellate cultures, followed by null amplification of 16S regions as verification to prove a culture is axenic, and subsequent high-performance liquid chromatography (HPLC) analysis to show if toxin is still produced in the axenic culture [19–21]. Results of these studies have been contradictory.

Currently, consistent culturing of dinoflagellate cultures with antibiotics is a common practice to select for organisms of interest and maintain monoclonal conditions. Eukaryotic cultures can be maintained with antibiotics to decrease the effects of bacterial populations on downstream analysis such as sequencing efforts, toxin analysis, and translation rate studies [22]. Although antibiotic use in dinoflagellate culturing can be useful, there is concern about bacterial antibiotic resistance allowing for re-growth of bacterial populations, as well as the impact antibiotics can have on the growth of the dinoflagellate culture.

We propose a new approach to investigate bacterial contributions to dinoflagellate cultures, by way of full bacterial genome assembly from long-read sequencing to identify pathways present in those bacterial populations, determine microbial taxonomic composition, and estimate relative abundance [23,24]. Here, we use this approach in a toxin-producing *Amphidinium carterae* CCMP1314 culture with a long history of laboratory growth in the presence of carbenicillin, kanamycin A, and spectinomycin antibiotics. This approach provides a potentially more complete, unbiased measure of bacterial diversity and metabolism within the *A. carterae* culture with and without ongoing antibiotic treatment.

Amphidinium carterae was chosen for this study firstly because of its cosmopolitan appearance in nature: it is one of the most common species found in sediments in multiple ecosystems [25–27]. Cultures of *A. carterae* are also easy to maintain and are known to grow to relatively high density compared to other dinoflagellate species [28,29]. This species is also often used as a model athecate photosynthetic peridinin-pigmented dinoflagellate due to a smaller genome compared to other free-living dinoflagellate species, and because it is a relatively early diverging toxic species [30].

In this study we have found populations of bacteria in an *A. carterae* culture that have thrived in the presence of antibiotics, identified antimicrobial genes within these populations, and observed how the dinoflagellate culture responds when reverted back to antibiotic-free growth conditions.

2. Results

2.1. Bacterial Growth

The growth of *A. carterae* cultures grown both with and without antibiotics was found to be similar during both lag and log phases, with daily maximum growth rate being about $63 \pm 9\%$ and $65 \pm 9\%$ for both treatments, respectively. Maximum cell densities were similar, with the antibiotic-free culture reaching $142,166 \pm 15,382$ cells mL⁻¹ and the antibiotic-treated culture reaching $126,000 \pm 1311$ cells mL⁻¹. Growth differed between antibiotic and control treatments once the stationary phase began. In the case of the antibiotic-treated culture, cell growth became stagnant and cell density remained relatively the same, while the cultures grown without antibiotics began to show significant decreases in cell number and signs of lysing and death under microscopy (Figure 1A). Cells within the antibiotic-free culture appeared to form aggregates, and dinoflagellate cells imaged at the beginning of stationary phase in the antibiotic-free cultures displayed mass lysing and the appearance of rod-shaped bacteria overwhelming the culture (Figure 1B), alluding to algicidal properties. These bacteria were not observed by microscopy in the antibiotic-treated cultures.

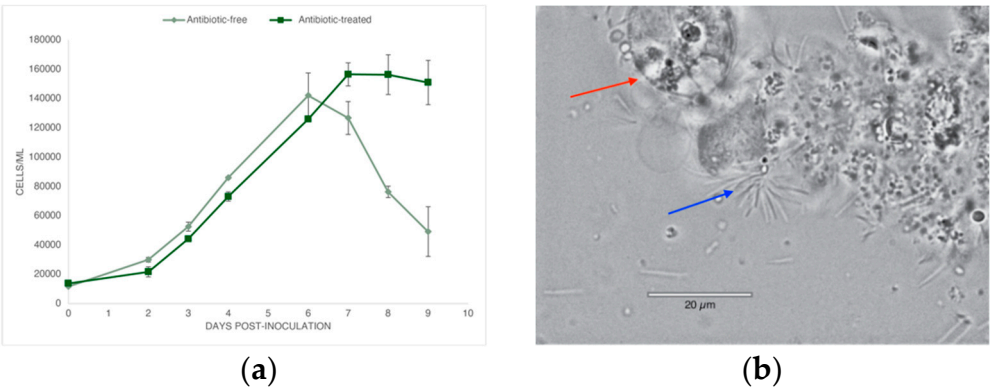


Figure 1. *Amphidinium carterae* growth with and without antibiotics (a) growth curves of *A. carterae* cultures; (b) Image of lysed *A. carterae* (red arrow) taken at 100X, and presence of rod-shaped bacteria (blue arrow).

2.2. Bacterial Identification

From the *A. carterae* cultures, metagenome assembly of 15 bacterial contigs from 2.9 to 6.0 Mb were identified based on 16S rDNA identity (Table 1). All 15 were single contig genomes and the *Flye* assembly reported 14 of the 15 being circular (all but *O. alexandrii*). Nearest neighbors of the 16S sequences found in these genomes gave consensus to their identification from 98 to 100% identity (Supplementary Figure 1A). The bacteria present included 10 Alphaproteobacteria, 1 Gammaproteobacteria, 3 Bacteroidia, and 1 Planctomycetota. Based on the very high (>98%) full-length 16S rDNA identity to previously described species the names of these high identity 16S rDNA matches are used as an operational taxonomy for the remainder of the text.

Within the 15 genomes between 2,890 and 5,758 genes were annotated; of these 43-70% had functional assignments according to BV-BRC (Supplementary Table 2A). Genome quality was qualified as good for all of the genomes, with CheckM Completeness ranging from 77.5% to 100%, and a majority of the genomes showing above 90% completeness [31]. CheckM Contamination scores were below 5% for all the genomes as well.

Table 1. Bacterial genome characteristics.

NCBI Sample Title	length	AT%
<i>Labrenzia</i> sp. strain ac12	6,053,707	40.9
<i>Hoeflea alexandrii</i> strain ac4	4,829,646	38.4
<i>Algihabitans albus</i> strain ac2	4,739,774	34.3
<i>Ahrensia marina</i> strain ac1	4,424,055	42.8
<i>Seohaecicola saemankumensis</i> strain ac14	4,275,009	36.6
<i>Roseovarius mucosus</i> strain ac13	3,969,289	38.9
<i>Rhodophyticola porphyridii</i> strain ac11	3,902,216	35.8
<i>Oceaniradius stylonematis</i> strain ac10	3,664,187	35.3
<i>Oceanicaulis alexandrii</i> strain ac9	2,992,841	37.7
<i>Nitratireductor</i> sp. strain ac15	2,917,504	39.8
<i>Marinobacter adhaerens</i> strain ac5	4,424,055	42.9
<i>Cyclobacterium xiamenense</i> strain ac3	5,806,256	51.6
<i>Marivirga tractuosa</i> strain ac6	4,787,102	65.3
<i>Muricauda</i> sp. strain ac8	4,366,883	58.0
<i>Phycisphaeraeaceae</i> SM1A02 strain ac7	3,415,114	34.9

¹ 16S sequence identification found using both the SILVA ACT service and NCBI BLASTn feature [32]. [‡] Contig 2139 was 'Unclassified' according to SILVA but identified by NCBI as an Alphaproteobacteria.

2.2. Bacterial Abundance

In the antibiotic-free cultures 52.2 % of the total bases and 49.9 % of reads mapped to the 15 bacterial genomes while in antibiotic-treated treated cultures 39.8% of total bases and 33.5% of reads mapped to bacteria (Figure 2a). Ten of the 15 bacterial genomes identified account for 90% of the total bacterial reads mapped in both conditions (Figure 2b) and the remaining five taxa accounted for <10% of total bacterial reads.

All 15 bacteria were detectable at some level in all the batches of sequencing data, but the relative abundance differed substantially for 7 bacterial genomes. Under antibiotic treatment *R. mucosus* was the most abundant single bacterium (at 53%) and then decreased to 6.16% without antibiotics. Without antibiotics, *Muricauda* sp., *Marivirga tractuosa*, *Ahrensia marina*, and *Seohaecicola saemankumensis* rose to the top 90% of the bacterial reads. Finally, when antibiotics were in use, populations of *O. stylonematis* and *C. xiamenense* were found in the top 90% of bacterial reads but were not abundant under antibiotic treatment.

The antibiotic-free and antibiotic-treated cultures both included 3 taxa at roughly similar proportions: *SM1A02*, *O. alexandrii*, and *A. albus*. When cultures were grown without antibiotics, the abundance of *SM1A02* and *O. alexandrii* slightly increased by 2.6 and 1.3%, while *A. albus* slightly decreased by 2.8%. The remaining 5 bacterial genomes were present at low levels in both culture conditions.

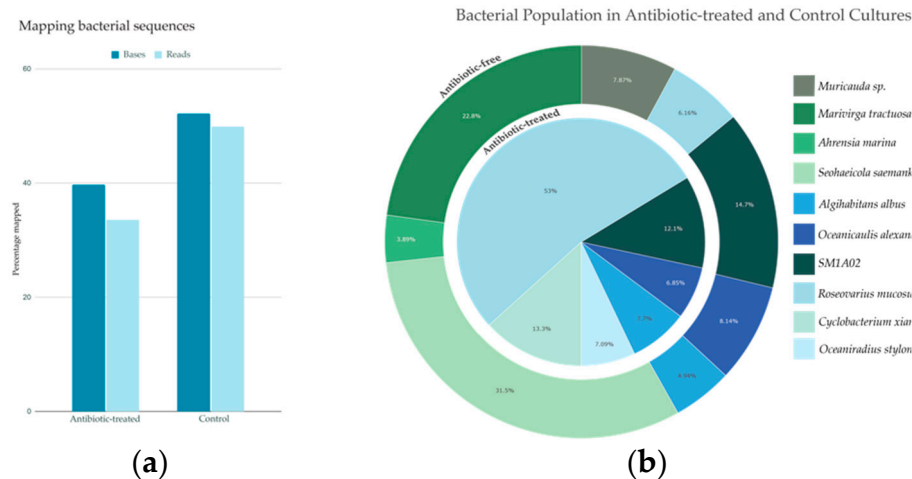


Figure 2. Bacterial populations with and without antibiotics (a) Percent of reads and bases mapping back to bacterial genomes from both the antibiotic-free and antibiotic-treated cultures, aligned using minimap2; (b) Bacterial abundance based on read mapping in the antibiotic-free and antibiotic-treated cultures.

2.3. Bacterial Metabolic Pathways

The metabolic abilities of the bacterial genomes were assessed using the KEGG database, showing a wide variety of potential pathways (Figure 3, Supplementary Table 3A). Many of the bacterial genomes have potential pathways for the production of amino acids, such as arginine, proline, serine, and threonine. Basic metabolic pathways such as carbohydrate metabolism and ATP synthesis were identified. Some of the bacterial genomes, including *R. mucosus*, *R. porphyridii*, *H. alexandrii*, *O. stylonematis*, *A. marina*, and *A. albus* were found to potentially contain photosynthesis machinery for photosystem II, as well as some carbon fixation pathways. The *Labrenzia* sp. genome, although the largest bacterial genome assembled, was also in low abundance in the antibiotic-treated culture and contained part of the reductive phosphate pentose cycle and the Calvin cycle, but not PSII.

Nitrate reduction pathways were found in 3 of the bacterial genomes, and denitrification machinery was identified only in the *M. tractuosa* genome. The *Nitrateductor* sp. found in low

abundance the antibiotic-treated culture, was negative for nitrate-reduction pathways despite the genera name, which aligns with prior research [33].

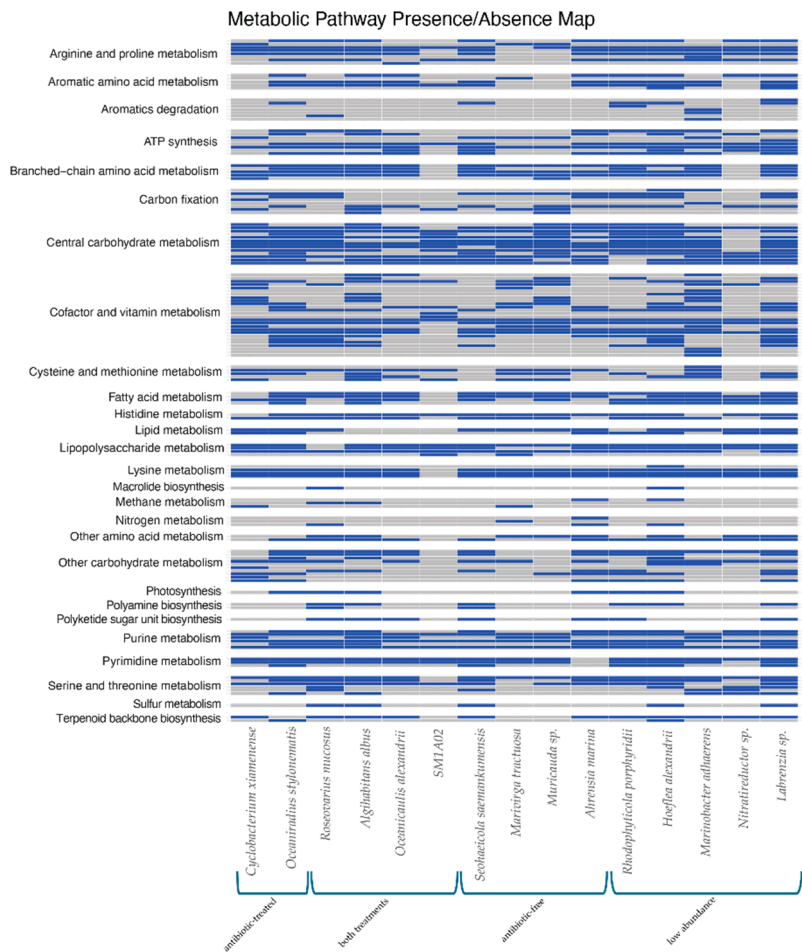


Figure 3. Functional traits identified via CARD based on the KEGG database [34]. Specific pathway names for each row are listed in Supplementary Table 3A.

2.4. Secondary Metabolite Synthesis

The assembled bacterial genomes were found to contain regions associated with the production of a variety of secondary metabolite compound classes, including building-block lactone structures such as butyrolactones, betalactones, and hserlactones (Figure 4). Terpenes and aromatic compound synthase genes were the major natural product occurring amongst the genomes. Some polyketide synthases (PKSs) and non-ribosomal peptide synthases (NRPSs) were identified, but none were predicted to participate in the biosynthesis of larger chemical structures according to antiSMASH.

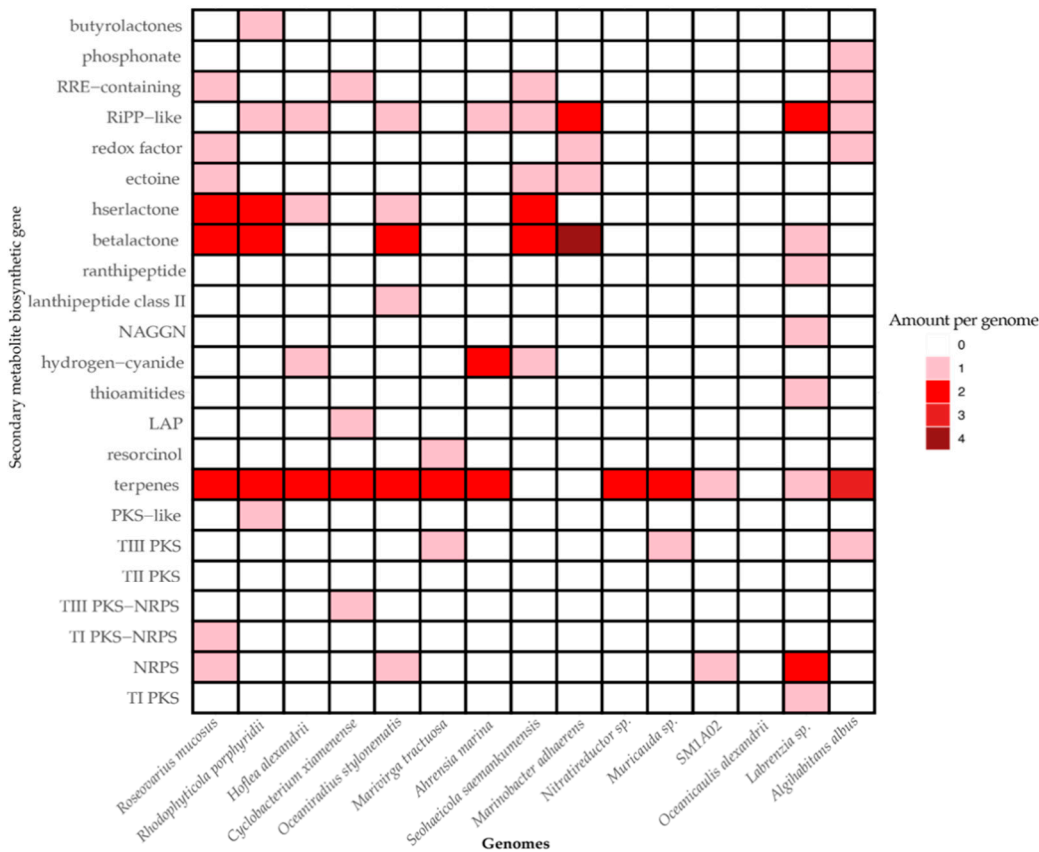


Figure 4. Secondary metabolites present in the bacterial genomes, identified by antiSMASH [35].

2.5. Antibiotic-Resistance

Generally, all of the bacterial genomes had antibiotic resistance genes in the CARD database search (Figure 5A). Interestingly, all of the genomes have a large number of resistance genes to tetracycline. The antibiotic resistance genes in the bacterial genomes found in the antibiotic-treated cultures do not appear to indicate a strong pattern of drug class resistance to the antibiotic cocktail used. Most of the antibiotic resistance genes were part of antibiotic efflux systems, especially in the assembled *Labrenzia* sp. genome (Figure 5B). This was generally followed by mechanisms including drug target alterations, reduction of drug permeability, drug inactivation, drug target protection, and drug target replacement.

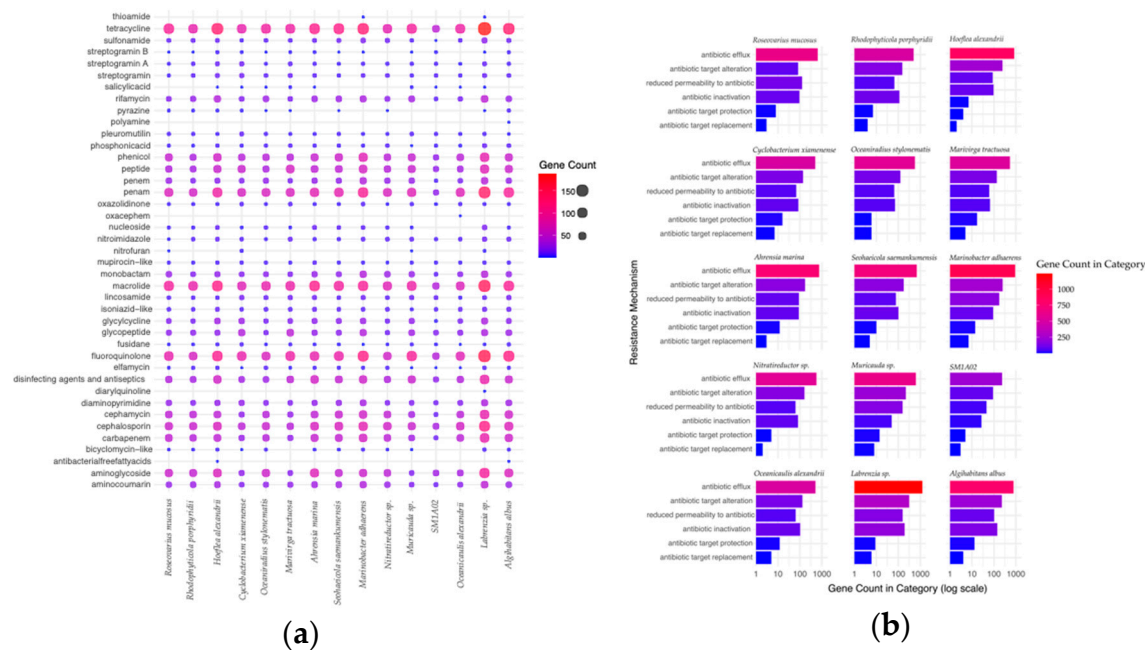


Figure 5. Antimicrobial resistance in assembled microbiome (a) Antimicrobial resistance genes within each assembled bacterial genome (b) Specialty genes identified from the CARD database [36].

The dominant bacterial genome in the antibiotic-treated culture, *R. mucosus*, contained 13 resistance genes that were either completely unique to the taxa, or only shared with other bacterial genomes found in the treated culture (Supplementary Table 2A). Of those 13, nine had the potential to confer resistance to carbenicillin, kanamycin A, and/or spectinomycin (Figure 6). Three of these genes encoded potential beta-lactamases, which inactivate beta-lactam antibiotics by hydrolyzing the peptide bond of the beta-lactam ring and may confer resistance to carbenicillin. One of the genes is similar to the efflux subunit of the AmrAB-OprM multidrug efflux complex, which impairs aminoglycoside accumulation. Lastly, 5 genes in the *R. mucosus* genome were identified as potential aminoglycoside acyltransferases, which may catalyze the AcCoA-dependent N-acetylation of amino groups on aminoglycoside molecule (such as kanamycin A and spectinomycin).

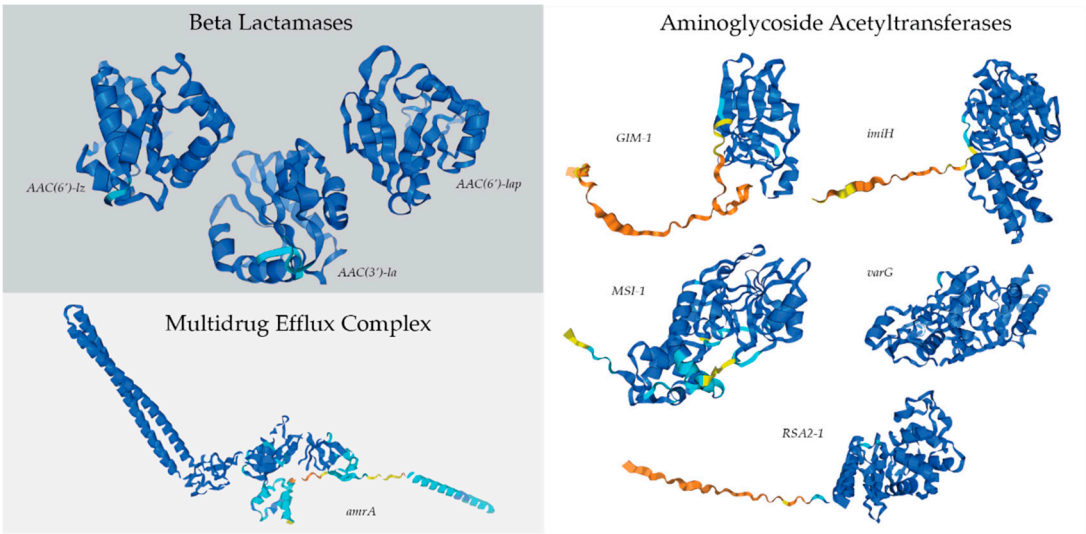


Figure 6. Antibiotic resistance genes protein structure predictions created by AlphaFold, unique to the assembled *Roseovarius mucosus* genome [37,38].

3. Discussion

Of the 15 genomes assembled every genome has a high (>95%) identity 16S rDNA match to an existing annotated sequence suggesting that the culture does not contain any entirely novel bacteria. Many of the assembled genomes were identified as bacterial species that have previously been observed as co-existing with dinoflagellates. Interestingly, the Planctomycetota bacteria assembled was identified as *SM1A02*, an uncultured strain associated with many dinoflagellate cultures [39]. This species' genome has previously been reconstructed using metagenomic assembly and binning. Research by Baker et al. found two of these *SM1A02* genomes to be 2.6 and 2.9Mb, while we have assembled a 3.4Mb genome here. Most recently, an assembly of *SM1A02* was produced from a *Karlodinium* culture which agrees with our genome length of 3.4Mb [40]. *SM1A02* is thought to likely be an anammox bacteria – efficient at nutrient removal, specifically through anaerobic ammonium oxidation [41]. This ability to oxidize ammonium to nitrogen gas may have an impact on the close association with dinoflagellate species.

Roseovarius mucosus was also identified, similar to a species found with the dinoflagellate *Alexandrium ostenfeldii*. In prior research, genes in *R. mucosus* were found that may play crucial roles in the interrelationship of the bacterium and dinoflagellate, such as genes for dimethyl sulfoniopropionate (DMSP) utilization. Research on the close interactions of DMSP-degrading *Roseobacter* species with DMSP-producing dinoflagellates are well-established [42]. Our metabolic results show *R. mucosus* likely has pathways for thiosulfate oxidation, supporting these past findings [43]. The ability for thiosulfate oxidation may also have a connection to the common sulfation of toxic amphidinol products produced by *A. carterae* or more broadly for sulfur cycling within the cultures [44,45]. The pathway for assimilatory nitrate reduction found in the *R. mucosus* genome has been observed as a potential nitrogen source by some [46,47]. The pathway for polyamine biosynthesis for putrescine and spermidine was also found in the *R. mucosus* genome, which may play an essential role in dinoflagellate growth [48–50]. The presence of PSII and other carbon-fixing pathways also aligns well with prior research of this species [51].

Hoeflea alexandrii and *Oceanicaulis alexandrii* were discovered with a dinoflagellate species of *Alexandrium* as well as bacterial species *Labrenzia alexandrii* and *Nitratireductor alexandrii*, which may be closely related to the *Labrenzia* and *Nitratireductor* species assembled here [52–55]. *Marinobacter adhaerens* has been found in close association with *Pyrodinium*, another toxin-producing dinoflagellate [56]. *Muricauda* species have been previously associated with *Amphidinium* as well [57]. A *Seohaecicola* species genome was recently assembled from a culture of *Karlodinium*, another toxin-producing dinoflagellate species [40]. Some species such as *O. stylonematis*, *A. s albus*, *C. xiamenense* and *R. porphyridii* were discovered in association with other kinds of microalgae such as diatoms and red algae [58–62].

Some species found only in the antibiotic-free cultures may be opportunistic due to the nutrient availability caused by dinoflagellate senescence. Both the assembled *R. porphyridii* genome and the *H. alexandrii* genome were found in very low abundances in the antibiotic-free culture. The *R. porphyridii* species in a genus of purple non-sulphur bacteria known to be halophilic and have the ability to perform photosynthesis [58]. Thiosulfate oxidation pathway genes were also found in the *H. alexandrii* genome.

The transition from antibiotic treatment to untreated is unlikely to have resulted in the introduction of new bacteria to the culture as all taxa were seen and could be at least partially assembled in sequencing of either antibiotic treated or untreated culture. Thus, the microbiome shift is more likely due to stronger growth of some species over others when antibiotics were present or absent rather than recruitment of novel species during culture changes, which were performed under sterile conditions.

Previous studies have demonstrated that use of the KAS-antibiotic treatment (with kanamycin A, ampicillin, and streptomycin) can be used to favor pigmented bacterial species [63]. Similar mechanisms may be why we saw such a shift towards bacterial populations with PSII and carbon fixation systems with the use of our antibiotic cocktail, from 12.5% to 50% of the highly abundant bacterial species having PSII pathways with the antibiotic-treatment. The *R. mucosus* identified in past

dinoflagellate cultures, which dominated the antibiotic-treated microbiome in this experiment, has been shown to contain bacteriochlorophyll *a* [51]. There is also evidence of dinoflagellates protecting certain pigmented bacterial populations from antibiotics as the pigmented bacteria may be protecting the microalgal cells from light stress via carotenoid production, which was previously shown to be produced by multiple assembled bacterial species (Figure 4) [63]. In prior research regarding coral symbiont dinoflagellate *Symbiodinium*, the bacterial microbiome was observed to support the dinoflagellate's PSII yield and decrease the production of reactive oxygen species (ROS) [64].

Antimicrobial resistance appears to be generally common amongst bacterial populations found in dinoflagellate cultures. In the case of the assembled genomes here, all of them had hundreds of potential antibiotic-resistance genes that likely allow their broad prevalence (Figure 5). The reasons for why some bacteria were found to survive better in antibiotic-treated or antibiotic-free conditions could be due to multiple causes. One may be that the minimum inhibitory concentration of the antibiotic used may differ from the actual concentration tested [65]. This may be due to the mechanism of resistance, or the antibiotic's resistance to degradation (such as in the case of carbenicillin compared to ampicillin) [66]. The specific genes found within each of these resistance groups may have varying efficacy against the antibiotics as well. In the case of *R. mucosus*, which best-endured the antibiotic-treatment used here, it may be that one of the nine antibiotic-resistant genes has greater efficacy over one or more of the antibiotics used compared to the machinery found in the other genomes (Figure 6).

The extent to which the bacteria from the assembled genomes are mutual, commensal, or deleterious to the *A. carterae* population is still obscure. The fact that the dinoflagellate population significantly decreased and showed signs of mass lysis without antibiotics leads us to believe that at least one abundant bacterial species in the antibiotic-free culture is likely the cause. Prior research on antibiotic effects on dinoflagellate growth have shown various results. In some cases, dinoflagellates appear to require their associated microbiomes to survive [11,67]. In the case of the antibiotic cocktail used here, the growth results align with previous observations of the antibiotic-treated *Amphidinium* cultures having a slightly extended growth phase and the ability to maintain higher densities of dinoflagellate cells [22]. Based on our microscopy analysis, we suspect the 10µm length rod-shaped bacteria that began to accumulate around the start of mass-cell-lysis is likely harmful to the dinoflagellates and may possess some algicidal properties. We could deduce the culprit may be *Marivirga tractuosa* or *Seohaecicola saemankumensis* due to the increased abundance found in the antibiotic-free culture (Figure 2B), as well as prior descriptions of this species being rod-shaped. Cells of *M. tractuosa* can be between 10-50µm length, while *S. saemankumensis* has been shown to be up to 5µm in length [68–70]. The *M. tractuosa* genome lacks many main metabolic pathways, such as amino acid biosynthesis suggesting this species requires resources gained from the lysed dinoflagellate cells. The *M. tractuosa* genome was also found to have complete denitrification pathways, which may contribute to a loss of bioavailable nitrogen in the culture [71]. The Coenzyme M pathway alludes to methanogenic abilities and production of methane, and potentially the use of dinoflagellate-released DMSP as a precursor [72,73]. Harmful algal blooms have been observed to precede methane increases in aquatic environments, which may be in part due to the shift in microbial community [73].

Prior research has been contentious over the secondary metabolite synthesis potential of dinoflagellate microbiomes, and contrasting results have identified toxin production as a product either of the bacterial community or the dinoflagellate cells themselves [13–17,74–77]. The fifteen apparently complete genomes assembled from this culture likely represent the bulk of prokaryotic diversity due to the extent of our sequencing and the production of full, well-covered genomes. Any missing diversity would likely be in very low abundance to evade detection and are not likely to be present at a level to contribute to toxin biosynthesis. Similarly, the gene annotation provides a potentially complete picture of the culture metabolic potential. However, a large fraction of predicted genes was unannotated, likely due to imperfect prediction of protein coding genes as well as knowledge gaps of every possible bacterial pathway. Our genome analysis into secondary metabolite synthesis has shown no evidence of potential bacterial origin for a processive multidomain PKS gene responsible for the amphidinol toxins associated with our *A. carterae* culture. Since multidomain,

processive PKS genes are very large open reading frames rich in easily defined conserved domains these genes would be unlikely to have been missed in the genomes described here. Several multidomain PKS / NRPS genes derived from bacteria are present broadly across core dinoflagellate transcriptomes which generally express a surfeit of domains associated with toxin production and lipid synthesis whether or not they are document toxin-producing species [18]. More research could be done to see what effect metabolic pathways, such as thiosulfate oxidation, may have on toxin production. Although it seems most likely that *A. carterae* independently synthesizes amphidinols, the bacterial populations may contribute resources for the task, such as acetate [45,78,79]. Of the bacterial genomes assembled, only *M. adhaerens* was shown to have a complete pathway for a phosphate acetyltransferase–acetate kinase pathway which produces acetate from acetyl-CoA (Figure 3). *Oceaniradius stylonematis*, *S. saemankumensis*, *R. porphyridii*, *H. alexandrii*, and the *Labrenzia* sp. all had complete phenylacetate degradation pathways to produce acetyl-CoA which may serve as a precursor to acetate synthesis, and *O. stylonematis*, *S. saemankumensis*, *R. porphyridii*, *R. mucosus*, *A. albus*, *O. alexandrii*, *M. adhaerens*, and the *Labrenzia* sp. had complete pathways for leucine degradation to acetyl-CoA as well.

The microbiome of algal species has been shown to contribute necessary vitamins and products to dinoflagellate species, the most recognized being cyanocobalamin (B12) [80]. Based on our metabolic findings, the introduction of B vitamins into dinoflagellate growth media does not appear to be redundant with the biosynthetic abilities of the microbiome. The vitamins added to our ESAW media preparation include biotin (H), B12, and thiamine (B1) to increase growth rate and final yield [81]. Of the bacterial genomes assembled, only the *M. adhaerens* genome had a pathway identified to synthesize biotin, and this species was in very low abundance. The only highly abundant species with aerobic and anaerobic pathways for the synthesis of B12 were *R. mucosus* and *O. stylonematis*, which were significantly more abundant in the antibiotic-treated cultures, and their decline without antibiotics may have been a factor in the cell mortality of *Amphidinium* as the nutrients in the culture diminished over the log phase. Only *A. albus* within the highly abundant bacterial species had a pathway for vitamin B1 synthesis, and yet this was only through a salvage pathway which utilizes precursors or similar compounds in the surrounding media for B1 biosynthesis [82].

The Planctomycetota bacteria *SM1A02* is the only assembled genome in the *Amphidinium* culture to have full pathways for menaquinone (vitamin K2) biosynthesis. Although vitamin K1 is conventionally known as a redox cofactor in plants and green algae, vitamin K2 can also be a secondary electron acceptor of PSI in some algal and archaeal species [83]. Vitamin K2 can also shuttle electrons between different respiratory complexes in anaerobic respiration or aerobic respiration in a microaerophilic environment [84]. The effect of vitamin K2 bioavailability for dinoflagellate species remains to be seen.

4. Materials and Methods

4.1. Cell Culturing

Cultures of *Amphidinium carterae* were grown in ESAW artificial marine media with a salinity of 20 ppt supplemented with f/2 nutrients without silicates at a starting concentration of 10K cells mL⁻¹ and allowed to acclimate for 14 days in 14:10 h light:dark period in 100 μmol photons m⁻² sec⁻¹ at 25°C [81]. Cultures have been continuously grown with an antibiotic cocktail of 100 μg/mL carbenicillin, 50 μg/mL kanamycin A and 50 μg mL⁻¹ spectinomycin (“antibiotic-treated”). For analysis, some cultures were weaned off growth in the presence of antibiotics over a month before subsequent DNA extraction (“antibiotic-free”). Cell counts were done using a Scepter 3.0 Cell Counter (Figure 1).

4.2. DNA Extraction and Long-Read Sequencing

For each dinoflagellate culture, the cells were spun down at 10,000 × g for 10 minutes to form cell pellets. Cell pellets were then resuspended in 0.1M EDTA and 0.5% SDS with 200 µg proteinase K and allowed to incubate for 10 minutes at 50°C. Following this incubation, equal 16.6% volume of 2% CTAB and 5M NaCl were added and once again the sample was allowed to incubate at 50°C for 10 minutes. After this, the solution was mixed 1:1 with chloroform and allowed to sit at room temperature for 10 minutes. The sample was then spun for 10 minutes at 10,000 × g and the aqueous layer was transferred to a new tube and mixed with binding buffer from the DNA Clean & Concentrator kit (Zymo Research). Aliquots of 750ul were spun through the columns, followed by washing and finally elution in 20-40 µL of water. Small fragments were filtered out using the PacBio SRE/SRE-XL kit according to manufacturer's directions. A high molecular weight DNA library was then prepped using the Nanopore whole genome sequencing kit (SQK-LS114), followed by sequencing on a MinION, GridION, or PromethION device (Supplementary Table 1A).

4.3. Genome Assembly

Following sequencing, DNA data in pod5 files were basecalled by Oxford Nanopore's Dorado basecaller (7.2.13) using the dna_r10.4.1_e8.2_400bps_sup@v4.3.0 model. Sequences from the antibiotic-free culture runs or antibiotic-treated runs were pooled and assembled using the Flye *de novo* assembler (2.9.1-b1780), filtering for sequences over 200 bases [85]. Bacterial contigs were identified using BLASTn for 16S sequences from NCBI's 16S RefSeq or 16S microbial ribosomal databases. Bacterial matches from each assembly were identified, and duplicate matches between the antibiotic-free and antibiotic-treated runs were compared, and the most complete bacterial genomes were chosen for further analysis.

4.4. Phylogenetic Analysis

Once the bacterial genomes were identified, 16S sequences were extracted and identified using SILVA's Alignment, Classification, and Tree (ACT) service according to the global SILVA alignment for rRNA genes, as well as through NCBI's BLASTn feature [32]. A consensus of the two was chosen based on match percentage. A phylogenetic tree was constructed using RaxML including 10 nearest neighbors identified by SILVA ACT from the database (Supplementary Figure 1A).

4.5. Read Abundance Mapping

To quantify the relative abundance of bacterial genomes within the antibiotic-free and antibiotic-treated cultures, reads from each condition were aligned to the single unified set of bacterial contigs using minimap2, using ordinary minimizers as seeds [86]. Alignment SAM files were converted to BAM using Samtools, then sorted, indexed, and finally coverage and stats files were created [87]. Genomes with greater than 90% coverage were considered present in the cultures, and abundance was calculated by the percent of reads mapping to that genome out of the total bacterial reads mapped in each condition.

4.6. Metabolic and Secondary Metabolites Analysis

Metabolic and biogeochemical functional trait profiles for each bacterial genome were assessed using METABOLIC-G using the KEGG database for pathway identification [34]. Genome sequences were also input into antiSMASH for secondary metabolite biosynthetic gene clusters search [35].

4.7. Antimicrobial Resistance Analysis

The Comprehensive Antibiotic Resistance Database (CARD) Resistance Gene Identifier (RGI) software was used for the prediction of antibiotic resistance genes in the bacterial genomes [36]. Perfect, Strict and Loose hits for genes were allowed but partial genes were excluded. Protein

predictions of antimicrobial resistance genes of interest were created using the AlphaFold Google DeepMind AI system [37,38]

5. Conclusions

Although *A. carterae* may be grown without antibiotics, the viability of the culture and subsequent sequencing analyses may benefit from their use. We observed die off of cells in the *A. carterae* cultures during the end of the log phase without antibiotics present, likely due to proliferation of one of the more antibiotic-sensitive bacteria identified. We also found a decrease in the proportion of bacterial reads sequenced with the use of antibiotics. As novel sequencing technologies, such as efficient long-read sequencing, becomes more readily available, the use of techniques to target sequences of interest will allow for more productive sequencing efforts. The data suggest both comprehensive and quantitative microbial genome sequencing can be accomplished from this culture, which could in future be expanded to work with in situ sequencing of dinoflagellate blooms.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org, Figure S1: 16S Phylogenetic Tree; Table S1: Sequencing Runs; Table S2: Basic Stats on Bacterial Genomes; Table S3: METABOLIC Pathway Identifiers; Table S4: *Roseovarius* Unique AMR Genes;.

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Conflicts of Interest: The authors declare no conflicts of interest.

Appendix A

Table A1. Nanopore sequencing runs.

Antibiotic-Free			Antibiotic-Treated		
Date	Reads	Device used	Date	Reads	Device used
2/1/2024	86,499	GridION	5/10/2023	122,303	MinION
2/2/2024	62,172	GridION	5/12/2023	59,113	MinION
2/3/2024	1,615	GridION	5/13/2023	180,995	MinION
3/15/2024	295,191	GridION	5/16/2023	222,574	MinION
3/17/2024	188,915	GridION	5/17/2023	152,303	MinION
3/18/2024	152,194	GridION	10/30/2023	3,345,955	PromethION
Total Reads		786,585			4,083,243
Total Bases		23,622,147,065			151,572,689,672

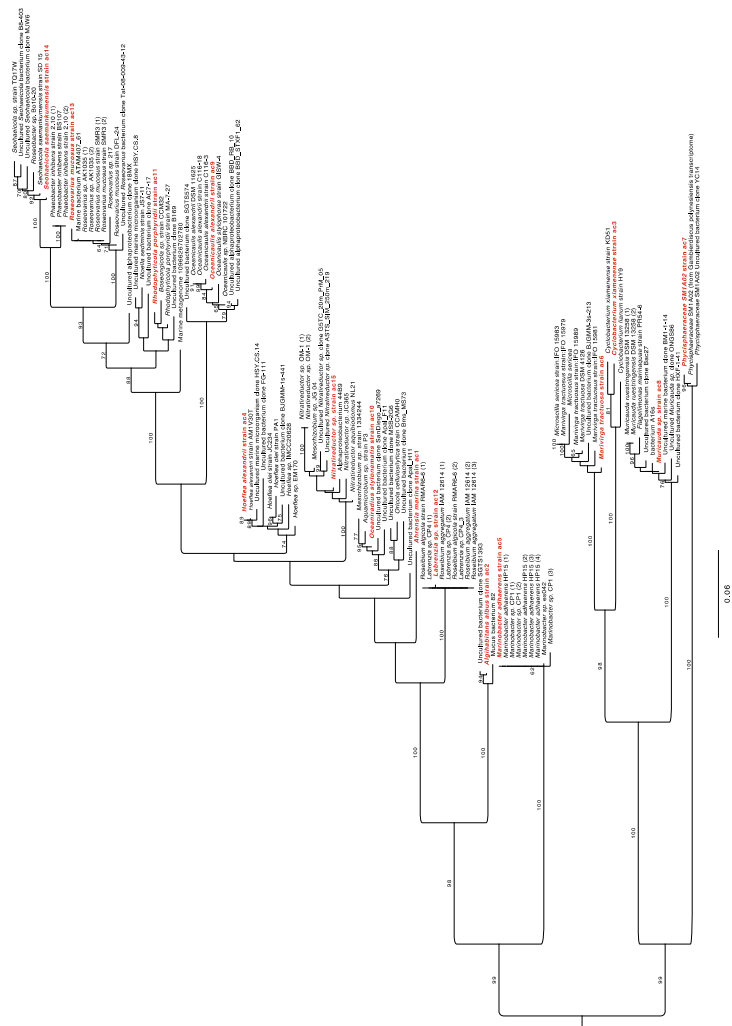


Figure A1. Phylogenetic tree including bacterial 16S sequences found in the *A. carterae* microbiome. Tree was generated using SILVA ACT Service using RaxML, the GTR model, and the Gamma rate model, with a minimum sequencing identity of 0.95 and including 10 neighbors.

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