Long-Term Stability of Bacterial Associations in a Microcosm of Ostreococcus tauri (Chlorophyta, Mamiellophyceae)

Sophie Vacant††, L. Felipe Benites††, Christophe Salmeron‡, Laurent Intertaglia‡, Manon Norest†, Adrien Cadoudal†, Frederic Sanchez†, Carlos Caceres* and Gwenael Piganeau*

1 Integrative Biology of Marine Organisms (BIOM), Sorbonne University, Centre National de la Recherche Scientifique, Oceanological Observatory of Banyuls, Banyuls-sur-Mer, France, 2 Sorbonne Université, Centre National de la Recherche Scientifique, Observatoire Océanologique de Banyuls, FR3724, Banyuls-sur-Mer, France

Phytoplankton–bacteria interactions rule over carbon fixation in the sunlit ocean, yet only a handful of phytoplanktonic–bacteria interactions have been experimentally characterized. In this study, we investigated the effect of three bacterial strains isolated from a long-term microcosm experiment with one Ostreococcus strain (Chlorophyta, Mamiellophyceae). We provided evidence that two Roseovarius strains (Alphaproteobacteria) had a beneficial effect on the long-term survival of the microalgae whereas one Winogradskyella strain (Flavobacteriia) led to the collapse of the microalgal culture. Co-cultivation of the beneficial and the antagonistic strains also led to the loss of the microalga cells. Metagenomic analysis of the microcosm is consistent with vitamin B12 synthesis by the Roseovarius strains and unveiled two additional species affiliated to Balneola (Balneolia) and Muricauda (Flavobacteriia), which represent less than 4% of the reads, whereas Roseovarius and Winogradskyella recruit 57 and 39% of the reads, respectively. These results suggest that the low-frequency bacterial species may antagonize the algicidal effect of Winogradskyella in the microbiome of Ostreococcus tauri and thus stabilize the microalga persistence in the microcosm. Altogether, these results open novel perspectives into long-term stability of phytoplankton cultures.

Keywords: picophytoplankton, mutualism, symbiosis, Ostreococcus, heterotrophic bacteria, vitamin B12, secretion systems

INTRODUCTION

Bacterial–phytoplankton interactions in the sunlit ocean fuel the biological carbon pump (Field et al., 1998) and are fundamental for our understanding of the base of the food web in marine ecosystems (Azam and Malfatti, 2007). The interactions between bacteria and phytoplankton are multifarious and may span the spectrum of relationships from mutualistic (Amin et al., 2015; Choix et al., 2018; Cooper et al., 2019) or opportunistic (Pinto et al., 2021) to antagonistic (Fukami et al., 1997; Mitsutani et al., 2001; Sohn et al., 2004; Wang et al., 2010). Mutualistic interactions are generally driven by reciprocal needs of both taxa specific bacteria and phytoplankton partners (Mönnich et al., 2020). These requirements encompass essential trace elements, nutrients (Amin et al., 2015), and vitamins, such as in the production and acquisition of the B vitamins...
analyzed the microcosm to investigate total bacterial diversity combinations of bacterial strains. Second, we sequenced and well as the dynamics between the microalga and the three between the microalga and each individual bacterial strain as of the short-term and long-term dynamics (up to 231 days) We first performed co-culture experiments to identify the nature of the short-term and long-term dynamics (up to 231 days) form of methionine synthase (METE) (Helliwell et al., 2011). the effect on microalgae growth or long-term stability requires genome sequencing of phytoplanktonic eukaryotes has unraveled the microalgae Ostreococcus tauri (Mamiellophyceae, Chlorophyta), a photosynthetic picoeukaryote which has been previously isolated from a Mediterranean lagoon (Courties et al., 1994) and the NW Mediterranean Sea (Grimslay et al., 2010). During exponential growth phase, the microalgae outnumber the bacteria, whereas the bacteria may outnumber the microalgae at a 50:1 ratio during the stationary phase and even more significantly so during the decay phase (Lupette et al., 2016). The advances in genome sequencing of phytoplanktonic eukaryotes has unraveled an unexpected genomic diversity of associated bacteria (Abby et al., 2014a; Rosana et al., 2016; Rambo et al., 2020). However, precise knowledge about the mutualistic, opportunistic, or antagonistic nature of the interaction and the estimation of the effect on microalgal growth or long-term stability requires co-cultivation of the microalgae and the bacterial partners (Amin et al., 2015; Behringer et al., 2018; Johansson et al., 2019; Lian et al., 2021; Pinto et al., 2021).

In our study, we took advantage of a microcosm containing O. tauri and a bacterial microbiome without external input, pour ainsi dire “in lockdown,” which had maintained the microalga for more than 1 year, to characterize the pairwise interaction between the microalga and the three bacteria isolated from this microcosm. Like many phytoplanktonic microalgae, O. tauri is auxotrophic for vitamin B12 as it requires vitamin B12 for growth and its genome does not encode the B12-independent form of methionine synthase (METE) (Helliwell et al., 2011). We first performed co-culture experiments to identify the nature of the short-term and long-term dynamics (up to 231 days) between the microalga and each individual bacterial strain as well as the dynamics between the microalga and the three combinations of bacterial strains. Second, we sequenced and analyzed the microcosm to investigate total bacterial diversity and the relative frequency of the different bacterial species present. We also investigated the genetic complementarity of the bacterial metagenome-assembled genomes (MAGs) for genes that may inform about the nature of the interaction with the microalgae: the genes involved for vitamin B12 synthesis and for the presence of bacterial secretion systems.

MATERIALS AND METHODS

Phytoplankton and Bacterial Strain Isolation From the Microcosm

A microcosm experiment was started in triplicate with O. tauri RCC4221 100-ml cultures in L1 media in 200-ml closed flasks (Sarstedt T75 ref 83.3911) opened weekly for sampling. The microcosm, culture, and co-culture experiments were performed at 15 μmol m⁻² s⁻¹ with shaking (135 rpm) in 12:12 light–dark conditions at 15°C. After initial discoloration of the culture, as previously observed when O. tauri cultures are not reinoculated with fresh media (Lupette et al., 2016), the culture regained the typical green color of O. tauri cultures after 1 month. Following 1 year of sustained green coloration, the identity of the microalgae was checked with strain-specific primers (Grimslay et al., 2010) and the long-term stability of O. tauri RCC4221 was confirmed. The bacteria were isolated from the microcosm by streaking an aliquot of the culture on marine agar (MA) Petri dishes (Difco 2216) and incubated at 20°C in the dark. Three different single colonies among the most dominant morphotypes were picked and subcultured two times on MA plates until getting pure cultures. Then, each selected strain was transferred onto marine broth (MB) tube at 20°C, 100 rpm in the dark. After 72 h of growth, 3 ml of these cultures was used for cryopreservation in 5% dimethylsulfoxide or 35% glycerol, put into a −80°C freezer, and added to the Banyuls Bacterial Culture Collection (as BBCC2900, BBCC2901, and BBCC2902, hereafter B2900, B2901, and B2902). About 1 ml of this resting liquid culture was pelleted for DNA extraction and 16S rDNA sequencing.

Axenic O. tauri cultures were obtained by adding 1% antibiotics to cultures at 10⁶ cell concentration in L1 ASW media as previously described (Sanchez et al., 2019). To investigate the effect of the co-culture of O. tauri on bacterial growth, we compared the temporal dynamics of bacteria in co-cultures and in a media without O. tauri, hereafter coined exudate media. The media experiments were prepared as follows: exponentially growing cultures of O. tauri in L1 medium were filtrated through 0.02 μm to keep O. tauri exudates neither larger than 20 nm particular organic matter nor microalga or bacterial cells.

The co-culture experiments were performed in 10-ml glass tubes as follows: 0.6 ml of bacterial cultures (at a cell concentration between 10⁸ and 10⁹ cells ml⁻¹) was added to 6 ml of axenic microalga culture (4 × 10⁷ cells ml⁻¹) grown in L1ASW media.

Cytometry Measurements

For flow cytometry counts of microalgae and free-living bacteria, 0.05 ml of culture was sampled, diluted at 1:10–1:10,000 and fixed for 15 min in the dark with a final concentration of electron microscopy–grade glutaraldehyde of 0.25% and Pluronic F-68 of
0.01% (Marie et al., 2014), flash-frozen in liquid nitrogen, and stored at −80°C until the analysis. Cell counts were performed with a BD FacsCanto II Flow Cytometry System [3-laser, 8-color (4-2-2), BD-Biosciences] equipped with a 20-mW 488-nm coherent sapphire solid-state blue laser. Accurate analyzed volumes and subsequent estimations of cell concentrations were calculated using Becton-Dickinson Trucount™ beads. Phytoplankton and bacterial cells were discriminated and enumerated according to their side scatter properties (SSC) for both and red fluorescence (>670 nm) due to chlorophyll pigments or green fluorescence due to SYBR Green I staining of the bacterial DNA [1:10,000 final concentration (Marie et al., 1997)], respectively. Data were acquired using DIVA software provided by BD Biosciences.

Metagenomics of Microcosm and 16S rDNA Sequencing From Bacterial Isolates

DNA extraction and purification for 16S rDNA sequencing of B2900, B2901, and B2902 were carried out with the Wizard® Genomic DNA Purification Kit (Promega) according to the manufacturer’s instructions. PCR and 16S rRNA gene sequencing were done as previously described (Fagervold et al., 2013) using the BIO2MAR platform facilities. Universal bacterial primers 27F and 1492R were used for PCR amplification. PCR products were cleaned up with AmpliClean Magnetic Bead PCR Clean-up Kit (NimaGen). Cleaned amplicons were sequenced with internal 907R primer using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and cleaned up with D-Pure Dye Terminator Removal kit (NimaGen). The cycle sequencing products were loaded into an AB3130xl genetic analyzer (Life Technologies). Partial 16S rDNA sequences of these three strains were assembled with metaspades version (Nurk et al., 2017) with rDNA containing contigs, and the complete 16S rDNA sequence was annotated with RNAmmer (Lagesen et al., 2007). Second, the reference genes and corresponding amino acid sequences involved in the adenosylcobalamin (vitamin B12), and biotin and niacin pathways were compiled from Warren et al. (2002); Helliwell et al. (2016), and Cooper et al. (2019) and the Uniprot Knowledge Database (Boutet et al., 2007) and are listed in Supplementary Table 1. The presence or the absence of a gene was inferred from best BLASTN (16S rDNA) or TBLASTN (protein-coding genes) hit from the reference gene set onto the assembly with an e-value threshold <10^-5.

The complete assemblies (available on 01/10/2021) of bacterial genomes that belong to the genera identified from the 16S rDNA were downloaded from GenBank: 86 Roseovarius, 75 Winogradskyella, 30 Balneola, and 68 Muricauda. Each contig from the metagenome was affiliated to the genus of the best blast hit (BBH) against this bacterial assemblies by BLASTN (e-value threshold <10^-5) (Altschul et al., 1990). The coverage of each contig was estimated by aligning the trimmed PE reads onto the assembly with BWA (bwa-mem2-2.0 version) (Li and Durbin, 2010) and SAMtools (Li et al., 2009). MAGs were obtained by binning contigs with BBH against assemblies of the same genus with similar coverage and GC content.

Each MAG was subsequently annotated with Prokka (Seemann, 2014). The predicted protein sequences were searched for secretion system components using the Macromolecular System Finder approach (Abby et al., 2014b) adapted for the detection of flagella and bacterial secretion system components in the TXSScan tool (Abby and Rocha, 2017) implemented on the Pasteur Institute Galaxy browser with default parameters.

Data Analysis

The dynamics of the microalgae and bacteria in the cultures were summarized by calculating the mean ± standard deviation (SD) of the minimum and maximum concentration of cells and their day of occurrence from the values obtained for each

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1https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/

2https://galaxy.pasteur.fr/
replicate. Moreover, we calculated the reproductive rate and the daily change in the concentration of microalgae (mean ± SD) between the maximum and the minimum concentration of cells and throughout the entire experiment for bacteria. In the case of the subculturing of the initial microcosm, we calculated the initial local maximum concentration of microalgae instead of the global maximum. We compared the average values in each co-culture with those in the axenic culture of *O. tauri* with a t-test. In addition, to better appreciate the temporal dynamics of the microalgae and bacteria and to facilitate the visual comparison among treatments, we fitted local regression curves to the observations of concentration against time. To this end, we used the function `geom_smooth` of the R library ggplot2 (Wickham, 2011).

To analyze the effect of the bacteria on the temporal dynamics of the microalgae, we fitted segmented regression models within each culture type separately using segmented R library (Muggeo, 2008). We focused on the time interval comprised between the maximum and the minimum *O. tauri* concentrations. We considered the natural logarithm of the concentration of microalgae as response variable and time as predictor. In this way, (1) we were able to identify different temporal trends within the time interval analyzed and (2) the estimates of the slope had a biological meaning, as they corresponded to the intrinsic growth rate ($r$):

$$r = \frac{\ln \left( \frac{N_f}{N_i} \right)}{t_f - t_i},$$

where $N_i$ and $N_f$ are cell concentrations at initial ($t_i$) and final ($t_f$) times, respectively. Then, we compared the breakpoint, i.e., the time at which the trend changed, and the slopes estimated for the axenic culture of the microalgae (control treatment) with those obtained for each co-culture of microalgae and bacteria (or combination of bacteria strains) by looking at the overlap of the 95% confidence intervals (CIs). We removed five observations of *O. tauri* concentrations because they corresponded to either (1) samples with zero flow cytometry counts that were followed by non-zero abundances or (2) samples with less than 10 counts preceded and followed by samples with zero counts. In the former case, concentrations were likely different from zero but no counts were detected, whereas in the latter case, cell counts very likely corresponded to flow cytometry noise. Anyway, the exclusion of these observations does not affect the interpretation of results. All graphs and statistical analyses have been performed in R version 4.1.0 (R Core Team, 2021).

### RESULTS

**Ostreococcus tauri** Cultures Thrive in the Company of the Microbiome in the Microcosm

The *O. tauri* cultures inoculated in 200 ml L1 media and left without any external input maintained the typical light green coloration for 1 year. Subsequent sampling of this microcosm during 50 weeks (Figure 1) revealed a stable concentration

\[ C_M = 10.40 \times 10^6 \text{ cells ml}^{-1}\] and a slightly increasing concentration of bacteria ($C_B$) up to $C_B = 41.00 \times 10^7 \text{ cells ml}^{-1}$, which corresponded to a 40:1 bacteria-to-microalgae ratio (Figure 1).

To preserve the initial microcosm to proceed to a long-term monitoring, we decided to replicate the microcosm by subculturing 1 ml into tubes containing 3 ml of sterile L1 ASW media. This resulted in a change of the microalgae–bacteria dynamic and equilibrium (Figure 2). The concentration of the microalgae reached $C_M = 1.04 \times 10^7 \text{ cells ml}^{-1}$ within 2 weeks, whereas the bacteria reached the value observed in flasks after 22 weeks. However, and contrary to what had been observed in the original microcosm, there was a slight increase in the concentration of the microalgae after day 79 (reproductive rate = 1.01 ± 0.00, corresponding to 0.05 ± 0.02 × 10^6 cells ml⁻¹ day⁻¹) and bacteria during the entire experiment (reproductive rate = 1.02 ± 0.00, corresponding to 1.94 ± 0.24 × 10^6 cells ml⁻¹ day⁻¹). As a result, the bacteria to microalgae ratio ranged from 7:10 to 58:1 along this experiment. In conclusion, while the stability of the microalgae concentration observed in the original microcosm could not be strictly reproduced in the subcultures as the bacterial/microalgae ratio increased from 40:1 to 58:1, both the microalgae and the bacteria could be maintained at high concentrations over the complete 231 days of the experiment (Figure 2).

**Some Bacteria Have Beneficial Effects Whereas Other Have Deleterious Effects on the Persistence of the Microalgae**

To assess the role of individual bacterial strains of the bacterial community of the microcosm, we isolated three strains and proceeded to co-culture experiments with *O. tauri* cultures treated with antibiotics. Long-term removal of 100% of the bacteria in an *Ostreococcus* culture below 10⁴ cells ml⁻¹ is delicate to achieve, and this is likely to be due to bacterial

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**FIGURE 2** Concentrations of *O. tauri* and bacteria during 33 weeks in a subculture of the initial microcosm. Dots represent observed concentrations. Solid lines represent the temporal dynamics of the concentrations predicted by fitting local regression curves. Shaded areas represent the 95% CIs. Note that a log₁₀ scale is used in y-axis.
Microalga collapsed after 39 ± first week after starting the culture. The axenic culture of the smaller than 1% of the signal of the microalgae throughout the concentrations until day 20 (culture in exudate media, co-culture led to a decay in bacterial oscillated between 0.64 ± 62 ± minimum C concentration of the microalgae, followed by a decrease to a Roseovarius whereas the co-culture of the microalgae with either of the Winogradskyella Table 1 growth (Figures 3B,C and Table 1): an initial 2–3 days stable concentration of the microalgae, followed by a decrease to a minimum $C_M = 0.05 \pm 0.03 \times 10^6$ cells ml$^{-1}$ (B2900, day 59 ± 4) and $C_M = 0.05 \pm 0.01 \times 10^6$ cells ml$^{-1}$ (B2902, day 62 ± 4), and a subsequent increase to reach concentrations that oscillated between 0.64 ± 0.61 × 10⁶ cells ml$^{-1}$ (B2902, day 103 ± 4) and 14.86 ± 5.18 × 10⁶ cells ml$^{-1}$ (B2900, day 78 ± 7). The concentration of bacteria increased at a mean reproductive rate = 1.02 ± 0.00 (B2900, 5.87 ± 0.47 × 10⁶ cells ml$^{-1}$ day$^{-1}$) and 1.01 ± 0.00 (B2902, 2.86 ± 0.34 × 10⁶ cells ml$^{-1}$ day$^{-1}$) and reached maximum $C_B = 1393.33 \pm 90.74 \times 10^6$ cells ml$^{-1}$ (B2900, day 183 ± 12) and 660 ± 36.37 × 10⁶ cells ml$^{-1}$ (B2902, day 190 ± 12) (Table 2).

In sharp contrast to the co-culture with Roseovarius, the co-culture of O. tauri and Winogradskyella strain B2901 leads to the loss of the microalgae population after 36 days (Figure 3D and Table 1). The decrease of O. tauri in the co-culture with Winogradskyella was even faster than the decrease observed in O. tauri axenic cultures before day 29, as the slope coefficient for the relationship between cell concentration and time is 24% lower and the 95% CIs of the slopes do not overlap (Table 3 and Supplementary Figure 1).

**Effect of the Microalga on the Bacteria**

We further investigated the effect of the microalga on the bacteria by comparing the dynamics of the bacteria with and without (exudate media) the microalga. For the two Roseovarius strains, co-culture and culture in exudate media led to initial growth (Figures 4A,B). For Winogradskyella, as opposed to culture in exudate media, co-culture led to a decay in bacterial concentrations until day 20 (Figure 4C), at which point the microalga decayed below 10^6 cells ml$^{-1}$ (Figure 4C). After day 20, the concentration of Winogradskyella increased to reach a plateau once the microalgae have died. As a conclusion, the microalgae and its exudate promoted the growth of Roseovarius, whereas the microalgae had a negative effect on the growth of Winogradskyella. Altogether, these observations suggest that the Roseovarius–O. tauri interactions are mutualistic and that the Winogradskyella–O. tauri interactions are antagonistic.

**Combining Antagonistic and Mutualistic Bacteria Does Not Reestablish Long-Term Survival of Microalga**

We further investigated whether the antagonistic effect of the Winogradskyella strain could be compensated by the addition of the beneficial Roseovarius strains. This was not the case as, whenever the Winogradskyella strain was added into a co-culture experiment, the concentration of the microalgae would reach null values within 36 days (Table 1 and Figure 5). As a conclusion, the long-term stability of the microalgae in the microcosm experiment cannot be reproduced with the three isolated strains but with either one or the combination of the two Roseovarius B2900 or 2902 strains. Therefore, it is likely that additional bacteria are tempering with the antagonistic effect of Winogradskyella present in the microcosm.

**Metagenomic Insights Into the Total Bacterial Diversity Within the Microcosm**

The assembly of the microcosm led to 1324 contigs (total 58.8 Mbp). Following the removal of the contigs aligning to O. tauri nuclear or organellar genomes (refer to section “Materials and Methods”), the bacterial diversity of the microbiome was inferred from 678 contigs (total 16.5 Mbp, average contigs length: 24.3 kbp, 240 contigs with length >1 kbp adding up to 16.3 Mbp). Screening this assembly for 16S rDNA confirmed the presence of Roseovarius and Winogradskyella sequences, which were 100% identical to the partial 16S rDNA Sanger sequencing performed on the bacterial isolates B2900, B2901, and B2902. The complete 16S rDNA of Roseovarius and
Winogradskyella was extracted from the metagenome assembly and also the 16S rDNA sequence of two additional lineages: *Muricauda* and *Balneola*. Interestingly, and without surprise, the BBH of these 16S rDNA sequences against GenBank has all been sampled from the marine environment, which includes a strain isolated from the culture of a diatom microalgae (Table 4). Taxonomic affiliation of the metagenome onto available assemblies assigned to these four bacterial genera led to 3.1 (Winogradskyella) to 4.8 Mb (*Muricauda*) MAG assemblies (Table 5). The MAG coverage and GC content statistics clearly separated *Roseovarius* (60% GC) and Winogradskyella (35% GC) affiliated contigs to the *Muricauda* + *Balneola* cluster (Figure 6). The *Roseovarius* MAG assembly shared a very high sequence identity (>99.9% nucleotide identity over >500 kbp) with a genome assembly affiliated to *R. mucosus* strain 85A, which has been isolated from the culture of a diatom microalgae, whereas the MAGs affiliated to Winogradskyella, *Balneola*, and *Muricauda* shared up to 86% nucleotide identity with sequences available from GenBank (Table 5). The percent of reads affiliated to each genera is thus 57% to *Roseovarius*, 39% to Winogradskyella, 1% to...
Balneola, and 2% to Muricauda (Table 5). The relative coverage of each MAG can, in turn, be used to estimate the relative frequency of each strain, that is, 49% of Winogradskyella, 47% of Roseovarius, 2% of Muricauda, and 1% of Balenola.

Metagenomic Insights Into the Identity of the Vitamin B12 Producer and the Presence of Secretion Systems

The search for genes encoding for the niacin, biotin, and adenosylcobalamin pathways suggests the presence of a complete adenosylcobalamin (vitamin B12) pathway in the Roseovarius MAG with 18 genes detected (cobA, cobI, cobJ, cobM, cobF, cobK, cobL, cobH, cobB, cobO, cobQ, cobU, cobP, cobD, cobS, cobV, CobC, and cobT, Supplementary Table 2). As for the niacin and biotin pathways, which have been demonstrated to be incomplete from a Dinoroseobacter strain depending on O. tauri for niacin and biotin synthesis (Cooper et al., 2019), none of the MAGs seem to contain the complete gene complement for both pathways. The complete gene pathway for biotin has been identified in the Muricauda MAG, whereas it is incomplete in the Roseovarius, Balenola, and Winogradskyella MAGs (Supplementary Table 2). However, MAGs may not correspond to complete genome assemblies, so that the absence of a gene is not as informative as its absence from a complete genome assembly. Interestingly, available genome data from other strains suggest that the biotin pathway is complete in some Roseovarius and Balenola strains, that the niacin pathway is complete for some Muricauda strains, and that the vitamin B12 pathway is complete in some Roseovarius strains (Supplementary Table 1). As a conclusion, gene content analysis of the MAGs suggested that the Roseovarius strains present in the microcosm provide the microalgae O. tauri with vitamin B12.

Protein secretion systems are complex molecular machineries that translocate proteins through the outer bacterial membrane and sometimes through the membrane of an eukaryotic cell (Denise et al., 2020). The screening of the four MAGs for secretion systems (Abby and Rocha, 2017) did not allow the identification of the T4SS candidate gene complement within the MAGs. However, we identified the candidate genes for T1SS in all four MAGs, for T9SS in the Winogradskyella and
Table 4: Description of the four complete 16S rDNA sequences extracted from the metagenome.

| 16S rDNA accession | Length (bp) | BBH* (accession) | Identities | Origin of BBH |
|---------------------|-------------|-------------------|------------|---------------|
| OK396682            | 1456        | Roseovarius mucosus strain SMR3 (CP020474.1) | 1455/1456 | Isolated from a culture of the Diatom Skeletonema marinoi |
| OK396703            | 1512        | Muricauda marina (NR_157633) | 1481/1482 | Isolated from marine snow of Yellow Sea (Su et al., 2017) |
| OK396683            | 1514        | Winogradskyella exilis (NR_116736) | 1448/1514 | Isolated from a starfish (Ivanova et al., 2010) |
| OK396702            | 1523        | Balneola vulgaris (NR_042991) | 1371/1474 | Isolated from the North-Western Mediterranean Sea (Agogué et al., 2005) |

BBH, best blast hit against GenBank. *BBH from uncultured isolates has been excluded.

Relative concentration of these four bacteria exists in the microbiome here.

Table 5: Description of the four MAGs assembled from the microcosm.

| Roseovarius MAG | Winogradskyella MAG | Balneola MAG | Muricauda MAG |
|-----------------|----------------------|--------------|---------------|
| Total length (Mb) | 4.7 | 3.1 | 3.2 | 4.8 |
| Nb of contigs | 79 | 43 | 32 | 71 |
| GC (%) | 60.3 | 35.2 | 41.6 | 41.6 |
| Coverage | 845.1 | 884.8 | 29.7 | 37.8 |
| BBH accession | JAH66P010000002.1 | CP019388.1 | LXYG01000014.1 | JAFLNE01000001.1 |
| BBH length (Mb) | 0.58 | 3.3 | 0.32 | 1.1 |
| Total length of BBH assembly (Mb) | 4.8 (215 contigs) | 3.3 (1 complete genome) | 3.6 (20 contigs) | 4.3 (20 contigs) |
| BBH name | Roseovarius mucosus | Winogradskyella sp. | Balneola sp. | Muricauda sp. |
| BBH origin | Culture of Seminavis robusta strain 85A (unpublished) | Seawater (unpublished) | Isolated from a culture of Emiliania huxleyi (Rosana et al., 2016) | Seawater (unpublished) |
| Maximum alignment/contig length (kb) | 528/677 | 698/695 | 11/272 | 168/208 |
| Identity over maximum aln (%) | 99.98 | 86.2 | 85.2 | 85.5 |

BBH, best blast hit by BLASTN.

Muricauda MAGs, and the candidate genes involved in the flagella within the Roseovarius MAG (Supplementary Table 3). We thus conclude that the Roseovarius strain may be motile, as observed in many Rhodobacteraceae (Bartling et al., 2018), though additional gene expression analyses would be required to check whether these genes are indeed expressed within the microcosm.

Discussion

Of the Importance of Long-Term Co-culture Experiments

We have isolated novel bacterial strains from a stable microcosm experiment started with a non-axenic O. tauri culture and provided evidence of the individual effects of these isolates on the microalgal growth and on long-term stability. The two Roseovarius isolates can be considered to be from the same species, as they share an identical 16S rDNA sequence, and the co-culture experiments demonstrated that they have a beneficial effect on the microalgal long-term survival. Analysis of the gene content of the Roseovarius MAG from the microcosm suggests that the Roseovarius strains are the unique producers of vitamin B12 in the microcosm, whereas O. tauri may provide niacin. However, there is no evidence of a type four secretion system (T4SS), whereas T4SS have been recently demonstrated as required for establishing a beneficial effect of another Rhodobacterales, Dinoroseobacter, on the growth rate of a dinoflagellate (Mansky et al., 2022). Unlike Roseovarius, Winogradskyella has a deleterious effect on the microalgal growth and long-term survival, which accelerates the decrease in the concentration of microalgae by 24% during the first 29 days of the co-culture ($R = 0.65$ vs. $0.86$, for co-culture vs. axenic conditions, respectively; Table 3 and Supplementary Figure 1). The analyses of the gene content of the Winogradskyella MAG suggested that it encodes a T9SS, which provides either a means of movement called gliding motility or a weapon for pathogenic bacteria (Lasica et al., 2017). This complex has so far only been identified within the Bacteroidetes phylum (Abby and Rocha, 2017) to which Balnoela, Muricauda, and Winogradskyella belong to.

To our knowledge, phytoplankton–bacteria co-culture experiments are only exceptionally monitored for more than 30 consecutive days, with the notable exception of a 200 days Synechococcus–Roseobacter co-culture experiment (Christie-Oleza et al., 2017). Our study demonstrates the importance of
long-term experiments as the first 15 days of co-culture may wrongly suggest stable concentrations of microalgae. Indeed, the evidence of the collapse of the microalgae populations in co-culture with both *Roseovarius* and *Winogradskyella* could only be observed after 15 days (Figure 5).

Obviously, the microalgal and bacterial cells will accumulate mutations and evolve over the course of long-term experiment (Krasovec et al., 2017). Interestingly, we observed that the number of bacterial cells tended to increase (slightly) over the course of the experiment (Figures 1, 2), whereas the number of microalgae only increased in the subcultured microcosm (Figure 2). Given that there is no external nutrient input into the system, this tendency suggests ongoing adaptation to the available resources in the microcosm.

The ratio of heterotrophic bacteria to microalgae at the end of both the initial microcosm (40:1) and the subculture of the microcosm (58:1) may be compared with the fraction of the photosynthetic pico-eukaryotic vs. heterotrophic bacteria fraction in the natural environment. This ratio can be estimated by cytometry and has been estimated to vary between 9:1 and 216:1 at the Station d’Observation Laboratoire Arago (SOLA, 42°29’N, 03°08’E) throughout the sampling performed during 2019 every 2 weeks (David Pecqueur, personal communication). Nevertheless, absolute concentrations in our experiments were markedly higher than in SOLA (bacteria range = 0.08 × 10⁶–0.22 × 10⁶ cells ml⁻¹; picoeukaryotes range = 0.49–13.90 × 10³ cells ml⁻¹), and this is likely the consequence of the initial higher availability of nutrients in the L1 culture media when the microcosm experiment was started. Alonso-Sáez et al. (2007) also reported concentrations of heterotrophic bacteria 1–2 orders of magnitude higher than those of picocyanobacteria and autotrophic picoeukaryotes during a monthly sampling carried out in 2003–2004 in the North-Western Mediterranean Sea. In terms of carbon biomass, heterotrophic bacteria are usually less abundant than phytoplankton in coastal waters, although the proportion of bacteria increases with the oligotrophy of the system and its biomass is frequently higher than that of phytoplankton in open oceans (Gasol et al., 1997).

**From the Laboratory to the Environment: Is the *Ostreococcus–Roseovarius* Coexistence Prevalent in the Environment?**

*Roseovarius* strains have been previously reported to be present in algal cultures, which include *O. tauri* cultures (Abby et al., 2014a). *Roseovarius* sp. MS2 strain commonly grows in cultures of the macroalgae *Ulva mutabilis*, where it takes advantage of the dimethylsulfoniopropionate (DMSP) released by the macroalgae and in turn releases compounds that promote the proper development of the macroalgae (Kessler et al., 2018). A previous 4 day co-culture of *Roseovarius mucosus* strain SMR3 and *Skeletonema marinoi*, a centric diatom, demonstrated that this bacterial strain stimulated the growth rate of the microalga (Johansson et al., 2019).

*Roseobacter*, a group belonging to the same order as *Roseovarius* (i.e., Rhodobacterales), is common in coastal waters and their abundances are correlated with Chla concentrations at a global scale, which could suggest an association with phytoplankton communities (Alonso-Sáez et al., 2007; Wietz et al., 2010; D’Ambrosio et al., 2014). In this regard, it was recently
reported that Rhodobacterales usually represented 5–10% of total prokaryotic abundance in surface waters in the Western Mediterranean Sea during mid spring, when phytoplankton bloom occurs (Sebastián et al., 2021).

The global analysis of 313 TARA Ocean metagenomes from 68 stations for taxon co-occurrence based on barcodes from the 18S rDNA and 16S rDNA sequences identified 36 robust associations involving *Ostreococcus* (Lima-Mendez et al., 2015). *Ostreococcus* concentration was positively associated with another eukaryotic taxa 35 times, whereas the only robust co-occurrence with a bacterial taxa was with the genus *Rhodopirellula*. It is important to note that the TARA Ocean sampling sites included mostly open ocean waters and that the corresponding communities sequenced did not contain sequence data affiliated to *O. tauri* but to two divergent sister lineages *O. lucimarinus* and *O. spp* RCC809 (Leconte et al., 2020). So, while the *Roseovarius*–*Ostreococcus* association has not been detected from the metagenomes analyzed in the Lima-Mendez et al. (2015) study, this association may be revealed in future metagenomic studies that include coastal sites, where Mamiellophyceae, which include *Ostreococcus*, have been found to be more prevalent (Tragin and Vaulot, 2018). Alternatively, there may be no need for a taxonomic constraint on mutualistic *Ostreococcus*–Bacteria associations, but rather a metabolic constraint. Indeed, a recent closed microbial community experiment (de Jesús Astacioa et al., 2021) provided evidence of metabolic but not taxonomic constraints on long-term persistence of different heterotrophic bacterial communities with the freshwater green algae *Chlamydomonas reinhardtii*. This metabolic redundancy between taxonomically diverse bacterial lineages may be invoked more generally to explain previous reports of a lack of overlap between bacteria–diatom associations observed in culture collections as opposed to bacteria–diatom associations observed in the natural environment (Crenn et al., 2018).

### Possible Applications of Bacteria for Long-Term Stability of Microalgae Culture

Co-cultivation of microalgae and bacteria may have application for biomass production of microalgae. Indeed, specific bacterial strains may be used to (1) increase algal biomass or (2) limit productivity loss due to contamination by an antagonistic bacterial or (3) lyse the microalga as part of the harvesting process with the addition of an antagonistic bacteria at the end of the growth phase (Lian et al., 2018). Obviously, these developments require precise knowledge of the interactions between specific microalgae–bacteria pairs (Lian et al., 2018).

As *Ostreococcus* cultures left without subculturing are lost upon 4–5 weeks, the *Ostreococcus* cultures are maintained by subculturing 200 µl in 10 ml fresh sterile L1 culture media in transparent tubes for every 3 weeks. The experimental evidence of the beneficial effect of *Roseovarius* on *O. tauri* RCC4221 is opening promising venues in microalga husbandry as it could decrease the frequency of subculturing and, thus, the risk of contamination by antagonistic bacteria or cross-contamination between strains during the subculturing process.

### DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. This data can be found here: partial 16S rDNA sequences of these three strains were completed with metagenomic contigs and full length 16S rDNA sequences were submitted to GenBank under accession numbers: OK396682, OK396683, OK396702, and OK396703. Metagenome Assembled Genomes of the microbiome and raw data are available from PRJNA797933.

### AUTHOR CONTRIBUTIONS

GP planned the experiments. SV performed the co-culture experiments and drafted the first version of the manuscript. SV, MN, AC, and CS performed the cytometry monitoring. MN, AC, FS, and SV were responsible for cultures. FS was responsible for DNA extraction. LI isolated, performed 16S rDNA sequencing, and provided the cultures of bacteria isolated from microcosm. LFB and GP performed the bioinformatic analyses of metagenomes. SV and CC performed the statistical analyses. LFB, CC, and GP wrote the final version. All authors contributed to manuscript editing.

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### SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.814386/full#supplementary-material
REFERENCES

Abby, S. S., Touchon, M., De Jode, A., Grimsey, N., and Pignaneau, G. (2014a). Bacteria in Ostreococcus tauri cultures – friends, foes or hitchhikers? Front. Microbiol. 5, 505. doi: 10.3389/fmicb.2014.00505

Abby, S. S., Neron, B., Ménager, H., Touchon, M., and Rocha, E. P. C. (2014b). MacSyFinder: a program to mine genomes for molecular systems with an application to CRISPR-Cas systems. PLoS One 9:e110726. doi: 10.1371/journal.pone.0110726

Abby, S. S., and Rocha, E. P. C. (2017). Identification of protein secretion systems in bacterial genomes using MacSyFinder. Methods Mol. Biol. 1615, 1–21. doi: 10.1007/978-1-4939-7033-9_1

Agouèt, H., Casamayor, E. O., Bourrain, M., Obernosterer, I., Joux, F., Henrdl, G. J., et al. (2005). A survey on bacteria inhabiting the sea surface microlayer of coastal ecosystems. FEMS Microbiol. Ecol. 54, 269–280. doi: 10.1016/j.femsec.2005.04.002

Alonso-Sáez, L., Balagué, V., Sà, E. L., Sánchez, O., González, J. M., Pinhassi, J., Agogué, H., Casamayor, E. O., Bourrain, M., Obernosterer, I., Joux, F., Herndl, G. J., et al. (2005). A survey on bacteria inhabiting the sea surface microlayer of coastal ecosystems. FEMS Microbiol. Ecol. 54, 269–280. doi: 10.1016/j.femsec.2005.04.002

Bell, W., and Mitchell, R. (1972). Chemotactic and growth responses of marine phototroph-heterotroph interactions. Appl. Environ. Microbiol. 26, 589–595. doi: 10.1128/AEM.26.3.589

Boutet, E., Lieberherr, D., Tognolli, M., Schneider, M., and Bairoch, A. (2007). BLAST from the command line: a fast检索 tool. Nucleic Acids Res. 35, W159–W162. doi: 10.1093/nar/gkm369

Grimsley, N., Pequin, B., Bachy, C., Moreau, H., and Piganeau, G. (2010). Cryptic sex in the smallest eukaryotic marine green alga. Mol. Biol. Evol. 27, 47–54. doi: 10.1093/molbev/msp203

Hellwell, K. E., Lawrence, A. D., Holzer, A., Kadahl, U. J., Sasso, S., Kräutler, B., et al. (2016). Cyanobacteria and eukaryotic algae use different chemical variants of vitamin B12. Curr. Biol. 26, 999–1008. doi: 10.1016/j.cub.2016.02.041

Hollwell, K. E., Wheeler, G. L., Leptos, K. C., Goldstein, R. E., and Smith, A. G. (2011). Insights into the evolution of vitamin B12 auxotrophy from sequenced algal genomes. Mol. Biol. Evol. 28, 2921–2933. doi: 10.1093/molbev/msr124

Ivanova, E. P., Christen, R., Gorshkova, N. M., Zhukova, N. V., Kurienko, V. V., Crawford, R. J., et al. (2010). Winogradskyella exilis sp. nov., isolated from the starfish Stellaster equestris, and emended description of the genus Winogradskyella. Int. J. Syst. Evol. Microbiol. 60, 1577–1580. doi: 10.1099/ijs.0.012476-0

Johansson, O. N., Pinder, M. I. M., Ohlsson, F., Egardt, J., Töpel, M., and Clarke, A. K. (2019). Friends with benefits: exploring the phycosphere of the marine dinoflagellate Skeletonema marinoi. Front. Microbiol. 10:1828. doi: 10.3389/fmicb.2019.01828

Kessler, R. W., Weiss, A., Kuegler, S., Hermes, C., and Wichard, T. (2018). Cryptic vitamin B12 biosynthesis in bacterial genomes using MacSyFinder. BMC Genomics 19:1103. doi: 10.1186/s12862-018-4946-6

Krasovec, M., Eyre-Walker, A., Sanchez-Ferandin, S., and Pignaneau, G. (2017). Macroagal-bacterial interactions: role of dimethylsulfiniopropionate in microbial gardening by Ulva (Chlorophyta). Mol. Ecol. 26, 1808–1819. doi: 10.1111/mec.14472

Krause, P. J., Dürich, K. C., Fuchs, B., and Pedrós-Armengol, A. (2009). Heterotrophic bacteria in the ocean. Annu. Rev. Mar. Sci. 1, 201–229. doi: 10.1146/annurev.marine.010908.163807

Leconte, J., Benites, L. F., Vannier, T., Wincker, P., Piganeau, G., and Jaillon, O. (2011). Insights into the evolution of vitamin B12 auxotrophy from sequenced algal genomes. Mol. Biol. Evol. 28, 2921–2933. doi: 10.1093/molbev/msr124

Limosn, N., Pequin, B., Bachy, C., Moreau, H., and Piganeau, G. (2010). Cryptic sex in the smallest eukaryotic marine green alga. Mol. Biol. Evol. 27, 47–54. doi: 10.1093/molbev/msp203

Ludwig, W., Schlemp, T., and Helgason, A. K. (2019). Friends with benefits: exploring the phycosphere of the marine dinoflagellate Skeletonema marinoi. Front. Microbiol. 10:1828. doi: 10.3389/fmicb.2019.01828

Muyzer, G., DeSantis, T. Z., Guardzero, A. L., Ryabin, T., Hall, V., et al. (2003). Diversity and complexity analysis of microbial communities based on 16S rRNA sequences. In: Microbial Diversity and Function, 2nd ed. (Eds: D. T. Williams, R. E. Colwell, and M. J. Schlesinger) (Blackwell, Oxford), pp. 119–147.
Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., et al. (2009). The sequence Alignment/Map format and SAMtools. Bioinformatics 25, 2078–2079. doi: 10.1093/bioinformatics/btp352

Lian, J., Schimmel, P., Sanchez-Garcia, S., Wijffels, R. H., Smidt, H., and Sipkema, D. (2021). Different co-occurring bacteria enhance or decrease the growth of the microalga Nanochloropsis sp. CCAP211/78. Microb. Biotechnol. 14, 1159–1170.

Lian, J., Wijffels, R. H., Smidt, H., and Sipkema, D. (2018). The effect of the algal microbiome on industrial production of microalgae. Microb. Biotechnol. 11, 806–818. doi: 10.1111/1751-7915.13296

Lima-Mendez, G., Faust, K., Henry, N., Decelle, J., Colin, S., Carcillo, F., et al. (2015). Determinants of community structure in the global plankton interactome. Science 348:1262073. doi: 10.1126/science.1262073

Lupette, J., Lami, R., Krasovec, M., Grimsley, N. H., Moreau, H., Pignane, G., et al. (2016). Marinobacter dominates the bacterial community of the Ostreococcus tauri phycosphere in culture. Microb. Symbioses 7:1414. doi: 10.3389/micro.2016.01414

Mansky, J., Wang, H., Ebert, M., Hättrig, E., Jahn, D., Tomasch, J., et al. (2022). Features of the opportunistic behaviour of the marine bacterium Marinobacter Muricauda sp. nov., isolated from marine snow of Yellow Sea. Microb. Symbioses 85, 962–968. doi: 10.1002/cyt.a.22517

Mitsuhashi, I., Yamashita, K., Kataguchi, H., Kato, J., Ueno, S., and Ishida, Y. (2001). Analysis of algicidal proteins of a diatom-lytic marine bacterium Pseudoalteromonas sp. strain A25 by two-dimensional electrophoresis. Physiologia 40, 286–291.

Mönich, J., Tébenn, J., Bergemann, J., Case, R., Wohlrab, S., and Harder, T. (2020). Niche-based assembly of bacterial consortia on the diatom Thalassiostra rotula is stable and reproducible. ISME J. 14, 1614–1625. doi: 10.1038/s41396-020-0631-5

Muggeo, V. M. (2008). Segmented: An R package to fit regression models with broken-line relationships. R News 8, 20–25.

Myklestad, S. M. (1995). Release of extracellular products by phytoplankton with special emphasis on polysaccharides. Sci. Total Environ. 165, 155–164.

Not, F., Siano, R., Kooistra, W. H. C. F., Simon, N., Vaulot, D., and Probert, I. (2012). “Chapter one – Diversity and ecology of eukaryotic marine phytoplankton,” in Advances in Botanical Research. Genomic Insights into the Biology of Algae, Vol. 64, ed. G. Pignane (Cambridge, MA: Academic Press), 1–53.

Nurk, S., Meleshko, D., Korobeynikov, A., and Pevzner, P. A. (2017). metaSPAdes: a new versatile metagenomic assembler. Genome Res. 27, 824–834. doi: 10.1101/gr.213959.116

Pinto, J., Lami, R., Krasovec, M., Grimaud, R., Urios, L., Lupette, J., et al. (2021). Features of the opportunistic behaviour of the marine bacterium Marinobacter algicola in the microalga Ostreococcus tauri phycosphere. Microorganisms 9:1777. doi: 10.3390/microorganisms901777

Quast, C., Pruesse, E., Yilmaz, P., Gorkin, J., Schweer, T., Yarza, P., et al. (2013). The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Res. 41, D590–D596. doi: 10.1093/nar/gks1219

R Core Team (2021). R: A Language and Environment for Statistical Computing. Vienna: R Foundation for Statistical Computing.

Rambo, I. M., Dombrowski, N., Constant, L., Erdner, D., and Baker, B. J. (2020). Metabolic relationships of uncultured bacteria associated with the microalgae Gambierdiscus. Environ. Microbiol. 22, 1764–1783. doi: 10.1111/1462-2920.14878

Rosana, A. R. R., Orata, F. D., Xu, Y., Simkus, D. N., Bramucci, A. R., Boucher, Y., et al. (2016). Draft genome sequences of seven bacterial strains isolated from a polymicrobial culture of coccolith-bearing (C-Type) Emiliania huxleyi M217. Genome Announce. 4:e00673–16. doi: 10.1128/genomeA.00673–16

Sanchez, F., Grefroy, S., Norest, M., Yao, S., Moreau, H., and Grimsey, N. (2019). Simplified transforma? On of Ostreococcus tauri using polyethylene glycol. Biol. Genes 10:399. doi: 10.3390/biogenes1005399

Sebastian, M., Ortega-Retuerta, E., Gomez-Consarnau, L., Zamanillo, M., Alvarez, M., Arístegui, J., et al. (2021). Environmental gradients and physical barriers drive the basin-wide spatial structuring of Mediterranean Sea and adjacent eastern Atlantic Ocean prokaryotic communities. Limnol. Oceanogr. 66, 4077–4095. doi: 10.1002/lno.11944

Seemann, T. (2014). Prokka: rapid prokaryotic genome annotation. Bioinformatics 30, 2068–2069. doi: 10.1093/bioinformatics/btu153

Sohn, J. H., Lee, J.-H., Yi, H., Chun, I., Bae, K. S., Ahn, T.-Y., et al. (2004). Kordia algicida genosp. nov., sp. nov., an algicidal bacterium isolated from red tide. Int. J. Syst. Evol. Microbiol. 54, 675–680. doi: 10.1099/ijs.0.002689-0

Su, Y., Yang, X., Wang, Y., Liu, Y., Ren, Q., and Zhang, X.-H. (2017). Muricarda marina sp. nov., isolated from marine snow of Yellow Sea. Int. J. Syst. Evol. Microbiol. 67, 2446–2451. doi: 10.1099/ijs.0.019992

Suminto, and Hirayama, K. (1997). Application of a growth-promoting bacteria for stable mass culture of three marine microalgae. Hydrobiologia 358, 223–230.

Tragin, M., and Vaulot, D. (2018). Green microalgae in marine coastal waters: the Ocean Sampling Day (OSD) dataset. Sci. Rep. 8:4020. doi: 10.1038/s41598-018-32338-w

Wang, X., Li, Z., Su, J., Tian, Y., Ning, X., Hon, H., et al. (2010). Lysis of a red-tide causing alga, Alexandrium tamarense, caused by bacteria from its phycosphere. Biol. Control 52, 123–130.

Warren, M. J., Raux, E., Schubert, H. L., and Escalante-Semerena, J. C. (2002). The biosynthesis of adenosylcobalamin (vitamin B12). Nat. Prod. Rep. 19, 390–412. doi: 10.1039/b10896f

Wickham, H. (2011). ggplot2: WIREs Comput. Stat. 3, 180–185. doi: 10.1002/wics.147

Wietz, M., Gram, L., Jergensen, R., and Schramm, A. (2010). Latitudinal patterns in the abundance of major marine bacterioplankton groups. Aquat. Microb. Ecol. 61, 179–189. doi: 10.1111/j.1560-2953.2006.01819.x

Winneppenincx, B., Backeljau, T., and De Wachter, R. (1993). Extraction of high molecular weight DNA from molluscs. Trends Genet. 9:407. doi: 10.1016/0168-9525(93)90102-n

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