Quorum sensing regulates ‘swim-or-stick’ lifestyle in the phycosphere

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Summary

Interactions between phytoplankton and bacteria play major roles in global biogeochemical cycles and oceanic nutrient fluxes. These interactions occur in the microenvironment surrounding phytoplankton cells, known as the phycosphere. Bacteria in the phycosphere use either chemotaxis or attachment to benefit from algal excretions. Both processes are regulated by quorum sensing (QS), a cell–cell signaling mechanism that uses small infochemicals to coordinate bacterial gene expression. However, the role of QS in regulating bacterial attachment in the phycosphere is not clear. Here, we isolated a Sulfitobacter pseudonitzschiae F5 and a Phaeobacter sp. F10 belonging to the marine Roseobacter group and an Alteromonas macleodii F12 belonging to Alteromonadaceae, from the microbial community of the ubiquitous diatom Asterionellopsis glacialis. We show that only the Roseobacter group isolates (diatom symbionts) can attach to diatom transparent exopolymeric particles. Despite all three bacteria possessing genes involved in motility, chemotaxis, and attachment, only S. pseudonitzschiae F5 and Phaeobacter sp. F10 possessed complete QS systems and could synthesize QS signals. Using UHPLC–MS/MS, we identified three QS molecules produced by both bacteria of which only 3-oxo-C16:1-HSL strongly inhibited bacterial motility and stimulated attachment in the phycosphere. These findings suggest that QS signals enable colonization of the phycosphere by algal symbionts.

Introduction

Phytoplankton constitute the foundation of the marine food web as they are responsible for nearly half of primary production on Earth (Field et al., 1998). Through their ability to carry out photosynthesis, phytoplankton transform atmospheric carbon dioxide gas to organic matter (Simon et al., 2009) that is assimilated and remineralized by heterotrophic bacteria (Pomeroy, 1974; Burkhardt et al., 2014). In exchange, bacteria produce essential factors (e.g., vitamins) (Kazamia et al., 2012; Bertrand et al., 2015) to support the growth of phytoplankton (Amin et al., 2012). Cumulatively, phytoplankton-bacteria symbiosis is believed to play an important role in nutrient availability and major biogeochemical cycles (Buchan et al., 2014; Durham et al., 2019).

Several bacterial lineages have been consistently observed to co-occur with phytoplankton, such as members of the Roseobacter group, Gamma- and Flavobacteria (Wagner-Döbler and Biebl, 2006; Teeling et al., 2012). Particularly, members of the Roseobacter group (hereafter roseobacters) have been shown consistently to form symbiotic relationships with phytoplankton (Amin et al., 2012). For example, roseobacters are adept at acquiring and assimilating phytoplankton metabolites (Miller et al., 2004) in exchange for a variety of cofactors. Ruegeria pomeroyi has been shown to assimilate organic sulfur compounds from the diatom Thalassiosira pseudonana in exchange for production of cobalamin, which is required for diatom growth (Durham et al., 2015). Sulfotobacter pseudonitzschiae SA11 produces the hormone indole-3-acetic acid (IAA) to enhance cell division of the diatom Pseudo-nitzschia multiseries, which leads to an increase in carbon export by the diatom and the exchange of diatom-derived organosulfur compounds and bacterial ammonia (Amin et al., 2015). Other phytoplankton such as the cocolithophore, Emiliania huxleyi, display different morphologies in response to endogenous IAA (Labeeuw et al., 2016) and exhibit enhanced cell division by roseobacters-derived...
Quorum sensing (QS) is one of the best-studied signaling mechanisms for bacterial cell-to-cell communication. Many bacteria have been shown to carry out QS by secreting small signalling molecules, known as auto-inducers, to assess changes in bacterial populations and to coordinate gene expression among a whole population (Waters and Bassler, 2005). In Proteobacteria, the primary class of autoinducers is acyl-homoserine lactones (AHLs), which are synthesized by an autoinducer synthase (LuxI) and perceived by an autoinducer regulator (LuxR) (Case et al., 2008). Bacteria use AHLs to regulate functions that are beneficial to carry out collectively, such as virulence, motility, and biofilm formation (Hammer and Bassler, 2003; Daniels et al., 2004; Antunes et al., 2010). Indeed, several Roseobacter-group bacteria use AHLs to regulate motility, virulence, biofilm formation, and nutrient acquisition when associated with marine snow (Gram et al., 2002; Hmelo et al., 2011), red algae (Gardiner et al., 2015), and sponges (Zan et al., 2012). The addition of exogenous AHLs produced by bacterial epibionts to colonies of the cyanobacterium *Trichodesmium* leads to an increase in alkaline phosphatase activity and consequently phosphorus acquisition (Van Mooy et al., 2012). In contrast, the lack of complete QS systems often leads to the inability of bacteria to attach to hosts. For example, a luxR-type gene knock-out strain of the *Roseobacter*-group member *Nautilia italica* R11 was unable to form biofilms or attach to the multicellular red alga *Delisea pulchra* (Gardiner et al., 2015). Despite these examples, there is little direct evidence showing how QS regulates bacteria-phytoplankton interactions or how QS influences inter- and intraspecies interactions and behaviour between bacteria within microbial consortia in the phycosphere. In this study, we examine how QS influences bacterial behaviour in the phycosphere of the ubiquitous diatom, *Asterionellopsis glacialis*, and whether QS provides an advantage to beneficial bacteria relative to other bacteria. Here, we hypothesize that QS regulates motility and attachment of beneficial bacteria, which may enhance their access to phytoplankton nutrients over non-beneficial bacteria.
Results and discussion

Bacterial attachment and influence on diatom physiology

The diatom *A. glacialis* is a ubiquitous species that has been isolated from almost every major water body around the world (Korner, 1970; Kaczmarska *et al*., 2014) and has recently been shown to be one of several abundant and widely distributed groups of diatoms in the open ocean from the Tara Oceans expedition (Malviya *et al*., 2016). In addition, *A. glacialis* often forms blooms and dense patches worldwide (Karentz and Smayda, 1984; Franco *et al*., 2016) that are characterized by high DOM secretions (Abreu *et al*., 2003), making this diatom an ideal model system to examine interactions with bacteria. Here, we characterize AHL molecules produced by bacteria isolated from the phycosphere of *A. glacialis* and examine the influence of these AHLs on the ability of beneficial and opportunistic bacteria to colonize the phycosphere of *A. glacialis*.

Bacterial isolation

To examine bacterial attachment and influence on diatom chain length in the phycosphere, three bacterial strains were isolated from xenic *A. glacialis* and classified using the 16S rRNA gene sequence identity as *Sulfotobacter pseudonitizschiae* F5 (Rhodobacteraceae; >99% similarity to *S. pseudonitizschiae*), *Phaeobacter* sp. F10 (Rhodobacteraceae; 97% similarity to *Phaeobacter galiacensis*) and *Alteromonas macleodii* F12 (Alteromonadaceae; 99% similarity to *A. macleodii*) (Supporting Information Fig. S2). The *Roseobacter* group (Rhodobacteraceae) is one of the most important groups of marine bacteria that primarily colonize both biotic (e.g., phytoplankton) and abiotic surfaces (e.g., marine snow) (Gram *et al*., 2002; Dang *et al*., 2008) and can comprise up to 25% of the marine bacterial community in some regions (Wagner-Döbler and Biebl, 2006). They have been shown to form a substantial component of the *A. glacialis* microbial consortium based both on 16S rRNA gene amplicon sequencing (Behringer *et al*., 2018) and shotgun metagenomics (Shibl *et al*., 2020). Mining the 16S rRNA gene sequences of the microbial community of *A. glacialis* recovered after 20 days of isolation from the field (Behringer *et al*., 2018), we recovered reads that display 100% sequence identity to the 16S rRNA gene of *Phaeobacter* sp. F10 and *A. macleodii* F12 and 99% sequence identity to *S. pseudonitizschiae* F5, indicating our bacterial isolates belong to the natural population of xenic *A. glacialis* and that members of this population persist through time under laboratory culturing conditions.

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**Co-culture of bacterial isolates with the diatom**

To test whether these bacteria attach to *A. glacialis*, co-cultures of each bacterium with the diatom were grown in batch cultures, and growth and attachment of both partners were monitored using microscopy. When co-cultured with *A. macleodii* F12, the specific growth rate (µ) of the diatom did not exhibit significant changes relative to axenic controls (µ\text{axenic} = 1.02 ± 0.05 d⁻¹; µ\text{co-culture} = 1.03 ± 0.02 d⁻¹) (Supporting Information Fig. S3A). Likewise, the growth of the diatom did not vary significantly when co-cultured with *Phaeobacter* sp. F10 (µ\text{axenic} = 0.81 ± 0.02 d⁻¹; µ\text{co-culture} = 0.84 ± 0.04 d⁻¹) (Supporting Information Fig. S3B). In contrast, *A. glacialis* co-cultured with *S. pseudonitzschiae* F5 exhibited a 27.6% increase in µ relative to axenic controls (µ\text{axenic} = 0.76 ± 0.03 d⁻¹; µ\text{co-culture} = 0.97 ± 0.03 d⁻¹) (Supporting Information Fig. S3C). In all co-cultures, bacteria exhibited ~3 orders of magnitude increase in cell density, indicating uptake of diatom-derived organic matter (Supporting Information Fig. S3D). Surprisingly, co-cultures of *S. pseudonitzschiae* F5 with *A. glacialis* showed a significant increase in longer diatom chains than axenic cultures, similar to observations with the xenic diatom (Supporting Information Fig. S1C). This observation may be a byproduct of the enhanced growth of *A. glacialis* with *S. pseudonitzschiae* F5 or a result of a more complex mechanism of interaction.

*Sulfobacter pseudonitzschiae* strains were first isolated from cultures of the toxigenic marine diatom *P. multiseries* obtained from the North Atlantic Ocean and the Pacific Northwest, with a model strain first coined as *Sulfobacter* sp. SA11 (Amin et al., 2015). Subsequently, several additional *S. pseudonitzschiae* strains were isolated from the diatoms *P. multiseries*, *Skeletonema marinoi* and *A. glacialis* originating from the Atlantic Ocean, the Swedish coast, and the Persian Gulf, respectively (Hong et al., 2015; Töpel et al., 2019). These repetitive recoveries of nearly identical bacteria (>99% 16S rRNA gene sequence similarity) from three genera of diatoms that originated from starkly different locations with large variations in temperature, salinity and nutrients indicate that *S. pseudonitzschiae* is a globally distributed bacterium that may be a true symbiont of diatoms. *S. pseudonitzschiae* SA11 enhances the growth rate of the diatom *P. multiseries* by 19%–35% compared with axenic controls partially due to the activity of the hormone IAA, which *S. pseudonitzschiae* biosynthesizes from diatom-derived tryptophan. Both organisms also exchange organosulfur compounds and ammonia to complement each other’s metabolism (Amin et al., 2015). In this study, *S. pseudonitzschiae* F5 enhanced the growth rate of the diatom *A. glacialis* by 27.6% (Supporting Information Fig. S3C). Further genome sequencing and annotation showed that *S. pseudonitzschiae* F5 also possesses three complete pathways for IAA biosynthesis from tryptophan (Supporting Information Table S1), suggesting it may use the same strategy as *S. pseudonitzschiae* SA11 to enhance diatom growth.

Compared with *Sulfobacter*, *Phaeobacter* species are known to colonize marine macro- and microalgal surfaces (e.g., *Ulva australis*, *Thalassiosira rotula*) (Rao et al., 2006; Thole et al., 2012). *P. inhibens* has been shown to control bacterial community assembly in the phycosphere of *T. rotula* (Majzoub et al., 2019). *P. inhibens* also produces the hormone IAA to promote the growth of the coccolithophore, *E. huxleyi*, similar to *S. pseudonitzschiae* (Segev et al., 2016). *P. gallaeciensis* has been shown to lyse senescent *E. huxleyi* by producing algicidal compounds known as roseobacticides (Seyedsayamdost et al., 2011). Although *Phaeobacter* sp. F10 did not enhance the growth rate of *A. glacialis* (Supporting Information Fig. S3B), metatranscriptomic analysis of a near-identical metagenomically assembled genome from the *A. glacialis* strain A3 microbial consortium indicates that *Phaeobacter* sp. F10 is also a symbiont of *A. glacialis* (Shibl et al., 2020).

Alteromonadaceae are widespread marine opportunistic copiotrophs (López-Pérez et al., 2012) that display algicidal activities against phytoplankton during algal blooms (Mayali and Azam, 2004). The *Pseudoalteromonas* and *Alteromonas* genera are known to effectively metabolize the organic matrix surrounding diatom frustules, exposing the silica shell to increased dissolution by the surrounding water (Bidle and Azam, 2001) and show strong algicidal activity by releasing dissolved substances (Mayali and Azam, 2004). For example, *A. colwelliana* shows growth inhibition and algicidal activity against the diatom Chaetoceros calcitrans (Kim et al., 1999). *A. macleodii* have been shown to degrade a variety of algal TEP and exopolysaccharides (Koch et al., 2019) that may enable them to benefit from phytoplankton-derived carbon without contributing to phytoplankton metabolism. In addition, *A. macleodii* has been shown to compete for nitrate with the diatom Phaeodactylum tricornutum in the presence of organic carbon (Diner et al., 2016).

**Bacterial attachment in the phycosphere**

In order to test which bacterial strains attach to the diatom or TEP, we removed free-living bacterial cells from co-cultures of each strain with the diatom using gravity filtration through a 3-μm membrane filter and stained TEP with alcian blue, and diatom and bacterial nucleic acids with SYBR Green I (Fig. 1). Both symbiotic strains, *S. pseudonitzschiae* F5 and *Phaeobacter* sp. F10, displayed strong attenuation onto filters, while no attached *A. macleodii* F12 were observed (Fig. 1D–F). The composite images of bright field and fluorescence showed a strong attachment

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preference to diatom TEP of *S. pseudonitzschiae* F5 and *Phaeobacter* sp. F10 in co-cultures with *A. glacialis*, similar to observations in xenic cultures (Fig. 1G,H). Surprisingly, *A. macleodii* F12 did not show any attachment capacity to *A. glacialis* or TEP (Fig. 1) despite its ability to degrade algal polysaccharides (Koch et al., 2019). These observations suggest an inherent mechanism that enables the roseobacters but not *A. macleodii* to attach to TEP. To shed more light on such mechanisms, we sequenced the genomes of all three isolates.

Genomic comparisons and AHLs identification

The genomes of the three strains were obtained by a combination of PacBio and Illumina sequencing as described in the methods. The GC contents of both *Roseobacter* group member genomes were more similar to each other (61.8% for *S. pseudonitzschiae* F5 and 60.0% for *Phaeobacter* sp. F10) than to *A. macleodii* F12 (44.9%) (Table 1), consistent with the phylogenetic similarities of *S. pseudonitzschiae* F5 and *Phaeobacter* sp. F10 compared to *A. macleodii* F12 (Supporting Information Fig. S2). To confirm the phylogenetic classification of the three strains based on the 16S rRNA gene, we compared the average nucleotide identity (ANI) between all three sequenced strain genomes and related publicly available genomes. Typically, an ANI value >95% between two genomes suggests they belong to the same species (Kim et al., 2014). The ANIs between *S. pseudonitzschiae* F5 and other *S. pseudonitzschiae* strain were >99% (accession numbers: GCF_000712315.1, GCF_013347125.1 and GCF_900129395.1) while the ANIs between *A. macleodii* F12 and other *A. macleodii* genomes were >96% (accession numbers: GCF_000808595.1, GCF_00172635.2 and GCF_001578515.1). On the other hand, the ANIs between *Phaeobacter* sp. F10 and six different *Phaeobacter* species were <86% (accession numbers: GCF_001888185.1, GCF_002891905.1, GCF_007923355.1, GCF_000154765.2, GCF_001251095.1 and GCF_000511385.1). These results further confirm the identity of *S. pseudonitzschiae* F5 and *A. macleodii* F12 and suggest *Phaeobacter* sp. F10 represents a new species.

The bacterium *S. pseudonitzschiae* F5 possesses the largest estimated genome size (5.1 Mb) compared with *Phaeobacter* sp. F10 (4.0 Mb) and *A. macleodii* F12 (4.7 Mb) (Table 1). Interestingly, *A. macleodii*
Table 1. Bacterial genome statistics.

| Statistic               | Sulfitobacter pseudonitzschiae F5 | Phaeobacter sp. F10 | Alteromonas macleodii F12 |
|-------------------------|-----------------------------------|---------------------|---------------------------|
| Genome size (bp)        | 5 087 289                         | 4 008 202           | 4 727 977                 |
| Number of contigs       | 17                                | 3                   | 5                         |
| Chromosome size (bp)    | 3 764 450a                        | 3 783 964           | 3 117 849                 |
| CDs                     | 4991                              | 3878                | 4654                      |
| GC content (%)          | 61.8                              | 60.0                | 44.9                      |
| N50 value               | 179                               | 153                 | 3 122                     |
| L50 value               | 3                                 | 1                   | 1                         |

*Sulfitobacter pseudonitzschiae* F5 chromosome size estimation was not possible due to high level of repeats in the genome. The size indicated is the size of the largest contig.

F12 possesses a 3.1 Mb chromosome and presumably several plasmids totaling ~1.6 Mb (Table 1), consistent with previous studies showing the presence of multiple megaplasmids in *A. macleodii* genomes (Cusick *et al.*, 2020). *S. pseudonitzschiae* F5 also had the greatest number of predicted genes (4991) compared with either Phaeobacter sp. F10 (3878) or *A. macleodii* F12 (4654) (Supporting Information Fig. S4A). A major difference between the three genomes was the apparent higher number of putative genes involved in membrane transport of substrates, particularly primary active transporters (e.g., amino acids, sugars) (Mishra *et al.*, 2014). For example, the relative abundance of putative membrane transporters in the *S. pseudonitzschiae* F5 genome is 10.50% compared with Phaeobacter sp. F10 (8.44%) or *A. macleodii* F12 (7.05%) when normalized to genome size, with primary active transporters being the most abundant in the roseobacters’ genomes but not in *A. macleodii* F12 (Supporting Information Fig. S4B). This observation suggests that both symbionts are more attuned to phytoplankton metabolites than *A. macleodii* F12.

Chemotaxis, motility, and attachment presumably contribute to successful colonization of phytoplankton surfaces. To examine the ability of all three bacteria to interact with the diatom phycosphere, we conducted a genome-wide comparison of genes involved in bacterial chemotaxis, motility, attachment, and quorum sensing. All three genomes contained four chemotaxis genes in a single operon-like structure (cheA, cheR, cheW, and cheY), while a fifth gene, cheB, was present in Phaeobacter sp. F10 and *A. macleodii* F12, but absent in *S. pseudonitzschiae* F5 (Fig. 2A and Supporting Information Table S2). CheAYW together mediate a signal transduction cascade that regulates flagellar motors, while CheB and CheR together regulate the methylation state of methyl-accepting chemotaxis proteins (Wuichet *et al.*, 2007). Flagellar genes were present in all three genomes, although *S. pseudonitzschiae* F5 and Phaeobacter sp. F10 also contained a suite of flagellar structure genes absent in *A. macleodii* F12 (fgN, fgK, fgJ, motB, and MotA/ToIQ/ExbB proton channel family protein). In addition, both *S. pseudonitzschiae* F5 and Phaeobacter sp. F10 contained 11 genes related to pilus formation in contrast to *A. macleodii* F12, which only contained a pilin *flp* gene, which is involved in pilus formation (Bardy *et al.*, 2003). For attachment, genes involved in exopolysaccharide production and biofilm formation were distributed throughout the genomes of the three bacteria (Fig. 2A and Supporting Information Table S2). These lines of evidence suggest that *S. pseudonitzschiae* F5, Phaeobacter sp. F10, and *A. macleodii* F12 have the ability to carry out chemotaxis, construct flagella and pili structures, and form biofilm. Despite this observation, *A. macleodii* F12 did not attach to *A. glacialis* or TEP produced by *A. glacialis* (Fig. 1).

Quorum sensing (QS) autoinducers (e.g., AHLs) have been widely shown to modulate important biological functions, such as biofilm formation and motility (Bassler, 2002; Waters and Bassler, 2005), indicating QS may be able to regulate bacterial ‘swim-or-stick’ lifestyles in the phycosphere and suggesting that *A. macleodii* F12 may lack a functioning QS system. Indeed, luxI-like genes that are responsible for biosynthesizing AHLs were only present in *S. pseudonitzschiae* F5 (2 homologues) and Phaeobacter sp. F10 (1 homologue), while *A. macleodii* F12 completely lacked apparent luxI homologues (Fig. 2B and Supporting Information Table S2). Indeed, luxI is rarely present in *Alteromonas* species. We found only two of 67 publicly available genomes belonging to the Alteromonadaceae contain putative AHL synthases. In addition to AHL biosynthesis, a transcriptional regulator, encoded by a luxR family gene, is required to perceive AHLs and coordinate gene expression among bacterial populations. All three genomes contained luxR family genes; while *S. pseudonitzschiae* F5 possessed four putative luxR-family genes and Phaeobacter sp. F10 possessed six, *A. macleodii* F12 possessed only one (Fig. 2B and Supporting Information Table S2). Typically, apparent luxR genes are found adjacent to or near a luxI gene on bacterial chromosomes in a single operon-like structure, such as in *S. pseudonitzschiae* F5 and Phaeobacter sp. F10 (Supporting Information Table S2). However,
some bacteria also have putative ‘solo’ luxR genes without associated putative luxI genes, which is the case in *A. macleodii* F12 and for the additional putative luxR homologues present in *S. pseudonitzschiae* F5 and *Phaeobacter* sp. F10. It has been hypothesized that bacteria possessing ‘solo’ luxR genes do so to detect and respond to exogenous signals from other bacterial populations (Hudaiberdiev et al., 2015) or that these solo genes represent the loss of QS function (Subramoni and Venturi, 2009). Some solo LuxR proteins have low specificity for AHL binding compared with LuxR proteins coupled with LuxI proteins (Subramoni and Venturi, 2009). For example, SdiA, a solo transcriptional regulator that is present in members of *Salmonella*, *Escherichia*, and *Klebsiella*, could bind seven different AHL molecules (Yao et al., 2006; Janssens et al., 2007). Potentially, the solo luxR genes in *A. macleodii* F12 and the roseobacters may respond to a wide range of AHLs from other bacteria.

To characterize AHLs produced by *S. pseudonitzschiae* F5 and *Phaeobacter* sp. F10, we purchased a suite of commercially available AHL standards (Supporting Information Table S3) and used ultra-high-performance liquid chromatography–tandem mass spectrometry (UHPLC–MS/MS) to identify potential AHLs in bacterial cultures. Three AHLs were recovered from *S. pseudonitzschiae* F5 and *Phaeobacter* sp. F10 pure culture supernatants with ionized parent masses of m/z 270.1700, 352.2480, and 354.2640 and were identified as 3-oxo-C10-HSL, 3-oxo-C16:1-HSL and 3-oxo-C16-HSL, respectively, using high-resolution m/z values, daughter-ion fragmentation and retention times (Fig. 3 and Supporting Information Fig. S5). No standard from the AHL library matched any metabolite in the supernatant of *A. macleodii* F12, consistent with its lack of luxI-like genes (Fig. 2). We attempted to measure AHLs in co-cultures of the roseobacters and the diatom but due to the significantly lower bacterial abundance in cocultures compared with pure bacterial cultures we were not successful in detecting AHLs. The identification of AHL production by diatom symbionts prompted examination of the influence of these signalling molecules on the ability of the symbionts to attach to diatom TEP.

**Fig. 2.** Chromosomal maps of bacterial isolate genomes showing distribution and localization of loci involved in attachment, motility, chemotaxis and quorum sensing. Tracks from the outermost to the center represent position on each chromosome, forward-strand protein-coding genes, reverse-strand protein coding genes, rRNA and tRNA genes, GC content and GC skew, respectively. Chromosomes of *S. pseudonitzschiae* F5, *Phaeobacter* sp. F10, and *A. macleodii* F12 are colour-labelled with red, blue and green, respectively.

A. Homologues related to chemotaxis, motility and attachment.

B. Homologues related to quorum sensing. Black line refers to a luxI gene homologues unique to *S. pseudonitzschiae* F5.

**Influence of AHLs on bacterial motility and biofilm formation**

Cell attachment is the first step in the process of biofilm formation, which requires motility for initial attachment to surfaces (Slightom and Buchan, 2009). Consequently, we examined the influence of AHLs on motility and biofilm formation since both functions are regulated by QS in bacteria (Hammer and Bassler, 2003; Daniels et al., 2004). All three AHLs and two QS inhibitors (QSI), 2(5H)-furanone and furanone C-30 (Ponnusamy et al., 2010; He et al., 2012), were used to test the motility and biofilm formation capabilities of *S. pseudonitzschiae* F5, *Phaeobacter* sp. F10, and *A. macleodii* F12. QSI were used to confirm that any phenotypes observed using AHLs were due to QS regulation and not byproducts of other processes. Since the *A. macleodii* F12 genome contained a putative solo luxR gene (Fig. 2B), we suspected *A. macleodii* F12 might still respond to AHLs produced by *S. pseudonitzschiae* F5 and...
Phaeobacter sp. F10 despite lacking the ability to synthesize AHLs.

The addition of 2 μM of each AHL to each bacterium showed statistically significant inhibition of motility in S. pseudonitzschiae F5 and Phaeobacter sp. F10 (P < 0.01) by all three AHLs, while both QSIs enhanced motility in Phaeobacter sp. F10 (Fig. 4A,B). Specifically, 3-oxo-C16:1-HSL exhibited the strongest inhibition of motility in S. pseudonitzschiae F5 by 78.2% (P < 0.001) compared with the two other AHLs (28.6% and 31.8%). Despite two AHLs, 3-oxo-C16:1-HSL and 3-oxo-C10-HSL, weakly inhibiting motility in A. macleodii F12, both QSIs also weakly inhibited its motility, suggesting A. macleodii F12 does not respond or weakly responds to roseobacters’ QS molecules (Fig. 4A, B). Bacterial motility is characterized by a circular swimming zone in motility assays or by dendritic morphology, which is typical for swarming motility caused by a locally restricted movement on agar plates (Michael et al., 2016).

To assess biofilm formation, a standardized crystal violet assay was conducted as described previously (O’Toole and Kolter, 1998). Interestingly, 10 μg ml⁻¹ of 3-oxo-C16:1-HSL significantly enhanced biofilm formation in S. pseudonitzschiae F5 and Phaeobacter sp. F10 by 26.7% and 211%, respectively (P < 0.05), while 3-oxo-C10-HSL and 3-oxo-C10-HSL did not influence biofilm formation in either bacteria (Fig. 4C). These results suggest each AHL molecule regulates different sets of functions. Similar observations have been shown previously (Su et al., 2018; Hou et al., 2019). In contrast, both QSIs inhibited biofilm formation of S. pseudonitzschiae F5 by 52.2% and 21.9%, respectively, relative to the control. None of the AHLs influenced biofilm formation of A. macleodii F12 (Fig. 4C).

These findings suggest that specific AHLs produced by diatom symbionts promote bacterial colonization of the phycosphere by enhancing the bacterial capacity to form biofilms and reduce motility, functions that are essential to successfully colonize the phycosphere. In contrast, A. macleodii F12 is unable to respond to these molecules or to effectively attach to TEP. Other mechanisms must also be functioning to further prevent opportunists, like A. macleodii F12, from benefitting from diatom exudates. One such mechanism is the regulation of microbial consortia by the eukaryotic host, A. glacialis. A. glacialis has recently been shown to release the unusual metabolites, rosmarinic acid and azelaic acid, in the phycosphere in response to bacteria (Shibl et al., 2020). Azelaic acid promoted the growth of both symbionts and concomitantly inhibited the growth of A. macleodii F12. Similarly, rosmarinic acid inhibited the motility of S. pseudonitzschiae F5 and Phaeobacter sp. F10 in an identical way to 3-oxo-C16:1-HSL, while upregulating motility in A. macleodii F12 (Shibl et al., 2020). Rosmarinic acid has been shown to interfere with QS in a plant pathogen (Hammer and Bassler, 2003), suggesting this molecule acts as a QSI. A similar mechanism of interfering with QS, a process known as quorum quenching, is used by the marine macroalga D. pulchra to inhibit swarming motility of Serratia liquefaciens by producing halogenated furanones (Rasmussen et al., 2000). Cumulatively, our observations suggest that S. pseudonitzschiae F5 and Phaeobacter sp.
F10 switch their free-living mode to a surface-attached mode to remain in the phycosphere by releasing AHLs. In addition, the adaptation of both strains to diatom metabolites (Shibl et al., 2020) ensures the success of these bacteria in the phycosphere relative to other bacteria that cannot colonize the phycosphere.

QS gene homology and organization in the Roseobacter group

To shed light on the prevalence of QS systems in the Roseobacter group, we constructed a phylogenetic tree of 52 sets of the LuxR-ITS-LuxI sequences (luxRI cassettes) in 32 Roseobacter group representative genomes. The tree revealed multiple conserved QS gene topologies that were distributed across the Roseobacter group (Fig. 5).

In Fig. 5, half of the roseobacters genomes contained more than one pair of luxRI (18/31) and more than two AHL molecules were identified in nine strains. It has been shown that >80% of bacteria in the Roseobacter group possess at least one luxRI gene cassette (Buchan et al., 2016). Phaeobacter species consistently appear to produce more than three AHLs using 2–3 luxI homologues (Fig. 5 and Supporting Information Table S4). For example, Phaeobacter gallaeciensis DSM 26640 produces up to 2(5H)-furanone and furanone C-30. Assays were conducted by inoculating 0.25% soft agar plates with each bacterium and incubating for 3 days. B. Percent motility of S. pseu-

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eight different AHLs via only three luxI homologues (Ziesche et al., 2015). Cude and Buchan (2013) provide a classification of the genetic makeup and organization of the luxRI gene cassettes and neighbouring genes, with the B topology (B group) being the most common cassette structure (Fig. 5). In our work, the B group contained 26 luxRI cassettes distributed in 26 genomes (Fig. 5). Both S. pseudonitzschiae F5 and Phaeobacter sp. F10 possess one cassette that belongs to this group, while a unique luxRI cassette only occurs in S. pseudonitzschiae F5. Interestingly, both S. pseudonitzschiae F5 and Phaeobacter sp. F10 strains produced the same three AHLs molecules (Fig. 5).

| Strains                                      | Gene Clusters | Length (bp) | AHLs       | Isolation source | MA References |
|----------------------------------------------|---------------|-------------|------------|------------------|---------------|
| Phaeobacter sp. F10                          | 3             | 1519        | A. glacioli (diatom) | Surface seawater | Y Y This study |
| Phaeobacter sp. F10                          | 3             | 1473        | A. glacioli (diatom) | Surface seawater | Y Y [1]       |
| Phaeobacter sp. F10                          | 3             | 1714        | A. glacioli (diatom) | Surface seawater | Y Y [2]       |
| Phaeobacter sp. F10                          | 3             | 1521        | A. glacioli (diatom) | Surface seawater | Y Y [3]       |
| Phaeobacter sp. F10                          | 3             | 1454        | A. glacioli (diatom) | Scalar | YY [3: 4] |
| Phaeobacter sp. F10                          | 3             | 1456        | A. glacioli (diatom) | Scalar | YY [3: 5] |
| Phaeobacter sp. F10                          | 3             | 1456        | A. glacioli (diatom) | D. polyester (red algae) | Y Y [7] |
| Phaeobacter sp. F10                          | 3             | 1456        | A. glacioli (diatom) | Blue Lagoon in Iceland | N Y [8] |
| Phaeobacter sp. F10                          | 3             | 1456        | A. glacioli (diatom) | M. laximaris (marine sponge) | YY Y [9] |

Fig. 5. Maximum-likelihood phylogenetic tree of 52 LuxR-ITS-LuxI loci and neighbouring genes in 32 roseobacterial genomes. Group B is highlighted by red branches at the top of the tree. Colour shades indicate the different groups of luxR/I cassettes. Bacteria isolated in this study are bold-faced. Length shows the sequence lengths of LuxR-ITS-LuxI loci. D = dendritic motility, M = motility, A = attachment. Y and N indicate whether a bacterium is able or unable to carry out each function. Isolation origin of bacteria highlighted in red indicates a biological host origin. The number of AHL molecules reported from each bacterium is provided, if known. Note that information on which cassette makes which AHL molecule is scarce. Some bacteria are present twice in the tree because they possess more than one Lux-like operon. Gene neighbourhood abbreviations: HK, histidine kinase; CHP, conserved hypothetical protein; TctA, TctA family transmembrane transporter; RND, RND multidrug efflux pump; AL, adenylosuccinate lyase; TF, trigger factor; Tran, transposase; TetR, TetR family transcriptional regulator; TR, transcriptional regulator. Bootstrap values >50% calculated from 100 iterations are shown at branch nodes. *Full references are listed in the Supporting Information.

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(Fig. 3 and Supporting Information Fig. S5), suggesting these molecules must be a byproduct of a homologous luxI, common to both S. pseudonitzschiae F5 and Phaeobacter sp. F10. This finding is consistent with previous studies showing that a single luxI-type gene is responsible for biosynthesizing more than one AHL molecule (Ortori et al., 2007; Hansen et al., 2015). LuxI proteins use S-adenosylmethionine (SAM) and acylated acyl-carrier protein (ACP) to catalyse the acylation and lactonization of AHL molecules (Churchill and Chen, 2011). Potentially, the luxI homologue common to S. pseudonitzschiae F5 and Phaeobacter sp. F10 does not discriminate well between different substrates and can accept acyl chains of varying lengths, enabling it to biosynthesize three AHLs. This lack of specificity could allow different AHL molecules produced by a single protein to bind different transcriptional regulators and thus regulate different functions. By regulating the availability of substrates needed to make each AHL, different AHLs may be produced depending on the environmental condition while continuously expressing the same AHL synthase (Yates et al., 2002). For example, different temperatures have been shown to influence the types and concentrations of seven different AHLs produced by one luxI in the fish pathogen Allivibrio salmonicida (Hansen et al., 2015). The diversity of substrates and selectivity levels of different luxI homologues also influence the proportions of various AHLs produced by roseobacters (Ziesche et al., 2018). S. pseudonitzschiae F5 and Phaeobacter sp. F10 were isolated from the same diatom, indicating horizontal gene transfer may be responsible for their identical production of AHLs. The second luxI gene in S. pseudonitzschiae F5 likely produces one or more other AHL molecules that we were not able to characterize due to the limitation of AHL mass spectrometry standards. As mentioned before, S. pseudonitzschiae F5 displayed a dendritic motility phenotype, which is absent in most roseobacters (Bartling et al., 2018). It is not clear what ecological advantage dendritic motility have on bacteria like S. pseudonitzschiae. Interestingly, two additional S. pseudonitzschiae strains displayed this phenotype previously (Bartling et al., 2018), indicating it is common in this species for unknown reasons.

Conclusions

Here, we have shown that two diatom symbionts use AHL molecules to inhibit their motility and enhance biofilm formation, processes that likely control their ability to attach to diatom TEP and thus colonize the phycosphere. In contrast, an opportunistic bacterium was incapable of attaching to diatom TEP and was found to lack the ability to synthesize AHL molecules. The ability of symbionts to switch their lifestyle from motile bacteria entering the phycosphere to permanent residents of this microenvironment is essential to marine bacteria that rely on phycosphere exudates to survive. This ‘swim-or-stick’ switch appears also to be partially controlled by diatoms that can make quorum sensing mimics, such as rosmaninic acid. Significant work is needed to delineate the importance of bacterial intraspecies signalling vs eukaryotic host interference in bacterial communication.

Experimental procedures

Diatom growth

Asterionellopsis glacialis strain A3 was deposited for this work in the National Center for Marine Algae and Microbiota (NCMA) under the accession number CCMP3542. Axenic A. glacialis strain A3 (CCMP3542) was generated as described previously (Behringer et al., 2018). All diatom cultures were grown at 22°C in a 12:12 h light/dark diurnal cycle (125 µE m⁻² s⁻¹) in semi-continuous batch cultures (Brand et al., 1981). Since in vivo fluorescence linearly correlates with cell numbers during exponential growth in batch cultures (Wood et al., 2005), diatom growth was monitored by measuring in vivo fluorescence of chlorophyll a (relative fluorescence units, RFU) using a 10-AU fluorimeter (Turner Designs, San Jose, CA, USA). Specific growth rates (μ) of diatoms were calculated from the linear regression of the natural log of RFU versus time during the exponential growth phase of cultures. The standard deviation of μ was calculated using values from biological replicates (n = 5 unless otherwise indicated) over the exponential growth period. The relationship between cell density and RFU of A. glacialis strain A3 can be formulated with the following equation: y = 14.958 × − 0.519, where y is cells/µL and x is the RFU value, calculated from the regression line of the linear portion of the growth curve. The adjusted R² value for this curve is 0.98 (P < 0.001).

Bacterial isolation, identification and phylogenetic analysis

To assess whether specific bacteria attach to A. glacialis strain A3, we cultivated bacteria from the xenic A. glacialis strain A3 culture as described previously (Shibl et al., 2020). Briefly, bacteria were isolated from the diatom at early stationary phase by serially diluting 0.5 ml 1000 times in sterile t/2 media (Guillard, 1975) and subsequently spreading 200 µl onto (i) marine agar plates, (ii) plates containing per litre of seawater: 15 g agar and 2 g carbon source (sodium succinate or glucose) and (iii) sterilized A. glacialis strain A3 culture suspension with 1.5% agar. Plates were incubated at 25°C in the dark for 3–7 days and single colonies with unique morphologies were re-streaked three times to eliminate
cross-contamination before storage at −80°C in 15% glycerol stocks.

Bacterial isolates were identified using direct PCR (Hofmann and Brian, 1991). Isolates were incubated in 3 ml marine broth at 25°C in the dark until optical density (600 nm) reached ~1.0; subsequently, 2 μl of each bacterium was used as a DNA template in PCR. The 16S rRNA gene from all bacteria was amplified by universal primers (27F, 1492R) as previously described (Amin et al., 2015). PCR products were purified by the Wizard PCR purification kit (Promega) and sequenced using Sanger sequencing (Apical Scientific, Malaysia). Sequences were aligned with 16S rRNA gene sequences from GenBank using ClustalW Multiple Alignment in BioEdit 7.0.9.0. A neighbour-joining (NJ) phylogenetic tree was constructed with BIONJ (Gascuel, 1997) using Kimura’s two-parameter model. Another tree was constructed using the maximum likelihood (ML) methods using PhyML 3.0 (Guindon et al., 2010) with GTR + R + I substitution model as determined by SMS (Lefort et al., 2018). The final 16S rRNA gene phylogenetic consensus tree was generated and edited using FigTree 1.4.2 (Rambaut, 2014). 16S rRNA genes of S. pseudonitzschiae F5, Phaeobacter sp. F10, and A. macleodii F12 were compared with the microbial community of A. glacialis recovered after 20 days of isolation from the field (Behringer et al., 2018) using the BLASTn tool in BLAST+ (Camacho et al., 2009). Average nucleotide identities (ANI) shared between the three isolates and their closely related species were calculated using the enveomics toolbox (http://enve-omics.ce.gatech.edu/ani/).

Co-culture generation and growth

For co-cultures, axenic A. glacialis strain A3 was inoculated from an acclimated, mid-exponential phase growing culture into 25 ml sterile f/2 media to achieve an initial diatom cell density of ~4000 cells mL⁻¹. Bacteria were grown in marine broth (ZoBell, 1941) in the dark overnight from a single colony at 26°C and shaking at 180 rpm. Cultures were centrifuged at 4000 rpm for 10 min followed by washing twice with sterile f/2 medium. Subsequently, cultures were used to inoculate axenic A. glacialis cultures at an initial bacterial density of ~1 x 10⁵ cells mL⁻¹. Bacterial counts in co-cultures were quantified by staining 1 ml fresh bacterial cells with 1x SYBR Safe stain (Edvotek Corp. USA), incubating stained samples in the dark at room temperature for 1 hour and using a CyFlow Space flow cytometer (Partec, Münster, Germany). Specific growth rates of diatoms were calculated as described above. Specific growth rates (µ) of bacteria were calculated from the linear regression of the natural log of cell counts from 2 to 6 days of cultures. The standard deviation of µ was calculated using values from biological replicates (n = 3 unless otherwise indicated).

Bright-field and fluorescence microscopy

To observe transparent exopolymeric particles (TEP) distribution and their attached bacteria, two staining steps were applied. Alcian blue was first used to stain TEP in diatom cultures. A 1-ml culture in mid-exponential phase was gently filtered by gravity onto 3-μm 25 mm polycarbonate membrane filters (Whatman) to remove free-living bacteria (~1 μm). Subsequently, 1 ml alcian blue in 0.06% glacial acetic acid (pH 2.5) was allowed to gently run down the tube wall onto the filter to stain samples for 10 min at room temperature (Long and Azam, 1996). Excess alcian blue was removed using a gentle wash with PBS buffer and gently filtered by gravity. Subsequently, filters that retained diatoms (~25 μm for a single cell), TEP particles and attached bacteria were fixed with Moviol-Sybr Green I as described previously (Lunau et al., 2005). Filters were visualized using a DMi6000B epifluorescence microscope (Leica) with a DMC2900 colour brightfield camera (Leica). Bright-field microscopy was used for TEP observation. Fluorescence microscopy was used for bacteria and diatom nucleic acid observations. L5 fluorescence filter was used for SYBR Green I staining of both bacterial and algal nucleic acids with an excitation wavelength range of 480–656 nm and an emission wavelength of 590 nm. Y5 fluorescence filter was used for algal chlorophyll autofluorescence with an excitation wavelength of 620 nm and an emission wavelength of 700 nm. Both bright-field and fluorescence images were merged using the LAS X software (Leica Microsystems, Germany). To observe diatom chain length, aliquots from A. glacialis cultures were directly placed onto microscopy slides and were visualized using the DMi6000B microscope described before. The chain length of axenic, xenic A. glacialis strain A3 and co-culture of the diatom with S. pseudonitzschiae F5 were quantified using an inverted microscope (Eclipse Ti-U, Nikon, Japan).

Bacterial genome sequencing and assembly

Genomic DNA of S. pseudonitzschiae F5, Phaeobacter sp. F10, and A. macleodii F12 were extracted from pure bacterial cultures using an E.Z.N.A. Bacterial DNA Kit (Omega BIO-TEK) according to the manufacturer’s instructions. DNA yield and quality were measured and checked using a Qubit 3.0 Fluorometer (Invitrogen; Life Technologies) and gel electrophoresis.

Genome sequencing of all three bacterial genomes was conducted using the Illumina and PacBio platforms at Apical Scientific Sdn. Bhd (Malaysia) for S. pseudonitzschiae F5 and Phaeobacter sp. F10 and at Novogene (China) for
A. macleodii F12. In brief, 100–1000 ng DNA was fragmented by acoustic disruption using a Covaris S220 system (Covaris, Woburn, MA) for Illumina sequencing. Subsequently, DNA libraries were built using a NEBNext™ Ultra™ II DNA Library Prep Kit (NEB E7645S/L, New England BioLabs®), and quantification and quality control of generated libraries were performed on an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). The libraries were then sequenced with TruSeq SBS Kit v4-HS reagents on an Illumina HiSeq 2500 platform (Illumina, San Diego, CA). For PacBio sequencing, DNA was sheared to ~15–20 kb using a Megarupter (Diagenode) and sequenced on a PacBio RS II system (Pacific Biosciences, Menlo Park, CA).

High-quality PacBio sub-reads were assembled by aligning the Illumina paired-end 150 bp reads with the Basic Local Alignment via Successive Refinement (BLASR) aligner, a tool that combines data from short read alignments with optimization methods from whole genome alignment (Chaisson and Tesler, 2012) and were trimmed using bbdruk, aligned with bbmap, and the resulting sorted bam file and the Pacbio consensus reference were used as input to pilon (–genomes genome.fasta, –bam input. bam) (Walker et al., 2014) for further error correction and polishing. The assemblies were further polished with arrow in Canu 1.7 with parameter corOutCoverage = 60 (Koren et al., 2017). The largest circular contig in each assembly represented the chromosome size for each bacterium. In addition, plasmid contigs were BLASTed against closely related genomes to confirm these were indeed plasmids. For S. pseudonitzschiae F5, we were not able to close the chromosome due to high number of repeats in the genome and thus exact chromosome size could not be determined (Table 1). The consensus reference was annotated by Prokka (Seemann, 2014) and RAST and checked for completeness by BUSCO (Simão et al., 2015). Circa 1.2.1 (http://omgenomics.com/circa/) was used to draw chromosomal circo plots and chord diagrams to compare the same gene locations in different genomes. All genome sequences and their annotations can be accessed at GenBank under the accession numbers WKFG01000000 and CP046140-CP046144.

Mining publicly available Alteromonas genomes for autoinducer synthase genes was performed on MicroScope (https://image.genoscope.cns.fr/microscope/home/index.php). Identification and classification of transporter proteins in the three bacterial genomes was done using 6097 membrane transport protein sequences downloaded from the Transporter Classification Database (TCDB) (Saier Jr et al., 2016). To this end, Gblast2 program (http://www.tcdb.org/labsoftware.php) with a cutoff e-value < 1e−20 was used and sequences with alignment scores <100 were removed. The chemotaxis, flagellar protein, pili, exopolysaccharides and quorum sensing related gene identities were confirmed by BLASTp against the model Roseobacter group strain R. pomeroyi DSS-3.

QS genes analysis

The luxR-like and luxI-like genes of S. pseudonitzschiae F5 and Phaeobacter sp. F10 were predicted by RAST and Prokka. Maximum likelihood phylogenetic trees of multiple copies of LuxI-like, genetically linked LuxR-like and their internal transcribed spacer (ITS) sequences from S. pseudonitzschiae F5, Phaeobacter sp. F10, and 30 publicly accessible Roseobacter group genomes were constructed using PhyML 3.0 (Guindon et al., 2010) with HKY85 substitution model, which was selected according to SMS (Smart Model Selection in PhyML) (Lefort et al., 2017). These 30 strains were chosen based on several criteria: (i) Strains must have published information about at least two of the following: AHLs molecules, motility or attachment lifestyles, (ii) all strains have whole genomes publicly available, and one of the following two criteria: (iii) Strains are phylogenetically related to S. pseudonitzschiae F5 or Phaeobacter sp. F10, or (iv) Strains are well studied as model roseobacters. The luxRI and ITS sequences of Pseudomonas aeruginosa PAO1 (RefSeq accession number NC_002516.2) were used as an outgroup. Phylogeny was tested with a fast likelihood-based method aBayes (Anisimova et al., 2011) with 100 bootstraps.

AHLs extraction and identification

To extract AHL molecules, S. pseudonitzschiae F5 and Phaeobacter sp. F10 strains were re- plated from glycerol stocks and subsequently single picked colonies were inoculated overnight in 25-ml marine broth at 26 °C in the dark with shaking at 180 rpm. Overnight cultures were subsequently transferred to 1 l sterile marine broth in triplicates and grown under the same conditions for 20 h. Finally, bacterial cells were removed by centrifugation (15 min, 7000 rpm) when the optical density (600 nm) reached ~1.0. To further remove residual bacteria, the supematant was further filtered through 0.2-μm polycarbonate membrane filters (Whatman, NJ, USA). Oasis HLB solid-phase extraction (SPE) cartridges (3 cc, 540 mg) were activated according to the manufacturer’s instructions and were subsequently used to remove organic molecules from the filtrates as previously described (Wang et al., 2017). Finally, extracts were eluted with 5 ml 0.1% (v/v) formic acid in methanol and dried using an evaporator (SpeedVac SC210A; Thermo Savant, Holbrook, NY, USA) and were stored at −20°C for subsequent UHPLC-MS/MS analysis. AHL standards (Supporting Information Table S3) were acquired from Cayman Chemicals and Sigma-Aldrich to

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help identify AHLs from *S. pseudonitzschiae* F5 and *Phaeobacter* sp. F10.

AHLs were analysed using an Agilent 1290 HPLC system coupled to a Bruker Impact II Q-ToF-MS (Bruker, Germany). Metabolites were separated using a reversed-phase separation method. In RP mode, medium-polarity and non-polar metabolites were separated using an Eclipse Plus C18 column (50 mm × 2.1 mm ID) (Agilent, USA). Chromatographic separation consisted of MilliQ-H2O + 0.2% formic acid (buffer A), and acetonitrile + 0.2% formic acid (buffer C) at a flowrate of 0.4 ml. The initial mobile phase composition was 90% A and 5% C followed by a gradient to 100% C over 18 min. The column was maintained at 100% C for 2 min followed by cleaning with isopropanol for 3 min. The column was allowed to equilibrate for 4 min using the initial mobile phase composition. Detection was carried out in the positive ionization mode with the following parameters: mass range = 50–1300 m/z measured at 6 Hz, ESI source parameters: dry gas temperature = 220°C, dry gas flow = 10 l min⁻¹, Nebulizer pressure = 2.2 bar, Capillary V = 3000 V, end plate Offset: 500 V; MS-ToF tuning parameters: Funnel 1 RF = 150 Vpp, Funnel 2 RF = 200 Vpp, ISCID Energy = 0 eV, Hexapole RF = 50 Vpp, Ion Energy = 4.0 eV, Low Mass = 90 m/z, Collision Energy = 7.0 eV, pre Pulse storage = 5 μs.

Data were processed and analysed with Metaboscape 3.0 (Bruker, Bremen). Processing was conducted with the T-Rex3D algorithm with an intensity threshold of 500 and a minimum peak length of 10 spectra. Spectra were lock-mass calibrated, and features were only created if detected in a minimum of three samples. The presence of a specific AHL was determined by comparing the high-resolution parent ion mass, daughter ion masses and retention times of the purchased standards relative to bacterial extracts.

**Bacterial motility and biofilm formation assays**

Bacterial motility assay was performed using semisolid (0.25% w/v) marine agar plates supplemented with a final concentration of 2 μM of each AHL or QS inhibitor (QSI) as described previously (Zan et al., 2012). The QSIs 2-(S)-furanone and (Z)-4-Bromo-5-(bromomethylene)-2-(S)-furanone (furanone C-30) were purchased from Sigma-Aldrich. *S. pseudonitzschiae* F5, *Phaeobacter* sp. F10, and *A. macleodii* F12 strains were grown in marine broth overnight and then were gently inoculated using a sterilized toothpick into the centre of the agar surface. Triplicate plates were incubated at 26°C for 3 days after which motility plates were observed using the Uvitec Cambridge Fire-reader imaging system. The proportion of motile area (percent motility) was measured using ImageJ software (http://rsb.info.nih.gov/ij/). Bacterial motility is characterized by a circular swimming zone in the motility assay or by dendritic morphology, which is typical of swarming motility caused by a locally restricted movement on agar plates (Michael et al., 2016).

Bacterial biofilm formation was quantified for *S. pseudonitzschiae* F5, *Phaeobacter* sp. F10, and *A. macleodii* F12 strains using the crystal violet assay (O’Toole and Kolter, 1998). Briefly, bacteria were cultured in marine broth overnight and then diluted to ~1 × 10⁵ cells ml⁻¹. 100 μl aliquots were transferred to a 96-well suspension culture plate (Greiner bio-one, CELLSTAR, Monroe, NC, USA). A previously optimized final concentration of 10 μg ml⁻¹ AHL or QSI were added to each well in triplicates (Ren et al., 2001; Zhu et al., 2019) while controls received no AHL or QSI addition. The plate was incubated at 26°C for 24 h and subsequently 25 μl of 0.1% crystal violet was added to each well and incubated at room temperature for 15 min. Wells were rinsed twice with 150 μl Milli-Q water to remove free-living bacteria and then dried at 60°C for 10 min. Finally, stained biomass was solubilized in 95% ethanol for 1 h and absorbance readings were measured using a plate reader (BioTek, Winooski, VT) at 600 nm. Unpaired t-test was used to compare the significant differences of control and other variables in bacterial swarming and biofilm formation.

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**Supporting Information**

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site:

**Appendix S1.** Supporting Information.

**Appendix S2.** Supporting Information.