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A study of biofilm formation in marine bacteria isolated from ballast tank fluids

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Abstract

Seawater-compensated fuel ballast systems maintain ship stability as fuel is spent but can introduce microorganisms that form biofilms, biodegrade fuel components, or enhance corrosion; all of which increase operating and maintenance costs. The aim of this study was to isolate planktonic bacteria from ballast tank fluids and taxonomically classify those that formed biofilm in culture. Twenty-two isolates were identified as belonging to seven genera based on 16S rRNA gene sequencing. Of the seven genera represented *Alteromonas*, *Pseudoalteromonas*, and *Brevundimonas* strains produced quantifiable biofilm in crystal violet assays. To test the hypothesis that the level of bulk nutrients would influence the extent of biofilm formation, isolates were grown in a conventional marine medium and marine medium supplemented with tryptone and yeast extract to represent standard and nutrient-replete media, respectively. While 80% of the *Pseudoalteromonas* strains produced 7 to 11-fold more biofilm in conventional medium vs. the supplemented medium, 64% of *Alteromonas* strains produced up to 50-fold less in the same medium. These results suggest that bulk nutrients influence the extent of biofilm formation in a taxa-specific fashion in these marine organisms. The sole *Dasania marina* isolate failed to display considerable biofilm growth in either media but was the only isolate to produce quorum sensing molecule(s), N-acyl homoserine lactone(s), in assays using an *Agrobacterium tumefaciens* reporter strain. Whether N-acyl homoserine lactones produced by *D. marina* could modulate biofilm formation in the other organisms isolated in this study would require further investigation.

Introduction

Biofilms are a form of fouling where microorganisms attach to a surface and produce a protective matrix that allows them to persist in hostile environments [1]. Several factors have been shown to influence the extent of biofilm formation including nutrient availability [2,3] and quorum sensing [4]. Biofilms present in marine systems can lead to increased maintenance costs or material damage [5]. For example, in maritime transport biofilm that accumulates on the hull of a ship promotes macrofouling by barnacles and mussels, and the increased drag increases fuel consumption by an estimated 30 to 50% [6]. Other examples of increases in biofouling-related maintenance costs include an annual increase of 2 to 4% due to fouling of ship ballast water [7] and 20 to 30% due to corrosion [8].

The Navy uses a compensated fuel ballast system in several classes of its ships, including cruisers and destroyers. Each system consists of a series of tanks connected in sequence and as fuel is spent seawater gets pumped in to compensate for the lost volume [9]. While this mechanism maintains ship stability during deployment, there can be unintended consequences to the ballast system infrastructure or fuel when seawater microorganisms are introduced. Several studies have demonstrated that some microorganisms can metabolize hydrocarbons present in fuels or enhance corrosion of the system [10-12].

The conditions for planktonic growth can be found within a ballast system [13] and include water, a permissible growth temperature and carbon source, and salt and nutrients which vary depending upon the route of the ship and type of water used for compensation [14]. In 2001, Drake *et al.* estimated that the ballast water from a ship can harbor up to 10^9 planktonic cells per L [15], and although the ballast water gets ejected during refueling operations, biofilm material remains attached to the walls of the system [16]. High-throughput DNA sequencing studies have shown that the composition and relative abundance of dominant taxa in bulk seawater differ from that within biofilms that form on surfaces submerged in seawater [17,18] suggesting that certain taxa, not all, contribute to biofilm formation. The aim of this study was to isolate planktonic bacteria from ballast tank fluids, classify those that formed biofilm in culture, and determine if the level of bulk nutrients in growth media influenced the amount of biofilm produced.

Materials and methods

Bacterial strains and growth conditions

Bacterial isolates were obtained from the ballast tank fluids of four US Navy ships in 2014: USS Sampson (seawater in tank for 8 months), USS Higgins (seawater in tank for 4-6 months), USS Bunker Hill (seawater in tank 4 months), and USS Lake Champlain (seawater in tank 1 week). Pooled ballast tank water was plated onto Marine Agar 2216 (BD Difco) within 48 hours of receipt and plates were incubated at 26°C. Isolated colonies were re-streaked and grown for a minimum of three transfers to obtain pure cultures. Freezer stocks were made in Marine Broth 2216 (BD Difco) (MB), a common medium for culturing heterotrophic marine organisms, supplemented with 20% glycerol and stored at – 80°C. Culturing of strains was carried out in MB and a nutrient-replete Marine Broth (MB/TY), which was MB supplemented with 5 g of tryptone and 4 g of yeast extract per L.

The reporter strain used for the detection of N-acyl homoserine lactones (AHL), *Agrobacterium tumefaciens* KYC55 (pJZ372, pJZ384,

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pJZ410) [19], was grown in Luria-Bertani broth (LB) or AT medium supplemented with spectinomycin (100 µg/mL) gentamycin (20 µg/mL) and tetracycline (2 µg/mL) [20]. This reporter strain detects a wide range of exogenous AHL of different lengths and compositions including 3-oxo-C4- to 3-oxo-C16-HSL and C4- to C18-HSL. The AHL-positive control strain, *Sinorhizobium meliloti* RM41, was grown in LB supplemented with 2.5 mM of $CaCl_2$ and 2.5 mM of $MgSO_4$.

Taxonomic classification of ballast tank isolates

To identify bacterial isolates, genomic DNA was extracted using the PureLink Microbiome DNA Purification Kit (ThermoFisherScientific, US) following the manufacturer's instructions. The 16S rRNA gene was amplified by PCR using the forward primer (fd1) 5'AGAGTTTGATCCTGGCTCAG3' and reverse primer (rp2) 5'ACGGCTACCTTGTTACGACTT3' set [21] and DreamTaq PCR Master Mix (ThermoFisherScientific). The PCR reactions were sequenced at the sequencing facility at the University of Texas at Austin using the forward and reverse primers listed above and 5'CAGCAGCCGCGGTAA3' (519F) [22]. The Basic Local Alignment Search Tool (BLASTN) was used to align and identify each sequence. Sequences were submitted to GenBank under the accession numbers MK250492-MK250514. The sequence identifications were verified using the Ribosome Database Project Naive Bayesian rRNA Classifier Version 2.11 [23].

Assays for biofilm production and planktonic growth

Strains were grown overnight on Marine Agar and individual colonies were inoculated into 4 mL MB or MB/TY and incubated at 26°C with shaking (100 rpm) for 2 d. The starter cultures were observed for the presence of ring and floating biofilms at 2 d and 7 d, respectively and were used to inoculate 96-well plates for the crystal violet assay.

Crystal violet assays were performed as described [24]. Briefly, starter cultures were seeded into 96-well round-bottom plates at 1:100 dilution (OD_{600} nm=1.0). The plates were incubated at 26°C for 2 d with shaking (100 rpm). The liquid culture was transferred to a 96-well flat-bottom plate to measure planktonic growth at OD_{600} using a Synergy HT microplate reader (Biotek Instruments, US), and the biofilm that remained attached to the wells of the plate was washed, stained with 0.1% crystal violet, and quantified by measuring the OD_{550} as described by O'Toole [24].

AHL detection

A modified version of the procedure by Joelsson [20] was used to recover AHL from the supernatant of bacterial cultures. Briefly, 4 mL of log-phase cultures grown in MB were centrifuged at 13,500 x g for 3 min and the supernatant was subjected to extraction with 4 mL of ethyl acetate. The residue was resuspended in 400 µL (10X extract) acetonitrile and stored in a glass vial at -20 °C.

A variation of the method by Cha [25] was used to detect AHL on solid media. Briefly, the AHL-reporter strain *A. tumefaciens* KYC55 was grown on LB agar with antibiotics at 26 °C for 2 d, and isolated colonies were inoculated into 0.6 mL AT medium with antibiotics and grown for 24 h. The cells were seeded into 20 mL of warm melted AT agar (0.8%) supplemented with antibiotics and 40 µL of 20 mg/mL X-gal and poured to make AT agar plates. Five µL aliquots from each 10X extract were spotted onto AT agar plates, and plates were incubated at 26°C for 24 h. To quantify AHL in broth cultures, β-galactosidase assays were conducted as described by Joelsson [20]. The 10X extract from *S. melilo*ti RM41 and 100 µM of the synthetic C8-HSL were included as positive controls.

Results and discussion

Identification of ballast tank isolates

Twenty-two bacterial isolates were classified into three dominant phyla, with all but two belonging to Gammaproteobacteria. SDH6 was classified as *Brevundimonas mediterranea* belonging to Alphaproteobacteria and SD20 was classified as *Bacillus adhaerens* belonging to the Firmicutes (Table 1). SD20 was identified as the only gram-positive bacterium among the cohort. Of the 21 gram-negative strains, eight of the Gammaproteobacteria were classified as *Alteromonas oceani* S35 and three as *A. tagae* BCRC 17571. Five isolates were assigned to the genus *Pseudoalteromonas*, two were identified as *Halomonas axialensis* and the remaining Gammaproteobacteria were single organisms from the genera *Dasania* and *Marinobacter*. These data are consistent with a recent high throughput sequencing study showing that the most abundant Gammaproteobacterial OTUs from ballast waters from five separate ships belonged to Alteromonadales, which encompass the *Alteromonas* and *Pseudoalteromonas* genera [26].

Biofilm production in MB and MB/TY

All isolates were initially screened for biofilm production by observing cultures grown in glass tubes (Figure 1). In doing so, two biofilm morphologies were observed: a ring and a floating disk, described by Mosharaf [27] as solid-air-liquid (SAL) and air-liquid

Table 1. Identification of bacterial isolates based on 16S rRNA gene classification

Isolate	Strain ID (GenBank closest match)	Maximum identity (%)	Accession No.
Gammaproteobacteria			
SD3	*Alteromonas oceani* S35	99.89	NR 159349.1
SD4	*Alteromonas oceani* S35	98.84	NR 159349.1
SD8	*Alteromonas oceani* S35	98.85	NR 159349.1
SD9	*Alteromonas oceani* S35	99.88	NR 159349.1
SD13	*Alteromonas oceani* S35	99.66	NR 159349.1
SD17	*Alteromonas oceani* S35	98.28	NR 159349.1
SD18	*Alteromonas oceani* S35	98.41	NR 159349.1
SD32	*Alteromonas oceani* S35	99.78	NR 159349.1
SD5	*Alteromonas tagae* BCRC 17571	98.93	NR 043977.2
SD7	*Alteromonas tagae* BCRC 17571	99.26	NR 043977.2
SD16	*Alteromonas tagae* BCRC 17571	100.00	NR 043977.2
SD1	*Pseudoalteromonas shioyasakiensis* SE3	99.57	NR 125458.1
SD10	*Pseudoalteromonas shioyasakiensis* SE3	99.57	NR 125458.1
SD31	*Pseudoalteromonas undina* NBRC 103039	99.89	NR 114191.1
SD1A	*Pseudoalteromonas tetraodonis* GFC IAM 14160	100.00	NR 041787.1
SD1B	*Pseudoalteromonas tetraodonis* GFC IAM 14160	100.00	NR 041787.1
SD2D	*Halomonas axialensis* Althf1	99.93	NR 026219.1
SD2E	*Halomonas axialensis* Althf1	99.86	NR 026219.1
SD1D	*Dasania marina* DMS 21967 KOPRI 20902	98.50	NR 043175.1
SDH2	*Marinobacter adhaerens* HP15	99.93	NR 037108.1
Alphaproteobacteria			
SDH6	*Brevundimonas mediterranea* V4.BO.10	99.85	NR 037108.1
Firmicutes			
SD20	*Bacillus aquimaris* TF-12	99.20	NR 025241.1

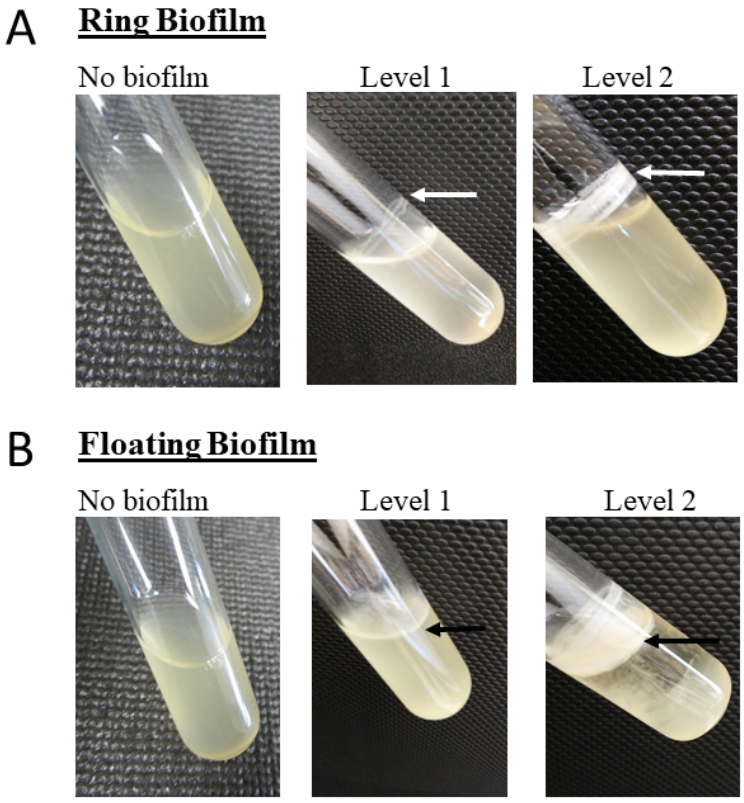

Figure 1. Images of biofilm formed in glass tubes. *panel A*, isolates were grown in MB or MB/TY for 2 d at 26 °C with agitation (100 rpm), and biofilms were observed and classified as level 1 (weak/thin biofilm) or level 2 (thick biofilm). *panel B*, strains were grown as described but were incubated further under static conditions for 7 d at 19 °C. Arrows point to biofilm

(AL) biofilms, respectively. The ring morphology adhered to the wall of the glass tube and formed at the interface between the air and liquid culture (Figure 1, *panel A*). The floating disk morphology was attached to the surface of the culture and may or may not appear to adhere to the tube (Figure 1, *panel B*). All isolates except for the *Marinobacter* strain formed at least one of the two types of biofilm observed (Table S1). *Alteromonas* strains produced thick ring and floating biofilms with the exception of SD16, one of the three *A. tagae* isolates. It did not produce a visible ring and produced only a weakly visible floating biofilm. The five *Pseudoalteromonas* strains also produced both morphologies, but to a lesser extent than the *Alteromonas* strains. Finally, the single strains of *Dasania*, *Marinobacter*, and *Bacillus* produced very little or no detectable biofilms in glass tubes (Table S1).

Sixteen of the 22 isolates produced quantifiable biofilm in crystal violet assays in either MB or MB/TY, with OD_{550} values greater than the media-only control (OD_{550}=0.04) (Figure 2). This included all *Alteromonas* strains (Figure 2, *panel A*), four of the five *Pseudoalteromonas* strains (Figure 2, *panel B*), and the sole *Brevundimonas* strain (Figure 2, *panel C*). The remaining six strains produced very little to no biofilm when grown in either media and included strains belonging to the genera *Halomonas, Dasania, Marinobacter,* and *Bacillus.*

Although there were eight *A. oceani* strains and all produced quantifiable biofilm material, there was wide variation in their abilities to do so (Figure 2). SD8 produced significantly more biofilm material than any of the other strains when grown in MB (OD_{550}=16.95±1.91). All but three isolates (SD4, SD17, SD18) produced more biofilm material when grown in MB vs MB/TY (Figure 2, *panel A*), with SD3 producing over 50-fold more. There was also variation in biofilm formation in the *A. tagae* isolates in that SD7 produced considerably more biofilm in MB than in MB/TY, and SD16, a weak biofilm producer, produced more biofilm in MB/TY. Interestingly, *A. tagae* (SD5), similar to SD7, produced more biofilm in MB (OD_{550} > 4), but the biofilm material was not consistently stable to withstand the washes and resulted in some replicates being weakly stained in the same experiment. This accounted for the high standard deviation observed for SD5 grown in MB media. Furthermore, quantification of crystal violet in nutrient-replete medium was difficult since this strain produced a gel-like "pellicle" akin to the Alphaproteobacteria member *Gluconacetobacter xylinus* [28], which was ejected during the wash steps. Floating biofilms have been extensively studied in gram-positive bacteria such as *Bacillus thuringiensis* [29] or *B. subtillus* [28], where components of the matrix which allow it to float consist of proteins that form amyloid-like fibers and a hydrophobic layer. It is unknown if the gel-like matrix formed by SD5 in nutrient-replete marine medium consisted of similar proteins. Nevertheless, it was one of the only isolates to form this type of floating biofilm in microtiter plates.

In contrast to the *Alteromonas* strains which generally produced more biofilm in MB than in MB/TY, three of the five *Pseudoalteromonas* strains produced 7- to 11-fold more biofilm in MB/TY (Figure 2). This included the sole *P. undina* strain (SD31) and the two *P. tetraodonis* strains (SD1A and SD1B). Of the two *P. shioyasakiensis* strains (SD1 and SD10), only one produced quantifiable biofilm and did so at equivalent levels in both growth media. That the majority of the *Alteromonas* isolates produced more robust biofilms in MB, while isolates belonging to *Pseudoalteromonas* did so in MB/TY, supports the hypothesis that the level of bulk nutrients influence the extent of biofilm formation, and that it did so in a taxa-specific fashion.

Finally, the sole *B. mediterranea* strain produced comparable levels of biofilm in both growth media (OD_{550}=4.74±0.78 and 4.72±0.69) but the lowest overall planktonic growth (OD_{600}) of all strains in MB (Table S2). The remaining five strains produced levels of biofilm that were comparable to background ($OD_{550} \leq 0.04$). For all isolates except SD5 (an *Alteromonas* strain), planktonic growth in the same cultures was higher in MB/TY than MB (Table S2).

AHL detection

Since quorum sensing is a variable that can influence biofilm formation and all of the biofilm-forming isolates in this study were gram-negative, they were subsequently screened on plates for the ability to produce AHL using the reporter strain *A. tumefaciens* KYC55. As shown in Figure 3, only *D. marina* (SD1D) produced AHL detectable by this reporter and did so in both growth media. Figure 3 *panel C* shows the controls used in this assay, demonstrating that the synthetic C-8 AHL and AHL produced by *S. meliloti* RM41 were detectable. When further experiments were performed to quantify β-galactosidase activity, the levels of activity of SD1D grown in either MB or MB/TY were comparable to levels from an extract of the positive control strain, *S. meliloti* RM41, and the synthetic C8-HSL (Figure 4). Also shown in the figure are extracts from SDH6 (*B. mediterranea*) grown in MB and MB/TY. This strain produced comparable biofilm in both types of media, however AHL were not detectable. Extracts from the other isolates were negative for AHL production by the reporter strain (data not shown). This negative result for the *Pseudoalteromonas* strains was not expected as extracts from *P. shioyasakiensis* and *P. tetraodonis* strains isolated from sediments in the Zhejiang Province demonstrated a positive response in reporter assays using a different *A. tumefaciens* A136 reporter strain that responds to C6- to C14-HSL [30]. This range is within that of the reporter strain used in this study. That some of the isolates produced AHL that were not detected by the reporter cannot be ruled out but was beyond the scope of this study. Indeed, Huang *et al.* concluded that AHL bioassay results are not always consistent among different screening methods [31]. However, only the *D. marina* isolate (SD1D), which failed to produce quantifiable biofilm under

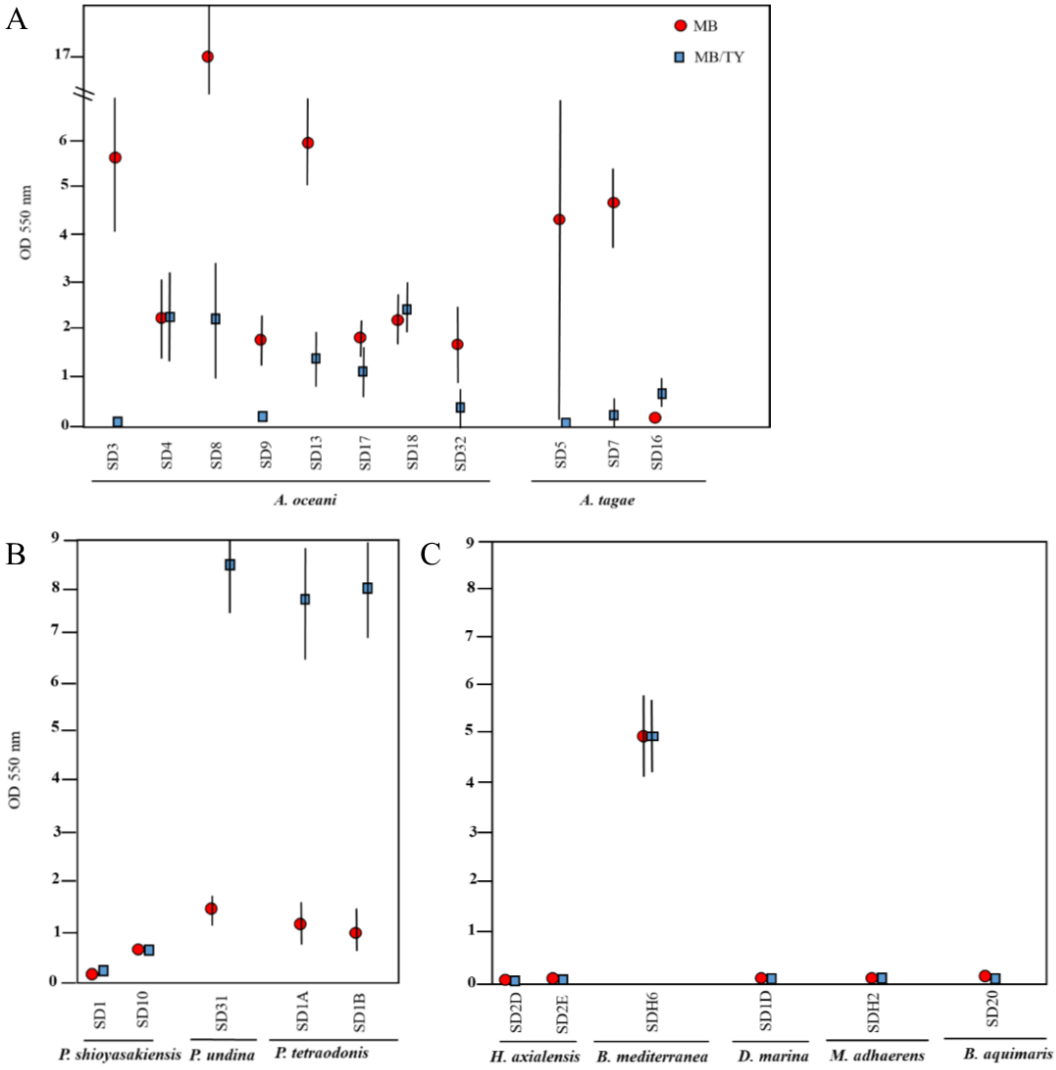

Figure 2. Biofilm quantification (OD 550 nm) in microtiter plates. Isolates were inoculated 1:100 (at an OD_{600} = 1) into microtiter plates, cultured for 2 d, and the biofilm material was stained as described in materials and methods. The OD_{550} for each strain was determined by subtracting the OD_{550} of the media-only wells. Data shown are representative of two independent experiments performed on different days. The results are the mean ± standard deviation (n = 8). Standard deviations below 0.2 are not shown

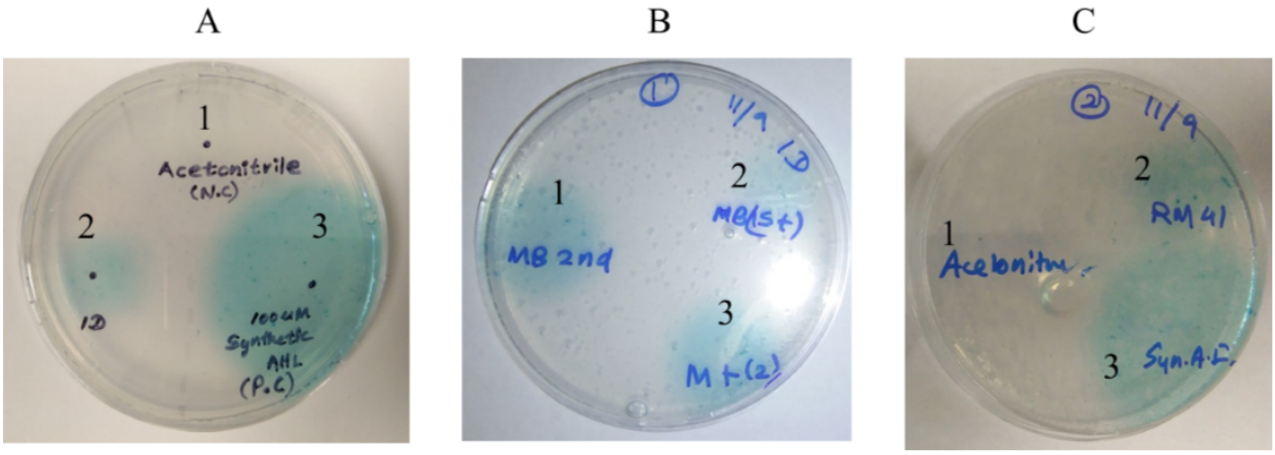

Figure 3. AHL screen using a reporter strain. The reporter strain *A. tumefaciens* KYC55 was seeded into warm AT medium containing antibiotics and X-gal as described in the materials and methods. 10X extracts from bacterial isolates were spotted onto AT agar plates. The turquoise zone is indicative of the presence of AHL. *panel A*, AHL production of SD1D grown in MB. 1 is acetonitrile (negative control), 2 is 10X extract from SD1D, 3 is synthetic C8-HSL (100 µM solution). *panel B*, AHL production of SD1D in MB (1) or MB/TY (2 and 3). *panel C*, AHL production in positive and negative controls. 1 is acetonitrile (negative control), 2 is 10X extract from *S. meliloti* RM41 (positive control extract), and 3 is synthetic C8-HSL (positive control)

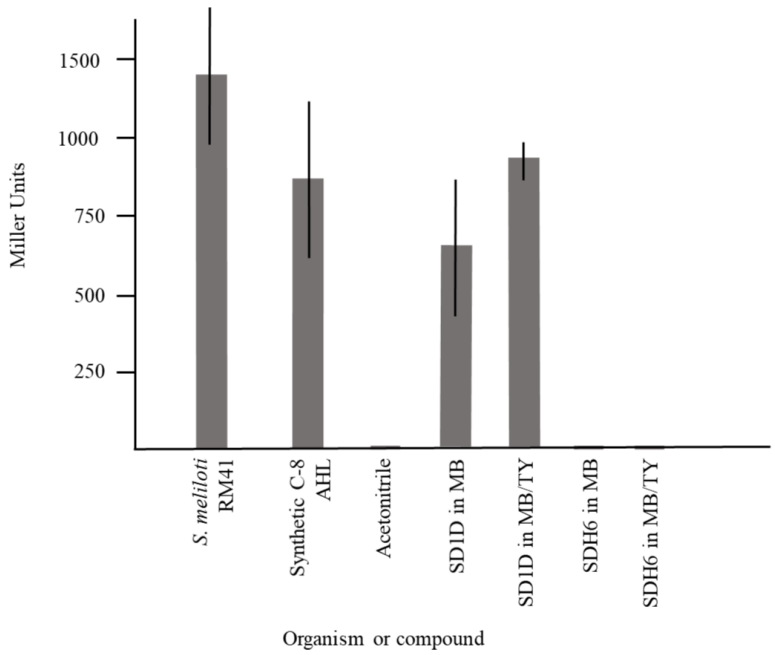

Figure 4. AHL quantification using β-galactosidase assays. One hundred microliters of the reporter strain *A. tumefaciens* KYC55 were seeded into 2 mL of AT medium. Ten microliters of C8-HSL (positive control) or 10X extracts were added, the culture was grown to an OD_{600} of 0.2 to 1, and β-galactosidase assays were conducted as described [20]. Data shown are the mean ± standard deviation (n = 2, two independent experiments). Standard deviations below 3 are not shown

both nutrient conditions, tested positive for AHL using the reporter. Whether or not AHL released by SD1D could have an impact on biofilm formation when co-cultured with the other isolates is currently being investigated.

Conclusion

Approximately 70% (16 of 22) of isolates recovered from ballast tanks produced quantifiable biofilms in at least one growth media tested. *Alteromonas* isolates displayed enhanced planktonic growth in nutrient-replete medium, however biofilm formation was reduced under these same conditions for 70% (8 of 11) of them, suggesting that increased bulk nutrients limit biofilm formation for this group. Indeed, studies in other biofilm-producing strains, including *Lysteria monocytogenes*, showed a similar trend [32]. Alternatively, and unexpectedly, Pseudoalteromonads produced more biofilm when bulk nutrients were in excess.

A recent study demonstrated that *Pseudoalteromonas* strains were both cultivable on hydrocarbon-enriched media and abundant in oiled mesocosms [33], consistent with *Pseudoalteromonas* strains being abundant in hydrocarbon-laden environments, such as ballast tanks that hold fuel. Furthermore, *Pseudoalteromonas, Alteromonas, Halomonas,* and *Marinobacter* strains were identified as hydrocarbon-

degrading bacteria enriched by the Deepwater Horizon oil spill in 2010 [34]. Whether or not these ballast fluid isolates can biodegrade fuel was beyond the scope of this study but is currently being investigated.

The reporter strain *A. tumefaciens* was selected for the screen since it detects a wide range of exogenous AHL [19] and those associated with biofouling include C4- to C12-HSL and 3-oxo-C6- to 3-oxo-C12-HSL [35]. AHL were not detected in culture extracts from the biofilm-forming isolates in this study, suggesting that strains recovered from ballast tanks may not use AHL for biofilm formation, use AHL that is out of the range of the reporter strain, or possibly use a different quorum sensing system altogether if at all. Whether AHL produced by the *D. marina* isolate could modulate biofilm formation is being investigated. To the authors knowledge, very little is known about *D. marina*, outside of its identification and characterization in 2007 [36]. Thus, the possibility of this organism using its quorum sensing system has yet to be explored and is of interest for future studies.

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Competing interests

The authors declare that they have no competing interests.

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