Bacterial aggregation assisted by anionic surfactant and calcium ions†

Narendra K. Dewangan, Nhi Tran, Jing Wang-Reed and Jacinta C. Conrad †*  

We identify factors leading to aggregation of bacteria in the presence of a surfactant using absorbance and microscopy. Two marine bacteria, Marinobacter hydrocarbonoclasticus SP17 and Halomonas titanicae Bead 10BA, formed aggregates of a broad size distribution in synthetic sea water in the presence of an anionic surfactant, dioctyl sodium sulfosuccinate (DOSS). Both DOSS at high concentrations and calcium ions were necessary for aggregate formation, but DOSS micelles were not required for aggregation. Addition of proteinase K but not DNase1 eliminated aggregate formation over two hours. Finally, swimming motility also enhanced aggregate formation.

1 Introduction

Aggregation of bacteria plays an important role in biofilm formation,1–5 wastewater treatment,6 stress protection,7,8 and biodegradation.9 A striking example of its significance arose during the 2010 Deepwater Horizon MC252 (DWH) oil spill: approximately 14% of the oil released during DWH accumulated in agglomerates of bacteria, organic matter, and hydrocarbons10 called marine oil snow (MOS). Because the density of MOS exceeds that of water, sedimentation of MOS was suggested as a mechanism by which oil was rapidly transported to the floor of the Gulf.11 During the DWH spill, a chemical dispersant (Corexit) was applied at the wellhead at a concentration of 19 ppm12 to break oil into droplets. Intriguingly, the effects of Corexit on MOS formation in marine waters remain vigorously debated, with contrasting reports suggesting that it enhances11 or suppresses14 MOS formation and, hence, affects the transport of oil through the water column.

The appearance of MOS during DWH suggests that surfactants can alter bacterial aggregation processes. Corexit, for example, can inhibit the precipitation of particles of natural organic matter that play an important role in MOS formation.15 As a second example, Pseudomonas aeruginosa PAO1 aggregates upon addition of sodium dodecylsulfate (SDS).16 Polymers and macromolecules also modulate aggregation of bacteria. Extra-cellular DNA (eDNA) produced by bacteria generates attractive Lifshitz-van der Waals and acid–base interactions17,18 that promote aggregation,19 whereas bacteria-produced mucin inhibits aggregation of motile bacteria.20 Addition of synthetic polymers can also drive aggregation via entropic depletion21–28 or enthalpic bridging2,17,18,26,29–35 interactions. Finally, surface structures such as pili,36 flagella,37,38 or curli can also cause aggregation in bacteria.39–41

Because the polymers, macromolecules, and surfactants that affect bacterial aggregation are often charged, ions in solution may alter aggregation processes. Calcium ions, as one example, appear at high concentrations in aquatic systems42 and are an essential component for maintenance of cell structure, motility, and cell differentiation.43 Divalent Ca2+ can form bridges between the negatively charged eDNA on the cell surface to enhance cell–cell aggregation.2 Cations can also screen the charges on cells, reducing electrostatic repulsion. Significantly higher concentrations of monovalent cations than divalent cations are required to destabilize micron-size bacteria; likewise, the separation distance between cells decreases as cation valency is increased.44 These selected examples suggest that bacterial aggregation in complex solutions can be driven by multiple physical factors. Despite its broad relevance, however, how the constituents of a complex fluid affect aggregation of bacteria (both in solution and near oil–water interfaces encountered in an oil spill scenario45) remains incompletely understood. Developing this understanding poses an especial challenge for non-model organisms to which genetic methods may not be easily applied.

Here, we investigated the effect of dioctyl sodium sulfosuccinate (DOSS), a surfactant used in oil spill response, and calcium ions on aggregation of two species of marine bacteria, Marinobacter hydrocarbonoclasticus SP17 and Halomonas titanicae Bead 10BA. We show that a decrease over time in the optical density of suspensions of bacteria in synthetic seawater in the presence of a
high concentration of DOSS reflects the formation of aggregates of bacteria, confirmed by microscopy experiments. We quantified the extent of aggregation by comparing the initial and final optical densities of the suspensions and the size and number of aggregates using fluorescence and confocal microscopy. Both DOSS and calcium ions are required for aggregation of these bacteria in synthetic seawater, mimicking the environment of a marine spill scenario. Three metrics for aggregation – the percentage decrease in optical density and the size and number of aggregates – increase concomitant with DOSS concentration in M. hydrocarbonoclasticus suspensions. Control experiments in saline solution revealed that this aggregation behavior is not due to the formation of DOSS micelles at high surfactant concentrations. Addition of DNase1 did not prevent aggregate formation over two hours, indicating that eDNA was not required for aggregate formation. Addition of proteinase, however, eliminated cell aggregation over two hours, suggesting that bacterial aggregation in this setting is dominated by extracellular proteins. Finally, we show that motile H. titanicae exhibit slightly greater aggregation compared to bacteria rendered nonmotile by shearing. Together, these results improve our understanding of bacterial aggregate formation in the presence of surfactants.

2 Materials and methods

2.1 Chemicals

Zobell marine broth 2216 (HiMedia lab), sodium pyruvate (Amresco), nutrient agar (Difco), diocyl sodium sulfosucinate (DOSS, ≥97%, Sigma-Aldrich), SYTO9 (ThermoFisher), sodium chloride (≥99%, BDH), magnesium chloride hexahydrate (≥99%, Alfa Aesar), magnesium sulfate heptahydrate (≥99.5%, Sigma-Aldrich), calcium chloride (Sigma-Aldrich), potassium chloride (≥99%, BDH), potassium nitrate (≥99%, EMD), dipotassium phosphate (≥98%, Sigma-Aldrich), tris(hydroxymethyl) amino methane (≥99.8%, Sigma-Aldrich), ammonium chloride (≥99.9%, Sigma-Aldrich), iron sulphate (≥99%, Sigma-Aldrich), DNase1 from bovine pancreas (Sigma-Aldrich), and sodium phosphate (≥99%, Sigma-Aldrich) were used as received.

2.2 Bacteria and growth conditions

Marinobacter hydrocarbonoclasticus SP17 (ATCC 49840) was obtained from Dr Douglas Bartlett (Scripps Institute of Oceanography, UCSD). The Bead 10BA strain is closely related to Halomona titanicae and hereafter is referred to by that name. It was isolated from samples collected at 1509 m in the Gulf of Mexico during DWH by Dr Romy Chakraborty and Dr Gary Anderson (Lawrence Berkeley National Laboratory) and received from Dr Bartlett. M. hydrocarbonoclasticus and H. titanicae plates were prepared by streaking bacteria from a frozen stock onto marine agar plates.46 The plates were incubated at 30 °C for 40 hours. Cultures were prepared by inoculating 20 mL of marine broth (37.4 g marine broth and 10 g sodium pyruvate in 1 L of Milliq water) from a single colony of bacteria from the plate and incubated for 20 h in an orbital incubator shaker (New Brunswick Scientific) at 200 rpm and 30 °C.

2.3 Cell aggregation assays

A cell culture (20 mL) grown to late exponential phase was centrifuged at 4000 g for 10 minutes in a Sorvall ST 16 Centrifuge (ThermoFisher Scientific). The supernatant was discarded and the cell pellet was resuspended in 5 mL of synthetic seawater (SSW). SSW was prepared by dissolving 12.1 g tris(hydroxymethyl) amino methane, 0.75 g KCl, 1.5 g calcium chloride, 3.47 g ammonium chloride, 6.16 g magnesium sulfate heptahydrate, 5.08 g magnesium chloride hexahydrate, and 35 g sodium chloride in 1 L of Milliq water.47 The ionic strength of this SSW, 990 mM, is somewhat higher than the value typically associated with seawater, ~750 mM, but was chosen to match earlier studies on M. hydrocarbonoclasticus.47,48 The pH of the solution was adjusted to 7.5 with 5 M HCl. We subsequently added 2 and 4 mL of aqueous solutions of iron sulfate (0.1% w/v) and sodium phosphate (10%, w/v), respectively. We prepared 3 mL of cell suspensions at an optical density (OD, measured at wavelength λ = 600 nm) of 0.6 with DOSS concentrations of 0, 10, 30, 60, 100, 200, 300, 400, 500, and 600 ppm in 15 mL centrifuge tubes. This concentration range was chosen partly based on earlier studies of biodegradation of crude oil in microcosm experiments,49–53 which used concentrations of Corexit 9500 as high as 144 ppm of Corexit 9500.49 We also examined much higher concentrations of DOSS in part to test the effect of surfactant micelle formation on aggregation, and confirmed that M. hydrocarbonoclasticus remained viable at DOSS concentrations of up to 600 ppm. The initial (t = 0, OD0) and final optical densities (t = 2 h, OD2) of the upper layer of each suspension were measured using a cell density meter (Lasco DSM-Micro). For time-dependent assays, the optical density of each sample was measured as a function of time in triplicate. The decrease in optical density is usually attributed to aggregation between bacteria and is referred as percentage aggregation, defined as %Aggregation = 100 × (OD0 − ODt)/OD0. We chose an incubation time of 2 h to investigate aggregation.55,56

To assess the effect of DNase1 on aggregation, cell suspensions of concentration 107 cells mL−1 were prepared at DOSS concentrations of 0, 200, and 600 ppm. DNase1 was added to suspensions to obtain a final concentration of 2 mg mL−1. The OD of the upper layer of each suspension was measured at 0 and 2 h.

To examine the effect of extracellular proteins on aggregation, a nonspecific protease, proteinase K, was added at a final concentration of 0.1 mg mL−1 57 to cell suspensions of concentration 109 cells mL−1 containing 0 ppm and 600 ppm DOSS. Images were acquired for each sample at 0 and 2 h using a VT-Infinity (Visitech, Sunderland, UK) confocal microscope equipped with a 40× oil-immersion lens (HCX PL APO, NA 1.25–0.75).

To investigate the effect of motility, 20 mL of H. titanicae suspension were sheared in a blender (Oster 6642) in liquify mode at high for 30 s to remove flagella.58 Subsequently, the suspension was centrifuged at 4000g for 10 minutes in a Sorvall
2.4 Quantification of bacterial aggregates

Samples were imaged using fluorescence microscopy and confocal microscopy to quantify aggregate number and size. A 500 μL aliquot of each sample was stained using 0.5 μL SYTO9 and incubated at room temperature for 2 minutes. A chamber to contain samples for imaging was created by placing a spacer (Sigma-Aldrich) on a glass slide (ThermoFisher). Subsequently, 20 μL of cell suspension was dispensed onto the chamber and covered with another cover slip. Bacteria were imaged using a fluorescence microscope (Leica Microsystems DM4000) and a VT-Infinity (Visitech, Sunderland, UK) confocal microscope with a 40× oil-immersion lens (HCX PL APO, NA 1.25–0.75).

At this magnification, necessary for imaging large aggregates, we were not able to resolve individual bacteria. At least six eight-bit images were acquired for each sample and the experiment was run in triplicate. Images were analyzed using ImageJ. Each image was converted into a binary image by assigning a value of 1 to all pixels that exceeded a threshold value of 2% of the maximum intensity (255). Subsequently, we extracted the number and area of each region that contained at least 100 pixels (9.5 μm², chosen by visual inspection) using the “Analyze Particles” tool. A sensitivity analysis of 15 images indicated that a change of ±0.1% in the threshold intensity did not alter the number of aggregates and a change of ±10 pixels in the threshold area changed the number of aggregates by ca. 10%. The aggregate number density ρ was calculated as the number of aggregates per unit surface area.

2.5 Surface tension and critical micelle concentration

The surface tensions of DOSS in SSW and in 58 g L⁻¹ NaCl were determined using the pendant drop method. The surface tensions of SSW and NaCl solutions were measured as a function of DOSS concentration using a Dataphysics OCA 15EC goniometer. Subsequently, we determined the critical micelle concentrations (CMC) of DOSS in SSW and in NaCl as the concentrations at which the slopes at low and high DOSS concentrations intersected (Fig. S1, ESI†).

2.6 Aggregation of bacteria in dispersed hydrocarbons

Cell cultures were prepared and harvested in a similar manner as for the aggregation assay. From the culture, a cell suspension at an OD₀ of 0.6 was prepared in SSW. Subsequently, we mixed 3 mL of the cell suspension and 100 μL of hexadecane with DOSS concentrations of 10, 60, 100, 200, 300, and 400 ppm in a 15 mL centrifuge tube. Each suspension was vortexed for 30 s, incubated at room temperature for 2 h, and imaged using a VT-Infinity confocal microscope (Visitech, Sunderland, UK).

3 Results and discussion

3.1 Bacteria aggregate over time in the presence of DOSS

We first examined the change in stability over time of suspensions containing M. hydrocarbonoclasticus, calcium chloride, and the surfactant DOSS in synthetic sea water. Turbidity measurements reveal that the optical density (OD) of the suspensions decreases over time, and the percent decrease in OD increases linearly as a function of time (Fig. 1a). The total percent decrease in OD is 27 ± 3% over 150 minutes for a suspension with an initial OD₀ of 0.6, corresponding to a cell concentration of 10⁸ cells mL⁻¹.

The suspensions remained visually homogeneous over this time scale, indicating that sedimentation likely did not markedly alter the cell and aggregate concentration (Fig. S2, ESI†). A similar decrease in optical density on short time scales was observed for Yersinia adhesin YadA-expressing E. coli and was attributed to an increase in aggregation.⁵⁴ Thus, we considered the effects of aggregation on turbidity measurements.

Fig. 1 (a) Aggregation of M. hydrocarbonoclasticus, measured as a percentage decrease in optical density (OD), as a function of time. (b) Number density ρ as a function of time. The DOSS concentration is 400 ppm, and the bacteria concentration is 10⁸ cells mL⁻¹ (OD₀ = 0.6). (b) insets show micrographs of aggregates at 10 min (left) and 150 min (right), with aggregates outlined in green. Error bars represent the standard deviation obtained from five independent cultures.
In optical density measurements, the transmitted light intensity reflects contributions from scattering from the constituents in suspension and depends on particle size and concentration and the incident wavelength, among other factors.\(^5^9\) For a monodisperse suspension of spherical particles of radius \(a\) with particle number concentration \(n\) that is illuminated over a path length \(L\), the transmitted light intensity is given by \(II_\text{tr} = \exp(-tL)\). Here the turbidity is given by \(\tau = nC_p\), where \(C_p\) is the scattering cross-section from a single particle and is proportional to the geometric cross-section via \(C_p = Qn \pi a^2\). The scattering coefficient \(Q\), however, is a non-monotonic function of particle size. Further, upon aggregation, the number concentration of scatterers decreases but their size (and distribution) increases. Thus, it is difficult to interpret measurements of transmitted intensity at a single incident wavelength in terms of a particle size,\(^5^9\) even prior to aggregation.

Hence, to gain information about the size and distribution of aggregates, we imaged suspensions containing DOSS as a function of time. Confocal micrographs reveal that aggregates of bacteria appear approximately 30 min after mixing (insets to Fig. 1b). From the images, we determined the areal number density \(\rho\) of aggregates in microscopy images as a function of time. The number density initially increases with time and approaches a plateau on time scales exceeding 1 h (Fig. 1b). Thus, we chose to examine suspensions on a time scale of \(t = 2\) h after mixing to investigate the effects of solution conditions on aggregation, consistent with other studies that examined aggregation over this time scale.\(^{55,56}\) Ref. 55, in particular, showed that bacteria aggregate on this time scale near oil–water interfaces.

### 3.2 State diagram and characterization of aggregates

We first examined the effect of cell concentration and DOSS concentration on aggregation (Fig. 3). Turbidity measurements, conducted at a fixed time of two hours after introduction of DOSS and reported as a percent decrease in OD, reveal that increasing the surfactant concentration leads to a more pronounced decrease in the optical density (Fig. 2). To confirm that the decrease in turbidity reflects an increase in aggregation, we also directly imaged the samples at the same two-hour time-point. Confocal micrographs reveal that \(M.\ hydrocarbonoclasticus\) bacteria aggregate as the concentration of DOSS is increased. At a fixed OD\(_0\), the number density \(\rho\) of aggregates generally increases as the DOSS concentration is increased. As the OD\(_0\) is increased from 0.03 to 1, lowering the average separation between cells, the concentration of DOSS required to observe a significant density of aggregates is slightly reduced.

The aggregate size also depends on OD\(_0\) and DOSS concentration (Fig. 4). At the two lowest values of OD\(_0\), increasing the DOSS concentration leads to a slight enhancement in the average size and a broader distribution (Fig. 4(a and b)). Large variation in the size of aggregates was also observed in mixtures of bacteria with polymers\(^2^4\) or leukocytes.\(^6^0\) In ref. 24, aggregation was driven by exopolymers produced by the bacteria. Increases in surfactant concentration have likewise been shown to promote exopolymer production;\(^6^1\) such an enhancement is consistent with the increase in aggregation observed in our experiments. Generally, aggregates are observed at lower DOSS concentrations at higher cell densities, consistent with the idea that decreasing the average separation between cells increases the likelihood of contact and hence aggregation. The aggregation behavior at the highest OD\(_0\) of 1.0, however, exhibits slightly different trends (Fig. 4(c)). Both the mean size and the breadth of the distribution are greatest at an intermediate DOSS concentration of 100 ppm, and decrease slightly upon further increase to 400 ppm.

### 3.3 Test of micelle-driven aggregation

Because aggregation requires a sufficiently large concentration of DOSS, we tested whether surfactant micelles were required for aggregation. This experiment was suggested by an earlier study that found that micelles drive aggregation of microscale droplets by inducing depletion attractions.\(^6^2\) In our experiments, the CMC of DOSS is below 30 ppm in both SSW and NaCl solution (the primary component of synthetic sea water), indicating that micelles are present at the higher DOSS concentrations. We therefore examined the aggregation behavior of \(M.\ hydrocarbonoclasticus\) bacteria in 58 g L\(^{-1}\) NaCl, whose ionic strength (991 mM) is close to that of synthetic sea water (990 mM), as a function of DOSS (Fig. S3, ESI†). These bacteria do not aggregate in 58 g L\(^{-1}\) NaCl, even at a DOSS concentration (600 ppm) that greatly exceeds the CMC. Thus, we conclude that aggregation in this system is not solely driven by depletion interactions induced by DOSS micelles.

### 3.4 Effect of calcium chloride

Calcium chloride is a component of synthetic seawater. Divalent cations such as Ca\(^{2+}\) have been shown to increase aggregation of bacteria in earlier studies.\(^2\) Thus, we examined the effect of calcium chloride on aggregation in our system. \(M.\ hydrocarbonoclasticus\) bacteria do not aggregate when calcium chloride is absent from the medium (Fig. 5a). The presence of CaCl\(_2\), however, is insufficient to drive aggregation without surfactant: at low concentrations of DOSS
(0 or 30 ppm), bacteria do not aggregate even at the highest CaCl₂ concentration of 3 g L⁻¹. Therefore, both DOSS and calcium chloride are essential for *M. hydrocarbonoclasticus* bacteria to aggregate in these conditions. The number density of aggregates grows as the concentration of DOSS (Fig. 5(a)) or calcium chloride (for DOSS concentrations of 100 and 400 ppm, Fig. 5(b)) is increased. For [DOSS] ≥ 100 ppm, both the average and maximum sizes of aggregates increase as calcium chloride concentration is increased (Fig. 5b).

That aggregation requires calcium chloride is consistent with previous studies that demonstrated the importance of divalent cations in aggregation and adhesion of bacteria. Ca²⁺ is known to enhance production of extracellular polymeric substances (EPS), composed of proteins, enzymes, nucleic acids, lipids, polysaccharides, and other compounds such as humic acids. Furthermore, divalent cations such as Ca²⁺ and Mg²⁺ can form crosslinks between extracellular substances and bacteria. Our experiments, however, are not able to distinguish whether CaCl₂ promotes aggregation by one or both of these mechanisms (enhancement of EPS production and crosslinking of EPS).

![Confocal micrographs of *M. hydrocarbonoclasticus* aggregates as a function of DOSS concentration (C, y axis) and initial bacteria optical density (OD₀, x axis). An OD₀ of 1.0 is equivalent to 1.7 × 10⁸ cells mL⁻¹. The scale bar is 20 µm. (b) State diagram for aggregation of *M. hydrocarbonoclasticus* as a function of DOSS concentration C and initial optical density OD₀. The color bar indicates the variation in the aggregate number density ρ. The dashed line indicates an approximate phase separation line. Ten images were analyzed for each sample. Each experiment was replicated at least two times.](image1)

![Size distribution of *M. hydrocarbonoclasticus* aggregates as a function of DOSS concentration at optical densities OD₀ of (a) 0.06, (b) 0.25, and (c) 1.0, shown as a violin plot. Red and black lines respectively indicate the median and mean of each size distribution. Ten images were analyzed for each sample. Each experiment was replicated at least two times.](image2)

![Aggregate (a) number density ρ and (b) size distribution as a function of DOSS concentration at 0, 1.5, and 3 g L⁻¹ calcium chloride. The bacteria concentration in each suspension is 10⁹ cells mL⁻¹. Ten images were analyzed for each sample. Each experiment was replicated at least two times.](image3)
3.5 Effect of extracellular polymeric substances

One component of bacterial EPS, eDNA,\textsuperscript{71} is able to be cross-linked by Ca\textsuperscript{2+}.\textsuperscript{2} Motivated by this earlier study, we examined the aggregation of \textit{M. hydrocarbonoclasticus} in the presence of an enzyme, DNase1, that can break phosphodiester bonds by hydrolysis.\textsuperscript{3} There was no significant change in aggregation of bacteria in the presence or absence of DNase1 up to a DOSS concentration of 600 ppm (Fig. 6). This result suggests that aggregation is not due to eDNA specifically, although Ca\textsuperscript{2+} may still form crosslinks or bridges between other EPS biopolymers such as alginate.\textsuperscript{72}

The EPS contains a variety of macromolecules besides eDNA. Thus, we also examined the effect of proteinase K, which digests proteins by breaking peptide bonds,\textsuperscript{73} on aggregation of bacteria. Clusters of bacteria are not present in suspensions containing DOSS to which proteinase K was added (Fig. 7(b)), but are present in its absence (Fig. 7(a)). Because these measurements were carried out at a single time point, we are not able to distinguish whether addition of proteinase K disrupted clusters or prevented their formation. Nonetheless, this result suggests that digestion of extracellular proteins disrupts the EPS used by bacteria to form aggregates under these conditions.

3.6 Effect of bacteria motility

Finally, we assessed the effect of motility on aggregation by quantifying the aggregation behavior of a motile bacterium, \textit{Halomonas titanicae}, that swims using multiple flagella. This bacterium can be rendered nonmotile through mechanical shearing, which removes its flagella.\textsuperscript{58} Optical density measurements reveal that motile bacteria aggregate more readily than nonmotile bacteria (Fig. 8(a) and Fig. S4, ESI\textsuperscript{†}). At a DOSS concentration of 600 ppm, where aggregation is most pronounced, the OD of suspensions of motile and nonmotile bacteria decreases by 40% and 30%, respectively, over 150 min. The number density of motile bacteria is slightly greater than that of nonmotile bacteria at intermediate DOSS concentration, whereas at high DOSS concentration the number density is within the experimental error (Fig. 8(b)). The average size of aggregates is identical within error for motile and nonmotile bacteria, but the distribution of aggregate size is much broader for motile bacteria (Fig. 8c). These results suggest that motility enhances aggregation of bacteria in this medium. This conclusion is consistent with an earlier study showing that motility and chemotaxis enhance aggregation of \textit{E. coli}.\textsuperscript{56} By contrast, aggregation of motile bacteria required higher concentrations of depletant polymer than in nonmotile bacteria, and was attributed to a reduction in the strength of the attraction due to motility.\textsuperscript{24,25} This comparison indicates that the effects of motility on aggregation depend sensitively on the nature of the interactions between bacteria.

As a first step to understand the differences in the aggregation of motile and nonmotile bacteria, we compare the differences in aggregate size to a physical model for the growth of marine bacterial aggregates over time.\textsuperscript{74} In the model of ref. 74, the change in the number of bacteria in an aggregate \(\text{d}N/\text{d}t\) is proportional to the (constant) ambient concentration \(C_b\), i.e. \(\text{d}N/\text{d}t = \beta C_b\), where the encounter rate kernel \(\beta\) represents the
(imaginary) volume of water from which bacteria are able to attach to the aggregate. The average swimming speed of *H. titanicae*, 10 μm s⁻¹, leads to an effective diffusivity of 33 μm² s⁻¹ whereas the diffusivity of nonmotile *H. titanicae* is 0.25 μm² s⁻¹.⁵⁸ At steady state, the encounter rate kernel for bacteria undergoing a random walk is β = 4πDRSh, where *R* is the aggregate radius, *D* is the diffusivity of bacteria, and *Sh* is the Sherwood number.⁷⁴ In the limit of slow sinking rate, appropriate for the small (~10 μm) clusters observed in our experiments, *Sh* can be taken to be 1 (i.e. dominated by diffusion). Thus, we expect dN/dt for motile bacteria to be ca. 100 times greater than that of nonmotile bacteria. In our experiments, however, there is not an appreciable difference in the size of motile and nonmotile aggregates after 2 h (Fig. 8(c)). The inability of this model to describe the size difference suggests that processes not included in the model, such as depletion of cells (such that *C* decreases over time) or chemotaxis, may limit the aggregate size.

### 4 Conclusions

We investigated aggregation of *M. hydrocarbonoclasticus* and *H. titanicae* in the presence of the anionic surfactant DOSS. In synthetic sea water, these marine bacteria continue to aggregate for at least 150 min in the presence of sufficiently high DOSS concentrations. Microscopic imaging revealed that the size distribution at a fixed time point, two hours after mixing, depends on the concentrations of both surfactant and bacteria. Both DOSS and calcium chloride are required for aggregation under these conditions. Although the DOSS concentrations exceed the CMC value, DOSS micelles do not drive the aggregation process. Addition of proteinase K inhibits aggregation, whereas addition of DNase1 does not prevent aggregate formation. Because addition of proteinase K inhibits aggregation, we speculate that aggregation is most likely mediated by protein-rich extracellular polymeric substances whose production is stimulated by the response to surfactant, perhaps as a stress response.¹⁶ Testing this idea directly by identifying genes in *M. hydrocarbonoclasticus* is challenging, because this organism is considerably less understood genetically than model bacteria and less amenable to genetic manipulation, but may become feasible with recently-developed vector delivery methods.⁷⁵ Finally, motility increases the number density of aggregates but not, apparently, their size over 2 h, in contrast with the predictions of the model of ref. 74. We speculate that depletion of cells from the suspension (not included in the assumptions of this model) may limit cluster size, and will test this idea in future work.

The marine oil snow that facilitated oil transport to the ocean floor during DWH contained both bacteria and dispersed oil.⁷⁶ Our preliminary experiments suggest that aggregates of bacteria may be able to form bridges between oil droplets to generate large flocs (Fig. S6, ESI†). Detailed studies of the role of surfactants on aggregation of bacteria in the presence of dispersed oil may shed light on the processes driving marine oil snow formation in the context of oil spill response. In addition, understanding of the physical mechanisms driving aggregation is important for bioremediation processes and in wastewater treatment.⁶ Finally, aggregation behavior can be exploited to
select adhering bacteria strains. Further studies to systematically identify the mechanisms driving aggregation under relevant conditions are thus likely to inform a wide range of biotechnological and environmental applications.

Conflicts of interest
There are no conflicts to declare.

Acknowledgements
This research was made possible in part by a grant from The Gulf of Mexico Research Initiative, and in part by the Welch Foundation (E-1869). Data generated for this paper is available on the Gulf of Mexico Research Initiative Information and Data Cooperative (GRIDIC) at https://data.gulfresearchinitiative.org (DOI: 10.7266/VFHNWQJY). We thank Ms Kelli Mullane and Dr Douglas Bartlett (UCSD) for providing the marine bacteria.

Notes and references