Aggregation of *Vibrio cholerae* by Cationic Polymers Enhances Quorum Sensing but Overrides Biofilm Dissipation in Response to Autoinduction

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ABSTRACT: *Vibrio cholerae* is a Gram-negative bacterium found in aquatic environments and a human pathogen of global significance. Its transition between host-associated and environmental lifestyles involves the tight regulation of niche-specific phenotypes such as motility, biofilm formation, and virulence. *V. cholerae*’s transition from the host to environmental dispersal usually involves suppression of virulence and dispersion of biofilm communities. In contrast to this naturally occurring transition, bacterial aggregation by cationic polymers triggers a unique response, which is to suppress virulence gene expression while also triggering biofilm formation by *V. cholerae*, an artificial combination of traits that is potentially very useful to bind and neutralize the pathogen from contaminated water. Here, we set out to uncover the mechanistic basis of this polymer-triggered bacterial behavior. We found that bacteria—polymer aggregates undergo rapid autoinduction and achieve quorum sensing at bacterial densities far below those required for autoinduction in the absence of polymers. We demonstrate this induction of quorum sensing is due both to a rapid formation of autoinducer gradients and local enhancement of autoinducer concentrations within bacterial clusters as well as the stimulation of CAI-1 and AI-2 production by aggregated bacteria. We further found that polymers cause an induction of the biofilm-specific regulator VpsR and the biofilm structural protein RbmA, bypassing the usual suppression of biofilm during autoinduction. Overall, this study highlights that synthetic materials can be used to cross-wire natural bacterial responses to achieve a combination of phenotypes with potentially useful applications.

Both natural and synthetic cationic macromolecules, such as cationic antimicrobial peptides, cationic polymers, and dendrimers, have been extensively reported as antimicrobial.1–3 Because of their positive charge, these polymers can efficiently bind the negatively charged envelope of Gram-negative and Gram-positive bacteria.1–6 At high concentrations and charge densities, these molecules have the potential to interfere with membrane integrity and decrease bacterial viability.1–3 However, antimicrobial activity is heavily dependent on the length and nature of the polymer and, more importantly, on the nature of the targeted microbe. At low concentrations, cationic polymers are still capable of causing bacterial aggregation by mediating electrostatic interactions but do so without significantly affecting bacterial membrane integrity and growth.7–12 We and others have previously reported that bacteria clustered by subinhibitory concentrations of cationic polymers display interesting phenotypes resembling those of biofilm communities.7–11 For instance, we have recently demonstrated that clustering of the diarrheal pathogen *Vibrio cholerae* with methacrylamides containing primary or tertiary amines leads to accumulation of biomass and extracellular DNA and repression ToxT-regulated virulence factors including choler toxin.12 However, what drives these phenotypic changes in response to polymer exposure remains unclear. Similarly, the marine bacterium *Vibrio harveyi* shows enhanced bioluminescence in response to polymer-mediated clustering.7,8,11 The bacterial components necessary to produce luminescence are encoded by the luxCDAEBE genes and subject to complex regulatory mechanisms. A major regulatory cascade controlling luminescence is quorum sensing. Because the regulators controlling luminescence are functionally conserved between *Vibrio* species, expression of *V. harveyi* luxCDAEBE genes can be used as a tool to probe quorum sensing in heterologous hosts.13

In *V. cholerae*, four parallel quorum sensing pathways operate,14 each governed by an autoinducer synthase, which produces a small, freely diffusible molecule that is released in the environment and can be sensed by a corresponding sensor/kinase component that controls the activity of the LuxU/LuxO phosphorelay. Of the four pathways, the LuxS/LuxPQ system, which produces and detects the interspecies autoinducer AI-2 (S-TMHF-borate) and the CqsA/CqsS system, which produces and senses the *Vibrio* specific autoinducer S-3-hydroxytridecan-4-one (CAI-1), are the best characterized. CqsA and LuxS synthesize the autoinducers, and the hybrid...
sensor/kinases CqsS and LuxQ sense and respond to their cognate autoinducers by dephosphorylating and inactivating the regulator LuxO. When extracellular autoinducer concentrations are low, LuxO is phosphorylated and activates the transcription of the small RNAs Qrr1−4, which in turn inhibits the expression of HapR, a master regulator controlling diverse cellular functions including luminescence, biofilm formation, and virulence. When the extracellular autoinducer concentration increases above a threshold, the sensor/kinases instead act as phosphatases, leading to dephosphorylation and inactivation of LuxO, ultimately allowing the expression of HapR. Consequently, when autoinducer concentration is high, HapR activates luminescence but suppresses virulence and biofilm genes. This regulatory mechanism is thought to mediate the dissipation of host-associated biofilms and enable transmission of *V. cholerae* from the intestine to the environment.

**RESULTS**

**Polymers Enhance Quorum Sensing in *V. cholerae***

First, we set out to test if clustering by the cationic polymers poly(N-[3-aminopropyl] methacrylamide) (P1, Figure 1A) and poly(N-[3-(dimethylamino)propyl] methacrylamide) (P2, Figure 1B) induced quorum sensing in *V. cholerae*. As previously shown and detailed here by N-SIM super-resolution microscopy, even low concentrations of polymers induced rapid clustering of bacteria without affecting bacterial viability (Figure 1C). For creating a fast and direct read-out for quorum sensing, the cosmid pBB1, which contains the luxCDABE luminescence genes from *V. harveyi*, was used to transform *V. cholerae* serogroup O1 biovar El Tor strain A1552 (serotype Inaba). The transformed bacteria were then grown in LB for 20 h, washed with artificial marine water (AMW), and adjusted to an OD of 0.2 in AMW alone or AMW containing polymers at concentrations ranging from 0.005 to 0.5 mg mL\(^{-1}\). In the absence of polymer, luminescence as a read-out of quorum sensing first decreased due to back-dilution of the culture from a high density overnight culture to OD 0.2 and then gradually increased due to accumulation of autoinducers, peaking at 4.5 h (Figure 1D and E, black traces). Interestingly, the quorum induction kinetics were significantly different in the presence of either P1 (Figure 1D) or P2 (Figure 1E) with initial luminescence being sustained and reaching a higher second peak at \(\sim 2.5\) h as opposed to 4.5 h in the absence of polymers. The magnitude of induction was higher in polymer-treated cultures (\(\sim 5\)-fold at peak quorum induction) compared to untreated cultures. Results were similar for the Ogawa serotype strain E7646 in that quorum sensing was initially sustained and then further enhanced (\(\sim 4\)-fold at peak quorum induction) by polymer-mediated bacterial aggregation (Figure 1F, G). In both strains, the magnitude of luminescence induction was independent of the polymer concentration used, suggesting a threshold response.
For assessing viable bacterial counts at the experimental end point, samples were serially diluted in high-salt buffer to disrupt clusters as previously described and plated. Similar numbers of colony-forming units were recovered from untreated or polymer-treated samples (Figure 1 H, I), suggesting that the presence of polymers did not affect bacterial viability or proliferation in agreement with our previous data. Taken together, our data demonstrate that these cationic polymers that cluster modulate the community behavior to give an accelerated and more robust autoinduction.

**Bacterial Density Shapes the Kinetics of Quorum Induction in Response to Polymer.** Because quorum sensing is usually tightly linked to bacterial density, we set out to explore how initial culture density affects quorum induction in the presence of polymers. *V. cholerae* carrying pBB1 as a quorum sensing reporter was adjusted to optical densities of 0.005, 0.05, and 0.5 in AMW alone or AMW containing 0.005–0.5 mg mL$^{-1}$ P1 or P2, and luminescence was monitored (Figure 2). The onset of autoinduction was not significantly modulated by the addition of polymers P1 or P2 to higher density cultures (OD$_{600}$ of 0.5), but an approximate 6- (P1) to 9-fold (P2) increase in peak luminescence was observed (Figure 2A, D). At lower culture densities, autoinduction of the untreated cultures was less pronounced (0.05) and eventually ceased (OD 0.005) because not enough autoinducer accumulated to reach the threshold concentration. In the presence of polymers, the initial quorum present in the culture was sustained, even in very dilute cultures (OD$_{600}$ of 0.5), but an approximate 6- (P1) to 9-fold (P2) increase in peak luminescence was observed (Figure 2A, D). At lower culture densities, autoinduction of the untreated cultures was less pronounced (0.05) and eventually ceased (OD 0.005) because not enough autoinducer accumulated to reach the threshold concentration.

In the presence of polymers, the initial quorum present in the culture was sustained, even in very dilute cultures, and the enhancement in peak luminescence was much more pronounced compared to that of untreated cultures (Figure 2B–F). Interestingly, clustering of very dilute cultures led to two peaks in luminescence with a gap of ∼4 h (Figure 2C, F). Of note, the luminescence was not a result of bacterial growth, which was negligible under the observed conditions (AMW) and over the time frame described, both at high and low initial densities (Figure 2G, H).

For the process of bacterial clustering and luminescence induction to be visualized, *V. cholerae* A1552 containing pBB1 was incubated in the presence of P1, P2, or AMW alone in glass-bottom plates. Bacteria were imaged every 30 min to simultaneously visualize clustering and luminescence induction. Both P1 and P2 bacterial clusters were observed within minutes and remained stable over the duration of the experiment (Figure 3). Luminescence appears to originate from and be restricted to bacterial clusters. Quantification of luminescence based on integrated pixel intensities showed a polymer-mediated enhancement of autoinduction (Figure 3H) in agreement with spectroscopic data.

**Polymer-Mediated Luminescence Is Not Because of Nutrient Starvation within Clusters.** Although autoinduction controls HapR and luminescence via the regulator
LuxO, other environmental cues, including nutrient availability, have been reported to feed into the LuxO signaling cascade and thus have the potential to affect luminescence. Nutrient sensing and the LuxO signaling pathway converge on the cyclic AMP (cAMP) receptor protein (CRP). During limitation of PTS sugars such as glucose, cAMP-CRP is capable of modulating LuxO activation by affecting the expression of autoinducer synthases. Because clustering of bacteria by polymers may limit nutrient access and induce starvation, we tested whether a CRP deletion strain would be capable of activating luminescence in the presence of polymers. The *V. cholerae* Δcrp strain was transformed with pBB1 to monitor luminescence. However, the culture produced very low levels of luminescence both in the presence and absence of polymers (Figure 4A, B), which is in agreement with previous work on

*V. fischeri* CRP. Although this suggests that cross-talk between CRP and LuxO signaling is a dominant cue for luminescence induction in *V. cholerae* as well, this made it unfeasible to determine whether the presence of polymers induced a state of carbon starvation, leading to luminescence induction via CRP. Instead, we repeated the experiment using induced a state of carbon starvation, leading to luminescence unfeasible to determine whether the presence of polymers would limit nutrient diffusion, as previously described that, at least under some conditions, quorum sensing may be subject to positive feedback, where quorum induction leads to increased production of one of the autoinducer synthases. Therefore, the enhancement in luminescence in response to polymers could be due to positive feedback as a result of the polymers increasing the local concentration of autoinducers above the threshold. Alternatively, the polymers could have a direct effect on the production of autoinducers. We set out to test this by establishing a reporter assay that allowed us to decouple quorum sensing from the production of autoinducers. For this assay, we used as a luminescence reporter a *V. cholerae* strain transformed with pBB1 that could sense both CAI-1 and AI-2 but could not produce either molecule (BH1578). This

![Figure 4. Polymer-mediated luminescence is not due to nutrient starvation within clusters.](image)

Figure 4. Polymer-mediated luminescence is not due to nutrient starvation within clusters. *V. cholerae* E7646 Δcrp containing the luminescence reporter pBB1 was grown for 16 h and then diluted to an OD_{600} of 0.2 in AMW alone (black) or AMW containing P1 (A) or P2 (B) at concentrations of 0.005 (green), 0.05 (blue), or 0.5 (red) mg mL^{-1}. Luminescence was recorded every 30 min for 10 h, and means ± SEM from at least three biological replicates are shown. (C) *V. cholerae* E7646 wild-type containing pBB1 was grown for 16 h and adjusted to an OD_{600} of 0.2 in AMW containing 1% glucose alone (orange) or in the presence of 0.5 mg mL^{-1} of P1 (light blue) or P2 (dark blue). Luminescence was recorded every 30 min for 10 h, and means ± SEM from at least three biological replicates are shown.

![Figure 5. Polymer-mediated enhancement of quorum sensing is mainly driven by CAI-1.](image)

Figure 5. Polymer-mediated enhancement of quorum sensing is mainly driven by CAI-1. *V. cholerae* A1552 wild-type (dark) and quorum sensing mutants containing pBB1 were grown for 16 h and then diluted into AMW to give equal cell densities and a total OD_{600} of 0.2. Strains were grown together in AMW alone (black) or AMW containing P1 at 0.005 (green), 0.05 (blue), or 0.5 (red) mg mL^{-1}. Luminescence was recorded every 30 min for 10 h, and means ± SEM from at least three biological replicates are shown. Mutants grown in coculture with the wild-type were (A) DH231 (ΔluxSΔcqsS) producing no AI-2, (B) WN1103 (ΔluxQΔcqsA) producing no CAI-1, (C) BH1578 (ΔluxSΔcqsA) producing no AI-2 or CAI-1, and (D) BH1651 (luxOΔDE).

**Polymer-Mediated Enhancement of Quorum Sensing Is Dominated by CAI-1.** In *V. cholerae*, at least four parallel quorum sensing pathways converge to control the activity of the quorum sensing regulator LuxO and thus quorum-regulated phenotypes including biofilm formation and virulence. We set out to test whether the polymer-mediated effect on quorum sensing was specific for any one pathway. *V. cholerae* wild-type strain A1552 was mixed with equal numbers of cells of quorum sensing mutants transformed with pBB1 either deficient in the production of AI-2 (DH231) or CAI-1 (WN1103) to give a final OD_{600} of 0.2. Additionally, the mutants were unable to sense the presence of AI-2 or CAI-1. Cultures producing less AI-2 produced a luminescence profile similar in shape and magnitude to quorum sensing proficient cells in response to polymer P1 addition (Figure 5A). In contrast, cultures producing less CAI-1 still had a comparable response profile upon addition of P1, but the magnitude of the luminescence enhancement was decreased (Figure 5B) compared to that of cultures producing less AI-2 (Figure 5A). In cocultures producing less of both autoinducers (containing strain BH1578), the luminescence response to polymer was abolished (Figure 5C). Similarly, cocultures containing a low-density locked mutant of the downstream quorum regulator LuxO (LuxOΔf7178) showed no luminescence induction upon addition of polymer P1 (Figure 5D). These data pointed at both autoinducers being involved in the quorum sensing enhancement in response to polymer with CAI-1 being the dominant inducer. Additionally, the luminescence response to the polymer seems to proceed through the canonical LuxO-dependent pathway.

Enhanced Quorum Sensing Is Driven by Enhanced Production of Autoinducers in Response to Polymers. It has been described that, at least under some conditions, quorum sensing may be subject to positive feedback, where quorum induction leads to increased production of one of the autoinducer synthases. Therefore, the enhancement in luminescence in response to polymers could be due to positive feedback as a result of the polymers increasing the local concentration of autoinducers above the threshold. Alternatively, the polymers could have a direct effect on the production of autoinducers. We set out to test this by establishing a reporter assay that allowed us to decouple quorum sensing from the production of autoinducers. For this assay, we used as a luminescence reporter a *V. cholerae* strain transformed with pBB1 that could sense both CAI-1 and AI-2 but could not produce either molecule (BH1578). This
reporter strain was exposed to supernatants from producer strains grown under different conditions to evaluate the effect of the polymer. Initially, we evaluated the assay by growing the reporter in the presence of supernatants harvested from wild-type V. cholerae or strains incapable of producing AI-2, CAI-1, or both autoinducers. Supernatant harvested from the quorum-proficient wild-type strain grown to high cell density triggered the highest level of luminescence in BH1578 (Figure 6A). The luminescence triggered by the AI-2-deficient strain was slightly decreased (Figure 6B), whereas luminescence was significantly decreased in response to supernatant from the CAI-1-deficient strain (Figure 6C) and was abolished in response to the strain deficient in both CAI-1 and AI-2 production (Figure 6D). Hence, the assay was capable of detecting different levels of autoinducers produced by a second strain.

We took this assay forward and harvested supernatants from wild-type cells exposed to AMW alone or AMW containing 0.005–0.5 mg mL⁻¹ polymers P1 or P2, and exposed the reporter strain to filtered supernatants to test if the levels of autoinducers produced by the wild-type strain in the presence of polymers were different. The reporter strain was not clustered under the assay conditions. Although wild-type cells exposed to AMW alone did not produce a detectable amount of autoinducer and thus no significant luminescence reading in the reporter strain, both polymers P1 and P2 enhanced the production of autoinducers by wild-type V. cholerae, leading to an increase in luminescence upon exposure of the reporter to the supernatants (Figure 6E, F). This data demonstrate that polymer exposure leads to enhanced production of autoinducers by the bacteria.

**Polymer-Mediated Quorum Induction Overcomes the Canonical Biofilm Dissipation Program in V. cholerae.** Contrary to many bacteria that use quorum signaling as a means to induce biofilm formation, in V. cholerae autoinduction promotes repression of biofilm production and dissemination via the regulator HapR. However, we had previously observed enhanced accumulation of V. cholerae upon prolonged exposure to cationic polymers, but whether this was accompanied by transcriptional changes at the level of biofilm production was not known. Thus, we grew V. cholerae containing transcriptional fusions to promoters regulating key biofilm components in the presence or absence of P1 and P2 (Figure 7). V. cholerae biofilms contain the structural protein RbmA and require the regulator VpsR, which controls the expression of the vps polysaccharide biosynthesis genes. Upon exposure to either P1 or P2, vpsR and rbmA were both significantly induced (Figure 7A, B), suggesting that upon polymer-mediated clustering, quorum sensing does not, as usual, suppress genes involved in biofilm production, but instead their transcription is enhanced. AphA, which is another direct target usually induced by VpsR, is suppressed in the presence of polymers (Figure 7C).

**DISCUSSION**

Traditionally, work on cationic polymers has been carried out with the development of antimicrobial materials as a main goal. However, recent work by our groups and others has demonstrated that such cationic polymers can be titrated against bacteria to achieve a charge balance that allows for the rapid and efficient clustering of bacteria but avoids membrane disruption and bacterial cell death.

The use of cationic polymers to induce rapid bacterial clustering in this way has proven as an interesting path to study effects of cell aggregation and crowding on bacterial physiology. Although such behaviors are often studied in batch cultures, by incubating bacterial cultures over a...
prolonged time, this means aggregation is accompanied by bacterial growth and eventually nutrient limitation, which makes it difficult to establish the primary cause of the observed phenotypes. In contrast, cationic polymers induce cell aggregation rapidly, within minutes, which allows us to study these phenomena independent of cellular proliferation and nutrient limitation.

We and others have previously observed that cationic polymers and dendrimers can, under certain conditions, trigger bioluminescence in the marine bacterium *V. harveyi*, suggesting they may induce or enhance quorum sensing.7,8,10,11 In a more recent study where we extended this work to the human pathogen *V. cholerae*, we observed that polymer-mediated clustering led to enhanced deposition of biomass and extracellular DNA, whereas it interfered with the induction of virulence genes in an infection model.12 Because virulence and biofilm production are both regulated by quorum sensing but are usually both regulated concurrently, the goal of this study was to test whether cationic polymers would trigger quorum sensing in *V. cholerae* and how this would affect downstream transcription of biofilm genes.

We used *V. cholerae* strains heterologously expressing the luxCDABE luminescence genes (on cosmid pBB1) from *V. harveyi* to be able to use luminescence as a direct readout for autoinduction. Over 16 h, *V. cholerae* would grow to high cell densities and as a result was strongly luminescent. Upon dilution into artificial marine water, cell density and autoinducer concentration would rapidly decrease, resulting in a decline in luminescence. After several hours, cells would eventually accumulate sufficient autoinducer to reach the quorum threshold and induce luminescence again. This behavior was observed in AMW alone (Figure 1) and is in agreement with commonly observed results from such experiments.14,21 In contrast, when cells were diluted into media containing polymers, they would undergo extensive clustering almost instantaneously, and luminescence readouts never dropped but instead further increased immediately (Figure 1), suggesting that clustering not only countered the dilution effect but further increased autoinducer concentration within the clusters. Interestingly, this behavior was observed over a broad space of cell densities (at least 2 orders of magnitude), including in dilute cultures that did not by themselves experience autoinduction (Figure 2C, F), suggesting that during clustering polymers create pockets containing strongly increased concentrations of autoinducers around bacterial aggregates.

We further demonstrated that both CAI-1- and AI-2-dependent quorum sensing cascades are activated in response to polymers and that clustering leads to an enhanced production of both autoinducers (Figures 5 and 6). The effect of the *Vibrio*-specific autoinducer CAI-1 dominated the clustering-driven luminescence phenotype (Figure 5) in line with previous results obtained for batch cultures of *V. cholerae* in rich medium.21

Some studies have hypothesized that luminescence could be a result of limited diffusion of nutrients in the polymer-mediated bacterial aggregates.19 Catabolite repression of luminescence has been reported for *V. fischeri*, where cAMP-CRP stimulates luxCDABE expression.22 However, this effect is alleviated by high concentrations of autoinducer.22 In our hands, CRP was essential for luminescence, both triggered by high cell density in the absence of polymers, in line with previous findings for an *E. coli Δcrp* mutant,20 as well as in response to polymer-induced clustering. Additionally, supplementation of the media with excess glucose did not quench luminescence even in the absence of autoinduction. This suggests that nutrient limitation within the clusters is not a major cue for luminescence induction but further underpins that cross-talk between nutrient sensing and quorum sensing pathways exists.

Finally, we followed up on our earlier observation that exposure to cationic polymers causes deposition of *V. cholerae* on inorganic surfaces and release of extracellular DNA, both hallmarks of biofilm formation.12 Here, we showed that this phenotype is the result of transcriptional activation of genes involved in biofilm production in response to polymer exposure. Biofilm induction may explain the enhanced resistance toward antimicrobials of bacteria that have been exposed to cationic polymers, as previously described by others.10 The expression of the biofilm regulator VpsR and the biofilm structural protein RbmA were both induced upon exposure to the polymers (Figure 7). This upregulation is in contrast to the canonical biofilm regulation where biofilm genes are repressed during autoinduction. VpsR is a master regulator of biofilm formation and a two component system response regulator. Although no cognate histidine kinase has been identified, VpsR is epistatic to the intracellular hybrid sensor histidine kinase VpsS.27 Induction of vpsR likely leads to the downstream induction of rbmA we observed here because rbmA is a direct target of VpsR regulation.28 However, vpsR induction in the presence of polymers seems to happen despite autoinduction, which should normally lead to suppression of vpsR. What is also different from a regular biofilm response is that VpsR in the presence of polymer fails to upregulate one of its other direct targets, aphA. We showed that, in contrast to this canonical response, aphA is strongly suppressed by the presence of polymers (Figure 7C). When Shikuma et al. identified VpsS as a regulator of VpsR, they established the existence of a pathway that proceeds from VpsS through the quorum regulators LuxU and LuxO and results in the VpsR-dependent activation of biofilm production independent of HapR.27 It may be that, in the presence of polymers, this pathway is active and dominates the effects of the CAI-1 and AI-2 pathways on biofilm. Unfortunately, the cognate signal activating VpsS is as yet unidentified.

## CONCLUSIONS

We showed here that clustering of *V. cholerae* in response to cationic polymers leads to autoinduction due to a rapid increase of local autoinducer concentration in the vicinity of aggregated bacteria. Moreover, we demonstrate that stimulation of further autoinducer synthesis is also observed and involves at least two of the four known quorum sensing systems, CAI-1 and AI-2. We speculate that the third quorum sensing pathway, which proceeds through the intracellular hybrid sensor kinase VpsS,28 is also activated and leads to the production of biofilm in response to polymer-driven aggregation. Our previous work together with the data presented here rules out membrane disruption and nutrient limitation within clusters, respectively, as cues leading to the phenotypes observed here. Our future work will aim to further dissect the pathway(s) triggered in response to polymer exposure to clarify whether VpsS is indeed involved and activated in response to polymers. Polymeric materials that inhibit bacterial dissemination, both mechanically and transcriptionally, may be useful for applications to enhance
wastewater treatment for the decontamination of water from *V. cholerae*.

### MATERIALS AND METHODS

**Bacterial Strains and Culture Conditions.** *V. cholerae* El Tor strains used in this study (Table 1) were derived from A1552 and E7956 wild-type; O1 biovar El Tor serotype Inaba

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**Plasmids**

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#### β-Galactosidase Assays.

**MATERIALS AND METHODS**

Polymerase chain reaction (PCR) was used to amplify the plasmid used in this study (Table 1).

**Luminescence Assays.** Luminescence assays were done using *V. cholerae* pBB1 transconjugants. The pBB1 cosmid was introduced into *V. cholerae* strains by triparental mating in the same conditions as for pRW50T. Overnight cultures of *V. cholerae* pBB1 were adjusted to an OD of 0.5, 0.1, and 0.01 in artificial marine water with 10 μg/mL of tetracycline. Polymers P1 and P2 were added at concentrations of 0.005, 0.05, and 0.5 mg mL⁻¹ in DMEM or AW in 200 μL final volume using a dark-wall clear-bottom 96-well plate. The plate was incubated up to 15 h at 37 °C with shaking at a 200 rpm, whereas luminescence and OD₆₀₀ were recorded every 30 min using a FLUOstar Omega plate reader. The following assays were done with bacterial cultures with OD₆₀₀ adjusted to 0.2. Cells were recovered after the assay and washed with high-salt PBS containing 200 mM NaCl to disrupt charge-based aggregation and plated onto LB with tetracycline to determine the viability. Plates were imaged using a BioRad Gel Doc XR System, and the images were processed with ImageJ.

**Super-Resolution Microscopy of Bacterial Clusters.** *V. cholerae* A1552 pBB1 was adjusted to 0.05 mg mL⁻¹ P1 in PBS for 1 h. Membrane integrity to be visualized, the sample was stained using the LIVE/DEAD BacLight Bacterial Viability Kit (Life Technologies) for 10 min at RT. The sample was mounted with ProLong Gold Antifade Mountant and covered with a coverslip. Images were taken on a Nikon Eclipse TE2000-U microscope. Image acquisition was done using Nikon NIS-Elements software, and final images were processed with ImageJ. Pixel intensity was determined from several clusters within frames using ImageJ.

**Luminescence Assays Using *V. cholerae* BH1578 pBB1 as a Reporter.** *V. cholerae* BH1578 pBB1 was used to determine the effect of polymers on the production of autoinducers. *V. cholerae* strains at an OD₆₀₀ of 0.2 were clustered with polymers at concentrations of 0.005, 0.05, and 0.5 mg mL⁻¹ in artificial marine water. Supernatants were recovered by centrifugation and used to resuspend *V. cholerae* BH1578 pBB1 previously adjusted to an OD₆₀₀ of 0.2 in 200 μL. Luminescence was measured at 37 °C using a FLUOstar Omega plate reader. Similarly, *V. cholerae* strains and *V. cholerae* BH1578 pBB1 were cocultured in 200 μL final volume, and polymers were added at concentrations of 0.005, 0.05, and 0.5 mg mL⁻¹ in artificial marine water. Both strains were adjusted to a final OD₆₀₀ of 0.1 each (0.2 total density). Incubation was done at 37 °C with shaking at 200 rpm, and luminescence and OD₆₀₀ were measured every 30 min using a FLUOstar Omega plate reader.

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**Notes**

The authors declare no competing financial interest.
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