Temporal Quorum-Sensing Induction Regulates Vibrio cholerae Biofilm Architecture

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Vibrio cholerae, the pathogen that causes cholera, also survives in aqueous reservoirs, probably in the form of biofilms. Quorum sensing negatively regulates V. cholerae biofilm formation through HapR, whose expression is induced at a high cell density. In this study, we show that the concentration of the quorum-sensing signal molecule CAI-1 is higher in biofilms than in planktonic cultures. By measuring hapR expression and activity, we found that the induction of quorum sensing in biofilm-associated cells occurs earlier. We further demonstrate that the timing of hapR expression is crucial for biofilm thickness, biofilm detachment rates, and intestinal colonization efficiency. These results suggest that V. cholerae is able to regulate its biofilm architecture by temporal induction of quorum-sensing systems.

Vibrio cholerae is a gram-negative, facultative pathogen that is the causative agent of cholera, a devastating diarrheal disease that affects millions of people in the developing world each year (5). Between epidemics, V. cholerae organisms live in marine, estuarine, and freshwater environments in association with zooplankton, phytoplankton, crustaceans, insects, and plants (3, 12). Various studies have suggested that biofilm-mediated attachment to abiotic and biotic surfaces may be important for V. cholerae survival in the environment (28, 29, 33).

Biofilm formation in V. cholerae is a multistep developmental process that is controlled by several regulatory pathways (28). The surface attachment of V. cholerae activates the transcription of the vps (Vibrio polysaccharide synthesis) genes that are responsible for synthesis of the VPS exopolysaccharide, the major component of the biofilm matrix (13, 24, 33). The regulation of VPS synthesis has been partially elucidated through the work of several groups. Environmental signals, such as monosaccharides, nucleosides, and bile salts, have been identified as activators of vps gene transcription and biofilm formation (10, 11, 13). VpsT, VpsR, and VieA are additional regulators of biofilm formation that respond to as-yet-unidentified environmental signals (2, 26, 31). In addition, quorum sensing also negatively regulates biofilm formation by repressing the expression of the vps operon (9, 34). Quorum sensing is a signaling process by which single-celled bacteria are able to produce and respond to small diffusible molecules called autoinducers, which accumulate as cell density increases and regulate the expression of a range of genes that control various physiological functions (6, 20, 27). The quorum-sensing system in V. cholerae has been shown to respond to at least two autoinducer molecules (21, 23, 34): CAI-1 and AI-2. CAI-1, whose structure is yet to be solved, is produced by CqsA and plays a major role in the regulation of biofilm formation. AI-2 is a furanosyl borate diester synthesized by LuxS that is also produced by many other bacteria (30). In contrast to CAI-1, AI-2 is largely dispensable in biofilm regulation (30). The accumulation of these autoinducers modulates the activity of a central regulator, LuxO, via the membrane receptors CqsS and LuxPQ (21). At low cell densities, LuxO actively represses the expression of another key quorum-sensing regulator, HapR, by activating the expression of a set of small RNAs which destabilize hapR mRNA (15, 16). At high cell densities, LuxO is inactivated and, thus, hapR expression is activated. Quorum-sensing-deficient mutants, such as the hapR and cqsA mutants, form thicker biofilms than do their wild-type isotypes (9, 34). These quorum-sensing-deficient mutants also experience difficulty in detaching from biofilm structures, and it was hypothesized that this may result in the decreased colonization efficiency observed in these strains (34).

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The Escherichia coli and V. cholerae strains used in this study are listed in Table 1 and were propagated in LB containing appropriate antibiotics at 37°C, unless otherwise noted. The cqsA-lacZ transcriptional fusion reporter was constructed by cloning the cqsA promoter region and lacZ of V. cholerae into pBRR1-MCS4 (14), and the construct was subsequently introduced into V. cholerae C6706lacZ. The inducible hapR plasmid was constructed by cloning the hapR coding sequence into pBAD24 (8), resulting in pZL37. The plasmids were then transformed into V. cholerae strains. To induce hapR, 0.1% arabinose was added to the medium at different time points. The hapR-Km′ transcriptional reporter was constructed by cloning a PCR-amplified 5′-end fragment of hapR overlapped with the promotorless kanamycin (Km) resistance gene into pGPT704. The resulting plasmid, pJJ255, was then introduced into V. cholerae and screened for homologous recombination events.

Biofilm formation assays. V. cholerae strains were grown on LB agar plates overnight and resuspended in LB broth at an optical density at 600 nm of ~0.6. Five milliliters of a 1:100 dilution of this suspension was then inoculated into 50-ml Falcon tubes containing 22-by-22-mm coverslips. Biofilms were formed on the coverslips at the air-liquid interface by allowing these cultures to stand for the time indicated at room temperature, which is close to the temperature experienced by V. cholerae in natural reservoirs (28). At the time points indicated, the coverslips were taken out and washed in phosphate-buffered saline buffer. Biofilm structures were disrupted by vortexing the coverslips in phosphate-buffered saline in the presence of glass beads (1 mm), and bacterial number was determined by serial dilutions. Biofilm development was quantified by crystal violet as described previously (35). Biofilm volume was estimated by measuring the aver-
TABLE 1. Strains and plasmids used in this study

<table>
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<tr>
<th>Strain or plasmid</th>
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<tr>
<td>Strains or plasmid</td>
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<tr>
<td>V. cholerae strains</td>
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<td>El Tor, wild type</td>
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<td>C6706lacZ</td>
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<td>JZV256</td>
<td>PhapR-Km' integrated in hapR locus</td>
<td>This study</td>
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<td>Plasmids</td>
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<tr>
<td>pBAD24</td>
<td>Cloning vector</td>
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<td>pJZ241</td>
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of the major quorum-sensing molecule CAI-1 in biofilms and planktonic cultures at different time points. Biofilm volume was estimated by measuring the average thickness of the biofilm structures using confocal laser microscopy (data not shown) (34). Figure 1A shows that over time, the concentration of CAI-1 produced by biofilm-associated cells was 10-fold higher than that in planktonic cultures. To test whether this higher concentration of CAI-1 was due to an increase in the transcription of the CAI-1 synthase gene cqsA, we introduced a plasmid containing a cqsA-lacZ transcriptional fusion into V. cholerae and assayed the β-galactosidase activity of biofilm-associated and planktonic cells. No significant difference in cqsA activity between biofilm and planktonic cells was detected (Fig. 1B), suggesting that biofilm cells do not produce more CAI-1 per se; rather, as the volume of biofilms is very small, the cell density of biofilms is much higher than that of free-living cells. Alternatively, it is possible that the biofilm matrix may restrict the diffusion of these small molecules to achieve a localized autoinducer concentration.

We then investigated the consequences of a high concentration of CAI-1 in biofilm cells. In general, when autoinducers
accumulate to their threshold concentrations, the quorum-sensing system is induced. Thus, it is possible that biofilm-associated cells reach their “quorum” earlier than do planktonic cells because of the faster accumulation of autoinducers in the biofilm. To test this possibility, we introduced a cosmid containing the V. harveyi luxCDABE operon, whose promoter can be activated by V. cholerae quorum-sensing systems (21), into V. cholerae and used light production as an indicator of quorum-sensing-dependent gene expression in planktonic and biofilm cells. Overnight cultures were diluted, and light production per cell was measured during subsequent growth. Figure 2A shows that planktonic cells produced light in a cell density-dependent pattern. This U-shaped curve is typical of a quorum-sensing-dependent phenotype, with the initial decrease in luminescence per cell resulting from the dilution of the culture causing a drop in the extracellular autoinducer levels to below the threshold concentration required for the stimulation of lux expression; subsequently, as cell density increases, lux is induced over time due to the accumulation of new autoinducers (21). Biofilm-associated cells, however, exhibited an earlier and higher induction of Lux activity, indicating that the quorum-sensing system is activated earlier and to higher maximum levels in V. cholerae biofilms than in planktonic cultures. In V. cholerae, quorum-sensing regulation acts through HapR, the key positive quorum-sensing regulator. We therefore examined hapR expression during biofilm growth. We fused the hapR promoter with a kanamycin resistance gene and integrated this construct into the V. cholerae chromosome by homologous recombination. The percentage of kanamycin-resistant cells during cell growth corresponded to hapR expression as judged by the hapR-lacZ reporter (data not shown). Moreover, both planktonic cells and biofilm-associated bacteria that were disrupted prior to kanamycin exposure, without the hapR-Km' insertion, were readily killed by kanamycin during growth (data not shown), indicating that kanamycin resistance was not due to any spontaneous mutations. The advantage of using this reporter is that it is very sensitive in detecting gene expression at the single-cell level. As expected, the percentage of kanamycin-resistant biofilm cells increased rapidly and hapR expression reached its maximal level after 8 h of growth, while the percentage of planktonic cells expressing hapR was much lower than that of biofilm cells (Fig. 2B). Taken together, these data suggest that the V. cholerae quorum-sensing system is activated earlier and to higher levels in biofilms than in planktonic cultures.

Previous studies have shown that quorum sensing negatively regulates biofilm formation in V. cholerae by repressing vps expression (9, 32, 34). To further investigate the impact of fast induction of quorum sensing in biofilm-associated cells on biofilm formation, we constructed a plasmid containing the hapR gene under the control of an arabinose-inducible PBAD promoter (8) and introduced this into a hapR deletion strain. The resulting strain, in addition to wild-type and hapR strains containing a vector control, was assayed for biofilm thickness after 18 h of growth. We added 0.1% arabinose to the medium at a number of different time points to artificially turn on quorum-sensing regulation at various times during the 18 h of growth. hapR mutants formed much thicker biofilms than those of wild-type strains (Fig. 3A), as previously demonstrated (9, 34). When hapR expression was induced at early time points (0 and 4 h) by the addition of arabinose, the resultant biofilms were similar to those formed by the wild-type strain (Fig. 3A). However, when hapR was induced in cultures after 8 h of growth, the biofilms formed were as thick as those observed with the hapR mutant, suggesting that the timing of hapR expression is critical for controlling biofilm formation. Interestingly, strains with early induction of hapR expression did not reduce biofilm formation, but rather formed wild-type-like biofilms. It is therefore possible that other cell density-dependent factors are involved in the process of HapR repression of biofilm formation. To further investigate the effect of the timing of hapR expression, we introduced a plasmid containing Pps-lux (11) into the above-mentioned strains and investigated how the timing of hapR induction affected vps expression. Again, when hapR was induced by arabinose at early time points, vps expression in biofilms was similar to that observed in wild-type strains (Fig. 3B). When hapR was induced later than 8 h, the expression of vps was comparable to that of the hapR mutant.

Quorum sensing negatively regulates biofilm formation in V. cholerae, preventing the formation of biofilms that are too
thick. It was hypothesized that thicker biofilms reduce the colonization efficiency of *V. cholerae* because it is difficult for bacteria to detach from overly thick biofilm structures and colonize new sites (34). As we have demonstrated that biofilm thickness is modulated by the timing of quorum sensing induction in *V. cholerae*, we predicted that the timing of hapR induction is also important for biofilm detachment and colonization efficiency. We thus measured the detachment rate of the hapR mutant containing a plasmid harboring P\(_{BAD}\)-hapR (pZL37) from biofilms when arabinose is added at different time points. Biofilms with the induction of hapR at early time points (0 to 4 h) displayed detachment rates similar to that of the wild type, while biofilms with hapR induction at 8 h or later showed lower detachment rates similar to that exhibited by the hapR mutant (Fig. 4A). The lower detachment rate from biofilms by late induction of hapR also correlated with colonization efficiency (Fig. 4B). When we performed infant mouse competition assays using wild-type biofilms and biofilms with hapR induced at different time points, we found that biofilms with late induction of hapR showed a greater-than-10-fold reduction in colonization capacity, similar to that of hapR biofilms (34). This suggests that bacterial cells may disperse more slowly from thicker biofilm structures in vivo, thus resulting in reduced colonization efficiency. This hypothesis remains to be proven.

In this study, we have shown that the *V. cholerae* quorum-sensing system was activated faster in biofilm-associated cells than in free-living bacteria, due to higher autoinducer concentration in biofilms. This action results in the formation of normal biofilm structures from which bacteria can rapidly disperse in order to efficiently colonize the host when necessary. This process could be critical since *V. cholerae* may enter hosts from environmental reservoirs in the form of biofilms. Evidence to support this process is provided by a study showing that the number of cholera cases in a Bangladeshi village...
dramatically declined when *V. cholerae*-associated macroparticles were removed from drinking water using a crude method of filtration through sari cloth (4). Furthermore, free-living *V. cholerae* are highly sensitive to low pH, while *V. cholerae* biofilms are more acid resistant, resulting in the hypothesis that this increased resistance to acid may promote survival during passage through the stomach (34). Upon reaching the intestine, it may be critical for *V. cholerae* to leave the biofilm structure in order to colonize the intestinal surface, as failure to do so reduces colonization efficiency (34). Therefore, an early induction of the quorum-sensing machinery in biofilms to tightly control biofilm thickness might be advantageous in promoting this process.

Quorum-sensing systems in *V. cholerae* are tightly regulated. In addition to autoinducer-regulated LuxO activity, other components are also involved in regulating quorum-sensing systems, including the VarS/VarA two-component sensory system that comprises an additional quorum-sensing-dependent regulatory input (15), the regulator VqmA that modulates *hapR* expression (18), and HapR autorepression (17). Our findings of early quorum sensing induction in biofilms may represent yet another regulatory mechanism to ensure appropriate temporal and spatial gene expression in *V. cholerae*.

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REFERENCES


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