Bacterial communication via quorum sensing has been extensively investigated in recent years. Bacteria communicate in a complex manner through the production, release, and reception of diffusible low molecular weight chemical signaling molecules. Much work has focused on understanding the basic mechanisms of quorum sensing. As more and more bacteria grow resistant to conventional antibiotics, the development of drugs that do not kill bacteria but instead interrupt their communication is of increasing interest. This study presents a method for analyzing bacterial communication by investigating single cell responses. Most conventional analysis methods for bacterial communication are based on the averaged response from many bacteria, masking how individual cells respond to their immediate environment. We applied a fiber-optic microarray to record cellular communication from single cells. Single cell quorum sensing systems have previously been employed, but the highly ordered array reported here is an improvement because it allows us to simultaneously investigate cellular communication in many different environments with known cellular densities and configurations. We employed this method to detect how genes under quorum regulation are induced or repressed over time on the single cell level and to determine whether cellular density and configuration are indicative of the single cell temporal patterns of gene expression.

The term “bacterial quorum sensing” describes communication between bacteria in their natural environment. Quorum sensing was first demonstrated in Vibrio fischeri and has since been shown to regulate multiple bacterial functions including proliferation, sporulation, genetic competence, production of virulence factors, and biofilm formation (1–3). Biofilms are associated with a large number of diseases and, upon formation, they often exhibit antibiotic resistance and can be difficult to eradicate using conventional antibiotics partially because communication levels and communication patterns can vary within a single biofilm (4). Bacteria communicate through the production, release, and reception of diffusible low molecular weight chemical signaling molecules, called autoinducers (AIs). AI molecules are always expressed at a low basal level and diffuse out of the bacteria into the surrounding cellular medium. Expression of quorum-controlled genes is negligible or absent at low cellular density. When the number of bacteria in an environment reaches a critical level, the AI in the medium exceeds a threshold concentration and binds to regulatory receptor proteins, and the AI-receptor complex induces or represses the expression of genes that are under quorum regulation (5–9).

The AI gene is usually one of the target genes under quorum control, creating a positive feedback loop for AI production, which causes a spike in AI expression. Quorum sensing is largely dependent on population density (10, 11), but several recent publications have shown that spatial distribution of the bacteria and mass transfer in the bacterial extracellular milieu are also important for bacterial communication (6, 8, 12).

Different bacteria use different AIs. Gram-negative bacteria communicate through acyl homoserine lactone (AHL) derivatives (Fig. 1), whereas Gram-positive bacteria communicate through oligopeptides. Different Gram-negative bacterial strains communicate through different AHLs. The AHLs differ in the acyl chain length and the substitution on the C3 position of the acyl chain. Both the AHLs and the oligopeptides are members of a family of autoinducers called (AI-1). AI-1 signaling molecules are used primarily to communicate between the same types of bacteria.

Another family of AIs called AI-2 is produced by both Gram-positive and Gram-negative bacteria and is thought to enable communication between different types of bacteria (5, 7, 9, 14–16). In a population of bacteria, the number of cells in the “on” and “off” state with respect to quorum sensing will vary with the AI concentration in the extracellular environment (5–7, 11). Studying communication between bacteria is important for understanding the mechanism of quorum sensing in different environments. Knowledge of these mechanisms can lead to different strategies for interrupting the communication and thereby preventing or disrupting the undesired results of quorum sensing (10, 17).

Most conventional analysis methods for quorum sensing are based on the averaged responses from thousands to millions of cells, masking how individual cells respond to their environment (10, 13, 18–21). Here, we present a novel method for investigating bacterial communication by simultaneously observing thousands of single cell responses. Previous publications have investigated quorum responses in single confined (22) or free (23) bacteria, but the number of cells that can be simultaneously interrogated is small, and the configuration of communicating cells cannot be controlled.

We applied a fiber-optic microarray system to detect single cell responses to cellular communication. Single bacterial cells were randomly distributed on the array, creating many different cellular configurations. The single cells were trapped in
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FIGURE 1. General AHL structure. The R group can be a methyl, hydroxyl, or an oxo-group, and the acyl chain length varies depending on the bacteria (13).

effects on the array, and their position on the array remained constant throughout the experiment. Cellular microenvironments with precise density and configuration were created where local fluctuations in AHL concentration were caused by cellular communication. The high density of the array allows us to simultaneously investigate many single cell temporal responses from cells in known cellular configurations. We demonstrate the ability to detect thousands of single cell quorum responses over time and show that, as suggested previously, the cellular configuration in a specific environment will affect the expression of quorum-regulated genes (6, 8, 12).

EXPERIMENTAL PROCEDURES

Materials

Luria Bertani (LB) broth and agar, ampicillin, polyethylenimine, polyacrylamide (PAA), thiamine, casamino acids, N-butryl-DL-homoserine lactone, N-hexanoyl-DL-homoserine lactone, N-(β-ketocaproyl)-DL-homoserine lactone, N-heptanoyl-DL-homoserine lactone, N-octanoyl-DL-homoserine lactone, N-(3-oxooctanoyl)-L-homoserine lactone, N-decanoyl-DL-homoserine lactone, and 2(5H)-furanone were purchased from Invitrogen. N-(3-oxooctanoyl)-L-homoserine lactone, and 2(5H)-furanone were purchased from Sigma-Aldrich. D-Glucose and hydrochloric acid were made from Difco minimal broth (Davis, containing 1 mg of casamino acids/liter, 2.5 mg of thiamine/liter, and 0.2% glucose).

Strains and Media

*Escherichia coli* harboring plasmid pJBA89 (Apr; pUC18NotluxR-P

LuxI-RBSII-ASV)-T0-T1) was a kind gift from Professor Soren Molin (Technical University of Denmark, Lyngby, Denmark). This strain expresses green fluorescent protein (GFP) in response to several AHL molecules. The strain does not produce AHL (10). Wild type AHL-producing *Serratia ficaria* (ATCC 33105) was purchased from the American Type Culture Collection (Manassas, VA).

The *E. coli* strain was grown in LB broth containing 100 µg/ml ampicillin at 30 °C in an incubator shaker (New Brunswick Scientific, Edison, NJ) at 300 rpm to a mid-log phase (A$_{600}$ = 1.0 measured on a Beckman DU 530 Life Science UV-visible spectrophotometer, Beckman Coulter). *S. ficaria* was grown in LB at 37 °C in an incubator shaker (New Brunswick Scientific, Edison, NJ) at 300 rpm to a mid-log phase (A$_{600}$ = 1.0 measured as above). The AHL-producing strain was labeled by incubating the strain with 50 µM SYTO 64 dye for 45 min in the incubator. At the time of analysis, the bacteria were diluted to the desired A$_{600}$ (~0.3 for the AHL producer and ~0.4 for *E. coli*, measured as above) in ABTG minimal medium. Minimal medium was made from Difco minimal broth Davis, containing 1 mg of casamino acids/liter, 2.5 mg of thiamine/liter, and 0.2% glucose.

Living Cell Array Fabrication

A highly ordered array of bundled optical imaging fibers (Schott, Elmsford, NY) containing ~50,000 individual fibers 3.1 µm in diameter was polished using a series of lapping films (30, 15, 9, 6, 3, 1, and 0.5 µm) on a Multiprep fiber polisher from Allied High Tech Products, Inc. (Rancho Dominguez, CA). The bundle was subsequently etched by placing one end in 0.025 M HCl for 1 min and 45 s, creating wells that were about 2.5 µm deep (26, 30). The etching was quenched by placing the bundle in deionized H$_2$O before it was briefly sonicated (~10 s) to remove residual debris from the etching process. The bundle was treated with 2% polyethyleneimine by placing a 2-µl drop of the polymer over the etched end of the fiber bundle and allowing the bundle to dry overnight. The etched end of the bundle was subsequently modified by attaching a 1-cm plastic sleeve (outer diameter 0.062 inch, inner diameter 0.031 inch) (Smallparts Inc., Miami Lakes, FL) to act as a vessel for bacterial suspensions. 10 µl of diluted bacterial suspension was placed on the fiber, and the fiber was centrifuged in an IEC Micromax RF (Thermo Electron) at 4000 rpm for 45 s, forcing cells into the wells. The AHL-producing strain was first placed on the fiber and then centrifuged. After centrifugation, residual liquid above the fiber was removed, and the *E. coli* culture was placed on the fiber, which was then centrifuged. Three different scenarios were employed in the quorum sensing analysis on the fiber-optic platform (see below).

Uninhibited Communication—The labeled AHL-producing strain, *S. ficaria*, and the AHL-sensing *E. coli* strain were both placed on the fiber as described above. After the fiber was centrifuged, any residual liquid above the fiber was carefully removed and replaced with ABTG minimal medium containing PAA. The PAA formed a gel cap over the fiber. The fiber was subsequently imaged as described below.

Synthetic AHL Response—A suspension of the *E. coli* strain was placed on the fiber, and the fiber was centrifuged as described above. PAA-doped medium was placed over the fiber as under “Uninhibited Communication,” except that synthetic AHL at different concentrations was added to the ABTG before the addition of PAA.

Inhibited Communication—The AHL-producing strain and the *E. coli* strain were placed on the fiber and covered with PAA-doped medium as explained under “Uninhibited Communication.” In addition, 2(5H)-furanone, a known communication inhibitor, was added to the ABTG medium at 410 nM before the addition of PAA.

Imaging of Fiber-Optic Array

The fiber was placed on an inverted microscope (model IX81, Olympus America, Melville, NY). A contrast image and three background images of GFP fluorescence were immediately taken using a CCD camera (Orca-ER, Hamamatsu, Bridgewater, NJ) at excitation wavelength 494 nm and emission wavelength 530 nm with 500-ms exposure (filter set 31003, Chroma Technology, Rockingham, VT). An image of SYTO 64 fluores-


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A fiber-optic microwell array was employed to investigate single cell quorum responses in many different cellular microenvironments and configurations. In these experiments, the temporal resolution of the single cell responses from cells in these different environments was monitored. Several publications report that cellular configuration, in addition to cell density, affects communication between bacteria (6, 8, 12). We utilized the highly ordered configuration of the fiber-optic bundle and our ability to distribute cells randomly on the bundle to simultaneously investigate many different cellular microenvironments. The different microenvironments were compared to determine the effects of both cellular density and configuration on communication. Cellular communication in different microenvironments has been previously reported (22), but precise control of cellular configuration as described here has not been reported.

To analyze quorum sensing in single cells, we employed a Gram-negative strain, S. ficaria, that produces an AHL as its quorum signaling molecule. An AHL-responsive E. coli reporter strain was used as the sensing strain. When the AHL concentration in the medium reached a threshold concentra-

cence at 590-nm excitation wavelength and 619-nm emission wavelength with 1-s exposure (filter set XF 102-2, Omega Optical, Brattleboro, VT) was then taken. The GFP expression in each E. coli was thereafter monitored over every 5 min using the same wavelengths as for the background images.

Data Analysis

A well map was constructed from the contrast image assigning each pixel coordinate in the images to a well on the fiber. The images of SYTO 64 fluorescence were overlaid the images of GFP fluorescence, and the positions of all AHL-producing cells and all E. coli were identified by identifying wells exhibiting SYTO 64 fluorescence and GFP fluorescence, respectively. An E. coli cell was identified by a well exhibiting fluorescence three times over the standard deviation above the background. For the AHL-producing cells, a formula had been predetermined for the expected number of AHL-producing cells on the fiber corresponding to the A_{600} of the bacterial suspension. To determine this formula, a series of experiments was performed, where solutions of AHL-producing cells at different optical densities were placed on the fiber and centrifuged. The number of resulting AHL-producing cells on the fiber was determined by identifying how many wells exhibited SYTO 64 fluorescence. Care was taken to ensure that any residual dye from the labeling process was removed to eliminate background fluorescence in empty wells. For each quorum sensing experiment, the expected number of AHL-producing cells on the fiber according to the optical density was known, and only the wells exhibiting the brightest fluorescence were identified as containing an AHL producer. The formula was used because in the quorum sensing experiments, some background from the labeling process was common, and using the formula prevented empty wells from being identified as occupied by an AHL producer. From the images, the AHL density and configuration in each 36-well microenvironment with E. coli in the center were obtained, and profiles of GFP fluorescence over time from each E. coli cell on the fiber were recorded. To ensure that the E. coli cells included in the data analysis exhibited activated quorum responses, only signals above 200 intensity units over the background were included. This number was on average 2–3 times higher than three times the standard deviation over the background. The data were analyzed using an in-house-developed image analysis program written in the Python programming language (Python Software Foundation, Hampton, NH). In the initial experiments, the fluorescence profiles were clustered into two clusters using k-means clustering, and ΔAHL as described in the text for each cluster was calculated. The sum of squares error for each cluster and the Euclidian distance between the two clusters were calculated in MATLAB (The MathWorks, Inc., Natick, MA). After the initial clustering, the data were further analyzed to determine the dependences of expression profiles on cellular density and cellular configuration in the immediate 36-well microenvironment. From all the temporal GFP fluorescence expression patterns in the dataset that were acquired, 3000 patterns were chosen at random. Each one of these target patterns was called k, and for each k pattern, k-means clustering of the entire dataset was performed to identify the 10 most similar GFP fluorescence patterns. This subset of 10 patterns was called s. The number and position of AHL-producing cells surrounding the E. coli expressing the target pattern k were then determined. This E. coli cell served as a reference for all E. coli expressing response patterns included in s. All E. coli cells in s were compared with the reference E. coli cell with respect to density and configuration of surrounding AHL-producing cells. Three measurements for each E. coli cell in s were calculated. The measurements were of the 1/0 variety; they were either true or false. The three tests were as follows. (i) Each E. coli in subset s was compared with the reference E. coli with respect to the number of surrounding AHL-producing cells in its microenvironment (36 wells as defined above). If an E. coli cell in s was surrounded by ±1 AHL-producing cells as compared with the reference E. coli cell, the test score was true (similar number of surrounding AHL-producing cells). For example, if the reference E. coli was surrounded by five AHL-producing cells, an E. coli in s that was surrounded by four, five, or six AHL-producing cells would receive a true test score. (ii) The second test determined whether the configuration of AHL-producing cells for each E. coli in s was similar to the configuration of AHL-producing cells surrounding the reference E. coli. The definition of similar configuration was one well position offset for each of the AHL-producing cells as compared with the reference E. coli cell. If an E. coli in s met this requirement, a true test result was recorded. (iii) To determine dependence on cellular density and configuration, tests i and ii were combined in this test. The test looked at the two previous tests, and if both test i and test ii were scored true, then this test received a true score. All other combinations resulted in a false score. All 3000 k patterns and their s datasets were examined for the set where the majority of responses received a true score. If more than 50% of the patterns in s were given a true score for test i, ii, or iii, k was considered to be positive. The percentages given in Table 1 are the number of positive k divided by the sample size of 3000.

RESULTS AND DISCUSSION

A fiber-optic microwell array was employed to investigate single cell quorum responses in many different cellular microenvironments and configurations. In these experiments, the temporal resolution of the single cell responses from cells in these different environments was monitored. Several publications report that cellular configuration, in addition to cell density, affects communication between bacteria (6, 8, 12). We utilized the highly ordered configuration of the fiber-optic bundle and our ability to distribute cells randomly on the bundle to simultaneously investigate many different cellular microenvironments. The different microenvironments were compared to determine the effects of both cellular density and configuration on communication. Cellular communication in different microenvironments has been previously reported (22), but precise control of cellular configuration as described here has not been reported.

To analyze quorum sensing in single cells, we employed a Gram-negative strain, S. ficaria, that produces an AHL as its quorum signaling molecule. An AHL-responsive E. coli reporter strain was used as the sensing strain. When the AHL concentration in the medium reached a threshold concentra-
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tion, the E. coli strain expressed GFP, which was under quorum control. The E. coli strain did not produce any AHL and functioned as a sensor/reporter. On the other hand, the S. ficaria AHL-producing cells communicated through both expression and reception of the AHL signaling molecules as described above. Because the GFP gene was under quorum control, GFP expression mimicked how an AHL-producing bacterium would express a quorum-controlled gene if it occupied the same position on the array as an E. coli cell. A fiber-optic array with ∼50,000 etched wells (3.1 μm wide and 2.5 μm deep) at the distal end was employed to analyze cell-cell communication. The wells on the array were sized to host a single bacterium, ensuring that the signal recorded from each well originated from a single cell. We labeled the AHL-producing strain with a red SYTO 64 dye and loaded them onto a microwell fiber array centrifugation. The GFP-expressing E. coli sensor strain was then loaded onto the fiber. Centrifugation forced the bacteria into the empty wells. The AHL-producing strain was first loaded on the fiber, followed immediately by the E. coli strain, to avoid the onset of GFP production while the bacteria were still in solution. We found that if the two strains were mixed in solution before centrifugation, GFP production in E. coli was induced before the cells were confined to the wells, thus not accurately reflecting the response of E. coli to AHL on the fiber. After placing the cells in the wells, the fiber was covered by nutrient medium with added PAA gel. The gel was added to slow down AHL diffusion into the bulk solution. The slower diffusion allowed better resolution of the temporal quorum response for each E. coli cell. Without the gel, the AHL rapidly diffused throughout the bulk medium, creating a uniform concentration of AHL. The slower diffusion in PAA created microenvironments containing different AHL concentrations on the fiber, enabling the comparison of the different configurations as described below. After placing the cells on the fiber, we employed a fluorescence microscope and a CCD camera to acquire a contrast image of the fiber, three background images of GFP fluorescence, and an image of SYTO 64 fluorescence to identify the S. ficaria cell locations. After loading and initial image acquisition, GFP fluorescence images were taken every 5 min for 24 h. We determined the location of each E. coli cell by identifying wells where the GFP fluorescence increased to more than three times the standard deviation above the background. A low level baseline expression of quorum-controlled genes can be seen in bacteria at low cell density (1); therefore, to ensure that E. coli were exhibiting a genuine quorum response, we only included E. coli exhibiting an increase in GFP fluorescence of at least 200 intensity units above the background in the data analysis.

The bacteria were randomly deposited on the fiber array, resulting in many different microenvironments due to the varying numbers and configurations of AHL-producing bacteria. We defined a microenvironment as the 36-well arrangement surrounding an E. coli sensor cell (Fig. 2). A 36-well microenvironment covered three concentric “rings” of wells around the central E. coli cell (see supplemental material for determination of the optimal microenvironment size). The density of cells in a microenvironment was defined as the number of cells in the 36 wells surrounding the central E. coli cell.

The experiment with both sender and receiver cells was the baseline case and was called the uninhibited scenario. In addition, we examined two other scenarios, a synthetic AHL scenario and an inhibited scenario. In the synthetic AHL scenario, only the E. coli sensor strain was placed on the fiber, and synthetic AHL was added to the PAA-doped nutrient medium above the cells. In the inhibited scenario, the AHL-producing bacteria and E. coli were both loaded on the fiber in the same manner as for the uninhibited experiments except that 2(5H)-furanone, known to weakly inhibit quorum sensing, was added to the PAA nutrient medium above the cells (16, 24, 25). GFP expression profiles from the different scenarios were then compared. The results are described and discussed below.

The E. coli strain had previously been employed in bulk experiments (10). Before investigating single cell quorum responses on our fiber-optic platform, we confirmed that the bacteria behaved similarly to these previous bulk measurements by performing quorum sensing tests both on the optical fiber bundle and in microtiter plates. We measured GFP expression when the E. coli strain was exposed to AHL-producing strains and different concentrations of synthetic AHL. We established that the E. coli responded to AHL by confirming increased GFP expression when exposed to increasing concentrations of AHL using both the fiber-optic and the plate reader methods (1, 10). We also ensured that the cells were able to communicate while confined to the wells. Communication between the cells causes an increase in AHL concentration over time. We confirmed communication between the AHL-producing cells by observing an increase in GFP expression over time from E. coli cells that were placed on the fiber with AHL-producing cells (Fig. 3). The E. coli cells alone did not produce significant levels of GFP. These results were in accordance with our microtiter plate experiments and previous quorum sensing studies (10, 17, 27) and confirmed that quorum sensing functioned similarly on the fiber as in conventional bulk analysis.

Upon confirming that the cells responded to AHL and that they communicated on the fiber-optic platform, we investigated whether additional information about cellular communication could be extracted from the single cell time-resolved data. In most conventional quorum sensing analysis methods,
the averaged response from thousands of cells is obtained, or data from a few single cells are obtained (18, 19, 22, 23). In these assays, a reporter protein is measured, where a high level indicates that the genes under quorum control are activated. The assays are easy to perform, and the ensemble results obtained are very useful for population studies; however, these data do not reflect how individual cells in the population respond. The single cell analyses described here provide insight into specific cellular quorum sensing responses and population heterogeneity, but a large number of single cells must be analyzed to obtain statistically meaningful data. By placing large numbers of individual cells on the fiber array, we were able to simultaneously extract thousands of single cell responses as temporal GFP expression profiles.

To analyze the data from the fiber-optic arrays, a custom-written image analysis program was used. This program enabled us to extract GFP expression profiles from individual E. coli cells. The GFP expression profile mimics the quorum-controlled gene response in a single responsive cell. In this custom program, the images of SYTO 64 fluorescence were overlaid with the time series of GFP fluorescence. We determined the number and location of all proximal SYTO 64-labeled AHL-producing bacteria within the 36-well microenvironment for each responding E. coli cell and assigned pixel coordinates in the images (see supplemental material). In each dataset, spatial coordinates were assigned for 3000 E. coli cells and their surrounding AHL producers.

GFP expression in E. coli as a function of cellular density was first investigated. We have previously reported that in a population of clonal cells in a homogeneous environment, the cells can respond with varying levels of reporter gene expression and that patterns of expression are indicative of the cellular response (28–30). We therefore hypothesized that the pattern of GFP expression would better reflect the cellular surroundings than detecting only the intensity of GFP fluorescence, as is common in most assays. To investigate the information contained in the temporal GFP expression patterns, we collected response patterns from all responding E. coli in a fiber experiment. We grouped E. coli exhibiting a similar GFP fluorescence intensity (within 200 intensity units) at similar times in the experiment (within 1 h). These groupings resulted in the division of each fiber experiment into several smaller datasets, and response patterns within each smaller dataset were analyzed. The E. coli cells included in each smaller dataset would appear
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to respond similarly if only the fluorescence intensities over time were analyzed. By expanding the parameters of the analysis to the GFP patterns, additional information obtained from the single cell responses could be identified. We performed these analyses for all three scenarios: uninhibited, synthetic AHL, and inhibited. We used the collected response patterns and performed clustering analysis to evaluate whether the GFP expression patterns of *E. coli* cells that are surrounded by a large number of AHL-producing cells are different from those of *E. coli* cells surrounded by a small number of AHL-producing cells. It has already been established that the level of GFP expression increases as the number of AHL-producing cells increases (5–9), but it has not been determined whether two *E. coli* cells expressing the same level of GFP will express different GFP patterns based on their surrounding cellular density. To ascertain whether there is information contained in GFP expression patterns, the fluorescence intensities of the temporal GFP fluorescence profiles were normalized between 0 and 1 and clustered using k-means clustering (31). The data were normalized to compare only the pattern of reporter gene expression, eliminating any differences due only to fluorescence intensity. In k-means clustering, the temporal GFP fluorescence profile from each *E. coli* cell was compared with all other profiles in the dataset. Profiles that were closest in Euclidian distance at each time point in the series were clustered. In these analyses, two clusters were generated for each dataset. For each cluster, a mean temporal GFP fluorescence profile was created. Euclidian distances were then used to describe the differences between the two mean profiles (32). If the two clusters were very different, the Euclidian distance between the two mean profiles was large. On the other hand, if the clusters were similar, the Euclidian distance between the two was small. Good quality clustering must be ensured to deduce meaningful conclusions from the data (32). We ensured that the data clustering was significant by employing standard statistical parameters (see supplemental material). In the uninhibited datasets, we found that the temporal profiles from *E. coli* clustered according to the density of surrounding AHL-producing cells in their microenvironment. This result suggests that the patterns of GFP expression, and therefore, the patterns of quorum signals experienced by the single cells, are dependent on the number of surrounding AHL-producing cells. The difference between cell responses was amplified as the difference in the number of AHL-producing cells surrounding the *E. coli* cell increased. For example, in one dataset analyzed using k-means, the mean GFP fluorescence profile from *E. coli* surrounded by an average of two AHL-producing cells was significantly different from a mean GFP fluorescence profile from *E. coli* surrounding by an average of five AHL-producing cells. In this example, the difference between the mean number of AHL-producing cells surrounding the *E. coli* cells in each cluster is three. The difference in the mean number of surrounding AHL-producing cells between the two clusters generated in k-means clustering will be referred to ΔAHL in the remainder of the study. As ΔAHL increased to four, five, six, etc., the Euclidian distance between the two mean GFP fluorescence profiles increased (see also supplemental Fig. 3). When we performed the same data analysis on datasets from the synthetic AHL experiments and the inhibited experiments, the mean profiles in the two clusters generated were similar. For inhibited experiments, the profiles did not cluster according to the number of AHL-producing cells surrounding the central *E. coli* cell. Fig. 4 is an example of different clustering in the three scenarios. Each curve in the graph represents the mean profile of all the temporal GFP fluorescence profiles in each cluster, and the number of surrounding AHL-producing cells listed on the graph is the mean number of AHL-producing cells surrounding each *E. coli* in each cluster. The data from each fiber experiment were divided into several smaller datasets. Therefore, each different experiment generated several clustering results; one example for each scenario is shown here. In Fig. 4, the ΔAHL for both the uninhibited and the inhibited scenarios is large (five). The two mean GFP fluorescence profiles generated were significantly different for the uninhibited case but were very similar for the inhibited case. In other instances where ΔAHL was small (less than three), the clusters generated for both the uninhibited and the inhibited scenarios were similar (supplemental Fig. 3). The similar fluorescence profiles in the synthetic AHL scenario illustrate that *E. coli* cells alone do not generate clustering similar to the uninhibited scenario. It is important to note that the main curves in Fig. 4 are not significantly different when comparing cells exhibiting similar GFP fluorescence intensities were clustered, demonstrating that the temporal profile of GFP expression is an indicator of bacterial communication in addition to the fluorescence intensity. When comparing cells exhibiting similar GFP intensity, the reporter gene expression pattern was clearly distinct when many communicating cells surrounded an *E. coli* cell as compared with when only a few communicating cells surrounded the sensor bacterium. If clustering was a result of the reporter *E. coli* cells responding in random patterns that could be grouped into distinct clusters, we should have observed the same clustering patterns in the synthetic AHL scenario. The inhibited scenario showed that when bacterial communication was inhibited, the GFP expression profiles from *E. coli* changed and did not cluster well. The distinct clustering was only seen when AHL-producing cells on the fiber were communicating freely and not when *E. coli* were exposed to synthetic AHL or when the communication was inhibited. It is therefore likely that the distinct clusters result from communication between the surrounding AHL-producing cells. Communication leads to sequential activation and deactivation of the quorum response, which resulted in the clustering results seen in the uninhibited scenarios. The ability to observe single cells in an array format enables us to monitor and compare real time activation or deactivation of genes under quorum control. These data can provide information about the duration and
intensity of quorum activation in response to different microenvironments. This information goes beyond conventional methods where an average *E. coli* response is detected.

After determining that the pattern of reporter gene expression correlated with cell density within a microenvironment, we investigated the effect of cellular configuration within the same microenvironment. We hypothesized that the configuration of AHL-producing cells would affect the reporter gene expression in *E. coli*, distinguishing microenvironments with the same AHL producer density from each other. Several research reports have emphasized the importance of bacterial configuration in addition to density in quorum sensing (6, 8, 12). This type of dependence on bacterial configuration is difficult to detect and analyze in most quorum sensing analytical methods. The fiber-optic array is ideal for studying the effect of bacterial configuration on cellular communication because many different configurations are created when the bacteria are randomly placed on the fiber. The high density of the array enables the simultaneous analysis of thousands of single cells. To investigate different bacterial configurations, datasets from the uninhibited and inhibited scenarios were chosen. The synthetic AHL scenario did not contain any AHL-producing cells and was therefore not included here. We wanted to see whether cells that were allowed to freely communicate exhibited dependence on cellular configuration as well as density. In the analyses described above, reporter gene expression pattern was dependent on cellular density; therefore, we wanted to expand the input parameters to include cellular configuration. Cellular density was defined above as the number of AHL-producing cells in a microenvironment, and cellular configuration was

![Graph showing clustering of temporal GFP fluorescence profiles from *E. coli* when exposed to different scenarios.](image-url)
defined as the location of these AHL-producing cells in the microenvironment. Cellular density and configuration are illustrated in Fig. 2, depicting two microenvironments with the same cellular density but different cellular configurations. A custom-written image analysis program was used where we randomly chose 3000 *E. coli* from each fiber experiment and determined the coordinates for each *E. coli* cell and its surrounding AHL producers within a 36-well microenvironment. Clustering analysis on the reporter gene profiles from the *E. coli* cells was then performed. The specific procedure of the statistical analysis is further described under “Experimental Procedures” as well as in the supplemental material. The desired output would inform us to what degree *E. coli* with the same pattern of reporter gene expression were also surrounded by a similar number of AHL-producing cells in similar configurations. We expected that the temporal expression of the reporter gene in *E. coli* for the uninhibited experiments would correlate to both the surrounding AHL density and the configuration of the AHL-producing cells. We expected that these correlations would be affected and weakened when communication was inhibited. To compare the two scenarios, we first confirmed that the distribution of bacteria on the arrays was the same in all experiments. We counted the number of surrounding AHL-producing cells within the 36-well microenvironment for all responding *E. coli* cells in several fiber experiments. We determined how many *E. coli* cells were surrounded by zero AHL-producing cells, one AHL-producing cell, two AHL-producing cells, etc. We expected the distributions to be similar for both the inhibited and the uninhibited scenarios because they were both prepared using the same protocol. Fig. 5 displays data from both scenarios, confirming that there was little difference in the distributions between the two experiments.

After confirming that the bacterial distributions were similar for all experiments, we analyzed the reporter gene expression profiles with respect to the number and configuration of AHL-producing cells within a microenvironment (see also the supplemental material). These analyses allowed us to determine whether the configuration of AHL-producing cells affected the
reporter gene expression in addition to the density of the cells in a microenvironment. In our analyses, six different datasets, each encompassing responses from 3000 *E. coli* cells, were included for both the uninhibited and the inhibited datasets. Table 1 shows the results from six uninhibited fiber arrays and six inhibited fiber arrays.

Reporter gene expression profiles from 3000 *E. coli* cells were chosen at random and clustered. For each response, the 10 most similar curves were identified in a subset of the data. In each of these subsets, the number and configuration of AHL-producing cells surrounding the *E. coli* cell were compared. In Table 1, the percentage of *E. coli* that were surrounded by AHL-producing cells in both similar configurations and similar numbers is shown. Similar was defined as an offset of 1 well with respect to configuration and ±1 bacterium with respect to number (see also the supplemental material). We see from the table that for the uninhibited scenario, there was a high percentage of similarity for both cell density and cell configuration. These results indicate that when the cells communicate, cells with similar configuration and similar density express genes under quorum control in a similar way, and not necessarily just with similar intensity. The results also demonstrate that the fiber-optic array can be employed to provide detailed information about cell-cell communication in a highly defined environment. For the inhibited scenario, we have already seen that the reporter gene expression profiles did not cluster well with respect to surrounding AHL-producing cells, and we see from these data that the overlap between dependence on cellular density and configuration was highly variable and uncorrelated. These inhibited scenario results suggest that when the communication between the bacteria is perturbed, an inconsistent quorum sensing response is detected, and the pattern in which the single cells express the reporter gene is altered. This observation would be masked in an ensemble analysis of communicating cells where the level of reporter gene expression is measured and can only be made when many single cells are simultaneously analyzed.

In summary, we utilized a highly ordered fiber-optic array and a custom image and data analysis program to evaluate bacterial communication in a cell population with single cell resolution. We employed a GFP-expressing *E. coli* sensor strain together with an AHL-producing Gram-negative strain and detected the modulation of AHL in many different cellular densities and configurations. The image analysis program enabled us to extract single cell temporal data and accurately determine cellular configurations from thousands of cells simultaneously. The fiber-optic array provides real time information about how genes under quorum control are induced or repressed as a result of AHL modulation. The array can also be used to investigate how different compounds affect the communication between cells. This information can be used in basic research of how cells communicate or for screening compounds that affect cellular communication. An important advantage of the fiber-optic array is that it enables the investigation of many single cells in different cellular configurations simultaneously. The cells are stationary but densely packed and are able to communicate through diffusion and reception of signaling molecules. The single cell data obtained from the fiber-optic array contain extensive information about cell responses in different microenvironments. We have shown here that reporter gene expression patterns in *E. coli* are affected by both cell density and cellular configuration. The results show that the intensity of the quorum response can be combined with the pattern of quorum response to more fully describe communication between cells. The information obtained from these single cell measurements goes well beyond conventional methods where only an average cellular response is measured.

Additional data analysis programs for the array have the potential to provide new ways of analyzing quorum sensing at the single cell level. A potential application is to perform a statistical analysis on lag time before and during quorum response and to determine the degree of quorum response with respect to cell density, configuration, and mass transfer in the medium. In addition, by comparing single cell data when employing different inhibition strategies, one may be able to determine whether an inhibitor interferes with the signal receptor, whether AHL expression is blocked at the expression level, or whether the signal molecule is degraded in the extracellular milieu before it reaches the signal receptor (16, 25). Different strategies of inhibition will likely result in different reporter gene patterns. Such analyses could provide detailed information about the nature of quorum sensing as well as the mechanism by which an inhibitor affects cellular communication.

**TABLE 1**

| Scenario | Dependence on cellular density and configuration % |
|----------|--------------------------------------------------|
| Uninhibited | 74 |
| Uninhibited 1 | 69 |
| Uninhibited 2 | 69 |
| Uninhibited 3 | 69 |
| Uninhibited 4 | 70 |
| Uninhibited 5 | 73 |
| Inhibited | |
| Inhibited 1 | 32 |
| Inhibited 2 | 43 |
| Inhibited 3 | 77 |
| Inhibited 4 | 46 |
| Inhibited 5 | 50 |
| Inhibited 6 | 77 |

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