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Localized Quorum Sensing in *Vibrio fischeri*

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Abstract

Quorum sensing is almost always regarded as a population density effect in three-dimensional bulk samples of bacteria. Here we create *two*-dimensional samples of *Vibrio fischeri* cells adhered onto glass surfaces to examine the effect of *local* population densities on quorum sensing. This is done by measuring the luminescent response. The 2-D bacterial populations enable us to simultaneously account for time and distance effects on quorum sensing, which were previously very challenging to access in typical three-dimensional bulk samples. Thus, we are able to consider quorum sensing in terms of signal diffusion. A diffusion model of quorum sensing signals guides the experiments and shows that for a given cell spacing (density) and diffusion time there exists a "true quorum"— a number of cells necessary for quorum sensing. We find that quorum sensing can occur locally in 2-D surface samples and is a function of cell population density as well as signal diffusion time.

Keywords: Cell–cell communication; Cell signaling; Diffusion model; Population dependence; Luminescence; AHL

1. Introduction

Over 30 years ago, Nealson reported that *Vibrio fischeri* produces an extracellular autoinducer, which accumulates as a function of cell density growth and activates luminescence at a threshold concentration [1]. This phenomenon was first called "quorum sensing" by Winans as a colorful way to describe the concept of autoinduction as being population controlled [2,3].

Quorum sensing occurs in *V. fischeri* via an autoinducer producer and receptor protein pair. Briefly, the LuxI protein produces autoinducer molecules, acylated homoserine lactones (AHL), which diffuse across the cell membrane into the surrounding medium. At a critical autoinducer concentration, interaction with the receptor protein LuxR is possible and binding occurs [4]. The LuxR–AHL complex is then able to bind to the promoter of the *V. fischeri* luminescence gene to activate transcription, resulting in light production. This light production is important in symbiotic relationships of *V. fischeri*, such as with the fish *Monocentris japonica* [5] and with the squid *Euprymna scolopes* [6]. Details on the regulation of gene expression by quorum sensing can be found in several reviews on the topic [7–11].

When quorum sensing was first observed, it was originally thought to be a special occurrence unique to just a few species. These included, for example, luminescence in *V. fischeri* [12], fruiting body formation in *Myxococcus xanthus* [13], and competence in *Streptococcus pneumoniae* [14]. It is now known that quorum sensing occurs in numerous species and in various forms. Typically, gram-negative bacteria use AHL [10] whereas gram-positive bacteria use a different type of autoinducer, oligopeptides [15]. There is yet another type of signal molecule, a furanosyl borate diester [16], now recognized, for example, in *Vibrio harveyi* [17] and *Escherichia coli* [18]. These furanosyl borate diesters make up the autoinducer 2 (AI-2) family of quorum sensing signals whereas AHL is referred to as AI-1. An organism can use multiple quorum sensing signals, networked in parallel (e.g., *V. harveyi*) or in series (e.g., *Pseudomonas aeruginosa*) [19]. Not only is quorum sensing an intraspecies activity, but it also occurs on an interspecies level. Bacteria use AI-2 to detect other species' populations [20,21], which allows for one species to be able to interfere and compete with another species' quorum sensing. For example, *E. coli* can inhibit *V. harveyi* luminescence, even at quorum-sized cell densities, by consuming AI-2 [21].

In 2002 Redfield hypothesized that autoinduction was diffusion controlled rather than population controlled and that it was not a co-operative action of a bacterial population, but rather a way for individual cells to gather information about

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the transport properties (e.g., diffusion and mixing) of the surrounding medium [22]. Redfield's hypothesis is plausible because the response to quorum sensing in many species is frequently a secretion, for example, of virulence factors [19,23], antibiotics [23], or extracellular polymeric substances [24,25], which are of benefit to an individual cell only if the secreted biomolecules stay local [22]. The "diffusion sensing" hypothesis, however, does not apply for all cases, such as with *V. fischeri* luminescence, which is not a secretory response to quorum sensing. It is, therefore, reasonable that quorum sensing is indeed due to both population and diffusion, and that different species developed quorum sensing based on one aspect (population or diffusion) of the possibly dualistic phenomenon.

The influence of diffusion on quorum sensing has not been thoroughly examined. The first pieces of experimental evidence came out of Basu et al.'s 2005 publication, which uses quorum sensing signal diffusion to form programmed patterns of cells [26]. *V. fischeri* autoinducer signals from sender cells elicit different fluorescent responses in receiver cells, which are genetically engineered to respond to different bands of signal concentrations with either red or green fluorescence, depending on the band-detect. Thus, plated mixtures of different band-detect cells surrounding a center disk of sender cells results in a bulls-eye pattern formation [26]. This work, however, was not directly interested in the effect of diffusion on quorum sensing and only bulk samples of cells of very high population densities were used.

We hypothesize that due to the physical phenomenon of AHL diffusion from surface cells, quorum sensing depends on both the *local* population density *and* the total time the AHL signals are allowed to diffuse from the cells. Traditionally, quorum sensing is studied in three dimensions in bulk suspensions in which cells are evenly distributed throughout the medium. In contrast, a local cell density occurs in a sample if cells are concentrated at a surface, perhaps with none in the planktonic form. At a smaller scale yet, heterogeneity in local cell density creates some areas that are more highly concentrated with cells than others.

To test diffusion effects on quorum sensing, we use *V. fischeri* as a model experimental system, vary surface cell density, and measure changes in luminescence with time. To aid in experimental design and also to mathematically examine diffusion in quorum sensing, an AHL diffusion model is used. Through diffusion modeling we show that surface cell densities formed via random adhesion and formed via patterned adhesion give similar AHL concentration profiles. The modeling also shows that the important factors to consider in quorum sensing are signal diffusion time, population density, and also population number. Luminescence experiments show that quorum sensing happens on a local scale and that signal diffusion has an effect. Larger cell concentrations and longer times result in more luminescence.

2. Materials and Methods

2.1. Cell culture

V. fischeri (ATCC Number 7744, Manassas, VA) was revived from a freeze-dried sample in Marine Broth (Difco 2216, Becton, Dickinson and Co., Franklin Lakes, NJ) and stocks were stored on Microbank solid porous bead carriers (Pro-Lab Diagnostics, Richmond Hill, ON) in a liquid nitrogen cryogenic refrigerator (Taylor-Wharton, Theodore, AL). Stock cells were revived in autoclaved sterile photobacterium broth (PB). The medium ingredients were based on Photobacterium Broth (Fluka 38719, Buchs, Switzerland) but modified to reduce precipitation, and thus, medium turbidity, which influences luminescence measurements. The PB medium contained, in g/L deionized (DI) water (Millipore, Billerica, MA): potassium dihydrogen phosphate (3.0 g/L), ammonium chloride (0.3), sodium chloride (30), iron(II) sulfate heptahydrate (0.01), magnesium sulfate heptahydrate (0.3), sodium β-glycerophosphate pentahydrate (23.5), tryptone (5), yeast extract (2.5), calcium chloride dihydrate (0.15), and sodium bicarbonate (1.0).

Once the revived culture reached the stationary phase of growth the cells were transferred to an absorbance of 0.01 at a wavelength of 600 nm measured in a Helios UV–vis spectrophotometer (Thermo Electron Corp., Waltham, MA), corresponding to a bulk cell density of $1.88 \pm 0.5 \times 10^6$ cells/mL. Harvest for experimentation from this subculture occurred near the start of the stationary phase when the luminescence per cell reached a maximum. All cell growth was in sterile culture tubes and flasks filled to a volume 20% of the total container volume to allow for proper oxygenation. Incubation conditions were 30 °C and 200 rpm on a radial shaker (Queue Systems, Parkersburg, WV).

2.2. Control of local (surface) cell density

The bulk cell density of harvested cells was fixed by dilution in PB and verified in the spectrophotometer. Bulk suspensions were transferred to a depth of 500 μm in a microwell (1.3-cm diameter) on a 24-well glass plate (MatTek, Ashland, MA). Before cell introduction, the glass plate surface was cleaned by sonication (VWR 550 T, West Chester, PA) with an Alconox soap solution, then sonication with 70% ethyl alcohol, and finally soaking in 6M hydrochloric acid for at least 1 h. Each step was followed by copious rinsing with DI water. Sterilization of the surface was ensured before the glass cleaning procedure was executed by exposing to ultra-violet light for at least 20 min.

Surface cell density was varied by using bulk suspensions of different cell densities and allowing those cells to settle and adhere to the glass for different times. Remaining bulk cells were removed by repeated gentle rinses using slow pipetting. Bulk suspension that was removed was replaced with fresh PB. This procedure (Fig. 1) was performed directly on the microscope stage, which was equipped with a stage plate custom machined in-house to hold the well plate. Vibrations were reduced with the microscope set on an optical table (Newport RS1000, Irvine, CA).

The samples were viewed with an inverted transmitted light microscope (Nikon Eclipse TE 2000-U, Melville, NY) with a 40× objective (Fig. 2). Sample depths were maintained at a depth of 500 μm by monitoring the glass-sample and sample-air interfaces' z-axis positions and adding fresh PB if necessary.