RESEARCH LETTER

Quorum vs. diffusion sensing: a quantitative analysis of the relevance of absorbing or reflecting boundaries

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Abstract

The consequences of the boundary conditions (signal reflecting vs. signal adsorbing) on bacterial intercellular communication were addressed by a combined physics and microbiology approach. A predictive biophysical model was devised that considered system size, diffusion from given points, signal molecule decay and boundary properties. The theoretical predictions were tested with two experimental agarose-gel-based set-ups for reflecting or absorbing boundaries. N-acyl homoserine lactone (AHL) concentration profiles were measured using the Agrobacterium tumefaciens NTL4 bioassay and found to agree with model predictions. The half-life of AHL was estimated to be 7 days. The absorbing vs. reflecting nature of the boundaries drastically changed AHL concentration profiles. The effect of a single nonreflecting boundary side was equivalent to a 100-fold lower cell concentration. Results suggest that the kinetics of signal accumulation vs. signal removal and their threshold-mediated phenotypic consequences are directly linked to the properties of biofilm boundaries, stressing the relevance of the diffusion sensing component in bacterial communication.

Introduction

The capability of bacteria to sense their self-emitted signal molecules and turning the information into action was originally referred to as quorum sensing (Fuqua et al., 1994). However, the phenomenon has been interpreted in different ways, including diffusion sensing (Redfield, 2002), where the function of signal secretion is assumed to be the evaluation of the diffusion of secreted products in the environment of a single cell. Further work integrated these views, leading to the concept of local efficiency sensing (Hense et al., 2007). Mathematical models have been developed to analyse such molecular signalling between cells (Dockery & Keener, 2001; Ward et al., 2001; Müller et al., 2006). Dulla and Lindow (2008) demonstrated that QS required only small cell numbers when confined by thin water films on dry leaves. Other studies addressed the optimal density at which QS is predicted to become advantageous (Pai & You, 2009). In microfluidic devices, confinement was shown to induce a QS response even for single cells (Boedicker et al., 2009). Spatiotemporal activation of cells has been modelled by Dilanji et al. (2012). Studying the transition to QS in Agrobacterium, Goryachev et al. (2005) showed that a much lower cell density was required in biofilms than in liquid cultures and deduced that QS could be used by bacteria to detect biofilm formation. Our prior work (Alberghini et al., 2009) introduced other conceptual views and terms (positional sensing, cluster sensing, cumulative gradient sensing).

Understanding N-acyl homoserine lactone (AHL) signalling in natural biofilms needs to account for the effects of the biofilm boundaries on the diffusion, reflection or removal of the signal molecules. Biofilms on submerged solid surfaces have different mass transfer properties on the surface facing side compared with the side facing the...
flowing fluid. Whether the signal reaches the activation threshold depends on AHL production and decay rates and the balance between loss and retention at the absorbing or reflecting system boundaries.

Here, we devised a biophysical model to predict the time-dependent concentration field of an AHL in an agarose matrix (mimicking natural biofilms). The biophysical model was verified by measuring AHL concentration profiles, which were consistent with the computed predictions in systems of different sizes and boundary conditions. The importance of boundary conditions was demonstrated by replacing one reflective with an absorbing boundary. Our results suggest that diffusion sensing contributes to cell-cell communication in biofilms.

**Materials and methods**

**Diffusion test set-up**

The AHL of choice was OHL (N-octanoyl-l-homoserine lactone, Fluka Chemie GmbH Buchs, Switzerland, molecular weight w_{OHL} = 227.3); we considered its diffusion in cylindrical agar discs with radius R and height h (the volume is V = πR^2h). Experimental data were obtained using two different sizes: a larger disc (R = 4.2 cm, V = 20 mL, h = 3.61 mm) obtained by pouring 20 mL of a 0.7% solution of molten agarose into plastic Petri dishes with a diameter of 8.4 cm and a smaller disc (R = 1.7 cm, V = 5 mL, h = 5.51 mm) by pouring 5 mL into mini Petri dishes with a diameter of 3.4 cm.

OHL was initially dispensed at the plate centre (10 μL of a 10 ng μL\(^{-1}\) solution, m_0 = 100 ng). Molecules diffuse in the disc, and their radial concentration profiles were detected after either 22 or 94 h, by an overlay of *Agrobacterium tumefaciens* NTL4 pZRL4 reporter cells (Shaw *et al.*, 1997) that are activated if AHL concentration thresholds are greater than a quorum threshold.

Our theory has three free parameters: the diffusion constant D, the degradation rate k and the quorum-sensing concentration threshold q, to induce a given bacterium. These three parameters can be optimized to better fit the prediction of Eqn. (S1) [in Data S1] to the experimental results obtained via the reporter-based assay as described below.

Some examples of concentration profiles obtained with this procedure are shown in Fig 1. We expect D, the effective AHL diffusion coefficient in agar, to be smaller than the diffusion coefficient (D_{H,0}) in water, which was estimated to be D_{H,0} = 4.9 × 10\(^{-10}\) m\(^2\)/s (Stewart, 1998, 2003). The half-life of AHL molecules, in the absence of bacterial cells at pH 7, was reported to be around 7 days (Englmann *et al.*, 2007) although it was earlier estimated to be 1 day (Schafer *et al.*, 2000).

**AHL dilution bioassay in microtitre plates**

To quantify the AHL concentrations at different distances from the centre at given incubation times, the overlay technique was complemented by a more quantitative method. Such modification overcomes a common limitation in the use of chromogenic AHL-reporting overlays when assessing diffusion gradients, which is the fact that once the signal concentration is above the QS threshold, no quantitative information on its actual con-
centration can be deduced. Instead of pouring an overlay of the reporter cells over the diffusion plates, cores of agarose (100 μL aliquots) were withdrawn with a plastic borer and transferred into 0.5 mL Eppendorf tubes, which were placed in a thermal block set at 95 °C for 15 min to melt the samples. As already discussed, and as confirmed by our analysis (see below), the AHL half-life, at room temperature and pH = 7, is rather long (7 days). Raising the temperature for 15 min is unlikely to induce a significant degradation of the C8-OHL signal molecules (Yates et al., 2000) and would not affect the relative concentrations between different samples because they were all treated in the same way. The only potential effect would be an overestimation of the quorum concentration threshold.

A series of twofold dilutions were performed by transferring 20 μL of molten agarose into an equal volume of water kept at the same temperature in the thermal block, and proceeding accordingly up to a 1 : 8 dilution. To reveal active concentrations within the serially diluted portions of the system, 10 μL of molten material was transferred into wells of a microtitre plate containing the Agrobacterium tumefaciens NTL4 reporter. The revealing microtitre plate had been prepared as follows. After following the same procedure for the overlay preparation described above, aliquots of 200 μL of A. tumefaciens suspension in molten AB agar with gentamycin and X-gal were dispensed into each of the wells of a sterile 96-well microtitre plate (Cellstar, Greiner bio-one, Kremsmunster, Austria). Upon solidification, 10 μL molten aliquots of the cores from the diffusion plates and their dilutions were layered over the agar in the wells, and the plates were incubated for 24 h at 30 °C. Digital images of the results were acquired directly on an Epson Perfection 1240U flatbed digital scanner. The whole experiment was repeated three times and found to be fully reproducible.

**Absorbing boundary conditions assembly**

To provide a set-up for absorbing boundary conditions, we modified a 96-well microtitre plate by piercing holes into the bottom of some of its wells by means of a rotating microblade (Fig. S1). Wells with holes in the bottom were temporarily sealed with sellotape to allow pouring of molten AB medium agarose (0.7%) with the suspended AHL-reporter A. tumefaciens NTL4 with X-Gal prepared as above but without gentamycin and supplemented with a suspension of an AHL-producing bacterial strain (Rhizobium leguminosarum bv. viciae A34). The latter was pregrown in TY medium for 24 h, centrifuged at 5300 g for 15 min, resuspended in 10 mL of sterile physiological salt solution (NaCl 0.98 g L⁻¹) and counted in a Petroff-Hauser chamber to calculate aliquots to be mixed along with the reporter strain in the molten agarose to obtain concentrations of 10⁴ and of 10⁶ cells mL⁻¹. The agar cylinders that resulted were 8 mm high and, upon removing the tape after gel solidification, offered an open circular bottom. This achieved the signal-absorbing condition upon placing the microtitre plate bottom into running water. The water container was slightly tilted and had a hole at the lower end and an inflow at the upper end to generate constant flow of water. A direct comparison with reflecting conditions was achieved using nonpierced con-
The incubation was carried out for over 5 days and monitored continuously for the onset of the blue colour brought about by X-Gal formation.

**Results and discussion**

The mathematical modelling of AHL diffusion predicted curves as the ones shown in Fig. 1. Plots represent expected concentration distributions upon placing a 10 μL drop of solution containing 100 ng of the pure molecule on the agarose gel matrix in the centre of plates of small (radius of 17 mm) or large size (radius of 42 mm) and allowing it to diffuse for 22 or 94 h, subject to degradation with rate $k$. The concentration profiles of serially halved concentrations (1:2, 1:4 etc.) are also shown. One critical characteristic is the boundaries. Once molecules reach the borders, they could be either absorbed or reflected, profoundly affecting the resulting concentration profiles. Although eventually all AHL molecules would be degraded anyway, this would happen after a decay time $1/k$ (typically days). Instead, boundaries of indefinitely absorbing nature would lead to a much faster decline of AHL concentrations, namely within the typical time $h^2/4D$ needed by an AHL molecule to diffuse from the disc centre to the top or bottom absorbing side at a distance $h/2$ (a couple of hours for our experimental setup). Conversely, in the case of reflecting walls, the concentration would become spatially uniform over time and slowly decrease with rate $k$. Such a situation would be reached earlier in smaller-sized systems than in larger-sized ones, as molecules would start bouncing back earlier from reflecting borders. In terms of absorption or reflection, the behaviour of a solid surface with respect to diffusing organic solutes is not known *a priori* as it depends on affinity properties of the specific material and, in case of absorbing behaviour, on the limits of its capacity to bind the compound.

Plots in Fig. 1 are modelled assuming the reflecting boundaries case. Thence, the small plate is predicted to reach flat concentration profiles earlier than the larger plate and to attain higher values, as the bounced molecules are confined within a smaller space. The critical concentration threshold $Q_s$ (orange broken line) required to induce QS serves as a reference cut-off line, to assess at which distance, time and system size, effective quorum is expected to be reached. The model predictions for absorbing boundaries were simply that AHL concentrations were well below the quorum activation threshold for all disc sizes, diffusion times and dilutions considered.

The behaviour of boundaries is a crucial issue applying to all contexts in which QS takes place, especially in biofilms, which are predominant in the environment (Costerton *et al.*, 2003). Evaluating whether the surface on which microbial biofilms develop acts as a ‘mirror’ for molecules or titrating them out becomes of primary importance. A dental plaque would encounter inducing concentrations depending on how reflecting tooth enamel is. In water-covered biofilms, underlying rock type and porosity condition the kinetics of signal perception. The same applies to biofilms colonizing artificial materials such as metal pipelines, implanted prostheses and manufactured material wherever wet conditions allow microbial development. The issue of diffusion sensing (self-perception of signals in narrow spaces) as opposed to quorum sensing (cell density assessment) is critically dependent on boundary properties.

To verify the reflective boundary model predictions, we used plastic Petri dishes of two sizes holding an agarose gel matrix on whose centre OHL was dispensed (Fig. 2). Distribution profiles were evaluated using *A. tumefaciens NTL4* in two ways: (1) to inspect the overall diffusion, plates were overlayed with the reporter after the two diffusion times. The blue front indicates where the concentration threshold for activation has been reached; and (2) on a parallel set of plates, instead of overlaying the reporter, small cylindrical agarose blocks were cored at increasing distances from the centre, melted and diluted to AHL concentration below the detection limit of the bioassay. For the large plate at 22 h, the QS activation front has not yet reached the edge as a colourless circular crown is still visible around the blue halo (Fig. 2). In the small plate instead, the signal spread by diffusion is more complete and a more saturated tint uniformly covers the plate. At 94 h, an above threshold concentration has reached the plate border also for the large disc.

This bioassay in itself does not provide strictly quantitative information, but signal concentrations above the threshold can be measured by stepwise dilution of samples from each zone, as the threshold is fixed. The detection limit was determined by the same assay to be 5 nM (Fig. S2). Agarose allows the dilution of molecules without extraction, by melting and mixing with corresponding volumes of hot water. Aliquots sampled at increasing distances from the origin verified the shape of the diffusion gradient predicted by the model (Fig. 2). As explained, three parameters can be chosen to fit the experimental profiles. Fig. 1 was obtained with the following fitted values: $D = 3.0 \times 10^{-10} \text{m}^2/\text{s}$, $k = 1/(7 \text{ days})$ and $Q_s = 5 \text{ nM}$. The theoretical prediction fits the observed profiles with good accuracy (see the predicted curves in Fig. 1 to observe at which times and distances they foretell the drop below the QS threshold broken orange line): for example, in the large plate at 22 h, only the first and the second distance (plate centre and area encompassing 10–15 mm) were predicted to have concentrations still above the threshold line for all the 3 dilu-
tions, and as Fig 2 confirms, the first two columns (A, B) were active, while those coming from cores taken at 25 mm or 40 mm (plate border) were colourless. All the other situations comply as well with the predicted profiles. The accurate correspondence between theoretical predictions and experimental evidence is crucially related to the choice of the free parameters. For instance, a value of $k = 1/(1 \text{ days})$, as estimated by Schaefer et al. (2000), would have implied much lower values of the AHL concentration profile after 94 h: in such a situation, for both sizes and all dilutions, induction would not have occurred. Therefore, our approach allows for an indirect measure of $k$, which is fully consistent with Englmann et al. (2007). Also the estimated values of $D$ (smaller than the diffusion coefficient of AHL in water) and $Q_s$ are congruent with expectations. In particular, the estimate of $Q_s = 5 \text{ nM}$ is close to the quorum threshold of the reporter strain determined with the bioassay using serial dilutions of AHL (Fig. S2).

If the borders were indefinitely absorbing, the outcome would have been radically different as no molecules would have bounced back and the concentrations in the small plates would not have been higher than in the large ones. More realistically, AHL molecules may be sequestered on plate walls only up to a certain surface saturating concentration, determined by the chemical properties of the material. After saturation has been reached, the walls would become reflecting. With the material used here, adsorption is negligible as the experimental data match the theoretical predictions that take only the reflecting behaviour into account.

For the experiments with an absorbing boundary, the set-up enabled continuous signal removal from the system (Fig. S1). As controls, wells with intact bottoms filled with the same mixture of bacteria submerged in agarose represented the reflecting conditions. The presence of producer, reporter and X-Gal in the gel enabled a direct monitoring of the effects in real time. At 96 h, the plate was imaged (Fig. 3). Absorbing conditions had a significant impact on the signal concentrations that built up in such a system. In open-bottom wells, in spite of the fact that the embedded producer cells had the same density as in the reflecting conditions control wells, the reporter response indicated a much lower AHL concentration. Note that the threshold colour response in the wells with $10^6$ cells mL$^{-1}$ under absorbing conditions was similar to the closed, reflective wells with $10^4$ cells mL$^{-1}$. Therefore, the effect of a non-reflecting boundary is about equal to the effect of a 100-fold lower cell concentration. It is also worth observing that in the absorbing boundary wells containing $10^4$ cells mL$^{-1}$, the signal was removed so effectively that the threshold of quorum sensing was never achieved. These data support the view of QS as a dynamic source of information for cells about the diffusion properties of a biofilm boundary.

In conclusion, using the highly sensitive $A. \text{tumefaciens}$ AHL bioassay, we demonstrated the strong effects that different boundary conditions have on AHL concentrations. The measured concentrations were in good agreement with the predictions of a mathematical model of AHL diffusion if it included decay of the AHL signal. The model enables calculation of concentration profiles with high spatial and temporal resolution. As a side result, we were able to give estimates for the diffusion constant and

**Fig. 2.** Results of the bioassay validating the predictive modelling. Petri plates, small and large, were supplemented with 100 ng OHL at the centre. OHL was allowed to diffuse for 22 h or 94 h and subsequently overlayed with the $A. \text{tumefaciens}$ reporter. Replicates of the same plates, not overlayed with the reporter, were used to sample aliquots at the positions shown by the broken circles. Stepwise twofold dilutions of the aliquots (corresponding capital or small letters) were transferred into the microtitre wells containing the reporter whose response is shown alongside.

**Fig. 3.** Results of the comparison between signal-reflecting and signal-absorbing conditions. Microtitre wells containing 8-mm-deep layers of embedded AHL-producing and AHL-reporting cells after 96 h of incubation under the conditions described in the text.
the degradation rate of AHL and for the quorum-sensing threshold of the reporter, which were in close agreement with values obtained using direct approaches. Our results highlight the hitherto underestimated role of the boundary conditions and geometry of the environment in quorum sensing.

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References


