

***In situ* quantitation of the spatial scale of calling distances and population density-independent *N*-acylhomoserine lactone-mediated communication by rhizobacteria colonized on plant roots**

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Abstract

We used computer-assisted microscopy at single cell resolution to quantify the *in situ* spatial scale of *N*-acylhomoserine lactone (AHL)-mediated cell-to-cell communication of *Pseudomonas putida* colonized on tomato and wheat root surfaces. The results of this *in situ* quantification study on close-to-natural surfaces challenge the conventional view of a quorum group requirement of high cell densities for this type of bacterial communication. *In situ* image analysis indicated that the effective 'calling distance' on root surfaces was most frequent at 4–5 µm, extended to 37 µm in the root tip/elongation zone and further out to 78 µm in the root hair zone. The spatial scale of these calling distances is very long-range in proportion to the size of individual bacteria. Geostatistical modeling analysis implicated the importance of AHL-gradients mediating effective communication between remote cells. We conclude that AHL-mediated cell-to-cell communication occurs not only within dense populations, but also in very small groups and over long ranges between individual bacteria, and therefore this cellular activity is more commonplace and effective than hitherto predicted. We propose that this cell-to-cell communication is governed more by the *in situ* spatial proximity of cells within AHL-gradients than the requirement for a quorum group of high population density.

Introduction

Various low molecular weight extracellular molecules mediate different types of cell-to-cell communication in the microbial world (Bassler, 2002). Those kinds of social interactions allow bacteria to detect each other as they colonize surfaces (Park, 2003). Gram-negative bacteria use *N*-acylhomoserine lactones (AHLs) to communicate with each other in natural environments. Above threshold concentrations, AHLs activate expression of certain genes to occupy their ecological niches, e.g., biofilm development, production of virulence factors or antibiotics, and persistence and viability on colonized host surfaces (Eberl, 1999; Kjelleberg & Molin, 2002; Hall-Stoodley *et al.*, 2004). AHL-mediated cross-talk can also be effective across species borders within microbial communities (Pierson *et al.*,

1998) and even between prokaryotes and eukaryotes (Joint *et al.*, 2002; Mathesius *et al.*, 2003). Because the bacterial traits activated by AHLs can be beneficial or harmful to hosts, identification of the attributes that govern cell-to-cell communication is very important and has major practical application in the design and implementation of synthetic antimicrobial strategies that expressly target microbial biofilms, aquatic environments and plant-microbe interactions (Fuqua *et al.*, 2001; Miller & Bassler, 2001).

It is generally thought that AHL concentrations exceeding the threshold are needed to activate genes, and their physiological functions occur only at high bacterial population densities. Hence, this specific type of microbial communication has become known as 'quorum sensing', functioning primarily as a sensor of high population density (Fuqua

et al., 1994), thus optimizing the expression of functions that are most beneficial when simultaneously performed by dense populations. This prevailing view is commonly based on measurements of gene expression within populations in stirred liquid suspension. However, despite its wide appeal, this quorum sensing paradigm has been recently challenged because the methods commonly used to detect it require high populations, and neither the need for group action nor the selective conditions required for its evolution have been demonstrated (Redfield, 2002; Shompole *et al.*, 2003; Alberghini & Squartini, 2004). Furthermore, many aspects of microbial physiology differ for cells colonized on surfaces, because in this case, the signaling molecules can disperse much faster and further than the attached population itself. For instance, spatial patterns of dispersion and active growth within discontinuous domains are major ecological determinants that govern microbial colonization of host surfaces, especially plants, and during early stages of biofilm development (Dazzo, 2004). One could theoretically predict that individual bacteria should benefit significantly if their sensory system could respond to small, local concentrations of the signal molecule, as that would communicate information about competitive and/or cooperative activities by a few neighbors before they reach high population densities in the cell's immediate microenvironment (Redfield, 2002). Here, we quantified the *in situ* spatial scale and local population requirements of AHL-mediated cell-to-cell communication during bacterial colonization of tomato and wheat root surfaces, and by so doing, tested the quorum sensing paradigm *in situ* at single cell resolution. This was done by taking advantage of recent technological developments in fluorescent reporter strain construction, computer-assisted confocal microscopy, digital image analysis and geostatistical modeling. The results obtained challenge the conventional view of a quorum group requirement of high cell densities for this type of bacterial communication. Portions of this work were presented at the International Rhizosphere Congress, 12–17 September 2004, in Munich, Germany (Gantner *et al.*, 2004).

Materials and methods

Bacterial reporter strains

The wild-type strain of *Pseudomonas putida* IsoF was originally isolated from the rhizosphere of tomato plants and produces four different *N*-acyl-L-homoserine lactones: 3-oxo-hexanoyl-homoserine lactone (3-oxo-C6-HSL), 3-oxo-octanoyl-homoserine lactone (3-oxo-C8-HSL), 3-oxo-decanoyl-homoserine lactone (3-oxo-C10-HSL) and 3-oxo-dodecanoyl-homoserine lactone (3-oxo-C12-HSL) (Steidle *et al.*, 2001, 2002). The 'AHL-source' strain derivative *Pseudomonas putida* IsoF was *rfp*-tagged by introduction of the plasmid pUT-Kan *rfp* (Tolker-Nielsen & Molin, 2000)

that constitutively expresses the red fluorescent protein (RFP) and therefore can be detected by fluorescence microscopy using the appropriate fluorescence optics. The 'AHL-sensor' construct, *Pseudomonas putida* F117 (pKR-C12) (Steidle *et al.*, 2001) has a mutation in its single copy of the *PpuI*-gene (Andersen *et al.*, 2001) that blocks its ability to synthesize AHLs under a variety of growth conditions, but contains an AHL-inducible reporter plasmid pKR-C12 with a green fluorescent protein (GFP)-encoding sensor cassette containing an AHL-regulated promoter and the *gfp*-gene (Andersen *et al.*, 2001). This AHL-reporter cassette responds with high specificity and differential sensitivity to defined AHLs (Andersen *et al.*, 2001; Riedel *et al.*, 2001; Steidle *et al.*, 2001). Within 30 min to 3 h after the F117 pKR-C12 reporter strain (Riedel *et al.*, 2001) is exposed to 3-oxo-C12-HSL above the threshold extracellular concentration of 20 nM, a sufficient number of signal molecules reenter the cell and complex with the promoter protein, allowing it to positively activate expression of *gfp* and produce enough green fluorescence to be detected (Riedel *et al.*, 2001; Steidle *et al.*, 2001, 2002). The version of GFP made by this sensor strain is short-lived because it is degraded fairly rapidly by the Clp protease inside the cell ($t_{1/2}$ = 30 min to 2 h), and therefore green fluorescence represents a fairly recent AHL-activated induction of *gfp*. Further genetic and biochemical characteristics of the recombinant F117 pKR-C12 strain relevant to its use as an AHL-sensor reporter strain are described in (Riedel *et al.*, 2001; Steidle *et al.*, 2001, 2002).

Experimental plant system

Seedlings of tomato (*Lycopersicon esculentum*) cultivar Micro-Tom (Bruno Nebelung, Everswinkel, Germany), a miniature cultivarset (Meissner *et al.*, 1997), were germinated from surface-sterilized seeds (5% sodium hypochlorite solution, 20 min) and grown axenically to exclude other organisms in sterile plastic boxes (PhytaTray[®], Sigma, Taufkirchen, Germany) filled with 250 g quartz sand (Dorsilt, Sakret, Ottobrunn, Germany) containing a mixture of 2–3.5 mm and 0.6–1.2 mm grains in the ratio of 4:1, respectively (Simons *et al.*, 1996; Gantner, 2003). Each planter was moistened with 20 mL of plant nutrient solution (Simons *et al.*, 1996) and incubated in climate controlled chambers illuminated with FR 96T12215 W cool white fluorescent lights (Sylvania, Philips, The Netherlands) delivering a photon flux of $120 \mu\text{mol m}^{-2} \text{s}^{-1}$ and programmed for a 14 h photoperiod at 25 °C light/20 °C dark cycle and 70% relative humidity. Tomato plants grown for 1 to 2 weeks were used for the experiments.

Inoculation and Incubation

The bacterial strains *P. putida* IsoF and F117 were grown separately overnight at 30 °C in nutrient broth medium

containing kanamycin (50 mg mL^{-1}) to a density of $1\text{--}3 \times 10^9 \text{ cells mL}^{-1}$, then harvested by centrifugation (4500 g , 10 min) three times in 10 mM MgSO_4 to remove media and antibiotics, resuspended to a density of $2 \times 10^9 \text{ cells mL}^{-1}$, and stored on ice. An inoculum of $500 \mu\text{L}$ of each was applied onto the tomato seedling roots, first with the AHL negative mutant followed by the AHL-producing strain. The planter boxes were closed with parafilm and returned to the growth chamber. Wheat seedlings (*Triticum aestivum*) were germinated from surface-sterilized seeds, inoculated with the reporter strains and grown in glass tubes under similar conditions. After 24 h incubation, the plant roots were carefully removed from the quartz sand while being kept moist with $1\times$ sodium phosphate buffer solution (10 mM sodium phosphate and 0.13 M sodium chloride at $\text{pH } 7.3$) and excised.

Three controls were included to validate the experimental system. One was a test for microbial contamination based on plating of axenic seedlings and sand samples on nutrient agar plates, followed by inspection for bacterial growth after overnight incubation at 30°C . A second control tested for the effects of the transgenic inserts in the otherwise isogenic strains (Steidle *et al.*, 2001, 2002) on their colonization ability when inoculated alone on plants. A third control tested for autoinduction by the sensor strain or its induction by plant-derived substances when inoculated alone on the plants and grown in gnotobiotic culture.

Microscopy, image analysis, geostatistics

Selected root pieces were transferred to slides, mounted in Citifluor (Citifluor Ltd., London, UK) to retard fluorescent photobleaching and covered with a coverslip. Georeferenced digital micrographs (each at 512×512 pixels) of the bacteria colonized on the root surface were acquired by laser scanning microscopy (LSM410, Carl Zeiss, Jena, Germany) in the epifluorescence confocal mode using a computer-controlled, high precision motorized stage allowing for acquisition and loss-less building of a mosaic image sequence. These individual georeferenced images were combined using Zeiss software (Ver. 3.85-Beta) into large (1536×1536 pixels) mosaic images to investigate the *in situ* distribution of the bacterial reporter strains at full, single-cell resolution on each root. Red fluorescence of RFP made constitutively by the reporter AHL-source strain *P. putida* IsoF was induced by excitation of the specimen using 543 nm laser light and detected with a 580 nm long pass filter. Green fluorescence of GFP made by the *P. putida* F117 pKR-C12 AHL-sensor strain in response to uptake of external AHL was induced at 488 nm and detected at wide band pass 510 nm . Additional z-scan confocal optisections were acquired in $2 \mu\text{m}$ increments to correlate the separation distances between cells of the AHL-source and AHL-sensor

strains *in situ* near the root tip where the bacteria were located in multiple planes of confocal focus.

In situ spatial distribution of the two bacterial populations on inoculated plant roots was performed by image analysis at single-cell resolution. The foreground objects of interest in the mosaic images were segmented by color and then analyzed by the Center for Microbial Ecology Image Analysis System (CMEIAS) (Liu *et al.*, 2001) operating in UTHSCSA ImageTool (Wilcox *et al.*, 1997). To measure the effective calling distance of AHL-mediated cell-to-cell communication, the linear distance separating the object centroids of each activated green fluorescent AHL-sensor cell and its nearest red fluorescent AHL-source cell neighbor on the root surface (excluding root hairs) was measured at $0.1 \mu\text{m}$ precision. Calling distances were not considered for bacteria attached to root hairs because their positions may have changed during mounting of the specimen.

The *in situ* spatial distribution of red, AHL-producing cells on the root surface was mathematically modeled by a geostatistical analysis (Palmer, 2002; Dazzo, 2004) to produce a kriging interpolation map of their local density and predicted connectivity to diffusing, radiating AHL gradients. For this analysis, georeferenced quadrat subsamples [17×13 pixels (width/height)] of fluorescent confocal images were made using a grid overlay and then analyzed by CMEIAS software to extract their centroid x, y coordinate positions and their local cell density (cells per quadrat subsample) as the z variate. The resultant georeferenced data were analyzed using GS+ software (Robertson, 2004) to test for connectivity of this regionalized variable to the spatial patterns of the AHL-producing cells over the defined spatial scale. After the best fit autocorrelation semivariogram model was computed, the corresponding two-dimensional kriging map was made to depict the interpolated centers of local cell density and predicted diffusion gradients of the signal molecule over the continuous spatial domain of the root surface.

Results and discussion

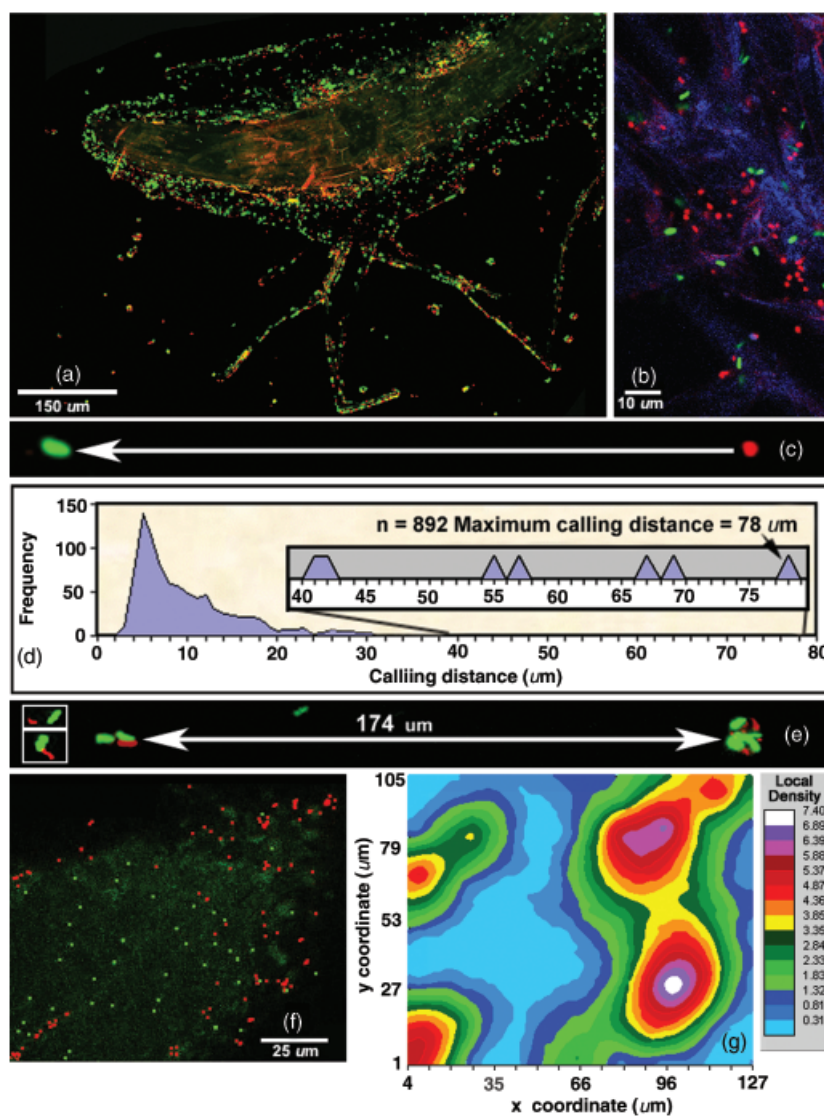
The first control experiment yielded no microbial growth on plates exposed to the axenic plants, indicating that these gnotobiotic culture systems were maintained free of extraneous microbes (including AHL-producers). The second control experiment indicated that the AHL-source and the AHL-sensor strains exhibited similar colonization patterns when inoculated alone on plant roots, indicating that neither production of nor ability to respond to AHL affected their colonization potential *per se*. In the third control experiment, the AHL-sensor strain did not fluoresce green when inoculated alone on the plants, thereby excluding a false-positive result due to autoinduction or activation by an AHL-mimicking compound of plant origin (Teplitski *et al.*,

2000). Furthermore, microscopic examination of inoculated plants revealed no evidence for endophytic invasion of the roots by either strain.

The AHL-sensor cells fluoresced green when both strains were inoculated together on the tomato roots, confirming previous results (Steidle *et al.*, 2001) and validating the use of this gnotobiotic culture/fluorescence microscopy system to examine bacterial communication *in situ*. Figs 1a and b are low and high magnification images, respectively, providing direct microscopical evidence that the AHL-source strain produces sufficient AHL signal molecules to activate GFP synthesis by the AHL-sensor strain on the root surface. Numerous spatially calibrated montage images were analyzed at single-cell resolution to quantify the spatial scale of

bacterial cell-to-cell communication *in situ*. Image analysis of the linear separation distance between each green AHL-activated cell and its closest red AHL-producing cell neighbor ($n = 892$, Fig. 1c) revealed the frequency distribution of calling distances on root surfaces shown in Fig. 1d. The majority of the activated sensor cells were in close proximity to signal-producing cells with an effective calling distance of 4–5 μm for AHL-mediated cell-to-cell communication *in situ* on the rhizoplane (Fig. 1d). The full range of effective calling distances measured in these studies extended to a distance of 37 μm in the root tip zone below the first root hair and to 78 μm on the root surface in the root hair zone where the best growth conditions for bacterial colonization occurred (Fig. 1d).

Fig. 1. *In situ* distribution of individual fluorescent *Pseudomonas putida* cells conducting *N*-acylhomoserine lactone (AHL) mediated cell-to-cell communication on tomato roots. (a–c, e, f) Confocal laser scanning micrographs of the root surface colonized by the red fluorescent IsoF 'source' strain that produces AHLs, and the green fluorescent F117 (pKR-C12) reporter 'sensor' strain activated by extracellular AHLs. Bar scales are indicated. In (c), the arrow depicts the *in situ* calling distance separating a green AHL-sensor cell from its nearest red AHL-source neighbor. (d) Frequency histogram of the range of calling distances for AHL-mediated communication between individual cells of the sensor and nearest neighbor source reporter strains on non-root-hair surfaces. The longer calling distances are indicated in the enlarged inset. (e) Cell-to-cell communication by neighboring AHL-source and AHL-sensor cells in small groups that are separated by long distances from each other and away from dense bacterial populations on the root surface. The white-bordered inserts near the left edge of the figure show two examples where the communicating foci consisted of one AHL-source and one AHL-sensor cell taken from areas separated by long distances from dense populations. (f) Posted locations of red AHL-source and green AHL-sensor cells colonized on a root tip, and (g) continuous spatial interpolation map of the predicted gradients of AHL signal molecules in the same region of the root as indicated in (f), based on a geostatistical block kriging analysis of the spatial distribution of each red (AHL-producing) cell weighted by its local spatial density (cells per quadrat subsample). Some of the important pseudocolored scaling of local density in the 2-dimensional kriging map (1g) is lost when presented have in the CMYK format; see the online version via <http://www.blackwell-synergy.com> to view the image in RGB format.



These calling distances are very long-range for bacteria considering that they are approximately 1–2 μm in length. We interpret this right-skewed frequency distribution of calling distances as a reflection of the *in situ* spatial scale of the AHL gradients that radiate out from sessile AHL-source cells on the root surface, enabling some individual AHL-sensor cells located considerable distances away to sense and respond to these signal molecules.

In addition, as little as one AHL-source cell could participate in cell-to-cell communication with AHL-sensor cells at remote locations even when these small foci of two or more cells were separated from dense bacterial populations on the root surface by very long distances, more than twice the usually measured furthest calling distance (e.g. Fig. 1e). Thus, one individual cell represents a sufficient quorum to produce and excrete enough signal molecules allowing it to communicate with and activate genes in neighboring cell(s) colonized on roots.

These results were corroborated by experiments with axenically grown young wheat plants, where the right-skewed frequency distribution of cell-to-cell calling distances had the same 4–5 μm mode ($n = 294$) and extended to a measured distance of 57 μm (data not shown). These findings indicate that AHL-mediated cell-to-cell communication can occur between individual bacteria over large areas of the root surface that extend far away from dense populations. This distribution of AHLs would significantly affect not only the ability of bacteria to respond to the presence of a few neighboring cells, but also may trigger beneficial responses on the underlying plant root itself, as host defense responses resulting in systemic resistance against fungal pathogens can be induced by AHLs under these experimental conditions (Hartmann *et al.*, 2004).

We used the powerful approach of geostatistics (Palmer, 2002) to measure the connectivity in regional variability of local cell density for the spatial distribution of red fluorescent (AHL-producing) cells in order to make statistically sound inferences of the scale of predicted AHL concentration gradients radiating out from these colonized sources. Figure 1f shows the georeferenced postings of individual red AHL-source and green AHL-sensor cells, conducting cell-to-cell communication *in situ* on a root tip. Semivariance analysis indicated that there was autocorrelation in spatial dependence of their local density, and an isotropic spherical geostatistical model made the best fit to the spatial distribution data extracted from this image, indicating that the local density of red AHL-source cells is spatially dependent over a range of separation distances up to 33.1 μm . This geostatistically predicted scale of maximum autocorrelated separation distance closely approximates the actual measured maximum *in situ* calling distance of 37 μm in the same region of the root. Figure 1g is a continuous interpolation

map derived from this spatial model of autocorrelated local density, which provides a vivid display of the predicted gradients of extracellular AHLs produced *in situ* (assuming that each source cell contributes equally). This geostatistically defendable kriging analysis (Palmer, 2002) clearly suggests that clusters of cells can produce AHL gradients that radiate out sufficiently to activate AHL-sensor cells located considerable distances away from these sources within the entire spatial domain, consistent in principle with the skewed frequency distribution of calling distances measured *in situ* (Fig. 1d). Of course, the three-dimensional scale of such AHL gradients in natural rhizospheres would be influenced by the extent of its adsorption to the root and to inanimate soil particles (e.g. clay and humic particulates), quenching by AHL-degrading enzymes made by the plant or other neighboring microbes, and resistance to diffusion by the irregular root substratum topography and/or viscous, discontinuous liquid films covering the root (Dong *et al.*, 2001; Schulz & Jørgensen, 2001).

In summary, this study of AHL mediated cell-to-cell communication quantified at single cell resolution indicates that (i) it does occur between individual bacteria at spatially discrete locations on colonized surfaces, (ii) the *in situ* spatial scale of the bacterial 'calling distance' measured for this type of communication can extend to 78 μm (a very long distance for a bacterium, proportionally equivalent to two players communicating with each other while located at opposite ends of a soccer field) in this environment, and (iii) single bacteria can communicate via AHLs even when separated from dense populations by very long-range distances. Direct *in situ* evidence is provided, indicating that even one individual bacterium can produce sufficient AHL signal molecules to communicate with another single bacterial cell neighbor. Based on these new, quantitative findings, we predict that AHL-mediated bacterial cell-to-cell communication is more commonplace and effective in nature than hitherto predicted, and that it is governed more by the *in situ* spatial proximity of cells within AHL concentration gradients than by a 'quorum' group requirement of high population density. Stated simply: individual bacteria can produce, sense and respond to AHL gradients made by their neighbor(s) during surface colonization, even when separated by long distances or from high population densities. As these cellular interactions can benefit individual cells (e.g. sensing their competitors or collaborators) (Redfield, 2002), they would be evolutionarily selected, thereby explaining why they are widespread in the microbial world.

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