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Sensitive Whole-Cell Biosensor Suitable for Detecting a Variety of *N*-Acyl Homoserine Lactones in Intact Rhizosphere Microbial Communities[▽]

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To investigate quorum sensing in rhizosphere soil, a whole-cell biosensor, *Agrobacterium tumefaciens*(pAHL-Ice), was constructed. The biosensor responded to all *N*-acyl homoserine lactones (AHLs) tested, except C_4 homoserine lactone, with a minimum detection limit of 10^{-12} M, as well as to both exogenously added AHLs and AHL-producing bacterial strains in soil. This highly sensitive biosensor reveals for the first time the increased AHL availability in intact rhizosphere microbial communities compared to that in bulk soil.

For many bacterial species, population behavior is controlled by quorum sensing (QS), a cell density-dependent secretion, detection, and response to small molecules (25, 29). Bacteria must reach a quorum to perform ecologically relevant and often vital functions, including biofilm formation, virulence, symbiosis, and extracellular enzyme production (5, 29, 32). Small spatial scales and the heterogeneity of microbial habitats make it difficult to study QS in nature by classical destructive methods, such as extraction of signals or signal producers; whole-cell biosensors address this problem by examining biogeography and ecology at microbial scales (2, 6, 14, 17).

QS in soil may indicate locations where signal molecules cannot disperse away from the producers (26), which is critical in stressful, nutrient-limiting environments. The patchy distribution of bacterial colonization and nutrient availability in soil and on plants (22, 23, 27) suggests that the expression of QS will be similarly heterogeneous. We thus developed a biosensor to examine QS in the rhizosphere, where root exudates quickly foster high microbial activity and altered community structure (19, 36), conditions that are likely to include the activation of QS in some populations.

The *Agrobacterium tumefaciens*(pAHL-Ice) biosensor was constructed as a transcriptional fusion of the *A. tumefaciens tra* box promoter region (*traG*-proximal region) with the *inaZ* reporter gene and includes a copy of the *A. tumefaciens N*-acyl homoserine lactone (AHL) receptor TraR gene on the same plasmid. The *tra* box promoter region was PCR amplified from *A. tumefaciens* C58 genomic DNA based on the published sequence (9), using primers designed with Primer3 (28). The *tra* box fragment was cloned into the broad-host-range vector pPROBE-KI′ upstream of the promoterless *inaZ* gene, which encodes an outer membrane-localized ice nucleation protein that catalyzes ice formation (16, 21). Using freeze-thaw transformation (12), pAHL-Ice was mobilized into *A. tumefaciens* strain C58C1 (strain C58 cured of its Ti plasmid) (34), and the resulting strain was designated *A. tumefaciens*(pAHL-Ice). Quantification of AHL-dependent ice nucleation was measured by a droplet-freezing assay (18).

Given that considerable cross talk between species using different AHLs may occur in a mixed microbial community, a biosensor that is relatively nonspecific in its detection of AHLs is a useful tool. *A. tumefaciens*(pAHL-Ice) responds to a wide range of AHLs (Fig. 1), exhibiting increased ice nucleation activity over 6 orders of magnitude with increasing concentrations of oxo-octanoyl homoserine lactone (oxo-C_8-HSL), the cognate QS signal (Fig. 1). The biosensor also exhibited increased ice nucleation activity with increasing concentrations of hexanoyl (C_6), ketocaproyl (oxo-C_6), octanoyl (C_8), decanoyl (C_{10}), dodecanoyl (C_{12}), and tetradecanoyl (C_{14}) HSLs (Fig. 1). No concentration of butyryl (C_4) HSL tested was sufficient to activate the biosensor above the background. Surprisingly, C_{14}-HSL, like oxo-C_8-HSL, activated the biosensor at very low concentrations (10^{-12} M) but conferred a level of ice nucleation that was only about twofold higher than the background. The ice nucleation activity of *A. tumefaciens*(pAHL-Ice) remained stable for up to 20 h when cells were incubated in the presence of oxo-C_6-HSL, and when they were transiently (5 min) exposed to oxo-C_6-HSL, biosensor ice nucleation activity peaked by 2 h and decreased with time (data not shown). These data suggest that the AHL biosensor is suitable for detecting QS in rhizosphere soils, where the signals are likely to vary strongly for species, concentration, and time.

Since soil microbes are generally considered carbon limited (1), it was important to establish that our AHL biosensor could function in bulk soil, which is considered a low-carbon environment. AHL levels in soil were altered by introducing two isogenic strains of *A. tumefaciens* differing in AHL production before inoculation with the biosensor. Wild-type *A. tumefaciens* strain C58 produces oxo-C_8-HSL, while C58C1 is cured of the Ti plasmid and is incapable of making AHLs. Soils were collected during the spring growing season from under the growing zones of the annual graminoid *Avena fatua* at the University of California Hopland Research and Extension

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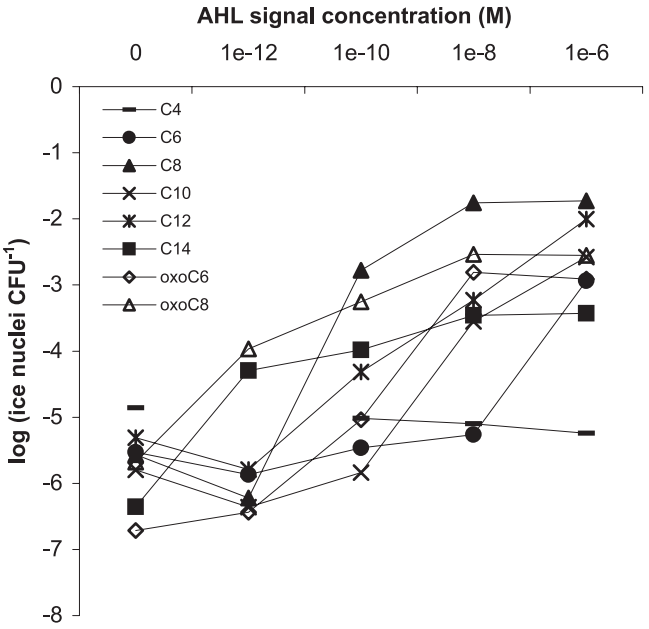

FIG. 1. Dose-response curves for a range of AHLs and the biosensor *A. tumefaciens*(pAHL-Ice) in minimal medium. In each case, the biosensor was grown to about 10^9 cells ml^{-1} and then split into 5-ml subcultures that already had a range of concentrations of one of the signals shown. Standard error bars were left off the graph for clarity, but standard errors were as follows: C_4, 0.172 to 0.371; C_6, 0.179 to 1.026; oxo-C_6, 0.08 to 0.946; C_8, 0.19 to 0.683; oxo-C_8, 0.435 to 0.97; C_{10}, 0.362 to 0.798; C_{12}, 0.254 to 0.582; and C_{14}, 0.168 to 0.638. Biosensor activity is reported as log(ice nuclei CFU^{-1}), and four biological replicates were performed for each concentration.

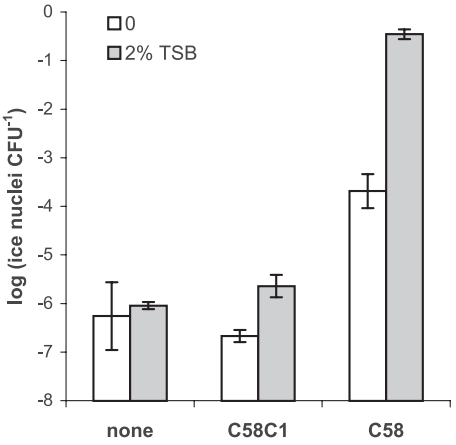

FIG. 2. The ability of the biosensor to respond to QS signals in soil was assessed by adding *A. tumefaciens* strain C58C1, which is cured of its Ti plasmid and cannot make QS signals, or the QS signal-producing *A. tumefaciens* strain C58. Bulk soil assays were conducted with soil slurries of about 0.5 g soil ml^{-1} in water alone (control) or in 2% tryptic soy broth (TSB) (nutrient addition), which were placed into tubes in which AHL stocks in ethyl acetate were previously dispensed and allowed to dry. For inoculation into soil, overnight cultures of biosensor cells in M9 medium were washed and resuspended in KPO_4 buffer at a concentration of 10^{10} cells ml^{-1}. Biosensor cells were added to the soil solution and incubated for 4 h. Soil slurries were then macerated to disperse cells before serial dilution and ice nucleation assays. Ice nucleation activities are reported as the logarithms of the numbers of ice nuclei measured per CFU of biosensor grown on selective plates. Error bars represent 1 standard error of the mean for four biological replicates.

Center (Hopland, CA). The *A. tumefaciens*(pAHL-Ice) biosensor responded to AHLs in soil and was not dependent upon exogenous nutrient addition (Fig. 2). Previous use of *A. tumefaciens* as a host for a nitrate biosensor demonstrated that *inaZ* and *gfp* reporter gene products are detectable in the bulk soil without the addition of nutrients (6), which was also the case here.

Nutrient additions that mimicked root exudates caused a slight increase of biosensor activity, either by increasing AHL production of the native soil bacterial population or by enhancing the biosensor's ability to respond to the endogenous AHLs in the soil. Root exudates stimulate growth of rhizosphere soil microbial communities (14, 33), estimated at about 10^{10} cells g^{-1} soil (11). Bacteria capable of QS are more common in the rhizosphere than in bulk soil (8), so they were likely stimulated by the 2% tryptic soy broth addition. While nutrient addition probably stimulated C58 AHL production as well as the responsiveness of the biosensor, exogenous nutrients were not necessary to detect AHLs.

To understand the capacity of the biosensor to respond to AHLs in heterogeneous soil environments, we examined the response of the biosensor to native rhizosphere soil compared to that to bulk soil as well as rhizosphere and bulk soils to which oxo-C_6-HSL was added. Rhizosphere studies were conducted in microcosms planted with *Avena fatua* (wild oat; Valley Seed Service, Fresno, CA). Two greenhouse experiments were conducted using soils collected at different times of year, planted with seeds from the same seed stock, as previously described (14). Both experiments resulted in higher ice nucleation activity in the rhizosphere than in bulk soil (Fig. 3A and B).

The biosensor responded when low (10^{-8} M) levels of oxo-C_6-HSL were added exogenously to bulk soil but not to rhizosphere soil (Fig. 3B), which may indicate enhanced rates of AHL degradation in the rhizosphere compared to those in bulk soil. QS signal quenching is common in many soils (35). Measured rates of 10^{-8} moles C_6-HSL h^{-1} g $soil^{-1}$ would be sufficient to reduce the low (10^{-8} M) but not the high (10^{-5} M) AHL levels to below the detection limit of our biosensor (Fig. 3B). AHL-degrading strains are found in the *Proteobacteria* and *Bacillus* groups (7, 13, 37), and *A. tumefaciens* harbors the lactonase gene *aiiB* on its Ti plasmid as well as *attM* on the At plasmid (4, 20). In our *A. tumefaciens* biosensor, the presence of *attM* on the At plasmid might have resulted in moderate AHL degradation, although a previous examination of C58 compared to the same strain without the At plasmid revealed only a 10% loss of signal via *attM* (4). These data suggest that while rhizosphere soil contains 10 times more AHLs than bulk soil, the standing AHL pool in the rhizosphere represents an equilibrium in a dynamic system of production, dilution, and degradation of signals.

Limitations of previously tested AHL biosensors precluded conclusions regarding the occurrence of QS in the rhizosphere compared to that in bulk soils. For example, a *Pseudomonas putida lasB*::*gfp* reporter strain was activated in nonsterile soil, indicating that at least 10^{-8} M oxo-C_{12}-HSL was present (31), although this study did not compare AHL levels in rhizosphere

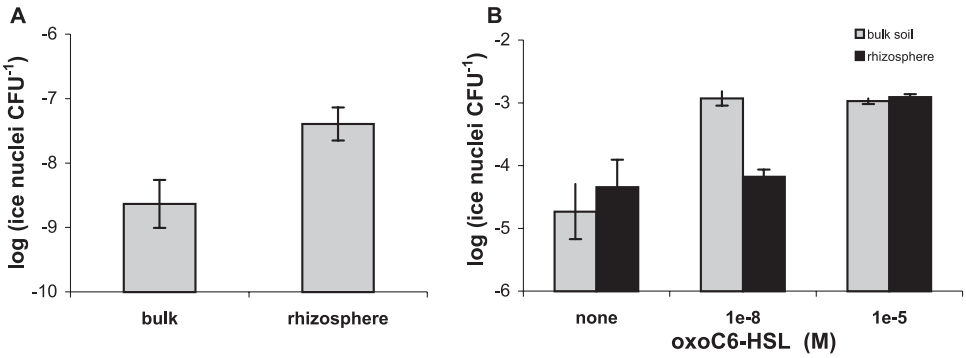

FIG. 3. (A) QS signal availability was measured in greenhouse microcosms, with roots and soils being spray inoculated with *A. tumefaciens*(pAHL-Ice) and ice nucleation activity being measured 12 h later. Background ice nucleation activity in soils was insignificant. Soils that adhered to the roots after gentle shaking were considered rhizosphere soil, while bulk soil was excised from intact microcosms at least 4 mm away from any roots. The difference in activities was significant (Student's *t* test; $P = 0.0012$). (B) To understand if the biosensor was capable of detecting increased signals, the QS signal oxo-C_6-HSL was added at 10^{-8} M and 10^{-5} M to growth chamber microcosms before the application of the biosensor. Error bars represent 1 standard error of the mean for three biological replicates corresponding to separate microcosms.

and bulk soils. Another study used a *gfp*-based *Vibrio fischeri* biosensor, stimulated by adding plant litter, to detect AHLs in compost soil (3), demonstrating that QS could be enhanced by adding a nutrient source. Interestingly, the AHL biosensor used in that study seldom detected AHLs in nonamended soils, revealing either its lack of sensitivity for AHLs, the relatively low levels of these signal molecules in soils lacking nutrient additions, or both.

The *A. tumefaciens*(pAHL-Ice) biosensor developed here is among the most sensitive AHL biosensors constructed, permitting examination of rhizosphere concentrations of AHLs for the first time. The analogous *A. tumefaciens traG*::*lacZ* biosensor has a minimum detection limit for a given AHL of at least 10-fold higher than that for *A. tumefaciens*(pAHL-Ice) (Fig. 1) (30). A *traI*::*lacZ* biosensor with *traR* overexpressed via the T7 promoter responds to oxo-C_8-HSL at concentrations down to 10^{-12} M (38). *A. tumefaciens*(pAHL-Ice) has the same lower detection limit due to the higher sensitivity of *inaZ* than that of *lacZ* (15, 18). The biosensor permits us to estimate rhizosphere AHL levels at 10^{-8} M oxo-C_8-HSL equivalents, in contrast to $<10^{-12}$ M for bulk soil (Fig. 3B); this is potentially 100-fold higher given the low response of the biosensor to noncognate AHLs (Fig. 1). These concentrations are as high as or higher than estimated signal concentrations in soil (10, 24) and agree well with reported levels of AHLs required to activate QS systems in vitro (29).

The heterogeneity of the soil environment makes biosensors powerful and essential tools for understanding QS in natural systems. QS is fast becoming understood as a central process influencing a myriad of plant-microbe interactions, and QS likely mediates microbial behaviors unique to the rhizosphere. The ice nucleation-based biosensor described here should be a useful tool for testing for AHL signaling in any natural environment, especially in heterogeneous soil environments.

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