

Title: Bacterial quorum sensing and nitrogen cycling in rhizosphere soil

Running head: Bacterial QS and rhizosphere N cycling

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## **Abstract**

Plant photosynthate fuels carbon-limited microbial growth and activity, resulting in increased rhizosphere nitrogen (N)-mineralization. Most soil organic N is macromolecular (chitin, protein, nucleotides); enzymatic depolymerization is likely rate-limiting for plant N accumulation. Analyzing *Avena* (wild oat) planted in microcosms containing sieved field soil, we observed increased rhizosphere chitinase and protease specific activities, bacterial cell densities, and dissolved organic nitrogen (DON) compared to bulk soil. Low-molecular weight DON (<3000 Da) was undetectable in bulk soil but comprised 15% of rhizosphere DON. Extracellular enzyme production in many bacteria requires quorum sensing (QS), cell-density dependent group behavior. Because proteobacteria are considered major rhizosphere colonizers, we assayed the

proteobacterial QS signals acyl-homoserine lactones (AHLs), which were significantly increased in the rhizosphere. To investigate the linkage between soil signaling and N cycling, we characterized 533 bacterial isolates from *Avena* rhizosphere: 24% had chitinase or protease activity and AHL production; disruption of QS in 7 of 8 eight isolates disrupted enzyme activity. Many  $\alpha$ -Proteobacteria were newly found with QS-controlled extracellular enzyme activity. Enhanced specific activities of N-cycling enzymes accompanied by bacterial density-dependent behaviors in rhizosphere soil gives rise to the hypothesis that QS could be a control point in the complex process of rhizosphere N-mineralization.

Key words: rhizosphere soil, extracellular enzymes, nitrogen cycle, bacterial quorum sensing, density-dependent bacterial group behavior

## Introduction

Nitrogen (N) is the most limiting nutrient to plant growth in temperate terrestrial ecosystems (Curl & Truelove, 1986; Vitousek & Howarth, 1991), in part because plants cannot access macromolecular organic N. The majority of soil N is organic, existing largely as chitin, proteins, lignoproteins and nucleic acids (Paul & Clark, 1996; Myrold, 1999). Plants can utilize some small molecular weight (MW) organic monomers but primarily rely on inorganic N (ammonium and nitrate), the products of N mineralization. The rate-limiting step in rhizosphere N mineralization is likely enzymatic conversion of high-MW polymeric organic N to low-MW organic N; this ultimately controls acquisition and uptake of N into the microbial and plant biomass (Phillips, *et al.*, 2003; Schimel & Bennett, 2004). Once soil N has been assimilated into microbial biomass, it can be mineralized as a result of predation (Clarholm, 1985), carbon starvation (Myrold, 1999), and/or water potential fluctuation or freeze thaw cycles (Kieft *et al.*, 1987). Integrated control of the multi-step conversion of macromolecular N to inorganic mineralized N is surprisingly poorly understood for soil generally and for rhizosphere soil in particular.

The rhizosphere is a zone of active interchange between plants and soil bacteria, where photosynthate fuels microbial growth and activity, ultimately resulting in the stimulation of N mineralization locally and thus net plant N assimilation (Hamilton & Frank, 2001; Herman *et al.*, 2006; Landi *et al.*, 2006). Microbes are primarily carbon limited in soil, (Lynch & Whipps, 1990); this limitation is partially relieved by exudates delivered by the root moving through soil space, converting bulk soil to rhizosphere soil. Root exudates provide abundant labile carbon, antimicrobial compounds, and hormones via root hairs and border cells (Brimecombe *et al.*, 2001; Bringhurst *et al.*, 2001; Farrar *et al.*, 2003). Rapid bacterial response to these exudates enables them to increase in number and also to become more active (Jaeger *et al.*, 1999; Lubeck

*et al.*, 2000; Herman *et al.*, 2006). This increased activity could include extracellular enzyme activity, which would enable enhanced depolymerization of soil organic N.

Bacteria in sufficiently dense populations perform many ecologically relevant behaviors that less concentrated cells do not, including extracellular enzyme production, exopolysaccharide production, biofilm formation and expression of virulence traits (Schauder & Bassler, 2001; Loh *et al.*, 2002; Bais *et al.*, 2006). Bacterial quorum sensing (QS) is a form of cell density-dependent population behavior, in which the production, detection, and response to specific small molecules regulate gene expression. While QS is common among all bacteria, only proteobacteria exhibit QS that is mediated by the signal molecules *N*-acyl-homoserine lactone (AHL) (Fray, 2002). AHL-producing proteobacteria have been found to be more common in the rhizosphere than bulk soil (Cha *et al.*, 1998; Elasri *et al.*, 2001), and proteobacteria constitute an estimated two thirds of many temperate plant rhizospheres (Hawkes *et al.*, 2007). Extractions of DNA from soil provide evidence for QS in the rhizosphere (Williamson *et al.*, 2005), and the potential for QS in the rhizosphere has been shown by inoculation of sterile soil (Steidle *et al.*, 2001). Two studies show direct evidence of QS in natural soil (DeAngelis *et al.*, 2007) and compost soil (Burmolle *et al.*, 2005) although the role of QS in control of soil processes has not been investigated in biologically intact soils.

QS-control of extracellular enzyme activity has been studied almost entirely within the context of pathogenesis, and is known mostly in pathogenic  $\gamma$ -Proteobacteria (*Pseudomonas aeruginosa* PAO1 (Passador *et al.*, 1993), *P. fluorescens* (Worm *et al.*, 2000), *Enterobacteria* spp (Rasch *et al.*, 2005), *Aeromonas hydrophila* (Swift *et al.*, 1999), *Erwinia carotovora* (Jones *et al.*, 1993; Pirhonen *et al.*, 1993), *Serratia* spp. (Eberl *et al.*, 1996), and *Vibrio* spp. (Croxatto *et al.*, 2002)) and  $\beta$ -Proteobacteria (*Burkholderia cepacia* (Aguilar *et al.*, 2003) and *Chromobacterium violaceum* (Chernin *et al.*, 1998)). The prevalence of QS-controlled secretion

of enzymes among pathogens (von Bodman *et al.*, 2003) and the prevalence of pathogens in soil (Berg *et al.*, 2005) suggests that bacterial QS may also be important in the context of soil N cycling.

Because roots affect large changes in soil bacterial growth and activity, and because of the prevalence of QS among bacteria, we set out to investigate QS in the rhizosphere. Growth of the California naturalized exotic *Avena fatua* (wild oat) in microcosms permits dissection of distinct root zones from bulk soil. We ask whether increased cell density in the rhizosphere enables expression of QS-controlled extracellular enzymes, releasing low molecular weight organic N as a result of soil polymer decomposition. We measured activities of the N-cycle enzymes chitinase and protease, as well as soil DON pools that may serve as intermediate substrates during the conversion of complex organic N to low-molecular weight, labile organic N. We also determined patterns of AHL concentrations and bacterial densities in zones of rhizosphere soil. In order to learn if bacteria in the rhizosphere are capable of producing QS-regulated extracellular chitinases and proteases, an extensive collection of bacteria isolated from field *Avena* rhizosphere soil was screened. The results reported here provide a spatial analysis of QS and N cycling in rhizosphere soil as well as incidence of QS-controlled extracellular enzyme activity in rhizosphere bacteria.

## **Methods**

### *Preparation of soils, plants and microcosms*

Soils were collected during the spring growing season from zones of the annual graminoid *Avena* at the University of California Hopland Research and Extension Center (Hopland, California). This soil is a medium-texture loam derived from hard sandstone and shale, classified as an ultic haploxeralf (Waldrop & Firestone, 2006). Soils were collected to a

depth of 10 cm, and upon transport to the lab were sieved to 2 mm and homogenized. Field moist water content and water holding capacity were determined, and soils were hydrated to 75% water holding capacity prior to packing into microcosms and planting.

Studies were conducted in microcosms planted with *Avena fatua* (Valley Seed Service, Fresno, CA). Seeds were germinated under a slow drip in darkness prior to planting. Microcosms (Figure 1) were composed and employed as previously described (Jaeger *et al.*, 1999). Plants were watered to water holding capacity with tap water every other day, and kept in the greenhouse under ambient light conditions. After 8 weeks of growth, the experimental chambers were filled, and soil harvested 7 days later. Bulk soil was excised from intact microcosms at least 4 mm away from any roots, while soils within 2 mm of the root were considered rhizosphere soil; root tip rhizosphere soil was 0-4 cm from the root tip, root hair rhizosphere soil was 4-8 cm from the root tip, and mature root rhizosphere soil was 8-16 cm from the root tip.

#### *Determination of total numbers of bacteria in soil*

Serial dilutions of soil were made in phosphate-buffered saline and sonicated; counts were made for 3 dilutions. Bacterial numbers were determined using the two-component stain BacLight Bacterial Viability Kit (Molecular Probes Inc., Eugene OR). Extractions were stained and viewed within 48 hours of sampling. Images were obtained using epifluorescence microscopy on a Leica DMRX fluorescence microscope (Leica Microsystems, Bannockburn, IL) with a 630x fluorescent oil-immersion lens. Three dilutions were prepared per sample and ten fields of view were counted per slide. Counts of live cells and dead cells were tabulated separately and total cells calculated by addition.

*Soil enzyme specific activity*

Soil enzyme activity was determined by adding 100  $\mu\text{l}$  of soil slurry (1 g soil in 100 ml 5 mM bicarbonate buffer) to wells of a 96 well plate containing 100  $\mu\text{l}$  of the substrate N-acetyl glucosamine-MUB (NAG-MUB). The fluorescence of each well was determined immediately after substrate addition and again after a 4 hour incubation at 27°C using a Fluorolog-3 spectrofluorometer (Horiba Jobin Yvon Inc., Edison NJ) with 365 nm excitation and 442 nm emission wavelengths. Rates of substrate degradation were calculated as difference in moles of MUB produced over time based on MUB standards. Enzyme activity for each treatment or replicate were normalized for the total number of cells to yield enzyme specific activity, reported as  $\text{pmol MUB h}^{-1} (10^9 \text{ cell})^{-1}$ .

Protease activity was measured using a modified ninhydrin assay (Rosen, 1957; Ladd & Butler, 1972). Because these incubations were of short duration, toluene was not used (Ladd & Butler, 1972). Briefly, 1% casein was added to soil samples and incubated at 100 rpm at 37°C. At each of two time points four hours apart, 1 ml aliquots of soil suspension were dispensed and cooled; supernatant was then recovered by centrifugation for 5 minutes at 16,200 x g. Color reagent (1:1 acetate-cyanide buffer to ninhydrin in DMSO) was added and incubated for 30 minutes at 60°C before adding 160  $\mu\text{l}$  of 50% isopropanol and determining absorbance at 570nm. Protease activity was calculated as the amount of casein converted to glycine based on amino acid standards. The specific protease activity was calculated by normalizing enzyme activity for total bacterial cells per sample and expressed as  $\text{pmol glycine (gln) h}^{-1} (10^9 \text{ cell})^{-1}$ .

### *Detection of AHLs using a biosensor*

Rhizosphere AHL availability was tested on plants of *Avena fatua* using the whole-cell biosensor *A. tumefaciens* pAHL-Ice (DeAngelis *et al.*, 2007). This whole-cell biosensor exhibits linearly increasing ice nucleation activity with increasing AHL in the range of  $10^{-8}$  to  $10^{-2}$  M for a variety of AHLs. Biosensor cells were grown overnight as a liquid culture in M9 minimal media with 0.4% glucose. Cells were washed once and resuspended in phosphate buffer to a concentration of  $10^{10}$  cells  $\text{ml}^{-1}$ . Soils were sprayed with biosensor suspension ( $0.5 \text{ ml g}^{-1}$  soil), corresponding to  $5 \times 10^{10}$  cells  $\text{g}^{-1}$  soil. Biosensor cells were recovered by macerating in phosphate buffer with 0.01% Tween-20. The number of ice nuclei per sample was estimated by a droplet freezing assay as described previously (Loper & Lindow, 1996) and were normalized for the number of biosensor cells determined by plating serial dilutions onto selective media.

### *Dissolved organic nitrogen analysis*

Total and low-MW dissolved organic nitrogen (DON) were measured using a persulfate digestion method. About 1 g soil per sample was taken from the rhizosphere near root hairs, as well as from bulk soil at least 4 mm from any roots. After recording the soil weights, soils were extracted with 50 mM  $\text{K}_2\text{SO}_4$  for 1 hour with shaking. Half of the filtrate was separated using Centriprep3 (Amicon Millipore) with a molecular weight cutoff of 3,000 Nominal Molecular Weight Limit (NMWL, equivalent to Daltons), yielding the low-MW DON samples. Once separated, each fraction was digested by persulfate oxidation to convert organic N to inorganic N. Digest controls consisted of 10 ppm alanine and MW separation controls were 10 ppm alanine, (molecular weight of 89.1 Da), plus 10 ppm bovine serum albumin (66,627 Da). Raw DON values were obtained by correcting for reagent blanks, mass loss, percent recovery, and starting concentrations of inorganic nitrogen. Soil DON and low-MW DON values were

obtained by subtracting undigested from digested inorganic nitrogen (ammonium plus nitrate plus nitrite) measured colorimetrically by flow-injection analysis (Lachat QC8000 flow injection analyzer, Lachat Instruments, Milwaukee, WI).

### *Isolation and physiological characterization of rhizosphere bacteria*

For culturing bacteria from the rhizosphere, 10 mM MES pH 5.5 soil suspensions were sonicated for 5 minutes and serial dilutions were then plated onto non-selective undefined and defined minimal media. Undefined media plates consisted of 10%, 1%, or 0.1% tryptic soy agar (TSA) or soil extract agar made by filtering autoclaved field soil. VL55 defined minimal media (Sait *et al.*, 2002; Joseph *et al.*, 2003) was combined with trace element solutions (Widdel, 1983; Tschech & Pfennig, 1984), vitamin solution (Janssen *et al.*, 2002), and one-twentieth dilutions of 5% carbon source (chitin, casein, xylan, pectin, soluble starch, or sodium acetate). Incubation times were extended for up to ten weeks so that slow-growing colonies could be isolated. Single colonies were re-streaked twice or until isolation was apparent. Pasteurization at 80°C for 10 minutes was employed to eliminate spore formers from further characterization. Positive pasteurization controls were *Bacillus atrophaeus*, *B. cereus*, and *B. thuringensis*, and negative pasteurization controls were *Erwinia herbicola*, *Serratia marsescens*, and *Pseudomonas aeruginosa*.

To test for extracellular chitinase or protease production, isolates were spotted onto overlay plates. The lower layer consisted of VL55 agar with no added C and 10 mg l<sup>-1</sup> cycloheximide; the upper layer consisted of colloidal chitin or hydrolyzed casein in 2% agar (Hsu & Lockwood, 1975). Plates were monitored for 10 days for zones of clearing indicative of extracellular enzyme activity. Chitinase activity was also tested fluorogenically (as above) on cell suspensions at 1.0 optical density 600 nm in 10 mM KPO<sub>4</sub> buffer. Isolates were tested for

AHL production using the whole-cell biosensors *A. tumefaciens* NT1 (ptraG:lacZ) (Cha *et al.*, 1998) and *C. violaceum* CVOblu (McClellan *et al.*, 1997). Isolates that activated one of the biosensors were screened further by extracting AHLs from late-log phase broth cultures with ethyl acetate, concentrating by evaporation to dryness, then resuspending in 20  $\mu$ l ethyl acetate and applying to TLC plates where agar overlays of bioindicator strains were performed in the same fashion.

Isolates were identified by sequencing PCR-amplified 16S ribosomal DNA using the primers 8F and 1492R (Wilson, 1990). Reactions were performed in a 50  $\mu$ l volume using Takara ExTaq as directed, with 3  $\mu$ M of each primer, 50  $\mu$ g BSA, and 2 units of DNA polymerase (Takara Mirus Bio Inc., Madison WI) for 25 cycles using 55°C annealing temperature. Sequencing was done using an Applied Biosystems GeneAmp 9700 sequencer. DNASTAR (DNASTAR, Inc., Madison WI) was used to assemble the contig, and chimera check was used on the Ribosomal Database Project II (Cole *et al.*, 2005) to ensure the integrity of the sequences. NCBI accession numbers are EU723088-EU723189 for 102 sequenced enzyme-producing isolates that were able to be sequenced and assembled into a phylogenetic tree (DeAngelis, 2006).

To prevent AHL accumulation in isolates, the plasmid pME6863 was introduced into isolates by electroporation. This plasmid harbors *aiiA* from *Bacillus* sp. A24 under the constitutive promoter  $P_{lac}$ ; *aiiA* encodes a lactonase capable of degrading a variety of AHLs including C4 and oxoC12-HSL (Reimann *et al.*, 2002), oxoC6, oxoC8 and oxoC10-HSL (Dong *et al.*, 2001), C6, C8 and C10-HSL (Ulrich *et al.*, 2004). Electroporation buffers were either 1 mM HEPES pH7.0, 1 mM MgCl<sub>2</sub>, and 175 mM sucrose; or 10% glycerol; or water. About 100 ng of plasmid was introduced to concentrated cells on ice in a 0.1cm cuvette, and 1.0-2.0 V, 25

$\mu$ FD, and 200 Ohms was then applied using a BioRad Gene Pulser (BioRad, Hercules CA). Positive transformants were verified by PCR. Degradation of AHLs was verified by ethyl acetate extraction of the cultures and use of the appropriate whole-cell biosensors, as above.

### *Statistical analysis*

Descriptive statistics were performed using JMPIN (SAS Institute, Inc., Cary NC). All analyses are based on four biological replicates, which correspond to individual microcosms containing 2-3 *Avena* plants each. Differences between root zones (bulk soil, root tip, root hairs, mature root) were calculated using oneway (unpaired) analysis of variance; p-values reported are for ANOVA with 3 degrees of freedom. The Tukey-Kramer HSD test was applied to rank the mean differences; lowercase letters indicate means that are not significantly different using a cutoff of 0.05. Pools of DON and low-MW DON were compared from rhizosphere (root hairs only) and bulk soil using a paired t-test. The distribution of cube-transformed, corrected DON measurements for all samples was normal based on the Shapiro-Wilk Test ( $p < 0.0001$ ).

## **Results**

### *Enhanced enzyme activity, bacterial density, and AHL signaling in rhizosphere soil.*

There were about ten times more total bacteria in active root zones than in bulk soil, while counts in soil adjacent to the mature root were intermediate to those in bulk soil and the active root zones (Figure 2A,  $p < 0.0001$ ). Similarly, we detected a ten-fold increase in AHL biosensor activity in the rhizosphere compared to the bulk soil (Figure 2B,  $p < 0.05$ ), by the whole-cell *A. tumefaciens* (pAHL-Ice) (DeAngelis *et al.*, 2007).

The specific activities (enzyme activity per cell) of chitinase and protease were generally higher in rhizosphere root zones compared to bulk soil. Patterns of enzyme activity were

different for the two enzymes. While chitinase specific activities were higher in the soil adjacent to the root tip and root hairs zones compared to bulk soil (Figure 2C,  $p < 0.0001$ ), protease activity per cell peaked at the root tip compared to adjacent bulk soil, (Figure 2D,  $p < 0.05$ ).

*More total and low-MW dissolved organic N in rhizosphere than bulk soil.*

Low-MW DON is defined here as DON smaller than 3 kDa, roughly corresponding to chitin polymers of 6 or fewer N-acetyl glucosamines, or to peptide chains with 10 or fewer amino acids. About twice as much total DON was found in the rhizosphere than in bulk soil (Figure 3,  $p < 0.05$ ). Low-MW DON was found to occur at levels indistinguishable from zero in the bulk soil, but comprised about 15% of the total DON in rhizosphere soil ( $p < 0.05$ )

*Isolation of microorganisms with QS-controlled exoenzyme production*

Bacteria were isolated and their phenotypes characterized to determine the frequency of extracellular enzyme production by rhizosphere bacteria and to what extent bacterial extracellular enzymes are under AHL-dependent QS control. After discarding colonies that produced aerial hyphae, we screened 533 bacterial isolates, of which 347 (65%) succumbed to pasteurization. The heat-sensitive strains were further tested for extracellular enzyme and AHL production. Extracellular enzyme activity (chitinase, protease, or both) was observed in 136 (39%) of the non-spore-forming isolates (Table 1). Of the chitinase producers, only 5 were originally isolated on plates with chitin as a sole carbon source, while of the protease producers 14 were originally isolated on plates with only casein. Only 128 of the 136 exoenzyme-producing isolates were able to be tested for AHL production; the remaining isolates were not amenable to liquid culture growth and/or ethyl acetate extraction of AHL. Of the 128 isolates tested, 30 (24%) produced AHLs detectable by our AHL biosensors (Table 1).

To assess the relationship between exoenzyme production and AHL accumulation in AHL-producing strains, we expressed a lactonase by transformation of *aiiD* into each isolate. Of the 30 strains demonstrated to produce AHL, we were able to successfully transform eight isolates with *aiiD* and quench AHL production (Table 2). Chitinase production was suppressed in 4 of the 6 chitinase-producing strains in which AHL accumulation was quenched. Protease activity was compromised in three *Burkholderias* in which lactonase was expressed, while that of *Caulobacter henrickii* sp. kmd\_084 was unaffected (Table 2). In some strains other traits such as pigment production, exopolysaccharide (EPS) production (manifested as shiny or gummy colonies), and biofilm formation (manifested as rings around culture tubes at the liquid-air interface) were incidentally observed to be dependent on AHL accumulation; we report these observations under the column “other” (Table 2) as these traits could possibly play a role in soil growth and survival.

## **Discussion**

Natural environments such as soil are not closed systems, so bacterial QS must depend on microscopic soil structure and biochemistry that would limit diffusion of signal molecules out of the microbial habitat (Redfield, 2002). Bacteria capable of QS typically produce a constant, low amount of QS signal, and a local threshold concentration of signal must accumulate before downstream genes are expressed. In this context, QS would function as a mechanism of microbial economics, enabling bacterial populations to limit production of extracellular enzymes until conditions were conducive to recovery of enzyme products, thus balancing the cost of extracellular enzyme production against the return of product (Koch, 1985). Economic models of microbial metabolism predict that enzyme production increases when simple nutrients are scarce and complex nutrients are abundant (Felse & Panda, 2000; Li & Roseman, 2004); this prediction

is supported by soil studies (Asmar *et al.*, 1994; Allison & Vitousek, 2005). A transcriptionally-based control mechanism such as QS would enable rapid up-regulation of enzyme production to mediate the quick changes in N cycling dynamics associated with conversion of bulk soil to rhizosphere soil; highly responsive changes in extracellular enzyme production are consistent with extracellular enzyme activity as the rate-limiting step in rhizosphere N mineralization (Schimel & Bennett, 2004). Our data suggest that further research is necessary to investigate the link between bacterial QS and rhizosphere N cycling.

Higher rhizosphere bacterial populations were associated with higher AHL levels near roots, suggesting increased density in soil spaces that permit the accumulation of QS signal (Figure 2A, B). Extracellular enzyme activity also increased (Figure 2C, D); if extracellular enzyme activity was constitutive and invariant relative to cell density, an increase in cell numbers should have produced only a proportional increase in enzyme activity per gram soil, and not one greater than the increase in cell numbers. A conceptual model of QS-controlled N cycling in the rhizosphere (Figure 4) can be distilled from our rhizosphere observations of AHL accumulation, extracellular enzyme activities involved in N cycling (Figure 2), and total and low-MW DON (Figure 3); units have been standardized for comparison. Combined chitinase and protease enzyme activities measured in this study could depolymerize  $13 \text{ ng N g}^{-1} \text{ soil h}^{-1}$ , a rate that accounts for a third of previous estimates of gross N mineralization ( $38 \text{ ng N g}^{-1} \text{ soil h}^{-1}$ ) measured in the same soils (Herman *et al.*, 2006). This suggests that we have captured a significant portion of N-cycling extracellular enzyme activity in the rhizosphere. The low-MW organic N pool in the rhizosphere measured here is  $31 \text{ ng N g}^{-1} \text{ soil}$ , compared to  $250 \text{ ng N g}^{-1} \text{ soil}$  of total DON. While measures of extracellular enzyme activity represent potentials only, and activities may vary with time (Waldrop *et al.*, 2000), the combined rate of depolymerization is consistent with the measured amount of low-MW DON, and suggests that this pool turns over

many times per day. These estimates agree well with other measures of N cycling (Herman *et al.*, 2006; Waldrop & Firestone, 2006), and recapitulate the importance of extracellular enzyme activity in rhizosphere N cycling.

In soil it is likely that chitinases and proteases originate from a variety of fungi and bacteria other than AHL-producing proteobacteria (Hodge, 1995; Paul & Clark, 1996); plants are also a potential source, though this is less-well documented. In our study, soil was sieved and homogenized before being packed into microcosms, likely reducing soil fungal biomass substantially, as fungi are sensitive to such disturbance (Balsler & Firestone, 2005). Because the maximum period for root growth into the experimental soil was only about 8 days, the increases in specific enzyme activities were most likely due to fast-growing bacteria.

Plant root exudates may also contribute to DON in the rhizosphere, though these pools are transient in the N budget; with actively growing plants, the net flow of nitrogen will always be from soil N to plant tissue. Previous work in our labs using a tryptophan-responsive whole-cell biosensor detected increased tryptophan in rhizosphere compared to bulk soil, with significantly more in older rhizosphere root zones and sites of lateral root emergence (Jaeger *et al.*, 1999). Amino acids comprise a component of root exudates, as can some enzymes. While root-derived amino acids could potentially fuel enhanced N mineralization in rhizosphere soil, depolymerization of macromolecular soil N must occur to provide a net N-gain to the plant.

The physiology of the root yields distinct root zone effects (Watt *et al.*, 2006), which is reflected in our data (Figure 2C, D). At the root tip, root border cells are the primary line of plant defense against pathogens, and may secrete substances to inhibit microbial activity (Farrar *et al.*, 2003). Jaeger and colleagues found the root tip to have the highest sucrose availability, decreasing as the root matured, though all root zones had higher sucrose than bulk soil; this study also used *Avena* planted to Hopland soil in the same microcosms, and so is highly analogous

(Jaeger *et al.*, 1999). Bringhurst and colleagues did not detect galactosides in the root tip zone, though they examined the legume *Medicago trunculata* in different soils (Bringhurst *et al.*, 2001). As the primary site of plant exchange with soil, the root hair zone is characterized by elevated root exudation of sugars and amino acids (Jaeger *et al.*, 1999; Bringhurst *et al.*, 2001), highest plant nitrate uptake (DeAngelis *et al.*, 2005), and highest bacterial cell numbers, extracellular chitinase activity, and AHL availability (Figure 2B). The mature root zone does not exude as much labile C, potentially causing C-limitation in the dense bacterial population that grew when the root was younger. The distinct chemical (AHL levels) and biological (specific enzyme activities) profiles of the root zones illustrate the dynamic temporal nature of the rhizosphere effect in soil microbial community function.

Since AHL abundance and total bacterial population sizes were similarly increased in the active rhizosphere root zones compared to mature root and bulk soil, AHL-producing cells are apparently among those responding to the root environment. There could potentially be bias in the rhizosphere-stimulated bacterial cell growth towards AHL- or extracellular enzyme-producing organisms. However, a high density microarray analysis of this rhizosphere soil community accompanied the work that we report here (DeAngelis, 2006), and showed that about 7% of the soil bacterial community responded to the presence of the root and included a diversity of taxa including substantial components of Proteobacteria, Actinobacteria, and Firmicutes (DeAngelis, 2006). Actinobacteria comprise a group of soil bacteria commonly associated with chitin utilization, while Proteobacteria and Firmicutes are more typically considered fast growers (Paul and Clark, 1996). The abundance and diversity of previously unknown AHL-producing taxa found in our study, especially  $\alpha$ -Proteobacteria, suggests a complex network of QS control of soil populations that is unexplored.

While our use of a culturing approach introduced bias in the types of organisms tested for AHL production and extracellular enzyme activity, there is still much value in the ability to examine the phenotypes of bacteria (extracellular enzymes controlled by QS). In addition, there exists a diversity of non-AHL QS signal mechanisms utilized within Proteobacteria and other Gram-negative bacteria, Gram-positive bacteria, and Actinomycetes (Miller & Bassler, 2001, Loh *et al.*, 2002), as well as diverse organisms that produce N cycling enzymes (Waldrop & Firestone, 2006). Extracellular enzyme activity was compromised in all but one isolates in which AHL accumulation was disrupted by AHL lactonase, suggesting that density-dependent control could be a common mechanism of regulating exoenzyme production among bacteria in soil.

Our QS-controlled extracellular enzyme-producing isolates were comprised of diverse taxa. The  $\alpha$ -Proteobacterial isolates (*Inquilinus* spp., *Rhizobium* spp., and *Agrobacterium* sp.) represent the first members of the class  $\alpha$ -Proteobacteria reported to have QS-controlled exoenzyme activity, as evidenced by AHL lactonase-blocked exoenzyme production (Table 2). Though the *Caulobacteria* sp. extracellular enzyme activity was not affected by lactonase, this is the first member of the order Caulobacteriales known to exhibit any QS-controlled behavior. QS has not been considered to be prominent in the  $\alpha$ -Proteobacteria as a group, but our results suggest that this behavior could be much more common among diverse  $\alpha$ -Proteobacteria than currently appreciated. The Burkholderiales are well known for having vast phenotypic diversity within a well-conserved 16S rRNA group (Coenye *et al.*, 2003), and this was confirmed in our studies (Table 2). In a separate study of this genus, 20 out of 44 *Burkholderia* isolates produced proteases, and all but 2 of these produced proteases in an AHL-dependent manner (Wopperer *et al.*, 2006). Burkholderias are prevalent in many rhizosphere soils, and also known for the diversity of QS-controlled extracellular enzymes they produce (Berg *et al.*, 2005). One isolate, *A.*

*rhizogenes* sp. kmd\_046, displayed increased chitinase activity after AHL quenching (Table 2), which may be related to the fact that Nod factors, which mediate early stages of interactions with plants, are lipo-chitin oligosaccharides (Spaink, 2000) and thus destruction of density-dependent signals may interfere with symbiotic association genes.

Clearly factors other than quorum sensing control N-cycling exoenzyme activity, including substrate availability, feedback mechanisms, and community composition. However, as a global regulator of cellular- and population-level gene expression, density-dependent quorum sensing provides an interesting potential control point for enzymes mediating organic N depolymerization and rhizosphere N mineralization.

### *Broader Implications*

Because of the well-known link between QS and plant disease (von Bodman & Farrand, 1995; von Bodman *et al.*, 2003) there is desire to target QS as a means of disease control (Dong *et al.*, 2001). Our results indicate that bacterial density-dependent behaviors might play a role in regulating soil N-cycling and plant N nutrition. Disruption of QS for disease control should consider the resultant effects on soil N cycling that might result from altering QS in native soil inhabitants.

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## Tables

Table 1. Iterative screening of bacterial rhizosphere isolates

screen	Number of isolates	Percent (%)	
round 1 – pasteurization at 80°C for 10 minutes of all initial isolates			
	533	-	... subjected to screen
	347	65.1	... succumbed to pasteurization
round 2 – extracellular enzyme activity of pasteurization-sensitive isolates			
	347	-	
	59	17.0	... extracellular protease activity only
	49	14.1	... extracellular chitinase activity only
	28	8.0	... both extracellular chitinase and protease activity
	136	39.2	... total extracellular enzyme-producing isolates
round 3 – QS signal AHL production of exoenzyme producing isolates			
	128 <sup>a</sup>	-	
	21	16.4	... AHL detected by <i>A. tumefaciens</i> ptraG:lacZ only
	8	6.3	... AHL detected by <i>C. violaceum</i> assays
	1	0.7	... AHL detected by both biosensors
	30	23.4	... total AHL signal-producing isolates

<sup>a</sup>Acyl-homoserine lactone, the protobacterial-specific quorum sensing signal molecules.

<sup>b</sup>Because not all isolates were amenable to liquid cultivation and/or ethyl acetate extraction necessary for this round of screening, we only tested 128 of the 136 total exoenzyme producing isolates for AHL production.

Table 2. Physiology of isolates with quenched quorum sensing via lactonase plasmid

	Protease <sup>a</sup>	Chitinase <sup>a</sup>	other
<i>Agrobacterium rhizogenes</i> sp. kmd_046	<i>n/a</i> <sup>b</sup>	↑	<i>n/a</i>
<i>Burkholderia</i> sp. kmd_093	↓	↔	<i>n/a</i>
<i>Burkholderia</i> sp. kmd_134	↓	↓↓	<i>n/a</i>
<i>Burkholderia</i> sp. kmd_085	↓	<i>n/a</i>	yellow pigment
<i>Caulobacter henricii</i> sp. kmd_084	↔	<i>n/a</i>	EPS, biofilm <sup>c</sup>
<i>Inquilinus ginsengisoli</i> sp. kmd_067	<i>n/a</i>	↓	EPS
<i>Inquilinus ginsengisoli</i> sp. kmd_475	<i>n/a</i>	↓↓	biofilm
<i>Rhizobium etli</i> sp. kmd_483	<i>n/a</i>	↓↓	biofilm

<sup>a</sup>Arrows denote elimination (↓↓), reduction (↓), increase (↑) or no change (↔) of exoenzyme activity measured by zones of clearing on substrate plates when lactonase plasmid was introduced; see Methods for details.

<sup>b</sup>The designation *n/a* in this column indicates that the trait was not present in the wildtype isolate, so the effect of quenching by lactonase is irrelevant.

<sup>c</sup>EPS manifested as shiny or gummy colony morphology compared to dry and wrinkly in the wildtype; Biofilm formation was observed as rings forming at the liquid-air interface at the sides of flasks or tubes during culture growth.

## Figure Legends

Figure 1. (A) Microcosms used in this study are constructed of plexiglass with dimensions 15 cm by 5 cm by 40 cm. Sieved (2 mm) homogenized soil is packed into the main chamber of the microcosms, and plants are grown from seedlings to maturity in the main compartment as shown. (B) After 6-8 weeks of growth, the microcosms are tipped to a 45° angle, and the solid divider separating the main chamber from the experimental chamber is replaced with a slotted divider, so that the roots will grow along the outside face of the microcosm. (C) A photograph of the microcosm shows the experimental chamber, with the roots visible; white lines denote the four soil types sampled: root tip, root hairs, mature root rhizosphere soil and bulk soil.

Figure 2. Functional characterization of three *A. fatua* rhizosphere root zones (root tip, mature root, and root hairs) compared to bulk soil. We used two independent measures of cell density, (A) total cell counts and (B) QS signal abundance using the whole-cell biosensor *A. tumefaciens* (pAHL-Ice). (C) Chitinase and (D) protease specific activities are reported as substrate converted g<sup>-1</sup> soil and converted to cell<sup>-1</sup> using total cell counts. Lowercase letters indicate means that are not significantly different by the Tukey-Kramer HSD test using a cutoff of 0.05. Error bars represent one standard error from the mean for four biological replicates equal to four microcosms.

Figure 3. (A) Total dissolved organic nitrogen (DON) was increased in the rhizosphere compared to bulk soil (paired t-test, p=0.0261). (B) Low-MW DON (paired t-test, p=0.0385) was not significantly different from zero in the bulk soil, while low-MW DON comprised almost 15% of

the total rhizosphere DON. Error bars are one standard error from the mean for 4 biological replicates.

Figure 4. A hypothetical model of rates and pools of N mineralization in the rhizosphere of *Avena fatua*. The possible effect of QS is shown based on calculated rates and pools of N cycling. Rate of extracellular enzyme activity is the sum of chitinase plus protease activities measured in rhizosphere soil as an average of activity measured in root tip and root hair rhizosphere zones. Gross N mineralization and  $\text{NH}_4^+$  pool from a previous study (Herman *et al.*, 2006). A schematic of the root (not to scale) displays the two extremes of hypothetical bacterial biogeography in the rhizosphere, where bulk soil bacteria exist as single cells (left of root) or primarily as microcolonies (right of root). Units in pools and rates have been converted to  $\mu\text{g N g}^{-1}$  dry soil and  $\mu\text{g N g}^{-1}$  dry soil  $\text{h}^{-1}$ , respectively.