Two Novel Bacterial Biosensors for Detection of Nitrate Availability in the Rhizosphere

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Kristen M. DeAngelis,† Pingsheng Ji,‡∥ Mary K. Firestone, and Steven E. Lindow*  
Department of Plant and Microbial Biology and Department of Environmental Science Policy and Management, University of California, Berkeley, California 94720

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The nitrate-regulated promoter of narG in Escherichia coli was fused to promoterless ice nucleation (inaZ) and green fluorescent protein (GFP) reporter genes to yield the nitrate-responsive gene fusions in plasmids pNice and pNgfp, respectively. While the promoter of narG is normally nitrate responsive only under anaerobic conditions, the L28H-fnr gene was provided in trans to enable nitrate-dependent expression of these reporter gene fusions even under aerobic conditions in both E. coli DH5α and Enterobacter cloacae EcCT501R. E. cloacae and E. coli cells containing the fusion plasmid pNice exhibited more than 100-fold-higher ice nucleation activity in cultures amended with 10 mM sodium nitrate than in nitrate-free media. The GFP fluorescence of E. cloacae cells harboring pNgfp was uniform at a given concentration of nitrate and increased about 1,000-fold when nitrate increased from 0 to 1 mM. Measurable induction of ice nucleation in E. cloacae EcCT501R harboring pNice occurred at nitrate concentrations of as low as 0.1 μM, while GFP fluorescence was detected in cells harboring pNgfp at about 10 μM. In the rhizosphere of wild oat (Avena fatua), the whole-cell bioreporter E. cloacae(pNgfp) or E. cloacae(pNice) expressed significantly higher GFP fluorescence or ice nucleation activity when the plants were grown in natural soils amended with nitrate than in unamended natural soils. Significantly lower nitrate abundance was detected by the E. cloacae(pNgfp) reporter in the A. fatua rhizosphere compared to in bulk soil, indicating plant competition for nitrate. Ice- and GFP-based bacterial sensors thus are useful for estimating nitrate availability in relevant microbial niches in natural environments.

A major part of the nitrogen cycle results from interactions between plants and soil microorganisms in the rhizosphere. As a critical compound in many transfers and transformations of nitrogen, nitrate is a labile, dynamic pool in rhizosphere N cycling. Nitrate is a significant source of plant N, and it can also be incorporated by a variety of microorganisms through assimilatory nitrate reduction (15) and is subject to denitrification. Nitrate cycling is a critical component in many transfers and transformations of N between plants and soil microorganisms in the rhizosphere. As a result, it cannot be accurately estimated by conventional means. Therefore, complementary methods with improved spatial resolution for the analysis of nitrate ion are desirable.

We report here the development of two whole-cell bacterial biosensors that are based on Enterobacter cloacae harboring nitrate-responsive reporter genes. These biosensors differ in their sensitivity and ease of application to soil systems. An ice nucleation reporter gene, inaZ, from the bacterium Pseudomonas syringae (17, 29) and a stable green fluorescent protein (GFP) reporter gene (36) were placed under the regulatory control of the promoter of Escherichia coli nitrate reductase gene (narG), which confers the reduction of the electron acceptor nitrate to nitrite. Since expression of nitrate reductase in E. coli occurs only under anaerobic conditions due to its secondary regulation by the transcriptional regulator Fnr, we provided cells with an altered fnr gene (22, 23) that enabled nitrate-inducible transcription by the narG promoter in the presence of oxygen. We provide evidence that both GFP and inaZ whole-cell bio-

* Corresponding author. Present address: Department of Plant and Microbial Biology, 111 Koshland Hall, University of California, Berkeley, CA 94720-3102. Phone: (510) 642-4174. Fax: (510) 642-4995. E-mail: icelab@socrates.berkeley.edu.
† Kristen DeAngelis and Pingsheng Ji contributed equally to this work.
‡ Present address: North Florida Research and Education Center, University of Florida, Quincy, FL 32351.
sensors are highly responsive to nitrate in culture and in the rhizosphere and that the population-level estimates of nitrate availability provided by the ice nucleation-based biosensors are complementary to the estimates of nitrate availability at the single-cell level provided by the GFP-based biosensor.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The bacterial strains and plasmids used are described in Table 1. Erwinia herbicola (Pantoea agglomerans) strain 299R is a spontaneous rifampin-resistant mutant of a culture originally isolated from pear leaves (3, 4). Enterobacter cloacae (EcCT501 R) is a spontaneous rifampin-resistant mutant of strain 299R (3, 4).

Preparation of genomic DNA. For routine studies, bacterial cells were grown in Luria-Bertani (LB) medium (44) at 37°C for E. coli or 28°C for Erwinia and Enterobacter strains except as otherwise stated. When cells were prepared for fluorescence measurements in culture or in the rhizosphere, cultures were grown aerobically in M9 minimal medium without shaking to deplete oxygen. Determination of ice nucleation activity of bacterial cultures was taken at different time intervals, and ice nucleation activity was analyzed by electrophoresis through a 2% agarose gel (Gibco BRL) and visualized after staining with ethidium bromide on a UV transilluminator.

For assessment of nitrate-regulated ice nucleation activity of E. herbicola 299R and E. coli DH5a containing pNARG-Ice, bacterial cultures grown overnight in LB broth amended with 50 μg/ml of kanamycin (LB-kan) were used to inoculate 250-ml flasks containing 50 ml of LB-kan or 250-ml bottles filled with LB-kan at a final density of approximately 10^6 CFU ml^-1. The LB medium was amended with or without 10 mM sodium nitrate or ammonium sulfate, and the flasks or bottles were incubated at 26°C with or without shaking (250 rpm). Samples of bacterial cultures were taken at different time intervals, and ice nucleation activity was measured by a droplet freezing assay of serial dilutions of the strains for routine studies, bacterial cells were grown in Luria-Bertani (LB) medium (44) at 37°C for E. coli or 28°C for Erwinia and Enterobacter strains except as otherwise stated. When cells were prepared for fluorescence measurements in culture or in the rhizosphere, cultures were grown aerobically in M9 minimal medium without shaking to deplete oxygen. Determination of ice nucleation activity of bacterial cultures was taken at different time intervals, and ice nucleation activity was analyzed by electrophoresis through a 2% agarose gel (Gibco BRL) and visualized after staining with ethidium bromide on a UV transilluminator.

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**TABLE 1. Bacterial strains and plasmids used in this study**

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Description</th>
<th>Reference(s) or source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Escherichia coli</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DH5a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DH5aR</td>
<td>Spontaneous Rif&lt;sup&gt;R&lt;/sup&gt; mutant of DH5a</td>
<td>This study</td>
</tr>
<tr>
<td>K-12 MG1655</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Erwinia herbicola</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>299R</td>
<td>Spontaneous Rif&lt;sup&gt;R&lt;/sup&gt; mutant of strain 299</td>
<td>3, 4</td>
</tr>
<tr>
<td><em>Enterobacter cloacae</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EcCT501</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EcCT501R</td>
<td>Spontaneous Rif&lt;sup&gt;R&lt;/sup&gt; mutant of EcCT501</td>
<td>This study</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pRK2073</td>
<td>Derivative of pRK2013 with a Tn7 insertion in the Km&lt;sup&gt;R&lt;/sup&gt; gene; Sm&lt;sup&gt;R&lt;/sup&gt;, Sp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>12, 15</td>
</tr>
<tr>
<td>pCR2.1-TOPO</td>
<td>PCR cloning vector with covalently bound topoisomerase I enzyme; Km&lt;sup&gt;R&lt;/sup&gt;, Ap&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>pPROBE-NI</td>
<td>Broad-host-range plasmid with a transcriptional fusion cassette containing a promoterless inaZ reporter gene; Km&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This lab</td>
</tr>
<tr>
<td>pPROBE-NT</td>
<td>Broad-host-range plasmid with a transcriptional fusion cassette containing a promoterless inaZ reporter gene; Km&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This lab</td>
</tr>
<tr>
<td>pPK434</td>
<td>1.6-kb HindIII-BamHI fragment of L28H-&lt;sup&gt;fnr&lt;/sup&gt;-&lt;sup&gt;inaZ&lt;/sup&gt; gene promoter in pACYC184; Km&lt;sup&gt;R&lt;/sup&gt;</td>
<td>21</td>
</tr>
<tr>
<td>pNARG-TOPO</td>
<td>pCR2.1-TOPO containing a 592-bp HindIII-EcoRI fragment of narG promoter; Km&lt;sup&gt;R&lt;/sup&gt;, Ap&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pSV1-dsRed</td>
<td>Constitutively expressing dsRed behind Kan promoter with pBBR replication; Sp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>B. Quijones</td>
</tr>
<tr>
<td>pNarG-Ice</td>
<td>narG-&lt;sup&gt;fnr&lt;/sup&gt;-&lt;sup&gt;inaZ&lt;/sup&gt; fusion plasmid containing a 1.6-kb HindIII-EcoRI fragment of narG promoter; Km&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pFNOR-TOPO</td>
<td>pCR2.1-TOPO with an inserted BamHI-XbaI fragment containing L28H-&lt;sup&gt;fnr&lt;/sup&gt;-&lt;sup&gt;inaZ&lt;/sup&gt; gene promoter cloned into pPROBE-NI; Km&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pNnice-&lt;sup&gt;fnr&lt;/sup&gt;-&lt;sup&gt;inaZ&lt;/sup&gt;</td>
<td>narG-&lt;sup&gt;fnr&lt;/sup&gt;-&lt;sup&gt;inaZ&lt;/sup&gt; fusion plasmid containing a 1.6-kb HindIII-EcoRI fragment of L28H-&lt;sup&gt;fnr&lt;/sup&gt;-&lt;sup&gt;inaZ&lt;/sup&gt; gene</td>
<td>This study</td>
</tr>
<tr>
<td>pNgfp</td>
<td>narG-&lt;sup&gt;fnr&lt;/sup&gt;-&lt;sup&gt;inaZ&lt;/sup&gt; fusion plasmid containing EcoRI and HindIII fragments of the gfp gene isolated from pPROBE-NT and inserted into pNarG-Ice</td>
<td>This study</td>
</tr>
</tbody>
</table>

<sup>a</sup> Rif<sup>R</sup>, Km<sup>R</sup>, Sm<sup>R</sup>, Ap<sup>R</sup>, and Cm<sup>R</sup>, resistance to rifampin, kanamycin, streptomycin, spectinomycin, ampicillin, and chloramphenicol, respectively.
bacterial cultures as described elsewhere (34). Bacterial population sizes were determined by plating appropriate dilutions of the cultures on LB agar (LA) amended with kanamycin, using a spiral plater (Spiral Systems, Inc., Cincinnati, OH). After 30 h of incubation at 28°C, colonies on the plates were counted, and the population size was used to yield cell-normalized estimates of ice nucleation activity.

(ii) pNice. A 1.6-kb BamHI-XbaI fragment containing the L28H-fnr structural gene in plasmid pPK434 was inserted into plasmid pNARG-TOPO to replace the BamHI-XbaI fragment containing the narG promoter (Fig. 1). The L28H-fnr gene, previously described as LH27-fnr, encodes Fnr with a single amino acid substitution that allows Fnr-dependent gene expression in the presence of oxygen (1, 23). The resulting plasmid contains a HindIII fragment harboring the L28H-fnr gene and was designated pFNR-TOPO. The HindIII fragment in pFNR-TOPO was then cloned immediately 5′/H11032 to the narG promoter in plasmid pNarG-Ice. The resulting plasmid was designated pNice (Fig. 1). pNice was introduced into E. herbicola 299R and E. cloacae EcCT501R by triparental mating as described above. Nitrate-regulated ice nucleation activity of DH5α/H9251, 299R, and EcCT501R containing pNice was assessed in M9 with various concentrations of sodium nitrate by using methods mentioned above.

(iii) pNgfp. The inaZ gene was removed from E. cloacae (pNice) by digestion with EcoRI and NotI (New England Biolabs) and replaced with a GFP reporter gene isolated as an EcoRI and NotI restriction digest fragment from pPROBE-NT (43) to produce pNgfp. This plasmid was transformed into E. coli strain DH5α by electroporation under standard conditions (44). The proper orientation of the cloned fragment was confirmed by restriction mapping of purified plasmids from individual colonies by using enzymes that cut asymmetrically in the plasmid.

(iv) pNgfp and pVS1-dsRed. In order to account for all biosensor bacteria applied to the rhizosphere, we introduced a compatible plasmid harboring a constitutively expressed red fluorescent protein (RFP) marker gene into the E. cloacae (pNgfp) reporter strain. E. cloacae (pNgfp) was electroporated with pVS1-dsRed, which contains the dsRed gene (M. Marco, unpublished data) downstream of a kanamycin promoter from E. coli conferring constitutive transcription in plasmid pVP61 (33) harboring a spectinomycin resistance gene. After electroporation into E. cloacae (pNgfp) by standard methods (44), the transformants were recovered on LA containing spectinomycin. Transformants containing pVS1-dsRed were identified by their pink colony color and were verified to have constitutive RFP expression and nitrate-dependent GFP expression by plating cells onto LA with or without 10 mM NaNO₃, fixing cells, and visualizing them by microscopy as described below.

To quantify nitrate-dependent GFP expression, single colonies of E. cloacae (pNgfp) (pVS1-dsRed) were inoculated into LB medium containing kanamycin and spectinomycin and incubated overnight. This overnight culture was used to seed a 100-ml culture of M9 minimal medium with kanamycin and spectinomycin. Cells were allowed to reach a density of 10⁹ cells ml⁻¹ before sodium nitrate was added, and GFP fluorescence of cells was monitored 2 and 4 h later, as described above.

Preparation of soil and plants. Soils were collected from the Red Hill, under the growing zones of the annual graminoid Avena fatua, 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 (7). Soils were collected to a depth of 10 cm and immediately processed for planting. Soil nitrate concentrations were reported to be 0.47 mg N kg⁻¹ soil at 0- to 10-cm depth during the spring growing season (7).

For testing of in situ performance of the biosensor cells containing pNice, soils were immediately sieved to 2 mm, homogenized, and mixed with fine quartz sand (1:1 by volume) before planting to improve drainage (19). Avena fatua (wild oat) and Phaseolus vulgaris (snap bean, cv. Bush Blue Lake 274) (Valley Seed Service, Fresno, CA) were grown in conical pots (Cone-Tainer Nursery, Canby, OR) that were cut vertically into two parts and reannealed with tape before planting to

FIG. 1. Schematic diagram of the construction of the pNice fusion plasmid containing the L28H-fnr gene. Cm⁰, Km⁰, and Ap⁰, resistance to chloramphenicol, kanamycin, and ampicillin, respectively. narGp indicates a 592-bp HindIII-EcoRI fragment of the narG promoter-regulatory region from the genome of E. coli K-12 strain MG1655.

VOL. 71, 2005 NOVEL BIOSENSORS DETECT NITRATE IN THE RHIZOSPHERE 8539
facilitate access to developing roots. The conical pots with seeds were incubated at room temperature until germination and then transferred to a greenhouse and watered daily with tap water. After about 10 days following germination, plants were moved to a growth chamber, maintaining temperatures of 22 to 25°C with a 12-h photoperiod and approximately 450 μmol photons/m² s⁻¹, and treated with 20 ml of either 10 mM or 0.1 mM sodium nitrate 2 h before inoculation with bacterial sensor strains; control plants received 20 ml of tap water.

Rhizosphere nitrate availability was also tested on plants of Avena fatua by using the E. cloacae pNgfp biosensor. Seeds were rinsed in tap water and pregerminated prior to being planted under a slow drip of tap water for 4 days in darkness. Once germinated, seeds were planted in Hopland field soil as described above. To prepare them for planting, soils were hydrated to 75% water holding capacity, corresponding to 15 ml sterile double-distilled water per 100 g field moist soil. Additional small microcosms were designed to create a rhizosphere environment that could be placed directly under a microscope, measuring 100 by 30 by 4 mm with one face made of a double-wide no. 1 coverslip (Proscitech, Australia). Soils were packed into microcosms and pregerminated seeds placed at the top and covered with a thin layer of soil. The microcosms were then inclined to a 45-degree angle relative to the horizontal, so that the roots would grow along the coverslip face of the microcosm; this setup is analogous to that used by Jaeger et al. (19). After 4 to 5 days of growth in growth chambers (16 h light days, approximately 450 μmol photons/m² s⁻¹, 20% humidity), seedlings were inoculated with bacterial biosensors.

Root inoculation and sampling of whole-cell sensor bacteria. E. cloacae sensor strain EcCT501R(pNice) was grown in M9 minimal medium with kanamycin and spectinomycin. Cells from the 48-h culture were harvested by centrifugation, washed twice with 10 mM sterile potassium phosphate buffer (PPB) (pH 7), and resuspended in PPB at a concentration of about 10⁶ cells ml⁻¹. The GFP fluorescence of individual cells was determined by microscopy. Washed cells were fixed in a solution of three parts 4% paraformaldehyde and one part phosphate-buffered saline. Fixed cells were then mounted on slides and examined by epifluorescence microscopy for GFP and RFP fluorescence.

TABLE 2. Ice nucleation activity of Erwinia herbicola 299R(pNarG-Ice) and Escherichia coli DH5α(pNarG-Ice) in the presence of various nitrogen-containing compounds under anaerobic and aerobic conditions

<table>
<thead>
<tr>
<th>Strainⁿ</th>
<th>Anaerobic</th>
<th>Aerobic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Without NO₃</td>
<td>With NO₃</td>
</tr>
<tr>
<td>299R(pNarG-Ice)</td>
<td>-3.23 ± 0.08</td>
<td>-0.72 ± 0.15</td>
</tr>
<tr>
<td>DH5α(pNarG-Ice)</td>
<td>-3.04 ± 0.06</td>
<td>-1.11 ± 0.12</td>
</tr>
</tbody>
</table>

ⁿ Bacterial cells were grown in Luria broth amended with 10 mM NaNO₃ or (NH₄)₂SO₄ as indicated.

b Ice nucleation activity was measured at −5°C at 12 h after inoculation. Data are the means from three replicate cultures ± standard errors of the means.

c ND, not determined.

RESULTS

Nitrate response of pNarG-Ice fusions. Nitrate-dependent ice nucleation was observed under anaerobic conditions in both E. coli DH5α and E. herbicola 299R harboring a transcriptional fusion of a 592-bp HindIII-EcoRI fragment containing the narG promoter-regulatory region to an inaZ reporter gene on plasmid pNarG-Ice. In LB medium amended with 10 mM sodium nitrate, the ice nucleation activities of E. coli(pNarG-Ice) and E. herbicola(pNarG-Ice) were 85- and 500-fold greater than those in LB medium without nitrate amendment under anaerobic conditions, respectively (Table 2). Both strains expressed ice nucleation activity that increased with increasing nitrate concentration in the range of 0.1 μM to
10 mM under anaerobic conditions (data not shown). Neither E. herbicola 299R nor E. coli DH5α(pNice) expressed detectable ice nucleation activity with or without parental plasmid pPROBE-NI under either anaerobic or aerobic incubation conditions (data not shown). The ice nucleation activity of E. herbicola(pNarG-Ice) or E. coli(pNarG-Ice) was not enhanced by nitrate addition under aerobic incubation conditions, nor was E. herbicola(pNarG-Ice) influenced by ammonium under either anaerobic or aerobic conditions (Table 2). In the presence of nitrate under aerobic incubation, both E. herbicola(pNarG-Ice) and E. coli(pNarG-Ice) expressed only low levels of ice nucleation activity (about 10⁻⁶ to 10⁻⁷ ice nuclei/CFU), which were about 100,000- to 1,000,000-fold lower than those in strains grown anaerobically (Table 2).

**Bacterial inaZ-based sensors regulated by nitrate under both aerobic and anaerobic incubation.** We expected that transcription of NarG should no longer be suppressed in the presence of oxygen in strains containing the mutant L28H- fnr gene. Under aerobic conditions, the ice nucleation activity of E. coli(pNice) was more than 100-fold higher in media amended with 10 mM sodium nitrate than in media lacking nitrate (about 10⁻⁶ ice nuclei/CFU and 10⁻⁷ ice nuclei/CFU, respectively) (Fig. 2A). The ice nucleation activity of E. coli(pNice) increased with increasing concentrations of nitrate in the medium in the range of about 10⁻⁷ to 10⁻³ M (Fig. 2B). These nitrate-mediated changes in ice nucleation activity were detectable within 2 hours after inoculation of the bacterium into nitrate-containing media, and little further increase in ice nucleation occurred during an additional 6-hour exposure (Fig. 2A). Since ice nucleation proteins are stable, continued cell growth was balanced by continued ice protein production in these cultures.

While pNarG-Ice conferred nitrate-dependent ice nucleation under anaerobic conditions in E. herbicola 299R, pNice did not confer expression of greater ice nucleation activity in E. herbicola 299R in either LB or M9 medium amended with 10 mM sodium nitrate, compared with media without added nitrate, under aerobic conditions (data not shown). While higher than that exhibited by E. herbicola(pPROBE-NI), the activity of E. herbicola(pNice) was nearly as low as that exhibited by E. herbicola(pNarG-Ice) under aerobic conditions, suggesting either that the modified Fnr did not function in E. herbicola 299R or that its effect was overcome by its native oxygen-dependent Fnr.
In order to develop a nitrate reporting strain that would be more environmentally competent than *E. coli* derivatives, pNice was introduced into the soil bacterium *E. cloacae* strain EcCT501R. Under aerobic conditions, EcCT501R(pNice) expressed much higher ice nucleation activity in M9 medium amended with 10 mM sodium nitrate (Fig. 2C) than did *E. coli* DH5α(pNice) (about 10^−3 ice nuclei/CFU and 10^−3 ice nuclei/CFU, respectively) (Fig. 2A). Strain EcCT501R(pNice) exhibited increased ice nucleation within 1 hour after exposure to nitrate under aerobic conditions, and 10,000-fold greater ice nucleation activity was expressed in M9 medium containing 10 mM sodium nitrate compared to that in medium without added nitrate (Fig. 2C). Under aerobic conditions, the ice nucleation activity of EcCT501R(pNice) increased with increasing concentrations of nitrate in M9 medium in the range of 10^−7 to 10^−3 M (Fig. 2D). A similar nitrate-responsive ice nucleation activity was seen under anaerobic conditions (Fig. 2D).

Assessment of nitrate availability in the rhizospheres of wild oat and snap bean by using an *inaZ*-based biosensor. In contrast to that of *E. cloacae* EcCT501R, the population size of *E. coli* DH5α declined rapidly after inoculation into the rhizosphere of snap bean (Fig. 3C). The population size of the *E. cloacae* biosensor strain remained at the relatively high numbers at which it was inoculated onto roots for over a day (Fig. 3A and 3B). For this reason, *E. cloacae* EcCT501R was used as a host strain for reporter gene fusions to evaluate nitrate availability in the rhizosphere.

Population sizes of EcCT501R(pNice) in the rhizospheres of wild oat (Fig. 3A) and snap bean (Fig. 3B) amended with 10 mM nitrate were only slightly higher than in those amended with 0.1 mM nitrate or without nitrate amendment. On snap bean roots, about 10^6.5 CFU were recovered per gram of rhizosphere soil amended with 10 mM sodium nitrate, compared to 10^6.3 CFU per gram of rhizosphere soil with lower nitrate concentrations (Fig. 3B), and this difference was not significant. Population sizes of EcCT501R(pNice) in the rhizospheres of wild oat and bean plants both remained relatively constant. Thus, we found no evidence for nitrate-mediated increases in populations of the biosensor strains over this short time period.

The ice nucleation activity of EcCT501R(pNice) was about 10^−2 ice nuclei/CFU at 3 hours after inoculation onto the rhizosphere of wild oat amended with 10 mM sodium nitrate, which was about 100-fold higher than that for roots grown in soil without nitrate amendment (Fig. 4), at both 3 and 6 h after inoculation of the biosensor bacteria. Since the ice nucleation activity of the cells used as inoculum was less than 10^−5 ice nuclei/CFU, it is clear that nitrate-dependent expression of ice nucleation occurred in both soils. The ice nucleation activity in the rhizosphere of wild oat amended with 0.1 mM sodium nitrate was also significantly higher than that with no nitrate amendment at 3 and 6 h after inoculation of the bacterium (Fig. 4). The ice nucleation activity of the nitrate biosensor in the rhizosphere of bean plants was similar at a given nitrate amendment to that for wild oat (data not shown).

Whole-cell GFP-based biosensors regulated by nitrate in culture. Since *E. cloacae* EcCT501R(pNice) exhibited a population-level nitrate-responsive ice nucleation activity and environmental competence in the rhizosphere, we developed a GFP-based variant to enable observations of nitrate availability to single cells as well as population-based estimates. The ice nucleation reporter gene was replaced in pNice to produce pNgfp. As expected, cells of EcCT501R harboring pNgfp ex-
hindered nitrate-dependent GFP fluorescence under both aerobic and anaerobic conditions. The expression of GFP in the presence of nitrate did not alter the growth of this strain (data not shown). Little GFP fluorescence was observed in cells of this strain in media lacking nitrate, but strong green fluorescence was observed in nitrate-containing media (Fig. 5). The GFP fluorescences of single cells grown in broth cultures containing a given concentration of nitrate were similar (Fig. 5). Because of the narrow range of GFP fluorescence of cells exposed to a given nitrate concentration, the GFP fluorescence of individual cells provides a relatively unambiguous estimate of its nitrate environment.

To enable the visualization of all cells of the nitrate biosensor and only cells of this strain, this strain was also transformed with pVS1-dsRed, a plasmid that is compatible with pNGfp and which harbors a constitutively expressed dsRed marker gene. This red-marked nitrate biosensor grew slightly slower than the strain without pVS1-dsRed but exhibited a nitrate-induced GFP fluorescence similar to that of EcCT501R(pNGfp) (Fig. 6). The measurement of GFP fluorescence in response to nitrate by using a fluorometer (Fig. 6) gives slightly different results than single-cell measurements using microscopy (Fig. 5); this could be due to the different methods used for detection. Little increase in GFP fluorescence was observed in cells grown in less than about $10^{-5}$ M nitrate, but fluorescence increased rapidly thereafter with increasing nitrate concentrations to about $10^{-2}$ M nitrate. The GFP fluorescence of cells exposed to $10^{-3}$ M nitrate was about 1,000-fold greater than that of control cells without nitrate exposure.

**Assessment of nitrate availability in the rhizosphere of wild oat by using a GFP-based NO$_3^-$ sensor.** The GFP-based nitrate biosensor was applied to roots to determine if these whole-cell biosensors would yield sufficient GFP fluorescence to detect nitrate in natural environments. Microcosms were treated with one of four treatments: nitrate alone, glucose alone, nitrate plus glucose, or water. The effect of each variable (nitrate, glucose, and root zone) on the incidence of induction of nitrate biosensor cells was calculated based on measured fluorescence per cell. Glucose-only and nitrate-only treatments did not have a significant effect and are omitted in Fig. 7. Two replicate experiments were conducted, and a Student $t$ test on the results ($P < 0.05$) determined that the two experiments’ results were not significantly different and could be combined into one large data set.

While we did not attempt to quantify GFP fluorescence in this experiment, it was obvious that there were three recognizably distinct populations of cells in the rhizosphere: those that were very strongly induced, exhibiting “very bright” GFP fluorescence; those that were only slightly induced, exhibiting “dim” but detectable GFP fluorescence; and those that were uninduced, having undetectable fluorescence. The fluorescence of very bright cells was apparent without any image adjustment, while the majority of cells were dim cells, unambiguously discernible only after image contrast adjustment. The bright cells had presumably sensed high local concentrations of nitrate; these were analyzed separately, either as a fraction of total cells with RFP fluorescence (Fig. 7B) or as a fraction of total cells with GFP fluorescence (Table 3), from the total dim plus bright fluorescent cells (Table 3 and Fig. 7A).

By this approach we found that nitrate has a strong effect on both the incidence of total cells responding detectably to nitrate and the percentage of highly induced cells (Fig. 7; Table 3). Location along the root significantly affected the proportion of highly induced cells but not that of cells as a whole that responded to nitrate by GFP fluorescence (Fig. 7A). The nitrate biosensor *E. cloacae* EcCT501R(pNGfp)(pVS1-dsRed) sensed 10 times as many regions of apparent high nitrate availability ($P = 0.0073$) in the bulk soil compared to the root hair and root tip regions of the rhizosphere in the absence of added nitrate (Fig. 7B). One-way ANOVA established significant differences ($F = 2.0582; P = 0.0524$) in overall nitrate availability between the root hairs without added nitrate and root hairs with added nitrate (Fig. 7A). One-way ANOVA also indicated significant differences ($F = 2.930; P = 0.0091$) in high nitrate availability between the bulk soil and rhizosphere, particularly root hairs and mature roots, when nitrate was added (Fig. 7B). Glucose treatment did not affect overall GFP fluorescence or induction of bright fluorescence.

Multiple regression analysis was used to build a model that would reflect the contributions of different factors to the incidence of detectable nitrate responsiveness of the nitrate biosensor and that proportion of highly responsive cells (Table 3). The variable that best fits the data is reported first, and the variable that fits the residuals of the data from the first variable is reported second, etc. For each iteration of the model, the $F$ statistic (as part of the whole-model $F$ test), $P$ value, and $R^2$ are reported (Table 3). This analysis shows that the only factors that contribute to GFP induction of the nitrate biosensor are nitrate and root zone, while glucose has a negligible effect on bacterial GFP expression of cells as a whole, and there is no interaction between added nitrate and glucose. Root zone contributed to induction of cells with very bright GFP fluorescence ($P = 0.0073$), as did nitrate and the cross product of nitrate and glucose ($P = 0.070$ and $P = 0.0743$), but not glucose alone (Table 3).

**DISCUSSION**

Transcription of a nitrate-inducible promoter by the root-colonizing bacterium *E. cloacae*, both in culture media and in the rhizospheres of wild oat and snap bean, was easily detected by an ice nucleation reporter gene system as well as a GFP reporter gene system coupled with a dsRed marker gene. Using the two different reporter systems, we were able to estimate the amount of nitrate available to rhizosphere bacteria. The *inaZ*-based nitrate biosensor expressed significantly higher levels of ice nucleation activity in the rhizospheres of wild oat and snap bean grown in soils amended with nitrate than in the unamended control (Fig. 4). These findings were in agreement with nitrate levels that were detected using the GFP marker reporter construct, which reported much higher levels of available nitrate in the bulk soil than in the rhizosphere (Fig. 7B).

Host strain survival in the rhizosphere was an important determinant of the success of our whole-cell biosensors. *E. coli* DH5α exhibited significant declines in population sizes in the rhizospheres of plants (Fig. 3C) and could not be used in this study. We directly applied the *E. cloacae* EcCT501R biosensor to roots and soils and incubated for 24 h, a period sufficient for *narG* induction. In Loper and Henkels’ study (32) of iron
availability using a pvd-inaZ ice nucleation reporter gene fusion, lack of growth in the rhizosphere did not hinder the usefulness of the inaZ reporter construct. Leveau and Lindow (27) pointed out that GFP fluorescence per cell is not only a function of the activity of the promoter but also a function of the rate at which the cell is reproducing; fast growth of the GFP biosensor population will result in dilution of the GFP signal upon cell division, causing an underestimate of the queued environmental signal. In our study, the population size of E. cloacae strain EcCT501R did not change significantly during the 24-hour course of our experiments (Fig. 3). Thus, such growth rate effects were probably not a contributing factor to the interpretation of the biosensors’ reports of bioavailable nitrate.

Because of the ability of the L28H-fnr gene product to overcome oxygen repression in E. cloacae EcCT501R, we used this strain for all in situ experiments (Fig. 3) (23, 42). E. coli DH5α exhibited narG promoter transcription and oxygen-dependent repression of NarG similar to those of EcCT501R (1, 23). Kiley and Reznikoff demonstrated that an Fnr mutant strain of E. coli containing the L28H-fnr gene product exhibited a 47-fold increase in beta-galactosidase with nitrate addition under aerobic conditions (23). E. herbicola 299R exhibited good nitrate induction of NarG under anaerobic conditions and has performed well in the rhizosphere as a whole-cell biosensor (3, 4, 18). However, the introduction of the L28H-fnr gene product did not overcome oxygen repression as did the E. coli DH5α and E. cloacae EcCT501R strains in this study. That the reporter strains are nitrate inducible aerobically is crucial to their application in soils, since in this application the microcosm soil was well aerated, and most applications would be under aerobic conditions (6, 18, 42, 46).

The inaZ reporter gene system is a sensitive and convenient tool for studies of nutrient conditions in natural environments such as the rhizosphere, partly because ice nucleation activity increases with roughly the square of ice protein content (29). This exponential increase in ice nucleation activity with increased transcription results in very high induction levels compared to other reporter genes. Out of a set of three sucrose-regulated reporter gene fusions made with GFP, inaZ, or lacZ, the inaZ reporter had the widest range of activity and had much higher sensitivity than GFP or lacZ (35, 41). The extremely low background level of ice nucleation activity in soil also makes inaZ a good reporter gene for use in the rhizosphere. Transcription of narG is induced about 18-fold (45) to 118-fold (2) by addition of 40 mM nitrate in culture media under anaerobic conditions. Given the exponential relationship between ice protein content and ice nucleation activity, we would expect about a 13,000-fold increase in ice nucleation activity in cells harboring the narG-inaZ gene fusions. Indeed, we observed about a 1,000- to 60,000-fold increase in ice nucleation activity upon addition of different concentrations of nitrate to E. coli and E. cloacae biosensor strains in culture (Fig. 2). Since even very low levels of gene expression are measurable using inaZ reporter gene fusions (34), this system often provides evidence of gene expression that is not detectable with other reporter genes (35). The inaZ-based nitrate biosensor was responsive to nitrate concentrations of as low as 10^{-7} M under both aerobic and anaerobic conditions, about 10-fold lower than that detected using the GFP-based biosen-

![FIG. 5. Histogram showing the fluorescence of individual cells of EcCT501R(pNgfp) grown in M9 minimal medium containing no nitrate (A), 0.1 μM nitrate (B), 10 μM nitrate (C), or 1 mM nitrate (D). After 4 h of incubation, cells were fixed, placed on slides, and immediately viewed with an epifluorescence microscope. The means (variances) of cells exposed to 0, 10^{-7}, 10^{-5}, and 10^{-3} M NaNO₃ were 7.171 (3.519), 27.25 (32.00), 277.4 (204.0), and 457.8 (362.3), respectively.](http://aem.asm.org/)
and the pNgfp sensor (Fig. 6), this is on par with the detection limit of the pNice sensor (Fig. 2) in situ detection of other nutritional responses of bacteria harboring a fusion of the narG containing different concentrations of nitrate. There was no detected GFP fluorescence without added nitrate (data not shown). OD, optical density unit.

FIG. 6. GFP fluorescence, determined using a fluorometer, of cell cultures of E. cloacae EcCT501R(pNgfp)(pVS1-dsRed) in minimal medium containing different concentrations of nitrate. There was no detected GFP fluorescence without added nitrate (data not shown). OD, optical density unit.

The GFP-based nitrate biosensor provides small-scale spatial information about nitrate availability near roots that is not possible to obtain with the ice nucleation biosensor (6, 27, 30). The use of dsRed as a marker gene enables recognition of all cells in a population of bioreporter cells, so that GFP fluorescence of individual cells can be resolved. However, high and variable background autofluorescence in the soil can make quantification of GFP fluorescence intensity difficult for some soils. Furthermore, subtracting high background levels of fluorescence from images of bioreporter strains in situ can artificially lower GFP fluorescence measurements and underestimate effector molecule concentrations. Relatively low background fluorescence in our soil allowed us to evaluate the availability of nitrate at the single-cell scale by using microscopy. We have also pooled the results and done statistical analyses to examine the availability of nitrate and the impact of root uptake on residual soil nitrate in soil near roots. This type of analysis eliminates some of the heterogeneity observed at the single-cell level, and it allows us to quantify larger spatial trends in nitrate availability at meso-spatial scales. Using the GFP reporter at the single-cell scale allows assessment of nitrate availability in soil microsites (niches) with dimensions of micrometers. Such resolution of soil bacterial environments at the niche scale is critical to understanding the in situ physiological ecology of soil bacteria.

Other nitrate or nitrogen biosensors have been constructed using different reporter genes, but each has its drawbacks to applications in the rhizosphere. An E. coli nitrate biosensor harboring a fusion of the narG promoter region of E. coli to the lux genes of Photobacterium luminescens (42) could detect nitrate at as low as 0.5 × 10⁻⁵ M in culture media. While this is on par with the detection limit of the pNice sensor (Fig. 2) and the pNgfp sensor (Fig. 6), this lux-based sensor functions only under anaerobic conditions. While reporter systems employing the bacterial luciferase genes luxAB have been used for in situ detection of other nutritional responses of bacteria in the rhizosphere (20, 26), the use of lux-based reporter gene systems in soil environments is confounded by soluble pigments and soil particles which can attenuate light emitted by bioluminescent strains, as well as a low level of metabolic energy of cells that may limit light production independently of transcription of narG (24, 30). In studies using a lux reporter gene for the detection of nitrogen starvation in the barley rhizosphere, there was much less bioluminescence of the reporter bacteria in unamended soil than in nutrient-amended soil (20). Although the reporter was designed to detect N limitation, it was unclear whether the lack of signal was due to general insufficient metabolic energy or specific N limitation (20).

In our study, nitrate concentrations in unamended soils were barely detectable using either the GFP- or the ice nucleation-based nitrate sensor, confirming that our soil-plant mesocosms initially provided a relatively nitrogen-limited environment (Fig. 4 and 7). Dim cells were similar in intensity to cultured cells seeing 10⁻⁷ M or less nitrate (Fig. 5). Low apparent nitrate availability, even after nitrate addition (Fig. 7), suggested that during the 12-h preincubation prior to sensor addition, nitrate was rapidly consumed by roots and rhizosphere microorganisms. Independent estimates of bulk field nitrate availability in this California grassland soil done by colorimetric analysis of KCl extracts of soil solutions found that concentrations of nitrate, while variable, were about 0.5 µg NO₃⁻ N g⁻¹ soil (about 100 µM) during periods of active plant uptake (7). The amount of nitrate available to the bioreporter in the rhizosphere in these studies was estimated to be between 0.1 and 1 µM in nonamended soils from measurements of nitrate-dependent ice nucleation activity in defined culture conditions (compare Fig. 2 and 4). We estimate the nitrate available to the E. cloacae strain in the rhizospheres of soils amended with 10 mM nitrate to be about 0.1 mM (Fig. 2 and 4). These biosensors thus report the biological availability of extremely dynamic nitrate pools. Based on the concentration of the nitrate solution sprayed on these root-soil systems, the GFP reports should have been dominated by brightly fluorescent cells. However, GFP reports from the rhizosphere in-
Table 3. Model of contributing effects of nitrate addition, root zone location, and glucose addition on E. cloacae(pNgfp) in soil microcosms

<table>
<thead>
<tr>
<th>Parameter and factor</th>
<th>$r^2$</th>
<th>$F$</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Total GFP-induced cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nitrate</td>
<td>0.0452</td>
<td>6.572</td>
<td>0.0114*</td>
</tr>
<tr>
<td>Root zone</td>
<td>0.0289</td>
<td>1.13579</td>
<td>0.2584</td>
</tr>
<tr>
<td>% Bright GFP cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Root zone</td>
<td>0.1389</td>
<td>4.2997</td>
<td>0.0073*</td>
</tr>
<tr>
<td>Nitrate</td>
<td>0.0395</td>
<td>3.3710</td>
<td>0.0416*</td>
</tr>
<tr>
<td>Nitrate $\times$ glucose</td>
<td>0.0383</td>
<td>3.2672</td>
<td>0.0743**</td>
</tr>
<tr>
<td>Glucose</td>
<td>0.0156</td>
<td>1.2974</td>
<td>0.2580</td>
</tr>
</tbody>
</table>

* For each iteration of the linear regression model, the $F$ statistic as part of the whole-model $F$ test, the $P$ value, and $r^2$ are reported. For percent total GFP induced cells, $n = 141$ and is percent (total GFP induced cells)/(total RFP cells). For total bright GFP cells, $n = 84$ and is percent (total GFP induced cells)/(total GFP cells). $*, P < 0.05; **, P < 0.10$. 

![Figure 7](https://example.com/figure7.png)

**FIG. 7.** GFP expression by E. cloacae EcCT501R(pNgfp)(pVS1-dsRed) in the rhizosphere of Avena fatua; error bars indicate one standard error. Two hours prior to application of sensors to the rhizosphere, microcosms were amended with the equivalent of 6.6 mM NaNO₃ plus 1.6% glucose (dark bars) or water (light bars). (A) Cells exhibiting any GFP fluorescence expressed as the total number of green fluorescing cells of any brightness divided by total number of red fluorescing cells ($\times 100$). (B) Cells exhibiting bright GFP fluorescence expressed as total number of very bright green fluorescing cells divided by total number of red fluorescing cells ($\times 100$). Statistically significant differences in either percent GFP induction or percent bright GFP induction are represented by different letters (for total percent induction, $n = 140$; for percent bright cells, $n = 84$).

dicted that plant/microbial uptake had rapidly depleted the added nitrate.

Based on measurement of fluorescence intensities of individual cells in the rhizospheres of nitrate-amended soils, we estimate that the weakly fluorescing cells in the rhizosphere sensed at most 1 $\mu$M nitrate. Strongly fluorescing cells were documented to occur at a concentration of 1 mM nitrate (Fig. 5). Assuming a soil water content of about 15%, the amount of nitrate added to the mesocosms would yield a soil solution nitrate concentration of about 6.6 mM. In cultures this concentration of nitrate induces high levels of GFP fluorescence that are nearly insensitive to further increases in nitrate concentrations. However, in soil receiving added nitrate, only a small subset of the bacterial population reported such high concentrations (ranging from 0.5% of the cells near the root tip to 3% of cells in the bulk soil). Thus, nitrate was not depleted from a small subset of soil microsites, microsites that were apparently isolated from active root uptake. Documentation by GFP reporters of the number and distribution of soil microsites diffusively isolated from plant root uptake is in itself an interesting result. Such heterogeneity may be very important for explaining the ability of microbes and plants to efficiently compete with one another for inorganic nitrogen (14, 25).

Examination of different root zones by using GFP biosensors for nitrate availability reveals that there can be higher nitrate available to soil bacteria in the bulk soil compared to those near roots. When nitrate concentrations were very low (no nitrate added), the root hairs were most active in uptake (Fig. 7A). After nitrate addition, soil near roots was rapidly depleted (Fig. 7B). More conventional methods for nitrate detection in the soil are unable to detect small-scale heterogeneity of nitrate availability in the rhizosphere. In other work, we have successfully documented the mesoscale patterns of mineralization and nitrification in the A. barbata rhizosphere (D. J. Herman et al., submitted for publication) by using $^{15}$N-based methods. Patterns of N mineralization and nitrification were found to vary substantially along the root, in part due to plant-microbe competition for inorganic N (5, 8). In the current study, we also observe mesoscale differences in nitrate uptake in different root zones, but in addition, we discerned microscale patterns of nitrate access and utilization by roots and microbes. Bacterial cell biosensors allow us to map the spatial patterns of interaction between bacteria and plant roots at a scale that reveals important understanding of the interactions occurring.

This is the first application of a whole-cell biosensor for the detection of nitrate availability in the rhizosphere. The use of a reporter system linked to nitrate reductase genes achieves high specificity and sensitivity while providing either mesoscale (millimeters) or microscale (micrometers) spatial resolution. Further studies that use nitrate biosensors to monitor the spatial and temporal patterns of nitrate availability in the root systems under natural environmental conditions will likely provide new insight into nitrogen dynamics at scales that will enable better understanding of root-microbe interactions.
REFERENCES


