APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Dec. 1997, p. 4920–4928 Vol. 63, No. 12
0099-2240/97/$04.00+0


# Distribution of Metabolic Activity and Phosphate Starvation Response of *lux*-Tagged *Pseudomonas fluorescens* Reporter Bacteria in the Barley Rhizosphere

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Received 31 December 1996/Accepted 3 October 1997

**The purpose of this study was to determine the metabolic activity of *Pseudomonas fluorescens* DF57 in the barley rhizosphere and to assess whether sufficient phosphate was available to the bacterium. Hence, two DF57 reporter strains carrying chromosomal *luxAB* gene fusions were introduced into the rhizosphere. Strain DF57-40E7 expressed *luxAB* constitutively, making bioluminescence dependent upon the metabolic activity of the cells under defined assay conditions. The DF57-P2 reporter strain responded to phosphate limitation, and the *luxAB* gene fusion was controlled by a promoter containing regulatory sequences characteristic of members of the phosphate (Pho) regulon. DF57 generally had higher metabolic activity in a gnotobiotic rhizosphere than in the corresponding bulk soil. Within the rhizosphere the distribution of metabolic activity along the root differed between the rhizosphere soil and the rhizoplane, suggesting that growth conditions may differ between these two habitats. The DF57-P2 reporter strain encountered phosphate limitation in a gnotobiotic rhizosphere but not in a natural rhizosphere. This difference in phosphate availability seemed to be due to the indigenous microbial population, as DF57-P2 did not report phosphate limitation when established in the rhizosphere of plants in sterilized soil amended with indigenous microorganisms.**

Several fluorescent *Pseudomonas* strains are able to promote plant growth either by producing growth-stimulating substances or through antagonism to plant pathogens (15, 19, 44). Introduction of beneficial pseudomonads into the rhizosphere may therefore be a way to improve agricultural production in an environmentally acceptable way. Studies of the distribution of introduced pseudomonads in the rhizosphere can provide useful information on their abilities to colonize the emerging root. However, knowledge of the factors that may affect their growth and survival, and their genetic and physiological responses to these fluctuating conditions, is scarce.

The rhizosphere environment is influenced by the input of organic material from the plant. A barley plant exudes from its roots 0.4 to 0.5 mg of organic substances into its surroundings during its first 10 days (39). The exudates consist mainly of low-molecular-weight carbon sources (including amino acids) readily available to microorganisms. Other nutrients, such as phosphorus, are also necessary to promote bacterial growth and survival (10), as phosphorus is present in, e.g., nucleic acids, lipopolysaccharides, and phospholipids.

Phosphorus can be present in soil as Ca, Al, or Fe precipitates, or it can be adsorbed to soil minerals. However, only soluble, inorganic phosphate is readily available to bacteria. Not only bacteria but also plants utilize phosphate around roots (17), and plants therefore compete with microorganisms for available phosphate in the rhizosphere. Several bulk analytical methods are available for the determination of phosphate content in soil. However, these methods are not able to determine the amount of phosphate that is actually available to microorganisms.

In general, bacteria respond to limiting phosphate concentrations by expressing a set of genes involved in the uptake and assimilation of this nutrient from the environment (11, 32). In the fluorescent pseudomonad *Pseudomonas aeruginosa*, some of these genes are known, e.g., the regulatory genes *phoU* and *phoB* (18) and the *oprP* and *phoA* genes, which encode a phosphate-specific porin and an alkaline phosphatase (9, 34), respectively. Little is known about phosphate starvation-induced genes in the soil bacterium *Pseudomonas fluorescens*, which contains several plant growth-promoting strains.

Transcriptional fusions between environmentally regulated promoters and reporter genes encoding a phenotype that is readily detected have been used to assess the availability of carbon sources and inorganic nutrients to cells residing in natural habitats (7, 25, 40). Reporter systems employing the bacterial luciferase genes *luxAB* are attractive, as they offer nonextractive in situ detection of gene expression in the environment by light-sensitive cameras (3, 38). By contrast, cells tagged with the beta-galactosidase gene *lacZ* or the ice nucleation activity gene *inaZ* have to be extracted from environmental samples prior to analysis (7, 25, 40).

Light emission from *luxAB* genes fused to a constitutive promoter depends on the cellular content of reduced flavin mononucleotide under conditions in which aldehyde and oxygen are in surplus. Therefore, bioluminescence from this type of reporter strain can provide a measure of the actual metabolic activity of the tagged strain (30).

We have previously identified several phosphate starvation-inducible loci in *P. fluorescens* DF57 (21). In the present study, we further characterized strain DF57-P2, which has an insertion of Tn*5*::*luxAB* in one of these loci, and used it as a reporter bacterium for phosphate availability in the barley rhizosphere. In addition, root colonization studies were performed with strain DF57-40E7, which expresses the *luxAB* genes constitu-

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tively. These experiments were performed to monitor the distribution of DF57 and to ensure that the bioluminescent response of the reporter strains was not impaired by, e.g., oxygen limitation or inadequate cellular reductant.

## MATERIALS AND METHODS

**Bacterial strains, culture media, and growth conditions.** We used two Tn*5*–*luxAB*-marked strains in these studies, *P. fluorescens* DF57-P2, reporting phosphate starvation (21), and the constitutive mutant *P. fluorescens* DF57-40E7. The cell-specific bioluminescence of DF57-40E7 decreases during phosphate starvation (ca. 50-fold after 5 days), whereas that of DF57-P2 increases more than 100-fold during phosphate starvation (21). The strains were cultured in Luria broth (LB) or in Davis minimal medium, as described previously (22), at 30°C on a rotary shaker at 200 rpm. Kanamycin (25 μg $ml^{-1}$) was used in both media routinely. For spread plating, LB was solidified by 1.5% Bacto Agar (Difco, Detroit, Mich.), and 20 μg of streptomycin $ml^{-1}$ and 20 μg of nystatin $ml^{-1}$ were included when appropriate. *Escherichia coli* HB101 (5) was used as the host for cloned *P. fluorescens* DF57 DNA. This strain was cultured in LB at 37°C on a rotary shaker, and 25 μg of kanamycin $ml^{-1}$ was added if plasmids containing Tn*5* were transformed into the strain.

**Cloning of the *lux*-tagged gene in DF57-P2.** The genomic DNA from *P. fluorescens* DF57-P2 was isolated as described by Kragelund et al. (21). Digestion of the purified DNA with *Eco*RI and self-ligation with T4 DNA ligase were carried out according to Sambrook et al. (35). The plasmid containing the Tn*5*-*lux* fragment (pP2) is able to replicate in *E. coli* due to an *oriV* in Tn*5* (43). This plasmid hence functions as a vector for the cloning of *P. fluorescens* genomic DNA in *E. coli*. The ligated circular DNA was transformed into *E. coli* HB101 by electroporation. The *E. coli* cells were made competent by washing exponentially growing cells in ice-cold water several times and resuspending them in 15% glycerol. The cells were stored at −80°C until used. Plasmid DNA (3 μl) was added to 200 μl of the thawed cells, and a 5-ms pulse of 2.48 kV and 200 Ω was applied (Bio-Rad gene pulser; Bio-Rad Laboratories, Hercules, Calif.). Immediately afterward, 1 ml of SOC medium (0.5% Bacto Yeast Extract, 2% Bacto Tryptone, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, and 20 mM glucose) was added and the cells were incubated at 37°C for 1 h before being plated on LB agar with kanamycin (to select for HB101 cells containing pP2). To verify that HB101 contained pP2, the plasmid was isolated by the alkaline lysis method according to Sambrook et al. (35) and Southern blotting was carried out as described previously (21).

**Sequencing.** The pP2 plasmid was purified with the Qiagen Plasmid Maxi Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. The dideoxy method with the T7 Sequencing Kit (Pharmacia Biotech, Hillerød, Denmark) and [α-$^{35}$S]dATP (Amersham, Birkerød, Denmark) was used to sequence the DNA flanking Tn*5* on pP2 according to the manufacturers' suggestions except that 25 μg of plasmid DNA was denaturated and 20 pmol of primer was used in the annealing procedure. First, primers complementary to sequences at the ends of Tn*5* were used (5′-TACTAGATTCAATGCTATCAATGAG-3′ and 5′-AGGAGGTCACATGGAATATCAGAT-3′). Then, new primers were made (DNA Technology, Århus, Denmark) on the basis of the obtained sequences. Both strands were sequenced, and data were analyzed with DNASTAR software (DNASTAR Inc., Madison, Wis.). Searches for DNA and protein homologies were carried out on the EMBL database and on all available protein databases via BioBase, Århus, Denmark.

**Growth rate, light emission, and starvation survival.** The growth rate as well as survival and light emission during carbon, nitrogen, or phosphate starvation of strain DF57-40E7 were determined as described for strain DF57-P2 by Kragelund et al. (21).

**Measurement of bioluminescence.** Bioluminescence from colonies and colonized roots (after removal of loosely adhering soil) was visualized by a Hamamatsu photonic camera system, model C2400-47 (Unit-One, Birkerød, Denmark), coupled to a Nikon 35-mm *f*0.85 macro lens. Cells were exposed to aldehyde by spreading 50 μl of *n*-decanal on the inside cover of a glass petri dish. Images were processed with Image-Pro Plus (Media Cybernetics, Silver Spring, Md.). No manipulations (background subtractions, etc.) were performed before the images were pseudocolored with a linear color scale as indicated by the color bars on the figures.

Bioluminescence from bacteria in liquid media was measured by luminometry (BioOrbit 1253; Struers KEBO Lab, Albertslund, Denmark). *n*-Decanal (3 μl of a 10% suspension in ethanol) was added to samples of 1 ml. The samples were vortexed for 15 s and, 3 min after addition of aldehyde, bioluminescence was measured three times for 10 s. Bioluminescence is expressed as relative light units (RLU).

**Extraction of the natural bacterial population from soil.** The soil used in all experiments was a loamy sand with 5.3 ppm of phosphorous taken from a field at the Royal Veterinary and Agricultural University, Tåstrup, Denmark (23). Soil samples of 10 g each were shaken at 200 rpm with 20 ml of sterile 0.9% NaCl at 4°C for 30 min. The soil was allowed to settle for 1 1/2 h before microorganisms were harvested from 10 ml of the supernatant by centrifugation at 12,000 × *g* for 8 min. The cells were washed twice with 0.9% NaCl and resuspended in 0.9% NaCl. Acridine orange direct counts (13) were used to determine the number of extracted bacteria (ca. $5 \times 10^7$ cells $ml^{-1}$). The extracted cells were further concentrated by centrifugation at 12,000 × *g* before they were used to inoculate autoclaved soil (see below).

**Colonization assays.** Colonization assays were initiated as described by Kragelund and Nybroe (23). Briefly, surface-sterilized barley seeds were inoculated in a cell suspension containing $10^9$ DF57-40E7 or DF57-P2 cells $ml^{-1}$ and planted in tubes with 50 g of soil. In this study, we used either autoclaved soil (23) or natural soil to obtain a gnotobiotic or natural rhizosphere system, respectively. Soil moisture was adjusted to 15% (wt/wt) with either sterile water, an extract containing natural bacteria to obtain $10^9$ cells g of $soil^{-1}$, or a cell suspension of DF57-P2 to obtain $10^8$ cells g of $soil^{-1}$. A water content of 15% corresponds to 80% of the field capacity of the soil (pF = 2 [determined by the Central Laboratory, Research Center Foulum, Foulum, Denmark]). The systems were incubated at 20°C (12 h dark/12 h light) for 7 days.

When the plants were harvested from the soil system, the root systems were shaken by hand to remove loosely adhering soil. For each plant, a bright-field and a dark-field image were obtained by the photonic camera (see above). Then, three individual roots were selected for further analysis. These were divided into 1-cm segments representing 0 to 1, 1 to 2, 2 to 3, 3 to 4, 4 to 5, 5 to 6, and 6 to 7 cm from the stem base. The three corresponding sets of segments were pooled, washed twice in 1 ml of sterile 0.9% NaCl by vortexing (23), and subsequently homogenized in 1 ml of 0.9% NaCl. For the purpose of this study, rhizosphere soil was defined as the material found in the washing fractions, while bacteria found in the root homogenate were assumed to represent rhizoplane and possible endorhizosphere populations. Bioluminescence was measured in all fractions by luminometry. Cell-specific bioluminescence was also determined in bulk soil samples. Rhizosphere and bulk soil samples contained comparable amounts of soil (ca. 15 mg per sample) to avoid differential quenching of bioluminescence.

CFU were determined by the drop plate method (14) in samples from gnotobiotic systems or by spread plating in samples from systems established in natural soil. Colonies were enumerated after incubation for 24 h at 30°C.

In one set of experiments, roots colonized by DF57-P2 in natural soil were soaked in 0.4% glucose and 7.5 mM $(NH_4)_2SO_4$ for 24 h prior to determination of bioluminescence and CFU. In another set of experiments, roots colonized by DF57-P2 in sterile soil were soaked in 250 mM phosphate buffer, pH 7.2, for 5 and 24 h prior to analysis.

**Reproducibility and statistics.** All colonization studies involving quantitative determination of bioluminescence by luminometry included two independent experiments, each performed in duplicate. Qualitative experiments demonstrating induction or repression of the phosphate limitation response were performed in triplicate.

Differences in cell-specific bioluminescence were tested by Student's *t* test performed on log-transformed data with the statistical program Sigma-Stat (Jankel Scientific, Erkrath, Germany). *P* values of $<0.05$ were considered necessary to establish statistically significant differences.

**Nucleotide sequence accession number.** The nucleotide sequence data have been deposited in the GenBank nucleotide sequence database under accession no. U59930.

## RESULTS

**Emission of bioluminescence by *P. fluorescens* DF57-40E7.** Bioluminescence of DF57-40E7 was not induced by osmotic stress (0.9 M NaCl), by low temperature (4°C), or by carbon, nitrogen, or phosphate starvation (data not shown). Light emission declined during starvation in a manner comparable to the decline observed for other mutants expressing bioluminescence during all investigated growth conditions (21). This suggested that the *luxAB* genes in DF57-40E7 are constitutively expressed and that bioluminescence is dependent on the metabolic activity of the tagged cells (29). Detectable bioluminescence was maintained for at least 10 days of starvation for carbon, nitrogen, or phosphate. Both strain DF57-40E7 and the wild type had generation times of 90 min in Davis minimal medium at 25°C. Furthermore, DF57-40E7 remained fully culturable for at least 10 days of starvation for C, N, or P as has previously been reported for the wild-type strain (21).

**Nucleotide sequence of the gene interrupted by Tn*5*-*lux* in *P. fluorescens* DF57-P2.** In order to identify the tagged gene in DF57-P2, 648 bp of chromosomal DNA flanking the transposon was sequenced. The sequenced region of the P2 plasmid contained part of a putative open reading frame from nucleotides 307 to 648 (Fig. 1A). The most likely translation start codon, GTG, was preceded by a probable ribosomal binding site, and a typical −10 region was located 160 nucleotides

**A**

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TGCCCTATGCATATCTTCAGACACCTCAAACGCGTGGTGACCGACTGGTTCCTGTGCGGCATGGTGCTCGCCACGC 76

TGCTGGCGTATTTCTTTCCCACCTTTGGTGCCAAGGGCGGTGCCATGCATGCCGAATGGGTGGTCAACATCGGCAT 152
        Pho box                                 Pho box             -10

CTTCGTGGTGTTCTTCCTGCACGGGGTCAACCTGTCCGGCGAGCAGATCCGCCACGGCCTGAAGAACATCCGGCTG 228


CACGTGATGGTGCAGGCGTTCACCTTTGGCGTATTTCCGTTGCTCTGGCTGCTCAGCAACTGGCTGCTGGGCAGCC 304
                                                                        RBS

AC GTG CCG GCG CTG CTG ATG CTG GGG TTC TTC TAC CTG TGC GCC CTG CCC TCG ACA ATT 363
   M   P   A   L   L   M   L   G   F   F   Y   L   C   A   L   P   S   T   I    19

TCG TCC TCG GTG GCC CTG ACC GGC AGT GCA AAA GGC AAC GTG CCG GCG GCG ATT CTC AAC 423
S   S   S   V   A   L   T   G   S   A   K   G   N   V   P   A   A   I   L   N    39

GCG AGC CTG TCC AGC GTA CTG GGG ATT TTC CTG ACC CCG TTG CTG GTC AGT TTC GTG GTC 483
A   S   L   S   S   V   L   G   I   F   L   T   P   L   L   V   S   F   V   V    59

GGC AGC GGC GCG GGT GGC ATC GAC CTG GGT TCA ACC TTG CTC GAC CTG TGC ATG ATG TTG 543
G   S   G   A   G   G   I   D   L   G   S   T   L   L   D   L   C   M   M   L    79

CTG CTG CCA CTG GTG CTG GGA CAG TTC CTG CGG CGC TGG CTG GCC GGT TTT TTC GGT CGC 603
L   L   P   L   V   L   G   Q   F   L   R   R   W   L   A   G   F   F   G   R    99

TAC AAA CGC TAC ACC AGC ATC ATC GAC AAA CTG GTG ATC CTG CT∇G 648
Y   K   R   Y   T   S   I   I   D   K   L   V   I   L   L     114
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**B**

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P2 Pho box          GTGCCATGCATGCCGAAT
P2 Pho box          CTGGCGTATTTCTTTCCC
oprP                CTGTCACAAAACCTTTCG
oprP                TTGCAGTCTCGGTGTCAC
oprO                CTGTAATGAAACATTTAT
oprO                ATGCAATTTGGCTGCAAT
E. coli consensus   CTGTCATA(A/T)A(A/T)CTGT(A/C)A(C/T)
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FIG. 1. (A) Nucleotide sequence from the region upstream of the Tn*5* insertion in the P2 gene and the deduced amino acid sequence. In the hypothetical promoter region, the Pho boxes, the −10 region, and the putative ribosome binding site (RBS) are underlined. The transposon insertion is indicated by ▽. (B) Putative Pho boxes in the P2 sequence aligned with the Pho boxes of the *oprP* and *oprO* genes in *P. aeruginosa* (36, 37) and with the *E. coli* consensus sequence. Shaded letters indicate matches with the shown *Pseudomonas* Pho boxes.

upstream. The P2 promoter contained a Pho box region 8 nucleotides further upstream (Fig. 1A), and a second putative Pho box was separated from the first by 19 nucleotides. The Pho boxes were identified on the basis of previously published Pho boxes from *Pseudomonas* spp. and the *E. coli* Pho box consensus sequence (Fig. 1B) (26, 37). The coding region of the sequence had a G+C content of 61% and typical *Pseudomonas* codon usage, with 81% G+C in the third codon position (42). The derived amino acid sequence showed 59% homology to a fragment of a hypothetical protein from the *xapB-lig* intergenic region in *E. coli*, as identified by Borodovsky et al. (4).

**Distribution and metabolic activity of strain DF57-40E7 in the rhizosphere.** Root colonization experiments with strain DF57-40E7 were performed to determine the distribution and metabolic activity of this strain in the barley rhizosphere. In 7-day-old gnotobiotic rhizosphere, the cell concentration in rhizosphere soil was between $8 \times 10^6$ and $2 \times 10^7$ CFU $cm^{-1}$ along the entire root system (Fig. 2A), whereas $4 \times 10^5$ to $9 \times 10^5$ CFU $cm^{-1}$ were more firmly associated with the root and found in the homogenate. This colonization pattern was comparable to that of the wild type (data not shown). Bioluminescence emitted by DF57-40E7 was observed along the entire root system (Fig. 3A), but we found considerable differences in cell-specific luminescence. In the rhizosphere soil, DF57-40E7 populations at the root tip on average emitted ca. $7 \times 10^{-7}$ RLU $CFU^{-1}$, which was significantly higher than values at the root base, ca. $1 \times 10^{-7}$ RLU $CFU^{-1}$ (Fig. 2A). The root base values resembled those observed from DF57-40E7 incubated 7 days in bulk soil (ca. $2 \times 10^{-7}$ RLU $CFU^{-1}$). Populations residing at the rhizoplane showed the reverse pattern: those at the root tip emitted significantly less bioluminescence than those at the base (Fig. 2A). Assuming an average cell-specific bioluminescence of $4 \times 10^{-7}$ RLU $CFU^{-1}$, the detection limit for light emission from DF57-40E7 corresponds to ca. $4 \times 10^3$ CFU cm of $root^{-1}$.

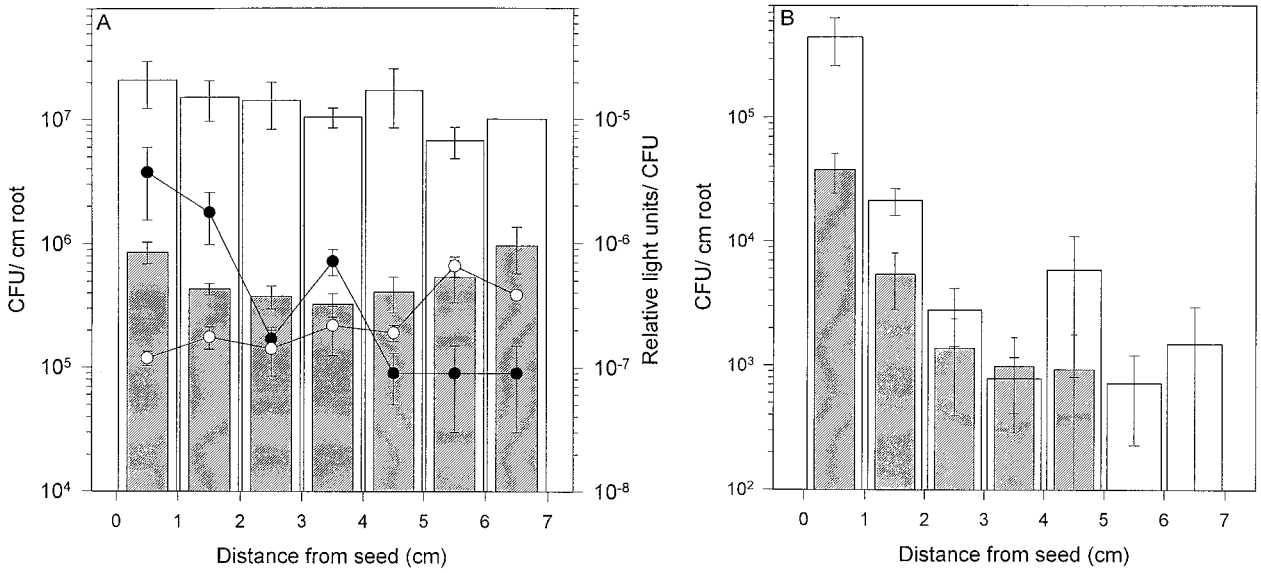


FIG. 2. Barley seeds were inoculated with *P. fluorescens* DF57-40E7 and planted in sterile soil (A) or natural soil (B). The population size of DF57-40E7 was determined as CFU (bars), and light emission (curves) was determined by luminometry in the rhizosphere soil samples (white bars, white symbols) and rhizoplane samples (grey bars, black symbols) at different parts of the roots. The data are presented as mean values ± standard errors.

In natural rhizosphere, the population of DF57-40E7 declined from ca. $4 \times 10^5$ CFU $cm^{-1}$ at the root base to ca. $10^3$ CFU $cm^{-1}$ at 7 cm from the base (Fig. 2B). Bioluminescence from DF57-40E7 was detected in the upper two root segments sustaining population densities above ca. $4 \times 10^3$ CFU $cm^{-1}$ (Fig. 3B). By luminometry, cell-specific bioluminescence values of $3 \times 10^{-7}$ and $1 \times 10^{-6}$ RLU $CFU^{-1}$ were recorded for these segments. In an additional experiment, DF57-40E7 was introduced both on seed and by soil inoculation. The four upper root fragments had population densities from $(3.2 \pm 0.8) \times 10^5$ to $(1.3 \pm 0.3) \times 10^5$ CFU $cm^{-1}$. The cell-specific bioluminescence varied from $(8.3 \pm 1.7) \times 10^{-7}$ to $(1.4 \pm 0.4) \times 10^{-6}$ RLU $CFU^{-1}$.

**DF57-P2 as a reporter for phosphate limitation.** DF57-P2 and DF57-40E7 established comparable population sizes in the gnotobiotic rhizosphere as shown by plate counts (compare Fig. 2A and Fig. 4A). The bioluminescence emitted by DF57-P2 demonstrated that phosphate limitation was encountered along the whole root system (Fig. 3C) and, in general, the variability of the cell-specific bioluminescence measurements was higher than that observed for DF57-40E7 (Fig. 4A). Assuming an average light output of $8 \times 10^{-8}$ RLU $CFU^{-1}$, the detection limit for DF57-P2 expressing the *lux* reporter system is ca. $2 \times 10^4$ CFU cm of $root^{-1}$.

To verify that DF57-P2 indeed reported phosphate limitation, gnotobiotic root systems colonized by DF57-P2 were transferred to phosphate buffer to reverse the conditions inducing bioluminescence. After 5 h, decreased luminescence was observed from roots transferred to phosphate buffer but not from roots transferred to 0.9% NaCl (Fig. 5), confirming that phosphate amendment turned off expression of the phosphate limitation response. Addition of phosphate buffer to roots colonized by the constitutive mutant DF57-40E7 led to a higher light output, showing that the phosphate amendment did not cause a nonspecific negative effect on cell activity (data not shown).

The cell distribution of DF57-P2 in natural rhizosphere was comparable to that of DF57-40E7 (Fig. 4B). Bioluminescence was not observed along the root either by photon counting imaging (Fig. 3D) or by luminometry, suggesting that DF57-P2 did not experience phosphate limitation. In these experiments the cell numbers of DF57-P2 were close to the detection limit for bioluminescence. To obtain a higher rhizosphere population we introduced DF57-P2 both on seed and by soil inoculation. We thereby obtained a population size of $4 \times 10^6$ to $2 \times 10^5$ CFU $cm^{-1}$ on the four root segments in proximity to the root base. This was above the detection limit for bioluminescence, but no light emission from DF57-P2 was detected. We therefore conclude that our failure to detect bioluminescence from DF57-P2 in natural rhizosphere was not caused by limited sensitivity of the detection system.

If DF57 did not experience phosphate limitation in a natural rhizosphere, it might be possible to induce a state of phosphate depletion. For example, adding nitrogen and carbon sources to the soil might lead to a balanced microbial growth response and hence to consumption of phosphate. When roots colonized by DF57-P2 were harvested from natural soil and incubated in a medium containing carbon and nitrogen sources, the cell concentration of DF57-P2 increased significantly (Table 1). Due to the high cell numbers, the average cell-specific bioluminescence could be determined to $4.2 \times 10^{-9}$ RLU $CFU^{-1}$, a level indicative of a noninduced reporter system.

The difference in phosphate availability between sterile soil and natural soil could hypothetically be an effect of sterilization or of the indigenous microbial population. To distinguish between these possibilities, microorganisms extracted from natural soil were added to the sterilized soil before barley seeds coated with DF57-P2 were planted. The presence of an indigenous microbial population inhibited root colonization by DF57-P2 compared to a gnotobiotic system, but, at the root segment proximal to the base, the population size of DF57-P2 was $7.4 \times 10^5 \pm 3.6 \times 10^5$ CFU $cm^{-1}$, or ca. 40 times above the detection limit for bioluminescence. As we did not detect light emission by the reporter bacterium, it appears that the presence of an indigenous microbial population prevents phosphate starvation of *P. fluorescens* DF57.

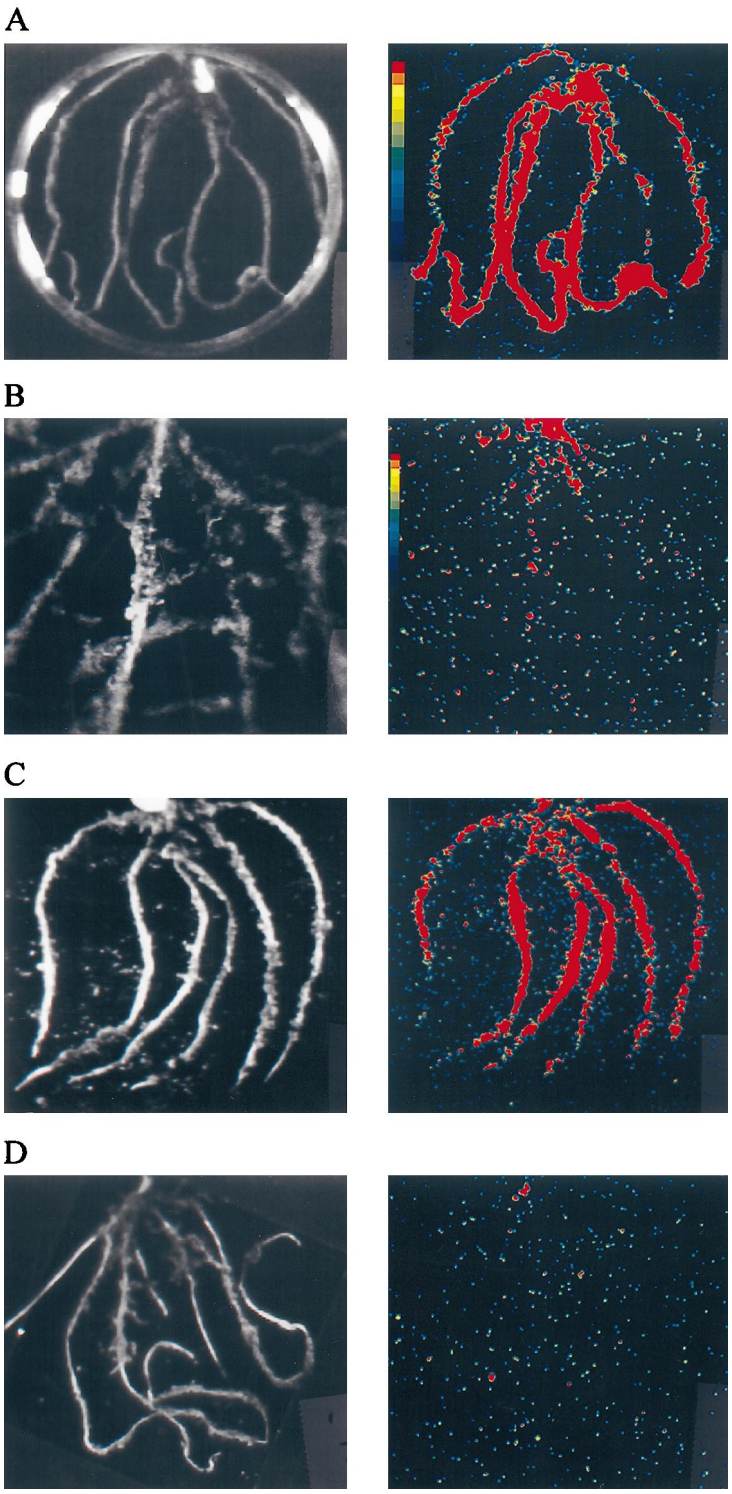


FIG. 3. Bright-field (left panels) and bioluminescence (right panels) images of 7-day-old barley root systems colonized by *P. fluorescens* DF57-40E7 or DF57-P2. (A) Seed inoculated with strain DF57-40E7 and planted in sterile soil; root lengths were ca. 7 cm. (B) Seed inoculated with strain DF57-40E7 and planted in natural soil; root lengths were ca. 15 cm, and the upper 5 cm of the roots are shown. (C) Seed inoculated with strain DF57-P2 and planted in sterile soil; root lengths were ca. 7 cm. (D) Seed inoculated with strain DF57-P2 and planted in natural soil; root lengths were ca. 15 cm. Representative plants are shown.

## DISCUSSION

**Root colonization by *lux*-tagged *P. fluorescens* DF57.** In this study we performed root colonization experiments with two *lux*-tagged reporter strains, DF57-40E7 (bioluminescence dependent on metabolic activity when aldehyde and $O_2$ are available) and DF57-P2 (bioluminescence expressed only during phosphate limitation, but also dependent on metabolic activity when aldehyde and $O_2$ are available). In gnotobiotic systems

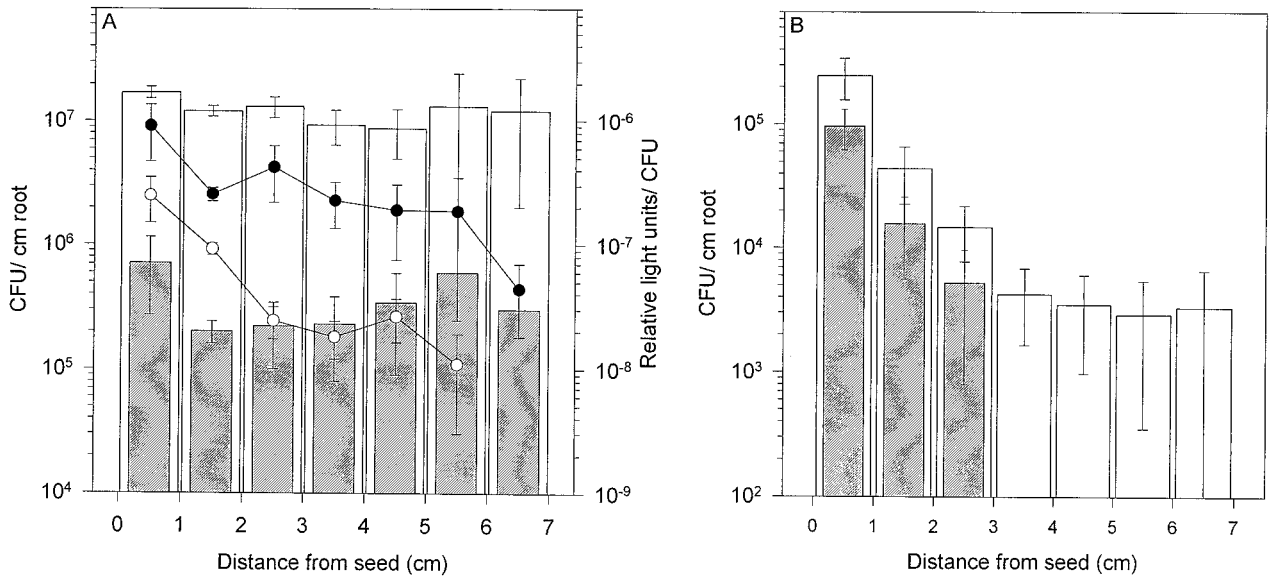


FIG. 4. Barley seeds were inoculated with *P. fluorescens* DF57-P2 and planted in sterile soil (A) or natural soil (B). The population size of DF57-P2 was determined as CFU (bars), and light emission (curves) was determined by luminometry in the rhizosphere soil samples (white bars, white symbols) and in the rhizoplane samples (grey bars, black symbols) at different parts of the roots. The data are presented as mean values ± standard errors. The detection level for bioluminescence (when induced) by luminometry measurements is ca. $2 \times 10^4$ CFU cm of root$^{-1}$. Bioluminescence was not detected in natural soil.

the cells of both DF57 reporter strains were equally distributed along the root, whereas in natural soil systems the population decreased gradually towards the root tip, as seen for other pseudomonads (2, 3, 24), probably due to competition from the indigenous microorganisms (23). The detection limit for bioluminescence emitted by the *lux*-tagged strains in the rhizosphere was comparable to that reported for other chromosomal *lux* inserts in *Pseudomonas* spp. (1, 8).

**Metabolic activity of *P. fluorescens* DF57-40E7 in the barley rhizosphere.** DF57-40E7 populations in 7-day-old rhizosphere generally expressed higher cell-specific luminescence than did populations in bulk soil. The results suggest, therefore, that on average the cells were more active in the rhizosphere than in the bulk soil. This may reflect that *P. fluorescens* strains are able to exploit a number of simple carbon sources present in exudates (39). In contrast, the cellular activity of a *Flavobacterium* strain, a poor competitor for root exudates, that was isolated from bulk soil was lower in the rhizosphere than in the bulk soil (12, 31).

The average cell-specific light emission of DF57-40E7 varied among different locations in the rhizosphere. For example, populations from the rhizosphere soil had higher activity at the root tip than at the root base. A comparable distribution of metabolic activity has been reported for the total bacterial population in pine rhizosphere soil by a dehydrogenase activity assay (31) and was ascribed to high rhizodeposition at the young root tips.

In contrast, the rhizoplane population of DF57-40E7 exhibited the lowest cell activity at the root tip, suggesting that growth conditions differed from those in the rhizosphere soil. This notion is supported by previous observations of a selection of specific bacterial subpopulations by different rhizosphere habitats (28, 41). The metabolic activity of bacteria inhabiting the rhizoplane at the root tip may be limited for nutrients, substrates, or electron acceptors by competition with the active plant cells in this area (16). Furthermore, noxious substances released by the plant cells may be able to inhibit the metabolic activity, and hence bioluminescence, of a constitutive reporter strain (33). Possibly, a panel of reporter bacteria sensing different stresses may be useful to address the factors in which the rhizoplane and rhizosphere soil differ.

**Nucleotide sequence of the gene interrupted by Tn*5-luxAB* in strain DF57-P2.** Strain DF57-P2 has previously been shown to express bioluminescence exclusively during phosphate starvation (21). The deduced N-terminal amino acid sequence of the gene interrupted by Tn*5-luxAB* shows 59% homology to the N terminus of a hypothetical *E. coli* protein identified by computer-assisted analysis of intergenic nucleotide sequences (4). Neither the function nor the regulation of the *E. coli* protein have been described. However, two putative Pho boxes appear in the promoter region of the P2 gene. They have 10 and 15 nucleotides in common with the *E. coli* consensus sequence of 18 nucleotides. This is comparable to homologies of Pho boxes identified in *E. coli* and *P. aeruginosa* (26, 37), suggesting that the *P. fluorescens* P2 gene is a new member of the phosphate regulon. Experiments with fluorescent pseudomonads, including *P. fluorescens*, have shown that genes activated by phosphate limitation are generally expressed at phosphate concentrations below 35 to 100 μM (7, 11, 20, 27).

**Phosphate availability in the rhizosphere.** In gnotobiotic rhizosphere systems, DF57-P2 reported phosphate starvation through emission of bioluminescence that was repressed by the addition of phosphate to the root system. Hence, DF57-P2 indeed reports limitation of this nutrient in the rhizosphere, an important finding as promoter activities in complex environments may be determined by signals different from those identified in the laboratory (6). We were able to show a large variability in the phosphate starvation response among cells residing in different locations along the root. This variability was predicted by de Weger et al. (7), who demonstrated phosphate limitation in the gnotobiotic rhizosphere by a *lacZ*-tagged reporter strain but were not able to distinguish between cells in different locations due to the limited sensitivity of the *lacZ* system.

The DF57-P2 reporter strain was not induced in a natural rhizosphere, indicating that the cells did not encounter phos-

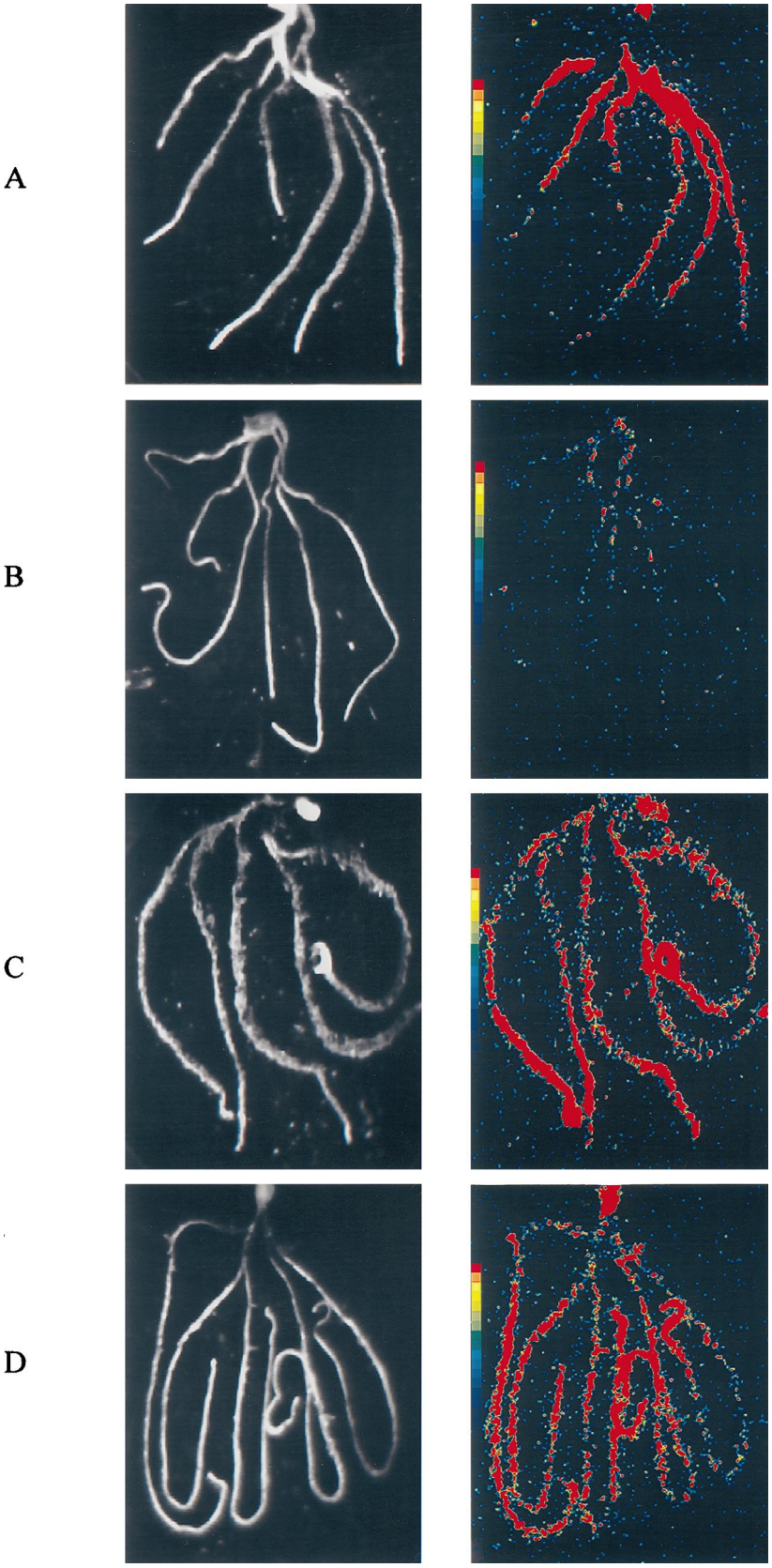


FIG. 5. Barley seeds were inoculated with *P. fluorescens* DF57-P2 and planted in sterile soil. Bright-field exposures (left panels) and bioluminescence images (right panels) were taken immediately after harvesting plants (A and C). Afterwards, roots from the plant in panel A were placed in phosphate buffer (B), and roots from the plant in panel C were placed in 0.9% NaCl (D). Representative plants are shown.

TABLE 1. Population size and cell-specific bioluminescence of strain DF57-P2 in natural rhizosphere and in natural rhizosphere after amendment with carbon and nitrogen sources[a]

| Root fragment[b] | Natural rhizosphere | | Amended rhizosphere | |
|---|---|---|---|---|
| | Population size ($10^5$ CFU $cm^{-1}$) | Cell-specific luminescence (RLU $CFU^{-1}$) | Population size ($10^7$ CFU $cm^{-1}$) | Cell-specific luminescence ($10^{-9}$ RLU $CFU^{-1}$) |
| 1 | 2.6 ± 0.4 | BD[c] | 2.5 ± 0.7 | 4.9 ± 2.8 |
| 2 | 2.3 ± 0.6 | BD | 2.7 ± 1.4 | 4.5 ± 1.9 |
| 3 | 1.8 ± 0.4 | BD | 2.3 ± 0.8 | 3.9 ± 0.8 |
| 4 | 1.9 ± 0.7 | BD | 2.0 ± 0.6 | 3.5 ± 1.4 |

[a] Data are means ± standard errors.
[b] Numbered in order of increasing distance from base.
[c] BD, below limit of detection.

phate limitation. A comparable observation was made by de Weger et al. for another *Pseudomonas* phosphate starvation reporter strain (7), but those authors were unable to exclude the possibility that their negative results were caused by the inadequate sensitivity of their reporter system. We do not find it likely that sensitivity problems affected our conclusions, as we obtained population sizes of DF57-P2 in natural rhizosphere well above the detection limit for bioluminescence of ca. $2 \times 10^4$ cells $cm^{-1}$. Furthermore, addition of a carbon-nitrogen nutrient mixture to roots colonized by the strain resulted in cell growth, suggesting that indigenous phosphate sources were not limiting. This result is in agreement with the lack of induction of the phosphate limitation reporter system in carbon-nitrogen amended systems.

The presence of an indigenous microbial population reduced the phosphate starvation response of DF57-P2 cells. It likewise caused a decrease in the DF57-P2 population, but that population was maintained at 40 times above the detection limit for bioluminescence on the root segment proximal to the root base. Hence, the indigenous microbial population might increase phosphate availability through production of phosphatases or organic acids, and/or phosphate might be mobilized by protozoan grazing.

Previous applications of biological sensors have demonstrated that pseudomonads may not experience severe iron limitation in the bean rhizosphere (25) and that *Pseudomonas* responds to root exudates in the wheat rhizosphere (40). Hence, biological sensors appear to be a useful tool for studies of nutrient conditions in rhizosphere environments. However, due to the physiological heterogeneity of bacteria inhabiting natural environments, there is a need for further development of the reporter systems to allow single-cell studies.

## ACKNOWLEDGMENTS

This study was supported by Danish Agricultural and Veterinary Research Council grant 9313839, the NOVO Nordisk Foundation, and the Danish Biotechnology Program.

We acknowledge the skilled technical assistance of Anita Jørgensen and Lene M. Nielsen and thank Bjarne M. Stummann and Frans J. de Bruijn for help and advice during the cloning and sequencing experiments.

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