Use of Phosphate-Reporter Bacteria to Study Phosphate Limitation in the Rhizosphere and in Bulk Soil

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Many relevant bacterial-plant interactions occur in the rhizosphere of the plant, but the conditions under which these interactions take place are largely unknown. In this study, we focused on the phosphate availability in the rhizosphere and in bulk soil. Using a promoterless Tn*lacZ*, four phosphate-reporter strains of *Pseudomonas putida* strain WCS358 were constructed that respond to phosphate limitation by the production of β-galactosidase. These strains did not respond to other nutrient-limiting or stress conditions. In the phosphate-reporter strain LP7, showing the highest levels of β-galactosidase activity upon phosphate-limited growth, β-galactosidase was induced when the level of phosphate dropped below 30–35 μM. When cells of this strain were growing under gnotobiotic conditions in the rhizosphere of potato, tomato, or radish plants, or in bulk soil or sand, they sensed phosphate limitation as judged from the significant increase in β-galactosidase activity in these cells. This study showed that reporter bacteria can be used to report on the growth conditions in the rhizosphere and in bulk soil or sand. From these results, it can be predicted that the use of a combination of different reporter bacteria responding to various conditions (e.g., limitation for nitrogen, carbon, or iron) will reveal the growth conditions in ecologically relevant niches.

Microbe-plant interactions in the rhizosphere have a great impact on agriculture; e.g., pathogens can harm crop plants, and plant-beneficial bacteria can increase yields (Lugtenberg *et al.* 1991). To control these interactions in an agronomically attractive way, better knowledge of the mechanisms involved is required; however, such a study is hampered by our poor knowledge of the conditions in the rhizosphere. Another limiting factor is the lack of appropriate techniques to approach this niche. Novel methods to detect bacteria in the environment have recently been developed (e.g., de Weger *et al.* 1991; Sharma and Signer 1990; Steffan and Atlas 1988). However, techniques to unravel the conditions under which microbes are growing in the rhizosphere are still to be developed. In this paper we describe a novel molecular approach to study these conditions.

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Phosphorus is an essential element for cells. In bacteria, it is present in nucleic acids, phospholipids, lipopolysaccharides, and various metabolites present in the cytoplasm. Several bacteria (e.g., *Escherichia coli, Bacillus* spp., *Pseudomonas* spp.) possess a special system that enables the bacterium to efficiently scavenge the phosphate present in its surroundings (Torriana-Gorini *et al.* 1987). This system is induced as soon as the bacteria experience a phosphate limitation, and it includes specific uptake proteins, alkaline phosphatase(s), and phosphate-binding proteins (Torriana-Gorini *et al.* 1987).

In soils, phosphate is retained either by precipitation as Ca-, Fe-, or Al-phosphates or by adsorption to these cations at the surface of soil minerals (Sample *et al.* 1980). Various chemical analyses are in use to assess the phosphate contents in soil; however, these methods do not reveal which portion of the phosphate is actually available to microorganisms. This study addresses the question of whether the levels of phosphate that are available to microorganisms in the rhizosphere or in the soil are limiting or sufficient.

We made use of phosphate-limitation-inducible promoters to construct "phosphate-reporter bacteria," in which the reporter-gene *lacZ* is turned on only under phosphate-limited conditions. After reisolation of these reporter bacteria from soil or from the rhizosphere, the level of the indicator enzyme, β-galactosidase, indicates whether the bacteria have been growing under phosphate-limiting or phosphate-sufficient conditions.

RESULTS

Isolation of phosphate-reporter strains.

Pseudomonas putida strain WCS358 was mutagenized using the promoterless Tn*lacZ* construct B20 (Simon *et al.* 1989). Individual mutants were screened on X-Gal-containing phosphate-deficient and phosphate-sufficient HBM minimal-medium plates. Out of 2,500 mutants, five (LP7, LP10, LP11, LP17, and LP18) were found in which β-galactosidase was induced on the phosphate-deficient plate and not on the phosphate-sufficient plate. Another mutant, designated LC2, was kept because of its high constitutive expression of β-galactosidase activity.

Characterization of the phosphate-reporter strains.

Insertions of Tn*lacZ* in these mutants were analyzed by Southern blotting of digests of *Eco*RI, *Bam*HI, and *Hin*dIII of the total DNA. The plasmid carrying B20 was used as a probe and showed different hybridizing bands in each of the three digests of strains LP7, LP10, LP11, and LP18, indicating that the mutations are located on different fragments. Strain LP17 showed a hybridization pattern identical to that of strain LP7 in each of the three digests, suggesting that the Tn*lacZ* was inserted in the same DNA fragment in these two strains (data not shown).

When the cells were grown to stationary phase in the minimal HBM medium containing different levels of phosphate, all mutants turned on β-galactosidase in the medium containing 0.4 mM phosphate (Fig. 1), but the levels of induction varied. For mutants LP10, LP11, and LP18, low levels of β-galactosidase were induced, in contrast to strains LP7 and LP17, which showed high levels of β-galactosidase under phosphate-limited growth. Strain LC2 showed high levels of β-galactosidase at all phosphate levels (Fig. 1).

We examined whether β-galactosidase is also induced in these mutants under other growth conditions, such as iron, nitrogen, or carbon starvation, or high-osmotic conditions. It appeared that under none of these conditions β-galactosidase was induced in any of the LP mutants. This result strongly suggests that the *lacZ* gene in these mutants is induced exclusively by phosphate limitation. LC2 induced high levels of β-galactosidase activity under all these conditions.

The production of alkaline phosphatase was not affected in any of the mutants. Tests on serial dilutions of phosphate in HBM medium plates containing either X-phosphate, the substrate for alkaline phosphatase, or X-Gal, the substrate for β-galactosidase, resulted in all four mutants in the induction alkaline phosphatase or β-galactosidase at similar phosphate concentrations (0.3–0.4 mM) in the medium. This suggests that the promoters regulating β-galactosidase production in these mutants are coregulated with one of the best-studied phosphate-limitation-inducible proteins, alkaline phosphatase.

Analysis of the cell envelope proteins in the wild-type strain WCS358 did not reveal any proteins that were induced under phosphate-limited growth. The mutants did not differ from their parent strain in cell envelope protein pattern. Growth curves in phosphate-poor and phosphate-rich media (minimal medium HBM or complex medium PPM) showed that growth of the four mutants was not affected compared with that of the wild-type strain WCS358 (data not shown). Neither of these results revealed in which genes the Tn*lacZ* had been inserted. The mutants can be used as phosphate-reporter strains in the rhizosphere, since 1) the affected gene products appear not to be crucial for growth under phosphate-poor conditions, and 2) the reporter gene is most likely specifically induced by phosphate limitation. For rhizosphere studies we selected strain LP7, since it has the highest level of β-galactosidase induction after phosphate-limited growth.

To test whether rhizosphere pseudomonads experience phosphate limitation at the same phosphate levels as the phosphate-reporter strain LP7, a series of 20 rhizosphere pseudomonads was tested for the phosphate concentration that induces alkaline phosphatase, a representative of one of the phosphate-limitation-inducible proteins. The strains were streaked on a series of HBM plates containing increasing amounts of phosphate and supplemented with the substrate for alkaline phosphatase, X-phosphate. After 1 day of growth, alkaline phosphatase could not be detected in two strains at any concentration tested, but in most of the remaining strains (90%), including strain LP7, alkaline phosphatase was induced on plates supplemented with phosphate up to 0.3 or 0.4 mM. Alkaline phosphatase was not induced on plates supplemented with phosphate at concentrations of 0.5 mM or higher.

The actual phosphate concentration at which β-galactosidase is induced in strain LP7 was determined by taking samples from logarithmic cultures in PPM medium over a time course. These cells were assayed for their β-galactosidase activity, and the phosphate concentration in the culture supernatant was determined. This was done for PPM cultures

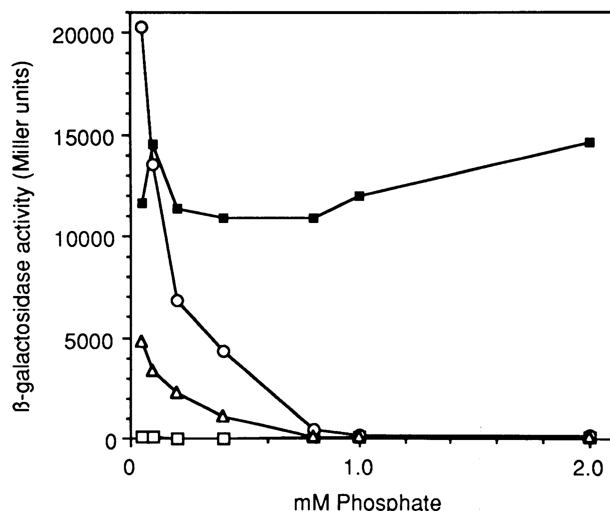


Fig. 1. β-Galactosidase activity of stationary-phase cells of the wild-type strain WCS358 (□) and mutant strains LP18 (Δ), LP7 (○), and LC2 (■) at various phosphate concentrations in HBM minimal medium. For mutants LP7 and LP17, similar curves were obtained, whereas the curves of mutants LP10 and LP11 were similar to the one of mutant LP18.

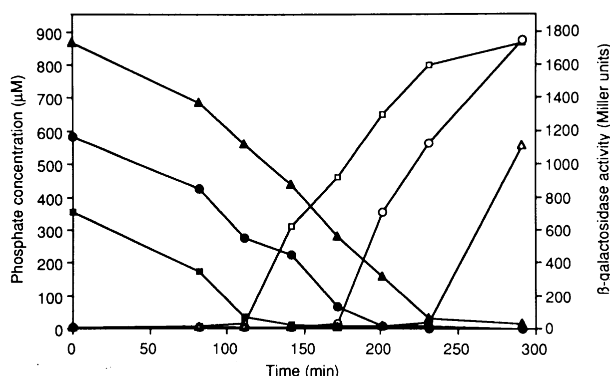
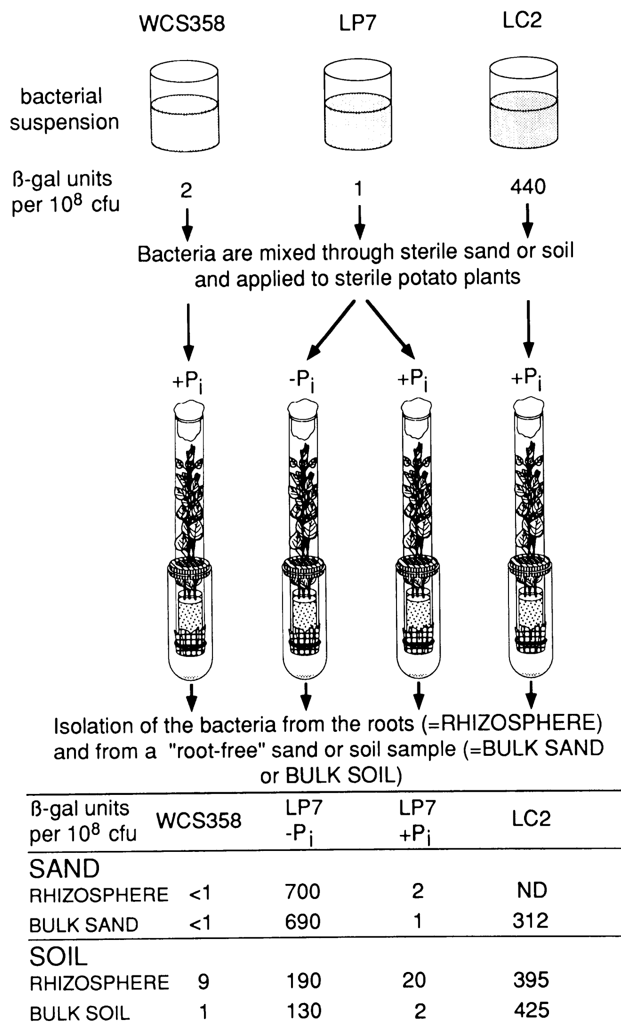


Fig. 2. β-Galactosidase activity of cells of strain LP7 and the phosphate concentration in μM during growth. At time zero, cells of strain LP7 were inoculated into PPM medium without any added phosphate (□, ■), or supplemented with 0.2 mM (○, ●) or 0.4 mM (Δ, Δ) phosphate. At various times during growth, samples were taken from the cultures, and the β-galactosidase activity of the cells (open symbols) and the phosphate concentration in the filtered supernatant medium (closed symbols) were determined. In each of these three cultures, the β-galactosidase activity in the cells increased as soon as the phosphate concentration dropped below 30 to 35 μM.

without added phosphate and for cultures supplemented at 0.2 or 0.4 mM. In these three cultures, β-galactosidase activity was induced in the cells of LP7 as soon as the phosphate level dropped below 30–35 μM (Fig. 2).

Use of the phosphate-reporter strain LP7 in a gnotobiotic system.

For every plant assay, the phosphate-reporter strain LP7 was grown in phosphate-rich medium (PPM or HBM), thus preventing the expression of β-galactosidase at the beginning of the experiment (Fig. 3). These cells were applied to plants growing in phosphate-rich or in phosphate-poor sand or soil. To test the background levels of the β-galactosidase assay in the samples, the wild-type strain WCS358 was used as a negative control in all experiments. As a positive control, plants bacterized with the β-galactosidase-constitutive mutant LC2 were used (Fig. 3).



ß-gal units per 10^8 cfu	WCS358	LP7 $-P_i$	LP7 $+P_i$	LC2
SAND				
RHIZOSPHERE	<1	700	2	ND
BULK SAND	<1	690	1	312
SOIL				
RHIZOSPHERE	9	190	20	395
BULK SOIL	1	130	2	425

Fig. 3. Schematic representation of the plant experiments performed using the gnotobiotic system. The β-galactosidase activities of the wild-type strain WCS358, the phosphate-reporter strain LP7, and the constitutive mutant LC2 were determined at the beginning of the experiment and after reisolation of the cells from the rhizosphere and bulk sand (upper part of the table) and from the rhizosphere and bulk soil (lower part of the table). This depiction represents four experiments. ND = not determined.

The number of cells reisolated from the rhizosphere or the bulk sand after 2 wk in the greenhouse varied between 10^6 and 10^7 cfu/ml of PBS. The β-galactosidase assays on the bacteria isolated from the rhizosphere or sand from the tubes bacterized with mutant strain LC2 showed that β-galactosidase could be detected in these sandy samples (Fig. 3). This was also demonstrated for the phosphate-reporter bacteria reisolated from the rhizosphere and bulk sand in the phosphate-poor system ($-P_i$). In these cells β-galactosidase had been induced 300- to 700-fold (Fig. 3), while the same cells reisolated from the rhizosphere or sand in the phosphate-rich system ($+P_i$) showed levels of β-galactosidase similar to those obtained for the wild-type strain, i.e., background levels (Fig. 3).

When sterilized soil was used instead of sand, the number of bacteria reisolated from the rhizosphere and the bulk soil again varied between 10^6 and 10^7 cfu/ml of PBS. β-Galactosidase activity could be detected in the suspensions of the constitutive mutant LC2 (Fig. 3). The suspensions isolated from the non-phosphate-supplemented soil ($-P_i$) containing the phosphate-reporter strain showed a 10- to 60-fold increase in β-galactosidase activity compared with the suspensions isolated from the phosphate-rich soil ($+P_i$, Fig. 3).

These results showed that phosphate-reporter bacteria can be used to sense the availability of phosphate in the rhizosphere, in sand as well as in soil. In the various experiments performed in this gnotobiotic system, the phosphate reporter always showed a much higher β-galactosidase activity in the phosphate-poor system than in the phosphate-rich system, but the levels of this increase varied between 10 and 700 times.

The phosphate-reporter strain was also tested in the rhizosphere of tomato and radish plants in the gnotobiotic system containing the clay soil. In both plants, the phosphate-reporter strain showed a significant increase in β-galactosidase activity in the natural soil compared with that in the phosphate-rich soil (data not shown).

Use of the phosphate-reporter strain LP7 in a non-gnotobiotic system.

A similar set of experiments was performed in the non-gnotobiotic system. First, natural clay soil from the Flevopolder was used. When bacterized potato stem cuttings were planted in this nonsterile soil, approximately 10^5 to 10^6 cfu/ml was reisolated from the rhizosphere. No significant differences in β-galactosidase between the phosphate-reporter bacteria or the wild-type cells reisolated from the phosphate-rich or the non-phosphate-supplemented soil or rhizosphere were found. This was not due to failure of the β-galactosidase assay in these natural soil samples since β-galactosidase could be detected in the rhizosphere or soil suspensions of strain LC2 (data not shown). Background levels in these samples were higher than in the gnotobiotic system (approximately 60–90 units/10^8 cfu).

When nonsterile sand was used instead of natural soil, higher numbers of phosphate-reporter cells were reisolated (approximately 10^6 to 10^7 cfu/ml), and again an induction in β-galactosidase activity was found for the suspensions containing the phosphate-reporter strain isolated from the phosphate-poor system, whereas the suspensions from the phosphate-rich system showed background levels of β-galactosidase (Table 1).

DISCUSSION

The plant-growth-promoting, root-colonizing *Pseudomonas putida* strain WCS358 was used to construct phosphate-reporter bacteria. The promoterless Tn*lacZ* construct B20 (Simon *et al.* 1989) was used to isolate four independent mutants that produced β-galactosidase under phosphate-limiting conditions. The Tn*lacZ* in these mutants most likely was inserted into different genes, all of which are induced by phosphate limitation. It is known that some genes or operons induced by phosphate limitation are also induced by other adverse conditions (Groat *et al.* 1986; Nyström *et al.* 1992). However, the β-galactosidase activity in these mutants was only induced by phosphate limitation and not by other stress conditions like osmotic stress, or limited iron, carbon, or nitrogen. This indicates that the β-galactosidase activity in these mutants is regulated specifically by phosphate, which makes them ideal tools to study phosphate availability in the rhizosphere.

Characterization of the mutants did not reveal in which phosphate-regulated gene or operon the Tn*lacZ* was inserted. The mutants did not differ from the wild-type strain WCS358 in 1) growth rate in phosphate-sufficient or deficient medium, 2) alkaline phosphatase production, or 3) composition of cell envelope proteins. In full-grown colonies of the four mutants, β-galactosidase as well as alkaline phosphatase was induced on plates containing 0.4 mM phosphate or less. This suggests that the promoters regulating β-galactosidase in these mutants as well as the promoter for alkaline phosphatase are subject to the same regulatory mechanism.

In the majority of a series of 20 rhizosphere pseudomonads, including strain LP7, alkaline phosphatase was induced on plates containing 0.3 or 0.4 mM phosphate. Although growth variations between the different strains may slightly influence the outcome of this assay, the result indicates that there are no major differences between the phosphate concentrations that induce β-galactosidase in phosphate-reporter LP7 and those that induce alkaline phosphatase in the majority of rhizosphere pseudomonads. This shows that strain LP7 is representative of tested rhizosphere pseudomonads with respect to sensing phosphate limitation.

These assays with colonies on plates do not indicate the precise phosphate concentration that is limiting to the cells. Using cells in liquid culture, we found that as soon as the level of available phosphate dropped below 30–35 μM, the β-galactosidase activity was turned on (Fig. 2). This level of phosphate is thus the actual phosphate concentration that is experienced as limiting by the cells. Cells in a colony on a plate will start to produce β-galactosidase as soon as the available phosphate on their growth spot drops below that level.

Table 1. β-Galactosidase activity of cells of *Pseudomonas putida* strain WCS358, the phosphate-reporter strain LP7, or the constitutive β-galactosidase-producing strain LC2 re-isolated from a non-gnotobiotic system containing sand[a]

		LP7[b]		
Substrate	**WCS358[b]**	**$-P_i$**	**$+P_i$**	**LC2[b]**
Rhizosphere	20	730	13	1,300
Bulk sand	20	940	5	2,000

[a] β-Galactosidase activity is expressed as units/10^8 cfu.
[b] Cells were re-isolated from the rhizosphere or from the bulk sand.

The phosphate-reporter strain appeared to be a valuable tool to detect phosphate-deficiency in the rhizosphere of different plants as well as in bulk soil. In the gnotobiotic system phosphate-reporter cells reisolated from the rhizosphere of potato plants and from the bulk sand or soil showed a significant increase in β-galactosidase activity compared with the same cells reisolated from phosphate-rich sand or soil. Similar results were obtained for the rhizospheres of tomato and radish. These experiments show that bacteria growing in the rhizosphere and bulk clay soil from an agricultural field experience a phosphate limitation. Chemical analyses on samples of this soil revealed a water-soluble phosphate content of 0.003%. At a moisture content of 13%, this amount may reflect a concentration of approximately 2.4 mM phosphate. This level of phosphate is not limiting for rhizosphere pseudomonads. However, during cell growth phosphate will be used, resulting in a local reduction in available phosphate concentration. Our results, showing induction of β-galactosidase in the phosphate-reporter cells reisolated from the soil or the rhizosphere, indicate that the cells had been growing in niches where the available phosphate had dropped below the inducing concentration of 30–35 μM. This implies that not all cells in the rhizosphere or bulk soil or sand experience phosphate limitation at the same time, depending on the niche where they are growing. This variation between cells may cause the surprising differences between the levels of β-galactosidase induction that were found in different experiments.

Unfortunately, in a non-gnotobiotic system using natural clay soil, no significant induction in β-galactosidase activity was observed in the phosphate-reporter cells reisolated from the rhizosphere or bulk soil. This negative result was not due to a failure of the β-galactosidase assay, since the cells of the constitutive mutant LC2 could be detected in the soil samples. A possible explanation may be that little phosphate limitation is experienced by the phosphate-reporter cells in the nonsterile natural soil, e.g., because the endogenous microbial population may increase the availability of phosphate for *Pseudomonas*. The resulting low β-galactosidase content, combined with the somewhat higher background level of β-galactosidase in clay soil, and the somewhat lower numbers of cells that were reisolated from the nonsterilized

Table 2. Characteristics of the natural soil used in the plant experiments[a]

Component	Percent
Particle distribution	
0–2 μm	25.3
2–16 μm	12.8
16–50 μm	20.1
50–105 μm	6.9
105–150 μm	0.2
150–2,000 μm	34.7
Organic matter	0.6[b]
$CaCO_3$	5.4[b]
Water-soluble phosphate	0.003[b]
pH	7.5

[a] The soil is a clay soil from the Flevopolder in the Netherlands mixed with sand as described in Materials and Methods. The analysis was done by the Bedrijfslaboratorium voor grond- en gewasonderzoek in Oosterbeek, the Netherlands.
[b] Values given are percentages of oven-dried soil.

soil may explain the negative result of our trials using natural soil.

Independent from our approach, Lindow and Loper (Lindow 1992; Loper *et al.* 1993) reported on a similar method using the ice-nucleation gene of *P. syringae* as a reporter gene to study iron availability in the rhizosphere. This system also functions under nonsterile soil conditions and is more sensitive than our system, which is a definite advantage over our system. However, the method using β-galactosidase does not require special equipment and is very fast and easy to perform. It is a very valuable and easy method for determining the growth conditions in systems where 10^7 cfu or more cells can be reisolated, e.g., under gnotobiotic or semisterile growth conditions. With lower numbers of reisolated cells (10^5 to 10^6 cfu), this method will also be valuable, provided the induction of β-galactosidase is very strong. This is illustrated by our experiments using natural soil, where 10^5 to 10^6 cfu was reisolated and where the β-galactosidase activity of the constitutive high-level-producing mutant strain LC2 could be detected in the soil samples. The use of other reporter bacteria that respond to other limitations, e.g., to iron, nitrogen, or carbon, or to pH variation or osmovariation will increase our knowledge of the conditions in selected niches.

MATERIALS AND METHODS

Bacteria and growth conditions.

Relevant characteristics of the potato root isolate *Pseudomonas putida* WCS358 have been described elsewhere (de Weger *et al.* 1986; Geels and Schippers 1983). A Tn*5*-labeled derivative of strain WCS358 was used for studies in the rhizosphere and soil (de Weger *et al.* 1989). *Pseudomonas* strains, assumed to represent the pseudomonad population in the rhizosphere, were isolates from the rhizosphere of potato, wheat, and cotton. These strains were provided by the following colleagues: B. Schippers, Baarn, the Netherlands; J. van der Wolf, Wageningen, Netherlands; J. Kloepper, Auburn, AL; D. Weller, Pullman, WA; J. Fredrickson, Richland, WA; and M. Ryder, Adelaide, Australia. *Escherichia coli* strain S17-1, harboring the promoterless Tn*lacZ* construct B20 (Simon *et al.* 1989), was provided by R. Simon. For phosphate limitation studies, *Pseudomonas* strains were grown at 28° C for 16 hr under vigorous aeration in the following complex or minimal medium: the complex proteose peptone medium (PPM; Cheng *et al.* 1970) was supplemented with 0.2 mM phosphate (phosphate-deficient) or 2.0 mM phosphate (phosphate-sufficient). The HEPES (sodium *N*-2-hydroxy-ethylpiperazine-*N*′-2-ethanesulfonate)-buffered minimal medium (HBM, Hancock *et al.* 1982) was supplmented with 10 μM $FeCl_3$ (to prevent iron limitation) and 0.1 mM phosphate (phosphate-deficient) or 1.5 mM phosphate (phosphate-sufficient). Growth conditions limiting for iron and nitrogen or growth under high-osmotic conditions were studied on the following solid media: phosphate-sufficient HBM without added iron (Fe-limited plates); phosphate-sufficient HBM containing 0.2 mM instead of 0.7 mM $(NH_4)_2SO_4$ (N-limited plates); phosphate-sufficient HBM medium with 0.55 M NaCl or 0.5 M sucrose (high-osmotic plates). The effect of carbon starvation was tested in liquid PPM containing 0.02% instead of 0.6% glucose. The antibiotics nalidixic acid and kanamycin were used in final concentrations of 25 μg/ml and 100 μg/ml, respectively. 5-Bromo-4-chloro-3-indolyl-β-*d*-galactoside (X-Gal, 40 μg/ml) was used as an indicator for β-galactosidase activity. Alkaline phosphatase production was determined on HBM plates containing 5-bromo-4-chloro-3-indolyl-phosphate (X-phosphate, 40 μg/ml). If necessary, media were solidified with 1.8% (w/v) agar (Difco Laboratories, Detroit, MI).

Isolation of phosphate-reporter bacteria.

A transposon mutagenesis procedure, using the promoterless Tn*lacZ*-B20 construct was performed as described previously (Marugg *et al.* 1985). The mutants were screened for β-galactosidase activity induced under phosphate limitation by streaking individual colonies on phosphate-sufficient and phosphate-deficient minimal medium (HBM) plates supplemented with X-Gal.

Quantitative colorimetric assays.

The β-galactosidase activities of bacteria were quantitatively determined using the substrate *O*-nitrophenyl-β-galactopyranoside (ONPG) according to the method of Miller (1972). Culture supernatants filtered through a 0.4-μm membrane filter were used to measure the phosphate concentration in the medium of a logarithmic culture. Total phosphate was determined using the Aquamerck phosphate test (Merck BV. Darmstadt, Germany), which is based on the formation of the blue phosphate-molybdate complex.

Cell envelope analysis.

Cell envelopes of cells grown in HBM medium were isolated by differential centrifugation after disruption of the cells by ultrasonic treatment (Lugtenberg *et al.* 1975). Cell envelope proteins were solubilized in the sample buffer for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and subjected to SDS-PAGE as described previously (Lugtenberg *et al.* 1975; de Weger *et al.* 1986). Proteins were stained with fast green.

DNA analysis.

Total DNA was extracted as described by Maniatis *et al.* (1982). DNA was digested with restriction endonucleases (*Bam*HI, *Eco*RI, and *Hin*dIII), electrophoresed in 0.7% agarose gels and transferred to an enhanced chemiluminescence (ECL) Hybond-H^+ membrane (Amersham International, Amersham, UK). For hybridization, the plasmid containing the B20 construct (Simon *et al.* 1989) was labeled with horseradish peroxidase. Hybridization and detection of the peroxidase-labeled probe was performed as described in the ECL manual (Amersham).

Gnotobiotic assay.

In the assay, either a quartz sand or a clay soil taken from agricultural fields in the Flevopolder, the Netherlands, were used (Fig. 3). The quartz sand varied in diameter from 0.1 to 0.3 mm and was washed with water, autoclaved (2 hr at 120° C), and dried before use. Two parts clay soil from the Flevopolder was mixed with 1 part sand. The characteristics of the resulting soil are given in Table 2.

Phosphate-reporter bacteria or control strains (10^8 cfu/ml) were harvested and resuspended in phosphate-deficient (no

phosphate) or phosphate-sufficient (4 mM phosphate) plant nutrient solution containing 5 mM $Ca(NO_3)_2$, 5 mM KNO_3, 2.0 mM $MgSO_4$, and micronutrients (Hofland *et al.* 1989). These suspensions were mixed through the sand to obtain a 10% moisture content. The clay soil was sterilized by gamma radiation (^{60}Co, 2.5 mrad) and brought to a 13% moisture level by addition of a bacterial suspension (10^8 cfu/ml) in water (phosphate-deficient) or in 20 mM phosphate solution (phosphate-sufficient). The soil or sand was put into a sterile glass cylinder capped with cotton (see Fig. 3). On top of the sand or soil column, 2 ml of water agar was pipetted to prevent desiccation. Sterile potato plantlets (cultivar Bintje) were cultivated in culture vessels (type GA7; Magenta Corp., Chicago, IL) on medium as described by Murashige and Skoog (1962), final pH 5.8, supplemented with 2.0% sucrose and solidified with 0.8% agar. Freshly cut plantlets were dipped into the appropriate bacterial suspension (10^8 cfu/ml) and planted in the agar layer in the tube. After 2 wk in the growth chamber (18° C, 16 hr light), bacteria were reisolated from the root system by mixing the roots vigorously in phosphate-buffered saline (PBS; i.e., 0.9% NaCl in 10 mM phosphate, pH 7.2). A sample of 5 g from the bulk sand or soil was also taken and mixed vigorously in PBS. From the suspensions, a sample was diluted and plated on King's medium B containing kanamycin (100 μg/ml) using a Spiral plater (model CU, Spiral System Instruments, Bethesda, MD). Two milliliters of the rhizosphere suspension was assayed in a β-galactosidase assay. The β-galactosidase activity of the suspensions is expressed as β-galactosidase units per 10^8 cfu.

The experiment was also performed using surface-sterilized tomato and radish seeds instead of sterile potato plantlets. After surface-sterilization by the method of van Peer (1990), the seeds were allowed to germinate on King's medium B plates, and after 2 days they were bacterized and planted in the tubes containing soil sterilized by radiation.

Non-gnotobiotic assay.

For the non-gnotobiotic assay, 10 nonsterile potato stem cuttings (cultivar Bintje) with roots approximately 2 cm long were dipped for 1 min in the appropriate bacterial suspension (approximately 10^8 cfu/ml) and planted in polyvinyl chloride tubes (11.5-cm diameter, 13-cm long). These tubes were either filled with bacterized sand or with bacterized natural clay soil (Table 2). The sand was bacterized by the addition of phosphate-deficient (no phosphate) or phosphate-sufficient (4 mM phosphate) plant nutrient solution containing phosphate-reporter strains or the control strains (10^8 cfu/ml) up to a moisture content of 20%. The natural clay soil was bacterized by the addition of a bacterial suspension (10^8 cfu/ml) in water (phosphate-deficient conditions) or 20 mM phosphate (phosphate-sufficient conditions) up to a moisture level of 18%.

These tubes were placed on a layer of wet vermiculite in the growth chamber (18° C, 16 hr light). After 10–14 days, the roots and samples from the bulk sand or soil were taken and handled as described above.

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