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Marking the Rhizopseudomonas Strain 7NSK$_2$ with a Mu d(*lac*) Element for Ecological Studies

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The mini Mu element Mu dII1681, which contains the *lac* operon genes and a kanamycin resistance gene, was inserted in the chromosome of plant growth-beneficial *Pseudomonas aeruginosa* 7NSK$_2$ to construct a marked strain (MPB1). In MPB1, β-galactosidase is permanently expressed under the culture conditions used. The MPB1 strain could be recovered with an efficiency of about 100% from a sandy loam soil on 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside medium containing sebacic acid and kanamycin. The limit of detection is about 10 CFU/g of soil. A detailed comparison was made between the wild-type strain 7NSK$_2$ and the Mu dII1681-containing MPB1 strain. The results showed that no genes essential for growth, siderophore production, survival in sterile and nonsterile conditions, plant growth stimulation, or root colonization had been damaged in the MPB1 strain, which means that MPB1 can reliably be used for ecological studies in soil. MPB1 survived well at 4 or 28°C but died off relatively rapidly in air-dried soil or at subzero temperatures. In these conditions, however, the MPB1 strain did not completely disappear from the soil but survived at a very low level of about 100 CFU/g of soil for more than 3 months. This observation stresses the need for very sensitive counting methods for ecological studies and for the evaluation of released microorganisms. Maize was inoculated with MPB1 via seed inoculation or soil inoculation. Upon seed inoculation, only the upper root parts were effectively colonized, while soil inoculation resulted in a complete colonization of the root system.

The fluorescent rhizopseudomonas strain 7NSK$_2$ is a plant growth-beneficial bacterium which, upon seed inoculation of various cereals and vegetables, can give an increase in growth, ranging from 10 to 25% under greenhouse conditions (13–15; M. Höfte, K. Y. Seong, E. Jurkevitch, and W. Verstraete, Plant Soil, in press). To monitor this organism after introduction into the soil, a selective marker is needed that does not interfere with the ability of the strain to survive and promote plant growth.

Very often, mutants resistant to the antibiotics rifampin and nalidixic acid are used to monitor the introduced bacterial populations (8, 11, 24). Recently, however, Compeau et al. (5) demonstrated that spontaneous rifampin resistance may not be an innocuous mutation in some pseudomonads. Also, the background resistance of soil microorganisms to rifampin and nalidixic acid can be relatively high, which limits the sensitivity of this method (7).

Marker systems based on immunological techniques are relatively insensitive, with a lower limit of detection of about 10^3 bacteria per gram of soil (6, 19) and may not distinguish between viable and nonviable cells (9).

The use of gene probes, especially the polymerase chain reaction, to detect bacteria in the environment seems to be promising (28) but is laborious and certainly not suitable for all laboratories interested in the ecology of soil microorganisms, since specialized equipment and radioactive labeling are needed. This means that special precautions in handling, storage, and disposal are required, while the use of nonautoradiographic labeling results in a reduction in sensitivity (9). Drahos et al. (7) developed a marker system based on the expression of the *Escherichia coli lac* operon genes into *Pseudomonas fluorescens* strains, enabling them to cleave the chromogenic substrate X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and to grow on a minimal lactose medium. In this report, a simple marker system is described, also based on the expression of *lacZ*, by introducing a Mu d2(*lac*) element into the chromosome of 7NSK$_2$.

Mu d1 and Mu d2 elements were originally designed to study transcriptional and translational fusions. Mu d2 elements lack a promoter and an initiating codon for *lacZ*. Expression of *lacZ* (with the production of a hybrid β-galactosidase protein) occurs when the Mu d2 element inserts itself downstream of (and in phase with) the promoter and start codon of an appropriate host gene (1). Mu d1-mediated transcriptional fusions to chromosomal promoters were previously described for *Pseudomonas putida* (27).

This report describes the use of the Mu dII1681 element as a selective marker for the fluorescent strain *Pseudomonas aeruginosa* 7NSK$_2$. Mu dII1681 was constructed by Castilho et al. (1) and has the *E. coli lac* operon genes and a kanamycin resistance gene substituted for internal Mu sequences. Mu dII1681 is defective for lytic growth but still has the Mu transposition genes. The behavior of the strain marked with Mu dII1681 is compared with that of the wild-type strain in vitro and in the soil, and the usefulness of the marked strain for ecological studies is demonstrated.

MATERIALS AND METHODS

Bacterial strains. The wild-type fluorescent strain *Pseudomonas aeruginosa* 7NSK$_2$ is a plant growth-beneficial rhizobacterium (13–15; M. Höfte et al., in press) isolated from the roots of barley (13). The *E. coli* donor strain MS1479 (F$^-$ *thi* Δ*lac*) contains the plasmid pMGB537 (R388::Mu dII1681) (26) and was a gift from J. A. Shapiro (University of Chicago). R388 is an IncW plasmid which encodes resistance to trimethoprim (Tp^r) and sulfadiazine (Su^r). Mu dII1681 contains the *lac* operon genes, encodes resistance to kanamycin (Km^r), is A$^+$B$^+$, and has a thermosensitive Mu *cts* 62 repressor. *lacZ* is not expressed in the pMGB537 plasmid.

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MPB1 was constructed by introducing Mu dII1681 into the chromosome of $7NSK_2$.

Media. *P. aeruginosa* $7NSK_2$ was routinely cultured in liquid modified King B medium (MKB; per liter, 5 g of Proteose Peptone no. 3 [Difco], 1.5 g of $MgSO_4 \cdot 7H_2O$, 1.2 g of K_2HPO_4, and 2 ml of glycerol [pH 7.2]). Enumeration of MPB1 populations from soil was carried out on modified M9 (18) medium (MM9); glucose was omitted, 2 g of sebacic acid [$COOH\text{-}(CH_2)_8\text{-}COOH$] per liter was used as a selective carbon source, and 20 mg of X-gal per liter and 200 mg of kanamycin per liter were also added. X-gal was prepared as a 1% solution in dimethyl formamide. MM9 plates were always incubated at 28°C. Enumeration of $7NSK_2$ populations from soil was carried out on solid MKB (sterile soil or populations of $>10^5$/g in live soil) and on 869 medium (containing, per liter, 10 g of tryptone, 5 g of yeast extract, 5 g of NaCl, and 1 g of glucose) supplemented with 3 mM $ZnCl_2$ for populations of $<10^5$/g in live soil. $ZnCl_2$ was added prior to autoclaving, and the pH was adjusted to 7. The 869 plates with Zn^{2+} were incubated at 37°C to increase selectivity. Doubling times were determined in 869 broth or in the minimal 284 gluconate medium (21).

Matings. Donor and recipient strains were grown exponentially at 30°C in 869 broth. Plate matings were carried out as described previously (17). Briefly, mixed spots of donor and recipient strains were grown overnight on 869 agar at 28, 40, or 41°C. Spots were suspended in 1 ml of 10^{-2} M $MgSO_4$ solution, diluted, and spread onto 869 medium supplied with chloramphenicol, kanamycin, and X-gal. Chloramphenicol (25 mg/liter) was added to counterselect the *E. coli* donor strain. Direct selection for $7NSK_2$ cells, in which β-galactosidase is produced via a translational fusion, was carried out by adding, per liter, 1,000 mg of kanamycin and 30 mg of X-gal. Sensitivity to trimethoprim and sulfadiazine was tested on a minimal 160 glucose medium (30) supplied with, per liter, 1,000 mg of trimethoprim or 1,000 mg of sulfadiazine.

Plasmid DNA, chromosomal DNA, and probe preparation. Miniscale plasmid isolation was carried out by the method of Kado and Liu (16) or by the alkaline lysis method (18). Large-scale chromosomal DNA extraction was carried out by the triton-lysozyme method (4). For probe preparations, the plasmid pMGB537 was isolated by the alkaline lysis method, digested with *Bam*HI, and separated on a 0.8% agarose gel. The 6.7-kilobase *Bam*HI fragment (*lac* operon) was removed from the gel via glassmilk elution with the aid of a Geneclean kit (Bio 101 Inc.) according to the protocol of the manufacturer. The fragment was ^{32}P labeled with a multiprime DNA labeling system (Amersham Corp.) following the suggested protocol.

Preparation of granulates. MPB1 or $7NSK_2$ was grown to stationary phase in 200 ml of MKB medium. Four hours prior to centrifugation, 1 g of glutamic acid per liter was added to the culture. After centrifugation at $10,000 \times g$, cells were washed in 0.1 M $MgSO_4$ and suspended in 2.5 ml of 0.1 M $MgSO_4$ plus 0.01% peptone and 2.5 ml of a 2% carboxymethyl cellulose solution. The suspension was mixed with 20 g of sterile talc, dried overnight in a laminar flow hood, ground, and stored at 4°C until used. One gram of granulated bacteria contained 10^8 to 10^9 viable cells.

Survival and recovery studies. Survival and recovery studies were carried out in loamy sand soil (from Tiegem, Belgium; pH 6.5, 1.7% carbon). The field moisture content at field capacity (F.C.) was 24% (expressed on oven-dried soil). Before use, the soil was sieved through a 2-mm-mesh screen. Glass jars containing 100 g of soil (0.75 F.C.) were covered with polyethylene foil to prevent drying. When necessary, the test soil was sterilized by autoclaving twice with 1 day in between. Soil was inoculated with a bacterial suspension in physiological solution containing a known amount of bacteria. Each treatment was carried out in duplicate. At fixed times, 10-g samples were taken and transferred to a flask containing 90 ml of physiological solution. Flasks were shaken for 1 h on a rotary shaker at 28°C. Serial 10-fold dilutions were plated out in duplicate.

Seed inoculation. A 200-ml culture of MPB1 in the stationary phase was centrifuged at $10,000 \times g$, and the cells were suspended in 10 ml of 0.1 M $MgSO_4$ and 2 ml of 2% carboxymethyl cellulose solution. This suspension was used to inoculate 25 maize seeds (previously sterilized with 3.5% NaOCl).

Root colonization study. Plastic pots (diameter, 8 cm; height, 17 cm) were filled with 900 g of Tiegem soil (0.75 F.C.). MPB1 was introduced by seed inoculation or soil inoculation (9 g of granulates per 900 g of soil). Pots were provided with two maize seeds, placed in a greenhouse, and watered daily with demineralized water until 0.75 F.C. was reached. After 1 and 2 weeks, the pots were cut into halves to obtain a top part and a bottom part. Roots, seeds, and soil were collected separately. Roots, together with the adhering rhizosphere soil were weighed and transferred to a flask containing 90 ml of physiological solution; the same was done for soil free of roots and for seeds. The suspensions were shaken at 150 rpm for 1 h at 28°C. Serial 10-fold dilutions were prepared and plated out in triplicate on MM9 medium. After preparing the dilution series from the roots plus rhizosphere soil suspension, roots were taken out, washed thoroughly with sterile demineralized water, dried with sterile blotting paper, and weighed. The cleaned roots were cut into small pieces. One part was used to determine the dry weight of the root sample. The other part was macerated, and an adequate amount of physiological solution was added. Serial 10-fold dilutions were prepared and plated out in triplicate on MM9 medium. Plates were incubated for 3 days at 28°C.

Pot experiments. Tiegem soil was pretreated with sugars and amino acids as previously described (14). Bacteria were mixed with the soil in a granulated form (1%, wt/wt). Control pots were inoculated with autoclaved granulates. Maize seeds of the cultivar "Adonis" were surface sterilized with NaOCl. Five seeds were sown in each pot. Each treatment had five replicates. After emergence, the number of plants per pot was reduced to four. The pots were placed in a greenhouse with a day temperature of 25°C and a night temperature of between 18 and 20°C. The light intensity was 120 microeinsteins/m^2 per s with an irradiation time of 16 h. The plants were watered daily with demineralized water. Plants were harvested after 4 weeks and dried at 105°C for 24 h. After harvest, bacteria were recovered from the roots as described above. The $7NSK_2$ bacteria were counted on MKB medium; MPB1 was enumerated with the selective MM9 medium.

Siderophore production. Siderophore production (pyoverdin) was determined by fluorescence measurements. The supernatant of the bacterial culture was diluted in Tris buffer (pH 7.4) (dilution depends on siderophore concentration, usually 1/50). Tris buffer as such served as a control. The relative intensity of fluorescence was measured at 460 nm while exciting at 405 nm with a Sequoia-Turner spectrofluorimeter. A quinine sulfate solution in H_2SO_4 (pH 2) was used as an internal standard.

A calibration curve was made for the direct conversion of

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TABLE 1. Transfer of pMGB537 to 7NSK$_2$ at different mating temperatures

Mating temperature (°C)	Plasmid transfer frequency per recipient cell	Frequency of blue cells per recipient cell
28	1.7×10^{-7}	$<7.5 \times 10^{-9}$
40	4.7×10^{-6}	1.8×10^{-8} (0.38%)
41	4.4×10^{-5}	1.6×10^{-7} (0.36%)

relative fluorescence units in micromolar pyoverdin, based on the 1:1 stoichiometry of the Fe(III)-pyoverdin complex. For this purpose, the supernatant of cultures with a known fluorescence was saturated with iron(III), put overnight at 4°C, and centrifuged at 12,000 × *g* on the next day to remove excess iron. The iron bound to pyoverdin was determined by the α,α′-dipyridyl method (22).

RESULTS

Introduction of foreign genetic material in 7NSK$_2$. The fluorescent strain *P. aeruginosa* 7NSK$_2$ appeared to be a natural restriction-positive strain. At a mating temperature of 28 or 37°C, the transfer frequency of broad-host-range plasmids like RP4 or R388 from *E. coli* to 7NSK$_2$ was extremely low (not more than 10^{-7} to 10^{-6} transconjugants per receptor strain) (data not shown).

When matings were carried out at 41°C (a sublethal temperature for 7NSK$_2$), transfer frequencies increased considerably (200 to 1,500 times, depending on the plasmid).

Isolation of Mu dII1681 containing 7NSK$_2$ cells. The transfer frequencies of the plasmid pMGB537 (R388::Mu dII1681) to 7NSK$_2$ at different mating temperatures are given in Table 1. Selective plates containing 869 medium supplemented with, per liter, 1,000 mg of kanamycin, 25 mg of chloramphenicol, and 20 mg of X-gal were incubated at 28°C. About 0.4% of the transconjugants turned blue on X-gal medium. Fourteen different blue colonies, named MPB1 to MPB14, were selected for further study. Nine colonies appeared to be Km^r Tp^s Su^s, three colonies were Km^r Tp^r Su^r, and 2 colonies were Km^r Tp^r Su^s. The absence or presence of the pMGB537 plasmid in the blue colonies was verified by miniscale plasmid extraction. No plasmids could be detected in the Km^r Tp^s Su^s colonies. The presence of the pMGB537 plasmid could be demonstrated in the colonies that were Tp^r Su^s or Tp^r Su^r. In these colonies, Mu dII1681 had found a plasmid promoter by rearrangement, as the plasmid, the kanamycin resistance, and the blue color were rapidly lost in the absence of selection pressure.

Four strains in which transposition of Mu dII1681 into the chromosome of 7NSK$_2$ had occurred and which turned deep blue on X-gal media were retained as possible marker strains. After repeated transfer in liquid 869 medium without kanamycin, only MPB1 and MPB6 produced a stable blue color. These two strains were added to a nonsterile sandy loam soil and were reisolated after a few days. MPB1 still formed intense blue colonies on X-gal medium, while the MPB6 colonies were much fainter. Finally, MPB1 was the strain selected for further investigation.

Southern analysis. To verify whether Mu dII1681 has only one insertion site in the chromosome of MPB1, the chromosomal DNA of MPB1 was digested with *Eco*RI, *Hin*dIII, and *Bst*EII and separated by agarose gel electrophoresis (0.8%). The gel was blotted onto nitrocellulose and screened by Southern hybridization with the 6.7-kb *Bam*HI fragment (*lac* operon) of pMGB537 (^{32}P labeled). *Eco*RI-digested chromosomal DNA of 7NSK$_2$ was used as a control. The autoradiogram showed that Mu dII1681 has indeed only one insertion site in MPB1 (Fig. 1).

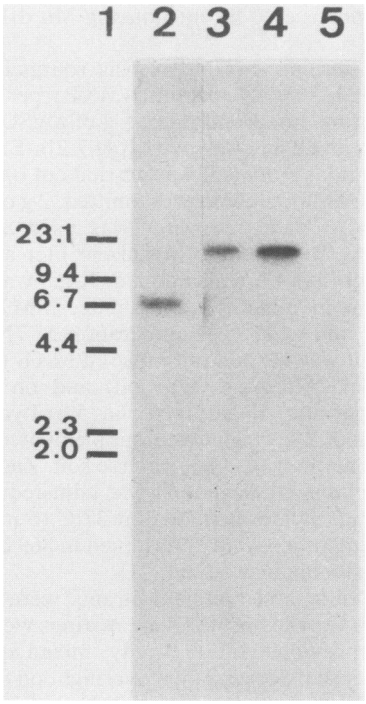

FIG. 1. Southern DNA hybridization of the chromosomal DNA of MPB1 with the 6.7-kb *Bam*HI fragment of pMGB537 (contains *lacYAZ*) (26). Lanes: 1, λ *Hin*dIII digest; 2, *Bst*EII digest; 3, *Hin*dIII digest; 4, *Eco*RI digest; 5, chromosomal DNA of the wild-type strain 7NSK$_2$, *Eco*RI digest.

Specific growth rates. The maximum specific growth rates of 7NSK$_2$ and MPB1 on 869 medium are, respectively, 0.84/h and 0.88/h (doubling time of 49 versus 47 min) at 30°C. In the minimal 284 gluconate medium, the maximum specific growth rates are 0.52/h for 7NSK$_2$ and 0.54/h for MPB1 (doubling time of 80 versus 76 min).

Siderophore production of MPB1 versus 7NSK$_2$. With the aid of siderophore deficient mutants of 7NSK$_2$, it could be demonstrated that siderophore production (pyoverdin) is at least partly responsible for the plant growth-beneficial capacities of 7NSK$_2$ (M. Höfte et al., in press). Furthermore, stress factors, like heavy metals, induce pyoverdin production by fluorescent *P. aeruginosa* 7NSK$_2$ on 869 medium (M Höfte, L. Diels, M. Mergeay, and W. Verstraete, Abstr. Pseudomonas Meet. Am. Soc. Microbiol. 1989, Palmer House Hotel, Chicago, abstr. no. I11, p. 17). Therefore, the ability to produce pyoverdin on different media was compared for the 7NSK$_2$ and MPB1 strains. In iron-limiting MKB medium, 7NSK$_2$ had a maximum pyoverdin production of 225 μM (± 21 μM) compared with 223 μM (± 13 μM) for the MPB1 strain. In 869 broth supplied with 3 mM Zn^{2+}, 7NSK$_2$ had a maximum pyoverdin production of 50 μM (± 4 μM) compared with 53 μM (± 0.3 μM) for the MPB1 strain. No significant differences in pyoverdin production between 7NSK$_2$ and MPB1 could be detected, whether the pyoverdin production was induced by iron limitation or by the presence of a heavy metal like Zn^{2+}.

Recovery efficiency of MPB1 from live soil. If MPB1 is to be used as a marker strain for ecological studies in live soil, it is

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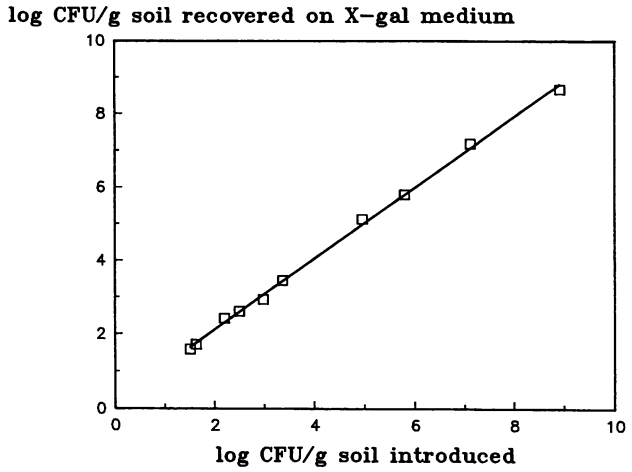

FIG. 2. Recovery efficiency of the *P. aeruginosa* MPB1 from nonsterile Tiegem soil (sandy loam). MPB1 was recovered on the selective MM9 medium.

important that MPB1 can be reliably recovered from the soil. For this purpose, a selective medium was developed, i.e., MM9, based on the minimal M9 medium (18) supplemented with sebacic acid [COOH-$(CH_2)_8$-COOH] and, per liter, 20 mg of X-gal and 200 mg of kanamycin to eliminate background growth. Among the fluorescent pseudomonads, sebacic acid can only be used by *P. aeruginosa*, and in odd cases by *P. fluorescens* biovar III and *P. putida* biovar B. It can not be used at all by members of the family *Enterobacteriaceae* like *E. coli* (23).

It was found that X-gal plates always have to be prepared freshly, as X-gal is unstable; colonies do not turn blue on old (>2 days) 869 X-gal plates, while in minimal media, counting efficiencies decrease drastically on old plates. Different dilutions of an MPB1 suspension with a known amount of bacteria (counted on MKB medium and MM9 medium) were added to 100 g of Tiegem soil. The number of MPB1 bacteria recovered from soil with the aid of the MM9 medium was compared with the number of MPB1 bacteria that was initially added. The results are represented in Fig. 2. Figure 2 shows that MPB1 can be very reliably recovered from soil. The recovery efficiency was 97%. The lower limit of detection is about 10 CFU/g of soil if 0.5 ml of a 1/5 dilution is plated out. Lower bacterial densities can be detected if more plates are used.

Plant growth-promotive capacities. The plant growth-promotive capacities of $7NSK_2$ and MPB1 were compared in a 4-week pot experiment with the maize cultivar "Adonis" as a test plant. In order to see a clear-cut plant growth-promotive effect, Tiegem soil was pretreated with sugars (660 mg of C per kg of soil) and amino acids (70 mg of N per kg of soil) as previously described (14) and was incubated for 3 weeks at 28°C before use. Prior to being sowed, bacteria were mixed with the soil in a granulated form. The initial cell density per gram of soil was 9.5×10^5 CFU for MPB1 and 10^6 CFU for $7NSK_2$. Soil inoculation with *P. aeruginosa* $7NSK_2$ resulted in a significant ($P = 0.05$) increase of the maize dry weight by 12.2%. The MPB1 strain gave a significant increase ($P = 0.05$) of 11.3%.

The plant growth-promotive capacities of $7NSK_2$ did not appear to be impaired by the chromosomal Mu dII1681 insertion. After harvest, MPB1 and $7NSK_2$ bacteria were recovered from the maize roots. For both strains, the number of bacteria on the roots was about 2×10^6 CFU/g (dry weight) of roots.

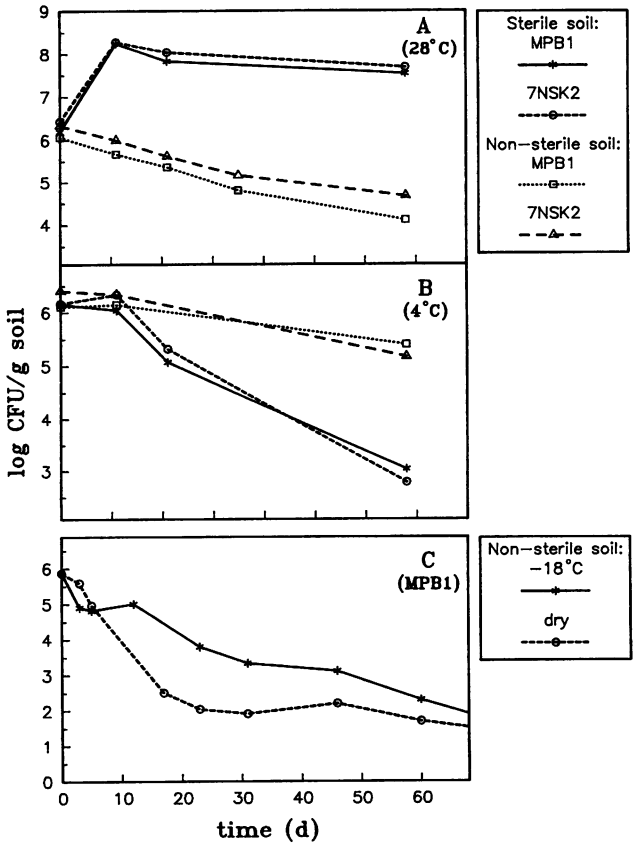

FIG. 3. (A) and (B) Survival of MPB1 and $7NSK_2$ at incubation temperatures of 4 and 28°C in sterile and nonsterile soils. MPB1 was recovered on MM9 medium; $7NSK_2$ was recovered on 869 agar supplied with 3 mM $ZnCl_2$. (C) Survival of MPB1 in dry conditions (air-dried soil, 2.25% moisture) and at −18°C.

Survival of MPB1 versus $7NSK_2$ in sterile and normal soils at two different temperatures (4 and 28°C). An important characteristic of a successful plant growth-beneficial strain is its ability to survive over a longer period of time in soil. By studying the survival of MPB1 and $7NSK_2$ in sterile and live soils at two different incubation temperatures, it was verified that Mu dII1681 had not inserted itself in a gene important for the viability of the $7NSK_2$ strain (at 4°C, the strains are not biochemically active; at 28°C, the strains are biochemically active). The survival of both strains was monitored over a 10-week period. MPB1 bacteria were recovered from the soil with the MM9 medium. The wild-type strain, which is naturally resistant against Zn^{2+}, could be recovered with 869 medium supplied with 3 mM $ZnCl_2$. This medium, on which the $7NSK_2$ strain produces strongly fluorescent colonies, is selective enough to detect about 10^3 CFU/g of soil, when plates are incubated at 37°C. The results are given in Fig. 3A and B.

The survival of MPB1 and $7NSK_2$ followed the same pattern in sterile and nonsterile soils at 4 and 28°C. At 28°C, $7NSK_2$ and MPB1 decreased at a rate of about 0.3 $\log_{10}$ CFU/g of soil per 10 days in nonsterile soil. In sterile soil, both strains multiplied and reached a maximum density of about 2×10^8 CFU/g of soil and then decreased very slowly at a rate of 0.1 $\log_{10}$ CFU/g of soil per 10 days.

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TABLE 2. Numbers of MPB1 bacteria in the soil and roots of maize after 7 and 14 days of growth

Incubation period (days)	Inoculation form[a]	No. of CFU in:						
		Top part of pot				Bottom part of pot		
		Soil[b]	Rhizosphere[b]	Roots[c]	Seeds[d]	Soil[b]	Rhizosphere[b]	Roots[c]
7	Seed	1.4×10^6	9.1×10^6	1.6×10^7	2.2×10^8	1.1×10^3	1.1×10^3	5.5×10^3
	Soil	3.7×10^6	8.0×10^6	3.1×10^7	1.0×10^8	5.3×10^6	5.8×10^6	5.5×10^6
14	Seed	1.0×10^6	5.0×10^6	8.6×10^7	2.1×10^7	1.1×10^4	1.1×10^4	1.7×10^5
	Soil	1.9×10^6	7.6×10^6	6.6×10^7	1.4×10^7	4.4×10^6	4.1×10^6	2.4×10^7

[a] MPB1 was introduced by seed inoculation or soil inoculation. Seed inoculation initial density, 1×10^{10} MPB1 per seed; soil inoculation initial density, 1.1×10^7 MPB1 per gram of soil.
[b] Per gram.
[c] Per gram (dry weight) of root.
[d] Per seed.

At 4°C, the $7NSK_2$ and MPB1 strains were not biochemically active. They neither grew nor multiplied at this temperature. This resulted in a relatively rapid decrease of about 0.4 $\log_{10}$ CFU/g of soil per 10 days in sterile soil. In nonsterile soil, however, both strains maintained more or less their inoculated titer and decreased only very slowly (0.1 $\log_{10}$ CFU/g of soil per 10 days) over a 10-week period. No significant differences in survival could be detected between $7NSK_2$ and MPB1 at any moment.

Survival of MPB1 under unfavorable conditions. Since there were no significant differences in survival between the $7NSK_2$ strain and the MPB1 strain, MPB1 was used to study the survival under unfavorable conditions in which numbers of MPB1 bacteria might drop below the detection limit of the selective medium, on the basis of heavy metal resistance (896 plus 3 mM Zn^{2+}) which was used to reisolate the $7NSK_2$ strain.

The survival of MPB1 was monitored over a 10-week period in a soil allowed to dry out (moisture content, 18% [0.75 F.C.] at time of inoculation; 2.25% [air dried] 2 days after inoculation and during the rest of the experiment) and in a soil incubated at −18°C. The MPB1 strain died off relatively quickly in the air-dried soil during the first 2 weeks (2 $\log_{10}$ CFU/g of soil per 10 days), followed by a much slower decline during the next 8 weeks (about 1 $\log_{10}$ CFU/g of soil per 10 days) (Fig. 3C). The MPB1 bacteria did not completely disappear from the soil but maintained themselves at a very low density. The die-off rate of MPB1 bacteria incubated at −18°C was about 0.7 $\log_{10}$ CFU/g of soil per 10 days. After 15 weeks, the air-dried soil still contained 5 MPB1 CFU/g of soil, while in the soil incubated at −18°C, bacterial numbers had dropped below 1 CFU/g of soil (data not shown). When the air-dried soil was supplied with water until 0.75 F.C. was reached, MPB1 numbers increased to 7×10^2 CFU/g of soil in 2 days. Even after 1 week of incubation at room temperature, MPB1 numbers in the soil, which was initially incubated at −18°C, were still below 1 CFU/g of soil.

Behavior of MPB1 in the rhizosphere of maize. Since MPB1 has the same root colonizing capacities as $7NSK_2$, MPB1 was used to compare the effect of seed or soil inoculation with the final distribution of the introduced beneficial bacteria in the soil and rhizosphere. Maize seeds were sown in cylindrical pots with a height of 17 cm in which roots could develop vertically. Numbers of MPB1 bacteria in the soil, in the rhizosphere, and on the roots were determined after 7 and 14 days of growth. The results are represented in Table 2. The results reveal that upon seed inoculation, only the upper root parts were effectively colonized. Colonization of the lower root parts was rather poor, even after 2 weeks of growth. Soil inoculation, however, resulted in complete and effective colonization of both the upper and lower root parts ($\approx 1 \times 10^7$ CFU/g of root, dry weight).

DISCUSSION

The plant growth-beneficial strain *P. aeruginosa* $7NSK_2$ turned out to be highly restriction positive. Its restriction system, however, could be circumvented by increasing the mating temperature to 41°C. This temperature also permits transposition of the Mu dII1681 element. Holloway (12) already reported the absence of restriction in *P. aeruginosa* strains grown at 43°C.

The plasmid pMGB537 is not very stable in $7NSK_2$, as it was quickly lost in the absence of selection pressure. As Mu dII1681 is lacking a promoter for the *lac* operon, an external promoter is required. This promoter is likely of chromosomal origin, although fusion with a plasmid promoter might have occurred prior to the transposition event. In MPB1, β-galactosidase, the gene product of *lacZ*, is permanently expressed under the culture conditions used and does not need induction by lactose. This means that when a carbon source other than lactose is used for the recovery of the MPB1 strain from soil, very little interference will occur from other β-galactosidase-producing soil microorganisms. By the method of Drahos et al. (7), which relies on growth on lactose media, this interference does occur, as about 10^3 nonfluorescent bacteria per gram of soil are capable of lactose utilization and formation of blue colonies on X-gal media. In order to enhance selectivity, Drahos et al. (7) had to add the rifampin-resistant phenotype to the *lacZY*-expressing cells. In our system, sebacic acid or any carbon source instead of lactose can be used, and furthermore, kanamycin can be added to eliminate totally background colony formation by other soil bacteria. It was demonstrated by Southern DNA hybridization that Mu dII1681 was inserted only once in the MPB1 chromosome. Extensive use of MPB1 in both in vitro experiments and soil (greenhouse experiments) have demonstrated that Mu dII1681 is very stably maintained.

The same pMGB537 plasmid has been used to insert Mu dII1681 in the chromosome of plant growth-beneficial *P. fluorescens* ANP15 (M. Höfte, unpublished results). In this strain, pMGB537 was transferred with a frequency of 3.7×10^{-6} per recipient. Blue colonies appeared with a frequency of 0.7%. Also, in this strain, R388 was unstable and quickly

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lost in the absence of trimethoprim and sulfadiazine selection pressure.

In this report, a detailed comparison has been made between the wild-type strain $7NSK_2$ and the Mu dII1681-containing MPB1 strain. The results show that no genes essential for growth, siderophore production, survival, plant growth stimulation, or root colonization have apparently been damaged by the Mu dII1681 insertion. This means that MPB1 can reliably be used for ecological studies in nonsterile soils.

The MPB1 strain could be recovered with an efficiency of about 100% from the sandy loam soil of Tiegem by shaking this soil for 1 h in physiological solution before making a dilution series. The survival of $7NSK_2$ and MPB1 in sterile and nonsterile soils incubated at 28°C (Fig. 3A) is in agreement with the observations of other authors (5, 10, 29). It is not known, however, why the $7NSK_2$ and MPB1 strains died off relatively quickly in sterile soil incubated at 4°C (Fig. 3B) while in a nonsterile soil incubated at 4°C, bacterial numbers remained virtually unchanged over a 10-week period. Sterilization of the soil seems to have removed a protective agent against the cold or to have liberated a toxic compound. In any case, observations showing better survival in nonsterile conditions are quite rare and deserve further examination.

The relatively rapid die off of MPB1 in an air-dried soil is typical for *Pseudomonas* spp., which are known to be drought susceptible (3). It is interesting, however, that the MPB1 strain did not completely disappear from the soil but survived at a very low but still detectable level (<100 CFU/g of soil) for more than 3 months. With the Mu dII1681 marker, population densities below 100 CFU/g of soil can easily be detected. Bacteria are apparently able to maintain themselves in soil at densities which are well below the detection limit of commonly used enumeration methods. The latter stresses the need for very sensitive detection systems in order to assess the ecology of microorganisms, in general, and of genetically modified microorganisms, in particular. The *P. aeruginosa*-related strain MPB1 is more susceptible to subzero temperatures than are strains like *Pseudomonas syringae* pv. *glycinea* which survived at its initial density over a 160-day period in a soil incubated at −12°C (24) or *P. fluorescens* ANP15 which kept its initial density for more than 14 weeks in Tiegem soil incubated at −18°C (Jan Boelens, unpublished results). It is possible that the inability to grow at 4°C makes the MPB1 strain more vulnerable to subzero temperatures.

The study about the behavior of MPB1 in the rhizosphere of maize (Table 2) revealed that upon seed inoculation, only the upper root parts were effectively colonized. The MPB1 bacteria were apparently not able to follow the fast-growing maize roots. The MPB1 bacteria that were found in the bottom part of the pots were probably transported with the vertical water flow in soil, as the soil moisture content was adjusted daily to 0.75 F.C. Parke et al. (25) and Chao et al. (2) have demonstrated that the water flow has an important influence on the distribution of *Pseudomonas* sp. in the rhizosphere. Also, soil biota, such as microarthropods and nematodes, might have played a role in the passive distribution of the bacteria. Soil inoculation with granulated MPB1 bacteria resulted in a very efficient root colonization. MPB1 bacteria established themselves in more or less the same numbers on the upper and lower root parts. In the upper part, a clear rhizosphere and spermosphere effect could be noticed as a result of the release of nutrients by the plant. These results demonstrate that the spread of MPB1 bacteria along the length of the root is insignificant and that colonization of the roots occurs mainly from the soil adjacent to the roots. In plant experiments carried out in pots (height, 8 cm) containing 500 g of soil, the difference between soil inoculation and seed inoculation will not be so clear cut, as plant roots will soon start to turn round as they only grow vertically for a very short period. In field plots, however, soil inoculation might provide a far better root colonization than does seed inoculation.

In conclusion, it can be stated that Mu dII1681 is a very reliable and stably maintained marker for *P. aeruginosa* $7NSK_2$. With the aid of the MPB1 strain, a lot can be learned about the survival and spread of the plant growth-beneficial strain *P. aeruginosa* $7NSK_2$ in soil. MPB1 is also a kind of model strain to assess the risks involved in the deliberate release of microorganisms, containing a genetically engineered DNA sequence (in this case, Mu dII1681) integrated in the chromosome. This report already indicates that Mu dII1681 is quite stably maintained. It is our intention, however, to investigate whether (and with what frequency) Mu dII1681, integrated in the chromosome of $7NSK_2$, can be transferred to other microorganisms by, for instance, the mechanism of retrotransfer, brought about by broad-host-range plasmids like RP4 (20).

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