In recent years, the interest in the use of bacteria for biological control of plant-pathogenic fungi has increased. We studied the possible side effects of coating barley seeds with the antagonistic strain *Pseudomonas fluorescens* DR54 or a commercial fungicide, imazalil. This was done by monitoring the number of indigenous *Pseudomonas* organisms and actinomycetes on barley roots during growth in soil, harvest after 50 days, and subsequent decomposition. Bacteria were enumerated by traditional plate spreading on Gould’s S1 agar (*Pseudomonas*) and as filamentous colonies on Winogradsky agar (actinomycetes) and by two quantitative competitive PCR assays. For this we developed an assay targeting *Streptomyces* and closely related genera. DR54 constituted more than 75% of the *Pseudomonas* population at the root base during the first 21 days but decreased to less than 10% at day 50. DR54 was not successful in colonizing root tips. Initially, DR54 affected the number of indigenous *Pseudomonas* organisms negatively, whereas imazalil affected *Pseudomonas* numbers positively, but the effects were transient. Although plate counts were considerably lower than the number of DNA copies, the two methods correlated well for *Pseudomonas* during plant growth, but after plant harvest *Pseudomonas*-specific DNA copy numbers decreased while plate counts were in the same magnitude as before. Hence, *Pseudomonas* was 10-fold more culturable in a decomposition environment than in the rhizosphere. The abundance of actinomycetes was unaffected by DR54 or imazalil amendments, and CFU and quantitative PCR results correlated throughout the experiment. The abundance of actinomycetes increased gradually, mostly in numbers of DNA copies, confirming their role in colonizing old roots.

Public concern about chemical pesticides has fostered an interest in application of bacteria for biological control to protect agricultural crops against pathogenic fungi (16). There is still a lack of knowledge concerning environmental risks of such microbial inoculants. Introduced bacteria may outcompete a certain indigenous subpopulation for nutrients and space. For example, the biocontrol strain *Pseudomonas fluorescens* CHA0 (36) displaced a part of the indigenous *Pseudomonas* population for a short period after application, probably because of competition for the same ecological niche in the rhizosphere (26). Further, toxic compounds produced by introduced strains may affect sensitive nontarget microorganisms.

*P. fluorescens* DR54 was isolated in Denmark from a sugar beet rhizosphere and is effective towards preemergence damping-off disease caused by such different fungal pathogens as *Pythium ultimum* and *Rhizoctonia solani* in laboratory pot experiments (28). The antifungal active compound viscosinamide has been isolated from DR54 and is believed to be the main agent of the biocontrol properties of the strain (29, 39).

When studying possible side effects of seed coating with a biocontrol strain, the effects of the biocontrol organism must be compared to the effects of the normally applied fungicide. In Denmark, the seed coat fungicide most used to protect winter and spring barley is the commercial formulated fungicide Fungazil A. It contains the active ingredient imazalil, which is a sterol biosynthesis inhibitor (3), at a concentration of 50 g liter$^{-1}$. In Denmark, the recommended dose of Fungazil A is 1 ml kg of seed$^{-1}$.

In this study, we focused on possible side effects of *P. fluorescens* DR54 and imazalil on two important bacterial groups in soil: *Pseudomonas* and actinomycetes. The actinomycete group is a very broad phylogenetic group of gram-positive bacteria with a high GC content. Traditionally, actinomycetes have been defined as bacteria with a filamentous, fungus-like growth form, but sequence analysis of 16S rRNA has shown that bacteria with more traditional growth forms, such as the coryneform bacteria and *Micrococcus*, also belong to the group (6). In temperate, well-drained soils with neutral to alkaline pH, the genus *Streptomyces* is often the dominating actinomycete genus, constituting around 95% of the filamentous actinomycetes as determined by plate spreading (44). The gram-negative genus *Pseudomonas* is now a well-defined genus, which formerly was known as fluorescent pseudomonads or as *Pseudomonas* rRNA homology group I (17).

Both groups are important in the degradation of organic matter, although with different life strategies. *Pseudomonas* strains are mostly associated with fresh organic matter, rich in easily degradable compounds (14, 20, 34, 38), while actinomycetes traditionally are considered to be most active late in the decomposition process, where they are strong competitors for complex organic compounds (4, 19). *Pseudomonas* typically has a considerably higher occurrence in the rhizosphere than in bulk soil (25, 34), while actinomycetes have been found to have
both higher and lower occurrences in rhizosphere soil than in bulk soil, depending on the plant species (5, 25).

The knowledge of the ecology of *Pseudomonas* and actinomycetes, however, is based mainly on traditional cultivation methods. Since only a small minority of the bacterial cells in soil are culturable (43), it is important to evaluate and compare results based on culturing with DNA-based methods. Olsen and Bakken (31) suggested that the ecological significance of culturable cells is large, as they represent 80 to 90% of the bacterial biovolume. The unculturable cells are a blend of species that we cannot culture and species with some cells that are in an unculturable state because of (e.g.) stresses (33).

We used the quantitative *Pseudomonas*-selective PCR method (14), developed an actinomycete (mainly *Streptomyces*)-selective PCR, and compared selective CFU counts with the amounts of specific DNAs from the two groups.

### MATERIALS AND METHODS

#### Mesocosm setup

The soil used was a sandy loam soil from the Royal Veterinary and Agricultural University experimental field station in Høje Taastrup, Denmark. Soil properties are as follows: sand 45.9%; silt 18.8%; clay 3.0%; organic matter, 4.8% (dry weight); water holding capacity 18.9% (dry weight). Soil was sampled in September 1998 and stored at 4°C for 2 weeks before sieving (4 mm). Macronutrients were added in the following concentrations (milligrams of nutrient kilogram of soil): N, 40; P, 8.6; K, 23; Mg, 10. Micronutrients were added at the following concentrations: (micrograms of nutrient kilogram of soil): B, 100; Mn, 100; Zn, 100; Cu, 3; Mo, 2. The soil was thoroughly mixed and distributed in mesocosms for growth of barley plants.

The mesocosms consisted of nontransparent plastic tubes with a diameter of 6 cm and a length of either 70 or 35 cm. Each tube was cut into two halves lengthwise; the halves were held together by strong waterproof tape, and at the bottom end was closed with polyethylene mesh to hold back the soil. This enabled destructive sampling of the mesocosms by opening lengthwise and taking out the soil core. The long tubes contained 2.4 kg of soil, and the short tubes contained 1.15 kg of soil. After addition of soil to the mesocosms, the soil water content was adjusted to 80% of water holding capacity and the soil was incubated at 10°C for 5 days. The mesocosms were placed in a climate chamber with light from four halogen-mercury 150-W lamps (HQI-TS NDL; Osram Sylvania, Danvers, Mass.), with a light intensity of about 500 μkat m⁻² s⁻¹ in a 16-h-light, 8-h-dark cycle. During the light period the temperature in the growth chamber was 15°C, and during the dark period it was 10°C. After 1 day, seeds were sown. A batch of spring-barley seeds (*Hordeum vulgare* type Lamia) was coated with either imazalil in the form of Fungazel A (Cilus, Herlev, Denmark) or *P. fluorescens* DR54. DR54 was chromosomally marked with green fluorescent protein (GFP) (30), which enabled us to distinguish it from the indigenous *Pseudomonas*. The resulting mutant, DR54-BN14, did not differ from the wild type in various physiological tests and barley root colonization (30). Imazalil was sprayed on seeds in the recommended dose (1 ml per kg of seeds) by means of a type HEGE 11 rotator (Hans Ulrich Hege Maschinenbau, Waldenburg, Germany). At the day of sowing, DR54 was applied to seeds by adding 170 seeds to 120 ml of washed overnight culture with 2.0 × 10⁹ cells ml⁻¹ (Luria-Bertani medium supplemented with 0.10% glucose [22], washed twice in 0.01 M phosphate buffer [pH 7.4]). The cell suspension was gently shaken for 30 min before sowing, which resulted in 5 × 10⁷ CFU of DR54 per seed sown. In parallel to this treatment, untreated seeds were sown in clean phosphate buffer for 30 min before sowing. Imazalil-coated seeds were sown dry, but 20 μl of phosphate buffer was pipetted on top of each seed. This corresponded to the mean amount of phosphate buffer adhering to DR54-coated seeds and control seeds.

Three seeds were sown per mesocosm at a 3-cm depth. Mesocosms with different treated seeds were placed randomly in the climate chamber. Throughout the experimental period mesocosms were regularly weighed and rewatered to maintain the water content. Where all three seeds germinated, one was removed, leaving two plants per mesocosm. For the first sampling occasions more seeds were sown to ensure enough rhizosphere soil for analysis. For the first four sampling occasions (days 4, 7, 10, and 14), the short tubes were used, and for all other sampling occasions the long tubes were used. After 50 days, above-ground plant material was removed to mimic harvest, initiating decomposition of the roots.

#### Sampling

Sampling. Sampling was done 4, 7, 10, 14, 21, 35, 50, 63, 91, and 112 days after sowing. On each sampling occasion three replicate mesocosms from each of the treatments were destructively sampled. The rhizospheres of the two plants were pooled. At all sampling days rhizosphere samples from the upper 5 cm of the root system were collected, and at days 14, 35, 50, 63, and 91, 2.5 cm of root tips from the root tips were also taken. Bulk soil from mesocosms with untreated seeds was analyzed on days 0, 10, 21, 50, and 112 by sampling ca. 0.5 g of root-free soil. Rhizosphere samples of approximately 30 cm of root with adhering soil and 0.5-g bulk soil samples were aseptically transferred to glass tubes containing 6 ml of phosphate buffer and mildly sonicated in an ultrasonic water bath for 30 s (Branson 5210; Merck Eurolab, Albertslund, Denmark). This soil suspension was used for selective spiking and quantitative PCR. Sampled roots were weighed, as well as roots alone. Furthermore, the actual water content of the soil in each tube was measured.

#### Plate counts

The number of culturable *Pseudomonas* organisms was counted on the *Pseudomonas*-selective (15, 18) Gould’s SI medium (9) amended with 50 mg of the fungal inhibitor delovict (containing 50% natamycin and 50% lactose) dissolved in 10 ml of methanol liter⁻¹. Fifty microliters of appropriate dilutions was plate spread and incubated at 20°C for 3 days. Three replicate plates were counted per sample, and the total number of colonies and the DR54 GFP-positive colonies were determined under blue light in a microscope.

Filamentous actinomycetes were counted on Winogradsky agar (containing, per liter, 5.0 g of KH₂PO₄, 2.5 g of MgSO₄·7H₂O, 2.5 g of NaCl, 0.05 g of MnSO₄·H₂O, 0.085 g of FeCl₃·7H₂O, and 18 g of Bacto Agar [Difco, Detroit, Mich.]) amended with 25 mg of natamycin dissolved in 10 ml of methanol liter⁻¹ to inhibit fungal growth. Fifty microliters of appropriate dilutions was spread on agar plates. Three replicate plates were counted per sample after 4 weeks of incubation at 20°C. Filamentous actinomycetes were recognized by their colony morphology.

#### Quantitative PCR

DNA extraction and purification from the soil samples were done with the Fast Soil purification kit (Bio 101, Vista, Calif.) in accordance with the manufacturer’s instructions (1). One DNA extract was used per sample to determine the DNA target number. This DNA extraction procedure decreased the number of intact actinomycete spores to 25 ± 3% of the initial amount. This was determined by counting suspensions of spores from *Streptomyces lavendulae* DSM 40009T and *Streptomyces phialocephalum* DSM 40446T before and after bead beating. Following this treatment, the content of the bead beater tube was resuspended and beads were removed by centrifugation, leaving spores in the supernatant. Quantitative competitive PCR on *Pseudomonas* was done as described elsewhere (14). Essentially, an internal standard, which is a shorter fragment with primers identical to the native DNA in the ends, was used as competitive template DNA in the PCR. Each PCR tube contained a total volume of 25 μl with 17.8 μl of DNA-free water, 2.4 μl of AmpliTaq Gold polymerase (PE Biosystems, Norwalk, Conn.), 2.4 μl of bovine serum albumin (Amersham Pharmacia, Uppsala, Sweden), 1 μl of deoxyxynucleoside triphosphate mixture (PE Biosystems), 0.12 μl (0.1 mM) each of the *Pseudomonas*-specific primer PSMc (2) and of the Bacteria-specific 9-27 primer (37) (Life Technologies, Roskilde, Denmark), 0.12 μl of AmpliTaq Gold polymerase (PE Biosystems), and 1.0 μl of sample as template. The specificity of PSMc was verified on 8 August 2000 on the RDP database Project (RDPhase database (21)). Furthermore, the PCR products from the imazalil treatment after 91 days were cloned using the TOPA TA Cloning Kit (Invitrogen, Carlsbad, Calif.). Clones containing the entire insert were identified by PCR using the *Pseudomonas*-specific primers as described above. The inserts in 10 clones were PCR amplified using the primers M13F and M13R as recommended by the manufacturer and were sequenced at the sequencing facility at GATC GmbH (Konstanz, Germany). All sequences aligned within the group of *Pseudomonas* and relatives when tested in the RDP database (21). Linear dilutions of the internal standard were used, and ethidium bromide-stained band intensities on the gels were quantified by a UV-visible-light recording camera using the Multi-Analyt software (Bio-Rad, Hercules, Calif.).

A quantitative competitive PCR selective for *Streptomyces* and related actinomycetes was developed. The master mix used was as described above except for the amount of DNA amplifying primer. The primer set was designed to target *Streptomyces DSM 40446T* (CCGGCCTA 3'), which is reported to be specific for detection of actinomycetes, and the reverse primer R513 (5' CCGCGGCTGGCAGCGACGTA 3'), which was used in combination with the PCR conditions used for the imazalil treatment after 91 days were cloned using the TOPA TA Cloning Kit (Invitrogen, Carlsbad, Calif.).
treatments with the control and the effect of time on the population development, and interaction between the two factors. To compare antifungal treatments, ANOVA or GLM in SAS 6.12 for the effects of sampling time, antifungal log transformed and analyzed by a two-way analysis of variance by the procedure CATMOD. The internal standard were 0.2, 0.4, 0.6, and 0.8 in the PCR template they were 0.28, 0.51, 0.71, and 0.82, respectively, in the PCR product. This was taken into account when calculating the number of actinomycete-specific DNA copies in the rhizosphere near the root base (Fig. 2). The DR54 treatment resulted in significantly higher numbers of CFU and DNA copies of Pseudomonas at days 4 and 7, compared to the control. This was not surprising, as both methods include DR54, which was inoculated at $5 \times 10^7$ CFU per seed. If the DR54 CFU counts are subtracted from the total Pseudomonas counts, a marked inhibition of the indigenous Pseudomonas is seen the first 21 days. Nevertheless, the appearance of the indigenous Pseudomonas on Gould’s S1 plates has most likely been suppressed to some degree by the emergence of fast-growing DR54 colonies at samplings when the number of these colonies was high. This results in a false picture of the suppression. However, at days 10 and 14 both Pseudomonas CFU and DNA show no difference between treatments (Fig. 2), when DR54 still constituted ca. 80% of the CFU. If the inhibition of indigenous Pseudomonas on the plates was pronounced, we would expect a higher number of Pseudomonas DNA copies in the DR54 treatment group at these samplings. This was not the case. Therefore, the data suggest that DR54 in a period of ca. 2 weeks displaced a part of the indigenous Pseudomonas population. Similar results were found by Natsch et al. (26) for P. fluorescens CHA0, which probably competed for the same ecological niche as the indigenous Pseudomonas in the rhizosphere.

Four days after sowing, imazalil caused a significantly higher level of indigenous Pseudomonas compared to the untreated control. This was seen both by CFU, which showed six times more Pseudomonas colonies than the control, and quantitative PCR, which showed three times more Pseudomonas-specific DNA copies (Fig. 2B). As Pseudomonas is known to be able to degrade many xenobiotic compounds (35), Pseudomonas bacteria may have been active players in the degradation of the root segments but were pooled for the whole root. We found no GFP-positive colonies in any rhizosphere samples of untreated or imazalil-treated plants, confirming that there was no contamination by GFP-positive colonies.

Effects of P. fluorescens DR54 and imazalil on the indigenous Pseudomonas and actinomycete populations. Both DR54 and imazalil transiently affected the indigenous Pseudomonas population in the rhizosphere near the root base (Fig. 2). The DR54 treatment resulted in significantly higher numbers of CFU and DNA copies of Pseudomonas at days 4 and 7, compared to the control. This was not surprising, as both methods include DR54, which was inoculated at $5 \times 10^7$ CFU per seed. If the DR54 CFU counts are subtracted from the total Pseudomonas counts, a marked inhibition of the indigenous Pseudomonas is seen the first 21 days. Nevertheless, the appearance of the indigenous Pseudomonas on Gould’s S1 plates has most likely been suppressed to some degree by the emergence of fast-growing DR54 colonies at samplings when the number of these colonies was high. This results in a false picture of the suppression. However, at days 10 and 14 both Pseudomonas CFU and DNA show no difference between treatments (Fig. 2), when DR54 still constituted ca. 80% of the CFU. If the inhibition of indigenous Pseudomonas on the plates was pronounced, we would expect a higher number of Pseudomonas DNA copies in the DR54 treatment group at these samplings. This was not the case. Therefore, the data suggest that DR54 in a period of ca. 2 weeks displaced a part of the indigenous Pseudomonas population. Similar results were found by Natsch et al. (26) for P. fluorescens CHA0, which probably competed for the same ecological niche as the indigenous Pseudomonas in the rhizosphere.

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- **TABLE 1. Strains tested in the Streptomyces-targeting PCR**

| Species                  | Strain          | PCR product |
|--------------------------|-----------------|-------------|
| Actinomycetes            |                 |             |
| Streptomyces lavendulae  | DSM 40069<sup>T</sup> | +           |
| Streptomyces diastaticus | DSM 40496<sup>T</sup> | +           |
| Streptomyces griseoflavus| DSM 40456<sup>T</sup> | +           |
| Streptomyces albus       | DSM 40313<sup>T</sup> | +           |
| Streptomyces sp.         | DSM 40533       | +           |
| Streptomyces albidoflavus| DSM 40555<sup>T</sup> | +           |
| Corynebacterium glutamicum| DSM 20300     |             |
| Rhodococcus sp.          | Our isolate     |             |
| 20 filamentous actinomycete isolates<sup>a</sup> | Our isolate     |             |
| Arthrobacter globiformis | DSM 20124<sup>T</sup> | –           |
| Micrococcus luteus       | DSM 20030<sup>T</sup> | –           |
| Nonactinomycetes         |                 |             |
| Flavobacterium ferrugineum| DSM 30193<sup>T</sup> | –           |
| Cytophaga arvensicolor   | DSM 3095        | –           |
| Bacillus cereus          | ATCC 14579<sup>T</sup> | –           |
| Pseudomonas fluorescens  | DR54            | –           |

- **a** isolated from the same soil using water agar.

RESULTS AND DISCUSSION

Fate of *P. fluorescens* DR54. In the rhizosphere near the root base of DR54-treated plants, the strain had a high relative occurrence (>75% of the total *Pseudomonas* population) during the first 21 days, but from day 50 onwards it constituted less than 10% (Fig. 1). In the rhizosphere of root tips, DR54 constituted less than 0.1% of the total *Pseudomonas* population at all sampling days (data not shown), which shows that DR54 is not a successful root colonizer, in agreement with the results of Normander et al. (30). Those authors divided a barley rhizosphere into three parts, upper (near the seed), middle, and lower, and found that the number of DR54 organisms was decreasing from the upper to the lower rhizosphere during the first 14 days of rhizosphere development. Natsch et al. (27) monitored the survival of *P. fluorescens* CHA0 in wheat rhizosphere, in which the abundance decreased from ca. $10^6$ root system$^{-1}$ to $10^5$ root system$^{-1}$ in 42 days. However, the results were not for

![FIG. 1. *P. fluorescens* DR54 on root base rhizosphere during growth, harvest, and decomposition of barley. The proportion of cultivable DR54 to the total number of CFU on Gould’s S1 agar is shown. Error bars show standard errors of the means.](http://aem.asm.org/Downloaded from http://aem.asm.org)
fungicide. Another possibility is that they benefited from the displacement of other taxa, leading to improved conditions for *Pseudomonas*. In the only study of effects of imazalil on bacteria, Fisher and Hayes (7) found that the numbers and function of *Rhizobium trifolii* were unaffected by imazalil at field concentrations. However, it is not possible to draw general conclusions from their observation and hence to determine what effects the compound had indirectly or directly on *Pseudomonas*. There were significantly fewer *Pseudomonas* CFU at day 35 in the imazalil treatment group compared to the untreated control (Fig. 2A). These changes are probably repercussions from the effects seen after 4 days and show the risk of side effects of imazalil and DR54 on soil bacteria.

Imazalil and DR54 did not affect the actinomycete population near the root base (data not shown). Therefore, the treatments are considered replicates for presentation of actinomycete CFU and specific DNA copies (Fig. 3). Likewise, the treatments did not affect the numbers of CFU of the two bacterial groups around root tips (see Tables 2 and 3). The difference in effects between *Pseudomonas* and actinomycetes demonstrates that side effects can be specific to certain taxa, and hence it is recommended not to monitor large groups like *Bacteria* exclusively when studying side effects.

**Succession of Pseudomonas.** The root base rhizosphere represented a habitat changing from young to old rhizosphere to a decomposing hot spot. Across this gradient, a succession in abundance of *Pseudomonas* and actinomycetes was observed. *Pseudomonas* numbers in untreated samples in rhizosphere around the root base increased significantly from young to 50-day-old rhizosphere, with respect to both culturable organisms (Fig. 2A) and the amount of specific DNA copies (Fig. 2B). Furthermore, the level of *Pseudomonas* CFU in bulk soil throughout the experiment and the level of *Pseudomonas* DNA copies in bulk soil at day 0 (Table 2) were significantly lower than those in the root base rhizosphere. This supports the view that *Pseudomonas* bacteria are rhizosphere competent (25, 34) and hence confirms that the agar plate results in the literature are not biased by cultivation. We found fewer *Pseudomonas* organisms in the root tip rhizosphere (Table 2) than at the root base, probably because the root tips represented young rhizosphere in which the *Pseudomonas* population had not yet proliferated.

After the plant shoot was removed at day 50 and until day 91, *Pseudomonas* CFU in the root base rhizosphere were not significantly different (Fig. 2A). However, the number of *Pseudomonas*-specific DNA copies (Fig. 2B) was significantly reduced right after the plant shoots were removed. In rhizosphere samples ca. 1,500 DNA copies per CFU were found, while around decomposing roots ca. 250 DNA copies per CFU were found (Fig. 2). In soil, low culturability (0.1 to 10%), depending on medium, incubation time, and conditions, has been reported (43), but to our knowledge this has not been studied for *Pseudomonas* alone. Nevertheless, *Pseudomonas* introduced into bulk soil or rhizosphere of barley or wheat is approximately 0.1 to 100% culturable (12, 30, 40, 41). We consider it unlikely that the smaller amount of DNA present...
around decomposing roots is due to changed DNA extraction efficiency compared to rhizosphere samples. However, it has not been possible to find studies of the efficiency of extraction of Pseudomonas in different physiological states or for different species within the genus. The number of 16S ribosomal gene copies in the genomes of the Pseudomonas strains tested varies from four to six (11, 32). Thus, it is unlikely that changes in Pseudomonas diversity have influenced the quantitative PCR results markedly. The decrease in the number of DNA copies following harvest is thus considered to be due to a decrease in the number of Pseudomonas organisms. Hence, this indicates that fewer Pseudomonas cells are present around decomposing roots than in the rhizosphere but that a higher fraction of them are culturable. Johnsen et al. (14) reported a ratio of ca. 10 DNA copies per CFU using the same methods as us. However, this was during the decomposition of 12-day-old barley roots, which are considerably less lignified than the roots in this study. Therefore, differences in the environments presumably control Pseudomonas culturability.

Succession of actinomycetes. In the rhizosphere near the root base, the CFU of filamentous actinomycetes showed a small but significant increase from day 4 to 63 (Fig. 3A). Compared with Pseudomonas, the actinomycete abundance was stable, though, as found in wheat rhizosphere by Miller et al. (25). Late in the decomposition process (days 91 and 112), the number of CFU was significantly higher. This is in accordance with the classical view that actinomycetes persist during the microbial succession beyond the initial phase of the degradation process because of their ability to penetrate and solubilize many polymers (24). Because of the fungus-like growth form, CFU from filamentous actinomycetes originate both from spores and from fragmented hyphae. CFU will probably often overrepresent the spores, because fragmentation of vegetative hyphae into single cells is not possible. A harsh fragmentation treatment will break vegetative hyphae into nonviable fragments (23). About 95% of the counted colonies of filamentous actinomycetes can be expected to belong to the genus Streptomyces (44).

The numbers of actinomycete CFU in bulk soil and root tip rhizosphere were in the same range as observed in the root base rhizosphere (Table 3). This is in contrast to the results of Miller et al. (25), who found that actinomycete numbers in wheat rhizosphere were ca. 10-fold higher than those in bulk soil, but many investigations have shown that this varies between plant species (see, e.g., references 5 and 25).

The results of the quantitative PCR showed an increase in actinomycete-specific DNA from young to older rhizosphere (Fig. 3B). After harvest, a significant decrease in specific DNA was observed, followed by a significant increase in the late decomposition phase, even though this increase was not as distinct as for the CFU. The level of specific DNA in bulk soil and root tip rhizosphere was the same as in the root base rhizosphere (Table 3), supporting the CFU results.

For actinomycetes, there are two differences between the CFU and quantitative PCR methods. The first concerns the specificity of the two methods. Because the vast majority of CFU counted on agar plates most likely are Streptomyces, the quantitative PCR method was directed towards Streptomyces rather than towards the whole gram-positive, high-GC group. This group was the target in the work of Heuer et al. (13), who used the same primers. Our protocol was more stringent, as we raised the annealing temperature from 65 to 72°C. DNAs from all tested soil isolates of filamentous actinomycetes and Streptomyces strains, but also DNAs from strains from a few other actinomycete groups, were amplified by the PCR protocol used.

Second, the specific DNAs conceivably represent spores and hyphae more equally, even though the DNA contents in and extraction efficiencies from spores and hyphae may differ (8). We found that the great majority of spores from two Streptomyces strains were broken with the bead beating method used in this experiment (data not shown). Assuming that the specific DNA represents spores and hyphae more equally than CFU, the larger increase in the rhizosphere seen by quantitative PCR can be explained by actively growing mycelia, colonizing the
cortex in old roots (42), or a population of actinomycetes not detected on agar plates.

Concluding remarks. In general, our quantitative PCR results confirm the plate counting results and hence the conceptions of the ecology of Pseudomonas and actinomycetes in the rhizosphere. Thus, quantitative PCR has confirmed that Pseudomonas spp. are fast colonizers whereas actinomycetes colonize roots at later stages. However, the increased culturability of Pseudomonas following barley harvest and the increase in actinomycete-specific DNA in the rhizosphere stress that plate spreading alone leaves out important information. These changes may be the result of a rise of in situ Pseudomonas and actinomycete activity. DR54 and imazalil transiently affected changes may be the result of a rise of in situ Pseudomonas and actinomycete activity. DR54 and imazalil transiently affected changes may be the result of a rise of in situ Pseudomonas and actinomycete activity. DR54 and imazalil transiently affected.

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