ABSTRACT

Maintaining threshold populations of inoculum microorganisms in the soil environment is important for such practical applications as biocontrol, plant growth-promotion, bioremediation, and nodulation. However, because of both technical and labor constraints in monitoring bacterial viability in nonsterile soils, few studies have reported on survival kinetics, particularly in relation to subtle alterations in soil acidity-related factors. A genetically modified strain Pseudomonas putida R20/AacZY or Rhizobium leguminosarum bv. trifolii 1625Ta/gusA was introduced into conditioned, nonsterile Gilpin (fine loamy, mixed mesic, Typic Hapludult) silt loam soil, limed at four low levels (pHw = 4.71, 4.81, 4.92, and 4.99) or derivative soil solutions with highly correlated (R² ≥ 0.81) chemical properties. Immediate declines in viability of both strains were found in all soils, reaching 0.1 to 1% initial colony-forming unit (CFU) g⁻¹ soil in 35 h for P. putida and in 68 h for R. leguminosarum bv. trifolii. Death rate constants (kd) for both strains were directly related to lime level (soil pH), although differences were not significant (P > 0.05) for the rhizobium. Use of soil solutions gave similar responses for both strains, but over much shorter incubation times. As with soils, kd values for both strains in soil solutions were directly related to lime level (solution pH). In both soil and solution experiments, survival (kd) was negatively correlated (R² ≥ 0.914) with pH and basic cation (Ca and Mg), and positively correlated (R² ≥ 0.933) with Al, concentrations. This relationship of viability to soil solution chemistry was broadly confirmed for both bacterial strains by use of fluorescent probes, suggesting increased cell membrane damage at lower pHs. These results demonstrate not only the alternative utility of using soil solutions, rather than nonsterile soils, for bacterial viability assessments, but also the positive effect of low-level liming (−0.28 pH unit increase) on survival of beneficial root-colonizing bacteria in acidic soils.

INTRODUCING BENEFICIAL BACTERIA into the soil environment has been of interest to microbiologist for many years, most notably the use of rhizobia as inocula for legumes. More recently, other bacteria have become important for purposes of enhancement of crop growth, bioremediation, and biocontrol. For all these uses, threshold populations are typically required. As a consequence, there has been renewed interest in developing methods for predicting their survivability, that is, their saprophytic competency, based on the properties of both the bacterium and soil.

It has often been observed that laboratory-grown, heterotrophic bacteria, introduced into soils, very seldom grow, and typically decline in number over time. In the absence of soil toxicity factors (and other stresses such as temperature, moisture, etc.), the major reason appears to be the lack of an easily oxidizable C source for growth, that is, the oligotrophic state of most natural soils (Williams, 1985; Roszak and Colwell, 1987; Morita, 1988). And, when toxic soils (here defined as being low in pH/Ca and possibly high in Al) are employed, populations decline more rapidly. This detrimental effect of acidic, nonsterile soils on survival has been amply demonstrated for such root-colonizing bacteria as the saprophytic pseudomonads (Zechman and Casida, 1982; Van Elsas et al., 1986, 1991; Aae et al., 1988; Compeau et al., 1988; Heijnen et al., 1993; Gu and Mazzola, 2001) and symbiotic clover rhizobia (Heynen et al., 1988; Postma et al., 1991; Heijnen et al., 1992, 1993; Hirsch, 1996; Watkin et al., 2000).

Although there is considerable literature on the beneficial effects of liming acidic soils on legume nodulation, no information exists on liming (essentially, Ca and pH increases) effects on rhizobia survival, per se, in soils. But, there is a large body of literature on pH and Ca (and Al) effects on rhizobia growth. Nearly all of these studies, however, have been done with solution growth cultures (i.e., amended with some easily oxidizable C source), rather than in soils (Munns, 1968; Wood et al., 1984a, 1984b; Howieson et al., 1992; Flis et al., 1993; Reeve et al., 1993; Glenn et al., 1997; Watkin et al., 1997; Dilworth et al., 1999). For pseudomonads, no literature exists on the effect of liming on survival in soils, nor on pH and/or Ca effects in solution growth cultures. The generalization from these studies, applied to soils, is that the more acidic the soil, and the less Ca present, the more toxic it is to introduced rhizobia (and, presumably to pseudomonads), and that the presence of Al exacerbates the situation.

To the best of our knowledge, no reports, other than a cursory examination by Staley and Voigt (2000), have appeared in the literature concerning the effect of subtle (low-level) liming of acidic soils on pseudomonad and rhizobium survival kinetics. Also, there appears to be no information available on their survival kinetics in soils, compared with soil solutions (as opposed to solution growth cultures). Determining this relationship is important because of the heterogeneous nature of soil, since soil may present a significantly different chemistry to introduced bacteria than that to which they are exposed in soil solutions derived from them (Van Elsas and Van Overbeek, 1993).

To address these issues, we employed two test systems. The first system consisted of an acidic (pHw 4.71; moderate Al concentration) soil, typical of abandoned pasture soils of the Appalachian hill-land region, in
laboratory microcosms. Liming at several low-levels was performed to simulate minimal management inputs, commonly practiced in the region. The second system consisted simply of soil solutions derived from the lime-treated soils. The viable population of the introduced bacterial strain, either a pseudomonad or rhizobium, in soils or soil solutions was then monitored over time by agar spread-plating to determine their survival kinetics. Although determination of viability by plate counts is a widely used method for assessing this complex, and hence, rather poorly defined state, newer and more direct methods exist. One such method employs fluorescent probes with high, but different, affinities for nucleic acids (DNA and RNA). Because the detrimental effects of acidic soil conditions, and the alleviating effects of liming, on the maintenance of the structural integrity of pseudomonads and rhizobia has not been previously reported, we employed these strains in an attempt to determine the nature of acidic soil toxicity at the cellular level, as well as to confirm the results of our soil solution/plate count experiments.

The specific objectives of this work, employing genetically modified (mutant) strains of a pseudomonad and rhizobium as model saprophytic and symbiotic root-colonizers, respectively, were to: (i) demonstrate the innocuous nature of transpositional insertion of lacZ and gusA into the genome of Pseudomonas putida and Rhizobium leguminosarum bv. trifolii parental strains, respectively, (ii) determine the effect of low-level liming (pH 4.71–4.99) of a nonsterile, conditioned soil on the survival kinetics of the strains in soils, compared to soil solutions derived from them, (iii) seek correlations between the survival kinetics in soils and soil solutions and their chemical composition, and (iv) confirm the liming effects on survival in soil solutions at the cellular structural level by use of fluorescent probes.

MATERIALS AND METHODS

Bacterial Strains and Media

The wild-type strain, Pseudomonas putida R20, was originally isolated from the lima bean (Phaseolus lunatus L.) rhizosphere (Osborn et al., 1989), while the wild-type strain, Rhizobium leguminosarum bv. trifolii (hereafter referred to as R. trifolii) 162S7a, was originally isolated from nodules of white clover (Trifolium repens L.) (Nitrigin, Inc., Milwaukee, WI). Both bacterial species are typical root-colonizing, heterotrophic, gram-negative bacilli. The mutant strain of P. putida (LR0101; Ap’, Tc’), carrying the lacZ insert, was derived by triparental mating, using pMON7197 as the donor plasmid (Drahos and NH4Cl, 1.0 g; MgSO4 cycline at 100, 50, and 3 mg L

Introduction of gusA into the genome of Pseudomonas putida and Rhizobium leguminosarum bv. trifolii parental strains, respectively, was done by carefully adding 12 drops (0.6 mL total) of the antibiotic, X-glc/Cy50Sp50 (Wilson, 1996). Bacterial inocula for all the experiments were prepared from liquid cultures, grown at 25°C under aerobic conditions to the early stationary phase in 250-mL side-arm Erlenmeyer flasks containing 100-mL of PF or YES broth. The cells were harvested by centrifugation (6400 × g for 10 min; 25°C), followed by two, 0.1-volume washes with cold (iced), distilled water, and centrifugations at 5°C. For the soil/plate count experiments, the concentrations of the cell suspensions were adjusted with cold, distilled water to an optical density (OD580 nm) = 0.40 in a colorimeter (Model 20D+; Thermo Spectronic, Rochester, NY). For the soil solution/plate count experiments, the cell suspensions were similarly prepared, except that the final concentrations were adjusted to OD = 0.10. For the soil solution/fluorescence experiments, the cell suspensions were likewise prepared, except that the final concentrations were adjusted to OD = 0.50, followed by centrifugation at 5°C and resuspension in cold, distilled water in half the original volume (OD = 1.0). Before the final OD readings, suspensions were filtered through a sterile, non-absorbent cotton pad to remove any visible cell clumps still remaining from culturing. Inocula prepared in this manner typically yielded titers of 9.5 × 10^8 and 4.9 × 10^9 CFU mL^-1 for the pseudomonad, and 2.1 × 10^8 and 10 × 10^9 CFU mL^-1 for the rhizobium, for the OD = 0.10 and 0.40 suspensions, respectively. These washed, adjusted cell suspensions were held on ice and used for inoculation within 30 min after the final step in their preparation.

Low-level Limed Soils and Soil Solutions

The soil was a Gilpin silty loam collected from the Ap horizon (0–15 cm) of an abandoned pasture, consisting primarily of poverty grass (Dantonia spicata L.) and sweet vernal grass (Anthoxantum odoratum L.). While still in the field-moist condition, dolomite lime was added to four equal lots (well mixed before splitting) equivalent to 0, 0.79, 1.69, or 2.59 Mg ha^-1. After pH equilibration to 4.71, 4.81, 4.92, and 4.99, the lots were sieved (2-mm mesh), air-dried, and stored as previously described (Staley, 2002). Before initiating the soil experiments, 1 kg of each pH soil was rewetted (~33 kPa or 30% soil moisture) with distilled water and conditioned by incubation in closed, double, plastic bags at 5°C for 2 days, then at 25°C for 1 day, to allow moisture equilibration and activation of the native microflora. In preparation for soil solution extraction, 1 kg of each pH soil was re-wetted (~33 kPa) and incubated as above, except that only a 1-d incubation at 25°C was used. Soil solutions were then extracted by centrifugation of the distilled water-equilibrated soils (Elkhathib et al., 1987), prefiltered (0.45 μm porosity), filter-sterilized (0.22-μm porosity), and frozen (~20°C) until used.

Soil Incubations and Plate Count Determinations

For the soil/plate count experiments, 10.0 g (air-dried basis) of rewetted/conditioned soil was added to 100-mL milk dilution bottles and evenly dispersed over the bottoms. Inoculation was done by carefully adding 12 drops (0.6 mL total) of the adjusted cell suspension (OD = 0.40), evenly, over the soil surfaces. The bottles were loosely capped and incubated at 25°C. At 1.5, 12, 20, 27, and 35 h for the pseudomonad, and 1, 8, 20, 26, 46, and 68 h for the rhizobium, single bottles at each lime level were removed from the incubator and destructively sam-
Viable cell enumeration of the introduced bacterium was initiated by transferring the soil in each bottle to a plastic stomacher bag, then adding 15 mL of phosphate-salt solution (PSS: Na$_2$HPO$_4$ 2.8 g; KH$_2$PO$_4$ 0.43 g; NaCl 7.2 g L$^{-1}$; pH 7.2) at 25$^\circ$C. The soil slurry was vigorously paddled for 30 s in a Stomacher (Model STO-80; Tekmar Co., Cincinnati, OH), then poured into another sterile, milk dilution bottle. Ten milliliters of PSS was added to the bag to rinse the small amount of remaining soil into the bottle. The bottle was added 75 mL of PSS, after which the diluted slurry was vigorously shaken for 1 min and allowed to stand for 5 min. Portions (1 mL) of the suspensions from the center of the bottles were removed and serially diluted in PSS. For the pseudomonad, appropriate dilutions were spiral-plated (Model D-2; Spiral Biotech, Norwood, MA) on BMX/Cy50Sp50 agar. For each dilution, the number of CFU was determined from the average of five agar plates. Typical, blue colonies of the pseudomonad and rhizobium on these selective media were visually counted after 5- to 6- and 8- to 10-d incubation at 25$^\circ$C, respectively. Colony counts, appropriate dilution factors and soil dry masses were used to calculate the CFU g$^{-1}$ soil, air-dry basis.

**Soil Solution Incubations and Plate Count Determinations**

For the soil solution/plate count experiments, 6 $\mu$L of the adjusted bacterial cell suspensions (OD $= 0.10$) were added to single, sterile test tubes containing 6 mL of soil solution (1/1000 dilution) at 25$^\circ$C. The tubes were capped and suspensions thoroughly mixed by vortexing for 5 s, followed by replacement in a waterbath at 25$^\circ$C. At 0, 2, 4, 6, 8, 12, 16, and 20 h for both the pseudomonad and rhizobium, 60 $\mu$L were aseptically transferred to 12 mL of sterile PSS (1/200 dilution) at 25$^\circ$C, vortexed for 5 s, then serially diluted in sterile PSS at 25$^\circ$C. Dilutions were spiral-plated on nonselective media, PF for the pseudomonad and YES for the rhizobium. Colonies were visually counted after 1 d incubation for the pseudomonad and 7 d for the rhizobium, respectively. Determined volume constants, appropriate dilution factors and soil dry masses were used to calculate the CFU mL$^{-1}$ soil solution.

**Soil Solution Incubations and Fluorescence Determinations**

For the soil solution/fluorescence experiments, 300 $\mu$L of the adjusted cell suspensions (OD $= 1.00$) were added to single, sterile test tubes containing 6 mL of soil solution (1/1000 dilution) at 25$^\circ$C. The tubes were capped and suspensions thoroughly mixed by vortexing for 5 s, followed by replacement in a waterbath at 25$^\circ$C. At 0.1 and 26 h for the pseudomonad, and 22 and 45 h for the rhizobium, vortexing for 5 s and subsampling was done, followed by continuing static incubation at 25$^\circ$C. Subsamples (0.9 mL) were added to Eppendorf tubes containing 2 $\mu$L of both Syto 9 (green fluorescent stain) and propidium iodide (red fluorescent stain) (LIVE/DEAD BacLight Kit; Molecular Probes, Eugene, OR). After 1 h of staining in the dark at room temperature, the suspensions were filtered onto 0.2-$\mu$m porosity, black, polycarbonate membranes (Osmonics, Livermore, CA). The membranes were then mounted on glass slides, flooded with antifade (Slowfade, Light; Molecular Probes, Eugene OR), slip-covered, and assayed by fluorescence spectroscopy. Three fields, selected at random, were observed for each slide with a 20X objective, without oil immersion. Excitation at 470 nm was provided with a Xenon-arc lamp, adjusted to 70 to 75 W, followed by scanning over a range of ~300- to 500 nm. The fluorescence emission spectra were determined with a PTI fluorescence system (PhotoTechnologies International, Lawrenceville, NJ), employing an XF31 filter set (Omega Optical, Inc., Brattleboro, VT), coupled to a Model JMT-2 epifluorescence microscope (Olympus, Melville, NY). The ratio of the integrated intensities of emission spectra between 520- to 545-nm (green) and 630- to 670-nm (red), detected by individual PMTs, was interpreted as the percentage of live (undamaged membranes) to dead (damaged membranes) cells.

**Statistical Analyses**

Death rate constants ($k_d$) were calculated from linear regressions of log transformed data, followed by analysis of the homogeneity of slopes. Significant differences in $k_d$ were determined by the use of pairwise contrasts ($P \leq 0.05$). Correlations and regressions were determined in SAS System for Windows, 2001, Release 8.02 (SAS Institute, 2001).

**RESULTS**

**Phenotypes of Wild-type and Mutant Strains**

Because marking the strains used in this study involved insertions of lacZY and gusA into the chromosomes of *P. putida* and *R. trifolii*, respectively, the possibility exists that some important function(s) may have been disrupted. In an attempt to determine this, a number of phenotypic characteristics of the wild-types, and their derivative mutants, were examined. For *P. putida*, of all 21 physiological tests in the API 20 NE (bioMerieux Industry, Hazelwood, MO) system, only the hydrolysis of *p*-nitrophenyl-$\beta$-D-galactopyranoside (PNPG) differed between the wild-type and mutant strains. Because this compound is an analog of lactose, its utilization by the mutant (lacZY-marked) strain, and not the wild-type strain, was expected. No differences for any of the API tests were found between the *R. trifolii* wild-type and mutant (gusA-marked) strains. Generation times, determined by optical density measurements in complex or defined media, were likewise unaffected ($P > 0.05$) by the insertions into either of the bacterial species, although there was some indication of a small increase in both mutants. Values for the *P. putida* wild-type and mutant were, respectively, 62 and 68 min in complex media (PF) and 128 and 144 min in defined MG media (same as ML but glucose substituted for lactose). Values for the *R. trifolii* wild-type and mutant were, respectively, 146 and 180 min in complex media (YES) and 330 and 360 min in defined media (BM). In addition, 13 antibiotics were examined for their ability to inhibit growth of either the wild-type and mutant strains. With the exception of spectinomycin (Sp) for the *R. trifolii* mutant, no differences were found. This exception was anticipated, since the transposon insertion for gusA carried Sp$^+$ as its selectable marker. Taken together, these results suggest that genetic modification of the mutant strains, by chromosomal insertion of either lacZY into *P. putida* or gusA into *R. trifolii*, had occurred in regions that are likely unimportant for the functioning of their normal regulatory and metabolic pathways.

**Soil and Soil Solution Chemical Properties**

Selected chemical properties of the Gilpin silt loam soils (Lime 0–3) used in this study, and of the soil solu-
tions derived from them, are given in Table 1. Over the range of soils, low-level liming had the anticipated effect of increasing pH, consistently, up to ~0.28 unit overall. Exchangeable Ca and Mg concentrations were, likewise, increased by 50 and 220%, respectively, whereas exchangeable Al was decreased by 25%. Base saturation was also consistently increased up to 53%. Overall, these properties identify all four soils, particularly the more acidic ones, as being stressful for forage legume growth and root bacterization. The chemical properties of the soil solutions mimicked those of the soils from which they were derived. Solution pH was consistently increased, up to ~0.34 unit overall, while Ca and Mg concentrations similarly responded to low-level liming, being increased by 32 and 180%, respectively. Aluminum concentrations were decreased by 30% over the range of soil solutions. Correlations coefficients ($R^2$) between these soil and soil solution parameters were 0.99, 0.98, 0.99, and 0.81 for pH, Ca, Mg, and Al, respectively.

### Soil Survival and Kinetics

Between a two- to three-log decrease was observed for *P. putida* R20/lacZY over the 35-h assay period, with the soils receiving lime allowing consistently improved survival (Fig. 1A). Statistical analysis of the responses revealed the death rate constants ($k_d$) to be significantly ($P \leq 0.05$), and consistently, related to lime level (soil pH), with constants ranging from 0.051 to 0.101 (Table 2).

For *R. trifolii* 162S7a/gusA in soils (Fig. 1B), survival was similar to that of the pseudomonad, but the rhizobium took nearly twice as long for a smaller (one- to two-log) decrease in viability (Fig. 1B). Nonetheless, survival appeared to be consistently improved with increasing soil lime levels. Statistical analysis of the responses revealed, however, that the rhizobial $k_d$ values were consistently, although not significantly ($P > 0.05$), related to soil pH, with constants ranging from 0.019 to 0.029 (Table 2).

### Soil Solution Survival and Kinetics

Survival of both bacteria in the soil solutions generally mimicked the results from the soil experiments. However, differences in survival were obtainable over much shorter assay periods for both species (6 vs. 35 h and 20 vs. 68 h for *P. putida* R20/lacZY and *R. trifolii* 162S7a/gusA, respectively), relative to soil solution pH. For *P. putida* R20/lacZY in soil solutions (Fig. 2A), between a 0.5- to 2-log decrease was found by the end of the experiment, with survival consistently related to soil solution pH. Statistical analysis of the responses revealed

### Table 1. Selected chemical properties of Gilpin silt loam soils and derivative soil solutions used in study.†

<table>
<thead>
<tr>
<th>Treatment‡</th>
<th>pHw</th>
<th>Ca (mg L$^{-1}$)</th>
<th>Mg (mg L$^{-1}$)</th>
<th>K (mg L$^{-1}$)</th>
<th>Na (mg L$^{-1}$)</th>
<th>Al (mg L$^{-1}$)</th>
<th>H (mg L$^{-1}$)</th>
<th>CEC§</th>
<th>Base Sat.¶</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lime 0</td>
<td>4.71</td>
<td>1.04 (25.1)</td>
<td>0.22</td>
<td>0.25</td>
<td>0.01</td>
<td>2.01 (48.4)</td>
<td>0.62</td>
<td>4.15</td>
<td>36.6</td>
</tr>
<tr>
<td>Lime 1</td>
<td>4.81</td>
<td>1.25 (29.0)</td>
<td>0.38</td>
<td>0.26</td>
<td>0.01</td>
<td>1.70 (39.4)</td>
<td>0.71</td>
<td>4.31</td>
<td>44.1</td>
</tr>
<tr>
<td>Lime 2</td>
<td>4.92</td>
<td>1.41 (30.9)</td>
<td>0.56</td>
<td>0.25</td>
<td>0.01</td>
<td>1.59 (34.8)</td>
<td>0.75</td>
<td>4.57</td>
<td>48.8</td>
</tr>
<tr>
<td>Lime 3</td>
<td>4.99</td>
<td>1.56 (34.4)</td>
<td>0.71</td>
<td>0.25</td>
<td>0.01</td>
<td>1.50 (33.0)</td>
<td>0.50</td>
<td>4.54</td>
<td>55.9</td>
</tr>
</tbody>
</table>

† All soil values given on an oven-dry (105°C) basis; Ap horizon (0- to 15-cm sample depth); plant residues removed; collected 28 Apr 1998.
‡ Lime rate 0, 1, 2, 3 equivalent to 0.0, 0.79, 1.69, and 2.59 Mg ha$^{-1}$, respectively, of limestone.
¶ CEC = $\Sigma$ (Ca + Mg + K + Na + Al + H); % base saturation = $\left[\Sigma$ (Ca + Mg + K + Na) / CEC$\right] \times 100$.
§ Values in parentheses are % CEC.
the $k_d$ values to be significantly ($P \leq 0.05$) related to pH, with constants ranging from 0.078 to 0.279 (Table 2).

Between a 0.25- to 0.5-log decrease was observed for *R. trifolii* 162S7a/*gus*A by the end of the experiment (Fig. 2B), with the extent of survival consistently related to soil solution pH. Statistical analysis of the responses revealed the $k_d$ values to be significantly ($P \leq 0.05$) related to soil solution pH (unlike for the soil experiment), with constants ranging from 0.008 to 0.029 (Table 2).

Table 2. Death rate constants ($k_d$) for *P. putida* R20 (*lacZY*) and *R. trifolii* 162S7a (*gus*A) strains as affected by soil lime levels (pH).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>$k_{d1}$</th>
<th>$k_{d2}$</th>
<th>$R^2$</th>
<th>$k_{d1}$</th>
<th>$k_{d2}$</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lime 0</td>
<td>0.101 a</td>
<td>0.950</td>
<td>0.029 a</td>
<td>0.920</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lime 1</td>
<td>0.178 b</td>
<td>0.760</td>
<td>0.023 a</td>
<td>0.960</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lime 2</td>
<td>0.064 bc</td>
<td>0.960</td>
<td>0.021 a</td>
<td>0.900</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lime 3</td>
<td>0.051 c</td>
<td>0.940</td>
<td>0.019 a</td>
<td>0.930</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soil solution</td>
<td>Lime 0</td>
<td>0.279 a</td>
<td>0.960</td>
<td>0.029 a</td>
<td>0.960</td>
<td></td>
</tr>
<tr>
<td>Soil solution</td>
<td>Lime 1</td>
<td>0.222 b</td>
<td>0.980</td>
<td>0.020 b</td>
<td>0.820</td>
<td></td>
</tr>
<tr>
<td>Soil solution</td>
<td>Lime 2</td>
<td>0.102 c</td>
<td>0.890</td>
<td>0.012 bc</td>
<td>0.710</td>
<td></td>
</tr>
<tr>
<td>Soil solution</td>
<td>Lime 3</td>
<td>0.078 c</td>
<td>0.860</td>
<td>0.008 c</td>
<td>0.850</td>
<td></td>
</tr>
</tbody>
</table>

*Values for $k_d$ within matrix type with same letter are not significantly different ($P > 0.05$) by use of pairwise contrasts.

Relationship of Survival to Soil and Soil Solution Chemistry

Correlations of $k_d$ with the chemical properties of the soils, and soil solutions derived from them, were significantly ($P \leq 0.05$) related to pH and most exchangeable cations (Table 3). Constants for both the soil and soil solutions were negatively correlated ($R^2 \geq 0.914$) with pH and basic cation (Ca and Mg) concentrations, and positively correlated ($R^2 \geq 0.933$) with Al concentrations.

Confirmation of Survival in Soil Solutions Using Fluorescent Probes

As evidenced in Fig. 3A, the ratio of green-red fluorescence was directly related to the viability (% survival) of *P. putida* R20/*lacZY* ($R^2 = 0.98$), when considered over the course of the experiment. More particularly, viability was consistently related to the pH of the soil solution at both assessment times. At the 0.1-h sampling time, viability was 63, 64, 85, and 93% in the Lime 0 to Lime 3 solutions, respectively, while at the 26 h sampling time, viability was 7, 10, 20, and 28%, respectively.

Likewise, the fluorescence ratio was directly related to the viability of *R. trifolii* 162S7a/*gus*A ($R^2 = 0.83$), when considered over the course of the experiment (Fig. 3B). Relative to soil solution pH, ratios were nearly consistently increased with increasing pH at each sampling time (22 and 45 h). However, resolution of these differences for the rhizobium by plate counts was not as distinct as for the pseudomonad, suggesting differences in these two bacterial species in their susceptibility to soil acidity-related toxic factors.

DISCUSSION

Most of the past work that attempted to determine soil bacterial survival was done in soils that had been treated in some manner, so as to eliminate the large, indigenous (background), microfloral population. This was done, primarily, to make assessment possible without the requirement for a selection medium, which traditionally has been difficult, or even impossible to obtain. However, sterilization of soils invariably results in some soil chemical changes, regardless of the process employed. Although employing nonsterile soils alleviates this concern, enumer-
ation of the introduced strain necessitates some selection strategy to separate it from the background population. Traditionally, antibiotic resistances have been used as selection strategies, but their use is always occasioned by concerns about altered saprophytic competence, since they are typically deletion mutations.

In the present work, we have employed several methodological strategies in an effort to overcome these concerns. For instance, we used a nonsterile, rewetted and conditioned soil for the soil studies, as well as for extraction of the soil solutions. We suggest that this more nearly reflects the biology and chemistry of the soil as taken from the field than sterile and/or unconditioned soil. Also, we employed the exogenous genes (lacZY and gusA) as selection markers for the introduced strains. Although one can never be certain that these chromosomal insertions (mutations) are innocuous, the results presented here strongly suggest that this is the case. Other workers, employing these same genetic markers for pseudomonads (Hattemer-Frey et al., 1990; Kluepfel et al., 1991; Parke et al., 1992; Hartel et al., 1994) and rhizobia (Wilson et al., 1991; Kang et al., 1997; Khan et al., 1999) have presented evidence affirming this suggestion.

Another methodological objective was to develop a more efficient, in terms of time and labor, procedure for determining survival kinetics in soils than the traditional agar spread-plating technique. To this end, we examined bacterial survival in soil solutions that were derived from the nonsterile and conditioned soils. The comparability of our results in soils (Fig. 1) and soil solutions (Fig. 2) for both bacterial species suggests that the chemistry they are encountering in the soil solutions closely mimics that in the soils. The consequence of this finding is that we now have an aqueous matrix that yields comparable results, at less expense, and which can be filter-sterilized, thus allowing the use of wild-type strains without markers.

To our knowledge, this is the first detailed report on the effect of liming at low levels on bacterial survival kinetics in soil solutions. In a previous report (Staley and Voigt, 2000), we showed similar low-level liming effects on rhizobium survival (R077 wild-type) in soil solutions derived from fresh and rewetted soils from the same field site. Differences in collection and treatment dates between this soil and the one used in the present study likely accounted for the small differences found in their chemical properties. Regardless, no quantitative effects or correlations with the soil solution chemical properties were determined. The only other report utilizing soil solutions (extracts) to monitor bacterial survival is that of Postma et al. (1988). Interestingly, they found nearly a log increase in the population of R. trifolii within ~2 d, after which the population remained stable for nearly 2 mo. An explanation for their results is likely that the soil solution was not only derived from an alkaline (pH 7.2), loamy sand soil after sterilization by radiation, but that it was from a phosphate filtrate of autoclaved soils, which undoubtedly solubilized oxidizable C.

All of the studies on the effect of pH on bacterial survival in acidic soils, of which we are aware, have been done with different soil types or soils with different management histories. Because of different biological (such as antagonists, predators) and physical (such as clay content) properties, it is difficult to separate these influences from purely chemical ones. Our suggestion that we have investigated only soil chemical effects on survival is based on the following. First, we used a single soil and relatively short incubation times (particularly in the soil solution experiments). Second, the consistency of the high correlations between pH and exchangeable cations and death rate constants (k) in both the soil/plate count and soil solution/plate count experiments (Table 3) suggests that biological and physical properties were not confounding factors. Finally, the literature reports, in which solution growth cultures have been exclusively used, consistently demonstrate the beneficial effect of Ca/Mg, and the detrimental effect of Al, on survival and/or growth of rhizobia. Similar effects on pseudomonads, closely related to rhizobia, would also be expected. Taken together, the last two points also suggest that other chemical constituents in the soil solution milieu, such as tannins and organic acids, are relatively unimportant as determinants of bacterial survival, at least in our Gilpin silt loam soil.

The results of the soil solution/fluorescence experiments broadly confirmed the soil solution/plate count
experiments, at least for the pseudomonad. Although we found the fluorescence ratio to be linearly related to viability (percent survival) for both the pseudomonad and rhizobium (Fig. 3), comparison with our soil solution/plate count results (Fig. 2), suggests a more qualitative than quantitative nature for the fluorescence method. For instance, from the regression line for the pseudomonad at Lim 0 (Fig. 2A), viability after 0.1 h was calculated at 90%, whereas it was calculated at 63% by the same time from the regression line in Fig. 3A. Likewise, from the regression line for the rhizobium at Lim 0 (Fig. 2B), viability after 22 h was calculated at 20%, whereas it was calculated at 88% by the same time from the regression line in Fig. 3B. A possible explanation for the pseudomonad discrepancy is that many of the cells had significant membrane damage, yet not to the extent that it could not be repaired on resuscitation on complex media. We can offer no explanation for the dissimilar fluorescence ratio results for the rhizobium, except to note that most rhizobia produce a heavy capsule of polysaccharide, which may have resulted in some cell clumping, whereas pseudomonads typically do not (Holt et al., 1994). Thus, although the fluorescence method is much less laborious than spread plating, its more qualitative nature and apparent bacterial species specificity, coupled with the high cost of the microscopic equipment required, will likely preclude its use by most researchers interested in determining bacterial survival kinetics in soil solutions.

CONCLUSIONS

The root-colonizing, pseudomonad and rhizobium strains not only failed to grow in a pH 4.71 Gilpin silt loam soil from an abandoned pasture, but died rapidly. Their rate of death (k), whether determined by agar spread-plating of inoculated soils or soil solutions, was directly related to small lime additions (up to pH 4.99). Survival was highly correlated with pH, Ca, Mg, and Al concentrations in both the (whole) soils and derivative soil solutions. Reduced exchangeable cation and increased Al concentrations, rather than organic constituents, likely accounted for the bacterial toxicity of these acidic soils. The use of filter-sterilized soil solutions, as surrogates for soils, represent a simple and efficient means for assessing survivability of bacteria destined for release into the soil environment and negates the requirement of marked bacterial strains, with their attendant questions of saprophytic competency. Perhaps more importantly, use of soil solutions offers a means for monitoring initial molecular rearrangements (even at the gene expression level) of introduced bacterial strains as they adapt to the soil chemical environment.

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