APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Mar. 1991, p. 649–654
0099-2240/91/030649-06$02.00/0

Vol. 57, No. 3

Population Dynamics of *Rhizobium leguminosarum* Tn*5* Mutants with Altered Cell Surface Properties Introduced into Sterile and Nonsterile Soils

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Received 1 June 1990/Accepted 12 December 1990

The influence of cell surface properties on attachment to soil particles and on population dynamics of introduced bacteria was studied in sterilized and nonsterilized loamy sand and silt loam. *Rhizobium leguminosarum* RBL5523 and three Tn*5* mutants (RBL5762, RBL5810, and RBL5811) with altered cell surface properties were used. Cellulose fibrils were not produced by RBL5762. Both RBL5810 and RBL5811 produced 80 to 90% less soluble exopolysaccharides and RBL5811 had, in addition, an altered lipopolysaccharide composition. In sterilized soil the total number of cells as well as the number of particle-associated cells of RBL5523 and RBL5810 were, in general, higher as compared with cell numbers of RBL5762 and RBL5811. Differences between strains in percentage of particle-associated cells in sterilized soil were only found at high inoculum densities, when populations increased little. In the nonsterilized silt loam, final population sizes, as well as numbers of particle-associated cells, of the parental strain (RBL5523) were higher than those of strains with altered cell surface properties after 56 and 112 days of incubation. But in general, differences in survival among the strains were not very marked. The importance of association with soil particles or aggregates for the survival of introduced cells was affirmed by the pronounced increase of the percentage of particle-associated cells during incubation in nonsterilized as well as sterilized soil. However, no clear relation among altered cell surface properties, particle association, and survival was found.

Bacteria introduced into soil have to cope with harsh conditions in the soil and to compete with well-adapted indigenous organisms (3, 22). An important attribute for survival of bacteria might be their association with soil particles or aggregates. For example, 90% of the indigenous rhizobia were found to be associated with soil particles (16), and extensive procedures (sonication, blending, and shaking) for dislodging indigenous soil bacteria demonstrate the strength of their association. As for introduced bacteria, the relative number of particle-associated cells increased with time after their introduction (1, 16, 18, 20).

Association with soil particles or aggregates can be due to enclosure in pores or attachment to soil particles. Enclosure in pores can be manipulated by varying the moisture content prior to inoculation (18, 20, 30). The impact of attachment, i.e., irreversible contact between bacterial cells and soil surfaces, might be examined by using bacteria with altered cell surface properties. Mutants with altered properties such as absence of cellulose fibrils, exopolysaccharide (EPS), or lipopolysaccharide (LPS) have been used for studying the infection process of rhizobia (5, 6, 9, 14, 24). However, the effect of these properties on attachment and on population dynamics in bulk soil are unknown. In nonsterilized soil not only the attachment, but also biotic interactions might be influenced by altered cell surface properties. Speculations have been made about the protective function of capsular and extracellular polysaccharides against predators and bacteriophages (7, 10, 25).

In the present work, the effect of an altered cell surface on particle association and population dynamics of introduced bacteria was studied in sterilized as well as nonsterilized soil. A strain of *Rhizobium leguminosarum* and three Tn*5* mutants were used as model organisms. High and low inoculum densities were applied to sterilized soil to obtain different numbers of dividing cells, which might influence attachment of cells to soil particles. In survival studies in nonsterilized soil, inoculations were made at two different initial moisture contents to establish differences in association of the inoculated cells with soil due to enclosure in pores, according to Postma et al. (20). Total cell numbers and numbers of particle-associated cells were determined.

MATERIALS AND METHODS

Soils. Two arable soils, a loamy sand and a silt loam (19, 20, 26), were air dried to 8 and 20% moisture content (approximately -10^3 and -10^4 kPa), respectively, sieved <2 mm, and stored at 4°C. Part of the soil was sterilized by gamma irradiation (4 Mrads), and sterility was tested by dilution plating on nutrient agar (3.25 g of Oxoid nutrient broth and 13 g of agar in 1,000 ml of water, pH 7.2). Prior to use, the nonsterilized loamy sand and silt loam were further dried to moisture contents of 1.5 and 5% (-10^5 kPa), respectively.

Bacterial strains. Strain RBL5523 (21) is derived from *R. leguminosarum* biovar *trifolii* strain LPR5039, which was cured of its Sym plasmid (12). In a rifampin-resistant derivative of LPR5039, the *R. leguminosarum* Sym plasmid pRL1::Tn*1831* was cured by selecting for transfer of spectinomycin (21). Strains RBL5762, RBL5810, and RBL5811 are Tn*5* mutants of RBL5523, obtained by transposon mutagenesis as described by Smit et al. (24), following the method of Beringer et al. (2). The strains were chosen for their altered cellulose fibril, EPS, or LPS properties (Table 1). Each

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TABLE 1. Surface properties of bacterial strains used in studies of the association of cells with soil particles or aggregates

Strain	Cellulose fibrils	EPS	LPS	Contact angle[a]	Electrophoretic mobility[a]
RBL5523	+	+	+	40°	−2.6
RBL5762	−[b]	+	+	24°	−2.5
RBL5810	+	−[c]	+	40°	−2.2
RBL5811	+	−[c]	−[d]	44°	−0.6

[a] Data obtained from van Loosdrecht: contact angle and electrophoretic mobility (10^{-8} m V^{-1} s^{-1}) are a measure of adhesion of bacterial cells to surfaces (28, 29).

[b] Diminished fluorescence on plates containing a minimal medium (RMM) with kanamycin and 0.02% calcofluor white under UV light and no extracellular fibrils detected by electron microscopy (24).

[c] Colonies with nonmucoid appearance on YMA and a reduction of soluble EPS production of 80 to 90% (wt/wt) as compared with RBL5523 (3a).

[d] Colonies lacking the O-antigen-containing LPS species determined by the method of de Maagd et al. (5).

mutant contained only one Tn*5* insertion and the location of Tn*5* was not in the Sym plasmid, as determined by the method described by Smit et al. (24). The impaired EPS production of RBL5810 and RBL5811 was due to different mutations, since complementation for EPS production occurred with different clones from an LPR5039 cosmid bank (3a).

Growth rate. The strains were cultured in yeast extract-mannitol broth (YMB) (11) supplemented with 50 mg of streptomycin per liter. Erlenmeyer flasks (250 ml) containing 50 ml of YMB without streptomycin were inoculated so that approximately 10^4 cells per ml were obtained upon inoculation. For each strain, two such batch cultures were incubated on a reciprocal shaker at 15°C. Cell numbers were determined daily by dilution plating on yeast extract-mannitol agar (YMA) (11) supplemented with 100 mg of cycloheximide per liter.

Colony morphology. To assess the ability of the strains to produce EPS under different nutritional circumstances, the strains were cultivated on plates containing solid media with different C/N ratios and colony morphology was monitored after 6 to 10 days at 28°C. Tryptone yeast agar (5.0 g of tryptone [Oxoid], 3.0 g of yeast extract [Oxoid], 1.0 g of $CaCl_2 \cdot 2H_2O$, and 13 g of agar in 1,000 ml of water), YMA, and soil agar were used. Soil agar was composed by adding 50 g of gamma-irradiated (4 Mrads) loamy sand or silt loam to 100 ml of sterilized water agar (13 g of agar per 1,000 ml of water).

Stability of antibiotic resistance markers. Inocula cultured in the presence of streptomycin and cells reisolated from both sterilized soils after an incubation of 54 days at 15°C were tested for their antibiotic resistance by replica plating. From each combination of strain, soil, and inoculum density, at least 100 colonies from YMA without antibiotics were transferred to YMA with 200 mg of streptomycin, 100 mg of spectinomycin, and 20 mg of rifampin per liter.

Sterile soil experiment. Inocula of the four strains were cultured in YMB supplemented with 50 mg of streptomycin per liter. After 2 days of incubation at 29°C on a rotary shaker, the cells were washed by centrifugation (7,000 × *g*, 15 min) and suspended in sterile deionized water. Two inoculum densities (approximately 10^7 and 10^9 cells per g of dry soil) were used to inoculate glass cylinders containing 10 g (dry weight) of sterilized loamy sand or silt loam. During inoculation, the moisture content of both soils was adjusted to −10 kPa (loamy sand, 16%, and silt loam, 40% moisture). The loamy sand was mixed with a spatula, and in the silt loam the added moisture spread by capillary forces only. After incubation for 1, 14, 33, and 54 days at 15°C in a moisture chamber to prevent drying of the soil, duplicate soil portions were washed five times with sterile deionized water as described by Postma et al. (20). The soil remaining after the washing procedure was shaken thoroughly (10 min, 280 rpm) with sterile gravel (diameter, 2 to 4 mm) in 95 ml of 0.1% sodium pyrophosphate to dislodge particle-associated bacteria. Rhizobia in both suspensions were enumerated by dilution plating on YMA supplemented with 100 mg of cycloheximide per liter so that total numbers and numbers of particle-associated cells were determined. The amount of remaining soil was weighed after filtration and drying at 105°C.

Nonsterile soil experiment. Glass cylinders containing 10 g (dry weight) of loamy sand or silt loam were prepared. To manipulate the distribution of the inoculated rhizobia, deionized water was added to part of the soil portions, producing soil portions corresponding to -10^5 and -500 kPa (loamy sand, 1.5 and 6.5%, and silt loam, 5 and 30% moisture, respectively). The soil portions were incubated in a moisture chamber for 2 days at 4°C. The added water in the silt loam spread by capillary forces only, whereas the loamy sand was mixed with a spatula prior to incubation. The soil portions were then inoculated with rhizobia cultured and washed as described and added in as much deionized water as was needed to obtain 1×10^7 to 4×10^7 cells per g of dry soil and final moisture contents corresponding to −10 kPa (loamy sand, 16%, and silt loam, 45% moisture). Again, only the loamy sand was mixed with a spatula, and both loamy sand and silt loam portions were incubated for 1 day at 4°C and thereafter at 15°C. Bulk density was approximately 1.0 g/cm^3 for both soils. Total rhizobial numbers and numbers of particle-associated cells were determined in duplicate soil portions with the washing procedure (20) after 1, 28, 56, and 112 days of incubation. The amount of remaining soil was weighed after filtration and drying at 105°C. YMA supplemented with 100 mg of streptomycin, 50 mg of spectinomycin, 20 mg of rifampin, 100 mg of cycloheximide, and 50 mg of benomyl per liter was used to enumerate the introduced strains. This combination of biocides was shown to have no influence on rhizobial numbers, but resulted in a sufficient suppression of indigenous microflora to enumerate 10^3 rhizobia per g of dry soil.

Statistical analyses. Effects of strain variation, incubation time, inoculum density, or initial moisture content, with all interactions, were analyzed by analysis of variance. In addition, these factors were analyzed for day 1 and for the two last sampling dates separately. The logarithm of the response variable was used, since proportional effects on bacterial numbers and percentages were studied, and the variance of replicates appeared to be stable on the log scale. Least significant differences (LSD) were calculated for significant levels $\alpha = 0.05$. When percentages are presented instead of the logarithmic values, least significant quotient ($= 10^{LSD}$) values are given.

RESULTS

Properties of bacterial strains. Generation times of strains RBL5523, RBL5762, and RBL5811 were 10 to 11 h in YMB at 15°C, whereas RBL5810 had a generation time of 19 h. Strains RBL5523, RBL5762, and RBL5811 reached population levels of 2×10^9 cells per ml in YMB in 8 days, but

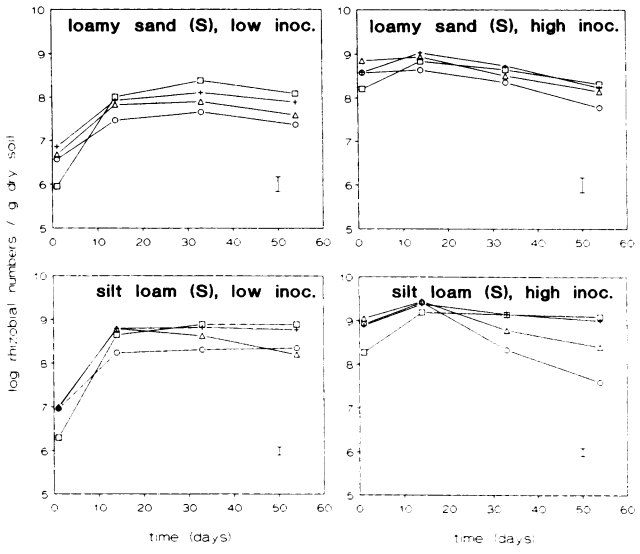

FIG. 1. Total cell numbers of four *R. leguminosarum* strains introduced into sterilized loamy sand and silt loam at low and high inoculum densities. Symbols: +, RBL5523; ○, RBL5762; □, RBL5810; △, RBL5811. Bars indicate least significant difference for $\alpha = 0.05$.

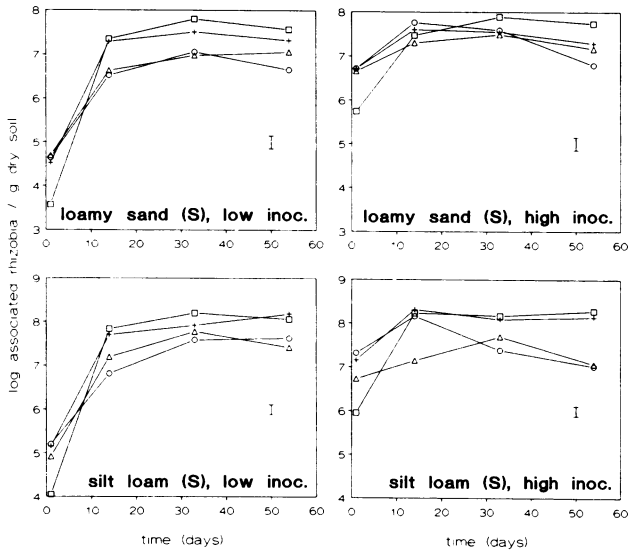

FIG. 2. Numbers of particle-associated cells of four *R. leguminosarum* strains introduced into sterilized loamy sand and silt loam at low and high inoculum densities. Symbols: +, RBL5523; ○, RBL5762; □, RBL5810; △, RBL5811. Bars indicate least significant difference for $\alpha = 0.05$.

RBL5810 started to flocculate after 9 days when only 10^8 cells per ml were present.

Strains RBL5523 and RBL5762 formed mucoid colonies on YMA (high C/N ratio) as well as on soil agar, but not on tryptone yeast agar (low C/N ratio). Organic matter content and amount of total N of the soil indicated that the C/N ratio of the soil agar should be approximately 14. Colonies of RBL5810 and RBL5811 were nonmucoid on all solid media tested.

After incubation for 54 days in sterilized soil, replica plating showed that the four strains had retained their antibiotic markers. Only in the treatment in which 10^7 RBL5811 cells per g of dry soil were introduced into sterilized silt loam did 2% of the colonies not grow on the medium with streptomycin, spectinomycin, and rifampin.

Sterile soil experiment. After introduction into sterilized soil, populations of the four strains increased during the first period of incubation (Fig. 1). When approximately 10^7 cells per g of dry soil were introduced, cell numbers increased, in general, by factors of 32 and 64 in the loamy sand and the silt loam, respectively. At the higher inoculum density (10^9 cells per g of dry soil), increases were only two- and fourfold, respectively. After 33 and 54 days of incubation, when populations were stabilized or started to decrease, differences in total numbers due to the inoculum density had disappeared in the silt loam, but in the loamy sand population levels were still somewhat higher at the higher inoculum density than at the low inoculum density. Strains RBL5523 and RBL5810 showed in most cases significantly higher final population levels (mean values at days 33 and 54) than RBL5762 and RBL5811 (Fig. 1).

The number of particle-associated cells increased strongly between days 1 and 14 (Fig. 2). Strains RBL5523 and RBL5810, which survived best, also showed higher numbers of particle-associated cells than RBL5762 and RBL5811 at days 33 and 54.

One day after inoculation, particle-associated cells, as a proportion of the total number of cells, were not significantly affected by the inoculum density and were only 0.4 to 2.5% of the total. The percentage of particle-associated cells increased up to 8 to 28% during 33 days of incubation. Thereafter, the percentage of particle-associated cells stabilized. Interestingly, there were significant differences in percentage of particle association between strains when high inoculum densities were applied (Table 2). However, after applying low inoculum densities, the percentage of particle-associated cells was not significantly different for the four strains and, in general, values were higher than at the high inoculum densities (Table 2).

The amount of soil left after the entire washing procedure was approximately 9.4 g for the loamy sand and 7.5 g for the silt loam. Incubation time and bacterial strain did not significantly influence these values.

Nonsterile soil experiment. Introduction of the four strains into both nonsterilized soils resulted in a decrease of cell numbers (Fig. 3). One day after inoculation, differences in moisture content prior to inoculation did not influence total numbers (Fig. 3). However, after 112 days, survival levels of RBL5523 and RBL5811 were higher in the silt loam with a low moisture content than in that with a high moisture content prior to inoculation. In the silt loam (both initial moisture contents) RBL5523 survived better than the Tn*5* mutants. In the silt loam with a high initial moisture content, significantly lower numbers of RBL5811 cells survived as compared with RBL5762 and RBL5810 (Fig. 3). Survival levels in the loamy sand differed little; the only exception was RBL5762 inoculated at the high initial moisture content.

One day after inoculation there was, in general, a significantly higher number of particle-associated cells in both soils at lower than at higher initial moisture contents (Fig. 4). At day 28, the number of particle-associated cells was similar to the initial level. This was followed by a steady decline during the rest of the experiment. After incubations of 56 and 112 days, the numbers of particle-associated cells (Fig. 4) showed a similar pattern as shown by the total numbers (Fig. 3): the same sequences in cell numbers of strains were found

TABLE 2. Percentages of particle-associated cells of four *R. leguminosarum* strains introduced with high and low inoculum densities in sterilized soil and with low (1.5 and 5%) and high (6.5 and 30%) initial moisture contents in nonsterilized soil

Strain	% of particle-associated cells[a]							
	Sterilized soil[b]				Nonsterilized soil[c]			
	Loamy sand		Silt loam		Loamy sand		Silt loam	
	Low	High	Low	High	1.5%	6.5%	1.5%	6.5%
RBL5523	26.7^{bc}	10.2^{a}	19.7^{c}	11.2^{b}	46.8^{b}	35.9^{b}	63.2^{e}	51.0^{d}
RBL5762	22.5^{b}	14.2^{ab}	17.9^{bc}	19.3^{bc}	45.1^{b}	53.2^{b}	36.7^{c}	27.2^{b}
RBL5810	28.3^{bc}	21.9^{b}	17.5^{bc}	13.1^{bc}	40.6^{b}	38.8^{b}	39.4^{c}	25.1^{b}
RBL5811	20.9^{b}	10.1^{a}	15.7^{bc}	8.2^{a}	22.7^{a}	21.2^{a}	28.6^{b}	15.2^{a}
LSQ[d]	1.67		1.67		1.48		1.23	

[a] There were no interactions of incubation time with the strains and inoculum densities or initial moisture contents. Superscripts a, b, c, d, and e indicate significantly different values ($P < 0.05$).
[b] Mean values of days 33 and 54.
[c] Mean values of days 56 and 112.
[d] LSQ, Least significant quotient ($\alpha = 0.05$).

and the differences due to initial moisture content were again present in the silt loam for strains RBL5523 and RBL5811.

One day after inoculation, the percentage of particle-associated cells was significantly higher in the soils inoculated at the lower moisture content. The percentage of particle-associated cells increased from only 0.1 to 4.2% up to 20 to 60% in 56 days. Thereafter, the percentage stabilized. In the loamy sand no differences due to initial moisture content were detected, whereas in the silt loam the percentage of particle-associated cells was higher for each strain at the lower than at the higher initial moisture content (Table 2). In both soils RBL5811 had significantly lower percentages of particle-associated cells as compared with the other three strains. In the silt loam, at both initial moisture contents, RBL5523 had significantly higher percentages of particle-associated cells than the other strains.

The amount of soil left after the entire washing procedure was approximately 9.7 g for the loamy sand and 8.5 g for the silt loam. Incubation time and bacterial strain did not significantly influence these values.

DISCUSSION

Association with soil particles, as a result of attachment to surfaces of soil particles or enclosure in soil pores, has been shown to influence the survival of introduced bacteria in soil (20). In the present study, *R. leguminosarum* mutants with altered cell surface properties were compared for possible differences in capacity to attach to soil particles. Comparable final population sizes were expected for the different strains within one sterilized soil, since each soil system is suggested to have its own "biological space" (15) with a distinct capacity to maintain a certain population (13, 17). The four strains, which originated from the same parental

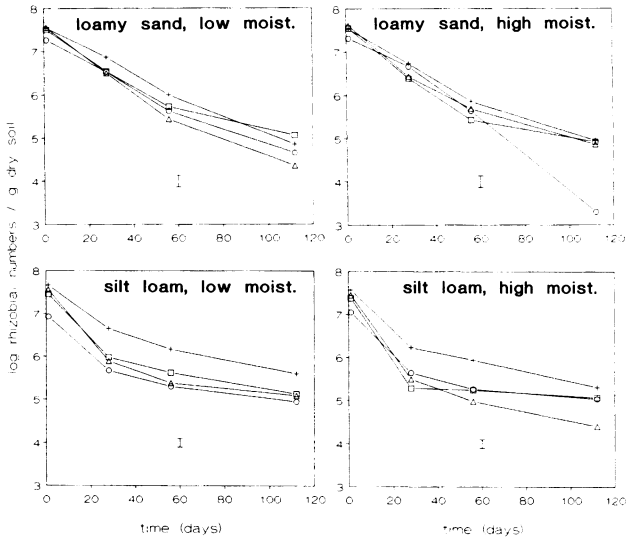

FIG. 3. Total cell numbers of four *R. leguminosarum* strains introduced into nonsterilized loamy sand and silt loam with low and high moisture contents prior to inoculation. Symbols: +, RBL5523; ○, RBL5762; □, RBL5810; △, RBL5811. Bars indicate least significant difference for $\alpha = 0.05$.

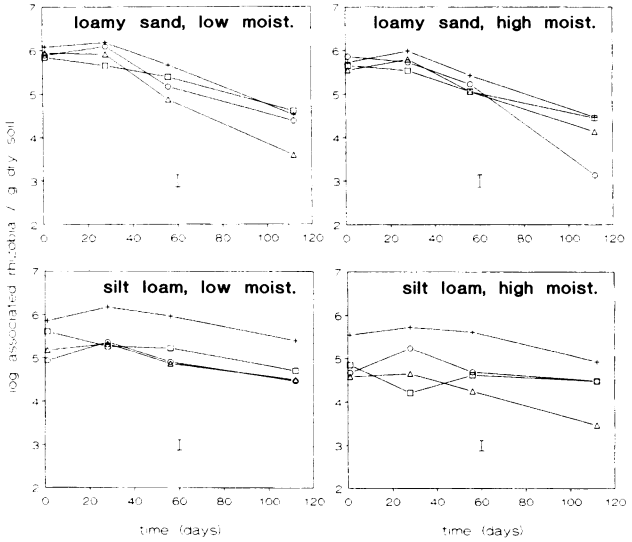

FIG. 4. Numbers of particle-associated cells of four *R. leguminosarum* strains introduced into nonsterilized loamy sand and silt loam with low and high moisture contents prior to inoculation. Symbols: +, RBL5523; ○, RBL5762; □, RBL5810; △, RBL5811. Bars indicate least significant difference for $\alpha = 0.05$.

strain, reached similar population levels in batch cultures with YMB, except for RBL5810, which flocculated at lower population levels. However, in the sterilized soils, RBL5523 and RBL5810 had, in general, higher final population sizes than RBL5762 and RBL5811.

In the nonsterilized soil, where more pronounced differences were expected due to the influence of biotic factors, differences between strains were not very clear. Only the parental strain (RBL5523) showed, in the nonsterilized silt loam, consistently higher final population sizes than the strains with altered cell surface properties. It was noteworthy that RBL5810, which had a much slower growth rate, also established well. Growth rate of mutants is often examined as a control; however, it is not known to what extent cell division takes place in nonsterilized soil, and the ability of cells to survive in nutrient-poor media might give more relevant information about ecological fitness in this situation.

The importance of particle association was affirmed in several ways. (i) In sterilized as well as nonsterilized soil, the percentage of particle-associated cells increased drastically during incubation, similar to previous results (18, 20). (ii) In sterilized and nonsterilized soil, final population sizes of the four strains followed the same pattern as the number of particle-associated cells. Moreover, in the nonsterilized silt loam the differences in numbers of particle-associated cells were more pronounced and were detected earlier than the differences in the total population size, showing that the population size was influenced by the number of particle-associated cells and not vice versa. (iii) In nonsterilized silt loam, higher percentages of particle-associated cells due to the moisture content prior to inoculation resulted in a higher survival rate. (iv) Strain RBL5811, which had the lowest percentage of particle-associated cells in both soils, was one of the poorer survivors, whereas the better survival of RBL5523 in nonsterilized silt loam corresponded with the highest numbers and percentage of particle-associated cells. However, association with soil particles is a result of attachment to soil surfaces and enclosure in soil pores, and the relative contributions of particle attachment versus enclosure are not clear.

Differences between strains in percentages of particle-associated cells were only found in sterilized soil when population sizes had increased two to four times during incubation. When population sizes increased 32 to 64 times, no differences between strains were found, and the percentage of particle-associated cells was generally higher than when populations increased little, suggesting that cell proliferation had more effect on association than the alterations of the cell surface of *R. leguminosarum*. A higher percentage of attachment of bacterial cells growing logarithmically than with stationary-phase cells was also detected in sand columns (4).

The differences between the strains in final population size and numbers of particle-associated cells could not be explained by EPS production or adhesion properties. The two EPS-impaired strains, RBL5810 and RBL5811, reacted differently. Inconsistent results on attachment (1) as well as predation (23) of EPS-producing bacteria have been found previously. Concerning the measured contact angle and electrophoretic mobility of the four strains (Table 1), RBL5811 was expected to have the highest adhesion values to glass, polystyrene surfaces, and soil particles (27, 29). In the soil experiments, however, RBL5811 was the strain with the lowest percentage of particle-associated cells. However, adhesion is only a first reversible step in the attachment to surfaces and attachment is only partly responsible for association of bacteria with soil. Bacteria can be enclosed in pores between or within aggregates. Similarly, DeFlaun et al. (4) mentioned that columns with natural soil filtered rather than measured attachment of bacteria.

Production of EPS has been suggested to play a role in attachment of bacteria to soil particles (1, 8), and speculations have been made about the protective function of capsular and extracellular polysaccharides against predators and bacteriophages (7, 10, 25). However, no convincing data could be found in the literature for these speculations, and also the present results of the Tn*5* mutants with altered cell surface properties did not elucidate the role of EPS in attachment to soil particles or protection of bacteria from predation. The importance of particle association for the survival of introduced bacteria in soil was affirmed, but none of the results gave evidence of the role of attachment. It might be that, in soil, at least for certain bacterial strains, enclosure in pores is of more importance than attachment to surfaces.

ACKNOWLEDGMENTS

We are grateful to M. C. M. van Loosdrecht for advice and information about bacterial adhesion, J. H. Oude Voshaar for help with the statistical analyses, and C. Waalwijk and A. J. B. Zehnder for useful discussions and critical reading of the manuscript.

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