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Fate and Activity of Microorganisms Introduced into Soil

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INTRODUCTION

Introduction of specific bacteria (and fungi) into soils has been performed in agricultural practice for decades. The main purposes of these releases are (39, 166) (i) to supply nutrients to crops; (ii) to stimulate plant growth, e.g., through the production of plant hormones; (iii) to control or inhibit the activity of plant pathogens; and (iv) to improve soil structure. Other, more recent, objectives for the introduction of microorganisms into soil are the mineralization of organic pollutants (bioremediation of polluted soils [96]) and the bioaccumulation or microbial leaching of inorganics (21, 45).

A variety of bacteria have been used in soil inoculations intended to improve the supply of nutrients to crop plants. *Rhizobium* species have been successfully used worldwide to permit an effective establishment of the nitrogen-fixing symbiosis with leguminous crop plants (19, 20). Other nitrogen-fixing symbionts, such as *Frankia* spp., have also been successfully introduced into soils (132). On the other hand, large areas of arable land in Russia, Australia, India, and Great Britain have been inoculated with nonsymbiotic nitrogen-fixing bacteria such as *Azotobacter*, *Azospirillum*, *Bacillus*, and *Klebsiella* spp. with the aim of enhancing plant productivity (89). In addition, phosphate (P)-solubilizing bacteria such as *Bacillus* and *Paenibacillus* (formerly *Bacillus*) spp. have been applied to soils to specifically enhance the phosphorus status of plants (25, 35). The stimulation of P supply to crops has also been the main purpose of the deliberate release of many mycorrhizal fungi.

The term "plant-growth-promoting-rhizobacteria" (PGPR) has been coined to encompass bacteria with plant growth-stimulating activity resulting from several different mechanisms (39). The production of plant growth-stimulating hormones and the suppression of minor plant pathogens by various mechanisms have been suggested to represent the main activities of PGPR. The best-known microorganisms with PGPR activity are bacteria belonging to the group of fluorescent *Pseudomonas* species (82), and a wealth of literature has accumulated on the mechanisms underlying their plant growth-promoting activity, as well as on their ecological performance (126). In addition to strains of the genus *Pseudomonas*, other bacteria such as *Bacillus* and *Azospirillum* spp. have been indicated as effective PGPR organisms (4, 28, 109a), the latter with potential for application in tropical climate soils (109a).

A third impetus to the emerging inoculant technology is the current necessity in many countries to control soil-borne plant diseases by biological means (11, 40, 44). Successful applications have included both bacteria and fungi. For instance, nonpathogenic *Agrobacterium radiobacter* has been used against crown gall disease, nonpathogenic *Fusarium* spp. have been used against *Fusarium* wilts (2), *Pseudomonas fluorescens* has been used against *Pythium* damping-off of cotton (74) as well as take-all disease of wheat (136, 164), and *Verticillium biguttatum* has been used against *Rhizoctonia solani*-induced potato damage (142). These antagonistic organisms most probably exerted their action directly on the pathogen. More recently, the induction by fluorescent pseudomonads of systemic resistance against soil-borne pathogens such as *Fusarium* spp. in plants was shown to be a promising control strategy (6a).

Microorganisms may also play a role in the formation of soil aggregates and hence in the stabilization of soil structure (108), and introductions of strains with such characteristics may be feasible. For instance, the introduction of polysaccharide-producing microorganisms, if these become established and function, might improve soil structure due to the aggregative action of polysaccharides (90). Bacteria such as *Paenibacillus polymyxa* have been assessed for their ability to stabilize soil aggregates, and the potential of these species for this application was suggested (58).

Bacterial inoculants are also potentially useful for enhancing

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the rate of degradation of xenobiotic compounds in soil, as well as for causing bioaccumulation or mobilization of inorganic compounds (45). For instance, the mineralization of pentachlorophenol in soil can be accelerated by introducing *Mycobacterium chlorophenolicum* or *Sphingomonas chlorophenolica* strains in the presence of various compounds, such as activated carbon and/or polyurethane, that control the interaction among soil, the inoculant organism, and the xenobiotic (96, 97). Moreover, the rhizosphere of *Phaseolus vulgaris* exerts a stimulative effect on the degradation of 2,5-dichlorobenzoate by a *Pseudomonas fluorescens* strain (36), which highlights the potential that plant roots have in microbe-mediated remediation of polluted soils.

Several releases of microorganisms into soils, notably those of rhizobia, have been successful; i.e., they resulted in the colonization of soil and plant roots to a level sufficiently high for the intended purpose. However, many failures or inconsistencies in achieving the objective have been reported as well (1, 145). These failures have raised concerns about the perspective of the great practical potential offered by microbial releases into soils. A key factor involved in the lack of success has been the rapid decline of the size of populations of active cells, to levels ineffective to achieve the objective, following introduction into soil.

The effective level of the density and activity of introduced microorganisms depends on the ecological conditions required by the application. When the introduced microorganisms are to be involved in a process which will give them a selective (nutritional or spatially protective) advantage in the soil system, only a minimal number of active cells is initially necessary for the application to be effective. Selection occurs, for instance, in the symbiosis of *Rhizobium* species with leguminous plants. Thus, only 300 rhizobial cells per seed have been reported to be required for optimal nodulation (53). Rhizobia can also be ecologically favored by amending soil with specific substrate. For instance, ground soybean added to soil enhanced the viable numbers of a specific *Bradyrhizobium japonicum* serogroup 1,000-fold, but the nodulation behavior of this subgroup in competition with other groups was hardly affected (160). Furthermore, when mineralization of xenobiotic compounds is desired and only the introduced microorganisms are able to produce the enzymes needed to utilize a specific compound as a source of energy, only minimal initial levels of the organism are often necessary. Thus, a 2,4,5-trichlorophenoxyacetic acid (2,4,5-T)-degrading organism, *Burkholderia cepacia* AC1100, was found to be selected from initially undetectable population densities upon application of 2,4,5-T to soil (29). A rapid increase of the AC1100 population size was noted concurrently with 2,4,5-T degradation activity, after which the population size fell rapidly to low levels.

However, when such an ecological selectivity or advantage is unlikely to occur in soil, maximum numbers of active inoculant cells are commonly required for the application to be successful. This is most often the case for biological control of plant pathogens based on competition for substrate and/or specific ecological niches, on predation or parasitism, on the production of antibiotics or related pathogen-inhibiting or -killing compounds, or on a combination of the above. In addition, applications of microorganisms for other purposes without clear ecological selectivity, e.g., the improvement of soil structure, require effective population levels. As soil is a heterogeneous system with a mixed biota under fluctuating local conditions, temporal and spatial aspects pertaining to the introduction should be critically evaluated for each release. Whereas some applications, e.g., the use of microorganisms in the bioremediation of soil, are relatively independent of time and space, others are necessarily time or space dependent. Time dependency is certainly an important factor in the biological control of soil-borne plant pathogens, since these often become manifest only at certain stages of plant development or under certain climatic conditions. In addition, there is often a clear dependency on the site in soil. Hence, in an effective plant pathogen control strategy, the activity of the introduced pathogen-antagonistic or -inhibiting organisms has to be synchronized with the peak, in time and space, of pathogen activity. For instance, *Pythium* spp., major soil-borne plant pathogens that cause seed rot and pre- and postemergence damping-off in a broad range of host plants, are known to attack plants at the seedling stage as well as at the change from the vegetative to the germinative stage. Therefore, the biological control agents used against this pathogen should be at maximum numbers and activity in these two discrete stages of plant development (74). Indeed, the lack of biocontrol efficacy of introduced *P. fluorescens* during late stages of potato plant development is probably correlated with the reduced populations of fluorescent pseudomonads in the potato rhizosphere at this stage (52, 98). Hence, the maintenance of sufficient activity of an inoculant population over a prolonged period after release often represents the main hurdle in the successful use of microbes as control agents of soil-borne plant diseases. Furthermore, efficient introduction into soil during the growing season is a major technical constraint.

For each introduction, abiotic soil factors such as texture, pH, temperature, moisture content, and substrate availability need critical assessment, since these largely determine the survival and activity of the introduced microorganisms (59). These factors are discussed briefly (Table 1). The response of the inoculant to the prevailing soil conditions depends on its genetic and physiological constitution. Thus, the effects of the aforementioned soil factors on the introduced microorganisms will differ in accordance with the ability of the inoculant to cope with adverse and fluctuating conditions, to survive, and to remain active.

In this review, we focus on the mechanisms that cause the decrease of numbers and activity of microorganisms following their introduction into soil and on the possible ways to improve the effectiveness of microbial inoculants in soil. Major issues involved in the effects of soils on the biotic and abiotic interactions between the inoculant and its environment are discussed. Since it is not possible to address all the effects of soil on all microbial inoculants, a selection has been made among organisms, particularly bacteria, relevant to agricultural practices. Whereas some effects described for these organisms may also hold true for other bacteria or soil conditions, this is not a generality.

POPULATION DYNAMICS OF INOCULANT BACTERIA IN SOIL

Population Size Declines and Their Causes

In general, population sizes of bacteria decline more or less rapidly following introduction into a natural soil, and growth of introduced populations in microbiologically undisturbed soils is a rare phenomenon. This growth/survival-inhibitory effect of soil has been called soil microbiostasis (71). It has been attributed to the scarcity of available nutrient sources to microbes in soil and the hostility of the soil environment to incoming microbes due to a myriad of adverse abiotic and biotic factors. Population declines have thus been observed for a wide variety of newly introduced bacteria irrespective of the source of their original isolation. For instance, declines were observed for

TABLE 1. Factors influencing bacterial survival in soils[a]

Origin	Factor	Effect
Biotic	Predation	Population size decrease
	Competition	Population size decrease/antagonistic effect on plant pathogens
	Root growth	Release of organic compounds, enhancing survival
Abiotic	Clay minerals	Protection against predation
	Water tension	High tension: water shortage, high osmolarity; low tension: anaerobism, increased nutrient availability by diffusion
	Organic carbon	Selection for copiotrophic or oligotrophic species; limited organic carbon results in starvation and reduction in activity
	Inorganic nutrients (N, P)	Limitation results in starvation
	pH	Selection for species; release of nutrients (e.g., P) or toxic compounds (e.g., Al^{3+}).
	Temperature	Metabolic activity as well as biotic (predatory) pressure affected.
	Chemicals (toxic waste)	Inhibition of sensitive organisms; selection of biodegradative, resistant, or tolerant forms

[a] Data from references 14, 144, and 148.

typical soil bacteria such as fluorescent pseudomonads (148); *Flavobacterium*, *Alcaligenes*, and coryneform spp. (104); and *Rhizobium* spp. (30, 117). Van Elsas and coworkers (146, 148) calculated from data reported in the literature that the decline in numbers of fluorescent *Pseudomonas* spp. could vary between 0.2 and 1.0 log unit per 10 days, depending on the soil type, the bacterial strain, and its genetic background.

The physiological characteristics of the inoculant organism determine to a great extent its fate and activity in soil. Hence, different species will show different responses, in terms of survival and activity. In certain cases, such responses are predictable given the intrinsic physiological characteristics of the inoculant strains used. For instance, immediately following introduction of a genetically marked *Bacillus subtilis* strain into field soils, the inoculant populations were shown to decline to levels approximately similar to the numbers of spores initially present in the inoculum (144). The populations remained at this level and mainly in the spore form over a period of 120 days. In the same experiment, a steady decline of a nondifferentiating *P. fluorescens* inoculant strain was observed. Furthermore, Thompson et al. (138) showed that an (oligotrophic) *Arthrobacter* inoculant survived for a longer period and in greater numbers than did a more copiotrophic *Flavobacterium* strain, in both planted and unplanted soil in three separate field experiments.

The physiological traits that play a role in the capacity of inoculant bacteria to colonize soil and survive are often not well known. Therefore, a thorough selection procedure is required when searching for effective inoculants. This is of prime importance for rhizosphere colonization when substrate utilization is probably a key factor, since the enhanced availability of substrate is the distinguishing factor between bulk soil and rhizosphere. Nijhuis et al. (104) used a stepwise procedure to select 4 bacterial strains from 420 that had originally been isolated from the rhizosphere of grass, for their capacity to colonize grass roots in large numbers over a prolonged period. These four strains showed a distinct pattern of colonization of different regions of the root system (which are known to be different in physiological terms [33]). One of the strains, identified as a *Flavobacterium* sp., occurred in significantly larger numbers in the rhizoplane of older root parts, whereas an *Alcaligenes* strain was more numerous on young root parts than on older ones (Fig. 1). Liljeroth et al. (88) showed that bacteria that occur preferentially on the tip of wheat roots are physiologically different from bacteria that occur on the older root parts (root base). In particular, the potential to use specific substrates, i.e., simple sugars and organic acids versus more complex compounds, was found to be a key factor which distinguished the root tip isolates from the root base isolates. The difference in ecological preference of the *Flavobacterium* and *Alcaligenes* species may be attributed to such differences in substrate specificities.

Factors Determining Inoculant Survival in Soils

Several mechanisms have been suggested to be main causes of the decline of microbial inoculant populations in soil (47, 146). Besides the intrinsic physiological characteristics of the organisms, abiotic and biotic soil factors play an important role. Abiotic soil factors (e.g., textural type, pH, temperature, and moisture) exert their (direct) effect on inoculant population dynamics by imposing stresses of various natures on the cells (47, 146). They can also act indirectly, by affecting the activity of the indigenous soil microflora. This topic has been extensively reviewed previously (see, e.g., references 14, 112, and 146) and is not the main focus of this review. Some key findings are summarized in Table 1.

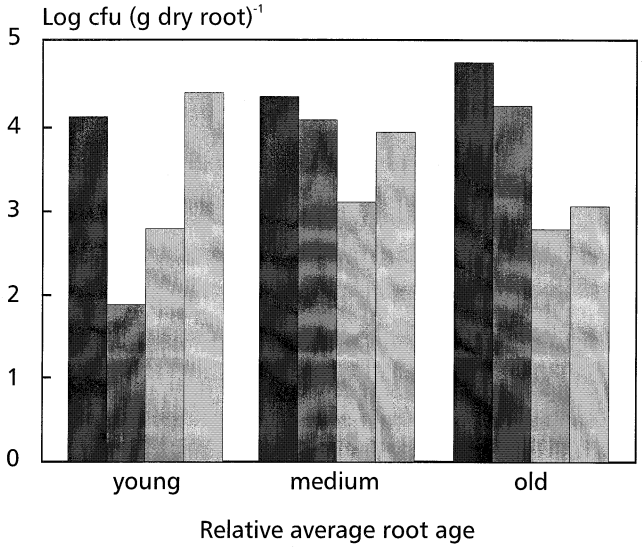

FIG. 1. Grass root colonization by four bacterial species (*B. cepacia* P2, *Flavobacterium* sp. strain F4, *P. fluorescens*, and *Alcaligenes* sp. [from left to right for each group of root ages]) introduced into a loamy sand soil via seed inoculation in relation to the age of the root segment. Reprinted from reference 104 with permission.

In addition to abiotic soil factors, other main causes of inoculant population declines are biotic (Table 1). The key argument that validates this contention is the observation that a population decline is often absent in sterilized soil (see, e.g., reference 67). At low inoculum levels in sterile soils, increases in population sizes can even be observed (117). This thriving of microbial populations following introduction into sterilized soils and their decline in nonsterilized soils (67) points to biotic factors, annihilated in the sterilization process, that play a major role in controlling the population dynamics of introduced bacteria in soils.

Cutler (34) first demonstrated that bacterial populations introduced into sterile soils together with predatory protozoa were reduced in size. The reduction of the population size of bacterial inoculants due to grazing by protozoa in soil has been confirmed in a number of more recent studies (37, 62, 67, 169, 170). A correlation was found between the decline in populations of individual bacterial strains and the activity and increase in the abundance of protozoa in soil (67, 170). Hence, protozoa play an important role as regulators of microbial inoculant population sizes in soil. As far as we can see, it is unknown whether there is specificity in the protozoan grazing on microorganisms and at what level this specificity is operational. However, Sinclair and Alexander (128) suggested that slow-growing bacterial species may be at a disadvantage in heavily grazed systems, since they will suffer greater population size reductions as a result of grazing than will fast-growing bacterial species, which have the capacity to regenerate rapidly in response to the enhanced nutrient availability brought about by protozoan grazing. On the other hand, it is possible that carbon-starved cells that reduced their size and entered a generalized stress-resistant form (153) will be less prone to digestion by protozoa than cells in exponential growth, which might limit the rate of their extinction.

Another biological factor, in line with the predation process, is the competition between inoculant and indigenous populations for available substrate and biological space. Postma et al. (120) performed a study in which sterilized soil portions were inoculated either with a mixture of bacterial cells, with a protozoan flagellate, or with a mixture of bacteria and flagellates. Soil portions were subsequently inoculated with a *R. leguminosarum* bv. trifolii strain. The total numbers of rhizobial cells were hardly influenced by the presence of either the mixture of bacterial cells or the flagellates. However, when both protozoa and the bacterial mixture were present, the numbers of rhizobial cells decreased. Moreover, the presence of other microorganisms also hindered rhizobial cells in the colonization of soil particles and aggregates, possibly exerting an effect on nutrient availability or indirectly by affecting protozoan activity. Hence, indigenous microorganisms competing for available resources represent a second biotic factor that can adversely affect incoming microbes (Table 1).

Effect of Soil Type on the Fate of Bacterial Inoculants

In a field study with genetically modified bacteria (144), a key difference in the survival patterns of introduced fluorescent pseudomonads in two different soil types was observed. Under similar prevailing climatic conditions, the inoculant revealed higher survival levels in finer-textured (clayey) than in coarser (sandy) soils. Moreover, Elliott et al. (46) showed that trophic interactions in soil, including nematode-protozoan-bacterium interactions, are influenced by the soil type as reflected in the pore space distribution. Colonization of soil particles and aggregates is assumed to be vital to ultimate inoculant survival in soil (65). The effects of, for instance, clay surfaces in conferring protection upon bacteria have been convincingly documented by Marshall (92). Recently, the survival of *Azospirillum brasilense* in 23 soil types was indeed found to correlate with soil clay content, in addition to other factors (9, 10). Furthermore, Foster (49) showed that upon chloroform treatment of soil, microorganisms were found only in mucigel deposits or in the interior parts of soil micropores. These soil microhabitats were suggested to be impenetrable by chloroform; hence, they constitute protective sites where microorganisms survive adverse conditions. In addition, Vargas and Hattori (159) clearly showed that in the presence of a cointroduced grazing protozoan species, the survival of inoculant bacteria localized in the interior parts of 1- to 2-mm soil aggregates was far better than that of cells present at external aggregate sites. This suggested that cells localized in the interior parts were physically protected from grazing by protozoa, presumably due to their localization in soil pores with small necks.

If soil pore space provides microorganisms with sites protective to soil fauna-feeding processes, including predation by protozoa, it is vital to the survival of introduced microorganisms to reach these protective sites in large numbers soon after inoculation. Microbial cells, upon introduction into soil, are probably not immediately localized in protective soil sites. Since the possibilities for directed movement of bacteria in soil are limited (49), specific action may be needed to localize as many inoculant bacteria as possible at protective sites. Postma et al. (116) showed that manipulation of the introduction method affected the localization of inoculant bacteria in protective microhabitats in soil. In their experiments, recipient soil was dried to different moisture levels prior to addition of bacterial cells. The bacterial cells would reach the soil pore space with the water of the inoculum. In the case of moist soil at the moment of introduction, the water present in small, potentially protective pores was assumed to act as a physical barrier to entry of cells, resulting in the localization of the inoculum in the relatively large pores. In initially dry soil, a greater proportion of smaller pores were not water filled and hence could be reached by the inoculant cells with the incoming water. This implies that bacterial cells introduced into initially wet soil would be accessible for predation to a larger extent than would cells introduced into initially dry soil. Indeed, after 60 days of incubation under the same moisture conditions (equalized after introduction of the inoculant cells), the survival of rhizobial populations introduced into the initially dry soils was significantly better than that of rhizobia that entered the originally wet soils. Based on the same approach, Wright et al. (169, 170) examined the effect of the soil ciliate *Colpoda steinii* on the survival of *P. fluorescens* located in pores with different neck diameters. Again, pores with small necks (diameter, <6 μm) were shown to confer a greater level of protection against protozoan predation upon the inoculant cells than were pores with necks 6 to 30 μm in diameter. Thus, spatial compartmentalization in soil is an important phenomenon which can be used to enhance the survival of inoculants.

Soils of different texture differ in particle size composition and, thus, in pore size distribution. Hence, pore size distribution strongly determines the fate of introduced microorganisms, and differences in the behavior of bacteria released into different-textured soils may be related to differences in the protective pore spaces present in these soils. In the aforementioned field experiment (144), a *P. fluorescens* inoculant strain was introduced into loamy sand and silt loam soils and monitored over the growing season (120 days). Monitoring was later extended to a period of 3 years. The results in Fig. 2 confirmed the enhanced inoculant survival in the finer-textured soil since

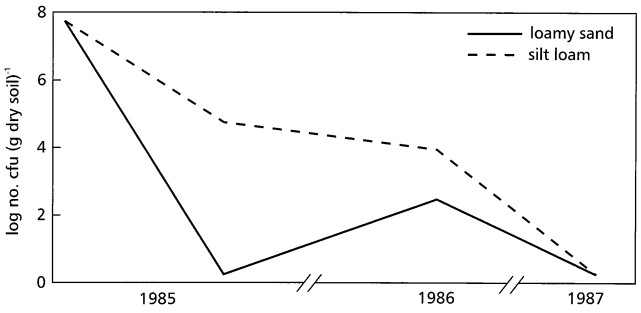

FIG. 2. Survival of *P. fluorescens* (kindly provided by B. Schippers, Willie Commelin Scholten Phytopathological Laboratory, Baarn, The Netherlands) introduced into a silt loam and loamy sand under field conditions. Data from reference 144.

the decline of the inoculant numbers was greater in the sandy soil used than in the silt loam soil.

The pore size distribution in soils can be quantified by the use of soil moisture curves, and this may represent an adequate tool for the prediction of inoculant fate in different soil types (38). Postma and van Veen (118) formulated the concept of soil pore neck categories with distinct differences as microhabitats for introduced bacteria (Fig. 3). Earlier studies (120) on the survival of bacteria in pores with necks of different sizes were the basis for the definition of total, accessible, habitable, and protective pore spaces. The concept that pores with necks of <0.8 μm are too narrow to be accessible for introduced rhizobial cells was based on work by Kilbertus (78). Pores with neck diameters of >0.8 μm but <3 μm would be protective, since they are accessible to bacterial inoculants yet largely inaccessible to bacterial predators.

Heijnen and van Veen (68) correlated the survival of a rhizobial inoculant with the pore size classes present in soil. The survival of rhizobial cells 57 days after introduction, S_{57} (expressed as the number of surviving cells of a 2.5×10^7 to 5×10^7 CFU g^{-1} soil inoculum) was described by using the amount of soil moisture, P, which can theoretically be held by pores with the appropriate pore neck diameters of <3 μm, 3 to 6 μm, and 6 to 15 μm. The following equation was obtained:

$$S_{57} = 6.98 + 0.04P_{<3\ \mu m} + 0.92P_{3\text{–}6\ \mu m} - 1.92P_{6\text{–}15\ \mu m}$$

This relationship accounted for 98% of the variance. The positive signs indicate that the respective pore size classes are protective for introduced bacteria and positively contribute to survival of the introduced bacteria. Although the aforementioned categories of pore sizes were slightly different from the ones formulated by Postma and van Veen (118), the basic concepts were in line with each other. Thus, the use of the concept of different pore size classes provides the basis for adequate prediction of the fate of bacteria introduced into different soils. A mathematical model useful for predicting the fate of introduced microorganisms in soil was based on this premise (143). The model further used the concept of incompatibility between resident and invading organisms and accidental migration of cells during water and nutrient fluxes. It adequately predicted the decline of introduced populations depending on the available protective pore space, nutrient availability, and the presence or absence of antagonistic microorganisms. Realistic parameter settings allowed for the fitting of inoculant survival curves obtained in the field, where these processes presumably take place (143).

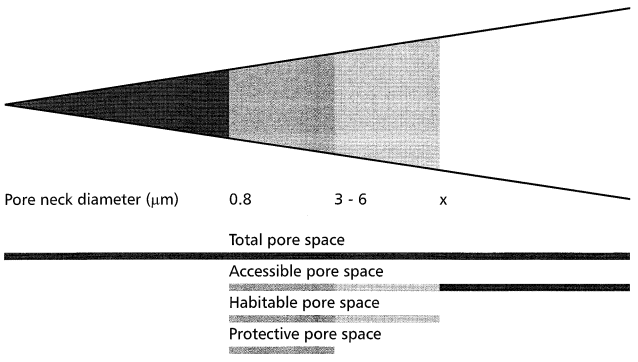

FIG. 3. Conceptual scheme of pore space categories in soils. The pore space is determined from the moisture characteristic curves; x relates to the maximum pore neck diameter of pores filled with water at a certain pF value. Reprinted from reference 118 with permission.

The effect of soil type on the fate of introduced bacteria also corroborates the known effect of soil type on the microbe-mediated turnover of organic matter in soils (see, e.g., reference 157). Turnover of organic matter has been observed to be slower in finer-textured soils than in coarser ones. This effect has been explained by the different extents to which soils offer physical protection to organic matter against degradation by microbes and subsequent turnover of microbial biomass, in which protozoan grazing plays an important role (86).

However, several aspects should be thoroughly considered when applying any model based on soil pore size distributions. First, the methodology to determine the pore size distribution by the use of soil moisture characteristics has drawbacks, as indicated by Smiles (130) and Darbyshire et al. (38). Theoretically, there is another pending problem. Postma and van Veen (118) determined the maximum number of rhizobial cells that could theoretically occupy the volume and/or the surface of the pore space of the accessible, habitable, and protective pore space categories. An approximately 10-fold larger population than that actually observed, in the order of 10^{10} to 10^{11} cells per unit of soil, could theoretically occupy the pore space of two soils. Therefore, other factors, such as the spatial diversity and localization of the habitable and protective pore size categories, are likely to be important for their accessibility and hence for the survival of introduced bacteria as well as substrate availability inside and out of the available pore space.

Substrate Availability in Soil

The availability of nutrients to inoculants in most environments, such as water and soil systems, is often low (57, 59, 65, 99, 112, 148). Indeed, soil not directly under the influence of plant roots has been characterized as a "grossly oligotrophic" environment for its inhabitants (115). The main reason for this gross limitation of nutrient (mainly carbon) supply is the unavailability of the organic carbon present in soil, either due to its recalcitrance (resistance to degradation) or to its location in soil sites that are not easily reached by soil microorganisms. Substrate availability (in terms of quantity and quality) is largely determinative for the biological carrying capacity of a soil, i.e., the biomass that can be supported or maintained. This can be concluded from observations on the population dynamics of microbes introduced into sterilized soils. Sterilization of soil by γ irradiation results in an increase in substrate availability (C, N, and P sources) due to the killing of soil organisms. Besides this, a minor effect on soil chemistry is expected. Postma et al. (119) showed that the population size of *R. leguminosarum* bv. trifolii introduced into two different γ-irra-

TABLE 2. Methods for determination of the physiological condition of inoculant bacterial cells or populations in soil

Criterion	Method	Parameter assessed
Culturability	Plate count versus immunofluorescent-cell or most-probable-number PCR count	Detection of cells which have lost the ability to multiply in the presence of externally applied nutrients
Cellular enlargement by nutrient application	Kogure method; application of nutrients in combination with cell replication inhibitor (nalidixic acid)	Ability to respond to a supply of nutrients
Cell length	(Immunofluorescence) microscopy, image analyzer	Cellular morphology to indicate the physiological condition
Stress resistance	Challenge by externally applied stress factors	Development of cross-protection to stress factors by exposure to soil conditions
Cellular activity	Viability stains such as redox dyes	Presence of membrane potential as an indicator of cellular activity

diated soils was independent of the initial inoculum density but dependent on the soil type. This observation corroborates those described in the experiments of Bennett and Lynch (12), in which three bacterial species, independent of their inoculum size, colonized sterilized barley roots up to similar final population sizes. King and Parke also found that introduced biocontrol *B. cepacia* cell populations colonized roots of peas to characteristic densities (79). The final population sizes probably represented the carrying capacity (defined as the ecological "space" available for maintenance and persistence) of the rhizosphere, in terms of nutrient supply and physical colonizable space, for this organism. Nannipieri et al. (103) suggested that each soil ecosystem has its own distinctive biological space with respect to the maximum level of microbial biomass and enzymatic activity. It has therefore been suggested that the final population size in sterilized soil observed by Postma et al. (119) represented the capacity of the soil to sustain a bacterial population in terms of substrate availability. However, this concept can be challenged since it does not take into account any putative spatial limitations to soil colonization or the serendipitous occurrence of hostile sites which block further colonization. Also, the concept is rather crude and difficult to apply in a comparative fashion for diverse specific bacterial groups, as differences in substrate preference between different groups are not taken into account. Nevertheless, it is likely that the population size of any microbial inoculant in soil will ultimately be limited by the availability of degradable substrates, next to that of freely colonizable space.

PHYSIOLOGICAL STATUS OF INTRODUCED MICROBIAL CELLS IN SOIL

The concerted activity of the individual microbial inoculant cells following introduction into soil generally determines the overall effectiveness of a microbial release. Hence, the effectiveness depends on the physiological status of the introduced microbial population throughout its life in soil, which in turn depends on the effects of prevailing conditions in the soil, including those of stress. Such stress conditions are, for instance, a generalized scarcity of easily available nutrients (e.g., C, N, and P sources), physical factors such as oxygen or water supply or limitation, high osmotic or matric tension (134), and, in particular, fluctuations in these conditions which are typical for the soil environment. Chemical factors such as extreme pH values or noxious chemical compounds may also play a role. Table 1 lists these factors and their effects on bacterial populations. It is difficult to assess the distribution patterns of such stress conditions in soil at a microscale level; however, they are likely to be heterogeneous and locally variable (49, 130, 148). Evidence for microscale heterogeneity in soil with respect to substrate oxidation was recently provided by using community BIOLOG substrate utilization patterns (61). A chemical stress condition may be prevalent at a particular soil site but absent only a short distance away. Therefore, the physiological status of individual microbial cells in a population in soil is probably not uniform (148, 155), and the physiological response of an inoculant population to soil conditions is the sum of the responses of individual cells which are determined by the effects reigning at the soil microhabitat level. The response of inoculants can be said to be more or less homogeneously affected in soil only when very dominant factors prevail, for instance when a soil becomes excessively dry, wet, cold, or warm.

The overall physiological status and activity of inoculants, e.g., as biopesticides or as detoxifying agents for contaminated soils, is of prime interest for the efficacy of the introduction. Moreover, if potential hazard is associated with the expression of (heterologous) genes and this gene expression is linked to overall cellular physiology, the physiological status of individual cells is of interest for biosafety considerations in releases of genetically modified microorganisms into soil. Therefore, the availability and development of methods to determine physiological parameters of both individual microbial cells and total microbial populations in soil are important. The physiological response of microbial inoculants to soil conditions can also be used in another way, because the physiological status of inoculant cells will reflect the actual stress status at a particular soil site. Hence, microbiosensors based on (promoterless) reporter gene insertions may be used to probe the local nutrient limitation or stress status of a soil or rhizosphere. For instance, the usefulness of a modified *Pseudomonas* strain to assess phosphate-limiting conditions in soil has been shown by de Weger et al. (42). The use of microbiosensors is important since, in spite of the easy assessment of essential nutrients in bulk and rhizosphere soils by soil chemistry methods such as the ones based on substrate-induced respiration (151), it is difficult to ascertain levels of nutrients or other compounds at particular soil sites such as microsites in the rhizosphere (91, 148). Knowledge about the availability, distribution, and diffusion of nutrient sources is essential to predict the in situ fate and activity of bacterial inoculants (143).

To ascertain the in situ physiology of inoculant cells in soil, a suite of methods based on cell extraction and subsequent microscopic observation is currently available (18). Table 2 lists some of these methods. Perhaps the simplest tool for determining the physiological status of bacterial cells introduced into soil is the assessment of variations in cell lengths by epifluorescence microscopy (117, 153). Cellular activity can be more precisely assessed via the presence of a measurable membrane potential by using redox dyes like 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl tetrazolium chloride (INT) (106, 171) or 5-cyano-2,2-ditolyl tetrazolium chloride (CTC) (70, 123, 168). Substrate responsiveness, assessed by the increase in cell size

in the presence of nutrients and a cell division inhibiting antibiotic like nalidixic acid (19, 43, 83, 102, 110, 111, 122, 165), is a tool which specifically addresses the capacity of cells to induce a growth response to substrate. Finally, assessment of the cellular development of resistance to different stress factors provides information about the physiological status at the population level (54, 55, 75, 76, 153). All of these methods have been successfully applied to determine the physiological status of bacterial populations both under in vitro (laboratory) conditions and in oligotrophic environments like soil and water, as discussed below.

Cell Size Measurements

A simple method to determine the physiological condition of microorganisms in soil is by cell size and biovolume measurements via microscopy of soil smears, eventually in combination with image analysis (16, 18). The method has been successfully applied to assess indigenous bacteria in soil (16), which often appear as minute (dwarf) forms (5). However, it is less suitable to assess the activity of microbial inoculants.

Generally, gram-negative inoculant species tend to decrease their cell size after exposure to stress conditions (99, 100). A series of papers in the last 10 years has convincingly shown cell size reductions in *Vibrio* spp. when exposed to oligotrophic conditions in aquatic systems (see, e.g., references 80 and 81). Other gram-negative bacteria such as the fish pathogen *Aeromonas salmonicida* showed similar responses. Oligotrophy can also affect the shape of (nondifferentiating) introduced cells. Holmquist and Kjelleberg (73) demonstrated that exponentially growing rods of *Vibrio* spp. which were starved for carbon, nitrogen, or phosphorus or a combination of these nutrients turned into small cocci, filaments ranging up to 25 to 30 μm, swollen rods containing poly-β-hydroxybutyrate (PHB), or small rods, respectively. A decrease in cell size has also been observed for typical soil bacteria like *R. leguminosarum* bv. trifolii (20, 117), and *P. fluorescens* (153) under conditions of nutrient scarcity such as are encountered in soil (115). The mean length of the *R. leguminosarum* inoculant cells decreased from 2.05 μm before introduction to 1.66 and 1.61 μm in loamy sand and silt loam soils, respectively, after 5 to 6 days of incubation. As no evidence for capsule size reduction was obtained, this suggested a reduction of cytoplasmic volume. The relative decrease in cell length during incubation in soil was similar to the decrease in cell length in a nutrient-poor liquid medium, which points to the importance of oligotrophy for this phenomenon rather than to soil physical factors. Reduction of bacterial cell size can thus be regarded as an adaptational response to nutrient stress (57). In a recent field microplot experiment, evidence was also obtained, via immunofluorescence, for the occurrence of small forms of introduced *P. fluorescens* cells (156a).

To differentiate introduced from indigenous soil bacteria in natural (nonsterilized) soil, specific tools, like fluorescent antibodies (17, 117) or oligonucleotide probes directed to the introduced strain (3, 64, 139), are essential to mark the inoculated cells. Since the use of antibodies gives rise to a halo around the bacterial cells, which may lead to an overestimation of the cell size, the use of fluorescently labelled oligonucleotide probes directed to strain- or species-specific regions of the 16S or 23S rRNA has been suggested to be advantageous for cell size determinations. However, it may still be difficult to obtain sufficient signal intensity inside the cells, because cells in soil are often nutrient deprived and may contain a limited number of ribosomal targets (63).

Bakken and Olsen (6) showed a relationship between culturability and cell size of bacteria in soil. Only 0.2% of the minute bacterial cells (cell diameter, <0.4 μm) extracted from a clay loam were shown to be culturable. Culturability increased as the cells became larger: 10% of the cells with diameters of 0.4 to 0.6 μm and 30 to 40% of the cells with diameters of >6 μm could produce colonies on agar plates. Hence, for the indigenous bacteria, culturability on agar media apparently was directly related to the initial cell size. However, it remains uncertain whether introduced cells after a cell shrinkage response to soil are commonly viable and substrate responsive, and thus may be directly active under soil conditions (32, 124).

Direct Activity Measurements

The metabolic activity of individual cells in soil can be measured by their transformation of tetrazolium salts into fluorescent pigments which are detectable by epifluorescence microscopy (18). By using compounds like INT or CTC (123), the intracellular accumulation of insoluble INT- or CTC-formazan crystals can be observed when an intact membrane potential is present (INT or CTC cell counts). Such metabolically active cells, as well as cells lacking formazan crystals, can be specifically detected by staining with a strain-specific fluorescent antiserum, as performed by Heijnen et al. (70). Hence, identification of metabolically active cells in a specific population in soil is possible. An alternative approach to determining cellular activity is based on the utilization of substrate by cells recovered from soil in the presence of nalidixic acid (direct viable count [DVC]) (83). Nalidixic acid inhibits DNA synthesis without affecting other metabolic activities of cells. Gram-negative bacteria will continue to grow without dividing, resulting in elongated forms. Hence, cells that are not able to form colonies on plates but are, however, still active can be counted. By using the DVC method, Pedersen and Jacobsen (113) showed that the culturability on agar media of *Enterobacter cloacae* and *Alcaligenes eutrophus* inoculants dropped by 3 log units in air-dried soil from that in moist soil whereas the substrate responsiveness of the inoculant determined by the DVC method only dropped by 2 log units. Total (immunofluorescence) cell counts were hardly affected. Hence, cellular activity assayed by the DVC method can reveal the proportion of cells, both culturable and nonculturable, that is responsive to substrate and presumably still able to utilize it. The method can be combined with an antibody- or oligonucleotide probe-based approach to allow for the assessment of the level of substrate responsiveness in a specific inoculant population in a mixed soil microbial community (70). In a recent study on the fate of *Flavobacterium* sp. strain P25 in bulk and wheat rhizosphere soils, the metabolic activity of the introduced population was shown to decline upon prolonged residence in soil, and after 2 weeks the activity in rhizosphere soil was higher (in terms of the percentage of active cells) than that in the bulk soil (70). Remarkably, a larger number of actively metabolizing cells was present in bulk soil than in rhizosphere soil during the initial 2 weeks in soil. CTC-reducing activity declined with time for inoculant populations in both bulk and rhizosphere soils. No difference in response was observed between the DVC and CTC methods, although the two methods assess different aspects of the physiology of the cell. Therefore, the decrease in activity of the inoculant cells probably represents a generalized physiological response to stressful soil conditions rather than a specific effect on either nutrient uptake or cell membrane potential.

Culturability of Inoculant Cells

Cells that have lost the capacity to multiply on culture media but still have a measurable membrane potential or show an increase in size as a response to substrate can be regarded as viable but nonculturable (32, 124). The viable-nonculturable paradigm is, however, wrought with uncertainties, as pointed out by Akkermans et al. (1). Many microorganisms may simply not grow when taken from the environment because our current understanding of conditions that favour their cell division is too limited. Although viable but nonculturable cells in soil are likely to play a role in processes like nutrient turnover, the breakdown of xenobiotics, root colonization or infection, and eventually cell division in later stages of colonization, the exact role remains uncertain. This is also true for cells which have lost both culturability and their response to substrate. Nonculturable cells of *P. fluorescens* R2f have been observed 1 year after the release of this strain into agricultural drainage water (156) as well as in soil (146, 149) and with *R. leguminosarum* bv. trifolii after prolonged residence in soil (72, 117). To investigate whether nonculturable forms can be resuscitated by the presence of easily degradable substrate, mannitol was applied to *R. leguminosarum* bv. trifolii-containing soil 40 days after introduction. This resulted in an increase in the culturable cell number without affecting the total cell counts as measured by immunofluorescence (Fig. 4). The magnitude of the effect was dependent on the level of mannitol added. Possibly, viable but nonculturable cells were reactivated by the addition of mannitol, enabling these to again develop colonies on the selective agar used. The nature of this physiological upshift at the molecular level is unknown, but it may range from an overall effect on the cellular energy status to an effect more specific for the ability of the cells to develop a colony on agar plates. Alternatively, a relatively small culturable subpopulation may increase rapidly in size (much as described for dormant *Micrococcus luteus* cells [161]) up to the biological carrying limit of the soil system.

The physiological status of the nonculturable cells thus determined remains uncertain, since it is unknown whether, in general, they are capable of becoming active and divide or are in a transition stage to cell death. Resuscitation of in situ-induced nonculturable cells, e.g., by application of cold or nutrient stresses in soil, is possible. However, to ascertain this, one should be absolutely certain that the total population is nonculturable and that the apparent resuscitation is not the result of simple outgrowth of a few remaining viable cells (48, 163). Further, it remains uncertain whether this resuscitation of nonculturable populations in soil is representative of the response of bacteria introduced into soil. The molecular response of bacteria to different stress conditions can be diverse (107), and after application of a single stress condition it may be different from the adaptation at the molecular level evoked by stress conditions which are naturally occurring in soil.

Culturability of introduced cells represents an indirect measure of cellular activity. It can be assessed by using selective plating, i.e., selecting for a marker present in the introduced microbial cells (149, 150). Spread plating is perhaps the simplest method which assesses microbial culturability. However, it is not very sensitive, since cells that show only a limited number of divisions (produce microcolonies) are commonly missed. Therefore, Binnerup et al. (15) developed an agar slide method which permitted the microscopic observation of microcolonies formed. Hence, assessment of the physiological diversity, with respect to substrate responsiveness, of soil microbial populations was possible. van Vuurde et al. (158) designed a method based on pour plate culturing which also enhanced the culturability of soil bacteria as compared to that of populations plated via spread plating. The combination of this method with a reaction to a specific antibody or with specific PCR conferred the required specificity for the target bacterial groups to be monitored (149).

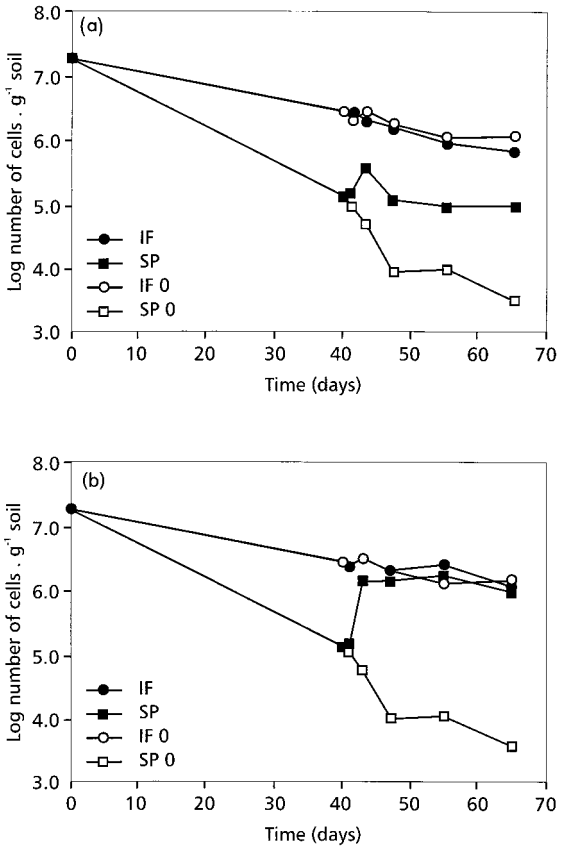

FIG. 4. Plate counts and immunofluorescence counts as indicators of culturable and total numbers of *R. leguminosarum* bv. *trifolii* introduced into a loamy sand soil. (a) Addition of 0.4 g of mannitol/g of soil on day 40. (b) Addition of 2.0 g of mannitol/g of soil on day 40. Abbreviations: IF, specific immunofluorescence cell counts; SP, selective CFU counts. Solid symbols (IF, SP) show counts in mannitol-amended soil; open symbols (IF 0. SP 0) show counts in unamended soil. Data from reference 72.

Physiological and Molecular Responses of Inoculant Cells

Measuring the viability of bacteria introduced into soil by either direct or indirect means without knowing the ecological impact of physiologically different conditions seems pointless. It is therefore important to gain more insight in the molecular processes taking place in the inoculant cells following their release into and exposure to soil. As one of the main stress conditions in bulk soil is thought to be nutrient (carbon) starvation (65, 115, 148), an understanding of the response of inoculants to this condition may be key to understanding their ecological fate in soil. The in vitro molecular responses of a limited number of species to stress conditions like carbon or generalized nutrient starvation are relatively well known (54, 55, 60, 93, 101, 107). For instance, from in vitro experiments with *Escherichia coli*, *P. putida*, and *P. fluorescens*, it is known that when exposed to carbon starvation, these bacterial cells can develop cross-protection to other stress factors like ethanol, moderate heat, and osmotic and oxidative stress (54, 55, 75, 76). However, information on such adaptive processes ob-

tained in natural soil has been scarce. Therefore, *P. fluorescens* R2f cells exponentially grown or carbon starved prior to introduction were recently tested in soil (154). Exponential-phase cells showed a progressively increasing resistance to the four stress factors during residence in soil, whereas the level of resistance of introduced carbon-starved cells remained constant and high. No difference in inoculant survival was observed between the two treatments. Addition of glucose to soil together with the exponential-phase cells resulted in enhanced population levels but in decreased resistance to the four stress factors. From this study, it was concluded that *P. fluorescens* inoculant cell populations adapted to carbon starvation did not show enhanced survival in soil and that external input of substrate into soil (comparable to the rhizosphere) resulted in decreased stress resistance. This last conclusion was substantiated by data obtained with strain R2f in the rhizosphere of wheat plants grown in the field. These cells showed reduced stress resistance in wheat rhizosphere soil in comparison with cells in the corresponding bulk soil (156a).

Carbon respiratory activity was used by Colbert et al. (31) to determine the metabolic activity of a *P. putida* strain carrying a salicylate-degradative plasmid in soil. The exotic substrate salicylate was added, which was specifically degraded by the introduced *P. putida* strain, resulting in enhanced amounts of respired carbon corresponding to increasing cell numbers. However, when all salicylate was utilized and respiration had dropped to its original level, cell numbers remained unchanged for several days. This suggested that large numbers of originally metabolically active cells were rapidly transformed into inactive ones, enabling them to survive.

Reporter gene technology is very useful in studies on the metabolic status of introduced bacteria in soil, and various different selectable or elective markers have been proposed or used (26, 114, 121, 133). For instance, Fravel et al. (50) used a bioluminescent reporter gene and photography to identify regions of colonization of the rhizosphere by *Enterobacter cloacae* and found a correlation between bacterial cell number and bioluminescence. Meikle et al. (94, 95) also used bioluminescence to assess the ability of luminescence-marked cells of *P. fluorescens* to regain activity following starvation in soil. Viable cell concentrations correlated well with final potential luminescence values. Studies with *lac*Z as a reporter gene controlled by a carbon starvation-induced promoter in *P. fluorescens* R2f in natural soil revealed that *lac*Z gene expression increased shortly after introduction of the cells into soil (152). Apparently, the introduced cells indeed sensed carbon-limited conditions in soil, and this resulted in a physiological response which involved the induction of the carbon starvation-inducible reporter gene. In a similar fashion, de Weger et al. (42) reported on the use of phosphate limitation-induced bacteria which allowed for the detection of phosphate limitation in soil and rhizosphere.

The amount of rRNA in cells can be used as another indicator of metabolic activity, since the number of ribosomes correlates loosely with growth or metabolic rate. For example, for aquatic systems Kramer and Singleton (84) showed a decrease in rRNA content in *Vibrio* cells to 10 to 26% of the original value 15 days following the onset of starvation. Addition of substrate resulted in actively respiring bacterial cells, as demonstrated by an increase in the rRNA contents of the natural communities (85). Such studies are not yet known for soil systems, and it is likely that similar studies in soil will be more difficult to perform.

Understanding the bacterial response to soil environmental stresses at the molecular level is of great importance in predicting and manipulating bacterial activities in soil. Based on the foregoing, differences in bacterial activities between bulk and rhizosphere soils can often be expected. However, to understand the effects of soil on its microbial inhabitants, it is very important to gain a still better and more refined insight in the factors controlling bacterial activities in specific soil sites, e.g., inside soil aggregates or at their outer surface, in small soil pores (<6 μm), and at different sites (young root parts versus older parts) in the rhizosphere soil or at the root surface. This is certainly an important and promising field for future work.

IMPROVEMENT OF THE ESTABLISHMENT AND EFFICACY OF MICROBIAL INOCULANTS IN SOIL

The efficacy of microbial releases can often be optimized by aiming for a maximization of numbers of inoculant cells as well as of (specific) metabolic activity per cell in the "right" soil niche at an adequate point in time. These factors may be linked in that the same ecological factors (e.g., availability of specific or general nutrients, favorable water content, and temperature) often enhance both bacterial survival and activity. With respect to the ecology of the inoculant in soil, two strategies can be put forward which determine the behavior of released strains, i.e., the presence and use of predictable ecological selectivity and the provision of ecological protection by using carrier materials. These approaches are treated in the following sections.

Ecological Selectivity

Ecological selectivity, i.e., the selection of an inoculant strain by some unique feature in the soil ecosystem, represents an important tool for effective establishment of an active inoculant cell population in soil. It can be brought about by selectivity with respect to antibiosis/inhibition or to substrate use. Several examples of promising approaches have been presented. Bashan (8) and Li and Alexander (87) showed that ecological selectivity worked in the rhizosphere by using the concept of microbial antibiosis. They temporarily suppressed the competing or antagonistic indigenous microflora by using an antibiotic, streptomycin, in conjunction with an antibiotic-resistant inoculant strain. The resulting inhibition of microbiostatic activity by the indigenous microflora allowed for the establishment of the inoculant populations at higher levels than those obtained in controls without added antibiotics. Similarly, a transposon Tn*5*-carrying *P. fluorescens* strain was shown to be selected by streptomycin in the soil and rhizosphere (23), based on the streptomycin resistance determinant provided by Tn*5*. Ecological selectivity can also be based on the use of a specific substrate which is unavailable to a majority of other soil microorganisms. Devliegher et al. (41) showed the effectiveness of this principle, in the use of a combined addition of specific detergents as carbon sources and detergent-degradative *Pseudomonas* spp. added to soil. Treatment of soil with certain detergents resulted in a 100- to 1,000-fold increase in the detergent-adapted inoculant populations and significantly enhanced the colonization of maize roots. Senoo et al. (127) also showed that *Sphingomonas paucimobilis* able to degrade γ-1,2,3,4,5,6-hexachlorocyclohexane (γ-HCH) was prevalent in soils treated with γ-HCH but not in untreated soils. Moreover, Nishiyama et al. (105) later showed that an introduced *S. paucimobilis* strain also had an ecological advantage in γ-HCH-treated soil as compared to untreated soil. An active population of the inoculant strain (with the unique capacity to aerobically utilize γ-HCH as the sole carbon source) was well established in a soil exposed to γ-HCH prior to bacterial inoculation. In soils without prior additions of γ-HCH, the strain

disappeared rapidly after introduction. Top (138a) extended the concept of ecological selectivity to plasmid-borne genes which conferred the capacity to degrade 2,4-dichlorobenzoate (2,4-D) to their hosts. In 2,4-D-treated soils, organisms able to degrade 2,4-D became rapidly apparent as a result of gene transfer from an inoculant organism which survived poorly in the system, followed by clonal selection of successful transconjugants, whereas such prevalence was not noted in untreated soil (138a). Thus, ecological selectivity can be conferred both upon organisms (complements of an array of metabolic capabilities) and upon specific genes carried by varied hosts in different genetic backgrounds.

However, in many cases, an ecological advantage for the inoculant organism is not available or not known. Yet, the great potential of ecological selectivity for the survival and efficacy of microbial inoculants for an array of important objectives, including crop protection and soil sanitation, often will urge its full exploration. In particular, further studies on the specificity of interactions between roots and microbes may provide promising information. As mentioned above, Liljeroth et al. (88) found that specific populations of bacteria could be characterized as typical root tip or root base bacteria, which were discernible on the basis of their capability to utilize specific substrates. The use of bacteria specializing in the colonization of specific regions of the root at times that fit the spatial-temporal dynamics of root pathogens may provide a solid basis for the successful use of microbial inoculations for crop protection or plant growth stimulation. This requires not only more knowledge on the population dynamics of specific bacteria but also a better understanding of root exudation processes (33). Goldmann et al. (56) indicated that certain plant species provided specific compounds, which could be utilized as sole C or N sources by bacteria containing plasmids with the necessary genetic code for the enzymes involved in the metabolism of the compounds. They called these compounds nutrient mediators. Similar mechanisms and compounds are known to play a role in the symbiosis of *Rhizobium* spp. with leguminous plants and in the interaction of *Agrobacterium* spp. with their host plants. The latter organisms stimulate the production of such compounds after infection of the host plant. Moreover, Wilson and Lindow (167) applied nutrient mediators (nutrient resource partitioning) to the phytosphere. A salicylate-degrading *P. putida* strain was shown to be fitter in the salicylate-treated phyllosphere of *Phaseolus vulgaris* than was its near-isogenic nondegrading counterpart (167).

There are currently several attempts to also promote directed changes in the rhizosphere populations of several plants by exploiting the possibility of the production of unique compounds by plant roots and of specific beneficial microorganisms capable of using these compounds (109). Beneficial plant-microbe interactions might thus be established, resulting in the concept of "biased" rhizospheres as proposed by F. J. de Bruijn and coworkers (Michigan State University, East Lansing). Further development of nutrient mediators and the genetic codes involved in their metabolism provides a powerful tool for improvement of the ecological competence of microbial inoculants through genetic engineering. Moreover, knowledge of other key physiological characteristics that determine bacterial survival and adaptation in soil may also lead to methods for genetic manipulations directed to improve the survival of bacteria introduced into soil. van Elsas and van Overbeek (148) speculated on these traits in a review on the responses of bacteria to soil stimuli, in particular physiological and genetic responses to root exudation and carbon limitation. They showed the existence of genes that could be activated by specific root exudates. In particular, a strong promoter was identified in *P. fluorescens*, which was specifically induced by proline present in the rhizosphere of gramineous plants (154). This induction was found to be prevalent in the rhizosphere of wheat both in soil microcosms and in the field (156a) and, further, in that of maize and grass. The use of such promoters in the rhizosphere provides a strategy to selectively induce strong in situ expression of a beneficial (e.g., biocontrol) gene, which is an example of ecological selectivity at the molecular level. Plant-induced expression of a biocontrol gene in the rhizosphere or rhizoplane is advantageous, in particular in the control of plant-pathogenic fungi, because this habitat is the prime site where infection of plants by soil-borne fungi starts. Specific inoculant cell activity would therefore take place only when required, which avoids an unnecessary metabolic load in cells not present at the target site.

Use of Carrier Materials

When ecological selectivity is not possible or applicable and maximization of the total inoculant activity in soil, i.e., the achievement of maximal numbers of active cells, is considered to be necessary, this should be obtained in some other way. Since natural soil commonly represents a hostile environment to inoculant cells (71), the use of inoculant formulations involving carrier materials for the delivery of microbial cells to soil or the rhizosphere is an attractive option. Carrier materials are generally intended to provide a (temporarily) protective niche to microbial inoculants in soil, either physically, via the provision of a protective surface or pore space (creating protective microhabitats), or nutritionally, via the provision of a specific substrate (140, 145, 162). They can be used to formulate inoculant bacteria for delivery either directly to soil or as a seed dressing for inoculation of the rhizosphere and/or rhizoplane. An optimal carrier should provide favorable conditions for survival as well as functioning of the inoculant cells, resulting in a sufficiently long shelf life as well as improved survival and activity in soil. The carrier should, further, be nontoxic and nonpolluting and have a constant quality. It should also allow an accurate release of microbial cells to the target sites in soil or rhizosphere and might even be used to inhibit the dispersal of inoculant cells to adjacent soil sites or to groundwater in cases when such spread is undesirable (140).

A wide range of carriers prepared from natural materials, e.g., peat, clay, and plant-derived compounds, have been tested and used. In particular, peat has been the carrier of choice in the *Rhizobium* inoculant industry (24, 125, 137, 162), and a significant improvement of inoculant effectiveness has often been noted in comparison with nonpeat inoculants (13, 125, 137, 162). Many applied aspects concerning the stability, moisture retention, pH, quality, reliability, and shelf life of the formulated inoculants have been extensively studied and reviewed (125, 137, 162). A discussion of these topics is beyond the scope of this review. Instead, in the following discussion, we address some concepts important in the further development and use of carrier materials.

Peat is a natural complex material subjected to variability in quality and composition, and problems with the consistency of peat-based rhizobial inoculant formulations, as reflected in poor efficacy of nodulation, have been reported (140, 145). Hence, strategies for a more consistent and reliable inoculant formulation based on defined carrier materials have been sought. As the montmorillonite-type clays can protect bacteria in soil via the creation of protective microhabitats, one possible strategy has been to formulate bacteria in clay-based carriers. Lyophilized rhizobial cells formulated in bentonite clay showed superior survival in soil compared to cells directly

added to soil (66), showing the feasibility of this approach. Montmorillonite clay coats have also been commercially tested and used to establish microbial inoculants on seeds for rhizosphere inoculations. However, the rhizosphere has classically been inoculated with other dry formulations based on carboxymethylcellulose or even talc applied onto the seeds (27). One drawback of the use of such dry formulations is the sensitivity of many bacteria, e.g., fluorescent pseudomonads (66), to the formulation process. Further, for soil inoculation, rather large amounts of clay were theoretically needed to establish a suitable density of the inoculant, which affects the physical properties of soil and can be impractical (66).

Carriers for the immobilization of bacterial or fungal (yeast) cells or enzymes for industrial use have been based on defined organic polymers forming porous matrices (77, 129). Calcium alginate, agarose, and k-carrageenan have most often been employed as the polymeric materials of choice (140), whereas polyurethane has also been used (22, 96, 135). The advantages of these carriers are their defined nature, their nontoxicity (generally), the ability to add compounds that either enhance cellular physiology or aid in assimilating the compounds to be degraded, and the general accessibility due to the relatively free diffusion of gases, liquids, or dissolved compounds. Although these carriers have proven their value in applications in aqueous media, allowing relatively free exchange via diffusion of substrate and metabolic products through the aqueous phase (129), their use in soil has occurred only in the last decade (7, 51, 140, 145). For this purpose, small beads consisting of the polymeric matrix containing the inoculant cells in fresh or even dried form have been tested (7, 140, 147). The beads can be prepared by mixing bacterial cells from culture with the prepolymeric material in solution as well as with any compatible additives needed, followed by polymerization and extrusion through an orifice to form beads (7, 129, 140). Following bead formation, a cell recovery or growth step is often included (7, 147). Inoculant cells are thus encapsulated in a porous organic matrix in beads 1 to 3 mm in diameter (7, 147). As such beads are likely to be less effective than smaller ones in establishing a well-spread inoculum in soil as well as in the degradation of pollutants (due to a less optimal surface-to-volume ratio), a microbead (diameter, <50 μm) production method which might alleviate these potential limitations was recently suggested (135). Pentachlorophenol-degrading *Flavobacterium* cells encapsulated in these beads were as catabolically active as were unencapsulated cells. A further potentially important development is the possibility of producing multilayered beads, e.g., those composed of an agarose core containing the inoculant cells covered with a polyurethane protective shell. Such beads have been suggested for use in the bioremediation of pentachlorophenol-containing aquifers (135).

Encapsulation of *P. fluorescens* cells in alginate beads supplemented with bentonite clay and skim milk resulted in excellent survival of the inoculant in soil (140, 147). Beads that had been dried prior to introduction into soil even revealed an outgrowth or resuscitation of inoculant cells upon exposure to soil, probably following rehydration of beads (147). Also, these beads could serve as inoculation sites for the rhizosphere of wheat, and high levels of colonization were achieved even if the beads were 1 cm away from developing roots (141, 147). The alginate/bentonite clay microhabitats also protected the inoculant cells against adverse conditions such as drought and the presence of lytic bacteriophages (131, 141) and allowed their root colonization activity for a long period. Next to the (degradable) alginate matrix, the bentonite additive may have formed a physical barrier between the inoculant cells and the surrounding soil environment (69).

Further improvement of the efficacy of carrier materials should be based on a greater understanding of specific ecological conditions, in terms both of the physical-chemical state and of the biological conditions that soil provides to incoming microbes and the conditions offered by the carrier and required for the application. For instance, microorganisms intended to serve as inoculants for the rhizosphere or rhizoplane, should in many cases avidly colonize plant roots when introduced into soil. For certain applications with highly rhizosphere-competent bacteria, the root may become sufficiently colonized by an inoculant strain applied as a seed dressing. However, if root colonization is insufficient or if roots are to be colonized at high densities at different sites in soil as well as different points in time, it can be advantageous to establish a network of colonization "foci" in soil, from which nearby developing roots can be colonized. As outlined, this can be achieved by using inoculant cells encapsulated in, for instance, alginate beads, because such beads represent local reservoirs for (slow) release of high levels of inoculant cells (7, 140, 141, 147). In this case, the survival of cells in the beads in soil is a critical parameter. On the other hand, if cells are introduced into soil to perform a function in bioremediation, they may be introduced encapsulated in alginate or κ-carrageenan beads or even in polyurethane or mixed particles, thus establishing "hot spots" in soil where inoculant cell densities are high (22, 96). In this case, the hot spots established may act as catalytic sites where the biodegradation process takes place, provided that the local conditions with respect to pollutant transport and level (rate of diffusion), other microbial nutrients, and abiotic factors (local humidity and pH) are favorable. As indicated above, additives in the carrier used, e.g., active carbon, clay, or a source of proteins or micronutrients, might assist in providing these locally favorable conditions.

CONCLUSIONS AND PERSPECTIVES

In general, a proper characterization of target soils and rhizospheres as habitats for introduced microbes, as well as adequate strategies to enhance the inoculant performance by using the concept of ecological selectivity or carrier materials, is key to the successful application of beneficial microorganisms to soil. As climatic conditions, soils, plants, and microorganisms are all variable and/or diverse, there is no general rule for how introductions into soils can be optimized. However, it is clear that since soil generally represents a hostile environment to microbial introductions and since microbial cells in soil are subjected to a range of adverse abiotic and biotic conditions, the success of the application of microbes depends to a large extent on how favorable to its survival and functioning the target environment is or can be made, in terms of either natural or induced ecological selectivity or available protective niches.

The effects of soil on the physiology and ecology of introduced microorganisms are still poorly understood at the microscale (pore) level. Future research in this area should aim for a better understanding of the in situ physiology of inoculant cells, as well as for possible ways to manipulate it. The advanced molecular techniques now available should assist us in doing exactly this. For instance, the use of reporter genes inserted either randomly or directly into the bacterial genome allows the specific detection and possible enhancement of in situ gene expression in inoculant cells. As responses to in situ stresses and other triggers of gene expression become better understood in relation to conditions that reign locally and as

potential differences in the response due to cellular localization become evident, avenues for the exploitation of these insights may become visible.

In addition, the dynamics of the inoculant population, i.e., the interactions of the inoculants with and response to their environment (predation, antagonism, and death/growth), should be assessed at the soil microhabitat level. Whereas it has been difficult to detect bacterial cells in soil via 16S rRNA targeted in situ hybridization (63), progress has recently been made, by using confocal laser scanning microscopy and specific oligonucleotide probes, in the detection of *Azospirillum* inoculants in the rhizosphere (64). This area of research, i.e., the assessment of where and in which numbers inoculant cells are localized in soil, how dynamic this situation is in relation to prevailing and local soil conditions, and where inoculant cells are able to grow, is another important area of study.

On the applied side, and given the history of failures or variabilities of previous microbial releases, it is interesting to test the concept of application of mixtures of ecologically diverse strains with similar functions instead of single strains. Such consortia might consist either of mixtures of completely natural strains or of different strains into which similar functions had been engineered. This way, beneficial functions might be expressed more continually in a soil or rhizosphere system, even under ecologically different and/or variable conditions. One possible example would be the use of two or more ecologically different bacteria, e.g., a copiotrophic avid root colonizer and an oligotroph, that both carry a beneficial (e.g., bioremediation) gene expressed under conditions that prevail along the root. The copiotrophic organism might provide biodegradative activity along the young plant roots, whereas the oligotroph might do so along the older root parts.

Obviously, the greatest problem in soil inoculations for beneficial purposes is the general obstinacy of the soil ecosystem, which normally acts as a buffer against incoming microorganisms. In cases of strong ecological selectivity for the inoculant organism, this recalcitrance of the system can be overcome and the selected organism can become established and active. For unselected or poorly selected inoculants, the greatest chances for success of microbial introductions might occur when the normal homeostasis of the system is (temporarily) disturbed, resulting in an alleviation of anti-invader pressure. The possibility of using these windows of opportunity for releases of organisms without ecological selectivity has not been fully explored. Alternatively, these inoculants should be established in soil after formulation in protective (carrier) materials, and this technology is flexible and adaptable to the physiological needs of many organisms.

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