Reviews

Azospirillum as a potential inoculant for agriculture

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***Azospirillum* sp. contribute to increased yields of cereal and forage grasses by improving root development in properly colonized roots, increasing the rate of water and mineral uptake from the soil, and by biological nitrogen fixation. A better understanding of the basic biology of the *Azospirillum*–root interaction, aided by the application of genetic engineering techniques, may lead to greater efficiency in its use as a biofertilizer.**

For most of this century, researchers have been trying to establish beneficial associations between microorganisms and plants, particularly around the surface of roots, in the rhizosphere[1]. In any given soil–plant environment there is a particular microfloral population that is selected (enriched) by compounds excreted by the roots which serve as carbon and energy sources for the microorganisms. The location, density and composition of these populations change constantly, depending on the stage of plant and root development, root depth, soil moisture, differential flow of photosynthates to roots during light and dark periods, and other factors. Obviously, this is an extremely complex environment, making it very difficult to introduce and establish large numbers of laboratory-cultured beneficial organisms. Nevertheless, inoculation with symbiotic nitrogen-fixing bacteria such as *Rhizobium* in legumes[2] and actinomycetes in trees[3] has been successful in promoting yields by supplying most of the nitrogen needs of the plant and thus reducing the need for costly nitrogen fertilizers. In these cases, the bacteria infect and proliferate inside the root tissue, thus avoiding competition for nutrients with other rhizosphere organisms.

Some of the most promising organisms, capable of colonizing roots in large numbers and exerting beneficial effects on plants, belong to the genus *Azospirillum*[4]. These nitrogen-fixing bacteria were brought to attention in 1974 by J. Döbereiner and her colleagues in Rio de Janeiro, Brazil[5]. They colonize mainly forage and grain grasses and are readily isolated from the rhizosphere. Forage and grain grasses are the main source of food in the modern world. Since they cannot fix nitrogen in symbiotic associations with *Rhizobium*, to obtain high yields, it is necessary to fertilize the soil with high amounts of nitrogen fertilizer. It is estimated that only 50% of the applied nitrogen fertilizer is used by the plants, most of the remainder being lost by denitrification or leaching; hence the importance of *Azospirillum* for promoting more efficient use of applied fertilizer and enriching the soil with nitrogen fixed in association with the roots. Results from field experiments carried out since 1974 have shown that *Azospirillum* inoculation does promote yield under certain environmental and soil conditions, but in some areas (such as Florida and Wisconsin) crop yield increases have not been consistent[6–9]. Also, the potential contribution of biological nitrogen fixation to improving yield is smaller than was expected[10].

The *Azospirillum*–root association, also known as associative symbiosis, has been extensively studied, mainly in forage and grain grasses, by various research groups throughout the world[5,11]. Research has focused on taxonomy[4], understanding unique physiological properties of the bacterium[12], genetics[6], ecology, colonization and effects on the rhizosphere[13,14,15], and (most important) the evaluation of the ability of *Azospirillum* to increase yields under field conditions[7,8].

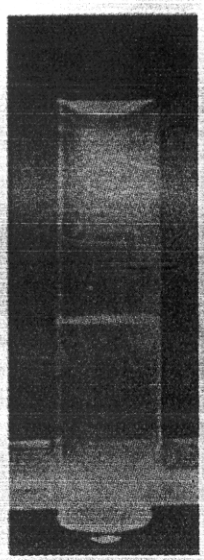

Fig. 1. A growth pellicle (band) formed one minute after mixing a suspension of Azospirillum brasilense *in a tube of semi-solid agar. Azospirillum moves aerotactically towards a self-created oxygen gradient. At the site of the pellicle, the amount of O_2 consumed by cell respiration equals the rate of oxygen diffusion.*

Properties and mechanism of action

So far, three species of *Azospirillum* have been recognized: *A. brasilense*, *A. lipoferum* and *A. amazonense*[4,5]. They are Gram-negative vibrion-shaped rods, 1 μm in diameter, very motile and possess a long, polar flagellum for swimming and, occasionally, peritrichous flagella for swarming on surfaces. The cells change shape and size with culture age, and produce cysts. *Azospirillum* species grow well on organic acids such as malate and succinate but vary in their ability to use different

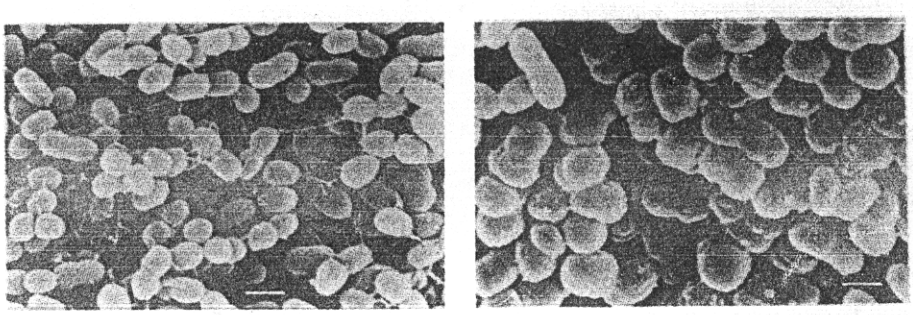

Fig. 2. Scanning electron micrographs of Azospirillum brasilense *Cd containing about (a) 5% (b) 40% poly-β-hydroxybutyrate of cell dry weight. Bar = 1 μm.*

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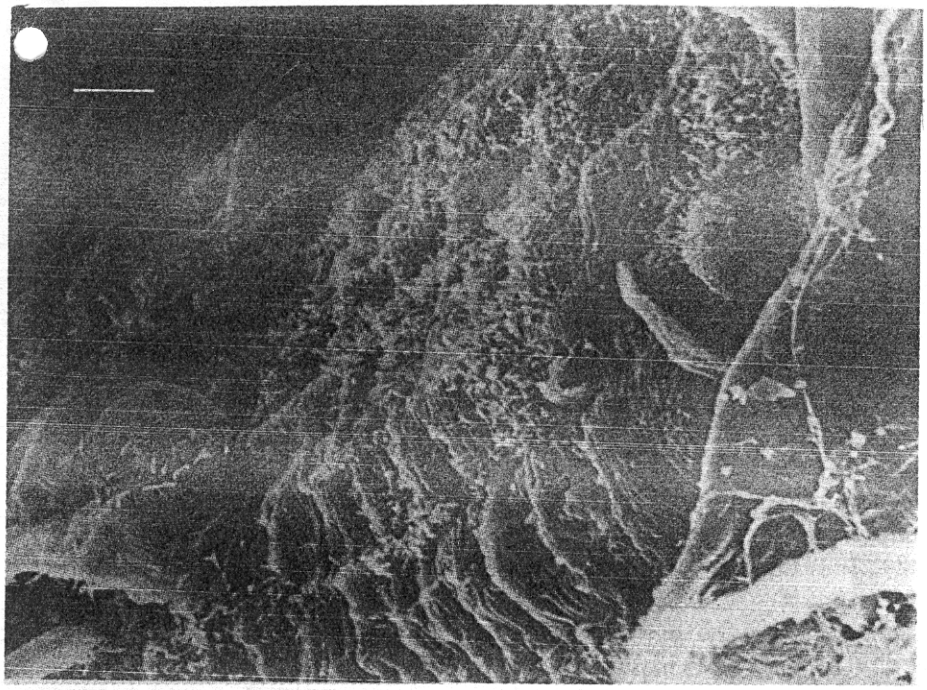

Fig. 3. Scanning electron micrograph of bacterial cells colonizing the root elongation zone of hydroponically grown wheat roots, 72 h after inoculation with 1 ml suspension containing 10^5 ml^{-1} Azospirillum *cells. Bar = 10 μm.*

sugars and amino acids as carbon and energy sources. They are very versatile, and possess a wide array of metabolic pathways for obtaining energy (reductant, ATP) and intermediates (as blocks for synthesis of other compounds) from organic acids, sugars and amino acids[4,12].

All azospirilla are nitrogen-fixing bacteria with nitrogenase properties comparable to those of other nitrogen fixers[6]. Regulation of nitrogenase in the presence of ammonia resembles that of photosynthetic bacteria such as *Rhodospirillum rubrum*, where the Fe-protein component of nitrogenase is activated by an activating factor[16]. Azospirilla can use NH_4^+, NO_3^-, amino acids and N_2 as N sources for growth. They can grow under anaerobic (NO_3^- as acceptor of electrons, denitrification), microaerobic (N_2 or NH_3 as nitrogen sources) and fully aerobic conditions with combined nitrogen only (NH_3, NO_3^-, amino acids)[4,12]. Since they apparently do not possess any special mechanisms for protection of their nitrogenase, they can only fix nitrogen under microaerobic conditions of very low dissolved-oxygen tension (5×10^2 Pa). The bacteria are preferentially microaerophilic, actively moving towards self-created gradients of oxygen concentration (Fig. 1). This aerotactic response takes only seconds to be detected[16,17]. *Azospirillum* responds chemotactically to gradients of attractants such as organic acids and amino acids[18]. Cells of *Azospirillum* accumulate large amounts (up to 50% of cell dry weight) of the storage compound poly-β-hydroxybutyrate used under conditions of starvation as a carbon and energy source for growth and nitrogen fixation[19] (Figs 2a, 6).

Although rhizosphere bacteria such as *Pseudomonas* sp., *Beijerinckia* sp. and *Azotobacter* sp. have some of the physiological properties outlined above, the possession of all the properties by *Azospirillum* make it well adapted for colonization and proliferation in the constantly changing oxygen and carbon gradients of the extremely competitive environment of the rhizosphere.

Studies with inoculated plants in the field have shown that *Azospirillum* comprises 1–10% of the total bacterial numbers in the rhizosphere. Its contribution to the bacterial biomass is even larger because azospirilla are larger (1.0 × 3.0 μm) than the more common rhizosphere pseudomonads (0.5 × 1.5 μm).

Although not conclusive, microscopic studies have indicated that azospirilla proliferate mainly on the root elongation zone and on the bases of root hairs (Figs 3, 4). They are found embedded in the mucigel, apparently colonizing intercellular spaces of the root cortex, and even in the vascular system[14].

Observations of roots of seedling corn, wheat, sorghum and other grasses show a very marked effect of *Azospirillum* inoculation on root length, surface area and branching. The effects vary with inoculum concentrations and environmental conditions. Inoculation with 10^5–10^6 cells per plant increases root elongation, root surface area, and the length of the elongation zone, whereas inoculum of 10^8–10^9 cells shortens the root elongation zone (Fig. 5). In all cases, inoculated mature plants clearly show a more highly branched, developed root system, whether grown hydroponically (Fig. 6), in pots with soil, or in the field[13,20] (Fig. 7).

It is not known if the changes in root morphology and development are caused by plant growth-promoting substances (auxins, kinetins, gibberillins) produced by the bacterium, as has been detected in pure culture, or by the plants as a response to *Azospirillum* colonization. This remains to be investigated, by developing methodology for detecting plant growth-promoting substances *in vivo*.

It has been demonstrated that *Azospirillum*-inoculated plants take up minerals (N, P and K) from solution at faster rates than controls[20,21], and, consequently, inoculated plants in the field accumulate dry matter, N, P and K in their stems and leaves at higher rates[22]. The minerals are then transferred to the developing panicles (sorghum) and ears (wheat), resulting in higher yields.

In addition, we have evidence that under water stress inoculated plants

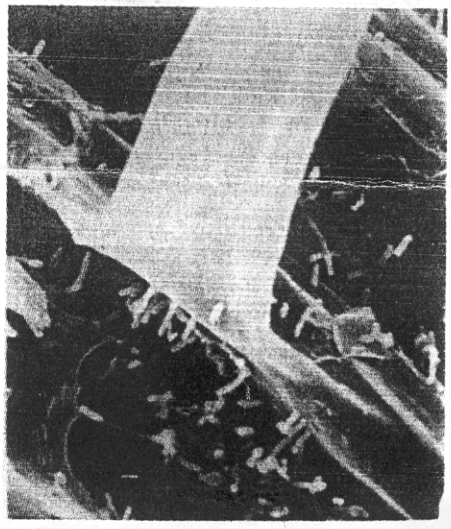

Fig. 4. Scanning electron micrograph of bacterial cells colonizing the base of a root hair of hydroponically grown maize roots, 24 h after inoculation with 1 ml suspension 10^5 ml^{-1} Azospirillum *cells. Bar = 1 μm.*

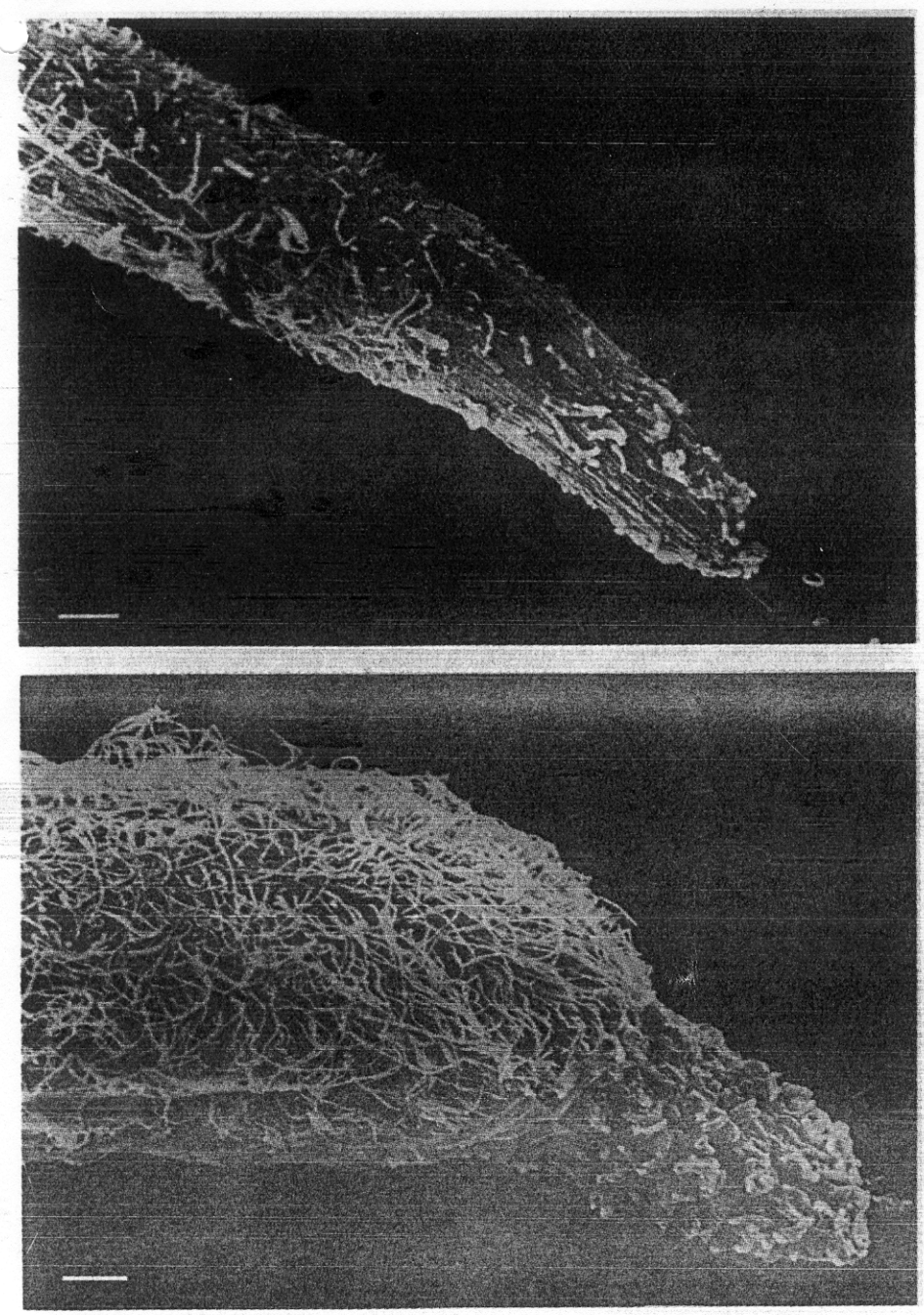

Fig. 5. Scanning electron micrograph showing the effect of Azospirillum *inoculation with 10^8 cells ml^{-1} on root tip morphology of* Sorghum. *Note a much shorter root elongation zone with proliferation of root hairs in inoculated (bottom) root than in control (top). Bar = 100 μm.*

show improvement in the water content of the plants: significantly less pressure is needed to pull out water (leaf water potential) from inoculated leaves compared to controls; increased stomatal conductance to gases (measured by steady state porometry) indicates that in the presence of water the stomata are more open; and lower canopy temperature (measured by infrared thermometry) shows more water in inoculated plants. Moreover, accumulated dry matter in the vegetative parts of the plants, and its translocation for seed filling depends on adequate water content.

All the above observations lead us to conclude that *Azospirillum*-inoculation benefits plant growth and increases yields of forage and grain grasses by improving root development, mineral uptake and plant–water relationships.

It has been postulated that biological nitrogen fixation by *Azospirillum* in association with roots may contribute significant amounts of nitrogen to the plant, thus potentially saving valuable N-fertilizers. Indeed, greater nitrogen fixation activities as measured by the acetylene reduction assay were detected in inoculated plants than in non-inoculated controls[5,7,11,14]. Higher nitrogen fixation rates were detected near or at flowering under conditions of high temperature and soil moisture. However, incubation of plants in a $^{15}N_2$ (a stable isotope) atmosphere and measuring by the ^{15}N-isotope dilution technique showed that only a very small proportion of the apparently fixed N_2 was incorporated into the plant[10]. It has not been possible to demonstrate N gains in inoculated plants above those initially present in soil[9,23]. The above measurements have shown that biological nitrogen fixation by *Azospirillum*–root associations in the field contribute some N to summer grasses and cereals and to the soil, in itself a very positive phenomenon. However, the quantitative accuracy of the measurements remains controversial.

Inoculation methods

Field inoculation presents complex problems. First is the problem of survival and viability of bacteria in the constantly changing soil environment. Second is the problem of application, i.e. the choice of inoculation method, time of application (at sowing, after germination or at emergence) and inoculum size that will bring the bacteria in contact with emerging roots. The most efficient *Azospirillum* inoculant preparation used so far is produced by mixing

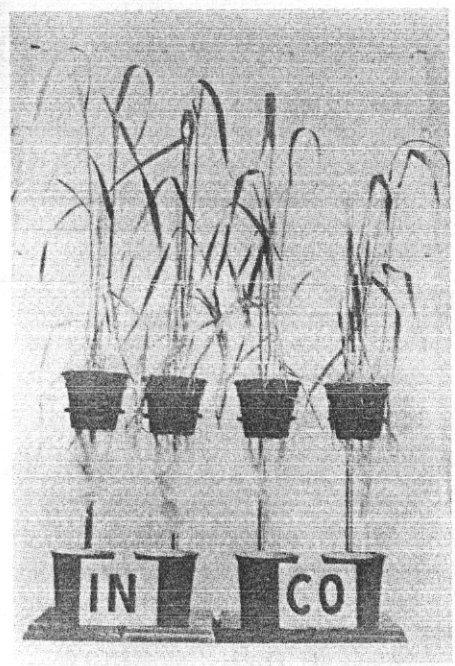

Fig. 6. Effect of Azospirillum *inoculation on root and shoot development of wheat in hydroponic systems. IN = inoculated, CO = control.* (Reproduced with permission from Ref. 20.)

Azospirillum suspensions with finely sieved, sterilized peat. Peat is highly adsorptive, easy to process, non-toxic to *Azospirillum* and *Rhizobium*, easy to sterilize and an inexpensive carrier[24]. Under proper storage, cells survive in large numbers, adsorbed to peat for at least six months. Upon application in the field, bacteria are released from the carrier for colonization of the emerging roots[24]. The inoculant can be used: as a powder for seed pelleting by mixing with the seeds before sowing; by dripping a peat inoculant suspension in the furrow; or as granular preparations applied in the furrow at sowing. All these methods are widely used for *Rhizobium* inoculation of legumes[24].

In most of the successful field experiments, the inoculant was applied and then followed by irrigation or rain in moist soil, thus ensuring colonization. Inoculant preparations and inoculation technologies must be improved. For example, applying bacteria in granular preparations or embedded in hard-to-degrade polysaccharide gels ensures survival of the inoculum and good colonization of roots in soils that may remain dry or cold for up to several weeks before root growth. (In extensive agriculture of the North American corn (maize) belt, farmers sow the field at the first available opportunity without controlling immediate seed germination.)

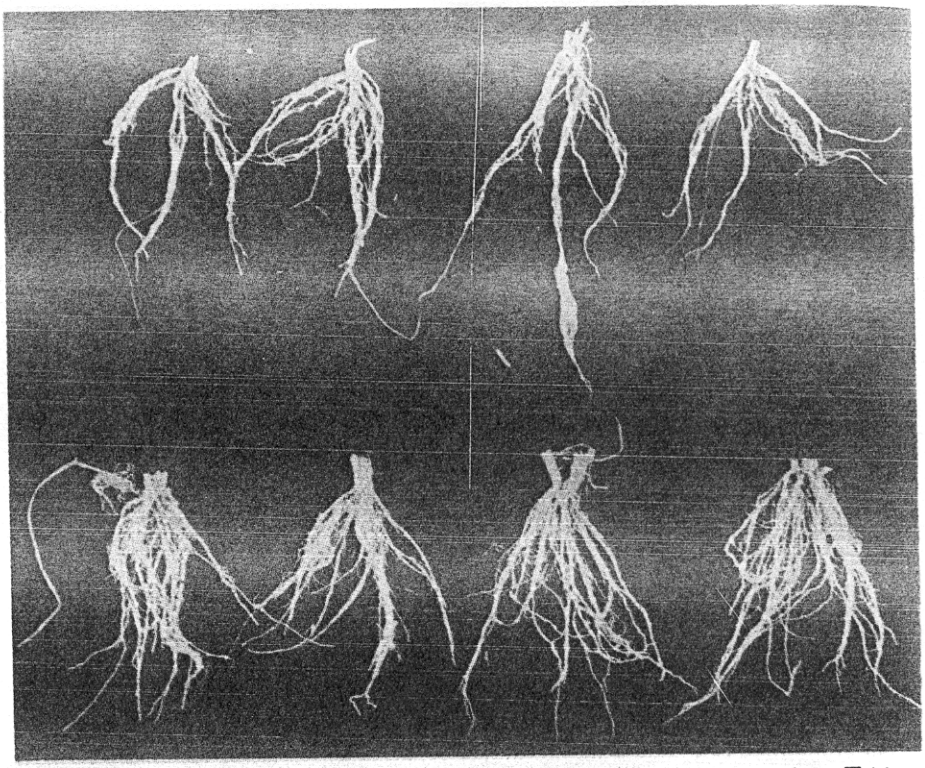

Fig. 7. Effect of Azospirillum *inoculation on root development of field-grown wheat (*Triticum aestivum *cv. Miriam). Roots were taken with a core sampler.* Bottom = *inoculated;* Top = *control.*

Positive effects on yields have been reported in about 65% of field inoculation experiments carried out in the past ten years in many parts of the world, under different fertilization levels and diverse environmental conditions[7,8,9,22]. However, in some locations, inoculation has not shown any significant effect[9]. As explained above, to affect root development and function, the roots must be colonized with optimal populations of *Azospirillum*. Failure is most likely in heavy soils, which are rich in organic matter, where *Azospirillum* may be adsorbed and must compete with large microbial populations for colonization of roots. Field inoculation experiments with *Azospirillum* have been carried out in Israel on a farm scale. Plots were large (60–100 m^2) and the number of replicates sufficient for statistical analysis[7,22]. Summer inoculation of maize, sorghum, *Panicum miliaceum* and *Setaria italica* resulted in greater yield increases than did winter inoculation of spring wheat.

In the summer crops, 75% of the experiments showed significant yield increases, whereas in spring wheat the success rate was 50%. In general, the most marked effects of inoculation were obtained at intermediate levels of N-fertilizers[7,8,9,22]. 'Intermediate levels'[7] is an arbitrary designation because it depends on the amount of available combined N in the soil before fertilization, and on rates of mineralization and nitrification in a particular soil. Therefore, when comparing inoculated and non-inoculated plots, the largest differences in yield were obtained when the soil was properly (but not excessively) fertilized. In soils with very low levels of fertilization, there was a positive but generally less marked influence. The same held true for excessively fertilized soil. This implies that the use of *Azospirillum* will be less important in very rich soil where plants reach their maximum growth potential, or in very poor soils without added fertilizer.

Improvement of *Azospirillum*–plant association by genetic manipulation

Work must proceed in several directions to make the widespread commercial use of *Azospirillum* possible. One of the major obstacles is the lack of a basic understanding of the physiological and morphological factors involved in the association. We have some preliminary evidence that processes such as the specific attraction of *Azospirillum* to roots, the colonization of specific sites on the root surface, and the hormonal

Glossary

mucigel – gelatinous material at the surface of roots grown in normal non-sterile soils. It includes plant mucilages, bacterial cells and their metabolic products, and colloidal mineral and organic matter from the soil.

acetylene reduction assay – nitrogen fixation can be directly measured by reduction of acetylene to ethylene by the enzyme nitrogenase, followed by gas chromatographic separation of ethylene and its assay by the sensitive flame ionization technique.

^{15}N-isotope dilution technique – fixed nitrogen enriched with ^{15}N is added to the soil. As it will constitute the only source of nitrogen for plants not inoculated with *Azospirillum*, such plants will accumulate ^{15}N to relatively high levels. Plants that can fix N_2 from the air (not enriched with ^{15}N) in addition to using fixed nitrogen from the soil will have a lower ^{15}N concentration, and from this isotope dilution the amount of N_2 fixed can be calculated. The stable isotope ^{15}N is measured by mass spectrometry.

effects of *Azospirillum* on roots, are important. However, they are not well understood. Also methods for quantitative estimation of the contribution of nitrogen fixation in the field must be developed.

To improve the various components of the system, we must determine the genetic characteristics of the bacterium and the plants involved. Gene regulation and expression of well defined gene systems is less known in soil bacteria as compared to the genetically better-known *E. coli* and *Klebsiella pneumoniae*, and methods for working with those bacteria have to be worked out for *Azospirillum*. The emphasis so far in nitrogen-fixing bacteria and in *Azospirillum* has been on the mapping and cloning of the *nif* genes coding for nitrogenase itself and for the processes controlling and aiding nitrogenase activity[2,6]. Most of the work on genetics of *Azospirillum* has recently been reviewed[6]. Homology with *K. pneumoniae nif* DNA (HDK and A) was detected. A fragment of *Azospirillum nif* HDK cluster was cloned in *E. coli*. Mutants with *Nif*$^-$ phenotype were isolated. They were impaired in glutamine synthetase or glutamate synthase activity[6].

The most recent development in *Azospirillum* genetics is the ability to produce, by transposon site-directed mutagenesis, mutants impaired in the MoFe-protein of nitrogenase[6] and incapable of fixing nitrogen, but with the rest of the genome remaining intact as in the wild type. This type of mutant is ideal for the study of mechanisms involved in the *Azospirillum*–root associations. Such mutants impaired in one property but possessing all the remaining genes encoding for functions related to colonization and survival can be used as a model to study the competitive environment of the rhizosphere.

From the physiological data that have accumulated on *Azospirillum*[4,8,12] it is evident that we need to produce target site transposon mutants to understand factors affecting the association with plants. This will be possible once the phenotype (purification of enzymes involved, morphological changes, etc.) is known and the genes are cloned. The mutants should lack or possess in excess the characteristics listed below. Only very limited work on this subject has been done in *Azospirillum* and other characteristics may emerge from research.

(1) Chemotactic and motility genes. Substantial advances have been made in the characterization of genes involved in chemotaxis of *E. coli* and *Salmonella*[25]. *Azospirillum* respond chemotactically to attractants such as organic acids and sugar, substances composing root exudates[18].

(2) Aerotaxis genes. *Azospirillum* follows self-created gradients of diminishing dissolved oxygen in a matter of seconds. This property may aid colonization of roots[17].

(3) Genes involved in production and utilization of the storage material poly-β-hydroxybutyrate, which may be involved in survival and proliferation of *Azospirillum* in the rhizosphere[19]. Some mutants have been obtained for *Rhizobium*[26].

(4) Genes involved in production of auxins, cytokinins and other plant growth factors. IAA-overproducing mutants of *Azospirillum* have been obtained by mutagenesis and their effect on roots assessed[27]. They do not seem to affect root morphology to a greater extent than the wild type.

(5) Genes involved in production and activity of pectic enzymes that may aid penetration of *Azospirillum* to intercellular spaces of the root cortex, and may soften the middle lamellae, thus favouring mineral and water uptake by the roots[11,28].

(6) *Nif*$^-$ mutants and *nif* constitutive mutants. These are used to assess nitrogen fixation potential for improving yields in plants colonized by them[6].

(7) Improved plants, in this case cultivars, that will excrete more specific nutrients (carbon and energy sources) that can be used by *Azospirillum* to enrich their populations and increase energy-requiring nitrogen fixation, can be engineered. Also, assuming that there are binding proteins (lectins) that aid the specific adsorption of *Azospirillum*, it is feasible to select plants or cultivars that produce more of these binding proteins. Recognition of *Azospirillum* by plant roots has been shown to be associated with substances sensitive to proteases[15]. However, these substances (proteins) have not yet been purified or characterized. If *Azospirillum* elicits root proliferation, there may be plants and cultivars that are more responsive than others. The mechanism(s) by which *Azospirillum* causes proliferation of roots is not yet known.

Knowledge accumulated from basic investigations of *Azospirillum*–plant associations aided by modern genetic techniques can be applied to the production of other inoculants important to agriculture, such as phosphate solubilizers, plant growth-promoting pseudomonads, mycorrhizal fungi, microorganisms that reduce damage caused by pathogens and pests, biocontrol agents of soil-borne fungi, microbial insecticides, microbial herbicides and microbes that compete with ice nucleating bacteria, preventing frost damage to leaves.

Economic impact and potential use

The costs of producing, marketing and applying *Azospirillum* inoculants are comparable to those of *Rhizobium*. Farmers in the USA pay up to $12.50 for inoculating one hectare of soybeans. However, mass production for the more extensive forage and grain grass crops, which comprise about 85% of the cultivated area of the world, may convert *Azospirillum* inoculants into a much less expensive product.

The USA cultivates about 28.3 million hectares of maize, South Africa 6 million, and France, Italy and Spain 3 million hectares. In these countries, an average of 150 kg N-fertilizer is applied per hectare. Other countries like Brazil, Mexico, Argentina, India and China cultivate 52.5 million hectares of maize, using much less fertilizer and obtaining lower yields.

The data accumulated from field studies show that *Azospirillum* (providing that the roots have been properly colonized) can be of use in both intensive and extensive agriculture in developed countries and in extensive agriculture in less developed countries. In the former, fields can be fertilized with 30 – 50% less fertilizer, the farmer obtaining yields in *Azospirillum*–inoculated fields comparable and even slightly higher than those of fully fertilized fields. In the case of properly fertilized fields there will be increases of 10–15% in yield. In less developed countries, *Azospirillum* inoculation may improve the use of the low amounts of available fertilizer, thereby improving yields by 15–20%. Those fields will still have to be fertilized in the following season. *Azospirillum* could be used efficiently in crops that are not commonly fertilized or irrigated in semi-arid zones, where inoculation improves the water status of the plant. In crops such as non-irrigated sorghum

in Israel, we have consistently obtained yield increases of 15–20% above controls in inoculated plots[7,22]. Favorable responses to *Azospirillum* – inoculation have been obtained in experiments carried out under soil conditions comparable to those in Israel. Irrigated fields were inoculated with freshly prepared inoculants, and bacteria properly colonized the emerging roots.

Much work remains to be done before *Azospirillum* is converted into a widely used product with consistent effects on yield. We must understand the basic association and overcome technical problems of inoculation under a wide variety of soil conditions.

The benefit to mankind and the economic potential makes it a worthwhile task.

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Computer-aided design in protein engineering

Tom Blundell and Michael J. E. Sternberg

The amino acid sequence of a protein can be engineered genetically to yield a molecule with modified or novel properties of clinical or industrial importance, through knowledge of the relationships between sequence, three-dimensional structure and function. Interactive computer graphics can display and model the structural information revealed by protein crystallography. In the absence of a suitable crystal structure, computer methods must predict protein conformation from sequence. The most powerful approaches for structure prediction are based on sequence homology or a more general analogy with known crystal structures. Improvements in these methods will require access to data bases containing the basic motifs of protein architecture.

Recent advances in DNA technology have enabled a range of modifications to be engineered[1] into the amino acid sequence of a protein. Site-directed mutagenesis, generally using oligonucleotide mismatch priming, can alter one or a few specific amino acids in a molecule, as in the pioneering work of Fersht, Winter and coworkers[2] on tyrosyl-tRNA synthetase. The use of restriction enzymes and ligases enable a gene to be produced incorporating segments from different molecules, so generating a hybrid polypeptide chain. For example hybrid interferon molecules[3] have been produced with residues 1–62 from α-interferon A and the remaining residues from interferon D. Beyond this, chimaeric molecules[4] have been made by splicing different parts of molecules, such as hapten-specific antibodies in which the Fc portion has been replaced with an active enzyme. Ultimately the most ambitious projects are to design useful proteins with novel sequences.

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The applications of these techniques have consequences for both fundamental and industrial research. Site-specific mutants can be used to test structure–function hypotheses by introducing new amino acids in positions thought to be responsible for substrate or receptor recognition. The methods will be used to make new or improved proteins of clinical interest (hormones, monoclonal antibodies, plasminogen activators) or of industrial value (new catalysts and metal scavengers) by optimizing the thermal stability, pH optimum, catalytic efficiency or specificity of the proteins.

For a rational approach to protein engineering, the conformation of the molecule and its modes of interaction with its ligands and with its environment must be understood at the atomic level. This article describes a concerted approach to this using protein crystallography, interactive computer graphics, databases and other forms of numerical modelling.

Protein crystallography

The best guide to select residues for mutagenesis is provided by a three-