IMPROVING THE PLANT-GROWTH PROMOTING ABILITY OF *AZOSPIRILLUM BRASILENSE* BY GENETIC MANIPULATION

by

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Abstract

Certain plant growth promoting bacteria, by hydrolyzing 1-aminocyclopropane-1-carboxylic acid, ACC (the precursor of ethylene), through the action of ACC deaminase, regulate the production of ethylene in plants, promoting root elongation and decreasing the deleterious effects caused by stress ethylene. *Azospirillum*, a PGPB that promotes the growth of numerous plant species, does not produce ACC deaminase.

A. brasilense Cd transformed with the ACC deaminase gene (*acdS*) from *Enterobacter cloacae* UW4 did not show any ACC deaminase activity. To overcome the lack of expression of *acdS* in *Azospirillum*, the wild type promoter of *acdS* was replaced with the *lac* promoter, cloned in the broad host range plasmid pRK415, and transferred into *Azospirillum brasilense* Cd and *A. brasilense* Sp245.

The roots of tomato and canola seedlings were significantly longer in plants inoculated with *A. brasilense* Cd/pRKLACC transformants (*acdS* under the control of the *lac* promoter) than those in plants inoculated with the non-transformed strain. In the case of wheat seedlings, inoculation with *A. brasilense* Cd transformants did not promote root growth. The difference in plant response (canola and tomato versus wheat) is attributed to the higher sensitivity to ethylene of canola and tomato plants as compared to wheat plants.

Inferring that the promoter of a tetracycline resistance gene (Tet^r) might impose less metabolic load on *A. brasilense* Cd cells than the *lac* promoter, *acdS* was fused to the Tet^r gene promoter by PCR, cloned in pRK415, and transferred into *A. brasilense* Cd.

A. brasilense Cd/pRKLACC transformants showed higher ACC deaminase

activity than the Cd/pRKTACC transformants (*acdS* under the control of the Tet^r promoter). However, the Cd/pRKLACC transformants showed a decrease in IAA synthesis, a lower growth rate, and a decreased ability to survive on the surface of tomato leaves as compared to the Cd/pRKTACC transformants.

Fresh and dry shoot weight of flooded tomato plants (*Lycopersicum esculentun* Mill. cv. Heinz 1439 VF) inoculated with *A. brasilense* Cd/pRKTACC was higher than plants inoculated with *A. brasilense* Cd/pRKLACC or non-transformed *A. brasilense* Cd. The level of epinasty of the same plants inoculated with *A. brasilense* Cd/pRKTACC was lower than in plants inoculated with *A. brasilense* Cd/pRKLACC or non-transformed *A. brasilense* Cd. Experiments on tomato plants exposed to *Fusarium oxysporum, Pythium aphanidermatum,* or *Pseudomonas syringae* and inoculated with *A. brasilense* Cd/pRKTACC or non-transformed *A. brasilense* Cd suggested that both the transformed and the non-transformed strain have biocontrol properties against the phytopathogens. The better protection provided by *A. brasilense* Cd/pRKTACC (when inoculated on tomato leaves) against pathogen attack suggest that the ability of the transformants to break down ACC deaminase lowers the levels of ethylene evolved by the plant as a response to pathogen attack. Further experiments are required to confirm this hypothesis.

The possibility of using *A. brasilense* Cd/pRKLACC in mangrove reforestation programs is discussed.

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Dedication

This thesis is dedicated to all those Mexican women who dare to go after their dreams and give themselves the right to pursue their own goals, and not those imposed by tradition.

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Glossary

ACC	1-aminocyclopropane-1-carboxylic acid
acdS	The ACC deaminase structural gene
cfu/mL	Colony forming units per mL
DF	Minimal medium designed by Dworkin and Foster (1958)
DRAT/DRAG	Dinitrogenase reductase ADP-ribosyltransferase and dinitrogenase reductase-activating glycohydrolase
IAA	Indole-3-acetic acid
IPTG	Isopropylthio-ß-D-thiogalactoside
ISR	Induced systemic resistance
LRP	Leucine responsive regulatory protein
OAB	Minimal medium designed by Okon et al. (1977).
PAF	Pseudomonas F agar medium (Merck, Darmstadt, Germany)
PGPB	Plant growth promoting bacteria
SEM	Scanning electron microscopy
TAE buffer	Tris acetate/EDTA electrophoresis buffer
TE buffer	Tris-EDTA buffer
WGA	Wheat germ agglutinin

1. Introduction

Annually, more than 100 million tons of nitrogen fertilizers and more than 90 million tons of potash and phosphate fertilizers are used worldwide to increase the yield of crop plants. Despite their efficiency in promoting crop yield, they can, under certain circumstances, pollute the environment and contribute to a number of human and animal health problems (Glick et al., 1999).

The potential negative environmental impact of the large scale use of chemical fertilizers together with their increased cost has prompted a number of scientists worldwide to consider the possibility of substituting chemical fertilizers with microbial, self-propagating sources of important plant nutrients. In recent years, researchers have turned their attention to promising bacterial species that occupy the rhizosphere, i.e., the area surrounding plant roots. The use of bacterial inoculants in agriculture is predicted to increase as the necessity to protect the environment and the consumer leads to reduction in chemical inputs. For example, the annual pesticide market based on bacterial inoculants represented approximately around $600 million US dollars in 1998, and the potential *Rhizobium* market in the USA is 1000 million dollars (Stacey and Upchurch, 1984). Current techniques of modern molecular biology provide necessary tools to develop new or improved strains for use in agriculture (Glick et al., 1999; Okon and Vanderleyden, 1999).

1.1 Plant growth-promoting bacteria

Free-living soil bacteria that are beneficial to plants are often referred to as plant growth-promoting bacteria or PGPB (Bashan and Holguin, 1998) and

include a number of different bacteria such as *Azotobacter* (Pandey et al., 1989a, b) *Azospirillum* (Bashan and Levanony, 1990), *Enterobacter* and *Klebsiella* (Haahtela and Korhonen, 1985), pseudomonads (Glick et al., 1999), *Acetobacter* (Cavalcante and Döbereiner, 1988), *Serratia* (Ruppel, 1991), and *Bacillus* (Chanway and Holl, 1991). While many of these bacteria bind to soil particles and are found in soil aggregates, many more interact specifically with the roots of plants. They are present in high concentrations around the roots of plants, presumably because of the presence of high levels of nutrients (especially small molecules such as amino acids, sugars and organic acids) that are exuded from the roots of most plants, and can be utilized by the bacteria to support their growth and metabolism (Glick et al., 1999).

PGPB can affect plant growth in two ways, indirectly or directly. The indirect promotion of plant growth occurs when PGPB lessen or prevent the deleterious effects of one or more phytopathogenic organisms. The direct promotion of plant growth by PGPB entails either providing the plant with a compound that is synthesized by the bacterium or facilitating the uptake of certain nutrients from the environment (Glick, 1995a). The mechanisms of direct growth promotion can be nitrogen fixation, solubilization of minerals such as phosphorus, production of siderophores, synthesis of phytohormones, synergetic interaction with other beneficial rhizosphere microorganisms (Gaskins et al., 1985) or the synthesis of some enzymes that can modulate plant growth and development by influencing the levels of plant hormones (Glick et al., 1994a, b). Moreover, a particular PGPB can stimulate plant growth through one or several of these mechanisms (Glick, 1995a).

1.2. The PGPB *Azospirillum*

Azospirillum was first isolated by Beijerinck in 1925 from nitrogen-poor sandy soil in the Netherlands, although it was not brought into prominence until 1976 when Döbereiner and Day reported its wide distribution in the rhizosphere of several tropical grasses as well as its capability to fix nitrogen. Since then *Azospirillum* has been isolated from the roots of numerous wild and cultivated grasses, cereals, and legumes and from tropical, subtropical, and temperate soils worldwide (Bashan and Levanony, 1990).

Azospirillum inoculation on field grown plants can increase the total yield from 10 to 30% (and even more under greenhouse conditions), having positive effects on various plant growth parameters. Above ground responses in cereal and non-cereal plants to *Azospirillum* inoculation can include increases in: total plant dry weight, the amount of nitrogen in shoots and grains, the total number of tillers and ears, the number of spikes and the number of grains per spike, grain weight, plant height and leaf size, and germination rates. In addition, a marked inoculation effect on development of the root system such as on root surface and volume has been observed (Bashan and Levanony, 1990). Inoculation of plants with two or more PGPB including *Azospirillum* can often promote plant growth more than inoculation with only one bacterial strain. For example, inoculation of legumes with both *Rhizobium* and *Azospirillum*, yielded positive effects for the plants including early nodulation, increased number of nodules, higher N_2 fixation rates, and a general improvement of root development, as compared to plants inoculated only with *Rhizobium* (Okon and Vanderleyden, 1999).

However, positive results with *Azospirillum* inoculation in the field are difficult to repeat, even when experiments are apparently performed identically. An evaluation of worldwide data accumulated over a 20 year period dealing with field inoculation experiments with *Azospirillum*, showed a 30-40% occurrence of failure (Okon, 1985). Unfortunately, the factors responsible for the irregularity in plant response have not been identified, the reasons being mainly because basic features of the plant-*Azospirillum* interaction are still unknown. This inconsistency in results can be attributed to variations in soil (Bashan et al., 1995), plant and microfloral components, the microbial composition of the rhizosphere and the bacterial community interactions (Janzen and McGill, 1995), plant growth phase, growth phase of the bacterial inoculum (Okon and Labandera-Gonzalez, 1994), weather, or other unidentified factors.

Despite the inconsistent results in the field, *Azospirillum* is being used in several regions of the world to inoculate cultivars of different species of plants, enhancing yields, while lowering requirements for chemical fertilizers (Okon and Vanderleyden, 1999). The possibilities offered by *Azospirillum* inoculants on cereals (an increase of 5-30% yield in 60-70% of reported experiments (Okon and Labandera-Gonzalez, 1994), may open a new market of millions of dollars in the agrochemical industry (http://www.inra.fr/Internet/Centres/Dijon/cost/inoculant.html).

During the last decade it has been repeatedly shown that *Azospirillum* has the potential for agricultural exploitation (Bashan, 1999). *Azospirillum*, with its metabolic versatility, is well suited to competition and harsh conditions which exist in the rhizosphere and in soil. It fixes nitrogen, reduces nitrate, can form cystlike structures under unfavorable conditions which may contribute to its

survival in temperate areas (Lamm and Neyra, 1981), produces melanin (Givaudan et al., 1993), poly-ß-hydroxybutyrate (Sadasivan and Neyra, 1985), plant hormones (Patten and Glick, 1996), vitamins (Rodelas et al., 1993), and anchors itself to roots with the help of fibrillar material (Bashan et al., 1991) thus facilitating nutrient uptake both by the plant and the bacteria. Furthermore, chemotactic motility of *Azospirillum* towards several plant elaborated attractants present in plant root exudates render this bacterium competent in the microbial rhizosphere (Bashan and Holguin, 1994). In addition, *Azospirillum* is aerotactic, meaning that cells can sense and move toward oxygen at concentrations in the range of 3-5 μM, which is optimal for the nitrogenase complex of these microorganisms (Okon and Vanderleyden, 1999).

Recent data indicate that *Azospirillum* has no preference for crop plants or weeds, or for annual or perennial plants, and can be successfully applied to plants that have had no previous contact with *Azospirillum* in their roots (Bashan and Holguin, 1997). *Azospirillum* is found mainly in tropical and subtropical regions, however, isolations from cold and temperate places like Finland (Haahtela et al., 1981) and the states of New York and New Jersey (De Coninck et al., 1988; Lamm and Neyra, 1981) and Ulumgol, Korea, (where the temperature is maintained below 4°C all year long) (Oh et al., 1999) have also been reported.

1.2.1 Adhesion

Adhesion is in most cases a prerequisite for PGPB to stimulate plant growth, as well as for biocontrol PGPB to protect plants from diseases (Bashan and Holguin, 1997; Schippers, 1988). A secure attachment of *Azospirillum* to the plant roots allows the bacteria to have better access to plant exudates and

prevents them from being washed away by water. The attachment also helps the plant to access bacterially excreted substances which diffuse into the intercellular spaces of the root cortex (Bashan and Holguin, 1997; De Troch and Vanderleyden, 1996).

The two step process proposed by Marshall (1986) for the attachment of soil bacteria to soil particles may occur in all bacteria with the attachment capacity to different substrates (Matthysse, 1996). First, in an initial reversible phase, equilibrium is being established between the attractive and repulsive forces on the interacting surfaces. It allows the cells to sense the surface and detach easily if the conditions are not appropriate. The second phase of attachment is a time-dependent phenomenon, that is presumably mediated by the production of extracellular bacterial polysaccharides or bacterial structures such as fimbriae, also called adhesins or pili. Both exopolysaccharides and fimbriae may help the bacteria to overcome the initial electrostatic repulsion barrier between the cell and a surface (Glick et al., 1999).

Bacterial, like most eukaryotic cells, have a net negative charge on their outer surface. If most surfaces in the environment are also negatively charged, how can two negatively charged surfaces come together? In some cases, the presence of inorganic ions may mask the repulsion of these electrical double layers, allowing two surfaces having negative charges to approach one another. With *Pseudomonas fluorescens* E6-22, incorporation of either $MgCl_2$ or $CaCl_2$ in the inoculum resulted in a three-fold increase in the binding of the bacterium to radish seedlings roots compared to when these cations were not added (James et al., 1985). Similar divalent cation effects have been reported to promote the adherence of a marine pseudomonad to glass (Stanley, 1983). The cations

involved in these studies may be neutralizing the repulsion of two negatively charged layers, thus allowing them to approach (James et al., 1985).

Once the bacterium has overcome the physicochemical forces that hinder the initial stage of adhesion, a much tighter binding is established. The most prominent participants in the second stage of bacterial adhesion to a surface are extracellular polysaccharides or bacterial structures such as fimbriae. The production of exopolysaccharides is a very common trait among bacteria and is probably a determinant of a successful colonization of any surface. In addition, exopolysaccharides may be involved in cell aggregation and their synthesis may increase the chances of survival for the bacteria under desiccation or nutrient deprived conditions (as in biofilms) (Glick et al., 1999).

The two distinct stages of attachment mentioned above have been described for *Azospirillum*. The first phase, or the adsorption phase, consists of reversible weak binding in which hydrophobic proteins present in the cell's surface or the polar flagellum are probably involved (De Troch and Vanderleyden, 1996). Studies of *Azospirillum* cell walls showed that the bacterial cells are mildly hydrophobic, and hydrophobic proteins might be involved in this first stage of attachment (Castellanos and Bashan, 1996). In the second phase of attachment or anchoring of *Azospirillum* to roots, the bacteria become irreversibly bound by the production of long fibrils of unknown nature and a large amount of mucigel-like substances (Bashan et al., 1991). The nature of the binding material is still obscure (De Troch and Vanderleyden, 1996). However, it is believed that anchoring in *Azospirillum* is mediated by a bacterial exopolysaccharide similar to 1,4-ß- and 1,3-ß- polysaccharides that binds to the fluorescent dye, calcofluor. Michiels et al. (1991) showed that *Azospirillum*

brasilense non-fluorescent mutants lost the ability to form flocs (macroscopic clumps embedded in a matrix of polysaccharide material) and to anchor to wheat roots, indicating that the calcofluor-binding polysaccharide may be necessary for flocculation and anchoring to roots (Burdman et al., 1998).

Azospirillum preferentially colonizes the elongation and root-hair zones, probably because they constantly exude low molecular weight compounds that can act as bacterial nutrients (Bashan and Levanony, 1989). The pattern of colonization and ability of *A. brasilense* strains to colonize plant roots seems to be strain specific. Schloter and Hartman (1998) found that from three different *A. brasilense* strains, strain Sp245 showed the highest and most persistent potential for colonizing wheat roots. This strain could also be detected in the inner root tissue up to a concentration of 10^6 cells/g dried root, and formed microcolonies in intercellular spaces. It has been suggested that pectinolytic or other polymer degrading enzymes, which may be specifically induced by components of root exudates, may facilitate the colonization of the root interior (Schloter and Hartmann, 1998). De Troch et al. (1992) found characteristic differences in the exopolysaccharide and lipopolysaccharide composition of the *Azospirillum brasilense* strains Sp245 and Sp7 which may contribute to the different colonization patterns observed (Schloter and Hartmann, 1998).

Claims of *Azospirillum* specificity for certain cereals species are documented (Bashan and Levanony, 1990). However, Bashan and Holguin (1995) showed that under controlled conditions, the bacteria colonized the root systems of 64 plant species belonging to 19 different botanical families, showing no preference for crop plants or weeds, or for annuals or perennials. This lack of specificity in the ability of *Azospirillum* to colonize plants is probably related to its

versatile chemotactic response. There are no reports of chemotaxis of *Azospirillum* spp. to a specific plant (De Troch and Vanderleyden, 1996). The chemotactic response of 15 different *A. lipoferum* and *A. brasilense* strains isolated from the roots of different C3 and C4 crop plants, showed a general chemotactile behaviour rather than a specific host dependent response (Fedi et al., 1992). However, it is not known if successful root colonization by *Azospirillum* implies plant growth promotion. Besides living roots, *Azospirillum* can also attach to dead roots, soil particles and polystyrene. The population level of *Azospirillum* colonizing polystyrene remained constant in a period of three days (10^4 cfu/60 mm^2 surface), and increased significantly after treating the surface with root exudates (Bashan and Holguin, 1993).

1.2.2. Cyst production

In the presence of a suitable carbon and energy source, and under nitrogen starvation, high oxygen tension and water stress conditions, *Azospirillum*'s vibroid cells can develop into round, non-motile, highly refractile encapsulated forms (called C forms) rich in poly-ß-hydroxybutyrate (Bastarrachea et al., 1987). Contrary to what is known about spores and commonly known cysts, these C forms are not dormant, since they were found capable of fixing nitrogen (Okon and Itzigsohn, 1992), of having a high nitrate reductase activity and an active ammonia assimilating pathway (Ueckert et al., 1991). Cyst cells of *Azospirillum* were found to have glutamine synthetase activity (Narula and Kleiner, 1995). Heavy capsulation gives C forms a particularly adhesive nature, so that thousands of them aggregate in a matrix of

polysaccharides material, forming large macroscopic clumps in liquid cultures (Sadasivan and Neyra, 1985).

1.2.3. Signal molecules

In the *Rhizobium*-legume symbiosis, as well as in other plant-bacterial interactions including *Agrobacterium* and phytopathogens, there exists at the molecular level an elaborate system of communication in which the bacteria can trigger the transcription of plant genes or, vice versa, root released compounds can activate the transcription of bacterial genes (Glick et al., 1999). For example, the *Rhizobia nod* genes are induced by flavonoids released by legume roots (Matthysse, 1996). In bacteria, phytopathogen genes involved in virulence can be induced by a broad range of compounds present in root exudates (Arlat et al., 1992). In the *Agrobacterium*-plant interaction, acetosyringone, released from the site of a wound in a plant, induces the expression of the virulence genes *virL* and *virM* which are involved in the transfer of oncogenic DNA from the bacterium to the host plant cell (Kalogeraki and Winans, 1998).

The interaction between plants and PGPB was probably favoured by natural selection due to the advantages it provided for both bacteria and plants. Thus it seems reasonable that a finely regulated system of communication between both members of the interaction has evolved that optimizes the use of available resources.

In spite of the incipient information in regard to cross-talk between plants and PGPB, the information available on other plant-bacteria interactions implies that an elaborate system of communication may exist between PGPB and plants,

especially under nutrient poor conditions, where a wise use of the restricted amount of resources is crucial.

Several studies involving the use of the reporter gene *lacZ* in PGPB, have shown that root released compounds can induce promoter activity in situ (van Overbeek and Elsas, 1995). Two dimensional polyacrylamide gel electrophoresis of the total proteins of *Azospirillum brasilense* Sp7 showed that the bacteria respond to the presence of wheat, maize, bean, and alfalfa root exudates by induction of the synthesis of some non-identified proteins (Van Bastelaere et al., 1993).

Wheat germ agglutinin (WGA) a graminae lectin, may function as a signal molecule in the *Azospirillum*-plant association. This lectin binds to cells of *A. brasilense* and *A. lipoferum* (Del Gallo et al., 1989) and when incorporated into an *A. brasilense* Sp245 culture, it promoted nitrogenase and glutamine synthetase activity and ammonia excretion by the bacteria (Antonyuk et al., 1993). The effect may be due to N-acetyl-D-glucosamine specificity of wheat germ agglutinin, since its preincubation with 10% N-acetyl-D-glucosamine caused the disappearance of the effects. A N-acetyl-D-glucosamine sugar side chain present in a 32-kDa protein of *Azospirillum lipoferum*, is presumed to be the receptor responsible for the binding of WGA. The WGA-receptor complex generated stimuli that led to elevated transcription of the *nifH* and *nifA* genes and of the *glnBA* gene cluster in *Azospirillum lipoferum* (Karpati et al., 1999).

Briefly exposing wheat roots to *A. brasilense* Cd significantly enhanced proton efflux from the roots five hours after inoculation (Bashan and Levanony, 1991). Similarly, inoculation of soybeans or cowpea roots with *A. brasilense* Cd increased proton efflux and also changed the phospholipid content of the cowpea

plant membranes inoculated with the same bacterium. The nature of the signal responsible for this plant response is unknown (Bashan and Levanony, 1991).

1.2.4. How does *Azospirillum* help plants?

Among the proposals for explaining *Azospirillum*'s mode of action are: hormonal activities, nitrogen fixation, undefined signal molecules modulating plant metabolism like enhanced proton efflux from roots and changes in membrane potential, nitrite production, and enhanced mineral uptake (Bashan and Levanony, 1990). As there isn't sufficient evidence to support the notion that one of these mechanisms is solely responsible for plant growth promotion, an additive hypothesis was proposed (Bashan and Levanony, 1990). The authors claim that the net beneficial effect of the plant upon *Azospirillum* inoculation is the result of a combination of all the factors mentioned beforehand, which participate either simultaneously or in succession.

1.2.4.1. Nitrogen fixation

Nitrogen fixation, or the reduction of N_2 to ammonia, is common among microorganisms (Young, 1992). Such microorganisms, called diazotrophs, utilize the same basic biochemical machinery for nitrogen fixation, which is carried out by the nitrogenase system. The genes involved in nitrogen fixation have a very ancient origin, perhaps predating all of today's organisms. Nitrogen fixation must have first occurred approximately 3 billion years ago under an anaerobic atmosphere. Later on, the evolution of oxygenic photosynthesis by cyanobacteria posed a problem for nitrogenase because of its high sensitivity to oxygen (Young, 1992).

The function and synthesis of nitrogenase are expensive metabolic processes requiring a considerable fraction of the cellular pool of energy and metabolites (each electron transfer is accompanied by the hydrolysis of two ATP molecules) (Oelze and Klein, 1996). Hence, as a cellular strategy to economize energy, nitrogenase is subject to strict control by the intracellular concentration of ammonium and other forms of combined nitrogen and is activated only under nitrogen deficient conditions (Oelze and Klein, 1996).

1.2.4.1.1. Nitrogen fixation genes

Nitrogen fixation is catalyzed by the nitrogenase complex which includes dinitrogenase (MoFe protein, *nif*DK gene products), containing the active site of dinitrogen reduction, and dinitrogenase reductase (Fe protein, *nif*H gene product) supplies the reducing power to the dinitrogenase (Vande Broek and Vanderleyden, 1995).

Formation of a functional nitrogenase in *A. brasilense* is primarily controlled at the level of transcription of the nitrogenase structural genes (*nif*HDK operon) which occurs only under nitrogen-limiting microaerobic conditions (Vande Broek and Vanderleyden, 1995). Nitrogenase activity is also regulated at the post-translational level by two mechanisms. The dinitrogenase reductase ADP-ribosyltransferase/dinitrogenase reductase activating glycohydrolase (DRAT/DRAG) system, which involves reversible nitrogenase inactivation via ADP ribosylation in response to micromolar concentrations of ammonia (Zhang et al., 1992). A second post-translational regulatory mechanism of nitrogenase activity independent of the DRAT/DRAG system has been revealed, although it is still not understood (Zhang et al., 1996).

The nitrogen fixation or *nif* genes in *Azospirillum* are located in at least four different genomic regions spanning a minimum of 65 kb of DNA (Singh et al., 1989). Expression of the 15 to 20 genes involved in nitrogenase synthesis and activity requires the transcriptional activator NifA. NifA is a regulatory DNA binding protein that binds to enhancer sequences located upstream of gene promoters related to nitrogen assimilation and nitrogen fixation (Vande Broek and Vanderleyden, 1995). NifA, in concert with sigma-54-RNA polymerase, initiates transcription of the nitrogenase genes *nif*HDK and other nitrogen related genes.

In *A. brasilense nifA* is transcribed in the presence of ammonia, however, NifA activity is prevented. Arsène et al. (1996) showed that in the presence of ammonia the N-terminal domain of NifA inhibits the activity of the whole NifA protein, and that the polypeptide P_{II} prevents the inactivation of NifA. The mechanism by which P_{II} blocks the inactivation of NifA is unknown; however, it is believed that only when P_{II} is present in its uridylylated form can it control NifA activity. In *Klebsiella pneumoniae* P_{II} is uridylylated in response to the ratio between the concentrations of 2-ketoglutarate and glutamine (key intermediates of nitrogen metabolism) and, although it has not yet been proven for *Azospirillum* spp., this model is nevertheless attractive (Holguin et al., 1999).

Glutamine synthetase (encoded by *glnA*) together with glutamate synthase (encoded by *gltB*), are involved in the main route of assimilation of newly fixed nitrogen in diazotrophic bacteria, and provide cells with the key intermediates of nitrogen metabolism, i.e., glutamine and and glutamate. In *A. brasilense*, *glnA* is adjacent to *glnB* (P_{II}) in the operon *glnBA*. The trasncription of

*gln*BA depends on three different, selectively used and nitrogen-regulated promoters (de Zamaroczy et al., 1993).

1.2.4.1.2. Does *Azospirillum* contribute nitrogen to the plants?

Due to *Azospirillum*'s close association with cereals and its ability to fix atmospheric nitrogen, it was initially believed that the counterpart of *Rhizobium* in legumes had been found for cereals: by exploiting the capabilities of *Azospirillum* it would be possible to supply nitrogen to economically important crops (Klingmüller, 1982). Further research suggested that the contribution of bacterially fixed nitrogen to the plant was minimal, and that a positive growth response by an inoculated plant did not necessarily mean that *Azospirillum* was passing the products of biological nitrogen fixation to the plant (Bashan and Levanony, 1990). It was argued that other factors besides nitrogen fixation such as phytohormone production, enhanced mineral uptake, and signal molecules that affect plant metabolism, were contributing to improved plant growth. However, recent data suggests that nitrogen fixation by *Azospirillum* may contribute significant amounts of nitrogen to plants under certain environmental conditions and during specific stages of development (Reviewed in Glick et al., 1999). Inoculation of plants with *Azospirillum* spp. produced the highest yield when the levels of chemical nitrogen fertilization were sub-optimal (Bashan and Levanony, 1990). Studies with nitrogen labeled with ^{15}N showed that under nitrogen-free conditions, *Azospirillum lipoferum* strain N-4 contributed about 66% of the total nitrogen in wetland rice plants (Malik et al., 1997). Saad et al. (1999) found that inoculation of sweet potato with *Azospirillum*, was more effective

when initial nitrogen fertilizer at one third of the recommended rate was applied to the plants.

It is possible that under nitrogen deficient conditions it is selectively advantageous for the plant and the bacteria to establish a mutualistic association involving a tight control of the use of the available resources. In legumes, plants that are given high levels of nitrogen fertilizer, plants that have not yet formed nodules do not produce them, and those that already have bacteroid-filled nodules, decrease the amount of nitrogen fixed and even allow the nodule to senesce. It may be that when adequate nitrogen is available in the environment, it is selectively advantageous for the plant not to pass glucose on to bacteria (Glick et al., 1999; Mauseth, 1995).

In summary, the interest in biological nitrogen fixation as a PGPB mechanism for promoting plant growth has been revived. The significant advances made in recent years in our understanding of the mechanisms by which environmental fixed nitrogen influences nitrogenase synthesis and activity as well as the mechanisms involved in ammonium assimilation and excretion, can lead to the development of strategies that can induce and enhance the transfer of nitrogen fixed by the bacterium to the plant (Colnaghi et al., 1997). Some examples already in evaluation are nitrogen-excreting mutants, and the development of nodule-like structures or para-nodules in cereals (Christiansen-Weniger, 1998). A promising venue is the use of endophytic diazotrophs as vectors for providing nitrogen to the plants (Sturz and Nowak, 2000).

1.2.4.2. Plant growth hormones

Phytohormones influence many cellular functions in plant tissues and are important regulators of plant growth and development. The phytohormones auxins have been implicated in the orientation of root and shoot growth in response to light and gravity, in differentiation of vascular tissue, in apical dominance, in initiation of lateral and adventitious roots, in stimulation of cell division, and in elongation growth in stems and roots (For a review, see Glick et al., 1999). Regulation of these processes by auxin is believed to involve auxin-induced changes in plant gene expression (Vande Broek et al., 1999). Over the past ten years, a number of plant genes that are transcriptionally induced by auxin, and that may play roles in one or more of these processes, have been cloned and characterized (Sitbon and Perrot-Rechenmann, 1997).

In addition to plant factors that influence the levels of auxin within the plant such as de novo synthesis, degradation, and conjugate formation and hydrolysis, auxin secreted by microbes can contribute to a plant's endogenous pool. The capacity to produce the auxin IAA or indole-3-acetic acid, is widespread among plant-associated bacteria (Patten and Glick, 1996). It has been suggested that 80% of bacteria isolated from the rhizosphere can produce IAA (Loper and Schroth, 1986). Several of these bacteria are involved in plant pathogenesis, while others, like *Azospirillum*, have plant growth promoting capabilities (Glick et al., 1999).

At least five pathways for auxin biosynthesis have been identified in rhizobacteria. However, two tryptophan dependent pathways appear to predominate: the indole-3-acetamide pathway and the indole-3-pyruvic acid pathway. Several pathways for IAA synthesis may be present in one bacterium,

and the manner to which bacterial auxin impacts on the plant is probably governed by the biosynthesis pathway the bacteria employs (Patten and Glick, 1996). The indoleacetamide pathway seems to be the primary route for IAA synthesis in strains classified as pathogenic. Because bacterial IAA synthesis by this route is generally constitutive, it may be that high levels of IAA exceed the capacity for IAA metabolism by a host plant. In contrast, PGPB synthesize IAA mainly via the indolepyruvic acid pathway, which is inducible and probably subject to stringent regulation by plant metabolites (Patten and Glick, 1996).

In *A. brasilense*, radiolabeled precursor feeding experiments support the existence of multiple IAA biosynthesis pathways (Prinsen et al., 1993). The low specific radioactivity of IAA compared with indoleacetamide following [^{3}H]-indoleacetamide feeding suggests that less than 0.1% of IAA is synthesized via the indoleacetamide pathway. When [^{3}H]-tryptophan was added to the bacterial culture medium 10% of the IAA was produced by a non-indoleacetamide, but tryptophan-dependent, pathway. Inferring from these results, roughly 90% of IAA synthesis occurs independently of tryptophan, form an as yet unidentified pathway (Prinsen et al., 1993).

IAA production has been proposed to account for the beneficial effect promoted by the plant-*Azospirillum* association. The reason being that *Azospirillum* causes changes in root parameters which are generally attributed to IAA. These are an increase in the production of root hairs and lateral roots, branching of root hairs, and enhancement in the rates of cell division and differentiation in meristematic tissues (Tien et al., 1979). Inoculation of wheat seedlings with an *A. brasilense* Nif$^-$ IAA$^+$ morphotype enhanced the number and length of lateral roots as compared to the wild type. However, inoculation with a

Nif⁻ but low IAA producer, did not elicit any response from the plant (Barbieri et al., 1986). Sometimes, external application of synthetic IAA imitated the positive effects of *Azospirillum* on root development and morphology. The effect of *Azospirillum* inoculation on root elongation of wheat plants and on branching of wheat root hairs was mimicked by the application of IAA. In other cases the application of the synthetic hormone did not simulate the positive effects induced by *Azospirillum* (Bashan and Holguin, 1997).

Besides IAA, *Azospirillum* is a producer *in vitro* of other phytohormones including gibberellins and cytokinins (for a review see Bashan and Holguin, 1997) which may be contributing to the positive effect on plants. The production of gibberellins by *Azospirillum brasilense* has been related to root growth promotion (Fulchieri et al., 1993) and to improved water absorption and nutrient uptake (Kapulnik et al., 1985). Gibberellin GA3 had similar effects to inoculation with *A. lipoferum* on promotion of root growth, specially in increasing root hair density (Piccoli and Bottini, 1994).

The response of the plant to microbial IAA is complex and subject to different levels of regulation. Factors like the location of the biosynthesis genes and their regulatory sequences, the presence of enzymes involved in converting free, active IAA into a conjugated, inactive form, and the interaction between microbe and plant, are involved in determining levels of IAA expression (Patten and Glick, 1996). To complicate matters even further, many of the effects of auxin on plants, whether from application of synthetic or bacterial IAA, may be a consequence of interactions between IAA and other phytohormones, especially ethylene and cytokinins (Glick et al., 1999). For example, expression of the enzyme 1-aminocyclopropane-1-carboxylic acid (ACC) synthase, which catalyzes

the conversion of S-adenosylmethionine (SAM) to ACC, the immediate precursor to ethylene, is regulated by IAA (Theologis, 1986).

Reports of growth inhibition in plants caused by inoculation with high inoculum levels of *Azospirillum* may be due to this interaction between ACC synthase and IAA. Several studies (Bashan, 1986; Yahalom et al., 1990) have shown that the morphological changes induced by *Azospirillum* in the plant root system, are directly related to inoculum concentrations: higher than optimal levels (10^5-10^6 cfu/mL) had inhibitory effects, while low bacterial doses had no effect (for a review see Bashan and Levanony, 1990). High concentrations of bacterially synthesized IAA may enhance ACC synthase activity and thereby increase the amount of ACC in the plant, and hence its level of ethylene (Kende, 1993). Yahalom et al. (1990) found that in burr medic seedlings (*Medicago polymorpha*), exposure to *Azospirillum* at a concentration of 10^9 cfu/ml caused a 40% increase in endogenous ethylene production by the roots as well as a decrease of 58% in root length, as compared to non-inoculated plants. A less concentrated inoculum did not increase ethylene production. The inoculation of the seedlings with compounds excreted by the bacterium into the growth medium (IAA was detected in the dialysate) also increased root length. However, a concentrated dialysate reduced root elongation in a manner similar to IAA at 10^{-5} M (Yahalom et al., 1990). These results suggest that inoculation of *Azospirillum* in high concentrations, results in an elevated production of IAA, which in turn induces ethylene synthesis by the plant, thus inhibiting root development.

Small changes in the level of bacterial IAA can dramatically alter the response of the plant to the PGPB. A *P. putida* GR12-2 mutant which secreted

four times the level of IAA synthesized by the wild type, inhibited root length in canola seedlings, while other mutants which secreted only two or three times more IAA than the wild type, promoted root elongation. These results are consistent with the existence of a threshold level of exogenous IAA that can be added to plants (Xie et al., 1996).

As for the interaction between IAA and cytokinins, it is now recognized that these phytohormones interact to regulate a variety of physiological processes in plants and that these interactions may be antagonistic (Glick et al., 1999). Our understanding of the effect of bacterial auxin on plants is thus further confounded by the production of cytokinins by many PGPB including *Azospirillum* (Reviewed in Glick et al., 1999).

1.3. Ethylene

Ethylene has a profound influence on the growth and development of plants: it is involved in several developmental stages including seed germination, fruit ripening, flowering, senescence, root initiation, stem and root growth, epinastic curvature, and in hermaphroditic plants it is involved in sex determination (Abeles et al., 1992; Penrose and Glick, 1997). It influences cell division and cell elongation thus promoting the elongation of growing stems, roots and other organs. Ethylene can induce physiological changes in plants like increases in respiratory enzymes, stomatal closure, the regulation of the synthesis of compounds such as chlorophyll, phenolic compounds and flavonoids and regulation of IAA levels and activity. During much of plant growth and development, ethylene production is low but during senescence and ripening large quantities are synthesized. Biosynthesis of ethylene by plants is

also accelerated during biological and environmental stresses and pathogen attack (Abeles et al., 1992).

In higher plants, ethylene is produced from L-methionine via the intermediates, S-adenosyl-L-methionine (SAM) and 1-aminocyclopropane-1-carboxylic acid (ACC) (Yang and Hoffman, 1984). The enzymes involved in this metabolic sequence are SAM synthetase, which catalyzes the conversion of methionine to SAM; ACC synthase, which is responsible for the conversion of SAM to ACC and 5′-methylthioadenosine (MTA); and ACC oxidase which further metabolizes ACC to ethylene, carbon dioxide, and cyanide (Glick et al., 1999).

ACC is a key intermediate in the biosynthesis of ethylene. At present, 46 different ACC synthases have been reported. Members of the multigene family of ACC synthases are differentially and independently regulated, some of them by stress (Fluhr and Mattoo, 1996). In tomato plants ACC synthase is encoded by at least nine genes and six of them are auxin inducible (Zarembinsky and Theologis, 1993).

ACC oxidase belongs to a family of enzymes characterized by requirements for Fe(II) and ascorbate, and loose substrate specificity. Its activity is induced by ACC during fruit ripening, flower senescence and mechanical wounding and in response to elicitors, novel fungal proteins and tobacco mosaic virus. Similar to ACC synthase, ACC oxidase also exists as a multi-gene family (Glick et al., 1999).

The levels of ethylene in plants can increase in response to stress inflicted by chemicals, temperature extremes, water stress, ultraviolet light, pathogen attack, and other trauma-causing factors. These responses in the plant can

enhance its survival under adverse conditions, e.g., production of antibiotic enzymes and phytoalexins (Glick et al., 1999). However, high levels of ethylene in plants can inhibit growth, cause wilting, abscission, and premature ripening and senescence, which in turn may lead to significant losses of fruits and vegetables (Glick et al., 1999).

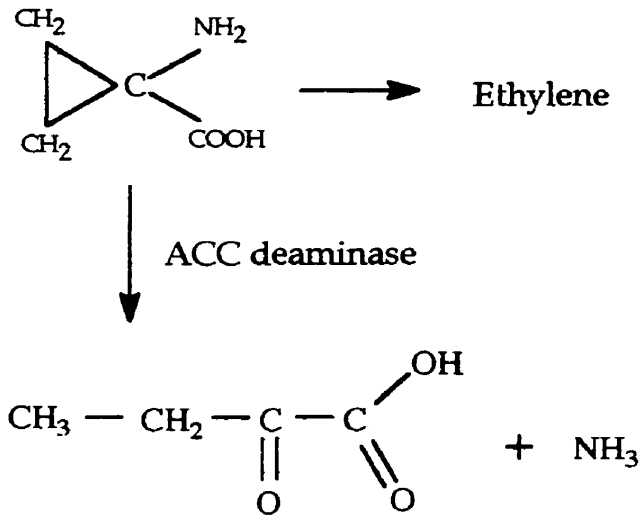
Many of the symptoms of a diseased plant may arise as a direct result of the stress imposed by infection from a pathogen. That is, a significant portion of the damage to plants infected with fungal phytopathogens occurs as a result of the response of the plant to the increased levels of stressed ethylene. Not only does exogenous ethylene often increase the severity of a fungal infection but, as well, inhibitors of ethylene synthesis can significantly decrease the severity of a fungal infection (Glick and Bashan, 1997).

1.3.1 ACC deaminase

Some microorganisms are capable of degrading ACC, the immediate precursor of ethylene, into ammonia and α-ketobutyrate through the action of the enzyme ACC deaminase (Fig. 1) (Hall et al., 1996). This microbial enzyme not found in plant tissues, has been detected in the fungus *Penicillium citrinum*, in the yeast *Hansenula saturnus*, and in a number of other bacterial strains, all of which originated from the soil either as soil sample isolates or as microbes typically found in the soil. While ACC deaminase has been found only in microorganisms, there are no microorganisms that synthesize ethylene via ACC (Penrose and Glick, 1997).

ACC deaminase genes have been cloned in plant tissues as an approach to ameliorate the negative effects of high levels of ethylene in plants. Some

Fig. 1. ACC deaminase breaks down ACC, the precursor of ethylene in plants, into α-ketobutyrate and ammonia.



α-ketobutyrate

examples of positive results derived from expression of ACC deaminase in transgenic plants are: considerable delay in senescence of tobacco flowers, delay in the initiation of ripening of detached tomato fruit, increase in the duration of the tomato ripening process, and significant reductions in the necrotic lesions of tomato plants challenged with the pathogens *Xanthomonas campestris* pv *vesicatoria* and *Verticillium dahliae* Kleb (reviewed in Glick et al., 1999).

In a number of different plants, ethylene stimulates germination and breaks the dormancy of the seeds (Esashi, 1991), but if the level of ethylene following germination is too high, root elongation is inhibited (Jackson, 1991). It has been suggested that certain PGPB can regulate the production of ethylene in developing seedlings by hydrolyzing ACC through the action of ACC deaminase. There are at least two direct consequences that are the result of lowering the level of ACC within the plant: a reduction of the amount of plant ethylene and a decreased extent of ethylene inhibition of plant seedling root elongation. The following sequence of events has been postulated to take place on the root surface colonized with a PGPB having ACC deaminase activity (Glick et al., 1998). The bacterium synthesizes and secretes IAA in response to tryptophan and other small molecules present in root exudates. IAA is taken up by the plant and besides stimulating plant cell proliferation and/or elongation, IAA also induces ethylene synthesis by stimulating the activity of the enzyme ACC synthase to convert S-adenosylmethionine to ACC (Kende, 1993). A significant portion of the ACC is exuded through the roots along with other amino acids, organic acids, sugars and other compounds. PGPB have been found to stimulate root exudation. For example, *Azospirillum lipoferum* and *A. brasilense* stimulated root exudation of rice seedlings up to 36% as compared to non-

inoculated plants (Heulin et al., 1987). The exudates are taken up by the bacteria and ACC is hydrolysed by the enzyme ACC deaminase to ammonia and α-ketobutyrate. The uptake and subsequent hydrolysis of ACC by the bacterium decreases the amount of ACC outside the plant; in order to maintain the equilibrium between internal and external ACC the plant must exude increasing amounts of ACC (Glick et al., 1998). Several reports give evidence to support the model (Glick et al., 1994a, 1994b, 1995a; Hall et al., 1996; Li et al., 2000; Xie et al., 1996).

ACC deaminase is probably an essential component of the mechanism that many PGPB strains use to promote plant growth (Glick, 1995a). It is possible that lowering the level of ACC, and hence the level of ethylene in the plant, triggers metabolic responses which result in an overall benefit to the plant. Besides increasing root elongation, the PGPB *Pseudomonas putida* GR12-2 promoted root fresh weight, protein content, the activity of root glutamine synthetase (involved in the assimilation of ammonia) and phosphoenolpyruvate carboxylase, probably involved in priming the Krebs cycle in the roots of the developing seedlings (Caron et al.,1995).

The ability of *P. putida* GR12-2 to decrease the level of ethylene within the plant may ameliorate the effects on plants provoked by stress ethylene. *P. putida* GR12-2 promoted the development of canola seedlings (root and shoot lengths, fresh and dry weights, and chlorophyll and protein contents of shoots) growing in saline soil or exposed to cold night temperatures (Glick et al., 1997). Inoculation of canola seeds with *Kluyvera ascorbata* SUD165- a bacterium exhibiting ACC deaminase activity, isolated from metal-contaminated wetlands, protected the plants from growth inhibition caused by the presence of a high

concentration (mM) of nickel (Burd et al., 1998). Inoculation of transgenic canola plants (containing the ACC deaminase gene) with the PGPB *Enterobacter cloacae* CAL2 that exhibits ACC deaminase activity, resulted in improved growth of canola plants in the presence of arsenate, compared with non-transformed canola or transgenic plants not inoculated with the bacterium (Nie, 2000).

The PGPB *Pseudomonas fluorescens* CHA0 transformed with the ACC deaminase gene from *Enterobacter cloacae* UW4, protected cucumber plants from the pathogen *Pythium ultimum* (Wang et al., 2000).

Considering that *Azospirillum* spp. strains do not have to ability to hydrolyze ACC (Holguin and Glick, 2000), we postulate that the transfer of an ACC deaminase gene into *Azospirillum* will help the bacteria to lower the levels of ethylene within the plants raised a) indirectly through IAA synthesis or, b) as an ethylene stress response. The expression of the ACC deaminase gene in *Azospirillum* a) may improve plant root and shoot growth parameters, b) may alleviate the inhibition of plant growth caused by high inoculum numbers, or by the presence of high bacterial numbers attached to the root, c) will help in reducing the variability of plant response imposed by different levels of bacteria colonizing the roots, d) will improve *Azospirillum* competitiveness in the rhizosphere, and e) will ameliorate the negative effects on plants caused by stress ethylene under flooding conditions or under pathogen attack.

Recent data showing that *Azospirillum* is capable of attachment to nearly every root system, suggests that *Azospirillum* has the potential to colonize mangrove roots. Mangroves are tropical and subtropical trees that are the major component of coastal marine-lagoon ecosystems and serve as feeding, spawning and reproductive areas for numerous economically and ecologically

important marine species (Rico Gray, 1993). Mangroves in semi-arid tropics are considered to be nutrient deficient ecosystems, containing very low levels of nitrogen and phosphorus. Because of these environmental factors, together with salt stress, mangroves in semi-arid tropics, following clearcutting, hardly every revegetate (Cintron et al., 1978). Because mangroves are being deforested on an alarming scale (Honculada-Primavera, 1993) it is imperative to consider strategies for their preservation and reforestation.

It was previously observed that mangrove seedlings grow better after inoculation with the diazotrophic filamentous cyanobacteria *Microcoleus chthonoplastes* (Toledo et al., 1995). Based on this observation, it was reasoned that mangrove seedlings might benefit from inoculation with PGPB as do many other plant species. Considering that the PGPB potential of the native microflora of semi-arid mangroves is unknown, a mangrove-PGPB association using well-known, non-specific, salt tolerant *Azospirillum* strains was proposed.

2. Materials and Methods

2.1 Bacterial strains and growth conditions

The *Azospirillum* strains used in this study were *A. brasilense* Cd ATCC 29710, *A. brasilense* Sp245 (Baldani et al., 1986) and *A. halopraeferens* AU10 (provided by B. Reinhold-Hurek, Max-Planck Institute, Marburg, Germany). *Escherichia coli* DH5α (Hanahan, 1983) was the host strain for the ACC deaminase gene from *Enterobacter cloacae* UW4 in pUC18 (Shah et al., 1998). *Escherichia coli* DH5α is a recombinant-deficient suppressing strain. The plant growth promoting bacteria *Enterobacter cloacae* CAL2 and *Enterobacter cloacae* UW4 have ACC deaminase activity and were isolated from the rhizosphere of tomato plants in King City, CA, USA and from the rhizosphere of reeds in the North Campus of the University of Waterloo, Waterloo, ON, Canada, respectively (Glick et al., 1995; Shah et al., 1998). *Pseudomonas putida* AC8 was isolated from the leaves of the wild flower *Arctium minus* from Waterloo, Ontario, Canada, and has ACC deaminase activity.

The *Enterobacter cloacae* strains were grown on either solid or liquid tryptic soybean broth (TSB) medium (Difco Laboratories, Detroit, Mich.) at 30°C, or on M9 minimal medium (Miller, 1972; $MgSO_4$, 2 mM; $CaCl_2$, 0.1 mM; thiamine, 1 mM; glucose, 0.2% w/v; g/L, Na_2HPO_4, 6; KH_2PO_4, 3; NaCl, 0.3) supplemented with either 37 mM NH_4Cl or 3.0 mM ACC (Calbiochem-Behring, La Jolla, Calif.). *Escherichia coli* DH5α was grown in Luria broth (Difco) at 37°C or in M9 minimal medium. *Pseudomonas putida* AC8 was grown in TSB medium at 30°C. *A. brasilense* Cd, *A. brasilense* Sp245, and *A. halopraeferens* were grown in Nutrient Broth (Sigma Chemical Co., St. Louis MO, USA) supplemented with 1% NaCl in

the case of the latter, or in OAB medium (Okon et al., 1977) at 30°C; (g/L: DL-malic acid, 5; NaOH, 3; $MgSO_4 \cdot 7H_2O$, 0.2; $CaCl_2$, 0.02; NaCl, 0.1; NH_4Cl, 1; yeast extract, 0.1; $FeCl_3 \cdot 6H_2O$, 0.02; mg/L: $NaMoO_4 \cdot 2H_2O$, 2; $MnSO_4$, 2.1; H_3BO_3, 2.8; $Cu(NO_3)_2 \cdot 3H_2O$, 0.04; $ZnSO_4 \cdot 7H_2O$, 0.24; 900 mL distilled water. Buffer solution, g/L: K_2HPO_4, 6; KH_2PO_4, 4; 100 mL distilled water, pH, 6.8). For the ACC deaminase assays, the growth media was supplemented with 3.0 mM ACC and when indicated with isopropylthio-ß-D-thiogalactoside (IPTG).

2.2. Isolation of flower and leaf-colonizing bacteria with ACC deaminase activity

Plants of the species *Melilotus officinalis, Lotus corniculatus, Trifoleum pratense, Trifoleum hybridum, Viccia cracca, Hypericum perforatum, Chrysanthemum leucanthemum, Arctium minus,* and *Apocynum androsaemifolium,* were harvested from the area around Columbia Lake and the Laurel Creek Conservation Area in Waterloo, Ontario, Canada. Flowers or leaves were cut and washed twice with non-sterile distilled water, followed by two washings with sterile distilled water under gentle stirring. The flowers or leaves were then placed in Erlenmeyer flasks containing Pseudomonas F (PF) medium (Merck, Darmstadt, Germany) and incubated under agitation of 200 rpm at 30°C. After 96 hours, the bacteria were pelleted and washed twice with 0.08 M phosphate buffer solution supplemented with 0.05 M NaCl, pH 7.2 (phosphate buffer saline solution, PBS) and resuspended in Dworkin and Foster (1958) (DF) salts medium with $(NH_4)_2SO_4$ as a nitrogen source: g/L, KH_2PO_4, 4; Na_2HPO_4, 6; $MgSO_4 \cdot 7H_2O$, 0.2; $(NH_4)_2SO_4$, 2; glucose, 2; gluconic acid, 2; citric acid, 2; 0.1 mL, trace elements solution; 0.1 ml of a 36 mM $FeSO_4 \cdot 7H_2O$ solution; 1 mL of vitamins solution; 0.1

mL of a 68 mM $CaCl_2 \cdot 2H_2O$ solution. Trace elements solution: g/L, H_3BO_3, 0.1; $MnSO_4 \cdot H_2O$, 0.1; $ZnSO_4 \cdot 7H_2O$, 1.25; $CuSO_4 \cdot 5H_2O$, 0.8; MoO_3, 0.1; vitamins solution: mg/L, biotin, 40; nicotinic acid, 100; thiamine, 100. After incubation for 48 hours, the bacteria were washed twice with PBS, resuspended in DF medium with ACC (3 mM) as the only source of nitrogen, and grown for another two days under the same conditions. Finally, the bacteria were washed once with PBS, streaked onto DF solid medium supplemented with 3.0 mM ACC and incubated at 30°C until visible colonies appeared. The isolates were then tested for ACC deaminase activity as described in section 2.8. and sent for identification by acid fatty analysis to Microbial ID, Inc., Newark, Delaware, USA.

2.3. DNA manipulation

2.3.1. Restriction endonuclease digestion

For restriction enzyme digestion, the DNA samples were incubated with the restriction enzyme and its buffer according to instructions from the manufacturer (Boehringer, Mannheim, Germany). For a double enzyme digestion, the restriction buffer was chosen according to the table in the Roche Molecular Biochemicals catalogue (2000, p. 154). The digestion was carried out in a total volume of 20 μL that contained 100 ~ 1000 ng of the DNA sample, 2 μL of the appropriate 10X buffer, and 2 units of the enzyme. After mixing the digestion cocktail was incubated for 1 h at the temperature specified by the manufacturer. Once digestion was complete, the enzymes were inactivated by phenol purification or by heating for 10 min at either 65°C or 85°C as outlined in the manufacturer's instruction.

2.3.2. DNA agarose gel electrophoresis

Gels were prepared with 1% agarose (Bio-Rad Laboratories, Hercules California, USA) and 0.5 μg/mL of ethidium bromide in 1X Tris-acetate buffer (TAE) (50X: 242 g Tris base, 57.1 ml glacial acetic acid, 100 ml 0.5 M EDTA (pH 8.0). The DNA molecular weight markers VIII and X (Boehringer, Manheim, Germany) were used as standards. The gels were run at 100 volts in an electrophoresis tank filled with TAE buffer. The DNA was visualized under UV light in a Video Documentation System for gel electrophoresis, (Imagemaster R VDS, Amersham Pharmacia Biotech, Quebec city, Canada).

2.3.3. Plasmid isolation

Plasmid DNA was routinely prepared by the alkaline lysis miniprep method (Sambrook et al., 1989). An overnight culture in a rich medium (such as Luria Broth for *Escherichia coli* or Nutrient Broth for *A. brasilense*) supplemented with the appropriate antibiotics, was distributed into 1.5 mL microcentrifuge tubes, and centrifuged at 16,000 x g for one minute to pellet the cells. The supernatant was removed and the pellet was resuspended in 200 μL of solution I (50 mM glucose, 25 mM Tris-HCl pH8.0, 10 mM EDTA pH 8.0). Next, 300 μL of freshly prepared solution II (0.2 N NaOH, 1% SDS) was added and the tube content was mixed five times and incubated on ice for 5 minutes. The suspension was then neutralized with 300 μL of ice-cold solution III (3.0 M potassium acetate, pH 4.8). The tube was inverted several times and put on ice for 5 minutes. The mixture was then centrifuged at 16,000 x g for 10 min, and the resulting supernatant was transferred to a new tube. RNase A (Sigma Chemical Co. St. Louis MO, USA) was added to a final concentration of 20

μg/mL and the tube was incubated at 37°C for 20 minutes. After the RNase treatment, the supernatant was extracted twice with a chloroform:isoamyl-alcohol solution (24:1 v/v). The plasmid DNA was precipitated by adding an equal volume of 100% isopropanol followed by incubation for 1 hour at -20°C and centrifugation at 16,000 x g for 20 minutes. The DNA pellet was then washed with 500 μl of ice-cold 70% ethanol and centrifuged for 5 minutes. The supernatant was removed and the pellet was dried under vacuum for 3 minutes. After drying, the plasmid DNA was resuspended in 20 μL of TE buffer (10 mM Tris-HCl, pH 8.0; 0.1 mM EDTA, pH 8.0) and stored at -20°C.

2.3.4. Transformation of *Escherichia coli* cells

Escherichia coli DH5α cells were transformed with plasmid DNA by treatment of the cells with $CaCl_2$ as described in Sambrook et al. (1989). To prepare competent cells 300 μL of an overnight *Escherichia coli* DH5α culture were inoculated in 30 mL of Luria Broth, and incubated at 37°C for 2-4 h, until the absorbance at 600 nm was read between 0.1 and 0.2. The cells were then transferred to an ice-cold centrifuge tube and kept on ice for 10 minutes, after which the cells were centrifuged at 5000 rpm for 5-10 minutes at 4°C. The supernatant was discarded and the cells were resuspended in 15 mL of ice cold 100 mM $CaCl_2$ and stored on ice for 30 minutes, centrifuged, resuspended in 3 mL of ice-cold 100 mM $CaCl_2$ and kept at 4°C for up to one week, since further storage of the cells significantly decreased their competence for transformation. For transformation of competent cells, 100 μL of cells were transferred to 10 μL of the ligation reaction contained in a 1.5 mL microcentrifuge tube. The contents of the tube were gently mixed and the tube was placed on ice for 20 minutes.

After that, the cells were heat shocked for 90 seconds in a water bath at exactly 42°C, being careful not to shake them. The cells were immediately put on ice for 2 minutes. A 900 µL aliquot of SOC medium (Sambrook et al., 1989) (per liter: 985 mL deionized H_2O, 20 g of bacto-tryptone, 5 g of bacto-yeast extract, 0.5 g of NaCl, 10 mL of a 250 mM solution of KCl, and 5 mL of a sterile solution of 2 M $MgCl_2$; Sambrook et al., 1989) was then added to the cells, followed by 1.5 h incubation at 37°C with shaking (approximately 150 rpm). The cells were pelleted by centrifugation at 3,000 rpm for 10 minutes, resuspended in 200 µL of SOC medium, plated on two plates, and incubated overnight at 37°C. Transformants were selected after 12-14 h growth at 37°C on Luria Base solid medium containing the appropriate antibiotic. In order to screen for the presence of inserts in the vectors pGEM-T, pUC19 or pRK415, 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) (0.1 mM) and IPTG were spread over the surface of LB plates. Incubation for more than 14 hours, renders false positives: the enzyme involved in the hydrolysis of the β-lactam ring in ampicillin is secreted into the medium and cells without resistance to ampicillin are able to utilize the enzyme and grow on ampicillin-containing medium (Sambrook et al., 1989).

2.3.5. Triparental mating

Plasmid DNA was transferred from *Escherichia coli* DH5α to *A. brasilense* strains Cd and Sp245 by triparental mating using pRK2013 in *Escherichia coli* HB101 as the helper plasmid (Figurski and Helinski, 1979). The broad-host-range plasmids pRK415 (10.5 kb, tetracycline resistant, IncP group) (Keen et al., 1988), pGSS15 (11.4 kb; IncQ group; tetracycline and ampicillin resistant) (Barth et al., 1981), pRKACC (a 3.8 kb fragment containing both the ACC deaminase gene

and its promoter region from *Enterobacter cloacae* UW4 cloned in pRK415; Shah et al., 1998), pRKLACC (the ACC deaminase gene under the control of the *lac* promoter), and pRKTACC (the ACC deaminase gene under the control of the Tet^r promoter) were transferred from *Escherichia coli* DH5α to *A. brasilense* Cd and Sp245. Conjugation was carried out as follows: The recipient, donor, and helper strains were grown overnight in rich media, washed once with phosphate buffer, resuspended in rich media and mixed in a volume (mL) ratio of donor/helper/recipient of 1:1:8 (no transconjugants were obtained using lower ratios). The bacterial mixture was filtered under vacuum through a 0.45 μMeter-filter (Millipore Corporation, Bedford MA, USA). The filter was then placed on LB solid medium and incubated overnight at 30°C. The following day, the filters were resuspended in OAB minimal medium (section 2.1) supplemented with the appropriate antibiotic and incubated under shaking conditions for 1 hour, followed by serial dilutions and plating. Transconjugants of the *A. brasilense* strains were selected following growth for three days at 30°C on OAB minimal medium supplemented with 3.0 mM ACC or with 19 mM NH_4Cl in the presence of 20 μg/mL tetracycline (for *A. brasilense* pRK415 derived transconjugants) or of 100 μg/mL ampicillin (for the *A. brasilense* pGSS15 derived transformants). The identity of the plasmid and the insert were confirmed following plasmid isolation by restriction enzyme digestion and agarose gel electrophoresis.

2.3.6. DNA purification

2.3.6.1. DNA phenol purification

After increasing the volume of the DNA containing solution 15X with double distilled H_2O, the DNA was extracted with a mixture of

phenol:chloroform:isoamyl alcohol (25:24:1 v/v) adding half the volume of the total volume in the reaction. After centrifugation for 2 minutes to separate the phases, a 3M sodium acetate solution (pH 5.2) was added to give a final concentration of 0.3M, followed by 100% ethanol (twice the volume of the whole reaction). The reaction was stored at -70°C for 1 h, centrifuged at 16,000 x g for 20 min, washed with 70% ethanol, centrifuged for 5 min, and dried under vacuum for 5 min. The DNA pellet was then resuspended in sterile double-distilled water or in TE buffer.

2.3.6.2. Purification of PCR products from agarose gels

DNA was extracted from agarose gels using either the GENECLEAN® II kit (Bio 101, Inc., La Jolla, Calif., USA) or the DNA Extraction kit (MBI Fermentas Inc., Burlington, ON, Canada).

PCR product reactions were loaded onto an agarose gel (15 cm) and run long enough (approximately 3 hours) to allow sufficient separation between bands. DNA bands were visualized using ultraviolet light, after which the band of interest was cut out of the gel using a razor blade. The excised band was placed into a 1.5 mL microcentrifuge tube and weighed. The agarose was dissolved by adding three volumes of saturated NaI solution followed by incubation at 50°C for 10 min, or until the agarose was completely dissolved. Five µL of resuspended glassmilk or silica powder suspension was added to the DNA-containing NaI solution. The tube was inverted gently several times and then placed on ice for at least 5 min to allow binding of the DNA to the matrix. The glassmilk or silica powder/DNA complex was pelleted by centrifugation for 5 sec, and the supernatant was discarded. The pellet was then washed three

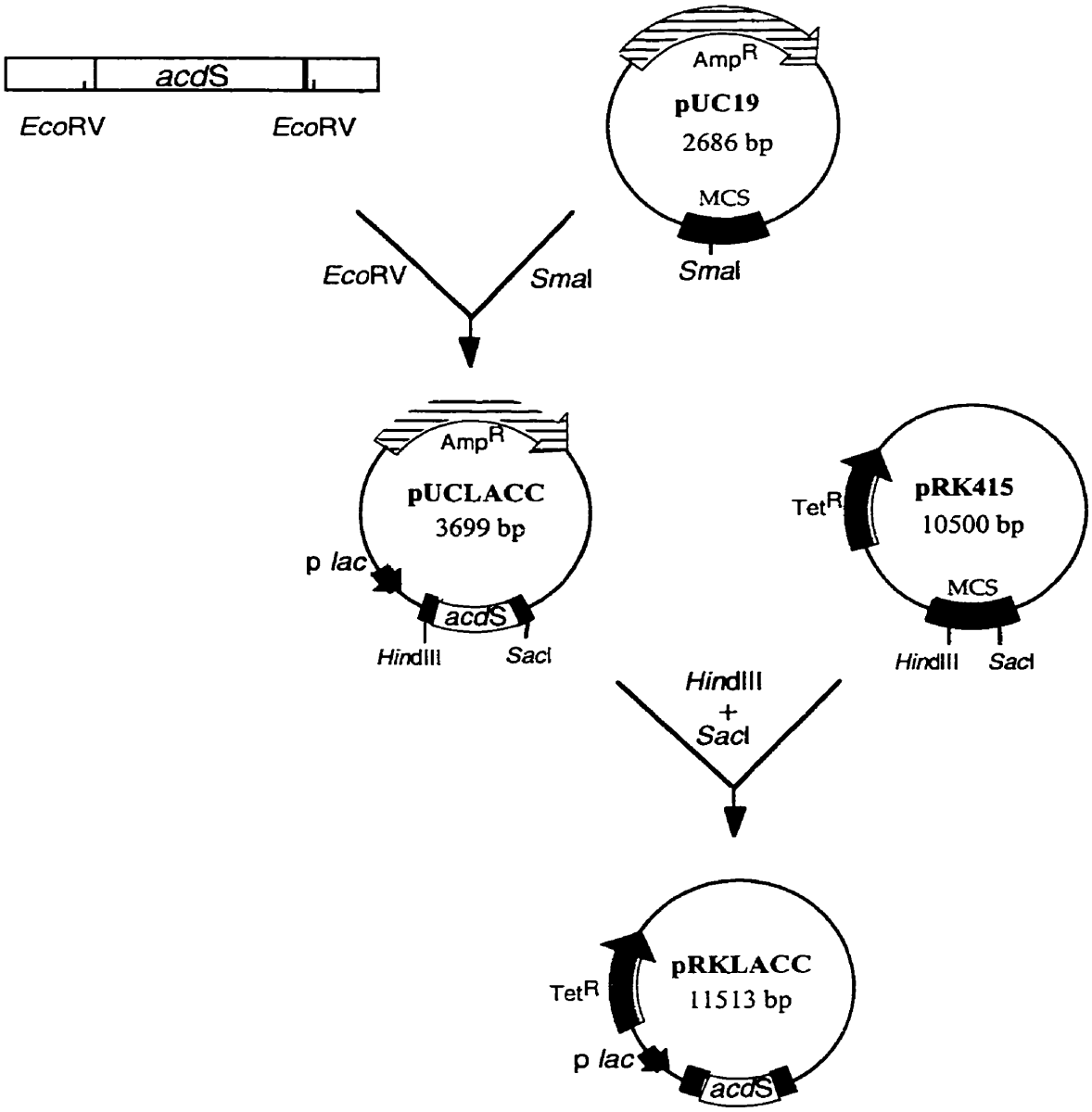
times with 500 μL of ice-cold wash buffer (provided by the manufacturer). During each wash, the pellet was resuspended gently in the wash buffer, centrifuged for 5 seconds and the supernatant removed. On the last wash, any remaining liquid was removed with a micropipette. The DNA was eluted from the silica powder/DNA complex by resuspending the pellet in 5 μL of sterile double distilled water and incubating it at 50°C for 5 minutes. After incubation, the tube was centrifuged for 1 min, and the supernatant was transferred into a fresh tube. The DNA still present in the pellet was eluted two more times with 5 μL of sterile double-distilled water, followed by brief centrifugation and recovery of the supernatant. To remove silica particles that may have been transferred with the supernatant, the tube was spun again briefly, and the supernatant transferred into a new tube.

2.4. Cloning of the ACC deaminase gene with the *lac* promoter

A 3.8 kb DNA fragment from *Enterobacter cloacae* UW4 that includes the ACC deaminase gene (*acd*S) and an 855 bp upstream region containing the *acd*S promoter region, was previously cloned in plasmid pUC18 and named p4U2 (Shah et al., 1998).

The ACC deaminase structural gene (*acd*S) (Shah et al., 1998) was excised from plasmid p4U2 without its native promoter by digestion with *Eco*RV (Fig. 2). The 1174 bp fragment containing *acd*S was inserted into the *Sma*I site within the multiple cloning site of pUC19 and the resulting plasmid, designated as pUCLACC, was introduced into *Escherichia coli* DH5α by transformation. The size and orientation of the insert was determined by restriction enzyme digestion and agarose gel electrophoresis. Analysis of transformants with ACC

Fig. 2. The ACC deaminase structural gene, *acdS*, was excised from its wild type promoter, and cloned into pRK415 under the control of the *lac* promoter.


acdS
EcoRV
EcoRV
AmpR
pUC19
2686 bp
MCS
SmaI
EcoRV
SmaI
AmpR
pUCLACC
3699 bp
p lac
acdS
HindIII
SacI
TetR
pRK415
10500 bp
MCS
HindIII
SacI
HindIII
+
SacI
TetR
pRKLACC
11513 bp
p lac
acdS

deaminase activity showed that, as expected, they contained *acd*S immediately downstream from the *lac* promoter.

Once it had been demonstrated that *acd*S could be expressed in *Escherichia coli* under the control of the *lac* promoter, *acdS* was cloned into the broad host range plasmid pRK415 (Fig. 2). To do this the *Hin*dIII-*Sac*I fragment of pUCLACC, was ligated to pRK415 digested with the same restriction enzymes, and transformed into *Escherichia coli* DH5α. One clone with high ACC deaminase activity, similar to that of *Enterobacter cloacae* UW4, was named pRKLACC and transferred from *Escherichia coli* DH5α into *A. brasilense* Cd and Sp245 by triparental mating.

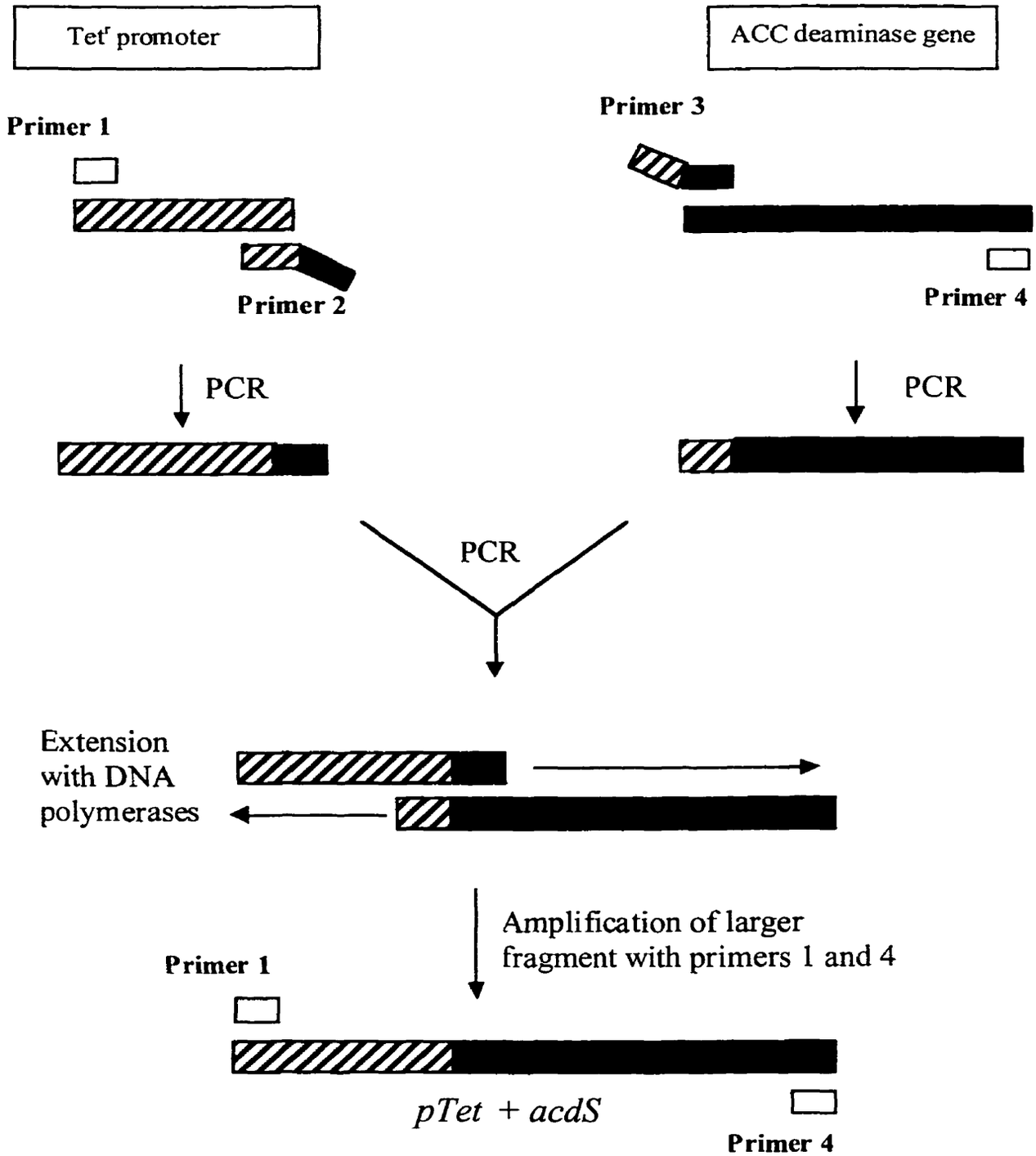
2.5. Cloning of the ACC deaminase gene under the control of the Tet^r promoter.

The ACC deaminase structural gene *acdS* was fused with the constitutive promoter of the tetracycline resistance gene by overlap extension (Ho et al., 1989) using the polymerase chain reaction, PCR (Rashtchian, 1995).

2.5.1. Overlap extension using the polymerase chain reaction

The first step in this procedure involves the generation of DNA fragments (the promoter region of the Tet^r gene and the ACC deaminase structural gene, *acdS*) with complementary regions using independent PCR reactions (Fig. 3). In the second step, the two fragments generated in the first PCR reactions are fused together in a subsequent PCR reaction. The overlap allows one strand from each fragment to act as a primer for the other strand and extension of this overlap results in the desired product.

Fig. 3. Fusion of the ACC deaminase structural gene, *acdS*, with the constitutive promoter of the tetracycline resistance gene by overlap extension using PCR


Tet^r promoter
ACC deaminase gene
Primer 1
Primer 3
Primer 2
Primer 4
PCR
PCR
PCR
Extension
with DNA
polymerases
Amplification of larger
fragment with primers 1 and 4
Primer 1
pTet + acdS
Primer 4

The oligonucleotide primers used in the experiments were as follows:

(1) 5'-CCC GAA **AGG TAC** CGC CTC ACG -3'

(2) 5'-CG ATT CAG GTT CAT ACT CGC TGC CTT ACT GCG TTA GC -3'

(3) 5'-GC AGT AAG GCA GCG AGT ATG AAC CTG AAT CGT TTT GAA -3'

(4) 5'-GCA ATC **TCG CAT GC**A TGG CGG-3'

The design of the primers was based on the Tet^r gene promoter region found in plasmid pBR322 (Bolivar et al., 1977) and the sequence of *acdS* from *Enterobacter cloacae* UW4 obtained by Shah et al., (1998). Primers 1 and 2 were used to amplify the promoter region of the Tet^r gene, using linear plasmid pBR322 (Bolivar et al., 1977) as template. Primer 1 was designed to include the *Kpn*I restriction site AGG TAC marked in bold in the above sequence. The first 14 bases of primer 2 are complementary to the first 14 bases of the ACC deaminase gene. To amplify the ACC deaminase structural gene, *acdS*, primers 3 and 4 were used, with pRKACC (carrying the ACC deaminase gene of *Enterobacter cloacae* UW4 cloned in the broad-host-range plasmid pRK415; (Shah et al., 1998) as a template. The first 17 bases of primer 3 are complementary to the first 17 bases of the Tet^r promoter region. Primer 4 was designed to include the *Sph*I restriction site TCG CAT GC marked in bold. The ribosome binding site in the promoter region of the Tet^r gene was modified from CAG GCA CCG TGT to AAG GCA GCG AGT (enclosed in a rectangle in the above sequence)

with the purpose of enhancing the ability of the mRNA to bind to the ribosome and thus enhance translation of the product. To generate the fused product *pTet-acdS*, a third PCR reaction was run using primers 1 and 4 and the gel purified PCR products, *pTet* and *acdS*, as templates.

The PCR reactions designed to generate either the ACC deaminase gene or the *pTet* region were as follows: A final reaction volume of 20 µL included 12.5 µL of sterile double-distilled H_2O, 2 µL of 10X buffer with $MgSO_4$, 2µL of dNTP's (2 mM), 1 µL of each primer (40 pmol/µL), 1 µL of template DNA (1 ng/µL), and 0.5 µL (5 units/µL) of PWO DNA polymerase (Boehringer Mannheim, Germany). Pwo DNA polymerase has 3'-5' exonuclease proofreading activity allowing a 3-fold increase in the fidelity of DNA synthesis compared to *Taq* DNA polymerase. After the reaction mixture was added, the content of each PCR reaction tube was mixed and centrifuged briefly, and 30 µL mineral oil was added on top of the mixture to avoid evaporation. Amplification was started from denaturation at 94°C for 5 min, and then conducted with thirty cycles consisting of 1 min at 94°C, 1 min at 52°C and 1 min at 72°C. Once this was completed, the reaction proceeded at 72°C for 5 min followed by cooling at 4°C.

Once the amplification of *pTet* and *acds* was confirmed by agarose gel electrophoresis, these PCR products were purified from the gel using a DNA extraction kit (section 2.3.6.2) and utilized as DNA templates to generate the fused product *pTet-acds*S. When phenol-purified PCR products were used as templates, the PCR reaction allowed not only amplification of the fused product, *pTet-acds*S but of *acdS* by itself, without the *pTet* region. It is possible that phenol purification did not wash off the primers from the first PCR

reactions, thus allowing amplification of the non-desired product.

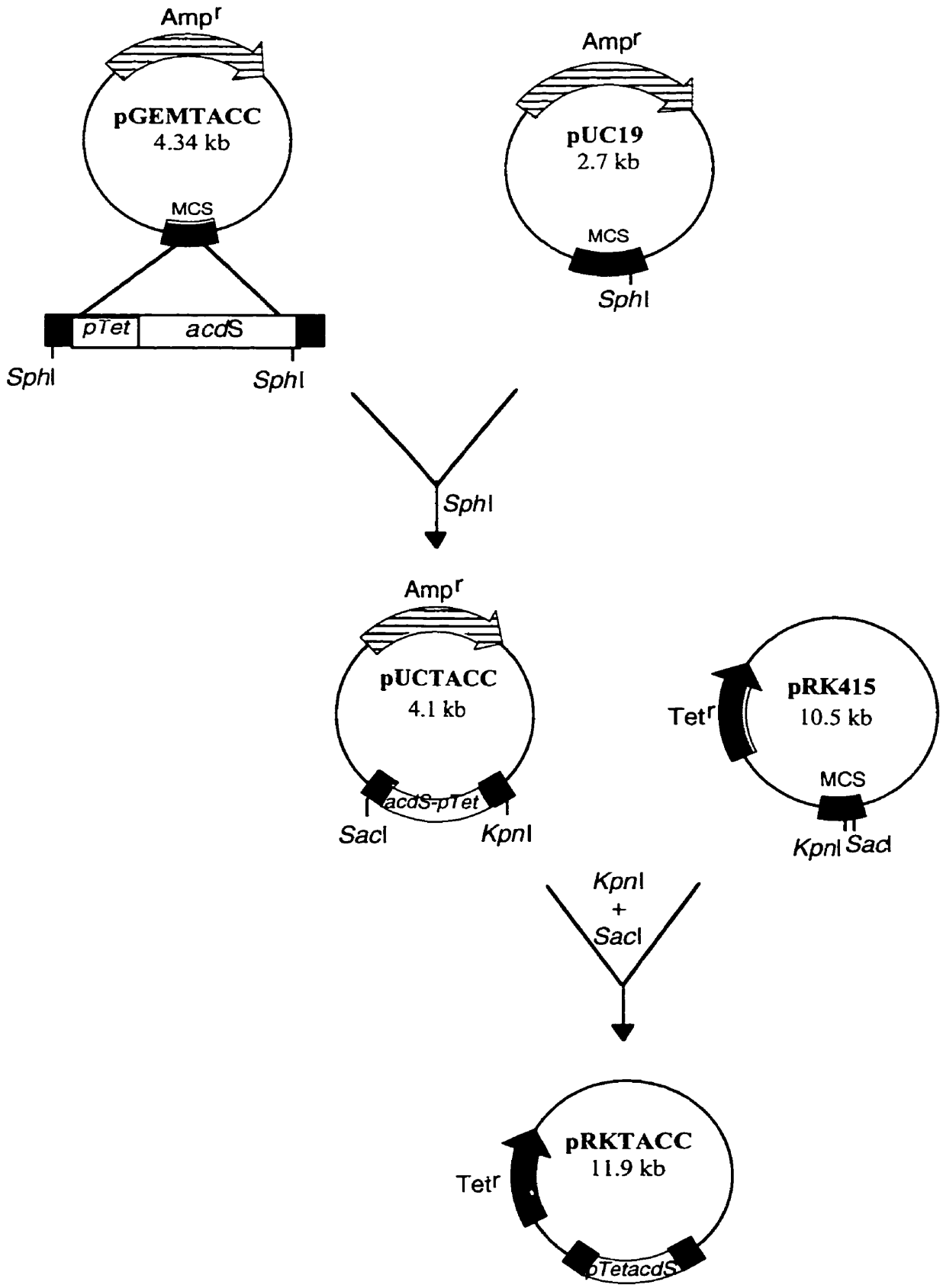
The PCR reaction to generate *pTet-acdS* was as follows: A final reaction volume of 50 µL included 35 µL of sterile double distilled H_2O, 5 µL of 10X concentrated buffer with 17.5 mM $MgCl_2$, 1 µL dNTP's (2mM), 3 µL of primer 1 and 3 µL of primer 4 (40 pmol/µL), 1 µL of the DNA solution containing the PCR product *acdS* (100 ng/µL), 1 µL of the *pTet* PCR product (100 ng/µL), (amplification of the fused product was not accomplished when lower concentrations of the template DNA were used) and 0.75 µL (3.5 units/µL) of Expand™ Long Template PCR system (Boehringer, Mannheim, Germany) composed of an enzyme mix containing thermostable *Taq* and Pwo DNA polymerases). Amplification was started from denaturation at 94°C for 5 min, and then performed with thirty cycles consisting of 1 min at 94°C, 2 min at 52°C and 3 min at 68°C. Once this was completed, the reaction proceeded to 72°C for 7 min followed by cooling to 4°C.

The polymerase chain reaction was performed in a Perkin Elmer Cetus DNA thermal cycler using synthetic oligonucleotides synthesized by MOBIX, McMaster University, Hamilton, ON as primers. In designing oligonucleotide primers an attempt was made to minimize the possible extent of intra- and inter- primer homology. The following web site was used: http://www.biotech.iastate.edu/Facilities/DSSF/primer_design.html)

2.5.2 Cloning of the fused PCR product *pTet-acdS* in pRK415

The PCR product reaction containing the ACC deaminase gene fused to the Tet^r promoter, *pTet-acdS*, was gel purified and ligated to the vector pGEM®-T (Fig. 4) (Promega, Madison, Wis., USA) according to the

Fig. 4. Cloning of *pTet-acdS* in pRK415. pGEMTACC represents *pTet-acdS* cloned in pGEM®-T, while pUCTACC and pRKTACC stand for *pTet-acdS* cloned in pUC19 and in pRK415, respectively.


Amp^r
pGEMTACC
4.34 kb
MCS
pTet
acdS
SphI
SphI
Amp^r
pUC19
2.7 kb
MCS
SphI
SphI
Amp^r
pUCTACC
4.1 kb
acdS-pTet
SacI
KpnI
Tet^r
pRK415
10.5 kb
MCS
KpnI SacI
KpnI
+
SacI
Tet^r
pRKTACC
11.9 kb
pTetacdS

manufacturer's specifications. The pGEM-T vector is a convenient system for the cloning of PCR products. It contains 3′ terminal thymidine overhangs at the insertion site that improve the efficiency of ligation of a PCR product into the plasmids by preventing recircularization of the vector and providing a compatible overhang for PCR products generated by certain thermostable polymerases like *Taq*. The *Taq* polymerase usually adds a single adenosine to the 3′ ends of PCR products (Mezei and Storts, 1994). The Expand™ Long Template PCR System employed for the generation of the fused fragment *pTet-acdS*, is composed of a mix of *Taq* and Pwo DNA polymerases, and thus generates 3′ single Adenosine overhang products mixed with blunt-end fragments. Ligation of *pTet-acdS* with the pGEM-T vector was successful using the PCR product purified either from the gel or purified by phenol-chloroform. However, it was observed that ligation was more effective when using the gel-purified PCR product. Successful ligation was obtained when the molar ratio of insert DNA to vector DNA was kept at a three or five to one ratio.

Due to incompatibility of restriction sites between the vector pGEM®-T and pRK415, *pTet-acdS* had to be cloned first to pUC19: *pTet-acdS* was excised from plasmid pGEM-T by digestion with *Sph*I (Fig. 4) and ligated with T4 DNA ligase as specified by the manufacturer (Boehringer, Mannheim, Germany) to pUC19 digested also with *Sph*I. *pTet-acdS* was then excised from pUC19 by double digestion with *Sac*I and *Kpn*I and ligated to pRK415 digested with the same restriction enzymes. The presence and size of the insert was determined by restriction enzyme digestion and agarose gel electrophoresis.

Before ligation all digestion products were purified with phenol-chloroform. The ligation mixture was used directly for transformation of *E. coli*

DH5α without inactivating T4 DNA ligase. The plasmid carrying *pTet-acdS* in pRK415 was named pRKTACC and transferred from *Escherichia coli* DH5α into *A. brasilense* Cd by triparental mating (section 2.3.5.).

2.6. DNA sequencing

Preparation of DNA for sequencing was according to the instructions from the MOBIX Central Facility, McMaster University, Hamilton, Ont., Canada. Plasmid DNA was isolated by the alkaline lysis miniprep method (section 2.3.3.). The isolated plasmid DNA was further purified using phenol-chloroform (section 2.3.6.1.). With these preparations it is ensured that no salt or organic compounds are in the final product. Removal of salt and organic compounds is facilitated by a number of washes with 70% ethanol and the final DNA pellet is resuspended in water as opposed to TE buffer. The concentration of the DNA used for sequencing was kept higher than 200 $ng \cdot \mu L^{-1}$, and sequenced with primers that anneal to the T7 or SP6 RNA polymerase promoters present in the pGEM-T vector which flank a multiple cloning site within the α-peptide coding region of the enzyme β-galactosidase.

DNA sequencing was performed at the MOBIX Central Facility, McMaster University, Hamilton, Ont., Canada. Sequencing reactions utilized the modified Taq-FS enzyme from Perkin-Elmer with fluorescently labeled dideoxy-terminator chemistry (Prober et al., 1987). The sequencer, an Applied Biosystems 373A Stretch, was used for running the gel and detecting the fluorescent bands. Sequence data was analyzed using the Macintosh program DNA Strider v. 1.2 (Marck, 1988).

2.7. Computer analysis of DNA promoter sequences

The upstream region of the gene encoding the regulatory protein AcdR that regulates transcription of the ACC deaminase gene, *acdS*, in *Enterobacter cloacae* UW4, was compared to promoter sequences in the *Azospirillum*'s genome by using the program LALIGN (Huang and Miller, 1991). This program finds the best local aligments between two sequences. To compare the upstream region of *acdR* with DNA sequences from prokaryotic and eukaryotic organisms, an alignment analysis was performed using BLAST (Stephen et al., 1997).

2.8. ACC deaminase assay

The *Enterobacter cloacae* strains, CAL2 and UW4, non-transformed *A. brasilense* Cd and Sp245 (in both log and stationary phases, *Escherichia coli* DH5α, and the *Azospirillum* strains transformed with the plasmids pRKACC (Shah et al., 1998), pRKLACC (carrying *acdS* under the control of the *lac* promoter cloned in pRK415), and pRKTACC (carrying *acdS* under the control of the Tet^r promoter cloned in pRK415), were grown in rich medium (tryptic soy broth medium, TSB, for *Enterobacter cloacae*, Luria Broth for *Escherichia coli*, and Nutrient Broth for the *Azospirillum* strains) for 18 hours. In the case of cells transformed with pRKLACC IPTG (0.8 mM) was added to the cultures to determine the requirement of this compound for induction of the *lac* promoter. The cultures of non-transformed cells were then harvested by centrifugation, washed with 0.1 M Tris-HCl (pH 7.5) and incubated for another 18 hours in minimal medium containing ACC (3 mM) as the sole source of nitrogen. The bacterial cells (transformed and non-transformed) were again collected by

centrifugation and resuspended in 0.1 M Tris-HCl (pH 8.5). To prepare the cell lysates the bacteria were broken either by sonication with a Branson Sonifier Cell Disruptor, (model 200, Branson Sonic Power Company, Danbury, CT, USA), or vortexed vigorously with 5% (v/v) toluene. The lysate was assayed for ACC deaminase activity as described by Honma and Shimomura (1978), by measuring the amount of α-ketobutyrate produced by the enzyme. α-ketobutyrate is derivatized to a phenylhydrazone by the reagent 2,4-dinitrophenylhydrazine (Sigma Chemical Co., St. Louis MO, USA), followed by the addition of NaOH, which develops the phenylhydrazone into a yellow colour. The concentration of α-ketobutyrate in the mixture is determined by measuring the absorbance at 540 nm and comparing the values to a standard curve of α-ketobutyrate.

The protein concentration in the cell lysates was determined by the method of Bradford (1976).

2.9. IAA assay

IAA was detected colorimetrically using Salkowski's reagent (Gordon and Weber, 1951) in the supernatants of *A. brasilense* Cd, *A. brasilense* Cd/pRKLACC, and Cd/pRKTACC cultures, grown at 30°C in nutrient broth or OAB minimal medium with or without 0.5 g/L tryptophan, and 20 mg/mL tetracycline in case of the transformants. The latter assay, which depends on the development of a red colour in a $FeCl_3$-acid solution, is reasonably sensitive and specific for IAA (Glickmann and Dessaux, 1995). The cultures were centrifuged, 1 mL of the supernatant removed to a small glass test tube, followed by 4 mL of Salkowski's reagent (add 150 mL of concentrated H_2SO_4 to

250 mL of double-distilled water, keeping the container on ice; add 7.5 mL of 0.5 M $FeCl_3$). The sample was vortexed briefly and incubated at room temperature for 20 minutes. The concentration of IAA was determined by monitoring the absorbance at 535 nm and comparing the values to a standard curve of indole-3-acetic acid ranging from 1-40 μg/mL.

2.10. Gnotobiotic root elongation assay

The effect of various bacterial strains on the elongation of plant seedling roots was determined as described by Lifshitz et al. (1987). Canola seeds (*Brassica campestris* cv. Reward), tomato seeds (*Lycopersicum esculentum* Mill. cv. Heinz 1439 VF, and Hard red spring wheat seeds (*Triticum aestivum* cv. Quantum), provided by Stokes Seeds Ltd. (St. Catharines, ON, Canada), stored at 4°C, were surface disinfected by immersion in 1% sodium hypochlorite for 5 minutes, and then thoroughly rinsed with sterile distilled water immediately prior to use. Once cleaned, equal numbers of seeds were incubated for 2-3 hours at 30°C with 10 mL of either 0.03 M $MgSO_4$ or a bacterial suspension in 0.03 M $MgSO_4$ prepared by growing the bacteria overnight in their respective growth medium, washing the cells twice with 0.03 M $MgSO_4$, resuspending them in the same solution and adjusting the concentration of cells as required by diluting the bacterial suspension in 0.03 M $MgSO_4$ prior to incubation with the seeds. Inoculum levels ranging from 10^7-10^9 cfu/mL were used since plants treated with lower levels did not respond to *A. brasilense* Cd or *A. brasilense* Cd/pRKLACC or Cd/pRKTACC inoculation.

Eight seeds were placed in each seed-pack growth pouch (125 by 157 mm; Mega International, MN, USA) that had been filled with 10 mL of distilled water

and autoclaved before the addition of the seeds. Ten pouches were used for each treatment. The pouches were incubated upright in a plastic tray partially filled with enough water to cover the bottom. The tray was covered with Saran Wrap™ and aluminum foil. After seed germination the aluminum foil was removed. All plants were grown in a growth chamber at a temperature of 25°C, with a light intensity of 130 μmol/m^2/s for 14 h and 10 h of darkness for five (wheat and canola) or six days (tomato).

For dry weight measurements, each plant was dried separately in an oven at 70°C for 24 h. Water content in plants was calculated by subtracting fresh weight from dry weight.

2.11. Pot-grown plants

Tomato seeds were treated with bacteria as described in section 2.10. and grown in Pro-Mix 'BX' general-purpose growth medium (Premier Horticulture, Ltd, Rivière du Loup, Canada) or vermiculite (Vil Vermiculite, Inc., Montreal, Canada) in a growth chamber at 25 or 26°C with an average day time light illumination of 130 μmol m^{-2} s^{-1} for 14 h and 10 h of darkness. Plants were germinated in 60 x 5.5 cm boxes and transferred to 1 L pots (15 cm high) after two weeks of growth. Plants grown in Pro-Mix soil were watered only with tap water, while plants grown in vermiculite were watered with tap water and every ten days with 1/2 strength Hoagland's solution (Hoagland and Arnon, 1938). For 1 L of full Hoagland's solution prepare 1 M solutions of $NH_4H_2PO_4$, KNO_3, $Ca(NO_3)_2$, and $MgSO_4$, and add 1, 6, 4, and 1 mL of each solution, respectively. Add 1 mL of the microelements solution and 1 mL of a 0.5%

(w/v) iron sulfate solution. Microelements solution (g/L): H_3BO_3, 2.86; $MnCl_2 \cdot 4H_2O$, 1.81; $ZnSO_4 \cdot 7H_2O$, 0.22; $CuSO_4 \cdot 5H_2O$, 0.08; $H_2MoO_4 \cdot H_2O$, 0.09.

2.11.1. Inhibition of plant growth caused by inoculation with high inoculum levels of *Azospirillum*

Tomato seeds were disinfected as described in section 2.10. and imbibed for two hours in a suspension of *A. brasilense* Cd or *A. brasilense* Cd/pRKLACC cells containing 10^8 cfu/mL. The seedlings were grown for two weeks at 25°C in a growth chamber under the conditions specified in section 2.11.

2.11.2. Flooding experiments

Plants grown for 5 weeks in pots with vermiculite were transferred to pots containing Pro-Mix 'BX', and inoculated with 60 mL of a bacterial suspension containing 10^8 cfu/mL. After three days the pots holding the plants were covered by a plastic bag and put inside another pot of equal dimensions. The plants were flooded with distilled water up to the internode level and incubated at 26°C for 4 days under the conditions described in section 2.11. The level of flooding was maintained throughout the experiment by adding distilled water as required.

2.11.2.1. Epinasty

Epinasty was calculated by determining the change of dimensions in the angle enclosed between the petiole and the main stem of the plant. The sketches were first drawn on paper held close to the plants, taking care not to

damage them. Afterwards, using a ruler, two lines were drawn: one perpendicular to the main stem and another one from the base of the petiole to the base of the leaf. The angle between the latter line and the perpendicular line was then measured with a protractor. A total of six angles were measured for each plant. The measurements were then pooled together to calculate the average.

2.11.2.2 Chlorophyll determinations

Tomato leaves were cut in pieces of approximately 3 x 3 cm, weighed, transferred into plastic tubes containing 7 mL of dimethyl sulphoxide, and incubated at 65°C until the leaves looked bleached. After adjusting the volume of the solution to 10 mL with dimethyl sulphoxide, the samples were read in a spectrophotometer at 645 and 663 nm. If the absorbance values were greater than 0.7, the extract was diluted 50% with dimethyl sulphoxide.

2.12. Attachment of *Azospirillum* to mangrove roots.

A. halopraeferens AU10 and *A. brasilense* Cd are known for their high salt tolerance (3% and 2% salt, respectively) (Hartmann et al., 1991). Since tropical seawater contains approximately 3.3% salt, it was hypothesized that the ability of these two salt tolerant strains to associate with mangrove roots was highly plausible.

Black mangrove (*Avicennia germinans* (L.) Stern) propagules (dispersal vegetative units) collected from a mangrove ecosystem in Baja California Sur, Mexico (Holguin et al., 1992), were axenically grown in glass beakers containing sand, at 22 ± 1°C in a growth chamber at a light intensity of 50 μmol m^{-2} s^{-1} at 12-h light:dark photoperiods. Each beaker was supplemented with the

mineral salt solution plus vitamins of the Murashige and Skoog (1962) medium containing 25 g sodium chloride/L and the following: (mg/L): NH_4NO_3, 1650; KNO_3, 1900; KH_2PO_4, 170; $MgSO_4 \cdot H_2O$, 370; $CaCl_2 \cdot 2H_2O$, 440; $FeSO_4 \cdot 7H_2O$, 27.8; Na-EDTA, 37.3; H_3BO_3, 1.55; $MnSO_4 \cdot H_2O$, 4.22; $ZnSO_4 \cdot 7H_2O$, 2.15; KI, 2; $NaMoO_4 \cdot 2H_2O$, 0.0735; $CuSO_4 \cdot 5H_2O$; 0.125; $CoCl_2 \cdot 6H_2O$, 0.125; glycine, 0.02; myoinositol, 1; nicotinic acid, 0.005; pyridoxine monochloride, 0.005; thiamine hydrochloride, 0.001. After one month the plants were transferred to flasks containing seawater and *Azospirillum brasilense* Cd or *A. halopraeferens* AU10 at a concentration of 10^6 cfu/mL and grown for eight days under the conditions specified above.

2.12.1. Scanning electron microscopy

Samples of root tissue were excised from test plants for eight consecutive days and prepared for SEM observation as follows. The roots were fixed with 2.5% glutaraldehyde (Sigma Chemical Co., St. Louis, MO, USA) in 0.2 M cacodylate buffer, pH 7.2, for 2 h at 28°C under a vacuum of 760 mm Hg. The samples were rinsed once in the same buffer. Then, the roots were dehydrated by passage through increasing concentrations of ethanol in water. The final wash was in 100% acetone. The samples were dried in a critical point dryer (Denton, DCP-1, Cherry Hill, NJ, USA) in a CO_2 atmosphere. The dried samples were affixed to stubs with conductive, self-sticking adhesive tabs and coated with 30 nm gold film (Polarum, Watford, UK) before being examined by SEM (Hitachi S-570, Japan) at 60 kV.

2.13. Adhesion of *Azospirillum* to tomato leaves

Nutrient-poor soil (3% organic matter; 0.05% total nitrogen) from BCS, Mexico (Bashan et al., 1999) was mixed with vermiculite in a ratio of 1:1 (v/v), autoclaved three times for 30 min each time to eliminate the spores of any microorganisms, saturated with sterile distilled water, poured into 300 mL Erlenmeyer flasks and used as a growth medium for aseptic tomato seeds. The flasks containing the plants were covered with a double layer of parafilm (replaced every three days to allow air exchange) and put in a growth chamber under the conditions specified in section 2.11 at 26°C. Once the tomato seedlings reached the second leaf stage, the leaves were inoculated with 100 µL of a bacterial suspension using a micropipette and the plants were incubated for another 120 hours. To prepare the bacterial suspension *A. brasilense* Cd, *A. brasilense* Cd/pRKLACC, *A. brasilense* Cd/pRKTACC, *A. brasilense* Sp245/pRKLACC, and *Pseudomonas putida* AC8 cells were grown overnight in LB medium, washed twice in a phosphate buffer, pH 6.8, 0.05 M, supplemented with 1.92 mM NaCl, 20 mM fructose and 18.7 mM NH_4Cl, and resuspended in the same buffer solution at a concentration of 10^7 cfu/mL. According to Bashan and Holguin (1993) these conditions allow the bacteria to produce abundant amounts of fibrillar material required for attachment.

Half of the leaves were used for examination by scanning electron microscopy, and the other half for counting the number of cells attached to the leaves by the dilution plate-count method.

2.13.1. Scanning electron microscopy

After 24, 48, and 96 h of incubating the plants, the leaves were cut, rinsed and vortexed in a phosphate buffer, pH 6.8, 0.05 M, and fixed in glutaraldehyde (2.5%) in the same buffer. The leaves were dehydrated by passage through increasing concentrations of ethanol in water and prepared for examination by SEM as described in section 2.12.1.

2.13.2. Bacterial counts on leaves and roots of tomato plants.

For counting the number of cells attached to leaves or roots, the sample was rinsed twice with 0.03 M $MgSO_4$, vortexed, and sonicated for three minutes using light sonication (Branson Sonifier Cell Disruptor, (model 200, Branson Sonic Power Company, Danbury CT, USA). The sample was then serially diluted and counted by the plate-count method on solid rich medium supplemented, when necessary, with the appropriate antibiotic.

2.14. Survival of *Azospirillum* in soil

Pro-Mix 'BX' soil spread evenly in Petri dishes, was saturated with sterile distilled water and inoculated with 12 mL of a bacterial suspension prepared as described in section 2.10., adjusting the concentration of cells to 10^7 cfu/mL. The petri dishes were incubated in the growth chamber under the conditions specified in section 2.11. at 26°C for a period of 1.5 months. The soil was kept moist throughout the experiment by adding sterile distilled water when required. The survival of bacteria in soil was determined by bacterial counts using the plate-count method.

2.15. Biocontrol properties of *A. brasilense* Cd/pRKTACC

The fungal pathogens *Phythium aphanidermatum* and *Fusarium oxysporum* f sp. *lycopersici* were grown in PDA medium for 2 and 7 days at 28°C and 24°C, respectively. The bacterial pathogen *Pseudomonas syringae* pv. *tomato* was grown overnight in King's B medium (King et al., 1954) at 28°C under shaking conditions, washed twice with 0.85% NaCl, and resuspended in the same solution adjusting the concentration of the cells to 10^7 cfu/mL.

Azospirillum brasilense Cd and Cd/pRKTACC were grown for 18 hours in TSB medium at 30°C, washed twice in a 0.05 M phosphate buffer (pH 6.8) supplemented with NH_4Cl_2 (18.7 mM), NaCl (2 mM) and fructose (20 mM), and resuspended in the same buffer adjusting the concentration of cells to 10^7 cfu/mL.

2.15.1. Biocontrol properties of *A. brasilense* Cd/pRKTACC when inoculated on the surface of tomato leaves

Leaves of five weeks-old tomato plants (*Lycopersicon esculentum* Mill. Cv. FND 902) were cut and placed in petri dishes containing filter paper (Whatman #1) saturated with sterile distilled water. The leaf surface was spread with the solution of *A. brasilense* Cd or *A. brasilense* Cd/pRKTACC with a cotton swab imbibed in the bacterial solution. The leaves were incubated for 3 days in a growth chamber at 28°C during the day and 21°C during the night. After the incubation period the leaves were infected with the fungal pathogens by placing on the leaf a plug of the pathogen. The leaves were inoculated with *P. syringae* pv. *tomato* by syringe pressure (volume: 1 mL). After incubation for 2 days the lesions in the leaves were analyzed.

2.15.1. Biocontrol *properties of A. brasilense Cd/pRKTACC* when inoculated on the roots of tomato seedlings

Tomato seeds (*Lycopersicum esculentum* Mill. cv. Heinz 1439 VF), treated as described in section 2.10. were imbibed for one hour in a bacterial suspension of *A. brasilense* Cd or *A. brasilense* Cd/pRKTACC (at 10^7 cfu/mL), and incubated on solid agar (2%, w/v) at 28°C. After germination (three days) the seedlings were suspended for 40 minutes in a solution prepared by blending an agar plug of the pathogen with 50 mL of sterile distilled water. The seedlings were then sown in 60 x 5.5 cm boxes and grown in the greenhouse for three weeks.

3. Results

3.1. Isolation of leaf and flower colonizing bacteria with ACC deaminase activity

The strategy for isolation of leaf and flower colonizing bacteria with ACC deaminase activity consisted in inoculating the petals or leaves in PAF rich media followed by transfer of the culture to DF minimal medium supplemented with 3.0 mM ACC as the only source of nitrogen. Six bacterial isolates were obtained; although assaying for ACC deaminase activity showed that only two of the six isolates had any appreciable activity; One of the isolates was obtained from the petals of the wild flower *Hyerpicum perforatum,* growing in the area around Columbia Lake in Waterloo, Ontario, while the other came from the leaves of the wild flower *Arctium minus* growing in the Laurel Creek Conservation Area in Waterloo, Ontario. Fatty acid analysis of the strain isolated from *Hypericum perforatum* identified it as belonging to the genus *Pseudomonas putida,* with a similarity index of 0.874 (a similarity index higher than 0.5 is considered a good match according to Microbial ID, INC, the company that identified the strains), and was thus named *P. putida* HP6. Fatty acid analysis of the strain isolated from *Arctium minus* was also identified as belonging to the genus *P. putida* with a similarity index of 0.910, and was thus named *P. putida* AC8. Observation by light microscopy of *Pseudomonas putida* HP6 growing on solid DF medium supplemented with NH_4Cl showed that the bacteria are motile rods, 1-2 μm long, < 1.0 μm in diameter, and the cells are found in pairs. *Pseudomonas putida* AC8 cells are also motile rods, 1.5-3 μm long and ~1.0 μm in diameter. Some of the cells associate in long strands, up to 10 μm in length. The

ACC deaminase activity of both strains was similar to *Enterobacter cloacae* UW4 (~20 μmoles of α-ketobutyrate/mg of protein/h).

3.2. ACC deaminase activity in non-transformed *A. brasilense* Cd and Sp245

Non-transformed *A. brasilense* Cd and Sp245 cells, cultured in nutrient broth or OAB minimal medium, showed no detectable ACC deaminase activity (Table 1) in neither the log nor stationary phase.

3.3. Selection of a broad-host-range plasmid for transfer of the ACC deaminase gene into *A. brasilense* Cd

Two different broad host range plasmids, pGSS15 and pRK415, were introduced into *A. brasilense* Cd by conjugation. *A. brasilense* Cd/pGSS15 transformants showed a significant decrease in growth rate on nutrient broth as compared to either *A. brasilense* Cd/pRK415 transformants, or non-transformed *A. brasilense* Cd (Fig. 5). This notwithstanding, all three strains eventually attained the same final cell density. *A. brasilense* Cd/pGSS15 transformants displayed an exceptionally long lag phase (typically around three days) and often decreased viability in culture. The observed differences in cell growth probably reflect a debilitation in cell function after transformation with pGSS15 but not after transformation with pRK415 (Glick, 1995b). For this reason plasmid pRK415 was chosen over pGSS15 for transformation of *A. brasilense* Cd.

3.4. Expression of the ACC deaminase gene in *A. brasilense*

Escherichia coli strains harboring pRKACC showed high ACC deaminase activity, comparable to that in *Enterobacter cloacae* UW4 from which the ACC deaminase gene was isolated (Table 1). However, when pRKACC (for a description see section 2.3.5) was transferred to *A. brasilense* Cd and Sp245, the *A.*

Fig. 5. Growth of *A. brasilense* Cd cells, either non-transformed or transformed with pRK415 or pGSS15, on nutrient broth.

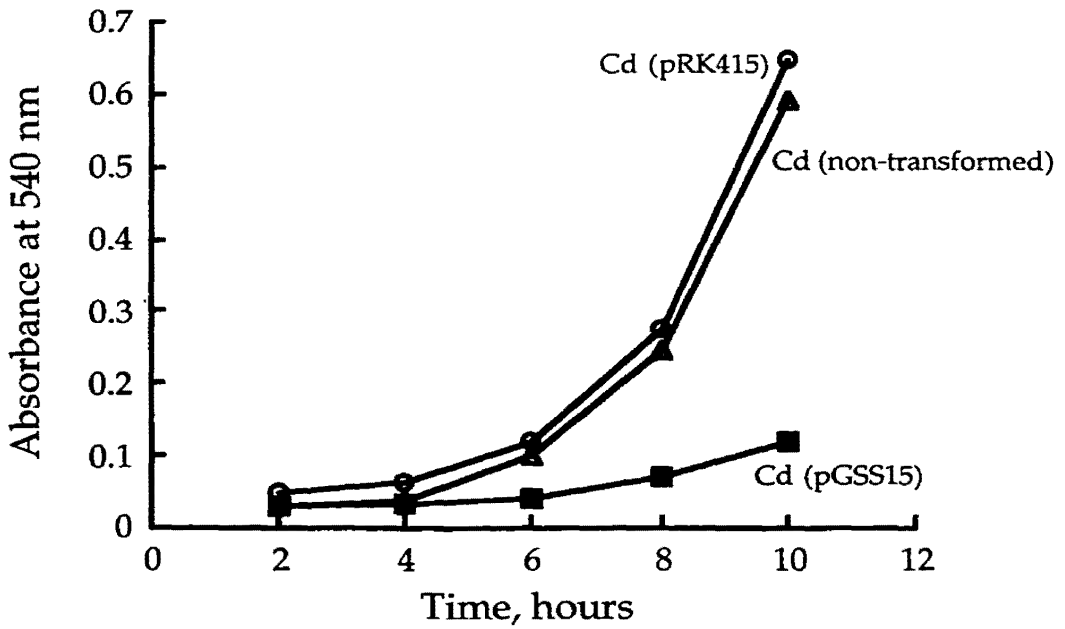

0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Absorbance at 540 nm
Cd (pRK415)
Cd (non-transformed)
Cd (pGSS15)
0
2
4
6
8
10
12
Time, hours

Table 1. ACC deaminase activity (expressed as μmoles α-ketobutyrate/mg protein/h) in different *A. brasilense* strains compared to *Enterobacter cloacae* UW4 and *Escherichia coli* pRKACC.

ACC deaminase was induced by resuspending late log phase cells in minimal medium (OAB for the *A. brasilense* strains and M9 for *Enterobacter cloacae* and *Escherichia coli*) supplemented with 3 mM ACC. The cell lysate was prepared by breaking the cells by sonication as described in Methods. The limit of detection of the ACC deaminase assay is $\geq$ 0.5 nmoles of α-ketobutyrate in a sample. Numbers followed by different letters denote significant statistical difference at $P \leq 0.05$ (Student's t-test).

Enterobacter cloacae UW4	*Escherichia coli* DH5α (pRKACC)	*Escherichia coli* DH5α	*A. brasilense* Cd	*A. brasilense* Sp 245	*A. brasilense* Cd (pRKACC)	*A. brasilense* Sp245 (pRKACC)
2.43±0.04 a	1.74±0.06 b	0	0	0	0	0

brasilense transformants did not have any measurable ACC deaminase activity (Table 1). To test for the presence of an inhibitor of ACC deaminase activity, the cell lysate of *A. brasilense*, Cd was concentrated in a SpeedVac Concentrator (Savant Instruments, Hicksville, N.Y) and added separately to a lysate prepared from the cells of *Enterobacter cloacae* CAL2, previously shown to have a high level of ACC deaminase activity. The addition of various amounts of *A. brasilense* lysate to the CAL2 lysate did not alter the ACC deaminase activity of CAL2 (Table 2).

3.5. Computer analysis of DNA promoter sequences

The ACC deaminase gene from *E. cloacae* is subjected to a complex system of regulation which involves the regulatory protein AcdR and the participation of at least two promoter regions (Grichko and Glick, 2000) (Fig. 6). Inferring that *A. brasilense* might be incapable of recognizing the *acdR* promoter, an analysis of this region was performed looking for similarity to other promoter sequences present in prokaryotes or in the *Azospirillum* genome. An alignment search for the upstream region of the regulatory protein AcdR performed with BLAST gave no significant similarity to other prokaryotic promoter sequences present in the databases. However, two DNA fragments rich in adenine showed significant similarity to other adenine rich sequences present in the eukaryotic genomes of *Caenorhabditis elegans* and *Homo sapiens* (Fig. 7). Comparison with LALIGN of the *acdR* upstream region to promoter sequences in the *Azospirillum*'s genome gave no significant similarity between any of the sequences.

3.6. Cloning of the ACC deaminase gene under the control of the *lac* promoter

To overcome the apparent lack of expression of ACC deaminase in the

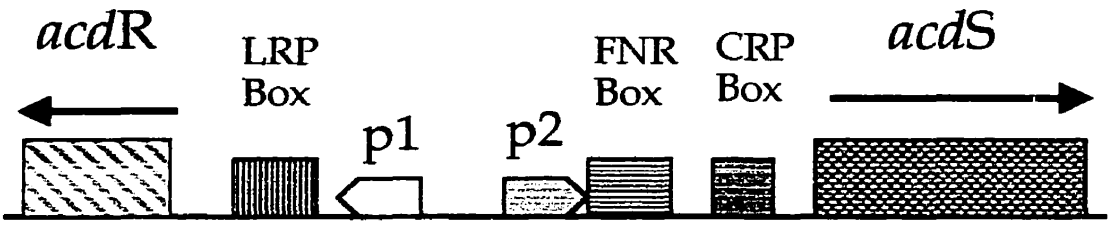
Table 2. Effect of a lysate from *Azospirillum brasilense* Cd pRKACC cells on the ACC deaminase activity of *Enterobacter cloacae* CAL2. ACC deaminase activity is expressed as μmoles of α-ketobutyrate/mg protein/h. The cell lysates were prepared by breaking down the cells with sonication as described in Methods. Numbers followed by different letters denote statistically significant differences at $P \leq 0.05$ in one way ANOVA.

* Protein concentration = 3.14 mg/mL

** Protein concentration = 8.72 mg/mL

Enterobacter cloacae CAL2	*Enterobacter cloacae* CAL2 * + *A. brasilense* Cd/pRKACC lysate** 9 : 1	*Enterobacter cloacae* CAL2 + *A. brasilense* Cd/pRKACC lysate 2 : 3	*Enterobacter cloacae* CAL2 + H_2O 2 : 3
4.13±0.08 b	4.11±0.07 b	2.58±0.08 a	2.53±0.05 a

Fig. 6. Model of regulation of the ACC deaminase gene in *Enterobacter cloacae* UW4 according to Grichko and Glick (2000).



*acd*R: LRP-like ACC deaminase regulatory protein

*acd*S: ACC deaminase structural gene

LRP Box: Possible binding site for an LRP protein

FNR Box: Possible binding site for an FNR (fumarate and nitrate reduction) protein

CRP Box: Possible binding site for a cAMP receptor protein

Fig. 7. Results of an alignment search performed with BLAST of the *acdR* upstream region. $_{p}$*acdR* stands for the promoter region of *acdR* from *Enterobacter cloacae* UW4

```
pacdR     aaaaacaaaacactaacgttatt
C.E.1     aaaaacaaaacacaaacgttatt
C.E.2     aaaaacaaaacacaaacgttatt
H.S.1    taaaaacaaaacactaa-------
H.S. 2   taaaaacaaaacactaac------
T.B.     taaaaacaaaacactaa-------
**************************************************

pacdR      ttaagaaaaaacctgaaaattt
C.E.3      ttaagaaaaaacctgaaaa---
C.E.4            aaaaaacctgaaaa---
C.E.5           gaaaaaacctgaaaattt
C.E.6            aaaaaacctgaaaattt
H.S.3          agaaaaaacctgaaaatt-
```

Abbreviations:

$_p$*acdR*: sequence in the promoter region of *acdR* from *E. cloacae* UW4.
C.E. 1: *Caenorhabditis elegans* cosmid F42G8.
C.E. 2: *C. elegans* cosmid B0218
C.E. 3: *C. elegans* cosmid F58A6
C.E. 4: *C. elegans* cosmid K07A9.
C.E. 5: *C. elegans* cosmid BE10
C.E. 6: *C. elegans* cosmid F07G6
H.S. 1: *Homo sapiens* clone DJ1178G13
H.S. 2: *H. sapiens* mRNA
H.S. 3: *H. sapiens* chromosome 21q22.2
T.B.: *Trypanosoma brucei* RNA gene

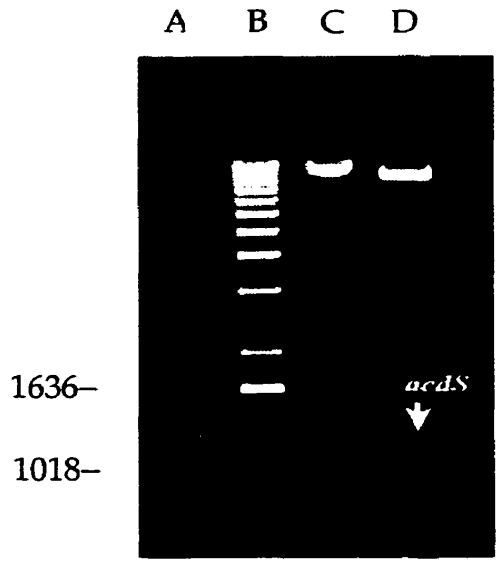
Azospirillum transformants we decided to put *acdS* under the control of the *lac* promoter (Fig. 2). We inferred that in *A. brasilense* the *lac* promoter would not be regulated by lactose as it is in *Escherichia coli* since *A. brasilense* does not grow on lactose (Tarrand et al., 1978) and therefore *A. brasilense* is unlikely to encode a *lac* repressor protein.

The ACC deaminase structural gene was cloned into the multiple cloning site of pUC19 downstream from the *lac* promoter (Fig. 2). The *Escherichia coli* DH5α cells transformed with the resulting plasmid pUCLACC, showed ACC deaminase activity in the presence of the inducer isopropylthio-ß-D-thiogalactoside, IPTG, demonstrating that *acdS* could be expressed under the control of the *lac* promoter. The gene *acdS*, was then sub-cloned into pRK415 under the control of the *lac* promoter and the *Escherichia coli* DH5α cells were transformed with the resulting plasmid pRKLACC. After verifying that the *Escherichia coli* DH5α/pRKLACC transformants had ACC deaminase activity, pRKLACC was transferred from *Escherichia coli* DH5α to *A. brasilense* Cd and *A. brasilense* Sp245 by conjugation and the transconjugants analyzed by restriction endonuclease digestion and gel electrophoresis (Fig. 8).

3.7. ACC deaminase activity of *Azospirillum brasilense* Cd and Sp245 transformed with pRKLACC

The *A. brasilense* transconjugants, *A. brasilense* Cd/pRKLACC and *A. brasilense* Sp245/pRKLACC, showed high ACC deaminase activity similar to that observed in *Enterobacter cloacae* UW4 (Fig. 9). No significant difference was found between the ACC deaminase activity of *A. brasilense* Cd/pRKLACC and that of *A. brasilense* Sp245/pRKLACC. As expected, ACC deaminase expression in *A.*

Fig. 8 DNA gel electrophoresis of plasmid DNA isolated from *A. brasilense* Cd/pRKLACC transformants. Lane D, which corresponds to *A. brasilense* Cd/pRKLACC digested with *Sac*I and *Hind*III, shows a band of approximately 1200 bp (marked with an arrow) located between 1636 and 1018 bp that corresponds to *acdS*.



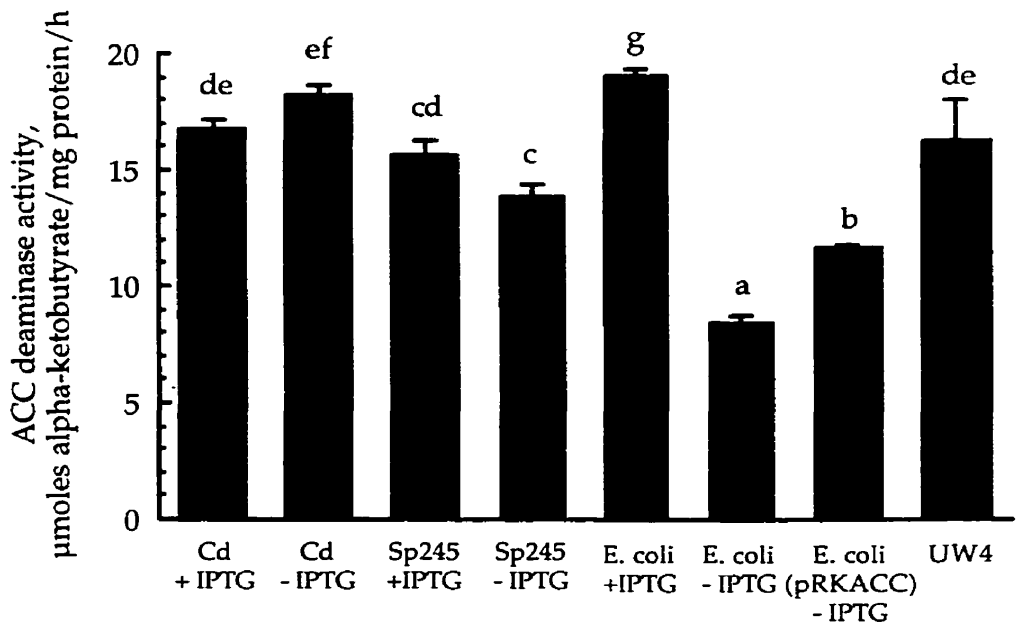
A. Non-transformed *A. brasilense* Cd digested with *Sac*I and *Hind*III.

B. DNA molecular weight marker

C. *A. brasilense* Cd/pRKLACC digested with *Sac*I

D. *A. brasilense* Cd/pRKLACC digested with *Sac*I and *Hind*III.

Fig. 9. ACC deaminase activity in *A. brasilense* Cd, *A. brasilense* Sp245 and *Escherichia coli* DH5α transformed with pRKLACC. *Escherichia coli* DH5α/pRKACC carries *acd*S under the control of its native promoter. The ACC deaminase activity of *Enterobacter cloacae* UW4 is given for comparative purposes. Columns marked with different letters differ significantly at $P \leq 0.05$ in a one way ANOVA.


ACC deaminase activity,
µmoles alpha-ketobutyrate/mg protein/h
20
15
10
5
0
de
ef
cd
c
g
a
b
de
Cd
+ IPTG
Cd
- IPTG
Sp245
+IPTG
Sp245
- IPTG
E. coli
+IPTG
E. coli
- IPTG
E. coli
(pRKACC)
- IPTG
UW4

brasilense transformants was not regulated by IPTG in contrast to the regulated expression of this enzyme in the *Escherichia coli* transformants.

3.8. IAA synthesis in *A. brasilense* Cd/pRKLACC transformants

Cultures of *A. brasilense* Cd/pRKLACC in OAB minimal medium supplemented with tryptophan showed a significant reduction in IAA concentration compared to cultures of non-transformed *A. brasilense* Cd (50 µg/mL against 70 µg/mL) (Fig. 10B). Similarly, the cell density in cultures of *A. brasilense* Cd/pRKLACC was significantly lower compared to cultures of *A. brasilense* Cd (absorbance of 1.4 against 1.7) (Fig. 10A). However, for cultures grown in nutrient broth containing tryptophan, no significant difference in IAA concentration or cell density was found between *A. brasilense* Cd/pRKLACC or *A. brasilense* Cd (Figs. 10C, 10D). IAA was not detected in cultures of *A. brasilense* Cd or *A. brasilense* Cd/pRKLACC grown in OAB medium without tryptophan.

3.9. Elongation of canola seedling roots following inoculation with *A. brasilense* Cd/pRKLACC.

Roots of canola seedlings inoculated with *A. brasilense* Cd/pRKLACC cells were significantly longer (up to 20%) than did non-inoculated seeds or seeds inoculated with non-transformed *A. brasilense* Cd (Fig. 11). *Escherichia coli* DH5α transformed with pRKACC induced root elongation in canola seeds to the same extent as *Enterobacter cloacae* CAL2 or *A. brasilense* Cd/pRKLACC.

Fig. 10. IAA concentration and cell density in *A. brasilense* Cd and *A. brasilense* Cd/pRKLACC cultures grown in OAB medium with tryptophan (A and B) or in nutrient broth with tryptophan (C and D). Bars represent standard error values.

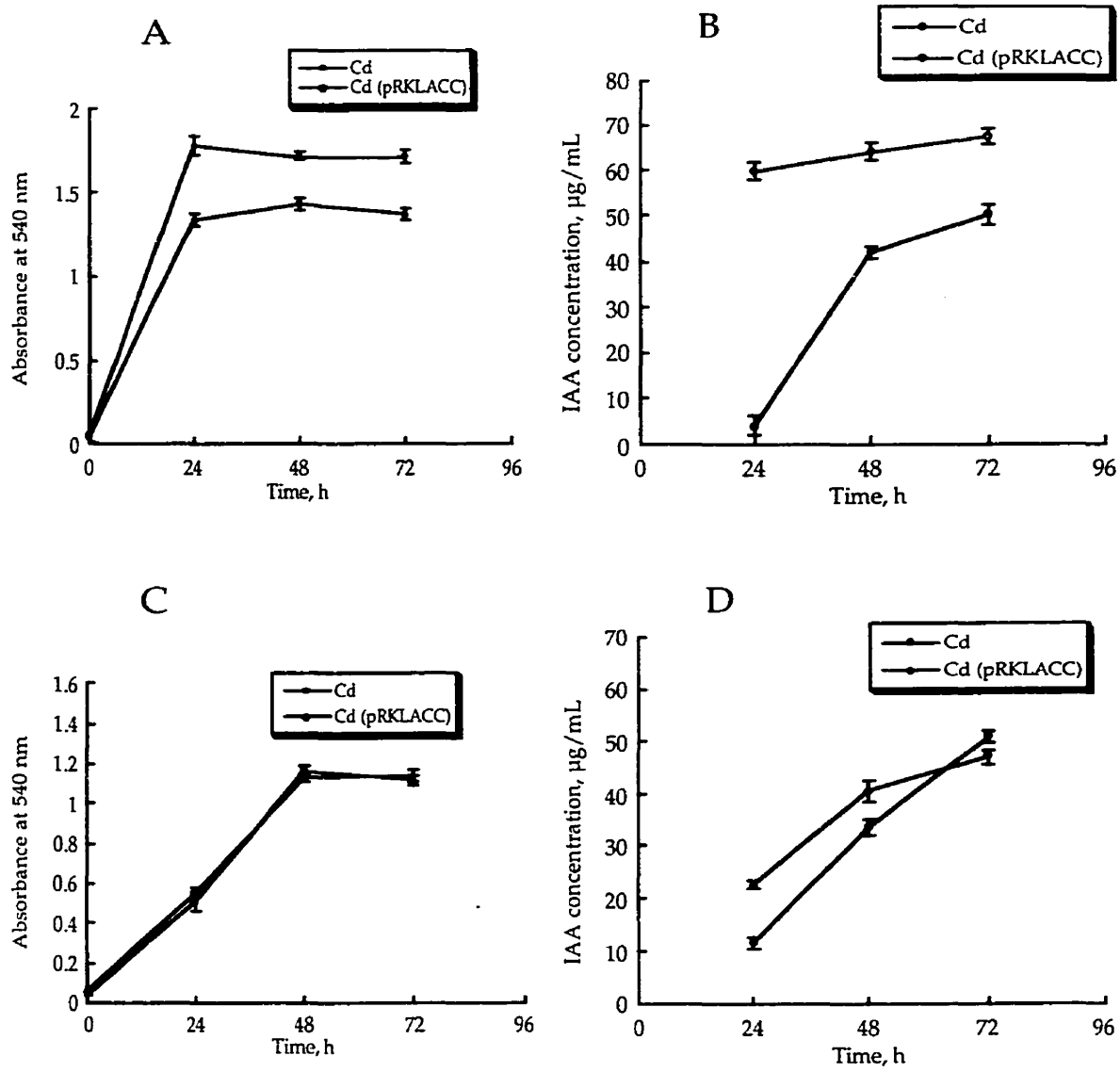
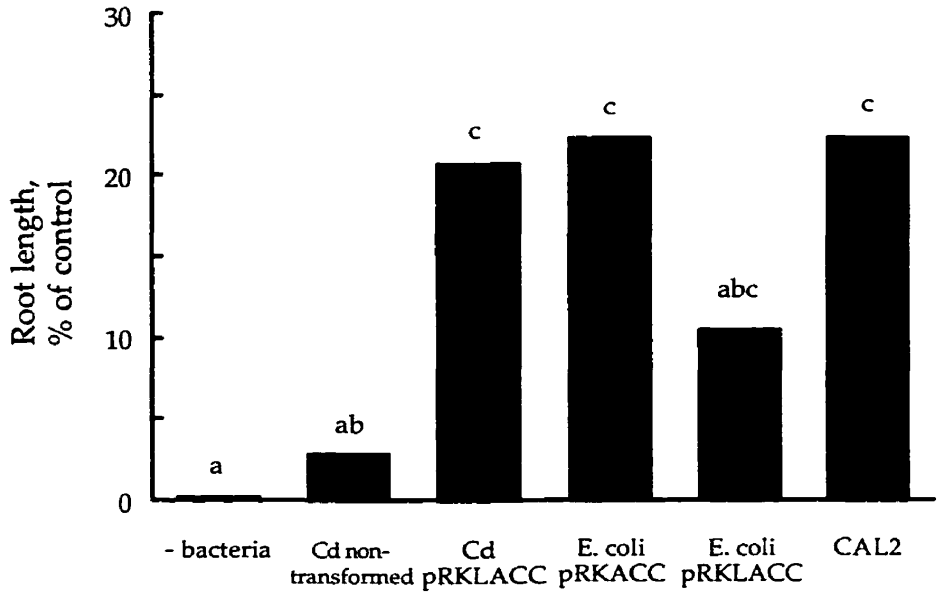

A
Cd
Cd (pRKLACC)
Absorbance at 540 nm
Time, h
B
IAA concentration, µg/mL
C
D

Fig. 11. Root length of canola seedlings, non-treated (- bacteria), or inoculated with *A. brasilense* Cd, *A. brasilense* Cd/pRKLACC, *Escherichia coli* DH5α/pRKACC, *Escherichia coli* DH5α/pRKLACC, and *Enterobacter cloacae* CAL2. The inoculum concentration of all strains was $1X10^7$ cfu/mL. The values on the y axis represent the percentage of increase from the control, which was considered as 0% (31.8 mm). Columns marked with different letters differ significantly at $P \leq 0.05$ in a one way ANOVA. N=80 for each treatment.


Root length, % of control
30
20
10
0
a
ab
c
c
abc
c
- bacteria
Cd non-transformed
Cd pRKLACC
E. coli pRKACC
E. coli pRKLACC
CAL2

3.10. Elongation of tomato seedling roots following inoculation with *A. brasilense*/Cd pRKLACC.

Tomato plants inoculated with *A. brasilense* Cd/pRKLACC at an inoculum concentration of 10^7 cfu/mL, had significantly longer roots (25% increase) compared to non-treated plants or plants inoculated with the non-transformed strain (Fig. 12). Wild type *A. brasilense* induced root elongation in tomato only when the inoculum level was increased to 10^8 cfu/mL. At this inoculum density, the length of tomato roots inoculated with *A. brasilense* Cd/pRKLACC was still significantly greater (15% increase) than plants inoculated with the wild type strain.

3.11. Elongation of wheat seedling roots following inoculation with *A. brasilense* Cd/pRKLACC.

In the case of wheat, no significant difference in shoot or root fresh weight (Figs. 13A, 13B), shoot dry weight (Fig 13C), or total plant water content (Fig. 13E) was observed between plants treated with *A. brasilense* Cd/pRKLACC and plants treated with non-transformed *A. brasilense* Cd. Nevertheless, plants treated with *A. brasilense* Cd/pRKLACC at an inoculation level of 10^7 cfu/mL, promoted the dry weight of roots compared to plants inoculated with non-transformed *A. brasilense* Cd at a concentration of 10^8 cfu/mL in which root dry weight was inhibited by the bacterium (Fig. 13D). The water content in all treated plants was significantly increased as compared to non-inoculated plants (Fig. 13E).

Fig. 12. Root length of tomato seedlings, non-treated (-bacteria), or inoculated with *A. brasilense* Cd and *A. brasilense* Cd/pRKLACC, at different concentrations of inoculum. Cd - plasmid stands for non-transformed cells and Cd + plasmid stands for cells transformed with pRKLACC. The numbers represent cfu/mL. The values on the y-axis represent the percentage of increase from the control, which was considered as 0% (76.3 mm). Columns marked with different letters differ significantly at $P \leq 0.05$ in one way ANOVA. N=80 for each treatment.

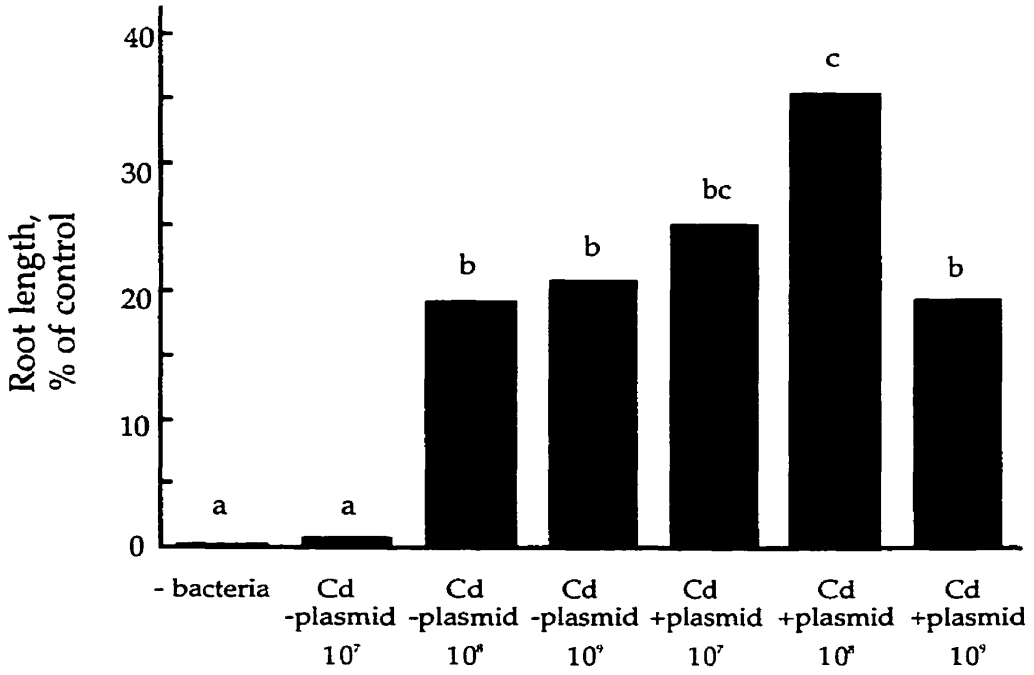
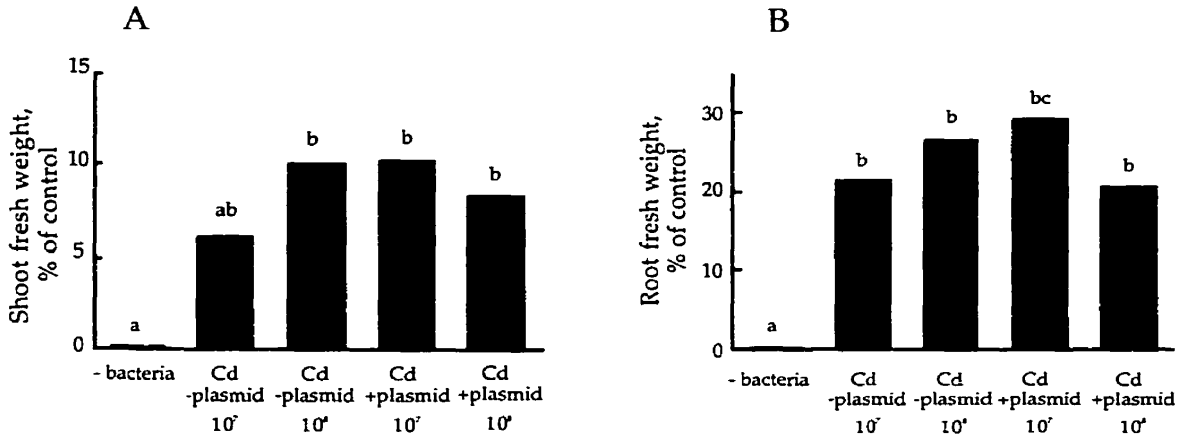
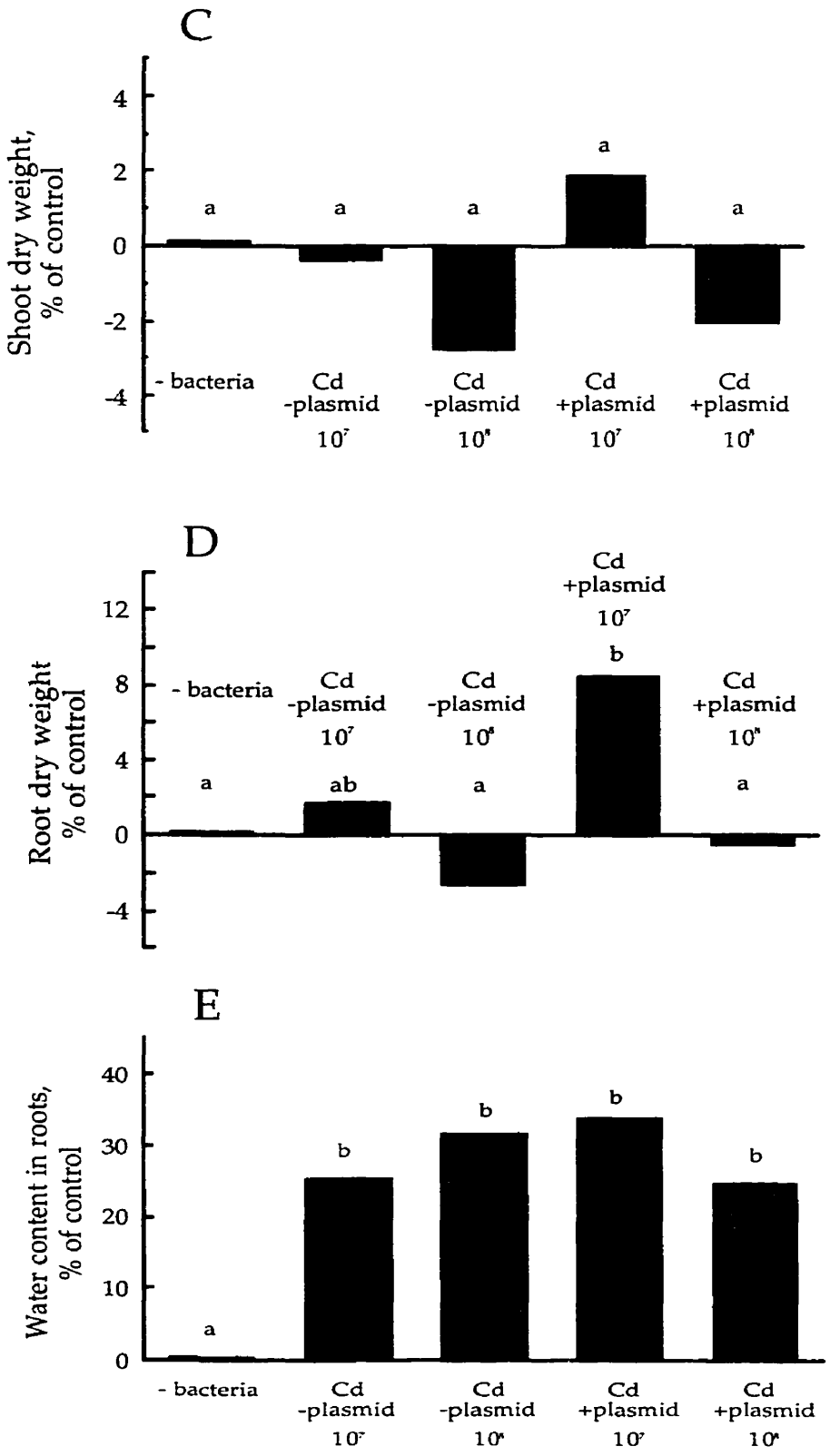

Root length, % of control
40
30
20
10
0
a
a
b
b
bc
c
b
- bacteria
Cd -plasmid 10^7
Cd -plasmid 10^8
Cd -plasmid 10^9
Cd +plasmid 10^7
Cd +plasmid 10^8
Cd +plasmid 10^9

Fig. 13. Shoot and root fresh weight (A and B), dry weight (C and D) and water content (E) of wheat plants inoculated with *A. brasilense* Cd (Cd - plasmid) and *A. brasilense* Cd/pRKLACC (Cd + plasmid) at different concentrations of inoculum. The values on the y-axis represent the percentage of increase from the control, which was considered as 0%. Shoot and root fresh weight values for the control are 98.5 and 54.31 mg respectively. Shoot and root dry weight and water content values for the control are 21.58, 7.90, and 46.4 mg, respectively. Columns marked with different letters differ significantly at $P \leq 0.05$ in one way ANOVA. N=80 for each treatment.


A
Shoot fresh weight, % of control
15
10
5
0
a
ab
b
b
b
- bacteria
Cd -plasmid 10⁷
Cd -plasmid 10⁸
Cd +plasmid 10⁷
Cd +plasmid 10⁸
B
Root fresh weight, % of control
30
20
10
0
a
b
b
bc
b
- bacteria
Cd -plasmid 10⁷
Cd -plasmid 10⁸
Cd +plasmid 10⁷
Cd +plasmid 10⁸


C
Shoot dry weight, % of control
4
2
0
-2
-4
a
a
a
a
a
- bacteria
Cd -plasmid 10⁷
Cd -plasmid 10⁸
Cd +plasmid 10⁷
Cd +plasmid 10⁸
D
Root dry weight % of control
12
8
4
0
-4
a
ab
a
b
a
- bacteria
Cd -plasmid 10⁷
Cd -plasmid 10⁸
Cd +plasmid 10⁷
Cd +plasmid 10⁸
E
Water content in roots, % of control
40
30
20
10
0
a
b
b
b
b
- bacteria
Cd -plasmid 10⁷
Cd -plasmid 10⁸
Cd +plasmid 10⁷
Cd +plasmid 10⁸

3.12. Inoculation with *Azospirillum brasilense* Cd/pRKLACC of tomato seedlings grown in pots.

Pot experiments of fifteen days old plants inoculated with *A. brasilense* Cd/pRKLACC showed an increase in root and shoot dry weight (10 and 6%, respectively) compared to plants inoculated with the non-transformed strain (Fig. 14). However, the differences between both treatments were not statistically significant.

3.13. Inhibition of plant growth caused by inoculation with high inoculum levels of *Azospirillum*.

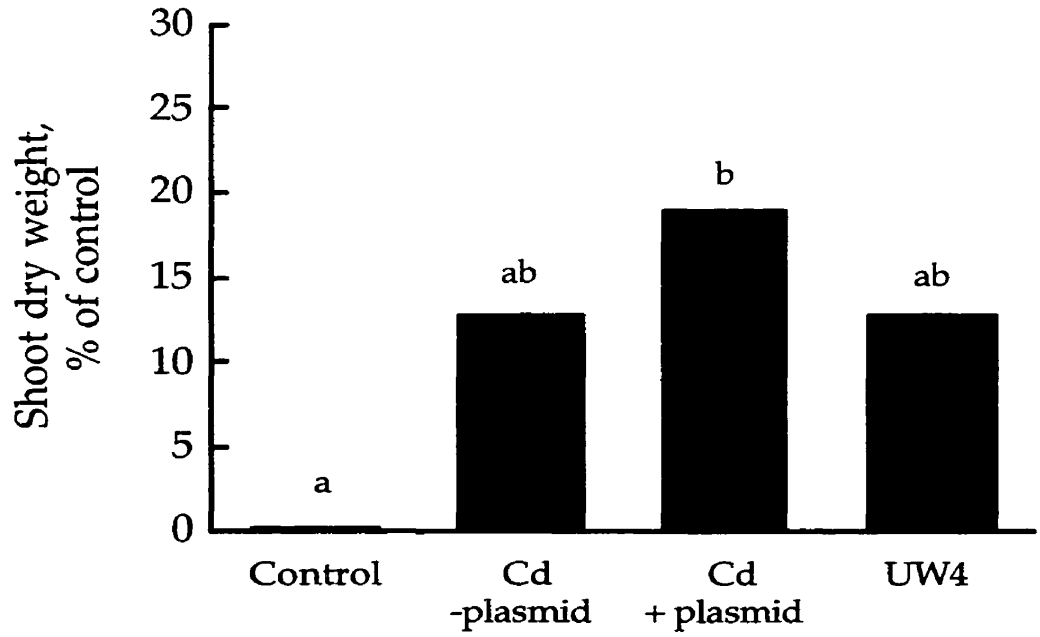
The dry weight of roots of tomato plants grown in pots for two weeks was inhibited when inoculated with non-transformed *A. brasilense* Cd at an inoculum density of 10^8 cfu/mL (Fig. 15). Interestingly, treatment of tomato plants with *A. brasilense* Cd/pRKLACC at the same inoculum level did not inhibit plant growth.

3.14. Cloning of the ACC deaminase gene under the control of the tetracycline resistance promoter.

The ACC deaminase gene, *acdS*, was fused with the promoter of the tetracycline resistance gene, *pTet*, by overlap extension using PCR. The first step of the procedure involved two PCR reactions; one to amplify the structural gene *acdS* and the other one to amplify *pTet* (Fig. 16). The second step in the procedure involved a third PCR reaction to generate the fused fragment *pTet-acdS* (Fig. 17). Running by electrophoresis of the PCR products *acdS* and *pTet-acdS* on an agarose gel show that the bands (Fig. 16) correspond to the predicted sizes (1171

Fig. 14. Shoot and root dry weights (A and B) of 15 days old tomato plants inoculated with *A. brasilense* Cd, *A. brasilense* Cd/pRKLACC, or *Enterobacter cloacae* UW4. Shoot and root dry weight values for the control are 47.4 and 11 mg respectively. Columns marked with different letters differ significantly at $P \leq 0.05$ in one way ANOVA.

A


Shoot dry weight,
% of control
30
25
20
15
10
5
0
a
ab
b
ab
Control
Cd
-plasmid
Cd
+ plasmid
UW4

B

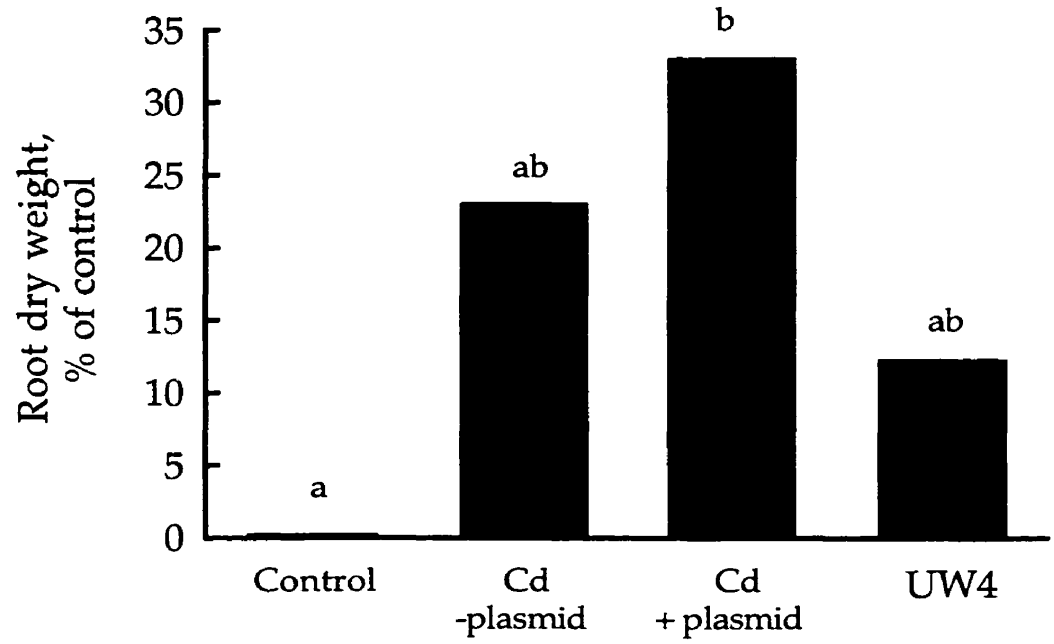

Root dry weight,
% of control
35
30
25
20
15
10
5
0
a
ab
b
ab
Control
Cd
-plasmid
Cd
+ plasmid
UW4

Fig. 15. Dry weight of roots of two weeks old tomato plants inoculated with *A. brasilense* Cd or *A. brasilense* Cd/pRKLACC at an inoculum density of 10^8 cfu/mL. Columns marked with different letters differ significantly at $P \leq 0.05$ in one way ANOVA.

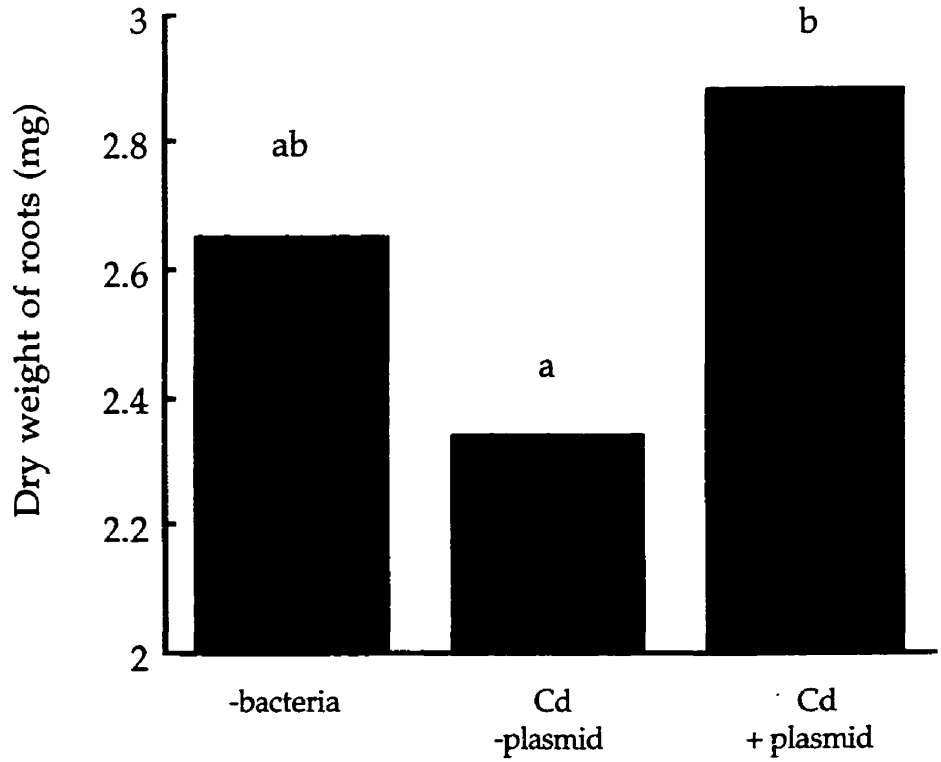
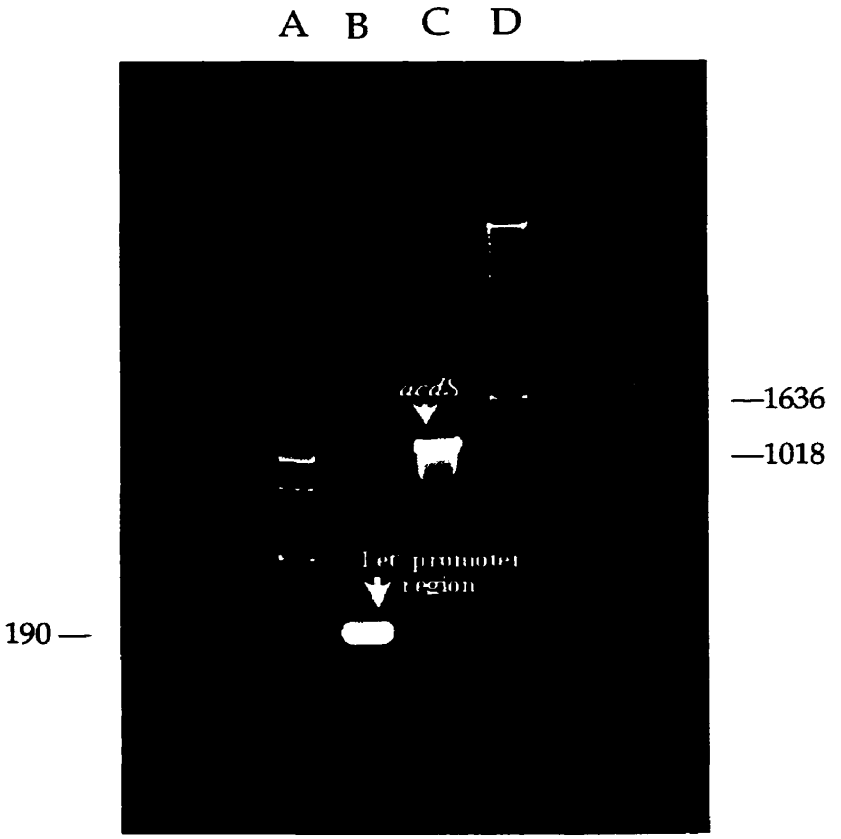

Dry weight of roots (mg)
3
2.8
2.6
2.4
2.2
2
ab
a
b
-bacteria
Cd
-plasmid
Cd
+ plasmid

Fig. 16. Amplification of the ACC deaminase structural gene *acdS* and the tetracycline resistance promoter region *pTet* by PCR. Lane B shows a band (194 bp) marked with an arrow that corresponds to *pTet,* while lane C shows a band marked with an arrow (1171 bp) that corresponds to *acdS*.



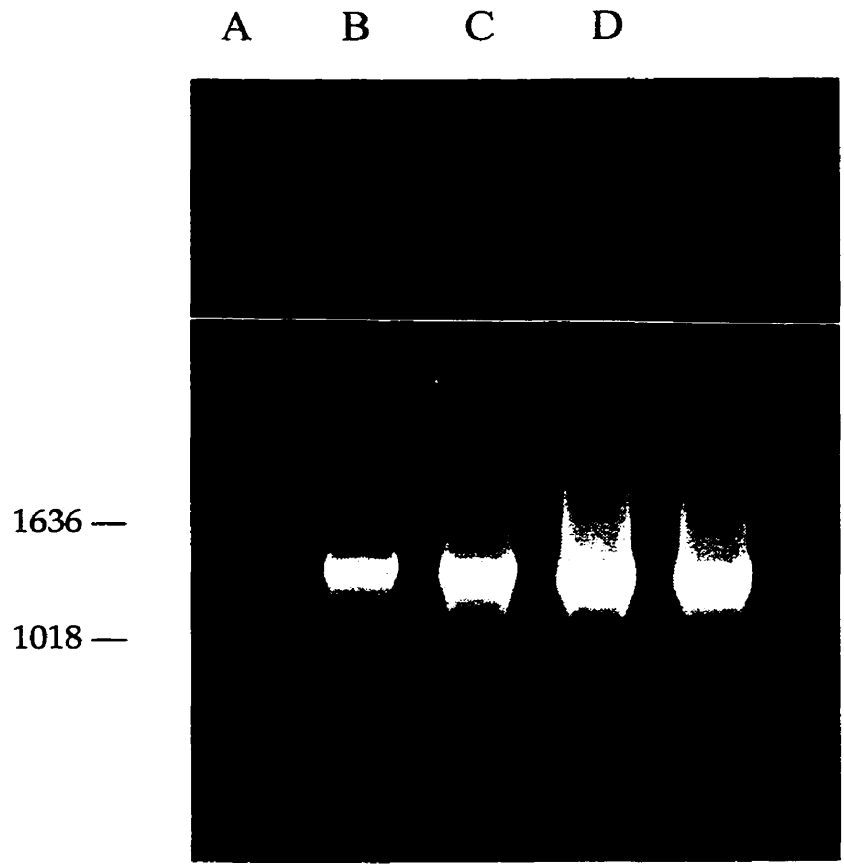
A. DNA molecular weight marker

B. Amplification by PCR of the tetracycline resistance promoter region

C. Amplification by PCR of the ACC deaminase gene, *acdS*.

D. DNA molecular weight marker

Fig. 17. Amplification by PCR of *pTet-acdS*. Lanes B, C, and D show a band (1334 bp) located between 1636 and 1018 bp (arrow in lane A) that corresponds to the fused product *pTet-acdS* .



A. DNA molecular weight marker

B, C, D. Amplification by PCR of *pTet-acdS*

and 194 bp, respectively) of the DNA fragments. Similarly, gel electrophoresis of the PCR product *pTet-acdS* shows that the predicted size for the fused product (1334) corresponds to the size of the fragment shown in the gel (Fig. 17). It should be noted that the size of the fused fragment *pTet-acdS* does not equal the sum of 1171 and 194 (1365 bp), since, as mentioned in section 2.5.1., the primers used to generate *pTet-acdS* overlap in 31 bp, resulting in a smaller fused fragment (1334 bp).

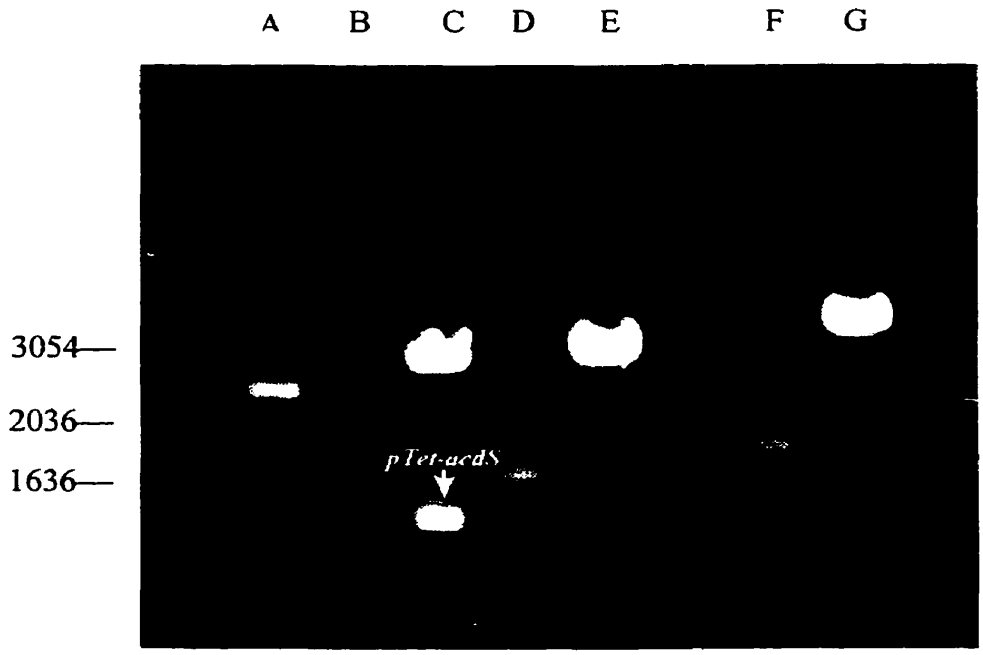
3.14.2. Cloning of the *pTet-acdS* in pRK415

The fused fragment *pTet-acdS* was first cloned in pGEM®-T (Fig. 18), a vector that contains 3′ terminal thymidine overhangs. This system is suitable for the cloning of PCR products because their 3′ adenosine overhangs produced when amplified with certain thermostable polymerases like *Taq*, are compatible to the thymidine overhangs in the vector pGEM-T. Due to an incompatibility of endonuclease restriction sites between the pGEM-T vector and pRK415, pTet-acdS was cloned first into pUC19 (Fig. 19) and then into pRK415. The resulting clone pRKTACC was then transferred to *Escherichia coli* DH5α and then to *Azospirillum brasilense* Cd (Fig. 20) by conjugation.

3.15. Growth of non-transformed *Azospirillum brasilense* Cd, Cd/pRKLACC, Cd/pRKTACC, and Cd/pRK415 in OAB minimal medium.

Non-transformed *A. brasilense* Cd reached the log phase earlier than *A. brasilense* transformed with pRKTACC, pRKLACC, or pRK415 (Fig. 21). However, after 16 hours, *A. brasilense* Cd/pRKTACC and Cd/pRK415 attained

Fig. 18. DNA gel electrophoresis of vector pGEM-T containing the insert *pTet-acdS* in Lane C shows a band (~1400 bp) marked with an arrow that corresponds to the insert *pTet-acdS*. The band above (~3000 bp), also in Lane C, corresponds to linear plasmid pGEM-T. Lane A shows un-digested pGEM-T, containing the insert, while Lane C shows un-digested plasmid pGEM-T without the insert.



A. Non-digested pGEM-T containing the insert *pTet-acdS*.

B. DNA molecular weight marker

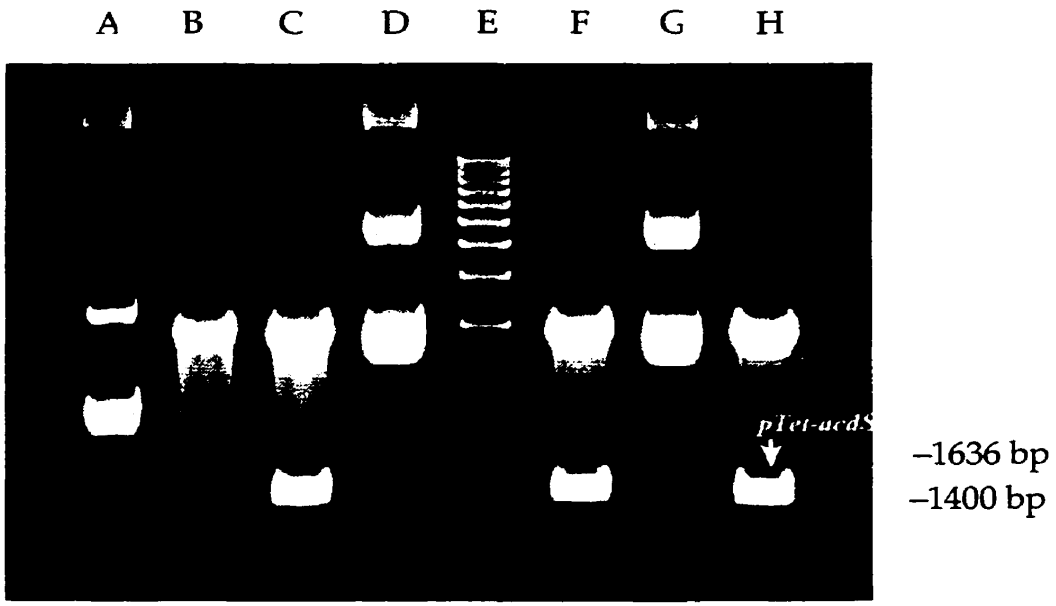
C. PGEM-T with the insert pTet-acdS digested with *Sal*I and *Apa*I

D. Un-digested pGEM-T without the insert *pTet-acdS*

E. pGEM-T without the insert digested with *Sal*I and *Apa*I

F Un-digested pGEM-T containing an unidentified insert

Fig. 19. DNA electrophoresis of pUCTACC. Lanes C, F, and H show that the insert *pTet-acdS* (marked with an arrow) is excised from pUCTACC when digested with *Sac*I and *Kpn*I. Lanes D and G show un-digested pUCTACC while lane A shows un-digested pUC19.



A. Non-digested pUC19

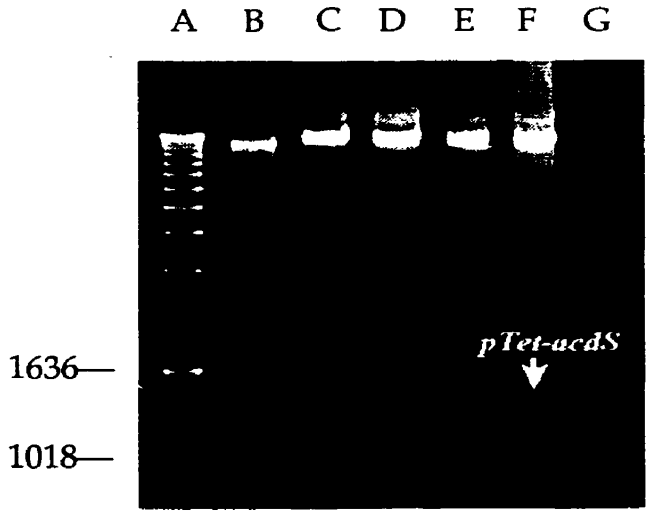
B. pUC19 digested with *Sac*I and *Kpn*I

C, F, H. pUCTACC digested with *Sac*I and *Kpn*I

D, G. Non-digested pUCTACC

E. DNA molecular weight standard

Fig. 20. DNA electrophoresis of pRKTACC isolated from *Azospirillum brasilense* Cd/pRKTACC transformants. As shown in lanes E and F, the insert *PTet-acdS* was excised from pRKTACC when cut with *Kpn*I and *Sac*I.



A. DNA molecular weight marker.

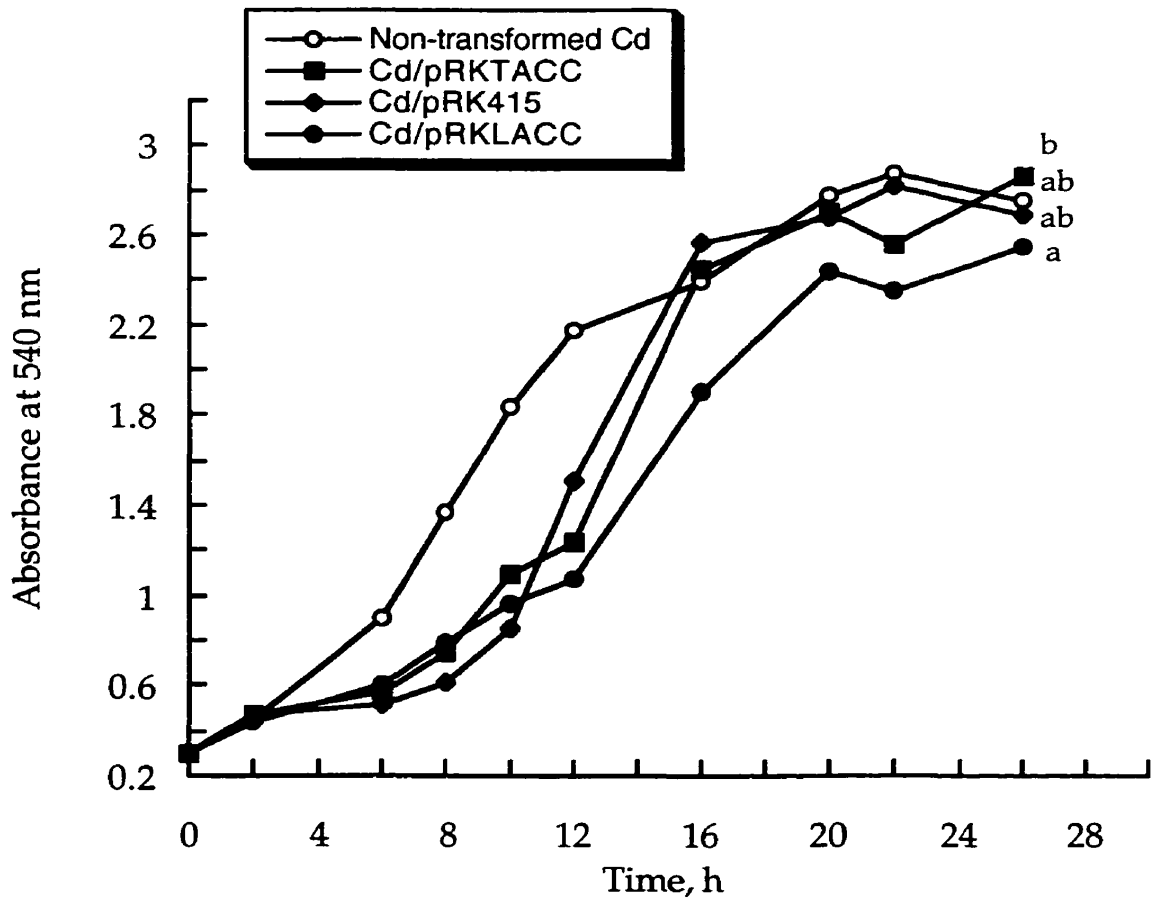
B. pRK415 cut with *Sac*I

C,D. *A. brasilense* Cd/pRKTACC digested with *Sac*I

E,F. *A. brasilense* Cd/pRKTACC cut with *Kpn*I and *Sac*I

G. Non-transformed *A. brasilense* Cd

Fig. 21. Growth of the *A. brasilense* Cd transformants and wild type in OAB minimal medium. Treatments (at 26 h) marked with different letters vary significantly at $P \leq 0.05$ in one way ANOVA.


Non-transformed Cd
Cd/pRKTACC
Cd/pRK415
Cd/pRKLACC
Absorbance at 540 nm
3
2.6
2.2
1.8
1.4
1
0.6
0.2
0
4
8
12
16
20
24
28
Time, h
b
ab
ab
a

the same cell density as the non-transformed strain. As for *A. brasilense* Cd/pRKLACC, after 26 hours of growth, its cell density was significantly lower than the cell density of pRKTACC.

3.16. ACC deaminase activity of *A. brasilense* Cd/pRKLACC and Cd/pRKTACC.

The ACC deaminase activity in *A. brasilense* Cd/pRKTACC was significantly lower than in Cd/pRKLACC (Fig. 22). No significant difference was found between the ACC deaminase activity in Cd/pRKLACC and *Enterobacter cloacae* UW4.

3.17. IAA synthesis of non-transformed *A. brasilense* Cd as compared to the transformants Cd/pRKLACC, Cd/pRKTACC and Cd/pRK415.

Cultures of *A. brasilense* Cd/pRKLACC in OAB minimal medium supplemented with tryptophan showed decreased amounts of IAA compared to cultures of non-transformed *A. brasilense* Cd, *A. brasilense* Cd/pRKTACC and Cd/pRK415 (Fig. 23). This reduction prevailed during the whole incubation period. No significant differences were found in the concentration of IAA between *A. brasilense* Cd/pRKTACC, Cd/pRK415, or non-transformed *A. brasilense* Cd cultures.

Fig. 22. ACC deaminase activity in *A. brasilense* Cd/pRKLACC, *A. brasilense* Cd/pRKTACC and *Enterobacter cloacae* UW4. Columns marked with different letters differ significantly at $P \leq 0.05$ in a one way ANOVA.

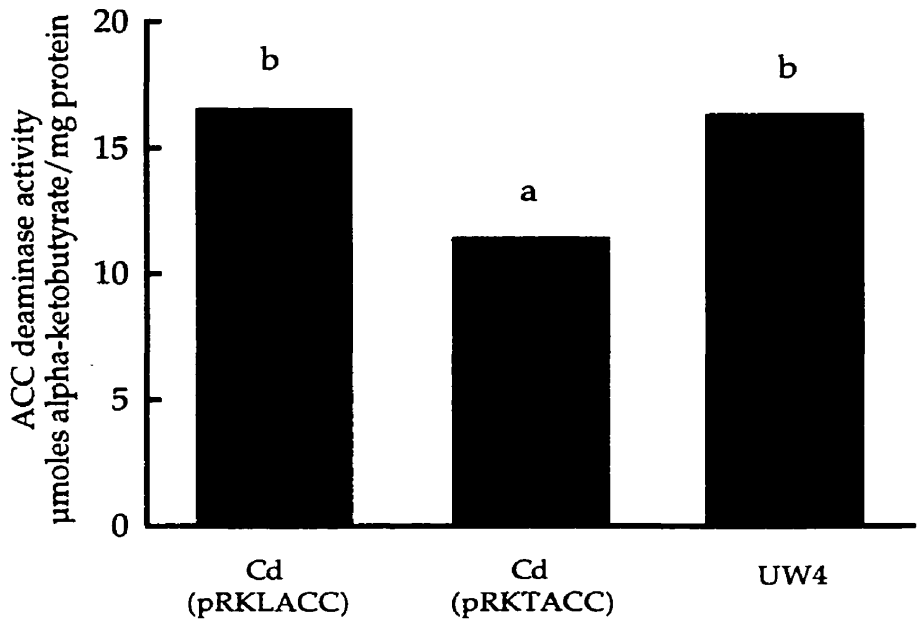
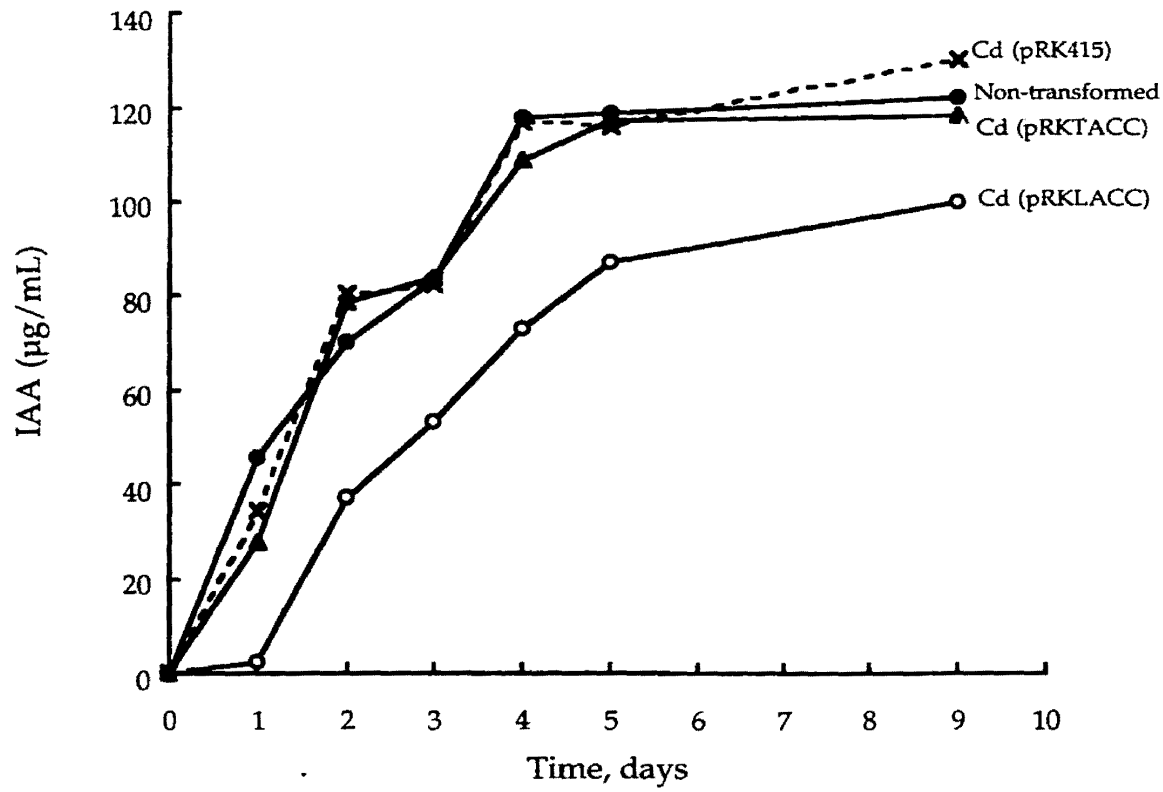

ACC deaminase activity
µmoles alpha-ketobutyrate/mg protein
20
15
10
5
0
b
a
b
Cd
(pRKLACC)
Cd
(pRKTACC)
UW4

Fig. 23. IAA concentration in *A. brasilense* Cd, Cd/pRKLACC, Cd/pRKTACC, and Cd/pRK415 cultures grown in OAB medium with tryptophan.


140
120
100
80
60
40
20
0
IAA (µg/mL)
0
1
2
3
4
5
6
7
8
9
10
Time, days
Cd (pRK415)
Non-transformed
Cd (pRKTACC)
Cd (pRKLACC)

3.18. Survival in soil of *A. brasilense* Cd as compared to the transformed strains Cd/pRKLACC, Cd/pRKTACC and Cd/pRK415

Analysis of survival in soil showed that the cell density of *A. brasilense* Cd/pRKLACC increased during the first ten days, after which it abruptly declined (Fig. 24). As for Cd/pRKTACC, its population level remained stable during the first 20 days after which it declined and subsequently (after 30 days) stabilized. The cell density of Cd/pRK415 declined during the first 20 days after which it remained relatively stable. In contrast, the population level of non-transformed *A. brasilense* Cd did not suffer abrupt changes and remained relatively steady during the whole period of incubation.

3.19. Flooding of tomato plants inoculated with non-transformed *A. brasilense* Cd, Cd/pRKLACC, and Cd/pRKTACC.

Treatment of flooded tomato plants with non-transformed A. brasilense Cd, Cd/pRKLACC, and Cd/pRKTACC significantly increased fresh weight (Fig. 25A,B) and dry weight (Fig. 25C,D) of roots and shoots as compared to non-treated plants. Flooded plants treated with Cd/pRKTACC showed significantly higher fresh and dry weight of shoots (Fig. 25A,C) as compared to plants treated with non-transformed *A. brasilense* Cd. However, no significant differences in shoots fresh or dry weight were found between plants treated with Cd/pRKTACC and Cd/pRKLACC, or plants treated with Cd/pRKLACC and the non-transformed strain. Plants treated with Cd/pRKTACC showed a significantly higher root fresh weight as compared to plants treated with

Fig. 24. Survival of *A. brasilense* Cd, Cd/pRKTACC, Cd/pRKLACC, and Cd/pRK415 in soil during a period of 40 days. The curves in the graph were plotted using a smooth curve fit by KaleidaGraph™ 3.0.

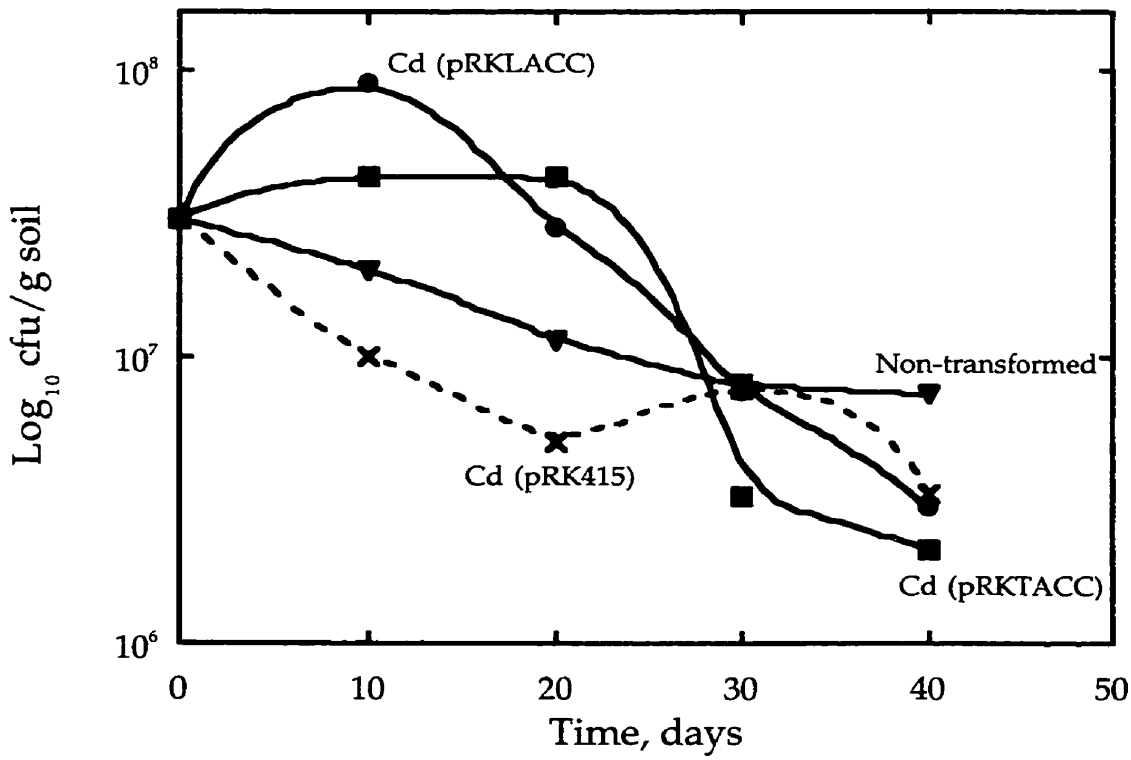
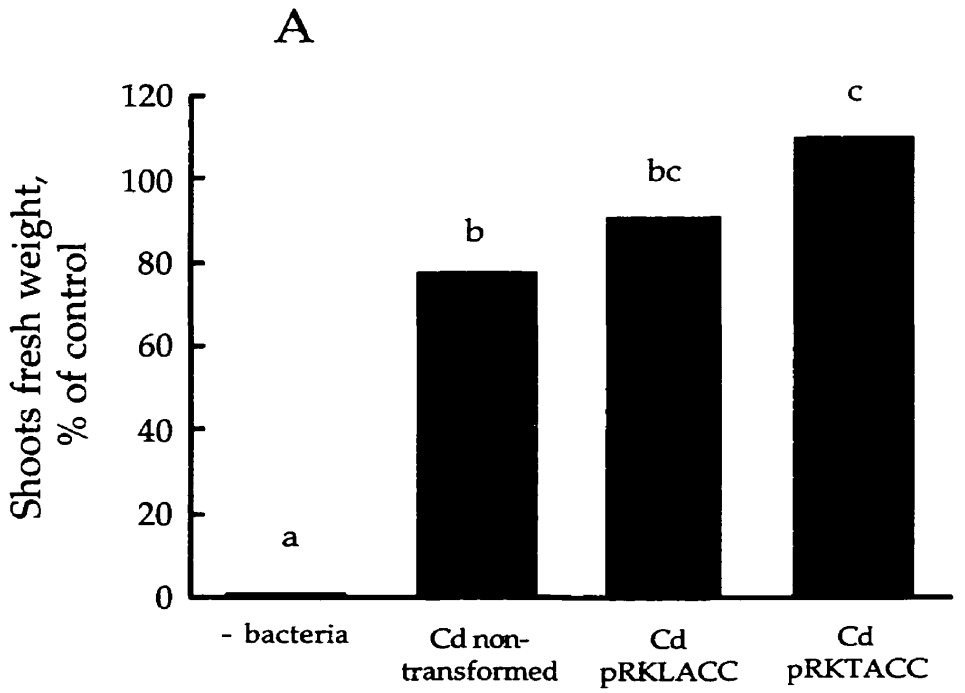
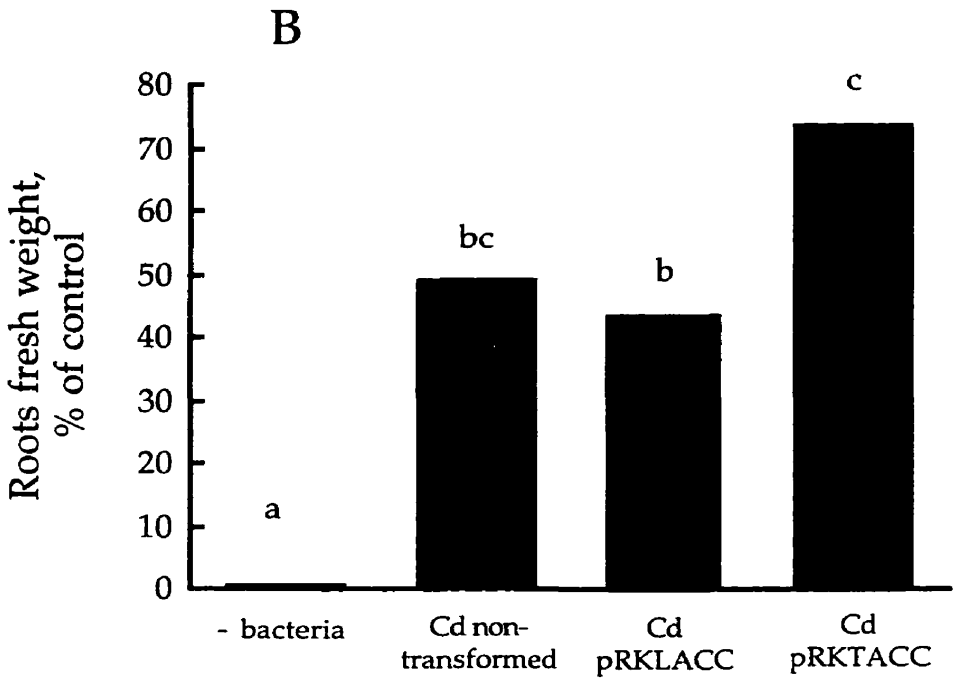
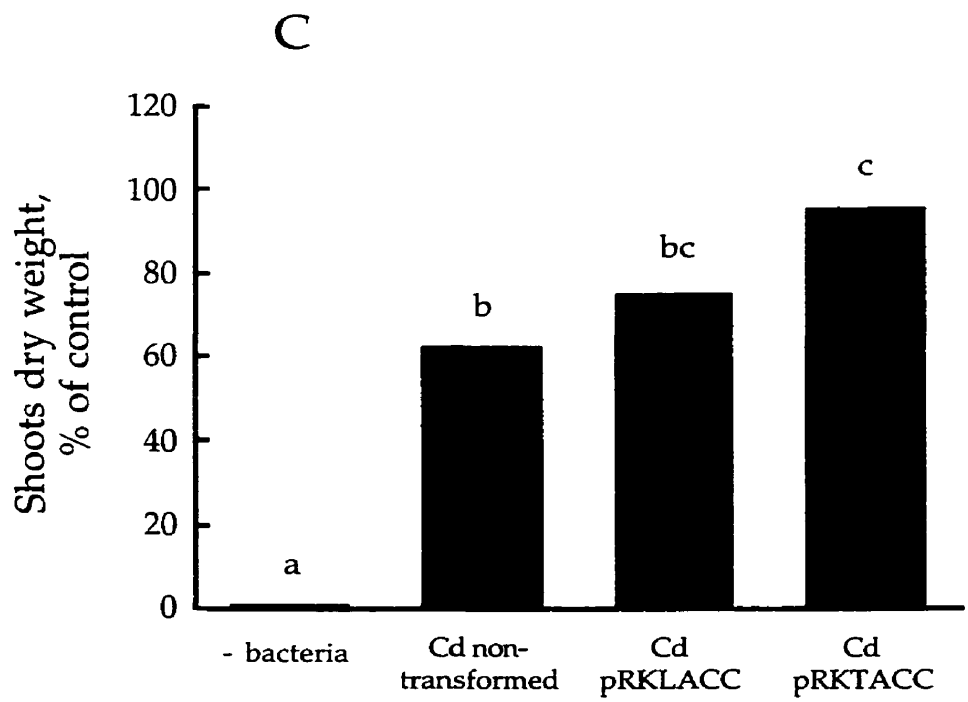
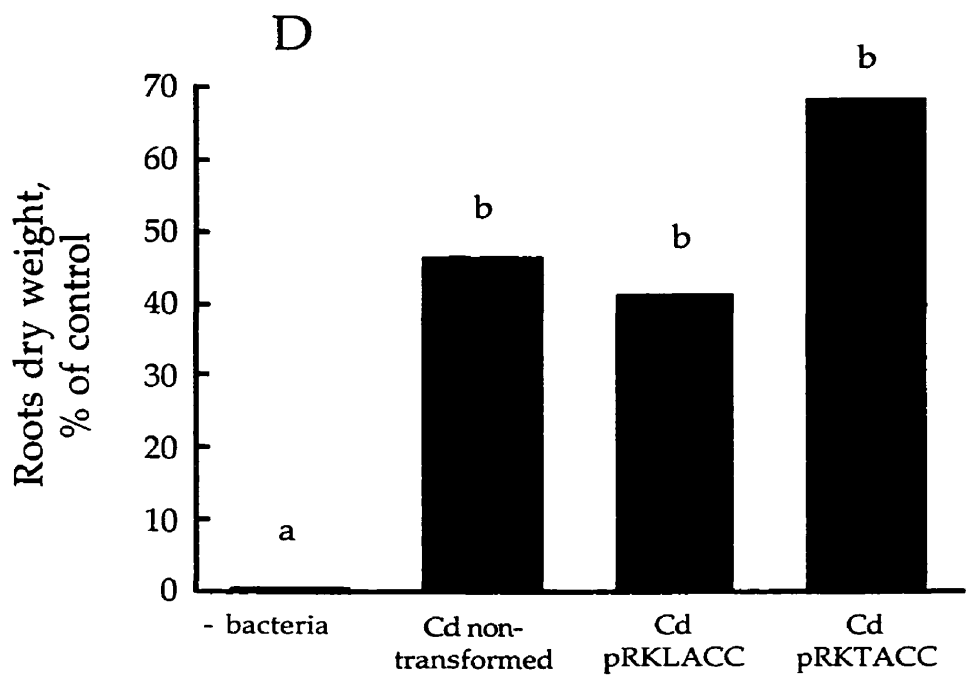

Cd (pRKLACC)
Non-transformed
Cd (pRK415)
Cd (pRKTACC)
10^8
10^7
10^6
Log_{10} cfu/g soil
0
10
20
30
40
50
Time, days

Fig. 25. Fresh (A,B) and dry weight (C,D) of shoots and roots of flooded tomato plants treated with non-transformed *A. brasilense* Cd, Cd/pRKLACC, and Cd/pRKTACC. Shoots and roots fresh weight values for the control are 5.43 and 1.37 g respectively, while the shoots and roots dry weight values for the control are 0.7 and 0.136 g respectively. Columns marked with different letters differ significantly at $P \leq 0.05$ in one way ANOVA.


A
Shoots fresh weight, % of control
120
100
80
60
40
20
0
a
b
bc
c
- bacteria
Cd non-transformed
Cd pRKLACC
Cd pRKTACC


B
Roots fresh weight, % of control
80
70
60
50
40
30
20
10
0
a
bc
b
c
- bacteria
Cd non-transformed
Cd pRKLACC
Cd pRKTACC


C
Shoots dry weight, % of control
120
100
80
60
40
20
0
a
b
bc
c
- bacteria
Cd non-transformed
Cd pRKLACC
Cd pRKTACC


D
Roots dry weight, % of control
70
60
50
40
30
20
10
0
a
b
b
b
- bacteria
Cd non-transformed
Cd pRKLACC
Cd pRKTACC

Cd/pRKLACC (Figs. 25B and 26). However, no significant differences in roots fresh weight were found between plants treated with Cd/pRKTACC and the non-transformed strain.

3.19.2. Epinasty in flooded tomato plants

Epinasty in flooded plants was more pronounced in non-treated plants and in plants inoculated with non-transformed *A. brasilense* Cd than in plants treated with Cd/pRKTACC (Figs. 27-29). The results can also be observed graphically (Fig. 27): The differences in epinasty between the treatments are more pronounced after 48 hours of flooding; plants treated with Cd/pRKTACC show a significantly lower degree of epinasty than non-treated plants or plants treated with the non-transformed strain.

3.19.3. Concentration of chlorophyll in flooded tomato plants, non-treated, treated with Cd/pRKLACC, Cd/pRKTACC, or with non-transformed *A. brasilense* Cd

The concentration of chlorophyl a and b was significantly higher in plants treated with *A. brasilense* Cd/pRKTACC (~20%) as compared to non-treated plants, plants treated with wild type *A. brasilense* Cd or with *A. brasilense* Cd/pRKLACC (Fig. 30). Inoculation of plants with either *A. brasilense* Cd/pRKLACC or non-transformed *A. brasilense* Cd did not have an effect on the concentration of chlorophyll in the plants.

3.20. Attachment of *A. brasilense* to tomato leaves

Inoculation of *A. brasilense* in leaves of axenically grown tomato plants showed that *A. brasilense* has the ability to attach to the leaves of tomato plants and can survive on the surface of the plant for a period of at least up to 14 days

Fig. 26. Roots of flooded tomato plants treated with *A. brasilense* Cd/pRKLACC or Cd/pRKTACC.

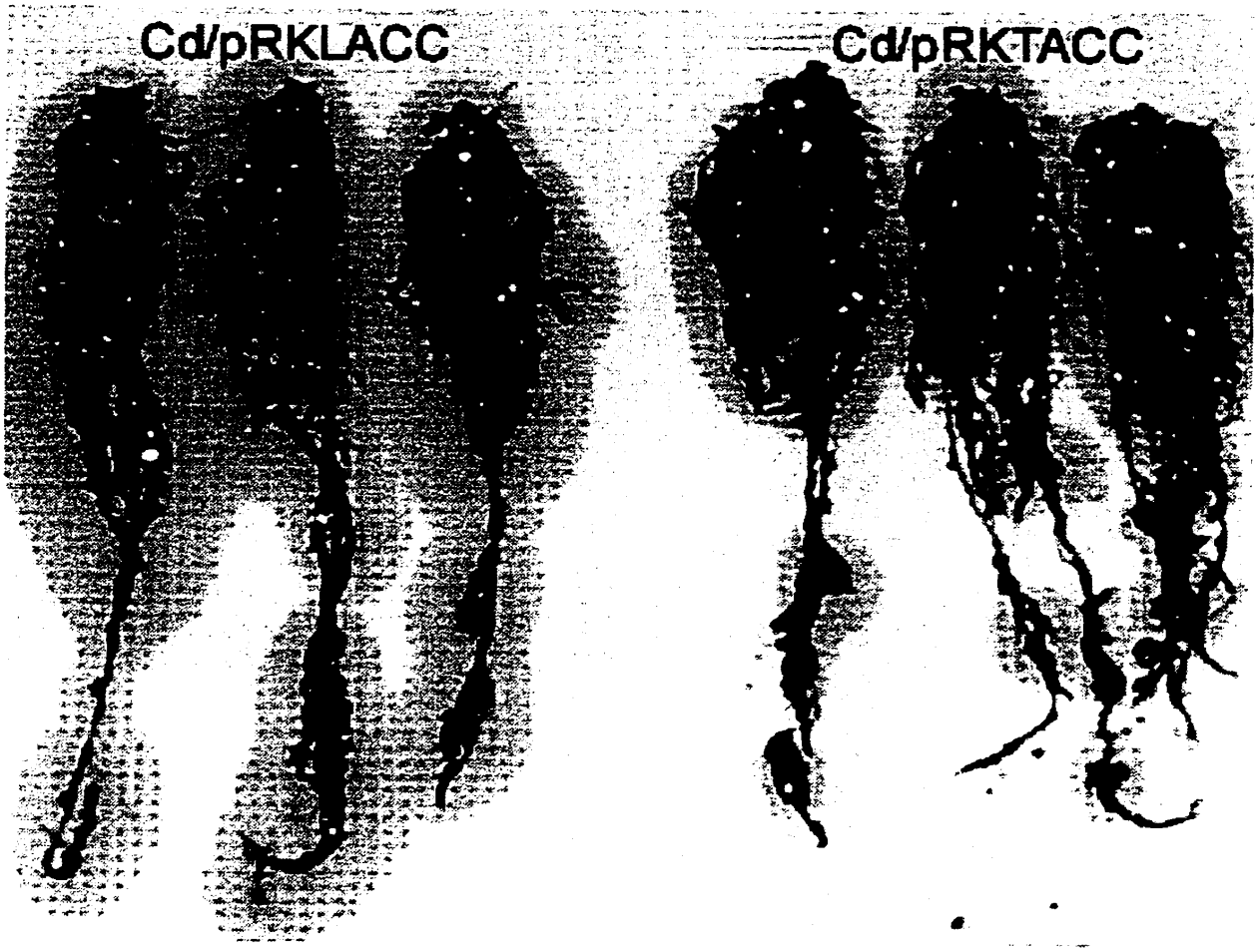

Cd/pRKLACC
Cd/pRKTACC

Fig. 27. Epinasty in flooded tomato plants measured as the change in the angle measured in degrees between the main stem and the petiole.

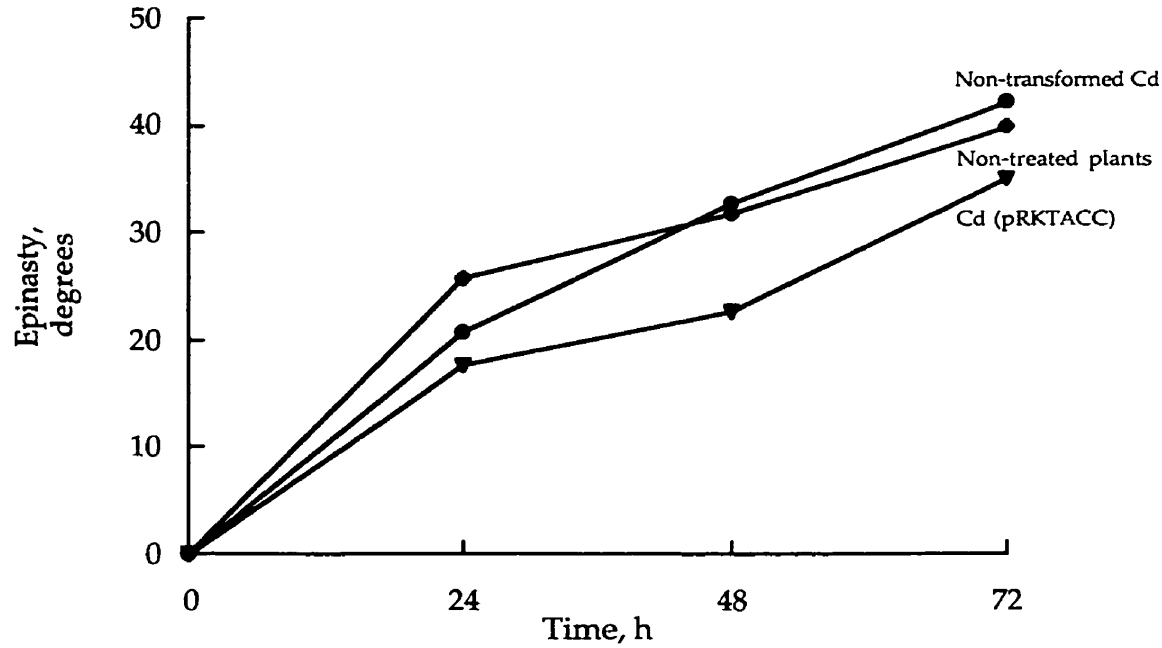

50
40
30
20
10
0
Epinasty, degrees
0
24
48
72
Time, h
Non-transformed Cd
Non-treated plants
Cd (pRKTACC)

Fig. 28. Comparison of epinasty in tomato plants treated with Cd/pRKTACC and non transformed *A. brasilense* Cd after two day of flooding.

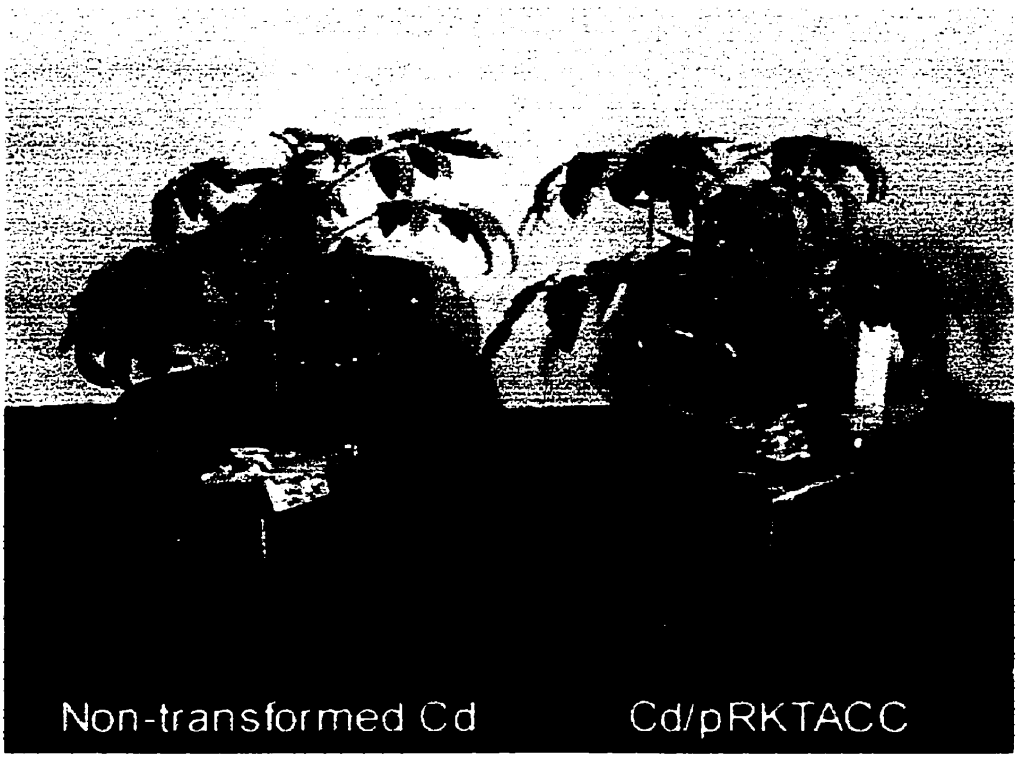
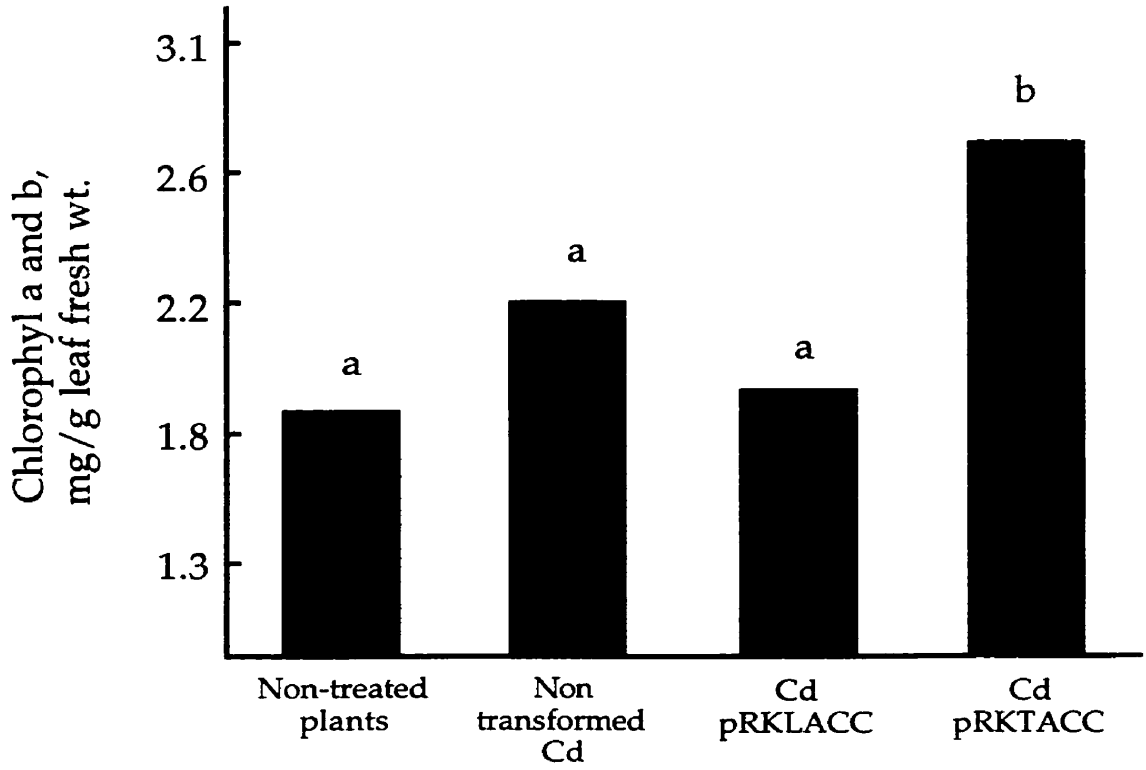

Non-transformed Cd
Cd/pRKTACC

Fig. 29. Comparison of epinasty in tomato plants treated with *Azospirillum brasilense* Cd/pRKTACC and non-treated plants after two days of flooding.

Non-treated plants
Cd/pRKTACC

Fig. 30. Concentration of chlorophyl a and b in flooded tomato plants treated with non-transformed *A. brasilense* Cd, *A. brasilense* Cd/pRKLACC, or *A. brasilense* Cd/pRKTACC


Chlorophyl a and b,
mg/g leaf fresh wt.
3.1
2.6
2.2
1.8
1.3
a
a
a
b
Non-treated plants
Non transformed Cd
Cd pRKLACC
Cd pRKTACC

(Fig. 31). The values for cell density of non-transformed *A. brasilense* Cd and Cd/pRKTACC were similar throughout the duration of the experiment. However, the cell density of Cd/pRKLACC decreased continuously with time, while the cell density of Sp245/pRKLACC declined from the fourth day and them remained stable from day six until the end of the experiment.

Observations by Scanning Electron Microscopy (Figs. 32-37) complemented with bacterial counts on agar plates (Fig. 31) show that all strains were capable of establishing on the leaf surface after one day of inoculation. Colonization of the leaves by *A. brasilense* cells (transformed and non-transformed strains) was performed by cell aggregates (Figs. 32-36), forming patches of cells across the leaf surface. Although attachment also occurred by individual cells (Fig 35), it was not the predominant pattern of colonization. In contrast, colonization by *Pseudomonas putida* AC8 was not in patches but in uninterrupted strands of cells (Fig. 37). All of *A. brasilense* strains, as well as *P. putida* AC8, produced fibrils that connected the cells to each other and to the surface of the leaf (Figs. 32-37). No bacteria were found colonizing non-inoculated plants (Fig. 38), suggesting that the bacteria in figs. 32-37 are indeed the introduced strains.

Cells of Cd/pRKLACC and *P. putida* AC8 were sometimes found embedded in a sheath (Figs. 33 b and 37 d). This material of unknown nature concealed the cells, making it difficult to detect and visualize the bacteria when taking the photographs (Fig. 33 b). Only one of the strains, Sp245/pRKLACC, was found colonizing a leaf pore opening or the area around it (Fig. 36 a). The pore opening has been covered by bacteria, and, since the leaf samples contained pores in either fashion (open or closed) (Fig. 38 a, c), it is impossible to discern if

Fig. 31. Survival of *A. brasilense* Cd, *A. brasilense* Cd/pRKLACC, *A. brasilense* Cd/pRKTACC, and *A. brasilense* Sp245/pRKLACC, on the surface of tomato leaves. The results are the average values from two experiments.

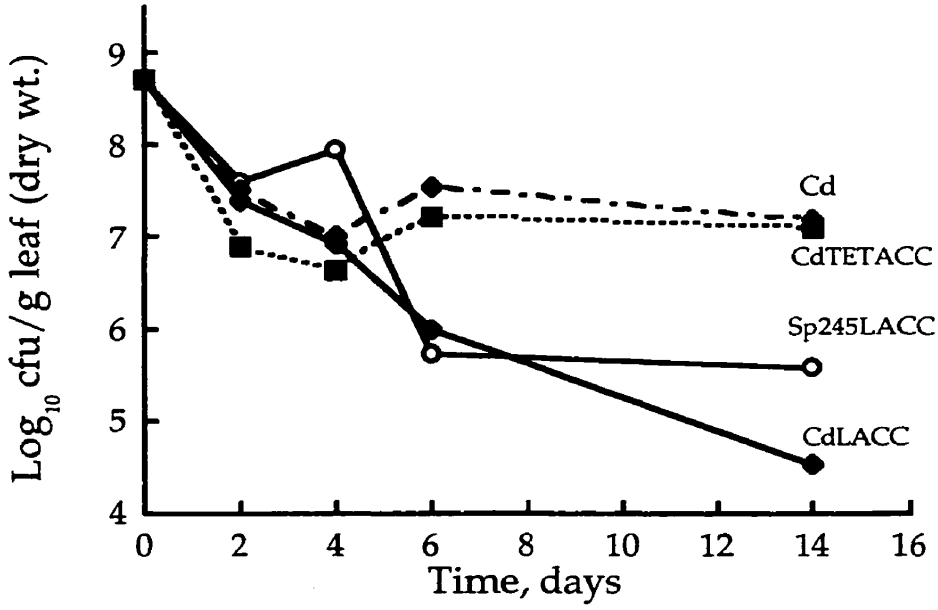
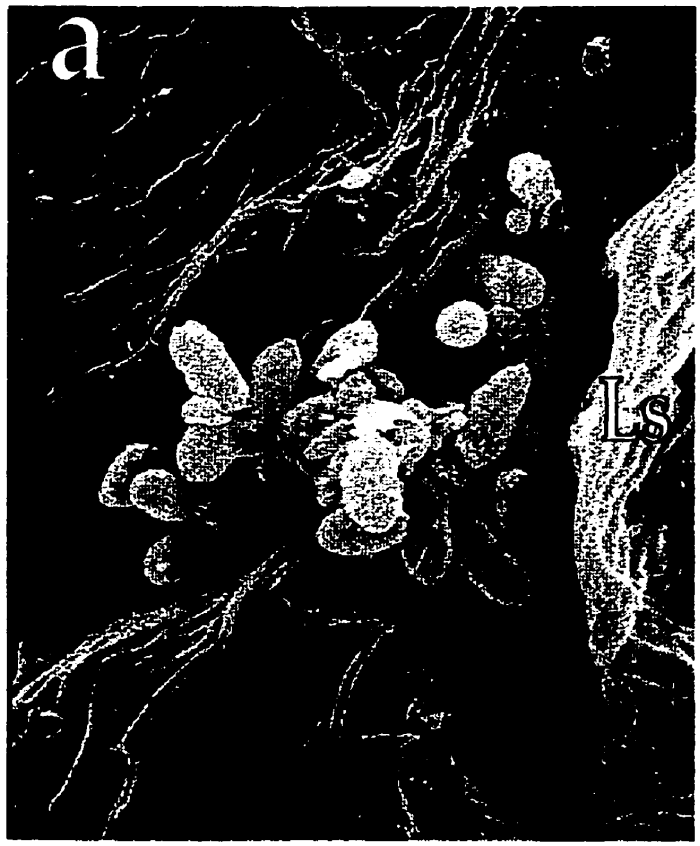

Log10 cfu/g leaf (dry wt.)
9
8
7
6
5
4
0
2
4
6
8
10
12
14
16
Time, days
Cd
CdTETACC
Sp245LACC
CdLACC

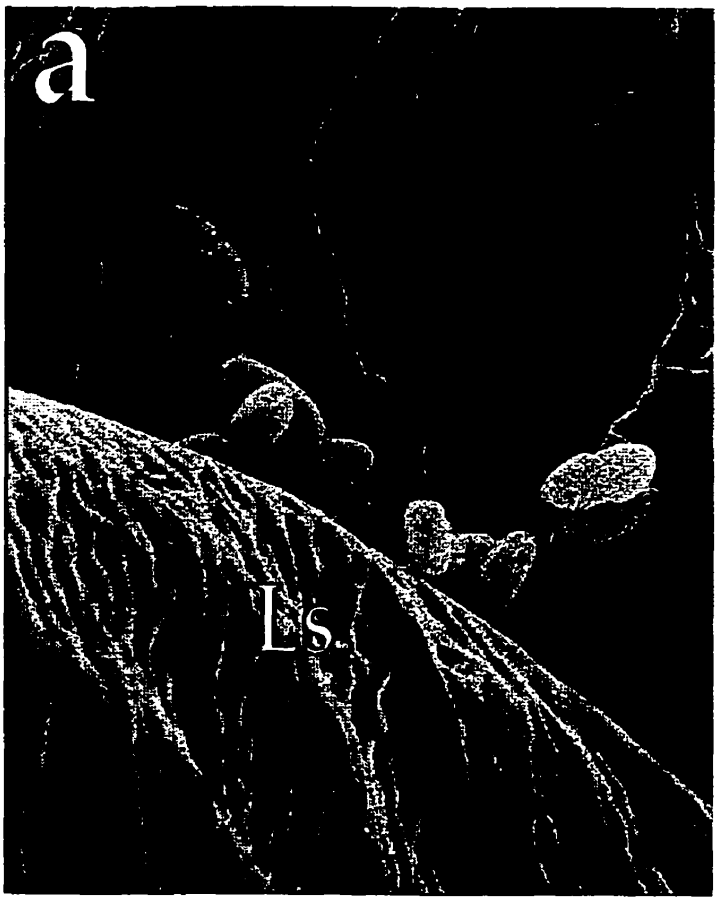
Fig. 32 Colonization of tomato leaves by non-transformed *A. brasilense* Cd two days after inoculation. a and b: Bacterial aggregates showing a fibrillar material connecting the cells to each other and to the leaf surface. Abbreviations: Ls, leaf surface; Fm, fibrillar material. Bar on (a) represents 6 μm.

Fig. 33 Colonization of tomato leaves by *A. brasilense* Cd/pRKLACC. a: Cell aggregates colonizing orifices on the leaf surface one day after inoculation. b: after four days of inoculation a sheath covered the cells. Abbreviations: Ls, leaf surface, s, sheath. Bar on (a) represents 3.75 μm.


a
Ls

b
s

Fig. 34 Colonization of tomato leaves by *A. brasilense* Cd/pRKTACC one day after inoculation (a) and (b). b: magnification of (a) showing the cells attaching to each other by fibrils (arrow). Abbreviations: Ls, leaf surface. Bars on (a) and (b) represent 4.3 and 1.76 μm, respectively.


a
Ls

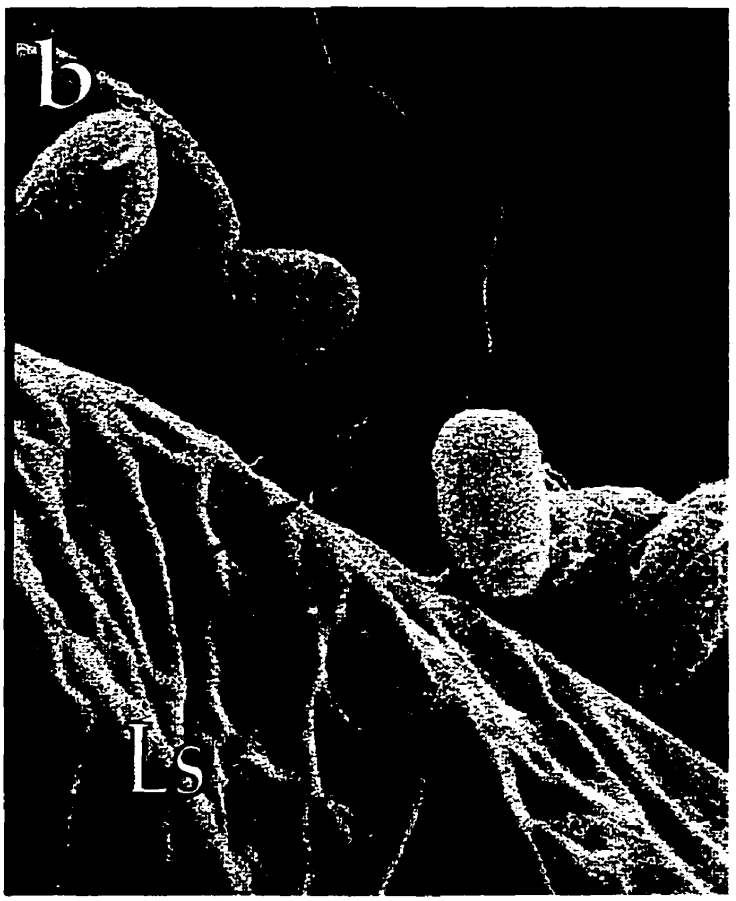
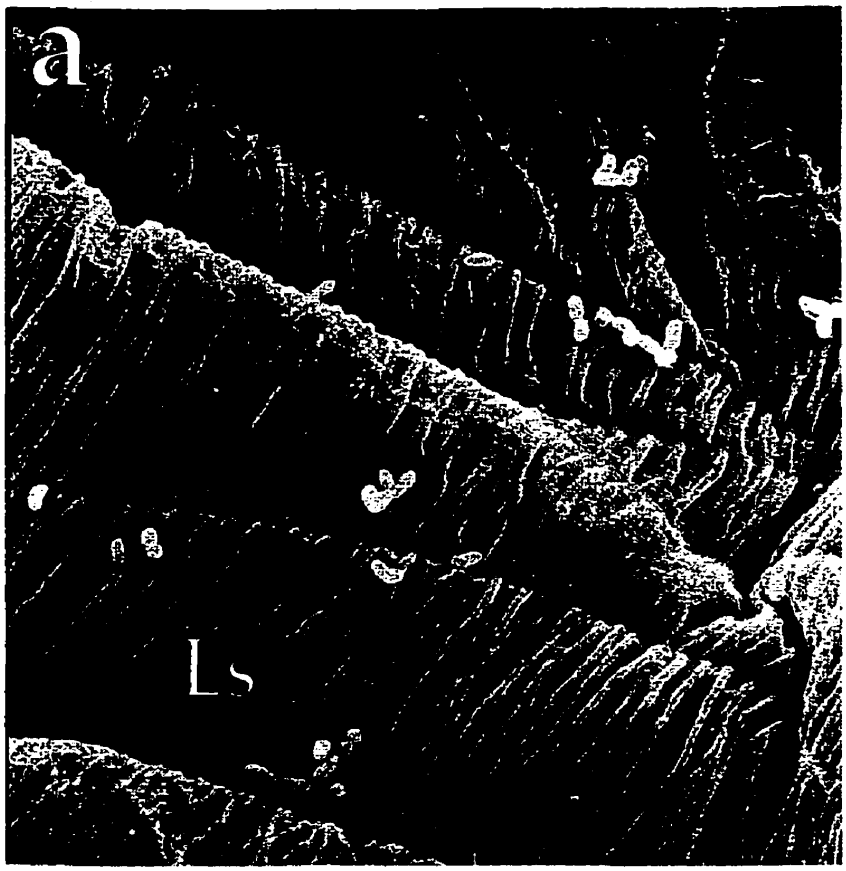

b
Ls

Fig. 35 Colonization of tomato leaves by *A. brasilense* Cd/pRKTACC one day after inoculation. a: Single cells and cells aggregates colonizing the leaf surface. b: one single cell attached to the leaf surface by short and long fibrils (arrows). Abbreviations: Ls, leaf surface; Bars on (a) and (b) represent 8.8 and 4.3 μm respectively.


a
Ls

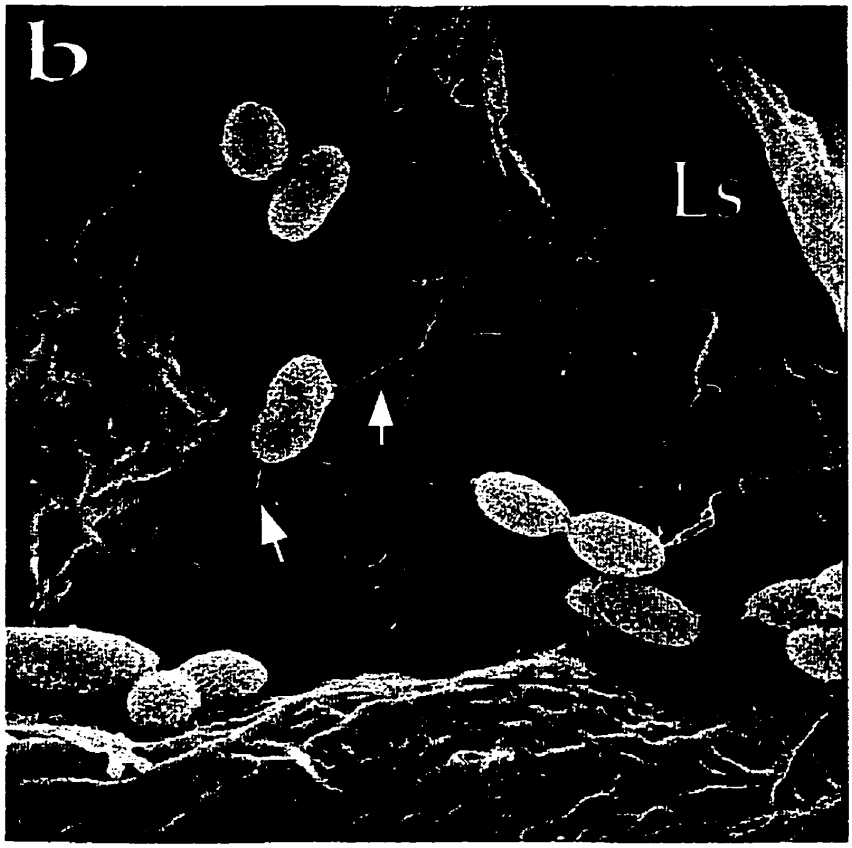

b
Ls

Fig. 36 Tomato leaf colonization by *A. brasilense* Sp245/pRKLACC after one day of inoculation. a: Cell aggregate colonizing a pore opening. c: Magnification of (b) showing colonization of the leaf surface by a bacterial aggregate. Abbreviations: Ls, leaf surface; p, pore. Bars on (a) and (c) represent 10 and 3 μm respectively.

a
P
Ls

b

c

Fig. 37 Tomato leaf colonization by *Pseudomonas putida* AC8. a: Colonization after two days of inoculation showing uninterrupted strands of cells along the leaf surface. b: Magnification of (a). c: Bacteria connected to each other by fibrils (arrows). d: The bacteria appear embedded in a sheath. Abbreviations: Ls, leaf surface; s, sheath. Bars on (a) and (c) represent 8.8 and 1.5 μm respectively.

a
Ls

b

c

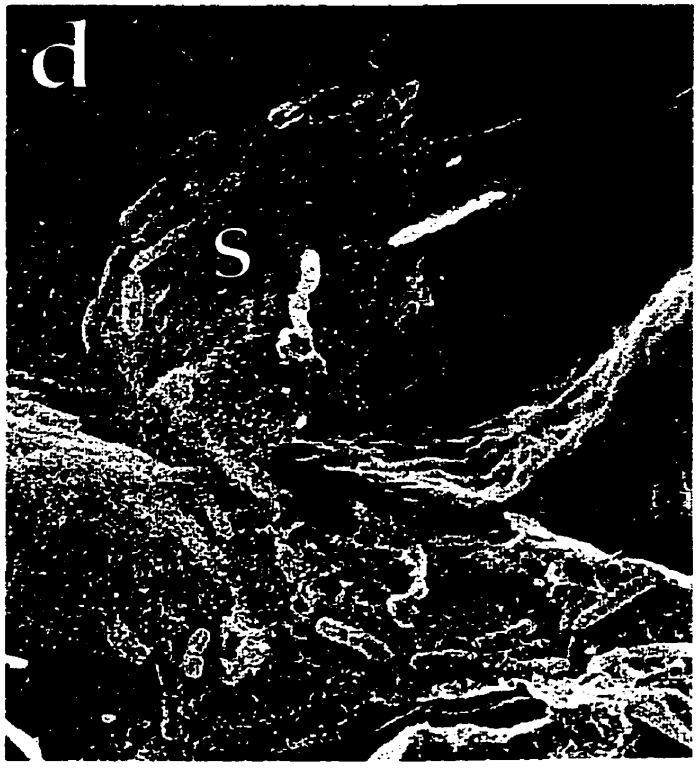
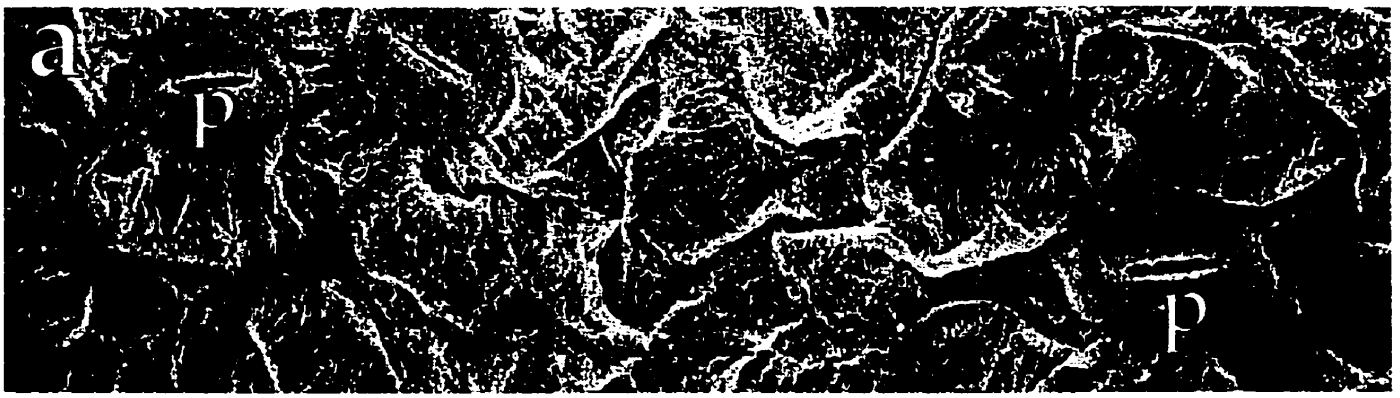
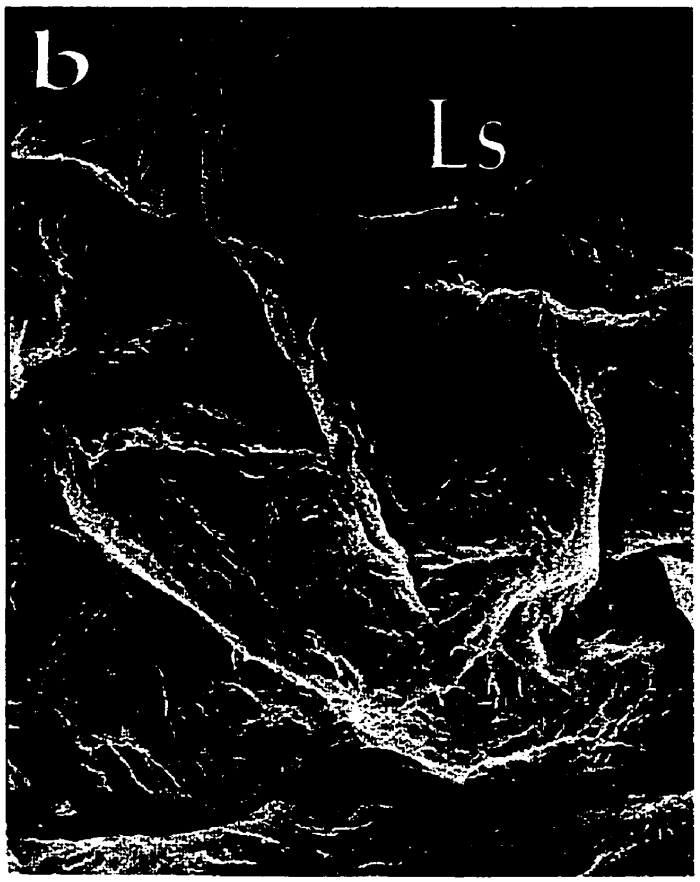
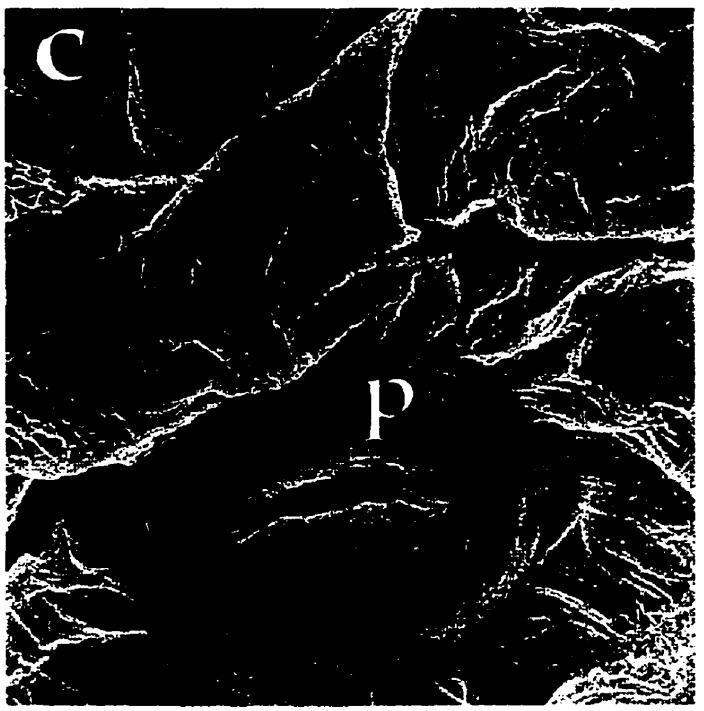

d
S

Fig. 38 Leaf surface of non-inoculated tomato seedlings. a: Leaf surface showing open pores. c: Leaf surface showing a closed pore. Bars on (a) and (c) represent 30 and 12 μm respectively.


a
p
p


b
Ls


c
p

the bacteria were colonizing an open or a closed pore. The number of cfu/leaf area was not calculated.

Every treatment consisted of four plants, each grown separately in a flask. One plant out of the four was randomly chosen for analysis on SEM. Each sample analyzed consisted of one leaf also randomly chosen.

3.21. Biocontrol properties of *Azospirillum brasilense* Cd/pRKTACC against *Fusarium oxysporum*

Tomato leaves infected with *Fusarium oxysporum* and treated with *A. brasilense* Cd/pRKTACC were less wilted and had less necrotic tissue than leaves treated with either non-transformed *A. brasilense* Cd or not treated with *A. brasilense* (Fig. 39). The number of bacterial cells on the surface of the leaves was not determined.

Treatment of tomato seedlings with *A. brasilense* Cd or Cd/pRKTACC of tomato seedlings prior to infection with *F. oxysporum* increased the survival of the plants to 95.8 and 75% respectively (Table 3), while plants treated only with *F. oxysporum* had a survival rate of 50%. Surprisingly, no differences were found between the fresh and dry weight of plants treated with *F. oxysporum* and the plants treated with non-transformed *A. brasilense* Cd (Fig. 40). However, *F. oxysporum* infected seedlings treated with Cd/pRKTACC had higher fresh and dry weight than plants treated with the non-transformed strain (although the differences were not statistically significant).

Fig. 39. Leaves of tomato plants infected with *Fusarium oxysporum* sp. *lycopersici*, *F. oxysporum* and A. *brasilense* Cd, or *F. oxysporum* and Cd/pRKTACC.

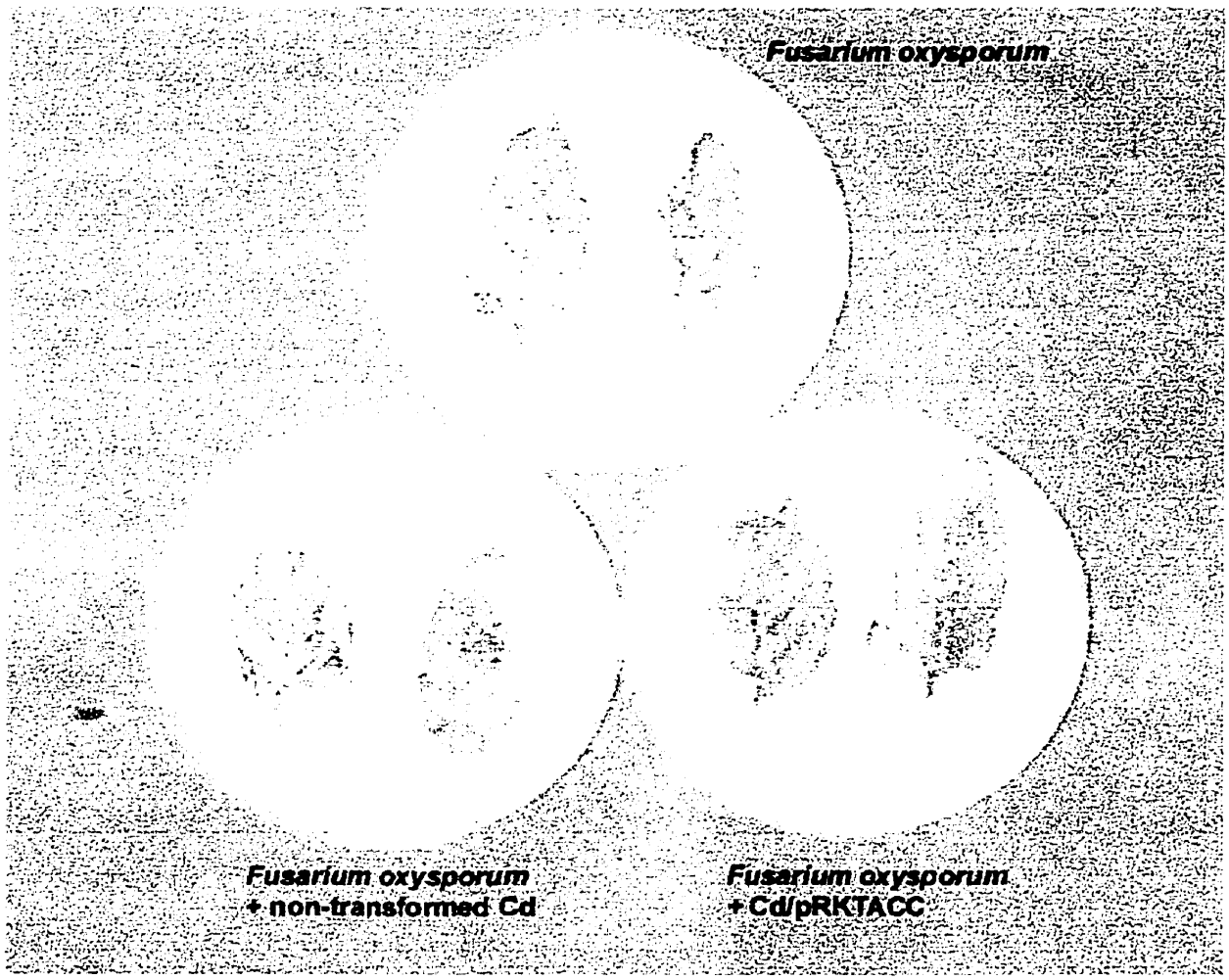
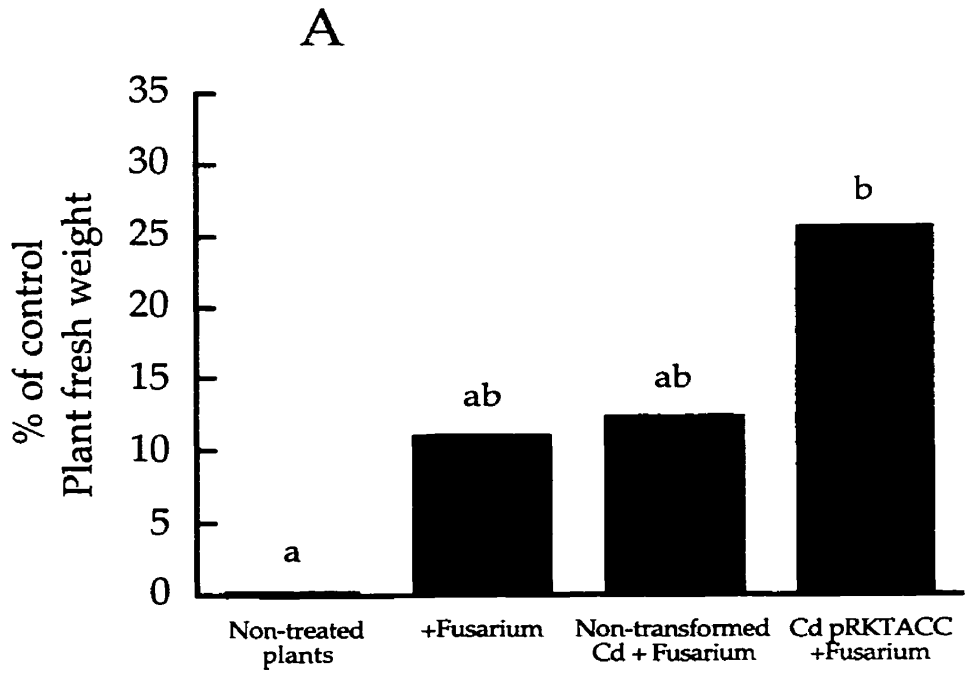
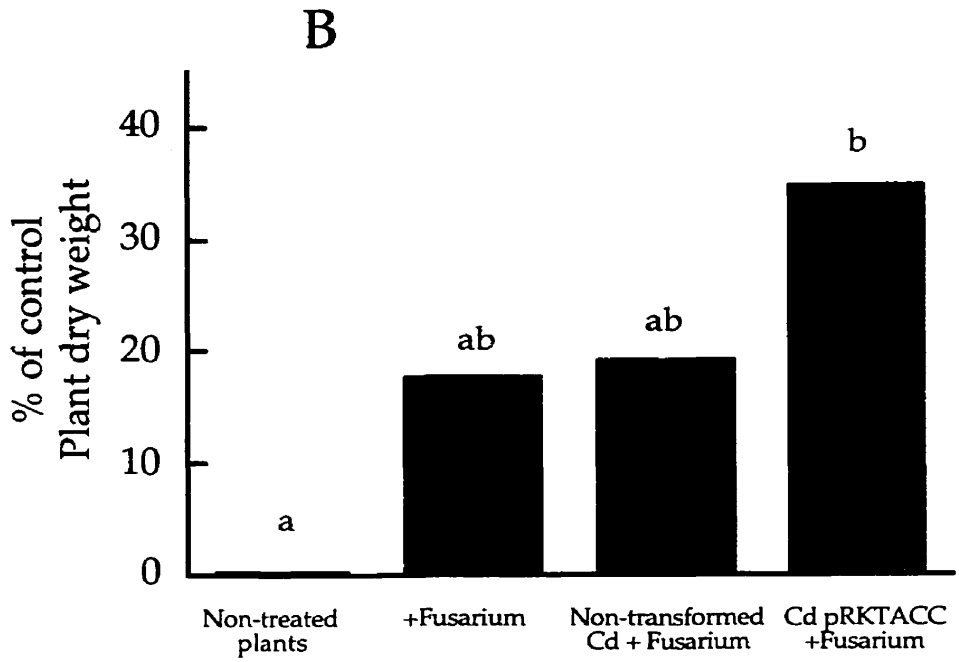

Fusarium oxysporum
Fusarium oxysporum
+ non-transformed Cd
Fusarium oxysporum
+ Cd/pRKTACC

Table 3. Survival (% of plants that developed from a total of 24 germinated seedlings) of tomato seedlings treated with *Fusarium oxysporum* sp. *lycopersici*, *F. oxysporum* and *A. brasilense* Cd, or *F. oxysporum* and *A. brasilense* Cd/pRKTACC.

Treatment	% of developed plants
Non-treated plants	66.7
+ *Fusarium*	50.0
Non-transformed Cd + *Fusarium*	95.8
Cd (pRKTACC) + *Fusarium*	75.0

Fig. 40. Fresh (A) and dry weight (B) of three weeks old tomato plants treated with *Fusarium oxysporum* sp. *lycopersici, F. oxysporum* and non-transformed *A. brasilense* Cd, or *F. oxysporum* and *Azospirillum brasilense* Cd/pRKTACC. Fresh and dry weight values for the control are 0.69 g and 44.8 mg respectively. Bars labeled with different letters differ significantly at $P \leq 0.05$ in one way ANOVA.


A
% of control
Plant fresh weight
35
30
25
20
15
10
5
0
a
ab
ab
b
Non-treated plants
+Fusarium
Non-transformed Cd + Fusarium
Cd pRKTACC +Fusarium


B
% of control
Plant dry weight
40
30
20
10
0
a
ab
ab
b
Non-treated plants
+Fusarium
Non-transformed Cd + Fusarium
Cd pRKTACC +Fusarium

3.22. Biocontrol properties of *Azospirillum brasilense* Cd/pRKTACC against the pathogen *Pythium aphanidermatum*.

Treatment of tomato seedlings with *P. aphanidermatum* killed all the plants. However, inoculation with *A. brasilense* Cd or Cd/pRKTACC previous to addition of the pathogen increased survival of the plants in 20.8 and 12.5% respectively (Table 4).

Inoculation of tomato seedlings with *A. brasilense* Cd or Cd/pRKTACC prior to infection with *P. aphanidermatum* decreased fresh weight of plants 24.4 and 12% respectively, as compared to non-infected plants (Fig. 41). Interestingly, plants treated with Cd/pRKTACC seemed to have higher fresh weight than plants treated with non-transformed *A. brasilense* Cd (the results are not significantly different). The number of bacterial cells was not determined.

3.23. Biocontrol properties of *Azospirillum brasilense* Cd/pRKTACC against the bacterial pathogen *Pseudomonas syringae*.

Treatment of tomato leaves with *A. brasilense* Cd/pRKTACC prior to inoculation with the pathogen *P. syringae*, decreased the percentage of necrotic tissue (around 30%), as compared to leaves treated with non-*transformed A. brasilense* Cd or leaves treated only with the pathogen (Fig. 42).

3.24. Colonization of mangroves by *A. brasilense* Cd and *A. halopraeferens* AU10

After three days of inoculation, the population of *A. brasilense* Cd was composed mainly of small colonies in which the cells were connected to one another by short fibrils (Fig. 43 c, d, arrows). In addition, some individual

bacteria were observed where these bacteria were also connected to the root surface by fibrils.

For *A. halopraeferens,* the colonization pattern was different from that observed with *A. brasilense. A. halopraeferens* was a better colonizer of the root surface. After three days of bacterial inoculation of mangrove seedlings, most bacteria were found as single cells embedded in a thick mucilaginous sheath on the root surface (Fig. 44 a, b). In addition, many bacteria were detected beneath the sheath (Fig. 44 c, arrows). In numerous areas along the roots, a halo (Fig. 44 d, thin arrows) surrounding the bacteria was detected.

Table 4. Survival (% of plants that developed from 24 germinated seedlings) of tomato seedlings treated with *Pythium aphanidermatum, P. aphanidermatum* and non-transformed *A. brasilense* Cd, or *P. aphanidermatum* and *Azospirillum brasilense* Cd/pRKTACC. The results are from one experiment.

Treatment	% of developed plants
Non-treated plants	66.7
+ *Phythium*	0
Non-transformed Cd + *Phythium*	20.8
Cd (pRKTACC) + *Phythium*	12.5

Fig. 41 Fresh weight of tomato plants treated with *Pythium aphanidermatum, Pythium aphanidermatum* and *Azospirillum brasilense* Cd, or *Pythium aphanidermatum* and Cd/pRKTACC. Fresh weight value for the control was 0.7g. Bars labeled with different letters differ significantly at $P \leq 0.05$ in one way ANOVA. The results are from one experiment.

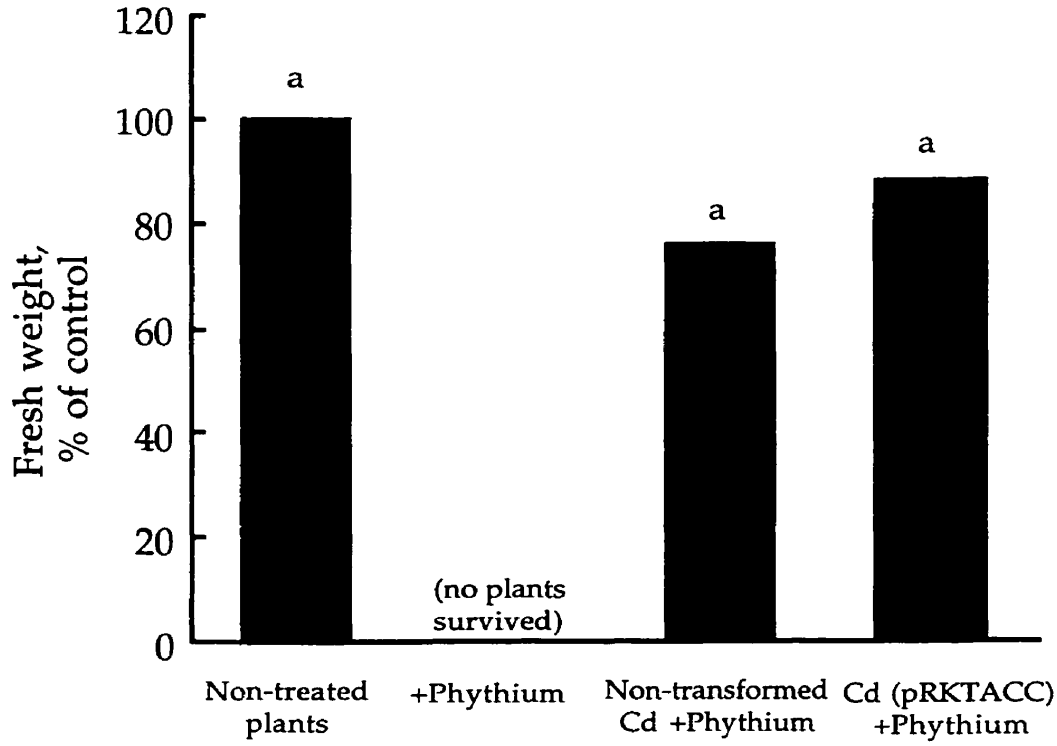

120
100
80
60
40
20
0
Fresh weight, % of control
a
a
a
(no plants survived)
Non-treated plants
+Phythium
Non-transformed Cd +Phythium
Cd (pRKTACC) +Phythium

Fig. 42. Leaves of tomato plants infected with *Pseudomonas syringae, P. syringae* and non-transformed A. *brasilense* Cd, or *P. syringae* and Cd/pRKTACC.

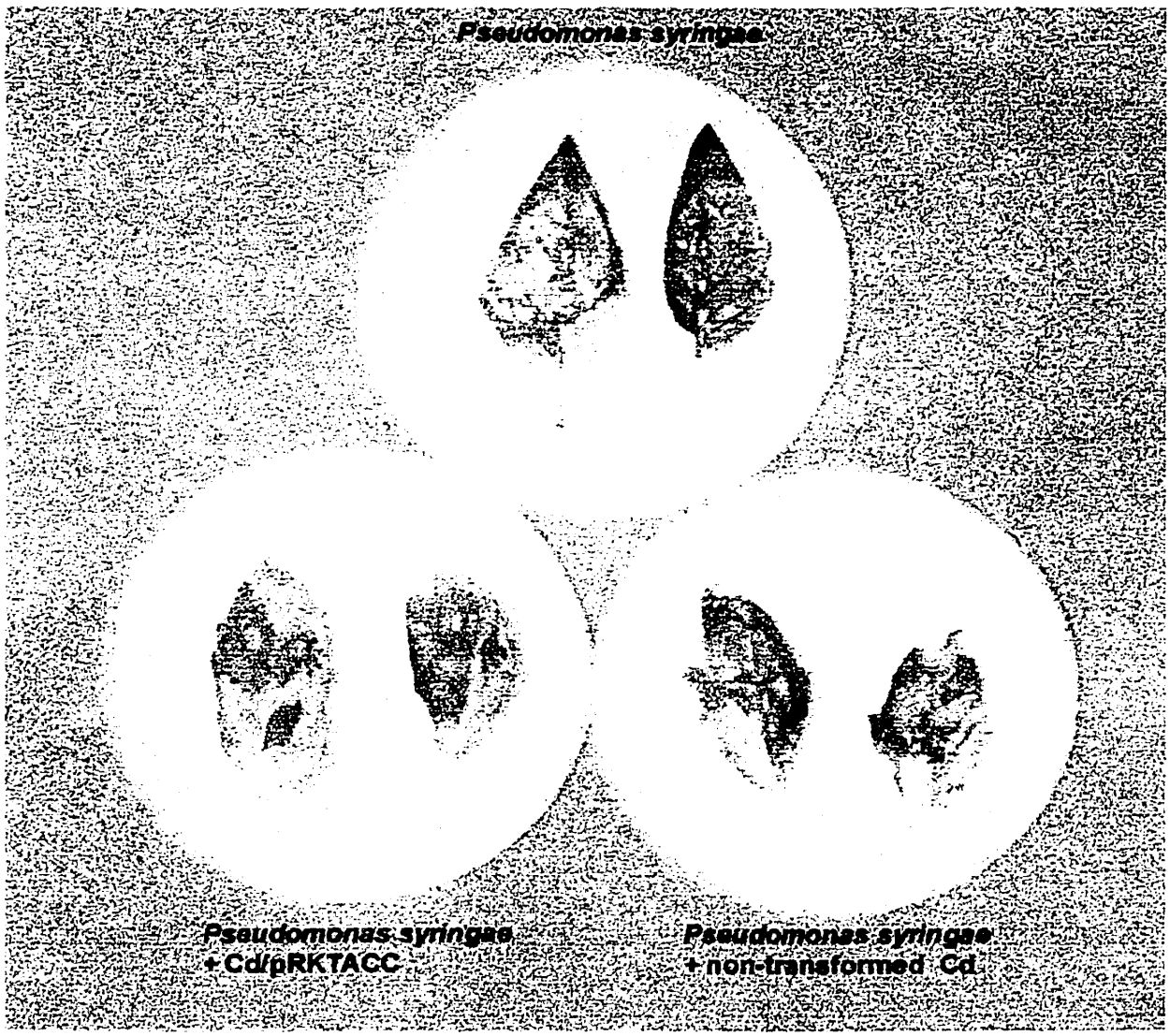

Pseudomonas syringae
Pseudomonas syringae
+ Cd/pRKTACC
Pseudomonas syringae
+ non-transformed Cd

Fig. 43 Root colonization of the mangrove *Avicennia germinans* (L) Stern by *A. brasilense* Cd three days after inoculation. a: Non-inoculated roots. b: magnification of (a) showing no bacteria on the root surface. c and d: Small colonies exhibit short (c) and long (d) fibrils (arrows). e: A single cell attaches to the root surface by fibrils (arrows). Abbreviations: Ab, *Azospirillum brasilense* Cd; Rs, root surface; bars represent 100 μm (a), 10 μm (b), and 1 μm (c,d,e).

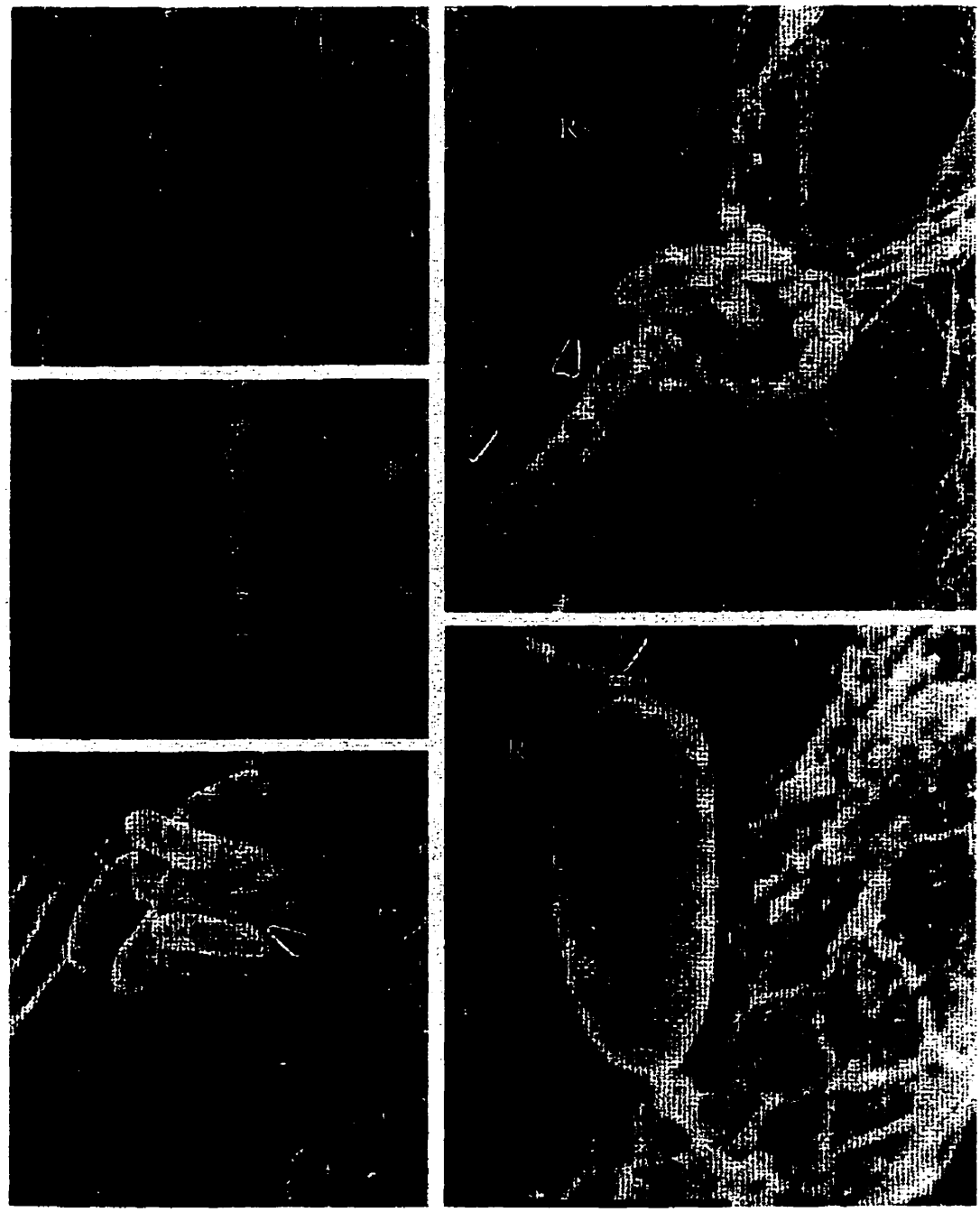
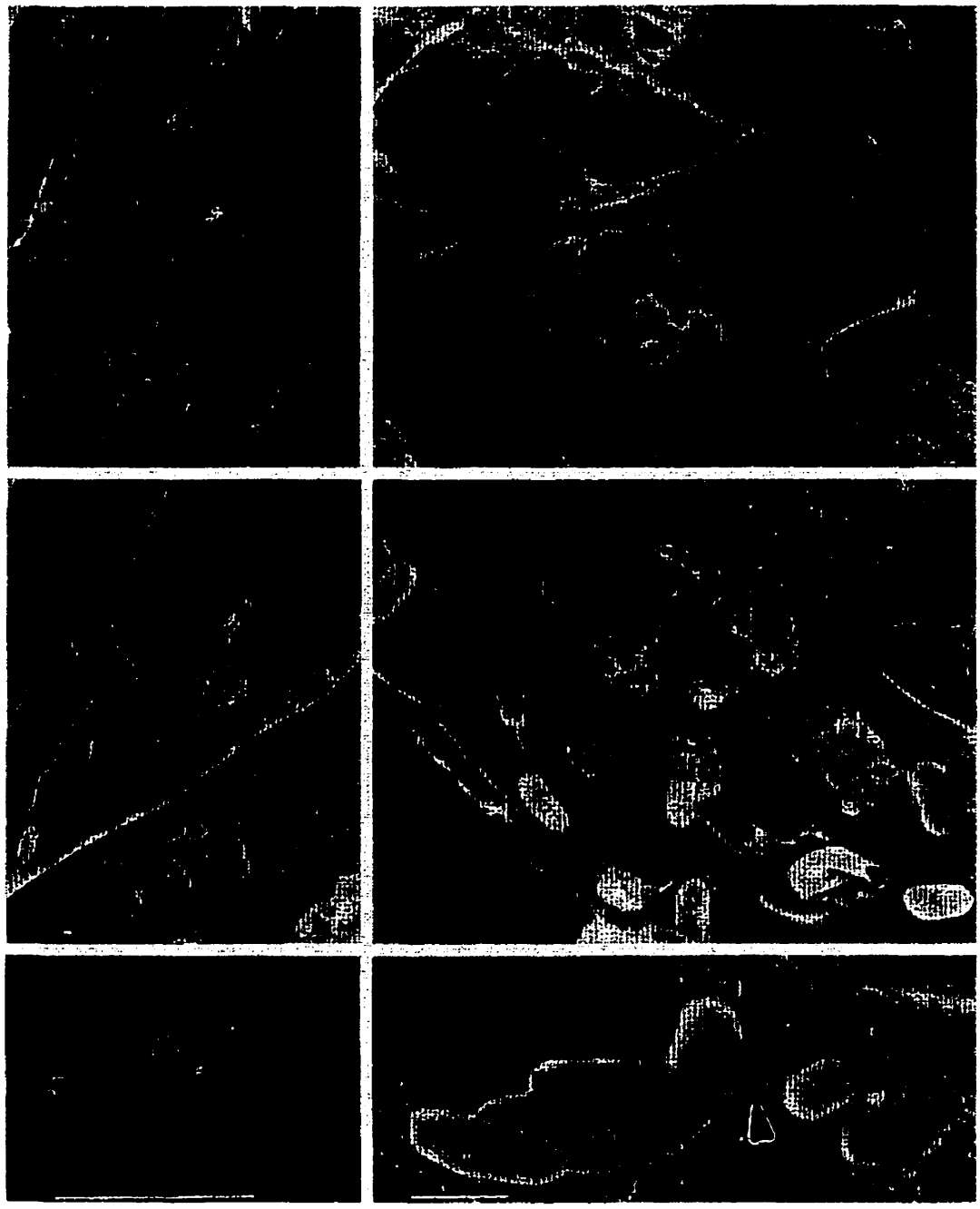


Fig. 44. Root colonization of the mangrove *Avicennia germinans* (L.) Stern by *A. halopraeferens* AU10 three days after inoculation. a, b: Single cells embedded in a thick mucilaginous sheath. c: single cells reside under the sheath (arrows). d: halos in the sheath (thin arrows) surrounding the bacteria, which are connected to the surface by fibrils (thicker arrows). e: small bacterial aggregates on the root surface. f: magnification of (e) showing fibrils (arrow) connecting the aggregate to the root surface. Abbreviations: Ah, *Azospirillum halopraeferens* AU10; Rs, root surface; s, sheath, h, halo. Bars represent 10 μm (a), 5 μm (b,c,e), and 1 μm (d,f).



4. Discussion

4.1. Isolation of leaf and flower colonizing bacteria with ACC deaminase activity

Plant growth promoting bacteria can be found not only in the rhizosphere, but on stems (Wong et al., 1994), leaves (Beattie and Lindow, 1999), flowers (Johnson and Stockwell, 1998), or in plant tissues internal to the epidermis (Glick et al., 1999). Currently, ACC deaminase activity is considered to be a ubiquitous phenomenon in soil bacteria with the ability to promote plant growth (Glick et al., 1995). However, prior to the work described in this thesis, no attempts had been made to determine the presence of PGPB with ACC deaminase activity from plant structures such as leaves or flowers. Considering that ACC can be synthesized on petals (Mor et al., 1985) and leaves (Fuhrer, 1982) it was inferred that finding bacteria with ACC deaminase activity on these plant structures would be highly plausible since the ability to degrade ACC would favorably select for their presence over other epiphytes. Since the medium utilized in the isolation procedure enriched for pseudomonads it was not a surprise to find that the two strains isolated from flowers and leaves belong to the genera *Pseudomonas*. Glick et al. (1995) utilized the same strategy for the isolation of PGPB with ACC deaminase activity from rhizosphere soil samples that originated from different geographical regions.

4.2. Selection of a broad-host-range plasmid for transfer of the ACC deaminase gene into *A. brasilense* Cd

To select a broad-host-range plasmid for transfer of the ACC deaminase gene into *Azospirillum* two vectors were considered: pRK415 (IncP) and pGSS15 (IncQ). IncP and IncQ plasmids have been previously and successfully used for transformation of *Azospirillum brasilense* (Holguin et al., 1999). Plasmid pRK290, a derivative of RP4 (Vande Broek et al., 1989) was able to replicate in a wide range of *Azospirillum* strains. Thus, it was assumed that pRK415 would replicate in *Azospirillum* since this plasmid is also a derivative of RP4.

Transformation of *A. brasilense* Cd with pGSS15 resulted in a significant decrease in its growth rate compared to either non-transformed *A. brasilense* Cd or to *A. brasilense* Cd transformed with plasmid pRK415 (Fig. 5). Previous studies have shown that the physiology of bacteria transformed with broad-host-range plasmids such as pGSS15 that encode the synthesis of large amounts of foreign protein (e.g. ß-lactamase), can be dramatically altered. This debilitation, which can affect the ability of a bacterium to fix nitrogen or to synthesize siderophores, has been attributed to the imposition of a metabolic load on the transformed cells as a consequence of the synthesis of high levels of foreign proteins (Glick, 1995 b; Glick et al., 1985; Hong et al., 1995). Another reason for the debilitation in pGSS15 transformants may be consequence of the higher copy number of IncQ plasmids compared to IncP plasmids (Barth et al., 1981). Consequently, to avoid debilitated *Azospirillum* transconjugants, pRK415 was chosen over pGSS15 as a vector for transferring the ACC deaminase gene into *A. brasilense.*

4.3. Expression of the ACC deaminase gene in *Azospirillum*

Escherichia coli cells harboring pRKACC (*acdS* and its regulatory region cloned in pRK415) showed high ACC deaminase activity, comparable to that in *Enterobacter cloacae* UW4 (Table 1). When pRKACC was transferred into *A. brasilense* Cd and Sp245, the transconjugants did not have any measurable ACC deaminase activity. To test for the presence of an inhibitor of ACC deaminase activity, the cell lysate of *A. brasilense,* Cd was concentrated and added separately to a lysate prepared from the cells of *Enterobacter cloacae* CAL2, previously shown to have a high level of ACC deaminase activity. The addition of various amounts of *A. brasilense* lysate to the CAL2 lysate did not alter the ACC deaminase activity of CAL2 (Table 2).

Expression of the *acd*S gene in *Enterobacter cloacae* UW4 is controlled by a complex regulatory system that involves the participation of a member of the leucine-responsive regulatory protein (LRP) family (Grichko and Glick, 2000) (Fig. 6). In *E. coli,* LRP is a global regulatory protein and controls more than 40 genes and operons involved in diverse cellular processes (Napoli et al., 1999). LRP-like proteins have recently been reported to be major transcriptional regulators in many bacteria (Friedberg et al., 1995). In *Enterobacter cloacae* UW4, the gene for an LRP-like protein is encoded upstream of *acdS* on the opposite strand. Analysis of the LRP-like protein upstream region revealed an A/T rich region similar to the degenerate consensus sequence to which LRP proteins bind (Rhee et al., 1996). In this A/T rich region 12 out of the 18 nucleotides that are deemed to represent the LRP binding site are identical to the LRP binding region from *Enterobacter aerogenes* (Grichko and Glick, 2000). In addition, the upstream region of *acd*S in *Enterobacter cloacae* UW4 DNA contains an FNR (fumarate and

nitrate reduction regulator) binding consensus sequence (Grichko and Glick, 2000). FNR is a transcriptional activator for many anaerobically regulated genes (Jordan et al., 1997). While a detailed understanding of the mechanism of transcriptional regulation of the *Enterobacter cloacae* UW4 *acd*S gene remains elusive, it is nevertheless clear that regulation of this gene involves a complex set of interactions with several regulatory proteins (Fig. 6).

The *A. brasilense* pRKACC transformants showed no measurable activity of ACC deaminase in spite of the fact that the transconjugants contained the entire upstream region of *acdS*, including the gene for the LRP-like protein. Moreover, this construct was readily expressed in *E. coli* (Table 1) and has been expressed in other gram-negative bacteria such as *Pseudomonas putida* (Shah et al., 1998) and *P. fluorescens* (Wang et al., 2000). Comparisons of 16S rRNA sequences provide a measure of phylogenetic relatedness between bacteria, *Pseudomonas*, *E. coli*, and *Azospirillum* are classified as proteobacteria (bacterial species traditionally thought of as gram-negative bacteria). However, *Pseudomonas* and *E. coli* are classified within the Beta subdivision, while *Azospirillum* is classified within the Alpha subdivision (Prescott et al., 1996). It is possible that *A. brasilense*, being distantly related from *Escherichia coli* and *Pseudomonas* spp., lacks some of the proteins that are essential for the expression of *acd*S. Alternatively, the *A. brasilense* strains may be incapable of recognizing the promoter of the *Enterobacter cloacae* LRP-like protein. Sequence alignment analysis of the LRP-like protein upstream region with *Azospirillum* promoters regions performed using LALIGN, Local Pair Alignment (Huang and Miller, 1991) or with non-characterized *Azospirillum* gene upstream regions, showed little identity between the regulatory sequences. A search for similarity between the LRP-like protein

upstream region and any other DNA sequences present in the data bank showed that two short sequences with runs of A's in $_{p}$*acdR* (Fig. 7) are similar to sequences found in *Caenorhabditis elegans* and in *Homo sapiens*. The latter sequences probably represent intron regions which are usually rich in runs of A's and T's (Zhang, 1998). This A/T rich region is not the LRP binding region detected by Grichko and Glick (2000) (Fig. 6).

The lack of expression of the *acd*S gene in the *A. brasilense* transconjugants could be due to competition for the same DNA binding site between the LRP-like protein and NifA; NifA acts as a DNA binding protein that normally recognizes and binds to two overlapping upstream activator sequences (UAS's) with the consensus sequence TGT-N_{10}ACA (Passaglia et al., 1995). Analysis of the upstream region of the *Enterobacter cloacae* UW4 LRP-like protein gene revealed the presence of one UAS with this consensus sequence (Li and Glick, submitted for publication). Because NifA normally binds to two overlapping UAS it could be argued that one UAS would not be sufficient for stable binding; however, it has been shown that NifA does not require two overlapping UAS to activate the *nif*H promoter (Passaglia et al., 1995). Furthermore, even though NifA is inactive in the presence of oxygen, its binding activity is still functional (Passaglia et al., 1998).

4.4. Cloning of the ACC deaminase gene under the control of the *lac* promoter

To overcome the lack of expression of *acdS* in *Azospirillum*, the gene was cloned in pRK415 under the control of the *Escherichia coli lac* promoter and transferred into *A. brasilense* Cd and Sp245 via *Escherichia coli* by conjugation. The

transconjugants showed high ACC deaminase activity, similar to that exhibited by *Enterobacter cloacae* UW4 (Fig. 9). As expected, *acdS* expression in *A. brasilense* Cd or Sp245 was not induced by IPTG, an inducer of the *lacZ* gene in *Escherichia coli*. No significant differences were found between the ACC deaminase activity of *A. brasilense* Cd and *A. brasilense* Sp245, although the ACC deaminase activity of the latter was lower in the absence of IPTG.

4.5. Elongation of seedling roots followed by inoculation with *A. brasilense* Cd transformed with pRKLACC

In a number of different plants, ethylene stimulates germination and breaks the dormancy of the seeds (Esashi, 1991); however, if the level of ethylene following germination is too high, root elongation is inhibited (Jackson, 1991). Plant growth promoting bacteria with ACC deaminase activity lower the levels of ACC by breaking it down to α-ketobutyrate and ammonia; the bacteria, by lowering the levels of ACC reduce the level of ethylene in plants and thereby prevent impairment of root growth (Glick et al., 1998; Li et al., 2000). This bacterial stimulation of root elongation could enhance the survival of seedlings, especially during the early stages of growth (Glick, 1995 a).

To determine if the expression of an ACC deaminase gene in *A. brasilense* could contribute to lowering the levels of ethylene in the early development of plants, canola, tomato and wheat seeds were inoculated with *A. brasilense* Cd transformed with an ACC deaminase gene. The gnobiotic root elongation essay has proven to be very useful for analyzing the effects of high levels of ethylene in the early development of plants (Jackson, 1991).

The transfer of *acdS* gave both *A. brasilense* Cd and *Escherichia coli* the ability to induce root elongation in canola plants (Fig. 11). This presumably occurs as a result of the acquired ability of these bacteria to break down ACC, the precursor of ethylene. It has been previously shown that transfer of *acdS* to PGPB exhibiting no inherent ACC deaminase activity gave the bacteria the ability to promote root elongation (Shah et al., 1998; Wang et al., 2000). In this work, root elongation in tomato seedlings inoculated with non-transformed *A. brasilense* Cd was enhanced only when inoculated with 10^8 cfu/mL (Fig. 12); lower inoculum densities appeared to have no effect on tomato roots. Similar results were found by Hadas and Okon (1987) who reported that the optimal *A. brasilense* Cd inoculum level for tomato was $>10^8$ cfu/mL. However, seedlings treated with *A. brasilense* Cd/pRKLACC did respond to an inoculum level of 10^7 cfu/mL. This result indicates that root elongation of tomato seedlings was probably induced by the ACC deaminase activity in *A. brasilense* Cd transconjugants. When inoculated with *A. brasilense* Cd/pRKLACC at 10^8 cfu/mL, the length of seedling roots increased significantly compared to seedling roots inoculated with the same density of non-transformed cells. This suggests that *A. brasilense* Cd possesses inherent mechanisms for plant growth promotion and that the acquisition of ACC deaminase activity, and hence the ability to break down ACC enhances its ability to promote root elongation.

In the case of wheat, plants inoculated with *A. brasilense* Cd/pRKLACC did not respond differently from plants inoculated with non-transformed bacterial cells (Fig. 13 A, B, C, E). This probably reflects the lower sensitivity of wheat compared to canola and tomato to ethylene (Hall et al., 1996). However,

these results do not necessarily imply that wheat plants grown under different conditions or wheat plants in different growth stages will not respond to inoculation with *A. brasilense* Cd/pRKLACC.

It is interesting that inoculation of wheat plants with *A. brasilense* Cd/pRKLACC at a concentration of 10^7 cfu/mL promoted root dry weight whereas inoculation of wheat with non-transformed *A. brasilense* Cd at 10^8 cfu/mL (Fig. 13 D), inhibited root dry weight. Reports of wheat growth inhibition by high levels of *A. brasilense* Cd inoculum are widespread in the literature (for a review see Bashan and Levanony, 1990). In this work, the differences in root dry weight between non-treated plants and plants inoculated with high inoculum levels of *A. brasilense* Cd are not statistically significant. However, the response of the plant to high inoculum levels of *Azospirillum* seems to be more dramatic in longer term experiments (Bashan, 1986).

4.6. Inhibition of plant growth caused by inoculation with high inoculum levels of *Azospirillum*

Root dry weights of two week-old tomato plants treated with high inoculum levels of non-transformed *A. brasilense* Cd were inhibited (Fig. 15), while those plants inoculated with high levels of *A. brasilense* Cd/pRKLACC were not. IAA is known to induce ACC synthase (Kende, 1993), thereby increasing the concentration of ethylene in plants. The lack of inhibition in growth of tomato plants after inoculation with *A. brasilense* Cd/pRKLACC might reflect the ability of these bacteria to break down the ACC induced by bacterial IAA. In this regard, inactivating the *ipdC* gene in *A. brasilense* Sp245, encoding a

key enzyme in the indole-3-pyruvic acid pathway, drastically reduced the impact of this strain on wheat root development (Dobbelaere et al., 1999). Inoculation of wheat plants with high levels (10^8-10^9 cfu/mL) of an *ipdC* mutant of *A. brasilense* Sp245 did not inhibit root growth as observed for the wild type strain. The effect of inoculum concentration on root morphology could be mimicked by applying increasing concentrations of IAA to the seeds.

4.7. IAA synthesis in *A. brasilense* Cd/pRKLACC transformants

In minimal medium, the presence of pRKLACC in *A. brasilense* Cd can reduce the growth rate and IAA synthesis of the bacterium by approximately 25% (Fig. 10 A, B). However, in nutrient broth, *A. brasilense* Cd/pRKLACC cells showed the same ability to produce IAA as the non-transformed bacterium (Fig. 10 C, D). These results suggest that synthesis of ACC deaminase imposes a metabolic load on *A. brasilense* Cd only when nutrients are scarce and thus energy sources are limited. To infer that pRKLACC interferes with the ability of *A. brasilense* Cd to synthesize IAA when associated with the root system would be premature. Recent evidence shows that the biosynthesis of IAA under *in vitro* conditions can not be extrapolated to *in situ* conditions: A transcriptional fusion of *ipdC* to an ice nucleation reporter gene in the epiphytic bacterium *Erwinia herbicola* showed that expression of *ipdC* was increased 1000-fold on pear flowers as compared to liquid cultures in minimal medium with or without tryptophan (0.02% final concentration) (Brandl and Lindow, 1997). Interestingly, the ice nucleation activity of cells grown in solid medium was higher than in cells grown in liquid medium of the same composition; this increase in expression was comparable to the expression levels detected on plants. Furthermore, the ice

nucleation activity was increased upon exposure of the cells to low water availability. IAA in epipythic bacteria may play a role in adaptation to conditions experienced on plant surfaces. Exogenously applied auxins have been demonstrated to stimulate the release of monosaccharides and oligosaccharides from the plant cell wall. It thus seems reasonable to assume that bacterial IAA production benefits the producing cells by similarly increasing nutrient availability in the vicinity of bacteria (Brandl and Lindow, 1997). The expression of *ipdC* may be involved in the recognition of, or adaptation to, conditions related to growth on a surface; the response to low water availability seems logical since free water may frequently be absent in the phylloplane (Brandl and Lindow, 1997).

Other factors that complicate our understanding of the mechanisms involved in IAA synthesis of *Azospirillum* when associated to plants are that several pathways for IAA synthesis are found in *Azospirillum brasilense,* and that scant data is available regarding the regulation of IAA synthesis in this bacterium (Patten and Glick, 1996; Prinsen et al., 1993; Vande Broek et al., 1999).

4.8. Cloning of the ACC deaminase gene under the control of the tetracycline resistance promoter

The *Escherichia coli lac* promoter is considered fairly strong, allowing high levels of transcription of a cloned gene (Snyder and Champness, 1997). Inferring that a construct including the ACC deaminase gene under the control of a constitutive promoter weaker than the *lac* promoter might impose less metabolic load on *Azospirillum,* it was decided to clone *acdS* under the control of a tetracycline resistance promoter. Previous works showed that this tetracycline

resistance promoter was recognized by *Azospirillum* (Holguin et al., 1999). As expected, vector pRKTACC, which included the *acdS* gene under the control of the Tet^r promoter, did not affect the growth rate and IAA synthesis of *A. brasilense* Cd as compared to pRKLACC, which decreased the cells growth rate and the ability of *Azospirillum* to synthesize IAA (Figs. 21, 23). However, it must be mentioned that measurements on absorbance for determining growth curves can be misleading, since dead cells are also being measured.

4.9. Survival of *Azospirillum* transformants in soil and in roots

The survival in soil of all *A. brasilense* Cd transformants, including *A. brasilense* transformed with pRK415 which did not contain *acdS*, decreased with time. However, the survival of non-transformed *A. brasilense* Cd remained relatively stable during the duration of the experiment (Fig. 24). Surprisingly, the survival of *Azospirillum* in the bulk soil (i.e., the fraction not affected by root activity), is a neglected subject in the literature and little is known about it (Bashan, 1999). Additionally, the existing reports of survival of *Azospirillum* in soils show disparity. The lack of consistency may be due to inadequate assessment of soil types used in the experiments concomitant with a lack of knowledge of soil factors affecting the survival of PGPB. A survival study of inoculated (10^7-10^8 cfu g^{-1} soil) *A. brasilense* Cd in Brazilian and Canadian soils showed that in most soils the population declined rapidly within the first two weeks and settled at a level of about 10^5 cfu/g soil. In Israeli soils from arid, semiarid, or mountainous regions, viability of *A. brasilense* rapidly declined or the population disappeared completely below detectable levels within 35 days after inoculation (Bashan, 1999). In contrast, *A. brasilense* populations in the arid

soils of Baja California (México) remained stable or even increased during the first 45 days of inoculation. In the latter study, clay content, N, organic matter, and water holding capacity were positively correlated with *A. brasilense* viability (Bashan et al., 1995).

Persistence of *Azospirillum* spp. in the soil is crucial in some field applications, i.e., for perennial plants and trees (Bashan, 1999). Considering the poor survival of *A. brasilense* Cd/pRKTACC in soil, the use of these transformants under such conditions would probably require multiple inoculations. Another possibility would be integration of the introduced ACC deaminase gene into the host bacterial chromosomal DNA. It has been observed that such an approach can minimize the impact of the metabolic load and enhance the stability of the transformed host cell (Glick and Pasternak, 1998).

An assessment of the survival of non-transformed *A. brasilense* Cd, Cd/pRK415, Cd/pRKLACC and Cd/pRKTACC in tomato one month after inoculation gave similar values for all cells (cfu/g dry weight roots: 4×10^5, 3.5×10^5, 2.7×10^5, and 3.3×10^5, respectively). This level of colonization in *Azospirillum* is within the average values reported by other authors (Bashan and Levanony, 1990).

4.10. Flooding of tomato plants inoculated with non-transformed *A. brasilense* Cd, Cd/pRKLACC, and Cd/pRKTACC

Different types of perturbations in plants can be environmentally generated and can create stress conditions that make the plants more responsive to ethylene already in the tissue, or cause the plants to synthesize additional ethylene thereby inducing ripening, senescence, or abscission (Abeles et al.,

1992). Inoculation of plants with plant growth-promoting bacteria that have ACC deaminase activity may ameliorate some of the effects of these stresses on plants. In this regard, the bacterium *Pseudomonas putida* GR12-2, which produces ACC deaminase, promoted the development of canola seedlings growing in saline soil or exposed to cold night temperatures (Glick et al., 1997), and the bacterium *Kluyvera ascorbata* SUD165, which also produces ACC deaminase, promoted plant growth in the presence of high levels of nickel in the soil (Burd et al., 1998).

Flooding is another type of environmental stress that can increase the levels of ethylene within the plants. In this work inoculation of tomato plants with *A. brasilense* Cd/pRKTACC protected the plants against flooding by decreasing the levels of epinasty (Figs. 27-29). It has been demonstrated that epinastic growth of the petioles is a response to accelerated rates of ethylene synthesis. English et al. (1995) found that under flooding conditions, tomato plants transformed with an antisense construct to one isoform of an ACC oxidase gene suffered less degree of epinasty. It has been found that in roots of flooded tomato plants, ACC synthesis continues or is enhanced, whereas oxidation to ethylene is blocked by the absence of oxygen (English et al., 1995). Some of the accumulated and un-metabolized ACC is then transported, in the transpiration stream, to the aerial tissues. Here, the presence of oxygen allows ACC oxidase-mediated conversion to ethylene in amounts sufficient to promote epinasty (Bradford and Yang, 1980; English et al., 1995). In this work, inoculation of tomato plants with *A. brasilense* Cd/pRKTACC, besides reducing epinasty during flooding conditions, increased the shoot fresh and dry weight as compared to plants inoculated with non-transformed *A. brasilense* Cd (Fig. 25). An increase in the levels of ethylene in plants may result in growth inhibition,

promotion of senescence of plant organs as well as necrosis of plant tissues (Fluhr and Mattoo, 1996). The inoculation of plants with *A. brasilense* Cd/pRKTACC probably reduced the level of ACC and hence the level of ethylene in the shoots thus leading to higher fresh and dry weight as compared to plants inoculated with non-transformed *A. brasilense* Cd.

Inoculation of tomato plants with non-transformed *A. brasilense* Cd or *A. brasilense* Cd/pRKTACC, decreased the root/shoot ratio of the plants in both fresh and dry weight (data not shown). An analysis of data from literature (Bashan and Dubrovsky, 1996) on shoot and root mass of crop grasses inoculated with *Azospirillum* spp., revealed that inoculation with *Azospirillum* spp. decreased the root/shoot ratio in 50% of the cases. The authors conclude that *Azospirillum* spp. participates in the partitioning of dry matter (both carbon compounds and minerals) at the whole plant level by affecting root functions.

In this work tomato plants inoculated with *A. brasilense* Cd/pRKTACC had a higher chlorophyll content than plants inoculated with *A. brasilense* Cd/pRKLACC or non-transformed *A. brasilense* Cd (Fig. 30). Glick et al. (1997) found that the chlorophyll content in shoots of canola plants inoculated with *Pseudomonas putida* GR12-2 which exhibits ACC deaminase activity, was higher than in plants treated with its mutant lacking ACC deaminase activity or in non-treated plants.

Generally speaking, the effect of *A. brasilense* Cd/pRKLACC on flooded tomato plants was not significantly different from non-transformed *A. brasilense* Cd. This means that flooded plants inoculated with *A. brasilense* Cd/pRKLACC are not more protected from flooding than are plants inoculated with non-transformed *A. brasilense* Cd. Poor survival of *A. brasilense* Cd/pRKLACC in

roots does not explain the inability of *A. brasilense* Cd/pRKLACC to protect plants against stress ethylene, since both *A. brasilense* Cd/pRKLACC and *A. brasilense* Cd/pRKTACC showed similar rate of survival in the roots (~10^5 cfu/g dry wt. roots).

4.11. The effect of *Azospirillum* on plants grown under non-optimal nutrient conditions

In flooding experiments, the differences in plant growth parameters between non-treated tomato plants and plants treated with either non-transformed or transformed *A. brasilense* Cd were striking (Figs. 25, 29). It has been previously observed that the effect of *Azospirillum* inoculation on plants is more pronounced when the plants are not grown under optimal nutrient conditions, as was the case in this work. The effect of *A. brasilense* on plants was diminished when the proportion of organic matter in the soil exceeded 1% (Fallik, et al., 1988). Soils rich in organic matter may provide enough nutrients for the plant and the effect of bacterial inocula is therefore obscured (Bashan, 1999). Addition of a bacterial mixture composed of *Azospirillum brasilense, Azotobacter chroococcum,* and *Klebsiella pneumoniae* to cucumber seeds in soil without nitrogen amendment, increased leaf area (22%) root dry weight (83%), shoot dry weight (30%), and nitrogen content (48%) as compared to non-inoculated plants (Hassouna et al., 1998). Sweet potato plants grown in sandy soils and inoculated with *Azospirillum* strain UPMB14, with 1/3 of the nitrogen recommended for sweet potato production, produced higher root yield, and had higher nitrogen content in the roots and leaves than plants given the normal amount of nitrogen fertilizer. Inoculation with *Azospirillum* was more effective when initial nitrogen

fertilizer at one third of the recommended rate was applied one week after planting. This approach resulted in a 74% increase in yield over the control (Saad et al., 1999). Young cacti, inoculated with *Azospirillum brasilense*, and grown in eroded urban soil (85% basalt rock, 2.8% organic matter, and 0.05% nitrogen content) had a survival rate of 76% after 3.5 years, while only 3 out of 63 non-inoculated plants survived after 2 years (Bashan et al., 1999). Piccoli et al. (1999) found that at high concentrations of nitrogen, while there was still rapid bacterial growth, gibberellin production by *A. lipoferum* was inhibited, as seen in cultures of the fungi *Gibberella fujikuroi* and *Fusarium moniliforme* in which gibberellin production begins once nitrogen is exhausted in the medium.

The interaction between PGPB and plants, although not strictly symbiotic, is intricate and complex, and probably started to develop since the time plants appeared on the planet (Prescott et al., 1996). It is quite possible that PGPB and plants have evolved mechanisms to induce expression of genes involved in a better exploitation of mutual resources (Sturz and Nowak, 2000). It thus does not seem surprising that when plants are grown under non-optimal growing conditions the ability of bacteria to help the plants is enhanced.

4.12. Attachment of *Azospirillum* to tomato leaves.

All *Azospirillum brasilense* strains, including both non-transformed and transformed strains, showed capacity to attach to tomato leaves (Figs. 32-37). There is evidence suggesting that bacteria can modify their environment to enhance colonization of plants, such as by increasing local nutrient concentrations or by producing a layer of extracellular polysaccharides. This

habitat modification may occur on the surface of leaves, as well as in the leaf interior (Beattie and Lindow, 1999).

The scanning electron microscopy micrographs show that all *Azospirillum* strains tend to colonize the leaves forming aggregates. Studies on bacterial colonization of leaf surfaces demonstrate that bacteria do not occur in a uniform pattern, but are localized in particular sites, the aggregated nature of bacterial cells of plant surfaces being a conspicuous feature (Beattie and Lindow, 1999).

The micrographs in this work (Figs. 32-37) reveal strands of amorphous material that emanate from and between bacterial cells on leaves. In these images, bacteria are closely packed but clearly embedded in an amorphous material that is presumably composed of exopolysaccharides of bacterial origin. Exopolysaccharides may anchor cells to the leaf surface and prevent cells from desiccation. The retention of water in the highly hygroscopic polysaccharide matrix should increase the water available to the bacteria. For example, purified exopolysaccharide from several phytopathogens induced persistent water soaking after introduction by infiltration into leaves (Beattie and Lindow, 1999).

It is possible that in this work, the use of a minimal medium with fructose and ammonium chloride as carbon and nitrogen sources, respectively, contributed to the ability of the cells to attach to the leaf surface. Burdman et al. (1998) found that growth of *A. brasilense* Cd in such a medium resulted in flocculation (formation of aggregates visible to the naked eye) after 24 h. These cells were rich in poly-β-hydroxybutyrate which is known to contribute to the survival of the cells under nutrient and water stress conditions (Sadasivan and Neyra, 1985). Pereg-Gerk et al. (1998) found that in *A. brasilense* Cd the

production of capsular polysaccharides, the flocculation process in culture, and the colonization of root surface are related factors, controlled by one gene, *flcA*.

Many bacteria with the ability to flocculate have been shown to produce extracellular fibrils that are efficient in polymer bridging; such fibrils are of a cellulosic nature in many species (Beattie and Lindow, 1999). The detailed composition and structure of *Azospirillum* exopolysaccharides are still unknown. It was found that these polysaccharides are fluorescent in the presence of calcofluor, suggesting the presence of cellulosic 1,4 β-glucans. Del Gallo (1989) suggested the involvement of a cellulosic polysaccharide in aggregation of *Azospirillum*; however, no cellulose fibrils were detected in *A. brasilense* strain Cd by Madi and Henis (1989).

After one day of inoculation, the aggregates of *A. brasilense* Cd/pRKLACC were visible in the scanning electron microscopy samples. However, after four days of inoculation the cells were covered by a sheath and were difficult to visualize (Fig. 33). Dufrêne and Rouxhet (1996) found that the cell surface composition varies throughout the growth cycle of *Azospirillum brasilense*: As the cells went from logarithmic to stationary phase there was an increase in cell adhesiveness accompanied by an increase in cell surface protein and cell surface hydrophobicity. These characteristics were correlated with an increase in cell adhesiveness to glass and polystyrene.

The pattern of attachment to tomato leaves of *A. brasilense* strains as compared to *Pseudomonas putida* AC8 was different (Figs. 32-37). The *A. brasilense* strains attached mainly as cell aggregates and it was common to find them colonizing crevices on the surface of the leaf. However, *P. putida* cells colonized the leaf surface in an uninterrupted fashion forming continuous strands of cells.

AC8*A. brasilense* Sp245/pRKLACC was the only strain found colonizing a leaf pore or stomata (Fig. 36). According to Beattie and Lindow (1999), in studies using electron microscopy, one of the most common sites of bacterial colonization was at stomata. Stomata can be used by pathogenic bacteria as a means of entry into a plant (Romantschuk and Bamford, 1986). By using strain specific monoclonal antibodies against *A. brasilense* Sp245, Schloter and Hartmann (1998) detected *A. brasilense* Sp245 in the inner root tissue forming microcolonies in intercellular spaces. Colonization of the inner root tissue by strain *A. brasilense* Sp245 was previously suggested by Baldani et al., (1986) and by Y. Bashan (personal communication). In this work, the possibility of internal colonization of tomato leaves by *A. brasilense* Sp245/CdpRKLACC was not explored further.

There is evidence to suggest that IAA is involved in the epiphytic fitness of some PGPB. The secretion of IAA by the bacterium may modify the microhabitat of epiphytic bacteria by increasing nutrient leakage from plant cells: enhanced nutrient availability may better enable IAA-producing bacteria to colonize the phyllosphere (Brandl and Lindow, 1998). In this work survival of A. brasilense Cd/pRKLACC on the surface of tomato leaves steadily decreased from the start of the inoculation. On the contrary, the population of *A. brasilense* Cd/pRKTACC remained steady throughout the experiment. It is possible that the decreased ability of *A. brasilense* Cd/pRKLACC to synthesize IAA in vitro (Fig. 23), which probably resulted from the imposition of a metabolic load on the transformed cells (see section 4.2.), was reproduced on the leaf surface, thus reducing the ability of the bacterium to survive. Brandl and Lindow (1998) showed that when wild type *Erwinia herbicola* and an IAA deficient mutant

produced by homologous recombination were inoculated simultaneously onto bean plants and pear flowers, the IAA deficient mutant could not compete against the wild type and reached significantly lower population sizes.

4.13. Plant pathogen biocontrol properties of *Azospirillum*

During pathogen attack, in an attempt to limit infection and spread of the pathogen, diverse defense mechanisms characterized by an increase in ethylene evolution by the plant are activated. This response to pathogen attack may cause damage to the plant since it can lead to cellular disruption, electrolyte leakage, spread of necroses and foliar disruption (Lund et al., 1998).

The synthesis of plant ethylene usually occurs in two distinct phases after pathogen attack (Abeles et al., 1992). In the first phase a small amount of plant ethylene is synthesized from the plant's preexisting pool of ACC, usually within a few hours after pathogen infection, and this may be one of the signals that turns on the plant's response to limit pathogen proliferation (Greenberg, 1997). In the second phase, the synthesis of ACC increases significantly and leads to the production of a high level of ethylene usually about three days following pathogen infection, which probably contributes to the plant damage caused by the pathogen infection (Abeles et al., 1992).

It has been recently demonstrated that ethylene synthesis and ethylene sensitivity are critical for foliar disease development in tomato (Lund et al., 1998). Foliar disease symptoms in tomato caused by *Pseudomonas syringae* were greatly reduced in an ethylene insensitive mutant (a single base substitution in the N-terminal coding region of the gene Le-ETR3 which encodes an ethylene receptor) plants in comparison with the wild-type. The authors found that the

population size of the pathogen in the mutant and in the wild type was the same; thus, a decrease in bacterial population could not account for the reduced foliar disease development. In other experiments, foliar disease symptoms were greatly reduced in one tomato line deficient in ethylene synthesis (because it contains an ACC deaminase transgene under the control of a constitutive promoter) as compared with the non-transformed cultivar (Lund et al., 1998).

In this work we hypothesized that the utilization of rhizosphere or phyllosphere bacteria with the ability to decrease the concentration of ethylene evolved by a plant as a response to pathogen attack would ameliorate some of the damage to plants caused by this susceptible response and would increase the chances of survival of the plant. This can be considered as an alternative method to preventing disease symptoms without controlling phytopathogens per se.

Tomato leaves treated with *A. brasilense* Cd/pRKTACC and after three days infected with *F. oxysporum* or *P. syringae* , visually appeared to be in a better state of health than non-treated leaves or leaves treated with non-transformed *A. brasilense* Cd (Figs. 39, 42). These preliminary results suggest that the transfer of the ACC deaminase gene into *A. brasilense* Cd gave this PGPB the ability to ameliorate some of the deleterious effects of pathogen attack by decreasing the concentration of ethylene in the plant.

The fact that the mechanism employed by *A. brasilense* Cd/pRKTACC to reduce foliar disease confers tolerance of bacterial pathogen infections may be considered a disadvantage in that the pathogen can still proliferate. However, it can be argued that since pathogen populations are not affected, selective pressure on the pathogen population for increased virulence is likely minimal or absent (Lund et al., 1998).

In the case of tomato seedlings infected with *F. oxysporum* or *Pythium aphanidermatum*, treatment of the seedlings with either non-transformed *A. brasilense* or *A. brasilense* Cd/pRKTACC substantially increased the survival of the plants (Tables 3, 4). These results suggest that the mechanisms involved in helping the plants by either of the *A. brasilense* Cd strains, were due to a competition effect or "competitive exclusion": early colonization of the roots by *Azospirillum* impeded colonization by the pathogen, thus preventing its establishment on the roots and subsequent infection. Non-treated plants, without any bacteria colonizing its roots, were readily exposed to colonization by the pathogen. The results also show that non-transformed *A. brasilense* Cd surpassed *A. brasilense* Cd/pRKTACC in the ability to exclude the pathogen from colonizing the roots.

Competitive exclusion is probably the most important mechanism by which antagonistic microorganisms achieve suppression of the pathogen (Johnson and Stockwell, 1998) and it is generally accomplished by a preceding inoculation of the biocontrol strain. For example, the commercially available biocontrol strain *Pseudomonas fluorescens* A506 suppresses growth of *Erwinia amylovora* (the cause of fire blight on pear and apple blossoms) when applied 72 h prior to inoculation with the pathogen; the bacterium does not provide any protection to the plant when inoculated simultaneously with the pathogen (Johnson and Stockwell, 1998).

The ability of wild type *A. brasilense* Cd to reduce disease symptoms can be also explained by induction of induced systemic resistance by the strain. Induced systemic resistance, or ISR, is a relatively uncharacterized form of induced disease resistance in plants triggered by non-pathogenic, root-colonizing

rhizobacteria. Triggering of ISR by rhizobacteria produces an unidentified translocatable signal that activates plant defense responses as yet not identified. The induced resistance in plants is not only restricted to the inoculation site of the bacteria but extends to the above-ground plant parts and is effective against different types of pathogens (Pieterse and Van Loon, 1999).

Induced systemic resistance is phenotypically similar to the better characterized mechanism of disease suppression called systemic acquired resistance. The main differences between the two mechanisms of disease suppression are that the latter is salicylic acid dependent and is mostly induced by pathogens (Pieterse and Van Loon, 1999).

It is possible that *Azospirillum brasilense* Cd has the ability to induce ISR in plants. In order to prove that resistance is induced and that it is truly systemic, it must be shown that inducing rhizobacteria are absent from the site of challenge with the pathogen and that the inducing bacterium and the challenging pathogen remain spatially separated for the duration of the experiment. The best evidence for PGPB mediated ISR is in experiments which show that the rhizobacterium does not antagonize the pathogen in culture (Van Loon et al., 1998).

ACC deaminase activity by PGPB may be involved in ISR. It is known that ethylene sensitivity in plants is crucial for ISR to develop. Inoculation of wild type *Arabidopsis* with *Pseudomonas fluorescens* WCS417, a PGPB with induced systemic resistance activity, protected the plants against bacterial speck (Van Loon et al., 1998). However, inoculation of an *Arabidopsis* ethylene insensitive mutant with the same strain did not protect the plants against the disease. Application of ACC induced resistance against *P. syringae* pv. *tomato* to the same level as the rhizobacterial strains (Van Loon et al., 1998). However, in the second

stage of pathogen attack the production of ethylene usually increases causing damage to the plant. The ability of the bacteria to break down ACC through ACC deaminase activity may help the plant to keep the levels of ethylene under control. Pieterse et al. (2000) monitored the levels of local and systemic levels of ethylene in ISR-expressing plants and were not able to detect changes in ethylene production. These results were interpreted by the authors as a lack of increase in ethylene synthesis. However, they could also be explained by the break down of ACC through ACC deaminase activity. This assumption is reinforced by the fact that upon induction of ISR and concomitant addition of ACC, the *Arabidopsis* plants developed a higher ACC-converting capacity.

In this work, no difference was found in dry and fresh weight between plants infected with *Fusarium* and plants treated with non-transformed *A. brasilense* Cd (Fig 40). These results suggest that this pathogen probably affects the plants mainly on the first days of development, and once the plant is able to pass that critical stage (which possibly involves the second peak of ethylene usually synthesized three days following pathogen attack), the pathogen no longer damages the plant, and, singularly, is even capable of promoting the growth of the plant to the same extent as wild type *A. brasilense* Cd.

Regarding the effect of *A. brasilense* Cd/pRKTACC on the fresh and dry weight of tomato plants infected with *Fusarium*, the results, although not statistically significant, indicate that plants treated with *A. brasilense* Cd/pRKTACC are better off than plants treated with wild type *A. brasilense* Cd, probably due to the ability of *A. brasilense* Cd/pRKTACC to break down ACC.

Introduction of an ACC deaminase gene into *P. fluorescens* CHA0 improved its ability to protect cucumber against *Pythium* damping off, and

potato tubers against *Erwinia* soft rot (Wang et al., 2000). However, contrary to what was found in this work, the authors reported that in the tomato-*Fusarium* system, the transformed strains with ACC deaminase genes provided the same protection against *Fusarium* crown and root rot as their parental strains. Different from *Azospirillum*, the parental bacterial strain *P. fluorescens* CHA0 has a broad spectrum of biocontrol activity against plant root diseases. It produces siderophores as well as the antimicrobial compounds hydrogen cyanide, 2,4-diacetylphloroglucinol and pyoluteorin. The inherent properties of this strain to suppress disease may be masking the effects of reduced ethylene levels caused by ACC deaminase activity. Another explanation could be related to lack of an adequate concentration of phytotoxins produced by *Fusarium*. Phytotoxins produced by some pathogens, like *Fusarium*, are critical for host recognition and infection. It is possible that *P. fluorescens* CHA0, by means of its inherent biocontrol activities, reduced the growth of *Fusarium*, and thus the concentration of phytotoxin did not reach the level required to elicit a major production of ethylene by the plant.

Another factor that should be considered when interpreting the results on plant treatments with bacteria having ACC deaminase activity, is the type of promoter utilized to express this gene. Considering that the expression of the ACC deaminase gene under the control of its native promoter requires 10-12 hours, bacteria transformed with this construct would not be able to significantly decrease the first ethylene peak evolved following pathogen attack (Wang et al., 2000). However, since *A. brasilense* Cd was transformed with a constitutive promoter, the bacterium would be able to consume immediately the ACC

involved in generating the first ethylene peak, thus avoiding deleterious effects on the plants right from the start of the attack.

The focus of research on *Azospirillum* inoculation has been mainly on its plant growth promoting abilities, and, as far as we know, the ability of *Azospirillum* to suppress plant disease has not been explored. An exception is one paper by Hassouna et al. (1998) that reports on the ability of *Azospirillum* to reduce the growth of pathogenic fungi. The authors reported that incubation of fungi in a 48 days old *Azospirillum brasilense* liquid culture reduced the dry weight of mycelium of *Fusarium oxysporum* f. sp. *lycopersici* by 90-96% and that of *Pythium* sp. by 71-95% as compared to pure fungal cultures. Since addition of a culture filtrate had the same effect on the fungi the authors speculate that the bacteria produce an agent that antagonizes the fungi. The nature of the agent was not further explored.

The regulation of the mechanisms involved in plant response against pathogen attack is complex and convoluted. Recent evidence indicates that plants can activate distinct defense responses tailored to specific types of parasites. For example, the defense mechanisms mediated by either salicylic acid or the growth regulators jasmonic acid and ethylene are utilized differentially against pathogens with contrasting modes of attack. The ethylene-jasmonic acid dependent defense response is activated by pathogens that kill plant cells to obtain nutrients. In contrast, the salicylic acid dependent response is triggered by a pathogen that obtains nutrients from living plant tissue (McDowell and Dangl, 2000). It will be interesting to determine the participation of ACC deaminase in these previously mentioned plant response mechanisms as well as in induced systemic resistance elicited by some PGPB.

4.14. Colonization of mangrove roots by *A. brasilense* Cd and *A. halopraeferens*

The colonization pattern on mangrove roots for *A. brasilense* is characterized by the presence of fibrillar material interconnecting cells and aggregates (Fig. 43). The same pattern of colonization observed in this work was seen in *A. brasilense* Cd associated to roots in tomato and cereal plants (Bashan et al., 1991). Anchoring of bacterial cells to the plant surface by a network of fibrillar material is probably a unique feature of *Azospirillum* root colonization. It was also observed that attachment to *A. brasilense* Cd to sand particles was by multistranded fibrils (Bashan et al., 1988). On the other hand, the colonization pattern of *A. halopraeferens* was different from *A. brasilense* Cd, since the cells were mainly found embedded in a mucilaginous sheath (Fig. 44). A similar sheath was observed after colonization of mangrove roots with cyanobacteria (Toledo et al., 1995). At present, little is known regarding the nature or the origin of this sheath. It may be a mixture of root mucigel and bacterial exopolysaccharides. It seems quite reasonable that the colonization of the surface and root interiors as well as the embedding in the mucilage layer results in an efficient osmoprotection, because *Azospirillum* itself has only a limited potential for cellular osmoprotection by osmolyte production (Hartmann et al., 1991).

The ability of *Azospirillum* to colonize mangrove roots together with its ability to attach to leaves shows the versatility of this bacterium in regard to its attachment capacity. No differences were observed in the pattern of colonization of *A. brasilense* Cd on leaves as compared to roots. Although on roots, cells of *A. brasilense* Cd reached a higher level (10^8 cfu/g dry wt.) (Puente et al., 1999) as compared to the level reached on leaves (10^7 cfu/g dry wt.). These results are

probably due to the higher concentration of nutrients on roots available to the bacteria.

Positive interactions between endophytes and their host plants can result in a range of beneficial effects which are similar and complementary with those reported from non-endophytic PGPB. Endophytes offer the twin benefits of being acclimated to their hosts, and present at seedling development and rhizosphere initiation (Sturz and Nowak, 2000). The utilization of endophytic PGPB as vectors for providing beneficial metabolites to the plants is a promising alternative for sustainable crop production systems.

Natural reforestation in mangrove ecosystems is usually very inefficient. As part of a very dynamic system subject to tidal movements and fluctuations in salinity and water availability, mangrove seedlings are continuously being exposed to highly variable conditions, which results in a very low rate of survival of the seedlings. Inoculation of mangrove seedlings with *Azospirillum* transformed with ACC deaminase activity would probably increase significantly the survival of the plants and should be considered as a promising venue in mangrove reforestation programs. We suggest that the ACC deaminase gene should be chromosomally integrated into *A. brasilense* Cd before being used in the field.

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