PLANT BENEFICIAL BACTERIA

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Bacteria associated with the plant rhizosphere may have beneficial effects on plant growth by providing nutrients and growth factors, or by producing antibiotics and siderophores, which antagonize phytopathogenic fungi and bacteria. There is considerable experimental support for the idea that plant growth promoting bacteria may be used as bio-fertilizers or biological disease control agents to increase agricultural yields. Recent advances in our understanding of the molecular biology of the systems responsible for plant growth stimulation are opening the way to strain improvement by genetic engineering.

During seed germination and plant growth, a number of soil bacteria and fungi become intimately associated with the developing rhizosphere. Many of these are soil saprophytes, doing little harm to healthy tissue. However, others cause serious diseases, being pathogenic on a variety of plants of commercial importance. The third class are bacteria living in close association with the plant rhizosphere, deriving benefit from root exudates and in turn having beneficial effects on the plant. There are a number of possible mechanisms for this, and, according to circumstances, one or more may be at work at the same time. The effects fall into two categories according to whether the bacteria benefit the plant directly or indirectly by antagonizing a phytopathogen or removing a growth inhibitor. This review summarizes recent advances in our understanding of the molecular biology and genetics of plant growth-promoting bacteria.

DIRECT BENEFICIAL EFFECTS

Direct beneficial effects arise when the bacteria provide the plants with useful products. These will be treated quite briefly here as they have been the subject of several recent reviews[1–3]. The most studied case is that of bacterial fixation of atmospheric nitrogen. Several types of nitrogen fixing bacteria are known; these may be free living (eg. *Klebsiella pneumoniae*[1]), or associated with the roots of either leguminous plants (eg. *Rhizobium meliloti*[1]) or monocots such as grasses, wheat and maize (*Azospirillum brazilense*[2,3]). It is well established that the effect of nitrogen fixation is most important for the leguminous plants whereas the benefit to non-legumes may be only small. The beneficial effect of *Azospirillum* may derive, not from its nitrogen fixing properties, but from the stimulating effect on root development, probably caused the production of auxins, gibberelin and cytokinin-like substances[2,3]. In addition many soil bacteria may enhance the mineral uptake of the plant, for example by the solubilization of phosphate in the soil[4]. Bacteria having direct beneficial effects on the plant may have considerable potential as biofertilizers.

INDIRECT BENEFICIAL EFFECTS

Indirect beneficial effects of rhizosphere colonizing bacteria may happen by the removal of deleterious microorganisms or chemicals from the environment. The most obvious way in which this can occur is by antagonism of phytopathogens, which are common in the soil under certain circumstances.

Antagonism of phytopathogens. There is a large body of evidence suggesting that monoculture of crops leads eventually to decreased yields and that one of the reasons for this is an increase in fungal and bacterial phytopathogens specific to that crop (Table 1). The problem can be cured by crop rotation, thereby denying the fungal pathogens a suitable host for a period of time, so that their numbers in the soil decrease[5]. Thus, the disease take-all of wheat and barley (caused by *Gaeumannomyces graminis*) can be effectively controlled by crop rotation with non-susceptible potatoes, corn or alfalfa. Curiously enough, however, the severity of take-all also decreases after about 4 years of monoculture. This is an acquired property of the soil due to changes in the microbial population and the soil is then said to be "suppressive" to take-all. While there may be many reasons for soil suppressivity, it is clear that the levels of fluorescent *Pseudomonas* antagonistic to *Gaeumannomyces graminis* are higher in the rhizospheres of wheat from suppressive than non-suppressive soil[6]. This suggested that plant beneficial *Pseudomonads* may be acting to suppress phytopathogenic fungi and/or bacteria (reviewed[7]).

There is now considerable experimental support for this view. Wheat yield increases of 27% have been obtained in field trials by treatment of wheat seeds with fluorescent *Pseudomonas* which protect against take-all in soil infested with *G. graminis*[8]. In other field trials, with sugar beet, fluorescent *Pseudomonads* have been shown to give root weight and sugar yield increases of 13% over a 3 year period[9]. Similarly, treatment of potato seed tubers with fluorescent *Pseudomonads* increased tuber yields by 70% compared to the untreated controls[10,11]. Protection by *Pseudomonads* against specific cotton pathogens such as *Thielavropsis basicola*, *Alternaria* sp., *Verticillium dahliae*, *Fusarium* sp. and *Pythium ultimum* has been demonstrated[12,13]. More recently, in greenhouse trials, fluorescent *Pseudomonads* have also been shown to increase plant emergence (64%) and plant growth (700%) of potato pieces infected by the soft rot bacterium *Erwinia caroto-*

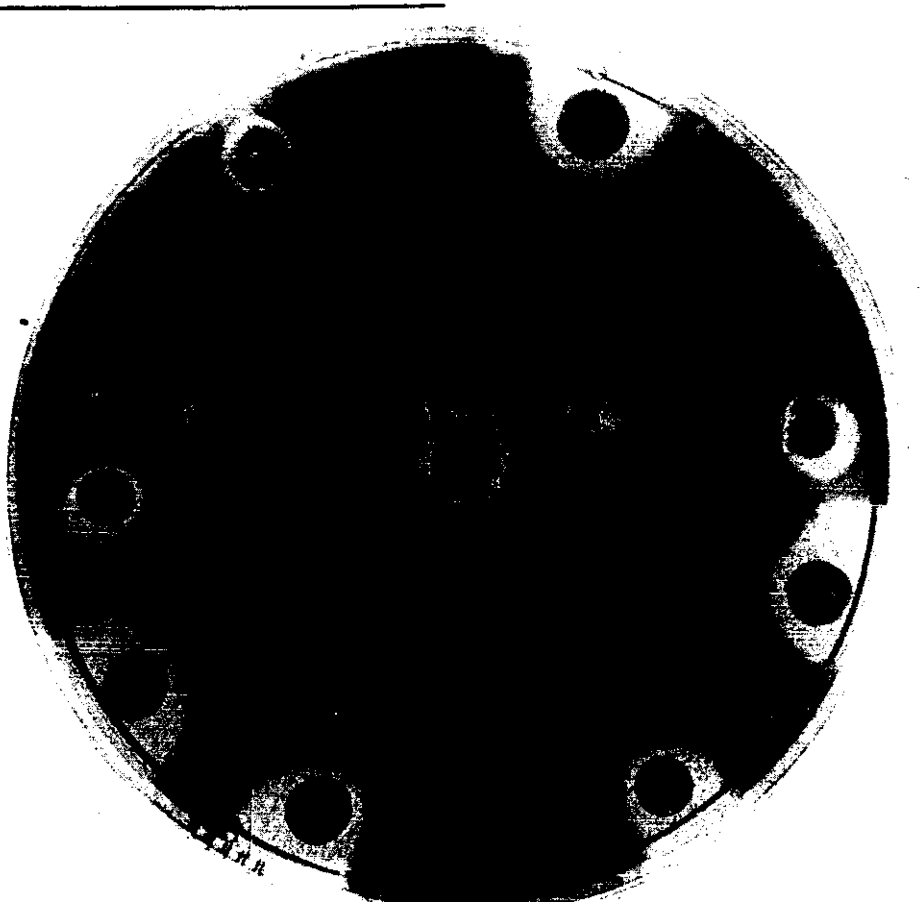
vora[14,15]. In the field, potato yield increases of 11.7% and 10.2% were obtained in two consecutive years, while the soft rot potential of the tubers also decreased[16]. In many cases the specific pathogen being antagonized by the rhizosphere *Pseudomonads* may be difficult to define under field conditions[11,17]. Indeed, it has been shown that a variety of minor pathogens (*Enterobacter*, *Klebsiella*, *Citrobacter*, *Flavobacterium*, *Achromobacter*, *Arthrobacter*, *Pseudomonas*) have harmful effects on plant growth, root elongation and seed germination. These deleterious effects can be prevented by inoculation with plant growth promoting bacteria[17].

The use of beneficial rhizobacteria for suppression of phytopathogens and increasing plant yield has, therefore, been amply demonstrated in experimental systems, including long term field trials. Studies have been carried out on the methods of treatment[8,9,11] and on the survival and root colonization of the *Pseudomonads* in the field[18,11]. Beneficial rhizobacteria would therefore seem to offer considerable advantages, particularly in soils infested with pathogens, and the time is probably ripe for their widespread use.

Mechanism of antagonism of phytopathogens. A great many rhizosphere bacteria (chiefly *Pseudomonas fluorescens* and *Pseudomonas putida*) have been isolated and screened for inhibition of phytopathogenic fungal or bacterial growth. More than half of these isolates inhibited one or several of the phytopathogens tested[15,19]. It has been known for a long time that *Pseudomonads* are capable of production of a variety of antibiotics[20]. Though relatively few of these have found application in medicine, it is now known that many act against phytopathogenic fungi and bacteria. On the other hand, fluorescent *Pseudomonads* owe their name to the production of fluorescent siderophores, which are high affinity Fe^{+++} chelators that specifically enhance their acquisition of iron[21,22] by binding to membrane bound siderophore receptors. This highly efficient iron scavenging mechanism is thought to compete with that of the fungal phytopathogens, thereby creating an iron-deficient environment deleterious to fungal growth[23]. It seems likely that, in nature, both antibiotics and siderophores participate in soil suppressiveness and the antagonism of phytopathogens. This is very difficult to demonstrate in the field and consequently most experiments have concentrated on laboratory experiments showing fungal inhibition in petri-dishes, where the experimental conditions can be well controlled.

Inhibition of phytopathogens by antibiotics. Many fluorescent *Pseudomonads* produce both antifungal antibiotics and siderophores. However, the latter can be suppressed by growth on iron rich medium (siderophore production being induced by iron limitation). In contrast, antibiotics are the product of secondary metabolism and their synthesis is stimulated by iron-rich low phosphate medium[20,15]. The situation is further complicated by the ability of some strains to produce more than one antibiotic. Thus, in the pioneering work of Howell and Stipanovic[12,13], *Pseudomonas fluorescens* strain (Pf-5) produced two different antibiotics: pyrrolnitrin, active against *Rhizoctonia solani*, *Thielavropsis basilicola*, *Alternaria* sp and *Verticillium dahliae*, and pyoluteorin, active against *Pythium ulti-*

The photograph shows *in vitro* inhibition of the wheat and barley fungal pathogen, *Gaeumannomyces graminis*, by the common soil bacterium, *Pseudomonas fluorescens*. This and other plant-beneficial microbial associations may be exploited in disease-control programs and in the production of biofertilizers.



mum. The bacterium was effective in the protection of cotton seeds against damping off by both *Rhizoctonia solani* and *Pythium ultimum*. The same strain was later shown to inhibit *Erwinia carotovora* in the petri-dish and to inhibit soft rot with *Erwinia carotovora* inoculated potato pieces[15]. The inhibition, in this case, is probably due to antibiotic production (and not to a high affinity siderophore produced by the same strain) since it occurred in iron rich medium. Another strain of *Pseudomonas fluorescens* 2-79 was able to suppress take-all in wheat[8], probably by producing an antibiotic identified as a dimer of phenazine carboxylic acid. This antibiotic was able to inhibit the growth of several fungi (including wheat pathogens *Gaeumannomyces graminis*, *Rhizoctonia solani* and *Pythium aristosporum*) and bacteria[24].

An initial step has recently been made towards the manipulation of antibiotic biosynthesis in rhizobacteria. More than 30 mutants of *Pseudomonas fluorescens* HV37a were isolated, which were unable to produce an unidentified antibiotic causing inhibition of *Pythium ultimum*. The use of cosmid gene banks, in a wide host range vector, enabled identification, by complementation, of five gene classes, located over a distance of 20 kb of DNA[25].

Similarly, other workers have studied a *Pseudomonas syringae* isolate which, after stem inoculation, is able to protect elm trees against the pathogenic fungus *Ceratocystis ulmi*. This fungus is the causal agent of Dutch elm disease which has killed millions of elm trees, worth billions of dollars. The bacterium produces an unidentified antibiotic able to inhibit the fungus in petri dish assays. The role of this antibiotic in plant protection was shown using transposon Tn*903* induced mutants, unable to synthesize the antibiotic. Initial steps were also made to clone some of the genes for antibiotic synthesis[26].

Inhibition of phytopathogens by siderophores. Most plant beneficial bacteria produce siderophores, fluorescent pigments with a high affinity for Fe^{+++}[21,23] and the iron-siderophore complex is recognized by specific receptors[27,28]. This may result is severe iron limitation in the rhizosphere and could limit the growth of fungi (*Gaeumannomyces graminis*; *Fusarium oxysporum*) and bacteria (*Erwinia carotovora*; *Pseudomonas syringae*)[23]. It is clear that siderophores are an important mechanism in the inhibition of both fungal and bacterial phytopathogens[11,15,27,29] and the effect has been reproduced using partially purified siderophores[30] and a synthetic iron chelating agent ethylenediaminedi-O-hydroxyphenylacetic acid[31].

More recently, the genes coding for the pseudobactin siderophore biosynthesis in *Pseudomonas* sp. B10 have been isolated from a cosmid gene bank by complement tion of mutants defective in siderophore productio The complementation patterns suggested a minimum 12 genes arranged in four gene clusters. This la number of genes is reasonable, given the complex stru ture of pseudobactin which is a linear hexapeptide, L-lysine-D-threo-β-hydroxyaspartic acid-L-alanine-D-allo-threonine-L-alanine-D-Nδ-hydroxyornithine, in which the N^8-OH nitrogen of the ornithine is cyclized with the C-terminal carboxyl group, and the N^{ϵ}-amino group of the lysine is linked via an amide bond to a quinoline derivative, which is responsible for the yellow-green fluorescence of both pseudobactin and *Pseudomonas* sp. strai B10[21]. The pseudobactin receptor gene of *Pseudom* B10 was similarly cloned, but in this case, by complemen ing a different, phytopathogenic, *Pseudomonad* susceptible to iron starvation by pseudobactin. The complemented strain was insensitive to pseudobactin, since it is now able to acquire Fe^{+++} from the ferric pseudobactin previously causing its inhibition[27,28].

The genes for siderophore (pseudobactin 358) biosynthesis have also been cloned from a different plant-beneficial *Pseudomonas putida* WCS358, as with *Pseudomonas* B10 many genes are involved (more than 7 genes least 5 gene clusters)[33,34].

Inhibition of crown gall by agrocins. Crown gall is a disease of roots and stems of many dicotyledonous plants, with a major economic impact on fruit trees, such as peach, apple, cherry, nuts and grapevines, and on ornamental plants such as rose trees. It is caused by *Agrobacterium tumefaciens* carrying a large (>200 kb) tumour inducing Ti plasmid[35]. Upon infection of wounded plants a 20 kb T-DNA segment of the Ti plasmid is transferred to the plant, becomes integrated into the DNA and subsequently causes tumour formation. The T-DNA segment induces the plant tumours to produce opines which are then used by the bacterium as sole carbon and nitrogen source.

In 1972, Kerr and co-workers reported a method of biological control of crown gall[36-38]. This method has been highly (through not invariably) successful and has now been used on a worldwide scale to contain the disease[5,39]. The method is preventative rather than curative and involves dipping or spraying the young plants or seeds with a non-pathogenic bacterium *Agrobacterium radiobacter* var. *radiobacter*. This bacterium produces agrocin 84 (an antibiotic specific for certain strains of *Agrobacterium*) in the form of a 6-N-phosphoramidate of a nucleo-

TABLE 1 Common Phytopathogens.

Phytopathogen	Disease	Host range
Fungi		
Fusarium solani sp.	Root rot, vascular wilt, pea wilt	Bean, peas and cotton
Gaeumannomyces graminis	Take-all	Wheat and barley
Phytophthora sp.	Damping-off, feeder root disease, root rot	Many species of conifers, orchards, ornamentals, soy beans.
Pythium sp.	Seed decay, damping-off, crown rot, feeder root disease	Sugar beet, potatoes and many other plants
Rhizoctonia solani	Seed decay, damping-off, root rot	Sugar beet, potatoes and many other plants
Thielaviopsis basicola	Black root rot	Tobacco, cotton, bean, many others
Verticillium dahliae	Wilt	Cotton and many other plants
Ceratocystis ulmi	Dutch elm disease	Elm trees
Bacteria		
Agrobacterium tumefaciens	Crown gall	Peach, apple, cherry, grape, nut, rose trees and many other plants
Corynebacterium sp.	Wilt, leaf blight, potato tuber rot	Beet, alfalfa, corn, wheat and many other plants
Erwinia carotovora	Soft rot	Potatoes, carrots, tomatoes and many other plants
Pseudomonas syringae	Halo blight, wildfire disease	Beans, tobacco and many other plants

tide analogue[40] which probably acts by inhibiting DNA synthesis[41]. Agrocin 84 is only effective against *A. tumefaciens* strains carrying nopaline type Ti plasmids and loss of the plasmid causes loss of sensitivity showing that agrocin 84 sensitivity is encoded by the Ti plasmid[42]. More recently wider host range agrocin producing strains have been isolated, which act against *A. tumefaciens* strains carrying nopaline, octopine and agropine Ti plasmids[43,44].

Agrocin 84 biosynthesis by *A. radiobacter* is encoded by a 48 kb plasmid[45] and loss of this plasmid resulted in loss of ability to control crown gall when inoculated at the same time as an agrocin sensitive pathogen, *A. tumefaciens*. However, protection could be obtained when the host plant was inoculated with the plasmid cured *A. radiobacter* 24 hours prior to infection with the pathogenic *A. tumefaciens*. This suggests that other factors such as competition for infection sites may also be important for biological control[46].

The molecular basis of agrocin production, agrocin immunity and agrocin sensitivity are beginning to be understood. The 48 kb pAgK84 plasmid was subjected to transposon Tn*5* mutagenesis and the genetic determinants coding for agrocin 84 biosynthesis were mapped. The genes for agrocin production were then cloned by complementation of the transposon induced mutants. Five complementation groups were found on a 14 kb DNA segment of the pAgK84 plasmid. Two separate loci have been identified on plasmid pAgK84 that encode immunity to its own agrocin[47]. Recently, the plasmid pAg396 coding for agrocin 84 has been transferred to *Rhizobium meliloti*[48]. It will be interesting to know whether this confers biological control properties against root tumour formation by *Agrobacterium tumefaciens*.

Competition for an ecological niche. Phytopathogenic bacteria occupy a particular ecological niche and the idea has been invoked that deliberate application of a non-pathogenic mutant of the same species may cause competition so that, if the relative numbers are favorable the phytopathogen could be excluded. Such a mechanism has already been mentioned above to explain the ability of agrocin defective *A. radiobacter* to cause limited protection from *A. tumefaciens*[46]. It has been hypothesized by Steven Lindow that deliberate release of non-pathogenic mutant *Pseudomonas syringae* could compete with plant pathogenic *Pseudomonas syringae* to prevent frost damage. *Pseudomonas syringae* causes frost damage to plants by making an ice nucleation protein that initiates ice crystalization at temperatures not normally favourable to ice formation. It is envisaged that deliberate release of mutant *Pseudomonas syringae*, from which the ice nucleation protein gene has been deleted, could prevent frost damage. This proposition has been the subject of considerable controversy since it involves deliberate environmental release of genetically modified bacteria on a large scale. While bacteria have been released in the past on a large scale (in the cases of *Agrobacterium radiobacter* and *Bacillus thuringiensis*), the proposition has met with enormous opposition from environmental lobbyists (reviewed[49]). Thus, it has only recently been given approval by the Environmental Protection Agency, after five years of delay. It should be noted that the *P. syringae* ice minus experiment involves a strain from which a deleterious gene has been deleted thereby rendering it more safe than the phytopathogen from which it was derived. More controversy can be expected over experiments where the bacteria proposed for release have been modified by genetic engineering. Work is presently underway to develop methods to assess the hypothetical risks of deliberate environmental release of recombinant micro-organisms and to monitor their fate following re-

New plant beneficial bacteria by genetic engineering. Genetic engineering has the potential to improve existing plant beneficial bacteria. However, it also could create organisms with new combinations of desirable traits not presently existing in nature. An attempt in this direction has been made by workers at the Monsanto company. Many micro-organisms produce compounds with insecticidal activity, and of these the best known is *Bacillus thuringiensis* which has been used commercially, for many years, without ill effects[52]. *B. thuringiensis* δ-toxin is a protein, produced in crystalline form in the bacterium, active against many insect species, including gypsy moths, inchworms, hornworm and cabbage looper. In the field the δ-toxin is sensitive to biodegradation and inactivation by sunlight and persists only a few days. It was rationalized that increased efficiency would arise if the toxin was made *in situ* by root colonizing bacteria. Accordingly, it was cloned into the transposon Tn*5* and transposed to strains of *Pseudomonas fluorescens* able to colonize corn roots. Cloning in Tn*5* offers the dual advantages of increased stability (compared to plasmids) and decreased horizontal transmission to other soil bacteria[53]. The possibility of transmission to other bacteria was later further diminished by cloning into a transposition defective Tn*5* transposon[54]. An application to field test the recombinant *Pseudomonas fluorescens* has been made to the EPA but not yet granted.

In experiments of this type, where it is desired to give rhizobacteria new or improved characters, great attention must be paid to the choice of bacterial host. Certainly good rhizosphere colonization will be necessary and virtually nothing is known about this at the molecular level. It has recently been shown that non-motile flagella-less mutants of a plant growth stimulating *Pseudomonas fluorescens* had impaired root colonization ability[55]. Other factors may include chemotaxis, adhesion, pectinases, cellulases, and the ability to survive under adverse soil conditions.

A unique kind of soil depolluting bacterium has been described by the team of Ananda Chakrabarty. Using a chemostat, and continuous cultivation for 8–10 months, these workers were able to 'evolve' a strain of *Pseudomonas cepacia* (AC1100) able to degrade 2,4-5-trichlorophenoxyacetic acid (2,4,5-T), a synthetic herbicide previously considered completely recalcitrant to biodegradation. It was demonstrated that application of this strain to soil contaminated with 1,000 ppm 2,4,5-T reduced the content of the herbicide by more than 98% within one week, enabling lettuce plants (sensitive to 10–15 ppm) to grow on the contaminated soil[56]. It should be noted that *P. cepacia* AC1100 evolved in a highly artificial environment and does not compete well with natural soil bacteria, being rapidly lost from the soil in the absence of 2,4,5-T. These experiments demonstrate the principle that treatment with suitable bacteria may transform heavily polluted soil to its original condition.

CONCLUSION

The prospects for improved agriculture, by the use of plant growth stimulating bacteria as biofertilizers or biological control agents, seem excellent. In almost all of the examples included in this report, the molecular biology of the systems is being developed and can be expected to lead to the improvement of existing strains by genetic engineering. The advantages would include better yield, lower costs, more liberty for the farmer in the choice of crop rotation, and reduced dependence on fertilizers and man-made fungicides. It would be expected that natural means of pathogen control would be favoured, by the environmentalists, over man-made chemicals; though, in reality, the opposite seems to be true. Indeed public resistance, particularly in the United States, Denmark and

Germany, is probably one of the main factors limiting investment and development in this area. It is to be hoped that the efforts currently being made by the environmental protection agencies to identify and quantify the hypothetical risks will result in a better informed public opinion on this subject.

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