Molecular Approaches for Improved Biological Control of Plant Diseases

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Summary: Environmental and consumer concerns and subsequent protective legislation have focused scientific interest on the development of microbial inoculants as environmentally acceptable methods of replacing or decreasing the use of chemical pesticides for crop protection. While potential biocontrol agents have been identified among fungal, actinomycete and bacterial species, molecular biology techniques are being increasingly employed to enhance the efficacy of these natural isolates. Such techniques include the development of improved inoculants capable of enhanced biocontrol metabolite production or with the ability to synthesise combinations of these metabolites. The exploitation of microbial inoculants as effective biocontrol agents involves the introduction of large numbers of microorganisms in to the soil environment. Ensuring that these procedures are compatible with sustainable, economically viable and environmentally friendly agricultural practice has involved monitoring and assessing the impact of microbial inoculants on phytopathogens and indigenous beneficial microflora.

Biological control of plant pathogens

Phytopathogenic fungi and nematodes, mediating a number of plant diseases, cause significant losses in crop yields annually. However, continued environmental and public health concerns related to the widespread use of chemically synthesised pesticides have resulted in protective legislation governing the use of some pesticides. With further stringent controls expected in the near future (e.g. 1998 levels of fumigant methyl bromide use must be less than those of 1991), scientific interest has focused on the development of microbial inoculants for crop protection (Cook et al. 1995; Dunne et al. 1996, 1997a; Van Veen et al. 1997). Biological control, exploiting the naturally-occuring negative interactions between beneficial disease-suppressive microorganisms and phytopathogens, represents an effective and environmentally acceptable strategy for the replacement, or decrease, of chemical plant protection measures (Cook et al. 1995; Handelsman and Stabb 1996). Evaluation of the mechanisms mediating microbial plant protection has resulted in the identification of antifungal factors such as siderophores, a variety of secondary metabolites and hydrolytic enzymes (Becker and Cook 1988; O’Sullivan and O’Gara 1992; Fenton et al. 1992; Geremia et al. 1993; Dowling and O’Gara 1994; Kobayashi et al. 1995; Flores et al. 1997; Cronin et al. 1997a, 1997b; Dunne et al. 1997b) produced by fungi, actinomycetes and bacteria. Plant protection by bacterial inoculants may also occur due to the induction of plant resistance mechanisms (Leeman et al. 1995; Ryals et al. 1996; Van Wees et al. 1997).

The effective application of microbial inoculants as biological control agents against soilborne pathogens involves the introduction of large numbers of microorganisms into the rhizosphere. This environment is extremely complex and is subject to diverse physical and chemical fluctuations, in addition to intense microbial activity (Handelsman and Stabb 1996). Introduced microbial inoculants must therefore be capable of remaining ecologically competent while maintaining stable levels of biocontrol activity. The ecological impact of
introduced metabolically active inoculants may influence the indigenous microbial community, affecting levels of pathogenic fungi and cyst and root knot nematodes in addition to arbuscular mycorhizal fungi and other beneficial microflora. Extensive analysis of the ecological effects of introduced inoculants is essential in determining their compatibility with sustainable, economically viable and environmentally friendly agricultural practices.

Biocontrol of pathogenic fungi and cyst nematodes by bacterial extracellular enzyme production

*Pythium ultimum*, a fungal plant pathogen causing damping-off of sugarbeet, and the potato cyst nematode *Globodera rostochiensis* are responsible for extensive damage to crop yields. Traditional crop protection measures against these pests have included crop rotation and the incorporation of chemical treatments into seed coatings. However, the exploitation of fungal or bacterial biocontrol agents, producing hydrolytic enzymes, has been proposed as an alternative protection strategy (Mankau 1980; Spiegel et al. 1991; Segers et al. 1994, 1995, 1996; Chernin et al. 1995; Dunne et al. 1997a). Bacterial strain *Stenotrophomonas maltophilia* W81 was isolated from the rhizosphere of sugar beet grown in a *Pythium*-suppressive soil (Dunne et al. 1997b). Using assay procedures described by Fenton et al. (1992), *S. maltophilia* W81 proved capable of inhibiting growth of the pathogenic oomycete *P. ultimum* under *in vitro* conditions (Dunne et al. 1997b). *S. maltophilia* W81 is unable to produce the fluorescent siderophores or secondary metabolites (e.g. phloroglucinol) often associated with bacterial biocontrol inoculants (Dunne et al. 1997a). However, the strain does produce copious amounts of the hydrolytic extracellular enzymes, protease and chitinase.

Further evaluation of the biocontrol efficacy of *S. maltophilia* W81 in soil naturally infested with *Pythium* spp. demonstrated that this biocontrol agent confers protection on sugarbeet seeds against colonisation by *Pythium* species (Dunne et al. 1997b). As *Pythium* sporangia respond rapidly to seed exudates (Nelson et al. 1988) and infect young plants within hours of their introduction into soil (Stasz et al. 1980) this early implementation of seed protection further translated into increased emergence of healthy plants under soil microcosm conditions (Dunne et al. 1997b).

Transposon mutagenesis of *S. maltophilia* W81 resulted in the isolation of an extracellular enzyme-deficient mutant, W81A1, unable to inhibit growth of *P. ultimum* under laboratory conditions. When applied as a seed coating, mutant W81A1 also proved incapable of conferring protection against *Pythium* colonization of treated sugarbeet seeds and subsequent damping-off disease in *Pythium*-infested soil (Dunne et al. 1997a, 1997b). Furthermore, genetic complementation and biochemical assessment demonstrated that the antifungal ability exhibited by *S. maltophilia* W81 is mediated by the enzymatic degradation and disruption of the *P. ultimum* cell wall (Dunne et al. 1997b).

In similar fashion, disruption of the structural integrity of the cyst nematode *Globodera rostochiensis* egg shell by purified commercial hydrolytic enzymes, particularly combinations of chitinases and proteases, can result in decreases in the emergence of healthy juveniles *in vitro* (Dunne et al. 1997a; Cronin et al. 1997c). The chitinolytic bacterial strains *Stenotrophomonas maltophilia* M1-12 and *Chromobacterium* UP1, like *S. maltophilia* W81, were isolated from the soil environment (Cronin et al. 1997c). When assessed under *in vitro* conditions, using procedures previously described by Cronin et al. (1997a), both bacterial isolates significantly decreased the ability of *G. rostochiensis* to hatch (Cronin et al. 1997c). Evaluation of *S. maltophilia* M1-12 and *Chromobacterium* strain UP1 under natural soil
conditions also resulted in significantly reduced levels of juvenile cyst nematode hatch (Cronin et al. 1997c).

In conclusion, current studies investigating the biocontrol activity of the antifungal, lytic enzyme-producing strain *S. maltophila* W81 against cyst nematodes may result in the development of a microbial inoculant capable of providing an alternative means of plant protection against multiple pests.

**Biocontrol of plant pathogens through antifungal metabolite production**

Soils which are suppressive to crop diseases such as take-all of wheat, black root rot of tobacco, Fusarium wilt of tomato and *Pythium*-induced damping-off of sugarbeet have been identified from many diverse geographic locations (Weller et al. 1988; Shanahan et al. 1992; Tamietti et al. 1993; Keel et al. 1996; Raaijmakers et al. 1996). Further studies have demonstrated the involvement of fluorescent pseudomonad production of antifungal secondary metabolites such as phenazines (Pierson and Thomashow 1992; Thomashow and Weller 1992), pyrrolnitrin (Homma et al. 1989), pyoluteorin (Howell et al. 1980; Kraus and Loper 1995) and c-acetylphloroglucinols (Shanahan et al. 1992; Keel et al. 1992) in suppression of root pathogens. In particular, 2,4-diacylphloroglucinol (Phil) producing strains appear to be especially abundant in soils that are naturally suppressive to certain diseases (Keel et al. 1996). For example, Harrison et al. (1993) estimated that at least 20% of the fluorescent pseudomonads isolated from *G. graminis* var. *tritici* infected roots grown in a take-all suppressive soil had a phenotype characteristic of Phil producers. However, while Phil is seen to have a broad inhibitory spectrum the exact mechanism of action of this compound is still to be determined.

*Pseudomonas fluorescens* strain F113, isolated from the rhizosphere of field grown sugarbeet, is capable of inhibiting the growth of the fungus *Pythium ultimum in vitro* and confers protection on sugarbeet against damping-off disease under natural soil microcosm conditions (Fenton et al. 1992). In addition to a fluorescent siderophore, the *P. fluorescens* F113 produces a number of secondary metabolites including 2,4-diacylphloroglucinol, hydrogen cyanide (HCN) and an iron-regulated protease. Characterisation of the transposon-induced mutant F113G22, defective only in its ability to produce Phil, revealed that it had lost the ability to inhibit fungal growth *in vitro* (Shanahan et al. 1992) and the ability to protect sugarbeet seeds against damping-off *in vivo* (Fenton et al. 1992). Further *in vitro* and microcosm assays implicated Phil production by F113 in the control of the cyst nematode *G. rostochiensis* and the soft rot potato pathogen *Erwinia caratovora* (Cronin et al. 1997a, 1997b).

The genetic locus containing the biosynthetic genes involved in the production of Phil has been cloned and characterised from a number of strains (Fenton et al. 1992; Bangera and Thomashow 1996). We have previously reported that a 6 kb genomic DNA fragment containing the Phil biosynthetic genes was sufficient to complement the F113G22 biosynthetic mutant (Fenton et al. 1992). Sequence analysis of this clone and of the biosynthetic locus of *Pseudomonas fluorescens* strain Q2-B7 identified a number of genes involved in the production of Phil (Cook et al. 1995; Delany et al. unpublished). To date, six genes have been implicated in Phil biosynthesis. Of these, four have proved essential for the biosynthesis of the metabolite, and the other two may be involved in regulation of production and transport of the molecule out of the cell (Thomashow et al. 1996; Delany et al. unpublished).

Phil is thought to be synthesised via a polyketide pathway, with monoacetylphloroglucinol (MAPG) as the final precursor intermediate (Shanahan et al. 1992). A putative
pathway for the synthesis of MAPG has been documented previously (Mann et al. 1987). The enzymatic activity responsible for the conversion of MAPG to Phl is coded for on the 6kb biosynthetic clone of F113 (Shanahan et al. 1993).

Research on antifungal metabolite production by pseudomonads has largely focused on understanding the biosynthetic and regulatory mechanisms of these strains. Generation of strains which have consistent performance and improved biocontrol activity through genetic modification may be essential to the effective and successful use of microbial inoculants. We have previously shown that the 6 kb biosynthetic clone of F113 is capable of conferring biocontrol ability when introduced into non-Phl producing Pseudomonas strain M114 (Fenton et al. 1992). This strategy not only allows the construction of novel biocontrol agents but also provides the opportunities for the improvement of currently available strains through the combination of multiple biocontrol traits.

The fungal pathogen Pythium ultimum affects gene expression in Pseudomonas fluorescens F113

Communication between members of the same species, as well as other species, is a major factor influencing the establishment and survival of organisms in their specific ecological niche. In many well characterized examples, such as the Agrobacterium-plant interaction and Rhizobium-legume symbiosis, the interaction involves a complex exchange of signal molecules (Clarke et al. 1992; Long 1996). This molecular communication can trigger responses and activate specific genes determining symbiosis, pathogenesis or disease resistance.

Fungal pathogens grow on a limited number of preferred hosts. Mechanisms mediating this host specificity involve the production of host-selective toxins and specific elicitors which allow recognition of the pathogen by the plant and the induction of the plant defense system (Kamoun et al. 1994; Knogge 1996). For successful pathogenesis, the fungal pathogen must elude the plant defense mechanisms, possibly through the production of suppressor molecules (Knogge 1996).

The regulation of bacterial physiological processes including bioluminescence, antibiotic production and expression of virulence factors in pathogens are regulated in a cell-density dependent manner through the accumulation of AHLs (quorum sensing: Salmond et al. 1995; Swift et al. 1996). However, recent reports have demonstrated that gene expression in one strain can be induced by signals produced by another. For instance, phenazine production in one strain of Pseudomonas aureofaciens may be influenced by signals produced by an alternative strain (Pierson and Pierson 1996). Such cross-talk indicates a high level of complexity in the interactions between organisms established in the same ecological niche, and that the production of diffusible molecules is a key factor in the ability of one organism to influence and regulate gene expression in another.

Studies in our laboratory have recently been extended to include the investigation of signalling between bacteria and fungi (Fedi et al. 1997). In order to determine whether differential gene expression could be detected in P. fluorescens F113 in response to the presence of the fungal pathogen Pythium ultimum, a library of random transcriptional gene fusions were constructed by transposon Tn5-LacZ mutagenesis. The reporter mutant bank was then screened in the presence and absence of the fungus, resulting in the isolation of five classes of mutants whose β-galactosidase activity is lowered in the presence of a diffusible factor released by the oomycete. F113 genes encoding for biological control traits would be an obvious target for phytopathogenic down-regulation. However, when assayed in vitro the
reporter mutants continued to inhibit fungal growth, although under soil conditions 3 of the mutants were impaired in their ability to colonize the sugarcane rhizosphere. These results indicate that *Pythium ultimum* is capable of affecting genes involved in the rhizosphere competence of *P. fluorescens* F113, possibly affecting its ability to establish itself in the environment and therefore overcoming the biocontrol activity of this antagonistic rhizobacterium (Fedi et al. 1997). These results allow an understanding of interspecies communication and have important implications for the development of effective biocontrol inoculants.

**Ecological impact of microbial inoculants**

The large-scale use of soil inoculants as biocontrol agents poses important ecological questions. A prerequisite for effective biocontrol of soilborne plant diseases is the ecological competence and the aggressive colonisation of the rhizosphere by the introduced biocontrol microbial spp. However, the introduction of large numbers of biological control agents may cause disruption of the abundance and diversity of indigenous microbial populations and this impact must be studied and understood. Studies have therefore assessed the effects of introduced microbial inoculants on resident bacterial and mycorrhizal populations (Barea et al. 1996; Tobar et al. 1996; Natsch et al. 1997; Niemann et al. 1997). In our laboratory, *P. fluorescens* F113 and the isogenic Phi-deficient mutant F113G22 were inoculated onto sugarcane seeds singly and in combination so that both strains competed with one another in the rhizosphere (Carroll et al. 1995). Sugarcane plants were grown under soil microcosm conditions for a period of 27 days, after which the sugarcane seedlings were removed. The soil was then resown with uninoculated seeds for nine subsequent cycles of growth of new seedlings. Results indicated that there were no significant differences in colonization or long-term survival between the *P. fluorescens* F113 wild-type and the transposon-induced mutant, suggesting that the ability of strain F113 to produce Phi does not provide an advantage in terms of ecological competence or colonization in the sugarcane rhizosphere. In addition, while the biocontrol ability of *P. fluorescens* F113 requires establishment of the strain in the rhizosphere, this does not result in a lasting perturbation of the resident culturable bacterial microbiota.

Further evaluation of wild-type F113 under field conditions assessed the influence of the biocontrol inoculant on control of *Pythium*-induced damping-off of sugarcane and on levels of selected indigenous soil microorganisms. A spontaneous rifampicin resistant derivative of *P. fluorescens* F113 was introduced as a sugarcane inoculant and its effects evaluated by direct comparison with untreated and chemical fungicide controls (Moënne-Loccoz et al. 1997). Introduced at a level of 6.0 log CFU per sugarcane seed the inoculant was below detection limits at 6 months after sowing and was not found to have had any significant effects on the total numbers of culturable aerobic bacteria in the sugarcane rhizosphere (Moënne-Loccoz et al. 1997). One year following release, strain F113Rif was found to be capable of colonizing the rhizosphere of uninoculated red clover sowed at the same site. As the introduction of large numbers of biocontrol agents may effect non-target indigenous soil microorganisms contributing to natural suppression of pathogens or to disease fertility, experiments were performed to assess the effects of F113Rif on *Rhizobium leguminosarum* biovar trifolii (Meade et al. 1985) and its ability to effectively nodulate red clover (Moënne-Loccoz et al. 1997). Results demonstrated that there were no significant differences in any of the plant performance parameters assessed (e.g. total foliage biomass, nitrogen content of the clover foliage, etc.) between plots previously inoculated with F113, the commercial fungicides or an untreated control. In addition, there were no significant effects on the degree of nodulation
between the three treatments. These observations suggest that inoculation of sugarbeet seeds with *P. Fluorescens* F113 did not affect the resident population of *Rhizobium leguminosarum* bv. *trifolii* from a functional aspect.

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**Literature**


