Pseudomonas for biocontrol of phytopathogens: from functional genomics to commercial exploitation

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Pseudomonas spp. that can colonise the roots of crop plants and produce antifungal metabolites represent a real alternative to the application of chemical fungicides. Presently, much research is aimed at understanding, at the molecular level, the mechanisms that enable *Pseudomonas* strains to act as efficient biological control agents. This approach is facilitating the development of novel strains with modified traits for enhanced biocontrol efficacy. However, without solving some inherent problems associated with the effective delivery of microbial inoculants to seeds and without knowledge on the biosafety aspects of novel biocontrol agents, the commercial potential of *Pseudomonas* spp. for plant disease control will not be realised.

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Abbreviations
BCA   biological control agent
IVET   *in vivo* expression technology
PPP   plant protection product
Phl   2,4-diacetylphloroglucinol
QS   quorum sensing
GMO   genetically modified organism

Introduction

There is increasing public concern regarding the continued use of agrichemicals that are damaging to human health or the environment. Such concerns are driving the search for more environmentally friendly methods to control plant disease that will contribute to the goal of sustainability in agriculture. Biotechnology has the potential to contribute enormously to this goal. By the year 2005 it is expected that biotechnology activity in the European Union will be valued at approximately Euro 250 billion, with particular growth predicted within the agrifood sector.

Soil-borne, non-pathogenic bacteria with the ability to antagonise fungal phytopathogens and thus prevent plant disease represent a realistic alternative to chemical fungicides. Consequently, the scientific literature contains a vast body of research on many soil bacteria with biocontrol abilities. These bacteria are known by several generic names, including biological control agents (BCAs), plant growth promoting rhizobacteria (PGPR) and biopesticides. Because of their catabolic versatility, their excellent root-colonising abilities, and their capacity to produce a wide range of antifungal metabolites, the soil-borne fluorescent pseudomonads have received particular attention. In addition, some *Pseudomonas*

BCAs have been shown to elicit a disease-resistance response in crop species, a phenomenon known as induced systemic resistance (ISR) (reviewed by van Loon *et al.* [1] and Pieterse and van Loon [2]). This dual activity of *Pseudomonas* BCAs (i.e. direct antagonism of phytopathogens and induction of disease resistance in the host plant) further highlights their potential as plant protection products (PPPs). In this review, we discuss the mechanisms by which *Pseudomonas* BCAs control plant disease, the strategies exploited to increase their biocontrol efficacy, and the industrial and regulatory issues that must be addressed before BCAs are developed for widespread use in agriculture.
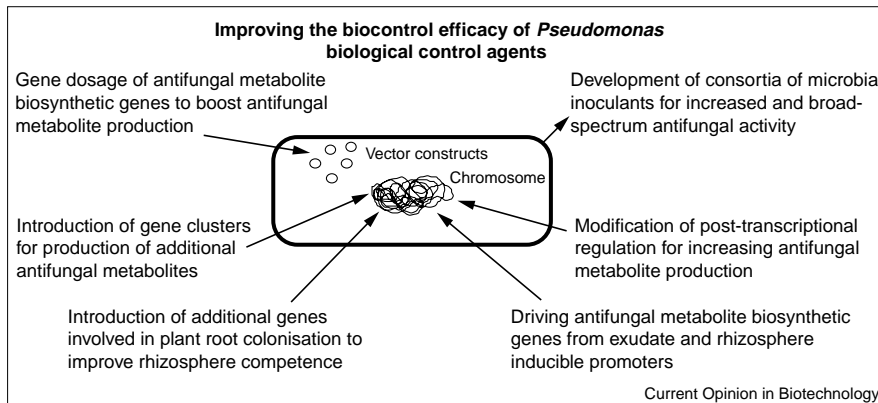
Biocontrol ability of fluorescent *Pseudomonas* isolates

Pseudomonas spp. and suppressive soils

Suppressive soils are soils in which phytopathogenic fungi are unable to persist or are present but fail to induce severe disease symptoms on susceptible crops. This phenomenon, although rare, has been well characterised and there is strong evidence that disease suppression is the result of the presence of certain rhizobacteria with antifungal activity. Several studies have demonstrated that *Pseudomonas* strains with the ability to produce the antifungal metabolite 2,4-diacetylphloroglucinol (Phl) can be isolated at high frequencies from soils suppressive to black root rot of tobacco and take-all disease of wheat [3,4]. The development of sensitive methods for the *in situ* detection of Phl has strengthened the link between Phl-producing pseudomonads and suppressive soil. Raaijmakers *et al.* [5] recently reported that Phl is produced on the roots of wheat grown in soils suppressive to take-all disease, caused by *Gaeumannomyces graminis* var. *tritici* (Ggt), but not in non-suppressive soils from which Phl-producing pseudomonads could not be isolated. Other crop plants that appear to select for bacterial antagonists include maize, in which a high proportion of the rhizoplane (i.e. root surface) *Pseudomonas* population (~15%) contained the Phl biosynthetic genes (monitored by the presence of the *phlD* gene) compared with a low proportion (<0.65%) in non-rhizosphere soil [6•].

Given the ecological importance of Phl production, it is not surprising that for many *Pseudomonas* BCAs biocontrol efficacy has been irrefutably linked to the production of this antimicrobial metabolite [3,7,8]. In addition to Phl production, other secondary metabolites including pyoluteorin, pyrrolnitrin, and phenazines have been linked to biocontrol [9–12]. Non-secondary metabolite antifungal compounds have also been described in *Pseudomonas* BCAs. Nielsen *et al.* [13•] identified a cell-surface molecule with both biosurfactant properties and antifungal activity.

Figure 1

Improving the biocontrol efficacy of *Pseudomonas* biological control agents

Gene dosage of antifungal metabolite biosynthetic genes to boost antifungal metabolite production

Development of consortia of microbial inoculants for increased and broad-spectrum antifungal activity

Vector constructs
Chromosome

Introduction of gene clusters for production of additional antifungal metabolites

Modification of post-transcriptional regulation for increasing antifungal metabolite production

Introduction of additional genes involved in plant root colonisation to improve rhizosphere competence

Driving antifungal metabolite biosynthetic genes from exudate and rhizosphere inducible promoters

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Schematic diagram depicting the strategies being followed to develop improved biocontrol strains of *Pseudomonas*.

Biochemical analysis of the compound showed it to be a newly described bacterial cyclic lipodepsipeptide, designated viscosinamide, which has subsequently been implicated in the control of *Pythium ultimum* in soil microcosm studies [14].

Requirement of rhizosphere competence for biocontrol efficacy

If a *Pseudomonas* strain cannot adequately compete within the environment of the rhizosphere and colonise the root surface then it will not be an effective BCA. It is important therefore to investigate bacterial colonisation and gene expression *in situ* in the rhizosphere. Recently, green fluorescent protein (GFP) and bioluminescence techniques have been employed effectively to investigate these issues. GFP technology, together with confocal laser scanning microscopy (CLSM), has facilitated the detection of single bacterial cells and has revealed that *Pseudomonas* BCAs often form microcolonies on the roots of crop plants [15,16]. Bioluminescence is a complementary technique for *in situ* monitoring of bacteria and has been used to monitor metabolically active *Pseudomonas putida* cells in the rhizosphere [17,18].

Substantial efforts have been made to identify genes required for key rhizosphere function(s). Given that the rhizosphere is a complex and ever changing environment, it is not surprising that a diverse array of genes have been shown to play an important role in plant root colonisation. To date, genes involved in nutrient acquisition, motility, chemotaxis, adhesion, secretion and stress response have been implicated in the colonisation ability of *Pseudomonas* strains. In addition, some unexpected functions that are important for rhizosphere performance have been identified. For example, using insertional mutagenesis, Dekkers *et al*. [19] demonstrated the importance of a site-specific recombinase for colonisation and speculated that this may be involved in the generation of functionally different subpopulations, allowing the strain to occupy various ecological niches. Furthermore, a recent study demonstrated

that the introduction of multiple copies of the site-specific recombinase gene (*sss*) into two other *Pseudomonas* strains resulted in enhanced colonisation ability of tomato roots in gnotobiotic systems [20•]. In another study, *in vivo* expression technology (IVET) was employed to identify genes specifically expressed within the rhizosphere [21••]. Interestingly, a gene identified in that study showed homology to type III secretion system genes, a finding which may suggest a more intimate and specific interaction between plants and associated beneficial bacteria.
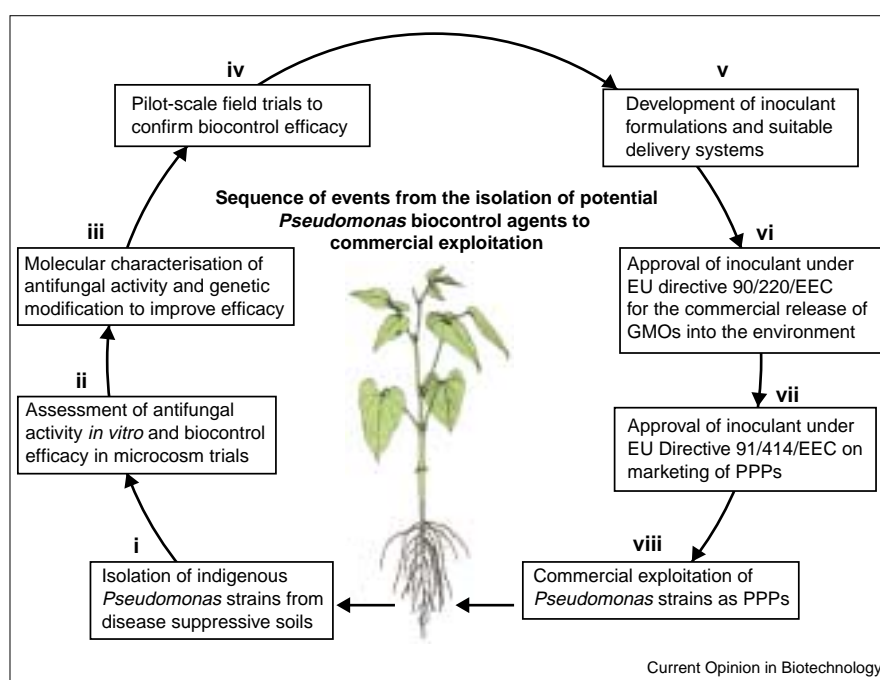
In addition to colonisation, the ability to compete for nutrients with indigenous microbial populations within the rhizosphere is an essential trait required for effective biocontrol of soil-borne phytopathogens. On a more subtle level, but no less crucial, there is evidence for interspecies signalling that may influence the rhizosphere competence of *Pseudomonas* BCAs. Fedi *et al*. [22] demonstrated that the phytopathogen *Pythium ultimum* produces diffusible factors that can down-regulate genes important for the survival of the biocontrol agent *P. fluorescens* F113 in the sugarbeet rhizosphere. Subsequent analysis demonstrated that two of the down-regulated genes mapped within separate ribosomal RNA (*rrn*) operons [23], whereas a third was identified as *gltB*, the gene encoding the large subunit of glutamate synthase (LM Smith and F O'Gara, unpublished data). More recently, Schnider-Keel and colleagues [24•] have demonstrated that the fungal toxin fusaric acid (FA), produced by the phytopathogen *Fusarium oxysporum*, can repress expression of the Phl biosynthetic gene *phlA*, confirming earlier reports that FA can inhibit Phl production by *P. fluorescens* [25]. These examples demonstrate the complex nature of interactions within the rhizosphere, and highlight the importance of conducting rhizosphere competence/biocontrol experiments in natural soil as well as in laboratory-based gnotobiotic systems.

Improving the biocontrol efficacy of *Pseudomonas* BCAs

There now exists incontrovertible evidence associating the biocontrol efficacy of *Pseudomonas* strains with the production

Figure 2

Schematic diagram showing the sequence of
events from the isolation of *Pseudomonas*
strains from disease suppressive soils,
through their development and improvement,
to their marketing as plant protection products
(PPPs). The scheme is relevant to the
European Union where scientific data
requirements detailed within specific
European Union directives are required for
bioinoculant registration.

Sequence of events from the isolation of potential
Pseudomonas biocontrol agents to
commercial exploitation

iv Pilot-scale field trials to confirm biocontrol efficacy

v Development of inoculant formulations and suitable delivery systems

iii Molecular characterisation of antifungal activity and genetic modification to improve efficacy

vi Approval of inoculant under EU directive 90/220/EEC for the commercial release of GMOs into the environment

ii Assessment of antifungal activity *in vitro* and biocontrol efficacy in microcosm trials

vii Approval of inoculant under EU Directive 91/414/EEC on marketing of PPPs

i Isolation of indigenous *Pseudomonas* strains from disease suppressive soils

viii Commercial exploitation of *Pseudomonas* strains as PPPs

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of antifungal secondary metabolites. One strategy to develop
improved biocontrol strains is to use genetic modification to
enhance this activity (see Figure 1 for schematic diagram
depicting the strategies for improving the biocontrol efficacy
of *Pseudomonas* spp.). To a certain extent, this involves the
construction of strains that produce increased levels of
antimicrobial metabolites. More significant, however, is the
recent focus on developing strains in which the timing of
production is altered. This is crucial because, in general,
Pseudomonas spp. only produce antifungal metabolites at high
cell densities during the late logarithmic or stationary phase of
growth. If the relevant biosynthetic genes are uncoupled from
their regulatory controls, however, this may facilitate early
production of antifungal metabolites, offering immediate
protection to crop seeds/seedlings.

Control of gene expression at the transcriptional level is
recognised as a primary mechanism for modulating the
production of secondary metabolites. In this regard, the
use of alternative σ factors has received considerable atten-
tion. It is proposed that the housekeeping factor (σ^D), the
heatshock factor (σ^H) and the stationary phase factor (σ^S)
play roles in regulating the production of particular
secondary metabolites [26–28]. Specific transcriptional
activators/repressors may also regulate the transcription of
secondary metabolite biosynthetic genes. The PhlF
protein, which is expressed from the Phl locus, represses
transcription of the *PhlA–D* operon, which comprises genes
encoding proteins that direct the synthesis of Phl [29•,24•].
Mutation of *phlF* in a *P. fluorescens* strain increased Phl
production *in vitro* during the early logarithmic phase of
growth. Similarly, overexpression of *phlA–D* resulted in Phl

overproduction and, concomitantly, enhanced biocontrol
efficacy against *P. ultimum* in laboratory microcosm trials
[30]. In recent years, an important role for quorum sensing
(QS), defined as cell-density-dependent control of gene
expression, in regulating the production of secondary
metabolites in pseudomonads has been described.
Predominantly, this work has focused on *P. aeruginosa*
([31,32••]; reviewed by de Kievet and Iglewski [33•]).
Characterisation of QS in *P. aurofaciens* [34,35], along with
the discovery that *P. fluorescens* F113 produces a number of
N-acylhomoserine lactones [36•], indicates that QS also
has significance for biocontrol strains of *Pseudomonas*.

In pseudomonads, a second level of control of the produc-
tion of many secondary metabolites has been recently
elucidated. This new regulatory cascade operates at the
post-transcriptional level. Regulation is effected through
the global regulators GacS/GacA, which are the environ-
mental sensor kinase and response regulator of a
two-component system conserved in many Gram-negative
bacteria (reviewed by Haas *et al.* [37••]). Two complemen-
tary studies have shed light on the mechanism of this
post-transcriptional regulation in strains of *P. fluorescens*. It
was established that the translation of genes encoding pro-
teins involved in the biosynthesis of HCN and a protease
was GacA-dependent, and that this dependence operates
via a translational repressor protein (RsmA/PrpA) [38••].
Independently, it was found that expression of a regulatory
RNA, called PrrB, was dependent on the GacS/GacA
system; furthermore, overexpression of PrrB restored
secondary metabolite production to a *gacA* mutant [39••].
On the basis of these data, and on previous studies with

PrpA and PrrB homologues in *Erwinia carotovora* and *Escherichia coli* (RsmA/RsmB and CsrA/CsrB, respectively), it is postulated that GacA positively regulates the expression of PrrB, which in turn sequesters the repressor protein PrpA, thus allowing the translation of mRNAs encoding biosynthetic proteins for secondary metabolite production.

The clustered organisation in *Pseudomonas* of many of the biosynthetic genes responsible for production of antifungal secondary metabolites facilitates the construction of strains with the potential to synthesise a range of antifungal metabolites ([40•,41•,42,43]; reviewed by Haas *et al.* [37••]). The data now emerging on post-transcriptional control mechanisms, however, illustrate additional considerations that must be taken into account when introducing new genes into biocontrol strains. Reprogramming both transcriptional and post-transcriptional regulation of these genes may be necessary to achieve optimal production of secondary metabolites.

Requirements for the exploitation of *Pseudomonas* BCAs
Inoculant delivery systems
Although the vast body of research on *Pseudomonas* BCAs deals with their capacity to control soil-borne fungal pathogens, there has been limited success developing commercially viable products (see Figure 2 for schematic diagram depicting requirements for commercial exploitation of *Pseudomonas* biocontrol agents). According to the records of the United States Department of Agriculture there are fewer than ten *Pseudomonas* inoculant products on the market for the control of fungal phytopathogens (http://www.barc.usda.gov/psi/bpdl/bpdlprod/bioprod.html). Inconsistency under field conditions has often been cited as the principal reason preventing the commercial use of many BCAs. An equally important, if not over-riding bottleneck, however, is the lack of suitable inoculant formulations that allow *Pseudomonas* cells to survive for long periods under storage at concentrations high enough to afford biocontrol [44]. Current seed coating and pelleting procedures require a drying step, which often results in considerable reductions in inoculant viability [45,46]. Evidence suggests that the addition of nutrients to seed pellets may be a useful strategy for improving inoculant survival [46]. Furthermore, carbon sources and minerals have been shown to have an important role in antifungal metabolite production by *Pseudomonas* BCAs, suggesting that nutrient amendments to formulations may also be a useful strategy for improving biocontrol efficacy [47]. Without doubt, however, further research is required on the development and optimisation of microbial inoculant formulations, which will be compatible with current seed coating technologies. Furthermore, because survival during seed coating/pelleting and during storage at ambient temperatures is critical for the development of microbial inoculant products, it seems logical that these traits should form an integral part of any screening process for the selection of new *Pseudomonas* BCAs.

Impact of *Pseudomonas* BCAs on non-target organisms
Before *Pseudomonas* spp. can be registered as plant protection products (PPPs), they must be assessed for their effect on human health and the environment. For example, the European Union directive 91/414/EEC (http://europa. eu.int/comm/food/fs/ph_ps/pro/legal/dir91-414-eec_en.pdf), which deals with the placing of PPPs on the market, requires that biocontrol strains undergo a stringent testing procedure analogous to the registration process in place for chemical fungicides. Essentially, a comprehensive scientific dossier is required to evaluate whether the BCA or its metabolites pose any toxicological and ecotoxicological risk. Furthermore, a separate scientific dossier is required for the use of genetically modified strains. The data requirements contained in Annex II of European Union directive 90/220/EEC (http://europa.eu.int/eur-lex/en/lif/dat/1990/en_390-L0220.html), which deals with the deliberate release of genetically modified organisms (GMOs) into the environment, demand the provision of comprehensive details on the nature of the GMO, including genetic stability, capacity for gene transfer and impact on non-target organisms, before commercial use [48]. It is the latter requirement that is potentially the most demanding and, consequently, is receiving the most scientific attention.

Two approaches have been taken to determine the impact of *Pseudomonas* BCAs on non-target organisms: assessment of the impact on specific microbial species (usually beneficial and symbiotic species), and assessment of the impact on total microbial populations. With regard to the arbuscular mycorrhizal fungi, which form symbiotic relationships with the majority of land plants, several studies have demonstrated that both wild-type and genetically modified Phl-overproducing *Pseudomonas* BCAs do not interfere with symbiosis [49–51]. There is evidence to suggest that *Pseudomonas* BCAs can affect the growth and subsequent nodule occupancy of certain *Sinorhizobium meliloti* strains in gnotobiotic systems [52]. Within commercial-scale field trials, however, a *Pseudomonas* BCA did not affect nodulation or nutrient levels in the foliage of a red clover rotation crop [53,54], again demonstrating the necessity of conducting impact analysis experiments within agronomically relevant parameters.

The impact of the wild-type *P. fluorescens* strain CHA0-Rif and a Phl- and pyoluteorin-overproducing derivative CHA0-Rif/pME3424 on the total indigenous culturable bacterial and fungal populations in the cucumber rhizosphere has been investigated. Compared with untreated plants, Natsch *et al.* [55] demonstrated that neither BCA affected the frequency of dominant bacterial groups, whereas Girlanda *et al.* [56] observed a detectable influence on the culturable fungal population. It is important to note, however, that the observed impact was smaller than the effect of growing cucumber repeatedly in the same soil.

As the majority of soil microorganisms cannot be isolated on laboratory media, a reliance on culture-dependent

approaches for impact assessment has been questioned. Lottmann *et al.* [57•] used a culture-independent approach for the assessment of bacterial diversity and demonstrated that inoculation of genetically modified lysozyme-producing potatoes with a lysozyme-tolerant *P. putida* strain did not affect the rhizosphere bacterial population. Again within this study, the over-riding influence of the plant on the microbial population was shown; the age of the plant and not the presence of the microbial inoculant had a significant impact on the rhizosphere bacterial population.

Conclusions

It is now over 30 years since *Pseudomonas* spp. were first recognised as potential BCAs. Within this period, and particularly within the past five years, intense scientific research has given rise to several well-characterised *Pseudomonas* BCAs that have now become model strains for understanding regulatory mechanisms in Gram-negative bacteria. Although the understanding of such mechanisms is of considerable scientific interest in its own right, it is envisaged that the application of this knowledge will lead to the development of *Pseudomonas* BCAs with improved reliability and efficacy. In addition, the role of intraspecies and interspecies signalling is proving a fruitful area of scientific research with equally relevant applications. For example, our increasing understanding of the role of N-acyl homoserine lactone signal molecules in antifungal metabolite production and the identification of promoters that can be induced or boosted in the rhizosphere is providing new approaches for the development of novel biocontrol agents. Some fundamental challenges remain, however. These include the development of consortia of BCAs for increased and/or broad-spectrum antifungal activity and the development of universal formulations to increase inoculant survival during seed coating and storage. In addition, future marketing of *Pseudomonas* inoculant products as environmentally friendly alternatives to chemical fungicides will depend on the generation of biosafety data required for the registration of biocontrol agents.

Acknowledgements

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