Investigating a Microbial Fungicide to Enhance Biological Control of Plant Disease

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ABSTRACT

The antibiotic, 2, 4-diacetylphloroglucinol (Phi), is produced by a range of naturally isolated fluorescent pseudomonads, found in disease suppressive soils. The natural isolate, *P. fluorescens* F113, protects pea plants from the pathogenic fungus, *Pythium ultimum*, by reducing the number of pathogenic lesions on the plant's roots. This beneficial effect was however, outweighed by the F113 causing an overall reduction in the emergence of the pea plants in the infected soil. The gene locus responsible for the Phi production was shown to be functionally conserved between the *P. fluorescens* F113 and another Phi producing organism, *P. fluorescens* Q2-87. Following identification of this functional sequence homology, the genes were isolated from F113, by optimised, long PCR. The 6.7-kb gene cluster was inserted into the chromosome of a non-pathogenic *P. fluorescens*, SBW25, which can effect biological control against the plant pathogen, *Pythium ultimum* through competitive exclusion of the fungus, by means of its strong colonising competence. The insertion was a targeted, homologous recombination designed to insert the Phi coding genes, from the F113, into a non-essential, lacZY coding region of the SBW25 chromosome. The transformed strains of SBW25 assumed two different morphological appearances. The morphological changes were noted at a ratio of 1:1 of normal morphology and altered morphology. Transformation of SBW25 with the Phi locus without this repressor element led to transformants with only normal morphology. All transformants were able to suppress *P. ultimum* through antibiotic production following the Phi transformation. However, the fitness of the transformants was reduced in flask culture, at 30°C, against the un-transformed SBW25. The organisms transformed with the entire Phi locus were seen to clump together in the culture media. The strain transformed with the Phi locus lacking the repressor element behaved normally. When inoculated on pea seedlings, the strain containing no repressor element behaved similarly to the F113, causing lower pea seed emergence. A transformant containing the entire Phi genetic locus had not lost its environmental competence on the pea roots, maintaining a high population, but was unable to maintain a high population in the surrounding soil.
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**Glossary**

All organisms described throughout the experimental chapters are *Pseudomonas fluorescens*.

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<th>Term</th>
<th>Description</th>
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<td>Phenolic antibiotic that has inhibitory effects on the plant pathogen, <em>Pythium ultimum</em>.</td>
</tr>
<tr>
<td>Damping-off</td>
<td>Seedling disease in a variety of crop plants.</td>
</tr>
<tr>
<td>F113</td>
<td>Isolated from the roots of sugar beet plants in Ireland. F113 is naturally capable of producing the antibiotic 2,4-diacetylphloroglucinol (Phi).</td>
</tr>
<tr>
<td>F113G22</td>
<td>Tn5 mutant of F113, unable to produce the antibiotic, Phi.</td>
</tr>
<tr>
<td>F113OP</td>
<td>Genetically modified derivative of F113. Contains a plasmid, pCUGP, which increases the production of the antibiotic, Phi, in comparison to strain F113.</td>
</tr>
<tr>
<td>Pa2</td>
<td>Abbreviation of SBW25EeZY-6KX.</td>
</tr>
<tr>
<td>Pa24r</td>
<td>Genetically modified derivative of Pa2 (SBW25EeZY-6KX). Pa24r contains an insertion of the Phi genetic locus minus a repressor element.</td>
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<tr>
<td>Phi</td>
<td>Abbreviation of 2, 4-diacetylphloroglucinol.</td>
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<tr>
<td><em>Pythium ultimum</em></td>
<td>A fungal pathogen and the causative agent of damping-off disease.</td>
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<td>Q2-87</td>
<td>Isolated from the roots of wheat plants in the USA. Q2-87 is naturally able to produce the antibiotic, 2,4-diacetylphloroglucinol (Phi).</td>
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<td>Rhizosphere soil</td>
<td>Only refers to soil closely associated or attached to plant roots.</td>
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<td>SBW25</td>
<td>Isolated from the phyllosphere of sugar beet in England. SBW25 does not produce any antibiotics that have been attributed to biological control.</td>
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1. CHAPTER 1
1.1. **BIOLOGICAL CONTROL**

For a full appreciation of the scope and the success of biological control an historical perspective will be examined. The earliest examples are now described as 'classical biological control' and are mainly associated with insect pest management. There is debate as to when the earliest biological control program was used to protect important human crop plants from predatory damage. However, there are records that as early as 3000 years ago, in Egypt, pest control was performed through the practice of crop rotation. In China, citrus pests were managed through the introduction of *Oecophila smaragdina*, ants, for hundreds of years (DeBach and Rosen, 1991).

Biological control can be defined as the use of parasitoid, predator, pathogen, antagonist or competitor populations to suppress a disease or pest population. Pest control has, historically, benefited from successful applications of biological control. Biological control can either be enhanced by engineering by man to achieve these aims or can occur naturally through environmental pressures. Historically, arthropod predation first revealed the commercial benefits of biological control. These early forms of pest management involved the introduction of predatory arthropods for the control of weeds and unwanted insects.

Since the first and second world wars, the introduction of powerful and broad acting pesticides turned attention away from the use of natural pest management. This chemical approach was extremely effective in preventing plant disease, and increasing plant yields. This strategy of pest management does not require information on the ecological origins of pests and the repeated application of these effective chemicals was seen to improve crop production. The knock-on effects of this strategy are being felt today as the limitations of this management approach are revealed.

*Problem with synthetic chemical pesticides*

The problems of the synthetic pesticide arise when they fail to control the target pest and lead to the introduction of a secondary pest. This is seen in the cases of pesticide resistance. This arises through differential survival of the target pest population to the toxic agent. Pesticide producers have three options. The introduction of a new
pesticide through research and development, application of higher initial doses of pesticide or addition of multiple active ingredients. The common factor affecting each option is the increased cost of the application together with increasing the environmental and human exposure to toxic compounds. None of these options are desirable to pesticide producers and hence the increased commercial interests in alternative forms of pest management.

The extensive and long term use of pesticides has led to concerns surrounding residue levels on plants destined for human consumption, deleterious effects on non target species and contamination of waterways. With an increasing appreciation of the balances and perturbations of the soil and plant ecosystems, pesticide manufacturers are under increasing pressures to guarantee the safety of their chemicals. Increased awareness of the effects of pesticides has encouraged a better understanding of the natural communities associated with crops. Modern techniques coupled with this awareness have led to the discovery of novel methods of natural pest management.

The world-wide application of biological control was documented and strategically used in the early to mid-parts of this century, often to replace ineffective powerful and broad acting pesticides. The earliest cases of biological control were primarily targeted at insect pests that infect the foliar cover of crop plants. These were traditionally protected by the application of chemical agents. An example is the protection of apple orchards from lepidoptera pests using lead arsenate in the 1920s. Lead arsenate was soon replaced by organophosphates (OP) and organochlorines (OC) as outbreaks of hitherto harmless species began to occur, notably the European red mite, *Panonychus ulmi* (Massee, 1958). These outbreaks arise because of a common occurrence seen with broad acting chemicals in that they not only destroy the target pests but also natural enemies of potential pests.

These newer chemicals were soon to receive their first limitation as resistance developed in target pests. In the 1950s a program commenced in Nova Scotia, to attempt to integrate a biological control strategy for the elimination of spider mites in conjunction with the use of conventional chemical pesticides. Selective ryania were used instead of OC and OP. However, the codling moth, *Cydia pomonella* and the
spider mites soon developed resistance to the chemical pesticides and the ryania were also seen as relatively ineffective. Through the 1960s and 1970s newer chemicals were developed such as acaricides to control the mite pest problem but again resistance was developed and the chemicals became ineffective. The irony of the future success of the biological control program was that it was the selective pressure of using these chemical pesticides that led to the emergence of biological control organisms that could protect apples from the resistant European spider mite. An integrated approach to protect apple orchards involved isolation of spider mites natural enemies, *Typhlodromus pyri*, that had themselves become resistant to the chemical pesticides. The use of this resistant predator together with the chemical pesticide on apple orchards that had been previously untreated led to a successful crop protection system where resistance was slow to build in the target pest (Way and van Emden, 2000).

In the early 1980s effective and cheap chemical pyrethroids were replacing the use of selective OP and carbamate resistant mites but again the chemical met with limitations as concern grew over its use in Western Europe due to high residual toxicity in food matter and the development of resistance. Currently work is being carried out on new chemicals that are harmless against this major predator of disease causing mites, *T. pyri*, and formulations of *Bacillus thurengiensis* (Bt) and pyrethroids have been found to be selective against the *T. pyri* but effective against the disease causing lepidoptera (Hardman *et al.*, 1995).

This is an example of a system of biological control when it is all too easy to use chemical agents. Repeatedly the limitations of their use in preventing crop damage is seen through acquired resistance of the disease causing species and the outbreak of new pests through destruction of natural predators of non-pest species. Perhaps for historical reasons citrus plants have received the greatest interest in terms of case study description.

In 1888 the vedalia beetle, *Rodolia cardinalis*, was introduced into California from Australia to combat cottony cushion scale, *Icerya purchasi*. This was considered a
landmark of Biological control as the cottony cushion scale was responsible for almost destroying the citrus industry in California. The introduction of a natural predator, the vedalia beetle, *Cryptochetum iceryae*, was successful in eliminating the pest from these economically important crops within just one season of its introduction. Although today the vedalia beetle is not commercially available, this is mostly due to the natural abundance of the organism following its introduction in the U.S over a hundred years ago.

Today, the vedalia beetle is still the best method of controlling cottony cushion scale. Vedalia are very fast growing, completing four generations in the time it takes cottony cushion scale to complete one generation. Insecticides such as ‘Malathion’ and ‘Supracide’ are often not as effective as vedalia beetle and are disruptive to the natural enemies needed for California red scale control. The insect growth regulators ‘Esteem’ or ‘Applaud’ treatments will kill cottony cushion scale, but their effect is slow, 1-2 months rather than 2 weeks with the vedalia beetle. In addition, the use of the insect growth regulators has caused an outbreak of cottony cushion scale in 1998/1999 in the San Joaquin Valley, as they are toxic to the vedalia beetle. The crops onto which the chemicals were added were protected from cottony cushion scale, but surrounding crops did not receive a high enough dose to kill the plant disease, however, it was sufficient to kill the vedalia beetle (Grafton-Cardwell, 2001).

This has been one of the few success stories of biological control and has been coined as “cottony cushion scale syndrome” (Hoy, 2000). Although the author’s description may not be universally known, the principle behind its name is. Cottony cushion scale control by the vedalia, lady-beetle, was inexpensive, permanent and highly effective. The program has since been used throughout the world in the protection of citrus from this disease. The ‘syndrome’ arises from the belief of researchers and research agencies that classical biological control should all act in this rapid, inexpensive, perpetual and fully effective manner. Of course this has been shown as almost an unique success for biological control.

These two examples have provided some historical and developmental perspective to biological control. In the apple industry effective chemicals are continually
manufactured. However, their limitations are soon seen. The continuing struggle between the use of naturally resistant predatory mites and broad acting chemicals is one that many biological control programs have faced. The citrus industry shows the successful example of an early attempt to manage pests using introduced predatory insects. It shows the potential for biological control but also the difficulty that researchers are faced with when attempting to apply these key principles to other crops using new control strains.

Perhaps the largest strides forward in biological control have come from the use of Bt and has put in perspective the limited commercial scope of biological control today. The biopesticide market still accounts for less than one percent of the crop protection market (Navon, 2000). Bt has been the leading biopesticide against lepidopterus pests since 1959. Since 1990 however, major advancements in the use of Bt have emerged, essentially from three strategies; natural and recombinant Bt products developed to broaden the insect host range; new formulations based on conventional or genetically engineered encapsulation of the toxin; knowledge and management of insect resistance to Bt.

There have been constraints on the use of Bt products such as narrow host range, oral route of toxicity, bollworm and borer larvae avoid Bt by penetrating into plants, solar irradiation inactivates crystal protein, wash off by rain reduced micro dose on plants, phyllophanes and allochemicals can inactivate Bt protein activity. Bt development had begun in the 1950s based on the spore crystal complex but only in the 1970s after the HD-1 strain of sub species *kurstaki* was isolated was Bt commercially viable, as this was active against a large number of moth species (Dulmage, 1970). Many thousands of strains have now been discovered of Bt and show a wide spectre of activity against insects particularly lepidoptera but also dipteria and coleoptera (flies and beetles). The toxic agent to these pests is a delta endotoxin that is produced by Bt spores. The monomeric form varies from 27000 to 135000 Mr, which is cleaved in the insect gut to the smaller active toxin. In the 1980s the majority of Bt had been isolated from insects or material closely associated with insects. The late 1980s and 1990s it has been recognised that Bt are ubiquitous in soil and highly active strains have been isolated in a wide variety of environmental samples (Martin and Travers, 1989).
Application of Bt is through conventional chemical ground spraying. This requires large water volumes and is relatively ineffective as the formulation is allowed to drip into the plant soil off the leaves. The use of air sprays has been successful in Israel against the *Boarmia selenaria* (Navon, 2000) an insect pest of avocado crops and *Anarsia lineata* in almond orchards (Roltsh et al., 1994). Since the endotoxin is coded by a single gene, which is usually carried on a plasmid it has been relatively easy to locate, clone and express these genes from a number of strains of Bt and express them in other organisms. To date the genes have been expressed in tomato and tobacco, however there is evidence that resistance can be developed by the lepidopterus pests they aim to control (Stone et al., 1989).

To date there are many commercial applications against Bt in many crops. Table 1.1 shows a list of the Bt producing strains, companies, target pests and crops that are commercially in operation around the world (Navon, 2000).

The focus thus far has been on classical biological control and pest management. This is a fair summary as the major success both commercially and scientifically has come from this area of the biological control program. Many biological control agents however have been isolated that can protect plants from disease that is not caused by insects but instead by soilborne microorganisms. The effectiveness of these biological control agents has not received the attention of the pest control formulations, as there is a shorter history of its research. Over the past 25 years biological control in the soil has begun to emerge as an important area of disease management of crop plants.

The best examples of biological control by microorganisms are crop rotation, organic amendment and control through growth in suppressive soil. As with biological control on plant leaves, plant soil biological control involves the disease suppression through the sustained interaction between the plant, the pathogen, the biological control agent, the microbial community and the physical environment. Soilborne disease biological control is particularly complex as this takes place within a dynamic environment at
Table 1.1

<table>
<thead>
<tr>
<th>Bt strain</th>
<th>Company</th>
<th>Product</th>
<th>Target pests</th>
<th>Crop</th>
</tr>
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<tbody>
<tr>
<td><strong>Natural</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kurstaki HD-1</td>
<td>Abbott Laboratories</td>
<td>Biobit, Dipel, Foray</td>
<td>Lepidoptera</td>
<td>Field and vegetable crops, greenhouse, orchard fruits and nuts, ornamentals, forestry, stored products</td>
</tr>
<tr>
<td>Kurstaki HD-1</td>
<td>Thermo Trilogy Corp</td>
<td>Javelin, Steward, Thuricide, Vault Bactospine, Futura</td>
<td>Lepidoptera</td>
<td></td>
</tr>
<tr>
<td>Kurstaki</td>
<td>Abbott Laboratories</td>
<td>Able, Costar Florbac, Xantari</td>
<td>Lepidoptera, armyworms</td>
<td>Row crops</td>
</tr>
<tr>
<td>Kurstaki</td>
<td>Therma Trilogy</td>
<td>Able, Costar</td>
<td>Lepidoptera</td>
<td></td>
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<tr>
<td>Aizawai</td>
<td>Abbott Laboratories</td>
<td>Trivid</td>
<td>Lepidoptera</td>
<td></td>
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<tr>
<td>Tenebrionis</td>
<td>Abbott Laboratories</td>
<td>Novodor</td>
<td>Lepidoptera</td>
<td>Potato, tomato, eggplant</td>
</tr>
<tr>
<td>Tenebrionis</td>
<td>Thermal Trilogy</td>
<td>Trident</td>
<td>Coleoptera</td>
<td>Potato, tomato, eggplant</td>
</tr>
<tr>
<td>Kurstaki</td>
<td>BioDalia</td>
<td>Bio-Ti</td>
<td>Lepidoptera</td>
<td>Avocado, tomato, vineyards, pine forest, Date palms</td>
</tr>
<tr>
<td>Kurstaki</td>
<td>Rimi</td>
<td>Bitayon</td>
<td>Lepidoptera</td>
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<td>Galleriae</td>
<td>Tuticori Alkali Chemicals &amp; Fertilisers Ltd</td>
<td>Spicturin</td>
<td>Lepidoptera</td>
<td>Cruciferous crop plants</td>
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<td>YB-1520</td>
<td>Huazhong Agric.</td>
<td>Mainfeng</td>
<td>Lepidoptera</td>
<td>Row crops, fruit trees</td>
</tr>
<tr>
<td>CT-43</td>
<td>Huazhong Agric.</td>
<td>Shuangdu</td>
<td>Lepidoptera, Coleoptera, Diptera</td>
<td>Row crops, garden plants, forests</td>
</tr>
<tr>
<td><strong>Genetically modified</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aizawai recipient Kurstaki donor</td>
<td>Thermo Trilogy</td>
<td>Agree, Design</td>
<td>Lepidoptera</td>
<td>Row crops</td>
</tr>
<tr>
<td>5-endotoxin encapsulated in <em>Pseudomonas fluorescens</em></td>
<td>Mycogen</td>
<td>MVP, MATTCH, MTRACK</td>
<td>Coleoptera, Lepidoptera</td>
<td>Row crops, potato, tomato, eggplant</td>
</tr>
<tr>
<td>Kurstaki recipient</td>
<td>Ecogen</td>
<td>Raven</td>
<td>Lepidoptera</td>
<td>Row crops</td>
</tr>
<tr>
<td></td>
<td>Ecogen</td>
<td>Leptinox (recombinant)</td>
<td>Lepidoptera, armyworms</td>
<td>Turf, hay row crops, sweet corn</td>
</tr>
</tbody>
</table>

List of commercially available Bt. Adapted from Navon *et al.* (2000)

the interface of the root and soil known as the rhizosphere. This term was coined by Hiltner in 1904 to describe specifically the interaction between the bacteria and legume roots (Lynch, 1990). The rhizosphere is typified by rapid change and high microbial communities.
These complexities have limited the use of biological control by microorganisms in the soil environment. The biological control in soil can be split into three areas: interaction with the microbial community; interaction with the plant and; interaction with the pathogen.

This concept will become important during the experimental sections of this thesis where a biological strain that naturally interacts with the plant will be modified to interact with the pathogen as well in an attempt to enhance biological control. Interaction with the microbial community has been shown by Schippers (1993) through the suppression of *Fusarium* wilt in radish through alteration of the natural fungal community following inoculation with a fluorescent pseudomonad.

*Bacillus cereus* strain, UW85, inoculation can induce dramatic changes in the composition of cultural bacterial communities on soya bean roots in the field (Gilbert et al., 1993). The effect is to make the root look like soil by encouraging soil bacteria to reside in the roots. This effect is known as camouflaging and in this example is believed to lead to the reduction in the pathogen around the roots as its preference is to colonise root and not soil microfloras.

Interaction with the plant by the biological control strain also plays an important role in disease suppression. The colonisation of plants' roots or leaves seems an essential prerequisite for successful biological control as a strong correlation is emerging between the biological control and root colonisation (Chin-A-Woeng et al., 2000; Schippers et al., 1987; Bull et al., 1991; Raaijmakers et al., 1998).

Induced resistance has been noted following microbe plant interaction. An example is the effect of inoculation with *Pseudomonas fluorescens* CHA0, a natural producer of pyoverdin which induces the release of proteins involved in systemic acquired resistance (SAR). This in essence involves the accumulation of salicylic acid which plays a role in the SAR suite of genes in the plant. The effect is the reduction of susceptibility to viral pathogens (Maurhofer et al., 1994).
Preparations of lipopolysaccharide from the surface of *P. fluorescens* have been shown to induce SAR and prevent *Fusarium* wilt as effectively as the living bacterial population itself (Leeman *et al.*, 1995).

Interaction with the pathogen directly is often the most common method of disease control and the method upon which most current research has focused. Namely protection through antibiotic production. This will be discussed in detail later in the chapter as this is an important approach to disease suppression in this thesis. In principle the advantage of biological control antibiotics is that resistance is developed more slowly than with synthetic pesticide use. This is because often more than one antibiotic is produced thus requiring the pathogen to generate multiple resistance. The second is that delivery is often more precise reducing selective pressures imposed by the widely dispersed use of synthetic pesticides.

This was demonstrated in a commercially successful manner with the biological control strain *Agrobacterium radiobacter*, the biological control agent responsible for preventing crown-gall caused by the pathogen *Agrobacterium tumefaciens*. The disease suppression is mediated through the antibiotic agrocin-84 produced by *A. radiobacter* by the plasmid pAgK84. This plasmid also contains the resistance element for resistance to agrocin-84. Mobilisation had occurred which resulted in the pathogenic agent *A. tumefaciens* becoming resistant to agrocin-84 through the acquisition of the resistance element on pAgK84. Commercially this problem has been overcome through the removal of the mobilisable element from plasmid pAgK84 however, the potential for this transfer of resistance remains a concern for other biological control models (Stockwell, 1996; Panagopoulos *et al.*, 1979).

Competition for nutrients is another method by which a biological control strain can act directly on the pathogen. The best understood mechanism is iron competition. Iron is an abundant element in soil but is only available as ferric oxide. Siderophores, including salicylic acid, pyochelin and pyoverdin, chelate iron and other metals, which mop up trace metals and contributes to disease suppression by conferring a competitive advantage to the biological control agent for the limited supply of the minerals in natural habitats (Loper and Henkels, 1997). Siderophores are low
molecular weight compounds that are produced under usually, iron-limiting conditions. The main evidence for the role of siderophore mediated biological control is through the work by Kloepper and Schroth (1981), but subsequent work using Tn5 mutants designed to inhibit pyoverdin production have shown that siderophore production can inhibit *Fusarium oxysporum* (Becker and Cook, 1988) and *Gramminis graminis* var. tritici (Weller et al., 1988).

The final major mechanism for plant biological control of soilborne pathogens in the role of microparasitism. The best examples of this form of biological control has come from the effect of the biological control fungi of the *Trichoderma* species. The parasitism has been shown to occur through communication between the pathogen and the *Trichoderma* species as there is a species specific mechanism for the coiling of the biological control strain around the pathogen. This was first demonstrated by Dennis and Webster in 1971, when it was shown that *Trichoderma* would not coil around threads made to replicate the mycelium of *Pythium ultimum*. The mechanism of parasitism is performed through the secretion of a battery of lytic enzymes that degrade the pathogen. Two strains that exhibit the greatest potential for commercialisation through his mechanism of biological control are *Gliocladium* and *Trichoderma*.

*Gliocladium virens* has become commercially successful (Lumsden and Walter, 1995) however the lack of reproducibility of the effects have been a major limitation to widespread commercial use.

To place the success of biological control into a commercial perspective a review was published in 1993 (Powell and Jutsum) which stated that in 1991 biological control agents accounted for just 0.5% of the total agrochemical market. Most of these sales were from the bio-insecticides and not against soilborne pathogens. Of these 90%, were attributed to the *Bacillus thurengiensis*, from which the live strain is rarely used and often either the gene products from genetically modified plants or from application of a formulation of the isolated endotoxin. In this case it is hard to include this as true biological control as the application is tending towards conventional chemical application. In 1991, the total agrochemical sales world-wide was $26,000
million, of which biological products accounted for $120 million, with true, non-Bt, biological control accounting for $10 million, just 0.04%. The amount of biofungicides sold worldwide in 1991 was just $1 million.

This thesis shall concentrate entirely on a method of disease crop protection using an anti fungal agent naturally produced in soils that can lead to the protection of plants against seedling diseases.

**Current perspectives of commercial biological control using microorganisms**

In recent years, there has been an increasing commercial and environmental interest in the use of natural microbial and plant metabolites for protecting crops against soilborne pathogens. This represents a niche of biological control concerned mainly with disease control. The identification of naturally disease suppressive soils has led to the isolation of numerous microbial strains that protect plants against a wide range of microbial plant pathogens. The realisation of the commercial potential for biological control for integrated crop management began with the first commercially available formulations. The United States Environmental Protection Agency (EPA) has given approval for the marketing of the following antifungal biological control strains. Bacterial biological control protection has been achieved using naturally isolated species. *Agrobacterium radiobacter* marketed as Galltrol-A (AgBioChem Inc), Nogall and Diegall (Care Technologies), and Norbac 84C (New Bioproducts Inc.).

*Bacillus subtilis* marketed as Epic and Kodiak (Gustafson Inc.), Rhizo Plus (KFZB Biotechnik GmbH), Serenade (AgraQuest Inc.) and System3 (Helena Chemical Co.); *Burkholderia cepacia* marketed as Intercept (Soil Technologies Corp.).

*Pseudomonas syringae* marketed as Bio-save100, Bio-save 110 (Eco Science Corp.); *Streptomyces griseoviridis* marketed as Mycostop (Kemira Agro).

Fungal formulations have also been commercially marketed for the purpose of crop protection by biological control. *Ampelomyces quisqualis* marketed as AQ10 (Ecogen Inc.). *Candida oleophila* has been marketed as Aspire (Ecogen Inc). *Coniothyrium*
minitans marketed as Contans (Prophyta Biologischer Pflanzenschutz GmbH) and KONI (Bioved Ltd.). *Fusarium oxysporum* marketed as Biofox C (S.I.A.P.A) and Fusaclean (Natural Plant Protection). *Gliocladium virens* marketed as SoilGard (Thermo Trilogy). *Gliocladium catenulatum* marketed as PreStop and Primastop (Kemira Agro). *Phlebia gigantea* marketed as Rotstop. *Pythium oligandrum* marketed as Polygandron. *Trichoderma harzianum* and other spp marketed as BioFungus and BinabT (Bio-Innovations AB), RootShield (Bioworks Inc.), Trichodex (Makhteshim Chemical Works), Trichopel (Agrim Technologies), Trichoject, Trichodowels, Trichoseal and Trichoderma 2000 (Mycontrol Ltd.).

Fluorescent pseudomonads encompass the largest single group of microorganisms detected in disease suppressive soils. At genus level, fluorescent pseudomonads have already proven themselves commercially viable. Formulations of *Pseudomonas fluorescens* have been marketed, *BlightBan A506* (Plant Health Technologies); used to protect against the fruit plant pathogen, *Erwinia amylovora*; and *Conquer* (Sylvan Spawn Laboratories) used to protect against bacterial blotch disease of mushrooms caused by *P. tolaasii*.

The specific area of biological control that has received the most attention in recent years has been the production of the antifungal metabolite 2,4-diacetylphloroglucinol (Phl) by natural isolates of fluorescent pseudomonads.

This secondary metabolite has received this interest as it naturally suppresses a broad range of fungal pathogens when grown in the presence of a wide selection of economically important crop plants. In these disease suppressive soils, it is considered as the major determinant of natural disease suppression, with 20% of pseudomonads isolated from take-all suppressive soils being able to produce Phl (Harrison *et al.*, 1993).

To date, four separate secondary metabolites produced by fluorescent pseudomonads have been identified as major inhibitors of pathogen disease on crop plants. The group consists of phenazines and the polyketides, pyoluteorin, 2,4-diacetylphloroglucinol, and pyrrolnitrin. The structures and genetic elements controlling each of these four
biosynthetic metabolites have been elucidated in recent years, and are described in detail later in this chapter.
1.2. **ANTIFUNGAL PRODUCTION BY FLUORESCENT PSEUDOMONADS**

*Phenazines*

Phenazines represent a group of antibiotic compounds that are cyclic, nitrogen containing and pigmented (Figure 1.1).

![Phenazine-1-carboxylic acid](image)

**Figure 1.1** Phenazine-1-carboxylic acid

They are produced through the shikimic pathway and are the major determinant in the ability of certain bacteria to suppress the pathogenic fungus *Gaeumannomyces graminis* var. *tritici*. *P. fluorescens* 2-79, isolated from disease suppressive soils in Lind, Washington State, has been found to suppress, take-all of wheat, through the production of phenazine-1-carboxylic acid (PCA). Another rhizosphere strain seen to suppress *G. graminis* var. *tritici* is *P. aureofaciens* 30-84 isolated from soil in Kansas, where take-all has declined, also through the same mechanism of production of PCA (Mazzola and White, 1994).

The biosynthetic locus responsible for phenazine production in strain 30-84 has been described by Pierson *et al.* (1995). He reveals five contiguous open reading frames (*phzFABCD*) and two additional genes *phzRI* belonging to the N-acyl-homoserine lactone mediated gene regulation. This form of genetic control is described later.
**Pyoluteorin (Pit)**

Pyoluteorin (Figure 1.2) is produced by three characterised fluorescent pseudomonads, *P. fluorescens* Pf-5, CHAO and S272 (Howell and Stiponovic, 1980; Maurhofer et al., 1994; Yaun et al., 1998).

![Pyoluteorin](image)

**Figure 1.2 Pyoluteorin**

Each of the fluorescent pseudomonad strains, CHA0, Pf-5 and S272, has disease suppressive qualities against the fungal pathogen *Pythium ultimum*, the major determinant of the seedling disease known as damping-off. Pyoluteorin is highly toxic to *P. ultimum*, in vitro, as described by Defago et al. (1990) and Maurhofer et al. (1992). There has been uncertainty as to the exact contribution of this antibiotic in disease suppression in vivo, as both strain Pf-5 and CHA0 also produce Phl and are also able to suppress *P. ultimum*.

Pyoluteorin is produced via the polyketide pathway. Polyketides are synthesised via a successive condensation reactions of short chain carboxylic acids in a process similar to fatty acid biosynthesis. Proline and acetyl-CoA are predicted as being the initial precursor for the assembly of the final molecule shown in Figure 1.2. These predictions are made by Bender et al. (1999), on the basis of the sequence analysis of the ten genes that map within the *Pit* biosynthetic gene cluster of Pf-5. The biosynthetic pathway has, in part been elucidated, and so far ten genes have been
isolated that form a cluster responsible for Plt production (Nowak-Thompson et al., 1999).

**Pyrrolnitrin**

Pyrrolnitrin (Figure 1.3) is an additional antibiotic produced by strains *P. fluorescens* CHA0 and Pf-5.

![Figure 1.3 Pyrrolnitrin](image)

The metabolite has been shown through transposon mutagenesis of the biosynthetic genes to inhibit the growth of the plant pathogen *Pyrenophora tritici-repentis* the causative agent of tan spot of wheat (Corbell and Loper, 1995). The Tn5-mutant, Pf-5, unable to produce pyrrolnitrin lost the suppressive ability against *P. tritici-repentis*, whilst still maintaining the production of the other metabolites Phl and pyoluteorin (Sarniquet et al., 1994).
2,4-diacetylphloroglucinol (Phl)

Diacetylphloroglucinol is a phenolic bacterial and plant metabolite with antiviral, bacterial, antifungal antihelminthic and phytotoxic properties (Figure 1.4). Phl, as with Plt is thought to be synthesised via the polyketide pathway. The mechanism of action of phloroglucinol derivatives is unclear, however, the two acyl and three hydroxyl groups on the phenolic nucleus, are all considered as groups active in the suppression process (Fujimoto et al., 1995). Phl production is thought to take place via the polyketide pathway, with monoacetylphloroglucinol a probable biosynthetic intermediate (Shanahan et al., 1993). The precursor for the assembly of Phl is believed to be acetyl-CoA through analysis of sequence data from the biosynthetic gene cluster responsible for Phl production in *P. fluorescens* Q2-87 (Bender et al., 1999).

![2,4-diacetylphloroglucinol](image)

**Figure 1.4 2,4-diacetylphloroglucinol**

2,4-diacetylphloroglucinol — broad spectrum fungicide

The biological control agent, 2,4-diacetylphloroglucinol has been shown to suppress seedling diseases found in economically important crops. These include the diseases of root rot of wheat, caused by *Fusarium oxysporum*; black root rot of tobacco, caused by *Thielaviopsis basicola* (Defago et al., 1990; Keel et al., 1990, 1992); damping-off of sugar beet, caused by *Pythium ultimum* (Fenton et al., 1992, Shanahan et al., 1992); damping-off of cotton, caused by *Pythium ultimum* and *Rhizoctonia solani* (Kraus and Loper, 1992); blotch of wheat, caused by *Septoria tritici* (Levy et al., 1992) and take-

Standard laboratory screens of fluorescent *Pseudomonas* spp. from a diverse geographic origin show they are able to suppress these plant pathogens. Notable strains of fluorescent *Pseudomonas* spp. that produce Phl are CHAO, F113, Q2-87, Pf-5 and S272. Attempts have been made with each of these strains to genetically characterise the genetic locus and the controlling elements responsible for the Phl production by these strains. This is discussed later in this chapter.

Summary of the strains and the metabolites known to be responsible for biological control of known plant pathogens are shown in Table 1.2.

**Table 1.2**

<table>
<thead>
<tr>
<th>Biological control strain</th>
<th>Phenazines</th>
<th>Pyoluteorin</th>
<th>Pyrrolnitrin</th>
<th>2,4-diacetylphloroglucinol</th>
<th>Pathogen</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHAO</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td><em>P. ultimum</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td><em>R. solani</em></td>
</tr>
<tr>
<td>F113</td>
<td></td>
<td></td>
<td>+</td>
<td></td>
<td><em>P. ultimum</em></td>
</tr>
<tr>
<td>Q2-87</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td><em>G. graminis</em></td>
</tr>
<tr>
<td>Pf-5</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td><em>G. graminis</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td><em>P. tritici repentis</em></td>
</tr>
<tr>
<td>S272</td>
<td></td>
<td></td>
<td>+</td>
<td></td>
<td><em>P. ultimum</em></td>
</tr>
<tr>
<td>30-84</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td><em>G. graminis</em></td>
</tr>
<tr>
<td>2-79</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td><em>G. graminis</em></td>
</tr>
</tbody>
</table>
1.3. **Genetics of 2,4-Diacetylphloroglucinol Biosynthesis**

Tn5 mutagenesis and complementation studies have been used to clone a 6 kb genomic fragment required for the biosynthesis of Phl from *Pseudomonas* sp. strain F113 (Fenton *et al.*, 1992). This recombinant plasmid, pUC203 containing this fragment, partially complemented a Phl⁻ Tn5 mutant, F113G22, derived from F113, to Phl⁺ phenotype. However, when this plasmid was used to transform eight other natural Phl non-producers, of *Pseudomonas* spp., only one strain conferred the ability to synthesise Phl. The authors believe this was because the cloned fragment did not containing the complete biosynthetic gene cluster for Phl production. The one strain that was transformed to the Phl⁺ phenotype may have already contained the missing element on its genome.

Similar studies have been used to identify two distinct loci, involved in Phl production, in *P. fluorescens* CHA0. An 11 kb fragment from the genome of *Pseudomonas* sp. strain CHA0 was shown to be able to partially complement the Phl⁻ Tn5 mutant, CHA625, derived from CHA0, to a Phl⁺ phenotype (Keel *et al.*, 1992).

A 22 kb genomic fragment was able to increase Phl production when introduced into the CHA0, natural Phl producer (Maurhofer *et al.*, 1995). The two fragments were shown to be both structurally and genetically distinct. Schnider *et al.* (1995) revealed the mechanism for the Phl overproduction imparted by the 22 kb fragment was due to the presence of the 2 kb sigma factor, *rpoD*, located within the fragment.

**Sequencing the biosynthetic gene cluster for Phl biosynthesis**

More recent characterisation of the genomic locus responsible for Phl biosynthesis has shown that a 6.5-kb region, of the *P. fluorescens* sp. strain Q2-87 genome, is sufficient to transform a non-producing strain of the *Pseudomonas* sp. into a Phl⁺ strain (Bangera and Thomashow, 1996). This study showed that the 6.5-kb fragment alone was capable of transforming all 13 Phl-non-producing recipient pseudomonads used in the study. The strains used were isolated from three different soil types and had no common characteristics that would suggest they would be predisposed to expressing the introduced biosynthetic locus.
Figure 1.5 A schematic representation of the biosynthetic gene clusters responsible for Phi biosynthesis in strain Q2-87 (from sequence data Bangera and Thomashow, 1996, transformed in VectorNTI, infomax).

The nucleotide sequence of the Phi biosynthetic locus has been published by Bangera and Thomashow (1996). It shows six genes, spanning a 6.7-kb region in the genome of Q2-87 (Figure 1.5). Transposon mutagenesis with Tn3HoHol led to the identification of a region spanning approximately 5 kb, that contained at least two divergently orientated transcriptional units, required for Phi production. The nucleotide sequence of the region shows six genes organised in three transcriptional units. Four genes comprise the operon phlACBD. Either side of this unit is genes phlE and phlF, which code for putative efflux protein and regulator protein respectively. Each gene has been characterised based on sequence homology (Table 1.3).
Table 1.3

<table>
<thead>
<tr>
<th>ORF</th>
<th>Predicted protein based on database homology</th>
<th>Accession No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>phlA</td>
<td>ACP synthase (<em>E. coli</em> FabH)</td>
<td>P24249</td>
</tr>
<tr>
<td>phlC</td>
<td>Mammalian SCPx (thiolase domain)</td>
<td>P32020</td>
</tr>
<tr>
<td>phlB</td>
<td><em>AcaC</em> from <em>P. furiosus</em></td>
<td>X85350</td>
</tr>
<tr>
<td>phlD</td>
<td>Calcone synthase family</td>
<td>S12224</td>
</tr>
<tr>
<td>phlE</td>
<td>PMF drug efflux proteins</td>
<td>M80252</td>
</tr>
<tr>
<td>phlF</td>
<td>Repressor proteins</td>
<td>X86780</td>
</tr>
</tbody>
</table>

Sequence homology showing predicted proteins from each of the ORF from the *P. fluorescens* Q2-87 Phi biosynthetic locus.

Numerous attempts have been made to optimise the biological control potential of each of the naturally isolated Phi producing strains. Hand-in-hand with this, research has attempted to understand the mechanisms by which Phi is synthesised and the full gene set responsible for its production. The sequence data from Q2-87 has helped to delineate the genes underlying the biosynthetic pathway, however studies of the sequence homology of the genes responsible for Phi production do not reveal a clear cut pattern for the synthesis of the Phi molecule. The larger transcript containing genes *phlA, phlC, phlB and phlD* from which a pattern of Phi synthesis can be elucidated. Gene, *phlA* has sequence homology to *E. coli, fabH*, which encodes the enzyme involved with polyketide synthesis, ketosynthase. Gene *phlC* shows homology with a thiolase located at the N-terminus of sterol carrier proteins. $\beta$-keto thioesters are intermediates in fatty acid synthesis and polyketide synthesis following the condensation of the precursor acetyl-CoA (Bender *et al.*, 1999). Gene, *phlB* shows homology with the gene involved with acetoacetyl-CoA synthesis, again a process involved in fatty acid synthesis, very similar to the polyketide pathway proposed for the production of Phi (Bangera and Thomashow, 1999, 1996; Bender *et al.*, 1999). The gene *phlD* is proposed as being responsible for the condensation and cyclisation steps that result in the formation of monoacetylphloroglucinol, the proposed precursor.
(Shanahan et al., 1993) molecule for the acetylation to diacetylphloroglucinol. Gene \textit{phlE} is involved in the mechanism for secreting the Phl from the bacteria, and may double as a resistance mechanism avoiding high concentrations of Phl accumulating within the cytoplasm. The gene \textit{phlF} has been proposed and recently confirmed as a repressor regulator of the Phl gene cluster (Delany \textit{et al.}, 2000; Schnider-Keel \textit{et al.}, 2000). The regulator gene is a common characteristic of biosynthetic operons and plays no part in the synthesis of the Phl molecule itself. Also divergent transcription is a common trait for biosynthetic regulation (Postle \textit{et al.}, 1984). Here the divergent transcription of the resistance element of tetracycline in Tn10 is described.

\textit{Genetic diversity of Phl producing organisms}

In 1996, Keel \textit{et al.} determined that part of the \textit{Phl} biosynthetic locus is conserved throughout all the Phl producing strains worldwide. This was achieved through using a 4.8-kb chromosomal DNA region from \textit{Pseudomonas} strain Q2-87, carrying the \textit{Phl} biosynthetic genes, as a probe. The probe successfully hybridised with all 45 Phl producing strains tested. These strains are well characterised for biological control in the U.S and Europe. The Phl producers were seen to show considerable phenotypic and genotypic diversity. Two phenotypically distinct groups were detected. The groups were split into those that produce Phl, hydrogen cyanide (HCN) and pyoluteorin and those that produce Phl and HCN only.

The 45 strains were further characterised by restriction fragment length polymorphism (RFLP) using \textit{EcoRV} and \textit{BamHI} to digest each strain’s genome. Amplified rDNA restriction analysis (ARDRA) fingerprints were also analysed as were rapid amplified polymorphic DNA (RAPD) fingerprints. From each of these tests, strain FI13 isolated from Ireland showed distinct polymorphism from all other natural producers tested.

Raaijmakers \textit{et al.} in 1997 showed they were able to show the same findings as Keel \textit{et al.} the previous year, by using the polymerase chain reaction (PCR) with primers designed to amplify the \textit{phlD} gene within the \textit{Phl} biosynthetic locus. This procedure showed that the \textit{phlD} gene is conserved throughout all the Phl producing strains that they tested.
Optimal conditions for Phi production

All Phi producers probed positively with the Q2-87 biosynthetic cluster suggesting that these genes are universally responsible for the antibiotic production. The broad geographical distribution and the genetic diversity seen through RFLP, ARDRA and RAPD fingerprinting of the strains suggests that although the Q2-87 – type gene locus may be present in all the Phi producing strains, it did not confirm that they are wholly responsible for Phi production. This is emphasised when conditions favouring Phi production are compared between three well-characterised strains.

In 1992, Shanahan et al. described the optimal conditions for in vitro production of Phi by strain FI 13. It was concluded that carbon source is critical to the production of high levels of the antibiotic. Strain FI 13 grown in media containing sucrose, mannitol or fructose produced high levels of Phi whereas media containing glucose or sorbose resulted in very low levels of Phi production. It was noted that bacteria grown in low nutrient broth to surface area ratios produced the highest quantity of Phi. This particular finding has been confirmed with the remaining Phi producing strains, with Phi production being highest when bacteria are grown on plates rather than in liquid culture (Shanahan et al., 1992; Bonsall et al., 1997).

In 1994, Nowak-Thompson et al., showed that the optimal carbon source for the production of Phi by strain Pf-5 was glucose. This is inconsistent with the findings of Shanahan et al., (1992) with strain FI 13, and reveals the complexity of Phi production by strains appearing to contain similar genetic Phi biosynthetic loci.

In 1999, a newly isolated natural producer of Phi was described by Nakata et al., in Japan. The strain, P. fluorescens S272, was shown to only produce Phi in either ethanol or glycerol and not in sucrose or mannitol. In addition, media amended with N-(3-oxodeconyl)-L-homoserine lactone considerably increased the production of Phi.
Although the genes responsible for the synthesis of Phl have been identified in strain Q2-87, and appear geographically conserved, there appear to be many additional factors that play an important role in the regulation of Phl biosynthesis, some of which appear strain specific and plant specific.

In 2000, Picard et al. showed through analysis of metabolite patterns and molecular typing that there is considerable diversity among Phl producing pseudomonads depending on plant species and soil type. The study was conducted on maize throughout one growing season and the results suggest that Phl producers are present throughout the plant life cycle in varying abundance, but that the genotypic diversity of these Phl producers is diverse and related to the plant age. The phlD genes from the Phl locus of these isolates were amplified by PCR and sequence homology was noted at >99% homology between the isolates. The genetic element responsible for Phl production therefore appeared to remain the same however the diversity of the strains that produced the Phl seems to alter with plant age. The major difference that occurs on plant ageing is the exudation from the plant roots and this is proposed to be the factor that has led to the divergence in Phl producers. The importance of plant nutrients in creating a differential selection pressure on natural Phl producers is an important finding from this in situ study. Although all strains tested were able to produce Phl, only a few were present throughout the entire plant life, through the varying nutrient conditions in the soil, indicating a trait that is positively selected for, throughout a plant’s life cycle.

McSpadden Gardener et al. (2000) attempted to generate a database to define the proportion of phlD+ isolates from wheat in take-all suppressive soils that contributed the greatest biological control protection to the wheat. Studies by Keel et al. (1996), Raaijmakers et al. (1999) and Picard et al. (2000) show that Phl producers are genetically diverse and therefore are likely to differentially contribute to plant protection. From the isolates recovered in this study a database was constructed showing that isolates from eight different soils from four separate geographical locations could be separated in to three phylogenetic groups following ARDRA analysis. It was noted that these groups showed a strong association with particular phenotypes when substrate utilisation was examined using Biolog assays. The authors
of this study admit that the Biolog assay used, utilised substrate that can distinguish microbial taxa rather than using substrate that would discriminate strains or groups that are likely to colonise the rhizosphere of particular plants. This could be achieved through simulation of the root exudates from particular plants into the Biolog assay to determine the groups that show the greatest potential for root colonisation *in situ*.

It is clear from these examples that determination of the genes responsible for Phi production do not in themselves lead to a full understanding of the diversity in the performance of strains containing these genes to protecting plants. There is clearly a large genotypic diversity in the strains that produce Phi and with that come differences in substrate utilisation and consequently survival and colonisation in different soil types and around different plants.

A study conducted in 1999 by Duffy and Defago, aimed to show which environmental conditions were important in enhancing biological control. The findings showed that there was a strain dependant pattern of antibiotic production and nutrient amendment. In strain CHA0, the addition of glucose led to an increase in the level of Phi produced. In strain F113 the addition of glucose led to no improved effect however sucrose caused an increase in Phi production. This supports work done by Shanahan *et al.* in 1992. The addition of zinc also led to a strain dependant increase in Phi production. The addition of inorganic phosphate led to the decrease in Phi production again in a strain specific manner. The significance of this work is to determine which of the numerous Phi producers will be able to act most effectively on specific plants grown in a particular soil. The enhancement and repression of Phi production on inoculation may also be beneficial. Phytotoxic effects seen with too high levels of antibiotic can be reduced through formulation with phosphates and an increase in production can be achieved through formulation with zinc. This work however accentuates the complexities of the factors that regulate secondary metabolite production even within the same species of organism.
1.4. **Regulation of Secondary Metabolite Production**

The successful biological control agent can protect susceptible plants in one of four ways. Competition for resources, site-exclusion, secondary metabolite production and microparasatism. The factors that control the balance between the pathogen and the biological control organism are often controlled by biotic and abiotic factors around the host plant. The competitive mechanism and the genetic control of this mechanism are important areas of research for a comprehensive understanding of optimal biological control.

Three separate regulatory pathways are believed to be responsible for adaptation to environmental stimuli in soil bacteria. A two component regulatory pathway alters gene expression in response to environmental signals. N-acyl-homoserine lactone (N-acyl HSL) mediated gene regulation responds to population dynamics, otherwise known as ‘quorum-sensing’. Finally, gene regulation mediated by sigma factors respond to an organism’s fitness and stress in the environment.

*Two component-mediated gene regulation*

The control of secondary metabolite production is partly controlled by external factors via two proteins. A transmembrane sensor-kinase protein will respond to direct contact with the environmental stimuli. Autophosphorylation at a specific histidine residue initiates the response causing the passage of a phosphorylation signal via the movement of the phosphate residue to the second protein within the bacterial cell. This causes the phosphorylation of the response-regulator protein at a conserved tyrosine residue. This induces a conformational change and enables the physical binding to DNA at a specific site. The site is often located within the promoter region of genes that are responsive to the two compartment regulatory control (Figure 1.6).
This is a universal process throughout the bacterial kingdom however in plant associated pseudomonads, sensor kinase and the response regulator proteins are called LemA and GacA, respectively (LemA termed GacS in *P. fluorescens*). The LemA and GacA regulation was identified through complementation studies of antibiotic producing organisms that through insertion mutation were unable to produce the antibiotic. Complementation of the mutants with genes homologous to known proteins of this pathway restored the organism ability to produce the antibiotics.

GacA (global antibiotic and cyanide control) was identified by Laville *et al.* (1992) in strain *P. fluorescens* CHA0. It was discovered that mutations in the pseudomonad gene, *gacA*, pleiotropically blocked the production of secondary metabolites Phl, HCN and pyoluteorin. Sequence data indicated that the GacA protein is a response regulator in the FixJ/DegU family of two component regulatory systems. It shows high homology to UvrY protein of *E. coli*, a member of the FixJ family of two component regulatory proteins. Complementation of the CHA0 gacA mutants with
plasmid pME3066 containing the cloned gacA loci from CHAO partly restored the wildtype CHAO phenotype. The sequence data of the GacA protein revealed a helix – turn – helix domain indicative of proteins able to bind DNA. This supported the role of GacA and GacA-like proteins as the response regulators responsible for DNA binding in the two-compartment regulation of secondary metabolite production.

In 1995, Corbell and Loper described the sensory component of this two component regulatory model. P. fluorescens Pf-5 mutated through Tn5 insertion lost the ability to produce Phi, HCN and pyoluteorin. This region was mapped, sequenced and denoted as apdA (antibiotic production gene). Complementation of the mutant strain with this region restored the ability to produce each metabolite. Sequence data of the region revealed close homology with known sensor regulators, lemA and repA encoding putative sensor kinases from P. syringae pv. syringae and P. viridiflava respectively. Sequence data additionally showed conservation of the histidine kinase domain that initiates the phosphorylation of the sensor-kinase in the two component regulation pathway. Constructs carrying the apdA gene were able to restore metabolite production to the mutant P. syringae pv. syringae, indicating a conservation of function between apdA and lemA. Further evidence supporting the multifaceted nature of metabolite control was the Pf-5 mutants were not phenotypically restored with the addition of the gacA construct able to restore the phenotype of the CHAO mutant.

Complementation of gacA and gacS (recently used to describe apdA-like genes in P. fluorescens), mutants of F113 was achieved by Aarons et al. (2000) through the addition of a previously uncharacterised locus that was shown to encode a small RNA molecule, PrrB RNA. The addition of the prrB gene in trans to either the gacA or gacS mutant F113, resulted in normal production of Phi and HCN, lost in both the gacA and gacS mutants. Mutation of the wild-type F113 at the prrB locus led to a significant reduction in the production of both Phi and HCN. The most significant observation was that the PrrB RNA is actually regulated through the gacA and gacS regulatory pathway. This was shown through northern blotting that PrrB transcripts were not present in either the gacA or gacS mutants. Also when cloned into a promoterless vector, prrB did not restore secondary metabolite production in either
the gacA or gacS mutants. The authors predict that the phenotypic complementation of gacA and gacS mutants by PrrB RNA suggests that PrrB may function as a regulator within the P. fluorescens GacA-GacS regulatory cascade.

An important study conducted by Duffy and Defago (2000) bridged the molecular knowledge of gacA-gacS regulation of secondary metabolites to a practical consideration of these genes. There are numerous problems in extrapolating the findings from successful biological control in the laboratory to application in the field. Not least are the practical problems of manufacture and scale up of the inocula for large scale application. This study found that scale up of CHA0 inocula resulted in the production of spontaneous mutants that were unable to produce the levels of secondary metabolites that the original seed wild-type could produce. This was attributable to the mutation of the gacA and gacS genes in the two-component regulatory pathway. They found that spontaneous mutants occurred at a frequency of 1% in all of almost 200 growth flasks. On industrial scale-up, the proportion of mutants rose to 61% in 500-ml fermentation vessels. It was noted that the level of biological control protection offered to cucumber plants was significantly reduced if just 10% of the inocula were mutants. At 50% mutants in the inocula, there was no observed protection of the cucumber. Duffy and Defago were interested in why such high levels of mutants appeared during scale up and to determine a way of controlling the problem. Although a conclusive answer to this question was not achieved, there was evidence that nutrient amendment could influence the selective pressures on the mutant population thus reducing their proportions in the fermenters with relation to the wild-type. The particular amendments that led to a reduction in mutants were the growth in 1/10 strength nutrient broth containing yeast extract (NBY) as opposed to full strength broth. The belief from the authors was that the nutrient concentration was not the cause per se for the increase in mutant proportions, but rather the osmotic potential of the media. Further amendments with the addition of minerals such as zinc, copper, cobalt, manganese, and ammonium molybdate also lowered the proportion of gacA-gacS mutants. Interestingly, these are the same minerals that lead to the increase in production of the secondary metabolites through the gacA-gacS pathway, as described previously by Duffy and Defago in 1999.
Although there is strong evidence for the presence of LemA (GacS)/GacA-like regulation of Phi and other metabolites useful in biological control, the environmental signals that directly activate this regulatory component are not known.

**N-acyl-homoserine lactone-mediated gene regulation**

Two-component gene regulation described earlier, shows metabolite control through direct stimulation by environmental substances. There has emerged evidence that metabolite expression can also be triggered by population dynamics in the rhizosphere. This process has become known as quorum sensing, mediated by N-acyl-homoserine lactone (N-acyl-HSL) and was described in the rhizosphere by Fuqua *et al.* (1994). The paradigm used to describe N-acyl-HSL mediated gene regulation is the control of bioluminescence in the marine symbiont, *Vibrio fischeri*. Luminescence is controlled by two divergently expressed operons. One operon encodes LuxI together with six structural proteins, LuxCDABEG. LuxI is responsible for the synthesis of an N-acyl-HSL, N- (3-oxohexanoyl)-L-HSL (OHHL), which is freely diffusible through the cellular membrane. In open seawater, the natural habitat of the bacteria, there is no build-up of this signal molecule within the bacteria. However, on colonisation of a fish organ, the signal molecule diffuses into the immediate surroundings where it remains thus reducing the concentration gradient across the bacterial membrane. With high bacterial colonisation, this external OHHL concentration increases rapidly. The OHHL signal molecule eventually ceases to diffuse from the bacteria and accumulates within the bacteria directly stimulating expression of the Lux operon regulator, luxR. This results in the expression of the structural genes, including luxAB that synthesise the enzyme luciferase, leading to luminescence. The process has also been referred as ‘autoinduction’ as the LuxR also acts to stimulate the further expression of luxI increasing the synthesis of OHHL.

This precise mechanisms for quorum sensing has not been described in fluorescent pseudomonads, but has been shown in other disease suppressive bacteria, notably *P. aureofaciens* 30-84 (Mazzola and White, 1994) responsible for the suppression of *G. graminis*, the pathogen that causes take-all of wheat. Disease suppression in this case is the result of the production of phenazine metabolites.
Pierson et al. (1995) described the gene locus responsible for phenazine production, in strain 30-84, as five contiguous open reading frames, *phzFABCD*. Two additional genes are necessary for the production of phenazine, *phzR* and *phzl*, encoding a repressor and *N*-acyl-HSL synthase, respectively. These two genes are analogous to *luxI* and *luxR* in *V. fischeri* (Wood and Pierson, 1996). The mechanism for the regulation of phenazine expression is believed to result from the accumulation of an *N*-acyl-HSL, hexanoyl-L-homoserine lactone (HHL) leading to the activation of the *phzR* product, inducing the expression of the *phzFABCD* operon and phenazine production. As with luciferase production in *V. fischeri* the expression of *phzR* results in the expression of *phzl* increasing HHL synthesis (Figure 1.7).

**Figure 1.7** (Taken from Pierson III et al., 1997) *N*-acyl-HSL mediated regulation of phenazine biosynthesis. Boxed L and G represents the putative LemA (also called GacS) and GacA two compartment regulatory system.

Within the past year an exciting new development has emerged that Phi production also undergoes autoinduction by the Phi molecule itself (Schnider-Keel et al., 2000). The end result is strikingly similar to that seen with the previous example of autoinduction by *N*-acyl-homoserine lactones. This Swiss research group, working with strain CHAO, developed a Tn5, insertion mutant unable to produce Phi. The region where the insertion took place was sequenced and identified as being locating
within an open reading frame of unknown function, designated \(phlH\). This was however, located adjacent to \(phlF\), the Phl repressor; and \(phlA\), the first gene of the Phl biosynthesis pathway.

The expression of the gene \(phlA\) was monitored through the construction of a \(phlA\)-'\(lacZ\) translational fusion. As expected, the production of Phl was mirrored by the expression of \(phlA\) in growth flask conditions, reaching a peak during late exponential phase. For the first time however, it was noted that the addition of exogenous Phl, to growth media which did not support Phl production in CHAO, led to an increase in the expression of \(phlA\). In addition, a \(phlA\) mutant of CHAO, unable to produce Phl, could be compensated by the addition of exogenous Phl, which led to production of Phl and the partial restoration of \(phlA\) expression.

The effect of Phl production was also investigated using a CHAO, \(phlF\) mutant. In this case the production of Phl was enhanced in comparison to the wild-type strain. The \(phlA\)-'\(lacZ\) fusion indicated that the expression of \(phlA\) was also increased and was found to be similar to that seen during increased Phl production in the wild-type with exogenously added Phl. Interestingly, the addition of exogenously added Phl to the \(phlF\) mutant led to no further increase in Phl production than the levels seen with the \(phlF\) mutant with no exogenously added Phl. In effect, the addition of Phl has no effect on \(phlF\) mutants and therefore the method of action of the Phl molecule is dependant on the presence of PhlF. The method by which \(phlF\) acts was shown by the Swiss group to be at a transcriptional level. This was indicated through the construction of a transcriptional fusion between \(phlA\) and \(lacZ\) (\(phlA\)-\(lacZ\)). Introduction of the \(phlA\)-\(lacZ\) fusion into the wild-type CHAO; the \(phlA\) mutant; and the \(phlF\) mutant, showed that in the \(phlA\) mutant, the expression of the \(phlA\)-\(lacZ\) fusion was four fold lower than the wild-type, and six fold higher in the \(phlF\) mutant during the late exponential phase of growth. These findings indicated that \(phlA\) expression is regulated positively by Phl and negatively by PhlF at the transcriptional level.

The action of PhlF as the repressor of Phl at the transcriptional level is supporter by the work of Delany \textit{et al.} (2000). This study showed that the PhlF protein binds to the
intergenic space between the \(phlA\) and \(phlF\) genes of the Phi biosynthetic locus. The role of PhlF as a transcriptional repressor of Phi production was shown through the introduction of high copy numbers of \(phlF\) in a F113 wild-type background. The results were the repression of Phi production and the repression of the expression of \(phlD\) transcription fusion by over 90%. A mutation in the \(phlF\) leads to an increase in Phi production \textit{in vitro}.

\textit{Sigma factor mediated gene regulation}

It has been described previously how external environmental molecules and bacterial community density can affect gene expression in the production of disease suppressive metabolites. Sigma factors have been shown to regulate the expression of at least four disease suppressive secondary metabolites, Phi, pyoluteorin, pyrrolnitrin and phenazines.

Sigma factors are polypeptides that make up part of the RNA polymerase holoenzyme. They provide RNA polymerase with the affinity for binding DNA and most importantly the specificity for binding particular promoters or particular regions within promoters. Depending on the organism’s growth state, different sigma factors will be synthesised to respond to the physiological requirements of that organism.

Two sigma factors have been identified which alter the expression of a number of important secondary metabolites in fluorescent pseudomonads. Sigma factor RpoD (\(\sigma^70\)) is the general housekeeping factor present through exponential growth and times of stress free growth. RpoS (\(\sigma^8\) or \(\sigma^{38}\)) is the sigma factor that is expressed when bacteria enter stationary phase during times of cellular stress. Schnider \textit{et al.} (1995) reported that the addition of multiple copies of the gene \(rpoD\) led to the increased production of Phi and pyoluteorin in \textit{P. fluorescens} CHA0.

Pfender \textit{et al.} (1993) reported sigma protein, RpoS, increasing pyrrolnitrin synthesis in \textit{P. fluorescens} Pf-5. Mutants of Pf-5 unable to synthesise RpoS, in addition to showing reduced survival in stress conditions (Sarniquet \textit{et al.}, 1995) could not produce pyrrolnitrin. The mutated strain however overproduced both pyoluteorin and Phi. The overexpression of Phi and pyoluteorin by CHA0 in response to additional
RpoD and in Pf-5 by removal of RpoS led Schnider et al. (1995) to speculate that the two sigma factors are competing for RNA polymerase during stationary phase. In addition, it seems clear that Phl and pyoluteorin are preferentially expressed during times of free growth whereas pyrrolnitrin is preferentially expressed during times of stress.

The delineation of this milieu of regulatory pathways and stimuli hold the key to developing an effective biological control strategy. Strains that can tolerate high population densities without evoking high levels of cell stress will be optimal for the production of Phl. The molecules responsible for triggering the two-component regulatory pathway need to be elucidated. An important consideration is to determine whether the signal is produced from the plant or the pathogen. If the signal molecule is from the plant exudate in response to pathogen attack, then the biological control strains may be limited to use on only one plant type as the signal may be unique to that species of plant. If the signal is from the pathogen then there is potential for the biological control strain to be used to protect multiple plant species against a common pathogen.

**Summary of the control of Phl biosynthesis**

An overview of the factors that appear to be involved in Phl biosynthesis and control of expression has been reviewed through interpretation of the evidence for the control and regulation of Phl production presented in this chapter. As new environmental and regulatory elements involved in Phl biosynthesis come to light, a delination of the events that control Phl biosynthesis have been summarised. Most recently, a review by Haas et al. (2000) has drawn together some of these factors.

The key to understanding the factors that regulate Phl synthesis is to understand why biological control in the laboratory is so rarely transferable to the field. We are increasingly able to understand the genetic factors that control Phl synthesis. The biosynthetic pathway has been elucidated in strain Q2-87 (Bangera and Thomashow, 1996), and been shown to be conserved throughout Phl producers world-wide (Keel et al., 1996; Raaijmakers et al., 1997; Picard et al., 2000). Common controlling elements have been determined, such as the Phl regulator, PhIF (Bangera and
Thomashow, 1996; Schnider-Keel et al., 2000; Delany et al., 2000); the major components of the two component regulatory pathway, GacA and GacS (Laville et al., 1992; Corbell and Loper, 1995); and the sigma factors that regulate Phl production depending on cell population dynamics (Pfender et al., 1993; Schnider et al., 1995; Sarniquet et al., 1995). GacA, PhlF, RpoD and RpoS, are DNA binding proteins and represent the endpoints of transcriptional regulation. Of them all, however, only PhlF has been shown to bind DNA that is spatially associated with the phi biosynthetic cluster (Delany et al., 2000). The discovery of these regulatory endpoints has not explained why Phl producers are heterogeneous in their production of Phl under a given set of laboratory and field conditions.

To appreciate this, the environmental factors that affect Phl production has been examined (Shanahan et al., 1992; Nowak-Thompson et al., 1994; Duffy and Defago, 1999; Nakata et al., 1999; McSpadden Gardener et al., 2000). This work revealed that there are strain specific responses, and that these are associated with the soil, plant-type and age of the plant from which the strains have been isolated. This is a complicating factor as the molecular delineation of Phl synthesis and control is seen as conserved throughout all the strains.

This is significant in such strain specific effects seen when differing nutrient sources are used to stimulate Phl production. In all Phl producers tested, glucose ubiquitously leads to the production of Phl (Duffy and Defago, 1999), except in strain F113, where glucose has no effect and sucrose is the preferred carbon (Shanahan et al., 1992; Duffy and Defago, 1999). Physiologically, this is because the F113 is the only Phl producing strain isolated from sugar beet, where the predominant carbon is sucrose from the plant exudate.

The molecular reasoning for this preference has not been explained. Whether the carbon sources act as signalling molecules, or purely as metabolic substrates is unclear. There is no evidence that the GacS, membrane sensory protein has an increased specificity for sucrose over glucose in F113. Similar findings have been described by Nakata et al. (1999) where a homoserine lactone substrate added to the growth medium caused an increase in Phl production in strain S272. There has been
no direct molecular evidence that indicates that this molecule acts directly on regulatory proteins in Phl biosynthesis in this strain, in the way that N-acylhomoserine lactones act in phenazine production in *P. aureofaciens* 30-84 (Mazzola and White, 1994).

It has been interesting to note that repressors of Phl production have a physiological significance. Schnider-Keel *et al.* (2000) describes the down regulation of Phl in CHA0 by the substrate, fusaric acid. Interestingly, this is a myotoxic metabolite produced by the pathogenic fungus, *Fusarium oxysporum*. However, the repression is not seen in the *phlF* mutant, CHA0. The study also showed that even the bacteria's own metabolites effect Phl production. Salicylic acid and pyoluteorin down regulate the production of Phl in CHA0 in the presence of the functional *phlF* gene. The importance of PhlF in the regulation of Phl appears more complex than described earlier in the role of Phl autoinduction by the Phl molecule itself. These findings have indicated that the metabolites, pyoluteorin, salicylic acid and fusaric acid act on PhlF to down regulate Phl production, whereas, the metabolite, Phl, acts on PhlF to derepress Phl production. Again the mechanism for this is unknown, however direct and competitive binding of these molecules to PhlF has been considered as one method. The physiological importance of this might be to firstly stop the autoinduction of Phl and secondly to allow the bacteria to adapt to its surroundings through the inversely related production of either pyoluteorin or Phl (Schnider-Keel *et al.*, 2000).

The work by Aarons *et al.* (2000) describing a small regulatory RNA molecule, PrrB RNA, in F113 may act, not as a transcriptional regulator in the GacS/GacA cascade, but as a post transcriptional regulator of Phl production. This evidence comes from structural and functional similarities between this PrrB RNA and a previously described translational repressor mRNA binding protein, RsmA. Blumer *et al.* (1999) showed that a null mutation of *rsmA*, would partially suppress a *gacS* defect in strain CHA0. The proposed function of this protein is the binding of mRNA at a ribosome binding site (RBS) thus preventing translations. The mutation of the RBS of *E. coli*, *lacZ* led to the translation of *lacZ* being under the full control of GacA and no longer the RsmA-like molecule. In addition, it was noted that a non-coding RNA molecule,
designated RsmB, could also prevent the suppression of translation by RsmA. This is believed to happen by binding RsmA thus preventing the interaction with the RBS on mRNA that prevents ribosome binding and translation.

Aarons et al. (2000) were able to show that the predicted secondary structure of PrrB contained eight stem-loops, which was similar to the structure of the RsmB regulator RNA, albeit, considerably smaller and containing little sequence homology. This together with the evidence that plasmids carrying prrB can complement gacA/gacS mutants of F113, and that prrB in a promoterless plasmid cannot complement gacA/gacS mutants supports the belief that PrrB acts to regulate secondary metabolite expression post transcriptionally.

The picture that comes out of these findings is that GacS initiates a response through interaction with an environmental stimulus. This is communicated through a phosphorylation cascade until GacA is stimulated, through a confirmational change to bind to DNA through its DNA binding helix. The belief in the past was that this took place within or adjacent to the cluster of genes that have been shown to be under the control of the GacS/GacA cascade, such as Phi. However no binding sites have yet been found, and it now seems certain that the area of DNA binding is probably at the promoters of post-transcriptional regulatory proteins such as those described previously such as either PrrB, RsmB. It seems unlikely that GacA acts to regulate RsmA because it is interesting to note that a mutant of gasA and rsmA (prrA) was able to produce Phi.

It has long been established that gacA mutants are unable to produce Phi. This suggests that GacA acts to increase the expression of RsmB and RsmA’s expression is controlled through another pathway. More work in this area will determine if the GacA acts directly on the expression of these molecules and whether PrrB is in fact a RsmAB like molecule. The identification of a RsmA-like molecule, PrrA, would be an important finding in determining this.
1.5. *Pseudomonas fluorescens* SBW25

**Isolation**

The focus of this study is the natural isolate *P. fluorescens* SBW25. This was identified as a rRNA group I, fluorescent pseudomonad and independently classified as a fluorescent non-pathogenic *Pseudomonas* somewhere between *Pseudomonas fluorescens* (LOPAT group IVb) complex, *P. aureofaciens* and *P. putida* (Application for approval for release or market, 1995). Since this classification, it is accepted that the isolate is classified as a *P. fluorescens*.

*P. fluorescens* SBW25 is a Gram negative, chemoheterotrophic, motile rod with polar flagella. It is plasmid free and an abundant member of the phyllosphere community. This bacterial species is believed to be non-pathogenic to humans, animals and plants and was not found antagonistic to other microbial inhabitants of the phyllosphere. SBW25 was isolated from the leaves of sugar beet plants grown at a field site, University Farm, Wytham, Oxfordshire (Thompson *et al.*, 1993).

*P. fluorescens* SBW25 was isolated from its natural environment following investigation of the natural microbiology associated with sugar beet (*Beta vulgaris*) over several seasons. It was found to be the most predominant microorganism dominating other microbial populations on both the phyllosphere and the root systems of the crop plants. Following the isolation of SBW25, the organism was genetically marked for the purpose of isolation and detection in laboratory experiments.

**Genetic markers**

Bailey *et al.* (1995) inserted two gene marker cassettes into the chromosome of the natural SBW25 isolate. A strategy was adopted to facilitate detection whilst minimising the possibility of gene exchange and metabolic disruption. Two distinct chromosomal sites were selected, approximately 1 Mbp apart on the SBW25 chromosome, site Ee and site –6– (Figure 1.9).
Site Ee was isolated from an *Eco*RI chromosome fragment from SBW25. The marker genes *lacZ* were inserted into a unique *Bgl*II site within the Ee site under the control of the *iucA* promoter isolated from pMON7117 (Barry, 1988). This EeZY fragment was transferred on a mobilisable suicide delivery plasmid and integrated into the chromosome of SBW25 at the Ee site by homologous recombination (Figure 1.8).

Site –6– was also isolated on an *Eco*RI chromosome fragment. The marker gene cassette containing kanamycin resistance gene and *xylE* genes were constructed and inserted into a unique *Bgl*II site within the –6– site. The 6KX fragment was delivered by electroporation and integrated into the –6– site of the SBW25 chromosome by homologous recombination (Figure 1.8).
Figure 1.8 Genetic marker cassettes showing insertion of xy/E / Km and lacZY into the chromosome of P. fluorescens SBW25

Figure 1.9 Chromosome map of P. fluorescens SBW25 showing the sites of insertion of genetic markers 1 Mb apart (Taken from Rainey & Bailey, 1996). Figure shows the site of insertion of the lacZY and the xy/E/km cassettes in to the SBW25 chromosome.
Fitness of genetically modified SBW25

The fitness of the modified organism was tested under limited conditions (Thompson et al., 1995) and was shown to be equally competitive with the wild-type. The mean doubling time, co-culture with the wild-type and persistence on leaves of plants sampled 56 days after seed inoculation revealed similar fitness characteristics.

A more comprehensive evaluation of the fitness of SBW25 and the modified strain containing the marker genes revealed a more detailed picture as to the fitness of the organism with and without the added marker genes (De Leij et al., 1998). The study took advantage of the natural isolate SBW25, and four modified derivatives of this strain. SBW25-6K, kanamycin marked at the -6- site; SBW25-6KX, kanamycin and xylE marked at the -6- site; SBW25EeK, kanamycin marked at the Ee site; and triply marked SBW25EeZY-6KX, with kanamycin and xylE marked at the -6- site with lacZY marked at the Ee site.

The study investigated the relative fitness of the four modified strains and the wild-type unmodified SBW25 in a variety of environmental models. Following inoculation to the leaves of wheat, the triply marked, SBW25EeZY-6KX was significantly outcompeted when compared to the wild type following combined, equal inoculation. There was however no significant difference between the proportion of the strains recovered when the triply marked SBW25EeZY-6KX and the SBW25-6KX were inoculated into the rhizosphere of pea or wheat. The fitness of the organisms was examined in both the soil and the rhizosphere of pea and wheat. There were no significant differences noted between the strains on the rhizosphere whereas in the surrounding soil, the proportion of the triply marked organism declined with respect to the single site modified strains.

The conclusion arrived at by the authors to explain these findings suggest that in regions of nutrient limitation such as soil, in comparison to rhizosphere, phyllosphere or nutrient broth, there is a reduction in the fitness of these modified organisms. The authors then describe the conflict of genetic modification that has no beneficial environmental effect, but effects a metabolic load on the host strain. The genes that have been inserted have no beneficial advantage to the SBW25 organism in the
environment. *LacY*, *LacZ* and *xylE* genes encode the lactose permease, β-galactosidase and catechol 2,3-dioxygenase, respectively. The constitutive expression of these genes may be metabolically expensive whilst not off setting this cost with an environmental advantage. The high nutrient levels of nutrient broth and the rhizosphere of pea and wheat impose a lower environmental stress on the microorganisms thus the difference in relative fitness has not been significant in these studies (Thompson *et al.*, 1995 and De Leij *et al.*, 1998).

Using SBW25 that has been modified at two distinct sites, the effect that the site of insertion has on environmental fitness can be determined. The strains SBW25EeK and SBW25-6K are both marked with kanamycin resistance gene but at chromosomal sites 1 Mbp apart, at Ee and -6-, respectively. Following inoculation wheat seeds with equal concentrations of the two modified strains, the populations in the rhizosphere were enumerated up to 35 days of plant growth. The results indicate that there is no difference in fitness between the strains and therefore these particular sites of insertion *per se* have played no role in the reduced fitness of the organism, SBW25EeZY-6KX, which has a gene insertion located at both sites.

Support of this finding is seen with a fitness study performed to assess the effect of SBW25 carrying a naturally derived and uncharacterised environmental plasmid, pQBR103 (Lilley and Bailey, 1997). This experiment utilised the triply marked SBW25EeZY-6KX, and a single marked variant, SBW25EeTc, a strain modified with the insertion of tetracycline resistance gene at the Ee site. Prior to the release of the plasmid-carrying organism, standardised population fitness of the two marked strains was performed. The results showed no significant difference in fitness of the two organisms in competition against each other on the rhizosphere or phyllosphere of sugar beet plants. This supports findings by De Leij *et al.*, 1998, indicating that in nutrient rich environments the triply marked strain can compete similarly to the singly marked strain. There appears to be no additional metabolic or biochemical burden on the SBW25 organism with the addition of the tetracycline resistance genes in comparison to the kanamycin marked strain used in the latter study.
The fitness of the SBW25 is reduced when harboring the naturally derived 330-kbp plasmid, pQBR103 even in the nutritionally rich phyllosphere and rhizosphere environment. In the rhizosphere the fitness of the plasmid carrying SBW25 is reduced, however, recovery is seen at around 150 days of plant growth. This effect has been replicated in greenhouse and field environments. A similar phenomenon is noted on the leaves of sugar beet plants in the field trial. It is suggested that the plasmid is clearly exerting a burden thus leading to the decline in population in comparison to the plasmid free strain. The recovery of the organism to similar population densities is due to a seasonal, temporal benefit that the plasmid gives the SBW25 late in the maturing plant (Lilley and Bailey, 1997a). This has been supported by earlier investigations that have shown that during mid-to late-season the number of natural transconjugants increase on the phyllosphere of sugar beet (Lilley and Bailey, 1997a). The plasmid confers beneficial traits (as yet unidentified) which are seasonally activated in relation to the maturity of the plant.

The findings of the studies by De Leij et al. (1998); Lilley and Bailey, (1997); indicate that genetic insertion into the chromosome at either site Ee or -6- decrease the fitness of SBW25 in low nutrient conditions but not in nutrient rich environments. The addition of a plasmid reduces the SBW25 fitness significantly even in nutrient rich environments; however, fitness can be regained once the plasmid delivers an environmentally beneficial trait.

The minimal impact on the fitness of the SBW25 strain following insertion at Ee and -6- site provide excellent locations for insertion of genes in future modifications. Because the marker genes that have been used are well characterised and commonly used as molecular biology tools, these regions themselves can act as regions of homology to deliver a targeted chromosomal insertion. This approach has two advantages, maintaining fitness and providing a method of selection of the new modification. Insertion at either site shall not disrupt vital housekeeping genes as the recombination can take place on the non-essential marker gene. The disruption of any of these marker gene locus through homologous recombination will act as a method of selection, either through loss of kanamycin resistance or loss of chromogenic marker gene through disruption of \textit{lacZY} or \textit{xylE}. 

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Environmental release

The efficacy and safety of genetically modified microorganisms released into the environment must be assessed. Although field trial application is the most realistic test of the impact that microorganism inocula has on the environment, greenhouse and microcosm trials remain excellent tools to evaluate specific environmental effects under controlled conditions. They also allow researchers to avoid the issues surrounding the deliberate release of genetically modified organisms in the environment.

SBW25EcZY-6KX, modified with kanamycin resistance gene, xylE and lacZY has been released at two sites in 1993, Wytham, Oxfordshire; and Horticulture Research International, Littlehampton. The release was made on sugar beet and wheat respectively, and the colonisation of the SBW25, dispersal, persistence, and gene transfer were investigated (Thompson et al., 1995).

Following seed inoculation SBW25 colonised both the rhizosphere and phyllosphere of both the wheat and sugar beet plants. This had already been shown in greenhouse studies, preliminary to the deliberate field release. Differences in the persistence of SBW25 from the greenhouse studies and field release were noted in the sugar beet trial where populations on the phyllosphere remained high in the greenhouse, they dropped off after about 500 days in the open environment. The reverse was noted in the wheat trial where greenhouse phyllosphere persistence was low but in the open field the population remained high with the organism surviving better.

Persistence and dispersal of the SBW25 in the sugar beet rhizosphere was monitored. The inocula remained closely associated with the inoculated plants and with time disappeared to undetectable limits from soil not associated with the plants. Dispersal was greater from the wheat plants with SBW25 detectable two meters from the inoculated plant. The reason for this disparity is believed to come from the different soil types at the two trial locations and the heavy rainfall during the wheat trail (De Leij et al., 1995, 1994).
There was no indication of the transfer of genetic material from the introduced SBW25 modified strain. The inoculation population was considered large enough and the detection method sensitive enough for any transfer events to have been detected (Thompson et al., 1995).

1.6. RATIONAL FOR THE AREA OF RESEARCH

The scientific rational for this study is concerned with the development of a strain of *P. fluorescens* that can be used for the purposes of biological control against the plant root pathogen *P. ultimum*, the causative agent of damping off of economically important crop plants. Throughout this introduction the various chemical methods of biological control, employed by *P. fluorescens* have been discussed. To achieve a commercially viable and efficacious approach researchers from various groups have attempted to characterise these mechanisms of biological control in order to understand and enhance the biological control properties. By far the most popular approach adopted by research groups working with these strains has been to complement known producers of the antibiotics with plasmid borne regions of the organisms’ own genome that lead to enhance the antibiotic production.

This investigation aimed to approach this problem from a different perspective. There is a good theoretical case for the use of an integrated strategy for biological control. This thesis shall examine the possibility for creating a strain of *P. fluorescens* capable of exhibiting biological control properties through production of the secondary metabolite, Phi and through competitive exclusion of the pathogen on the plant root system through greater competence on the rhizosphere.

A suitable strain for this strategy is the *P. fluorescens* SBW25. As described earlier in the chapter, this strain has been well characterised, both by researchers currently working at the University of Surrey and by research groups with whom the group have enjoyed close collaboration.

The choice of strain has come from findings indicating a strong colonising ability of this strain in the phyllosphere and rhizosphere of a variety of crop plants. This
provides a good mechanism for the delivery of the antibiotic to the root system. The high rhizosphere competence of SBW25 can enhance biological control through competitive exclusion of fungal pathogens from the plant roots. Findings by De Leij et al. in 1998 and Thompson et al. (1995), indicate that this rhizosphere competence has not been significantly decreased with the addition of multiple genetic markers with comparison to strains with a single marker. The addition of a variety of markers with no loss of competence makes the SBW25 an excellent tool for laboratory studies.

*Pseudomonas fluorescens* SBW25 also has advantages for future development in that the strain has been isolated from an UK agricultural site thus establishing its potential for use on agricultural crops for the purpose of biological control. The basis of its original isolation is its high colonisation of crop plants thus making it the organism of choice for inoculation of crops and delivery of antibiotic. It has been genetically marked with both visual markers and antibiotic markers providing a tool for extensive laboratory study. The genetically modified strain, SBW25EcXY6K, has undergone deliberate field release with permission from the Department of the Environment and is therefore undergone comprehensive genetic characterisation, including genome mapping.

The secondary metabolite, Phl, was chosen as the fungicide for transformation into the SBW25 strain as it is broad-acting, able to inhibit the growth of *P. ultimum*, a major economic pest, affecting multiple crop plants.

Through a European Union funded initiative that began in 1994, the University of Surrey has been working in partnership with Researchers at University College Cork, Ireland, where the Phl producing strain, F113 was originally isolated. Part of this collaborative work has enabled research to be carried out on the F113 strain at the University of Surrey and for information to be freely exchanged between groups.

The ability for strain F113 to produce Phl, and inhibit *P. ultimum* in plate bioassays has been documented (Shanahan et al., 1992). A derivative of F113 has been constructed that can over-produce Phl through transformation of the F113 strain with an ~8-kb region of the F113 genome on a plasmid system. As described earlier in the
chapter this region was unable to transform seven of eight non-Phl producer strains. This region was not, therefore, considered to be able to universally transfer the synthesis of Phl throughout other *P. fluorescens*.

At the time this study was beginning a group in at the Washington State University, Oregon, U.S, headed by Linda Thomashow, published a sequence of a region of the strain Q2-87 that could universally transfer the ability to produce Phl to non-producing *P. fluorescens* (Bangera and Thomashow, 1996).

The rational for this study, therefore, became to isolate the region of the F113 strain that was responsible for the production of the Phl antibiotic. Then to transfer it into the SBW25 strain in order to produce a strain of *P. fluorescens* that could exhibit biological control through antibiotic production and also competitive exclusion. It can then be investigated if the modified strain has enhanced biological control over naturally isolated strains that only possess one biological control attribute.
2.1. **Summary of Results**

The recombinant strain of *Pseudomonas fluorescens* F113:pGUCP (F113OP), carries extrachromosomal copies of the genes responsible for the production of 2,4-diacetylphloroglucinol (Phl). The amount of Phl produced by this strain in comparison to the wild-type F113, was increased 2.5 times *in situ*, from soil surrounding a 21-day microcosm trial. Against the fungal plant pathogen *Pythium ultimum*, the F113 and F113OP strains inoculation led to a reduction in the emergence of pea plants in comparison to non-inoculated control and Phl negative, Tn5 mutant, F113G22. Pea is not sensitive to synthetic Phl, with no reduction in emergence in the presence of 1000 x physiological levels. F113G22 developed significantly more *Pythium* induced pathogenic lesions than the control indicating possible inocula induced susceptibility to *Pythium* infection. Both F113OP and F113 reduced the *Pythium* induced lesions indicative of Phl biological control. It is concluded that there is the possibility that the reduction in emergence of pea plants inoculated with F113OP and F113, is caused by a combined effect of physical damage by the bacterial inocula together with high levels of Phl production.
2.2. INTRODUCTION

The use of microorganisms that possess biological control activity against fungal, plant root pathogens has proved to be commercially promising. Natural, disease suppressive soils have been found to contain populations of natural microorganisms, which are capable of the production of 2,4-diacetylphloroglucinol (Phi). Harrison et al. (1993) estimated that approximately 20% of fluorescent pseudomonads isolated from roots infected with *Gaeumannomyces graminis* var. *tritici* grown in take-all suppressive soil had a Phi\(^+\) phenotype. Keel et al. (1996) identified similar proportions of fluorescent pseudomonads with the Phi locus in *Thielaviopsis basicola*, suppressive soils, in Switzerland.

Phi has been shown to act as a broad-spectrum antibiotic produced by fluorescent pseudomonads from a broad geographical distribution. *Pseudomonas fluorescens* CHA0 has biological control activity against black root rot of tobacco (*T. basicola*) and take-all of wheat (*G. graminis* var. *tritici*) (Maufhofer et al., 1995). *P. fluorescens* Q2-87 also suppresses against take-all of wheat (Harrison et al., 1993) and *P. fluorescens* F113 against damping-off of sugar beet caused by *Pythium ultimum* (Fenton et al., 1992).

Efforts to enhance the biological control potential of these strains have concentrated on the addition of multiple copies of the Phi genes, on plasmid systems. The additional Phi production is intended to reduce the inocula required to maintain biological control efficacy, thus reducing the cost of the application to farmers. This is considered crucial if biological control is to become a viable alternative to the chemical fungicides in use at present.

Bonsall et al. (1997) reported overproduction of Phi in Q2-87 with addition of the plasmid construct pPhl5122, carrying a 6.5-kb fragment of Q2-87 genome. Production of Phi has also been increased through modification of a regulatory component that appears to regulate the expression of Phi. Schnider et al. (1995) introduced the *rpoD*
gene, encoding the housekeeping sigma factor σ^70, into CHA0, on plasmid, pME3424. This resulted in increased production of Phl in vitro.

Increasing the level of Phl production in natural biological control strain CHA0 has been shown to increase the biological control ability against *Fusarium oxysporum* f.sp *cucumerinum*, *Phomopsis sclerotioides* and *P. ultimum* on cucumber plants (Maurhofer et al., 1995; Schnider et al., 1995).

The aim of this experiment is to investigate the biological control potential of the over production of Phl by *P. fluorescens* F113:pCUGP. The comparison shall be made in relation to the natural biological control strain *P. fluorescens* F113 and the Phl negative mutant F113G22 (Tn5 insertion mutation) on pea plants inoculated with and without the seedling pathogen *P. ultimum*.
2.3. MATERIALS AND METHODS

**Soil description**
The soil used was a sandy loam of the Holiday Hills series, taken from Merrist Wood Agricultural College (Surrey), and had been under permanent pasture for at least 15 years. The analysis of the soil, conducted at the University of Surrey, was pH 5.4, particle ratio 10:9:81 clay: silt: sand respectively, and organic matter content 1.6 % by weight. The total NPK contents by weight were 0.124%, 0.033% and 0.861% respectively.

**Microbial strains and treatments**
Three strains of *Pseudomonas fluorescens* were used with different modifications. Strain *P. fluorescens* F113 that produces the antibiotic 2,4 diacetylphloroglucinol (Phi), and was marked with a lacZY gene cassette; a Phi negative derivative (strain *P. fluorescens* F113G22) produced by Tn5 mutagenesis (Shanahan et al. 1992) and a constitutive plasmid based Phi over-producer F113R:pCUGP (*P. fluorescens* F113OP) marked with a gus gene cassette.

The bacteria were grown on full strength, tryptone soya agar (Oxoid) for 3 days at 30°C. The bacteria were suspended in 10 ml of sterile, quarter strength Ringer's solution using disposable plastic plate spreaders to scrape off the bacterial mat, and colony forming units (c.f.u) were determined by direct counting and spread plates. Control plates (without bacteria) were also flooded with quarter strength Ringers solution and surface scraped with spreaders. The resulting suspensions containing 6x10⁹ c.f.u/ml of the bacteria were subsequently used to imbibe pea seeds (*Pisium sativum* var. Montana), at a ratio of one seed per ml, for 8 hours (stirred every 30 minutes) prior to planting, resulting in 2.8 x 10⁸ ± 0.4 x 10⁸ c.f.u per pea seed. No significant differences in inoculation potential between strains were observed.

*Pythium ultimum* (IMI 308273) was obtained from CABI Bioscience. Material from stock cultures was grown on plates of potato dextrose agar (PDA) at 25°C for 3 days
(primary plates). Four 5 mm disks were cut and placed in a flask containing: 95 g of sand, 5 g of organically grown processed oats and 20 ml of distilled water, all previously autoclaved twice. The flasks were incubated for 3 weeks at 25°C and mixed at 7-day intervals. After incubation, the *Pythium* media was homogenised in a blender and mixed with coarsely sieved soil at a concentration of 6% w/w, resulting in 5.6 c.f.u per g soil (determined by spread plates).

**Experimental design**

*Pythium* inoculated or uninoculated soil (150 g) was placed in experimental microcosms were constructed from 210-mm high polyacetate cylinders, slotted between the top and base of plastic 90-mm diameter Petri dishes creating semi-enclosed microcosms. Each treatment was replicated 5 times and each microcosm consisted of five imbibed seeds, planted at a depth of approximately 1-cm below the soil surface. Twenty five ml of water was added to each microcosm before they were placed in a random design into a growth chamber (Vindon Scientific) set at a 16-hour photoperiod with a day/night temperature regime of 21°C/15°C respectively. The relative humidity was maintained at 70% and the light intensity was 10,000 lux at shelf level (Figure 2.1).

*Figure 2.1* Growth chamber containing pea plant microcosms. Picture taken at the end of 21-days growth
Sampling and analysis

After 21 days of growth, the plants were harvested, the number of plants emerged were counted and the individual plant shoot and root weights measured. Subsequently the number of nodules, lateral roots and lesions along with the root lengths were measured for each plant per microcosm. Rhizosphere soil (closely associated with the plant roots) was collected by shaking soil closely associated with the roots over a 2-mm sieve and stored at 4°C until required on the same day. Soil from three OP treated microcosms and three *P. fluorescens* F113 microcosms was collected and used for extraction of Phl.

One gram of pooled fresh root samples, were taken from each microcosm and macerated in 9 ml Ringers solution using a pestle and mortar. One gram of rhizosphere soil from each microcosm was also suspended in 9 ml Ringers solution. Filamentous fungal populations were quantified by plating a ten fold dilution series of each root macerate or soil suspension onto 10% malt extract agar containing 100 ppm streptomycin and 50 ppm rose bengal. Plates were incubated at 20°C for 5 days before enumeration. P1 medium was used for the enumeration of indigenous root, fluorescent *Pseudomonas*. These plates were incubated at 25°C and enumerated after 5 days growth. To enable quantification of introduced *P. fluorescens* F113G22 and F113 strains, P1 medium (Katoh and Itoh, 1983) was amended with 50 ppm X-Gal upon which recovered *lacZY* modified *Pseudomonas* could be identified as blue colonies. Separate P1 plates amended with 50 ppm X-Gluc was used for the enumeration of the F113OP strain. Tryptone soya agar (10%) was used for the enumeration of total culturable bacteria and incubated for 7 days at 25°C.

Extraction of Phl

Phl was isolated from the rhizosphere of the pea plants on the principle of the method described by Bonsall *et al.*, (1997). Twenty grams of pea rhizosphere soil was added to 10 ml of Ringers used to wash the corresponding pea root. This was mixed in a 250 ml flask with 70 ml of 80% acetone acidified to pH 2.0 with 10% trifluoroacetic acid (TFA) and shaken (200 rpm) for 2 hours at room temperature. The 100 ml mixture was dispensed into a glass beaker, left to settle and evaporated to a volume of 14 ml, acidified to pH 2.0 with 10% TFA, extracted twice with 20 ml ethylacetate and
evaporated to dryness. Extracts were resuspended in 200 μl 35 % acetonitrile (ACN) and 0.1% TFA. The solution was centrifuged (15 000 rpm, 2 min) prior to analysis by HPLC.

*Plate bioassays.*
Plate bioassays were performed against a pathogenic fungus, *Pythium ultimum* grown on potato dextrose agar (PDA). Test bacterial colonies were spotted onto the plates 2 cm from the Petri dish edge. After placing the fungal plug at the centre of the dish, the plates were incubated at 20°C, for 4 days, until the fungus had grown to the edge of the dish. Evidence of fungal inhibition by the test bacteria was recorded.

*HPLC analysis*
For peak separation of the soil and root extract, a 10 cm x 4.8 cm reverse phase, Spherisorb C\(^{18}\) was eluted with solvent (1.0 ml per minute) using an isocratic mobile phase of 78% ACN, 0.1% TFA. The extract was manually injected into a 20 μl loop and absorbency was measured at λ\(_{270\text{nm}}\). Data was recorded using a PeakNet Chromatography Workstation, (Dionex). The separation of broth extract was performed using a 20 cm x 4.8 cm reverse phase, Spherisorb C\(^{18}\) column.

*Tolerance.*
Pea seeds were imbibed in solutions of three concentrations of synthetic Phi (O’Gara *et al.*, UCC, Ireland, personal communications). One hundred seeds were imbibed for each treatment and planted, 25 per seedling tray, in triplicate. Control groups were sewn in triplicate. Control seeds imbibed in Phi solvent, 75% methanol. Concentrations of 500 ng, 5 μg and 50 μg per seed of synthetic Phi (MW210) were used. This approximates to 10, 100 and 1000 times the natural Phi concentration in Quincy take-all suppressive soil (calculated against natural levels found in *Pythium* suppressive soils isolated in Quincy, USA, at 19.1 ng per gram of root, extracted to approximately 60% efficiency (Raaijmakers *et al.*, 1999)). Seed growth conditions were as described previously, and seed emergence was recorded after five days.
Statistical analysis

Data were analysed using SPSS for Windows (SPSS inc.) by means of a one way ANOVA and subsequently differences among treatments (multiple comparisons) were determined using least significant differences (LSD) between means as the post hoc test.
2.4. RESULTS

Plate bioassays

Figure 2.2 shows the effects of the strains, F113 and F113OP on *P. ultimum*. These effects were recorded from Potato Dextrose Agar (PDA) plates grown at 20°C, in the dark. These plate bioassays show qualitative differences between the amounts of *P. ultimum* inhibition between the two strains. No truly quantitative or comparative estimations are possible, as inoculation populations in these preliminary assessment experiments were not controlled. The F113OP shows a greater inhibition of the *P. ultimum* than the F113 as shown by the relative diameters of the zones of inhibition.

Figure 2.2 Strain F113OP and wild-type, F113, inhibiting the growth of *P. ultimum* on PDA plate. Photograph taken following 4- days growth at 20°C. Level of inhibition appears greater for F113OP indicated by the larger zone of clearance around the bacterial inoculum.
Emergence experiment with synthetic Phil

There were no significant differences between the three levels of the synthetic Phil concentrations (Figure 2.3).

![The effect of synthetic Phil on the emergence of pea seeds](image)

**Figure 2.3** The effect of high levels of synthetic Phil on the emergence of pea plants. Emergence recorded after 5 days of growth. Each treatment was performed in triplicate, and the mean percentage emergence from the three trays (n=75) was statistically analysed by one way ANOVA to compare the means. The concentration of Phil applied to the seeds prior to planting was calculated against natural levels found in *Pythium* suppressive soils isolated in Quincy, U.S, at 19.1 ng per gram of root, extracted to approximately 60% efficiency (Raaijmakers et al., 1999).
2.5. RESULTS OF PLANT GROWTH

The emergence of pea seeds is shown in Table 2.1 and graphically in Figure 2.4. In the control microcosms (no fungal agent) it was found that 100% (n=25) of the plants emerged across the F113, F113G22 and control treatments. 92% (23/25) of pea plants emerged in the F113OP treatment. In the *Pythium* treatment two major findings emerged. Plant emergence within the control and F113G22 treatments was significantly greater than emergence within the F113 and F113OP treatments. Overall, across all treatments a significant reduction in emergence was found within the *Pythium* condition compared to the control.

![Emergence of pea plants during 21-day microcosm trial in the absence and presence of *P. ultimum* Infection](image)

*Figure 2.4* Emergence of pea plants from microcosm trial treated with F113G22, F113, F113OP or control (no bacterial inocula). Plot showing the mean emergence of 25 plants divided into five replicate microcosms per treatment, each containing 5 plants (n=25). The *P. ultimum* data on the right of the chart shows seed emergence from microcosms with the addition of the pathogen, *P. ultimum* into the soil. Again, 5 plants per microcosm with each treatment replicated 5 times (n=25).
Table 2.1 Pea emergence, shoot and root weights as affected by Pseudomonas inocula and Pythium ultimum.

<table>
<thead>
<tr>
<th>Plant growth</th>
<th>Soil without Pythium</th>
<th>Soil treated with Pythium</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>F113G22</td>
</tr>
<tr>
<td>Emergence</td>
<td>1.00\textsuperscript{a}</td>
<td>1.00\textsuperscript{a}</td>
</tr>
<tr>
<td>Shoot (g)</td>
<td>1.16\textsuperscript{a}</td>
<td>1.17\textsuperscript{a}</td>
</tr>
<tr>
<td>Root (g)</td>
<td>0.88\textsuperscript{a}</td>
<td>0.80\textsuperscript{a}</td>
</tr>
<tr>
<td>s/r Ratio</td>
<td>1.34\textsuperscript{a}</td>
<td>1.51\textsuperscript{a}</td>
</tr>
</tbody>
</table>

\textsuperscript{1} Treatments: control, no inocula; F113G22, inoculated with lacZY marked Ph\textsuperscript{r} (Tn5 mutated) Ps. fluorescens F113G22; F113, inoculated with lacZY marked Ph\textsuperscript{r} P. fluorescens F113; F113OP, inoculated with gus marked P. fluorescens F113R:pCUGP (Ph\textsuperscript{r} over producer).

\textsuperscript{2} Emergence, proportion of seedlings emerged five days after sowing; shoot, mean shoot weight; root, mean root weight; s/r ratio, mean ratio of shoot weight to root weight.

\textsuperscript{3} Letters, within a row, indicate significant differences at \(p<0.05\) level. For emergence the mean of five microcosms is given (\(n=5\)). For shoot, root and s/r ratio, \(n\) is proportional to the emergence to a maximum of 25.
Table 2.2 Mean root length and number of lateral roots, nodules and lesions per root system as affected by *Pseudomonas* inocula and *Pythium ultimum*.

<table>
<thead>
<tr>
<th>Root measurements²</th>
<th>Soil without <em>Pythium</em>¹</th>
<th>Soil treated with <em>Pythium</em>¹</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>F113G22</td>
</tr>
<tr>
<td>Root length (cm)</td>
<td>16.96 a</td>
<td>14.34 b</td>
</tr>
<tr>
<td>Lateral Roots³</td>
<td>22.51 a</td>
<td>22.6 a</td>
</tr>
<tr>
<td>Lesions³</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Nodules³</td>
<td>17.33 a</td>
<td>18.64 a</td>
</tr>
</tbody>
</table>

¹ Treatments; control, no inocula; F113G22, inoculated with *lacZY* marked Phi' (Tn5 mutated) *P. fluorescens* F113G22; F113, inoculated with *lacZY* marked Phi' *Ps. fluorescens* F113; F113OP, inoculated with *gus* marked *P. fluorescens* F113R:pCUGP (Phi over producer).

² Lateral roots, mean number of lateral roots per root system; Nodules, mean no. of nodules per root system; lesions, mean no. lesions per root system; ND, none detected.

³ Letters, within a row, indicate significant differences at p<0.05 level, five roots per microcosm were measured (n=25).
Also in the absence of *P. ultimum*, other plant parameters were measured. On Table 2.1 and 2.2 the left-hand panel summarises the findings from these measurements. Shoot weight, root weight, root length, numbers of lateral roots and numbers of root nodules all remain statistically similar between the different bacterial inoculations.

The right hand panel of Table 2.2 shows the results from the remainder of the plant measurements from the *Pythium* infected treatment. The only significant differences are seen in the number of lesions on the plant roots. This is shown graphically in Figure 2.5. The lowest number of root lesions is seen in, F113OP, inoculated plants whereas the highest are seen with F113G22 inoculated plants. The F113G22 treated plants have significantly more lesions than all other treatments, with the greatest difference clearly being with the F113OP, *p*<0.0001, then the F113, *p*<0.001, and then the control, *p*<0.05.

![Figure 2.5](image)

**Figure 2.5** Mean number of root lesions from pea plants infected with *P. ultimum* following 21-days growth. Lesion numbers calculated from 5 roots per microcosm, each treatment replicated 5 times (n=25).
The remainder of the plant measurements in the right-hand panel, of Table 2.2, shows that no further significant differences between treatments are noted in root length, lateral roots and nodulation.

**Comparison between plants with and without *P. ultimum* infection**

Tables 2.1 and 2.2 show the results of the effects on plant growth with and without *P. ultimum* infection. Comparing the panel on the right (*P. ultimum* infection), and the left (non-infection) on each of these Tables, an assessment of the overall effect of the infection can be made.

Table 2.1 and Figure 2.4, shows that the emergence has been significantly reduced between infected and non-infected plants. The reduction between respective controls is significant, p<0.05, and shows a reduction of 24%.

This is the same for F113G22 treated plants. The F113 treated plants have reduced to a greater statistical significance, p<0.001 a fall of 44%, with F113OP being reduced by 48% in comparison to the F113OP in non-infected soils. Again a significant reduction, p<0.001.

Although there was seen to be no differences within groups of infected and non-infected soils for shoot weight and root weight, the difference between groups is seen as significant. The weights of both the shoot and roots is reduced by the *P. ultimum* infection, p<0.05.

In Table 2.2, root length of control plants in *P. ultimum* free soils is seen to be significantly greater than all *P. ultimum* infected plants (p<0.05), however, F113G22 and F113 in the *P. ultimum* free treatments have not changed significantly in relation to their infected counterparts.

The F113OP inoculated plants have a significantly lower root length than their respective *P. ultimum* free, comparator (p<0.05), and are also significantly shorter than the F113G22, F113 and control treatments, in the *P. ultimum* free soils (p<0.05).
Lateral roots and root nodulation follow a generally similar pattern to one another, between the soil treatments. They each show a higher number of their relative parameters in the non-infected soil than in the *P. ultimum* infected soil (p<0.05). The only deviation from this was seen with the lateral roots of the control treated plants. These remained statistically the same even during *P. ultimum* infection.
2.6. RESULTS OF MICROBIAL POPULATIONS

P. ultimum free soil

All microbial population counts are summarised in Table 2.3. The left-hand panel indicates microcosms that have not been infected with P. ultimum. The measurements were total bacteria populations in rhizosphere soil and root; total fungal populations in rhizosphere soil and root; introduced pseudomonad populations in rhizosphere soil and root; indigenous pseudomonad populations in rhizosphere soil and root.

The total bacterial population in the rhizosphere soil of the F113G22 treated plants has risen significantly in comparison to all other treatments. Otherwise, the bacterial populations of the root and rhizosphere soil have not been effected by the different inoculates.

There are no differences in fungal populations in either the root or the rhizosphere soil between any of the four treatments.

The introduced pseudomonad population in the roots shows no significant difference between the three inoculated treatments. However, the F113OP treated plants have a significantly higher (p<0.05) number of indigenous pseudomonads associated with them compared to the other treatments.

In the soil, the number of indigenous pseudomonads recovered is statistically similar in all treatments. The introduced pseudomonads however are significantly higher in the F113 treated plants.

P. ultimum infected soil

The right hand panel of Table 2.3 shows the summary of the results from the microbial counts made from plants grown in P. ultimum infected soils.

The total root bacteria are present in significantly higher numbers (p<0.05) in F113G22 inoculated plants compared to the other plant treatments. In the rhizosphere soil the F113G22 treated plants had a significantly larger bacterial population than
plants treated with F113 and the control. It was not found to be significantly different to the F113OP, however.

The total fungal population was significantly higher in the control and the F113OP treated plants in comparison to F113 and F113G22 treatments. The fungal populations in the rhizosphere soil remained statistically similar throughout the four treatments.

Introduced pseudomonad populations revealed that the F113OP treated plants has a significantly lower F113OP colony count than the other treatments on the plant roots. In the rhizosphere soil all inocula was recovered to statistically similar levels.

The indigenous population of the root was significantly higher in the F113OP treated plants than the F113. In the rhizosphere soil, the indigenous populations showed that the control has a statistically higher indigenous population than the F113G22, but remains similar to the F113OP and F113 treatments.

**Comparison between P. ultimum infected soil and non-infected soil**

In Table 2.3, the left panel shows microbial populations in the absence of *P. ultimum* infection and the right panel shows microbial populations in the presence of infection.

Total bacterial populations in both the soil and the roots appear not to have statistically different populations’ counts as a result of infection. The only treatment that shows statistical difference to its comparitor is the F113G22 in the infected microcosm. This has a larger population of bacteria associated with roots than the F113G22 in the un-infected microcosm.

The fungal population shows no consistent differences in populations between the infected and uninfected microcosm. Root fungal populations are statistically higher in the control and the F113OP treated, infected, plants, in relation to their comparitor in un-infected microcosms. In the case of fungal populations in the rhizosphere soil, both infected and uninfected microcosms reveal similar fungal populations for all treatments.
Introduced pseudomonads on the root are statistically higher in *P. ultimum* infected microcosms than the uninfected microcosm in both F113G22 and F113 treated soil. The F113OP population remained unchanged between the infected and the uninfected microcosms.

The introduced pseudomonads in the rhizosphere soil remain statistically similar between the *P. ultimum* infected soils than the soils that are un-infected. In the root, the indigenous pseudomonad population of the control has risen significantly in response to the *Pythium* infection. All other treatments have not changed significantly following the infection with *P. ultimum*. 
Table 2.3 Log$_{10}$ bacterial/fungal populations as affected by *Pseudomonas* inocula and *Pythium ultimum*.

<table>
<thead>
<tr>
<th>Populations</th>
<th>Soil without <em>Pythium</em></th>
<th>Soil treated with <em>Pythium</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>F113G22</td>
</tr>
<tr>
<td>Root bact</td>
<td>7.41$^b$</td>
<td>7.99$^b$</td>
</tr>
<tr>
<td>Soil bact</td>
<td>7.09$^b$</td>
<td>7.87$^a$</td>
</tr>
<tr>
<td>Root Fungi</td>
<td>3.91$^b$</td>
<td>3.78$^b$</td>
</tr>
<tr>
<td>Soil Fungi</td>
<td>3.98$^a$</td>
<td>4.10$^a$</td>
</tr>
<tr>
<td>Root Intr. pseu</td>
<td>ND</td>
<td>6.00$^b$</td>
</tr>
<tr>
<td>Soil Intr. pseu</td>
<td>ND</td>
<td>4.96$^b$</td>
</tr>
<tr>
<td>Root Ind. pseu</td>
<td>6.54$^b$</td>
<td>6.48$^c$</td>
</tr>
<tr>
<td>Soil Ind. Pseu</td>
<td>5.68$^b$</td>
<td>5.19$^b$</td>
</tr>
</tbody>
</table>

1 Treatments; control, no inocula; F113G22, inoculated with lacZY marked PhI (Tn5 mutated) *P. fluorescens* F113G22; F113, inoculated with lacZY marked PhI* P. fluorescens* F113; F113OP, inoculated with gus marked *P. fluorescens* F113R:pCUGP (PhI over producer).

2 Root bact, root bacteria/g root; Soil bact, soil bacteria/g soil; ind pseu, indigenous fluorescent pseudomonads/g root; int pseu, introduced fluorescent pseudomonads/g root; Root fungi, root fungi/g root; soil fungi/g soil.

3 Letters, within a row, indicate significant differences at p<0.05 level, (n=5).
2.7. IDENTIFICATION OF PHl BY HPLC

Phi isolation from soil

Table 2.4 summarises the peak separation data from extracts taken from the pea rhizosphere of plants inoculated with F113 and F113OP. Figure 2.6, shows HPLC traces of the six samples (one sample taken from three F113 inoculated microcosms and one sample taken from three F113OP inoculated microcosms). An additional sample is spiked with synthetic Phi to indicate a retention time for Phi separation of approximately 8.10 minutes. The trace from a rhizosphere extract from a F113G22 treated plant is superimposed for comparison of an extract of soil deficient in Phi.

| Table 2.4 |
|-----------------|-----------------|-----------------|
| Organism | Retention time (rt) | Peak Height | Peak area |
| Min | |
| F113 | 8.08 | 38206 | 2719864 |
| F113 | 8.07 | 28356 | 1457638 |
| F113 | 8.13 | 22780 | 652777 |
| F113OP | 8.13 | 74265 | 2630402 |
| F113OP | 8.07 | 56461 | 3097536 |
| F113OP | 8.13 | 41295 | 2883484 |

Phi extraction of 20 g of soil and washed root from three separate microcosms treated in either F113 or F113OP from the 21-day pea microcosm trial. Extracts separated by HPLC (20 µl injected from a total 200 µl suspension of the total extract). Peak heights and area calculated through integration using PeakNet chromatography software (Dionex).
Figure 2.6 HPLC traces that show the results of extraction from soil samples closely associated with the roots of 21-day-old pea plants. Phl retention time approx. 8.00 minutes. Blue traces indicate extract from F113OP inoculated pea. Green trace indicate extract taken from F113 inoculated pea. Red trace is the F113OP trace spiked with synthetic Phl. Pink trace shows the trace obtained from F113G22 inoculated pea (λ_{270nm})
The F113 and the F113OP were not statistically different in terms of the amount of Phi present per gram of sample taken.

The samples have been standardised against the populations of Phi producing bacteria present in the soil of each of the microcosms that the Phi was extracted and measured. The numbers of introduced pseudomonads isolated from both the root and rhizosphere soil are shown in Table 2.3. The values shown in Table 2.3 are the mean of the populations in the five replicate microcosms. Using population data, from individual microcosms, a precise correlate between the amount of Phi detected from individual microcosms and number of Phi producing bacteria in the respective microcosm was made. Table 2.5 summarises the population of Phi producing bacteria from each of the six microcosms together with the area under the curve (AUC) calculated from the individual HPLC traces from each of the microcosms.

<table>
<thead>
<tr>
<th></th>
<th>F113</th>
<th>F113</th>
<th>F113</th>
<th>F113OP</th>
<th>F113OP</th>
<th>F113OP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Log bacterial</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>population soil</td>
<td>6.00</td>
<td>5.84</td>
<td>5.60</td>
<td>5.48</td>
<td>5.70</td>
<td>5.95</td>
</tr>
<tr>
<td>and roots</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Log AUC Phi</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>production</td>
<td>6.43</td>
<td>6.16</td>
<td>5.81</td>
<td>6.42</td>
<td>6.49</td>
<td>6.46</td>
</tr>
<tr>
<td>Ratio (Phi/c.f.u)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.72</td>
<td>2.11</td>
<td>1.63</td>
<td>8.77</td>
<td>6.20</td>
<td>3.20</td>
</tr>
</tbody>
</table>
The bar chart in Figure 2.7, shows the comparison between the amount of Phi isolated from the soils of the six microcosms and the numbers of Phi producing bacteria isolated from each respective microcosm. This chart shows graphically that all F113OP treated microcosms have a high level of Phi. Fewer Phi producing colonies are required to achieve the maximal Phi that was detected from these microcosms. In the F113 treated microcosms, less Phi was present, with an increase in Phi with increased colony numbers.

**Relative comparison between Phi production and bacterial populations in individual microcosms**

![Bar chart showing Phi production and bacterial population comparison](image)

**Figure 2.7** Phi produced in six individual microcosms compared to the number of Phi producing organisms in those microcosms. Phi detected from 20 μl injection of a 200 μl total extraction suspension. Suspension extracted from 30 g of soil/root from 21-day old pea plants.
Figure 2.8 shows a comparison of the ratio of Phi producers and Phi detected from each of these six microcosms. The ratio of the amount of Phi produced per colony from the microcosms was calculated and shown in Table 2.5. Statistical analysis of the means of the F113 and F113OP Phl/c.f.u., shows the production per colony to be statistically (p<0.05) increased per colony in the F113OP population within these microcosms. Statistics calculated using Student t-test assuming unequal variance of the means.

Proportion of Phi per c.f.u of F113 and F113OP within 21-day old pea microcosm

Figure 2.8 Comparison of the mean production of the Phi antibiotic per c.f.u in F113 and F113OP treated microcosms (n=3).

The proportion of Phi that is produced by the F113OP is 6.056±2.78, compared to the F113 population which produced, 2.155±0.55 units of phl / c.f.u. The F113OP produces at least 2.5 times the amount of Phl than the F113 in planta.
2.8. **DISCUSSION**

In microcosms infected with *Pythium* both the F113 and the F113OP are seen to significantly reduce the emergence of pea plants in comparison to the control and F113G22 treatments. The reason for this may be the presence of Phl in these treatments in comparison to the F113G22 and control treatments. The tolerance experiment showed however that Phl alone does not exhibit toxicity to the pea plants.

Pea seeds, imbibed with synthetic Phl at concentrations 10, 100 and 1000 times physiological levels do not show any effects of toxicity leading to lowered seed emergence. Findings by Maurhofer *et al.* (1995) found that there was a correlation between the sensitivity of the plants to Phl and the toxicity imposed by a recombinant, OP strain of CHA0.

There may be a synergy between root damage and Phl production causing the reduced emergence in the Phl⁺ inoculated plants in the presence of *Pythium*.

Naseby *et al.* (1999) describes the increase in the organic acids released from pea plants inoculated with F113G22 and F113. The reason for this increased release of organic acids has been attributed to root leakage or exudation caused by root damage by the F113 and F113G22 inocula on the root of pea. This alone has not been shown to reduce emergence in the absence of *Pythium* in the experiment by Naseby *et al.* (1999).

Root damage alone cannot explain the pattern of emergence seen in the *Pythium* infected soil, as F113G22, behaves in the presence and absence of *Pythium* similarly to the control pea plants. Exposure of pea to high levels of Phl, alone is not phytotoxic, as shown in the tolerance experiment. The lower emergence is only evident with the Phl⁺ strains F113 and F113OP in the presence of *Pythium*.

The significant differences in lesions on the plant roots may indicate the *modas operandum*, of pathogenicity. The F113G22 has a significantly higher number of
lesions which are characteristic of *Pythium* infection. The control plant presents with statistically less and similar numbers to the biological control strains F113 and F113OP. The increased lesions seen on the F113G22 compared to the control are indicative of bacteria-inocula induced root damage predisposing the plant to *Pythium* affected lesions. The reduction of lesions seen on the F113 and F113OP treated plants suggest that *Pythium* infection is lower, even following the inoculum root damage which suggests that Phl produced by these strains is reducing the *Pythium* lesions. It would be interesting to determine if the effect of the reduction in emergence seen in plants inoculated with Phl⁺, F113 and F113OP, is due to a combination of root damage caused by the bacterial inocula combined with the Phl, which may be increasingly able to penetrate the plant roots. This could be determined through the inoculation of plants with F113G22 with the addition of synthetic Phl to compare whether emergence is reduced in comparison to the F113G22 inoculum alone.

If this were to show that Phl does indeed contribute to reduced emergence in the presence of an F113 delivery inocula then it may be worth pursuing the findings from the emergence rates seen in the non-*Pythium* infected soil.

The F113OP modified to overproduce Phl by constitutive plasmid expression reduced the plant emergence to 23/25 plants in comparison to 25/25 plants emerging in the other treatments. It may be interesting to conduct an experiment to determine if the F113OP does indeed significantly reduce plant emergence. At present this is impossible to tell as the numbers of plants per treatment do not generate a background for emergence in the control treatment and hence do not allow us to determine whether the F113OP emergence lies outside the expected natural variance of emergence for the control. If this were found to be the case, the findings from the Phl analysis from the soil of microcosms treated with F113 and F113OP may be interesting.

The F113OP was found to overproduce Phl in comparison to the wild-type F113 as the levels of Phl in the soil at the end of the experiment was approximately 2.5 times greater in F113OP treated microcosms than F113 microcosms. This standardisation is based on findings from Raaijmakers et al. (1999) indicating that Phl production by a
natural Phi producer, *P. fluorescens* Q2-87, is directly correlated to the populations of the Phi producer in the soil. As population densities at the early stage of seed germination are similar, as inoculation densities will still predominate, the level of Phi would be expected to be even greater around the F113OP inoculated seeds than the F113 inoculated plants.

If indeed the effect seen in this study is the result of a Phi overproduction causing plant toxicity, it would not be the first time. This effect has been noted with another Phi producer, CHA0 on the emergence of tobacco plants, sweet corn and cress (Maurhofer *et al.*, 1995). Again, in 1999, Moenne-Loccoz *et al.* (1999), described the effect of F113 inoculum preparation on biological control efficacy. In brief, the investigation aimed to add the F113 biological control strain to an EB™/vermiculite based seed pellet, for protection of sugarbeet against damping-off disease. If the F113 was washed in a sucrose asparagine (SA) broth prior to incubation into the EB™/vermiculite mix, the population of F113 that survives pelleting, storage and subsequent planting is significantly increased in comparison to F113 washed in Ringers. The result from these two forms of seed preparation on biological control efficacy of the inoculum is puzzling. The seeds inoculated with F113 and SA does not provide biological control and causes significantly lower emergence rate than that seen for the seeds inoculated with F113 washed in nutrient free Ringers, in the presence of pathogenic *Pythium*. The populations however are lower in the SA-free preparation than in the ineffective SA amended inoculum.

Although this ambiguity is not explained in the investigation, evidence from this study may indicate the role of Phi in reducing plant emergence. Sucrose asparagine medium, the nutrient supplement used, provides the optimal nutrient medium for the production of Phi by F113 (Shanahan *et al.*, 1992).

It appears that inoculating F113 with medium that induces high levels of Phi production can causes a reduction in the emergence of seeds. This compares to F113 inoculated seeds that do not have optimal conditions for Phi production. Again, it is noted that the enhanced Phi production has caused a toxic effect.
This effect seen with the possible reduced emergence in the F113OP strain in *Pythium* uninfected soil is worth investigating further.

The findings of this study have revealed some important possible routes of toxicity by Phl producing organisms and emphasised the importance of studying plant – microbe interactions when designing approaches to biological control. For the future direction of research in this area to proceed modifications need to be made to this approach to biological control using Phl.

These include the elimination of plasmid systems to aid the regulation of Phl production *in planta*. Excess Phl production has been the obvious line of research for enhancing the properties of biological control strains. The evidence of plant toxicity from both CHA0 and F113 modified to overproduce Phl will limit their potential commercial use to a few commercial crops that are not damaged by the inocula. Plasmid systems will inevitably give rise concerns about the risk of DNA transfer in the environment, and may prove a significant problem in these systems achieving commercial marketing authorisation.

The possibility of an integrated approach to biological control allowing low levels of Phl to act together with a secondary biological control mechanism to control plant disease should be considered. The primary method of biological control by overproduction of Phl has its limitations, so using physiological levels of Phl production with an alternative, non antibiotic approach, may widen the application of the biological control organism to multiple crops.

If plant damage is caused by the combination of the F113 and high levels of Phl production it will encourage us to consider an alternative approach to biological control with 2,4 diacetylphloroglucinol. The future aim of this work is to utilise physiological levels of Phl, produced by an alternative organism, *P. fluorescens* SBW25. This combination may be effective in the control of *P. ultimum* on the roots of pea without the pathogenic effects noted with the F113 organism. This approach also utilises an integrated approach to biological control. *P. fluorescens* SBW25, has been shown to exclude plant pathogens through competitive exclusion, through
aggressive colonisation of plant roots. Because of this additional method of pathogen inhibition, it is logical to conclude that any remaining pathogens would be successfully inhibited with lower levels of the antibiotic, Phl.
3. CHAPTER 3
3.1. **SUMMARY OF RESULTS**

The antibiotic 2,4 diacetylphloroglucinol is an important factor in biological control by fluorescent *Pseudomonas* spp. Of many soilborne diseases including take-all disease of wheat and damping-off of sugarbeet. PCR primers were designed that would anneal to the *Phl* biosynthetic gene locus of the U.S isolated, *Phl* producer, *P. fluorescens* Q2-87. These primers were targeted to the genomic DNA of *P. fluorescens* F113, a *Phl* producer, isolated from Irish soil. The PCR reaction resulted in PCR products the same size as that predicted from sequence data from the *P. fluorescens* Q2-87 strain, indicating that there is likely conservation of the *Phl* producing locus in these geographically diverse *Phl*+ organisms. Partial sequence analysis of these PCR generated fragments showed that there was >80% nucleotide sequence homology between the Q2-87 and F113, *Phl* production locus. Translational sequence analysis shows the conservation to be >93% for each of the regions sequenced. Approximately 35% of the amino acids that were conserved between the two strains did not have conserved nucleotide triplet codes. In the case where amino acids were not conserved there was a functional conservation of the amino acid. This occurred at a frequency of approximately 1% over the regions sequenced. A further 1% of amino acids showed no functional conservation and were considered non-conserved between the two geographically divergent strains. This evidence supports the hypothesis that the *Phl* locus sequenced from *P. fluorescens* Q2-87, isolated in the U.S, is functionally conserved in the Irish isolated, *P. fluorescens* F113, and is the candidate for the locus responsible for *Phl* production by this strain.
3.2. **INTRODUCTION**

*Pseudomonas fluorescens* strains F113, CHA0 and Q2-87 have each been shown to have disease suppressive qualities against a range of plant, fungal, pathogens. *P. fluorescens* F113 has been shown by Fenton *et al.* 1992 and Shanahan *et al.* 1992, to be suppressive of the plant pathogen *P. ultimum* and protects sugar beet from the seedling disease damping off. The mechanism of this protection has been shown through Tn5 mutagenesis to involve a 6 kb genomic region of the F113 genome responsible for the production of the secondary metabolite 2,4-diacetylphloroglucinol (Phi). *P. fluorescens* F113 strain that has a Tn5 insertion at this 6 kb region of the genome is unable to produce Phi, and does not inhibit the growth of *P. ultimum* in plate bioassays (Shanahan *et al.*, 1992). Complementation of Phi Tn5 mutant, with the 6 kb F113 genomic fragment restored the Phi production. Transformation into eight non-Phi producing strains resulted in only one strain able to produce the Phi metabolite (Fenton *et al.*, 1992). The authors of this work concluded that the 6 kb F113 genomic fragment used to transform these natural non-producing strains encoded some information for the biosynthesis of Phi but not the entire genomic biosynthetic pathway for universal production of Phi in all transformed non-producers.

*P. fluorescens* CHA0, isolated in Switzerland also possesses disease suppressive characteristics in soil. This strain produces both Phi and phenazines, both believed to be responsible for the disease suppressive ability of this strain (Defago *et al.*, 1990; Keel *et al.*, 1990, 1992). Complementation of a Tn5 mutant of CHA0, unable to produce Phi and no longer able to suppress *P. ultimum*, with an 11 kb CHA0 genomic fragment, showed partial restoration of suppressive properties and Phi production (Keel *et al.*, 1992).

The addition of a 22 kb fragment was able to increase Phi production of a wild-type strain CHA0. This effect was through sigma factor mediated expression and not through complementation of genes directly responsible for biosynthesis of Phi (Schnider *et al.*, 1995).
Chapter 3

*P. fluorescens* strain Q2-87, isolated from disease suppressive soils in the U.S, has been shown to suppress the wheat seedling pathogen, *Gaeumannomyces graminis*, the causative agent for take-all. Suppression has been shown to be through the production of Phl, as a Phl− mutant of Q2-87 was unable to suppress *G. graminis*. Vincent *et al.* (1991) revealed a 35 kb, Q2-87 genomic fragment, was capable of restoring Phl production to the Phl−, Tn5 mutant, Q2-87::Tn5-1.

Bangera and Thomashow (1996) further characterised this 35 kb, Q2-87 genomic fragment, to determine the genetic component that is responsible for restoring Phl production to the Phl− mutant. Mutagenesis of this 35 kb fragment with the transposon Tn3HoHoI, revealed that a region of 6.5-kb within the 35 kb fragment was responsible for conferring the ability to produce Phl in the Tn5 mutant. Further proof was obtained through the transformation of thirteen naturally Phl− fluorescent pseudomonads. All 13 were reported to have the ability to produce Phl following transformation with plasmid, pMON5122, containing this 6.5-kb fragment.

The nucleotide sequence for the genomic fragment from strain *P. fluorescens* Q2-87 has been published and submitted to the public access databases at the European Bioinformatics Institute (EBI), GenBank accession number, U41818. The nucleotide sequence of the region reveals six genes organised in three transcriptional units. Each gene has been characterised based on sequence homology with genes of known function also in the database. The cluster contains one transcriptional unit responsible for biosynthesis of Phl, and two separately transcribed units involved in repression of the operon and removal of the biosynthetic metabolite.

The aim of this investigation is to determine whether the genes responsible for Phl biosynthesis in Q2-87, isolated from U.S soils, have been conserved in the F113 strain isolated from Irish soils. Alone, this would provide interesting information as to the evolutionary divergence of these functionally related, geographically divergent organisms. The findings are important in the context of this thesis as a strategy for the isolation of the complete Phl biosynthetic locus from *P. fluorescens* F113. The relative lack of success when transforming natural non-Phl producers with a locus already isolated from the F113 strain by Fenton *et al.* (1992) with only one organism
in eight successfully transformed, suggests that the locus previously isolated is either incomplete or not universally functional. This is unlike the *P. fluorescens* Q2-87 genomic locus that conferred Phl production ability to all thirteen non producing organisms transformed (Bangera and Thomashow, 1996).
3.3. MATERIALS AND METHODS

Bacterial strain and growth conditions
The strain used in this study was Pseudomonas fluorescens F113 isolated from root hairs of a sugar beet plant in Ireland (Shanahan et al., 1992) and P. fluorescens SBW25 isolated from the phyllosphere of sugar beet, U.K (Thompson et al., 1993). The organism was maintained in Luria broth (LB), supplemented with antibiotic kanamycin (Km) 50 mg ml⁻¹ and grown at 30°C.

Primer design and synthesis
Primers were designed using the sequence data for the biosynthetic loci for Phl production of P. fluorescens Q2-87 (GenBank accession no. U41818) These are shown in Table 3.1. Primer pairs were designed so that the predicted fragments produced would include the whole Phl gene cluster. All primers were added 0.5 μg per 25 μl reaction. All primers were synthesised by GIBCO/BRL.

Table 3.1

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>G + C (%)</th>
<th>Position</th>
</tr>
</thead>
<tbody>
<tr>
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<tr>
<td>phl730</td>
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<td>phl410</td>
<td>GTCATCATTTATCCCGGCTCAA</td>
<td>48</td>
<td>410</td>
</tr>
</tbody>
</table>
Target DNA
Total genomic DNA was prepared from *P. fluorescens* strain F113 following 30 h incubation at 30 °C in 10 ml LB. DNA was extracted by rapid genome isolation technique adapted from Pitcher *et al.* (1989). 100 ng template DNA was used per 25 μl PCR reaction.

DNA polymerase
*Tag2000™* (Stratagene) is a recombinant *Taq* DNA polymerase isolated from the thermophilic bacteria, *Thermus aquaticus*. 2.5 U of *Tag2000™* was used per 25 μl PCR reaction.

Additional components
Optimisation of PCR buffer conditions was achieved using Opti-prime PCR optimisation kit purchased from Stratagene. For PCR products of 2 – 3 kb optimal conditions were found to be 10 mM Tris-HCl (pH 9.2), 1.5 mM MgCl₂ and 25 mM KCl. dNTPs were added at 0.2 mM (Promega).

PCR cycling conditions
All amplifications were performed in a Perkin-Elmer GeneAmp PCR System 2400™, using microAmp tubes (0.2 ml). All reactions were performed in 25 μl reaction volumes. Cycle conditions for primer pairs; phl7 and phl730; phl410 and phl2461; phl696 and phl2461; phl2392 and phl4373; and, phl4325 and phl7071 were as follows: initial denaturation at 94 °C for 2 min followed by 30 cycles of denaturation at 94 °C for 30 sec, anneal at 62 °C for 2 min and extension for 2.5 min at 72 °C. This was followed by a final extension of 7 min at 72 °C.

Analysis of PCR products
Agarose gel electrophoresis was used to analyse all PCR products. 5 μl of each PCR reaction mix was routinely analysed. 1.6 % agarose (NBL Biosciences) was used in all cases made in TAE buffer (40 mM Tris acetate (pH 8.3), 20 mM glacial acetic acid and 0.2 mM EDTA). Gels were run at 70 V, 85 mA. All gels were stained with
ethidium bromide and visualised under U.V illumination and photographed using a gel documentation system (UVP Imagestore 5000™).

*Automated DNA sequencing*

DNA was sequenced on an Applied Biosystems 373A™ automated sequencer using the dye terminator cycle sequencing method.

*Sequence analysis and multiple sequence alignment*

Sequence alignments were performed on the World Wide Web using the European Bioinformatics Institute EMBL database. Sequence alignment tool ClustalW, was used for all alignments.
3.4. **RESULTS**

PCR to show conservation of gene cluster between *Pseudomonas fluorescens* strains Q2-87 and F113.

The primer pairs chosen were based on the sequence data taken from *P. fluorescens* strain Q2-87 (Accession no. U41818). Primer pairs were chosen to encompass the entire operon responsible for Phl production in *P. fluorescens* Q2-87. The schematic diagram of the positions of each primer is shown in Figure 3.1a. Figure 3.1b shows the predicted PCR products that would result if the F113 template is conserved over this region with comparison to the known Phl biosynthetic gene cluster of strain Q2-87.
Figure 3.1a Schematic diagram of the Phi biosynthetic locus as revealed from sequence data from *P. fluorescens* Q2-87 (Acc. no. U41818). Coloured blocks show position of primer pairs used to anneal to the genome of *P. fluorescens* F113.

Figure 3.1b Schematic diagram of the PCR generated fragments from a template containing a Phi biosynthetic locus that is conserved with that from Q2-87. These fragments come from using primer-pairs described in Figure 3.1a.
Where possible, primers were designed to anneal within the open reading frame predicted from the database. These are regions where a high degree of sequence conservation would be expected. Primer pairs phl7071 and phl7 were designed outside the open reading frames to determine if strong conservation between Q2-87 and F113 was maintained in non-coding regions away from the genes directly responsible for Phl production in strain Q2-87.

The PCR products generated by each primer pair were analysed by agarose gel electrophoresis to determine their sizes. The PCR products were shown to be approximately 2000 bp, 2000 bp and >2200 bp for primer pairs phl410 – 2461 (Figure 3.2), phl2392 – 4373 and phl4325 – 7071 (Figure 3.3) respectively. Primer pair phl7 and phl730 resulted in no PCR generated product through the entire range of PCR conditions tested. Predicted and observed lengths are summarised in Table 3.2.

![Figure 3.2 Results of PCR using primer pair phl410 and 2461. Lane 1 and 2 show the results of the reaction using genomic DNA from P. fluorescens F113. Lane 3 shows the result of using genomic template of P. fluorescens SBW25. Marker is DNA from a lambda HindIII digest.](image-url)
Figure 3.3 Gel to show the DNA product of PCR using *P. fluorescens* F113 genomic template.

Lane 1 shows the product using the primer pair phl2392-4373, resulting in a ~2.0-kb PCR fragment.

Lane 2 shows the product from the reaction using primer pair phl4325-7071. The resulting product is >2.2 kb as indicated by the lambda HindIII digested marker.

### Table 3.2

<table>
<thead>
<tr>
<th>Primer pair</th>
<th>Predicted PCR product length (bp)</th>
<th>Actual observation from agarose gel -length (bp)</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>phi7 - phi730</td>
<td>749</td>
<td>No Product</td>
<td></td>
</tr>
<tr>
<td>phi410 - phi2461</td>
<td>2076</td>
<td>~2000</td>
<td>Figure 3.2</td>
</tr>
<tr>
<td>phi2392 - phi4373</td>
<td>2006</td>
<td>~2000</td>
<td>Figure 3.3</td>
</tr>
<tr>
<td>phi4325 - phi7071</td>
<td>2772</td>
<td>~2700</td>
<td>Figure 3.3</td>
</tr>
</tbody>
</table>

Predicted PCR product length of PCR products amplified using these primer pairs and the Irish isolate, F113 template. Predictions made from sequence data from the U.S isolated strain Q2-87.
Each of the PCR products was purified and part sequenced to confirm that the DNA amplified was from the *Phi* biosynthetic locus of *P. fluorescens* F113. The sequence data from each of the three PCR products is shown in Figures 3.4 to 3.9. They have been aligned against the sequence of the *Phi* coding region of the *P. fluorescens* Q2-87 (acc. no.U41818) using ClastalW alignment tool from the European Biotechnology Institute, Heidleberg.

Figures 3.4 to 3.9, show the results of sequence alignments between sequences taken from PCR generated fragments from the genomic template of strain F113 against the published *Phi* biosynthetic gene locus from strain Q2-87. All alignments are between 81 and 88% homologous to the sequence from Q2-87. The lowest homology is seen with regions sequenced using primers phl7071 and phl4975, with 81% homology to Q2-87. Sequence using primer phl3700 shows the greatest homology, of 88% over a 376-base stretch. The results are summarised in Table 3.3.

<table>
<thead>
<tr>
<th>Primer name (text Figure)</th>
<th>Length of sequence</th>
<th>Number of nucleotides that align Q2-87</th>
<th>Homology (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>phl2392 (3.4)</td>
<td>337</td>
<td>286</td>
<td>85</td>
</tr>
<tr>
<td>phl2461 (3.5)</td>
<td>309</td>
<td>266</td>
<td>86</td>
</tr>
<tr>
<td>phl3700 (3.6)</td>
<td>376</td>
<td>332</td>
<td>88</td>
</tr>
<tr>
<td>phl4325 (3.7)</td>
<td>346</td>
<td>302</td>
<td>87</td>
</tr>
<tr>
<td>phl4975 (3.8)</td>
<td>259</td>
<td>210</td>
<td>81</td>
</tr>
<tr>
<td>phl7071 (3.9)</td>
<td>309</td>
<td>250</td>
<td>81</td>
</tr>
</tbody>
</table>

Summary of alignment results of sequences taken from PCR generated products from strain F113 template against database sequence from strain Q2-87 (acc. no. U41818)
**Figure 3.4**: Comparison between nucleotide sequences from PCR generated product from *P. fluorescens* F113 and Q2-87 (GenBank acc. no. U41818). Sequence alignment performed on CLUSTALW sequence alignment tool (EBI).

*conserved nucleotide sequence*
Figure 3.5: Comparison between nucleotide sequences from PCR generated product from *P. fluorescens* F113 and Q2-87 (GenBank acc. no. U41818). Sequence alignment performed on CLUSTALW sequence alignment tool (EBI).
**Figure 3.6** Comparison between nucleotide sequences from PCR generated product from *P. fluorescens* FI13 and Q2-87 (GenBank acc. no. U41818). Sequence alignment performed on CLUSTALW sequence alignment tool (EBI).
**Figure 3.7:** Comparison between nucleotide sequences from PCR generated product from *P. fluorescens* F113 and Q2-87 (GenBank acc. no. U41818). Sequence alignment performed on CLUSTALW sequence alignment tool (EBI).
**Phi gene cluster with PCR products**

**Sequence comparison:**
PCR generated fragment from F113 and sequence data from Q2-87.

<table>
<thead>
<tr>
<th>PhIU41818</th>
<th>GACACTCTGGGACATAATGCGAGGCTGACATCGCTTTGTTGAGGCAGCCAGGCCCTTGCC1560</th>
</tr>
</thead>
<tbody>
<tr>
<td>4975</td>
<td>****</td>
</tr>
<tr>
<td>PhIU41818</td>
<td>AAGAGAGAGGGGTCGAAACAGGTGTGCTGGAATAGCAGATATGCGCTGGC1620</td>
</tr>
<tr>
<td>4975</td>
<td>****</td>
</tr>
<tr>
<td>PhIU41818</td>
<td>CTGTACATTCATCTTGCCATCAATCCGACAGCAGCAGATGGTCGTGCTTTCAAGGCC1680</td>
</tr>
<tr>
<td>4975</td>
<td>****</td>
</tr>
</tbody>
</table>

**PFU41818**

<table>
<thead>
<tr>
<th>PhIU41818</th>
<th>AATGCTGTTCTGTCGGAAGCCCAGCCCATTCTGACAGGAATGACCGGCTCTCCCTGGGG1740</th>
</tr>
</thead>
<tbody>
<tr>
<td>4975</td>
<td>****</td>
</tr>
<tr>
<td>PhIU41818</td>
<td>CGGTATGTTGTCAGCCACATCGGGCGCGAAGAAAG-CTGGCATGAAATGCGGATCA1799</td>
</tr>
<tr>
<td>4975</td>
<td>****</td>
</tr>
<tr>
<td>PhIU41818</td>
<td>CATTTCAGTACCACAAAGACAGTGGCGCAGCTCCCAGCTCCGGGTAGGACTCGGTCAAGT1859</td>
</tr>
<tr>
<td>4975</td>
<td>****</td>
</tr>
</tbody>
</table>

*conserved nucleotide sequence*

**Figure 3.8:** Comparison between nucleotide sequences from PCR generated product from *P. fluorescens* F113 and Q2-87 (GenBank acc. no. U41818). Sequence alignment performed on CLUSTALW sequence alignment tool (EBI).
**Figure 3.9.** Comparison between nucleotide sequences from PCR generated product from *P. fluorescens* FI13 and Q2-87 (GenBank acc. no. U41818). Sequence alignment performed on CLUSTALW sequence alignment tool (EBI).

*conserved nucleotide sequence*
The sequences described in Figures 3.4 to 3.9 were analysed to compare homology of the amino acid translation sequence. Figures 3.10 to 3.15 show the translation sequence of the nucleotide sequences in Figures 3.4 to 3.9. All translation alignments showed amino acid homology above 93%, with sequences from phl2392 and phl3700 showing 100 and 99% homology respectively. This compares with 85 and 88% respectively from direct nucleotide alignment with strain Q2-87. The summaries of these results are expressed in Tables 3.4 and 3.5. It was not possible to align translation sequence from primer phl7071, as it can be seen from the schematic diagram of the Phl locus (Figure 3.1ab), that this region lies outside the ORF for gene phlF of strain Q2-87.

Alignments are calculated in the following way. Number of conserved amino acids that have conserved triplet codes. Number of homologous amino acids that have different nucleotide triplet codes. Number of functionally homologous amino acids, although not directly homologous and the number of semi-functional amino acids that do not share triplet code.

The final column indicates amino acids that are functionally and structurally distinct from the strain Q2-87. The proportions have been amended not to include nonsense triplet codes that contained ‘N’-base in the original nucleotide sequence.
### Table 3.4

<table>
<thead>
<tr>
<th>Primer (text Figure)</th>
<th>Conserved amino acid and nucleotide</th>
<th>Conserved amino acid (not conserved nucleotide)</th>
<th>Functionally conserved amino acid</th>
<th>Functionally semi-conserved amino acid</th>
<th>No amino acid conservation</th>
</tr>
</thead>
<tbody>
<tr>
<td>phl2392 (3.10)</td>
<td>57/98 (58)</td>
<td>41/98 (42)</td>
<td>0/98 (0)</td>
<td>0/98 (0)</td>
<td>0/98 (0)</td>
</tr>
<tr>
<td>phl2461 (3.11)</td>
<td>65/98 (66)</td>
<td>29/98 (30)</td>
<td>2/98 (2)</td>
<td>1/98 (1)</td>
<td>1/98 (1)</td>
</tr>
<tr>
<td>phl3700 (3.12)</td>
<td>42/67 (63)</td>
<td>24/67 (36)</td>
<td>0/67 (0)</td>
<td>1/67 (1)</td>
<td>0/67 (0)</td>
</tr>
<tr>
<td>phl4325 (3.13)</td>
<td>67/101 (66)</td>
<td>27/101 (27)</td>
<td>1/101 (1)</td>
<td>2/101 (2)</td>
<td>2/101 (2)</td>
</tr>
<tr>
<td>phl4975 (3.14)</td>
<td>43/76 (57)</td>
<td>26/76 (34)</td>
<td>4/76 (5)</td>
<td>2/76 (3)</td>
<td>1/76 (1)</td>
</tr>
</tbody>
</table>

Summaries of the translation sequence alignment from PCR sequence data from strain F113 and database sequences of *Phi* biosynthetic cluster Q2-87 (acc. no.U41818). Figures are presented as alignments per total number of amino acids in sequence. Exclusions of non-amino acid coding triplets due to base-N in nucleotide sequence. Percentages are shown in brackets.

### Table 3.5

<table>
<thead>
<tr>
<th>Primer</th>
<th>Total conserved amino acids</th>
<th>Amino acid homology (%)</th>
<th>Nucleotide homology (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>phl2392</td>
<td>98/98</td>
<td>100%</td>
<td>85%</td>
</tr>
<tr>
<td>phl2461</td>
<td>94/98</td>
<td>96%</td>
<td>86%</td>
</tr>
<tr>
<td>phl3700</td>
<td>66/67</td>
<td>99%</td>
<td>88%</td>
</tr>
<tr>
<td>phl4325</td>
<td>94/101</td>
<td>93%</td>
<td>87%</td>
</tr>
<tr>
<td>phl4975</td>
<td>69/76</td>
<td>91%</td>
<td>81%</td>
</tr>
<tr>
<td>phl7071</td>
<td>NA</td>
<td>NA</td>
<td>81%</td>
</tr>
</tbody>
</table>

Summary of alignment homology scores from amino acid alignment and nucleotide alignments for respective sequences from strain F113 and strain Q2-87. Amino acid alignment is the sum of the homologous amino acids regardless of the nucleotide triplet sequence.
Figure 3.10: Comparison between translation sequences from PCR generated product from *P. fluorescens* F113 and Q2-87 (GenBank acc. no. U41818). Sequence alignment performed on CLUSTALW sequence alignment tool (EBI).
**Phi** gene cluster with PCR products

Sequence comparison:
PCR generated fragment from F113 and sequence data from Q2-87

**PFU41818**

<table>
<thead>
<tr>
<th>AA Sequence</th>
<th>Conserved with altered nucleotide sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>PFU41818</td>
<td>DLGLRTSTVQLPIAQLGCVAGAAAINRANDFASLSPDNHALIVSLFSSLCYQPQDTKLH 180</td>
</tr>
<tr>
<td>2461</td>
<td>DFXSRAPDNHVLIVSXEFSSLCYQPQXTKLH 31</td>
</tr>
<tr>
<td><strong>PFU41818</strong></td>
<td>AFISAALFGDAVENSMRADDQAPFGKIAKTSYGFLPDEHYIKYIYKDSGFTLDKAV 240</td>
</tr>
<tr>
<td>2461</td>
<td>AFISAALFGDAVENSMRADDQAPFGKIAKTSYGFLPDEHYIKYIYKDSGFTLDKAV 91</td>
</tr>
<tr>
<td><strong>PFU41818</strong></td>
<td>MNSIXDVAPMMEELMFETFNQHCAQNDFFPHTGGRKILDEVLQLDLGPAVISRDSL 300</td>
</tr>
<tr>
<td>2461</td>
<td>MNSIXDVAPMME- 105</td>
</tr>
</tbody>
</table>

*conserved aa sequence with altered nucleotide sequence
*conserved aa sequence with conserved nucleotide sequence
altered aa with conserved chemical function
altered aa with semi-conserved chemical function

**Figure 3.11:** Comparison between translation sequences from PCR generated product from *P. fluorescens* F113 and Q2-87 (GenBank acc. no. U41818). Sequence alignment performed on CLUSTALW sequence alignment tool (EBI).
**Figure 3.12:** Comparison between translation sequences from PCR generated product from *P. fluorescens* F113 and Q2-87 (GenBank acc. no. U41818). Sequence alignment performed on CLUSTALW sequence alignment tool (EBI).
**Figure 3.13:** Comparison between translation sequences from PCR generated product from *P. fluorescens* F113 and Q2-87 (GenBank acc. no. U41818). Sequence alignment performed on CLUSTALW sequence alignment tool (EBI).
Sequence comparison:
PCR generated fragment from F113 and sequence data from Q2-87.

Figure 3.14: Comparison between translation sequences from PCR generated product from *P. fluorescens* F113 and Q2-87 (GenBank acc. no. U41818). Sequence alignment performed on CLUSTALW sequence alignment tool (EBI).
3.5. **DISCUSSION**

*P. fluorescens* F113 is a natural producer of the antibiotic 2,4-diacetylphloroglucinol (Phi) and on plate assays against the plant pathogenic fungi, *Pythium ultimum* has a growth inhibition effect. *P. fluorescens* Q2-87 is also a natural producer of the antibiotic and displays similar effects in plate bioassays. The genes believed to be responsible for the production of Phi have been isolated and sequenced from the strain *P. fluorescens* Q2-87. *P. fluorescens* Q2-87 was isolated from plant suppressive soil in the U.S whereas *P. fluorescens* F113 was isolated from plant suppressive soils in Ireland.

This study set out to confirm that the genes identified in *P. fluorescens* Q2-87 were indeed present in *P. fluorescens* F113 and therefore prime candidates for the production of Phi in the *P. fluorescens* F113 strain.

PCR probes were designed with the sequence data taken from *P. fluorescens* Q2-87. The primers were intentionally designed to anneal within the open reading frames (ORFs) of the six gene operon from *P. fluorescens* Q2-87. These regions were considered to remain conserved throughout geographical distribution of *P. fluorescens* organism. Two primer phl410 and phl7071 were set to flank the coding region in order to include the entire coding region within the PCR products. The flanking regions chosen were as adjacent to the coding regions as was possible without designing poor quality primers, i.e. GC rich or containing long runs of a single nucleotide. Primers were also chosen that together would encompass the entire loci of the *Phi* cluster to confirm that the genes’ spatial arrangement on the chromosome is conserved between strains *P. fluorescens* Q2-87 and F113.

The PCR products obtained from the reaction using primer pairs phl410-2461, phl2396-4375 and phl4325-7071 were expected to be 2071 bp, 2000bp and 2771 bp respectively if the loci in *P. fluorescens* F113 was the same as that for *P. fluorescens* Q2-87. The results show that the products were of this size when visualised against a lambda HindIII marker following agarose gel electrophoresis.
Primer pairs phl7 and phl730 gave no PCR product through any of the PCR optimisation processes. The reason is either the PCR optimisation still did not provide optimal conditions for this pair of primers, or more likely that the DNA sequence is not conserved between the *P. fluorescens* Q2-87 and the *P. fluorescens* F113 strains at this region of the biosynthetic gene locus. When primer pairs phl7 and phl2461 were used in combination again no PCR product resulted suggesting that the region to which primer phl7 anneals is not conserved. This is concluded as primer phl2461 can anneal as it produces the correct product when paired with primer phl410.

The primer, phl7, has been designed to anneal 407 bp downstream of the coding gene cluster reported from sequence data from *P. fluorescens* Q2-87. There is a loss in conservation of the genome sequence between the *P. fluorescens* Q2-87 and F113 suggesting that this region has no advantageous importance to either organism. This hypothesis is supported by the absence of an ORF at this region with respect to the sequence analysis from *P. fluorescens* Q2-87. The lack of conservation of this region suggests that this region has no function in regulation of the gene cluster. This is supported by the absence of any known regulatory sequences responsible for protein binding in Q2-87.

The sequence data from the each PCR generated fragment shows that there is a high level of conservation between the *P. fluorescens* Q2-87 and *P. fluorescens* F113. For each region that was sequenced the conservation was high enough to support the hypothesis that PCR generated fragments created with primers designed to anneal to the *Phl* cluster of *P. fluorescens* Q2-87 generated amplified fragments of the *Phl* coding region of *P. fluorescens* F113. With the variation of sequence seen between the two strains it was important to consider the translation sequence that was generated from the PCR fragments. This was considered important to help eliminate the possibility of evolutionary artefacts that may have resulted in an almost identical, yet defunct, gene cluster in *P. fluorescens* F113.

Analysis of the translation sequence from each of the five coding regions shows that although variation is present with nucleotide sequence there is little variation in amino acid sequence. This suggests that there has been evolutionary divergence of the base
sequence between the two geographically separate isolates, however the selective environmental pressure for each organism has remained and functional amino acid sequence has been maintained.

The nucleotide sequence data from PCR product generated from primer phl2392 shows 41 of 99 triplets are different with respect to the sequence data from *P. fluorescens* Q2-87. Their translation sequence reveals that there is 100% conservation of the amino acid sequence. This functional conservation would suggest a divergent evolution between *P. fluorescens* Q2-87 and *P. fluorescens* F113. A similar pattern is seen with the remaining four sequences from PCR generated products. The sequence taken using primer phl3700 shows 24 of 89 amino acids that have altered codon sequences yet code identical amino acids. One amino acid change has taken place, aspartic acid (D) to glycine (G), however these two amino acids are said to be semi-conserved with respect to their chemical classification. The sequence taken from phl4325 shows 27 of 130 amino acids that have altered codon sequences that encode identical amino acids. There are four changes in amino acid sequence with respect to *P. fluorescens* Q2-87, leucine (L) to phenylalanine (F), which shows conservation of amino acid function and alanine (A) to glycine (G) and serine (S) to proline (P) which show semi-conserved amino acid function. Only one amino acid change, arginine (R) to cysteine (C) has no functional conservation.

Sequence data from primer phl4975 shows 26 of 104 amino acids have altered nucleotide sequences with respect to *P. fluorescens* Q2-87 and seven amino acids have not been conserved. Four of these have conserved chemical function with two being semi-conserved. Only one amino acid has no conservation. Sequence data from phl2461 generated PCR fragment shows 29 of 105 amino acids have altered nucleotide sequences, but encode identical amino acids. Four amino acids have not been conserved between F113 and Q2-87, however two have conserved chemical groups and one has semi-conserved groups. One amino acid is not conserved.

As base sequence has diverged, yet amino acid sequence has been conserved, it can be assumed that the region amplified by PCR contain the genes responsible for the Phl
production in *P. fluorescens* F113 as this region has undergone evolutionary adaptation yet maintaining the potential for functional output.
4.1. **Summary of the Results**

The work reported in chapter 3 of this thesis showed the sequence of the 2,4-diacetylphloroglucinol (Phi) biosynthetic locus to be functionally conserved between *P. fluorescens*, Q2-87 and *P. fluorescens*, F113 isolated from geographically distinct locations. Optimising PCR conditions led to the successful amplification of the entire 6.7-kb gene locus responsible for Phi production from the strain F113. The subsequent cloning into plasmid pMC1871 resulted in a construct capable of suicide delivery of the Phi genes into the *P. fluorescens* SBW25. This was possible through the process of homologous recombination. Through positive selection of the integrated plasmid, single, homologous recombination of the Phi genes was achieved, however, double recombination was not detected as it occurred at a frequency of <1 in $10^5$, and blue/white selection was the only system for selection of the double recombination event. The recombinants were genetically and phenotypically characterised and were shown as derivatives of the wild-type SBW25. Colony morphology of the transformants showed two morphologically distinct groups. One strain from each of the morphological groups were selected for further investigation and denoted as Pa21 and Pa25. The transformation was repeated with the Phi locus, minus the repressor element, and only colonies of a homogeneous morphology were, this time, recovered. One colony Pa24r' was chosen for further analysis. All transformants were tested for inhibition of *P. ultimum* and all showed fungal inhibition on PDA bioassay plates. The inhibition was not as great as that seen with the natural Phi producer, F113. HPLC analysis showed that each morphologically distinct colony produced Phi, at the same levels as produced by strain F113, when grown in Tryptone Soya Broth.
4.2. INTRODUCTION

The pseudomonad strain F113 has been shown by Shanahan et al. (1992) to produce 2,4 diacetylphloroglucinol (Phl) which is a secondary metabolite responsible for the strain’s ability to suppress *P. ultimum* a soilborne fungal, plant pathogen. Through previous studies described in chapter 2 it has been shown that Phl production by F113 on pea seedlings has a deleterious effect on these plants. This chapter aims to address a method for transferring the biological control properties of Phl production of F113 to another fluorescent pseudomonad, *P. fluorescens* SBW25, with the aim of achieving antibiotic mediated biological control, without the organism-specific, adverse effects, that were seen with *P. fluorescens* F113 inoculation.

Identification of the genes responsible for the production of the antibiotic, Phl, in *P. fluorescens* F113 through PCR and subsequent partial sequence analysis (chapter 3) has enabled us to form a strategy for the isolation of this useful biological control locus from strain F113.

Strain *P. fluorescens* SBW25 is a non-pathogenic plant fluorescent pseudomonad that was isolated from the phyllosphere of wheat in Oxfordshire, UK (Thompson et al., 1993). Microcosm trials and field release trials involving inoculation of SBW25 onto both pea (De Leij et al., 1998), wheat (De Leij et al., 1998, 1995; Thompson et al., 1995) and sugarbeet (Thompson et al., 1995) has shown this strain to be non-pathogenic.

*Pseudomonas fluorescens* SBW25 has been shown to be the major microbial coloniser of wheat and sugar beet following field trial application and sampling of natural environments (Thompson et al., 1993). Naseby et al. (2000) have shown that *P. fluorescens* SBW25 colonises pea seedlings more strongly than the biological control strains *P. fluorescens* F113, Q2-87 and CHAO. Although *P. fluorescens* SBW25 does not produce any secondary metabolites that can be attributed to biological control, the organism can protect plants from *P. ultimum* infection. The author has attributed this to the organism’s strong colonisation ability and biological
control through competitive exclusion of an ecological niche or subsequent limitation of nutrient supply to competing fungal pathogens.

Following the confirmation of the conservation of the Phl locus in F113 and Q2-87 a strategy to isolate the gene cluster from F113 was developed. This chapter describes a method for PCR optimisation to isolate a large biosynthetic cluster from F113 whilst maintaining its functionality with the use of innovative ‘long-PCR’ technology.

Polymerase chain reactions (PCR) have become one of the most important and widely used tools in molecular biology due largely to the discovery and application of thermal stable DNA polymerases, such as Taq DNA polymerase. Regular PCR has two disadvantages however: the fidelity is relatively low and the size of the target DNA fragments is relatively short.

The aim of this investigation is to amplify the entire 6.7-kb Phl biosynthetic locus from *P. fluorescens* F113 with the use of primers designed using sequence data from *P. fluorescens* Q2-87. The primers have already been shown to anneal at the biosynthetic locus from results obtained from chapter 3.

Conventional PCR using Taq polymerase under a wide range of PCR optimisation was shown to be unsuccessful in the amplification of this region from the F113 genomic template. Barnes (1994) comments that this is a common problem with conventional PCR using Taq polymerase. Although the primer/template interactions and template quality and sequence uniquely determine the success of PCR reactions, it is common to encounter failure of PCR using Taq polymerase when templates exceed 3-5 kb in length.

Barnes (1994) also speculates that the reason for failed PCR using Taq polymerase is that misincorporated nucleotides reduce the efficiency of amplifying long targets. A mismatched 3'-terminal base may cause prematurely terminated strand synthesis. Even low levels of nucleotide misincorporation estimated for Taq DNA polymerase at frequency of ~1 in 10-50,000 bases will affect long sequences.
It has been shown by Barnes (1994) and Lundberg et al. (1991) that the use of proofreading thermostable polymerases mixed with Taq non-proofreading polymerase can increase the length and yields of PCR products. Thermostable polymerases that contain 3' to 5' exonuclease activity have been shown to increase the fidelity of PCR by reducing the error rate to 1/30th of that of Taq (Mattila et al., 1991).

Recently the advent of commercial formulations have made 'Long-distance' PCR an increasingly popular method for isolation of large gene loci. However there still remain major limitations to its use through the scarcity of universal protocols and success with only well characterised templates. The potential is evident with amplifications of 22 kb of β-globin from human genomic DNA by Cheng et al. (1994) and 9-23 kb amplification of human inserts from recombinant λ plaques (Cheng et al., 1994). Barnes, in 1994, amplified a 6.7-kb region of λ DNA using a mix of Klentaq and pfu. When either pfu or Klentaq was used alone the reaction was unsuccessful.

Cloning and transformation of the PCR generated product into P. fluorescens SBW25 for the expression of Phi was the ultimate goal of this investigation. The technique of homologous recombination using a marked strain of SBW25, SBW25EcZY-6KX produced by Bailey et al. (1995) was used to ensure stable transformation.

Bailey et al. (1995) used homologous recombination to transform P. fluorescens SBW25 to express the marker genes. Chromosomal sites designated -6 and -Ee were cloned following total genomic digest of the SBW25 chromosome. The lacZY marker was cloned into region Ee and both the kanamycin resistance gene and xylE genes were cloned into region -6. Subsequent studies by De Leij et al. (1998) and Bailey et al. (1995) showed the organism’s fitness following insertion at these sites were not reduced in comparison to a strain that only had a single insertion at only one site.

The large, 2.5-kb lacZY region of the SBW25 chromosome, was chosen as a site for targeted integration of the Phi gene locus prepared by optimised ‘long’ PCR. Of concern is that random integration of the large, 6.7-kb, Phi coding region into the
SBW25 chromosome would cause a reduction in the organism's ecological fitness. Through targeted integration into the *lacZ*Y site, which is known not house essential genes for the organism's environmental fitness, the chances of reducing SBW25's rhizosphere competence is lowered. This would help to maintain the crucial aspect of SBW25's biological control capability.
### 4.3. MATERIALS AND METHODS

**Bacterial strains, plasmids and growth conditions**

Strains and plasmids used in this study are shown in Table 4.1. *Escherichia coli* DH5α was used as the host strain for all plasmids. All strains were maintained on Luria broth and grown at 30°C and 37°C for pseudomonads and *E. coli*, respectively, and supplemented with antibiotics kanamycin (Km) 50 mg ml\(^{-1}\) and tetracycline (Tc) 25 µg ml\(^{-1}\), where appropriate.

### Table 4.1

<table>
<thead>
<tr>
<th>Bacterial strain or plasmid</th>
<th>Relevant characteristics</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>E. coli</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DH5α</td>
<td><em>HsdR17(r- m</em>) supE44 thi-1 recA1 gyrA96 (naf) relA1* (lacZYA-argF)(_{196}) (lacZ M15)</td>
<td>Laboratory stocks</td>
</tr>
<tr>
<td><strong>P. fluorescens</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SBW25</td>
<td>Sugar beet leaf isolate</td>
<td>De Leij <em>et al.</em>, 1995</td>
</tr>
<tr>
<td>Pa2</td>
<td>Sugar beet leaf isolate with markers lacZY(^+), Km(^r) and xylE</td>
<td>Bailey <em>et al.</em>, 1995</td>
</tr>
<tr>
<td>Pa21</td>
<td>Transformants containing <em>Phl</em> Locus, Tc(^r), lacZY(^+), Km(^r)</td>
<td>This study</td>
</tr>
<tr>
<td>Pa25</td>
<td>Transformants containing <em>Phl</em> Locus, Tc(^r), lacZY(^+), Km(^r) (altered colony morphology)</td>
<td>This study</td>
</tr>
<tr>
<td>Pa24r(^-)</td>
<td>Transformants containing <em>Phl</em> locus minus repressor, Tc(^r), lacZY(^+), Km(^r)</td>
<td>This study</td>
</tr>
<tr>
<td>F113</td>
<td>Natural <em>Phl</em> producer isolated in Ireland</td>
<td>Shanahan <em>et al.</em>, 1992</td>
</tr>
<tr>
<td>F113OP</td>
<td>F113 containing plasmid pCUGP, containing extrachromosomal copies of <em>Phl</em> genes</td>
<td>O’Gara, personal communication</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pMC1871</td>
<td>Tc(^r), lacZ, oriC</td>
<td>Pharmacia</td>
</tr>
<tr>
<td>pMCPhl9</td>
<td>Tc(^r), <em>Phl</em>(^r), oriC</td>
<td>This study</td>
</tr>
<tr>
<td>pMCPhl9(^r)</td>
<td>Tc(^r), <em>Phl</em>(^r) (minus repressor), oriC</td>
<td>This study</td>
</tr>
</tbody>
</table>

Bacterial strains and plasmids used in this experiment
Growth was routinely measured at optical density 550 nm (OD$_{550}$). In addition, plating onto LB, monitored the number of cultivable cells.

Primers were designed using the sequence for the biosynthetic loci for *Phl* of *P. fluorescens* Q2-87 (acc. no. U41818) and are shown in Table 4.2. Primer pairs were designed so that the predicted fragments produced would include the whole *Phl* gene cluster. All primers were added 0.5 µg per 25 µl reaction. All primers were synthesised by GIBCO/BRL.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>G + C (%)</th>
<th>Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>phl2461</td>
<td>AACGTCGACCAGTACAATTGCCGATC</td>
<td>52</td>
<td>2461</td>
</tr>
<tr>
<td>phl4325</td>
<td>ACCACACAGGACGATGTCGTACTCA</td>
<td>52</td>
<td>4325</td>
</tr>
<tr>
<td>phl7071</td>
<td>GATATCGCTCATGGTGCACTTGTTCA</td>
<td>46</td>
<td>7071</td>
</tr>
<tr>
<td>phl410</td>
<td>GTCAATCGTATCCGCCAGCGTCAA</td>
<td>48</td>
<td>410</td>
</tr>
<tr>
<td>pMCF</td>
<td>Designed against <em>lacZ</em> region of plasmid pMC1871 — adjacent to <em>EclI</em>36II restriction site</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pMCB</td>
<td>Designed against <em>lacZ</em> region of plasmid pMC1871 — other side of <em>EclI</em>36II restriction site</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Target DNA**

Total genomic DNA was prepared from *P. fluorescens* strain F113 following 30 h incubation at 30 °C in 10 ml LB. DNA was extracted by rapid genome isolation technique adapted from Pitcher *et al.*, 1989. 100 ng template DNA was used per 25 µl PCR reaction.

**DNA polymerase**

*Taq2000™* (Stratagene) is a recombinant *Taq* DNA polymerase isolated from the thermophilic bacterium, *Thermus aquaticus*. 2.5 U of *Taq2000™* was used per 25 µl PCR reaction. *TaqPlus Long™* (Stratagene) was used for long and accurate PCR amplification. This is a polymerase mix purchased from Stratagene containing a mix of *Taq2000™* and cloned *Pfu* DNA polymerase exhibiting 3'-5' proof-reading
activity. 7.0 U of TaqPlus Long™ was used per 25 μl PCR reaction. 7.0 U of Expand High Fidelity™ (Roche).

**Additional components**

Optimisation of PCR buffer conditions was achieved using Opti-prime™ PCR optimisation (Stratagene). For PCR products of 2-3 kb, optimal conditions were: 10 mM Tris-HCl (pH 9.2), 1.5 mM MgCl₂ and 25 mM KCl. All four dNTPs were added at 0.2 mM (Promega). For PCR product of 6.7-kb optimal buffer conditions were: 10 mM Tris-HCl (pH 9.2), 3.5 mM MgCl₂ and 25 mM KCl. Further optimisation was required with the addition of 15 % glycerol (exclusive of glycerol in storage buffers) and the addition of Taq extender™ PCR additive (Stratagene) at 7 U per 25 μl reaction.

PCR cycling conditions. All amplifications were performed in a Perkin-Elmer GeneAmp PCR System 2400™, using microAmp tubes. All reactions were performed in 25 μl reaction volumes. Cycle conditions for primer pairs: phl410 and phl2461; and phl4325 and phl7071 were as follows: initial denaturation at 94 °C for 2 min followed by 30 cycles of denaturation at 94 °C for 30 sec, anneal at 62 °C for 2 min and extension for 2.5 min at 72 °C. This was followed by a final extension of 7 min at 72°C.

Cycle conditions for the amplification of the 6.7-kb fragment using primer pairs, phl410 and phl7071 were as follows: initial denaturation 80 °C for 1 min, followed by 30 cycles of denaturation at 94 °C for 10 sec, anneal at 62 °C for 30 sec and extension at 72 °C for 7 min. This was followed by a final extension of 10 min at 72 °C.

**Analysis of PCR products**

Agarose gel electrophoresis was used to analyse all PCR products. 5 μl of each PCR reaction mix was routinely analysed. 1.6 % agarose (NBL Biosciences) was used in all cases made in TAE buffer (40 mM Tris acetate (pH 8.3), 20 mM acetic acid and 0.2 mM EDTA). Gels were run at 70 V, 85 mA. All gels were stained with ethidium bromide and visualised under U.V illumination.
Chapter 4

**pMCPhl9 construct design**

Plasmid pMC1871 (Pharmacia) was used as a suicide insertion vector for the *Phl* coding genes generated by long high fidelity PCR from the F113 template. pMC1871 was digested with restriction endonuclease *Ecl136I* (Fermentas) to reveal a linearised 7.5 kb, blunt-ended DNA fragment. Both plasmid DNA and PCR generated 6.7 kb fragment were purified from crystal violet gels (Invitrogen). The PCR fragment was treated with *Pfu* DNA polymerase and treated with kinase (Roche). The plasmid ends were treated with calf-intestinal phosphatase (Pharmacia) to inhibit re-circularisation during ligation. Ligation was performed at 22°C for 16 hrs using T4 DNA ligase (Pharmacia). Vector to insert ratios were manipulated following concentration estimation against a known quantity of lambda *HindIII* marker. All cloning was performed in *E.coli* DH5α.

**pMCPhl1r construct design**

The repressor negative construct was made by the removal of the repressor gene from the long high fidelity PCR generated *Phl* gene cluster by restriction digest. Plasmid pMCPhl9 was digested with *Ecl136I* and *HpaI*. This revealed two fragments, 6848 bp and 6693 bp. The digestion mix was treated with calf intestinal phosphatase to prevent re-ligation of the pMCPhl9 construct. Following gel purification both fragments were ligated into *Ecl136I* and *HpaI* digested pMC1871. All colonies were picked and screened by the technique of rapid DNA lysis. Colonies of interest were selected on plasmid size. Primer probes targeted within the *Phl* gene cluster confirmed the presence of the *Phl* cluster.

**Selection by rapid DNA lysis**

The cloning site within the pMC1871 was situated within a promoterless *lacZ* gene, and it was therefore not possible to select for transformants by blue/white selection. Colonies containing possible correct constructs were consequently selected on plasmid size following rapid lysis of picked colonies. Individual Colony stabs were mixed in 15 μl of detergent and incubated at 48°C for 45 min. A 5 μl aliquot of the mix was loaded onto a dry agarose gel and DNA separated and visualised following gel electrophoresis. Colonies containing larger plasmids were analysed further. High
quality plasmid DNA was isolated using a QIAGEN mini-prep™ kit. The plasmids of interest were digested and sequenced in the region of the insertion site to reveal constructs containing the PCR fragment located within the pMC1871 vector.

**Transformation of SBW25**

SBW25 was grown overnight in 10 ml LB broth at 30°C until stationary phase and 10 ul used to inoculate 250 ml LB (shaken at 200 rpm at 20°C) until mid-exponential phase, optical density (O.D) $\lambda_{550} = 0.6$. After chilling on ice for 30 min, five aliquots in 50 ml Falcon tubes were centrifuged (3 K rpm, 20 min) at 4°C. After removal of the supernatant, the pellet was re-suspended in 50 ml chilled 15% sterile glycerol and the Falcon tubes centrifuged for 20 minutes 3 K rpm at 4°C. After removal of the supernatant, the pellet was re-suspended in 10 ml 15% glycerol, and all five pellets were pooled into one Falcon tube to make a total volume approximately 50 ml and the suspension centrifuged as before to remove the supernatant. The remaining pellet was re-suspended in 250 µl 15% glycerol, and kept on ice.

Pure plasmid DNA was prepared by ethanol precipitation to give a concentration ~1mg/ml. 45 µl of chilled SBW25 suspension was mixed with 5 µl (5 µg) of plasmid DNA in a chilled microfuge tube, and left on ice for 1-2 min. The entire 50 µl mix was then transferred to a chilled electroporation cuvette (0.2 cm gap, BioRad).

Electroporation was performed using a BioRad GenePulsar (0.8 ms, 2.5 kV, 200 Ω and 25 µFD). Within one minute of pulsing, the cells were suspended in 500 µl of SOB broth and gently mixed. The cuvette was placed at 30°C for 4 hr and then plated onto Tc+ LB plates and incubated for 48 hours at 30°C.

**Suicide plasmid integration**

Colonies with Tc+, Km+ and LacZY+ phenotypes were picked. Probing the colonies with PCR primers homologous to the Phi biosynthetic genes confirmed the presence of integrated plasmid in the SBW25 chromosome.
DNA from each of the colonies was prepared using a QIAGEN mini prep kit to confirm the absence of pMCPhl9 and pMCPhlr maintenance in SBW25.

**Phi detection using HPLC**

Bacteria were grown overnight or until they reach stationary phase used to inoculate 50 ml flasks of Tryptone Soya Broth (Oxoid) and shaken at 20°C for four days. 1.0 ml samples were taken and centrifuged (13 K rpm, 5 min). The supernatant was removed and mixed with equal volumes of ethyl acetate and following vortexing, centrifuged (13 K rpm, 1 min) and the ethyl acetate layer carefully removed. The procedure was repeated to give a second extraction. The ethyl acetate from both extractions was then pooled and evaporated to dryness. The residue was re-suspended in 200 μl, 78% acetylnitrile (ACN:H₂O v/v) with 0.1% trifluoroacetic acid (TFA).

For peak separation of the extract, a 20 cm x 4.8 cm reverse phase, Spherisorb C¹⁸ was eluted with solvent (1.0 ml per min) using an isocratic mobile phase of 78% ACN, 0.1% TFA. The extract was manually injected into a 20 μl loop and absorbency was measured at 270 nm.

Spiked sample was prepared from TSA flasks that had not been inoculated but had undergone incubation for 4 days at 20°C. Extraction was performed in the same way as described for the test cultures. Synthetic Phi, dissolved in the mobile phase was added to the final extract.

**Plate bioassays**

Plate bioassays were performed against a pathogenic fungus, *Pythium ultimum* grown on potato dextrose agar (PDA). Test bacterial colonies were spotted onto the plates 2 cm from the Petri dish edge. After placing the fungal plug at the centre of the dish, the plates were incubated at 20°C, for 4 days, until the fungus had grown to the edge of the dish. Evidence of fungal inhibition by the test bacteria was recorded.
4.4. RESULTS OF LONG PCR

Primer design
PCR primers were selected with two criteria in mind. The first was to ensure that all the genes responsible for the Phl+ phenotype are located within the two primers. The second is that the primers are able to anneal to the *P. fluorescens* FI13 template.

Primers phl410 and phl7071 flank the entire gene cluster responsible for Phl production in *P. fluorescens* Q2-87. The gene cluster of *P. fluorescens* Q2-87 spans from 414 - 6800. The PCR generated fragments produced with primer pairs phl410-2461 and phl4325-7071, were of the expected size based on sequence data from *P. fluorescens* Q2-87 (Acc. no. U41818) of ~2000 bp and ~2700 bp respectively (Figure 3.2 & 3.3). This confirms that primers phl410 and phl7071 can successfully anneal to the correct location of the *P. fluorescens* FI13 genomic template.

Enzyme selection
*Taq2000™* (Stratagene), was the polymerase of choice for PCR generated products using primers phl410-2461 and phl4325-7071. No PCR products were detected when this enzyme was used for the 6.7-kb amplification of the *P. fluorescens* FI13 genome with primer pair phl410-7071. *TaqPlus Long™* (Stratagene) proved successful for this amplification (Figure 4.1). To improve fidelity and yield of the amplification product new enzyme formulations were used. *Taq Precision™* (Stratagene) enabled the amplification of the *P. fluorescens* FI13 genome, as did *Taq Expand High Fidelity™* (Roche).
Figure 4.1 Lane 2 shows the 6.7-kb PCR fragment generated from the template of *P. fluorescens* F113 using primer pair phl410 and phl7071. These primers were chosen as they encompass the entire coding region of the *Phl* biosynthetic locus of *P. fluorescens* Q2-87. Thermostable DNA polymerase, *TaqPlus* Long™ (Stratagene) with additional optimisation was used for this successful amplification. Gel shows λ HinII marker in lane 1.

*Taq Expand High Fidelity*™ (Roche) increased the yield of the PCR product under exactly the same optimisation conditions compared to *TaqPlus* Long™ (Stratagene).

Using an enzyme formulation containing 100% proof-reading polymerase, *Pfu Turbo*™ (Stratagene) resulted in a failed PCR amplification. There was no success throughout the entire range of PCR optimisation.

**Rapid cycling**

It was possible to amplify the 2.7-kb fragment using *Taq2000*™ and primer pair phl4325-7071 using 0.5 ml PCR tubes and a BioRad thermocycler. Amplification of the 6.7-kb fragment was unsuccessful using this equipment, despite the entire range of PCR optimisation.
Altering the equipment to a Perkin-Elmer GeneAmp™ PCR System 2400 or 9600, using thin-walled, 0.2 ml PCR tubes did result in PCR amplification of the 6.7-kb fragment.

**Buffers**
Buffer conditions of each reaction were systematically manipulated for all enzymes and both PCR thermocyclers used. Only buffers at pH 9.2 resulted in a successful, 6.7-kb PCR product. The MgCl₂ and KCl concentrations were ideal at 3.5 mM and 25 mM respectively. Altering MgCl₂ concentrations to 1.5 mM still resulted in the generation of the 6.7-kb product; however, the reproducibility of the reaction was diminished. Lowering the MgCl₂ concentration led to non-specific bands and occasional total failure of the PCR reaction.

The KCl concentration appears critical, as increasing the concentration to 75 mM resulted in no PCR product, even with optimal pH 9.2 and MgCl₂ at 3.5 mM.

**Thermocycling conditions**
Using standard PCR cycling conditions, (initial denaturation 94°C for 2 min followed by 30 cycles of denaturation at 94°C for 30 sec, anneal at 62°C for 2 min and extension for 2.5 min at 72°C, followed by a final extension time of 7 min at 72°C), used to generate 2.7-kb fragment from *P. fluorescens* FI13 genome resulted in no PCR fragment when used to generate the 6.7-kb *P. fluorescens* FI13 fragment.

Addition of an initial step at 80°C for 1 min to replace the 2 min initial step at 94°C was essential to generate the 6.7-kb product. Decreasing the denaturation times at the beginning of each cycle from 30 sec to 10 sec was also necessary to generation of the product.

With an extension-time of 2.5 min per cycle there was no generation of the desired product. Increasing to 4.5 min was necessary to generate the product. Above 6 min extension-time, again no product was detected. The final extension time was
increased to 10 min, however there was no reduction in product yield from the usual final extension time of 7 min.

**Reaction volume**

Reaction volumes <25 μl were required when generating the 6.7-kb fragment. 50–100 μl volumes were suitable for amplifying the 2.7-kb Phi fragment under identical reaction conditions.

**Adjuncts**

It was necessary to add 15% glycerol to the reaction mix to amplify the 6.7-kb fragment. Addition of dimethylsulfoxide (DMSO), formamide, betaine or bovine serum albumin resulted in no PCR product being generated.

**DNA**

Only purified DNA templates led to the generation of the 6.7-kb fragment. Genomic DNA was purified as described in the method. DNA concentrations greater than 100 ng led to reproducible amplification of the 6.7-kb product. Lower DNA concentrations occasionally led to amplification of the 6.7-kb fragment, but this was not always reproducible.

DNA quality or quantity was not important when amplifying the 2.7-kb fragment from the *P. fluorescens* F113 genome, as even colony stabs into the reaction mix could act as a suitable template.

**4.5. RESULTS OF SUICIDE VECTOR CONSTRUCTS**

pMC1871 was selected as a suicide vector as its origin of replication was incompatible in *P. fluorescens* SBW25. A restriction map revealed a suitable site for cloning within a *lacZ* gene. This enabled approximately 1 kb of *lacZ* to flank the cloned PCR product following cloning. This *lacZ* region would facilitate homologous recombination into the *lacZY* marked SBW25 genome. The stages of the construction of pMCPhl9 (Figure 4.2).
Figure 4.2 The stages leading to the construction of pMCPhl9. Plasmid pMC1871 (Pharmacia) was digested with restriction enzyme EcoR1361I located centrally within the promoterless lacZ. Using primer pair phi410 and phi7071, a 6.7-kb fragment was produced from a PCR reaction from strain \textit{P. fluorescens} F113. Blunt-ended ligation was used to produce the 14165-kb vector, pMCPhl9.
The 6.7-kb PCR fragment was successfully cloned into the 7.5-kb pMC1871 vector following optimisation of the ligation conditions. Ligation at 22°C for between 2 and 16 hr using Pharmacia, Ready-to-go ligation mix. Vector to insert ratios of 1:200 were also required. Lowering insert concentration resulted in no suitable clones.

Through the entire ligation optimisation no clones were constructed when digested DNA or PCR generated DNA was purified from ethidium bromide gels. Purification from crystal violet gels was essential for constructs to be detected following ligation under optimum conditions.

Selection of the constructed plasmid was achieved by screening of total DNA. Figure 4.3 shows a typical gel with lane 11 showing a candidate colony with a larger plasmid. Following plasmid purification a 14-kb plasmid was observed (Figure 4.4). Primers were designed either side of the Eel 13611 site, on pMC1871, to confirm the insertion of the PCR generated Phi locus from F113, and show its orientation within the suicide vector (Figure 4.5)
**Figure 4.3.** Screening method employed to detect candidate colonies containing plasmids exceeding 10-kb. Lanes 1 to 14 show the total DNA of 14 colonies selected following transformation with ligation mix pMC1871 and the PCR generated Phi fragment. Lane 11 indicates a colony with a plasmid band in the region exceeding 10-kb, and considerably larger than the plasmids in the remaining colonies on this gel. Colony 11 was selected for closer plasmid analysis. Marker λ HindIII.
Figure 4.4 Lane 1, the undigested pMCPhl9 plasmid isolated from initial rapid screening. Lane 2, Linearised plasmid pMCPhl9 digested with restriction endonuclease, Ecl136I at a unique site. (Lane 3, 2x dilution of the undigested plasmid in lane 1).
Figure 4.5 Gels to show the result of primers against plasmid pMCPhl9 (lane 1 on both gels A and B) and plasmid pMC1871 (lane 2 on both gels).

Gel A shows PCR products produced from the reaction using primer phl4325, designed to anneal within the PCR generated insert, and primer pMCB, designed to anneal within the lacZ site of pMC1871, adjacent to the Ecl136I cloning site.

Gel B shows PCR products from the reaction using primer phl4325, designed to anneal to the PCR generated fragment, and primer pMCF, designed to anneal on the opposite side of the Ecl136I cloning site with respect to the pMCB primer.

The results from gel A confirm that the PCR generated products have been cloned in to pMC1871, indicated by the positive PCR reaction using primers pMCB and phl4325. The size of this PCR product is predicted at ~2.7-kb. The negative PCR reaction in the pMC1871 that does not contain the PhtI biosynthetic locus confirms the positive result. The orientation of the PhtI biosynthetic locus can be determined from these findings. Region 4325 of the PhtI insert lies ~2.7-kb from the site where the pMCB primer was designed to anneals on pMCPhl9.
Construction of the repressor negative construct is shown (Figure 4.6). *Ecl136II* was identified as the only restriction enzyme with a unique site within the repressor gene of the cloned *Phl*, PCR, fragment. Restriction digestion at this site revealed a single 14 kb band supporting the presence of the predicted unique site (Figure 4.4). A unique site was sought that could be used to cut the repressor from the pMCPhl9 plasmid, however no suitable site was present within the flanking *lacZ* region essential for the homologous recombination into SBW25. A *DraIII* site was expected to be unique and could be cut together with *Ecl136II*, to remove the entire *Phl*, PCR generated, fragment minus the repressor. Digestion of pMCPhl9 with *DraIII* resulted in multiple bands, indicating the presence of an additional, unexpected *DraIII* site located within the *Phl* biosynthetic cluster generated by PCR.
Figure 4.6 The stages leading to the construct of pMCPhl9. Plasmid, pMCPhl9 was digested with restriction endonuclease Ecl13611 and HpaI. The resulting fragments were cloned into an Ecl13611, HpaI digested, pMC1871 plasmid, resulting in a 13.3-kb, Phi repressor negative construct.

The two similar sized products from the initial pMCPhl9 digest are shown schematically in the diagram. No separation of these two fragments was attempted prior to ligation to the pMC1871-digested plasmid. Only the desired fragment (containing the Phi locus) would be stable once cloned into pMC1871, as the remaining fragment contains an additional, pMC1871 origin of replication.

Re-circularisation of the pMCPhl9, HpaI and Ecl13611 was prevented by removal of terminal phosphate groups.
As restriction mapping revealed no alternative unique sites, \textit{HpaI} was selected for restriction digest (Figure 4.7). Digestion of pMCPhl9 with \textit{Ecl136II} and \textit{HpaI} gives fragment sizes of 6848 bp and 6693 bp. The ethidium bromide gel photo shows the bands generated following this digest. For the purposes of cloning the fragments were purified from a crystal violet gel, however due to the poor resolution of this gel the bands could not be distinguished.

![Figure 4.7](image)

\textbf{Figure 4.7} Results of restriction digest of plasmid pMCPhl9 with restriction endonuclease \textit{HpaI} and \textit{Ecl136II}. The gel shows two bands \textasciitilde6.6-kb and \textasciitilde6.6-kb. Sequence prediction taken from pMCPhl9 indicates that the \textit{Phi} gene cluster is located in the smaller of the two bands i.e the band approximately equal to the 6557 band of the Lambda HindIII marker.

Together the two bands were purified from the crystal violet gel and a ligation reaction was performed with pMC1871, digested with \textit{Ecl136II} and \textit{HpaI} (5966 bp), to generate clones predicted to be 12814 and 12659 bp in size. Ten clones were selected for rapid lysis and confirmation of the presence of a 13 kb plasmid. These plasmids were annealed to primers targeted against the \textit{Phi} genes within the pMC1871. Eight of the ten clones were shown to contain the \textit{Phi} gene cluster shown
by bands being generated of the predicted size. These clones were taken for further analysis and named pPhl1r', pPhl2r' to pPhl8r'.

4.6. RESULTS OF SBW25 TRANSFORMATION

SBW25 strain PA2, marked with lacZY, Km' and xylE, were electroporated with the suicide, delivery plasmid, pMCPhl9. Colonies were selected on the ability to grow on Tc, Km and turn blue on X-gal amended LB plates.

Successful recovery of Tc' transformants was only possible when electroporating with 5 μg of plasmid DNA. Lower DNA concentrations resulted in no detectable colonies. A range of 10 ng, 100 ng and 1 μg were chosen initially following initially unsuccessful transformation with ~1ng of plasmid DNA. Increasing the plasmid concentration to 1 μg also resulted in no Tc' colonies. A decision was made to attempt the transformation by electroporation with the entire plasmid mix of 5 μg. The resulting transformation resulted in the presence of eleven colonies that exhibited the Tc' phenotype. The Nine colonies grew following transformation with pMCPhl9, all exhibiting a Tc', LacZY+ and Km' phenotype. Colonies 1, 3, 4, 6, 8 and 10, all appeared morphologically identical to the wild-type marked strain Pa2. Colonies 2, 5, 7, 9 and 11 all had morphology different to the parent strain, but identical to each other. These observations were based on colony size, morphology of the colony edges and surface texture. One colony from each of the morphological groups was chosen for further analysis, colony Pa21 and Pa25. Figure 4.8a and Figure 4.8b show the morphological change that has occurred on the surface of transformants Pa25 in respect to Pa21, which remains identical to the marked wild type, Pa2.

Amplification of genomic DNA from these colonies with PCR primers designed to anneal to the Phl genes resulted in positive bands in all the transformants tested (Figure 4.9).
**Figure 4.8a** Morphological changes of the transformants Pa25 (left) and Pa21 (right). Both have been derived from transformation of SBW25. Pa2, with pMCPhl9, containing the full biosynthetic locus for Phl production.

**Figure 4.8b** The same plates photographed as above, contrasting background to emphasise the colony changes seen at the edges of the individual colonies.
Figure 4.9 Amplification of DNA from SBW25, Pa2, transformed with plasmid pMCPhl9. PCR primers phl4325-7071, designed to anneal within the \textit{Phi} biosynthetic locus of plasmid pMCPhl9.

Lane 1: PCR reaction using F113 genomic template, +ve band at 2.7-kb
Lane 2 -9: PCR reaction using transformed SBW25, Pa21, Pa22, Pa23, Pa24, Pa25, Pa26, Pa27, Pa28, +ve band at 2.7-kb
Lane 10 & 11: PCR reaction using plasmid pMCPhl9, +ve band at 2.7-kb
Lane 12 & 13: PCR reaction using wild-type SBW25, no +ve band at 2.7-kb
M: lambda \textit{HindIII} marker
SBW25 marked with lacZY and Km', were transformed with the suicide plasmid pMCPhl'r', the Phl repressor negative construct. Again, 5 μg of plasmid DNA per electroporation was required for detectable transformation frequencies. Following the transformation, six colonies were selected based on Tc'/ Km' phenotype. These were amplified with primers directed against Phl coding genes. Colony 4 produced a positive band corresponding to Phl. This was designated as Pa24r', and used for further fitness experiments.

**Double homologous recombination**

Colony Pa21 and Pa25 were cultured for 4 days in LB at 20°C in 250 ml shaking flasks. The flasks contained no antibiotic amendment. The culture was serially diluted and spread on X-gal amended LB plates. Of ~100,000 colonies plated, all maintained blue colouration with no white colonies detectable. Pa25 and Pa21 all maintained their distinct morphological appearance. Fifty colonies of each were picked and spotted onto Tc' amended plates and all showed a Tc' phenotype.

No double homologous recombination was detected. All 50, of the 50 colonies selected still contained the Tc' phenotype therefore there were no revertants sampled. These would result from the complete loss of the plasmid pMCPhl9 under the non-selective growth conditions. Loss of the entire plasmid would result in both the Tc' gene and the Phl leaving the bacteria.

The presence of white colonies would indicate the double integration event, within the lacZY locus of the SBW25 chromosome, causing insertion mutagenesis and disruption of the blue phenotype. This would be the result of the loss of only the Tc' backbone of the pMCPhl9.

As 100,000 colonies were plated all of which were still blue, and Tc', an assumption can be made that the probability of the second recombination event is greater than 1 in $10^5$ transformations from single recombinant to double recombinant. Of the 50 colonies tested, all still maintained the single integration of pMCPhl9.
4.7. **RESULTS OF PLATE BIOASSAYS**

Plate bioassays of strains Pa21, Pa25 and Pa24r', on potato dextrose agar (PDA) against the fungal pathogen *P. ultimum* resulted in inhibition. This was a qualitative observation made against biological control inhibition of the SBW25, strain Pa2, not transformed with the *Phi* gene cluster (Figure 4.10).

The degree of inhibition is seen to be lower than that for the natural producer F113, on the PDA plates grown at 20°C.
4.8. **RESULTS OF HPLC ANALYSIS**

Following growth at 20°C for 4 days in TSB, growth medium from cultures containing strain F1130P, F113, Pa25 and Pa21 were extracted and detection of Phi was performed by HPLC. The trace (Figure 4.11) show the results of the detection. A spiked sample was added to compare peak separation times for the Phi in this HPLC system (retention time (rt) = 11 min). The results show that F1130P has produced the greatest quantity of Phi under these conditions, with Pa21 and F113 producing equally low quantities of Phi as each other. Strain Pa25 produces more detectable Phi under these growth conditions than Pa21 and F113, as indicated by the larger peak area and height of the peak (rt = 11 min).
Figure 4.11 HPLC traces of F113OP, F113 (green and blue respectively). This is compared to a sample of TSA media extracted and spiked with synthetic Phl (Red). Extracts of medium from Pa21 and Pa25 are shown by the black and pink traces respectively. All samples were grown in TSB, 20°C, and absorbency measures at $\lambda_{270}$.
4.9. Discussion

Long high fidelity PCR

The successful amplification of a 6.7-kb fragment direct from the genome of *P. fluorescens* F113 has highlighted the potential that Long PCR has in broadening its application away from well characterised templates. This paper has highlighted how a systematic approach can highlight and overcome difficulties when extending the use of optimised PCR to poorly characterised templates.

A considerable problem in this type of study is the lack of suitable controls and the lack of positive information that can be gained from a negative result. A positive amplification reaction on one template gives little information as to the conditions that will be suitable on a different template.

For this reason the control information for this study has come from PCR amplification on the same template. The controls were conventional PCR reactions of 2 kb and 2.7-kb amplifications covering the left and right sides of the 6.7-kb *Phl* cluster aimed at being amplified.

Primer design is important in any PCR reaction. For this reaction, two oligonucleotide primers were designed that flanked the entire *Phl* coding region of *P. fluorescens* F113. Two primers, phl410 and phl7071 flanked the *Phl* coding region for *P. fluorescens* F113 and following conventional PCR showed that acting together with primers phl2461 and phl4325, respectively, would lead to the generation of amplified DNA within the gene cluster of interest. This finding confirmed that the oligonucleotides, phl410 and phl7071, together, could act as primers for the amplification of the entire 6.7-kb region between them.

The thermostable polymerase *Taq2000™* (Stratagene) was able to amplify the control fragments, but when attempting to amplify the 6.7-kb fragment no product resulted. Using *TaqPlus Long™* (Stratagene) a mix of *Taq2000™* and a thermostable polymerase with 3'-5' exonuclease activity, *Pfu* polymerase, resulted in the successful amplification of the 6.7-kb cluster.
The limitation of the non-proof-reading polymerase Taq2000 on amplifying the 6.7-kb fragment is believed to be a mis-matching of nucleotides as it amplifies along a strand of DNA. Over 2-4 kb, the enzyme makes few mistakes and the reaction are able to continue along the entire length, allowing for the complete fragment to be amplified. Over longer distances the enzyme mis-matches a nucleotide and has no means to repair the error and the PCR reaction is stopped. Adding a proof-reading enzyme to the reaction allows the mistaken nucleotide to be removed by the exonuclease activity, and a correct nucleotide put in its place thus allowing the reaction to continue.

When *Pfu* alone was used in the reaction no product resulted. The reason for this could be the slow progress this polymerase makes along the template strand. The high temperatures, inherent in the PCR reaction, degrade and denature many of the components in the reaction mix. The longer it takes a polymerase to travel along a template the greater the chance that either the template becomes degraded or the primers thus halting the reaction.

The PCR equipment plays an important role in the successful amplification of long pieces of DNA. The control reaction could be successfully amplified in thick walled PCR tubes in reaction volumes up to at least 100 µl. The 6.7-kb fragment could only be amplified in 0.2 ml thin walled PCR tubes in a thermocycler that can switch between temperatures rapidly during the PCR reaction. Reaction volumes also had to be kept below 25 µl for reproducible, successful amplification.

The rapid transfer between temperatures ensures that the reaction mix is not subjected to high temperatures longer than the pre-programmed time. The high temperatures involved in the PCR reaction have already been mentioned in degrading templates and primers. Reducing the time spent at high temperatures shall maintain the concentration and integrity of these two components. With three temperature changes per amplification cycle, the rapid temperature transfer throughout 25 to 30 cycles results in a large overall reduction in time spent at temperatures exceeding 63°C during the entire reaction.
Reducing the volume in the tubes seems important for similar reasons. The lower volume allows rapid temperature changes to occur in the reaction tubes.

The pH of the reaction was optimised at a high pH, pH 9.2. This is believed to be important as it prevents the depurination of the template at high temperatures. Chu et al., 1986, showed that Taq DNA polymerase stops at sites of depurination. In vivo in E. coli, unexcised apurinic sites are thought to be lethal as the DNA polymerase cannot cross them (Brewer et al., 1990). Barnes, 1994, noted the importance in maintaining a high pH at denaturation temperatures. A Tris buffer of pH 8.55 at 35°C altered to a pH of 6.45 at 95°C. As the pH of the Tris buffer drops with increase in temperature, there is an increase chance of depurination taking place. It is therefore important to have a buffer at high enough pH at 25°C to ensure the repeated exposure to high temperatures in the PCR reaction shall not lead to excess depurination and therefore premature termination of the polymerising reaction.

The conditions of the reaction were further optimised to favour the generation of the long product. The denaturation times for the reaction were decreased so preventing degradation of the template, polymerase and primers. The addition of glycerol reduces the melting temperature of the DNA thus allowing the lowering denaturation temperatures. The glycerol also has the effect of stabilising the polymerase enzyme. The addition of large amounts of template DNA, 150 ng per 25 μl reaction, also appeared necessary due to the template’s suspected gradual degradation during the long periods at high temperatures. The extension time of the reaction was increased to allow time for the polymerase to travel the length of the desired fragment of DNA.
Electroporation

The limiting factor to the success of plasmid integration into the host, SBW25, chromosome is the frequency with which the DNA enters the host cell and the frequency of integration into the genome.

During this investigation attempts have been made to optimise where possible the probability for integration into the SBW25 chromosome. The larger the region of homology between the plasmid and the genomic DNA, the greater the frequency for integration. Plasmids pMCPhl9 and pMCPhlr', were both designed to contain a ~1000 bp lacZ region of homology, to the SBW25 lacZY locus, flanking each side of the cloned Phi insert.

The size of this lacZ, flanking region of homology, was considered adequate based on evidence from allelic exchange in Y. enterocolitica (Kaniga et al., 1991). Flanking regions of 1 kb and 1.5-kb were used to perform allelic exchange to insert a luxAB cassette. Frequency of transformation was between $10^{-4}$ and $10^{-6}$ per μg of DNA.

During this investigation it was considered that the 2 kb, lacZ region that flanks the Phi loci, was adequate for a frequency of insertion that could be detectable. The limiting factor for detection of transformants is, therefore, the frequency of integration of the DNA into the host cell.

Powell et al. (1993) transformed SBW25 with the Tc' gene by homologous recombination. The homologous regions that flanked the Tc' locus totalled 4 kb. The frequency of the transformation/integration of this fragment was 1 in $10^7$ recipients per μg of DNA.

Artiguenave et al. (1997) investigated the parameters for optimising transposon mutagenesis by electroporation of a P. fluorescens. They indicated that the transformation frequency of the plasmid carrying the Tn5 Km' gene, was approximately $10^3$-$10^4$ times the transposition frequency (frequency of insertion into
They were able to achieve transposition frequencies of $10^5$ per $\mu$g of plasmid (7.5-kb) DNA.

It is expected that the frequency of integration would be lower in the case of the integration of the $\Phi l$ locus flanked by 2-3 kb flanking region than the transformation performed by Powell et al., 1993. Also the integration frequency can be expected to be lower than that seen for the transposition frequency achieved by Artiguenave et al. (1997). This is due to transposons containing genetic elements (insertion sequences) that increase the frequency with which they integrate into bacterial genomes.

Two approaches were examined for high efficiency transformation. Chemical treatment of the SBW25 with DMSO to make the cells susceptible to heat shock and secondly, preparation for electroporation. It was seen that chemical transformation was not successful for obtaining transformants with integrated pMCPhl9 or pMCPhlr'. Preparing cells for electroporation was successful when large amounts of plasmid DNA were used for the transformation. In the order of 5 $\mu$g of DNA was required per reaction for transformation/integration of the plasmid at detectable levels.

Transformation frequency is calculated based on the number of transformation events per $\mu$g of DNA. Standard protocols for transformation of $E. coli$ by electroporation suggest the use of small amounts of DNA (1-10ng) to achieve the highest frequency of transformation. In these cases, $10^4$ transformants will be representative of a transformation frequency of $10^7$-$10^8$ transformants per $\mu$g of DNA. However increasing the DNA concentration to 1 $\mu$g for the transformation reaction will usually result in larger numbers of transformants, such as $10^5$, but the overall frequency of transformation is reduced from $10^7/10^8$, to $10^5$ transformations per $\mu$g DNA. In terms of transformation frequency the efficiency is likely to be reduced when transforming with larger quantities of DNA, however the actual numbers of colonies that are transformed is increased. This is only true whilst the DNA is below toxic levels for the target cells.
This theory is important when attempting to transform cells that have a relatively low competence for transformation with DNA. If transformation frequency falls below $10^4$ colonies per $\mu$g of DNA, then $<10$ colonies would be expected with DNA concentrations of 1-10 ng per transformation. To increase the number of recoverable transformants to detectable levels the DNA concentration must be increased, reducing the overall efficiency, but increasing the number of colonies that are transformed. In this case, $5 \mu$g was the lowest amount of DNA required to achieve detectable transformation.

In the case of this transformation, not only is the frequency of SBW25 transformation important, but also the subsequent frequency of plasmid integration into the SBW25 chromosome. Here, the transformation frequency resulted in $\sim$10 colonies, following transformation with $5 \mu$g of DNA. This represents a transformation/integration frequency of 2 colonies per $\mu$g DNA. This is indicative of a high transformation frequency, followed by an additional low integration frequency. Obviously DNA concentrations of up to 5 $\mu$g are not toxic to SBW25 in this electroporation protocol.

There were two additional compounding factors connected with the low efficiency of transformation/integration seen when plasmids pMCPhl9 and pMCPhlr were used to transform SBW25. The first was, the circularised plasmid used for the transformation, and the second problem was the large size of the plasmids used for the transformation. The plasmid size has a critical effect on the transformation efficiency of electroporation into *P. fluorescens* (Artiguenave *et al.*, 1997). It was found that the bigger the plasmid, the lower the transformation frequency. For the plasmid used in the Artiguenave study, a 7.5-kb resulted in a transformation frequency of $2.6 \times 10^7$ transformants per $\mu$g of DNA, whereas a 48-kb plasmid resulted in only $\sim 1 \times 10^4$ transformants per $\mu$g of plasmid DNA.

Electroporation of linearised DNA was the method chosen for the transformation of the SBW25 with Tc$^r$ and Km$^r$ genes (Powell *et al.*, 1993; Bailey *et al.*, 1995). This is believed to increase the frequency of transformation into the genome by homologous recombination through reduction of the stearic hindrance seen with circularised
plasmid DNA. Coiled or super-coiled plasmid is thought to 'hide' regions of homology within the coiled structure thus not presenting regions of homology as openly as when the plasmid has been linearised.

This method can only be used if the genes inserted can provide a mechanism of positive selection and is therefore often used when antibiotic resistance genes or heavy metal resistance genes are being inserted. Selection can then be aided through positive selection of this trait with elimination of non-resistant strains on appropriate selective media. This was the case with the introduction of the Km' and Tc' genes to SBW25 by Bailey et al. (1995) and Powell et al. (1993).

In this investigation the Phi insert does not exhibit a phenotype that can be positively selected on appropriate selective media. The addition of the Tc' on the suicide vector backbone was necessary in order to achieve the positive selection.

This formed the rational behind the 2-step homologous recombination method that was employed. The first step involved the single cross over of one flanking region with its corresponding region of homology on the chromosome of the SBW25. The circularised plasmid therefore remains attached to the chromosome by this flanking region. The genes contained on the plasmid can still be expressed within the host genome due to association with the chromosomal DNA. The Tc' gene on the backbone of the integrated plasmids pMCPhl9 and pMCPhlr' are expressed only once the single cross over with the host genome has occurred.

Without any integration, no Tc' is noted, as both plasmids are unable to replicate and survive due to having a non-compatible origin of replication in SBW25.
Morphological changes between transformants

The morphological change that occurred following transformation/integration of the pMCPhl9, containing the entire Phi locus was unexpected.

The insertion was designed to occur within the lacZY region of the SBW25, Pa2, chromosome. This region was homologous with a large 2 kb, lacZ region, on both the suicide plasmids, pMCPhl9 and pMCPhl9'.

It was evident, however, following the morphological differences between the pMCPhl9 transformants that the site of insertion is not the same in Pa21 and Pa25. It would be interesting to determine the sites of insertion using southern blotting to discover if the lacZY region in SBW25, Pa2, does act as a region of homology for the insertion of the suicide vector pMCPhl9. If the lacZY region does show integration then it would be interesting to determine at which point on the lacZ cluster the single cross over has taken place. Figure 4.12 shows the theoretical possibilities for integration of the pMCPhl9 into the lacZ region of SBW25 chromosome.
Figure 4.12 Schematic representation shows the integration of pMCPhl9 with the SBW25 chromosome. Curved blue arrows represent the point of cross over between homologous regions of pMCPhl9 and Pa2 at point A. Curved pink arrows show the area of cross over at respective regions B of pMCPhl9 and Pa2. Straight direction arrows show the direction of SBW25, Pa2, lacZY, promoter activity on the pMCPhl9 plasmid.

Single homologous recombination at site A would result in a disruption of the lacZ gene and the resultant transformant would become white with Tc' phenotype. The disruption occurs through the first read through from the lacZ promoter, through the Phi insert. The second read through is not disrupted through the lacZ on the plasmid backbone through the chromosomal lac operon, however the plasmid carries no lac promoter and therefore there will be not expression of lacZ. Cross over can theoretically occur at the same frequency at site B, where the lacZ will read through the promoter, and result in blue colonies which had a Tc' phenotype.

Knowing the site of insertion in this case would aid in the explanation of whether the site of insertion was the lacZY of the SBW25 site as designed; and if so, whether there is a lethal effect following integration at site A and hence no white colonies detected.

There still remains the unresolved peculiarity seen when an identical transformation of SBW25 is performed with the repressor negative pMCPhl' suicide plasmid. In this case none of the transformants show the altered morphology as seen with Pa25.
Through southern blot hybridisation, a comparison between the sites of insertion of the \( \Phi h \) cluster in Pa21, Pa25 and Pa24\(^r\) can be made. It is of considerable interest to determine whether there is another region of homology on the SBW25 chromosome that can account for the 50:50 split in morphology between for Pa21 and Pa25. From the amplification of SBW25, Pa2 DNA, using primer pairs designed to anneal adjacent to \( \text{phlF} \) and within \( \text{phlC} \) [\( \text{phl4325} \) and \( \text{phl7071} \) (schematic page *9)] of the \( \Phi h \) cluster, it was noted in, Figure 4.9, that the DNA from the parent SBW25, Pa2 and the DNA from transformants Pa21 and Pa25 all formed a 1 kb PCR product. As the primers were designed to anneal against the \( \Phi h \) locus it may suggest a region of the SBW25 chromosome that shows some homology to a region of the cloned \( \Phi h \) locus and therefore an additional site for homologous integration aside from the \( \text{lacZY} \) between the suicide vector backbone and Pa2 chromosome. There may be a region between the \( \Phi h \) insert and the Pa2 chromosome also.

Previous amplifications of the Pa2 chromosome, with primer pairs designed to anneal at \( \text{phlE} \) and \( \text{phlD} \) resulted in no PCR product (chapter 3). This would suggest either an evolutionary fragment of the \( \Phi h \) cluster or a related gene of similar homology as found in the \( \Phi h \) cluster. It would be interesting to determine if the region of homology is the repressor element itself. This region possesses a helix-turn-helix as described by Delany \textit{et al.} (2000), which is a conserved DNA binding motif. As such, there is a possibility that the genetic sequence is also highly conserved and that there are more than one DNA binding motif coding sequence expressed in SBW25 for the transcription of many other DNA binding regulatory proteins.

The insertion of the repressor, \( \text{phlF} \) negative construct to form, Pa24\(^r\), led to no morphology differences between transformant.

The change in morphology may be caused by the presence of the repressor. This may be to either the direct action of the repressor in SBW25, or more likely that it is causing the suicide plasmid to integrate at another site on the SBW25, Pa2 chromosome. This is seen with Pa21 and Pa25 having identical DNA added, however transformants having different morphologies. It was discussed that there may be two distinct sites for insertion into the Pa2, through homology with possibly the \( \text{lacZ} \) on
the backbone of pMCPhl9 and pMCPhlr' and possibly the phlF gene on the pMCPhl9. In order to find a common factor the sites for the insertion in Pa21, Pa25 and Pa24r' need to be determined by southern blotting.

The morphological changes that have taken place are of interest in themselves. It appears that surface changes have taken place as shown in Figures 4.8a and b. This may be important when considering the colonisation potential of these organisms in the rhizosphere. The polysaccharide coat of the soil bacterium provides the adhesion for fixing to plant roots and aggregating into micro colonies. This mechanism enables high bacterial densities to accumulate on plant roots, which allow effective biological control to occur. There is evidence that that bacterial population densities on roots are positively correlated to the ability to suppress disease in pathogen infected soil (Dekkers et al., 1998; Simons et al., 1996; Raaijmakers et al., 1999). It is of interest to see what the change in morphology has on the relative fitness of the organism in planta.
5. CHAPTER 5
5.1. **SUMMARY OF RESULTS**

The biosynthetic gene cluster responsible for the production of 2,4-diacetylphloroglucinol (Phi) was used to transform *Pseudomonas fluorescens*, SBW25BeZY-6KX, Pa2, to produce recombinant strains, Pa21, Pa25 and Pa24r' (*Phi* locus minus repressor gene). The growth characteristics of the strains Pa21 and Pa25 were altered in comparison to the parent strain Pa2 and the modified Pa24r' in liquid broth cultures, especially at higher temperatures of 30°C. Additionally, morphological changes were apparent in strain Pa25 in comparison to the wild-type strain. The level of inhibition of *P. ultimum* by all three modified strains was low on PDA bioassay plates in comparison to the natural Phi producer, F113, from where the Phi genes were originally isolated. *In planta* the colonising advantage of the modified strain with morphological changes, Pa25, was lost in comparison to the wild type, Pa2 on the pea plant roots and rhizosphere soil. The morphologically identical transformant, Pa21, maintained the colonising advantage in the root system, but colonised the soil poorly in comparison to the wild type, Pa2, control. Transformed SBW25 with the *Phi* locus, minus the repressor element, Pa24r' may have led to a lower emergence of plants, similar to that seen with F113, the natural producer, however this would need further investigation to conclude a statistical certainty. Transformants Pa21 and Pa25 had no deleterious effects on plant growth or development.
5.2. **INTRODUCTION**

Biological control as an economically viable addition to crop pest control is currently attracting major research interest. The environmental, economic and functional capabilities of traditional chemical fungicides and fumigants are currently being questioned. Regulators and public alike have expressed concern as to the levels of chemical protection applied to crops, as have farmers as to the economic cost of fungicide application.

It has emerged over the past five years that biological alternatives are viable for the protection of certain economically important crops. Some members of the family of fluorescent pseudomonads has revealed properties that have shown they can protect pea, sugar beet, tobacco, cucumber and tomato crops from a major fungal plant pathogen *Pythium ultimum*. This fungus is a major cause of damping off, a seedling disease that can greatly reduce the yield of infected crops. *Pseudomonas fluorescens* strains Q2-87, CHA0 and F113 have all been shown to produce the fungicide 2,4-diacetylphloroglucinol (Phl) which has been directly linked to the inhibition of the plant pathogen *P. ultimum*.

The *P. fluorescens* strain SBW25 isolated from the phyllosphere of sugar beet, has also been shown to protect pea plants from the pathogen *P. ultimum*, although it does not produce Phl or any secondary metabolites that can be attributed to this effect. Protection is attributed to the strong colonising ability of this organism on the roots of the plants, successfully competing against the pathogenic fungus.

One of the most promising approaches to delivering effective biological control agents is to enhance the natural biological control capabilities of an isolated organism with genetically introduced method of protection. *P. fluorescens* strains F113, Q2-87 and CHA0, all effect their biological control capability through production of the secondary metabolite, Phl. *P. fluorescens* SBW25, effects its biological control behavior through competitive exclusion of the pathogen *in planta*. This study has investigated the effect of integrating two distinct methods of biological control
through the modification of *P. fluorescens* SBW25 to contain the genes responsible for Phi production.

The most promising method for environmental microorganism modification has been chromosomal integration. Genetic material introduced in this way provides the greatest stability of the modification in comparison to a plasmid-based modification. With environmental release there is rarely a suitable selection pressure on the organism to maintain the plasmid.

Integration of genetic material has been previously shown to reduce the fitness of modified organisms through increasing genetic load and insertion-disruption of functional genes (De Leij *et al.*, 1998). This has been recorded in the *P. fluorescens* SBW25 strain marked with genetic markers randomly inserted into the chromosome. Even with this reduction in fitness, the marked *P. fluorescens* SBW25 still maintains its competitive colonisation advantage with respect to the natural Phi producers (Naseby and Lynch, 1998).

The targeted integration into the region of this organism’s chromosome, known not to effect the overall fitness, is the preferred approach when introducing genetic material. This can increase the stability of the insertion in comparison to a plasmid-based modification and allow the researcher to predict the site of insertion and therefore predict the effect on the organism that may result. The technique of homologous recombination can deliver genes to a known site within the organism’s chromosome by flanking the genes for insertion with a region of DNA homologous to that within the organism’s chromosome. The homologous regions will recombine, and the genetic material introduced shall be translated as a continuous piece of the organism’s own chromosome.

As part of a responsible research strategy following the genetic modification to *P. fluorescens* SBW25, the ecological fitness and perturbations *in planta* to both the plant and resident microbial communities were investigated.
5.3. **MATERIALS AND METHODS**

**Bacterial strains, plasmids and growth conditions**

The strains used in this study are shown in Table 5.1. All strains were maintained on TS Broth/agar (Oxoid) and grown at 30°C. Where appropriate, colonies were supplemented with the antibiotics kanamycin (Km) 50 mg ml\(^{-1}\) and tetracycline (Tc) 25 \(\mu\)g ml\(^{-1}\).

<table>
<thead>
<tr>
<th>Bacterial strain or plasmid</th>
<th>Relevant characteristics</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>P. fluorescens</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SBW25</td>
<td>Sugar beet leaf isolate</td>
<td>De Leij et al., 1995</td>
</tr>
<tr>
<td>Pa2</td>
<td>Sugar beet leaf isolate with markers lacZY, Km(^{r}) and xylE</td>
<td>Bailey et al., 1995</td>
</tr>
<tr>
<td>Pa21</td>
<td>Transformants containing Phi Locus, Tc(^{r}), lacZY, Km(^{r})</td>
<td>Chapter 4, this study</td>
</tr>
<tr>
<td>Pa25</td>
<td>Transformants containing Phi Locus, Tc(^{r}), lacZY, Km(^{r}) (altered morphology)</td>
<td>Chapter 4, this study</td>
</tr>
<tr>
<td>Pa24(^{r})</td>
<td>Transformants containing Phi locus minus repressor, Tc(^{r}), lacZY, Km(^{r})</td>
<td>Chapter 4, this study</td>
</tr>
<tr>
<td>FI13</td>
<td>lacZY, Km(^{r})</td>
<td>Shanahan et al., 1992</td>
</tr>
</tbody>
</table>

Growth was routinely measured at optical density 550 nm (OD\(_{550}\)). In addition, plating onto LB, monitored the number of culturable cells.

**Growth flask fitness experiment**

Bacterial cultures Pa2, Pa21, Pa24\(^{r}\) and Pa25 were grown to stationary phase (72 hours, 20°C), and 10 \(\mu\)l was used to inoculate 50 ml flasks containing TSB. These were incubated on a shaking incubation tray (180 rpm) at either 20°C or 30°C. Two millilitre of growth culture was taken at frequent time points during log phase and into stationary phase. One millilitre of the sample was used to measure optical density \(\lambda_{550}\) of the culture and the remaining 1 ml was serially diluted and plated for colony counts.
Plant growth fitness experiment

The overnight culture was used to inoculate full strength TSA for 3 days at 30°C. The bacteria were suspended in 10 ml of sterile quarter strength Ringer’s solution using disposable plastic plate spreaders to scrape off the bacterial mat and the colony forming units were determined. Control plates (without bacteria) were also flooded with quarter strength Ringer’s solution and the surface scraped with spreaders. The resulting suspensions containing $6 \times 10^9$ c.f.u / ml were subsequently used to imbibe pea seeds (*Pisium sativum* var. Montana), at a ratio of one seed per ml, for 4 hours (stirred every 30 minutes), resulting in between 2 and $4 \times 10^8$ c.f.u. per seed.

The experimental microcosms were constructed from 210 mm high acetate cylinders, slotted between the top and base of plastic 90 mm diameter Petri dishes creating semi-enclosed microcosms. Each treatment was replicated 5 times and each microcosm consisted of eight imbibed seeds, planted at a depth of approximately 1 cm below the soil surface. The soil was sandy loam of the Holiday Hills series, taken from Merrist Wood Agricultural College (Surrey), and had been under permanent pasture for at least 15 years. The analysis of the soil, conducted at the University of Surrey, was pH 5.4, particle ratio 10:9:81 clay:silt:sand respectively, and organic matter content 1.6% by weight. The total NPK contents by weight were 0.124%, 0.033% and 0.861% respectively.

A volume of 25 ml of water was added to each microcosm before they were placed in a random design into a growth chamber (Vindon Scientific) set at 16 hour photoperiod with a day/night temperature regime of 21°C/15°C respectively. The relative humidity was maintained at 70% and the light intensity was 10,000 lux at shelf level.

Sampling and analysis

After 21 days growth the microcosms were harvested, the following plant measurements were made; the plant shoot and root (wet and dry) weight, number of nodules, length of each root and the number of lateral roots. Rhizosphere soil (closely associated with the plant roots) samples were collected and stored at 4°C. The samples were subsequently used for microbial populations’ counts. One gram of soil from
each replicate was suspended in 9 ml Ringers solution, as was 1 g of mashed root from each replicate. Filamentous fungal populations were quantified by plating a ten fold dilution series of each root macerate or soil suspension onto 10% malt extract agar containing 100 ppm streptomycin and 50 ppm rose bengal. Plates were incubated at 20 °C for 5 days before enumeration. P1 medium (Katoh and Itoh, 1983) was used for the enumeration of root indigenous, fluorescent *Pseudomonas*, plates were incubated at 25°C and enumerated after 5 days growth. To enable quantification of the introduced *P. fluorescens* Pa2, Pa25 and Pa24r*, the medium was amended with X-gal (50 ug/ml). Tryptone soya agar (10%) was used for the enumeration of the culturable bacteria.

**Statistical analysis**

Data were analysed using SPSS for windows (SPSS inc,) by means of a one way ANOVA and subsequently differences between treatments (multiple comparisons) were determined using least significant differences (LSD) between means as the *post hoc* test.
5.4. RESULTS

Growth flask experiment.
Figure 5.1 and 5.2 show growth curves for the bacteria strains Pa2, Pa24r', Pa21 and Pa25 at 30°C and 20°C respectively.

Figure 5.1 Growth of strains Pa2, Pa21 (transformant) and Pa25 (transformant) and Pa24r' (transformant) in TSB, 30°C shaken at 180 rpm.

Growth rate and OD were similar for the Pa24r' strain (*Phl* minus the repressor) with relation to the Pa2, wild-type derived, marked strain. The Pa21, and Pa25 strains, transformed with the entire *Phl* biosynthetic locus were unable to achieve the high optical density seen with the Pa2. Instead the effect of ‘clumping’ was seen, resulting in low OD throughout the culture flasks. Clumping is described as the aggregation of biomass. In each flasks there were many separate clumps that had formed.
Figure 5.2 Growth of strains Pa2, Pa21 (transformant) and Pa25 (transformant) and Pa24r (transformant) in TSB, 20°C shaken at 180 rpm.

Strains Pa2 (wild-type derived), Pa21 (Phi transformed), Pa25 (Phi transformed) and Pa24r (containing the Phi locus minus the repressor gene), all show similar growth dynamics at 20°C. There is no evidence of ‘clumping’ as was seen with growth of the same strains at 30°C.
Plant growth

Seed emergence was significantly reduced in F113 treated plants compared to the control and Pa2, Pa21 and Pa25 treatments (Figure 5.3) (Table 5.2). There was 100% emergence (40/40) for the control, Pa2, Pa21 and Pa25. 93% emergence (37/40) for Pa24r', and 70% emergence (28/40) for the F113. The reduction in emergence seen for the Pa24r' is inconclusive in terms of statistical significance. This was noted in chapter 2 with the F113OP emergence. Such small reduction with comparison to no natural variance of emergence in the control does not allow us to statistically determine that the observed reduction is a statistically significant event. There were no significant differences between shoot or root weights between the treatments. The combined effect of this was that the shoot-to-root ratio was unchanged between the six treatment groups.

![Mean emergence of pea plants after 5 days of the 21-day microcosm trial](image)

**Figure 5.3** Emergence of pea plants after 5-days of the 21-day microcosm experiment. Five microcosms per treatment, each contain 8 plants per microcosm (n=40)
Table 5.2 Pea emergence, shoot and root weights as affected by *Pseudomonas* inocula.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>SBW25EeZY-6KX (Pa2)</th>
<th>Pa21</th>
<th>Pa25</th>
<th>Pa24r</th>
<th>F113</th>
</tr>
</thead>
<tbody>
<tr>
<td>Emergence</td>
<td>1.0a</td>
<td>1.0a</td>
<td>1.0a</td>
<td>1.0a</td>
<td>0.93b</td>
<td>0.7a</td>
</tr>
<tr>
<td>Shoot (g)</td>
<td>0.91a</td>
<td>0.91a</td>
<td>0.99a</td>
<td>0.98a</td>
<td>0.99a</td>
<td>1.30a</td>
</tr>
<tr>
<td>Root (g)</td>
<td>0.71a</td>
<td>0.74a</td>
<td>0.75a</td>
<td>0.83a</td>
<td>0.80a</td>
<td>0.83a</td>
</tr>
<tr>
<td>S/r Ratio</td>
<td>1.31a</td>
<td>1.23a</td>
<td>1.38a</td>
<td>1.20a</td>
<td>1.29a</td>
<td>1.24a</td>
</tr>
</tbody>
</table>

1 Treatments; control, no inocula; SBW25EeZY-6KX (Pa2) (*lacZY, Km*, *xyfE*); Pa21 [Pa2 transformed with pMCPHil9, (normal morphology)]; Pa25 [Pa2 transformed with pMCPHil9, (divergent morphology)]; Pa24r [Pa2 transformed with pMCPHir*, (*PhI* locus minus repressor gene)]; F113, *LacZY* marked natural *PhI* strain.

Emergence, proportion of seedlings emerged five days after sowing; shoot, mean shoot weight; root, mean root weight; s/r ratio, mean ratio of shoot weight to root weight.

Letters, within a row, indicate significant differences at p<0.05 level. For emergence the mean of five microcosms is given (n=5). For shoot, root and s/r ratio, n is proportional to the emergence to a maximum of 40.
Table 5.3 Mean root length, number of lateral roots and nodules per root system as affected by *Pseudomonas* inocula.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>SBW25 (Pa2)</th>
<th>Pa21</th>
<th>Pa25</th>
<th>Pa24r</th>
<th>F113</th>
</tr>
</thead>
<tbody>
<tr>
<td>Root length (cm)</td>
<td>19.85a</td>
<td>21.45ab</td>
<td>24.15b</td>
<td>22.15ab</td>
<td>20.36a</td>
<td>23.51ab</td>
</tr>
<tr>
<td>Lateral roots</td>
<td>20.27b</td>
<td>27.00a</td>
<td>22.65ab</td>
<td>23.67ab</td>
<td>24.65ab</td>
<td>21.00ab</td>
</tr>
<tr>
<td>Nodules</td>
<td>10.87c</td>
<td>15.11b</td>
<td>18.00a</td>
<td>13.92bc</td>
<td>13.00bc</td>
<td>14.78bc</td>
</tr>
</tbody>
</table>

Treatments: control, no inocula; SBW25EeZY-6KX (Pa2) [lacZY, Km', xylE]; Pa21 [Pa2 transformed with pMCP19, (normal morphology)]; Pa25 [Pa2 transformed with pMCP19, (divergent morphology)]; Pa24r [Pa2 transformed with pMCP19, (Pil locus minus repressor gene)]; F113, lacZY marked natural Pil' strain.

Lateral roots, mean number of lateral roots per root system; Nodules, mean no. of nodules per root system

Letters, within a row, indicate significant differences at p<0.05 level. Five roots per microcosm were measured (n=5).
the Pa21 modified treatment with respect to all other treatments. There was no significant difference in nodulation between the other treatment groups.

**Total microbial populations**

Shown in Table 5.4, and graphically in Figure 5.4, the total bacterial counts for plants treated with Pa25, F113 and Pa24r' on the roots was significantly decreased in comparison to the Pa2 and Pa21 treated plants. Soil bacterial populations were not significantly different between any of the treatments.

![Total bacteria population in 21-day old pea roots](image)

**Figure 5.4** Total bacterial counts isolated from 1 g of roots of 21-day pea plants. Figures taken from the mean of 5 microcosms from each treatment.
Table 5.4 Log bacterial populations as affected by *Pseudomonas* inocula.

<table>
<thead>
<tr>
<th>untreated</th>
<th>SBW25 (Pa2)</th>
<th>Pa21</th>
<th>Pa25</th>
<th>Pa24r</th>
<th>F113</th>
</tr>
</thead>
<tbody>
<tr>
<td>Root bacteria</td>
<td>8.46b</td>
<td>8.52a</td>
<td>8.63a</td>
<td>7.86a</td>
<td>7.96b</td>
</tr>
<tr>
<td>Soil bacteria</td>
<td>7.85a</td>
<td>7.38a</td>
<td>7.32a</td>
<td>7.62a</td>
<td>7.62a</td>
</tr>
<tr>
<td>Int pseudoroot</td>
<td>N/A</td>
<td>6.95bc</td>
<td>7.09a</td>
<td>6.75bc</td>
<td>6.69bc</td>
</tr>
<tr>
<td>Int pseudosum</td>
<td>N/A</td>
<td>6.55a</td>
<td>5.96a</td>
<td>6.23bc</td>
<td>6.55ab</td>
</tr>
<tr>
<td>Ind pseudoroot</td>
<td>6.83bc</td>
<td>6.18a</td>
<td>6.62abc</td>
<td>6.82bc</td>
<td>6.89bc</td>
</tr>
<tr>
<td>Root fungi</td>
<td>4.50a</td>
<td>5.01b</td>
<td>4.99bc</td>
<td>5.08b</td>
<td>4.99b</td>
</tr>
<tr>
<td>Soil fungi</td>
<td>5.24a</td>
<td>5.48b</td>
<td>5.21a</td>
<td>5.30a</td>
<td>5.38bc</td>
</tr>
</tbody>
</table>

Treatments; control, no inocula; SBW25EeZY-6KX (Pa2) [lacZY, Km', xylE]; Pa21 [Pa2 transformed with pMCPhI9, (normal morphology)]; Pa25 [Pa2 transformed with pMCPhI9, (divergent morphology)]; Pa24r [Pa2 transformed with pMCPhr', (Phi locus minus repressor gene)]; F113, laeZY marked natural Phi' strain.

Root bact, root bacteria/g root; Soil bact, soil bacteria/g soil; ind pseudor, indigenous fluorescent pseudomonads/g root; Int pseudor, introduced fluorescent pseudomonads/g root; Root fungi, root fungi/g root, soil fungi/g soil.

Letters, within a row, indicate significant differences at p<0.05 level, (n=5).

N/A - No bacterial inocula introduced in Control treatments; N/D - Plate count data missing.
**Pseudomonas populations**

Table 5.4 and Figure 5.5, show the effect of introduced pseudomonad population between the treatment groups. The numbers of Pa21 recovered was statistically greater than other modified Ph1 strains Pa25, Pa24r and F113. All SBW25 strains were recovered with statistically similar populations to the SBW25 Pa2. Both Pa25, Pa24r and F113 were shown not to be significant different in respect to their population on the root system.

![Introduced pseudomonad population in 21-day old pea roots](image)

**Figure 5.5** Number of introduced pseudomonads isolated per gram of root from 21-day old pea plants. Samples are the mean of 5 microcosms per treatment sampled.
In the soil the highest recovery of inocula was the Pa2 and Pa24r, which were both significantly greater than Pa21, F113. Pa25 was also recovered in significantly lower numbers than the Pa2 but was statistically similar (p>0.05) to the Pa24r (Figure 5.6).

**Figure 5.6** Number of introduced pseudomonads isolated per gram of soil from 21-day old pea plants. Samples are the mean of 5 microcosms per treatment sampled.
The indigenous pseudomonad root population also showed significant differences between treatments. Population Pa2 was significantly lower than all treatments except Pa21. Pa21, treated plants were, however, statistically similar in indigenous root populations (p<0.05) as the remaining treatments (Figure 5.7).

![Indigenous pseudomonad population in 21-day old pea roots](image)

**Figure 5.7** Number of indigenous pseudomonads isolated per gram of root from 21-day old pea plants. Samples are the mean of 5 microcosms per treatment sampled.

**Fungal populations**

All four strains of SBW25 showed a significant increase in root fungal population compared to the control and the F133 treatment. The F113 treatment showed no difference in respect to the control treatment. The soil fungal population was significantly increased with respect to all other treatments by Pa2. There were no differences in soil fungal populations between any of the other treatments.
5.5. DISCUSSION OF PLANT GROWTH

Growth flasks

The results for the growth flask experiment show that at 20°C there is clumping of colonies of the Pa21 and Pa25 strains and not in the Pa2 and the Pa24r. The eventual OD reached is also lower for these two strains. The difference is more marked in the flasks grown at 30°C. The reason for the low OD was due to 'clumping' of the Pa21 and Pa25 strains, a phenomenon not seen with either the Pa2 or Pa24r. Clumping has resulted in no homogenous mix of colonies throughout the broth, and consequently lower OD measurements.

This effect may be due to two reasons. The effect of stress has been noted to cause P. fluorescens to clump in broth cultures (De Leij, personal communication). Higher temperatures stimulating rapid growth may have caused stress to the organisms' transformed with the entire Phi locus. Temperatures up to 30°C have been seen to be ideal for rapid and competent growth of many P. fluorescens, including SBW25, as is seen with the wild type, Pa2 under similar conditions. The stress may have come either from the metabolic burden imposed by the addition of the biosynthetic locus or from the disruption of genes effecting growth competence by the genetic insertion.

With the recent unraveling of a common method of post-transcriptional regulation of secondary metabolites in P. fluorescens CHA0 and F113 (Blumer et al., 1999; Aarons et al., 2000), a possible route of cell stress can be seen. It is believed that Phi biosynthesis may be regulated post-transcriptionally by small RNA molecules that bind ribosome-binding sites of mRNA. In CHA0, RsmA is believed to block ribosome binding of mRNA from Phi transcription. The small RNA regulatory protein, RsmB is thought to be regulated through the GacA-GacS regulatory cascade and its presence binds RsmA thus preventing the blocking of Phi mRNA translation. It is not clear how specific these regulatory molecules are, but they appear ubiquitous throughout microorganisms, as they have been isolated in E. coli (Liu and Romeo, 1997). In SBW25, the addition of Phi genes may be causing an additional burden on the organism through having to produce additional quantities of these regulatory proteins. In addition, the trigger for the production of these molecules is not known, but being a part of the GacA-GacS regulatory cascade, it may be assumed that the
trigger is an environmental stimulus that triggers the cytoplasmic membrane bound, GacS.

Another possibility for the clumping may be due to another aspect of the regulation of production of Phl by these strains. Shanahan et al. (1992) showed that F113 produces the largest quantities of Phl when associated with a growth surface. In this case the use of granite chips in the growth medium increased Phl production. The formation of colony clumps is associated with biofilms, where organisms require contact to surfaces or each other in order to produce secondary metabolites. Again this is noticed in the regulation of phenazines through quorum-sensing (chapter 1). Essentially this is the mechanism by which the proximity of colonies to each other regulates the production of the secondary metabolite. The fact that the clumping is not detected in the repressor negative strain, Pa24r', may be because the organism has lost the ability to control the expression of the Phl biosynthetic locus, and hence the environmental factors which act as signals for this control become irrelevant to the organism.

Schnider-Keel et al. (2000) indicated that Phl actually acts to autoinduce the expression of Phl biosynthesis through blocking phlF binding to the Phl gene cluster. This is a positive feedback mechanism that in strain CHAO can be broken through the production of other secondary metabolites such as pyoluteorin. Pyoluteorin seems to compete for the phlF with Phl thus preventing Phl binding and preventing Phl repression. F113 does not produce pyoluteorin and no compound has yet been identified that can act in the same way as pyoluteorin to break this positive feedback loop.

Autoinduction of Phl may explain the findings of Shanahan et al. (1992) when it was observed that the greatest quantity of Phl was produced when bacteria were able to form dense populations on granite surfaces. This would ensure a high level of Phl around each bacterium that can repress the action of the phlF repressor and lead to high levels of Phl production.

Although there is nothing in this explanation to explain the molecular reasoning for the effect of clumping seen in Pa21 and Pa25 it is an advantageous process for the
high expression of Phl. The fact that this is not seen in the *phlF* strain, Pa24r', is difficult to explain in molecular terms at this stage. Physiologically, there is no need to clump or form high population densities to increase the concentration of Phl in the surroundings as there is no PhlF protein to repress Phl biosynthesis.

**Plant growth**

Plant growth measurements were taken to assess any differences that the modified strains of SBW25 would have on crop production. The emergence rate is indicative of early action of the inocula on the seed and emerging young roots and shoots. The results show that the strains modified with the entire gene cluster including repressor gene, show no differences from the marked wild-type derived, Pa2, whereas the repressor-less organism, Pa24r' is appearing to behave similarly to the wild-type natural producer, FI 13. This may support data in chapter 2 where higher levels of Phl production around the germinating seed can lead to a reduction in the emergence of the shoots in association with the bacterial inoculum. This has a great impact on crop yield as plants that had not emerge within 5 days never emerged throughout the 21-day trial. As with chapter 2, these reduced emergences need to investigated using larger seed numbers to achieve a background of variance for the control emergence so that significance of any observed reductions can be calculated.

The remaining measurements were taken destructively at the conclusion of the plants' 21-day growth. This gave an indication of any effects that the modified organisms have impacted on the plant throughout its development. Strain Pa21 showed signs of improving plant growth by increasing the root length in comparison to the control. The effect of increased nodulation seen with Pa21 is caused by the limitation of nitrogen received by the plant. The effect has been caused by the inocula, which has utilised the nitrogen, thus depriving the plant, causing the increase in nodulation to increase the amount of available nitrogen for the plant. This theory is supported by the high population of Pa21 isolated from the pea roots, with comparison to the levels of the inocula recovered for the remaining treatments.
Population counts

The effects of the modified strains were hypothesised as being minimal due to the non-pathogenic nature of the wild-type derived, Pa2 strain (De Leij et al., 1998). The most important differences between the strains are likely to be revealed when the colonising ability of the modified organisms was analysed. This gives an indication of the relative colonising fitness of each strain in planta.

Pa21 was present in higher numbers on the roots than the Pa24r, F113 and Pa25 organism. Both Pa21 and Pa2, were present in greater numbers than the F113. The reason for this might is the competitive nature of these organisms to colonise the root systems. The Pa2 has previously been shown by to colonise more strongly with respect to Phi producers Q2-87, F113 and CHA0 (Naseby et al., unpublished) and more strongly than F113 and F113G22 (Naseby and Lynch, 1998). This is believed to account for the biological control ability of the strain against the fungal plant pathogen, Pythium ultimum. If this high colonisation ability, unique to SBW25 has been significantly reduced by the modification, then a potential weapon against plant pathogens may have been lost.

The Pa21, with colony morphology the same as Pa2, has shown that it can maintain its population to that of the Pa2. The Pa25, modified strain, is appearing to behave similarly to the F113, with significantly lower colonisation than Pa21, which remains similar to the Pa2, and no difference to the F113, which is also significantly lower than the Pa21 and Pa2. Because the genes inserted are identical to those for Pa21, the reduction in fitness points away from a genetic load in itself. The difference in colonisation is possibly due instead to physical characteristics of the polysaccharide coat surrounding the bacteria. Colonisation of roots is characterised by the formation of microcolonies embedded in a bacterial mucigel on plant roots (Chin-A-Woeng et al., 1997; Normander et al., 1999). There is clearly a visual difference in the lipopolysaccharide coat of the two strains, Pa25 and Pa21 as indicated in the photographs in Figure 4.8.

The biggest change from root to soil population has been the modified strain Pa21, that has an altered ability to survive nutrient deficient conditions such as found in soil,
but has maintained the strong colonising characteristic of the wild-type derived Pa2, on nutrient rich roots.

The indigenous pseudomonad population also showed characteristics that would suggest a change in behaviour of the modified strains. Pa2 has significantly lower indigenous population in the root than the F113, Pa24r and control. This is indicative of a strong colonising presence of this strain being able to dominate and competitively exclude the resident populations, a key advantage for biological control. Pa21 is the only modified strain able to exclude the resident population to the same degree as the Pa2 unmodified strain. This trend suggests that it has, in part, maintained its competitiveness around the root systems of plants even with the introduction of the Phi locus, and as seen previously, does not impart pathogenic effects on the pea plant, as does the F113 strain.

Of all strains, Pa21, may possess the necessary qualities for effective integrated biological control, through maintaining its wild-type trait of competitive exclusion on the plant roots, whilst containing the genes from the F113 biological control strain for Phi biosynthesis.

Interestingly, an additional beneficial trait appears to emerge with the strain Pa21’s lowered competence in the rhizosphere soil. With fears of the spread of genetically modified organism, and persistence in the soil, this trait may be of some ecological and commercial benefit and becomes a candidate for further investigation.
6.1. **GENERAL DISCUSSION AND CONCLUSIONS**

In this chapter it is intended to draw together the major findings and observations that have been made throughout this Ph.D thesis.

At the outset of the project, the goal was to achieve an alternative approach to biological control using a combination of established microbial mechanisms: biological control through the production of natural antibiotics, secondary metabolites, and biological control through competitive exclusion of the pathogen.

The final result of this investigation was the construction of two strains of SBW25, Pa21 and Pa25 that have differing phenotypic characteristics following the insertion of the *Phi* coding locus from *P. fluorescens* strain, F113. The morphological change that has occurred appears to involve the outer layer, or polysaccharide layer of the bacterium Pa25.

Following this transformation it was noted that in the rhizosphere of pea the populations of the Pa21 were decreased significantly in comparison to the parental untransformed strain Pa2. However, on the roots the populations remained statistically similar. This is an exciting observation in that the colonisation ability is closely linked to the biological control capabilities of the microbial strain (Chin-A-Woeng *et al.*, 2000; Schippers *et al.*, 1987; Bull *et al.*, 1991; Raaijmakers *et al.*, 1998). Chin-A-Woeng *et al.*, (2000) describes for the first time the direct link between colonisation and ability to prevent plant disease. Through mutation of genes known to reduce colonisation such as site specific recombinase (*Sss*), motility and prototrophy for amino acids, the bacterium *P. chlororaphis* PCL1391, was prevented from controlling *Fusarium oxysporum* induced lesions on tomato, even when wild-type quantities of phenazine-1-carboxamide was still being produced.

Schippers *et al.* (1987) showed that inadequate colonisation leads to reduced biological control activity. Bull *et al.* (1991) showed that the number of take-all lesions was inversely proportional to the number of *Phi* producing organisms on the
root system of wheat. Raaijmakers et al. (1998) showed that a threshold population of Phi producing colonies, per gram of root, must be present on wheat to prevent pathogen induced take-all.

The reasons for this alteration in the phenotypic morphology of Pa25 in relation to Pa21 and the parent strain Pa2 are unknown, and to begin to discover this, the site of the Phi insertion must be determined in future work. It was noted that the strain Pa25 not only showed morphological abnormalities on the bacteria surface, but also that the colonisation of pea roots was significantly lower in comparison to the parent Pa2.

Dekkers et al. (1998) and Simons et al. (1996) described that mutations in the O-antigen of the lipopolysaccharide in Pseudomonas leads to reduced rhizosphere competence and reduced growth rate in comparison to the parental strain. This behaviour was noted in the strain Pa25 both in terms of rhizosphere competence on pea and growth rate in TSB flasks, compared to the parental Pa2 strain. Although it is speculative to assume the same O-antigen has been disrupted through the Phi integration, it is worth recognising the similarity in traits of deliberately mutated strains and the strains Pa21 and especially Pa25 in comparison the parent Pa2.

An encouraging aspect of this research has been the success in delivering the genes responsible for biological control to the SBW25 strain, Pa21, without significantly altering the competence on root systems in comparison to the parent Pa2. This strategy of exploitation of microbial competence in the rhizosphere has begun to receive the attention of numerous groups in the field of microbial biological control. Dekkers et al. (2000) reported on the genetic manipulation of P. fluorescens WCS305 and F113 through the addition of the site-specific recombinase gene (Sss) from P. fluorescens WCS365. The recombinant WSC305 and F113 increased their colonisation of the roots of tomato plants between 16-40 and 8-16 fold respectively. Significantly, strain WCS305 gained the ability to protect the tomato plant from the Fusarium pathogen following this genetic transformation.

In TSB growth flasks at 30°C the transformants Pa25 and Pa21 were reported to aggregate to form clumps. This is distinct from the behavior of the parent and wild-
type strains Pa2 and Pa1 respectively, under identical growth conditions. This clumping is possibly either a stress response to the addition of the genetic load or an altered trait of the polysaccharide coating of these transformants. Interestingly, clumping around root systems is a phenomenon that has been shown to be physiologically important in enhancing plant growth promotion.

Nakata et al. (2000), deliberately induced clumping, or flocculation, of the biological control strain S272. The natural soil isolate *P. fluorescens* S272, able to produce Plt and Phl, can increase the stem growth of water cultivated Kaiware radish when inoculated with a mixture of a polysaccharide flocculent from *Klebsiella pneumoniae*. The polysaccharide is thought to increase adhesion of the bacterium to the Radish roots thus increasing the growth promotion activity of the secondary metabolite antibiotics produced by S272.

The importance of aggregation is considered two-fold. As described above the physical binding of the biological control delivery agent to the roots seems to lead to a more direct delivery of the secondary metabolite. Secondly, Chin-A-Woeng et al. (1997) and Normander et al. (1999) have described that *P. fluorescens* growing on roots form discrete micro colonies that are embedded in a mucigel and closely associated with indigenous bacteria. Only about 1% of the root surface is believed to be inhabited by the biological control strains, however, successful biological control strains can still prevent the colonisation of plant pathogens. There is evidence that the formation of micro colonies can lead to cell-cell communication between bacterium resulting in an amplified affect of the biological control strains.

It was speculated that the location of insertion of the *Phl* coding genes was into at least two sites on the SBW25 chromosome. This is based on the two morphological appearances following identical transformation with the *Phl* construct, pMCPhl9. The feeling is that although the site of insertion may not be the intended site within the *lacZY* locus as intended by the suicide vector design, the alternative site of insertion probably has a region of homology with the suicide vector. It remains of interest that the transformation of SBW25 with the
construct, pMCPhlr', that contained the Phl construct, minus the repressor gene, only resulted in colonies that were unchanged from the parent Pa2 strain.

Delany et al. (2000) described the repressor gene from the F113 Phl gene cluster, as containing a conserved N-terminus helix-turn-helix, DNA-binding motif, as described by Brennan and Matthews (1989). This region showed a high level of amino acid conservation with other DNA binding proteins and as such could act as a region of homology between the Phl construct and genes encoding other DNA binding proteins on the SWB25 chromosome.

If this were the case then the possible sites for insertion could be the intended lacZY sites together with a site homologous to the Phl repressor gene in the pMCPhl9 transformants. As the pMCPhlr transformants lacked the repressor gene, then there would not be insertion at a repressor gene of the SBW25 chromosome that may explain why there is only one morphological group of transformants associated with pMCPhlr' transformants.

The Phl transformed strains Pa21, Pa25 and Pa24r' all produced inhibition of the P. ultimum on PDA, plate bioassay plates. The inhibition was similar in all three strains and there were consistently smaller zones of inhibition seen with these transformed strains than is seen with P. fluorescens F113. In shaking flasks containing TSB media the levels of Phl detected from Pa21, Pa25 and F113 were similar.

The fact that the repressor negative mutant, transformant Pa24r', did not produce a larger zone of inhibition than Pa21 and Pa25 was surprising. The gene phlF has long thought to act as a repressor of Phl synthesis (Bangera and Thomashow, 1996, 1999) and recently been shown to do so by Delany et al. (2000). Delany et al. (2000) demonstrated that the addition of extra chromosomal copies of the phlF element into P. fluorescens F113 led to the significant inhibition of Phl produced by the F113 strain. A mutation of the phlF gene led to de-repression of the Phl production, with an increase in Phl production in comparison to the wild-type, and lower growth rates of the organisms during the early exponential phase. Interestingly these findings indicate that the mutation of the repressor element actually only increases production during
the early stages of the growth phase. During the time when the majority of Phi is produced by F113, late exponential, the levels of Phi production are actually similar in both phlF⁺ and phlF⁻ variants.

The inhibitory mode of action of the PhlF repressor protein is unknown, however it is believed to act as a transcriptional regulator able to bind the intergenic region between the phlF locus and the phlA gene locus (Delany et al., 2000). The purpose of the PhlF protein was speculated as protecting the cells from high levels of Phi at the early stages of growth and does not have an effect later during late exponential growth.

The observation therefore that inhibition on plate bioassay plates was similar in all Phi transformant Pa21, Pa25 and Pa24r⁻, after 5 days growth, is probably explained by the observations made by Delany et al. (2000). The overall amount of Phi present on the bioassay plate after 5 days is likely to be similar between the transformants. The only difference that could be expected, if we extrapolate from the observations by Delany et al. (2000), would be during the first day when the colony growth was in its earliest stages. The repressor negative Pa24r⁻ would be expected to produce a larger quantity of Phi as was seen in the F113-phlF mutant. Typically in plate bioassays, the pathogen does not reach the biological control bacteria until 3 or 4 days following inoculation. By this time Phi levels in the media surrounding the biological control strains are probably very similar, as similarly high levels will have been produced over the past couple of days.

To look at the real affect of a Phi repressor negative strain, Pa24r⁻ on Phi production a time course experiment would need to be conducted where Phi levels could be monitored throughout the growth cycle.

A very important consequence of this information is what happens in situ, on the plant seeds when these strains are used as inocula prior to planting. In this investigation, when pea seeds were inoculated with F113 a reduction in emergence following 5 days growth was noted. The same did not occur with the transformants Pa21 and Pa25, nor the parent strain Pa2 or the non-inoculated control. The detrimental effect of Phi on plants inoculated with Phi producing organisms has been seen previously. Maurhofer
et al. (1995) noted that a Phi over producer, CHA0/pME3090, could reduce the level of disease on tobacco plant roots following infection with *Thielaviopsis basicola* but would severely reduce the growth of the tobacco plants. The same strain was also toxic to sweet corn. In both cases the effect is believed to arise from overproduction of Phi around the plants. In 1999, Moenne-Loccoz et al. indicated that strain F113 could, under specific conditions; reduce the emergence of sugar beet. In that case the F113 was mixed in a media that provided conditions for high Phi production. On removal of this media from the inocula, there was no reduction in plant emergence.

The effect of F113 and F113OP was seen to reduce the emergence of pea seeds in this investigation in *Pythium* infected soils. These treatments did result in fewer lesions than was noted in the plants treated with the F113G22 strain that does not produce Phi. The lesions themselves are caused by the *Pythium* pathogen, as was indicated by their appearance and their presence only in pathogen infected soil. However the fact that the F113G22 treated plants had considerably more lesions than the control, untreated plants indicate that some lesions are, in part, caused by the F113 inocula itself. It was clear from the work prior to this experiment that high levels of Phi was not toxic to the pea plants and therefore it is argued that the reduced emergence of pea plants in the infected soil is possibly due to a combination of *Pythium* and Phi only in association with the delivery inoculum, F113. To conclusively answer whether the F113 inoculum damaging the plant roots, as described by Naseby et al. (1999), makes the plant susceptible to Phi toxicity can be investigated through a dose ranging experiment similar to the tolerance experiment. A replica group of microcosms can be used with F113G22 inoculated seeds together with a range of exogenously added Phi.

In order to fully understand any effect that Phi might have on the plants, we must be aware of the time course concentration of Phi on inoculated seeds. This is a reiterated point made earlier, but is crucial to understand if genetic manipulation of Phi production is to be effective in biological control. When pea plants are inoculated with Pa24r and F113 in the last experiment of this thesis, there is a reduction in plant emergence. If the Pa24r is behaving like the F113-*phiF* mutant constructed by Delany et al. (2000) then the levels of Phi being produced during the early growth phase following inoculation onto the peas may be the detrimental factor causing the
poor seed emergence in comparison to the other transformed strain, Pa21 and Pa25. There has recently been a proof that the Phi acts to autoinduce its own production and this may take place through the \textit{phlF} gene described by Delany \textit{et al.}, (2000).

Schnider-Keel, \textit{et al.}, (2000) described that Phi can actually act as a signal to lead to the autoinduction of further Phi. The findings from the work from Schnider-Keel \textit{et al.}, (2000) suggests that the Phi molecule competes with the binding site for the promoter-binding region on the PhlF protein. This way, either the PhlF binds the promoter region of \textit{phi A}, or the Phi molecule, thus preventing binding to \textit{phi A} and preventing Phi production. In the \textit{phlF} mutant Pa24r', there is no PhlF protein therefore there is effectively continuous autoinduction as there is no possibility of binding to the \textit{phi A} promoter to prevent Phi production.

Picard \textit{et al.} (2000) revealed an interesting finding from natural soil environments around plants of varying ages. The finding revealed that the frequency of Phi producing bacteria is very low during the early stages of plant development and increase as the plant ages. This finding supports the nature of this project where we attempt to move away from the traditional approaches of maximising Phi production through removal of repressors or addition of multiple copies of the biosynthetic locus.

With the findings from Schnider-Keel \textit{et al.} (2000), nature appears to already have a method of producing large quantities of Phi, quickly, through autoinduction. Therefore, determining the stimuli that initiates Phi production may be a more subtle method of increasing the biological control potential of these strains, rather than adding massive inocula which are unable to adapt to their environment. Duffy and Defago (1999) went some way to achieving this through the identification of common nutrients, such as phosphates and zinc which repress and stimulate Phi production, respectively.

In chapter 4, one of the most technically demanding parts of the study was tackled. Using the finding of homology in chapter 3, a systematic approach to develop a method for PCR of long fragments was described. With PCR, a negative reaction gives very little information as to what the problems with the reaction were. With so
many variables influencing the success of amplifying long pieces of DNA it can be an extremely time consuming procedure to obtain a positive result.

The results and method section in chapter 4 detail the eventual successful outcome and the precise conditions for reproducibility for this reaction. In achieving this, a universal guide to approaching long PCR in poorly characterised templates can be addressed. From this experience it is recommended that attempts should be made to reduce the number of variables that require optimisation. These variables were reduced through the use of a control reaction. The control reaction was the amplification of shorter fragments using the same template and the same primers to generate two shorter fragments that encompassed the ends of the gene cluster. This confirmed that the primers would anneal to the template at a given temperature. The range of buffers that suited this primer/template complex was also narrowed down using this control method. Throughout this process it is important to optimise using the DNA polymerase formulation intended for the final Long PCR reaction. It was possible to generate some data through the use of this conventional PCR system that is not as critical to the reaction conditions as in the long PCR reaction. For example, non-specific primers may result in multiple bands, whereas during a long PCR reaction the result is likely to be zero amplification revealing blank, uninformative gels.

Temperature was the key to the success of the long PCR system, and reducing the temperature wherever possible is crucial to maximising the chances of a successful reaction. This can be achieved through using low reaction volumes, modern PCR thermocyclers, thin walled PCR tubes, and shortening denaturation times and adding a substance like glycerol, to lower the DNA melting temperature.

The first indication that an amplification reaction is proceeding is a homogenous DNA smear on the agarose gel following electrophoresis. Reducing the amount of polymerase at this point will reveal distinct bands, and conditions can be manipulated as with conventional PCR to achieve suitably stringent reaction conditions.
Cloning of the PCR generated Phl locus and the subsequent transformation of P. fluorescens SBW25 are described in chapter 4. Improvements to this process could be made through the incorporation of a positive selection mechanism throughout each step of the process. Initially cloning of the Phl fragment lacked positive selection, and colonies containing pMCPhl9 or pMCPhl1' were only selected following individual visualisation of total DNA to detect large plasmids.

The limiting factor without positive selection was seen with isolation of transformants that had undergone double homologous recombination. The single homologous recombination event did incorporate positive selection through the expression of Tc' marker. This event occurred at a frequency of 2 colonies per μg of DNA. Artiguenave et al. (1997), showed that integration of transposon Tn5 occurred at approximately 10^-4 times the transformation frequency.

A method of positive selection of the second step of the double homologous recombination must be employed. This will rely on a system where the loss of the plasmid backbone will result in positive identification of a bacterial strain.

One method would be the incorporation of a sucrose sensitive gene into the plasmid backbone. On incorporation through a single cross-over the bacteria become sensitive to sucrose in the culture media. This does not provide positive selection at this stage as this is achieved with an antibiotic resistance gene, such as Tc'.

On plasmid removal, the bacteria are no longer sensitive to sucrose and only those that have the lost the plasmid backbone, having undergone double recombination, shall survive. These colonies will require confirmation of the presence of the inserted Phl genes, as it is possible for the entire plasmid to detach from the chromosome, leaving bacteria revertants.
Future directions for research

Certain elements of this work have raised issues as to the difficulty and the unexpected effects of moving biosynthetic genes from one organism to another. Through limitations of time during this project, certain avenues of research had not been pursued or developed. Of particular interest to me would be the further characterisation of the repressor element and an understanding of the pleitropic effects it appears to have on the transformants ability to survive under differing conditions, the clumping and the morphology alterations.

In conjunction with this, it would be useful to discover the optimal conditions for growth and Phi production by the transformed strains. Without this, there is no little indication as to the physiological subtleties of Phi expression. Plate bioassays are limiting as they only encompass one condition, often limited to the optimal condition at which the pathogen survives.

Further analysis of the finding of the chapter 2 experiment, through quantification of Phi throughout the plant life, rather than just at the end. Although Phi levels were similar at the end of the experiment, there is no direct evidence of the levels at planting. This assumption, based on the findings at the end of the experiment, and results from Raaijmakers et al. (1999), that Phi production is proportional to colony numbers, is that at planting, the F113OP treated plants were subjected to higher concentrations of Phi than the F113 inoculated plants.

As mentioned during chapter 5, the high competence of Pa21 on the pea roots, coupled with the poor survival in the rhizosphere soil, may make transformant Pa21, an ideal candidate for contained, and targeted delivery of disease biological control.
7. REFERENCES


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