Biological control of *Pythium ultimum* by *Stenotrophomonas maltophilia* W81 is mediated by an extracellular proteolytic activity

Colum Dunne,^{1,2} Jer J. Crowley,¹ Yvan Moëgne-Loccoz,^{1†}
David N. Dowling,^{1‡} Frans J. de Bruijn² and Fergal O’Gara¹

Author for correspondence: Fergal O’Gara. Tel: +353 21 272097. Fax: +353 21 275934.
e-mail: f.ogara@bureau.ucc.ie

¹ Department of Microbiology, University College Cork, Cork, Ireland

² MSU-DOE Plant Research Laboratory and Department of Microbiology, Michigan State University, East Lansing, MI 48824, USA

Stenotrophomonas maltophilia strain W81, isolated from the rhizosphere of field-grown sugar beet, produced the extracellular enzymes chitinase and protease and inhibited the growth of the phytopathogenic fungus *Pythium ultimum* *in vitro*. The role of these lytic enzymes in the interaction between W81 and *P. ultimum* was investigated using Tn5 insertion mutants of W81 incapable of producing extracellular protease (W81M1), extracellular chitinase (W81M2) or the two enzymes (W81A1). Lytic enzyme activity was restored in W81A1 following introduction of a 15 kb cosmid-borne fragment of W81 genomic DNA. Incubation of *P. ultimum* in the presence of commercial purified protease or cell-free supernatants from cultures of wild-type W81, the chitinase-negative mutant W81M2 or the complemented derivative W81A1(pCU800) resulted in hyphal lysis and loss of subsequent fungal growth ability once re-inoculated onto fresh plates. In contrast, commercial purified chitinase or cell-free supernatants from cultures of the protease-negative mutant W81M1 or the chitinase- and protease-negative mutant W81A1 had no effect on integrity of the essentially chitin-free *Pythium* mycelium, and did not prevent subsequent growth of the fungus. In soil microcosms containing soil naturally infested by *Pythium* spp., strains W81, W81M2 and W81A1(pCU800) reduced the ability of *Pythium* spp. to colonize the seeds of sugar beet and improved plant emergence compared with the untreated control, whereas W81A1 and W81M1 failed to protect sugar beet from damping-off. Wild-type W81 and its mutant derivatives colonized the rhizosphere of sugar beet to similar extents. It was concluded that the ability of *S. maltophilia* W81 to protect sugar beet from *Pythium*-mediated damping-off was due to the production of an extracellular protease.

Keywords: biological control, *Pythium ultimum*, sugar beet, *Stenotrophomonas maltophilia*, protease

INTRODUCTION

The interactions between pathogenic or deleterious soil micro-organisms and the plant root take place in the rhizosphere, a complex environment in which those

detrimental micro-organisms are themselves subject to competition, antagonism, parasitism and/or predation by other soil organisms such as fungi, bacteria, viruses, nematodes and protozoa (Atlas & Bartha, 1993; Cook *et al.*, 1995). In certain soils, which have been referred to as suppressive, such interactions may be sufficient to prevent disease development (Scher & Baker, 1982; Alabouvette *et al.*, 1993). Disease suppression may also be achieved in other soils following the introduction of selected biological control agents into the soil or the

[†] Present address: Ecologie Microbienne du Sol, Université Claude Bernard (Lyon 1), 69622 Villeurbanne cedex, France.

[‡] Present address: Department of Applied Biology and Chemistry, Carlow Regional Technical College, Carlow, Ireland.

rhizosphere. Beneficial micro-organisms of interest for biocontrol of soil-borne pathogens and pests have been identified among fungi, actinomycetes and bacteria and they represent a promising alternative to the use of chemical pesticides for crop protection (Becker & Schwinn, 1993; Cook, 1993; Dunne *et al.*, 1996, 1997; Cronin *et al.*, 1997; Keel & Défago, 1997). In addition to their beneficial properties, a key feature of effective biocontrol agents is their ability to persist in soil and aggressively colonize the rhizosphere. Therefore, it has been proposed that isolation of biocontrol micro-organisms indigenous to an environment similar to that in which they will be required to function may ensure greater efficacy of plant protection (Cook, 1993).

Antibiosis is perhaps the most-studied mechanism of biocontrol in bacteria. Several secondary metabolites with antifungal properties have been identified, for example phenazines, pyrrol derivatives, hydrogen cyanide and phloroglucinols (reviewed by Dowling & O'Gara, 1994). Their contribution to the protection of plants against fungal root diseases by pseudomonads has been documented using genetic and biochemical approaches (Thomashow & Weller, 1988; Fenton *et al.*, 1992; Voisard *et al.*, 1994), and some of these compounds have been successfully quantified in the rhizosphere (Keel *et al.*, 1992). In addition, niche exclusion, competition for nutrients such as iron (mediated through production of fluorescent siderophores) or carbon sources, induced resistance, parasitism and extracellular lytic enzymes have been implicated in plant protection by bacteria (Lemanceau & Alabouvette, 1991; O'Sullivan & O'Gara, 1992; Chernin *et al.*, 1995; Kobayashi *et al.*, 1995; Leeman *et al.*, 1995; Keel & Défago, 1997).

Under *in vitro* conditions, exposure of phytopathogenic fungi to lytic enzymes such as chitinases, proteases, amylases or glucanases can result in the degradation of the structural matrix of fungal cell walls (Oppenheim & Chet, 1992; Lorito *et al.*, 1994). The role of lytic enzymes produced by the mycoparasitic fungus *Trichoderma harzianum* in biological control of fungal pathogens has been demonstrated (Geremia *et al.*, 1993; Haran *et al.*, 1996), and overproduction of an inducible extracellular protease by copy number effect resulted in enhanced biocontrol efficacy (Flores *et al.*, 1997). Transgenic biocontrol agents and plants with a conferred ability to produce antifungal chitinases have also been reported (Oppenheim & Chet, 1992). However, in contrast to the attention devoted to lytic enzymes produced by biocontrol fungi, comparatively fewer studies have focused on bacterial production of lytic enzymes for biocontrol of fungal pathogens, and only circumstantial evidence was provided in the latter to attribute observed biocontrol abilities to lytic enzyme production (Mitchell & Hurwitz, 1965; Fridlender *et al.*, 1993; Chernin *et al.*, 1995; Kobayashi *et al.*, 1995).

The diversity of biocontrol traits in most soil bacteria other than *Pseudomonas* spp., *Burkholderia* spp. and *Bacillus* spp. remains virtually untapped. In this study, a rhizosphere isolate obtained from field-grown sugar

beet, *Stenotrophomonas* (previously *Pseudomonas* and then *Xanthomonas*) *maltophilia* W81, was studied that inhibited the fungal pathogen *Pythium ultimum* under laboratory conditions. Mutant derivatives of W81 were developed through transposon insertion and were used to demonstrate that protection of sugar beet against *Pythium*-mediated damping-off by the wild-type bacterial strain was due to its ability to produce an extracellular protease.

METHODS

Isolation from the sugar beet rhizosphere of a strain of *S. maltophilia* antagonistic to *P. ultimum*. *S. maltophilia* strain W81 was isolated on S1 medium (Gould *et al.*, 1985) from the rhizosphere of sugar beet grown near Glanworth (Co. Cork, Ireland) at a field site chosen on the basis of its lack of history of damping-off disease of sugar beet. The strain was selected for its ability to inhibit growth of the fungal pathogen *P. ultimum* (obtained from the Commonwealth Mycological Institute, Egham, Surrey, England) on a range of solid media, i.e. Luria-Bertani (LB; Sambrook *et al.*, 1989) agar, sucrose-asparagine (SA; Scher & Baker, 1982) agar, potato dextrose agar (PDA; Oxoid), malt extract agar (MEA; Oxoid), and nutrient agar (NA; Oxoid), as follows. Strain W81 was grown overnight in 10 ml LB broth (28 °C, with shaking). The cells were washed twice in quarter-strength Ringer's solution (Oxoid), resuspended in 100 µl quarter-strength Ringer's solution and streaked onto the plates. After a 48 h incubation at 28 °C, plugs of *P. ultimum* freshly grown on SA agar were placed in the centre of the plates, which were then incubated at 28 °C for a further 3–5 d prior to assessing fungal growth.

Micro-organisms, plasmids and growth conditions. Bacterial strains and plasmids used in the study are described in Table 1. Strain W81 was identified as *S. maltophilia* (Swings *et al.*, 1983; Palleroni & Bradbury, 1993) based on the results of BIOLOG biochemical tests and fatty acid methyl ester analysis (DSM, Braunschweig, Germany). *S. maltophilia* W81 and its mutants were routinely grown in LB broth (at 28 °C), *Pseudomonas fluorescens* strain F113 in SA broth (at 28 °C) and *Escherichia coli* in LB broth (at 37 °C). Where appropriate, antibiotics were added to growth media at the following concentrations: streptomycin, 100 µg ml⁻¹; kanamycin, 25 µg ml⁻¹; tetracycline, 25 µg ml⁻¹; and chloramphenicol, 30 µg ml⁻¹. Bacteria were maintained in glycerol solutions at -20 °C. *P. ultimum* was kept on corn meal agar (Difco).

Plate assays for chitinolytic and proteolytic activity. Chitinolytic activity was assessed using a solid minimal media containing K₂HPO₄ (0.8 g l⁻¹), KH₂PO₄ (0.2 g l⁻¹), (NH₄)₂SO₄ (0.5 g l⁻¹), MgSO₄·7H₂O (0.2 g l⁻¹), CaCl₂·2H₂O (10 mg l⁻¹), FeCl₃·6H₂O (10 mg l⁻¹), ZnSO₄·7H₂O (1 mg l⁻¹), Casamino acids (2 g l⁻¹), purified agar (15 g l⁻¹) and an equal volume of a 1% (w/v) colloidal chitin suspension (Sigma). Proteolytic activity was studied on plates containing skim milk (100 g l⁻¹), yeast extract (1.5 g l⁻¹) and technical agar (15 g l⁻¹).

Bacteria were streaked directly onto the plates or, alternatively, 100 µl filter-sterilized cell-free supernatant of bacterial cultures (in SA or LB), obtained after concentration (× 500) using an Amicon ultra-filtration unit, were spotted onto the centre of the plates. The plates were incubated overnight at 28 °C. Both chitin- and skim-milk-containing plates are opaque and enzyme activity was identified by the development of a zone of clearing (halo) around the colonies and the cell-free supernatant spots.

Table 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
<i>S. maltophilia</i>		
W81	Wild-type, Sm ^r Km ^r Phl ⁻ Hcn ⁻ Flu ⁻ Chi ⁺ Prt ⁺	This study
W81M1	Tc ^r Prt ⁻ , Tn5-764cd mutant of W81	This study
W81M2	Tc ^r Chi ⁻ , Tn5-764cd mutant of W81	This study
W81A1	Tc ^r Chi ⁻ Prt ⁻ , Tn5-B50 mutant of W81	This study
W81A1(pCU800)	Tc ^r - Cm ^r -complemented mutant of W81A1	This study
<i>P. fluorescens</i>		
F113LacZY	Phl ⁺ Hcn ⁺ Flu ⁺ LacZY ⁺	Fedi <i>et al.</i> (1996)
<i>E. coli</i>		
HB101	<i>recA hsdB hsdM strA pro leu thi</i>	Boyer & Roulland-Dussoix (1969)
MC1061	<i>ara leu lacX74 galU galK hsdB hsdM strA</i>	Casadaban & Cohen (1980)
DH5 α	ϕ 80 <i>lacZ</i> AM15 Δ (<i>lacZYA-argF</i>)U169 <i>hsdR17 recA1 endA1 thi-1</i>	Sambrook <i>et al.</i> (1989)
LE392	F ⁻ , <i>hsdR514 supE44 supF58 lacY1 galT22 metB1 trpR55 λ^-</i>	Sambrook <i>et al.</i> (1989)
S17-1	F ⁻ , <i>pro recA1</i> RP4-2 integrated (Tc ^r ::Mu) [Km ^r ::Tn7(Sm Tc ^r)]	Simon <i>et al.</i> (1983b)
Plasmids		
pSUP106	Broad-host-range cosmid vector, IncQ Cm ^r Tc ^r	Simon <i>et al.</i> (1983a)
pCU800	pSUP106 with a 15 kb insert from W81	This study
pRK2013	Mob ⁺ Tra ⁺ Km ^r	Ditta <i>et al.</i> (1980)
pSUP102::Tn5-B50	Tc ^r	Simon <i>et al.</i> (1989)
pRL1063a	Km ^r LuxAB ⁺	Wolk <i>et al.</i> (1991)
pRL764cd	Tc ^r LuxAB ⁺	This study

Polyacrylamide gel assays for chitinolytic and proteolytic activity. Filter-sterilized concentrated cell-free supernatants obtained as described above were electrophoresed on denaturing and non-denaturing polyacrylamide gels (Sigma) in which 1% skim milk or 1% chitin had been incorporated. Following electrophoresis, and where appropriate, SDS was removed from the polyacrylamide gels by washing them in Triton X-100. The gels were then incubated overnight at 28 °C and stained with Coomassie brilliant blue R250 stain. After a brief period of destaining, bands of clearing corresponding to particular protein bands were observed. Where 1% chitin was incorporated into the polyacrylamide gels, chitinolytic activity was visible without the requirement for staining.

Biochemical quantification of extracellular proteolytic activity. Bacterial extracellular proteolytic enzyme production was quantified colorimetrically, using a modification of the procedure described by McKellar *et al.* (1981). The strains were grown overnight at 28 °C (with shaking). The cells were harvested by centrifugation at 3000 *g* for 10 min and resuspended in a buffer containing 1 M MOPS and 15 mM CaCl₂ (pH 6.5). One gram Hammarsten casein (BDH) was dissolved in 100 ml acetate phosphate buffer (pH 6.5) and the buffered casein was incubated at 28 °C for 1 h. The cell suspensions (0.5 ml) were added to 10 ml casein solution and the mixture was incubated at 28 °C with gentle agitation. Samples (1 ml) were aseptically removed at intervals and placed in 1.5 ml

Eppendorf tubes. The cells were centrifuged and 500 μ l supernatant transferred to new tubes. A volume of 500 μ l ice-cold 10% trichloroacetic acid was added, vortexed and allowed to precipitate for 30 min on ice. Precipitated material was removed by centrifugation, and 200 μ l supernatant added to 750 μ l potassium borate buffer (pH 9.5) and 400 μ l 0.1% 2,4,6-trinitrobenzenesulfonic acid (TNBS). Following incubation at 37 °C for 1 h to allow colour development, the reaction was stopped by addition of 800 μ l 1 M NaH₂PO₄ containing 18 mM Na₂SO₃. Colour development was read at λ = 420 nm (Beckman DU 640 spectrophotometer, path length 1 cm) within 20 min and related to glycine equivalents released using a standard curve.

Detection of siderophores, hydrogen cyanide and phloroglucinols. Production of siderophores was assessed on SA plates containing the iron chelator ethylenediamine-di(o-hydroxyphenylacetic acid) (EDDHA; 250 μ M) and on chrome azurol S (Schwyn & Neilands, 1987) indicator medium. Hydrogen cyanide production was assayed qualitatively, as described by Castric & Castric (1983). The polyketide 2,4-diacetylphloroglucinol was studied by HPLC analysis (Shanahan *et al.*, 1992).

Genetic manipulations. Mobilization of plasmids between *E. coli* and *S. maltophilia* W81 was performed using the helper plasmid pRK2013 (Ditta *et al.*, 1980), transformation of *E. coli* DH5 α -using the standard CaCl₂ heat-shock method, and

recombinant DNA manipulations as outlined by Sambrook *et al.* (1989). All digestions and ligations were done using enzymes from Promega or Boehringer Mannheim.

Tn5 mutagenesis of the Sm^r strain W81 was performed using pSUP102::Tn5-B50 (Tc^r; Simon *et al.*, 1983b, 1989) or with pRL764cd, a tetracycline-resistant derivative of pRL764 (Cohen *et al.*, 1997), as previously described by Kragelund *et al.* (1995). The resulting Tn5 insertion derivatives (Sm^r Tc^r) were screened for chitinolytic and proteolytic activities on plates, as described above. Mutants deficient in the production of protease (W81M1), chitinase (W81M2) or both enzymes (W81A1) were identified.

Restoration of W81A1 lytic enzyme activity was performed as follows. A genomic library of W81 was constructed using the IncQ broad-host-range cosmid pSUP106 (Simon *et al.*, 1983a), as described by Fenton *et al.* (1992). Ligation products were transfected into *E. coli* LE392 (Sambrook *et al.*, 1989) using the bacteriophage-head-based Packagene system (Promega). Resultant Cm^r Tc^r colonies were mated with W81A1, using HB101(pRK2013) to provide helper functions. Transconjugants were screened for restored chitinolytic and proteolytic activities, as described above, and W81A1(pCU800) with restored enzyme activity was obtained.

DNA-DNA hybridization procedures. Total genomic DNA was prepared by caesium chloride/ethidium bromide equilibrium density gradient centrifugation (Sambrook *et al.*, 1989). DNA was restricted with *Eco*RI and immobilized on Hybond-N+ nylon membranes (Amersham) by capillary blotting. DNA probes, i.e. Tn5-B50, Tn5-764cd and the pCU800 genomic insert, were isolated from agarose gels using a QIAEX extraction kit (QIAGEN). Probe DNA was radioactively labelled using a Primagene labelling kit (Promega). Hybridization experiments were completed, according to the manufacturer's recommendations, using an ECL Direct Nucleic Acid Labelling and Detection System (Amersham).

Sequence analysis. DNA flanking the inserted Tn5-764cd was cloned from the protease-deficient mutant W81M1 using a modification of the method described by Wolk *et al.* (1991). Total genomic DNA was prepared by caesium chloride/ethidium bromide equilibrium density gradient centrifugation (Sambrook *et al.*, 1989). DNA was digested using the restriction enzyme *Dra*I, which does not cut the transposon. The resulting fragments were self-ligated and transformed into *E. coli* DH5 α . DNA sequence flanking the inserted transposon was determined using unique primers (GENOSYS, Cambridge, UK) homologous to the left (5' TACTAGAT-TCAATGCTATCAATGAG 3') and right (5' AGGAGGT-CACATGGAATATCAGAT 3') ends of Tn5-764cd. These primers were designed based on previously described shorter primers (Black *et al.*, 1993; Fernandez-Pinas *et al.*, 1994). PCR fragments for automated sequence analysis were sequenced using an ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer) and the following conditions: 96 °C 30 s, 50 °C 15 s, 60 °C 4 min, for 25 cycles, and were analysed on an ABI PRISM 310 Genetic Analyser (Perkin-Elmer). Similarities in the DNA and protein databases were searched for using the BLAST algorithm (Altschul *et al.*, 1990).

Susceptibility of *P. ultimum* to commercial and W81-produced chitinases and proteases *in vitro*. The effect of commercial microbial chitinase and Proteinase K on *P. ultimum* was studied using mycelium grown for 3–5 d in liquid LB (at 28 °C, without shaking). The mycelium was collected using a sterile metal spatula, washed in quarter-strength Ringer's solution, resuspended in fresh LB broth

containing chitinase from *Serratia marcescens* (Sigma C-1525, EC 3.2.1.14) (0.5–1.0 units ml⁻¹) or Proteinase K (Sigma P-8044, EC 3.4.21.14) (0.5–1.0 units ml⁻¹) and incubated for 48 h at 28 °C.

Some of the samples were used for microscopic examination of the mycelium, either in wet mounts or after staining with cationic (methylene blue, crystal violet) or anionic (safranin, fuchsin acid, Congo red, eosin yellow) dyes. The remaining samples were used to study the ability of the fungus to grow, as follows. The mycelium was washed in quarter-strength Ringer's solution and placed in the centre of LB, PDA, MEA or NA plates. Fungal growth was assessed after incubating the plates for 7 d at 28 °C. In all assays, untreated fungal mycelium was used as a negative control.

The experiments were also performed using concentrated cell-free (filter-sterilized) culture supernatants of W81 and its mutant derivatives instead of commercial chitinase and protease. The procedures were identical to those described above.

Soil microcosm experiments. The ability of wild-type W81 and its mutant derivatives to protect sugar beet from damping-off disease was investigated in natural soil microcosms prepared using a sandy-loam soil obtained from the surface horizon of a field located near Brinny (Co. Cork, Ireland). The soil was chosen on the basis of (1) a history of damping-off disease of sugar beet at the site and (2) natural infestation of the soil by *Pythium* spp. [>1000 propagules (g soil)⁻¹], as shown using a *Pythium*-selective medium (Jeffers & Martin, 1986). The soil was sieved through a 0.5 cm mesh screen prior to preparing the microcosms (140 g soil per 7.5 cm diameter pot). Sugar beet seeds (cv. Accord) were inoculated by dipping them in cell suspensions (approximately 10⁶ c.f.u. per seed) of the bacteria studied. The latter included W81, W81M1, W81M2, W81A1, W81A1(pCU800) and the biocontrol agent *P. fluorescens* F113LacZY (Fedi *et al.*, 1996). Controls consisted of seeds dipped in quarter-strength Ringer's solution (untreated control) or a solution containing the synthetic fungicides Previcur N (propamocarb; Schering) at 20 ml (kg seed)⁻¹ and thiram at 7.5 g (kg seed)⁻¹ (commercial control). Nine seeds were sown per pot, at a depth of 1.5 cm. Soil water content was adjusted to 70% saturation of the soil porosity every 3 d by spraying with distilled water. The pots were incubated in a growth chamber (12 °C, 16 h photoperiod).

Infection of seeds by *Pythium* spp. was assessed daily during the first 7 d of the experiment, as follows. The seeds were removed from soil, washed in sterile quarter-strength Ringer's solution and incubated on the *Pythium*-selective agar of Jeffers & Martin (1986) at 28 °C for 48 h prior to scoring for the presence of *Pythium* spp. The percentage plant emergence was determined at 28 d. Colonization of the sugar beet rhizosphere by the seed inoculants was assessed by colony counts on LB containing streptomycin and kanamycin (for wild-type W81), streptomycin, kanamycin and tetracycline [for W81M1, W81M2, W81A1 and W81A1(pCU800)], or SA containing X-Gal (for F113LacZY). Maintenance of pCU800 in W81A1 was determined by replica plating onto LB agar containing streptomycin, tetracycline and chloramphenicol. No colonies were found on the selective media when studying plants from the untreated control (detection limit of 10² c.f.u. per root system).

Statistical aspects. All *in vitro* experiments were performed with three replicates of each treatment and were repeated at least three times. A randomized complete block design was used for the soil microcosm experiments, which were done four times. Seed infection by *Pythium* spp. was studied using ten seeds from each of three replications for each treatment at

each sampling time. Plant emergence was investigated using nine replications. Each replication of each treatment consisted of one pot with nine seeds per pot. Six root systems were used to assess rhizosphere colonization of each strain at each sampling time, as described previously (Carroll *et al.*, 1995). Percentages were arcsin-transformed and colony counts were log-transformed prior to analysis. Within each repeated experiment, analysis of variance was done at each sampling time using the General Linear Model procedures of the Statistical Analysis System (SAS Institute, Cary, NC, USA). When appropriate, differences between treatments were studied using Fisher's LSD test. All analyses were performed at $P = 0.05$ level. Similar results were obtained each time the soil microcosm experiments were run and representative results obtained in one of the four runs are shown in Table 2.

RESULTS

S. maltophilia W81 inhibits *P. ultimum* *in vitro* and produces the extracellular enzymes chitinase and protease

S. maltophilia W81 inhibited the growth of the fungal pathogen *P. ultimum* on the solid media LB agar, SA agar, PDA, MEA and NA. Colonies produced zones of clearing on plates containing chitin or casein, which is indicative of extracellular chitinase and protease activities, respectively. Extracellular chitinase and protease activities were observed even when readily available sources of carbon (i.e. glucose, sucrose, maltose, galactose, succinate, malate or fumarate) and/or nitrogen (i.e. ammonium sulphate) had been added to the chitin and skim milk plates, respectively. Production of extracellular protease and chitinase was not affected when the level of available iron in low-iron plates was increased by the addition of 100 μM FeCl_3 , despite the

fact that iron influences extracellular protease activity in certain rhizobacteria (Dowling *et al.*, 1996). Further characterization of W81 indicated that the strain does not produce the biocontrol metabolites 2,4-diacetylphloroglucinol or hydrogen cyanide. When grown on SA plus EDDHA or on chrome azurol S, W81 did not produce a siderophore (data not shown).

Construction of mutants of *S. maltophilia* W81 deficient in extracellular lytic enzyme production and cloning of Tn5-tagged loci

To determine if the inhibition of *P. ultimum* by W81 *in vitro* was linked to the ability of the bacterium to produce extracellular lytic enzymes, mutants of W81 deficient in the production of extracellular chitinase or protease were sought by mutagenesis with the transposons Tn5-B50 and Tn5-764cd. Approximately 15000 mutants were screened by replica plating for loss of lytic enzyme activity, and a Tn5-B50 induced mutant unable to produce either extracellular enzyme (W81A1) was identified (Table 2). Mutants incapable of extracellular protease production (W81M1) or extracellular chitinase production (W81M2) were only isolated following mutagenesis with Tn5-764cd (Table 2). The transposon Tn5-tagged locus of W81M1 was cloned from the genome, as described in Methods. A partial DNA sequence of the tagged locus was determined using primers corresponding to the left and right ends of the Tn5-764cd transposon.

Apart from lytic enzyme production, strains W81M1, W81M2 and W81A1 did not differ from W81 when compared using a variety of biochemical tests (aerobic acidification of glucose; production of catalase, oxidase,

Table 2. Evaluation of enzyme production by *S. maltophilia*, and biological control of *P. ultimum* under *in vitro* and natural soil microcosm conditions

Treatment	Enzyme production		Biological control		
	Lytic enzyme production	Proteolytic activity*†	Inhibition of <i>P. ultimum</i> growth <i>in vitro</i>	<i>Pythium</i> -free seeds after 1 d in soil (%)†	Emergent sugarbeet plants after 28 d in soil (%)†
Commercial control	—	—	+	100 ^a	72 (4) ^a
Untreated control	—	—	—	5 (5) ^b	38 (6) ^c
F113LacZY	—	—	+	90 (4) ^a	59 (4) ^b
W81	Chitinase, protease	6.5 (0.6) ^a	+	87 (9) ^a	64 (7) ^{ab}
W81M1	Chitinase	0.9 (0.6) ^b	—	20 (6) ^b	38 (8) ^c
W81M2	Protease	7.3 (0.8) ^a	+	84 (6) ^a	57 (3) ^b
W81A1	None	0.8 (0.2) ^b	—	30 (11) ^b	36 (4) ^c
W81A1(pCU800)	Chitinase, protease	6.4 (0.4) ^a	+	86 (8) ^a	58 (4) ^b

* One unit of activity corresponds to 1 nmol glycine equivalents released (ml cell-free supernatant)⁻¹ min⁻¹.

† Values given are means ($\pm$ SD). Within each column, values followed by a different letter are significantly different according to Fisher's LSD test at the $P = 0.05$ level.

Fig. 1. Inhibition of growth of *P. ultimum* *in vitro*. *P. ultimum* inoculated alone completely covered the PDA plate (a). Wild-type *S. maltophilia* W81 inhibited growth of the fungus (b). Mutant W81A1 was unable to inhibit fungal growth (c), unlike W81A1(pCU800) with restored lytic enzyme production (d).

gelatinase, casein protease, DNase; and assimilation of acetate, citrate, malate, glucose, mannose, maltose, N-acetylglucosamine and maltose) and growth curves in liquid LB and SA.

Sequence analysis

The amino acid sequences deduced from the primary DNA sequence were compared to sequences in the non-redundant protein databases at the National Centre for Biological Information (NCBI). Results indicated that the region in which Tn5-764cd has inserted in the protease-deficient mutant W81M1 shows 39% identity between residues 260 and 370 of the acrosin trypsin-like serine peptidase (S12968). Further shared similarities with other S8, S21, C1 and M4 peptidase families suggest that the insertion of Tn5-764cd caused a mutation in a gene encoding a proteolytic enzyme.

Construction of a cosmid library of *S. maltophilia* W81 and complementation of the mutant W81A1

Introduction of a *Bam*HI fragment of approximately 15 kb from a cosmid library of W81 into the chitinase- and protease-negative mutant W81A1 resulted in the generation of the complemented derivative W81A1(pCU800) with restored ability to produce the two extracellular lytic enzymes (Table 2). The presence of pCU800 in W81A1 also restored the ability to inhibit *P. ultimum* *in vitro* (Fig. 1). Southern hybridizations of W81M1, W81M2 and W81A1 using Tn5-764cd, Tn5-B50 and the 15 kb genomic insert as ³⁵S-labelled probes demonstrated that the complementing insert originated

from the same genomic region of W81 in which the transposon is inserted in W81A1. However, the three distinct mutant phenotypes associated with W81M1, W81M2 and W81A1 were due to transposon insertions into different genetic regions. Introduction of pCU800 into *P. fluorescens* M114 and F113, two chitinase-negative strains in which extracellular protease is iron-regulated, did not confer chitinolytic activity or iron-independent proteolytic activity.

Detection and quantification of extracellular lytic enzymes in cell-free culture supernatants of wild-type W81 and its mutant derivatives

Concentrated cell-free supernatants from SA and LB cultures of *S. maltophilia* W81, W81M1, W81M2, W81A1 and W81A1(pCU800) were used to further investigate the ability of W81 and its mutant derivatives to produce extracellular chitinase and protease *in vitro*. Concentrated cell-free supernatants from cultures of wild-type W81 displayed chitinase and protease activity on plates, in SDS-PAGE gels and in non-denaturing PAGE gels. In contrast, concentrated cell-free supernatants from cultures of the mutants W81M1, W81M2 and W81A1 did not display proteolytic, chitinolytic or either lytic enzyme activity, respectively. However, plate, gel and biochemical results indicated that chitinase and protease activity had been restored in the complemented derivative W81A1(pCU800). Quantification of extracellular protease activity in cell-free culture supernatants demonstrated that wild-type W81, W81M2 and W81A1(pCU800) produced similar levels of active enzyme (Table 2).

Role of lytic enzymes in inhibition of *P. ultimum* by *S. maltophilia* W81 *in vitro*

To determine whether lytic enzymes produced by W81 were responsible for the ability of the strain to inhibit *P. ultimum* *in vitro*, mycelium of the fungal pathogen was incubated in the presence of cell-free supernatants from cultures of W81, W81M1, W81M2, W81A1 or the complemented mutant W81A1(pCU800). Whereas fungal mycelium previously exposed to W81A1 or W81M1 cell-free culture supernatant (or from the control) displayed intact cell walls, the cell walls of mycelium incubated in the presence of cell-free culture supernatants from W81, W81M2 or the complemented derivative W81A1(pCU800) were partially or completely degraded and intracellular fungal constituents had been released from the hyphae (Fig. 2). Furthermore, there were differences observed between the samples of mycelium when microscopic examinations were performed using wet mounts, or after staining with the anionic dyes safranin, eosin yellow, acid fuchsin or Congo red. No differences were observed following staining of mycelium with the cationic dyes methylene blue or crystal violet.

Similar experiments were performed using commercial chitinase and protease instead of cell-free culture supernatants. Incubation of *P. ultimum* in the presence of

Fig. 2. Wet mounts of *P. ultimum* mycelium following incubation in cell-free culture supernatants of *S. maltophilia* W81 (a) or W81A1 (b). Whereas the mutants W81M1 or W81A1 deficient in proteolytic enzyme production had no detrimental effects on the fungal mycelium compared with the control (not shown), exposure to cell-free supernatants of wild-type W81, the chitinase-negative mutant W81M2 or W81A1(pCU800) resulted in disruption of the integrity of the cell wall and significant loss of intracellular constituents. Magnification $\times 1000$.

chitinase had no influence on the mycelium, but exposure of the fungus to protease resulted in the lysis of the hyphae similar to that obtained with cell-free culture supernatants of W81, W81M2 or W81A1(pCU800).

To confirm that the effect of W81 on *P. ultimum*, mediated through the production of extracellular protease, was fungicidal and not fungistatic, mycelium of the fungus previously incubated in the presence of cell-free culture supernatants of W81 or its mutants was washed and placed in the centre of plates containing LB agar, PDA, MEA or NA. Results indicated that fungal mycelium previously exposed to cell-free culture super-

natants of W81, W81M2 or W81A1(pCU800) failed to grow on the plates. In contrast, *P. ultimum* previously incubated in the presence of cell-free culture supernatants of W81A1, W81M1 or from the untreated control gave rise to fungal growth on each medium. In conclusion, the fungicidal effect of *S. maltophilia* W81 on *P. ultimum* *in vitro* involved a proteolytic extracellular enzyme.

Biocontrol ability of wild-type *S. maltophilia* W81 and its mutant derivatives in natural soil microcosms

The ability of *S. maltophilia* W81 and its mutant derivatives to control the extent of damping-off of sugar beet was investigated in soil microcosms containing soil naturally infested by *Pythium* spp. Whereas 95 % sugar beet seeds from the untreated control were colonized by *Pythium* spp. at 1 d after their introduction into soil, inoculation of seeds with W81 or W81M2 prior to sowing reduced the level of *Pythium* colonization to less than 20 % (Table 2). This beneficial effect was not found in the W81A1 or W81M1 treatments, but seed inoculation with the complemented derivative W81A1(pCU800) resulted in a level of *Pythium* colonization of seeds similar to that in the proteolytic wild-type W81 and W81M2 treatment.

Percentage plant emergence was assessed 28 d after sowing. Plant emergence was higher in the W81 and W81M2 treatments than in the untreated control, in the case of wild-type W81 reaching the level of protection achieved with synthetic fungicides in the commercial control (Table 2). The effects of W81 and W81M2 on damping-off disease was similar to that of *P. fluorescens* F113LacZY, a tagged derivative of the biocontrol strain F113 (Fedi *et al.*, 1996). Unlike W81, W81A1 and W81M1 had no influence on damping-off of sugar beet, but the complemented derivative W81A1(pCU800) had a positive effect on plant emergence, which was similar to that of wild-type W81. In conclusion, the results from the soil microcosm experiment indicated that the biocontrol capability of *S. maltophilia* W81 in the sugar beet–*Pythium* pathosystem involved the ability of the bacterium to produce an extracellular protease.

Colonization of the rhizosphere of soil-grown sugar beet by wild-type *S. maltophilia* W81 and its mutant derivatives

The seed inoculants *S. maltophilia* W81, W81M1, W81M2, W81A1 and W81A1(pCU800) colonized the rhizosphere of soil-grown sugar beet to similar extents during the 28-d-long experiment, with all inoculants exhibiting an approximately 10-fold decrease after the first 7 d. This indicates that for W81A1 and W81M1, the lack of protection against damping-off was not due to a reduction in ecological fitness in the rhizosphere. Plasmid pCU800 was maintained in W81A1 at levels higher than 95 %. Colony counts for W81 and its mutants were essentially similar to those of the biocontrol pseudomonad F113LacZY. At each sampling time, colonies were chosen at random from the plates used for colony

counts and their phenotype was investigated. All exhibited the same chitinase and protease activities on plates as those of the corresponding inoculants W81, W81M1, W81M2, W81A1 or W81A1(pCU800).

DISCUSSION

S. maltophilia is a typical soil inhabitant (Singer & Debette, 1993) most often found associated with plants (McInroy & Kloepper, 1994; Wilson & Lindow, 1994; Berg *et al.*, 1996). In the rhizosphere of rapeseed for instance, *S. maltophilia* is present at population levels of approximately 10^7 c.f.u. (g root)⁻¹ and represents a mean of 4% of the total culturable bacterial community (Berg *et al.*, 1996). These bacteria have been the focus of much attention due to their potential for bioremediation (Blake *et al.*, 1993). *S. maltophilia* has also been proposed for the protection of crops or turfgrass against fungal pathogens (Kobayashi *et al.*, 1995; Berg *et al.*, 1996) and as a bioherbicide against the weed downy brome (Mazzola *et al.*, 1995). High levels of proteolytic activity are a prominent feature of *S. maltophilia* (Singer & Debette, 1993).

The results presented in the current study indicate that *S. maltophilia* W81 can inhibit the growth of *P. ultimum* *in vitro* (Fig. 1). *S. maltophilia* W81, which does not produce fluorescent siderophores, HCN or 2,4-diacetylphloroglucinol (Phl) often associated with biocontrol pseudomonads, also has the ability to control the extent of damping-off of sugar beet in soil naturally infested by *Pythium* spp. (Table 2). Tn5-764cd insertion mutants of strain W81 were generated to investigate whether the biocontrol ability of the bacterium was linked to the production of extracellular lytic enzymes. These mutants proved incapable of extracellular protease (W81M1) or extracellular chitinase (W81M2) production. Cloning and sequence analysis of the genetic region into which Tn5-764cd had inserted in W81M1 confirmed that the mutation occurred in a protease structural gene. Another mutant (W81A1) proved unable to produce either extracellular enzyme until lytic enzyme activity was restored through introduction of 15 kb of cosmid-borne W81 genomic DNA (pCU800) (Table 2). In certain pseudomonads, regulation of extracellular enzyme and secondary metabolite production has been shown to involve two-component systems (Laville *et al.*, 1992; Hrabak & Willis, 1993; Gaffney *et al.*, 1994; Sacherer *et al.*, 1994). Since W81A1 is deficient in the production of both chitinase and protease enzymes, it may, therefore, prove to be a regulatory mutant.

Results obtained with W81, W81M1, W81M2, W81A1 and the complemented derivative W81A1(pCU800) under *in vitro* and natural soil microcosm conditions showed that the biocontrol ability of W81 was mediated by proteolytic enzyme production (Fig. 1, Table 2). Whereas commercial chitinase had no effect on *P. ultimum* *in vitro*, exposure of the fungus to commercial protease or the proteolytic cell-free culture supernatants of wild-type W81, W81M2 or W81A1(pCU800) resulted in degradation of proteinaceous cell-wall components

and leakage of cell constituents (Fig. 2). Irreversible loss of mycelial growth ability further indicated that the antagonism of W81 towards *P. ultimum* was due to extracellular protease production. This finding is supported by the fact that in Oomycetes, such as *P. ultimum*, the cell wall is essentially free of chitin but does contain proteinaceous material (Mitchell & Hurwitz, 1965; Haran *et al.*, 1996). When assessed in soil microcosm assays, wild-type *S. maltophilia* W81 and its mutant derivatives colonized the rhizosphere of sugar beet at similar population levels, indicating that the absence of biocontrol ability was not due to a reduction in ecological fitness. The role of lytic enzymes in the ability of some strains of *S. maltophilia* to control phytopathogenic fungi has been hypothesized but, in contrast to this study, without genetic evidence (Kobayashi *et al.*, 1995; Berg *et al.*, 1996).

Early implementation of biocontrol mechanisms is required for effective control of *Pythium* spp. as germination of sporangia takes place rapidly in response to seed exudates (Nelson *et al.*, 1988), allowing infection of the plant within hours of sowing (Stasz *et al.*, 1980). Seed inoculation with W81 reduced the level of colonization of the seed by *Pythium* spp. 1 d after sowing, from 95% (untreated control) to 13% (Table 2). The level of fungal colonization of the seed up until germination (i.e. 4–7 d after sowing) remained below 20% in the proteolytic W81, W81M2 and W81A1(pCU800) treatments. Furthermore, wild-type W81, W81M2 and W81A1(pCU800) effectively reduced the extent of damping-off of sugar beet in soil microcosms (Table 2). The lack of biocontrol ability displayed by W81A1 and W81M1 can be attributed to the absence of extracellular protease activity. The coefficient of correlation between colonization of seed by *Pythium* spp. and percentage plant emergence (i.e. -0.94) was statistically significant. However, as many as 38% of all seeds colonized by *Pythium* spp. in the untreated control escaped damping-off, an indication that fungal colonization of the seed/seedling is a prerequisite but is not sufficient for expression of the disease. In fact, 1 d after sowing, when studying crushed seeds previously surface-sterilized by sequential immersion in methanol and sodium hypochlorite and rinsed in sterile quarter-strength Ringer's solution, no internal infection of the seeds by *Pythium* spp. was found. The levels of plant protection conferred by the proteolytic bacterial treatments were similar to that achieved by *P. fluorescens* strain F113, the biocontrol activity of which is mediated through the production of the secondary metabolite 2,4-diacetylphloroglucinol (Fenton *et al.*, 1992).

The exploitation of biocontrol agents in the field is limited by the sensitivity of biocontrol mechanisms to environmental conditions. For instance, biological control mediated through siderophore production is restricted to low-iron soil conditions (Scher & Baker, 1982), whereas the majority of antimicrobial secondary metabolites require iron availability for production (Keel *et al.*, 1989). The current study indicates that the biocontrol properties of *S. maltophilia* W81 against *P.*

ultimum are mediated through production of an extracellular protease, which takes place under a wide range of *in vitro* conditions (including under high-iron conditions or in the presence of readily available carbon and nitrogen sources). These are obviously beneficial traits when considering the fact that W81, to be effective, needs to exert its biocontrol potential in the rhizosphere, a heterogeneous and fluctuating environment (Foster *et al.*, 1983) in which the availability of nutrients (e.g. iron; Loper & Henkels, 1997) may vary in space and time. In contrast, casein protease production is iron-regulated in many other rhizobacteria (Sexton *et al.*, 1996; Dowling *et al.*, 1996). In *S. maltophilia* strain C17, extracellular protease production is constitutive but repressed by the easily metabolizable substrate maltose, probably via catabolite repression (Singer & Dettle, 1993). However, repression by maltose does not occur in W81.

In conclusion, a reduction in damping-off of soil-grown sugar beet was achieved following seed treatment with *S. maltophilia* W81, which exhibits antagonism towards *P. ultimum* mediated by production of an extracellular protease. We propose that protease production may be useful in the construction of novel, genetically modified biocontrol inoculants. Further work will also evaluate the possibility of developing consortia of biocontrol agents in which strains with extracellular protease activity will be combined with others producing antifungal secondary metabolites.

ACKNOWLEDGEMENTS

The authors thank Pat Higgins and Liam Burgess for technical assistance. We are also grateful to A. Millcamp (MSU) for useful discussion. This work was supported in part by grants awarded by the Irish Science and Technology Agency Forbairt (SC-96-349) and the Biotechnology programmes of DGXII of the European Commission (BIO2-CT92-0084, BIO2-CT93-0196, BIO2-CT93-0053, BIO2-CT94-3001, BIO4-CT96-0027, BIO4-CT96-0181 and FMRX-CT96-0039) to F.O'G. and by the US Department of Energy (DE-FG 0290ER20021) and the NSF Center for Microbial Ecology (DIR 8809640) to F. J. de B.

REFERENCES

Alabouvette, C., Lemanceau, P. & Steinberg, C. (1993). Recent advances in the biological control of fusarium wilts. *Pestic Sci* 37, 365–373.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. (1990). Basic local alignment search tool. *J Mol Biol* 215, 403–410.

Atlas, R. M. & Bartha, R. (1993). *Microbial Ecology: Fundamentals and Applications*. Redwood City, CA: Benjamin Cummings.

Becker, J. O. & Schwinn, F. J. (1993). Control of soil-borne pathogens with living bacteria and fungi: status and outlook. *Pestic Sci* 37, 355–363.

Berg, G., Marten, P. & Ballin, G. (1996). *Stenotrophomonas maltophilia* in the rhizosphere of oilseed rape: occurrence, characterisation and interaction with phytopathogenic fungi. *Microbiol Res* 151, 19–27.

Black, T. A., Cai, Y. & Wolk, C. P. (1993). Spacial expression and autoregulation of *hetR*, a gene involved in the control of heterocyst development in *Anabaena*. *Mol Microbiol* 9, 77–84.

Blake, R. C., II, Choate, D. M., Bardhan, S., Revis, N., Barton, L. L. & Zocco, T. G. (1993). Chemical transformation of toxic metals by a *Pseudomonas* strain from a toxic waste site. *Environ Toxicol Chem* 12, 1365–1376.

Boyer, H. W. & Roulland-Dussoix, D. (1969). A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. *J Mol Biol* 41, 459–472.

Carroll, H., Moënné-Loccoz, Y., Dowling, D. N. & O'Gara, F. (1995). Mutational disruption of the biosynthesis genes coding for the antifungal metabolite 2,4-diacetylphloroglucinol does not influence the ecological fitness of *Pseudomonas fluorescens* F113 in the rhizosphere of sugarbeets. *Appl Environ Microbiol* 61, 3002–3007.

Casadaban, M. & Cohen, S. N. (1980). Analysis of gene control signals by DNA fusion and cloning in *Escherichia coli*. *J Mol Biol* 138, 179–207.

Castric, K. F. & Castric, P. A. (1983). Method for rapid detection of cyanogenic bacteria. *Appl Environ Microbiol* 45, 701–702.

Chernin, L., Ismailov, Z., Haran, S. & Chet, I. (1995). Chitinolytic *Enterobacter agglomerans* antagonistic to fungal plant pathogens. *Appl Environ Microbiol* 61, 1720–1726.

Cohen, M. F., Meeks, J. C., Cai, Y. A. & Wolk, C. P. (1997). Transposon mutagenesis of heterocyst-forming filamentous cyanobacteria. *Methods Enzymol* (in press).

Cook, R. J. (1993). Making greater use of introduced microorganisms for biological control of plant pathogens. *Annu Rev Phytopathol* 31, 53–80.

Cook, R. J., Thomashow, L. S., Weller, D. M., Fujimoto, D., Mazzola, M., Bangera, G. & Kim, D. S. (1995). Molecular mechanisms of defence by rhizobacteria against root disease. *Proc Natl Acad Sci USA* 92, 4197–4201.

Cronin, D., Moënné-Loccoz, Y., Fenton, A., Dunne, C., Dowling, D. N. & O'Gara, F. (1997). Role of 2,4-diacetylphloroglucinol in the interactions of the biocontrol pseudomonad strain F113 with the potato cyst nematode *Globodera rostochiensis*. *Appl Environ Microbiol* 63, 1357–1361.

Ditta, G., Stanfield, S., Corbin, D. & Helinski, D. R. (1980). Broad host range DNA cloning system for gram-negative bacteria: construction of a gene bank of *Rhizobium meliloti*. *Proc Natl Acad Sci USA* 77, 7347–7351.

Dowling, D. N. & O'Gara, F. (1994). Metabolites of *Pseudomonas* involved in the biocontrol of plant disease. *Trends Biotechnol* 12, 133–141.

Dowling, D. N., Sexton, R., Fenton, A., Delany, I., Fedi, S., McHugh, B., Callanan, M., Moënné-Loccoz, Y. & O'Gara, F. (1996). Iron regulation in plant-associated *Pseudomonas fluorescens* M114: implications for biological control. In *Molecular Biology of Pseudomonads*, pp. 502–511. Edited by T. Nakazawa, K. Furukawa, D. Haas & S. Silver. Washington, DC: American Society for Microbiology.

Dunne, C., Delany, I., Fenton, A., Lohrke, S., Moënné-Loccoz, Y. & O'Gara, F. (1996). The biotechnology and application of *Pseudomonas* inoculants for the biocontrol of phytopathogens. In *Biology of Plant-Microbe Interactions*, pp. 441–448. Edited by G. Stacey, B. Mullin & P. M. Gresshoff. St Paul, MI: International Society for Molecular Plant-Microbe Interactions.

Dunne, C., Delany, I., Fenton, A. & O'Gara, F. (1997). Mechanisms involved in biocontrol by microbial inoculants. *Agronomie* 16, 721–729.

Fedi, S., Brazil, D., Dowling, D. N. & O'Gara, F. (1996). Construction of a modified mini-Tn5 *lacZY* non-antibiotic marker

cassette: ecological evaluation of a *lacZY* marked *Pseudomonas* strain in the sugarbeet rhizosphere. *FEMS Microbiol Lett* 135, 251–257.

Fenton, A. M., Stephens, P. M., Crowley, J., O'Callaghan, M. & O'Gara, F. (1992). Exploitation of gene(s) involved in 2,4-diacetylphloroglucinol biosynthesis to confer a new biocontrol capability to a *Pseudomonas* strain. *Appl Environ Microbiol* 58, 3873–3878.

Fernandez-Pinas, F., Leganes, F. & Wolk, C. P. (1994). A third locus required for the formation of heterocysts in *Anabaena* sp. strain PCC7120. *J Bacteriol* 176, 5277–5283.

Flores, A., Chet, I. & Herrera-Estrella, A. (1997). Improved biocontrol activity of *Trichoderma harzianum* by overexpression of the proteinase-encoding gene *prb1*. *Curr Genet* 31, 30.

Foster, R. C., Rovira, A. D. & Cock, T. W. (1983). *Ultrastructure of the Root-Soil Interface*. St Paul, MN: American Phytopathological Society.

Fridlender, M., Inbar, J. & Chet, I. (1993). Biological control of soilborne plant pathogens by a β -1,3 glucanase-producing *Pseudomonas cepacia*. *Soil Biol Biochem* 25, 1211–1221.

Gaffney, T. D., Lam, S. T., Ligon, J. and 9 other authors (1994). Global regulation of expression of antifungal factors by a *Pseudomonas fluorescens* biological control strain. *Mol Plant-Microbe Interact* 7, 455–463.

Geremia, R. A., Goldman, G. H., Jacobs, D., Ardiles, W., Vila, S. B., Van Montagu, M. & Herrera-Estrella, A. (1993). Molecular characterisation of the proteinase-encoding gene, *prb1*, related to mycoparasitism by *Trichoderma harzianum*. *Mol Microbiol* 8, 603–613.

Gould, W. D., Hagedorn, C., Bardinelli, T. R. & Zablotowicz, R. M. (1985). New selective media for enumeration and recovery of fluorescent pseudomonads from various habitats. *Appl Environ Microbiol* 49, 28–32.

Haran, S., Schickler, H. & Chet, I. (1996). Molecular mechanisms of lytic enzymes involved in the biocontrol activity of *Trichoderma harzianum*. *Microbiology* 142, 2321–2331.

Hrabak, E. M. & Willis, D. K. (1993). Involvement of the *lemA* gene in production of syringomycin and protease by *Pseudomonas syringae* pv. *syringae*. *Mol Plant-Microbe Interact* 6, 368–375.

Jeffers, S. N. & Martin, S. B. (1986). Comparison of two media selective for *Phytophthora* and *Pythium* spp. *Plant Dis* 70, 1038–1043.

Keel, C. & Dfago, G. (1997). Interactions between beneficial soil bacteria and root pathogens: mechanisms and ecological impact. In *Multitrophic Interactions in Terrestrial Systems*, pp. 27–46. Edited by A. C. Gange & V. K. Brown. Oxford: Blackwell Scientific Publications.

Keel, C., Voisard, C., Berling, C. H., Kahr, G. & Dfago, G. (1989). Iron sufficiency, a prerequisite for the suppression of tobacco black root rot by *Pseudomonas fluorescens* strain CHA0 under gnotobiotic conditions. *Phytopathology* 79, 584–589.

Keel, C., Schnider, U., Maurhofer, M., Voisard, C., Laville, J., Burger, U., Wirthner, P., Haas, D. & Dfago, G. (1992). Suppression of root diseases by *Pseudomonas fluorescens* CHA0: importance of the bacterial secondary metabolite 2,4-diacetylphloroglucinol. *Mol Plant-Microbe Interact* 5, 4–13.

Kobayashi, D. Y., Guglielmoni, M. & Clarke, B. B. (1995). Isolation of the chitinolytic bacteria *Xanthomonas maltophilia* and *Serratia marcescens* as biological control agents for summer patch disease of turfgrass. *Soil Biol Biochem* 27, 1479–1487.

Kragelund, L., Christoffersen, B., Nybroe, O. & de Bruijn, F. J. (1995). Isolation of *lux* reporter gene fusions in *Pseudomonas*

fluorescens DF57 inducible by nitrogen or phosphorus starvation. *FEMS Microbiol Ecol* 17, 95–106.

Laville, J., Voisard, C., Keel, C., Maurhofer, M., Dfago, G. & Haas, D. (1992). Global, stationary-phase control in *Pseudomonas fluorescens* mediating antibiotic synthesis and suppression of black root rot of tobacco. *Proc Natl Acad Sci USA* 89, 1562–1566.

Leeman, M., van Pelt, J. A., den Ouden, F. M., Heinsbroek, M., Bakker, P. A. H. M. & Schippers, B. (1995). Induction of systemic resistance by *Pseudomonas fluorescens* in radish cultivars differing in susceptibility to fusarium wilt, using a novel bioassay. *Eur J Plant Pathol* 101, 655–664.

Lemanceau, P. & Alabouvette, C. (1991). Biological control of fusarium diseases by fluorescent *Pseudomonas* and non-pathogenic *Fusarium*. *Crop Prot* 10, 279–286.

Loper, J. E. & Henkels, M. D. (1997). Availability of iron to *Pseudomonas fluorescens* in rhizosphere and bulk soil evaluated with an ice nucleation reporter gene. *Appl Environ Microbiol* 63, 99–105.

Lorito, M., Peterbauer, C., Hayes, C. K. & Harman, G. E. (1994). Synergistic interaction between fungal cell wall degrading enzymes and different antifungal compounds enhances inhibition of spore germination. *Microbiology* 140, 623–629.

McInroy, J. A. & Kloepper, J. W. (1994). Studies on indigenous endophytic bacteria on sweet corn and cotton. In *Molecular Ecology of Rhizosphere Microorganisms*, pp. 19–28. Edited by F. O'Gara, D. N. Dowling & B. Boesten. Weinheim: VCH.

McKellar, R. C. (1981). Development of off-flavour in ultra-high temperature and pasteurized milk is a function of proteolysis. *J Dairy Sci* 64, 2138–2145.

Mazzola, M., Stahlman, P. W. & Leach, J. E. (1995). Application method affects the distribution and efficacy of rhizobacteria suppressive of downy brome (*Bromus tectorum*). *Soil Biol Biochem* 27, 1271–1278.

Mitchell, R. & Hurwitz, E. (1965). Suppression of *Pythium debaryanum* by lytic rhizosphere bacteria. *Phytopathology* 55, 156–158.

Nelson, E. B., Harman, G. E. & Nash, G. T. (1988). Enhancement of *Trichoderma* induced biological control of *Pythium* seed rot and pre-emergence damping-off of peas. *Soil Biol Biochem* 20, 145–150.

Oppenheim, A. B. & Chet, I. (1992). Cloned chitinases in fungal plant-pathogen control strategies. *Trends Biotechnol* 10, 392–394.

O'Sullivan, D. J. & O'Gara, F. (1992). Traits of fluorescent *Pseudomonas* spp. involved in suppression of plant root pathogens. *Microbiol Rev* 56, 662–676.

Palleroni, N. J. & Bradbury, J. F. (1993). *Stenotrophomonas*, a new bacterial genus for *Xanthomonas maltophilia* (Hugh 1980) Swings et al., 1983. *Int J Syst Bacteriol* 43, 606–609.

Sacherer, P., Dfago, G. & Haas, D. (1994). Extracellular protease and phospholipase C are controlled by the global regulatory gene *gacA* in the biocontrol strain *Pseudomonas fluorescens* CHA0. *FEMS Microbiol Lett* 116, 155–160.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Scher, F. M. & Baker, R. (1982). Effect of *Pseudomonas putida* and a synthetic iron chelator on induction of soil suppressiveness to *Fusarium* wilt pathogens. *Phytopathology* 72, 1567–1573.

Schwyn, B. & Neilands, J. B. (1987). Universal chemical assay for the detection and determination of siderophores. *Anal Biochem* 160, 47–56.

- Sexton, R., Gill, P. R., Jr, Dowling, D. N. & O'Gara, F. (1996). Transcriptional regulation of the iron-responsive sigma factor gene *pbrA*. *Mol Gen Genet* **250**, 50–58.
- Shanahan, P., O'Sullivan, D. J., Simpson, P., Glennon, J. D. & O'Gara, F. (1992). Isolation of 2,4-diacetylphloroglucinol from a fluorescent pseudomonad and investigation of physiological parameters influencing its production. *Appl Environ Microbiol* **58**, 353–358.
- Simon, R., Priefer, U. & Pühler, A. (1983a). Vector plasmids for *in vivo* and *in vitro* manipulations of gram-negative bacteria. In *Molecular Genetics of the Bacteria-Plant Interaction*, pp. 98–106. Edited by A. Pühler. Berlin & Heidelberg: Springer.
- Simon, R., Priefer, U. & Pühler, A. (1983b). A broad host range mobilisation system for *in vivo* genetic engineering: transposon mutagenesis in gram-negative bacteria. *Bio/Technology* **1**, 784–791.
- Simon, R., Quandt, J. & Klipp, W. (1989). New derivatives of transposon Tn5 suitable for mobilisation of replicons, generation of operon fusions and induction of genes in gram-negative bacteria. *Gene* **80**, 161–169.
- Singer, E. & Dobbie, J. (1993). Nutritional factors controlling exocellular proteinase production in a soil-isolated *Xanthomonas maltophilia* strain. *J Basic Microbiol* **33**, 113–121.
- Stasz, T. E., Harman, G. E. & Marx, G. A. (1980). Time and site of infection of resistant and susceptible germinating pea seeds by *Pythium ultimum*. *Phytopathology* **70**, 730–733.
- Swings, J., De Vos, P., Van den Mooter, M. & De Ley, J. (1983). Transfer of *Pseudomonas maltophilia* Hugh 1981 to the genus *Xanthomonas* as *Xanthomonas maltophilia* (Hugh 1981) comb. nov. *Int J Syst Bacteriol* **33**, 409–413.
- Thomashow, L. S. & Weller, D. M. (1988). Role of a phenazine antibiotic from *Pseudomonas fluorescens* in biological control of *Gaeumannomyces graminis* var. *tritici*. *J Bacteriol* **170**, 3499–3508.
- Voisard, C., Bull, C. T., Keel, C., Laville, J., Maurhofer, M., Schnider, U., Défago, G. & Haas, D. (1994). Biocontrol of root diseases by *Pseudomonas fluorescens* CHA0: current concepts and experimental approaches. In *Molecular Ecology of Rhizosphere Microorganisms*, pp. 67–69. Edited by F. O'Gara, D. N. Dowling & B. Boesten. Weinheim: VCH.
- Wilson, M. & Lindow, S. E. (1994). Coexistence among epiphytic bacterial populations mediated through nutritional resource partitioning. *Appl Environ Microbiol* **60**, 4468–4477.
- Wolk, C. P., Cai, Y. & Panoff, J. M. (1991). Use of a transposon with luciferase as a reporter to identify environmentally responsive genes in a cyanobacterium. *Proc Natl Acad Sci USA* **88**, 5355–5359.

Received 11 July 1997; revised 26 August 1997; accepted 3 September 1997.