Overproduction of an inducible extracellular serine protease improves biological control of *Pythium ultimum* by *Stenotrophomonas maltophilia* strain W81

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***Stenotrophomonas maltophilia* W81 can protect sugar beet against *Pythium*-mediated damping-off disease through the production of an extracellular protease. Here, the proteolytic enzyme of W81 was purified by anion-exchange chromatography and characterized as a serine protease. The purified enzyme was fungicidal against *Pythium ultimum* *in vitro*. Its synthesis was inducible by casein in W81, and mutagenesis of this strain using the luciferase (*luxAB*) reporter transposon Tn5-764cd resulted in the isolation of two mutant derivatives (W81M3 and W81M4) capable of producing significantly increased levels of extracellular protease in the presence of casein. Strain W81M4 also exhibited increased chitinolytic activity. The *luxAB* fusions in strains W81M3 and W81M4 were highly expressed in the absence of casein but not in its presence, suggesting that the corresponding loci were involved in down-regulating extracellular protease production. Extracellular protease production in the W81 wild-type strain and protease overproduction in mutants W81M3 and W81M4 were also induced in the presence of the autoclaved fungal mycelium. In soil microcosms naturally infested by *Pythium* spp., inoculation of sugar beet seeds with W81M3 or W81M4 resulted in improved biocontrol of *Pythium*-mediated damping-off disease compared with W81, and the level of protection achieved was equivalent to that conferred by chemical fungicides. The wild-type W81 and its mutant derivatives did not differ in rhizosphere colonization. Therefore, the improved biocontrol ability of W81M3 and W81M4 resulted from their capacity to overproduce extracellular serine protease.**

Keywords: plant protection, phytopathogenic fungi, bacterial inoculants, serine protease, overproduction.

INTRODUCTION

Phytopathogenic fungi, such as *Pythium* species (oomycetes) which mediate damping-off disease, cause persistent and significant losses in annual crop yields

(Agrios, 1997). Effective chemical control of pre-emergence damping-off can be achieved by the addition of synthetic fungicides to the seed prior to sowing, but these chemicals are of little use against post-emergence damping-off. Furthermore, public health and environmental concerns have focused scientific interest on the development of environmentally acceptable alternatives to chemical fungicides (Cook, 1993; Dunne *et al.*, 1996). Biological control represents such an alternative strategy by exploiting the negative interactions that occur between disease-suppressive micro-organisms and plant pathogens (Cook *et al.*, 1995; Handelsman & Stabb, 1996). The development of successful biocontrol strategies, which take into consideration the complexity

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Abbreviations: AEBSF, 4-(2-aminoethyl)benzenesulphonyl fluoride; Cm, chloramphenicol; Km, kanamycin; Rif, rifampicin; Sm, streptomycin; Tc, tetracycline; TNBS, 2,4,6-trinitrobenzenesulphonic acid.

of the soil environment and generate biocontrol agents with consistent performance, relies on a thorough understanding of the interactions between the fungal pathogen, its host and the introduced biocontrol agent, and the ecological impact of the microbial inoculant in the soil environment (Dowling *et al.*, 1996; Handelsman & Stabb, 1996; Fedi *et al.*, 1997; Lugtenberg & Dekkers, 1999).

Beneficial micro-organisms of interest for biocontrol of soil-borne pathogens and pests have been identified among fungi, actinomycetes and bacteria (Becker & Schwinn, 1993; Dunne *et al.*, 1997a; Keel & Défago, 1997) which produce a variety of antifungal factors such as siderophores (Becker & Cook, 1988; O'Sullivan & O'Gara, 1992), secondary metabolites (Dowling & O'Gara, 1994; Nielsen *et al.*, 1998; Bangera & Thomashow, 1999; Delany *et al.*, 2000) and hydrolytic enzymes (Dunne *et al.*, 1997b, 1998; Thrane *et al.*, 1997). Protection of plants by bacteria has also been successfully mediated by the induction of systemic plant resistance (Van Wees *et al.*, 1997).

Recent *in vitro* studies have demonstrated that the exposure of selected phytopathogenic fungi to lytic enzymes such as chitinases, proteases or glucanases can result in the degradation of the structural matrix of fungal cell walls (Lorito *et al.*, 1994; Dunne *et al.*, 1997b). Exposure of the fungus *Pythium ultimum*, an oomycete lacking significant amounts of chitin in its cell wall (Mitchell & Hurwitz, 1965; Dietrich, 1973), to endo-1,3- β -glucanase and cellulase enzymes purified from *Trichoderma harzianum* culture supernatants inhibited germination of encysted zoospores and prevented elongation of germ tubes (Thrane *et al.*, 1997). The role of a proteolytic enzyme produced by the mycoparasitic fungus *T. harzianum* in biological control of fungal pathogens has been clearly demonstrated (Geremia *et al.*, 1993; Haran *et al.*, 1996). Further studies have shown that overproduction of the inducible *T. harzianum* extracellular protease resulted in enhanced biocontrol efficacy against the fungal pathogen *Rhizoctonia solani* (Flores *et al.*, 1997).

The Gram-negative rod *Stenotrophomonas maltophilia* (formerly *Xanthomonas maltophilia*) is a typical soil inhabitant (Bollet *et al.*, 1995; Berg *et al.*, 1998) which has been the focus of scientific interest due to its potential for bioremediation (Boonchan *et al.*, 1998) and for biological control (Berg *et al.*, 1996; Jakobi *et al.*, 1996; Giesler & Yuen, 1998). *S. maltophilia* W81 is a sugar beet rhizosphere isolate capable of conferring protection against *Py. ultimum*-mediated damping-off (Dunne *et al.*, 1997b, 1998). Mutagenesis of *S. maltophilia* W81 with Tn5-764cd demonstrated that this ability is mediated by proteolytic enzyme production (Dunne *et al.*, 1997b). In Tn5-764cd-carrying mutants, *lux* reporter genes are under the control of resident promoters, enabling gene-expression studies by measurement of bioluminescence. Indeed, the original transposon Tn5-764 (Wolk *et al.*, 1991) and other related luciferase reporter-gene transposons have been used to identify inducible genes in *Pseudomonas* (Kragelund *et al.*, 1995),

Sinorhizobium (Milcamps *et al.*, 1998; Milcamps & de Bruijn, 1999) and cyanobacteria (Wolk *et al.*, 1991).

The objective of this study was to assess whether enhancement of the extracellular protease activity displayed by *S. maltophilia* W81 could result in improved suppression of *Py. ultimum*-mediated damping-off disease. To achieve this goal, Tn5-764cd derivatives of W81 displaying increased proteolytic activities were developed and compared with the wild-type *in vitro* and in natural soil microcosms grown with sugar beets.

METHODS

Micro-organisms, plasmids and growth conditions. Bacterial strains and plasmids used in the study are described in Table 1. Strain W81 was previously identified (Dunne *et al.*, 1997b) as *S. maltophilia* (Palleroni & Bradbury, 1993). *S. maltophilia* W81 and its mutants were routinely grown in Luria-Bertani (LB) broth (Sambrook *et al.*, 1989) at 28 °C, *Pseudomonas fluorescens* strain F113Rif in sucrose asparagine (SA; Scher & Baker, 1982) at 28 °C and *Escherichia coli* in LB broth (at 37 °C). Antibiotics were added to growth media at the following concentrations: rifampicin (Rif), 100 $\mu\text{g ml}^{-1}$; streptomycin (Sm), 100 $\mu\text{g ml}^{-1}$; kanamycin (Km), 25 $\mu\text{g ml}^{-1}$; tetracycline (Tc), 25 $\mu\text{g ml}^{-1}$; and chloramphenicol (Cm), 30 $\mu\text{g ml}^{-1}$. Bacteria were maintained in glycerol solutions at -20 °C. *Py. ultimum* was obtained from the International Mycological Institute (IMI 308273) and was maintained on corn meal agar (Difco).

FPLC isolation and identification of *S. maltophilia* W81-produced protease. *S. maltophilia* W81 and the mutant W81M1, which is unable to produce extracellular protease due to Tn5-*lux* insertion into the protease structural gene (Dunne *et al.*, 1997b), were grown overnight in a minimal medium containing 0.1% glucose and 1% skim milk, as described previously (Dunne *et al.*, 1997b). Cultures were centrifuged at 3000 g and supernatants were filter-sterilized and concentrated ($\times 20$) by filtration through Centricon spin columns with a 10 kDa cut-off (Amicon). Concentrates were dialysed against 50 mM Tris/HCl (pH 7.5) for 4 h and proteins were separated on a DEAE-Sephacel anion-exchange chromatography column (1.6 $\times$ 20 cm; Pharmacia) equilibrated with the same buffer. Bound proteins were eluted with a gradient between 0 and 500 mM NaCl in the same Tris/HCl buffer. Ten millilitre fractions were collected using the FPLC Gradifrac system (Pharmacia). Each fraction was assessed for protein content by measuring the absorbance at 280 nm. Fractions were assessed for proteolytic enzymic activity using the fluorimetric assay described by Twining (1984), whereby 80 μl FPLC-derived samples were incubated in the presence of 80 μl FITC-labelled casein at 28 °C for 2 h (reaction mix pH 6.5). Insoluble material was then removed by centrifugation at 3000 g. Eighty microlitres of each supernatant was then treated with 300 μl 5% TCA. Protein was allowed to precipitate at room temperature for a minimum of 1 h, and was then removed by centrifugation. Sixty microlitres of supernatant was neutralized by addition of 3 ml 0.5 M Tris, pH 8.5. Fluorescence was measured at 490 nm using a Beckman DU640 spectrophotometer and cuvettes of 1 cm pathlength. One thousand units of enzyme activity were arbitrarily equated to 1 unit of fluorescence. The observed proteolytic activity was characterized biochemically through the addition singly of 10 individual protease inhibitors [antipain dihydrochloride, bestatin, chymostatin, E-64, leupeptin, pepstatin, phosphoramidon, 4-(2-aminoethyl)-

Table 1. Bacterial strains and plasmids used in the study

Strain	Relevant characteristics	Source or reference
Strains		
<i>S. maltophilia</i>		
W81	Wild-type, Sm ^r Km ^r Phl ⁻ Hcn ⁻ Flu ⁻ Chi ⁺ Prt ⁺	Dunne <i>et al.</i> (1997b)
W81M1	Tc ^r Prt ⁻ , Tn5-764cd mutant of W81	Dunne <i>et al.</i> (1997b)
W81M2	Tc ^r Chi ⁻ , Tn5-764cd mutant of W81	Dunne <i>et al.</i> (1997b)
W81M3	Tc ^r Prt ⁺⁺ , Tn5-764cd mutant of W81	This study
W81M4	Tc ^r Chi ⁺⁺ Prt ⁺⁺ , Tn5-764cd mutant of W81	This study
W81A1	Tc ^r Chi ⁻ Prt ⁻ , Tn5-B50 mutant of W81	Dunne <i>et al.</i> (1997b)
W81A1(pCU800)	Tc ^r Cm ^r , complemented mutant of W81A1	Dunne <i>et al.</i> (1997b)
<i>Ps. fluorescens</i>		
F113Rif	Phl ⁺ Hcn ⁺ Flu ⁺ Rif ^r	Carroll <i>et al.</i> (1995)
<i>E. coli</i>		
HB101	<i>recA hsdB hsdM strA pro leu thi</i>	Boyer & Roulland-Dussoix (1969)
DH5 α	ϕ 80 <i>lacZ</i> Δ M15 Δ (<i>lacZYA-argF</i>)U169 <i>hsdR17 recA1 endA1 thi-1</i>	Sambrook <i>et al.</i> (1989)
Plasmids		
pCU800	IncQ Cm ^r Tc ^r broad-host-range cosmid pSUP106 with a 15 kb insert from W81	Dunne <i>et al.</i> (1997b)
pRK2013	Mob ⁺ Tra ⁺ Km ^r	Ditta <i>et al.</i> (1980)
pRL764cd	Tc ^r LuxAB ⁺	Dunne <i>et al.</i> (1997b)

benzenesulphonyl fluoride (AEBSF), EDTA and aprotinin; Boehringer Mannheim], each of which is capable of inhibiting the activity of specific families of proteolytic enzyme, and the subsequent assessment of the remaining enzymic activity, as described above. Each protease inhibitor was tested twice.

Susceptibility of *Py. ultimum* to W81-produced protease *in vitro*. *Py. ultimum* mycelium was prepared by growth in LB broth as described previously (Dunne *et al.*, 1997b). Collected mycelium was then resuspended in fresh LB broth containing commercial protease (EC 3.4.21.14, 0.5–1.0 units ml⁻¹; Sigma) or FPLC-derived samples of wild-type W81 or W81M1 supernatant and incubated for 48 h at 28 °C. Since W81 is chitinase-positive, the experiment was also performed using commercial chitinase from *Streptomyces griseus* (EC 3.2.1.14, 0.5–1.0 units ml⁻¹; Sigma).

As described previously (Dunne *et al.*, 1997b), treated mycelium was examined microscopically, both in wet mounts and following staining with anionic dyes. Remaining samples were assayed for fungal growth, as follows: *Pythium* mycelium was washed in quarter-strength Ringer's solution and placed in the centre of LB agar, potato dextrose agar (PDA; Oxoid), malt extract agar (MEA; Oxoid) or nutrient agar (NA; Oxoid). Growth of the fungus was assessed following incubation of the plates for 7 d at 28 °C. In all assays, untreated *Pythium* mycelium was used as a control and fungal growth was observed on each medium tested.

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 was performed using the standard CaCl₂ heat-shock method. Recombinant DNA manipulations were performed as outlined by Sambrook *et al.* (1989). All digestions and ligations were performed using enzymes from Promega or Boehringer Mannheim.

Tn5-764cd mutagenesis of the Sm^r strain W81 was performed following procedures previously described by Kragelund *et al.*

(1995), using pRL764cd. Tn5-764cd is a tetracycline-resistant derivative of pRL764 (Wolk *et al.*, 1991) developed to overcome the natural resistance of strain W81 to kanamycin (Dunne *et al.*, 1997b). The resulting Tn5-764cd insertion derivatives (Sm^r Tc^r) were screened for chitinolytic and proteolytic activities on solid media, as follows. Chitinolytic activity was assessed qualitatively using the solid chitin minimal medium described by Dunne *et al.* (1997b), and proteolytic activity was observed 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 either inoculated directly onto the solid media or, alternatively, 100 μ l filter-sterilized supernatant from bacterial cultures grown in SA or LB, obtained after concentration ($\times$ 500) using an Amicon ultra-filtration unit, were spotted onto the plates. These plates were incubated overnight at 28 °C. Both chitin- and skim milk-containing plates are opaque and enzymic activity was identified by the development of a zone of clearing (halo) surrounding the colonies and the cell-free supernatant spots. Mutants capable of overproducing protease (W81M3) or both chitinase and protease (W81M4) were identified.

Quantitative determination of extracellular chitinolytic and proteolytic activities. Chitinolytic and proteolytic activities were also quantified biochemically. Extracellular chitinolytic activity was determined colorimetrically, as described by Boller & Mauch (1988). Extracellular proteolytic activity was measured using a modification (described by Dunne *et al.*, 1997b) of the colorimetric procedure of McKellar (1981), whereby precipitated material resulting from proteolysis of Hammersten casein (BDH) buffered with 1 M MOPS and 15 mM CaCl₂, pH 6.5 reacts with 0.1% 2,4,6-trinitrobenzenesulphonic acid (TNBS), allowing colour development. Alternatively, the fluorimetric assay described by Twining (1984) was used (see above).

Induction of bioluminescence and/or proteolytic activity in Tn5-764cd mutants of *S. maltophilia* W81. Mutants of *S. maltophilia* W81 carrying Tn5-*lux* fusions were grown at

28 °C for 12 h in either LB broth or a minimal medium containing 0.1 % glucose (Dunne *et al.*, 1997b), in the presence of chitin, casein or *Py. ultimum* mycelium, to assess the latter for possible induction of bioluminescence or proteolysis. Proteolysis was measured using Twinning's fluorimetric assay (see above).

Bioluminescence was first assayed by placing 50 µl n-decylaldehyde (Sigma) on the inside cover of a glass Petri dish, which was then used to cover colony-containing plates for a period of 60 s. Luminescent colonies were then visualized using a Hamamatsu photonic camera system (model C1966-20; Photonic Microscopy) for a further 60 s, as described by Kragelund *et al.* (1995). Bioluminescence was then measured with a Lumac Biocounter M1500 by placing 2 ml bacterial culture into a glass cuvette and quantifying the resulting luminescence following the addition of 100 µl 5 % n-decylaldehyde in ethanol.

Growth rate determinations. Growth rates of *S. maltophilia* W81 and its mutant derivatives were determined in liquid LB incubated with aeration at 28 °C. At hourly intervals, 1 ml samples of the cultures were aseptically removed, OD₆₀₀ measured and cell numbers determined on solid LB containing Sm or Sm plus Tc for wild-type W81 or its mutant derivatives, respectively.

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 chosen is naturally infested by *Pythium* spp. [> 1000 propagules (g soil)⁻¹ detected using a *Pythium*-selective medium (Jeffers & Martin, 1986)] and conducive to disease development. Soil handling and microcosm preparation (140 g soil per pot) were as described by Fenton *et al.* (1992) and Moënné-Loccoz *et al.* (1999). Sugar beet seeds (cv. Accord) were inoculated by dipping them in cell suspensions of the bacteria studied (approx. 10⁶ c.f.u. per seed). The latter included wild-type W81; W81M1, deficient in protease production; W81M2, deficient in chitinase production; W81M3, capable of protease overproduction; W81M4, capable of overproducing both chitinase and protease; W81A1, unable to produce either lytic enzyme; W81A1(pCU800), with restored lytic enzyme production; and the biocontrol agent *Ps. fluorescens* F113Rif (Carroll *et al.*, 1995; Moënné-Loccoz *et al.*, 1998). The untreated control consisted of seeds dipped in quarter-strength Ringer's solution. The commercial control involved treating seeds with 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. Nine seeds were sown per pot. 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 at 1 d after sowing, as previously described (Dunne *et al.*, 1997b). Sugar beet 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. At 48 h the seeds were scored for the presence of *Pythium* spp. The percentage of 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 Sm plus Km (for wild-type W81), LB containing Sm plus Km plus Tc (for mutant derivatives of W81), or SA containing Rif (for F113Rif). Extracts were made, each from a whole root system with its closely adhering soil (Dunne *et al.*, 1998). 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 tests. All *in vitro* experiments were performed with three replicates of each treatment and were repeated at least three times. The effects of treatments were studied by analysis of variance and Fisher's LSD tests ($P < 0.05$).

A randomized complete block design was used for the soil microcosm experiments, which were performed on three independent occasions. Seed infection by *Pythium* spp. was studied using ten seeds from each of three replications for each treatment. 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 (Moënné-Loccoz *et al.*, 1999). Percentages were arcsin-transformed and colony counts were log-transformed prior to analysis. Within each run of the experiment, analysis of variance was performed at each sampling time using the General Linear model procedures of the Statistical Analysis System (SAS Institute Inc.). 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 completed and representative results (obtained in one of the three runs) are shown in Fig. 3.

RESULTS

Purification and characterization of the *S. maltophilia* W81 extracellular protease

The extracellular protease synthesized by *S. maltophilia* W81 was purified by DEAE-Sephacel anion-exchange chromatography. Protein content analysis resulted in the identification of two distinct peaks (Fig. 1a). Only fractions corresponding to the first peak (i.e. fractions 3–5) displayed proteolytic activity when assayed with FITC-labelled casein (Fig. 1b). Similar procedures were followed using strain W81M1, a transposon-induced mutant of W81 deficient in extracellular protease activity (Dunne *et al.*, 1997b). Its protein profile was similar to that of the wild-type strain, except for the distinctive first peak present in the W81 fractions which was absent in the mutant strain (Fig. 1a). Furthermore, none of the fractions obtained from strain W81M1 culture supernatant exhibited proteolytic activity (Fig. 1b). The extracellular proteolytic activity of the wild-type strain was characterized using a series of ten specific protease inhibitors. Only one of these inhibitors, AEBSF, affected the FITC-labelled-casein-degrading activity of the enzyme. This indicates that this extracellular protease produced by W81 is a serine protease.

Effect of the *S. maltophilia* W81-produced serine protease on *Py. ultimum* *in vitro*

Concentrated cell-free culture supernatant of strain W81 (but not that of mutant strain W81M1) caused degradation of the cell wall of *Py. ultimum* (Dunne *et al.*, 1997b). In the current study, we found that exposure of *Py. ultimum* mycelium to purified W81 serine protease resulted in cell-wall degradation (data not shown). Subsequent washing and plating of the treated mycelium

Fig. 1. Purification of a proteolytic enzyme by DEAE-Sephacel anion-exchange chromatography from culture supernatant of *S. maltophilia* W81. Analysis of the protein content of the collected FPLC fractions (a) shows that an obvious peak (fractions 3, 4 and 5) present with wild-type W81 ($\square$) is absent with protease-deficient mutant W81M1 ($\blacktriangle$). Fluorimetric assessment of the proteolytic activity of the collected fractions (b) demonstrates that enzymic activity is associated only with this peak.

on several solid media where *Py. ultimum* is routinely grown indicated that the effect of the serine protease was fungicidal. Degradation of the fungal cell wall and irreversible loss of growth ability were also observed when the experiment was performed using a commercial protease. In contrast, the non-proteolytic FPLC fractions obtained from strain W81, as well as all fractions from the protease-negative mutant strain W81M1, had no effect on the fungus. This confirms that the effect of W81 on *Py. ultimum* was mediated by its extracellular protease.

Transposon-induced mutants of *S. maltophilia* W81 displaying increased extracellular protease production

Mutants of strain W81 overproducing extracellular protease were obtained by mutagenesis with the transposon Tn5-764cd. Approximately 15000 Tc^r colonies were screened on solid medium containing casein and mutants with increased extracellular protease activity were apparent from the diameter of the haloes of casein hydrolysis surrounding colonies (Fig. 2a). The mutant strains W81M3 and W81M4 produced zones of hydrolysis with diameters approximately three times greater than that of the halo around the wild-type strain W81. When the colorimetric TNBS assay was used, it

Fig. 2. Proteolytic activity and bioluminescence of transposon-induced mutants of *S. maltophilia* W81. Mutants W81M3 and W81M4 capable of increased protease production are apparent in (a) as larger haloes caused by increased degradation of casein. These mutants are luminescent in the absence of a proteinaceous inducer (b) but are repressed in the presence of casein or *Py. ultimum* mycelium.

appeared that strains W81M3 and W81M4 produced approximately three times the amount of extracellular protease synthesized by strain W81 (Table 2). Strain W81M4 also displayed enhanced chitinase activity on chitin plates when compared with W81 (data not shown). Fluorimetric quantification experiments demonstrated that mutant W81M4 produced approximately three times as much chitinase as the wild-type strain (Table 2).

Mutant strains W81M3 and W81M4, in addition to two other Tn5-764cd mutants of W81 obtained in a previous investigation (W81M1 and W81M2; Dunne *et al.*, 1997b), were compared by Southern hybridization, using ³⁵S-labelled Tn5-764cd DNA as a probe (as described by Dunne *et al.*, 1997b). This experiment demonstrated

Table 2. Growth and extracellular proteolytic and chitinolytic activities exhibited *in vitro* by *S. maltophilia* W81 and its mutant derivatives

Standard deviations are given in parentheses. Within each column, values followed by a different letter are significantly different according to Fisher's LSD test ($P < 0.05$).

Strains	Growth rate in LB broth (h^{-1})*	Protease activity		Chitinase activity	
		Units†	% of wild-type level	Units‡	% of wild-type level
W81	1.28 (0.22)	6.5 (0.6) ^a		59.6 (8.0) ^a	
W81A1	1.18 (0.16)	0.8 (0.2) ^b	12 (3) ^a	2.5 (1.2) ^b	4 (2) ^a
W81A1(pCU800)	1.26 (0.19)	6.4 (0.4) ^a	99 (6) ^b	57.3 (8.0) ^a	96 (13) ^b
W81M1	1.17 (0.13)	0.9 (0.6) ^b	15 (9) ^a	61.3 (3.1) ^a	103 (5) ^b
W81M2	1.27 (0.17)	7.3 (0.8) ^a	112 (12) ^b	0 ^b	0 ^a
W81M3	1.13 (0.21)	19.2 (1.8) ^c	295 (28) ^c	67.7 (3.4) ^a	114 (6) ^b
W81M4	1.09 (0.21)	18.3 (1.2) ^c	282 (19) ^c	178.0 (3.3) ^c	299 (6) ^c

* All growth rates were statistically identical.

† TNBS colorimetric assay described by McKellar (1981): one unit of activity corresponds to 1 nmol glycine equivalents released (ml cell-free supernatant)⁻¹ min⁻¹.

‡ Unit of activity defined as $1000 \times A_{585}$ (ml cell-free supernatant)⁻¹ h⁻¹, as described by Dunne *et al.* (1997b).

Table 3. Effect of casein, colloidal chitin and autoclaved *Py. ultimum* mycelium on protease production by *S. maltophilia* wild-type W81 and its mutants and luminescence of Tn5-764cd-induced derivatives of W81

Standard deviations are given in parentheses. Within each column, values followed by a different lower case letter are significantly different according to Fisher's LSD test ($P < 0.05$). Within each row, values for protease activity and for luminescence followed by a different upper case letter are significantly different according to Fisher's LSD test ($P < 0.05$).

Strains	Control		In presence of casein		In presence of colloidal chitin		In presence of autoclaved <i>Py. ultimum</i> mycelium	
	Protease activity*	Luminescence†	Protease activity*	Luminescence†	Protease activity*	Luminescence†	Protease activity*	Luminescence†
W81	81 (12) ^{Aa}		141 (15) ^{Ba}		90 (11) ^{Aa}		123 (6) ^{Ba}	
W81A1	8 (3) ^{Ab}		5 (1) ^{Ab}		8 (1) ^{Ab}		7 (2) ^{Ab}	
W81A1 (pCU800)	84 (11) ^{Aa}		138 (11) ^{Ba}		83 (9) ^{Aa}		119 (10) ^{Ba}	
W81M1	7 (3) ^{Ab}	79 (22) ^{Aa}	11 (2) ^{Ab}	17900 (100) ^{Ba}	7 (4) ^{Ab}	70 (16) ^{Aa}	5 (1) ^{Ab}	5380 (990) ^{Ca}
W81M2	75 (14) ^{Aa}	57 (27) ^{Aa}	129 (11) ^{Ba}	39 (17) ^{Ab}	65 (9) ^{Aa}	48 (18) ^{Aa}	113 (6) ^{Ba}	34 (15) ^{Ab}
W81M3	88 (11) ^{Aa}	14900 (1000) ^{Ab}	254 (23) ^{Bc}	435 (103) ^{Bc}	97 (14) ^{Aa}	12500 (1500) ^{Ab}	167 (16) ^{Cc}	1300 (200) ^{Cc}
W81M4	91 (12) ^{Aa}	22300 (1300) ^{Ac}	229 (22) ^{Bc}	587 (121) ^{Bc}	147 (11) ^{Cc}	4100 (970) ^{Cc}	196 (19) ^{Bc}	754 (158) ^{Bd}

* FITC-labelled casein assay described by Twining *et al.* (1984): one unit of fluorescence arbitrarily equated to 1000 units of protease activity.

† Luminescence detected min⁻¹ in the presence of 100 µl 5% n-decylaldehyde.

that the corresponding sites of transposon insertion were located in four different regions of the genome (data not shown). This suggests that each of the mutants corresponds to the disruption of a distinct gene. Apart from the observed differences in levels of lytic enzyme production, the four Tn5-764cd mutants of strain W81 did not differ from the wild-type on the basis of growth in LB broth (Table 2) or when compared using a variety of biochemical tests (aerobic acidification of glucose; production of catalase, oxidase, gelatinase, DNAase;

assimilation of acetate, citrate, malate, glucose, mannose, maltose, *N*-acetylglucosamine and maltose; data not shown).

Induction of extracellular protease synthesis in *S. maltophilia* W81 and its mutant derivatives

The successful use of biocontrol agents requires a good understanding of patterns of expression of the corresponding biocontrol traits. In the case of extracellular

lytic enzymes, a key issue may be how the presence of inducers or target organisms influences enzyme synthesis. Under *in vitro* conditions, extracellular protease production by wild-type W81 increased in a similar fashion in the presence of casein or autoclaved *Py. ultimum* mycelium (Table 3), as observed in *T. harzianum* (Geremia *et al.*, 1993; Flores *et al.*, 1997). Similar results were obtained with the chitinase-negative mutant W81M2 and the W81A1 mutant strain carrying the plasmid pCU800 which restores lytic enzyme production (Dunne *et al.*, 1997b). Casein and autoclaved fungal mycelium did not induce proteolytic activity in the extracellular-protease-deficient mutants W81A1 and W81M1 (Dunne *et al.*, 1997b). In the absence of appropriate inducer, the two Tn5-764cd-induced mutants W81M3 and W81M4 produced similar amounts of extracellular protease as the wild-type W81 strain (Table 3), indicating that overproduction of the enzyme occurred only after induction. The increase in extracellular protease production by these mutant strains in the presence of autoclaved *Py. ultimum* mycelium was less than that induced by casein. In contrast with studies on the wild-type W81 strain, an increase in extracellular protease production (although less than with autoclaved fungal mycelium) was also observed when strain W81M4 was incubated in the presence of colloidal chitin (Table 3).

Effect of casein, chitin and autoclaved *Py. ultimum* mycelium on expression of loci inactivated by Tn5-764cd in mutants of *S. maltophilia* W81

Transposition of Tn5-764cd results in the generation of transcriptional fusions, in which the promoterless *luxAB* reporter gene cassette is regulated by resident promoters. We exploited this property to determine the effect of casein, chitin and autoclaved *Py. ultimum* mycelium on the expression of the loci inactivated by the transposon in mutant strains W81M1, W81M2, W81M3 and W81M4, with the objective of gaining insight into the regulation of extracellular protease production in strain W81. Bioluminescence data indicated that the W81M1 protease structural gene inactivated by Tn5-764cd was expressed at a much higher level in the presence of casein or autoclaved fungal mycelium (Table 3). In contrast, the *lux* gene fusions in the mutants W81M3 and W81M4 were significantly less luminescent in the presence of casein or autoclaved *Pythium* mycelium than in their absence (Fig. 2b and Table 3). Strain W81M4 also displayed reduced luminescence when incubated in the presence of chitin.

Biocontrol of *Pythium*-mediated damping-off of sugar beet by *S. maltophilia* W81 and its mutant derivatives in natural soil microcosms

In soil microcosms naturally infested by *Pythium* spp., inoculation of sugar beet seeds with wild-type W81, the chitinase-negative mutant W81M2 or the reference biocontrol agent *Ps. fluorescens* F113Rif (Carroll *et al.*, 1995; Moënne-Loccoz *et al.*, 1998) increased the per-

Fig. 3. Effect of *S. maltophilia* W81 and its mutant derivatives on damping-off disease of sugar beet in natural soil microcosms prepared with soil naturally infested by *Pythium* spp. A commercial treatment (i.e. synthetic fungicides Previcur N and thiram), an untreated control (i.e. seeds soaked in quarter-strength Ringer's solution), and the effect of the biocontrol agent *Ps. fluorescens* F113Rif were also studied. The percentage of seeds free from colonization by *Pythium* spp. at 1 d after sowing is shown in (a). The percentage emergence of sugar beet at 28 d after sowing is given in (b). Error bars represent standard deviations. In (a) and in (b), bars headed by a different letter are significantly different according to Fisher's LSD test ($P < 0.05$).

centage of seeds not colonized by *Pythium* spp. from 5% (in the untreated control) to between 80% and 90% at 1 d after sowing (Fig. 3a). The percentage of *Pythium*-free seeds was 98% or higher when seeds were inoculated with W81M3 or W81M4 or treated with chemical fungicides (commercial control). As in a previous investigation (Dunne *et al.*, 1997b), this beneficial effect

was not found in the protease-negative W81M1 treatment. The percentage of plants that emerged was assessed 28 d after sowing, and results showed that the two mutants W81M3 and W81M4 provided better protection against disease than W81, W81M2 or F113Rif (Fig. 3b). Furthermore, only the W81M3 and W81M4 treatments achieved levels of plant protection as high as that conferred by the use of chemical fungicides.

Colonization of the rhizosphere of sugar beet by the seed inoculants was assessed by colony counts on selective media throughout the 28 d experiment. At each sampling time, the population sizes of the strains in the rhizosphere were statistically identical (approx. $10^{4.5}$ c.f.u. per root system at 28 d). In each inoculated treatment, ten colonies were chosen at random from the plates used for counts at each sampling time and their phenotype was investigated. All exhibited similar extracellular chitinase and protease activities on plates as compared with those of the corresponding inoculants. No colonies were found on the selective media when studying plants from the untreated control (detection limit of 10^2 c.f.u. per root system).

DISCUSSION

In this study, an extracellular protease produced by *S. maltophilia* W81 was purified by DEAE-Sephacel anion-exchange chromatography using a protocol similar to that employed by Thrane *et al.* (1997) to isolate a *Pythium*-affecting endo-1,3- β -glucanase from *T. harzianum*. The purified enzyme was characterized biochemically as a serine protease, in agreement with previous results of amino acid sequence analysis suggesting that the W81 extracellular protease shares significant homology with an acrosin trypsin-like serine peptidase (S12968) (Dunne *et al.*, 1997b).

Since extracellular protease production appears to be responsible for the ability of strain W81 to control the extent of *Pythium*-mediated damping-off of sugar beet (Dunne *et al.*, 1997b, 1998), we hypothesized that superior biocontrol activity may be obtained by increasing production levels of this protease. In the current study, mutagenesis with the transposon Tn5-764cd resulted in the isolation of W81M3 and W81M4, two mutants producing approximately three times more extracellular protease than the wild-type W81 strain in the presence of casein (Table 2). Mutant W81M4 also displayed enhanced chitinolytic activity compared to W81, a property that may be useful for biocontrol of chitinase-sensitive pathogens and parasites of plants (Cronin *et al.*, 1997) but not of *Pythium* spp. (Dunne *et al.*, 1997b). A similar strategy of transposon mutagenesis resulted in the generation of mutants of biocontrol agent *Ps. fluorescens* CHA0 capable of overproducing the antifungal compound pyoluteorin (Schnider *et al.*, 1995).

Overproduction of extracellular protease by W81M3 and W81M4 did not take place in the absence of inducer. However, both W81M3 and W81M4 displayed strong luminescence under non-inducing conditions but not in

the presence of casein (Table 3), suggesting that the genes inactivated in these mutants may be involved in down-regulating extracellular protease production. In the case of W81M4, which also overproduces chitinase, the presence of colloidal chitin caused induction of extracellular protease production, although at significantly lower levels than that caused by casein. Therefore, the putative regulatory locus inactivated in W81M4 may be involved in down-regulation of both extracellular protease and chitinase production.

Tn5-764cd was used previously to generate mutant W81M1 deficient in extracellular protease synthesis (Dunne *et al.*, 1997b). Here, W81M1 exhibited strong luminescence in the presence of casein but not in its absence (Table 3), demonstrating that the structural gene involved in extracellular protease production in W81 was inducible by proteinaceous substrates. Furthermore, induction studies demonstrated that extracellular protease production by wild-type W81 increased considerably in the presence of casein (Table 3). In this study, casein was used in experiments as a substitute for proteinaceous components of the fungal cell wall. This approach was appropriate since a similar induction of extracellular protease production occurred when strain W81 and its protease-positive mutants were exposed to autoclaved *Py. ultimum* mycelium (Table 3).

Early implementation of biocontrol mechanisms is required for effective control of *Pythium* spp. as sporangia of the fungus respond quickly to seed exudates (Nelson *et al.*, 1988), leading to plant infection within hours of sowing (Stasz *et al.*, 1980). Therefore, the observation that compounds exuded by the pathogen or present in the cell wall can induce extracellular protease production in W81 and its mutants suggests that the mutants W81M3 and W81M4 overproducing extracellular protease may confer increased protection to sugar beet against *Pythium* spp. when compared with the wild-type. Inoculation of seeds with W81, W81M3 or W81M4 resulted in similar levels of *Pythium*-free seeds at 1 d after sowing, which is not surprising as the wild-type strain was already very effective at preventing early colonization of seeds by the fungus (Fig. 3a). However, both W81M3 and W81M4 displayed a higher disease-suppressive ability throughout the 28 d experiment as compared with the wild-type strain, and each of the two mutants was as effective as the cocktail of commercial fungicides (Previcur N and thiram) for plant protection (Fig. 3b). Such levels of plant protection were previously achieved only when using a combination of W81 and *Ps. fluorescens* F113Rif (Dunne *et al.*, 1998).

Overproduction of an inducible extracellular protease in *T. harzianum* resulted in reduced growth rate of the biocontrol fungus (Flores *et al.*, 1997). In the case of W81, however, wild-type and mutants displayed the same growth rate *in vitro* (Table 2) and a similar ability to colonize the rhizosphere of sugar beet in soil microcosms (data not shown). Therefore, the construction of W81M3 and W81M4 by transposon mutagenesis had no obvious negative effect in terms of their

ecological fitness in the rhizosphere. Moreover, the increase in biocontrol did not result from higher populations of the inoculants in the rhizosphere. In conclusion, the approach used in this study to develop improved biocontrol strains by increasing the production of the extracellular serine protease in *S. maltophilia* W81 was substantiated by significant enhancement in the suppression of *Pythium*-mediated damping-off of sugar beet in soil microcosms.

ACKNOWLEDGEMENTS

The authors thank Pat Higgins and Liam Burgess for technical assistance. We are also grateful to A. Milcamps (DOE Plant Research Laboratory, MSU) for useful discussion and Tadhg O'Sullivan (Dept of Microbiology, UCC), Abdallah Magboul and Therese Uniak-Lowe (Dept of Food Chemistry, UCC) for advice on enzyme purification. This work was supported in part by grants awarded by the Irish Science and Technology Agency Forbairt (SC-96-349 and SC-98-261) 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, FMRX-CT96-0039, BIO4-CT97-2227 and BIO4-CT98-0254) 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.

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Received 25 October 1999; revised 19 April 2000; accepted 8 May 2000.