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

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*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, 1993; Cook et al., 1995). Disease suppression may also be achieved in other soils following the introduction of selected biological control agents into the soil or the...
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 & Defago, 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 phlobaphenins (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 & Defago, 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; Friedlender 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

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristics</th>
<th>Source or reference</th>
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<tr>
<td><strong>Strains</strong></td>
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<tr>
<td><em>S. maltophilia</em></td>
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<tr>
<td>W81</td>
<td>Wild-type, Sm' Km' Phl' Hcn' Flu' Chi' Prt'</td>
<td>This study</td>
</tr>
<tr>
<td>W81M1</td>
<td>Te' Prt', Tn5-764cd mutant of W81</td>
<td>This study</td>
</tr>
<tr>
<td>W81M2</td>
<td>Te' Chi', Tn5-764cd mutant of W81</td>
<td>This study</td>
</tr>
<tr>
<td>W81A1</td>
<td>Te' Chi'+ Prt', Tn5-B50 mutant of W81</td>
<td>This study</td>
</tr>
<tr>
<td>W81A1(pCU800)</td>
<td>Te'- Cm'-complemented mutant of W81A1</td>
<td>This study</td>
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<tr>
<td><strong>P. fluorescens</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F113LacZY</td>
<td>Phl' Hcn' Flu' LacZY'</td>
<td>Fedi <em>et al.</em> (1996)</td>
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<td><strong>E. coli</strong></td>
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<tr>
<td>HB101</td>
<td>recA hsdB hsdM strA pro leu thi</td>
<td>Boyer &amp; Roulland-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dussoix (1969)</td>
</tr>
<tr>
<td>MC1061</td>
<td>ara leu lacX74 galU galK hsdB hsdM strA</td>
<td>Casadaban &amp; Cohen</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(1980)</td>
</tr>
<tr>
<td>DH5x</td>
<td>Δ80lacZAM15 Δ(lacZYA-argF)U169 hsdR17 recA1 endA1 thi-1</td>
<td>Sambrok <em>et al.</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(1989)</td>
</tr>
<tr>
<td>LE392</td>
<td>F', hsdRS14 supE44 supF58 lacY1 galT22 metB1 trpR55 λ'</td>
<td>Sambrok <em>et al.</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(1989)</td>
</tr>
<tr>
<td>S17-1</td>
<td>F', pro recA1 RP4-2 integrated (Te'::Mu) [Km':::Tn7(5m Tc')]</td>
<td>Simon <em>et al.</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(1983b)</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td>Simon <em>et al.</em></td>
</tr>
<tr>
<td>pSUP106</td>
<td>Broad-host-range cosmid vector, IncQ Cm' Te'</td>
<td>(1983a)</td>
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<td>pCU800</td>
<td>pSUP106 with a 15 kb insert from W81</td>
<td>This study</td>
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<td>pRK2013</td>
<td>Mob' Tra' Km'</td>
<td>Ditta <em>et al.</em></td>
</tr>
<tr>
<td>pSUP102::Tn5-B50</td>
<td>Te'</td>
<td>Simon <em>et al.</em></td>
</tr>
<tr>
<td>pRL1063a</td>
<td>Km' LuxAB'</td>
<td>Wolk <em>et al.</em></td>
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<tr>
<td>pRL764cd</td>
<td>Te' LuxAB'</td>
<td>This study</td>
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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 3,000 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 µl supernatant transferred to new tubes. A volume of 500 µl 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 µl supernatant added to 1.5 ml of 10% trichloroacetic acid and 0.5 ml of 4% (w/v) 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 2,4,6-trinitrobenzenesulfonic acid (TNBS). Following incubation at 37 °C for 1 h, the reaction mixture was added to 1.5 ml of 1.0 M NaH₂PO₄ containing 18 mM Na₂SO₄. 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 µM) and on chrome azurol S (Schwyn & Neilsen, 1987) indicator medium. Hydrogen cyanide production was assayed qualitatively, as described by Castric & Castric (1983). The polyketide 2,4,6-triacetylphloroglucinol 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* DH5α 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' strain W81 was performed using pSUP102::Tn5-B50 (Tc'; 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' Tc') 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 Packaging system (Promega). Resultant Cm' Tc' colonies were mated with W81A1, using HB101/pPK2013 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 EcoRI 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 Dral, which does not cut the transposon. The resulting fragments, which were self-ligated and transformed into E. coli DH5α, DNA sequence flanking the inserted transposon was determined using unique primers (GENOSYS, Cambridge, UK) homologous to the left (5' TACTGATATTCAATGCATAATCAG 3') and right (5' AGGAGGTCCAGTTGAAATCAGT 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) (0.5–10 units ml⁻¹) or Proteinase K (Sigma P-8044, EC 3.4.21.14) (0.5–10 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 per g soil)⁻¹, as shown using a Pythium-selective medium (Jeffers & Martin, 1996). 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 studyingplants from the untreated control (detection limit of 10⁸ 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
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 medium. No growth on SA plus EDDHA or on chrome azurol S, W81 did 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-B30 and Tn5-764cd. Approximately 15000 mutants were screened by replica plating for loss of lytic enzyme activity, and a Tn5-B30 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 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, phloroglucinol or hydrogen cyanide. When grown on SA plus EDDHA or on chrome azurol S, W81 did not produce a siderophore (data not shown).

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

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Enzyme production</th>
<th>Biological control</th>
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<tr>
<td></td>
<td>Lytic enzyme</td>
<td>Proteolytic</td>
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<tr>
<td></td>
<td>production</td>
<td>activity*†</td>
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<tr>
<td>Commercial control</td>
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<tr>
<td>Untreated control</td>
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</tr>
<tr>
<td>F113LacZY</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>W81</td>
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<td>6.5 (0.6)*</td>
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<td></td>
<td>protease</td>
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<td>W81M1</td>
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<td></td>
<td>protease</td>
<td></td>
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* One unit of activity corresponds to 1 nmol glycine equivalents released (ml cell-free supernatant)-1 min-1.
† Values given are means (± 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.
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 BamHI 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 wildtype 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...
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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 ×1000.

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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 supernatants 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 & Kloeper, 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, 1963; 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/seeding 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 & Debette, 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.

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