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Characterization of a Chitinase Gene from *Stenotrophomonas maltophilia* Strain 34S1 and Its Involvement in Biological Control

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A chitinase gene was cloned on a 2.8-kb DNA fragment from *Stenotrophomonas maltophilia* strain 34S1 by heterologous expression in *Burkholderia cepacia*. Sequence analysis of this fragment identified an open reading frame encoding a deduced protein of 700 amino acids. Removal of the signal peptide sequence resulted in a predicted protein that was 68 kDa in size. Analysis of the sequence indicated that the chitinase contained a catalytic domain belonging to family 18 of glycosyl hydrolases. Three putative binding domains, a chitin binding domain, a novel polycystic kidney disease (PKD) domain, and a fibronectin type III domain, were also identified within the sequence. Pairwise comparisons of each domain to the most closely related sequences found in database searches clearly demonstrated variation in gene sources and the species from which related sequences originated. A 51-kDa protein with chitinolytic activity was purified from culture filtrates of *S. maltophilia* strain 34S1 by hydrophobic interaction chromatography. Although the protein was significantly smaller than the size predicted from the sequence, the N-terminal sequence verified that the first 15 amino acids were identical to the deduced sequence of the mature protein encoded by *chiA*. Marker exchange mutagenesis of *chiA* resulted in mutant strain C5, which was devoid of chitinolytic activity and lacked the 51-kDa protein in culture filtrates. Strain C5 was also reduced in the ability to suppress summer patch disease on Kentucky bluegrass, supporting a role for the enzyme in the biocontrol activity of *S. maltophilia*.

Biological sources for plant disease control remain an important potential alternative to the use of pesticides. Biological controls that are based on introduced microbes, however, have been slow to develop due to inconsistencies in their performance (70). Such inconsistencies often result from a lack of understanding the mechanisms by which individual microorganisms function to control disease. It is often difficult to gain a complete understanding of how biocontrol agents control diseases, since many function through a variety of mechanisms. In such cases, identifying contributing mechanisms often requires a systematic approach that directly evaluates individual traits and their contributing roles to the overall operating mechanisms.

The lytic activity of bacteria is one of a number of mechanisms that has been implicated in biocontrol for several years (5, 13, 23, 26, 35, 38, 41, 45). Investigative studies on lytic activity among biocontrol agents have focused largely on the characterization of enzyme systems capable of degrading fungal cell wall components, of which chitinases are among the most intensively studied (4, 23, 26, 45, 58, 72, 73).

Molecular and biochemical characterizations have revealed that chitinases, similar to other glycosyl hydrolases, are modular in nature and can differ according to their structural organization. Enzymes can vary both within and between microbes, depending on the numbers, types, and positions of discrete binding and catalytic domains (reviewed in references 16 and 66). Domains that have been described within bacterial chitinases include chitin-binding domains (22, 42, 60, 61, 65), cadherin-like domains (42), and fibronectin type III domains (14, 65, 69).

Stenotrophomonas maltophilia represents a rhizosphere bacterial species of potential agronomic importance (7, 10, 11, 12, 15, 30, 31, 33, 34, 39). Traits of *S. maltophilia* associated with biocontrol mechanisms include antibiotic production (25, 44), extracellular enzyme activities such as protease and chitinase (10, 30, 44, 72), and rhizosphere colonization potential (10, 12, 30, 33, 34). To date, however, only a few biocontrol traits expressed by *S. maltophilia* strains have been molecularly characterized (10), and therefore information concerning gene structure and evaluation of biocontrol traits through direct mutagenesis procedures for this group is lacking.

S. maltophilia strain 34S1 was identified as a biocontrol agent for summer patch disease of Kentucky bluegrass (*Poa pratensis*) caused by the root-infecting fungus *Magnaporthe poae* (30). Several traits expressed by *S. maltophilia* strain 34S1 are thought to contribute to its biocontrol activity; however, the contributing roles for the various traits in disease control remain unclear. In this study, we describe the molecular characterization and structural organization of a single chitinase gene responsible for chitinolytic activity in *S. maltophilia* 34S1 and evaluate its contribution to biocontrol of summer patch disease.

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MATERIALS AND METHODS

Bacterial and fungal strains, vectors, and media. All bacteria were grown and maintained on Luria-Bertani (LB) agar (Difco). *S. maltophilia* 34S1 and *Burkholderia cepacia* M53 were grown at 30°C as described (30). The genomic cosmid

library, constructed in pLAFR3 (59), was maintained in *Escherichia coli* HB101 (Gibco-BRL). Subcloning experiments were performed using the vectors pUC118 (New England Biolabs) and pRK415 (27) and *E. coli* DH5α (Gibco-BRL) as the host strain. Strains containing plasmids were maintained on LB agar supplemented with appropriate antibiotics at the following levels unless otherwise noted: tetracycline (Tc), 12.5 μg/ml; kanamycin, 50 μg/ml; ampicillin, 50 μg/ml; and rifampin 100 μg/ml.

M. poae strain 73-15 (ATCC 64411) was maintained on potato dextrose agar (PDA) (Difco).

Construction of *S. maltophilia* genomic library and screening for chitinase activity. Total genomic DNA of *S. maltophilia* 34S1 was isolated using the sodium dodecyl sulfate (SDS) lysis procedure described previously (59). DNA was purified twice on a cesium chloride gradient, and 60 μg of DNA was partially digested with *Sau*3A before size-fractionating on a 10 to 40% glycerol gradient centrifuged at 194,000 × g for 33 h in a Beckman SW40 rotor. DNA fragments greater than 15 kb were collected, ligated to the *Bam*HI site in pLAFR3, and packaged into lambda phage using Gigapak Gold extracts (Stratagene).

No positive clones for chitinase activity were detected when the entire genomic library was initially screened in *E. coli* or any subsequent subcloning experiments regardless of fragment orientation and IPTG (isopropyl thiogalactopyranoside) induction. Therefore, chitinase activity was detected by mobilizing all clones into *B. cepacia* M53 by triparental matings (9), plating onto M9 minimal salts (53) in 0.7% agarose supplemented with 0.25% yeast extract (Difco), 2% colloidal chitin (36), and Tc (200 μg/ml), and screening for clearing zones around colonies.

Molecular characterizations and sequence analysis. Restriction digests, electrophoresis, ligations, and Southern hybridizations were performed as described (53). The nucleotide sequence of both strands was determined by fluorescence labeling with the Amplitaq FS fluorescence labeling kit (Applied Biosystems Inc.) run on an ABI model 373 automated sequencer. All nucleotide sequences and trace plots were analyzed using the DNA analysis programs of DNAStar (Lasergene).

Chitinase activity from the cosmid clone pXM7B5 was subcloned to a 2.8-kb *Xho*I-*Sac*I fragment by first ligating into the *Sal*I and *Sac*I sites located in the polylinker of pUC118 to form plasmid pXMC131. This subclone was excised as an *Hin*dIII-*Eco*RI fragment using restriction sites located in the vector and ligated into the low-copy-number, broad-host-range vector pRK415.

Saturation transposon mutagenesis of cosmid pXM7B5 contained in *E. coli* was performed using λ::Tn*5* as described (8). A cosmid clone containing Tn*5* in the coding region of the chitinase gene, designated pXM7B5::Tn*5*-1, was identified by screening for loss of chitinase activity in *B. cepacia* M53 on colloidal chitin agar. Restriction map and Southern blot analyses of this plasmid using sequences internal to Tn*5* as a probe positioned the transposon insertion in a 250-bp *Kpn*I fragment internal to the *chiA* open reading frame (see Fig. 1B). The mutation in pXM7B5::Tn*5*-1 was introduced into the genome of *S. maltophilia* 34S1 by marker exchange mutagenesis by the method of Ruvkun and Ausubel (51). A resulting mutant, designated strain C5, was verified by Southern blot analysis to contain Tn*5* in the 250-bp *Kpn*I fragment.

Isolation and characterization of extracellular chitinase. To characterize extracellular enzymes and chitinase activity in culture filtrates, all bacteria were grown in M9 salts broth supplemented with 0.25% yeast extract and 2% colloidal chitin. *S. maltophilia* transconjugants and *B. cepacia* transconjugants were grown in medium supplemented with Tc at 100 and 200 μg/ml, respectively. After 3 days of growth at 30°C, cells were pelleted by centrifugation (10 min, 7,500 × *g*), and culture filtrates were precipitated with 80% ammonium sulfate at room temperature for 60 min before centrifuging for 15 min at 16,900 × *g*. Pellets were resuspended in 1/1,000 the original volume and dialyzed in 10 mM Tris-HCl (pH 8.0).

Chitinase activity in culture filtrates was assayed by using *p*-nitrophenyl-*N,N′*-diacetylchitobiose (pNP) as a substrate similar to the method described (50). Values were standardized based on the cell densities of cultures.

SDS-polyacrylamide gel electrophoresis (PAGE) was performed as previously described (32). Nondenaturing (native) gels consisted of 10% acrylamide in 0.375 M Tris-HCl (pH 6.8). Chitinase activity was detected by UV light exposure of native gels containing 0.1 mM 4-methylumbelliferyl-β-D-*N,N′*-diacetylchitobioside [4-MU-$(GlcNAc)_2$] or 4-methylumbelliferyl-β-D-*N,N′*-triacetylchitobioside [4-MU-$(GlcNAc)_3$] (Sigma).

Chitinase was purified from *S. maltophilia* cultures by first centrifuging cells (10 min at 5,000 × *g*) and passing the culture fluid through a 0.45-μm membrane to remove particulates. The filtrate was buffered with 20 mM Tris-HCl (pH 8.0) with 1 mM phenylmethylsulfonyl fluoride included to inhibit proteases. Ammonium sulfate was added to a final concentration of 1 M, and the filtrate was applied to a 1-ml phenyl-Sepharose column (Amersham Pharmacia Biotech) equilibrated with 20 mM Tris-HCl (pH 8.0) buffer containing 1 M ammonium sulfate. Following washing with 20 ml of equilibration buffer, fractions were eluted by three × 1-ml washes of 20 mM Tris-HCl (pH 8.0), with stepwise 0.25 M decreases in ammonium sulfate, followed by three × 1-ml washes in 25% acetonitrile and a final elution in three washes with 1 ml of 50% acetonitrile. The protein concentration of each fraction was determined, and aliquots were run on SDS-PAGE. The purified protein was subjected to N-terminal Edman degradation peptide sequencing and confirmed as the *chiA* gene product by using Fastp web software (University of Virginia).

In vitro antagonism and biocontrol assays. For all biological assays, mutant strain C5 was compared directly with the wild-type strain 34S1; strain C5 complemented with the *chiA* subclone in pXM431 was not included in experiments because of plasmid instability over long periods of time. In vitro antagonism assays were conducted as previously described (29). Biocontrol assays for summer patch disease were conducted on Kentucky bluegrass var. Baron grown in a 4:1 mix of pasteurized sand to peat in 9-cm conical containers (30). Containers were inoculated with the fungal pathogen at a depth of 1.5 cm below the soil surface at the time that seeds were sown (approximately 60 seeds per container).

Bacteria were prepared as treatments by pelleting cells from overnight cultures grown in LB and suspending them in sterile H_2O to a concentration of 5×10^8 CFU/ml; 25 ml of the bacterial solution was applied to each container as a soil drench at 2 and 3 weeks after seeding. Plants were moved to the growth chamber set at 30°C and 70% humidity with 500 μE of light for 14 h, 4 weeks after seeding to induce disease.

Plants were rated for disease severity based on a visual assessment of the percentage of necrotic plants within a container, as described (30). Ratings were conducted every 3 to 4 days following movement of plants into the growth chamber (day 0). Each treatment was replicated 10 times (with each container representing a replicate) within an experiment, and the experiment was repeated five times.

The disease progress data followed a logistic pattern of increase over a 14- to 17-day period. For data analysis, disease ratings were transformed using the logit transformation, and a simple linear regression equation was calculated for each replicate. A slope and *y* intercept were estimated for each replicate. In addition, disease severity at day 10 was estimated for each replicate from the regression equations. The disease incidence at day 10, slope, and *y*- intercept were analyzed in a two-way analysis of variance using the three treatments as the first factor and the five experiments as the second factor.

S. maltophilia populations in the Kentucky bluegrass var. Baron rhizosphere were determined as previously described (30). Populations were sampled 1, 4, 7, 10, and 14 days after application of bacteria. All samples were dilution plated onto LB agar supplemented with rifampin. Samples were replicated three or five times, depending on the experiment, and the experiment was repeated three times.

Nucleotide sequence accession number. The nucleotide sequence of *chiA* was deposited in the Genbank database under accession no. AF014950.

RESULTS AND DISCUSSION

Cloning and nucleotide sequence analysis of the *chiA* gene from *S. maltophilia*. Screening the *S. maltophilia* 34S1 genomic library for chitinase activity in *B. cepacia* M53 resulted in the identification of two cosmid clones that caused bacterial colonies to clear colloidal chitin in agar within 5 days. Comparisons of restriction digest banding patterns indicated that the two cosmids had a region of overlap that was later confirmed to be homologous by Southern hybridizations (data not shown). One cosmid clone, designated pXM7B5, was selected for further characterization.

Chitinase activity from pXM7B5 was subcloned to a 2.8-kb *Xho*I-*Sac*I fragment in pXMC431 as described in Materials and Methods. The nucleotide sequence of the 2.8-kb DNA fragment revealed a large open reading frame, designed *chiA*, which encoded a predicted protein of 700 amino acids with a molecular mass of 72.4 kDa (Fig. 1A). Seven bases upstream of the predicted start ATG was the purine-rich sequence GGAG, resembling a putative ribosome-binding site. An exact 12-base inverted repeat, separated by a single nucleotide, that resembles a transcription termination sequence (AACGCCCCGGC

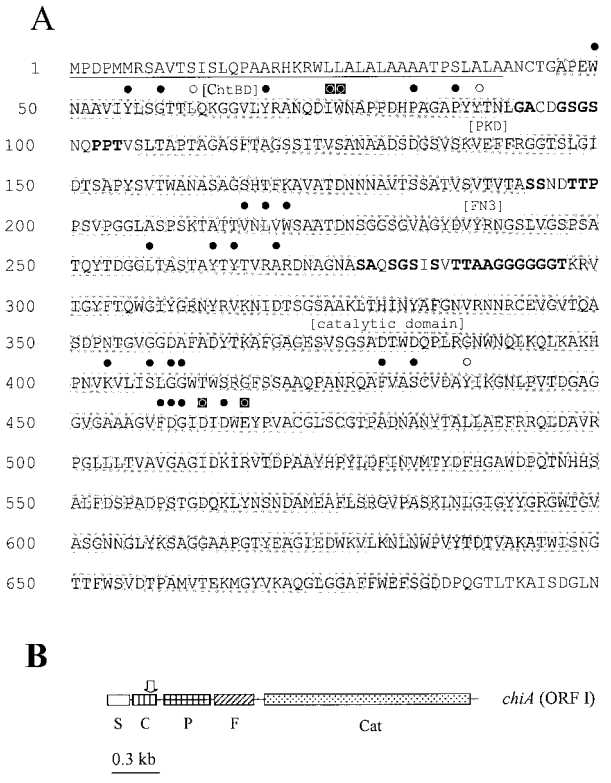

FIG. 1. (A) Deduced amino acid sequence of the *chiA* gene from *S. maltophilia*. Underlined residues represent signal peptide sequence; shaded residues represent specific domain regions identified in brackets above the sequence as the chitin binding domain (ChtBD), PKD domain (PKD), fibronectin type III domain (Fn3), and catalytic domain. Residues in bold represent amino acids common to domain linker regions. Solid circles above amino acid positions represent conserved identical residues, open circles represent conserved similar residues, and boxed circles represent residues essential for function. (B) Map of the *chiA* open reading frame (ORF I) with relative positions of the signal peptide sequence (S), chitin binding domain (C), PKD domain (P), fibronectin type III domain (F), and catalytic domain (Cat). The vertical arrow above the chitin binding domain represents the position of Tn*5* insertion.

ACTGCCGGGGCGTT) was identified 51 bases after the stop codon.

Structural organization of the *chiA* gene from *S. maltophilia* strain 34S1. Analysis of the first 41 residues of the deduced sequence of *chiA* indicated the presence of a signal peptide sequence (47) with a predicted cleavage site between two alanine residues (Fig. 1). Cleavage at this site results in a predicted protein with a molecular mass of 68 kDa. An RPS-BLAST search of the conserved-domain database identified a catalytic domain belonging to family 18 glycosyl hydrolases (20) at the C-terminal end of the protein between residues 298 and 685.

The web-based analysis tool SMART (http://smart.embl-heidelberg.de), which identifies domains based on sequence homology and tertiary structure (55, 56), was used to identify three additional domains within the deduced ChiA sequence: a type 3 chitin-binding domain (ChtBD3) located between residues 47 and 92, a polycystic kidney disease (PKD) domain located between residues 107 and 194, and a fibronectin type III (Fn3) domain between residues 201 and 278 (Fig. 1). Spanning each of the four domains were short stretches of sequences found to be rich in Gly, Ala, Pro, Ser, and Thr (Fig. 1A) that typically resemble domain linker regions (63).

Because of the modular nature of chitinases, similarity of the *chiA* sequence to other chitinase genes at the whole-gene level was low. Therefore, *chiA* was also compared to other database sequences by domain regions. For each domain, the six sequences showing the highest amino acid identity with the deduced *chiA* protein are listed in Table 1. Comparisons of related sequences between domains clearly showed variation in species origin, gene source, and mean percent identity to the deduced *chiA* sequence. These observations are consistent with the proposal that the modular nature of glycosyl hydrolases contributes to domain shuffling (14, 16, 60) and suggests that the various domains of *chiA* were recruited from a range of different species and gene sources.

Comparison of the deduced ChiA sequence with other catalytic domains revealed several conserved residues previously identified within family 18 glycosyl hydrolases, including those essential for function (60, 68) (Fig. 1A). The identity of ChiA with the six most related catalytic domains ranged between 46 and 54% in pairwise comparisons. In contrast, ChtBD3 had the lowest range of amino acid identity to related sequences in pairwise comparisons among the four domains found in ChiA, ranging between 30 and 49% (Table 1). Despite the lower identity, ChtBD3 also contained identical or related residues (Fig. 1A) considered well conserved for this domain type (2, 22). Higher similarities were observed to both the PKD and Fn3 domains than to ChtBD3. Overall, the Fn3 domain was observed to have the highest range of similarity to related sequences, ranging between 56 and 66% identity, and contained several previously identified conserved amino acids (1) (Fig. 1A).

PKD domains were first identified in *PKD1*, the primary gene responsible for polycystic kidney disease in humans. PKD domains are structurally characterized as β-sheets containing an immunoglobulin (Ig)-like fold, similar to Ig-like folds found in proteins and domains grouped within the Ig superfamily, which includes Fn3 (3). Thus, PKD domains are structurally similar to Fn3 domains and have been previously identified in bacterial sequences (46). Within chitinases, however, Fn3 domains are quite prevalent, while PKD-like domains have not been reported in the literature. Our results with Blast searches suggest that many glycosyl hydrolases, including chitinases, contain sequences similar to the PKD region from ChiA (Table 1).

Fn3 and PKD domains have been proposed to originate from animals (1, 3), suggesting the possibility that both domains may have been recruited through similar events by *S. maltophilia*. The likelihood of this event is further supported by the prevalence of clinical strains of *S. maltophilia*. Since the functions of the putative binding domains found in ChiA were not evaluated in this study, their roles in chitinolytic activity remain unclear. However, previous studies have demonstrated that such binding domains in chitinases, including ChtBD3 and Fn3, enhance chitinolytic activity (18, 42, 60, 61, 67). The structural similarities between Fn3 and PKD suggest that the latter would also contribute to such a role, but this remains to be experimentally proven.

TABLE 1. Pairwise comparison of deduced ChiA domains to homologous protein sequences

Domain and organism	Protein	Accession no.	Reference	Residue positions	% Identity
ChtB3 domain					
Xanthomonas sp.	Chitinase A	BAA36460	52	34–74	49
Deinococcus radiodurans	Hypothetical protein	D75274	71	176–210	34
Vibrio cholerae	Chitodextrinase	AAF96599	19	38–75	34
Arthrobacter sp.	Chitinase B	CAB62499	37	529–570	31
Vibrio furmissii	Chitodextrinase	P96156	28	39–75	30
Pseudoalteromonas sp.	Chitinase C	AAC79667	62	785–815	30
PKD domain					
Xanthomonas sp.	Chitinase A	BAA36460	52	104–159	66
Vibrio cholerae	Chitinase A	AAC72236	6	339–408	54
Vibrio cholerae	Chitinase A	AAC72236	6	182–250	48
Thermobifida fusca	Exocellulase E6	CAA20643	24	154–223	40
Streptomyces coelicolor	Cellulase	AAD39947	48	151–220	40
Vibrio cholerae	Chitinase	AAC72236	6	88–156	39
Fn3 domain					
Xanthomonas sp.	Chitinase A	BAA36460	52	185–244	66
Cellulomonas fimi	Exoglucanase B	AAB00822	57	798–874	60
Cellulomonas fimi	Exoglucanase B	AAB00822	57	895–971	60
Cellulomonas fimi	Exoglucanase A	P50401	57	577–654	59
Cellulomonas fimi	Endoglucanase D	AAA23089	40	454–530	56
Cellulomonas fimi	Endoglucanase D	AAA23089	40	550–626	56
Catalytic domain					
Janthinobacterium lividum	Chitinase 69	AAA83223	17	246–652	54
Doohwaniella chitinasigens	Chitinase 67	AAF21468		221–619	52
Streptomyces peucetius	Chitinase C	AAF43629		264–614	47
Streptomyces coelicolor	Chitinase C	BAA75644	48	243–594	47
Streptomyces lividans	Chitinase C	BAA02168	14	243–594	47
Streptomyces thermoviolaceus	Chitinase 40	JC2135	64	51–398	46

Characterization of chitinase activity produced by *S. maltophilia* strain 34S1 and construction of chitinase mutant strain C5. Strain C5, mutated within the *chiA* locus by marker exchange mutagenesis, was devoid of visible chitinolytic activity when screened on chitin agar medium. Quantitative analysis of chitinase activity in culture filtrates using pNP as a substrate verified that strain C5 was significantly reduced in activity. Activity was restored to levels comparable to that of the wild-type strain 34S1 when the mutant strain C5 was complemented with pXMC431 (Fig. 2).

Chitinase activity was analyzed in culture filtrates of *S. maltophilia* strains grown for 72 h in chitinase-inducing medium. Denaturing gels resolved a major protein band of approximately 51 kDa observed in culture filtrates of strains 34S1 (pRK415) and C5(pXMC431) that was absent in the culture filtrate of strain C5(pRK415) (Fig. 3A). Native protein gels containing either 4-MU-$(GlcNAc)_2$ or 4-MU-$(GlcNAc)_3$ resolved an identical major band of chitinase activity in culture filtrates of strains 34S1(pRK415) and C5(pXMC431), but not in culture filtrates of strain C5(pRK415) (Fig. 3B). Excision of the active band from native gels followed by electrophoresis on denaturing gels verified that chitinase activity corresponded in size with the 51-kDa protein observed in culture filtrates (data not shown).

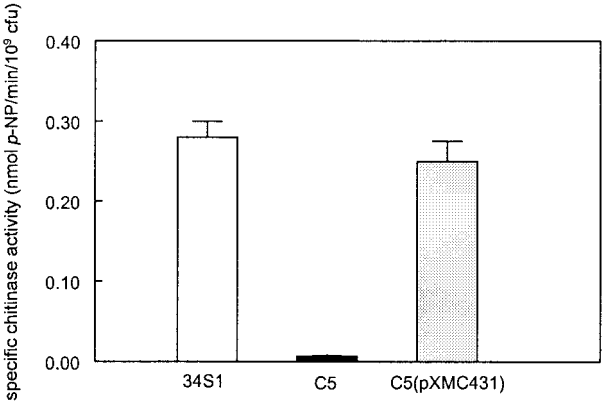

FIG. 2. Specific chitinase activity of *S. maltophilia* culture filtrates grown in M9 minimal salts medium supplemented with yeast extract and 2% colloidal chitin for 72 h. Activity was assayed using pNP as the substrate and standardized to 10^9 CFU for each culture. Data represents the mean of three replicates with standard deviation.

To verify that the 51-kDa protein is associated with chitinolytic activity and is indeed encoded by *chiA*, the protein was purified by hydrophobic interaction chromatography. Fractions containing chitinolytic activity resolved a single band identical in size to the major 51-kDa protein band observed in culture filtrates (Fig. 3C). N-terminal sequencing resulted in an identical sequence of the first 15 amino acids of the mature protein encoded by *chiA*, beginning at the proposed signal peptide sequence cleavage site at position 42. The size, however, is not consistent with the predicted size of the mature protein of 68 kDa based on the sequence, suggesting that degradation of the mature protein occurs in culture.

The observations resulting from mutagenesis and complementation studies are consistent with the indication that chitinolytic activity produced by *S. maltophilia* strain 34S1 is likely encoded solely by *chiA*. In another study investigating chitinases produced by *S. maltophilia*, chitinolytic activity appears to be due to a multienzyme system (73). However, this partic-

ular strain, C3, is physiologically distinct from *S. maltophilia* type strains, including strain 34S1; strain C3 produces multiple isoforms of chitinase and also expresses β-1,3-glucanase activity (72, 73). In our own investigations, strain C3 is taxonomically distinct from type strains of *S. maltophilia* and likely is positioned in a taxon separate from *Stenotrophomonas* (21; D. Y. Kobayashi, unpublished data).

Biological control of summer patch and rhizosphere populations by *S. maltophilia* strains 34S1 and C5. Comparison of the *chiA* mutant strain C5 with the wild-type strain 34S1 in in vitro antagonism assays using the fungus *M. poae* resulted in no obvious differences in growth inhibition of the fungus. In contrast, differences were observed between disease progress curves of summer patch on Kentucky bluegrass plants treated with mutant strain C5 compared with wild-type strain 34S1 over 14- to 17-day periods in five different experiments. Actual disease severity ratings are presented in Fig. 4 (for convenience, only four of the five experiments are presented). In all experiments, disease severity over time in plants treated with mutant strain C5 was less than in untreated control plants, but greater than in plants treated with strain 34S1. This intermediate response suggested that biocontrol activity was reduced but not completely lost in mutant strain C5.

The increase in disease severity, depicted as percent necrosis over time, followed a nonlinear relationship (Fig. 4). A logit transformation on disease severity values for all five experiments combined linearized the data, and simple linear regression equations were calculated. These transformed data fit a linear relationship very well (r^2 = 0.75 to 0.97 for all replications), and no interactions were found between experiment and treatment for any of the factors examined (disease severity, slope, or *y* intercept). Regressions resulting from the combined results of the five experiments confirmed that disease severity of plants treated with strain C3 was at an intermediate level between untreated control plants and plants treated with strain 34S1. Disease increased (as indicated by the slope of the regression) at a greater rate in untreated control plants compared to plants treated with the wild-type strain 34S1 or mutant strain C5; rates of disease increase for the latter two treatments were not significantly different (Table 2).

In contrast to the slope of the regression, the *y* intercept of the disease progress curve for plants treated with strain 34S1 was significantly less than that for plants treated with mutant strain C5 or the untreated control plants (Table 2). This observation indicated that onset of disease symptom production was significantly delayed in plants treated with strain 34S1 compared with strain C5 and untreated control plants. Since the rate of disease progression (slope) was not significantly different in plants treated with either strain, the difference in initial disease onset was the major factor contributing to differences in disease control. Similar results in disease progress curves were observed in previous studies when plants were treated with different concentrations of strain 34S1; rates of disease progress were not significantly different, but plants treated with higher concentrations of strain 34S1 were delayed in disease onset compared with plants treated with lower concentrations (30).

Disease severity at day 10 estimated from the regression equations showed significant differences among all three treatments, with the greatest disease severity occurring in untreated control plants, an intermediate level in plants treated with strain C5, and the lowest level of disease severity in plants treated with strain 34S1 (Table 2).

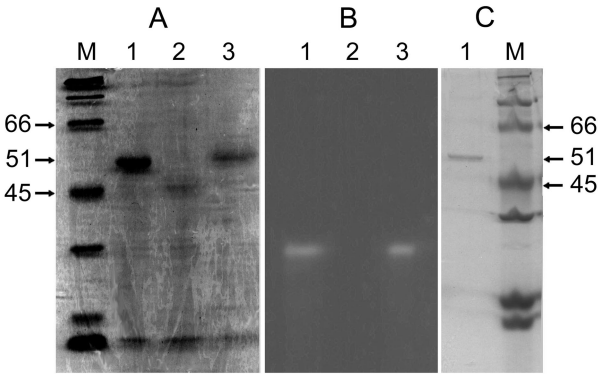

FIG. 3. Protein gel electrophoresis of *chiA* gene product expressed in *S. maltophilia* strains. (A) Coomassie-stained SDS-PAGE of extracellular proteins isolated from 3-day-old culture filtrates. Lane M, broad-range molecular size markers (in kilodaltons). Lane 1, wild-type strain 34S1; lane 2, chitinase mutant strain C5; lane 3, strain C5(pXMC431). (B) Chitinase active protein bands from culture filtrates detected in native gels using 4-MU-$(GlcNAc)_2$. (Gel is not in alignment with gels in panels A and B.) Native gels stained with 4-MU-$(GlcNAc)_3$ (not shown) produced identical activity banding patterns. Lane 1, wild-type strain 34S1; lane 2, chitinase mutant strain C5; lane 3, strain C5(pXMC431). (C) Coomassie-stained SDS-PAGE of chitinase protein purified from 3-day-old culture filtrate of *S. maltophilia* strain 34S1 by hydrophobic interaction chromatography. Lane 1, purified chitinase; lane M, broad-range molecular size markers.

In contrast to disease suppression, mutant strain C5 did not appear to be drastically affected in ability to colonize the rhizosphere of Kentucky bluegrass compared with strain 34S1 during sampling times known to influence biocontrol activity (30). After both strains 34S1 and C5 initially established at 10^8 CFU/g (dry weight) of rhizosphere sample, populations steadily decreased to similar populations below 10^6 CFU/g of sample on five observation dates over a 2-week period beginning at the time of application. No significant differences were detected between strains C5 and 34S1 on any of the five observation dates during this period in three separate experiments (data not shown).

Chitinolytic activity has been implicated to play a role in the biocontrol activity of several biocontrol bacteria, including *S. maltophilia* strains. However, direct molecular evidence for its role is lacking for many of these biocontrol systems. Mutation of *chiA* in *S. maltophilia* strain 34S1 abolishes chitinolytic activity and affects the ability of the strain to suppress summer patch disease, providing direct evidence for the role of this enzyme in biocontrol activity. Populations of mutant strain C5 were not significantly impaired in the turfgrass rhizosphere compared with the wild-type strain 34S1, providing further support that the enzyme functions directly in fungal antagonism. Because of plasmid instability problems, we were unable to evaluate biocontrol activity to mutants restored with chitinase. As a result, the possibility that the mutation had an indirect or polar effect(s) on other traits contributing to biocontrol cannot be excluded. The latter is less likely, since molecular analysis of *chiA* suggests that it is transcribed monocistronically.

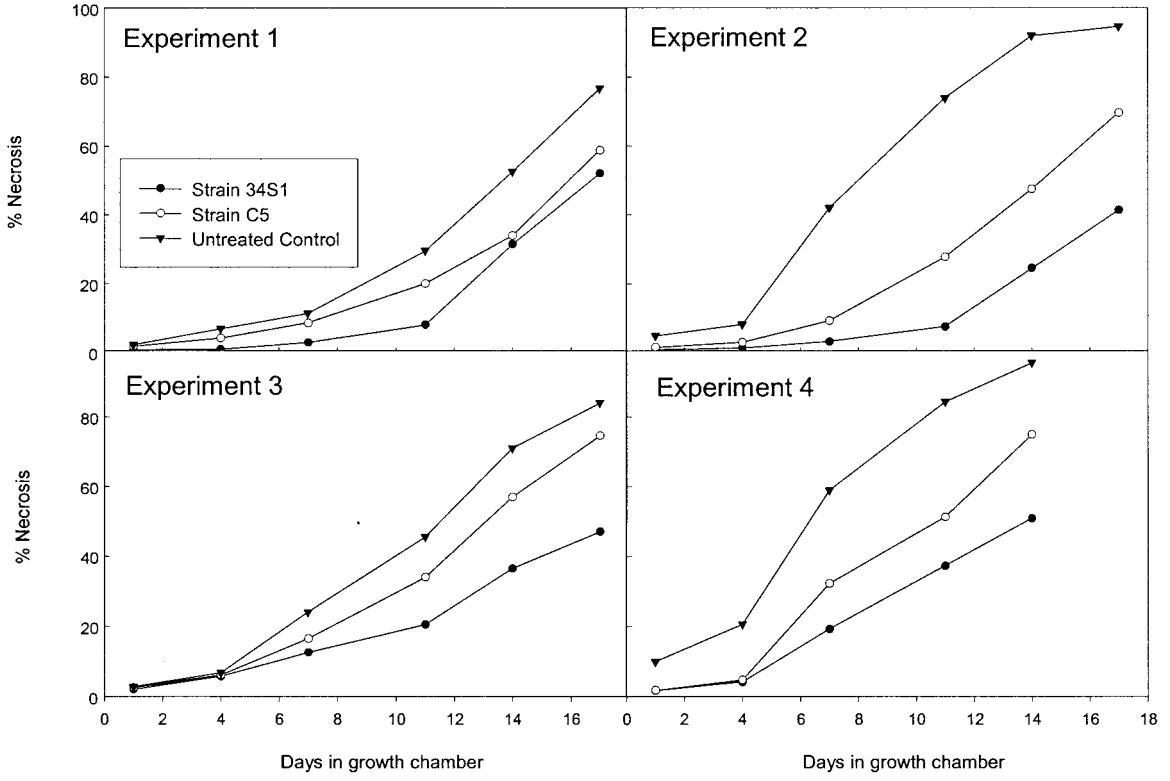

FIG. 4. Progression of summer patch disease severity on Kentucky bluegrass plants treated with *S. maltophilia* strain 34S1 and mutant strain C5. Progression of disease severity was plotted as average values estimated for percentage of necrosis within a container of plants over time. Four of five experiments are presented; statistical analysis conducted on the linearized data from all five experiments is presented in Table 2.

Combining evidence that chitinolytic activity functions in direct fungal antagonism with the observation that rate of disease progression was not affected in C5-treated plants compared with 34S1-treated plants, but also that disease onset was significantly delayed for the latter, it seems likely that 34S1 is better capable of reducing initial pathogen colonization of turfgrass plants. However, once initial infection occurs, disease progresses at similar rates.

Mutant strain C5 was not completely reduced in biocontrol activity, and thus it is evident that other biocontrol mechanisms are operating in this system. Other traits produced by *S. maltophilia* strain 34S1 could be contributing to antifungal antagonism in this system, including additional enzyme activities and antibiotic-like compounds. *Stenotrophomonas* spp. are known to produce several different types of extracellular enzymes and antibiotics that likely contribute to biocontrol activity among strains within this bacterial group. For example, Dunne et al. (10) found that the proteolytic activity of *S. maltophilia* contributes to biocontrol activity of sugar beet damping-off caused by *Pythium ultimum*. Nakayama et al. (43) characterized a new group of antibiotic-like compounds, xanthobactins, produced by *Stenotrophomonas* sp. strain SB-K88. In this study, purified xanthobactin A directly suppressed *Pythium* damping-off of sugar beet. As demonstrated with *Trichoderma harzianum* (54), it is possible that a synergistic, antifungal response results from the combined effect of chitinase with antibiotics produced by *S. maltophilia*.

TABLE 2. Results of linear regressions of summer patch disease on Kentucky bluegrass in greenhouse studies[a]

Treatment	Slope	*y* intercept	Disease severity
Strain 34S1	0.36*	−5.14*	−1.78*
Strain C5	0.41*	−4.63†	−0.86†
None (control)	0.61†	−4.26†	1.26‡

[a] Slope was averaged over the five experiments and describes the slope of the logit-transformed line. The *y* intercept was averaged over the five experiments and describes the logit-transformed line. Disease severity was calculated from the regression equation and represents logit-transformed disease severity after 10 days. Similar symbols within columns represent no significant difference.

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