Chitinases from the Plant Disease Biocontrol Agent, *Stenotrophomonas maltophilia* C3

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ABSTRACT

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Stenotrophomonas maltophilia strain C3, a biocontrol agent of *Bipolaris sorokiniana* in turfgrass, produced chitinases in broth media containing chitin. Chitinases were partially purified from culture fluid by ammonium sulfate precipitation and chitin affinity chromatography. The chromatography fraction with the highest specific chitinase activity was inhibitory to conidial germination and germ-tube elongation of *B. sorokiniana*, but it was less inhibitory than the protein fraction or the raw culture filtrate. The fraction exhibited strong exochitinase and weak endochitinase activity. Optimum temperature and pH for chitinase activity were 45 to 50°C and 4.5 to 5.0, respectively. Chitinase activity was inhibited by Hg^{2+} and Fe^{3+}, but not by other metal ions or enzyme inhibitors. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of the chromatography fraction revealed the presence of five protein bands of 25, 32, 48, 65, and 75 kDa. Partial amino acid sequences of the 32-, 65-, and 75-kDa proteins indicated that they are homologous to known bacterial chitinases. There was no homology found in the partial amino acid sequences of the 25- and 48-kDa proteins to any known chitinases. Five chitinase-active proteins were detected in the protein and chromatography fractions by activity gels, but when each protein was extracted and re-electrophoresed separately under denaturing conditions, only 32- or 48-kDa proteins were revealed. It was concluded that strain C3 produces at least two chitinases that are antifungal.

Plant disease control by chitinolytic bacteria has long been reported (17,28). Application of chitin to soils or foliage increased numbers of chitinolytic bacteria along with providing pathogen control (10,17). In addition, bacterial chitinase genes, when introduced into heterologous strains, provided antifungal activity (26, 29), or when introduced into plants, conferred increased disease resistance (15). However, few studies on bacterial agents have provided direct evidence, through analysis of mutants lacking chitinase activity or through assessment of extracted chitinase for biological activity, that chitinase had a role (4,14,26). We recently reported *Stenotrophomonas maltophilia* strain C3 to be a biocontrol agent, active in part through chitinase activity (38). The bacterial strain was effective in inhibiting germination of conidia of *Bipolaris sorokiniana* (Sacc.) Shoemaker, on grass leaves and, thereby, suppressing Bipolaris leaf spot in the field (36). Control efficacy was increased by applying colloidal chitin with the strain (36,37) or by applying cells of C3 with fluid from broth cultures (37). The latter strategy also provided effective field control of *Rhizoctonia solani* on turfgrasses (34) and rust (*Uromyces appendiculatus*) on common bean (35). As evidence that chitinase was involved in biological control, application of colloidal chitin to tall fescue with C3 increased chitinase activity on leaves over that of treatment with the bacterium alone (38). In addition, loss of chitinase production in C3 through spontaneous mutation resulted in loss of most of its antifungal activity. Furthermore, the degree to which various fractions of C3 broth culture fluid suppressed pathogen germination and infection of tall fescue was related to chitinase activity level in each fraction.

Besides strain C3, a number of *S. maltophilia* strains inhibit fungal growth or infection when applied to plants (2,5,6,10–12,

21), but there is no mechanism of antagonism that is common among these strains. Biological control by these strains has been attributed to induced resistance (6), antibiotics (21), and protease (5). Chitinase production was expressed in most strains of *S. maltophilia* evaluated in vitro by O'Brien and Davis (22), but only 1 of 11 *Verticillium*-inhibitory strains tested by Berg et al. (2) was chitinolytic, indicating that involvement of chitinase in fungal inhibition by *S. maltophilia* is strain specific. A direct connection between chitinase activity and biological control has not been shown for any *S. maltophilia* strain other than C3. Chitinase genes from strain 34S1 (GenBank Accession No. AF014950), a biocontrol agent of summer patch disease of turfgrass and from a strain with no reported biocontrol activity (GenBank Accession No. AF047411) were sequenced. No information is available, however, about the characteristics of chitinases produced by *S. maltophilia* or about the effect of its chitinases on fungal growth.

Our objectives in this study were to determine conditions for production of chitinases by *S. maltophilia* C3 in vitro and to characterize partially purified chitinases according to their antifungal activity, substrate specificity, and the influence of environmental factors on their activity. We also determined the number of the chitinases produced by C3, their molecular weights, and their similarity to other known chitinases.

MATERIALS AND METHODS

Culture conditions. Strain C3 was stored at –70°C in nutrient broth containing 10% glycerol. For each experiment, a fresh culture was grown on tryptic soy agar (Sigma Chemical Co., St. Louis) for 24 h at 25°C. The cells were suspended in 0.01 M potassium phosphate buffer (pH 7.0) to 5×10^9 CFU/ml. Broth media, in 200 ml volumes (pH 7.0), were seeded with 0.5 ml of the suspension and incubated at 25°C for up to 10 days with constant shaking. During the culturing period, samples were collected from each flask and used for determining bacterial numbers, protein concentration, and chitinase activity.

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The basic broth medium (19) was composed (gram per liter) of $(NH_4)_2SO_4$, 1.0; $MgSO_4 \cdot 7H_2O$, 0.3; KH_2PO_4, 1.36; and yeast extract, 0.5. Prior to autoclaving, the pH of the broth was adjusted to 7.4 with 1 N NaOH so the pH was 7.0 after autoclaving. Crude chitin (practical grade; Sigma Chemical) was added at 10 g/liter as the carbon source. In experiments comparing different carbon sources for chitinase production, glucose, colloidal chitin, and fungal cell wall preparations were tested at the same concentration. Colloidal chitin was prepared from crude chitin by the method of Roberts and Selitrennikoff (25). Cell walls of *B. sorokiniana* and *Pythium ultimum* were extracted from 7-day-old broth cultures in a minimal medium according to the procedure of Sivan and Chet (27).

Purification procedures. Cell-free culture fluid was prepared from 7-day-old broth cultures. Cells and other particulates were removed by centrifugation at $27,000 \times g$ for 25 min. The supernatant was filter-sterilized and stored at $-20°C$ before use. Proteins in the supernatant were precipitated by the addition of ammonium sulfate to achieve 85% saturation. After incubation with gentle stirring for 4 h, the precipitate was collected by centrifugation at $48,000 \times g$ for 25 min. For testing of the fraction for chitinase activity, the pellet was dissolved in 50 mM potassium phosphate buffer (pH 6.0), and dialyzed against the same buffer. For further chitinase purification, the pellet was dissolved in a minimum volume of 20 mM sodium bicarbonate buffer (pH 8.4) and dialyzed against the same buffer.

Chitinases were purified by affinity chromatography with chitin regenerated from chitosan (Sigma Chemical) by acetylation in aqueous methanolic acetic acid as described by Molano et al. (18). All purification steps were performed at 4°C unless noted otherwise. A column (15×2.5 cm) was filled with regenerated chitin, equilibrated with 20 mM $NaHCO_3$ (pH 8.4), and loaded with 8 ml of the dialyzed protein fraction. After incubation on ice for 1 h, the column was washed with 300 ml of 20 mM $NaHCO_3$ (pH 8.4) followed by 200 ml of 20 mM sodium acetate (NaAc) buffer (pH 5.5) to remove components bound nonspecifically to the chitin. Chitinases were eluted by washing the column with 300 ml of 20 mM acetic acid (HOAc) at pH 3.0. Each fraction was collected and concentrated to approximately 10 ml in a stirred pressure cell over a YM-10 membrane (Amicon, Beverly, MA). The NaAc and HOAc fractions were dialyzed against 10 mM NaAc at pH 5.5 and stored at $-20°C$.

Chitinase activity assay. All chitin oligomers used as substrates for chitinase assays were purchased from Sigma Chemical, and each contained more than 99% effective reagent. Chitinase activity in all fractions was measured by a colorimetric assay as described by Roberts and Selitrennikoff (25), with chitobiose linked to ρ-nitrophenol (ρNP-$(GlcNAc)_2$) as the substrate. In experiments to determine substrate specificity, ρNP-GlcNAc and ρNP-$(GlcNAc)_3$ also were used. A reaction mixture, containing 100 µl of a 0.5-mM solution of substrate in 50 mM potassium phosphate buffer (pH 6.0) and 10 µl of a fraction was added to duplicate wells of a microplate. After incubation at 37°C for 20 min, the reaction was stopped by adding 10 µl of 1 N NaOH to each well. The release of the chromophore ρ-NP from the substrate was measured at 410 nm in a microplate reader (Molecular Devices Corp., Sunnyvale, CA). One unit of enzyme activity was defined as the amount of enzyme that produced 1 µmol of ρ-NP per minute at 37°C. Specific activity of a test fraction was expressed as activity units per milligram of total protein, with protein content determined by the Bradford method (3) with bovine serum albumin as a standard.

Antifungal assay. All fractions were tested for effects on the germination of *B. sorokiniana* conidia and elongation of germ tubes. Prior to bioassay, the fractions were concentrated as described above, dialyzed against 50 mM potassium phosphate buffer (pH 6.0), and diluted with the same buffer to the same protein concentration. Suspensions of conidia were prepared as

previously described (36). Each test fraction (60 µl) was mixed with 20-µl conidial suspension in the wells of sterile depression slides. The slides were incubated in petri dishes lined with moistened filter papers for 8 h at room temperature. Conidia were examined for germination, and germ-tube length was measured with an ocular micrometer under a compound microscope (Optiphot; Nikon Inc., Melville, NY) at 410× magnification. Each treatment was repeated on six slides, and 100 conidia were examined per slide.

Effects of temperature, pH, metal ions, and inhibitors on chitinase activity. The influence of environmental factors on chitinase activity was investigated with the chitinase-active HOAc fraction from chitin affinity chromatography diluted in 50 mM potassium phosphate buffer (pH 6.0) to 0.003 units per 10 µl. To determine the influence of temperature on chitinase activity, mixtures of ρNP-$(GlcNAc)_2$ and the enzyme fraction were incubated for 20 min in microplates placed at 16 to 55°C. Effects of pH were assessed by the following buffers: sodium lactate at pH 3.0 and 3.5; sodium acetate at pH 4.0, 4.5, and 5.5; potassium phosphate at pH 6.0, 6.5, 7.0, and 7.5; Tris-HCl at pH 8.0 and 8.5; and glycine-NaOH at pH 9.0, 9.5, and 10.0. Metal ions (K^+, Mg^{2+}, Ca^{2+}, Zn^{2+}, Ni^{2+}, Co^{2+}, Mn^{2+}, Cu^{2+}, Fe^{3+}, Ag^+, and Hg^{2+}) and enzyme inhibitors (dithiothreitol, β-mercaptoethanol, and EDTA) were tested as 1- and 5-mM solutions in 50 mM potassium phosphate buffer (pH 6.0). Each test solution was mixed with the enzyme fraction in duplicate wells of a microplate. After incubation at 22°C for 1 h, residual chitinase activity in the mixture was measured by adding the substrate and expressed as a percentage of the activity determined in the absence of metal ions or enzyme inhibitors.

Characterization of chitinases by electrophoresis. Proteins in the protein fraction and the HOAc fraction from affinity chromatography were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and tested for chitinase activity according to the method of Chernin et al. (4) with some
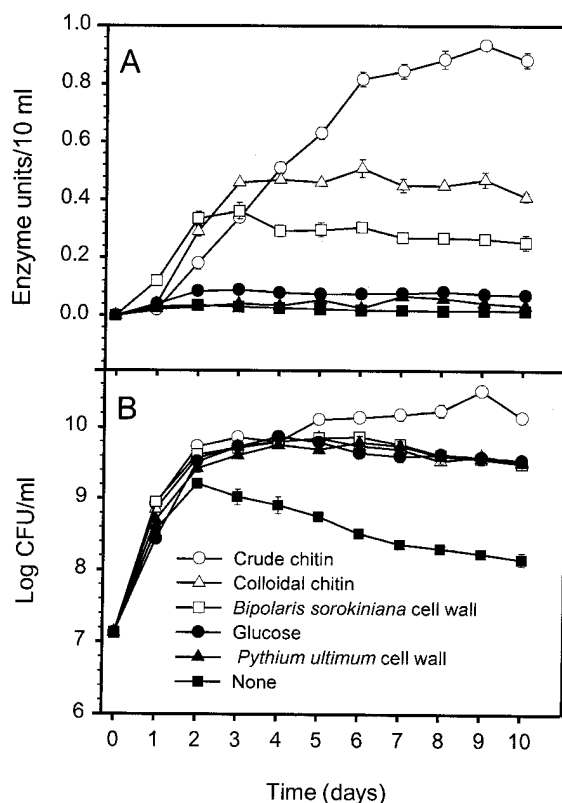

Fig. 1. A, Chitinase activity and **B,** population sizes of *Stenotrophomonas maltophilia* strain C3 in broth cultures with various carbon sources. Error bars denote standard deviation.

modifications. Samples were mixed with an equal volume of 2×
Laemmli sample buffer (13) containing 2% Triton X-100 (Sigma
Chemical), and the mixtures were heated at 50°C for 5 min. As
reported for other bacterial chitinases (4,31), C3 chitinases were
not affected by heating at 50°C in preliminary experiments. Electrophoresis was conducted in 8% polyacrylamide gels. After electrophoresis, SDS was removed to renature the proteins by incubating the gels in distilled H_2O for 10 min and then in 2.5% Triton
X-100 for 30 min at room temperature. Subsequently, chitinase
activity was detected by incubating the gels at room temperature
in 0.2 mM solutions of chitin oligomers linked to 4-methylumbelliferone (4-mu-GlcNAc, 4-mu-(GlcNAc)$_2$, and 4-mu-(GlcNAc)$_3$)
in potassium phosphate buffer. Gels were inspected for fluorescent
bands under ultraviolet light.

To determine the molecular weight of chitinase-active proteins,
fluorescent bands were excised from the activity gels, and each
band was ground separately in a minimum volume of 1.5×
Laemmli sample buffer. The samples were incubated at 85°C for
5 min and centrifuged at $27,000 \times g$ for 2 min. Each supernatant
was subjected to SDS-PAGE on a 12% polyacrylamide gel. The
resulting gel was stained with silver nitrate according to the
method of Merril et al. (16).

Sequence analysis of chitinases. Partial amino acid sequences
were determined for chitinase-active (32- and 48-kDa) proteins
detected in the protein fraction and for all proteins in the HOAc
fraction from chitin affinity chromatography. The proteins were
separated on 12% SDS-PAGE gels by the method of Laemmli
(13). The gels were washed for 5 min with 10 mM CAPS/NaOH
buffer (pH 11.0) containing 10% methanol, and the proteins were
transferred to polyvinylidene difluoride (PVDF) membranes with
a semidry electroblotter (Immobilon P; Millipore Corp., Bedford,
MA) at 100 mA for 20 min. The membranes were washed with
methanol, stained with 0.1% (wt/vol) Amido black in 40% methanol and 10% acetic acid for 1 min, and destained with several
changes of distilled water. The major stained bands were excised,
and the N-terminal amino acid sequence of the proteins were
determined at the University of Nebraska-Lincoln Protein Core
Facility (Biosystems Procise 494; Perkin-Elmer Applied Biosystems, Foster City, CA). Proteins that were blocked at the N terminus were subjected to enzymatic digestion and peptide separation
according to the method of Penheiter et al. (23). Ten bands of a
protein (≈30 μg) were cut from 12% SDS-PAGE separation gels
and digested with 0.3 μg of trypsin (Sigma Chemical) at 37°C for
20 h. The resultant peptides were fractionated on a 2.1×250 mm
C18 column (Model 218 TP52, VYDAC, Hesperia, CA) (1 column volume = 0.866 ml) with a linear gradient from 2% CH_3CN
in 0.1% trifluoroacetic acid to 60% buffer B (90% CH_3CN in
0.09% trifluoroacetic acid) over 15 column-volumes at 0.2 ml/min
on a high performance liquid chromatography (Microbore; ISCO,
Lincoln, NE). Absorbance was monitored at 214 nm, and peaks
were collected manually. Peptide fragments with high relative
peak absorbance were sequenced as described above and resulting

amino acid sequences compared for homology with proteins in the
databases. Sequencing of multiple fragments from the same protein was not pursued when a match of >15 residues was observed
for a given peptide.

Statistical analysis. Analysis of variance was applied to quantitative data. Percentage data (i.e., conidial germination and relative chitinase activity) were analyzed after arcsine transformation,
but because the results of the two methods were similar, only the
analysis of nontransformed data is presented. Fisher's LSD was
used for treatment mean comparisons.

RESULTS

Induction of chitinase production. Production of chitinase by
strain C3 was induced by chitin-containing substrates including
the cell wall of *B. sorokiniana*. Cultures in crude chitin had the
highest chitinase activity, reaching peak level after 9 days (Fig.
1A). In contrast, very little chitinase activity was detected when
glucose or *P. ultimum* cell wall was provided as a carbon source.
Although the increase in chitinase activity in the chitin-containing
cultures reflected the increase in cell density, chitinase activity
was not entirely related to growth of strain C3, because similar
cell growth occurred in cultures containing glucose and *P. ultimum*
cell wall (Fig. 1B).

Partial purification of chitinases. Following ammonium sulfate precipitation, 73% of the chitinase activity present in the raw
cell-free culture fluid was recovered, but the specific activity was
not increased (Table 1). Through chitin affinity chromatography,
over 90% of the contaminating proteins were removed by washing
with NaHCO$_3$ and NaAc solutions, although considerable chitinase
activity was eluted out with the NaHCO$_3$ fraction. The highest
total and specific chitinase activity levels were found in the HOAc
fraction; chitinases were purified over eightfold, and 37% of the
total chitinase activity was recovered.

Antifungal effects of chitinase fractions. All of the fractions
from chitin affinity chromatography, except the NaAc fraction,
inhibited *B. sorokiniana* conidial germination and germ-tube
growth as compared with the phosphate buffer control (Table 1).
The HOAc fraction that contained the highest specific chitinase
activity and the NaHCO$_3$ fraction that contained the lowest specific chitinase activity were equal in effect. Both fractions were
less inhibitory to *B. sorokiniana* than the raw culture fluid or the
protein fraction.

Substrate, pH, and temperature effects on chitinase activity.
The HOAc fraction from affinity chromatography exhibited highest chitinase activity when ρNP-(GlcNAc)$_2$ was used as the chitin
oligomeric substrate (OD$_{410}$ = 0.99). No yellow color was observed when using ρNP-GlcNAc (OD$_{410}$ = 0.01), and only a faint
yellow color was generated in the presence of ρNP-(GlcNAc)$_3$
(OD$_{410}$ = 0.14). Using ρNP-(GlcNAc)$_2$ as the substrate, the maximum activity in the HOAc fraction occurred at pH 4.5 to 5.0.
Almost no activity was detected above pH 9.5 (Fig. 2A). With pH

TABLE 1. Chitinase activity in, and inhibition of, *Bipolaris sorokiniana* by various purification fractions from chitin broth cultures of *Stenotrophomonas maltophilia* strain C3

Fraction[y]	Protein (mg)	Chitinase activity(units)	% Chitinase recovery	Specific activity	Chitinase purification (fold)	Antifungal activity[z]	
						% Germination	Germ tube (μm)
Culture fluid	56.18	8.11	100	0.14	1.0	10 c	2.1 c
Total protein	42.75	5.92	73	0.14	1.0	12 c	2.0 c
NaHCO$_3$	28.71	2.20	27	0.08	0.6	32 b	8.8 b
NaAc	0.60	0.28	3	0.48	3.4	78 a	15.6 a
HOAc	2.45	2.97	37	1.21	8.6	30 b	8.4 b
Phosphate buffer	…	…	…	…	…	81 a	18.7 a

[y] NaAc = sodium acetate; HOAc = acetic acid; and total protein was determined in the ammonia sulfate fraction of the culture fluid.

[z] Each fraction was adjusted to a protein concentration of 0.25 mg/ml prior to antifungal assay. The values are means of data from two repetitions of the experiment, with six replicates per treatment. Means in a column followed by the same letter are not significantly different at $P = 0.05$ according to LSD test.

kept at 6.0, hydrolysis of ρNP-(GlcNAc)$_2$ by the HOAc fraction was highest at 45 to 50°C (Fig. 2B). Some activity was evident at temperatures as low as 16°C, but it decreased rapidly above 50°C.

Effects of metal ions and reagents on chitinase activity. Most of the chemicals at 1 mM did not greatly affect chitinase activity, except Hg^{2+}, which caused 57% inhibition (Table 2). Hg^{2+} and Fe^{3+} at 5 mM completely eliminated activity. Ag$^+$, Mn^{2+}, Cu^{2+}, dithiothreitol, and β-mercaptoethanol at 5 mM inhibited enzyme activity from 15 to 25%, whereas K$^+$, Mg^{2+}, Ca^{2+}, Zn^{2+}, Ni^{2+}, Co^{2+}, and EDTA did not affect activity appreciably.

Protein analysis and chitinase detection by SDS-PAGE. Extracellular protein profiles of the extracts from chitin-amended cultures (Fig. 3, lanes 2 and 3) were different from those of glucose-amended cultures (Fig. 3, lane 1). As revealed by SDS-PAGE, a number of proteins were produced in chitin-amended cultures that were not apparent in glucose-amended cultures.

Chitinase activity in protein fractions from chitin-amended cultures was detected in activity gels. Using 4-mu-(GlcNAc)$_2$ as the substrate, five fluorescent bands were detected, with two bands in upper positions and three bands in lower positions (Fig. 4A, lane 2). When 4-mu-GlcNAc was used instead, no fluorescent bands were detected; when using 4-mu-(GlcNAc)$_3$, only faint fluorescence was observed at the upper gel position (data not shown). Regardless of the substrates used, no fluorescent bands were visible in the protein fractions from glucose-amended cultures (Fig. 4A, lane 1).
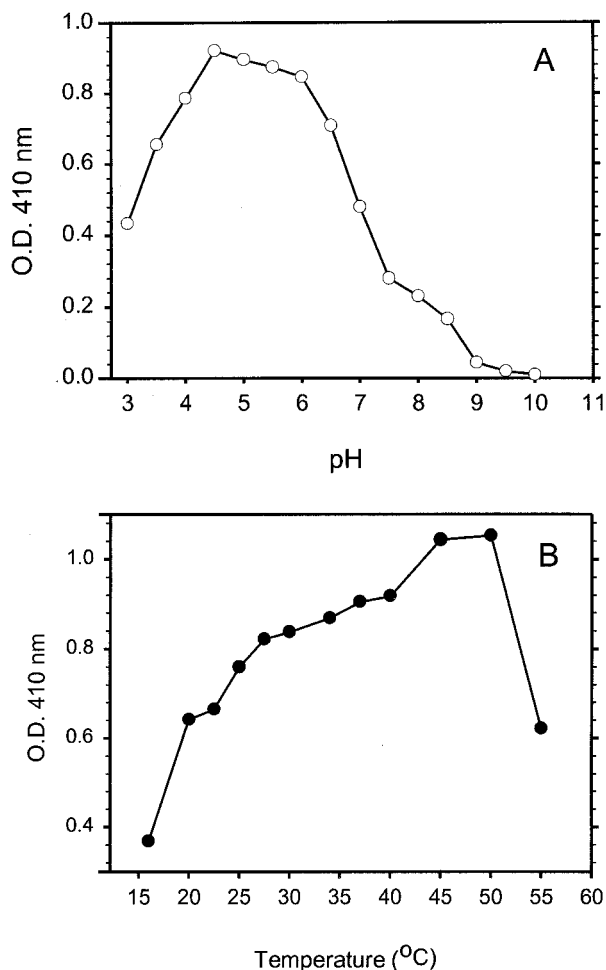
When the five fluorescent bands were excised individually and the extracted proteins subjected to SDS-PAGE under denaturing conditions, only two protein bands were apparent upon silver staining (Fig. 4B, lanes 1 through 5). The upper two and the lower three fluorescent bands (Fig. 4A, lane 2) in the activity gel appeared as 48-kDa (Fig. 4B, lanes 1 and 2) and 32-kDa (Fig. 4B, lanes 3 through 5) protein bands, respectively, in the denatured gel.

When the HOAc fraction was analyzed in activity gels with the protein fraction, the two fractions yielded the same five fluorescent bands (data not shown). When the fluorescent bands from the HOAc fraction were individually re-electrophoresed under denaturing conditions, each of the upper two fluorescent bands appeared as a 48-kDa polypeptide band, and each of the lower three

TABLE 2. Effects of metal ions and inhibitors on chitinases in acetic acid (HOAc) fraction from chitin affinity chromatography of chitin broth culture of *Stenotrophomonas maltophilia* strain C3

Chemical[y]	Residual chitinase activity (% phosphate buffer)[z]	
	1 mM	5 mM
Phosphate buffer	100	100
KCl	93	91
MnCl$_2$	98	87
MgCl$_2$	99	92
CuCl$_2$	106	87
CaCl$_2$	101	95
FeCl$_3$	98	0
NiCl$_2$	102	90
HgCl$_2$	43	0
CoCl$_2$	98	98
AgNO$_3$	97	75
ZnSO$_4$	99	102
EDTA	96	94
β-Mercaptoethanol	96	86
Dithiothreitol	98	88
LSD (0.05)	8	8

[y] HOAc fraction was incubated with each chemical at 1 or 5 mM for 1 h at 22°C before being assayed for residual chitinase activity.

[z] Activity of the HOAc fraction diluted in phosphate buffer was regarded as 100%. The values are means of data from two repetitions of the experiment, with three replicates per treatment.
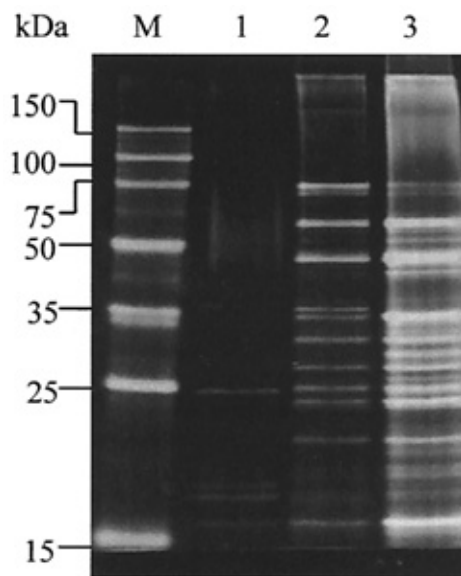

Fig. 2. A, Effects of pH and **B,** temperature on chitinase activity in the acetic acid (HOAc) fraction from chitin affinity chromatography of extracellular proteins produced by *Stenotrophomonas maltophilia* strain C3. For the pH experiment, chitinase activity was measured at 37°C for 20 min. For the temperature experiment, chitinase activity was measured at pH 6.0 for 20 min.

Fig. 3. A silver-stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12% polyacrylamide) of heated (85°C) extracellular proteins produced by *Stenotrophomonas maltophilia* C3 in different media. Lane M, molecular mass standards; lane 1, supernatant from a glucose-amended culture; lane 2, supernatant from a chitin-amended culture; and lane 3, ammonium sulfate precipitate from the supernatant of the chitin-amended culture.

fluorescent bands appeared as a 32-kDa polypeptide band (data not shown). When the whole HOAc fraction was electrophoresed under denaturing conditions, five silver-stained bands with molecular weights of 75, 65, 48, 32, and 25 kDa were detected (Fig. 5, lane 4).

N-terminal amino acid sequences. The N-terminal 15 amino acids of the 32-kDa chitinase from the protein fraction showed homology with internal sequences of chitinases from *S. maltophilia* 34S1, *Streptomyces thermoviolaceus*, *Amycolatopsis methanolica*, *Streptomyces splicatus*, *Streptomyces lividans*, *Janthinobacterium lividum*, and *Arthrobacter* spp. (Table 3). The highest homology was observed with an internal region (residues 369-383) of chitinase from *S. maltophilia* 34S1, with 12 of 15

residues identical. The N-terminal amino acid sequence of the 48-kDa chitinase from the protein fraction APVGT GQGTN QKHYQ was not homologous to any chitinase in the database. The N-terminal 15 amino acid sequences of the 32- and 48-kDa proteins from the HOAc fraction were identical to the respective same-size chitinases found in the protein fraction.

The sequence of N-terminal 15 amino acids of the 25-kDa protein from the HOAc fraction, A(C)NYV E(E)TRG VNYTC, was not homologous to any chitinase in the database. The N-termini of the 65- and 75-kDa proteins from the HOAc fraction appeared to be blocked. After trypsin digestion of these two proteins, internal peptide fragments T29 of the 65-kDa protein and T25 of the 75-kDa protein were subjected to N-terminal amino acid analysis. The 25 amino acids of the two fragments were identical VTDPG AYHQY LDFIN VMTYD FHGAW and homologous with known chitinases from both prokaryotic and eukaryotic organisms (Table 3). The highest homology was found to residues 518-542 of *S. maltophilia* 34S1 chitinase A, with 23 of the 25 amino acids identical. No other peptide fragments from the 65- and 75-kDa proteins were sequenced.

DISCUSSION

Strain C3 is unique in comparison with other strains of *S. maltophilia* that have been reported to be biocontrol agents against fungal plant pathogens (2,5,6,10–12,21), in that only C3 has been reported to have efficacy in controlling pathogenic fungi under field conditions (34–36). It also is unique in that its biological control activity was related, in this study, to the production of two chitinases. Strain 34S1, isolated from Kentucky bluegrass rhizosphere and active against *Magnaporthe poae* (10), is the only other biocontrol strain of *S. maltophilia* for which its chitinase has been characterized. 34S1 differs from C3 in that 34S1 excretes only one chitinase (D. Kobayashi, *personal communication*). The two strains also differ in colony morphology, extracellular protein profiles, and some other biochemical properties (Z. Zhang, *unpublished data*). The importance of chitinase as a mechanism of action in biological control by 34S1 has not been established.

We surmise that the chitinase-active HOAc fraction from chitin-affinity chromatography contained primarily chitinases, with small amounts of other proteins with strong affinity to the chitin matrix.
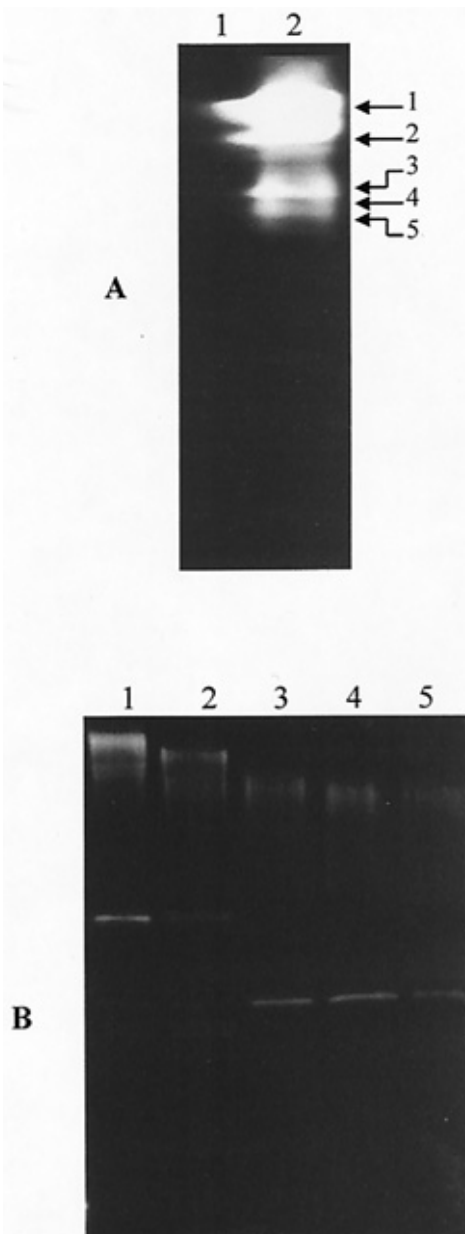

Fig. 4. A, A chitinase activity gel of extracellular proteins produced by *Stenotrophomonas maltophilia* C3, stained with 4-methylumbelliferyl-β-1,4-diacetylchitobiose. Lane 1, total protein from glucose-amended cultures, and lane 2, total protein from chitin-amended cultures. **B,** A silver-stained gel (12% polyacrylamide) containing fluorescent bands excised from activity gel **A** and electrophoresed separately. Lanes 1 to 5 each correspond to a fluorescent band indicated by an arrow in activity gel **A**, from top to bottom, respectively. Molecular mass standard was not loaded in the activity gel because proteins were not denatured as described by Laemmili (13), and therefore, their migrations in the gel were not totally dependent on their molecular weights.
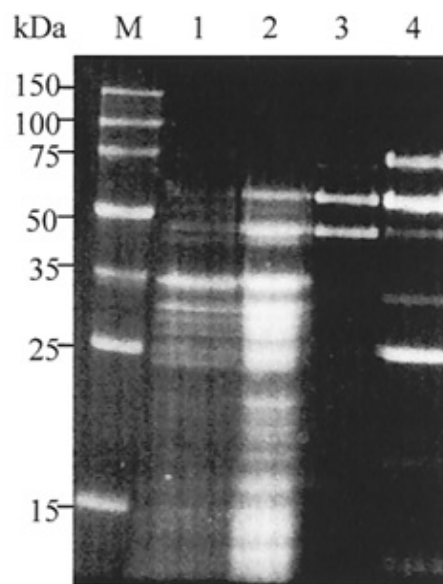

Fig. 5. A sodium dodecyl sulfate-polyacrylamide gel electrophoresis of fractions from chitin affinity chromatography of extracellular proteins produced by *Stenotrophomonas maltophilia* C3. Lane M, molecular mass standards; lane 1, total protein; lane 2, NaHCO₃ fraction; lane 3, sodium acetate fraction (NaAc); and lane 4, acetic acid fraction (HOAc).

In partially purified form, the chitinases inhibited *B. sorokiniana* conidial germination and germ-tube elongation in vitro. Although we did not have sufficient amounts of this fraction to confirm that chitinases alone could affect leaf spot development, our previous findings from experiments with C3 cells and culture fluid fractions showed that inhibition of conidial germination and germ-tube growth was directly correlated with disease suppression (36,37). Although the HOAc fraction had the highest level of specific chitinase activity, it was not as inhibitory to conidial germination and germ-tube growth as the raw culture extract or the protein fraction. The NaHCO3 fraction, with the lowest level of specific chitinase activity, was equal to the HOAc fraction in antifungal effect. These results support our previous findings, using culture filtrate fractions and chitinase-deficient mutants, which indicate the involvement of other metabolites in biological control in conjunction with chitinase (38). The other factors very likely could be other types of lytic enzymes including β-1,3-glucanase, protease, and lipase, all of which were produced in broth cultures (38). A low molecular-weight fraction of C3 culture fluid was slightly inhibitory to conidial germination and leaf spot development (38), suggesting that antibiotic compounds such as maltophilin (9) or xanthobaccins (21) might also be produced by C3.

The presence of five chitinase-active bands detected in the activity gels initially suggested that there may be five chitinases produced by C3 in vitro. Although the production of multiple chitinases has been reported for chitinolytic bacteria, for example, *Bacillus circulans* (31) and *Enterobacter agglomerans* (4), it seemed improbable that the relatively small bacterial genome could contain five distinct sets of genes to encode the same trait. The five individual activity bands appeared as only two proteins (32 and 48 kDa) when re-electrophoresed in denatured SDS-PAGE, leading us to conclude that C3 produces two chitinases. One possible explanation for the apparent reduction in numbers of proteins is that each of the chitinases, in its undenatured state, existed as multiple isoforms with different conformations, which migrated at different rates in the activity gels; heating at 85°C might have denatured all isoforms into one form (24,33).

When the HOAc fraction was heated at 85°C and subjected to SDS-PAGE, five protein bands were detected including the 32- and 48-kDA chitinases. The 65- and 75-kDa proteins are very likely forms of the same protein because of their identical partial amino acid sequence. The fact that the same peptide fragments from both proteins were sequenced was a coincidence. The two large proteins could be related to the 32-kDA chitinase because all of them were homologous to regions in chitinase A of *S. maltophilia* 34S1. If these proteins are related, the large proteins, by their size, may be products of dimerization of the 32-kDA chitinase. Alternatively, the 75-kDa protein could have been an uncleaved chitinase; in this case, the 32- and 65-kDa proteins may have been derived from the 75-kDa protein through enzymatic cleavage, because strain C3 also excretes proteases during growth (37). Proteolytic cleavage of portions of polypeptides from the N- and C-terminal ends of bacterial chitinases has been reported (7,33). The 65- and 75-kDa proteins were not represented by fluorescent bands on activity gels, and this may have been due to concentrations of the large proteins being too low to detect. The 25-kDa protein also was not apparent in the form of a fluorescent band in activity gels, and therefore, could be an inactive derivative of the 32- or 48-kDa chitinase, or it may have been an unrelated chitin-binding protein. Complete sequence information for each of the five proteins will be required to determine their exact relationships.

The 65- and 75-kDa proteins were homologous to chitinases from a wide variety of organisms: gram-negative and gram-positive bacteria, archea (*Pyrococcus kodakarensis*), braconid wasp (*Chelona* spp.), protozoa (*Entamoeba* spp.), and prawn (*Penaeus japonicus*) (Table 3). They also were homologous to some non-chitinolytic mammalian proteins (data not shown). These enzymes were listed as having catalytic domains in glycoside hydrolase family 18 (8). Because high sequence conservation is expected within the catalytic domain due to its important function, the sequenced portions of the 65- and 75-kDa proteins might be part of the catalytic domain. Except for chitinase A from *S. maltophilia* 34S1, the N-terminal amino acid sequence of the 32-kDa chitinase was homologous only to chitinases from gram-positive bacteria. Given the variable nature of that portion of chitinase molecules, the homology suggests that the 32-kDa chitinase might be acquired from unrelated bacteria through horizontal gene transfer. The 48-kDa chitinase appears to be unique because its N-terminal

TABLE 3. Amino acid sequence homology of the 32-, 65-, and 75-kDa proteins from *Stenotrophomonas maltophilia* strain C3 with other chitinases[w]

Organism[x]	Protein	Accession No.	Residue	Sequence[y]
Stenotrophomonas maltophilia C3	32-kDa chitinase	…	1-15	FQAGESVSGASDTWD
Stenotrophomonas maltophilia 34S1	Chitinase A	AF014950	369-383	-G-------SA----
Streptomyces thermoviolaceus	Chitinase	D14536	106-120	-T-DQ----QA----
Amycolatopsis methanolica	Chitinase	U31277	90-104	YT-AN--D-VA----
Streptomyces plicatus	Chitinase 63	M82804	299-313	YT-DQ--D-VA----
Streptomyces lividans	Chitinase C	D12647	298-312	YT-DQ--D-VA----
Arthrobacter spp.	Chitinase	AJ250585	131-145	YA-DK----KA---E
Janthinobacterium lividum	Chitinase	U07025	325-339	R-PKRT-D-QVIP--
Stenotrophomonas maltophilia C3	75-kDa protein	…	1-25 of T25[z]	VTDPGAYHQYLDFINVMTYDFHGAW
Stenotrophomonas maltophilia C3	65-kDa protein	…	1-25 of T29[z]	-------------------------
Stenotrophomonas maltophilia 34S1	Chitinase A	AF014950	518-542	----A---P----------------
Clostridium thermocellum	Chitinase	Z68924	221-244	-E-EI---------I----YS---
Janthinobacterium lividum	Chitinase	U07025	482-505	-E-AL-S--M-W--L-------G-
Chelonus spp.	Venom chitinase	U10422	200-216	-------L-------P-
Entamoeba histolytica	Chitinase	U78319	334-352	F---I-W--L----L-S-
Entamoeba invadens	Chitinase	U78320	341-359	F-K---W--L----L-S-
Pyrococcus kodakaraensis	Chitinase	AB024740	356-372	K---S--I----Y----
Arthrobacter spp.	Chitinase	AJ250585	408-424	K----V-M-S--L----
Streptomyces thermoviolaceus	Chitinase	D14536	238-261	--YAGAA--V-WY-------F---
Streptomyces plicatus	Chitinase 63	M82804	436-458	-Y-EASK-I-WY-------F---
Streptomyces lividans	Chitinase C	D12647	435-457	-Y-EASK-I-WY-------F---
Penaeus japonicus	Chitinase	D89751	325-347	-VP-LNR---W-A--------H-

[w] The 32-kDa chitinase was extracted from the protein fraction and from a chitin-affinity chromatography acetic acid (HOAc) fraction; the 65- and 75-kDa proteins were extracted from the HOAc fraction.

[x] Order of the organisms was based on decreasing homology of their chitinases to proteins from strain C3.

[y] Sequence alignments were performed using a BLAST search available through the ExPASy-Swiss database. Amino acid residues denoted by a dash (-) are identical to those of the 32-kDa chitinase or the 75-kDa protein from C3.

[z] T25 and T29 denote peptide fragments generated by trypsin digestion.

sequence was not homologous to that of any reported chitinase. None of the five proteins were homologous to another *S. maltophilia* chitinase (GenBank Accession No. AF047411), further indicating that there is diversity in chitinases produced by the species.

Factors affecting chitinase activity in *S. maltophilia* were investigated in this study for the first time. One factor was nutritional conditions for chitinase induction. Chitinase production by strain C3 in vitro was induced in the presence of chitin and *B. sorokiniana* cell wall. The lower levels of chitinase activity occurring in the presence of the cell wall rather than in the presence of chitin may be related to proteins associated with chitin molecules in the cell wall inhibiting contact between the enzymes and the substrate (1,32), or may have been due to the chitin content in the cell wall preparation being lower than in the purified substrates. *P. ultimum* cell wall, which does not contain chitin (1), was the least inductive carbon source. Crude chitin was more effective in inducing chitinase production by C3 than colloidal chitin, and this may have been due to residual proteins in crude chitin serving as growth factors or nutrients (11). In media lacking chitin, low levels of chitinase activity were still detected, suggesting that strain C3 constitutively synthesizes low amounts of chitinases during growth. A similar phenomenon was reported for *Bacillus circulans* (31).

Another factor investigated in this study was substrate specificity of the enzymes. The terms exochitinase and endochitinase have been coined for the enzymes that catalyze the release of chitobiose units from the nonreducing end of chitin chains and the random hydrolysis of chitin at internal sites, respectively (20). *N*-acetyl-β-1,4-glucosaminidase, which also acts in exosplitting mode, catalyzes the release of GlcNAc monomers from chitobiose or the chitin chain (20). The chitinases from strain C3 effectively cleaved trimeric chitin derivatives, cleaving chitobiose from chitin, but they were not capable of digesting dimeric chitin derivatives. The incubation of the activity gel in 4-mu-(GlcNAc)$_3$ generated faint fluorescence at the upper gel position, suggesting that the 48-kDa chitinase from C3 may also hydrolyze tetrameric chitin analog, but slowly. According to the current system of chitinase nomenclature, the 32-kDa chitinase is an exochitinase, and the 48-kDa chitinase is primarily of the exochitinase category, with only slight endochitinase activity. Neither chitinase has *N*-acetylglucosaminidase activity. These results were in line with findings provided by the HOAc fraction.

We also identified environmental factors that might affect chitinase activity. C3 chitinases were strongly inhibited by Hg^{2+}, suggesting the presence of a reactive cysteine in the active site. This is similar to chitinases from other bacterial species such as *Pseudomonas aeruginosa* (30). Except for Hg^{2+} and Fe^{3+}, the other ions tested had only slight effect on C3 chitinase activity. The partially purified chitinases exhibited an acidic pH optimum and were active at temperatures above 15°C and below 50°C. Taken together, these data suggest that strain C3 will secrete enzymatically competent chitinases in planta under field conditions.

Future studies on chitinase production by C3 will focus on purification of the individual enzymes and characterization of their structural and regulatory genes. Information on the regulation and expression of chitinases, in conjunction with other mechanisms, will contribute to a better understanding and eventual improvement of biological control by bacterial agents.

ACKNOWLEDGMENTS

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