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Cloning of a *Serratia marcescens* Gene Encoding Chitinase

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***Serratia marcescens*, a chitinase-producing microorganism, was shown to produce five unique chitinolytic proteins with subunit molecular masses of 21, 36, 48, 52, and 57 kilodaltons. A cosmid library of *S. marcescens* DNA was constructed in the broad-host-range cosmid pLAFR1 and screened in *Escherichia coli* for clones capable of degrading chitin. A total of four independent clones (22- to 27-kilobase inserts) were isolated, characterized by restriction endonuclease digestion, and shown to share a common 9.5-kilobase *Eco*RI fragment apparently encoding the same 57-kilodalton chitinase, the most abundant chitinase produced by *S. marcescens*. Chitinase expression from these constructs in both *E. coli* and *Pseudomonas fluorescens* 701E1 is apparently driven by an *S. marcescens* promoter. The significantly higher chitinase levels produced in *E. coli* relative to those in *P. fluorescens* 701E1 suggest that *E. coli* may recognize this promoter sequence more efficiently than *P. fluorescens*.**

Chitin, a polymer of *N*-acetylglucosamine (NAG), represents a major structural component of many agronomically important pests including insects, fungi, and nematodes (3, 23). The enzymatic digestion or deformation of the chitin component of these organisms by chitinase could present an effective method for their control. Furthermore, the production and delivery of chitinase to the specific site of infectivity by appropriate rhizoplane- or phyloplane-colonizing bacteria, such as the fluorescent pseudomonads, could present a novel method for biological control.

The addition of chitin to soil has been shown to reduce populations of fungal plant pathogens (18) and plant parasitic nematodes (14, 16). Chitin application leads to increased populations of chitinolytic bacteria, especially actinomycetes, and fungi. These increases are correlated with reductions in pathogenic fungi and nematodes and, more importantly, with the reduction of infectivity and, hence, crop damage (6, 14, 23). Although the evidence for the role of chitinase in fungal and nematode control is indirect, the correlation is strong and suggestive. Highly purified chitinase is essential to determine unequivocally its efficacy against fungi and nematodes. More importantly, the gene(s) encoding chitinase must be cloned, expressed, and stably maintained in rhizoplane- or phyloplane-colonizing bacteria to evaluate its in vivo efficacy.

Chitinases (EC 3.2.1.14) are a class of hydrolytic enzymes that are commonly found in bacteria, fungi, nematodes, insects, crustaceans, plants, and some vertebrates (21) and that degrade chitin by either an endolytic or exolytic mechanism. *Serratia marcescens* was selected as the source of chitinase for the studies described in this paper for the following reasons: (i) crude preparations of chitinases from *S. marcescens* are commercially available; (ii) an effective affinity chromatographic purification procedure for the *S. marcescens* chitinases has been reported (19); (iii) the gene(s) encoding these chitinases and their associated regulatory signals is likely to be recognized and expressed directly in *Escherichia coli*; (iv) preliminary data (20) suggest that the predominant *S. marcescens* chitinase is an endolytic enzyme that solubilizes chitin more rapidly than the exolytic enzymes and should, therefore, be more efficacious; and (v) *S. marcescens* chitinases hydrolyze "crystalline" chitin (20).

In this study, we describe: (i) the partial purification of *S. marcescens* chitinases, (ii) the identification of five unique proteins with chitinolytic activity that are produced by *S. marcescens*, (iii) the cloning of an *S. marcescens* gene encoding the most abundant *S. marcescens* chitinase, and (iv) the comparison of the relative levels of chitinase expression from various constructs in both *E. coli* and *Pseudomonas fluorescens* 701E1, a soybean root-colonizing pseudomonad.

MATERIALS AND METHODS

Bacterial strains and plasmids. The origins and relevant characteristics of the bacterial strains and plasmids used in this study are listed in Table 1. We thank E. Cabib, National Institutes of Health, Bethesda, Md., for *S. marcescens* QMB1466 and F. Ausubel, Harvard, Mass., for kindly providing pRMSL26 from which the pLAFR1 cosmid was derived by *Eco*RI restriction and ligation.

Media and cultural conditions. Exponential cultures were routinely grown (9) in Luria broth (LB) medium (17), with tetracycline (12.5 μg/ml) or kanamycin (50 μg/ml) added for plasmid-containing strains. Cell extracts were prepared as described previously (9) in 50 mM potassium phosphate buffer, pH 6.3 (buffer A), and were used as a source of chitinase.

Chitinase-positive clones were identified by screening Tc^r cosmid-containing strains on chitin overlay plates, prepared by overlaying a 25-ml agar base composed of nutrient agar (10 mg/ml) plus Bacto-Agar (10 mg/ml) with a 10-ml layer of the same mixture plus colloidal chitin (5 mg/ml [dry weight]). Colloidal chitin, prepared as described by Molano et al. (19), was homogenized with a Polytron (Brinkmann Instruments, Inc., Westbury, N.Y.) at maximum speed for 1 min before autoclaving to provide a homogeneous chitin suspension.

Chitinase assay. Chitinase activity was determined by using a modification of the radioactive method described by Molano et al. (19), in which insoluble chitin and the soluble chitin degradation products were separated by passage through a Gelman Acrodisc disposable filter assembly (pore size, 0.2 μm). Protein concentrations were determined by the dye-binding assay described by Bradford (5). Chitinase

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specific activities were expressed as micromoles of NAG equivalents formed per minute per milligram of protein. Nonspecific protease activity was measured in buffer A by the procedure described by Long et al. (12) with azocasein (Sigma Chemical Co., St. Louis, Mo.) as the substrate. Chitin deacetylase activity was measured in buffer A as described previously (1).

Chitinase purification. Chitinase was partially purified from crude, extracellular, commercial preparations of *S. marcescens* chitinase (United States Biochemical Corp., Cleveland, Ohio) by a modification of the affinity chromatographic procedure reported by Roberts and Cabib (22). One gram of crude, lyophilized chitinase was dissolved in 200 ml of buffer A, dialyzed at 4°C against 4 liters of the same buffer for 12 h, and dialyzed an additional 3 h against fresh buffer. The protein concentration of the dialyzed preparation was diluted to 0.67 mg of protein per ml, a concentration optimized for the efficient binding and elution of chitinase from colloidal chitin. Homogenized colloidal chitin slurry (7 mg of chitin per ml, dry weight) was mixed with an equal volume of the dialyzed chitinase at 4°C. The binding was rapid and approached 100% within 15 min (data not shown).

The chitin-bound chitinase was centrifuged for 20 min at 20,000 × *g*, washed with 900 ml of buffer A, suspended in 100 ml of buffer A, and incubated overnight at 30°C with shaking to allow the chitinase to solubilize the chitin, thus releasing the enzyme. Approximately 30% of the bound chitinase activity was eluted. The suspension was centrifuged to remove the particulate matter and dialyzed extensively against buffer A to eliminate the soluble chitin degradation products. Dialyzed chitinase was concentrated 20-fold in an Amicon (Lexington, Mass.) stirred-cell concentrator with a YM10 ultrafiltration membrane.

Approximately 8.4 mg of concentrated chitinase was loaded onto a column (1.5 by 90 cm) of Ultrogel AcA54 (LKB Instruments, Inc., Rockville, Md.) and eluted in buffer A at 10 ml/h. Fractions (1.5 ml) were collected and analyzed for chitinase activity. Column fractions were examined for protein composition by 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis (11). SDS-PAGE gels were stained either with Coomassie blue R250 (7) or by the silver staining method of Wray et al. (24).

Chitinase from recombinant cosmid clones was purified from cellular crude extract preparations by the chitin affinity method described above, except that colloidal chitin was added to the dialyzed crude chitinase at a ratio of 1 mg of chitin (dry weight) per 0.2 U of chitinase.

Preparation of antisera. A total of three female BALB/c mice were immunized with an intraperitoneal injection of Freund complete adjuvant containing 100 μg of 57-kilodalton (kDa) chitinase followed by two booster injections consisting of 100 μg of 57-kDa chitinase in Freund incomplete adjuvant at 3-week intervals.

Construction and screening of an *S. marcescens* cosmid library for chitinase expression. A cosmid library of *S. marcescens* QMB1466 chromosomal DNA was prepared in pLAFR1 essentially as described by Maniatis et al. (13). *S. marcescens* chromosomal DNA was isolated as described by Marmur (15). Cosmid pLAFR1 was isolated by a slight modification of the Birnboim and Doly procedure (4). Approximately 100 μg of plasmid DNA from 150 ml of overnight culture was recovered by chromatography with 0.5 g of NACS-52 resin in a 10-ml BioRad Econocolumn according to the instructions of the manufacturer (Bethesda Research Laboratories, Gaithersburg, Md.). *Eco*RI cleaved pLAFR1 and *S. marcescens* DNA were ligated, packaged into lambda heads in vitro, and used to infect *E. coli* SR193 cells, essentially as described by Maniatis et al. (13). Approximately 2,800 Tc^r clones were screened on chitin overlay plates at 30°C for chitinase-positive clones, as visualized by the chitin clearance around the colonies.

The individual *Eco*RI fragments from two chitinase-positive clones (pMON5027 and 5031) were subcloned into the *Eco*RI site of the broad-host-range plasmid, pMON5012 (Table 1), a derivative of pKT230 (2). There are two promoters that could potentially promote the expression of genes inserted into pMON5012 at the *Eco*RI site: the β-lactamase promoter (P*bla*) and a promoter near the origin of replication (designated P*ori*) that transcribes in the opposite orientation (D. J. Drahos, unpublished data). The resultant plasmids were transformed into *E. coli* MC1009 cells, transformants selected on LB plates containing kanamycin and chitinase-positive clones identified by using chitin overlay plates (see above).

Mobilization of chitinase cosmids into pseudomonads. Chitinase-positive cosmids were mobilized from *E. coli* SR193 into the soybean root-colonizing strain *P. fluorescens* 701E1 by the triparental mating procedure, using pRK2013 (8).

RESULTS

Purification of *S. marcescens* chitinases. A mixture of crude, extracellular chitinases was purified by the chitin affinity chromatographic method described above. The purification was monitored for contaminating hydrolytic activities, particularly nonspecific protease(s) and chitobiase activities, to determine the specificity of the chitin affinity method. Data in Table 2 showed that although only a two- to threefold increase in the chitinase specific activity was achieved, the relative specific activity of the contaminating nonspecific proteases was reduced approximately 150-fold. Likewise, the relative specific activity of chitobiase, the enzyme which converts the NAG dimer (chitobiose) to the NAG monomer, was reduced approximately 50-fold (M. J. Fiske, unpublished data).

The *S. marcescens* chitinase activities purified by the chitin affinity method were partially resolved by molecular

TABLE 1. Bacterial strains and plasmids

Bacterial strains or plasmids	Relevant genotype or phenotype	Source
Strains		
E. coli		
SR193	C600, *rpsL hsdR Leu⁻ Pro⁻ supE* (λ p*lac-5* Δ*B*am58-71 *c*I857)	S. G. Rogers
LE392	*hsdR supE44 supF58 lacY1 galK2 galT22 metB1 trpR55* λ⁻	S. G. Rogers
MC1009	Δ(*lacIPOZY*) *X74 galK galU* Δ(*ara leu*) *7697 rpsL recA*	M. J. Casadaban
P. fluorescens 701E1	Mucoid, fluorescent, pseudomonad field isolate	B. C. Hemming
Plasmids		
pLAFR1	IncP Tc^r λ *cos*⁺	F. Ausubel
pMON5012	IncQ Km^r *lacZ*⁺	D. J. Drahos
pRK2013	ColE1-Tra $(RK2)^+$ Km^r	D. R. Helinski

TABLE 2. Purification of *S. marcescens* chitinases

Enzyme	Vol (ml)	Total protein (mg)	Chitinase[a] (sp act)	Total chitinase (U)	Chitinase/ Protease[b] ratio
Crude	245	304	1.7	517	1
Chitin affinity[c]	22	37	4.1	152	143
Ultrogel AcA54[d] peak					
I	14.6	0.58	5.2	13	NA
II	12.7	1.14	5.2	28	NA
III	18.8	3.76	5.4	88	NA
IV	10.6	0.38	5.3	8.8	NA

[a] Chitinase specific activity is expressed as micromoles of NAG formed per minute per milligram of protein.

[b] Ratio of the specific activities for chitinase and protease. NA, Not assayed.

[c] Chitinase preparation after the chitin affinity step was concentrated from 450 to 22 ml by using an Amicon stirred cell with a YM10 membrane.

[d] Ultrogel step conducted with 5 of 22 ml. Total activity is extrapolated to 22 ml.

sieve chromatography with Ultrogel AcA54. Four reproducible peaks of chitinolytic activity were observed (Fig. 1). Each enzymatic peak correlates to a peak of one or two specific proteins. Peaks I, II, and IV correspond to proteins with monomeric molecular masses of 48, 36, and 52 kDa, respectively, as determined by SDS-PAGE analysis (Fig. 1). Peak III contains two major proteins with molecular masses of 21 and 57 kDa. Individual chitinolytic proteins were not completely separated either by these or other purification techniques that were examined (e.g., phosphocellulose, carboxymethyl cellulose, or DEAE-cellulose). The successful cloning of one or more of the genes encoding these activities should provide a more amenable and efficient approach to obtain large quantities of the individual proteins to characterize and compare their physical and enzymatic parameters.

Cloning of an *S. marcescens* gene encoding chitinase activity. An *S. marcescens* cosmid library was constructed in *E. coli* SR193 with *S. marcescens* QMB1466 chromosomal DNA and screened on chitin overlay plates to identify chitinase-producing clones. *S. marcescens* QMB1466 produced five chitinolytic proteins of similar size, based on SDS-PAGE analysis, to those purified from the commercial chitinase preparation (data not shown). Of the 2,800 Tc^r cosmids screened, six chitinase-positive clones were identified by the production of clear zones, visible against the translucent background of colloidal chitin (Fig. 2). These cosmid clones contain inserts that range in size from 22 to 27 kilobases (kb). Of these cosmids, two, designated pMON5031 and pMON5032, contain identical 24.5-kb inserts, as determined by *Eco*RI and *Bgl*II restriction analysis. A third clone (pMON5033) contains a similar insert minus a 2.0-kb *Eco*RI fragment. A total of four independent clones (pMON5027 [23.5 kb], 5030 [27.0 kb], 5031 [24.5 kb], and 5033 [22.5 kb]) express chitinase activity in *E. coli* SR193 on chitin overlay plates incubated at 30°C. *E. coli* SR193 containing pMON5027 produces the largest zone of clearing, whereas the same strain containing pMON5031 produces the

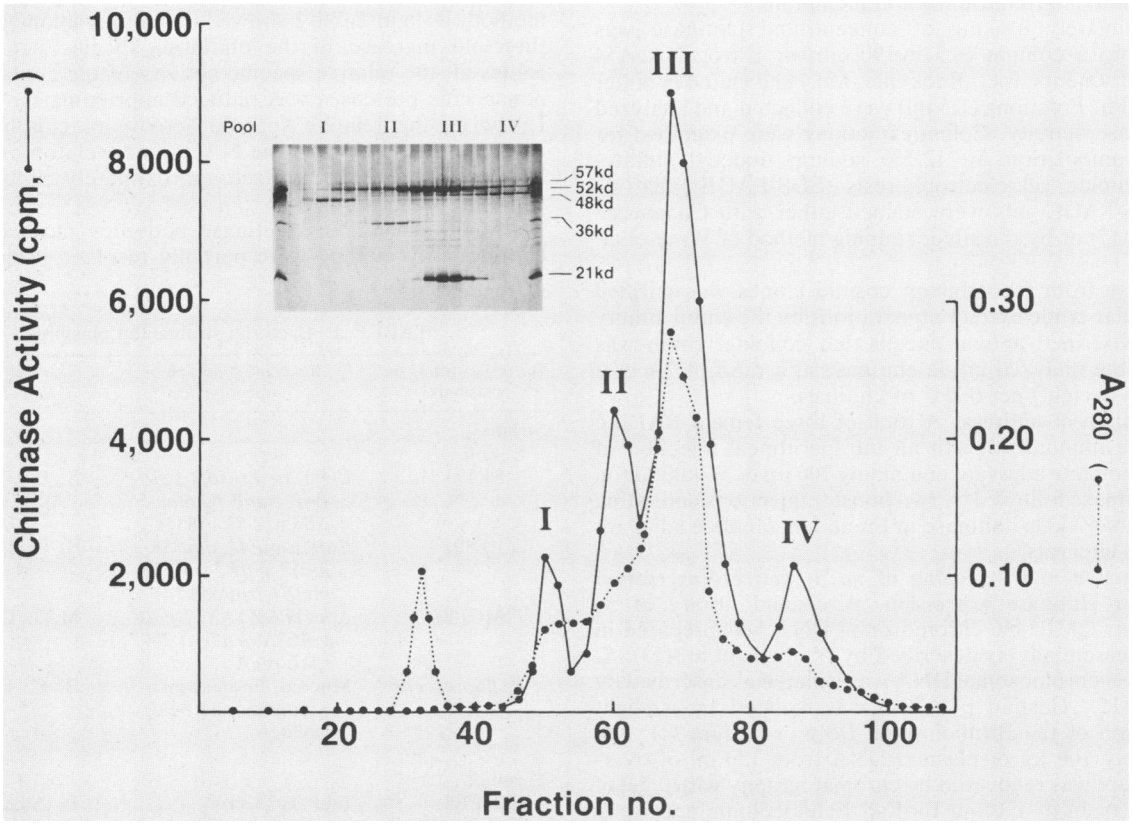

FIG. 1. Elution pattern of chitin affinity-purified *S. marcescens* chitinase activities from an Ultrogel AcA54 column. Conditions for chromatography and SDS-PAGE analysis (insert) are described in the text. Symbols: – – –, protein (A_{280}); ——, chitinase activity.

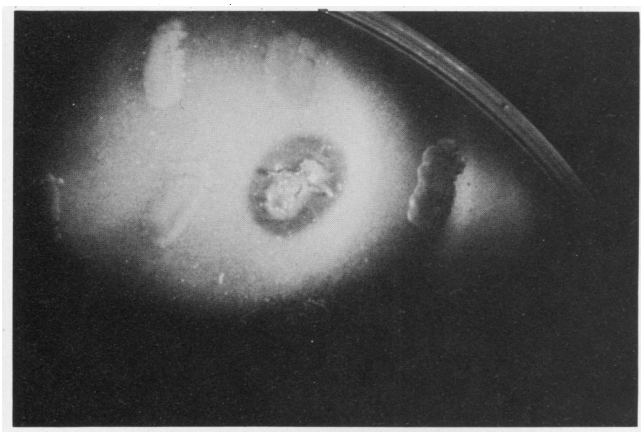

FIG. 2. Chitin clearance on chitin overlay plates by *E. coli* SR193(pMON5027) after 10 days of incubation at 30°C.

smallest (data not shown). All four cosmids were mobilized into *P. fluorescens* 701E1, and the resultant strains were analyzed for the rate of clearing on chitin overlay plates. In contrast to the results with *E. coli*, the pMON5031 construct produces the largest zone of clearing in *P. fluorescens* 701E1, and the pMON5027 construct produces the smallest (data not shown). However, *P. fluorescens* 701E1(pMON5031) required considerably longer time (>13 days) than *E. coli* SR193(pMON5027) (6 to 8 days) to produce a detectable zone of clearing. In vitro chitinase activities were determined for cultures of *E. coli* SR193 and *P. fluorescens* 701E1 containing either no plasmid, pMON5027, or pMON5031 to quantitate the levels of chitinase produced. Data in Table 3 confirm that *E. coli* SR193(pMON5027) produces higher chitinase activity than the same strain containing pMON5031. Chitinase activities of the *P. fluorescens* 701E1 constructs were below the level of detection for this assay.

*Eco*RI digestion of the four cosmids established that all four share a common 9.5-kb *Eco*RI fragment that encodes chitinase activity. This 9.5-kb *Eco*RI fragment was cloned from pMON5031 into pMON5012 in both orientations (pMON5035 and 5036). The levels of chitinase activity in *E. coli* LE392 strains containing pMON5035 and 5036 were comparable (Table 3). This suggests that chitinase production is independent of the insert orientation.

TABLE 3. Chitinase expression in *E. coli* and *P. fluorescens*[a]

Host	Plasmid	Insert size (kb)	Chitinase (sp act)[b]
E. coli SR193	None		<0.005
	pMON5027	23.5	0.018
	pMON5031	24.5	<0.005
E. coli LE392	None		<0.005
	pMON5035	9.5	0.026
	pMON5036	9.5	0.028
P. fluorescens 701E1	None		<0.005
	pMON5027	23.5	<0.005
	pMON5031	24.5	<0.005
	pMON5035	9.5	<0.005
	pMON5036	9.5	<0.005

[a] All strains were grown at 30°C in LB plus appropriate antibiotic to mid-exponential phase and chitinase activity measured (see the text).

[b] Chitinase specific activity is expressed as micromoles of NAG formed per minute per milligram of protein.

Purification of chitinase from cosmid clones. Chitinase was purified from each of the original, independent, cosmid clones. All four clones contain the common 9.5-kDa *Eco*RI fragment and produce the same 57-kDa chitinase. The chitinase purified from the SR193 strains containing pMON5027 and pMON5031 was compared with the mixture of chitinases purified from *S. marcescens* by SDS-PAGE analysis (Fig. 3). These data emphasize the tremendous purification efficiency of the chitin affinity chromatography procedure. Intracellular chitinase was purified 64-fold with a 41% yield from SR193(pMON5027) by this simple, one-step protocol to greater than 95% purity as determined by Coomassie blue-stained 12.5% SDS-PAGE analysis.

Number of independent *S. marcescens* chitinases. Data in Fig. 1 indicate that at least four proteins with chitinase activity and different subunit molecular weights can be separated by chromatography on Ultrogel AcA54. The appearance of a unique protein band on SDS-PAGE gels directly correlated with each of the four chitinase peaks, except peak III, in which two proteins (21 and 57 kDa) reached maximum levels. Although the correlation is strong and suggests that the 36-, 48-, and 52-kDa proteins are responsible for the observed peaks of chitinase activity, each chitinase peak was contaminated to various extents with the 57-kDa chitinase. Mouse polyclonal antibodies raised against the 57-kDa chitinase were used to neutralize the chitinase activity of the 57-kDa protein to ensure that the 36-, 48-, and 52-kDa proteins possess chitinase activity and to determine whether the 21-kDa protein has chitinase activity. Western blot analysis (Fig. 4) shows that the mouse anti-chitinase antibodies react specifically with the 57-kDa protein. No cross-reaction was observed with the 21-, 36-, 48-, and 52-kDa proteins. The antibody preparation totally neutralized the chitinase activity of the 57-kDa enzyme (Fig. 5). Chitinase activity from the four chitinase pools (Fig. 4)

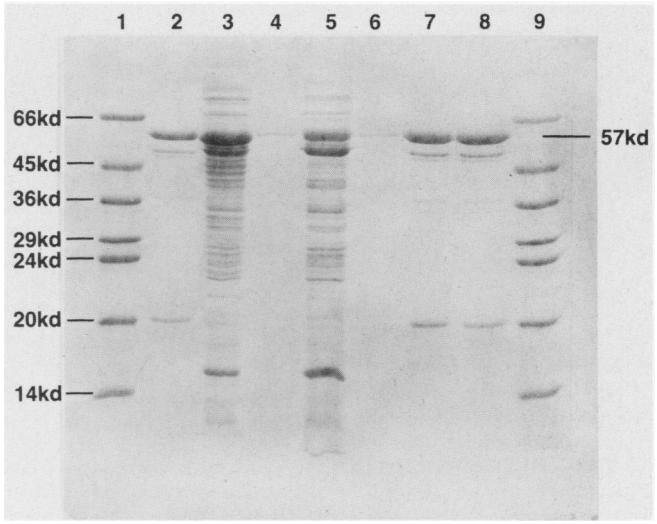

FIG. 3. SDS-PAGE analysis of chitinase purified from *E. coli* SR193(pMON5027) and SR193(pMON5031). Lanes 1 and 9, molecular weight markers; lanes 2, 7, and 8, chitin affinity-purified *S. marcescens* chitinases; lanes 3 and 4, SR193(pMON5031) crude and chitin affinity-purified proteins; and lanes 5 and 6, SR193(pMON5027) crude and chitin affinity-purified proteins.

was reduced by incubation in the presence of an excess of this antibody to the extent predicted by the level of the contaminating 57-kDa enzyme. The 29, 37, 89, and 5% reduction in chitinase activities observed for pools I, II, III, and IV, respectively, is in reasonable agreement with the levels of 57-kDa protein visualized in Fig. 4. The inability to totally neutralize the chitinase activity of these pools with the antibody raised against the 57-kDa chitinase, coupled with the column profile data presented in Fig. 1, strongly suggests that *S. marcescens* produces at least five different chitinolytic proteins with subunit molecular masses of 21, 36, 48, 52, and 57 kDa.

DISCUSSION

We showed that *S. marcescens* produces multiple chitinolytic proteins and have cloned the gene that encodes the previously purified (22) 57-kDa chitinase. A previous report suggested that *S. marcescens* may produce more than one chitinase; however, this assertion was based solely on the presence of two bands of chitinase activity on native PAGE gels (22). Data presented in Fig. 1 and 5 strongly suggest that *S. marcescens* produces five proteins with chitinase activity and subunit molecular masses of 21, 36, 48, 52, and 57 kDa. Chitinase activity was measured by solubilization of [^{3}H]chitin, labeled with [^{3}H]acetic anhydride. Because chitin deacetylase could also be active under the assay conditions used, all chitinase preparations were also assayed specifically for chitin deacetylase. No chitin deacetylase activity was observed even at a sensitivity that would detect an activity of 1% of that found for chitinase (data not shown). The function of these apparent multiple chitinases is unclear. They may either be required or act synergistically to attack native chitin. An unequivocal characterization of the function of an individual, as well as a combination of chitinolytic proteins, can only be ascertained with the purified enzymes. The cloning of the genes encoding these enzymes was pursued to address their function and to ascertain the fungicidal and nematicidal potential of individual proteins, as well as to engineer a chitinase-producing root-colonizing pseudomonad for fungal and nematode biocontrol testing.

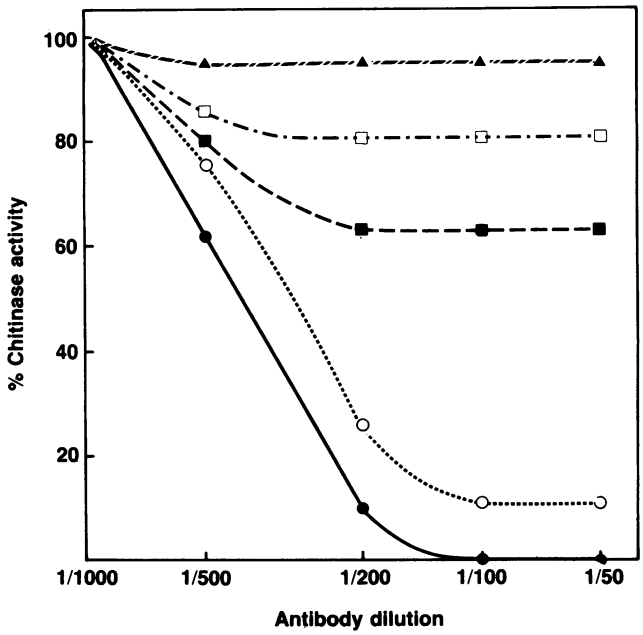

FIG. 5. Antibody neutralization of 57-kDa chitinase activity. Chitinase activity attributable to the 57-kDa chitinase from Ultrogel AcA54 pools I (□, 48 plus 52 kDa), II (■, 36 plus 57 kDa), III (○, 21 plus 57 kDa), IV (▲, 52 plus 57 kDa), and *P. fluorescens* 701E1(pMON5040) (●, 57 kDa) was neutralized by incubation with mouse anti-chitinase (57 kDa) polyclonal antibodies. Approximately 0.08 U of total chitinase activity was incubated with mouse antiserum at the indicated dilutions for 3 to 4 h at 4°C. Residual chitinase activity was measured and expressed as chitinase activity relative to the activity observed with the 1:1,000 dilution, because this dilution routinely stimulated chitinase activity relative to untreated controls.

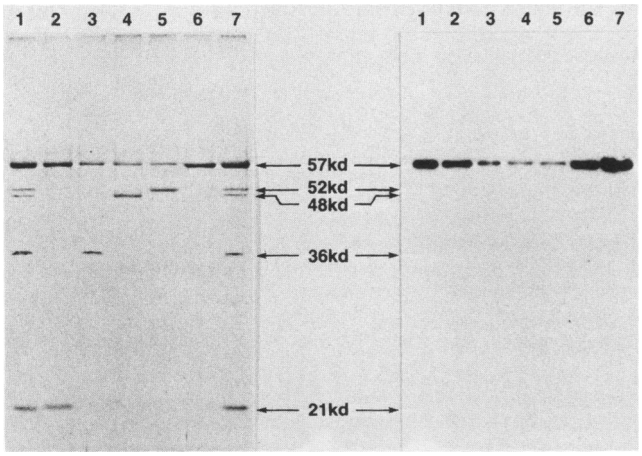

FIG. 4. SDS-PAGE analysis of *S. marcescens* chitinases and Western blot hybridization with mouse anti-chitinase (57 kDa) polyclonal antibodies. Samples of chitinase (0.08 U) were separated by 12.5% SDS-PAGE (7), electrophoretically transferred to a Gene Screen hybridization transfer membrane (New England Nuclear Corp., Boston, Mass.), and detected with mouse anti-chitinase antibodies (1:1,000 dilution) and ^{125}I-labeled protein A (Amersham Corp., Arlington Heights, Ill.). Lanes 1 and 7, *S. marcescens* chitin affinity-purified chitinases; lane 2, Ultrogel pool III (21 plus 57 kDa); lane 3, Ultrogel pool II (36 plus 57 kDa); lane 4, Ultrogel pool I (48 plus 57 kDa); lane 5, Ultrogel pool IV (52 plus 57 kDa); lane 6, 57 kDa chitin affinity-purified chitinase from *P. fluorescens* 701E1.

Only one gene encoding chitinase activity (the 57-kDa chitinase) was isolated from screening four to five genomic equivalents of cosmid clones. Based on cosmid size, approximately 800 pLAFR1 cosmid clones should represent greater than 95% of the genome of most bacteria (13). *S. marcescens* does preferentially synthesize the 57-kDa chitinase (Fig. 3). The inability to identify clones encoding the other chitinases could probably be attributed to the lack of sensitivity of the chitinase detection assay, assuming that the level of chitinase expression from the cosmids in *E. coli* reflects the relative expression levels of the chitinases in *S. marcescens*. Colonies of cells that carry the cosmids and produce the 57-kDa chitinase require 6 to 8 days at 30°C to solubilize sufficient chitin to be detected by zones of clearing on chitin overlay plates. Clones producing significantly lower enzyme levels may therefore be undetectable and require a more sensitive screen. This conclusion is supported by the inability of Horwitz et al. (10) to detect chitinase-positive clones by direct screening for chitin clearance. These investigators also cloned one or possibly two different *S. marcescens* genes encoding chitinase from the same strain, QMB1466, by screening for β-*N*-acetylhexosaminidase activity. The

gene(s) encoding chitinase is, fortuitously, physically linked to the gene encoding β-*N*-acetylhexosaminidase. The clone encoding the 57-kDa chitinase described in this manuscript cannot be directly compared with that reported by Horwitz et al. because neither the molecular weight of the gene product nor the size of the DNA fragment encoding chitinase was reported (10). However, Horwitz et al. have suggested that one of the chitinases may be located on a 3.6-kb *Sal*I/*Eco*RI fragment (10). Plasmid pMON5036, containing the 9.5-kb *Eco*RI insert encoding the 57-kDa chitinase, does not contain a 3.6-kb *Eco*RI/*Sal*I fragment (data not shown), which suggests that this chitinase activity is different than that previously described (10).

The independence of insert orientation on the expression level of pMON5035 and pMON5036 in *E. coli* LE392 indicates that chitinase synthesis in these constructs is driven from an *S. marcescens* promoter contained on the 9.5-kb insert, perhaps the chitinase promoter itself, and not from vector promoters. Data in Table 3 also suggest that sequences within the inserts of cosmids pMON5027 and 5031, but outside the 9.5-kb *Eco*RI fragment, apparently influence chitinase expression. *E. coli* SR193 containing pMON5027 produces significantly higher chitinase activity than the SR193(pMON5031) construct (Table 3), even though both appear to share the same 9.5-kb *Eco*RI insert, which, when subcloned into pMON5012, produces similar levels of chitinase on chitin overlay plates (data not shown). The significance of this observation is unclear. A more detailed structural analysis is also required to explain why the pMON5027, 5035, and 5036 constructs in *E. coli* consistently produce higher chitinase activities than the same constructs in *P. fluorescens* 701E1 (Table 3). The insertion of a stronger promoter upstream from the chitinase structural gene results in a higher level of chitinase activity in *P. fluorescens* than in *E. coli* (S. A. McPherson, D. J. Drahos, and R. L. Fuchs, manuscript in preparation), which suggests that the poor expression of the chitinase constructs in *P. fluorescens* 701E1 may be transcriptional in nature and not caused by the inefficient translation of the chitinase structural gene sequence. Based on this observation, stable, constitutive, high-level expression vectors are being constructed for the production of the 57-kDa chitinase in root-colonizing pseudomonads and will be used to determine the efficacy of these strains as biological control agents.

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