APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Mar. 1997, p. 834–839 Vol. 63, No. 3
0099-2240/97/$04.00+0

Molecular Cloning, Structural Analysis, and Expression in *Escherichia coli* of a Chitinase Gene from *Enterobacter agglomerans*

LEONID S. CHERNIN,[1]* LEONARDO DE LA FUENTE,[1]† VLADIMIR SOBOLEV,[3] SHOSHAN HARAN,[1] CONSTANTIN E. VORGIAS,[4] AMOS B. OPPENHEIM,[2] AND ILAN CHET[1]

The Otto Warburg Center for Biotechnology in Agriculture and Department of Plant Pathology and Microbiology, Faculty of Agriculture, The Hebrew University of Jerusalem,[1] and Department of Plant Genetics, The Weizmann Institute of Science,[3] Rehovot 76100, and Department of Molecular Genetics and Biotechnology, The Hebrew University-Hadassah Medical School, Jerusalem 91120,[2] Israel, and Laboratory of Biochemistry, Biology Department, Athens University, Panepistimiopolis, Kouponia, Athens 15701, Greece[4]

Received 27 June 1996/Accepted 20 December 1996

The gene *chiA*, which codes for endochitinase, was cloned from a soilborne *Enterobacter agglomerans*. Its complete sequence was determined, and the deduced amino acid sequence of the enzyme designated Chia_Entag yielded an open reading frame coding for 562 amino acids of a 61-kDa precursor protein with a putative leader peptide at its N terminus. The nucleotide and polypeptide sequences of Chia_Entag showed 86.8 and 87.7% identity with the corresponding gene and enzyme, Chia_Serma, of *Serratia marcescens*, respectively. Homology modeling of Chia_Entag's three-dimensional structure demonstrated that most amino acid substitutions are at solvent-accessible sites. *Escherichia coli* JM109 carrying the *E. agglomerans chiA* gene produced and secreted Chia_Entag. The antifungal activity of the secreted endochitinase was demonstrated in vitro by inhibition of *Fusarium oxysporum* spore germination. The transformed strain inhibited *Rhizoctonia solani* growth on plates and the root rot disease caused by this fungus in cotton seedlings under greenhouse conditions.

Many bacteria, fungi, and plants are able to hydrolyze chitin to oligomeric derivatives of *N*-acetyl-β-D-glucosamine (GlcNAc), disaccharides, and GlcNAc monomer (15). The structural genes encoding chitinases have been cloned from a wide variety of organisms, including bacteria (2, 8, 9, 17, 19, 20, 22). Chitinolytic bacteria from the genera *Aeromonas* (7) and *Serratia* (12) have been shown to be potential agents for the biological control of plant diseases caused by various phytopathogenic fungi whose cell walls contain chitin as a major structural component. Evidence that chitinases are responsible for this effect has been presented elsewhere (8, 16).

The soilborne *Enterobacter agglomerans* IC1270 has a broad spectrum of antifungal activity. This strain produces and secretes a number of chitinolytic enzymes, including two *N*-acetyl-β-D-glucosaminidases and an endochitinase. The importance of the chitinolytic enzymes in strain IC1270 biocontrol activity was demonstrated by using *Rhizoctonia solani* in cotton as a model and comparing the parent IC1270 strain with Tn*5* mutants deficient in chitinolytic activity (3). In addition, strain IC1270 produces pyrrolnitrin, an antibiotic previously found only in *Pseudomonas* spp., and this antibiotic activity was considered advantageous as an aid to the chitinases in their attack on fungal phytopathogens (4).

In the present study, the gene *chiA*, which encodes the endochitinase (E.C.3.2.1.14) designated Chia_Entag, was cloned, sequenced, and expressed in *Escherichia coli*. The deduced amino acid sequence of Chia_Entag was highly similar to that of Chia_Serma of *Serratia marcescens*, enabling homology modeling based on Chia_Serma's known three-dimensional structure. The transformed *E. coli* strain exhibited antifungal activity, including the ability to protect plants against disease caused by *R. solani.*

* Corresponding author. Mailing address: The Hebrew University, Otto Warburg Center for Biotechnology in Agriculture, Faculty of Agriculture, P.O. Box 12, Rehovot 76100, Israel. Phone: 972-8-9481128. Fax: 972-8-9468785. E-mail: CHERNIN@AGRI.HUJI.AC.IL.

† Present address: Department of Biochemistry, Biological Research Institute Clemente Estable, Montevideo 11600, Uruguay.

MATERIALS AND METHODS

Media and bacterial growth. Liquid or solid (1.5% [wt/vol] agar) Luria broth (LB) or Luria agar was used for bacterial growth. Potato dextrose broth or agar (Difco Laboratories, Detroit, Mich.) was used for the cultivation of fungi. To induce chitinolytic activity, *E. agglomerans* IC1270 was grown for 4 days at 30°C with aeration in liquid synthetic medium with 0.2% (wt/vol) colloidal chitin as the sole carbon source, prepared as described previously (3). *E. coli* JM109 and its derivatives were grown with aeration in LB for 24 h at 37°C. Ampicillin (100 μg/ml) was added to the growth media when needed. For the assessment of chitinolytic activity, recombinant *E. coli* was grown overnight in LB and then diluted 10-fold in fresh LB and grown at 37°C with aeration for an additional 3 to 4 h. The isopropyl-β-D-thiogalactopyranoside (IPTG) (Sigma Chemical Co., St. Louis, Mo.) solution was then added to a final concentration of 1 mM, and the cultures were incubated overnight.

PCR amplification. Two oligonucleotide primers were used in a PCR with genomic DNA isolated from *E. agglomerans* IC1270. The 5′-TATCCTCTCGG AATAA-3′ and 5′-GAATTCACTCAAACAATCT-3′ primers, designed on the basis of published sequence data of the chitinase A gene *chiA* of *S. marcescens* 27117 (GenBank accession no. LO1455), were identical to residues 521 to 536 (−27 to −12) of the sense strand and 2599 to 2618 of the antisense strand of the 2,618-bp sequence, respectively. A standard PCR was performed in a total volume of 25 μl, containing 1.5 mM $MgCl_2$, 200 mM (each) deoxynucleotide triphosphate (dATP, dCTP, dGTP, and dTTP), 10 pmol of each PCR primer, 1 U of *Taq* DNA polymerase (Promega Co., Madison, Wis.), and 10 ng of genomic DNA from *E. agglomerans* IC1270. The reaction mixture was overlaid with mineral oil, and thermal cycling was achieved in a PTC-100 programmable thermal controller (MJ Research Inc., Watertown, Mass.) according to the following program: 25 cycles of 94°C for 1 min, 54°C for 1 min, and 72°C for 1.5 min and a final extension at 72°C for 4 min. PCR products were resolved on a 0.8% agarose (Eastman Kodak Co., New Haven, Conn.) gel in Tris-acetate-EDTA buffer. The gels were stained with ethidium bromide at a final concentration of 1 μg/ml. The 1-kb ladder (Gibco/BRL, Grand Island, N.Y.) was used as a DNA marker. The PCR products were purified with a Qiaex gel extraction kit (Qiagen Inc., Chatsworth, Calif.).

Recombinant DNA techniques. Generally, standard protocols were used (1). The procedure used for isolating genomic DNA from *E. agglomerans* IC1270 was modified by an additional treatment with lysozyme (5 mg/ml, 1 h, 37°C) added to

Tris-EDTA buffer prior to sodium dodecyl sulfate (SDS) and proteinase K treatment. Restriction enzyme reactions were buffered as recommended by the manufacturers. The entire PCR product was cloned into a pGEM-T vector (Promega) under the control of the *lac* promoter. The ligation mixture was used to transform *E. coli* JM109, and hybrid clones were selected by a blue-white assay on LB agar plates, containing Amp (100 μg/ml), IPTG (0.5 mM), and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-thiogalactopyranoside) (80 μg/ml). The transformants were analyzed for the presence of plasmid DNA on a 1% agarose gel stained with ethidium bromide.

DNA sequencing and sequence analysis. The resulting clone, pCHITEa1, extracted from transformant N1 (JM109/pCHITEa1) was used directly for the double-stranded DNA sequencing reactions, performed at the DNA sequencing unit at the Weizmann Institute of Science (Rehovot, Israel), by dye terminator sequencing using a *Taq* DyeDeoxy Terminator Cycle Sequencing Kit and a DNA Sequencing Stretch (model 373; Applied Biosystems, Foster City, Calif.). Standard T7 and SP6 primers and a set of specific internal primers in two directions each, designed to overlap with and to match the end parts of the sequences determined in each previous step, were used for sequencing of the PCR product inserted into the pGEM-T vector. FACTURA 1.2.0 and Auto Assembler 1.3.1.0 programs (Applied Biosystems) were used to obtain the entire sequence of both strands of the inserted DNA, which was a total of 2,165 bp. DNA and deduced protein sequences were analyzed by using University of Wisconsin Genetics Computer Group (Madison, Wis.) sequence analysis software.

Protein structure modeling and analysis. A multiple-sequence alignment with Clustal W optimized by the Profile network from HeiDelberg (PHD) program (14) was used for homology modeling of Chia_Entag. The sequence alignment obtained was the basis for homology modeling using WHAT IF software (21). A model for the structure of Chia_Entag was built from that of Chia_Serma by the modeling procedure described by Chinea et al. (5) and de Fillipis et al. (6). Calculations of solvent-accessible surfaces (10) and contact surfaces between atoms (18) were used to locate each of the 69 amino acid residues that differed between these enzymes.

Preparation of crude secreted and intracellular proteins, assay of chitinolytic activity in solution, and detection of chitinolytic enzymes after gel electrophoresis. All procedures were essentially performed as described previously (3). The chromogenic and fluorescent derivatives of GlcNAc—*p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide (pNP-GlcNAc), *p*-nitrophenyl-β-D-*N*,*N*′-diacetylchitobiose [pNP-$(GlcNAc)_2$], 4-methylumbelliferyl-*N*-acetyl-β-D-glucosaminide (4-MU-GlcNAc), and 4-methylumbelliferyl-β-D-*N*,*N*′-diacetylchitobioside [4-MU-$(GlcNAc)_2$] (all from Sigma)—were used as substrates for a determination of chitinolytic activity in solution and after protein separation by SDS-polyacrylamide gel electrophoresis (PAGE). For determination of chitinolytic activity in solutions with chromogenic chitin derivatives, the standard reaction mixture contained approximately 20 μg of protein. The release of the chromophore pNP from the substrates was measured at 410 nm, and 1 U of enzymatic activity was defined as 1 μmol of pNP per μg of protein per h. The data from six independent experiments are significant at a P level of 0.05.

Assays of antifungal activity in vitro. A plate assay using *R. solani* as the test fungus was performed as described previously (3). The fungal spore germination assays were performed according to the procedure described by Lorito et al. (11) with minor variations, using *Fusarium oxysporum* f. sp. *meloni* as the test fungus and the crude secreted enzyme preparations from *E. agglomerans* IC1270, *E. coli* JM109/pCHITEa1, or *E. coli* JM109 as the test solutions. A stock suspension of ca. 10^6 conidia of the fungus/ml in 30% glycerol was prepared and kept at −12°C. Equal volumes (20 μl) of spore suspension, 3 × potato dextrose broth, and the test solution (about 80 U of chitinolytic activity) were mixed in sterile Eppendorf tubes. The tested solutions were replaced with sterile water in control samples. Tubes were incubated at 25°C for 24 h. A drop of the mixture from each tube was placed on a microscope slide, and the percentage of conidial germination was determined from the first 100 spores chosen at random.

Greenhouse assays. *R. solani* damping-off of cotton (*Gossypium barbardense* L. 'Pima') seedlings was chosen as the model to test the efficacy of *E. agglomerans* isolates as biocontrol agents, by using a previously described procedure (3). A seed cover layer was infected with a preparation of *R. solani* and mixed with a water suspension of the bacteria being tested (ca. 10^9 cells/kg of soil). The bacterial treatment was repeated daily. Each strain was tested in six replicates, and experiments carried out at 28 to 30°C were independently repeated three times. The disease incidence was determined after 12 to 15 days as the percentage of seedlings showing symptoms of root rot disease.

Nucleotide sequence accession number. The GenBank accession number for *chiA* of *E. agglomerans* is U59304.

RESULTS

Nucleotide and amino acid analyses of the cloned Chia_Entag. We cloned the chitinase of *E. agglomerans* IC1270 using two external primers whose design had been based on the *chiA* gene of Chia_Serma. The obtained PCR product was ligated into the *Sma*I restriction site of pGEM-T, and recombinant clones were isolated in *E. coli* JM109. A clone carrying the hybrid plasmid pCHITEa1 was selected for further studies.

The complete 2,165-bp sequence of the insert was determined, and the deduced amino acid sequence of Chia_Entag yielded an open reading frame of 1,686 nucleotides, coding for a protein of 562 amino acids with an estimated molecular mass of 60,880 Da (Fig. 1).

A database search revealed that the deduced Chia_Entag protein amino acid sequence was 87.7, 71.9, 52.2, and 32.2% identical to those of Chia_Serma, Chia_Aerca from *Aeromonas caviae* (17), Chia_Altso from *Alteromonas* sp. strain O-7 (20), and Chi1_Bacci from *Bacillus circulans* WL-12 (22) (Fig. 2). The precursor protein of Chia_Entag had a leader peptide identical to that of Chia_Serma (8) and Chia_Aerca (17), suggesting that it is also cleaved at the same site. Thus, the mature protein probably starts at Ala24 (position 1 in Fig. 2).

Computer analysis revealed 86.8% identity between the *chiA* genes of *E. agglomerans* and *S. marcescens*. In both genes, a Shine-Dalgarno sequence, AGGA, preceded the start codon by nine bases and a GC-rich inverted repeat structure began 16 bp beyond the stop codon (underlined in Fig. 1). However, the stop codons differed (UAG in *E. agglomerans chiA* vs. UAA in the Chia_Serma gene) and, due to the substitution of two AT pairs for GC pairs (underlined in Fig. 1), the loop structure located immediately in front of the cleavage site in the Chia_Serma gene (8) was not found in the Chia_Entag gene.

A model of Chia_Entag's three-dimensional structure was built based on the crystal structure of Chia_Serma described by Perrakis et al. (13) (Fig. 3). Calculations of solvent-accessible surfaces showed that 58 of the 69 Chia_Entag amino acid residues which differed from those of Chia_Serma (Fig. 2), are located on the protein surface. The other 11 residues (marked with asterisks in Fig. 2), whose solvent-accessible surface area was found to be equal to zero, were buried in the protein. Calculations of contact surfaces between side chains of residues which differed between Chia_Serma and Chia_Entag revealed six cases of simultaneous substitutions of a pair of residues which are in mutual side chain contact (data not shown). A cluster of amino acids (positions 385 to 405, marked by an arrow in Fig. 3) which exhibited no homology with the corresponding region of Chia_Serma (Fig. 2) probably forms two loops facing the solvent.

Chitinolytic activity of *E. coli* carrying the *chiA* gene of *E. agglomerans*. The chitinolytic activity found in the culture medium of the transformant JM109/pCHITEa1 was active in the hydrolysis of the trimeric pNP-$(GlcNAc)_2$, but not the dimeric pNP-GlcNAc (Table 1). These data indicate that the chitinolytic activity served as an endochitinase. A significantly decreased level of chitinolytic activity was found in the intracellular fraction of the transformant. No activity was detected in the secreted proteins or extracts of strain JM109 or its transformant carrying the pGEM-T vector.

To demonstrate directly that the transformed *E. coli* strain produced and secreted the Chia_Entag endochitinase, the chitinolytic activity of extracellular and intracellular proteins of JM109/pCHITEa1, renaturated following separation by SDS-PAGE, was examined and compared with that of the original *E. agglomerans* IC1270. The results obtained with the analog of trimeric chito-oligosaccharides, 4-MU-$(GlcNAc)_2$, showed that out of the three chitinolytic enzymes with apparent molecular masses of 89, 67, and 59 kDa which are present in the extracellular proteins of strain IC1270 (Fig. 4, lanes 3 and 4), only one, corresponding to the 59-kDa chitinase, was detected in the extracellular and intracellular proteins of JM109/pCHITEa1 (Fig. 4, lanes 1 and 5). Since this activity was not observed when the dimeric derivative of chitin, 4-MU-

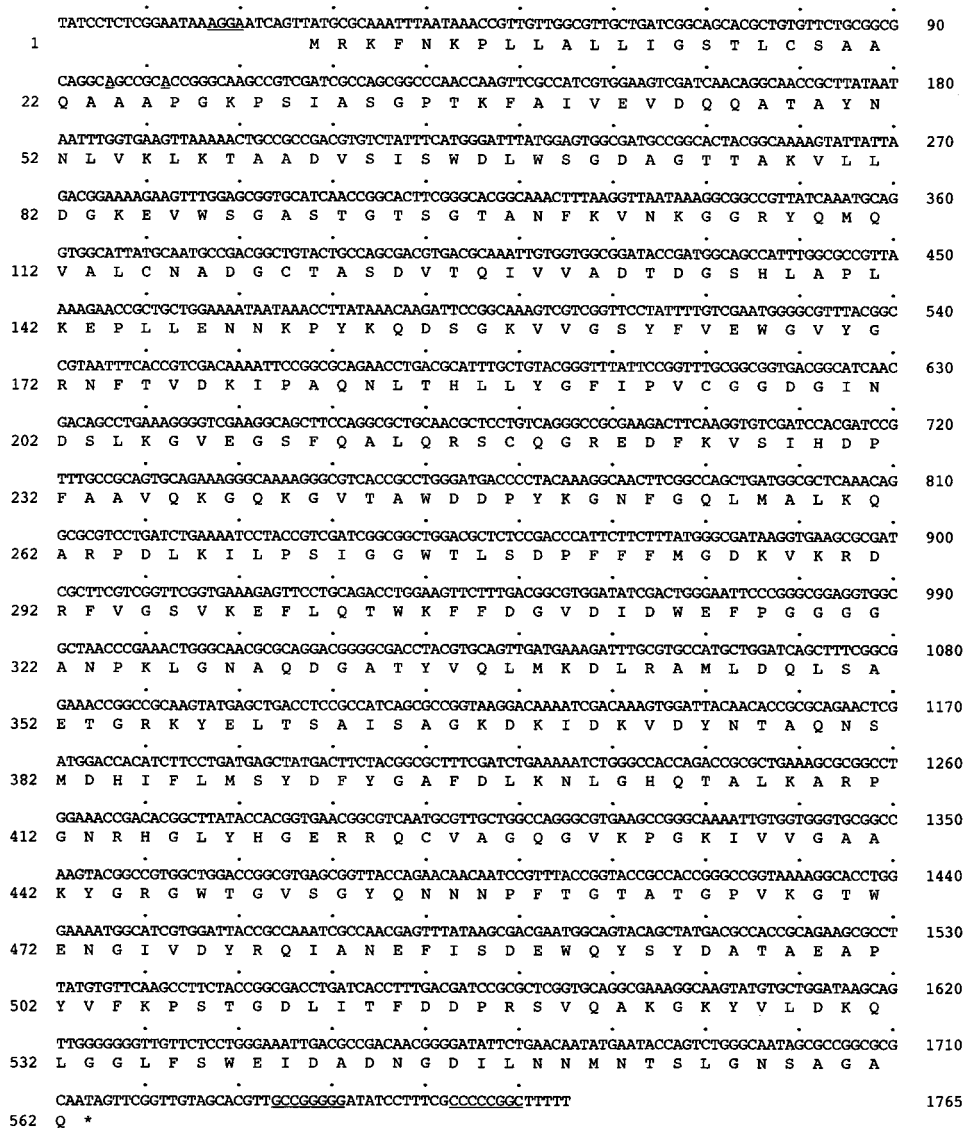

FIG. 1. Sequences of the *chiA* gene and its protein product, the Chia_Entag chitinase. The DNA sequence is numbered at right; the deduced amino acid sequence, given in single-letter code, is shown below the nucleotide sequence and numbered at left. The termination codon is marked with an asterisk, and the Shine-Dalgarno (SD) sequence (AGGA) and other features mentioned in text are underlined.

GlcNAc, was used as a substrate (data not shown), it was attributed to endochitinase. Similar to its counterpart in strain IC1270, this chitinase was heat resistant (Fig. 4, lane 2). An amorphous band of chitinolytic activity migrating at >100 kDa was observed in the secreted proteins (Fig. 4, lanes 1 and 3), disappeared following heating (Fig. 4, lanes 2 and 4), and was not found in the intracellular proteins (Fig. 4, lane 5). As has been assumed previously (3), this activity stemmed from an unresolved complex of the secreted chitinases. No bands of chitinolytic activity from the secreted proteins of *E. coli* JM109 were found (Fig. 4, lane 6).

Antifungal and biocontrol activity of the *E. coli* strain producing chitinase of *E. agglomerans*. The chitinolytic *E. coli* strain carrying the plasmid pCHITEa1 was tested for antifungal activity against the phytopathogenic fungus *R. solani* and was found to suppress the latter's growth on plates. The original strain IC1270 showed stronger suppression of the fungus, whereas almost no suppression was observed with the nontransformed *E. coli* strain (Fig. 5).

The effects of crude preparations of the chitinases secreted by strains *E. agglomerans* IC1270 and *E. coli* JM109/pCHITEa1 on spore germination of *F. oxysporum* f. sp. *meloni* were assayed. The percentage of spore germination inhibition was calculated by using the following equation (11): $\%I = (1 - \%S_t/\%S_c) \times 100$, where $\%I$ represents the percentage of inhibition and $\%S_t$ and $\%S_c$ represent the percentages of spores germinating in the treated preparation or in the control (addition of water instead of the crude proteins from the indicated strains), respectively. The results of each experiment are reported as the average of three replications, and the number of experiments is also given. The standard error was calculated at a P level of 0.05. The rate of spore germination in the control was 68.0% ± 9.2%. The *F. oxysporum* f. sp. *meloni* spore germination assay showed that in the presence of crude prep-

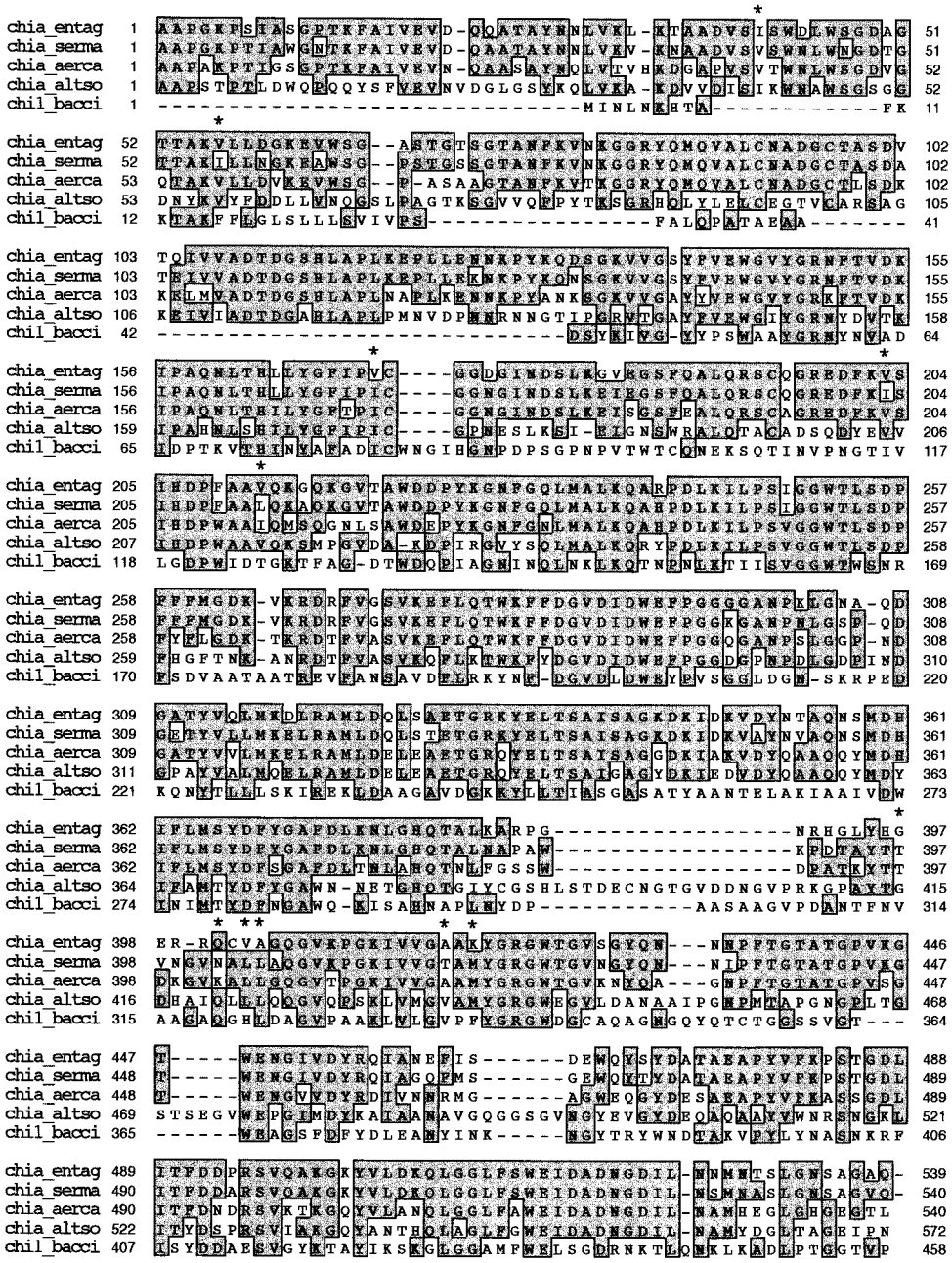

FIG. 2. Alignment of deduced amino acid sequences of some bacterial chitinases. The amino acid sequences of the chitinases from *E. agglomerans* (Chia_Entag, deduced from translation of the DNA sequence obtained in this study), *S. marcescens* (Chia_Serma, 1CTN Protein Data Bank entry), *Aeromonas caviae* (Chia_Aerca, deduced from translation of the GenBank DNA sequence, accession no. U09139), an *Alteromonas* sp. (Chia_Altso, from the Swiss-Prot database), and *B. circulans* (Chia_Bacci, from the Swiss-Prot database) were aligned by using the PileUp algorithm of the GCG package and the SeqVu V1.0.1 program (Garvan Institute of Medical Research, Sydney, Australia). Numbering starts from the first residue of the mature protein. Buried amino acid residues differing from the corresponding ones in Chia_Serma are marked with an asterisk.

arations of extracellular proteins produced by strains IC1270 and JM109/pCHITEa1, the rate of spore germination significantly decreased relative to the control (35.1% ± 3.1% and 21.3% ± 3.4%, respectively [from 11 and 10 experiments, respectively]). When the amount of extracellular proteins of the parent strain JM109 (about 8μg) equivalent to that in crude preparations of chitinases secreted by strains IC1270 and JM109/pCHITEa1 was added to the experimental mixture as a test solution, almost no difference from the control was found (6.8% ± 2.1% [from six experiments]).

Application of *E. coli* JM109/pCHITEa1 or *E. agglomerans* IC1270 to soil infested with *R. solani* reduced disease incidence (root rot) in cotton seedlings by about 60 and 75%, respectively, whereas the nontransformed *E. coli* JM109 strain was found to be ineffective (Fig. 6).

DISCUSSION

The recombinant strain *E. coli* JM109/pCHITEa1, expressing the *E. agglomerans chiA* gene, exhibited endo-mode chitin

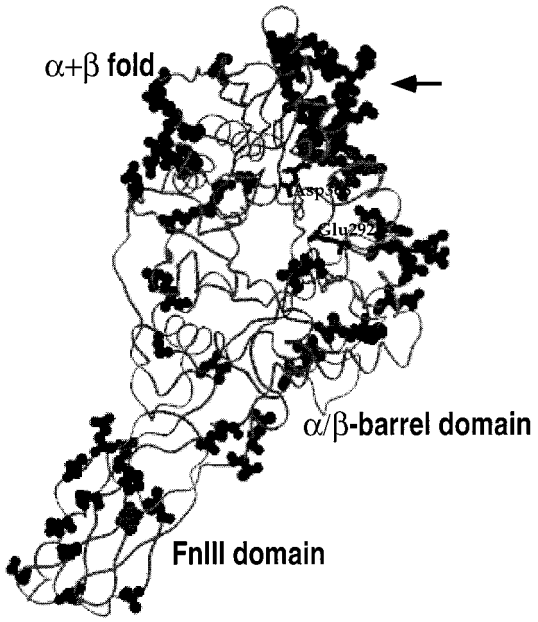

FIG. 3. Model of the three-dimensional structure of the mature Chia_Entag protein. Residues differing from those of Chia_Serma are presented as space-filling models (black, surface located; grey, buried). The residues Glu292 and Asp368, which belong to the major catalytic domain, are indicated as black ball-and-stick elements. The nonconserved region (residues 385 to 405) is marked with an arrow.

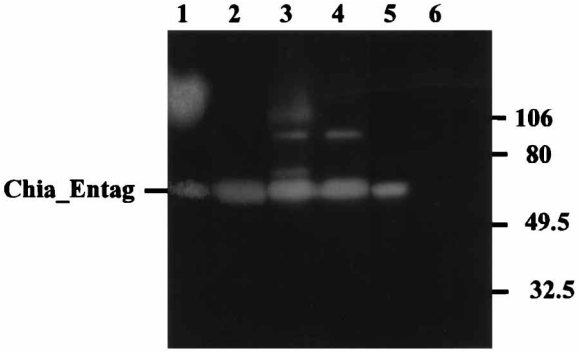

FIG. 4. Detection of chitinolytic activity of extracellular and intracellular proteins after separation by SDS-PAGE. 4-MU-$(GlcNAc)_2$ was used as the substrate. Extracellular proteins from *E. coli* JM109/pCHITEa1 (lanes 1 and 2), *E. agglomerans* IC1270 (lanes 3 and 4), and *E. coli* JM109 (lane 6) and intracellular proteins from JM109/pCHITEa1 (lane 5) are shown. In lanes 2 and 4, the proteins were heated at 55°C in sample buffer without 2-mercaptoethanol. The lanes contain protein in amounts as follows: 1 and 2, 40 μg; 3 and 4, 10 μg; 5 and 6, 80 μg. Protein size markers (in kilodaltons) are shown on the right.

splitting activity as determined by the release of the chromogenic pNP from trimeric but not dimeric chito-oligosaccharide analogs. The endochitinase produced by strain JM109/pCHITEa1 was revealed by SDS-PAGE as a single band corresponding in size to the endochitinase Chia_Entag found in strain *E. agglomerans* IC1270. Contrary to strain IC1270, the recombinant *E. coli* JM109/pCHITEa1 strain did not require colloidal chitin in the growth medium to produce the endochitinase. However, the chitinolytic activity found in extra- and intracellular proteins of the recombinant strain was significantly lower than that of the parent strain IC1270, suggesting higher expression of the *chiA* gene under the control of its original promoter in *E. agglomerans* cells. It is also worth noting that most of the endochitinase produced by the transformant was found in the extracellular fraction, whereas in the case of strain IC1270, the chitinolytic activity of the intracellular proteins was higher than that in the secreted proteins (3).

The *E. agglomerans* chitinase gene characterized in this paper has high DNA sequence homology (86.8%) with *chiA* encoding the chitinase A of *S. marcescens* (Chia_Serma). Identity between the two chitinases at the amino acid level was 87.7%. The predicted molecular mass of the 61-kDa precursor included a 3-kDa fragment (23-amino-acid leader sequence) of a signal peptide which has also been found in some other bacterial chitinases. This signal peptide is probably cleaved away during protein transport to the periplasmic space (8, 16, 17). Hence, the size of the mature Chia_Entag protein (58 kDa) estimated by amino acid sequence analysis is very close to the apparent molecular mass of 59 kDa estimated by SDS-PAGE (3).

A database search showed that the similarity between currently known bacterial chitinases correlates with the taxonomic position of *E. agglomerans*. Thus, the chitinases of *S. marcescens* and *E. agglomerans* are closer to those from *Aeromonas caviae* (the *Vibrionaceae* family) than to those of *Alteromonas* spp. (a group of aerobic marine bacteria) or to those of the gram-positive *B. circulans* (Fig. 2). These comparisons suggest that the levels of diversity between various chitinases correlate with the evolutionary distances between the bacteria that produce them.

In this paper, we present a model of Chia_Entag's three-dimensional structure based on the known crystal structure of Chia_Serma (13). The model revealed high structural similarities between these chitinases. Calculations of solvent-accessible surfaces for the Chia_Entag residues which differed from Chia_Serma revealed that most of them are located on the protein surface and therefore are not expected to lead to significant differences in the enzyme's overall three-dimensional structure. Both structures consisted of an all-β-strand amino-terminal fibronectin III (FnIII)-type domain, an α+β fold domain, and an α/β-barrel domain. In Chia_Serma, the

TABLE 1. Analysis of chitinolytic activity

Strain	Protein	U of chitinolytic activity (mean ± SE) with substrate:	
		pNP-GlcNAc	pNP-$(GlcNAc)_2$
E. coli JM109	Secreted	0	0
E. coli JM109/pCHITEa1	Secreted	0	11.7 ± 3.3
E. coli JM109/pCHITEa1	Intracellular	0.1 ± 0.02	3.6 ± 1.1
E. coli JM109/pGEM-T	Secreted	0	0
E. agglomerans IC1270	Secreted	103.2 ± 22.1	39.2 ± 8.4

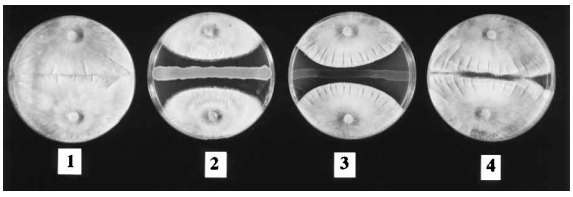

FIG. 5. Assay of antifungal activities on plates. A suspension of bacterial cells was seeded in a line through the center of a potato dextrose agar plate and incubated at 30°C for 24 h. Two agar disks from an actively growing culture of *R. solani* were placed on either side of the bacterial growth area, and the plates were incubated at 30°C for 4 days. Plates: 1, control (no bacteria); 2, *E. agglomerans* IC1270; 3, *E. coli* JM109/pCHITEa1; 4, *E. coli* JM109.

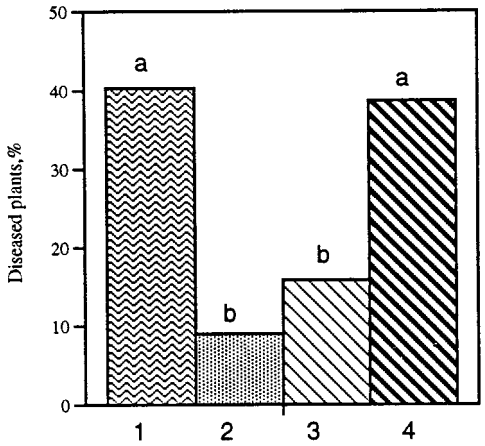

FIG. 6. Assay of antifungal activity against *R. solani* in cotton (*G. barbardense* L. 'Pima'). Column 1, control (plants irrigated only with tap water); columns 2 to 4, plants treated with water suspensions of *E. agglomerans* IC1270, *E. coli* JM109/pCHITEa1, and *E. coli* JM109, respectively. Values for columns labeled with the same letter are not significantly different at a P level of 0.05 according to Student's t test. Each value is the mean of three independent experiments with six replicates (pots) for each of the variants per experiment and 10 plants per pot.

first domain has been suggested to facilitate the binding of chitinase to chitin while the last domain is catalytic and retains conserved residues Glu315 and Asp391 (corresponding to Glu292 and Asp368 in Chia_Entag), being located in the active site (13). Contact surface calculations showed that residues which are in mutual contact in both structures were subjected to putative correlated replacement, probably further supporting the structural similarities between the two proteins. Almost no identity between these chitinases was found in the range of residues 385 to 405. However, based on the three-dimensional structure of Chia_Entag, these changes do not appear to be essential for the enzyme's activity, since they form an extension of the protein.

E. coli JM109/pCHITEa1, expressing the *E. agglomerans chiA* gene, acquired the ability to suppress growth of *R. solani* and spore germination of *F. oxysporum* f. sp. *meloni* in vitro and to protect cotton against disease induced by *R. solani* under greenhouse conditions. However, the transformant effected less significant fungal suppression in vitro than did the parent strain IC1270. This difference can be explained not only by the lower level of chitinolytic activity secreted by the transformant (probably because the *chiA* gene was cloned under the control of the relatively weak *lac* promoter) but also by the fact that the parent strain in addition to the endochitinase has been found to produce two other chitinolytic enzymes and pyrrolnitrin. Although this antibiotic activity has been found to be less important for the biocontrol of *R. solani* by strain IC1270 under greenhouse conditions (3), it is involved in suppression of the fungus on plates (4).

The results presented in this work confirm the role of chitinases in the antifungal activity of various microbial antagonists (7, 8, 11, 12, 16). The *E. agglomerans* chitinase gene could therefore be used to transform other bacteria or plants and provide them with the ability to control fungal phytopathogens.

ACKNOWLEDGMENTS

This research was supported by research grant 4022 to L.C. from the Ministry of Science and Arts, Israel; by the Ministry of Science and Arts, Israel, and the Commission of the European Communities (EC grant 937058 IL); by the Binational Agricultural Research and Development (BARD) Fund; by the Eshkol Fund of the Ministry of Science and Arts, Israel; and by the charitable fund of the Chais family. V.S. acknowledges the support of the Forschheimer and Wilstatter Centers at the Weizmann Institute of Science.

We thank T. Mehlman for help with DNA sequence assembly and P. Nuzzo for her help with the antifungal activity assays.

REFERENCES

1. **Ausubel, F. M., R. Brendt, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.).** 1994. Current protocols in molecular biology. John Wiley & Sons Inc., New York, N.Y.
2. **Chen, J. P., F. Nagayama, and M. C. Chang.** 1991. Cloning and expression of a chitinase gene from *Aeromonas hydrophila* in *Escherichia coli*. Appl. Environ. Microbiol. **57:**2426–2428.
3. **Chernin, L., Z. Ismailov, S. Haran, and I. Chet.** 1995. Chitinolytic *Enterobacter agglomerans* antagonistic to fungal plant pathogens. Appl. Environ. Microbiol. **61:**1720–1726.
4. **Chernin, L., A. Brandis, Z. Ismailov, and I. Chet.** 1996. Pyrrolnitrin production by an *Enterobacter agglomerans* strain with a broad spectrum of antagonistic activity towards fungal and bacterial phytopathogens. Curr. Microbiol. **32:**208–212.
5. **Chinea, G., G. Padron, R. W. W. Hooft, C. Sander, and G. Vriend.** 1995. The use of protein-specific rotamers in model building by homology. Proteins **23:**415–421.
6. **de Fillipis, V., C. Sander, and G. Vriend.** 1994. Predicting local structure changes that result from point mutations. Protein Eng. **7:**1203–1208.
7. **Inbar, J., and I. Chet.** 1991. Evidence that chitinase produced by *Aeromonas caviae* is involved in the biological control of soil-borne plant pathogens by this bacterium. Soil Biol. Biochem. **23:**973–978.
8. **Jones, J. D. G., K. L. Grady, T. V. Suslow, and J. R. Bedbrook.** 1986. Isolation and characterization of genes encoding two chitinase enzymes from *Serratia marcescens*. EMBO J. **5:**467–473.
9. **Kless, H., Y. Sitrit, I. Chet, and A. Oppenheim.** 1989. Cloning of the gene coding for chitobiase of *Serratia marcescens*. Mol. Gen. Genet. **217:**471–473.
10. **Lee, B., and F. M. Richards.** 1971. The interpretation of protein structures: estimation of static accessibility. J. Mol. Biol. **55:**379–400.
11. **Lorito, M., A. Di Pietro, C. K. Hayes, S. L. Woo, and G. E. Harman.** 1993. Antifungal, synergistic interaction between chitinolytic enzymes from *Trichoderma harzianum* and *Enterobacter cloacae*. Phytopathology **83:**721–728.
12. **Ordentlich, A., Y. Elad, and I. Chet.** 1988. The role of chitinase of *Serratia marcescens* in biocontrol of *Sclerotium rolfsii*. Phytopathology **78:**84–88.
13. **Perrakis, A., I. Tews, Z. Dauter, A. B. Oppenheim, I. Chet, K. S. Wilson, and C. E. Vorgias.** 1994. Crystal structure of a bacterial chitinase at 2.3 A resolution. Structure **2:**1169–1180.
14. **Rost, B., and C. Sander.** 1994. Combining evolutionary information and neural networks to predict protein secondary structure. Proteins **19:**55–72.
15. **Sahai, A. S., and M. S. Manocha.** 1993. Chitinases of fungi and plants: their involvement in morphogenesis and host-parasite interaction. FEMS Microbiol. Rev. **11:**317–338.
16. **Shapira, R., A. Ordentlich, I. Chet, and A. B. Oppenheim.** 1989. Control of plant diseases by chitinase expressed from cloned DNA in *Escherichia coli*. Phytopathology **79:**1246–1249.
17. **Sitrit, Y., C. E. Vorgias, I. Chet, and A. Oppenheim.** 1995. Cloning and primary structure of the *chiA* gene from *Aeromonas caviae*. J. Bacteriol. **177:**4187–4189.
18. **Sobolev, V., R. C. Wade, G. Vriend, and M. Edelman.** 1996. Molecular docking using surface complementarity. Proteins **25:**120–129.
19. **Toyoda, H., Y. Matsuda, S. Ikeda, T. Tamai, M. Morita, C. Kami, K. Yoshida, and S. Ouchi.** 1993. Cloning of a chitinase gene from a chitin-degrading bacterium and its application to control of plant disease, p. 393–400. *In* R. A. A. Mozzarelli (ed.), Chitin enzymology. European Chitin Society, Ancona, Italy.
20. **Tsujibo, H., H. Orikoshi, H. Tanno, K. Fujimoto, K. Miyamoto, C. Imada, Y. Okami, and Y. Inamori.** 1993. Cloning, sequence, and expression of a chitinase gene from a marine bacterium, *Alteromonas* sp. strain O-7. J. Bacteriol. **175:**176–181.
21. **Vriend, G.** 1990. WHAT IF: a molecular modeling and drug design program. J. Mol. Graph. **8:**52–56.
22. **Watanabe, T., K. Kobori, K. Miyashita, T. Fuji, H. Sakai, M. Uchida, and H. Tanaka.** 1993. Identification of glutamic acid 204 and aspartic acid 200 in chitinase A1 of *Bacillus circulans* WL-12 as essential residues for chitinase activity. J. Biol. Chem. **268:**18567–18572.