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Production and Processing of a 59-Kilodalton Exochitinase during Growth of *Streptomyces lividans* Carrying pCHIO12 in Soil Microcosms Amended with Crab or Fungal Chitin

ANNA P. VIONIS,[1] FRANK NIEMEYER,[1] AMALIA D. KARAGOUNI,[2] AND HILDGUND SCHREMPF[1]*

FB Biologie/Chemie, Universität Osnabrück, D-49069 Osnabrück, Germany,[1] and Institute of General Botany, University of Athens, GR-15784 Athens, Greece[2]

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***Streptomyces lividans*(pCHIO12), which carries the previously cloned *Streptomyces olivaceoviridis exo-chi*O1 gene on a multicopy vector, secretes a 59-kDa exochitinase, consisting of a catalytic domain (40 kDa), a central fibronectin type III-like module, and a chitin-binding domain (12 kDa). The propagation rate of *S. lividans* (pCHIO12) was higher in soil microcosms amended with fungal mycelia than in those containing crab chitin. Comparative biochemical and immunological studies allowed the following conclusions to be drawn. Within soil microcosm systems amended with crab shell chitin or chitin-containing *Aspergillus proliferans* mycelia, the strain expressed the cloned *exo-chi*O1 gene and produced high quantities of a 59-kDa exochitinase. The enzyme was preferentially attached via its binding domain to the pellet from soil or liquid cultures. In contrast, truncated forms of 47, 40, and 25 kDa could be easily extracted from soil. The relative proportions of the 59-kDa enzyme and its truncated forms varied depending on the source of chitin and differed in soil and in liquid cultures.**

After cellulose, chitin is the second most abundant polymer in nature, and about 10^8 tons is synthesized annually. Within the polymer, the *N*-acetylglucosamine chains are arranged in an antiparallel (α) or a parallel (β) fashion. Chitin is present in the exoskeleton of arthropods, coelenterata, nematodes, protozoa, and mollusca as well as in the cell walls of many fungi (20). It is hydrolyzed by chitinases, which have been characterized for various bacteria, fungi, and plants. In addition to a few species of the bacterial genera *Aeromonas* (6), *Bacillus* (31), *Cellvibrio* (34), and *Serratia* (7), nearly every *Streptomyces* species is chitinolytic (2, 3, 14).

Streptomycetes grow as substrate hyphae and, upon depletion of nutrients, differentiate to aerial mycelia and spores. Amendment of the soil with pure chitin or fungal mycelia led to a significant increase in streptomycetes (18, 33). Fungi are estimated to be the main contributors to soil biomass; they constitute about 70% of the total weight of the mass (1). Therefore, it has been assumed that streptomycetes play a major part in the decomposition of fungal and other chitins within soil (29, 33).

Several chitinolytic enzymes were identified from various streptomycetes, including *S. antibioticus* (11), *S. griseus* (28), *S. plicatus* (22), and *S. lividans* (19). With the help of a screening programme, *Streptomyces olivaceoviridis* was identified as the most efficient degrader of crystalline chitin (2), and five of its chitinases have been purified (24).

Previously, we had succeeded in cloning an exochitinase gene from *S. olivaceoviridis* (3). The exochitinase is encoded by an open reading frame (*exo-chi*O1) of 1,794 nucleotides, which in turn encodes a 62-kDa protein. Several domains could be predicted by hydrophobic cluster analysis. The N terminus (32 amino acids [aa]) shows characteristics typical of signal peptides. The signal peptide is followed by a region of 169 aa which has no similarity to any known protein. The adjoining region of 90 aa displays sequence similarities to the fibronectin type III (FnIII) module (5). The C-terminal part (336 aa) constitutes the catalytic domain, belonging to family 18 of glycosylhydrolases (8). The architecture of the deduced domains resembles that of the chitinases encoded by *chi*63 (*Streptomyces plicatus*) (22) and *chi*D (*Bacillus circulans*) (31).

The *exo-chi*O1 gene had been cloned in a *Streptomyces* multicopy vector, and the resulting construct, pCHIO12, had been transferred into *S. lividans* (3). During cultivation with crab chitin, *S. lividans*(pCHIO12) secreted high levels of a 59-kDa mature chitinase which was found tightly bound to the crystalline substrate and could be released only by high concentrations of guanidine hydrochloride. In the course of growth, a 47-kDa enzyme showing chitinolytic activity was detected in the supernatant, and antibodies raised against this protein cross-reacted with the 59-kDa enzyme. The truncated 47-kDa chitinase consists of the FnIII module and the adjoining catalytic domain (3, 4) (see Fig. 4). The FnIII region shows amino acid similarities to the fibronectin modules predominantly present in the DNA-binding domain, the cell attachment site, and the main heparin-binding site of fibronectin (25). It is interesting that an FnIII-like part, the function of which remains to be elucidated, has also been found within chitinase A1 from *B. circulans* (31), cellulase B from *Cellulomonas fimi* (17), and in several other bacterial carbohydrases, including amylases and a polyhydroxybutyrate depolymerase (5).

The 59-kDa and the 47-kDa forms of the exochitinase hydrolyze 4-nitrophenyl-chitobioside and native crab shell chitin best at 45 to 55°C and pH 7.3. Both purified enzymes cleave chitotriose, -tetraose, -pentaose, and -hexaose as well as 4-nitro-phenylchitobioside equally well and release nearly identical quantities of reducing sugars from colloidal chitin. In contrast, the 59-kDa enzyme generates considerably higher amounts of reducing sugars from crab shell chitin and fungal mycelia. Microscopic inspection showed that the 59-kDa enzyme, contrary to the 47-kDa form, converts native fungi to short fragments and protoplast-like elements. The data suggested that the presence of the binding domain is a prerequisite for efficient degradation of crystalline chitin (4).

* Corresponding author. Mailing address: FB Biologie/Chemie, Universität Osnabrück, Barbarastrasse 11, D-49076 Osnabrück, Germany. Phone: 49/541-969 2895. Fax: 49/541-969 2804. Electronic mail address: Schrempf@sfbbio1.biologie.uni-osnabrueck.de.

Here we analyze the growth and chitinolytic activity of *S. lividans* and *S. lividans* containing the cloned exochitinase (*exochi*O1) gene in a soil microcosm system. Biochemical and immunological studies revealed that high levels of the 59-kDa exochitinase were formed and that the processing of the enzyme to truncated forms depended on the culture conditions.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The chitinase overproducer *S. lividans* 66 carrying plasmid pCHIO12 (3), *S. lividans* 66 with the vector pIJ702 (10), and a rifampin-resistant mutant from the indigenous, newly identified Greek soil isolate *S. griseus* CAG17 were used for our studies. Spore suspensions were prepared by the method of Hopwood et al. (10) and stored in 10% glycerol at −20°C. To test for chitinase activities, strains were inoculated in minimal medium (10) supplemented with either 1% ground crab shell chitin or 1% autoclaved lyophilized *Aspergillus proliferans* mycelium (harvested in the logarithmic stage).

Soil microcosm experiments. The soil was obtained from Greece. The soil composition and the sieving procedure have been described previously (12). After sterilization (twice for 1 h), the soil was wetted to a water content of approximately 10% (wt/wt) (-210 kPa). It was distributed in sterile Falcon tubes and mixed with 1% ground crab shell chitin or 1% ground *A. proliferans* mycelium as appropriate. Soil aliquots of all nutritional regimens were separately inoculated with 10^5 spores of each strain per g and incubated as batch microcosms at 22°C. On days of sampling, one set of aliquots from each treatment were destructively sampled and treated as follows: three 1-g aliquots were extracted as described by Wellington et al. (32) for determination of total viable-cell counts; 10 g was extracted as previously described (9) for spore counts; 1 g was taken for extraction of extracellular enzymes (see below); 100 mg was used for direct separation of proteins present in soil by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE); 100 mg was used for direct immunological detection of chitinase by fluorescence microscopy (see below); and 100 mg was fixed for examination under the scanning electron microscope (see below). Inoculants were enumerated on RASS medium (9). For selective recovery of the two *S. lividans* strains, thiostrepton was added to the medium at a final concentration of 50 μg/ml. Selective enumeration of *S. griseus* CAG17 was carried out on RASS medium containing rifampin (50 μg/ml).

Extraction and enrichment of extracellular enzymes. Soil samples (1-g aliquots) were extracted with 50 mM Tris-HCl (pH 7.4; 1:2, wt/vol) on a rotary shaker at 4°C for 30 min and then centrifuged in a cooled bench centrifuge (Hettich) at 1,000 × *g* for 15 min to sediment soil particles. The supernatants were kept, and the pellets were reextracted twice as described above. The pooled supernatants were precipitated with $(NH_4)_2SO_4$ (90%, wt/vol) at 4°C overnight. The precipitated proteins were collected by centrifugation (25,000 × *g* for 30 min), suspended in 50 mM Tris-HCl buffer (pH 7.4; 5% of the original volume), and tested for chitinolytic activities, yielding values for extractable chitinase activities in the absence of soil (see below).

The proteins of supernatants from liquid cultures were gained in a similar fashion, as recently described by Blaak and Schrempf (4).

Enzyme assays. Enzyme activity was determined by measuring the release of 4-nitrophenol from nitrophenol-β-D-chitobioside at 410 nm (4). Assay mixtures (within a microtiter plate) amounted to a volume of 120 μl, consisting of 100 μl of 50 mM sodium citrate-phosphate, pH 7.5, 10 μl of 0.5 mM pNPC, and 10 μl of concentrated protein solution (obtained as described above).

In order to determine total chitinase activity in soil and in pure culture pellets, 100 mg of each sample was incubated with 500 μl of 50 mM sodium citrate-phosphate, pH 7.5, and 50 μl of 0.5 mM pNPC solution (in double-distilled water) at 37°C for 45 min. Then, 120 μl of the mixture was transferred to microtiter plates. Optical densities were read with an Elisa microtiter plate reader. All samples were measured twice.

Separation of proteins and Western blot analyses. Concentrated soil-protein extracts (see above), concentrated filtrates from liquid cultures, culture pellets, and soil (100 mg of each) were separated on 10% polyacrylamide gels containing SDS (15) and stained with Coomassie blue or silver stain. Alternatively, proteins were transferred to nylon membranes after SDS-PAGE, and immunodetection of proteins was performed as previously reported (3).

Fluorescence microscopy. Soil and pure culture samples were incubated with primary antibodies raised against the 47-kDa form of the exochitinase and subsequently treated with fluorescein isothiocyanate-labelled secondary antibodies (3). Inspection of the samples were carried out under UV light with a Zeiss-Axiovert 10 microscope fitted with a 50-W short-arc mercury lamp and filters for epifluorescence studies. Photographs were taken on Ilford HP5 400 ASA film.

Scanning electron microscopy. Soil and pure culture samples were fixed and dehydrated as described by Schlochtermeier et al. (26). After critical-point drying with a Balzers union CPD010 apparatus and sputter coating with gold using Polaron Equipment Ltd. SEM coating unit E5000, samples were examined with a Cambridge Stereoscan 250 scanning electron microscope, and microphotographs were made with Agfapan APX 100 film.

In situ hybridization. The oligonucleotide 5′-TATAGTTACCACCGCCGT-3′ corresponded to a region conserved within the 23S RNA gene from gram-positive bacteria with a high G+C content in their DNA (23). The oligonucleotide probe was commercially synthesized with a C6-TFA aminolinker [6-(trifluoracylamino-)-hexyl-(2-cyano-ethyl)-(*N*,*N*-diisopropyl)phosphoramidite] at the 5′ end and labelled with 5(6)-carboxyfluorescein-*N*-hydroxysuccinimide ester (MWG Biotech).

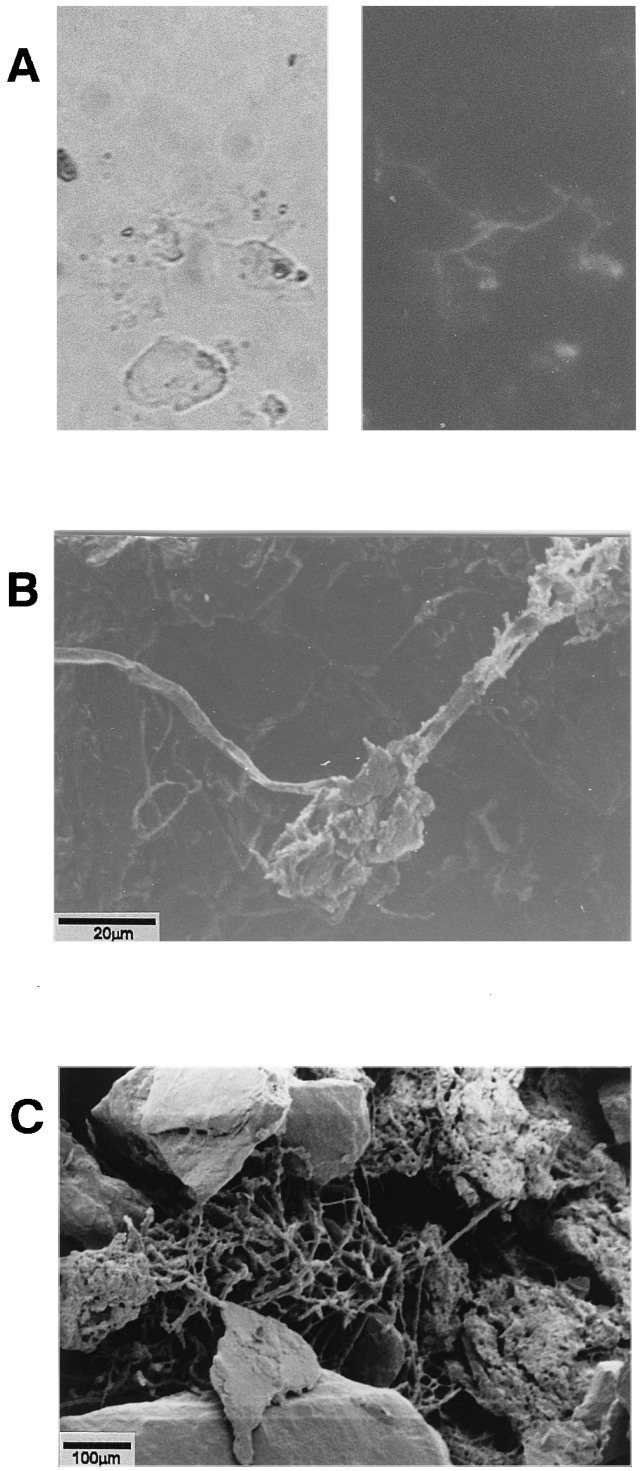

FIG. 1. Visualization of *Streptomycetes* mycelia. Within soil containing 1% fungal mycelia (A, left), hyphae of *S. lividans*(pCHIO12) were detected by hybridization with a specific oligonucleotide (targeted against a 23S rRNA region) and visualized by fluorescence microscopy (A, right). Scanning electron microscopy allowed direct observation of *S. lividans*(pCHIO12) in soil containing 1% *A. proliferans* mycelia (B) or 1% chitin (C).

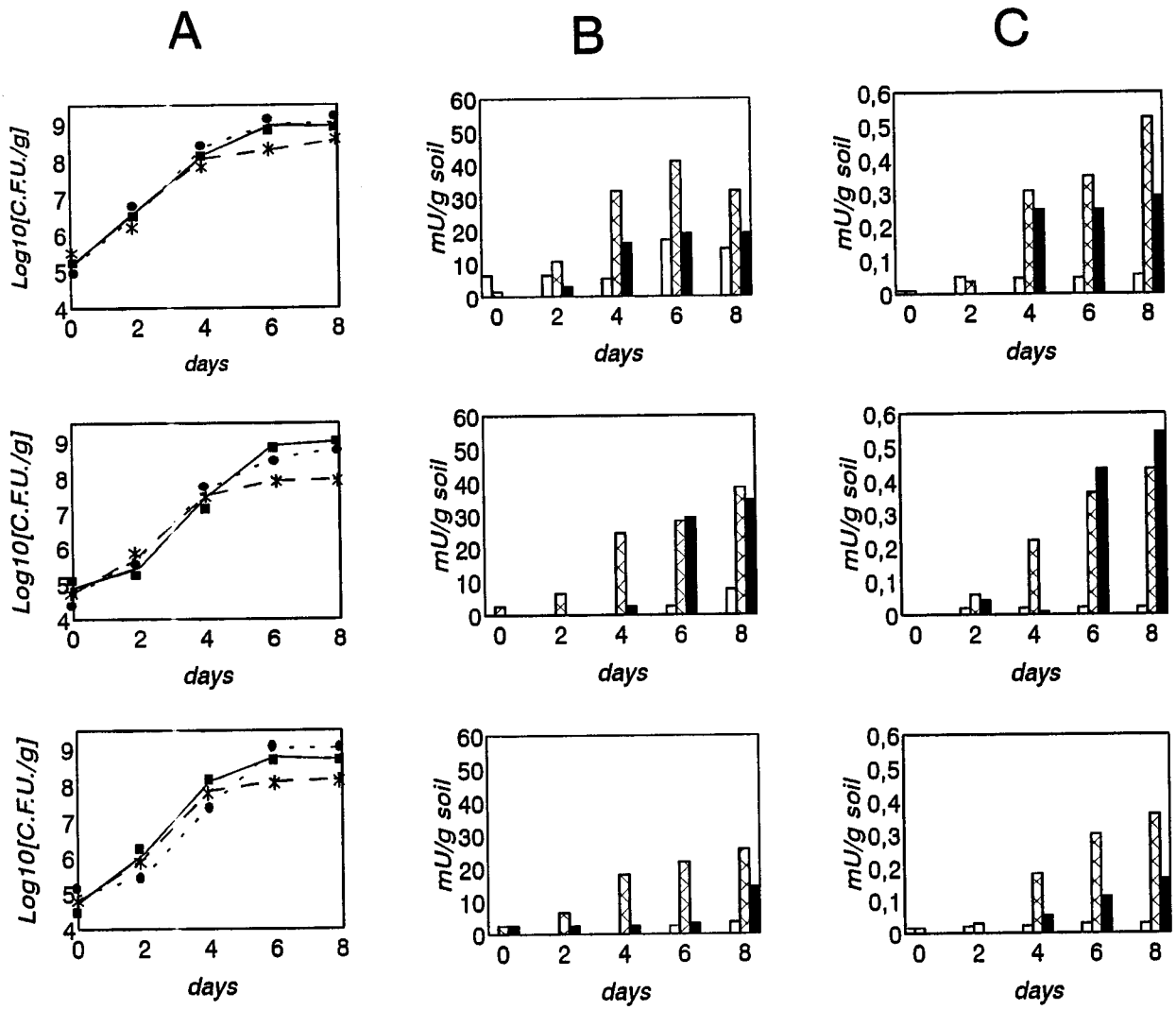

FIG. 2. (A) Population levels. Total viable-colony counts were determined for *S. griseus* CAG17 (top), *S. lividans*(pCHIO12) (middle), and *S. lividans*(pIJ702) (bottom) grown for up to 8 days in sterile soil without amendment (∗) or amended with 1% chitin (●) or 1% *A. proliferans* mycelia (■). (B and C) Chitinolytic activities. Total (B) and extractable (C) chitinolytic activities were assayed in samples from sterile soil that was not amended (open bars) or amended with 1% chitin (hatched bars) or 1% *A. proliferans* mycelia (solid bars) inoculated with *S. griseus* CAG17 (top), *S. lividans*(pCHIO12) (middle), or *S. lividans*(pIJ702) (bottom). Samples were analyzed every 2 days for up to 8 days of cultivation.

Pure culture and soil samples were washed with phosphate-buffered saline (PBS; 140 mM NaCl, 2.7 mM KCl, 10.1 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3) and fixed with ethanol as previously described (23). Samples (3 μl) of the fixed material were spotted on cleaned, gelatin-coated microscopic slides (23) and immobilized by drying at 46°C for 15 min. A series of immersions in 50, 80, and 96% (vol/vol) ethanol (3 min each) completed the fixation. Hybridization with the oligonucleotides mentioned above was carried out as described by Roller et al. (23).

Statistical analysis. Statistical analysis of the viable-count data and determination of minimum significant differences were done as previously described (12).

Chemicals. Chemicals for SDS gel electrophoresis were obtained from Serva, and molecular mass markers and substrates were from Sigma. Substances for immunological analysis were ordered from Dianova. All other chemicals were obtained from Merck or Sigma. Thiostrepton was a gift from S. J. Lucania (E. R. Squibb and Sons Inc., Princeton, N.J.), and rifampin was a gift from Linda Cavaletti (Lepetit, Milan, Italy).

RESULTS

Visualization of *Streptomyces* mycelia. The network of *Streptomyces* hyphae from *S. lividans*(pCHIO12) growing in soil amended with crab chitin or *Aspergillus* mycelia was visualized by scanning electron microscopy (Fig. 1B and C). By using fluorescently labelled oligonucleotides (generated from a region of the 23S rRNA gene [23]), the hyphae could be directly monitored in soil (Fig. 1A).

Survival of streptomycetes in soil containing crab or fungal chitin. The survival of *S. lividans* carrying the vector pIJ702 or pCHIO12, overproducing the exochitinase, was compared with that of *S. griseus* CAG17, an isolate indigenous to the soil used. After 6 days, viable colonies (ca. 10^9 CFU/g of soil) consisted predominantly of spores (data not shown), and the counts remained constant for up to 8 days. In soil containing 1% *A. proliferans* mycelia, the amount and proportions of viable-cell and spore counts corresponded to those described above. However, after 6 to 8 days, 10 times fewer spores were formed (data not shown), whereas the number of total viable colonies was identical to that when the soil had been mixed with crab chitin. In the first 2 days, the population levels of *S. griseus* in nonamended soil corresponded to those in soil containing chitin

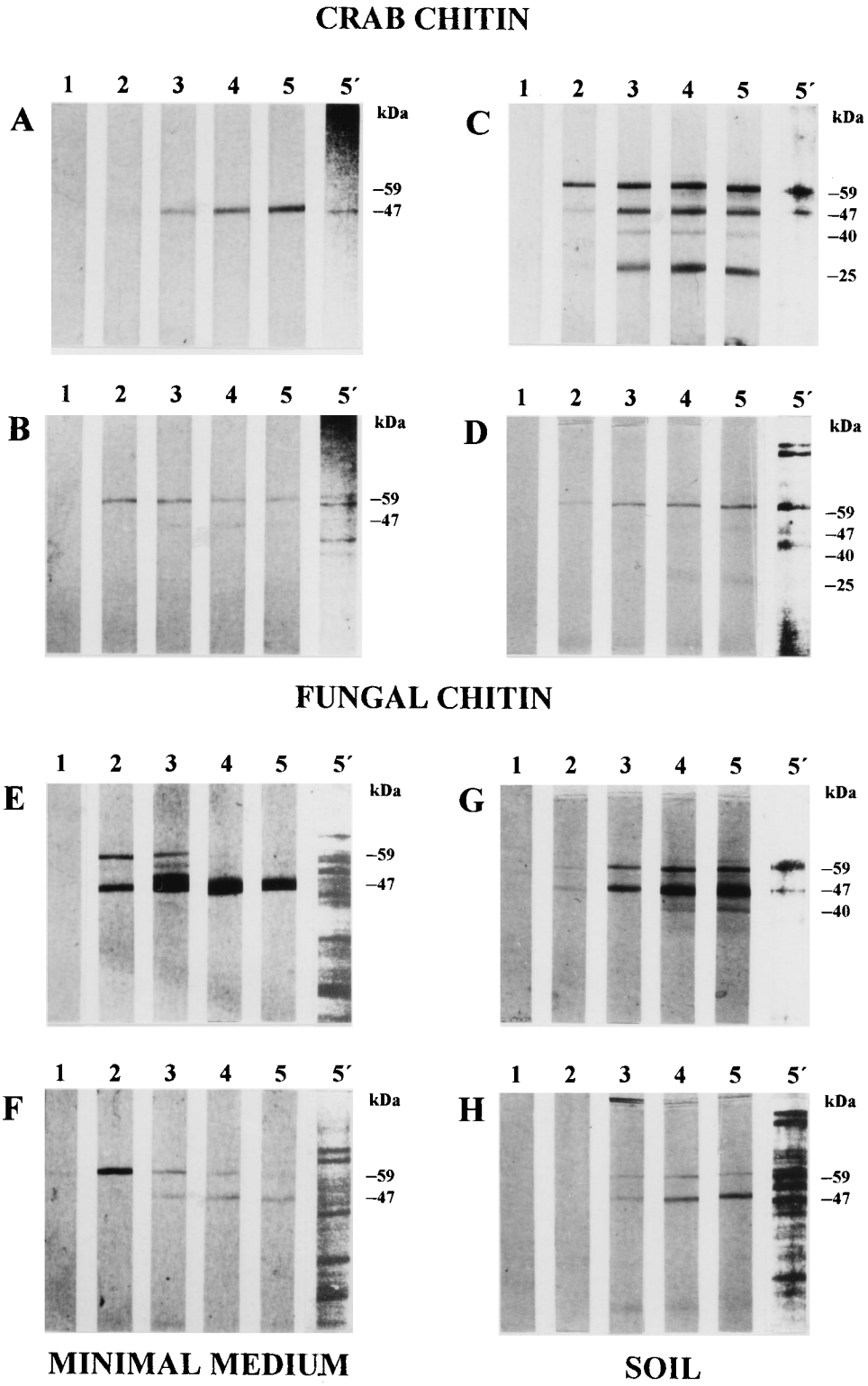

FIG. 3. Detection of chitinase and its truncated forms. *S. lividans*(pCHIO12) was cultivated in minimal medium or in soil supplemented with 1% crab shell chitin (A, B, C, and D) or 1% mycelia from *A. proliferans* (E, F, G, and H) for 0, 2, 4, 6, and 8 days (lanes 1 to 5, respectively). Proteins from culture supernatants (A and E), culture pellets (B and F), extracts from soil (C and G), and soil pellets (D and H) were separated by SDS-PAGE. After transfer of the proteins to nylon membranes, the production of the exochitinase was detected with antibodies previously raised against the 47-kDa form of the enzyme (3). Proteins obtained after 8 days of cultivation were also separated by SDS-PAGE and stained as a control (lanes 5′).

(Fig. 2A, top). This suggests that the nonamended, sterilized soil contained a sufficient amount of nutrients for the indigenous strain. However, the increase in population numbers after 4 days was less significant than that observed in the course of a cultivation in chitin-enriched soil, and spores as well as total viable colonies amounted to approximately 3×10^8 CFU/g of soil after 8 days of incubation.

S. lividans(pIJ702) (control) and *S. lividans*(pCHIO12) survived equally well in nonamended soil, and their population levels were about four times lower than that of the *S. griseus* strain. In soil to which crab chitin had been added, the counts of total colonies and of spores were nearly identical for the two strains and reached 6×10^8 to 7×10^8 CFU/g of soil (Fig. 2A, middle and bottom). It is interesting, however, that in the presence of *Aspergillus* mycelia, both *S. lividans* strains yielded approximately the same numbers of total viable colonies as the indigenous *S. griseus* strain.

Determination of total chitinolytic activity. After 4 to 8 days of cultivation, *S. griseus* CAG17 reached its highest total chitinolytic activity in the presence of ground crab chitin, with levels ranging between 30 and 40 mU/g of soil (Fig. 2B, top). Contrary to this, total chitinase activities amounted only to 15 to 18 mU/g of soil in the course of cultivation with fungal hyphae. Low levels of chitinolytic activity were observed in nonamended soil. The extractable activities were about 100 times lower than the corresponding total activities.

During the first 2 days in soil amended with crab chitin, the total chitinolytic activity of *S. lividans*(pCHIO12) rose steadily and, after 8 days, amounted to about 40 mU/g (Fig. 2B, middle). When the strain was inoculated into soil supplemented with fungal mycelia, the chitinolytic activities increased more slowly during the first 4 days of cultivation. After 6 to 8 days, however, they were comparable to those obtained in soil containing crab chitin. The corresponding activity of the control strain *S. lividans*(pIJ702) was lower; it reached about 28 and 15 mU/g of soil for soils amended with crab chitin and *Aspergillus* mycelia, respectively (Fig. 2B, bottom). As for the *S. griseus* strain, the total activities of *S. lividans*(pIJ702) and *S. lividans* (pCHIO12) were about 100 times higher than the extractable ones. However, when grown in the presence of fungal mycelia, the extractable chitinase activity of *S. lividans*(pCHIO12) was approximately 2.5 times higher than that of the control strain *S. lividans*(pIJ702) (Fig. 2C, middle and bottom).

Immunodetection of exochitinase. The chitinases of the *S. griseus* strain have not yet been purified and characterized. Therefore, we tested whether antibodies raised against the exochitinase encoded by pCHIO12 cross-reacted with concentrated extracellular chitin-inducible proteins from *S. griseus* and the *S. lividans* control strain. This was not the case. Thus, the following studies were performed with *S. lividans*(pCHIO12) only.

When *S. lividans*(pCHIO12) was grown in flasks containing minimal medium and 1% crab chitin, increasing quantities of the truncated 47-kDa chitinase were synthesized in the course of 8 days (Fig. 3A). The amount of the truncated 47-kDa enzyme detected in the culture filtrate was about 10 times higher when the strain grew in minimal medium supplemented with fungal mycelia instead of crab chitin (Fig. 3E). As expected from our previous studies, the 59-kDa form of the exochitinase, which contained the binding domain (3, 4), adhered firmly to the pellet consisting of chitin and *Streptomyces* hyphae clinging to it (Fig. 3B and F and Fig. 4). Though only about 19% of the cell wall from the *Aspergillus* hyphae consists of chitin (20, 21), the hyphae serve as the best chitinous substrate for the strain harboring the cloned exochitinase gene.

It is interesting that within soil amended with 1% crab shell chitin, considerably higher amounts of the large 59-kDa enzyme, the truncated 47-kDa enzyme, and a 25-kDa form and a small quantity of 40-kDa truncated protein could be extracted (Fig. 3C) than from the corresponding liquid culture. When soil samples (Fig. 3D) were applied directly to SDS-polyacrylamide gels, predominantly the 59-kDa enzyme was identified, and its maximal expression after 6 to 8 days corresponded to that obtained in liquid culture after 2 to 4 days (Fig. 3B).

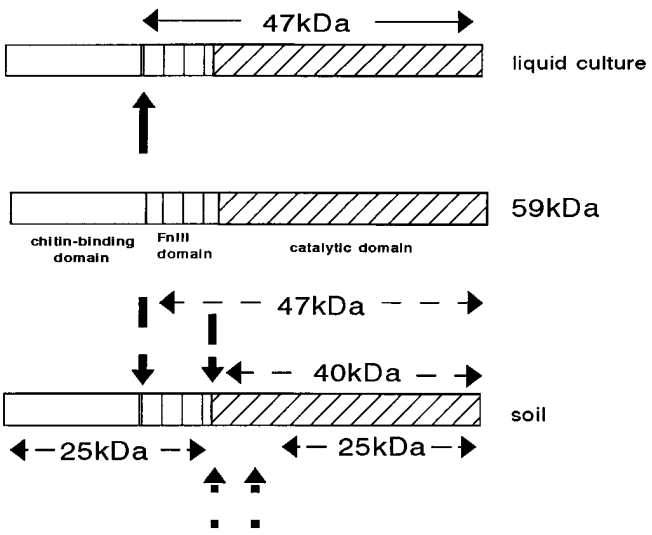

FIG. 4. Processing of the 59-kDa exochitinase. The exochitinase is secreted by *S. lividans*(pCHIO12) as a 59-kDa enzyme, which binds via its N-terminal binding domain (12 kDa) to chitinous substrates. As recently described, an extracellular protease (4) (solid vertical arrow) releases a truncated, catalytically still active form of the enzyme (47 kDa) to the medium during growth in liquid culture. In the course of the studies presented, we discovered that, in addition to the 47-kDa enzyme, shorter, truncated forms are generated during cultivation in soil. The predicted cleavage sites are indicated (broken vertical arrows, bottom)

In the supernatant of the liquid culture with 1% fungal mycelia as the sole nutrient, small quantities of the 59-kDa enzyme were present; the amount of the 47-kDa truncated enzyme was significantly higher, and after 4 to 5 days, it was the only one present (Fig. 3E). Compared with a liquid culture containing crab chitin (Fig. 3A), the presence of fungal mycelia led to an about 10-fold-higher level of the 47-kDa enzyme (Fig. 3E). In the same culture, the 59-kDa form was ascertained in the pellet (Fig. 3F). However, in the course of cultivation, its quantity declined, and after 5 days, it was no longer detectable.

When soil was amended with fungal mycelia, the extract contained a large proportion of the 47-kDa enzyme and the 59-kDa enzyme; in addition, a small part of a 40-kDa form of the enzyme was discovered (Fig. 3G). The corresponding soil pellet included considerable quantities of the 47-kDa form in addition to small amounts of the 59-kDa enzyme.

By immunofluorescence microscopy, the enzyme was shown to adhere strongly to the fungal mycelia. After the action of the chitinase overproducer, the *Aspergillus* mycelia contained holes in their cell walls (Fig. 5).

DISCUSSION

In the laboratory, bacteria are generally cultivated and studied as cell suspensions in defined liquid media. Within the natural environment, however, bacteria are exposed to continually changing environmental conditions in soil, sediments, and water. Soil is one of the most complex microbial habitats, differing from others by the dominance of solid surfaces and by spatial as well as temporal fluctuations in aqueous and gaseous

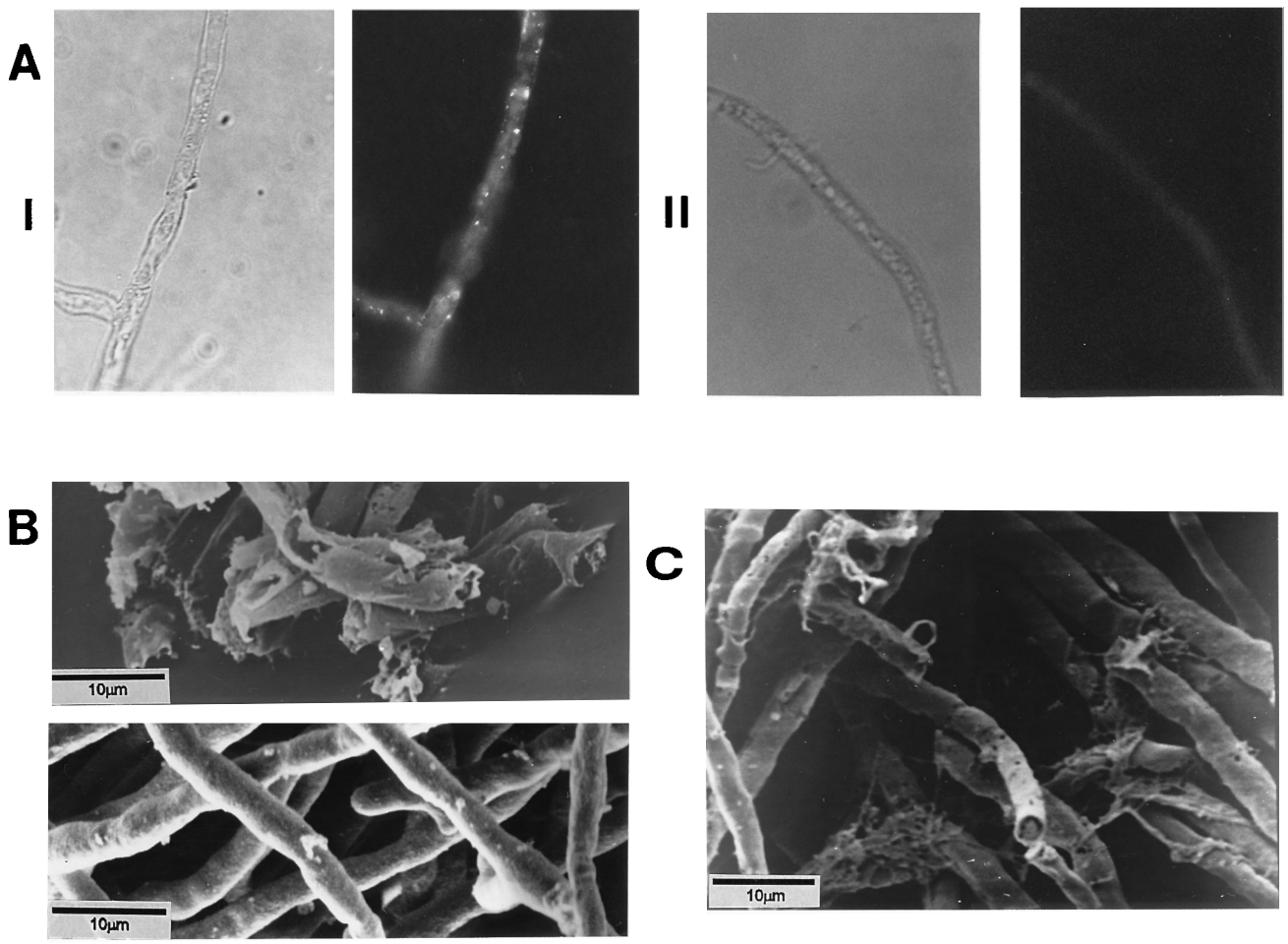

FIG. 5. Microscopic and immunological studies. Hyphae from *A. proliferans* were incubated with (I) or without (II) the 59-kDa chitinase, treated with antibodies raised against this enzyme, and inspected visually (AI and AII, left) or under UV light (AI and AII, right). (B and C) Scanning electron microscopy of *A. proliferans* hyphae grown in coculture with *S. lividans*(pCHIO12) revealed small holes within the chitin-containing cell walls of the fungal hyphae.

phases. Soil consists of solid particles which are associations of minerals (i.e., quartz, feldspar, and clay) and organic substances. Water, inorganic ions, low-molecular-weight organic molecules, and gases are dispersed between the solid particles (30). Within soil, in addition to diffusible metabolites, microbes will encounter macromolecules such as celluloses, xylans, chitin, and others.

Fungi are estimated to be the main contributors to soil biomass and to constitute about 70% of the total mass weight. Fungal cell walls are mainly decomposed by various *Pseudomonas*, *Bacillus*, and *Clostridium* spp. as well as by nearly all *Streptomyces* spp. (21). Earlier studies had shown that the number of chitinolytic streptomycetes was increased by amendment with mycelium of fungi. Since streptomycetes produce, in addition to chitinolytic enzymes, glucanases, proteases, and other exoenzymes (3, 14), it has been assumed that they play a major part in the decomposition of fungal material in soil (33).

Extracellular enzymes of microbial origin can be found free or bound to cell colloids (13, 16, 30). Therefore, they are much more difficult to assay than in pure cultures. Various enzymatic activities have been detected in soil, including oxidoreductases, hydrolases, and transferases (21).

To investigate the survival and chitinolytic activity of *Streptomyces* strains in chitin-enriched soil without continuously altering conditions, we used a batch soil microcosm system, the composition of which (minerals, pH, and type and concentration of ions) was determined earlier (12). The nonamended soil contained a sufficiently high level of nutrients to allow efficient enumeration of 10^5 and 10^4 CFU/g of soil for the indigenous *S. griseus* strain and the two *S. lividans* strains. The increase in viable-cell counts and the ratio of spores to viable cells varied, depending on the strain and the chitin source. All strains reached nearly identical (10^9 CFU/g of soil) and highest total viable-cell counts in soil amended with fungal chitin.

Like several other streptomycetes, the strains used for these studies produced several chitinases. Thus, the specific characteristics can be explored only by additional studies. Using antibodies, we succeeded in comparing and quantifying the expression of the previously cloned exochitinase (3) of the transformant *S. lividans*(pCHIO12) in pure culture and soil. The results of the studies allowed the following conclusions to be drawn. (i) The 59-kDa enzyme containing the binding domain is preferentially found in the pellet from soil and liquid cultures amended with crab chitin. (ii) The corresponding culture supernatants or soil extracts contain the truncated 47-kDa form of the enzyme (3, 4), previously reported to lack the binding domain, and small quantities of a 40-kDa protein, which likely corresponds to the catalytic domain and is probably

generated by proteolytic cleavage between the catalytic and the FnIII domain (Fig. 4, bottom). (iii) The relative proportions of the processing products vary with the source of the chitin. (iv) The 25-kDa protein was detected only in extracts from soil amended with crab chitin. As it is absent in the corresponding soil pellet, it is likely that the 25-kDa protein lacks the binding domain; it may have been generated by cleavage at different positions within the 59-kDa form or its truncated forms (Fig. 4).

Our previous studies (4) had clearly indicated that the binding domain links the enzyme to its high-molecular-weight substrate, which is then efficiently hydrolyzed. Moreover, we have now proved that the 59-kDa enzyme binds along the fungal hyphae (Fig. 5). Recently we had revealed that the truncated 47-kDa enzyme does not adhere to either colloidal or crystalline crab chitin, indicating that the FnIII domain is not essential for binding to crab chitin. Studies of truncated forms of the chitinase A1 from *B. circulans* allowed a similar conclusion (31). It is interesting that the 47-kDa enzyme is nearly absent from the pellet of soil and liquid cultures amended with crab chitin but present in pellets derived from cultures amended with fungal mycelia. Further binding studies (4a) indicate that the 47-kDa enzyme still interacts with fungal mycelia, although to a lesser degree than the 59-kDa form. These data suggest that the FnIII domain (still present in the 47-kDa truncated form) may assist in the interaction of the enzyme with fungal mycelia. Further studies are required to test this assumption.

We recently discovered a novel chitin-binding protein which lacks chitinolytic activity (27). This protein (CHB1) was induced in the presence of unsoluble chitin, and it has a high affinity to α-chitin (27, 35). We found that many streptomycetes produce a CHB1 homolog, forming a glue-like layer on fungal mycelia (6a). At present, we are investigating whether the protein mediates efficient adhesion of streptomycetes to chitinous material in soil. Earlier studies revealed that attached microorganisms are often more active than free cells (30). Other proteins mediating adhesion of spores or hyphae from streptomycetes to soil will be of further interest.

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