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Sequencing of a 1,3-1,4-β-D-Glucanase (Lichenase) from the Anaerobic Fungus *Orpinomyces* Strain PC-2: Properties of the Enzyme Expressed in *Escherichia coli* and Evidence that the Gene Has a Bacterial Origin

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A 971-bp cDNA, designated *licA*, was obtained from a library of *Orpinomyces* sp. strain PC-2 constructed in *Escherichia coli*. It had an open reading frame of 738 nucleotides encoding LicA (1,3-1,4-β-D-glucanase; lichenase) (EC 3.2.1.73) of 245 amino acids with a calculated molecular mass of 27,929 Da. The deduced amino acid sequence had high homology with bacterial β-glucanases, particularly in the central regions and toward the C-terminal halves of bacterial enzymes. LicA had no homology with plant β-glucanases. The genomic DNA region coding for LicA was devoid of introns. More than 95% of the recombinant β-glucanase produced in *E. coli* cells was found in the culture medium and periplasmic space. A N-terminal signal peptide of 29 amino residues was cleaved from the enzyme secreted from *Orpinomyces*, whereas 21 amino acid residues of the signal peptide were removed when the enzyme was produced by *E. coli*. The β-glucanase produced by *E. coli* was purified from the culture medium. It had a molecular mass of 27 kDa on sodium dodecyl sulfate-polyacrylamide gels. The K_m and V_{max} values with lichenin as the substrate at pH 6.0 and 40°C were 0.75 mg/ml and 3,790 μmol/min/mg, respectively. With barley β-glucan as the substrate, the corresponding values were 0.91 mg/ml and 5,320 μmol/min/mg. This enzyme did not hydrolyze laminarin, carboxymethylcellulose, pustulan, or xylan. The main products of lichenin and barley β-glucan hydrolysis were triose and tetraose. LicA represented the first 1,3-1,4-β-D-glucanase reported from fungi. The results presented suggest that *licA* of *Orpinomyces* had a bacterial origin.

Hemicelluloses, which include β-glucans, mannans, and xylans, are major constituents of plant cell walls. The mixed linked 1,3-1,4-β-D-glucans (β-glucans) form a major part of cell walls of cereals, such as oat and barley, and account for up to 70% of the cell wall in barley endosperm (11). β-Glucans consist of glucose units joined by β-1,4 and β-1,3 linkages and include lichenin or barley β-glucan. 1,3-1,4-β-D-Glucan 4-glucanohydrolase (β-glucanase; lichenase) (EC 3.2.1.73) cleaves β-1,4 linkages adjacent to β-1,3 bonds in glucans, yielding chiefly cellobiosyltriose and cellotriosyltetraose (2, 21). β-Glucanases have been found in several *Bacillus* species, including *B. subtilis* (45), *B. amyloliquefaciens* (28), *B. macerans* (9), *B. licheniformis* (38), *B. brevis* (39), and *B. polymyxa* (24), and in *Clostridium thermocellum* (53), *Fibrobacter succinogenes* (54), *Ruminococcus flavefaciens* (22), *Rhizobium meliloti* (4), and *Cellvibrio mixtus* (51). A cDNA clone encoding a β-glucanase has been isolated and sequenced from germinating barley (20). Unlike 1,4-β-D-endocellulases, which are widely distributed in bacteria, fungi, and plants, β-glucanases are known to be produced only by plants and bacteria (9, 20, 26).

Obligately anaerobic fungi are part of the natural microflora of the alimentary tracts of many herbivorous mammals (44). Since the first anaerobic fungus, *Neocallimastix frontalis*, was isolated in 1975 from the rumen of sheep (48), at least 13 different anaerobic fungi have been isolated from ruminant and nonruminant herbivores (13, 49). Based on morphological patterns, anaerobic fungi are divided into two groups, monocentric and polycentric (8). Anaerobic fungi produce highly active hydrolytic enzymes (7), they physically associate with the lignocellulosic tissues of plant fragments, and their hyphae penetrate the plant tissue in vivo (1). These facts indicate that the fungi are involved in degradation of plant biomass and play an important role in the rumen ecosystem. Several cellulases and xylanases have been cloned and sequenced from the monocentric fungi *Neocallimastix patriciarum* (6, 17, 23, 57) and *Piromyces* sp. (19) and from the polycentric fungus *Orpinomyces* sp. strain PC-2 (34, 35). Three mannanases have been cloned and sequenced from a *Piromyces* sp. (19, 43). The present work reports cloning and sequencing of a β-glucanase cDNA designated *licA* from *Orpinomyces* strain PC-2, and the characterization of the enzyme produced in *Escherichia coli* and *Orpinomyces* strain PC-2. This is the first report of a 1,3-1,4-β-D-glucanase from a fungus. Several observations suggest that the β-glucanase gene of *Orpinomyces* strain PC-2 has a bacterial origin.

MATERIALS AND METHODS

Fungal strains, culture conditions, and vectors. *Orpinomyces* sp. strain PC-2 was isolated and described by Borneman et al. (7); *Neocallimastix frontalis* EB188 was a gift from R. Calza (3). For enzyme production, the fungi were grown at 39°C for 6 days in 2-liter round bottles, each containing 1 liter of basic medium described by Barichievich and Calza (3) and 0.3% Coastal Bermuda grass. The medium was autoclaved for 30 min and then cooled under a stream of CO_2. Penicillin (334 U/ml), streptomycin sulfate (80 μg/ml), and chloramphenicol (10 μg/ml) were filter sterilized (pore size, 0.22 μm) and added just prior to inocu-

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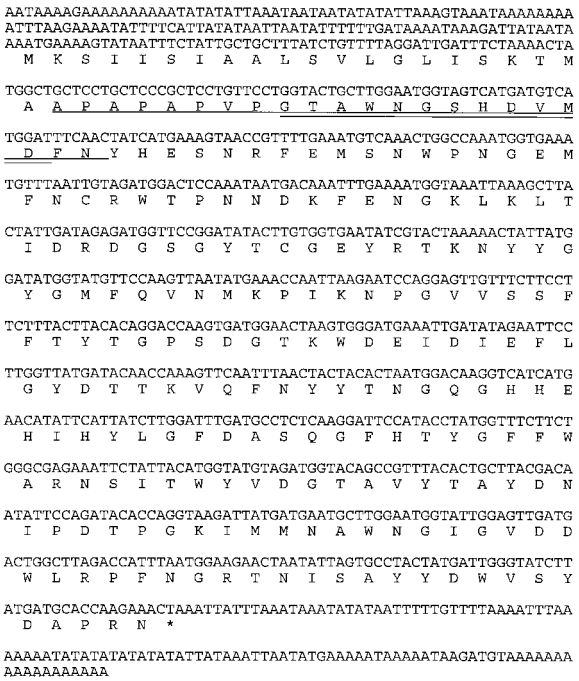

FIG. 1. Nucleotide sequence and deduced amino acid sequence of *licA* cDNA from *Orpinomyces* sp. strain PC-2 harboring pLIC6. The N-terminal 22-amino-acid sequence determined for the purified recombinant enzyme is underlined, and the N-terminal 12-amino-acid sequence determined for the purified enzyme from *Orpinomyces* strain PC-2 culture supernatant is underlined twice. The asterisk indicates the stop codon.

lation. *Escherichia coli* XL1-Blue, λZAPII, and pBluescript SK(−) were products of Stratagene Cloning Systems (La Jolla, Calif.).

Construction and screening of an *Orpinomyces* cDNA library. Extraction of RNA, purification of mRNA, and construction of a cDNA library of *Orpinomyces* sp. strain PC-2 were described previously (14).

The top agar of NZY medium (52) plates containing 5 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) and 0.2% lichenin (Sigma Co., St. Louis, Mo.) was used to isolate β-glucanase-producing plaques. After growth at 37°C overnight, plates were stained with 0.1% Congo red and destained with 1 M NaCl (5); yellowish haloes on red background were indicative of β-glucanase-positive plaques. Positive plaques were enriched by a second screening. Lambda phage was converted into pBluescript SK(−) by in vivo excision. Colonies positive on Luria-Bertani (LB) plates were inoculated into 5 ml of LB medium containing 50 μg of ampicillin per ml (LB-ampicillin medium). The cultures were grown at 37°C with shaking at 250 rpm, until an optical density at 600 nm (OD_{600}) of 1.5 was reached. Then 10 μl of 0.5 M IPTG was added to each culture which was then grown for another 5 h. Supernatant and cells were separated by centrifugation (5,000 × *g*, 10 min). The *E. coli* cells were resuspended in 50 mM sodium citrate buffer, pH 6.0, and broken by sonification with a Branson Sonifier 450 (Danbury, Conn.). The cell debris was spun down at 10,000 × *g*. After centrifugation, the clear supernatants were used for testing β-glucanase and carboxymethyl cellulase (CMCase) activities. pBluescript DNA was purified from cultures grown overnight in LB-ampicillin medium with the Wizard Maxipreps plasmid purification system (Promega, Madison, Wis.). Nucleotide sequences of insert DNA were determined with an automatic PCR sequencer (Applied Biosystems). Both universal and specific primers were used to sequence both strands of the inserts. Sequence data were analyzed by using the Genetics Computer Group program (version 8; University of Wisconsin Biotechnology Center, Madison) on the VAX/VMS system of the BioScience Computing Resource at the University of Georgia.

Analysis of genomic DNA. *Orpinomyces* sp. strain PC-2 was grown for 3 days in a basic medium (3) with glucose (0.4%, wt/vol) as the carbon source. Mycelium collected by passing the culture through three layers of cheesecloth was rinsed three times with water. Disruption of cells and isolation of chromosomal DNA were done by the methods of Rozman and Komel (50). The size and purity of the DNA were determined by electrophoresis on agarose (0.8%, wt/vol) gels and staining with ethidium bromide (52). Oligonucleotides used as PCR primers were 5′ATGAAAAGTATAATTTCTATTGC3′ and 5′TTAGTTTCTTGGTGC ATCATAAG3′, corresponding to opposite strands of the end regions of the open reading frame (ORF) of *licA* (see Fig. 1). By using these oligonucleotides as the primers and the genomic DNA and the cDNA library as the templates, PCR was performed on a 480 Thermal Cycler (Perkin-Elmer Co., Norwalk, Conn.). Amplification was performed for 30 cycles, with each cycle consisting of 90 s of melting at 95°C, 60 s of annealing at 42°C, and 60 s of extension at 72°C. PCR products were separated by electrophoresis on agarose gels (2.5%, wt/vol) and visualized by ethidium bromide staining (52).

Localization of β-glucanase in fractions of *E. coli*. For the localization of β-glucanase in cells and cultures of *E. coli*, the procedures of Neu and Heppel (46) and Cornelis et al. (16) were employed. The extracellular fluid and cells were separated by centrifugation at 13,000 × *g* for 10 min. Cells were washed twice in half the volume of the culture with 10 mM Tris-HCl, pH 8.0, and suspended in the same volume in a solution of 25% (wt/vol) sucrose containing 5 mM EDTA. The suspension was shaken for 10 min at room temperature. After centrifugation (5,000 × *g*, 10 min), the cells were resuspended in the same volume in a solution of ice-cold water and the suspension was shaken for 10 min at 4°C. After centrifugation (5,000 × *g*, 10 min), the supernatant was used as the periplasmic fraction. The pellet was resuspended in the same volume of 50 mM sodium citrate buffer, pH 6.0, and an aliquot was sonicated (four times at 7,000 cycles in a Branson Sonifier 450). The supernatant obtained after centrifugation at 5,000 × *g* for 10 min was used as the intracellular fraction. β-Galactosidase (52) and alkaline phosphatase (46) were used as cytoplasmic and periplasmic markers, respectively.

SDS-PAGE assay. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in Laemmli's buffer (32). The gels were stained for proteins with Coomassie brilliant blue R-250 (Sigma) or used for zymogram analyses. Enzyme samples were pretreated by incubating for 1 h at 40°C in SDS-PAGE sample buffer, and SDS-PAGs were run at 4°C. To enhance removal of SDS and recovery of enzymatic activity following SDS-PAGE, gels were prewashed in 50 mM sodium citrate buffer, pH 6.0, with 1% (wt/vol) bovine serum albumin (BSA) (40). β-Glucanase and CMCase activities were detected by the zymogram method of Béguin (5) with a overlay containing the appropriate substrate (0.2%, wt/vol) and agarose (2%, wt/vol) in 50 mM sodium citrate buffer, pH 6.0.

Enzyme assay. All enzyme assays were carried out in duplicate in 50 mM sodium citrate buffer at pH 6.0 and 40°C unless otherwise stated. β-Glucanase activity was assayed by mixing a 0.2-ml aliquot of appropriately diluted enzyme with 0.4 ml buffer containing 0.4% (wt/vol) lichenin or barley β-glucan (Sigma). The reaction was allowed to continue for 15 min and was terminated by the addition of 1.2 ml of 31 mM dinitrosalicylic acid reagent (42). The reaction tubes were then placed in boiling water for 5 min. Reducing sugars were measured by reading the absorbance at 550 nm. Glucose was used as the standard. The hydrolysis of other polysaccharides was tested by a procedure similar to that used for lichenin. One unit of enzyme activity was defined as the amount of enzyme required to release 1 μmol of glucose equivalent per min. Specific activity was expressed as units per milligram of protein. Protein concentration was determined by the method of Bradford (10) and the Coomassie protein assay reagent from Pierce Chemical Co. (Rockford, Ill.) in duplicate sets with BSA as the standard.

Enzyme purification. A culture of *E. coli* XL1-Blue harboring pLIC6 grown overnight was diluted 500-fold in LB-ampicillin medium and grown to an OD_{600} of approximately 1.5. After the addition of 1 mM IPTG, the culture was incubated for 6 h at 37°C. The clear supernatant was obtained by centrifugation (7,000 × *g*, 10 min) at 4°C. Four hundred milliliters of the supernatant was concentrated to a volume of approximately 30 ml by using an ultrafiltration cell (Amicon Co., Beverly, Mass.) equipped with a PM 10 membrane, and the buffer was changed to 20 mM potassium phosphate, pH 7.0. Ammonium sulfate was added to a concentration of 0.8 M. The solution was centrifuged (20,000 × *g*, 10 min) at 4°C to remove precipitated material and then loaded on a Phenyl Superose 10/10 (Pharmacia, Piscataway, N.J.) column equilibrated with 20 mM potassium phosphate (pH 7.0) containing 0.8 M ammonium sulfate. The β-glucanase was eluted first with a 200-ml linear gradient of ammonium sulfate from 0.8 to 0 M and then with 100 ml of distilled water. The β-glucanase solution was concentrated, and the buffer was changed to 20 mM piperazine-HCl, pH 5.5. The solution was applied to a Mono Q 5/5 (Pharmacia) column equilibrated with 20 mM piperazine-HCl buffer, pH 5.5. The β-glucanase did not bind to the column and was recovered by applying 5 column volumes of the buffer. The β-glucanase fraction was concentrated, and the buffer was changed to 20 mM sodium acetate, pH 5.0. The enzyme solution was applied to a Resource S column (Pharmacia). The enzyme did not adsorb to the column and was recovered by washing the column with 5 volumes of the buffer. Final purification was achieved by gel filtration on a Superdex 75 10/30 column (Pharmacia) equilibrated with 20 mM sodium phosphate, pH 6.0, containing 100 mM NaCl. Fractions containing β-glucanase were combined and stored at −20°C. Purification of the β-glucanase from supernatants of *Orpinomyces* sp. strain PC-2 cultures was done by the above procedure.

N-terminal amino acid sequencing of the proteins. Amino acid sequencing was done with protein samples obtained from SDS-PAGs. The proteins in the gels were transferred onto polyvinylidene difluoride membranes in a Mini Trans-Blot cell (Bio-Rad Laboratories, Richmond, Calif.). The transferred proteins were visualized by Ponceau S staining and excised with a razor blade. N-terminal amino acid sequencing was performed on an Applied Biosystems model 477A gas-phase sequencer equipped with an automatic on-line phenylthiohydantoin analyzer.

Enzyme characterization. The effects of pH on the activity and optimum pH of the β-glucanase were determined at 40°C with the following buffers: 0.1 M sodium acetate (pH 4.2 to 5.4), sodium phosphate (pH 5.8 to 7.8), and HEPES-NaOH (pH 8.2 and 8.6). Enzyme stability at different pH values was determined by measuring the residual activity after incubating the enzyme for 24 h at 4°C at pHs from 3.0 to 10.2 with the buffers above, glycine-HCl for pH 3.0 to 3.4, and piperazine-HCl for pH 9.4 to 10.2. The effect of temperature on β-glucanase was determined by assaying the enzyme at temperatures from 30 to 65°C. Thermostability was measured by incubating enzyme samples in 50 mM sodium citrate buffer, pH 6.0, for up to 24 h at temperatures from 40 to 60°C. The enzyme solution was chilled in an ice bath for 5 min and then examined by the standard assay at 40°C. In all these assays, lichenin was used as the substrate.

For determinations of K_m and V_{max} values, three different assays were performed at pH 6.0 and 40°C with lichenin and barley β-glucan as the substrates at concentrations from 0.02 to 1.0%. K_m and V_{max} values were obtained from Lineweaver-Burk plots.

For the analysis of lichenin and barley β-glucan degradation products, 5 U of purified recombinant β-glucanase was incubated with 5 mg of substrate in 1 ml of 50 mM sodium citrate buffer, pH 6.0, at 40°C. Samples were periodically withdrawn, and the reaction was stopped by placing the samples in boiling water for 5 min. A 10-μl portion of each sample was spotted onto a thin-layer chromatography (TLC) silica gel plate (Analtech, Inc., Newark, Del.) and chromatographed in a solvent system containing chloroform, glacial acetic acid, and water (6:7:1, vol/vol/vol) (33). Plates were sprayed with a reagent consisting of aniline (2 ml), diphenylamine (2 g), acetone (100 ml), and 85% H_3PO_4 (15 ml). The sugars were visualized by heating the plate for 15 min at 105°C (25). Glucose, cellobiose, cellotriose, cellotetraose, and cellopentaose were used as the standards.

Nucleotide sequence accession number. The nucleotide sequence of *licA* of *Orpinomyces* sp. strain PC-2 has been assigned accession number U63813 in the GenBank database.

RESULTS

Isolation of *licA*. A cDNA library of *Orpinomyces* sp. strain PC-2 was screened for plaques hydrolyzing lichenin. Initially 20 positive plaques were identified after screening 2×10^6 plaques. Seven of these plaques were randomly chosen for further enrichment and purification. The other 13 lichenase-positive plaques were not further investigated. Of the seven purified plaques, three showed β-glucanase activity without detectable CMCase activity. The other four exhibited both β-glucanase and CMCase activities and were confirmed to produce cellulases (35).

From this point, our work focused on the three plaques with only 1,3-1,4-β-D-glucanase. The three plasmids, pLIC2, pLIC3, and pLIC6, were 0.9, 1.0, and 1.0 kb long, respectively. Restriction mapping of the plasmids in vivo excised from the plaques revealed that they had similar restriction patterns (not shown). Sequencing of both ends of the inserts showed that they were from transcripts of the same gene (*licA*) differing in length at the 5′ end.

Nucleotide and deduced amino acid sequences of the *Orpinomyces* sp. strain PC-2 *licA*. The complete nucleotide sequence of *licA* derived from pLIC6 is shown in Fig. 1. It consisted of 971 bp, and it contained an ORF encoding a polypeptide of 245 amino acids with a molecular mass of 27,929 Da. A typical 18-mer poly(A) tail was found at its 3′ end. The putative translation start codon (ATG) for *licA* was assigned based on the facts that there were stop codons in all three frames preceding the ORF, there was no ATG codon upstream of the proposed ORF, and a typical signal peptide was located at the N terminus of the polypeptide (56). In addition, zymogram analysis and N-terminal sequencing of the recombinant β-glucanase (LicA) purified from *E. coli* supernatant and of the enzyme from *Orpinomyces* strain PC-2 discussed below confirmed this assignment. Anaerobic fungal mRNAs do not contain typical *E. coli* Shine-Dalgarno-like sequences for translation initiation. However, the sequence AGA, 10 bp upstream of the ATG start codon, may act as a ribosomal binding sequence. This sequence found in the xylanase gene (*xynA*) from *N. patriciarum* has been suggested to be a weak ribosomal binding sequence in *E. coli* (23).

The G+C contents of the entire sequence and the ORF of *licA* were 28 and 35.5%, respectively, and that of the 5′ and 3′ noncoding sequences was 4.3%, which is extremely low. The codon usage for *licA* is similar to other *Orpinomyces* strain PC-2 cellulase and xylanase genes (34, 35). Twenty-one codons were not utilized, and there was a marked preference for T in the wobble position (53% of all codons contained T at this position).

Homology of LicA with other β-glucanases. The deduced amino acid sequence of LicA was compared with other protein sequences in the SWISS PROT and GP data banks. A number of β-glucanases homologous to LicA were found in mesophilic and thermophilic bacteria including anaerobic ruminal bacteria. A high degree (>50%) of identity was found with β-glucanases from *Bacillus* species, *C. thermocellum*, and the carboxyl-terminal β-glucanase domain of xylanase/β-glucanase (XYLD) of *R. flavefaciens*. The identity with β-glucanase from *F. succinogenes* was 30.6%. In contrast, no significant sequence homology was found with barley β-glucanase (20). Comparison of sequences homologous with LicA of *Orpinomyces* (Fig. 2) revealed that the similarity between the β-glucanases is higher in the central and C-terminal regions than at the N-terminal ends.

No intron in *licA*. The ORF region of *licA* genomic DNA was amplified by PCR, and its size was compared with that amplified from the cDNA (not shown). DNA bands of equal sizes (0.75 kb) were visualized with either genomic DNA or the cDNA library as the template, demonstrating that *licA* is devoid of introns.

Expression and distribution of LicA in *E. coli* cultures. To study the expression and distribution of the β-glucanase synthesized in *E. coli* harboring the plasmid pLIC6, extracellular, periplasmic, and cellular fractions were isolated. A major portion (>95%) of the total activity was found in the extracellular supernatant fraction. The remaining activity was distributed between the periplasmic and cytoplasmic fractions (Table 1). The β-glucanase in the supernatant was visualized as a single band of about 27 kDa on SDS-PAGs by the zymogram technique (Fig. 3B). The β-glucanase band had no CMCase activity. It has been suggested that the production of β-glucanase in *E. coli* alters the permeability of the outer membrane and allows leakage of the periplasmic protein into the supernatant (12). This may be the case with the expressed *Orpinomyces* β-glucanase, as *E. coli* XL1-Blue harboring pLIC6 secreted about 7.5-fold-more protein (77.6 μg/ml) into the supernatant than did *E. coli* harboring the plasmid without *licA*.

Purification and N-terminal processing of the enzymes from *E. coli* and *Orpinomyces*. A summary of purification of the recombinant LicA from the supernatant of an *E. coli* culture is given in Table 2. The enzyme was purified about 23-fold to a specific activity of 3,650 U/mg and a yield of about 43%. Calculations based on the specific activity of the purified β-glucanase indicate that the recombinant LicA protein constituted about 4% of the secreted protein. Considering that the *E. coli* XL1-Blue is not a superexpression host strain for recombinant protein, the large amount of recombinant LicA detected in the supernatant indicated that the signal sequence of LicA may be efficiently processed by *E. coli*.

The β-glucanase purified from the supernatant of *Orpinomyces* sp. strain PC-2 culture was subjected to N-terminal sequence analysis. The sequence GTAWNGLHDVMD matched residues 30 to 41 (with one exception, i.e., L instead of S at residue 36) of the deduced amino acid sequence from the DNA. This demonstrated that a 29-amino-acid signal sequence

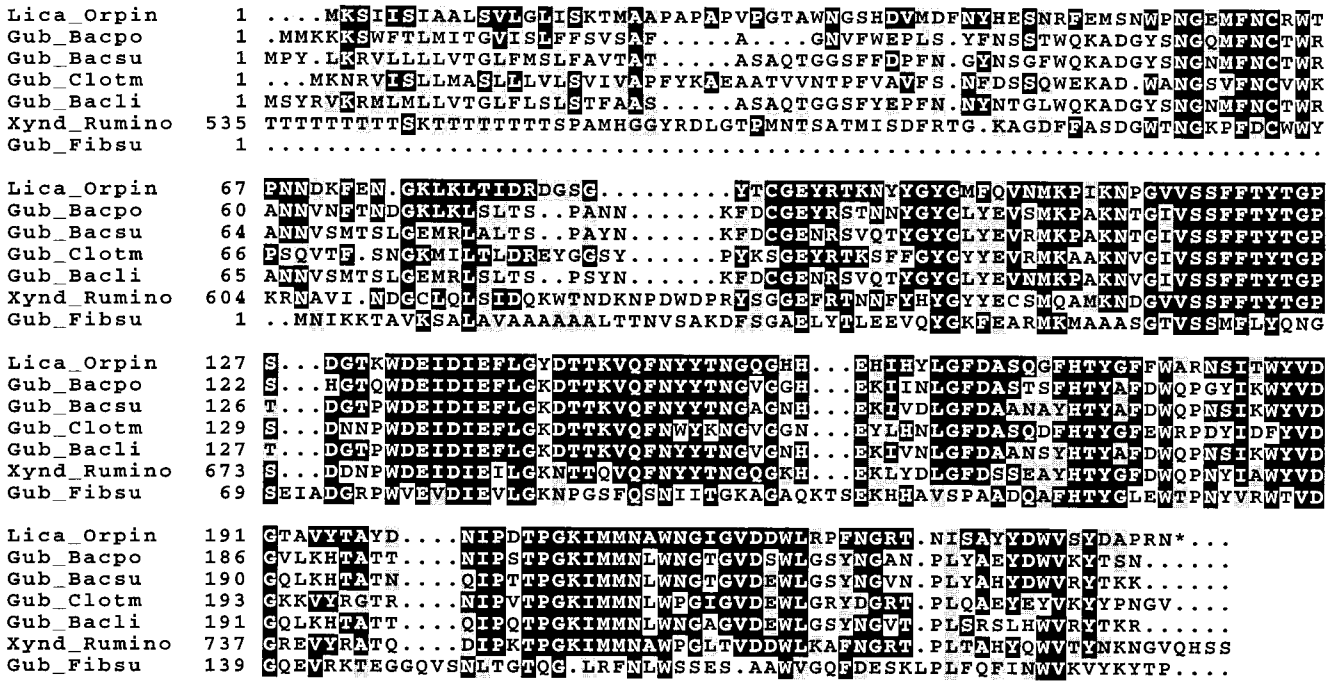

FIG. 2. Alignment of homologous β-glucanases with the LicA sequence. The sequences shown are *Orpinomyces* strain PC-2 (Lica_Orpin), *B. polymyxa* (Gub_Bacpo) (24), *B. subtilis* (Gub_Bacsu) (45), *C. thermocellum* (Gub_Clotm) (53), *B. licheniformis* (Gub_Bacli) (38), *R. flavefaciens* (Xynd_Rumino) (22), and *F. succinogenes* (Gub_Fibsu) (54).

had been removed to give the mature enzyme of 216 amino acids as produced by *Orpinomyces* (Fig. 1). The 22 N-terminal residues of the recombinant LicA isolated from the supernatants of *E. coli* were APAPAPVPGTAWNGSHDVMDFN. This sequence matched the deduced protein sequence (amino acid residues 22 to 43 [Fig. 1]) perfectly, which demonstrates that the enzyme is processed in *E. coli* with a 21-amino-acid signal sequence being removed to give a mature active enzyme of 224 amino acids.

Enzyme characterization. The purified recombinant LicA appeared as a single band on SDS-PAGs (Fig. 3). The apparent molecular mass of 27 kDa (Fig. 3) is consistent with the deduced molecular mass of the mature LicA (25.7 kDa) lacking the proposed signal peptide of 21 amino acid residues. The activity of β-glucanase was determined from pH 4.2 to 8.6 with lichenin as the substrate. A typical pH profile was obtained with an optimum between pH 5.8 and 6.2 and with approximately 80% activity at pH 5.4 and 7.0. The enzyme was stable for at least 24 h between pH 3.4 and 9.8 at 4°C. The effect of temperature on β-glucanase activity was determined in 50 mM sodium citrate at pH 6.0 from 30 to 65°C. Maximum activity was observed at 45°C, and activity decreased rapidly above 55°C. Thermal stability was investigated by incubating the enzyme at different temperatures for up to 24 h (Fig. 4). Almost no activity loss was observed at 40°C by incubation in the above buffer at pH 6.0 for 24 h, and 72 and 59% of the enzyme activity were retained after 24 h of incubation at 45 and 50°C, respectively. Inactivation occurred at 55°C with only 30% of the enzyme activity remaining after 1 h of incubation.

The recombinant LicA was specific for lichenin and barley β-glucan, which consist of mixed 1,3-1,4-β-D-linkages. No hydrolysis was observed with laminarin, pachyman, carboxymethyl cellulose (CMC), acid-swollen cellulose, Avicel, pustulan, arabinogalactan, mannan, araban, starch, xylan, pullulan, galactan, gum arabic, *p*-nitrophenyl-β-D-xyloside, PNP-β-D-glucoside, or PNP-β-D-cellobioside as a substrate. K_m and V_{max} values at 40°C and pH 6.0 obtained from Lineweaver-Burk plots for lichenin and barley β-glucan were 0.75 and 0.91 mg/ml and 3,790 and 5,320 U/mg of protein, respectively.

Hydrolysis products formed during the action of the purified recombinant LicA on lichenin and barley β-glucan are shown in Fig. 5. With lichenin, the major product was a triose, which migrated on TLC just ahead of cellotriose and was considered to be 3-*O*-β-cellobiosyl-D-glucose (18, 29). Minor products included a pentaose assumed to be 3-*O*-β-cellotetraosyl-D-glucose and a tetraose, 3-*O*-β-cellotriosyl-D-glucose, which migrated a little ahead of cellopentaose and cellotetraose. A minor component was a biose (laminobiose) migrating ahead of cellobiose. In contrast, the major products from barley β-glucan hydrolysis were triose and tetraose, reflecting the structural differences between lichenin and barley β-glucan (11).

LicA is not produced by *N. frontalis*. Zymograms were used to compare β-glucanase and cellulase activities in supernatants of *Orpinomyces* sp. strain PC-2 and *N. frontalis* cultures with lichenin as the substrate. A strong band of β-glucanase activity

TABLE 1. Distribution of recombinant LicA produced by *E. coli* pLIC6[a]

Time (h)	Distribution of activity			Total activity
	Extracellular	Periplasmic	Cytoplasmic	
2	11.7 (95.4)	0.46 (3.70)	0.11 (0.90)	12.3
4	20.0 (95.9)	0.70 (3.33)	0.16 (0.77)	20.9
7	20.7 (99.0)	0.11 (0.53)	0.10 (0.48)	20.9

[a] Activities were measured with lichenin as the substrate. The values represent units of β-glucanase activity per milliliter of *E. coli* culture. The values in parentheses are the percentages. The cultures were grown in 500 ml of LB-ampicillin medium for 10 h at 37°C before 1 mM IPTG was added. The cultures were then grown after induction for the stated time.

TABLE 2. Summary of purification of recombinant LicA from culture medium of *E. coli*

Step	Protein level (mg)	Activity[a] (U)	Sp act (U/mg)	Yield (%)
Supernatant	28	4,390	157	100
Phenol Superose	1.7	3,150	1,850	71.8
Mono Q	1.1	2,650	2,400	60.4
Resource S	0.82	2,420	2,950	55.1
Superdex 75	0.52	1,910	3,650	43.5

[a] Activities were measured with lichenin as the substrate.

appeared at approximately 27 kDa in enzyme preparations of *E. coli* containing pLIC6, purified recombinant LicA, and supernatant from *Orpinomyces* strain PC-2 culture, but the band was absent with CMC as the substrate, confirming that LicA is specific for lichenin (Fig. 6). In contrast, no equivalent β-glucanase activity band corresponding to the *Orpinomyces* strain PC-2 β-glucanase was detected in *N. frontalis* EB188 culture (Fig. 6), nor was any other band specific for β-glucanase, which indicates that this fungus does not produce a 1,3-1,4-β-D-glucanase. Multiple faint bands of high molecular masses were observed between 40 and 80 kDa in both *Orpinomyces* strain PC-2 and *N. frontalis* EB188 culture supernatants. They represent cellulases having the ability to hydrolyze lichenin.

DISCUSSION

Anaerobic fungi are now being recognized for their ability to produce hydrolytic enzymes that are effective in the degradation of plant biomass and for their role as important components of the microflora of the rumen ecosystem. Previous studies have demonstrated that 1,3-1,4-β-D-glucanase is present in bacteria and plants. This study demonstrates that it is also present in the anaerobic fungus *Orpinomyces* sp. strain PC-2. Biochemical properties of LicA from *Orpinomyces* strain PC-2 are typical of bacterial 1,3-1,4-β-D-glucanases. The activity of LicA is restricted to mixed linked β-D-glucans, such as lichenin and barley β-glucan, and no other hemicellulosic and cellulosic

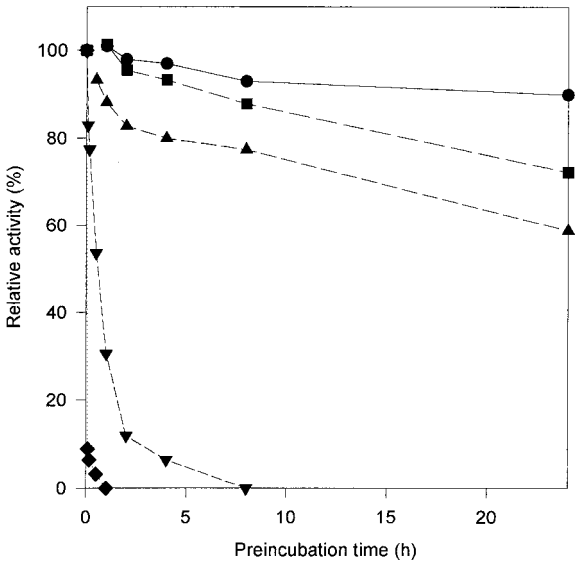

FIG. 4. Thermostability of purified LicA. Purified LicA was preincubated at 40°C (●), 45°C (■), 50°C (▲), 55°C (▼), or 60°C (◆).

substrates are hydrolyzed, which indicates that LicA is a true 1,3-1,4-β-D-glucan 4-glucanohydrolase (11). The hydrolysis products were similar to those described for β-glucanases from *B. subtilis* (29) and *R. succinogenes* (18).

The amino acid sequence of LicA is highly similar to those of bacterial β-glucanases. The motif DEIDIE (residues 133 to 138) is conserved in most bacterial β-glucanases. It is located in an active-site cleft, as suggested for β-glucanase from *B. licheniformis*, and it participates in catalysis (30). According to the classification of Henrissat and Bairoch (27), LicA should be placed in glycosyl hydrolysis family 16 which includes most bacterial β-glucanases. *Orpinomyces licA* gene is devoid of introns in its ORF region, and its flanking regions are of fungal origin (extremely AT rich). These results provide evidence for a possible common origin for *Orpinomyces* and bacterial β-glucanases. *Neocallimastix celB* gene (57) and *Orpinomyces* sp.

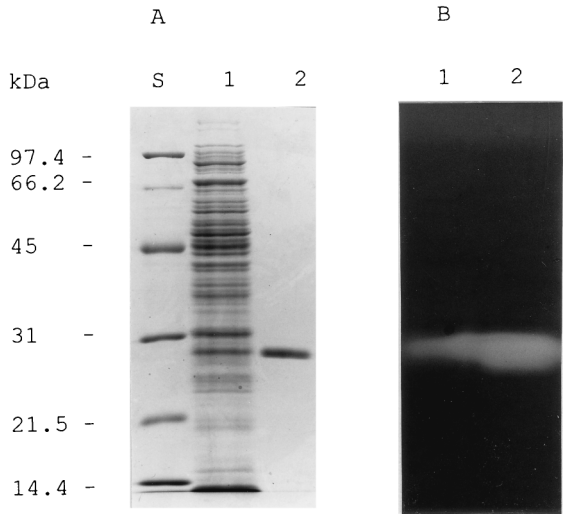

FIG. 3. SDS-PAG and zymogram of recombinant LicA. (A) Coomassie brilliant blue-stained SDS-PAG; (B) β-glucanase zymogram gel. Lane S, protein molecular mass standards; lanes 1, crude enzyme (40 μg); lanes 2, purified LicA (2 μg).

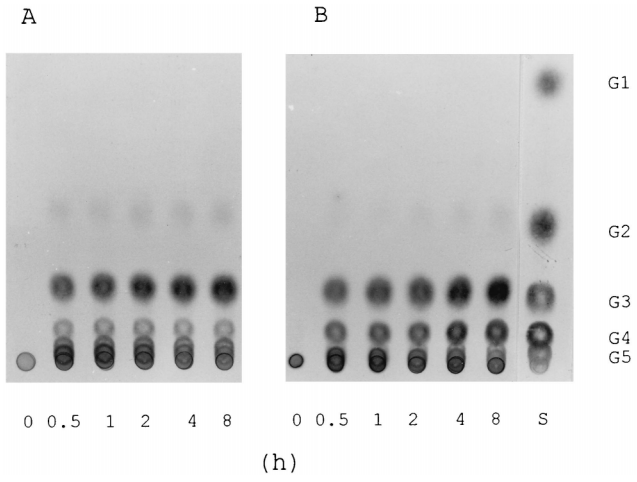

FIG. 5. TLC analysis of products of lichenin and barley β-glucan hydrolysis by purified LicA. Samples were taken at the times indicated. Lichenin (A) and barley β-glucan (B) were the substrates used. Glucose (G1), cellobiose (G2), cellotriose (G3), cellotetraose (G4), and cellopentaose (G5) were used as the standards (S).

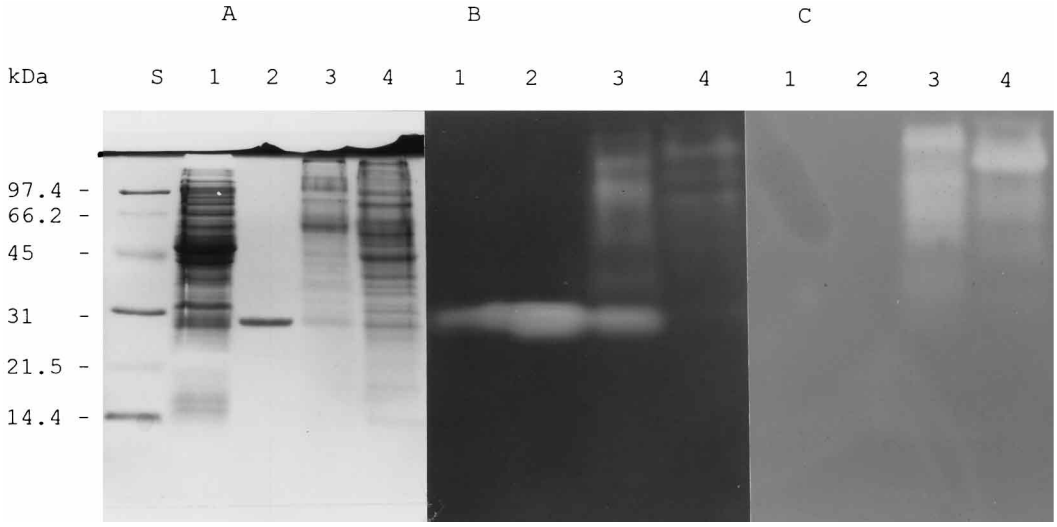

FIG. 6. Zymogram analysis of culture supernatants of *Orpinomyces* strain PC-2 and *N. frontalis* EB188 on lichenin or CMC. (A) Coomassie brilliant blue-stained gel; (B) β-glucanase zymogram gel; (C) CMCase zymogram gel. Lane S, protein molecular mass standards; lanes 1, crude enzyme sample (40 μg) of recombinant LicA; lanes 2, purified LicA (2 μg); lanes 3, culture supernatant of *Orpinomyces* strain PC-2 (25 μg); lanes 4, culture supernatant of *N. frontalis* EB188 (35 μg).

strain PC-2 *xynA* and *celB* genes (34) are also devoid of introns. The lack of introns in the hydrolase genes of anaerobic fungi is in contrast with the hydrolase genes of aerobic fungi, which usually have introns (31, 37). It has been suggested that there is gene transfer between ruminal bacteria and fungi and subsequent duplication of the genes, which encode endocellulases and xylanases (23, 34, 35). More recently, several ruminal fungal cellulases exhibited substantial homology with an aerobic fungal cellobiohydrolase (17, 35), suggesting that there may have been a common ancestral precursor of cellulolytic aerobic fungi and ruminal anaerobic fungi (17).

Most hydrolytic enzymes of anaerobic fungi reported so far have a noncatalytic repeated peptide domain (NCRPD) (19, 23, 34, 35). The NCRPDs of different enzymes may contain two or three repeats each consisting of 32 to 40 amino acid residues. There is high homology between the repeats regardless of the origin (monocentric or polycentric fungus) of the enzyme. NCRPDs are not needed for catalysis or for cellulose binding. It has been suggested that they function as docking domains in a fashion similar to that of the dockerin domains of catalytic enzymes present in the cellulosome of *C. thermocellum* (15, 19, 55). Enzymes from anaerobic fungi with the NCRPDs are now considered to be associated with large multienzyme cellulosome-like complexes. Evidence for such complexes in anaerobic fungi has been presented (19, 36). *Orpinomyces* LicA does not contain an NCRPD, which suggests that it is a free enzyme rather than a component of a multienzyme complex. The fact that it can be isolated in free form from the culture medium supports this view. Previously, a cellobiohydrolase was found in *N. patriciarum* that lacks an NCRPD but has a cellulose binding domain (17). It was assumed that this enzyme is not part of a complex. Anaerobic fungi appear to produce both a multienzyme complex and free hydrolytic enzymes for the degradation of plant tissue.

The signal sequence of 29 amino acid residues preceding the mature enzyme isolated from *Orpinomyces* and deduced from the DNA sequence contains all features associated with secretion signal sequences (47, 56). It has a basic amino acid (Lys) as the second N-terminal residue, which is followed by a 13-amino-acid stretch of which 10 are hydrophobic. The cleavage site, when the enzyme is secreted from *Orpinomyces*, is after the sequence PVP. Signal peptidases of anaerobic fungi have not been studied, and the specific sequences where they are cleaved have not been established. When the enzyme is secreted from *E. coli*, the cleavage occurs after the sequence TMA, which is an established site for a signal peptidase of *E. coli* processing an alkaline phosphatase (41). Apparently, the signal peptidases of *Orpinomyces* and *E. coli* have different specificities.

Monocentric and polycentric fungi effectively degrade plant material. They differ morphologically, and it is now apparent they differ by producing hydrolytic enzymes with different structures and activities. Several hydrolytic enzymes from the monocentric *Neocallimastix* and *Piromyces* species have more than one catalytic domain in a single protein (19, 23), a feature not found in any of the enzymes cloned and sequenced from the polycentric *Orpinomyces* (34, 35). The findings reported here of a 1,3-1,4-β-D-glucanase in *Orpinomyces* strain PC-2 and the lack of this enzyme in *N. frontalis* EB188 are additional distinctions between these fungi.

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