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The *Streptomyces reticuli* α-chitin-binding protein CHB2 and its gene

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When co-cultivated with chitin-containing fungi, *Streptomyces reticuli* secretes the chitin-binding protein CHB2. Microscopical and immunological investigations revealed that CHB2 acts like a glue to mediate the contact between the fungal and the *Streptomyces* hyphae. CHB2 was purified to homogeneity, and the sequence of its N-terminal amino acids was determined and used to deduce an oligonucleotide, which was then used to probe a subgenomic library. The *chb2* gene was cloned, sequenced and overexpressed. The deduced mature protein has a molecular mass of 18·6 kDa, and a large number of its amino acids are identical to those of CHB1 from *Streptomyces olivaceoviridis*. CHB2 effectively targets different types of α-chitin, but no other polysaccharide. The dissociation constant (K_d) for binding to purified crab shell chitin is 0·27 μM. Immunological studies suggest that homologues of CHB1 and CHB2 are secreted by streptomycetes while growing in the presence of α-chitin-containing substrates.

Keywords: *Streptomyces reticuli*, α-chitin-binding protein, CHB2

INTRODUCTION

Chitin, a polymer of *N*-acetyl-D-glucosamine, is a component of the exoskeleton of insects, mollusca, coelenterata, nematodes and protozoa, and of the cell walls of many fungi. Naturally occurring chitin varies in the length of its chains, which are arranged in an antiparallel (α) or in a parallel (β) fashion (Muzzarelli, 1977).

Streptomycetes are Gram-positive bacteria, some of which utilize chitin as their sole carbon and nitrogen source. Thus they can be easily enriched from soil on plates containing chitin as the only nutrient. The addition of chitin to soil leads to a massive increase in the number of streptomycetes. *Streptomyces* strains produce various chitinolytic enzymes, and a few of them have been isolated and purified (for a summary see Blaak *et al.*, 1993).

We discovered that *Streptomyces olivaceoviridis* secretes, in addition to chitinases, an inducible chitin-binding protein (CHB1) (Blaak & Schrempf, 1995; Blaak *et al.*, 1993; Beyer & Diekmann, 1985). This protein (18·7 kDa) lacks catalytic or antifungal activity, and it specifically targets α-chitin, but not β-chitin, chitosan or cellulose (Schnellmann *et al.*, 1994). The relative location of chitin in different biological samples can be determined by microscopy with fluorescein-labelled CHB1 or anti-CHB1 antibodies (Zeltins & Schrempf, 1995; Schnellmann *et al.*, 1994). The gene encoding *chb1* was characterized and overexpressed in *Streptomyces lividans* (Schnellmann *et al.*, 1994).

In this paper, we report that homologues of CHB1 are abundant among streptomycetes. Moreover we describe a second chitin-binding protein, CHB2, and its gene. Physiological and immunological investigations suggest that CHBs play a part in the interaction of streptomycetes and chitin-containing fungi.

The GenBank/EMBL/DDBJ accession number for the sequence reported in this paper is Y14315.

METHODS

Micro-organisms and their cultivation. The *Streptomyces* species *Streptomyces reticuli* 45 and *Streptomyces tendae* (H. Zähner, University of Tübingen), *S. olivaceoviridis* (H. Diekmann, University of Hannover) and *S. lividans* 66 (D. Hopwood, John Innes Institute, Norwich) were cultivated in minimal medium supplemented with chitin. All other *Streptomyces* strains (see Results) were obtained from the German culture collection (DSM, Braunschweig). The *S. lividans* transformant carrying the plasmid pCHB1 overproduced the

Table 1. Binding specificity of proteins

Binding of CHBs was detected by anti-CHB1 antibodies, followed by FITC secondary antibodies, as described in Methods. The amount of bound proteins was determined as outlined in Methods and in Fig. 5.

Substrate*	CHB2	CHB1
α-Chitin		
Crab shell	++	++
Hen lobster tendon	++	++
β-Chitin		
Diatom spike	−	−
Squid pen	+/−	+/−
A. nidulans (mycelia)	++	++
Chitosan	−	−
Cellulose, isolated from *Acetobacter* sp.	−	−

*Samples were obtained from the following sources: crab shell chitin, Sigma; hen lobster tendon and squid pen chitin and cellulose, H. Chanzy, Grenoble, France; diatom spike chitin, P. Kestler and G. Purschke, Osnabrück, Germany.

CHB1-binding protein and was cultivated as described previously by Schnellmann *et al.* (1994). *Neurospora crassa* and *Aspergillus proliferans* were grown in complete medium (Zeltins & Schrempf, 1995).

Chemicals and enzymes. Chemicals for SDS-PAGE were obtained from Serva. Molecular mass markers were bought from Sigma. Columns for FPLC were purchased from Pharmacia. Chitin from crab shells (practical grade; Sigma) was used after having been ground, and colloidal chitin was prepared according to Jeuniaux (1966). For kinetic studies, highly purified chitin powder from crab shells (Sigma) was utilized. Avicel, xylan and chitosan were obtained from Sigma. Other types of polysaccharides (Table 1), β-chitin, chitosan and cellulose, were kindly provided by H. Chanzy, Grenoble, France. Chemicals for DNA–DNA hybridizations were bought from Boehringer Mannheim, restriction enzymes were purchased from Gibco-BRL, Gelvatol (provided by G. Jeserich, Osnabrück) was supplied by Monsanto, and all other chemicals were obtained from Merck or Sigma. Thiostrepton was a gift from S. J. Lucania (Squibb).

Isolation and analysis of total DNA and plasmids. Chromosomal DNA of *S. reticuli* was isolated after growth in a sucrose-containing complete medium for 2 d, as described by Dittrich *et al.* (1991). The *Escherichia coli* plasmid pUC18 and the multicopy *Streptomyces–E. coli* shuttle vector pWHM3 (Vara *et al.*, 1989) were isolated according to Sambrook *et al.* (1989) and Hopwood *et al.* (1985), respectively. The DNAs were cleaved with various restriction enzymes according to the suppliers' instructions. Ligation was performed with T4 ligase (Sambrook *et al.*, 1989). Gel electrophoresis was done in 0·8–1% agarose gels using TBE buffer. Fragments were visualized under UV light after staining with ethidium bromide (Sambrook *et al.*, 1989).

Transformations. *E. coli* was transformed with plasmid DNA using electroporation (Sambrook *et al.*, 1989). *S. lividans* 66 protoplasts were transformed and regenerated as described by Hopwood *et al.* (1985). Transformants were selected using an overlay of 0·4% agarose containing 500 μg thiostrepton ml^{-1}.

DNA sequencing. For sequencing, specific DNA fragments were subcloned into pUC18/19. For the sequencing reaction, the standard primers of the *lacZ* system and synthesized oligonucleotides were utilized. Sequencing was done with an automated system (ALF express; Pharmacia). The sequence was analysed using the GENMON program (GBF) and reading frames were determined with the GCWIND program (D. Shields, Dublin). Sequence comparisons were done using the EMBL databases (Heidelberg).

Protein purification. *S. reticuli* or *S. lividans*/pCHB2 was cultivated in minimal medium supplemented with 1% ground chitin from crab shells (practical grade; Sigma) on a rotary shaker at 30 °C. After 5 d, the culture filtrate was subjected to filtration and then applied to a DEAE column (HR10/10; Pharmacia) previously equilibrated with 20 mM Tris/HCl, pH 9·0. Unbound protein concentrated by $(NH_4)_2SO_4$ precipitation (90%, w/v) was rechromatographed on a Phenyl Sepharose column, previously equilibrated with 20 mM Tris/HCl and 1·2 mM $(NH_4)_2SO_4$. Elution was performed in 20 mM Tris/HCl, pH 7·0, with a continuously decreasing concentration of $(NH_4)_2SO_4$ (from 1·2 to 0 M) at a flow rate of 1 ml min^{-1}.

SDS-PAGE, transfer of proteins and amino acid sequencing. SDS-PAGE was performed with 10% polyacrylamide gels in the presence of 0·1% SDS according to Laemmli (1970). If protein sequences were to be determined, the protein was blotted onto a PVDF membrane (Immobilon P; Millipore), as described by Schlochtermeier *et al.* (1992). The N-terminal sequences of the mature protein were determined by Edman degradation by R. Schmid, Universität Osnabrück, Germany.

Western blot analysis. Antiserum was obtained previously by immunization of a rabbit with the purified CHB1 mixed with Freund's complete and incomplete adjuvants (West *et al.*, 1990; Blaak *et al.*, 1993). Proteins were separated by electrophoresis in SDS-polyacrylamide gels (Laemmli, 1970) and transferred onto nylon filters (Fluorotrans, Pall). These were treated in blocking reagent (1% milk powder, 0·3% BSA) containing buffer (136 mM NaCl, 2·6 mM KCl, 1·4 mM K_2PO_4, 8·1 mM Na_2HPO_4, pH 7·1) for 30 min. The filters were then incubated overnight in the same buffer in which the antiserum was included (diluted 1:20000). After three washes in the buffer, the filters were treated with alkaline-phosphatase-conjugated goat anti-rabbit [IgGF(ab)$_2$] diluted 1:10000. Colour development was performed as described by West *et al.* (1990).

Analysis by immunofluorescence light microscopy. Small Petri dishes (for tissue cultures) were filled with 1·5 ml poly-D-lysine (molecular mass 50 kDa) solution [50 μg (ml H_2O)$^{-1}$] and kept open overnight. A suspension (0·1 ml) of ground chitin (1%) or fungal mycelia in buffer (136 mM NaCl, 2·6 mM KCl, 1·4 mM KH_2PO_4, 8·1 mM Na_2HPO_4, pH 7·1) was dropped in the middle of the coated Petri dish. After being dried for 3 h at room temperature, the dish was flooded three times with the buffer plus 1% BSA. The region where chitin was present was covered with 100 μl of a solution containing 0·5 μg of the 20 kDa protein and left at room temperature for 1 h. After three washes in the same buffer, a 1:100 dilution of the antiserum was added for 1 h, followed by another three washes in buffer. A fluorescein-labelled secondary antibody was added for 1 h. After three washes in buffer, the remaining layer was directly visualized using UV light, Kodak Ektachrome professional film and an Axiovert microscope.

Binding tests. Samples (40 μg) of CHB1 or CHB2 were mixed with 20 mg chitin powder derived from crab shells (Sigma; no.

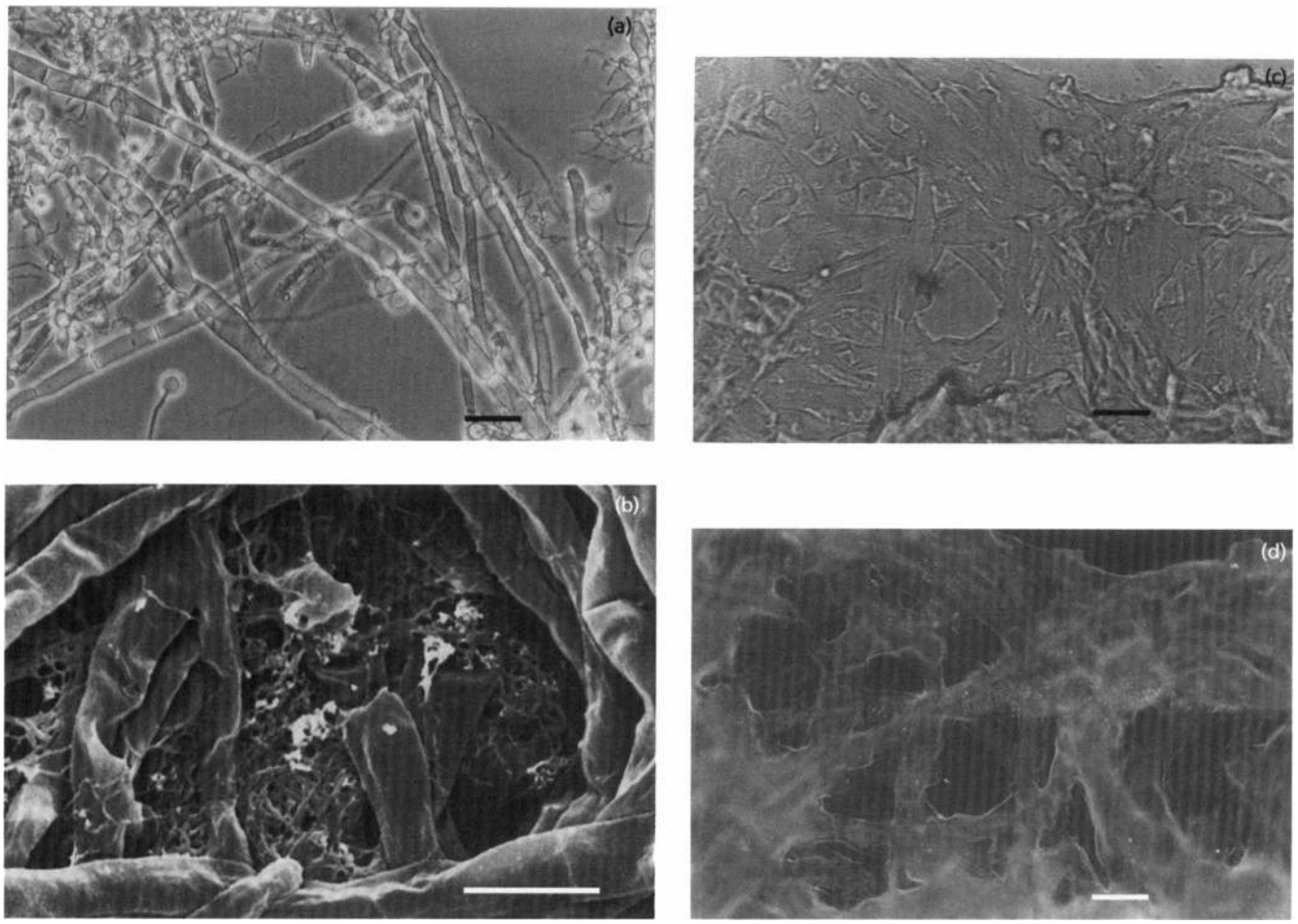

Fig. 1. Microscopical investigations. Co-cultures of *A. proliferans* and *S. reticuli* (producing CHB2) were analysed by (a, c) light and (b) electron microscopy. After treatment with anti-CHB1 antibodies, followed by secondary antibodies, the samples were inspected under UV light (d). Similar results were obtained when *S. reticuli* had been replaced by *S. olivaceoviridis* (producing CHB1), or when *A. proliferans* had been exchanged for *N. crassa*. Bars, 10 μm.

C3641) in 300 μl NaCl/P_i buffer (136 mM NaCl, 2·6 mM KCl, 1·4 mM K_2HPO_4, 8·1 mM Na_2HPO_4, pH 7·1) containing 1 M NaCl, and shaken for 16 h. After two washes with NaCl/P_i, the sample was centrifuged at 10000 *g* for 5 min. The supernatant was collected and the pellet was resuspended in NaCl/P_i buffer (Zeltins & Schrempf, 1997). Previously we had found that neither NaCl/P_i nor elevated NaCl concentrations removed CHB1 from chitin. The extensive washing procedure was applied to remove any remaining solution containing unbound protein (Zeltins & Schrempf, 1995). Identical volumes of the supernatant and the suspended pellet were mixed with loading buffer, heated for 10 min at 100 °C and loaded onto a 12% SDS-PAGE gel. Proteins were detected by staining with Coomassie blue solution (Laemmli, 1970) and, after transfer onto a nylon membrane (Fluorotrans, Pall), by antibodies raised previously against CHB1 (Schnellmann *et al.*, 1994). The corresponding cross-reacting bands were scanned (Cybertech densitometric unit) and the relative intensities were related to CHB1 (set as 100%, control).

Determination of dissociation constants. To reduce non-specific binding of proteins, highly purified chitin powder from crab shells (Sigma; no. C3641) (10 or 20 mg ml^{-1}) was washed twice with blocking buffer (see below), followed by two washes with distilled H_2O and one with 20 mM Tris/HCl, pH 7·0, 1 M NaCl. Samples of the prewashed chitin were suspended in 20 mM Tris/HCl, pH 7·0, 1 M NaCl, mixed with different quantities (0–20 μg ml^{-1}) of the chitin-binding protein and incubated on a rotary shaker (10 r.p.m.) for various time periods. Portions (20 μl) were removed and centrifuged (30 s, 14000 r.p.m.). The supernatant comprising unbound (free) protein (*F*) was diluted to less than 200 ng ml^{-1} and quantified by an ELISA test (see below). For each protein, solutions of known concentrations (0–200 ng ml^{-1}) were analysed simultaneously by ELISA to obtain a reference plot (Zeltins & Schrempf, 1997). The calculated difference between initially used total protein (*T*) and the unbound protein allows the determination of the quantity bound to chitin (*B*): $[B] = [T] - [F]$.

To obtain the dissociation constants, the data were analysed by double-reciprocal plots of $1/[B]$ versus $1/[F]$, as reported previously by Goldstein *et al.* (1993) and Gilkes *et al.* (1992).

The ELISA test was used to quantify protein. Microtitre plates were coated overnight with 50 μl of the protein to be analysed at various concentrations (0–200 ng ml^{-1}). After two washes with distilled H_2O, each well was washed with 250 μl blocking buffer (0·17 M H_3BO_4, 0·12 M NaCl, 0·05% Tween 20, 1 mM

EDTA, 0·25% BSA, 0·05% NaN_3, pH 8·5), followed by another three washes with distilled H_2O. Blocking buffer (50 µl) containing the polyclonal anti-CHB1 (1:2500 dilution) was added for 2 h. Two washes with distilled H_2O, one with blocking buffer, and again two with distilled H_2O were done. Then each well was incubated with 50 µl anti-rabbit IgG alkaline phosphatase conjugate (1:2500; Sigma) for 1 h, followed by further washes (twice with distilled H_2O, once with NaCl/P_i for 10 min, and again twice with distilled H_2O). To each well, 75 µl substrate buffer (3 mM *p*-nitrophenylphosphate, 0·05 M Na_2CO_3, 0·05 mM $MgCl_2$) was added for 20 min and the A_{405} was determined (EAR400AT, SLT-Labinstruments). All steps were performed at room temperature.

RESULTS

Occurrence of chitin-binding proteins

Several *Streptomyces* species, including strains of *Streptomyces albus*, *Streptomyces canescens*, *Streptomyces citrofluorescens*, *Streptomyces coelicolor* A3(2), *Streptomyces coelicolor* Müller, *S. lividans*, *Streptomyces parvulus*, *Streptomyces vinaceus*, *S. reticuli*, *Streptomyces rimosus* and *S. tendae*, as well as *S. olivaceoviridis* (positive control), were cultivated for 5 d in minimal medium supplemented with 1% ground prewashed (1 M HCl) crab shells, or colloidal chitin. The proteins from concentrated supernatants and the pellets (consisting of the insoluble substrate and the mycelia) were separated by SDS-PAGE and analysed for cross-reaction with antibodies raised previously against the purified chitin-binding protein (CHB1) from *S. olivaceoviridis* (Schnellmann *et al.*, 1994). Each of the tested strains produced an extracellular protein of about 18–19 kDa that cross-reacted with anti-CHB1 antibodies. A large portion was linked to the pellet of the cultures (data not shown). The quantities of the cross-reacting protein varied, depending on the strain. *S. reticuli* produced the highest amount (3 µg per 10 ml) of a cross-reacting protein, whereas *S. lividans* secreted about 15-fold less, a low value in comparison with other strains as well.

Microscopical and physiological studies

Additional investigations revealed that the *Streptomyces* hyphae were adhering to the insoluble substrate. As visualized by immunological studies, CHB1 or CHB2 formed a glue-like substance around the fungal hyphae (Fig. 1). The *Streptomyces* strains secreted the CHBs only when grown with ground crab shells, or with living or autoclaved mycelia from chitin-containing fungi (i.e. *A. proliferans*, *N. crassa*), but not in the presence of chitobiose or glucose.

Purification of the *S. reticuli* chitin-binding protein (CHB2)

S. reticuli produced relatively high amounts of a protein cross-reacting with anti-CHB1 when grown in the presence of ground crab chitin as the carbon and nitrogen source, but not with glucose (Fig. 2). The protein was isolated as outlined in Methods; from 900 mg present in the supernatant of a 3 l culture, 300 mg was recovered after final purification. The enriched mature protein, which cross-reacted with anti-CHB1 antibodies, was subjected to Edman degradation. Thirteen N-terminal amino acids could be determined (see below).

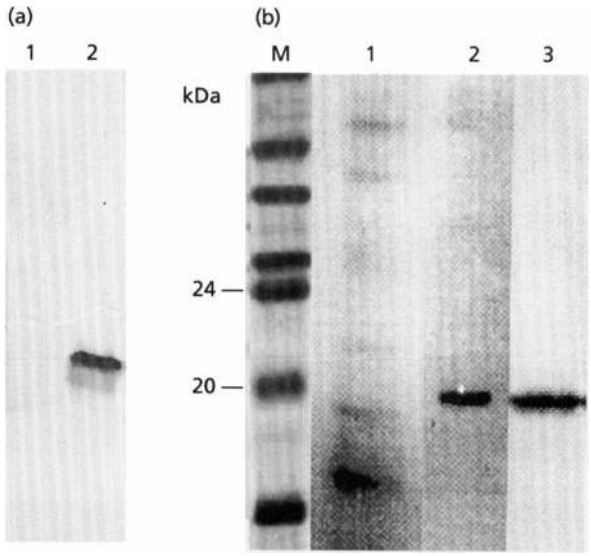

Fig. 2. (a) *S. reticuli* was grown in the presence of glucose (lane 1) or chitin (lane 2) as the sole carbon source. After concentration by $(NH_4)_2SO_4$ precipitation, extracellular proteins were separated by SDS-PAGE, transferred to a membrane and treated with anti-CHB1 antibodies. (b) Enrichment of CHB2. Proteins from the culture filtrate of *S. reticuli* (grown with ground crab chitin) were concentrated (lane 1) with $(NH_4)_2SO_4$ (90%, w/v) and, after filtration, applied to a DEAE column. Unbound proteins were chromatographed on a Phenyl Sepharose column. A sample of the peak fraction was analysed on SDS-PAGE gels, followed by staining with Coomassie blue (lane 2), and was tested for cross-reaction with anti-CHB1 antibodies (lane 3). The size markers are in lane M.

Identification of the *chb2* gene and its sequence

A specific oligonucleotide (5′-CACGGCATCACCGACCTCCCCGTCAGCCGGCAGAAG-3′) was deduced from the 13 aa, HGYTDLPISRQKM, determined at the N-terminus of the secreted mature CHB2 protein.

One *Sma*I (1·65 kb) and one *Bam*HI (2·3 kb) fragment from the *S. reticuli* total DNA hybridized with the oligonucleotide (data not shown). A mini-library of *Bam*HI fragments (2–3 kb) was constructed in pUC18 and screened for the desired *E. coli* transformants by colony hybridization. Several clones contained the identically sized *Bam*HI fragment of 2·3 kb, which hybridized with the oligonucleotide. The inserts of several plasmids were analysed by restriction enzymes and they proved to be identical. The insert of the plasmid pUCH20 (Fig. 3a) was subcloned and se-

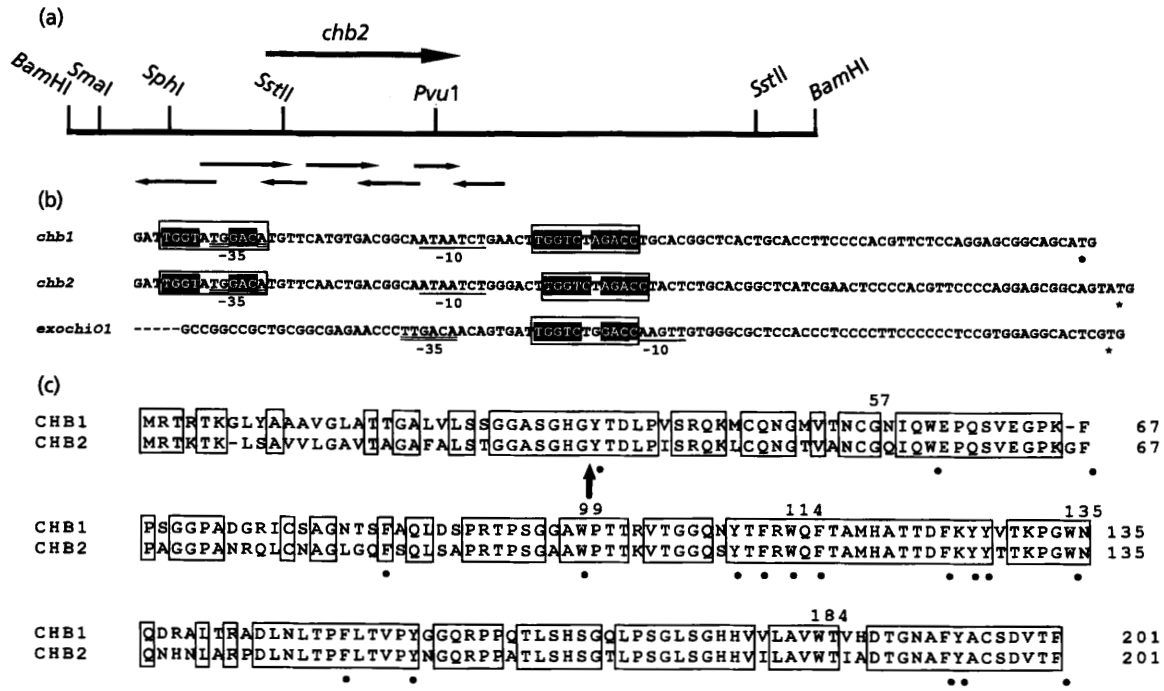

Fig. 3. (a) Characterization of the *chb2*-carrying DNA fragment. The 2·3 kb *Bam*HI fragment from the genomic *S. reticuli* DNA was cloned into pUC18, resulting in the formation of the plasmid pUCH20. Recloning in the bifunctional *Streptomyces–E. coli* vector pWHM3 (Vara *et al.*, 1989) entailed the formation of pCHB20. The arrangement of sequenced DNA stretches is indicated by arrows. (b) Comparative studies. The upstream regions of the *chb2*, *chb1* (Schnellmann *et al.*, 1994) and *exochiO1* (Blaak & Schrempf, 1995; Blaak *et al.*, 1993) genes were compared. The start codon of each gene is marked by an asterisk. The accession number in the EMBL database is Y14315. (c) The deduced CHB2 protein was aligned with the deduced CHB1 protein (Schnellmann *et al.*, 1994). Aromatic amino acid residues are marked by a dot. The end of the signal peptide is indicated by an arrow.

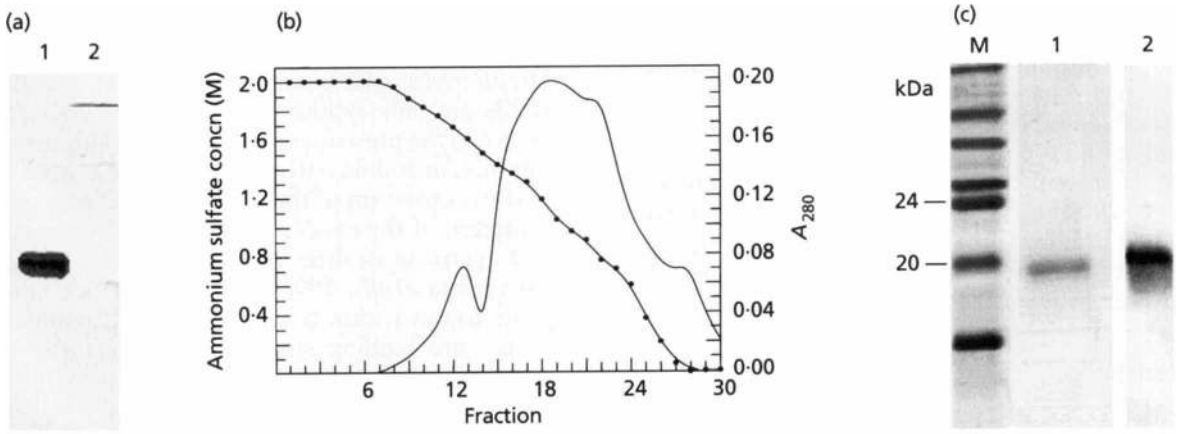

Fig. 4. Induction and overexpression of CHB2 and its purification. (a) *S. lividans*/pCHB20 (lane 1) and the control strain *S. lividans*/pWHM3 (vector) (lane 2) were grown in the presence of ground crab chitin, and extracellular proteins were tested for cross-reaction with anti-CHB1 antibodies, as described in Fig. 2. (b) Purification of CHB2. *S. lividans*/pCHB20 was cultivated in crab-chitin-containing medium. Proteins were concentrated as described in Methods. Protein fractions obtained after chromatography with a Phenyl Sepharose column were separated by SDS-PAGE. After transfer, cross-reactions with anti-CHB1 antibodies were tested for each fraction. ●, Ammonium sulfate concentration; —, absorbance. (c) The peak fractions were pooled and analysed on an SDS-PAGE gel, stained with Coomassie blue (lane 1), and analysed for cross-reaction with antibodies (lane 2). The size markers are in lane M.

quenced. The sequences of both strands of the overlapping fragments were established with universal primers and additional oligonucleotides.

The sequence of the complete reading frame (*chb2*) has a G+C content of 69·5 mol%. A start codon (ATG), a putative ribosome-binding site, the −10 and −35

regions of a possible promoter and a stop codon (TGA) could be identified.

Analysis of the deduced protein

The deduced protein (Fig. 3c) consists of 201 aa. The amino acids in positions 30–42 correspond to those determined for the secreted CHB2 protein. These data suggested that the first 29 aa comply with a signal peptide. An inspection of the hydrophobicity profile (not shown) revealed that a hydrophobic region (20 aa) follows the first 6 aa, three of which are positively charged. In addition, a cleavage site (ASG) typical of a signal peptide is present. The molecular mass of the deduced mature protein amounts to 18·6 kDa, which is in good agreement with the apparent molecular mass determined by SDS-PAGE. The deduced isoelectric point is 9·01.

Putative regulatory *cis* elements

The 11 bp motifs A and B are located a few base pairs downstream of the putative −10 region of the promoter and overlapping the −35 region. Motif A shares seven identical base pairs with motif B (similar motifs are situated upstream of the *chb1* gene). A and B were separated by 23 bp, 22 of which were identical with those of the corresponding region upstream of *chb1* (Fig. 3b).

Overexpression of the *chb2* gene

The 2·3 kb *Bam*HI fragment was ligated into the *Bam*HI-cleaved *Streptomyces–E. coli* multicopy vector pWHM3 (Vara *et al.*, 1989) and transformed into *S. lividans*. Several transformants carrying the correct construct were obtained. In contrast to the control strain *S. lividans* containing the vector pWHM3, they produced high levels of CHB2 when the strain was grown in the presence of ground crab chitin (Fig. 4a), but not with glucose. *S. lividans*/pCHB20 was chosen for further studies. The strain was shown to produce about 15 times more CHB2 than the progenitor *S. reticuli* strain. The CHB2 protein was purified to homogeneity (Fig. 4).

Characteristics of CHB2

CHB2 was purified as described in Methods. The homogeneous purified protein (Fig. 4) targets purified types of α-chitin, but not β-chitin, chitosan or cellulose (Avicel) (Table 1). It also had a high affinity for mycelia or conidia from chitin-containing fungi (i.e. *A. proliferans*, *N. crassa*).

Relative affinity of antibodies and binding test

As several amino acids within CHB2 differ (Fig. 3c) from those in CHB1, the relative affinities of antibodies were determined. Compared to CHB1 (100%), polyclonal anti-CHB1 antibodies (Schnellmann *et al.*, 1994) had a reduced affinity (~ 52%) for CHB2.

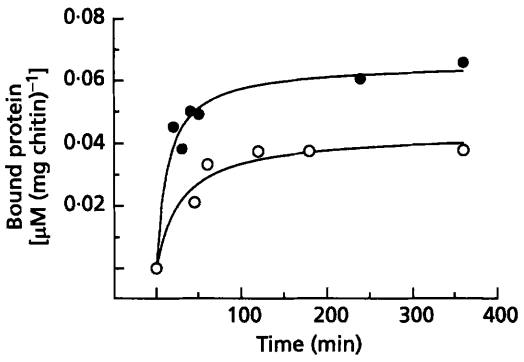

Fig. 5. Temporal course of protein binding. Ground crab shell chitin (20 mg) and 40 μg protein CHB2 (○) and protein CHB1 (●) were mixed in buffer, as outlined in Methods, and incubated at 4 °C for various time periods. Samples of 20 μl were removed and the unbound protein was determined in the supernatant by an ELISA test. The quantity of each of the proteins was calculated by their affinity to anti-CHB1.

Samples of CHB2 and CHB1 (control) were mixed with highly purified chitin powder from crab shells and the dependence of binding on the incubation time was determined. After 360 min, the capacity of CHB2 was 0·038 μM and that of CHB1 was 0·063 μM (Fig. 5). The dissociation constant of CHB2 was determined (see Methods) to be 0·2 μM.

DISCUSSION

The studies revealed that many streptomycetes secrete an α-chitin-binding protein. Like the *S. olivaceoviridis* exochitinase (exoChiO1) (Blaak *et al.*, 1993) and other *Streptomyces* chitinases (Miyashita *et al.*, 1997), the CHBs are only synthesized if *Streptomyces* strains are grown in the presence of pure chitin or chitin-containing samples, including native fungal mycelia. Motifs similar to those upstream of the *chb1* and *chb2* genes are found upstream of the *exochiO1* gene from *S. olivaceoviridis* and upstream of three chitinase genes from *S. lividans* (Miyashita *et al.*, 1997), but not in front of chitinase genes found within bacteria. Thus we assume that the motifs are binding sites for one or more regulatory protein.

The deduced protein CHB2 from *S. reticuli* consists of 201 aa, 77·7% of which are identical (Fig. 3c) to those of CHB1 (201 aa) from *S. olivaceoviridis* (Schnellmann *et al.*, 1994). It is interesting that the relative positions of all aromatic amino acid residues, including five tryptophan (W) residues, correspond in both proteins. The K_d value of CHB2 is 0·27 μM and it thus binds about 2·5 times less effectively than CHB1 (0·11 μM) (Zeltins & Schrempf, 1997) when purified insoluble crab shell chitin is used as substrate. Our spectroscopical investigations of CHB1 and its mutated derivatives have indicated that CHB1 is a highly flexible protein, lacking α-helices. Residues W134 and W184 are located in buried regions; W57, in contrast, lies in an exposed area. A replacement of W57

by leucine or tyrosine leads to minor topological changes, but entails a loss of binding capacity of about 90% and an increase in the K_d to 2·17. An exchange of the buried residues W134 and W184 induces conformational alterations and, in due course, a reduction of their binding affinities. Their K_d values amount to 0·91 and 0·26, respectively (Zeltins & Schrempf, 1997).

In their natural habitat, streptomycetes encounter different α-chitin types, which vary in the length of their *N*-acetylglucosamine chains, the degree of crystallinity and in their dependence on the presence of accessory inorganic compounds or proteins. As a consequence, the consistency of the chitinous layers in organisms such as arthropods (comprising crustaceans and insects), molluscs, nematodes, worms and fungi differs considerably (Muzzarelli, 1977). Within fungal cell walls, chitin is embedded in several types of other polysaccharides, including glucans. Thus it will be worthwhile analysing the relative affinities of CHB2 and CHB1, as well as of mutated proteins, to various types of chitin. It is expected that they may vary significantly. Consequently we speculate that the secreted CHBs from individual *Streptomyces* strains differ in their relative dissociation constants to various types of chitin. It will be interesting to study the competition for various types of chitin among streptomycetes secreting different CHBs in their natural habitat. In addition to mediating close contact to the chitin-containing organisms, CHB1 invades deeper layers of chitin (Zeltins & Schrempf, 1995). Therefore we plan to investigate whether CHBs also induce a loosening of chitinous layers. The degradation by the *Streptomyces* chitinases could thus be facilitated.

The identified *Streptomyces* CHBs are the first of their kind. They do not share any relevant amino acid identities with the chitin-binding domain of the chitinase A1 (ChiA1) from *Bacillus circulans* (Watanabe *et al.*, 1993), which interacts with colloidal chitin, or with the chitin-binding region of the *S. olivaceoviridis* exochitinase (exoChiO1). The latter binds equally well to crystalline α- and β-chitin, but not to colloidal chitin or another polysaccharide (Blaak & Schrempf, 1995). It will be of interest to elucidate the three-dimensional structure of the CHBs and to define the parameters that determine their highly specific recognition of α- but not β-types of chitin.

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