A Bacillus amyloliquefaciens ChbB protein binds β- and α-chitin and has homologues in related strains

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A small (19.8 kDa) protein was identified in Bacillus amyloliquefaciens ALKO 2718 cultures during growth in the presence of yeast extract and chitin, but not with glucose. The protein targets β-chitin best, then α-chitin, but barely any other polysaccharide. This described chitin-binding protein (ChbB) is the first of its type from a Bacillus strain and cross-reacts with antibodies raised against the Streptomyces α-chitin-binding protein CHB1. Using reverse genetics, the chromosomal chbB gene of strain ALKO 2718 was identified, cloned and sequenced. ChbB shares several motifs with the α-chitin-binding proteins CHB1 and CHB2 of Streptomyces and CBP21 of Serratia marcescens predominantly targeting β-chitin. Synthesis was repressed by glucose and the presence of cre boxes suggests catabolite control. Using PCR, Southern hybridization and anti-ChbB antibodies, the presence of a chbB gene, as well as of a ChbB protein homologue, was ascertained in several tested B. amyloliquefaciens strains, but not in Bacillus subtilis 168. Contrary to B. subtilis 168, all B. amyloliquefaciens strains secreted varying amounts of enzymic activity, degrading carboxymethyl chitin coupled with Remazol brilliant violet.

Keywords: chitin-binding protein, chbB gene

INTRODUCTION

Many members of the Bacillaceae secrete enzymes that allow them to degrade extracellular macromolecules. In addition to secretion of proteases, the ability to degrade polysaccharides is a common property. Various types of secreted amylase have been characterized from mesophilic (e.g. Bacillus amyloliquefaciens, Bacillus licheniformis) and alkaliphilic species (Vihinen & Mäntsalä, 1989; Priest, 1985). Other polysaccharide-degrading enzymes secreted by Bacillus species include different types of cellulases (Ito, 1997; Birsan et al., 1998; Sanchez-Torres et al., 1996; Blanco et al., 1998), xylanases (Blanco et al., 1999; Sabini et al., 1999) and lichenases (1,3-1,4-β-glucanases) (Borriss et al., 1990; Hoj et al., 1992; Schimming et al., 1992; Tabenero et al., 1994; Sanchez-Torres et al., 1996).

In contrast to the above polysaccharides, only a few Bacillus species are known to hydrolyse the second most abundant polysaccharide in nature, chitin, and its deacetylated derivative chitosan. B. amyloliquefaciens, Bacillus megaterium and Bacillus subtilis are counted among the degraders of shrimp shell waste (Sabry, 1992), although their detailed degradation properties have been rarely investigated (Frädberg & Schnürer, 1998). Chitinases have been identified within Bacillus cereus (Pleban et al., 1997; Trachuk et al., 1996), Bacillus circulans (Watanabe et al., 1992) and Bacillus thuringiensis (Sampson & Gooday, 1998). Much is known about B. circulans chitinases and their corresponding genes (Watanabe et al., 1992; Wiwat et al., 1999; Zeng et al., 1998). Chitosanases have been characterized from B. circulans (Mitsutomi et al., 1998; J. Saito et al., 1999), B. cereus (Kurakake et al., 2000) and some unidentified Bacillus species (Izume et al., 1992).

In contrast to members of the Bacillaceae, most Strepto-
myces species produce a multitude of chitinases (Robbins et al., 1988; Blaak et al., 1993; Blaak & Schrempf,
Cultures of respectively. The plasmids used were pGEM-T from Promega, M15(pREP4) were purchased from Stratagene and Qiagen, the latter was supplemented with either yeast extract (0 °C), 5% glucose, 2% peptone, 0.5% yeast extract (0 °C, 37 °C) ATCC 23842, IAM 1523, IFO 3034, IFO 3037, KA 63, N, P, SB OUT 8426 were purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany). The strains used, i.e. ATCC 15841, ATCC 23350, ATCC 23842, IAM 1523, IFO 3034, IFO 3037, KA 63, N, P, SB I, T, ZFL 14/4, ZF 178 and B. licheniformis 414p were from the IPK collection, and strains OUT 8419, OUT 8420, OUT 8421, OUT 8426 were purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany). The Escherichia coli strains XL-1 Blue and MI5(pREP4) were purchased from Stratagene and Qiagen, respectively. The plasmids used were pGEM-T from Promega, pQE16 from Qiagen and pUC18 from New England Biolabs. Cultures of E. coli or B. amyloliquefaciens were grown at 37 °C in TBY (Breves et al., 1997), or in Spizizen’s minimal medium (Anagnostopoulos & Spizizen, 1961), respectively. The latter was supplemented with either yeast extract (0.5%), ground crab shell chitin (1%), Avicel (1%), β-glucan (0.5%) or starch (1%). Transformants of E. coli XL-1, E. coli XL-1 Blue or MI5(pREP4) containing pQE16-based constructs were grown in the presence of ampicillin (100 μg/ml). To keep selection for the plasmid pREP4, kanamycin (20 μg/ml) was added during cultivation of E. coli MI5 containing this plasmid.

Isolation and analyses of DNA. Chromosomal DNA from B. amyloliquefaciens ALKO 2718 was isolated with pUC18 digested with EcoRI. The initial PCR amplification was performed with primers A and B designed according to the N-terminal amino acids from the mature 20 kDa protein after Edman degradation (see Results). Subsequent steps of RAGE were done using the primer pairs EF or GH and CD or IK, respectively (Fig. 2b). PCR was carried out with KlenTa Polymerase (Sigma) or the Expand Long Template PCR System (Boehringer Mannheim, now Roche). The PCR fragments were purified with a QIAEX gel elution kit (Qiagen) and cloned by using the pGEM-T vector kit (Promega). E. coli was transformed with plasmid DNA using CaCl₂ or electroporation (Sambrook et al., 1989). Using chromosomal DNA of B. amyloliquefaciens as well as primer L (corresponding to the Shine-Dalgarno sequence AAAG-AAGGGAG) and primer M (which replaces the stop codon of orf3 by a Bgl II site), the complete orf3 (about 650 bp) was amplified and the DNA fragment was cloned into the pQE16 vector, resulting in the plasmid pQEC1. Primers used are listed in Table 1. Plasmid pHBC1 was constructed using pHB201 (Bacillus Genetic Stock Center, Ohio State University, Columbus, OH, USA) after EcoRI and EcoRV digestion. The cbbB DNA fragment was isolated from pQEC1 after HindIII digestion and subsequently, after blunting and MinI digestion, ligated into the prepared vector DNA. Plasmid pHBC1 was transformed into naturally competent cells of B. subtilis 168 using standard conditions (Dubnau & Davidoff-Abelson, 1971).

DNA sequencing and analysis. DNA sequencing was performed by an automated system (ALF express; Pharmacia), using the recommended primers (T; promoter region and reverse) for the pGEM-T vector with the AutoRead sequencing kit (Pharmacia). Sequencing of pQE16 constructs was done using the primers recommended by Qiagen. Sequence analysis was performed with the computer software package of IntelliGenetics (Mountain View, CA, USA) and Lasergene software of DNASTAR (Madison, WI, USA). BLAST software (National Center for Biotechnology Information, Bethesda, MD, USA) was used for on-line database scanning.

Purification of the His-tag protein. Plasmid pQEC1 was transformed into E. coli MI5(pREP4) and overexpression was achieved following the procedure outlined in the QiAexpressionist handbook (Qiagen). The His-tag fusion protein was accumulated in the periplasm and was thus available by osmotic shock treatment. The washed cells were suspended in 30 mM Tris/HCl, pH 8.0, 20% sucrose, incubated on ice for 10 min, sedimented (8000 g, 15 min, 4 °C), resuspended in 5 mM ice-cold MgSO₄, pH 8.0, 10 min and centrifuged for 15 min at 4 °C. The supernatant was collected, equilibrated to 50 mM Na₂HPO₄, pH 8.0, 300 mM NaCl, 10 mM imidazole, and applied to an Ni-NTA column (Qiagen). After five washes, corresponding to the volume of the column, in the same buffer supplemented with 40 mM imidazole, proteins were released by increasing the imidazole concentration. The fusion protein was found to be released in the presence of 200 mM imidazole. The purity of the protein was analysed by SDS-PAGE and by immunodetection using anti-ChbB antibodies (see Fig. 4a).

Isolation of ChbB from B. amyloliquefaciens. The strain was streaked on LB agar medium and incubated at 37 °C overnight. Cells were collected from 10 plates and transferred to 200 ml Spizizen’s minimal medium (Anagnostopoulos & Spizizen, 1961) containing 1% ground crab shell chitin on a rotary shaker (200 r.p.m. for 4 h at 37 °C). After 30-fold dilution (final volume 6 l), cultivation was continued for 36 h. The growth cultures were inspected under UV light.

Cloning and transformation. Cloning of the orf3 DNA fragment (see Fig. 2) was done using the RAGE (rapid amplification of genomic ends) protocol for PCR cloning, as described previously (Hoang & Hofemeister, 1995). EcoRI-cleaved chromosomal DNA of B. amyloliquefaciens ALKO
was cooled to 4 °C and (NH₄)₂SO₄ (70% saturation) was added. After stirring for 2 h, the precipitate obtained was applied to a DEAE column (HR10/10, equilibrated with 20 mM Tris/HCl, pH 9–10) and eluted in the same buffer with continuously increasing concentrations of NaCl (from 0 to 0.5 M) for 1 h and incubated in the same buffer in the presence of antiserum (dilution 1:500) for 2 h. After two washes with TBS (20 mM Tris/HCl, pH 7.5, 500 mM NaCl containing 0.05%, v/v, Tween 20 and 0.2%, v/v, Triton X-100) and one wash with TBS only, the precipitate was obtained by centrifugation (18000 g, 30 min, 4 °C), suspended in 50 ml 20 mM Tris/HCl buffer, pH 9–10, and applied to a MonoS column equilibrated to pH 5–2. Proteins were eluted in the same buffer with continuously increasing concentrations of NaCl (from 0 to 0.5 M) at a flow rate of 0.6 ml min⁻¹. The fractions containing ChbB protein were collected and equilibrated to pH 8–0, 1 M (NH₄)₂SO₄, then applied to phenyl Superose (HR 5/5) at a flow rate of 0.6 ml min⁻¹ (see Fig. 4b).

**SDS-PAGE and amino acid sequencing.** SDS-PAGE was performed with 12.5% polyacrylamide gels and 0.1% SDS (Laemmli, 1970). For N-terminal amino acid sequencing proteins were transferred to a PVDF membrane (Immobilon P; Millipore) and subjected to Edman degradation using a model LF 3400 gas-phase sequencer (Beckman), followed by HPLC of the phenylthiohydantoin amino acids.

**Immunological studies.** Antiserum was obtained by immunization of a rabbit with the purified His-tagged ChbB protein isolated from an *E. coli* M15(pREP4) transformant containing the construct pQEC1. Proteins were separated by SDS-PAGE (Laemmli, 1970) and blotted onto a nitrocellulose membrane (Sartorius) or a nylon membrane (Fluorotrans; Pall). The membrane was treated with blocking buffer (1% BSA, 150 mM NaCl, 10 mM Tris/HCl, pH 7–5) for 1 h and incubated in the same buffer in the presence of antiserum (dilution 1:500) for 2 h. After two washes with TBS (20 mM Tris/HCl, pH 7.5, 500 mM NaCl containing 0.05%, v/v, Tween 20 and 0.2%, v/v, Triton X-100) and one wash with TBS only, the membrane was treated with alkaline-phosphatase-conjugated goat anti-rabbit secondary antibodies (diluted 1:10000) for 2 h. Colour development was performed in TBS buffer containing 0.33 mg Nitro blue tetrazolium chloride ml⁻¹ and 0.165 mg 5-bromo-4-chloro-3-indolyl phosphate (BCIP) ml⁻¹ (Sigma).

**Binding tests.**Five micrograms of the His-tag ChbB protein purified from *E. coli* or the purified ChbB protein secreted by *B. amyloliquefaciens* was mixed with 2 mg substrate in 50 μl of the indicated buffer (see below), containing 1% BSA, and shaken for 16 h at 4 °C or for 3 h at room temperature. The samples were centrifuged at 10000 g for 5 min, then each supernatant was collected. The pellet was washed twice in the same buffer and resuspended with 50 μl buffer. Each sample (supernatant or resuspended pellet) was mixed with loading buffer, heated for 10 min at 100 °C and analysed using 12.5% SDS-PAGE. The relative quantities of the protein were estimated after Coomassie staining or immunodetection (Zeltins & Schrempf, 1995) by scanning of corresponding bands and subsequent analysis using Scion Image software (Scion, MD, USA). To study the effect of pH, crab shell powder was mixed with ChbB protein in aliquots of various buffers adjusted to different pH values. Citrate/phosphate buffer (10 mM) was used for tests at pH 3–7, Tris/HCl buffer (10 mM) for pH 7–10. The effect of salt was tested after the addition of NaCl (0.5 or 1 M) to citrate/phosphate buffer (pH 7, 10 mM, 1% BSA) containing the chitosin sample. Various substrates, i.e. α-chitin (crab shells), β-chitin from Sepia and from *Siboglinum fjordicum*, chitosan, β-glucan (yeast), β-glucan (barley), cellulose from cotton linters and xylan (oat spells), were used to study the binding specificity of the ChbB protein in citrate/phosphate buffer (pH 7, 10 mM, 1% BSA).

**Analysis by immunofluorescence light microscopy.** A suspension (0.1 ml) of chitin (1%) (10 mM citrate/phosphate buffer and 1% BSA, pH 7.0) was centrifuged and washed three times with the buffer plus 1% BSA. The chitosin sample was then covered with 100 μl of a solution containing 0.5 μg ChbB

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**Table 1. Oligonucleotide primers used for PCR**

<table>
<thead>
<tr>
<th>Name</th>
<th>5′ → 3′ sequence*</th>
<th>Description</th>
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<tbody>
<tr>
<td>A</td>
<td>CAYGGNTAYATAHTTKGARCCNGT</td>
<td>Degenerate primer†</td>
</tr>
<tr>
<td>B</td>
<td>GTNWNSMGNCNTAYAGGGNGNC</td>
<td>Degenerate primer†</td>
</tr>
<tr>
<td>C</td>
<td>CTACCATCCGACGGGACCTGCAACGGG</td>
<td>Internal sequence nucleotides‡</td>
</tr>
<tr>
<td>D</td>
<td>TTGTCCAGATCTCCTCGTTGCAAGGC</td>
<td>Internal sequence nucleotides‡</td>
</tr>
<tr>
<td>E</td>
<td>ACTGGCAGCTACACCGGACCTAGGC</td>
<td>Internal sequence nucleotides</td>
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<td>F</td>
<td>ACTGGCAGCTACACCGGACCTAGGC</td>
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<td>H</td>
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<tr>
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<tr>
<td>Uni2</td>
<td>GTAACAGCCCGCAGT</td>
<td>Universal sequencing primer for pUC18</td>
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*The IUPAC-code was used to show alternative nucleotides; N denotes an inosine residue.
†Degenerate primers corresponded to the first 15 aa of the mature ChbB which were obtained after Edman degradation.
‡Primers designed for genomic walking. The relative positions of the primers within the cloned region are indicated in Fig. 2.
§Primers designed to subclone the *chbB* gene *(orf3; Fig. 2)* into the pQE16 vector, where primer M induced an extra BglII restriction site.
protein and left at room temperature for 1 h. After three washes in the same buffer, a 1:100 dilution of the antiserum [anti-His-tag ChbB antibodies (rabbit)] was added for 1 h, followed by another three washes in buffer. A fluorescein-labelled secondary antibody (rabbit) 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 Axiowert microscope.

**Chemicals and materials.** Chitin from crab shells (practical grade; Sigma) was used after grinding and colloidal chitin was purchased from Fluka. The chitinase was from Sigma. All other chemicals were obtained from Merck, Sigma or Serva. KlenTaq polymerase was from Sigma. Amersham. T4 DNA ligase, T4 DNA polynucleotide kinase, Klenow enzyme and the Expand Long Template PCR system were purchased from Boehringer Mannheim (now Roche). Qiagen. DEAE, MonoS and phenyl Superose (HR5/5) columns were from Pharmacia. Restriction enzymes were obtained from New England BioLabs (Beverly, MA). Tryptone and yeast extract were obtained from Difco Laboratories (Detroit, MI). Ni-NTA agarose was supplied by Qiagen. DEAE, MonoS and phenyl Superose (HR5/5) columns were from Pharmacia. Restriction enzymes were purchased from Boehringer Mannheim (now Roche). KlenTaq polymerase was from Sigma. Serratia marcescens chitinase was from Sigma. All other chemicals were obtained from Merck, Sigma or Serva.

**Test for chitinolytic activity.** Cultures were grown in Spizizen minimal medium with 0.5% colloidal chitin and 0.2% yeast extract at 37 °C for 24 h. The cell-free supernatant was used for enzyme testing. The test was performed using carboxymethyl chitin/Remazol brilliant violet (no. 04106; Loewe Biochemica) by mixing 0.1 ml citrate/phosphate buffer (0.2 M, pH 6.0) and 0.1 ml substrate (0.2% in water) with 0.2 ml of the respective culture supernatant or enzyme sample. The incubation was performed for 12 h at 37 °C. The reaction was stopped by adding 0.1 ml 1 M HCl and absorption was estimated at 600 nm. The plate assay was performed by streaking cells on TBY agar containing 2% colloidal chitin. The plates were incubated for 48 h at 37 °C and rinsed with KJ/J3 reagent. Chitin hydrolysis was recorded by haloes in zones of colony growth.

**RESULTS**

**Detection of a putative chitin-binding protein by B. amyloliquefaciens ALKO 2718**

During cultivation in TBY medium, B. amyloliquefaciens ALKO 2718 secretes several proteins, including an amylase (AmyAB) and a xylanase (Fig. 1). One of these proteins had an apparent molecular mass of about 20 kDa and the N-terminal amino acids HGYKPEPSVRAYMGA, which shared significant identity with the mature chitin-binding proteins CHB1 and CHB2 recently discovered in streptomycetes (Schnellmann et al., 1994; Kolbe et al., 1998). This 20 kDa protein cross-reacted moderately with antibodies raised against the Streptomyces binding protein CHB1 (see Fig. 4).

**Inducible synthesis of the putative chitin-binding protein**

B. amyloliquefaciens ALKO 2718 was grown in Spizizen’s minimal medium (Anagnostopoulos & Spizizen, 1961) in the presence of glucose, yeast extract, starch, cellulose (Avicel) or chitin. Under none of these conditions was chitinase activity recorded. However, the 20 kDa protein was synthesized in each of these media, except if glucose served as carbon source (Fig. 1a). Further analyses revealed that production of the 20 kDa protein in the medium with yeast extract was strongly reduced in the presence of glucose (Fig. 1b). The 20 kDa protein was mainly found in the supernatant (80%), but also adhering to chitin (about 20%), from which it could be detached by 3 M guanidinium hydrochloride (data not shown).

**Identification and characterization of the chbB gene and neighbouring genes**

Using total DNA of B. amyloliquefaciens ALKO 2718, the initial PCR amplification was performed with the primers A and B designed according to the determined N-terminal amino acid sequence from the mature 20 kDa protein (Fig. 2). Subsequent steps of the RAGE (rapid amplification of genomic ends) procedure were performed using the primer pairs EF or GH and CD or IK, respectively (Fig. 2, Table 1). Restriction fragments of total B. amyloliquefaciens DNA (cut with HindIII, HindIII or EcoRI) hybridizing with the labelled PCR fragment corresponded to those predicted from the restriction map of the amplified PCR product. Using chromosomal DNA of B. amyloliquefaciens, a fragment of about 4.5 kb was generated after PCR cloning and was sequenced. Three complete and two incomplete ORFs were found (Fig. 2a). The experimentally determined N-terminal amino acid sequence of the secreted 20 kDa protein (see first paragraph) corresponded to amino acid residues 28–43 of the protein deduced from
**Bacterial chitin-binding proteins**

![Restriction Map](image)

**Fig. 2.** Cloning strategy and map of the cloned DNA region. (a) Restriction map showing the orientation of the ORFs. *orf3* encodes the ChbB protein. (b) Sets of PCR primers (see Table 1) were used for stepwise PCR cloning of stretches (black lines) comprising a 4.5 kb chromosomal DNA region. (c) The predicted promoter, ribosome-binding site (RBS), translation initiation codon and the motifs (box 1–3) of *orf3* matching the cre binding site for the catabolite control protein CcpA are indicated.

![Alignment](image)

**Fig. 3.** Alignment of deduced chitin-binding proteins. Amino acid sequences: 1, A hypothetical 21 kDa protein from *Serratia marcescens* (G 974698); 2, the CBP21 protein from *Serratia marcescens* (Suzuki et al., 1998); 3, ChbB from *Bacillus amyloliquefaciens*; 4, CHB1 from *Streptomyces olivaceoviridis* (Schnellmann et al., 1994); 5, CHB2 from *Streptomyces reticuli* (Kolbe et al., 1998). Amino acids identical to ChbB (3) are shaded in black.

**orf3** (with a predicted signal peptide, 1–27) (Fig. 3). The predicted mature 19.8 kDa protein deduced from *orf3* shares 39, 37 and 45% identical amino acids with the previously identified chitin-binding protein CHB1 from *Streptomyces olivaceoviridis* (Schnellmann et al., 1994), CHB2 from *Streptomyces reticuli* (Kolbe et al., 1998) and CBP21 from *Serratia marcescens* (Suzuki et al., 1998), respectively. The *B. amyloliquefaciens* gene was named *chbB* (Fig. 3), as it encodes a chitin-binding protein (see above and below) from a *Bacillus* species.

About 140 nt upstream of *orf3*, a putative promoter (σ^A^) can be deduced, and palindromic sequences downstream of the gene could represent transcription terminators. Three motifs, TGAAGCGTGTTCAGCA (box 1), TGAGGCAGCTTTCTG (box 2) and AGACCCGGT-CAGCA (box 3) were found close to the proposed promoter (box 1), as well as within the gene about 50 (box 2) and 90 nt (box 3) downstream of the translation initiation codon ATG (Fig. 2c). These motifs matched the consensus WGNAASCIGWNNCA of the DNA
binding (cre) site for the catabolite control protein CcpA (Hueck et al., 1994).

The proteins deduced from the additional sequenced ORFs (Fig. 2a) share a high degree of similarity with proteins of unknown function, i.e. part of YnaE (incomplete Orf1), YvgO (Orf4) or part of YobB (incomplete Orf5), the genes of which are scattered in the B. subtilis 168 chromosome (Kunst et al., 1997). The part of the protein deduced from the sequenced orf2 (Fig. 2a) is similar to the deduced N5,N10-methylene-
tetrahydromethanopterin reductase from Staphylococcus aureus (accession No. U96107). It remains to be shown whether the B. amyloliquefaciens Orf2 protein corresponds to a functional reductase catalysing the reversible reduction of N5,N10-methylenetetrahydromethanopterin with the reduced F420 as electron donor, a characteristic feature of methanogenic archaea (Vaupel & Thauer, 1995) or whether it corresponds to another type of F420-dependent reductase.

Purification of the ChbB protein and the His-tag ChbB fusion protein

B. amyloliquefaciens was pregrown (see Methods) and after washing transferred to minimal medium supplemented with ground crab shell chitin (1%). Since the predominant part of the desired protein was found in the supernatant of the culture, the latter served as isolation source. Like the previously described CHB1 and CHB2 proteins (Schnellmann et al., 1994; Kolbe et al., 1998), the Bacillus protein does not bind to DEAE at a range of various pH values, including pH 9. The concentrated run-through of the DEAE column contained two dominant proteins of 20 and 24 kDa. The 20 kDa protein cross-reacted with anti-CHB1 antibodies and could be purified to near homogeneity by chromatography using MonoS material and citrate/phosphate at pH 5.2 (Fig. 4b). About 1 mg ChbB protein was gained per 1 l culture.

To gain larger amounts of the protein, the chbB gene was fused in-frame with six histidine codons, resulting in the construct pQEC1 (for details see Methods). After induction with IPTG, an E. coli transformant carrying pQEC1 produced larger quantities (2 mg per 500 ml culture) of the His-tag ChbB fusion protein (Fig. 4a). The latter was used to raise anti-ChbB antibodies, as well as for binding studies.

Binding specificities of the ChbB protein

The binding properties of the purified mature B. amyloliquefaciens ChbB protein were studied (Fig. 5). The quantities of the proteins, bound and/or unbound, were analysed by SDS-PAGE and, if necessary, the proteins were immunodetected. For initial studies anti-CHB1 antibodies were used; however, as their affinity was comparatively low, they were substituted by anti-

Fig. 6. Microscopical investigation. The B. amyloliquefaciens ChbB protein was mixed with α-chitin from crab shell (a), β-chitin from Siboglinum fjordicum (b) or β-chitin from Sepia (c) and treated with anti-ChbB antibodies, as described previously (Kolbe et al., 1998). Samples were inspected under UV light (top row) and visible light (bottom row) in the presence of the appropriate filter. As a control, each substrate was treated only with antibodies (not with ChbB); none was fluorescent (data not shown).
However, weak binding of β-chitin and reduced for α-chitin; however, weak binding of β-glucan from yeast and barley, and of crystalline cellulose was detected (Fig. 5c).

For visualization (detection of fluorescence) bodies newly raised against the His-tag ChbB protein (from E. coli, see above). For binding tests, ChbB (5 µg) was mixed with 2 mg of each substrate. The maximally bound ChbB was set as 100%. The pH optimum was established as 7 (Fig. 5a), and salt reduced binding to about 30% (0.5 M NaCl) or 50% (1 M NaCl) (Fig. 5b). ChbB showed a preference for β-chitin, less for α-chitin; however, weak binding of β-glucans from yeast and barley, and of crystalline cellulose was detected (Fig. 5c).

For visualization (detection of fluorescence) α- and β-chitin were treated with ChbB. Binding was detected with primary anti-ChbB antibodies, followed by secondary fluorescein-labelled antibodies. Fluorescence was most intense for β-chitin and reduced for α-chitin. No fluorescence was scored on the control chitin sample which had not been treated with ChbB (Fig. 6). After purification, the His-tag fusion protein ChbB obtained from the heterologous E. coli host was found to have identical binding characteristics (data not shown).

Abundance of homologues of the chbB gene and of the ChbB protein

To assess the abundance of the chbB gene, total DNA was isolated from several B. amyloliquefaciens strains and primers L and M were used for PCR detection. The PCR product of 12 out of 17 strains yielded a DNA fragment corresponding in size to ALKO 2718 (Fig. 7a). The DNA of some strains (IFO 3034, IFO 3037 and OUT 8421) induced the formation of a fragment of the same size, but in lesser quantities. The DNA of strains OUT 8420 and OUT 8426 did not yield a PCR product under standard conditions. All strains showed a hybridizing band which was either similar to ALKO 2718 or a different size. Neither a PCR product nor a hybridizing fragment was obtained with DNA of B. subtilis strains 168 and 6B 72. Moreover, using the anti-ChbB antibodies a protein similar in size to ChbB of ALKO 2718 was detected among proteins of several B. amyloliquefaciens strains grown in the presence of chitin. A corresponding protein was obtained with DNA of strains 168 and 6B 72. Moreover, using the anti-ChbB antibodies a protein similar in size to ChbB of ALKO 2718 was detected among proteins of several B. amyloliquefaciens strains grown in the presence of chitin. A corresponding protein was missing in cultures of B. subtilis 168 (data not shown).

Chitinolytic activity of B. amyloliquefaciens strains

On chitin-containing plates, the B. amyloliquefaciens strains OUT 8419, OUT 8420, OUT 8421, OUT 8426, IFO 3034 and IFO 3037 degraded chitin (clear zones), like strain B. licheniformis 41p, known to have chitinolytic activity (J. Hofemeister and others, unpublished).
Using the dye Remazol brilliant violet coupled with carboxymethyl chitin, the relative chitinolytic activities were compared. Strains ATCC 23842, KA63 and N showed only low activities, others (including ALKO 2718, ATCC 15841, ATCC 23350, IAM 1523, P, SBI, T and ZFL 14/A) had moderate activities, while strains IFO 3034, IFO 3037, OUT 8419, OUT 8420, OUT 8421 and OUT 8426 showed increased activities (Fig. 8); these were, however, lower than those of B. licheniformis 41p.

**Analysis of a B. subtilis transformants**

*B. subtilis* 168, which lacks a *cbbB*-like gene (Kunst *et al*., 1997), was transformed with plasmid pHBCI carrying the *cbbB* gene. Contrary to the progenitor strain, the transformants secreted a small (19.8 kDa) protein cross-reacting with anti-ChbB antibodies, indicating that the gene was transcribed and translated in the new host. Like the *B. subtilis* 168 host, the transformants lacked chitinolytic activity (Fig. 8).

**DISCUSSION**

During growth with a number of high-molecular-mass carbon sources (i.e. yeast extract, starch, cellulose or chitin), *B. amyloliquefaciens* ALKO 2718 secretes a small protein of 19.8 kDa (named ChbB). The ChbB protein adheres to chitin and moderately cross-reacts with antibodies previously raised against the *Streptomyces olivaceoviridis* chitin-binding protein CHB1 (Schnellmann *et al*., 1994). The experimentally determined N-terminal amino acids of ChbB share significant identity with those of the mature *Streptomyces* chitin-binding proteins CHB1 (Schnellmann *et al*., 1994) and CHB2 (Kolbe *et al*., 1998). Using reverse genetics, the *cbbB* gene was cloned and analysed. The ChbB protein (Fig. 3) deduced from the sequence shares 39, 37 and 45% amino acid identity with CHB1 from *Streptomyces olivaceoviridis* (Schnellmann *et al*., 1994), CHB2 from *Streptomyces reticuli* (Kolbe *et al*., 1998) and CBP21 from *Serratia marcescens* (Suzuki *et al*., 1998), respectively. Like the *Streptomyces* CHB1 and CHB2 proteins, ChbB interacts with α-chitin and in addition with β-chitin, a feature characteristic of the CBP21 protein from *Serratia marcescens*. The deduced ChbB protein shares several motifs with the *Streptomyces* CHBs, *Serratia marcescens* CBP21 and the Chi protein from another *Serratia marcescens* strain, the binding properties of which have not yet been investigated (Shin *et al*., 1996) (Fig. 3).

The *Bacillus* ChbB carries a tyrosine residue (Y) (see Fig. 3) corresponding in its location to the W57 residue in *Streptomyces* CHBs. The latter has been shown to be directly involved in the interaction with α-chitin (Zeltins & Schrempf, 1997). Its replacement by a leucine or a tyrosine residue also leads to nearly complete cessation of binding to α-chitin (Zeltins & Schrempf, 1997). The two *Serratia* proteins (CBP21 and Chi) also carry a tyrosine residue in this position, corresponding to W57 in CHB1 (Fig. 3) (Shin *et al*., 1996). It is interesting that the four additional W residues within the CHBs correspond in their relative positions to those present in ChbB and the *Serratia* CBP21 protein (W99, W114, W134 and W184; see Fig. 3). Strikingly, ChbB lacks all the cysteine residues which are found in the CHBs, CBP21 and Chi. We have shown (Svergun *et al*., 2000) that within CHB1 5–8 bridges are formed. Since ChbB lacks cysteine residues, 5–8 bridges stabilizing the topology cannot be formed and it is expected that the shape of ChbB is more flexible than that of CHB1.

ChbB does not display relevant amino acid identities with various types of accessory chitin-binding domains within chitinases from different organisms, including those from streptomycetes (Blaak & Schrempf, 1995; Saito *et al*., 1999) and *B. circulans* (Watanabe *et al*., 1992). Neither does ChbB share relevant common motifs with a recently discovered *Streptomyces tendae* protein (9.8 kDa) targeting chitin within various fungi (Bormann *et al*., 1999), with *Vibrio parahaemolyticus* chitovibrin (134 kDa) (Montgomery & Kirchman, 1994) assumed to mediate adhesion to chitin-containing organisms, nor with a small chitin-binding polypeptide (73 residues) from the haemocyte of horseshoe crab (Suetake *et al*., 2000). In nature chitin is very diverse in its organization [i.e. parallel (β) or anti-parallel (α) arrangement of N-acetylglucosamine chains, variable length, different degrees of crystallization] and its associated compounds (i.e. protein, inorganic substances or glucan). It is therefore not surprising that a number of proteins have evolved with subtle differences in recognition.

The effect of glucose on ChbB secretion (tested for *B. amyloliquefaciens* ALKO 2718) suggests that the expression of the *cbbB* gene is under catabolite control. This assumption is supported by the identification of cre boxes in the vicinity of a putative promoter (σ$^{'*}$) of the *cbbB* gene, thus indicating transcriptional repression (Hueck *et al*., 1994; Gösseringer *et al*., 1997; Stülke & Hillen, 2000).

In contrast to *B. subtilis* strains 168 and GB 72, all investigated *B. amyloliquefaciens* strains display varying levels of low and moderate chitinolytic activity and also secreted a protein of about similar size, cross-reacting with anti-ChbB antibodies. All *B. amyloliquefaciens* strains secreting a ChbB homologue share a DNA region with *S. marcescens* CBP21 and Chi. We have shown (Svergun *et al*., 2000) that within CHB1 5–8 bridges are formed. Since ChbB lacks cysteine residues, 5–8 bridges stabilizing the topology cannot be formed and it is expected that the shape of ChbB is more flexible than that of CHB1.

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In contrast to *B. subtilis* strains 168 and GB 72, all investigated *B. amyloliquefaciens* strains display varying levels of low and moderate chitinolytic activity and also secreted a protein of about similar size, cross-reacting with anti-ChbB antibodies. All *B. amyloliquefaciens* strains secreting a ChbB homologue share a DNA region which hybridizes with the *cbbB* gene. The different sizes of the hybridizing DNA fragments as well as the varying efficiency of PCR amplification (using only one set of primers and the same conditions) reflect some evolutionary divergence of homologues of the *cbbB* gene. Our sequence data showed that the *B. amyloliquefaciens* *cbbB* gene is situated next to genes of so far unknown function, which have counterparts in the *B. subtilis* 168 genome. There they are found scattered (Kunst *et al*., 1997), while a *cbbB* homologue could not be identified. It thus appears likely that acquisition of the *cbbB* gene by *B. amyloliquefaciens* leads to ChbB-mediated interaction with chitin-containing substrates (i.e. certain fungi and a number of chitin-containing organisms).
which are subsequently degraded by their chitinolytic activity. Therefore, B. amyloglucosidalis strains are, contrary to B. subtilis, expected to have a selective advantage in colonizing and hydrolysing chitin-comprising substrates in their natural habitats (i.e. soil and marine environments) (Gooday, 1990).

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**REFERENCES**


Hoang, V. & Hofemeister, J. (1995). *Bacillus* amyloglucosidalis possesses a second type I signal peptidase with extensive sequence similarity to other *Bacillus* SPases. Biochim Biophys Acta 1269, 64–68.


