The Bacillus subtilis 168 csn gene encodes a chitosanase with similar properties to a Streptomyces enzyme

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INTRODUCTION

Chitosans are a wide range of linear polysaccharides consisting of 1,4-β-linked N-glucosamine residues, partially substituted with N-acetyl groups to various degrees of acetylation. In nature, chitosan appears in significant amounts (around 30% of dry weight) in the cell walls of the Zygomycetes, a group of fungi comprising phytopathogens (Rhizopus) and opportunistic human pathogens (Mucor); chitosan is also present, although in smaller amounts, in the cell wall of other fungi and in some green algae such as Chlorella (Davis & Eveleigh, 1984). Chitosanases (EC 3.2.1.132) catalyse the hydrolysis of the glycosidic bonds of chitosan and have been used to obtain chitosan oligomers for clinical application as wound-healers, blood anticoagulants and haemostatic materials; chitosan oligomers have also been used as moisturizing agents, food and feed additives, waste-water treatment agents, fertilizers and seed coating fungistatic agents (Sandorf, 1989; Hirano, 1996).

An estimated 1–7% of heterotrophic soil bacteria synthesize chitosanases (Davis & Eveleigh, 1984) and chitosanase activities have been reported in a variety of microbial species and plants (reviewed by Somashekar & Joseph, 1996); genes encoding chitosanases have also been identified in the Chlorella PBCV-1 and CVK2 viruses (Lu et al., 1996; Yamada et al., 1997). Some chitosanases have been characterized and their amino acid sequences determined, such as the one from Fusarium solani f. sp. phaseoli SUF368 (Shimosaka et al., 1996) and a few others of bacterial origin, including those of Bacillus ehemensis EAG1 (Akiyama et al., 1999), Bacillus circulans MH-K1 (Yabuki et al., 1988), Streptomyces sp. N174 (N174 chitosanase; Boucher et al., 1992), Nocardioides sp. N106 (Masson et al., 1995) and ‘Matsuebacter chitosanotabidus’ 3001 (Park et al., 1999). The crystal structures of Streptomyces sp. N174 (Marcotte et al., 1996) and B. circulans MH-K1 (Saito et al., 1999) chitosanases are available.

This paper describes the isolation and expression of the csn gene from Bacillus subtilis 168 originally identified in our laboratory (GenBank accession no. X92868; Parro et al., 1997a). The gene was cloned and propagated in B. subtilis, and the chitosanase was overproduced, partially purified and biochemically characterized.

METHODS

Bacterial strains, plasmids and media. B. subtilis 168 (trpC2) cells were cultured in Luria Broth (LB) (Sambrook et al., 1989) or minimal medium, consisting of minimal salts solution (114 mM K₂SO₄, 62 mM KH₂PO₄, 44 mM KH₂PO₄, 34 mM sodium citrate, 0.8 mM MgSO₄·7H₂O; pH adjusted to 7 with 10% NaOH), supplemented with 0.4% (w/v) glucose, 50 μg l-tryptophan ml⁻¹, 0.2 mg l-glutamine ml⁻¹, 4 μg FeCl₃ ml⁻¹, 0.2 μg MnSO₄ ml⁻¹ and trace elements solution (42.6 μM CaCl₂, 12.5 μM ZnCl₂, 2.5 μM CuCl₂, 2.5 μM CoCl₂, 2.5 μM Na₂MoO₄·2H₂O). Kanamycin (10 μg ml⁻¹) or chloramphenicol (7.5 μg ml⁻¹) were added to the media when needed. Escherichia coli MC1061 (hsdR2 mcrB1 araD139 Δ(araABC–leu)7679 ΔlacX74 galU galK rpsL thi) was used as
a host for plasmid propagation. Plasmid pNR2 (Parro & Mellado, 1993) was used to propagate csn in high copy number. Plasmid pUCAT194 is a pUC19 derivative carrying the EcoRI fragment from pZA327 (a gift from J. C. Alonso) which contains the chloramphenicol resistance gene (cat) and was used as a vector for csn disruption.

DNA manipulation and PCR amplification. General recombinant DNA manipulation was carried out as described by Sambrook et al. (1989). Restriction endonucleases and DNA-modifying enzymes were from Promega and Boehringer Mannheim. B. subtilis 168 chromosomal DNA was used as a template for PCR amplification and long-range PCR (LR-PCR) amplification (Barnes, 1994; Cheng et al., 1994). DNA fragments were purified from low-melting-point agarose gels (LM3; Hispanagar) using Streptomyces coelicolor agarase, which was overproduced and purified in our laboratory (Parro et al., 1997b). Chromosomal DNA was obtained as described by Harwood & Cutting (1990). PCR and LR-PCR amplifications were carried out in an automated thermocycler (PTC-100; MJ Research). PCR amplification included a denaturation step at 95 °C for 3 min, followed by 30 cycles of incubation at 95 °C for 1 min, 55 °C for 1 min and 72 °C for 2 min; the reaction was ended by 10 min incubation at 72 °C. DNA fragments were amplified by using chromosomal DNA (500 ng) from B. subtilis 168 with 1 U EcoTaq polymerase (Ecogen) in the presence of 2 mM MgCl₂ and 40 pmol of each primer in a final reaction volume of 100 μl. To obtain DNA fragments longer than 2 kb, LR-PCR amplification was carried out using the GeneAmpXL kit (Perkin Elmer) and following the manufacturer’s instructions. The reaction included a denaturation step at 94 °C for 5 min, followed by 15 cycles of incubation at 94 °C for 30 s and 66 °C for 10 min, and 11 cycles of incubation at 94 °C for 30 s and 66 °C for 10 min with an increment of 15 s per cycle; the amplification was ended by 10 min incubation at 72 °C. DNA fragments were amplified from B. subtilis 168 chromosomal DNA (500 ng) with 20 U Tth DNA Polymerase (Promega) containing 1 mM Mg(CH₃COO)₂ and 40 pmol of each primer in a final reaction volume of 100 μl. For automatic DNA sequencing, a 373 DNA sequencer from Applied Biosystems and an Edit-View 1.0 DNA sequencer viewer (Applied Biosystems) were used.

Transcriptional analysis and RNA manipulations. Aliquots from the different cultures were lysed (Mellado et al., 1981) and total RNA was extracted as described by Kedzierski & Porter (1991). High-resolution S1 nuclease protection experiments were as described by Barthelmy et al. (1986), Sambrook et al. (1989) and Parro et al. (1998) using 50 μg total RNA. The DNA molecular size ladders were chemically derived (Maxam & Gilbert, 1980) from the same DNA fragment used as a probe in the experiments. Total RNA was transferred to nylon membranes (Hybond N+; Amersham) and used for Northern analysis as described by Sambrook et al. (1989). Nylon membranes were incubated overnight at 65 °C in 0.5 M sodium phosphate pH 7.2, 10 mM EDTA, 7% (w/v) SDS. A PCR internal fragment of the csn gene was amplified from genomic DNA with the oligonucleotides csn2 (5'-GGCGAG-GGCTATACATGGCGGAGGG-3') and csn1 (5'-GGCATATCCGATGTTTCTAGGG-3') as primers. The amplified DNA fragment (5 ng) was used as template to extend 10 pmol primer csn1 with 5 U sequencing grade Taq DNA polymerase (fmol DNA Cycle Sequencing System; Promega) in the presence of 1× fmol DNA Sequencing Buffer (Promega). The labelled DNA was used as a probe for Northern analysis.

Pulse–chase and Western blot experiments. One millilitre aliquots from different phases of cell cultures growing in defined medium were labelled with 100 μCi (37 MBq) [³⁵S]-methionine (Redivue Pro-mix L-[³⁵S] in vitro cell labelling mix; Amersham) in a 0.5 min pulse, following a procedure described previously (Parro & Mellado, 1994). A 1000-fold molar excess of non-radioactive methionine and cysteine were then added and the incubation continued; 100 μl aliquots were removed from the labelled cultures at 0, 0.5, 1, 2, 5 and 10 min after the pulse and the extracellular and intracellular labelled proteins were subjected to immunoprecipitation and analysis by SDS-PAGE (Laemmli, 1970). Proteins were immunoprecipitated as described previously (Parro & Mellado, 1994) with polyclonal antibodies raised against mature Csn extracted from acrylamide gels (Dunbar & Schwöbel, 1990). Samples treated with non-immune serum were always run in parallel as a negative control. Pulse–chase labelling experiments were repeated at least twice. Gels were exposed to Molecular Dynamics Storage Phospho Screens. Screens were scanned with a Molecular Imager FX (Bio-Rad) and relative amounts of radioactivity were determined with Quantity One version 4 software (Bio-Rad). ³H-Methylated molecular mass reference markers were obtained from Amersham.

For Western blot analysis, intracellular and extracellular proteins were fractionated by SDS-PAGE (Laemmli, 1970) and transferred to Immobilon PVDF membranes (Millipore) as described by Timmons & Dunbar (1990). Half of the transferred material was stained with 1% (w/v) Coomassie brilliant blue R-250 in 50% (v/v) methanol, 20% (v/v) acetic acid for 15 min. The other half of the transferred material was incubated with antibodies raised against mature Csn and peptides reacting with the antibodies were revealed by further incubation with 0.1 μCi (3.7 kBq) ml⁻¹ ¹²⁵I-labelled protein A from Staphylococcus aureus (Amersham), as described by Timmons & Dunbar (1990). Membranes were exposed to Agfa Curix RP2 film at −70 °C. Protein concentration in the different samples was determined as described by Bradford (1976), using standard bovine gamma globulin (Bio-Rad).

Chitosanase assay. Chitosanase activity was assayed as described by Boucher et al. (1992) using the neocuprine reagent (Dygert et al., 1965) for reducing sugar determination and 0.2% (w/v) chitosan flakes (practical grade; Sigma) dissolved in 50 mM sodium acetate buffer pH 5.7 as substrate. Activity was measured after 15 min incubation at 37 °C. One unit (U) of enzyme is defined as the amount of enzyme that liberated 1 μmol D-glucosamine equivalents min⁻¹ under the assay conditions. For chitosanase substrate specificity studies, the substrates were prepared as 2 mg ml⁻¹ solutions or suspensions in 50 mM sodium acetate buffer pH 5.5 and assayed as described above.

Chitosanase purification and analysis. Total protein from 100 ml culture medium of B. subtilis 168(pQCC10) in LB was precipitated at 80% saturation of ammonium sulfate at 4 °C. The precipitate was collected by centrifugation at 12000 g for 20 min, dissolved in 50 mM Tris/maleate buffer pH 7.3 (buffer A) and applied to a 45 × 1 cm Sephadex G-100 column (Pharmacia) previously equilibrated with the same buffer. The flow rate of the column was 15 ml h⁻¹. Fractions showing chitosanase activity were pooled (17.5 ml) and applied to an SP Fast Flow Sepharose 6 × 2.5 cm column (Pharmacia) previously equilibrated with the same buffer. The flow rate of the column was 15 ml h⁻¹. Unbound protein was washed from the column with buffer A containing 75 mM NaCl. Elution of chitosanase was carried out at the same flow rate in a step-wise manner with 30 ml buffer A containing 100, 150, 200, 250 and 300 mM NaCl. All purification steps were carried out at 4 °C. Fractions showing chitosanase activity were pooled and the
purified enzyme was stored at −20 °C in 50% (v/v) glycerol. The enzyme remained active without loss of activity for approximately 6 months. The N-terminal amino acid sequence of the purified mature chitosanase was determined by Edman degradation in a Procise 494 protein sequencer (Applied Biosystems).

Chitosanase sequences were retrieved from the NCBI GenBank database. The B. circulans MH-K1 chitosanase sequence was the version determined by Saito et al. (1999). Protein sequence comparison and analysis were carried out using the CLUSTAL W multiple sequence alignment program from the UWGCN package (version 1.7; Thompson et al., 1994). Sequence alignments were adjusted manually taking into account the structural relationships of chitosanases revealed by Saito et al. (1999). Phylogenetic analysis of the aligned sequences was performed using the maximum-likelihood analysis of the Phylogeny Analysis Using Parsimony (PAUP) program version 4.0 (Swofford, 1988) from the UWGCN package.

RESULTS AND DISCUSSION

The csn gene encodes a chitosanase

The csn DNA sequence was determined previously (Parro et al., 1997a) and was predicted to encode a chitosanase whose coding sequence is preceded by a putative RBS with a high degree of identity to the consensus RBS of B. subtilis. The putative pre-Csn is a 277 amino acid protein with a molecular mass of 31.5 kDa. The N-terminal sequence analysis predicted a cleavage site between Ala-35 and Ala-36. The putative pre-Csn is a 277 amino acid protein with a molecular mass of 31.5 kDa. The N-terminal sequence analysis predicted a cleavage site between Ala-35 and Ala-36. The putative pre-Csn is a 277 amino acid protein with a molecular mass of 31.5 kDa. The N-terminal sequence analysis predicted a cleavage site between Ala-35 and Ala-36. The putative pre-Csn is a 277 amino acid protein with a molecular mass of 31.5 kDa. The N-terminal sequence analysis predicted a cleavage site between Ala-35 and Ala-36. The putative pre-Csn is a 277 amino acid protein with a molecular mass of 31.5 kDa. The N-terminal sequence analysis predicted a cleavage site between Ala-35 and Ala-36.

Transcriptional and translational analysis of the csn gene

To assess if csn expression was subject to catabolite repression, B. subtilis 168 was grown in LB or minimal medium with different carbon sources: 0.4 or 1% glucose, 1% glycerol or 1% (w/v) mannitol. Aliquots of the different cultures were lysed and total RNA was extracted at mid-exponential, transition-to-stationary and stationary phases of growth as indicated in the Methods section. A 930 nt monocistronic transcript was detected by Northern blot analysis in all cases, transcription reaching its maximum at the transition-to-stationary phase (Fig. 2a), indicating that csn was temporally regulated and was not subject to catabolite repression. No catabolite repression was expected since no CRE elements (Miwa et al., 2000) were present in the csn regulatory region (Parro et al., 1997a). Csn activity was detected in the supernatant of all cultures (not shown), clearly indicating that no post-transcriptional catabolite repression existed. D-Glucosamine has been reported to induce chitosanase production in Streptomyces (Boucher et al., 1992; Price & Storck, 1975), but when 1% (w/v) D-glucosamine was used as carbon source it did not induce csn transcription in B. subtilis 168 (Fig. 2a).

Chitosan is not a good carbon source because it precipitates above pH 6.5. Nevertheless, Northern blot analyses were performed to detect chitosan induction of csn transcription in B. subtilis 168(pNR2) cultures growing in minimal medium in the presence of 0.4% glucose as carbon source that was either substituted or supplemented by 0.4% chitosan in the middle of the exponential phase. Bacterial growth and the csn transcription pattern were unaffected in the latter case (chitosan being ignored as carbon source by the bacteria in the presence of glucose), whereas when glucose was substituted by chitosan the bacterial growth became almost synchronized, with a considerably longer doubling time, resulting in only two generations more, and the csn transcription level diminished as the culture approached stationary phase (results not shown). B. subtilis 168(pNR2) was able to grow in minimal medium containing chitosan as the sole carbon source, but with eight-fold longer doubling time and a much lower cellular mass at stationary phase compared to cultures grown in the presence of glucose (results not shown).

The csn transcription initiation site was determined by high-resolution S1 nuclease protection experiments, using total RNA from B. subtilis 168 growing in minimal medium supplemented with 0.4% glucose. A 1355 bp PCR fragment containing part of the csn sequence was amplified by PCR from the B. subtilis genome using oligonucleotides 3661 (5′-GACATGATCTTGCCGATGGC-3′), derived from the yraK gene immediately preceding csn in the chromosome, and 4061 (5′-CAAA-
Fig. 1. (a, b) Amino acid sequence comparison of mature chitosanases from (a) *B. subtilis* 168 (Bsu), *Streptomyces* sp. N174 (Str) and *Nocardoides* sp. N106 (Noc); and (b) *B. subtilis* 168 (Bsu), *B. circulans* MH-K1 (Bci) and *B. ehimensis* (Beh). To take into account the structural relationships of chitosanases revealed by Saito et al. (1999), manual alignment was needed. Accepted conservative replacements for the manual alignments were I, L, V and M; D and E; A and G; R and K; S and T; F and Y. Asterisks indicate the conserved Glu and Asp residues equivalent to Glu-22 and Asp-40 from *Streptomyces* sp. N174 suggested to be essential for the chitosanase activity (Boucher et al., 1995). Identical and similar residues in all sequences are indicated by black and white circles, respectively. (c) Phylogenetic tree of the five chitosanases. Bootstrap replicate values derived from 1000 replications are indicated in square brackets. The relative numbers of substitutions per 100 residues are also indicated.
The transcription termination site of the chitosanase gene was also determined by S1 nuclease mapping. A 109–112 nt protected fragment was detected corresponding to a protected fragment was detected during the transition-to-stationary phase of growth (Fig. 3a). The relative amounts of precursor and mature forms were determined by densitometer scanning of the autoradiographs. The 15% of mature chitosanase that remained cell-associated after the 0–5 min pulse illustrates that the passage through the cell wall of B. subtilis is an active step during secretion as identified by Leloup et al. (1997) and Bolhuis et al. (1999).

**Construction of csn mutant and Csn overproducer strains**

To disrupt csn, a 280 bp DNA fragment from the central part of the csn coding sequence was amplified by PCR from the B. subtilis 168 chromosome using the csn2 and csn1 oligonucleotides as primers. The PCR product was purified and inserted into the unique, previously made blunt XbaI site of pUCAT194 to generate plasmid pUCSN1. After propagation in E. coli MC1061, pUCSN1 was used to transform B. subtilis 168. A transformant, BSCAT40, was selected with a single copy of pUCSN1 inserted into the chromosome, as confirmed by LR-PCR analysis using as primers 366I and 145d, derived from the csn2 and csn1 oligonucleotides as primers. The PCR product was amplified by PCR from the chromosome, using the csn2 and csn1 oligonucleotides as primers. The PCR product was purified and inserted into the unique, previously made blunt XbaI site of pUCAT194 to generate plasmid pUCSN1. After propagation in E. coli MC1061, pUCSN1 was used to transform B. subtilis 168. A transformant, BSCAT40, was selected with a single copy of pUCSN1 inserted into the chromosome, as confirmed by LR-PCR analysis using as primers oligonucleotides 366I and 145d, derived from the coding sequence of the csn gene.
Purification and characterization of chitosanase

Mature chitosanase was purified from stationary-phase supernatants of *B. subtilis* 168(pQC10) cultures grown in LB. In that phase of growth, *B. subtilis* 168(pQC10) accumulates approximately 60-fold more chitosanase activity than *B. subtilis* 168(pNR2) in the same culture conditions (results not shown). Mature Csn was purified as described in Methods. The purified enzyme, eluting from the SP-Fast Flow Sepharose column at 250 mM NaCl, was protease-free and almost 95% pure, as responding DNA sequences of the *csn* flanking genes, *yraK* and *yraM*, respectively (not shown). Western blot analysis of chitosanase production in *B. subtilis* BSCAT40, showed the complete absence of chitosanase (Fig. 3b).

A 2881 bp DNA fragment comprising *csn* and flanking regions was amplified by LR-PCR from the *B. subtilis* 168 chromosome using primers 366I and yraMR (5′-GCCTACTGGAAATAGTTCGGAG-3′), derived from the *yraM* gene. The amplified DNA fragment was purified and digested with *Dra*I to obtain a 1634 bp DNA fragment comprising the *csn* coding region plus the 625 bp preceding it and the 179 bp located behind it in the *B. subtilis* chromosome. The purified *Dra*I fragment was inserted into pNR2 through its unique *Sma*I site to generate the high-copy-number plasmid pQC10 that carried *csn* in the same relative orientation as the *cat* gene, as confirmed by DNA sequencing. *B. subtilis* 168(pQC10) produced chitosanase in considerably larger amounts than *B. subtilis* 168, as determined by Western blot assays (Fig. 3b), and propagation of pQC10 in the *csn* mutant *B. subtilis* BSCAT40 restored its ability to produce the enzyme at equivalent levels (Fig. 3b). *B. subtilis* 168(pQC10) was able to grow in minimal medium containing chitosan as the sole carbon source, whereas *B. subtilis* BSCAT40 did not (results not shown), as expected according to their relative levels of chitosanase production.
determined by SDS-PAGE (Fig. 4). N-terminal sequencing of the purified protein confirmed the predicted length (35 aa) of the leader peptide (not shown). The relative degree of purification of the *B. subtilis* Csn [specific activity 56.9 U (mg protein)\(^{-1}\), yield 33\%, and purification factor 1.83] was comparable to that of the N174 chitosanase, which was purified following a very similar procedure (Boucher et al., 1992).

The optimal pH (5-7) and temperature (60 °C) of the purified Csn resembled those of other characterized chitosanases (Somashekar & Joseph, 1996) and they are close to those of the N174 chitosanase (pH 5.5 and 65 °C; Boucher et al., 1992). Thermal stability of the enzyme was determined by preincubation at various temperatures (from 37 to 60 °C) in the absence of substrate for different periods of time (0–60 min) in 50 mM acetate buffer pH 5.7 and measurement of the residual activity. The enzyme was stable at 37 °C for 10 min but the stability rapidly decreased above 50 °C, and after 15 min at 60 °C activity dropped by almost 15%. As suggested earlier for other chitosanases (Pelletier & Sygush, 1990), the apparent discrepancy between the optimum temperature and the lability of chitosanase at 60 °C may reflect that the optimum temperature is related to the chemical reaction catalysed, whereas thermal stability refers to the lability of the protein structure. Enzyme activity was not affected by Ca\(^{2+}\) and Mg\(^{2+}\), but it was almost completely inhibited by Fe\(^{3+}\) and Cu\(^{2+}\) and inhibited by about 50% by Co\(^{2+}\), as determined when the different ions were added as 1 mM chloride salts to the reaction. Most of the chitosanases described are inhibited by heavy metal ions and part of the inhibition may be attributable to the chitosan having a strong tendency to form stable complexes with these ions (Yabuki et al., 1988).

Purified *B. subtilis* chitosanase can hydrolyse glycolchitosan at a similar rate (23% relative to 100% activity on solubilized Sigma chitosan) to that of the N174 chitosanase (35% of the maximal rate; Boucher et al., 1992). Although it has been reported that chitosanases from several sources (reviewed by Somashekar & Joseph, 1996) hydrolyse CM-cellulose and/or chitin to a different extent, Csn cannot do this, as also reported for chitosanases of *Streptomyces* sp. N174 (Boucher et al., 1992), *Nocardioides* sp. N106 (Boucher et al., 1992), *B. circulans* MH-K1 (Yabuki et al., 1988) and *B. eibensis* (Akiyama et al., 1999). Chitosan concentrations higher than 1 mg ml\(^{-1}\) inhibited Csn, as also happens with N174 chitosanase (Boucher et al., 1992). The apparent \(K_m\) for the *B. subtilis* 168 chitosanase, determined from a double reciprocal plot (not shown), was 0.110 mg ml\(^{-1}\) and its \(V_{max}\) was 66.3 U mg\(^{-1}\). These parameter values are similar to those of the N174 enzyme (\(K_m\) 0.088 mg ml\(^{-1}\), \(V_{max}\) 96.5 U mg\(^{-1}\); Boucher et al., 1992) but differ from those of *B. circulans* MH-K1 (\(K_m\) 0.63 mg ml\(^{-1}\); Yabuki et al., 1988) and *B. megaterium* (\(K_m\) 0.82 mg ml\(^{-1}\); Pelletier & Sygush, 1990).

From the results obtained it can be concluded that the *csn* gene of *B. subtilis* 168 encodes a chitosanase whose amino acid composition and functional characteristics are close to those of the Gram-positive bacterium *Streptomyces* sp. N174, despite the phylogenetic distance of their respective genera.

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