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

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The *Bacillus subtilis* 168 *csn* gene encodes a chitosanase. It was found that transcription of the *csn* gene was temporally regulated and was not subject to metabolic repression. Chitosanase synthesis was abolished in a *csn* mutant strain. Csn was overproduced in *B. subtilis*, partially purified and characterized. The deduced amino acid sequence, K_m, and optimal pH and temperature of the *B. subtilis* enzyme were closer to those of a chitosanase from *Streptomyces* sp. N174 than to those of chitosanases from other *Bacillus* strains.

Keywords: chitosanase, *Bacillus subtilis*, gene expression, catabolite repression

INTRODUCTION

Chitosans are a wide range of linear polysaccharides consisting of 1,4-β-linked D-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 (rewieved 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 ehimensis* 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.

Abbreviation: LR-PCR, long-range PCR.

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 (11·4 mM K_2SO_4, 62 mM K_2HPO_4, 44 mM KH_2PO_4, 3·4 mM sodium citrate, 0·8 mM $MgSO_4.7H_2O$; pH adjusted to 7 with 10 M NaOH), supplemented with 0·4% (w/v) glucose, 50 μg L-tryptophan ml^{-1}, 0·2 mg L-glutamine ml^{-1}, 4 μg $FeCl_3$ ml^{-1}, 0·2 μg $MnSO_4$ ml^{-1} and trace elements solution (42·6 μM $CaCl_2$, 12·5 μM $ZnCl_2$, 2·5 μM $CuCl_2$, 2·5 μM $CoCl_2$, 2·5 μM $Na_2MoO_4.2H_2O$). 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* Δ*lacX74 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 *Eco*RI 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 $MgCl_2$ 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 2·0 U *Tth* DNA Polymerase (Promega) containing 1 mM $Mg(CH_3COO)_2$ 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 Barthelemy *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′-GGCGAGGCTATACATGCGGACGGG-3′) and csn1 (5′-GGCATTATCCGATCGTTTCATGG-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 (3·7 MBq) [^{35}S]-methionine (Redivue Pro-mix L-[^{35}S] *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 & Schwoebel, 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). ^{14}C-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} ^{125}I-labelled protein A from *Staphyloccocus 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 I bovine gamma globulin (Bio-Rad).

Chitosanase assay. Chitosanase activity was assayed as described by Boucher *et al*. (1992) using the neocuproine 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(pQC10) 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 UWGCG 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-parsimony analysis of the Phylogeny Analysis Using Parsimony (PAUP) program version 4.0 (Swofford, 1988) from the UWGCG 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 Csn signal peptide length (35 aa), hydrophobicity (0·7; Kyte & Doolittle; 1982) and number of positive charges (4) are closer to those of the streptomycetes group (mean peptide length, $35{\cdot}5 \pm 7{\cdot}9$; mean hydrophobicity, $0{\cdot}56 \pm 0{\cdot}6$, mean number of positive charges, $3{\cdot}9 \pm 2{\cdot}4$; Edman *et al.*, 1999) than to those of *Bacillus* (mean peptide length, $28{\cdot}2 \pm 5{\cdot}6$; mean hydrophobicity, $0{\cdot}93 \pm 0{\cdot}4$, mean number of positive charges, $2{\cdot}0 \pm 0{\cdot}9$; Edman *et al.*, 1999). The estimated molecular mass for the 242 amino acid mature Csn was 27·4 kDa. The deduced amino acid sequence showed significant homology with chitosanases present in the GenBank database (Fig. 1), being closer to those from actinomycetes (*Streptomyces* sp. N174, 39% identity and 51% similarity; *Nocardioides* sp. N106, 37% identity and 49% similarity) (Fig. 1a) than to those from *Bacillus* (*B. ehimensis*, 21% identity and 28% similarity; *B. circulans* MH-K1 20% identity and 27% similarity) (Saito *et al.*, 1999; Fig. 1b). In the *B. subtilis* mature chitosanase, residues Glu-19 and Asp-35, equivalent to the Glu-22 and Asp-40 suggested to be essential for catalytic activity in N174 chitosanase (Boucher *et al.*, 1995), are conserved and equivalent residues are also present in other chitosanases (Fig. 1). The disulfide bridge between Cys-50 and Cys-124 present in the *B. circulans* MH-K1 (Saito *et al.*, 1999) and *B. ehimensis* enzymes is not conserved in the *Streptomyces* sp. N174 and *B. subtilis* chitosanases. The parsimony analysis of the primary structure of the *Streptomyces* sp. N174, *Nocardioides* sp. N106, *B. ehimensis*, *B. circulans* MH-K1 and *B. subtilis* 168 mature chitosanases produced a phylogenetic tree where the *B. subtilis* enzyme appeared closer to those of actinomycetes (Fig. 1c).

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 366I (5′-GACATGTACTTGTTCGG-GATGGC-3′), derived from the *yraK* gene immediately preceding *csn* in the chromosome, and 406I (5′-CAAA-

(a)

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                                      *                   *
Str   1 AGAGLDDPHKKEIAMELVSSAENSSLDWKAQYKYIEDIGDGRGYTGGIIGFCSGTGDMLE   60
Noc   1 AAVGLDDPHKKDIAMQLVSSAENSSLDWKSQYKYIEDIKDGRGYTAGIIGFCSGTGDMLD   60
Bsu   1 --AGLNKDQKRR-AEQLTSIFENGTTE--IQYGYVERLDDGRGYTCGRAGFTTATGDALE   56

Str  61 LVQHYTDLEPGNILAKYLPALKKVNGSASH--SGLGTPFTKDWATAAKDTVFQQAQNDER  118
Noc  61 LVADYTDLKPGNILAKYLPALRKVNGTESH--AGLASAFEKDWATAAKDSVFQQAQNDER  118
Bsu  57 VVEVYTKAVPNNKLKKYLPELRRLAKEESDDTSNL-KGFASAWKSLANDKEFRAAQDKVN  114

Str 119 DRVYFDPAVSQAKADGLR-ALGQFAYYDAIVMHGPGNDPTSFGGIRKTAMKKA-RTPAQG  176
Noc 119 DRSYFNPAVNQAKA-SLR-ALGQFAYYDAIVMHGPGDSSDSFGGIRKAAMKKA-KTPAQG  175
Bsu 115 DHLYYQPAMKRSDNAGLKTALARAVMYDTVIQHGDGDDPDSFYALIKRTNKKAGGSPKDG  174

Str 177 GDETTYLNAFLDARKAAMLTEAAHD--D-TS----RVDTEQRVFLKAGNLDLNPPLKWKT  229
Noc 176 RDEATYLKAFLAARKTVMLKEEAHS--D-TS----RVDTEQTVFLNAKNFDLNPPLKWKV  228
Bsu 175 IDEKKWLNKFLDVRYDDLMNPANHDTRDEWRESVARVDVLRSI-AKENNYNLNGPIHVRS  233

Str 230 --YGDPYVINS                                                   238
Noc 229 --YGDSYAINS                                                   237
Bsu 234 NEYG-NFVIK-                                                   242
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(b)

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                                           *                   *
Beh   1 ASPDENFSPETLQFLRDRTGLDGEQWNNI-MKLINKPEQDDLNWIKYYGYCEDINDERGY   59
Bci   1 ASPDDNFSPETLQFLRNNTGLDGEQWNNI-MKLINKPEQDDLNWIKYYGYCEDIEDERGY   59
Bsu   1 ------------------AGLNKDQKRR-AEQLTSIFENGTTE--IQYGYVERLDDGRGY   39

Beh  60 SIGIFGATTGGPRDTHPDGPELFKAYDAAKGAGNPSVE--GALKRLGINGKMKGSILEIK  117
Bci  60 TIGLFGATTGGSRDTHPDGPDLFKAYDAAKGASNPSAD--GALKRLGINGKMKGSILEIK  117
Bsu  40 TCGRAGFTTA----T-GDALEVVEVYTKAV-PNNKLKKYLPELRR--LAKEESDDTSNL-   90

Beh 118 DSEKVFCGKIKKLQNDPAWRKAMWETFYNVYIRYSVEQARQRGFTSALTIGSFVDTALNQ  177
Bci 118 DSEKVFCGKIKKLQNDAAWRKAMWETFYNVYIRYSVEQARQRGFTSAVTIGSFVDTALNQ  177
Bsu  91 ---KGFASAWKSLANDKEFRAAQDKVNDHLYYQPAMKRSDNAGLKTALARAVMYDTVIQH  147

Beh 178 GATGDSNTLQGL----LARSGSST----NEKTFLKKFHAKRTLVVDTNEYNQPPNGKN--  227
Bci 178 GATGGSDTLQGL----LARSGSSS----NEKTFMKNFHAKRTLVVDTNKYNKPPNGKN--  227
Bsu 148 GDGDDPDSFYALIKRTNKKAGGSPKDGIDEKKWLNKFLDVRYDDLMNPANHDTRDEWRES  207

Beh 228 --RVK-QWDTLLDMGKMNLKNVDAEIAQVTNWE---                          257
Bci 228 --RVK-QWDTLVDMGKMNLKNVDSEIAQVTDWEMK-                          259
Bsu 208 VARVDVLRSI-AKENNYNLNGPIHVRSNEYGNFVIK                          242
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(c)

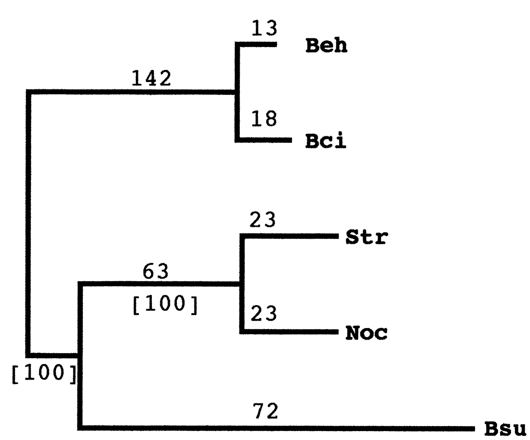

Fig. 1. (a, b) Amino acid sequence comparison of mature chitosanases from (a) *B. subtilis* 168 (Bsu), *Streptomyces* sp. N174 (Str) and *Nocardioides* sp. N106 (Noc); and (b) *B. subtilis* 168 (Bsu), *B. circulans* MH-K1 (Bci) and *B. ehimensis* (Beh). To take into account the structural relationships 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.

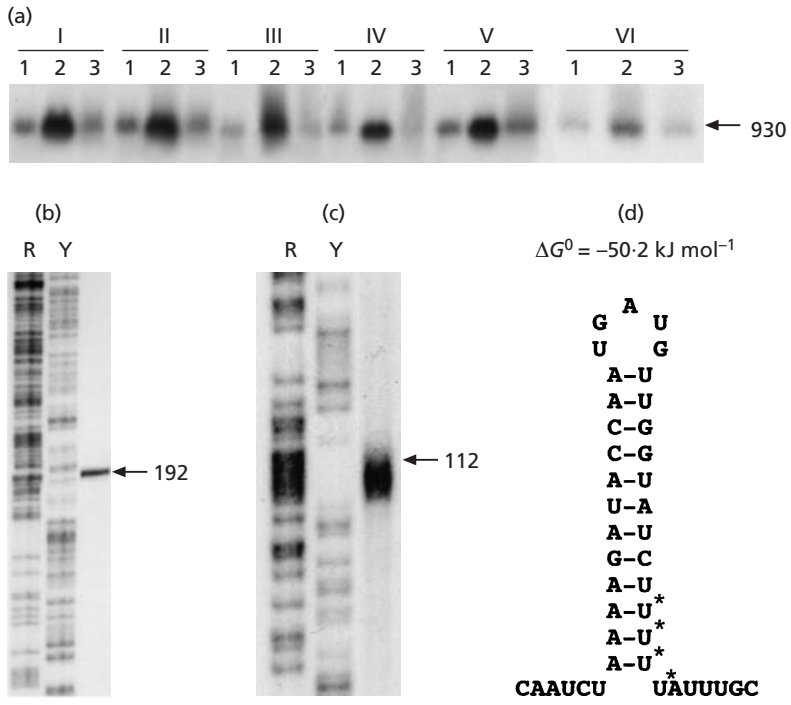

Fig. 2. Transcriptional analysis of the *csn* gene. (a) Northern blot analysis. Total RNA was extracted at mid-exponential (lane 1), transition-to-stationary (lane 2) and stationary (lane 3) phases of *B. subtilis* cell cultures growing in LB (lanes VI), or minimal medium supplemented with 0·4% glucose (lanes I), 1% glucose (lanes II), 1% glycerol (lanes III), 1% mannitol (lanes IV) or 1% D-glucosamine (lanes V). The transcript size (nt) is indicated. (b, c) Transcription initiation (b) and termination (c) sites for *csn* were determined by S1 nuclease mapping using 50 μg total RNA from *B. subtilis* cultures extracted at transition-to-stationary phase. The size of the protected fragments (nt) is indicated. Lanes R and Y contain the purine and pyrimidine sequence ladders, respectively, resulting from chemical degradation of the radioactively labelled probes run in parallel. (d) The stem–loop structure for the *csn* terminator. Asterisks indicate the nucleotides where transcription terminates.

GATACTTGTCAGCTGTTCCG-3′) and digested with *Hin*dIII. A 355 bp fragment thought to contain the *csn* promoter region was radioactively labelled at its unique 5′ blunt end and used as a probe. A 192 nt protected fragment was detected corresponding to a transcript starting at 33 bp upstream of the *csn* translation start codon (Fig. 2b). The deduced −35 and −10 regions of the *csn* promoter are separated by 17 bp and showed homology to those of the consensus *B. subtilis* σ^A promoters (Parro *et al*., 1997a)

The transcription termination site of the chitosanase gene was also determined by S1 nuclease mapping. A 384 bp DNA fragment was amplified by PCR from the *B. subtilis* genome using oligonucleotides csnt (5′-GAACAACTATAATCTAAACGGACC-3′), derived from the *csn* coding sequence, and 145d (5′-GACGG-AACAGTTTATACGCATGG-3′), derived from the *yraM* gene immediately after *csn* in the chromosome, and digested with *Hin*fI. The resulting 359 bp fragment was radioactively labelled at its unique *Hin*fI 3′ end and used as a probe. A 109–112 nt protected fragment was detected (Fig. 2c), locating the transcription termination site 65–68 nt downstream of the *csn* translation stop codon; the presence of more than one protected band could be due to the S1 nuclease nibbling effect previously described (Christie & Calendar, 1983; Mellado *et al*., 1986). The stem–loop structure predicted around the transcription termination site is depicted in Fig. 2(d).

To determine the secretion pattern of the chitosanase precursor, total intracellular polypeptides from mid-exponential, transition-to-stationary and stationary phases of growth from *B. subtilis* 168 cultures grown in minimal medium in the presence of 0·4% glucose were pulse-labelled with [^{35}S]methionine and chased with a 1000-fold molar excess of non-radioactive methionine and cysteine as described in Methods. The labelled proteins were incubated with antibody raised against the extracellular chitosanase and immunoprecipitated polypeptides analysed by SDS-PAGE. Chitosanase was only detected during the transition-to-stationary phase of growth (Fig. 3a), coinciding with *csn* transcription being more abundant (Fig. 2a). Pre-Csn was rapidly processed and secreted; within 2 min after the pulse-labelled pre-Csn was chased extracellular mature enzyme was detected (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 *Xba*I 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 *csn* through a single crossover event as confirmed by LR-PCR analysis using as primers oligonucleotides 366I and 145d, derived from the cor-

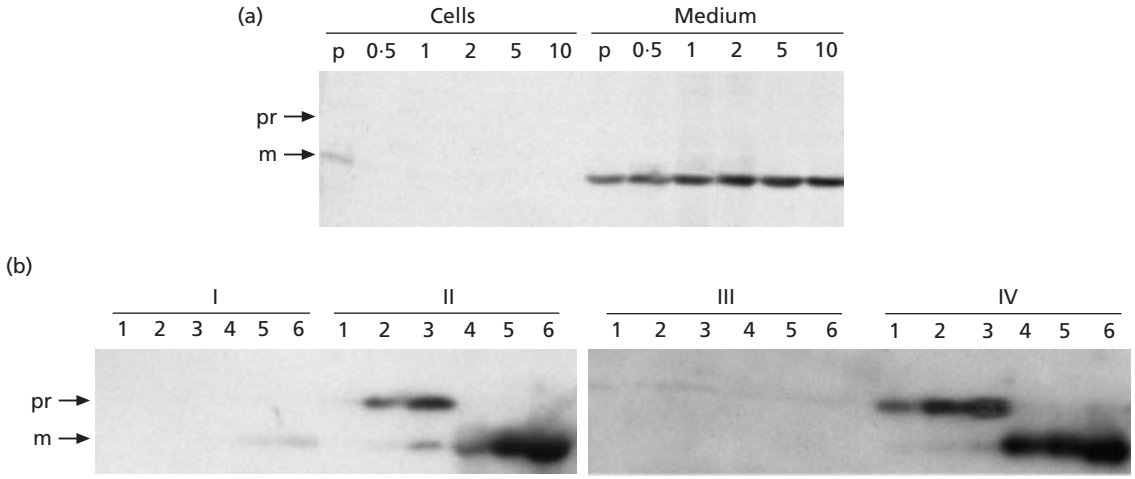

Fig. 3. Chitosanase synthesis. (a) *B. subtilis* cultures grown in minimal medium supplemented with 0·4% glucose were pulse-labelled for 0·5 min (p) and chased for 0·5, 1, 2, 5 or 10 min as indicated. Total intracellular ('Cells') and extracellular ('Medium') proteins were subjected to immunoprecipitation using antibodies raised against *B. subtilis* mature Csn, and analysed by 12·5% SDS-PAGE. (b) Western blot analysis of intracellular (lanes 1–3) and extracellular (lanes 4–6) chitosanase present in *B. subtilis* 168(pNR2) (lanes I), *B. subtilis* 168(pQC10) (lanes II), BSCAT40 (lanes III) and BSCAT40(pQC10) (lanes IV) cell cultures grown in minimal medium supplemented with 0·4% glucose at exponential (lanes 1 and 4), transition-to-stationary (lanes 2 and 5) and stationary (lanes 3 and 6) phases of growth. pr, pre-Csn; m, mature Csn.

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.

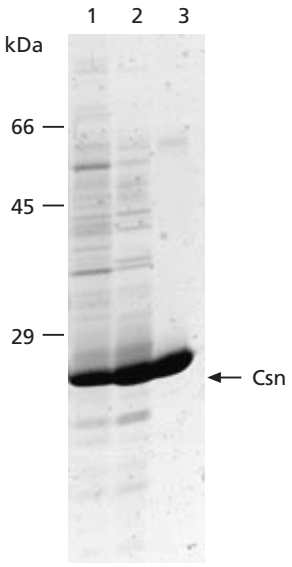

Fig. 4. Chitosanase purification. Coomasie-blue-stained 12·5% SDS-PAGE of total extracellular protein recovered after ammonium sulfate precipitation (lane 1), gel filtration through a G-100 Sephadex column (lane 2) and ion exchange by passage through a SP-Fast Flow Sepharose column (lane 3). A 50 μg sample of protein was loaded in each lane.

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 aproximately 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

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 glycol-chitosan 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. ehimensis* (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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