

Multiple genes involved in chitin degradation from the marine bacterium *Pseudoalteromonas* sp. strain S91

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A cluster of three closely linked chitinase genes organized in the order *chiA*, *chiB* and *chiC*, with the same transcriptional direction, and two unlinked genes, *chiP* and *chiQ*, involved in chitin degradation in *Pseudoalteromonas* sp. strain S91 were cloned, sequenced and characterized. The deduced amino acid sequences revealed that ChiA, ChiB and ChiC exhibited similarities to chitinases belonging to family 18 of the glycosyl hydrolases while ChiP and ChiQ belonged to family 20. ChiP and ChiQ showed different enzymic activities against fluorescent chitin analogues, but neither was able to degrade colloidal chitin. ChiA possessed chitinase activity but did not bind chitin; ChiB bound chitin but had no chitinase activity; ChiC possessed strong chitinase activity and also bound chitin. Production of ChiC in S91 appeared to be controlled by *chiA* expression, since insertion of a transposon into the ORF of *chiA* resulted in the loss of chitinase activity as well as loss of ChiC proteins in a chitinase-negative mutant. In *Escherichia coli*, ChiC appeared to be expressed from its own promoter.

Keywords: *Pseudoalteromonas*, glycosyl hydrolases, chitinase, chitobiase, chitin degradation

INTRODUCTION

Chitin is one of the most abundant and important sources of nutrients and energy in the marine environment (Gooday, 1990). It has been estimated that the production of chitin by copepods alone is several million tonnes annually, resulting in a continuous 'rain' of particulate organic matter through the water column (Yu *et al.*, 1991). Chitin accumulation in the environment is not observed, despite chitinous materials being relatively resistant to degradation (Jeuniaux *et al.*, 1986; Rudall & Kenchington, 1973). It appears that chitin is relatively rapidly recycled in most environments (Gooday, 1994). Degradation of chitin is an important microbial process returning nutrients to the marine environment. Chitinolytic bacteria play a critical role in this chitin recycling process (Gooday, 1994).

Enzymic digestion of chitin consists of two consecutive

steps. Chitinase (EC 3.2.1.14) hydrolyses the polymer form of chitin to small oligosaccharides, especially chitobiose (GlcNAc₂), the dimer of *N*-acetylglucosamine subunits. Chitobiase (β -*N*-acetylglucosaminidase, EC 3.2.1.52) hydrolyses GlcNAc₂ to yield the final product, *N*-acetylglucosamine (GlcNAc) (Cabib, 1987). Hydrolysis of the β -(1,4)-glycosidic linkage of chitin can proceed via exo- or endochitinase activity. These enzymes are found in a wide range of organisms. Individual chitinase genes have been cloned and sequenced from various micro-organisms, including bacteria such as *Serratia marcescens* (Brurberg *et al.*, 1994; Fuchs *et al.*, 1986; Jones *et al.*, 1986), *Aeromonas caviae* (Sitrit *et al.*, 1995), *Alteromonas* sp. strain O-7 (Tsujibo *et al.*, 1993b), *Vibrio harveyi* (Soto-Gill & Zyskind, 1984), *Vibrio furnissii* (Keyhani & Roseman, 1996) and *Bacillus circulans* WL-12 (Watanabe *et al.*, 1990, 1994a). Most chitinolytic bacteria appear to have more than one chitinase and while relatively little is known about the organization of chitinase genes, even less is known about their regulation. It is not known if bacterial chitinase genes occur in operons or are part of global regulatory systems such as regulons and stimulons.

Abbreviations: CAP, calf intestine alkaline phosphatase; MU, 4-methylumbelliferyl.

The GenBank accession numbers for the sequences reported in this paper are AF007894 (*chiA*), AF007895 (*chiB*), AF007896 (*chiC*), AF072375 (*chiP*) and AF072374 (*chiQ*).

We are interested in studying gene expression in *Pseudoalteromonas* sp. strain S91 in response to environmental conditions, particularly those relevant to biofilm communities (Stretton *et al.*, 1996; Techkarnjanaruk *et al.*, 1997). Expression of an individual *chi* gene in S91 was quantified by inserting a promoterless reporter gene, *lacZ*, under the control of the *chi* promoter (Techkarnjanaruk *et al.*, 1997). Another S91 strain, in which a promoterless *gfp* gene was fused to the same *chi* promoter, showed that this *chi* gene is expressed in cells growing as microcolonies during biofilm development on squid pen, a natural biodegradable substratum (Stretton *et al.*, 1998).

In order to investigate further the response of individual chitinase genes in S91 to environmental conditions, it was necessary to clone and characterize the bacterium's multiple chitinases. In the present study, five genes involved in chitin degradation in S91, including three closely linked genes, were identified and characterized. The sequence of the transposon-interrupted chitinase gene in S91CX (Techkarnjanaruk *et al.*, 1997) and S91CGFP (Stretton *et al.*, 1998) was completed and found to be involved in the expression of a downstream chitinase gene as well as production of a chitinase itself.

METHODS

Bacterial strains, plasmids and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *Pseudoalteromonas* sp. strain S91 was grown as previously described (Techkarnjanaruk *et al.*, 1997). *Escherichia coli* strains were grown at 37 °C in Luria-Bertani broth (LB) (Miller, 1972). Colloidal chitin was prepared from a practical-grade chitin (crab shells, Sigma), as described by Shihamara & Takiguchi (1988), and used to make chitin agar plates (Techkarnjanaruk *et al.*, 1997). Where appropriate, antibiotics and IPTG (Sigma) were used at the following concentrations: for S91, streptomycin at 200 µg ml⁻¹ and kanamycin at 600 µg ml⁻¹; for *E. coli*, ampicillin at 50 µg ml⁻¹, kanamycin at 50 µg ml⁻¹, chloramphenicol at 50 µg ml⁻¹ and IPTG at 120 µg ml⁻¹.

DNA manipulations. Standard molecular cloning techniques employed in this study (small- and large-scale plasmid DNA extraction, genomic DNA isolation, restriction enzyme digestion, agarose gel electrophoresis, DNA ligation and transformation) were performed as described by Sambrook *et al.* (1989) unless stated otherwise.

Genomic DNA library construction and screening. An S91 genomic library was constructed using the pUC118 plasmid vector, to identify S91 genes involved in chitin degradation. Genomic DNA of strain S91 was partially digested with *Sau*3A, and DNA fragments 3–7 kb in size were separated and purified using Wizard Magic DNA Cleanup System (Promega). The resulting DNA fragments were ligated into a *Bam*HI/CAP-pretreated pUC118 vector. The ligation mixture was transformed into competent *E. coli* DH5αF' IQ cells. The library was screened for clones producing chitinase and N-acetylglucosaminidase using the direct plate screening method as described below.

A second genomic library was constructed in a Lambda Zap Express vector (Stratagene) to isolate further genes involved in chitin degradation. Genomic DNA of strain S91 was partially

digested with *Sau*3A, and DNA fragments 4–10 kb in size were separated and purified using Wizard Magic DNA Cleanup System (Promega). The resulting DNA fragments were ligated into a *Bam*HI/CAP-pretreated Lambda Zap Express vector, packaged and allowed to infect *E. coli* XL-1 Blue MRF' cells as in the recommended procedure (Stratagene). The library was plated on NZY plates (Stratagene) overnight with appropriate dilutions. Mass excision of the library to form bacterial colonies was also undertaken using *E. coli* XL0LR, *E. coli* XL-1 Blue MRF' and the ExAssist helper phage following the manufacturer's instructions (Stratagene). Colonies or plaques were screened against fluorescent chitin analogues as described below.

Direct plate screening method using fluorescent chitin oligomer analogues. A direct plate screening method was modified from that described previously (McCreath & Gooday, 1992; Robbins *et al.*, 1988) to screen colonies for chitinase activity. The method involved growing either bacterial colonies or a lawn of plaques on appropriate agar plates. After overnight incubation, plates were overlaid with 0.7% agarose (5 ml) containing a mixture of fluorogenic chitin oligomer analogues (Sigma): 4-methylumbelliferyl-N,N',N''-triacylchitotriose [4-MU-(GlcNAc)₃] to detect endochitinase activity (200 µl of 250 µM); 4-methylumbelliferyl-N,N'-diacylchitobiose [4-MU-(GlcNAc)₂] to detect exochitinase activity (200 µl of 500 µM); and 4-methylumbelliferyl-β-D-N-acetylglucosamine [4-MU-GlcNAc] to detect N-acetylglucosaminidase/chitobiase activity (200 µl of 2 mM). Plates were incubated at 30 °C for 15 min. Colonies or plaques which possessed either exo- or endochitinase, or N-acetylglucosaminidase activity, showed a brightly fluorescent halo under UV light. All presumptive positive clones were tested against individual fluorescent chitin oligomer analogues to determine types of chitinase activity as well as against colloidal chitin. Colonies were spotted with 5 µl of either 4-MU-(GlcNAc)₃, 4-MU-(GlcNAc)₂ or 4-MU-GlcNAc and incubated at 30 °C for 10–15 min and then visualized under UV light.

Nucleotide sequence analysis. DNA sequences were obtained (using an automated sequencer, Applied Biosystems model 373, at a DNA sequencing facility at Westmead Hospital, Sydney, Australia) by directed sequencing with progressive oligonucleotide primers (Sambrook *et al.*, 1989) using plasmid DNA as templates. Both DNA strands were sequenced. Comparison of DNA sequences and amino acid sequences with sequences available in databases was performed using programs in the Genetic Computer Group (GCG) software package (University of Wisconsin) (Altschul *et al.*, 1990; Warren & States, 1993). The computation was performed through a computer link to the Australian National Genomic Information Service (ANGIS).

Protein electrophoresis. SDS-PAGE was carried out according to the procedure of Laemmli (1970). After electrophoresis was completed, renaturation of the enzymes (Trudel & Asselin, 1989) and detection of chitinase activity in the gel were carried out as described previously (Techkarnjanaruk *et al.*, 1997). Cellular protein fractions were prepared by forming spheroplasts of cell cultures as described by Koshland & Botstein (1980). Culture supernatants were collected and used as the extracellular fraction. Binding of chitinolytic proteins to prepared colloidal chitin was carried out by a modification of the method described by Wanatabe *et al.* (1990). Briefly, this involved incubating the periplasmic protein fraction with chitin particles for 5–10 min on ice. The mixture was washed with an excess amount of phosphate buffer pH 6.5 and centrifuged three times to remove non-specific binding proteins. The pellet of chitin particles was resuspended in

Table 1. Bacterial strains and plasmids used in this study

| Strain or plasmid | Relevant characteristics | Reference or source |
|------------------------------|--|--------------------------------------|
| Bacterial strains | | |
| <i>Pseudoalteromonas</i> sp. | | |
| S91 | Spontaneous mutant of wild-type S9, Sm ^R | Albertson <i>et al.</i> (1996) |
| S91CX | S91::mini-Tn10: <i>lac:kan</i> , Sm ^R Km ^R , LacZ ⁺ , chitinase-negative | Techkarnjanaruk <i>et al.</i> (1997) |
| S91CGFP | S91::mini-Tn10: <i>gfp:kan</i> , Sm ^R Km ^R , GFP ⁺ , chitinase-negative | Stretton <i>et al.</i> (1998) |
| <i>Escherichia coli</i> | | |
| DH5 α | F' ϕ 80 <i>dlacZ</i> Δ M15 Δ (<i>lacZYA-argF</i>)U169 <i>deoR recA1 endA1</i> <i>hsdR17</i> (r _k ⁻ m _k ⁺) <i>phoA</i> , <i>supE44-λ</i> ⁻ <i>thi-1</i> <i>gyrA96</i> <i>relA1</i> | Gibco-BRL |
| XL-1 Blue MRF' | Δ (<i>mcrA</i>)183 Δ (<i>mcrCB-hsdSMR-mrr</i>)173 <i>endA1 supE44 thi-1</i> <i>recA1 gyrA96 relA1 lac</i> [F' <i>proAB lacI</i> ⁿ Δ M15 Tn10 (Tet ^R)] | Stratagene |
| XL0LR | As XL-1 Blue MRF' except Su ⁻ λ ^R | Stratagene |
| Plasmids | | |
| pBK-CMV | Cloning vector, Km ^R | Stratagene |
| pBCSK ⁺ | Cloning vector, Cm ^R | Stratagene |
| pCHB1 | 5 kb insert in pUC119 containing an <i>N</i> -acetylglucosaminidase gene isolated from pUC118 plasmid library of S91, Ap ^R | This study |
| pCHT1 | 4.5 kb insert in pBK-CMV containing an exochitinase, endochitinase and <i>N</i> -acetylglucosaminidase gene isolated from Lambda-ZAP genomic library of S91 | This study |
| pCHT2 | 6 kb insert in pBK-CMV containing exo- and endochitinase gene isolated from Lambda-ZAP genomic library of S91 | This study |
| pCHT21 | Derivative of pCHT2: deletion of 1.5 kb <i>Eco</i> RI fragment | This study |
| pCHT22 | Derivative of pCHT2: deletion of a 2.5 kb <i>Pst</i> I fragment | This study |
| pCHT23 | Derivative of pCHT2: deletion of 2.5 kb <i>Pst</i> I fragment in pBCSK ⁺ in the opposite orientation to that in pCHT22 | This study |
| pCHT7 | As pCHT2 except DNA insert in the opposite orientation | This study |
| pCHT8 | 5.9 kb insert in pBK-CMV containing exo- and endochitinase gene isolated from Lambda-ZAP genomic library of S91 | This study |
| pCHT9 | 6.5 kb insert in pBK-CMV containing exo- and endochitinase gene isolated from Lambda-ZAP genomic library of S91 | This study |

protein loading buffer and heated by boiling for 3 min. Samples were analysed by SDS-PAGE chitinase activity gels (Trudel & Asselin, 1989).

RESULTS AND DISCUSSION

Isolation of chitinase genes from *Pseudoalteromonas* S91

Screening of the two S91 genomic libraries constructed in *E. coli* against chitin oligomer analogues yielded 12 fluorescent colonies from a total of about 10000. Each clone was then tested against each chitin oligomer analogue individually, and plasmid DNA from each clone was subjected to restriction enzyme analysis and cross-hybridization (data not shown). From these data, six unique recombinant clones were identified and their plasmids named pCHB1, pCHT1, pCHT2, pCHT7, pCHT8 and pCHT9. The ability of positive recombinant clones to degrade colloidal chitin was tested. *E. coli*/pCHT2, *E. coli*/pCHT7, *E. coli*/pCHT8 and *E. coli*/pCHT9 were able to degrade chitin as seen by clearing haloes on chitin-containing agar plates, whereas *E. coli*/pCHT1 and *E. coli*/pCHB1 were unable to degrade chitin (Table 2).

Sequence analysis of the *Pseudoalteromonas* S91 chitinase-encoding clones

Analysis of the DNA sequence of the 4.5 kb S91 insert in pCHT1 revealed a single ORF of 2583 nucleotides, designated *chiP*. This *chiP* ORF encoded a polypeptide of 861 amino acids with a calculated molecular mass of 96 kDa. The nucleotide sequence of the 5 kb S91 DNA insert in pCHB1 was determined. An ORF of 2352 bp, designated *chiQ*, was identified. The *chiQ* ORF encoded a polypeptide of 784 amino acids with a calculated molecular mass of 88 kDa.

Based on Southern hybridization, restriction enzyme analysis (data not shown) and DNA sequence information, physical maps of pCHT2, pCHT7, pCHT8 and pCHT9 were constructed as shown in Fig. 1(a). Plasmids pCHT2 and pCHT7 contained a DNA insert of 6 kb showing identical restriction enzyme sites. The orientation of the DNA insert in pCHT7 was in the opposite orientation to that in pCHT2 (Fig. 1a).

Sequence analysis of DNA inserts in pCHT2, pCHT8 and pCHT9 revealed overlapping inserts containing three ORFs with a total length of 7600 bp, organized in order *chiA*, *chiB* and *chiC* with the same transcriptional

Table 2. Activity of recombinant *E. coli* clones and derivatives against fluorescent chitin oligomer analogues and chitin

| Plasmid in recombinant clone | Size of DNA insert (kb) | Activity against chitin substrates | | | |
|------------------------------|-------------------------|---|--|------------------------------|---------|
| | | 4-MU-(GlcNAc) ₃ (endochitinase) | 4-MU-(GlcNAc) ₂ (exochitinase) | 4-MU-GlcNAc (chitobiase*) | Chitin† |
| pCHB1 | 5.0 | — | — | + | — |
| pCHT1 | 4.5 | + | + | + | — |
| pCHT2 | 6.0 | + | + | — | ++ |
| pCHT21 | 2.5 | — | — | — | — |
| pCHT22 | 3.5 | + | + | — | ++ |
| pCHT23 | 3.5 | + | + | — | ++ |
| pCHT7 | 6.0 | + | + | — | ++ |
| pCHT8 | 5.9 | + | + | — | + |
| pCHT9 | 6.5 | + | + | — | ++ |

* Chitobiase = *N*-acetylglucosaminidase.

† Chitin digestion as shown by a clearing zone around a colony grown on colloidal chitin agar: + or ++, clearing (activity); —, no clearing (no activity).

direction (Fig. 1a). pCHT2 contained a partial *chiA*, complete *chiB* and a truncated *chiC* ORF. The one complete ORF of 1578 bp, *chiB*, encoded a polypeptide of 525 amino acids (calculated molecular mass 56 kDa); the downstream truncated ORF of 2436 bp, *chiC*, encoded a truncated polypeptide of 813 amino acids and the upstream partial ORF of 1920 bp encoded partial *chiA* (Fig. 1a).

pCHT8 contained a full-length *chiA* of 3165 bp encoding a protein of 1054 amino acids (calculated molecular mass 113 kDa), which overlapped with, and extended further upstream of, the insert of pCHT2 (Fig. 1a). The DNA insert in pCHT9 contained the full-length *chiC* of 2610 bp encoding a protein of 869 amino acids (calculated molecular mass 93 kDa), which overlapped with, and extended downstream of, the insert of pCHT2 (Fig. 1a). A 740 bp region previously sequenced from the chitinase-negative, transposon-interrupted mutant, S91CX (Techkarnjanaruk *et al.*, 1997) was found to be identical with part of the sequence of *chiA*. The complete DNA sequence of *chiABC* revealed that the transposon had inserted into *chiA* at nucleotide 2284 from the ATG start codon.

Similarity searches were carried out between the deduced amino acid sequences of ChiA, ChiB, ChiC, ChiP and ChiQ of S91. ChiA, ChiB and ChiC showed homology to each other in specific regions as described below. Significant overall homology was found between ChiP and ChiQ, with 26% identity and 51% similarity at the amino acid level. No sequence homology of either ChiP or ChiQ to ChiA, ChiB and ChiC was found, either overall or over short regions. Nucleotide sequences upstream and downstream of *chiP* (pCHT1) and *chiQ* (pCHB1), upstream of *chiA* (pCHT8) and downstream of *chiC* (pCHT9) (at least 500 bp each way) showed no significant similarity to any chitinase genes in the

databases using the BLAST program (Altschul *et al.*, 1990) (data not shown).

Separation of *chiB* and *chiC* ORFs

Further subcloning of pCHT2 was carried out to separate the ORFs *chiB* and truncated *chiC*, resulting in pCHT21 and pCHT22 (Fig. 1b). Plasmid pCHT21 contained partial *chiA*, complete *chiB* and 930 bp of *chiC*, which had lost part of the putative catalytic as well as the whole of the chitin-binding domains (Fig. 1b). *E. coli*/pCHT21 showed no chitinase activity against fluorescent chitin substrates or colloidal chitin, in contrast to *E. coli*/pCHT2 and *E. coli*/pCHT22 (Table 2). Plasmid pCHT22 contained a functional, although truncated, 2436 bp of *chiC* (putative catalytic and chitin-binding-domains remained the same as in pCHT2) as well as a partial *chiB* (Fig. 1b). The presence of an S91 promoter capable of expressing in *E. coli* on the truncated *chiC* (pCHT22) was shown by reversing the orientation of the insert in relation to the *lacZ* promoter on the vector. This was achieved by subcloning the 3.5 kb insert DNA from pCHT22 into pBCSK⁺; the resulting plasmid, pCHT23 (Fig. 1b) also possessed chitinase activity (Table 2). This was consistent with the result found for pCHT9 (containing complete *chiC*), in which the direction of transcription of *chiC* was shown, by DNA sequence analysis, to be in the opposite direction to the vector's *lacZ* promoter (Fig. 1a); *E. coli*/pCHT9 was active against chitin analogues and was able to degrade colloidal chitin (Table 2).

Analysis of ChiP and ChiQ in *E. coli*

Proteins from the extracellular, periplasmic and cytoplasmic fractions produced by *E. coli*/pCHT1, encoding ChiP, were separated and analysed on duplicate SDS-

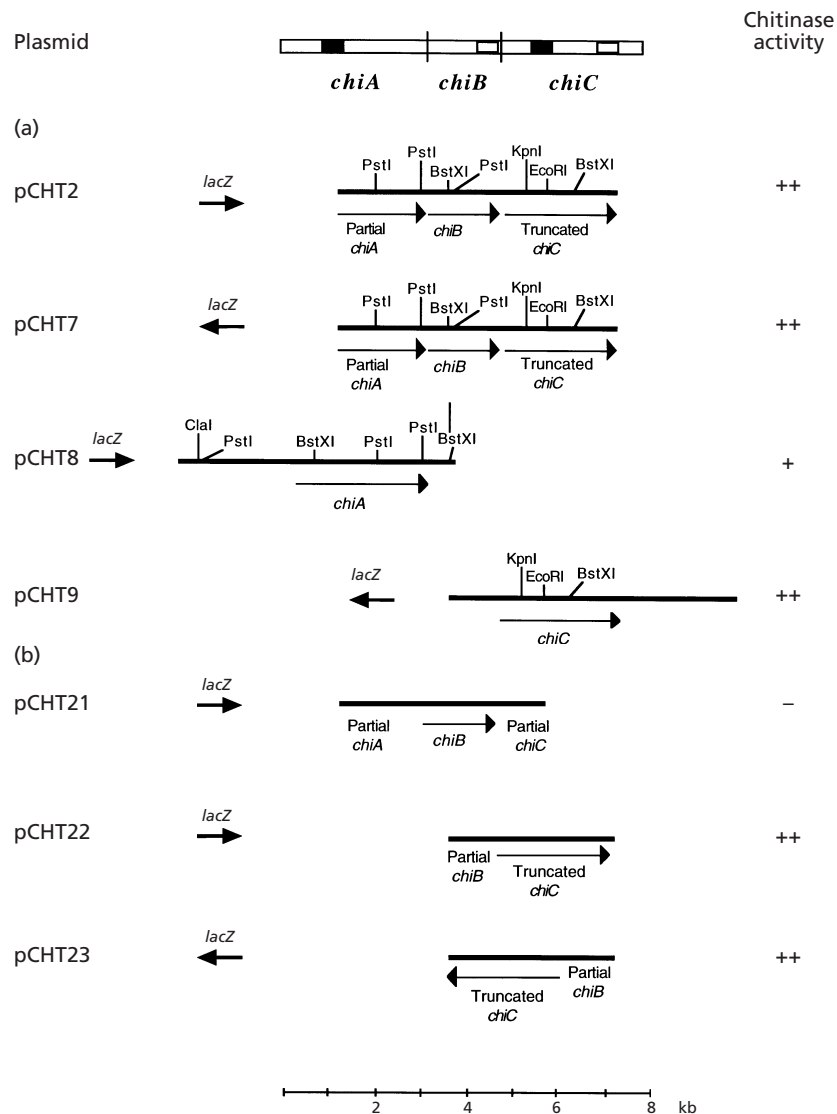


Fig. 1. Restriction maps of 591 DNA in (a) the chitinase-positive clones, pCHT2, pCHT7, pCHT8 and pCHT9, and (b) the pCHT2 derivatives pCHT21, pCHT22 and pCHT23. The direction of the cloning vector's *lacZ* promoter (arrow) is indicated. The location and direction of ORFs are shown by the arrows below the map. Putative catalytic (■) and putative chitin-binding (□) domains are shown.

PAGE gel sets. Putative ChiP was observed in the cytoplasmic and periplasmic fractions and none was detected in the extracellular fraction (data not shown). The periplasmic protein fraction contained a novel protein, putative ChiP, with a molecular mass of about 94 kDa (Fig. 2a, lanes 3, 5 and 7). The 94 kDa protein was active against MU-(GlcNAc)₃, 4-MU-(GlcNAc)₂ and 4-MU-GlcNAc (exochitinase, endochitinase and *N*-acetylglucosaminidase activity) as observed on the activity gel (Fig. 2b, lanes 3, 5 and 7).

Several attempts to isolate and locate *N*-acetylglucosaminidase activity in subcellular fractions of *E. coli*/pCHB1, encoding ChiQ, were unsuccessful. No *N*-acetylglucosaminidase activity could be detected in any fraction on activity gels, either with or without boiling

of protein samples, using 4-MU-GlcNAc. However, *E. coli*/pCHB1 colonies showed activity against this substrate in the agar plate assay (Table 2). Neither ChiP nor ChiQ was able to degrade colloidal chitin (Table 2).

Similarity searches with the deduced amino acid sequences in the databases revealed that ChiP and ChiQ showed significant homology to a number of related proteins in family 20 of the glycosyl hydrolases, either bacterial *N*-acetylglucosaminidases or eukaryotic hexosaminidases (Henrissat & Bairoch, 1993). ChiP showed highest identity (40%) and similarity (61%) to the chitobiase of *V. harveyi* (Soto-Gill & Zyskind, 1989). ChiQ showed highest identity (34%) and similarity (56%) to the *N*-acetylhexosaminidase of *V. furnissii* (Keyhani & Roseman, 1996).

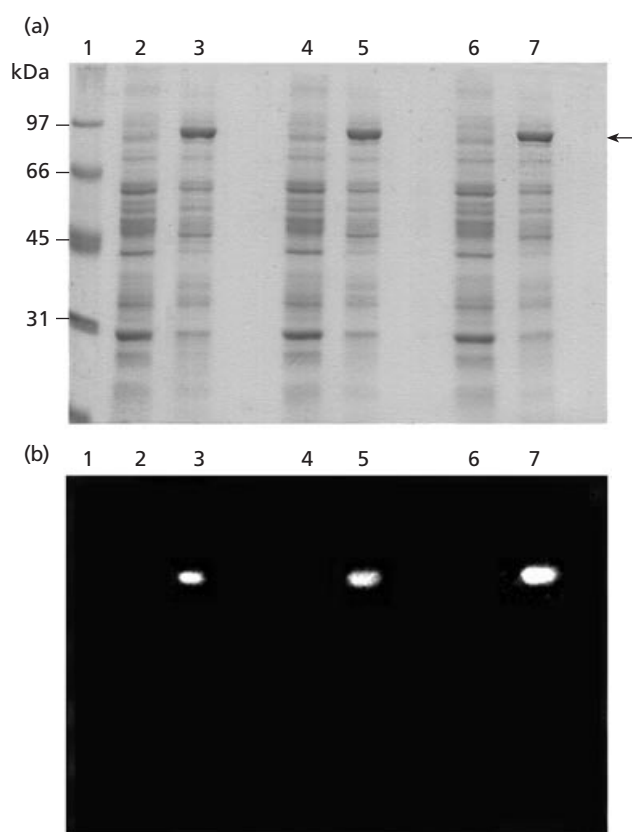


Fig. 2. SDS-PAGE analysis of periplasmic protein fractions of *E. coli*/pCHT1. (a) Coomassie brilliant blue stained 10% SDS-polyacrylamide gel. (b) Chitinase activity detected on the polyacrylamide gel. Samples (5 µg total protein) were the periplasmic protein fractions of: *E. coli* XL0LR host strain (lane 2) and *E. coli*/pCHT1 (lane 3) tested with 4-MU-(GlcNAc)₃; *E. coli* XL0LR (lane 4) and *E. coli*/pCHT1 (lane 5) tested with 4-MU-(GlcNAc)₂; *E. coli* XL0LR (lane 6) and *E. coli*/pCHT1 (lane 7) tested with MU-GlcNAc. Lane 1, molecular size standards: rabbit muscle phosphorylase *b* (97.4 kDa), bovine serum albumin (66.2 kDa), hen egg white ovalbumin (45.0 kDa) and bovine carbonic anhydrase (31.0 kDa).

Analysis of ChiA, ChiB and ChiC in *E. coli*

In order to determine the cellular location of chitinolytic proteins in *E. coli* carrying either *chiA* (pCHT8), *chiB* (pCHT21) or *chiC* (pCHT9), exponential-phase cultures were fractionated and the extracellular, periplasmic and cytoplasmic fractions were analysed separately by SDS-PAGE. Chitinase proteins were observed in the cytoplasmic and periplasmic fractions and none was detected in the extracellular fraction (data not shown). The periplasmic protein fraction from *E. coli*/pCHT8, encoding ChiA only, showed a relatively weak chitinolytic activity band of high molecular mass estimated about 120 kDa (Fig. 3a, b, lane 3). No protein band with chitinolytic activity was observed in the periplasmic protein fraction from *E. coli*/pCHT21, encoding a complete ChiB, although a novel protein of about 60 kDa was observed on the gel stained with Coomassie blue (Fig. 3a, b, lane 4). The periplasmic

fraction of *E. coli*/pCHT9, encoding complete ChiC only, showed two chitinolytic regions with relatively strong activity, with the larger molecular mass region possibly containing two bands (Fig. 3b, lane 5). On the gel stained with Coomassie blue, two novel protein bands of about 90 and 60 kDa were apparent in *E. coli*/pCHT9 (Fig. 3a, lane 5). No chitinase activity band was found in the periplasmic protein fractions from control strain *E. coli* XL0LR (Fig. 3a, b, lane 2).

The periplasmic protein fractions from *E. coli*/pCHT2, *E. coli*/pCHT21, *E. coli*/pCHT22 and *E. coli*/pCHT8 were also tested for their ability to bind to colloidal chitin. Two proteins, of about 80 and 60 kDa, were found in the periplasmic protein fraction of *E. coli*/pCHT2, encoding complete ChiB and truncated ChiC, after adsorption to chitin (Fig. 4, lanes 3 and 4). A protein of about 60 kDa was purified from the periplasmic protein fraction of *E. coli*/pCHT21, encoding the complete ChiB only, after adsorption to chitin (Fig. 4, lanes 5 and 6). The 80 kDa chitinolytic protein from the periplasmic fraction of *E. coli*/pCHT22, encoding truncated ChiC only, was also able to bind to chitin (Fig. 4, lanes 7 and 8). No chitin-binding protein was obvious in the periplasmic protein fraction of *E. coli*/pCHT8, encoding ChiA only, after adsorption to chitin particles (Fig. 4, lanes 9 and 10).

Sequence analysis

The S91 *chiABC* gene cluster encoded three chitinase proteins, each one having significant homology to chitinases belonging to family 18 of the glycosyl hydrolases (Henrissat & Bairoch, 1993). From the deduced amino acid sequence ChiA (113 kDa) was most homologous to the chitodextrinase of *V. furnissii* (Keyhani & Roseman, 1996) with 49% identity and 65% similarity and contained a putative catalytic domain in the middle region (Fig. 1a). This region was also highly conserved among catalytic domains of other bacterial chitinases, as detailed by Watanabe *et al.* (1993, 1994b). No sequence similarity to chitin-binding domains of other chitinases was found in the deduced amino acid sequence of ChiA (Fig. 1a, b), which was consistent with analysis of ChiA in *E. coli* (Fig. 4). At the amino acid level, the S91 ChiB showed significant similarity to known chitin-binding domains identified in some bacterial chitinases, as well as to the cellulose-binding domains of *Bacillus* sp. cellulases, CelA and CelB (family 5 of the glycosyl hydrolases) (Fukumori *et al.*, 1986), as described in detail for the ChiA of *V. harveyi* by Svitil & Kirchman (1998). No catalytic domain was found in the deduced amino acid sequence of the S91 ChiB (Fig. 1a), consistent with findings for ChiB in *E. coli* (Table 2, Fig. 3). The deduced amino acid sequence of ChiC (93 kDa) also showed significant homology to other chitinases, the highest being 66% identity and 79% similarity to the ChiA of *Alteromonas* sp. strain O-7 (Tsujiro *et al.*, 1993b). A putative catalytic domain and a chitin-binding domain were found in the S91 ChiC at amino acid residues 245–345 and 775–820,

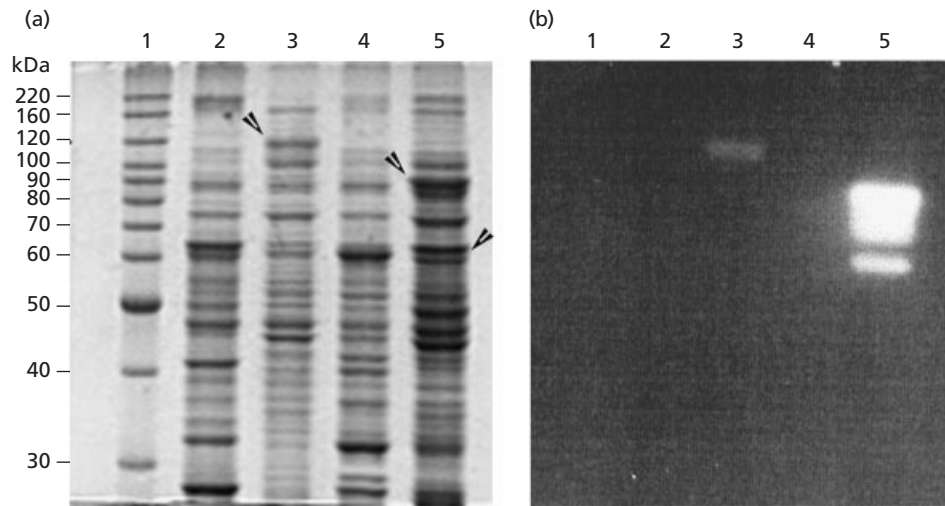


Fig. 3. SDS-PAGE analysis of periplasmic protein fractions of *E. coli* clones. (a) Coomassie brilliant blue stained 10% SDS-polyacrylamide gel. (b) Chitinase activity detected on the polyacrylamide gel using 4-MU-(GlcNAc)₃. Samples (5 µg total protein) were the periplasmic protein fractions of: *E. coli* XL0LR host strain (lane 2), *E. coli*/pCHT8 (*chiA*, lane 3), *E. coli*/pCHT21 (*chiB*, lane 4) and *E. coli*/pCHT9 (*chiC*, lane 5). Lane 1, Molecular size standards (Benchmark standard protein, Gibco-BRL).

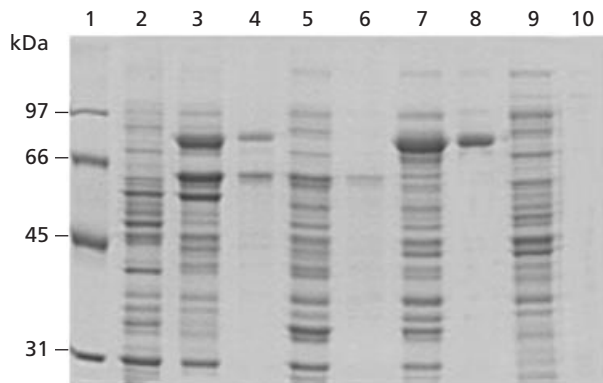


Fig. 4. Coomassie brilliant blue stained 10% SDS-polyacrylamide gel. Samples (5 µg total protein) were the periplasmic protein fractions of: *E. coli* XL0LR host strain (lane 2), *E. coli*/pCHT2 (lane 3), *E. coli*/pCHT2 after adsorption to chitin particles (lane 4), *E. coli*/pCHT21 (lane 5), *E. coli*/pCHT21 after adsorption to chitin particles (lane 6), *E. coli*/pCHT22 (lane 7), *E. coli*/pCHT22 after adsorption to chitin particles (lane 8), *E. coli*/pCHT8 (lane 9) and *E. coli*/pCHT8 after adsorption to chitin particles (lane 10). Lane 1, molecular size standards (details as for Fig. 2).

respectively (Fig. 1a), consistent with findings for ChiC in *E. coli* (Table 2, Figs 3 and 4).

Proteolytic processing of chitinase proteins after their secretion has been found to be common, for example, as shown for *B. circulans* WL-12 (Watanabe *et al.*, 1990) and *Alteromonas* sp. strain O-7 (Tsujibo *et al.*, 1993b), resulting in more than one chitinolytic protein being produced from one gene. The calculated molecular mass of the deduced amino acid sequence of ChiC was 93 kDa, whereas the chitinase activity gels of the

supernatant of S91 (76 and 64 kDa; Techkarnjanaruk *et al.*, 1997) and the periplasmic fractions of *E. coli* encoding ChiC (80 and 60 kDa; this study) showed at least two chitinase activity regions which were smaller in size. This is similar to the findings reported for *Alteromonas* sp. strain O-7 (Tsujibo *et al.*, 1993b). Protein analysis of purified ChiA (65 kDa) from the supernatant of *Alteromonas* sp. strain O-7 and from the periplasmic fraction of an *E. coli* clone producing ChiA (85 and 78 kDa) showed that the chitinase protein bands were smaller than the calculated molecular mass (87 kDa) of the complete sequence (Tsujibo *et al.*, 1993b). The N-terminal sequences of the *Alteromonas* sp. strain O-7 ChiA, Chi-85 and Chi-78 were identical, indicating that three forms of enzyme differing in size were the result of partial proteolysis occurring at the C-terminal region. Because of the high homology of the S91 *chiC* to the *Alteromonas* sp. strain O-7 *chiA* together with the similar results obtained from the protein analysis, it is possible that extracellular chitinases from S91 may also undergo similar proteolytic processing.

Catalytic domains

Multiple amino acid sequence alignment of S91 ChiA and S91 ChiC with other bacterial chitinases showed significant homology in several regions, particularly in the catalytic domain (data not shown). The residues Asp-197, Asp-200, Asp-202 and Glu-204 in the *B. circulans* WL-12 chitinase A (corresponding to Asp-474, Asp-477, Asp-479 and Glu-481 in the S91 ChiA and Asp-305, Asp-308, Asp-310 and Glu-312 in the S91 ChiC) have been shown to be essential for chitinase activity by site-directed mutagenesis (Watanabe *et al.*, 1993, 1994b). The putative catalytic domains of S91 ChiA and S91 ChiC contained all four appropriately spaced amino

acid residues as found in the catalytic domains of chitinase A1 of *B. circulans* WL-12 chitinase and ChiA of *Alteromonas* sp. strain O-7 (Tsujibo *et al.*, 1993a). Interestingly, the *V. furnissii* chitodextrinase, which had highest homology to the S91 ChiA but which contained only three of these residues, was active against some chitin oligosaccharides but not against chitin (Keyhani & Roseman, 1996). It is possible, therefore, that the lack of one of the four amino acid residues essential for catalytic activity may contribute to the inability of the *V. furnissii* chitodextrinase to degrade chitin.

Chitin-binding domains

The S91 ChiB and S91 ChiC proteins were able to bind specifically to colloidal chitin, although no chitinolytic activity was detected for S91 ChiB. The deduced amino acid sequence of ChiB showed no catalytic domain, which was in agreement with all chitinase activity tests being negative for *E. coli*/pCHT21 carrying *chiB* only (Table 2). Analysis of the deduced amino acid sequences of the S91 ChiB and S91 ChiC revealed regions with significant similarity to known chitin-binding domains of several chitinases including, for example, the C-terminal ends of the *Clostridium paraputrificum* ChiB (Morimoto *et al.*, 1997), the *B. circulans* WL-12 ChiA (Watanabe *et al.*, 1994a) and the *V. harveyi* ChiA (Svitil & Kirchman, 1998) proteins, which have been shown experimentally to have chitin-binding activity. No sequence similarity to chitin-binding domains of other chitinases was found in the S91 ChiA, consistent with the result that S91 ChiA, produced in *E. coli*, showed no chitin-binding activity.

The function of S91 ChiB, lacking chitinolytic activity, in chitin degradation is unclear, but it may involve helping bind S91 cells to chitin surfaces as previously shown for *V. harveyi* (Montgomery & Kirchman, 1993). These authors found that attachment of a chitinase-overproducing *V. harveyi* mutant to chitin was about twice as much as that of the wild-type and that the mutant overproduced a 53 kDa chitin-binding protein which showed no chitinase activity (Montgomery & Kirchman, 1993). The gene encoding this 53 kDa chitin-binding protein has not yet been identified. These data are consistent with those found for *Streptomyces olivaceoviridis*, in which a 20 kDa extracellular chitin-binding protein (CHB1) lacking chitinolytic activity has been identified (Schnellmann *et al.*, 1994). It was suggested that the function of CHB1 is to assist chitinase enzymes in hydrolytic digestion of bound chitin. Production of CHB1, which binds specifically to crystalline α -chitin, is induced by chitin (Schnellmann *et al.*, 1994). The CHB1 DNA sequence has been determined and its deduced amino acid sequence shows no apparent similarity to either catalytic or chitin-binding domains of family 18 of the glycosyl hydrolases but does show similarity to cellulose-binding domains of cellulases (Schnellmann *et al.*, 1994). Recently, a chitin-binding protein (CBP21, 21 kDa) which adsorbs to chitin but has no chitinase activity was isolated from *Serratia*

marcescens 2170 (Watanabe *et al.*, 1997). The gene encoding CBP21 was cloned, sequenced and found to be located approximately 1.5 kb downstream of a previously isolated chitinase, *chiB*, in *S. marcescens* 2170 (Suzuki *et al.*, 1998). CBP21 showed significant homology to the CHB1 of *Streptomyces olivaceoviridis*. CBP21 showed its highest binding activity to squid pen (containing β -chitin) followed by colloidal chitin and regenerated chitin (Suzuki *et al.*, 1998).

Organization and regulation of bacterial chitinase genes

Reports on the sequences of genes in the chitinase systems of *Serratia liquefaciens* (Joshi *et al.*, 1988), *Aeromonas* sp. no. 10S-24 (Shiro *et al.*, 1996), *Bacillus circulans* WL-12 (Watanabe *et al.*, 1992), *Serratia marcescens* 2170 (Suzuki *et al.*, 1998; Watanabe *et al.*, 1997) and *Pseudoalteromonas* S91 (this study), are all that is known about the organization of chitinase genes in bacteria. So far, only one study has identified genes involved in the regulation of chitin degradation (Joshi *et al.*, 1988). These proposed regulatory genes are part of a cluster of chitinase genes in *S. liquefaciens* (Joshi *et al.*, 1988), but no nucleotide sequence information or protein products of these genes have yet been reported. In a previous study, a chitinase-negative mutant, S91CX, unable to digest colloidal chitin was obtained by transposon mutagenesis of *Pseudoalteromonas* S91 (Techkarnjanaruk *et al.*, 1997). Two extracellular chitinolytic proteins, of about 76 and 64 kDa, present in culture supernatants of S91 grown in minimal medium with either chitin or *N*-acetylglucosamine, were obviously missing from culture supernatants of S91CX grown under the same conditions (Techkarnjanaruk *et al.*, 1997). It was thought that these proteins were produced by the gene interrupted by the transposon, particularly since 740 bp of sequence of the gene downstream of the transposon had high homology to other known chitinases (Techkarnjanaruk *et al.*, 1997). The present study showed that the transposon had interrupted the *chiA* gene in S91CX and the 76 and 64 kDa chitinolytic proteins missing in the culture supernatant of S91CX were probably the products of *chiC*, not *chiA*, meaning that transposon interruption of *chiA* resulted in loss of ChiC as well as ChiA. Since the S91 *chiA* gene was located upstream of the *chiB* and *chiC* genes, the simplest explanation for the loss of chitinase activity in S91CX would be that *chiABC* was an operon. However, results from analysis of the genes cloned individually showed that the S91 *chiC* gene was able to be expressed from its own promoter in *E. coli*/pCHT23 and *E. coli*/pCHT9 (Table 2). It is possible that the ChiA protein, showing chitinase activity in *E. coli*, regulated expression of *chiC* in S91 in some way. Further studies are under way to investigate the expression of the individual *chi* genes in S91.

Little is known about expression of individual chitinases, possibly because there are usually multiple genes in bacteria. Studies investigating activity of

chitinases generally assay the total activity of different enzymes which may be regulated differently, and the contribution of any one particular gene at a given time is not known. In order to investigate the expression as well as the contribution to chitin degradation of the individual genes, a promoterless reporter gene can be inserted under the control of each *chi* gene promoter. Techkarnjanaruk *et al.* (1997) used this approach to show that expression of a *chi* promoter in S91, now known from the current study to be the *chiA* promoter, is induced by colloidal chitin, GlcNAc, early starvation phase and increased CO₂ levels, is not catabolite repressed by glucose but is repressed by rich medium. Further, Stretton *et al.* (1998) showed that this *chiA* promoter appeared to be expressed strongly in S91CGFP cells growing in microcolonies on the surface of a natural biodegradable substratum, squid pen. Work is under way to clone a *gfp* reporter gene (Cormack *et al.*, 1996) under the control of the putative promoter regions from each of the *chiA*, *chiB* and *chiC* genes and transfer these constructs to S91 for quantitative expression studies. Also each wild-type S91 *chiA*, *chiB* and *chiC* gene is being subcloned to a suitable vector (Stretton *et al.*, 1998) and transferred to S91CX (Techkarnjanaruk *et al.*, 1997) to assess restoration of chitinase activity, and to S91 to assess over production of each gene product individually.

Svitil *et al.* (1997) suggested that *V. harveyi* produces different chitinase enzymes to hydrolyse different forms of chitin. Higher growth rates and more chitinase activity were observed when cells were grown on β -chitin from squid pen than on α -chitin from snow crab (Svitil *et al.*, 1997). *V. harveyi* was able to secrete at least 10 different chitinase enzymes depending on which type of chitin the cells were grown (Svitil *et al.*, 1997). Chitinase A and a chitin-binding protein (CBP21) of *S. marcescens* 2170 have different levels of binding activity toward different chitin types: squid pen (β -chitin) is the best substrate for binding CBP21, but not for chitinase A (Suzuki *et al.*, 1998). The current study found that, in *E. coli*, the S91 ChiC appeared to have stronger activity against colloidal chitin than the S91 ChiA did (Fig. 3). It is known that expression of *chiA* is induced more by GlcNAc than by colloidal chitin (Techkarnjanaruk *et al.*, 1997). Further, the S91 *chiA* gene promoter appears to express the reporter *gfp* gene strongly in cells growing on squid pen (β -chitin) (Stretton *et al.*, 1998). It is possible that the S91 chitinases also have different activities against different chitin substrates. Now that the individual *chi* genes of S91 have been cloned, it may be possible to construct further *chi*-reporter gene fusions with which the response of each *chi* gene to different chitin substrates can be quantified.

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