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Chitinase B from *Serratia marcescens* BJL200 is exported to the periplasm without processing

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A gene encoding a chitinase from *Serratia marcescens* BJL200 was cloned and expressed in *Escherichia coli* and *S. marcescens*. Nucleotide sequencing revealed an open reading frame encoding a 55·5 kDa protein of 499 amino acids without a typical signal peptide for export. The cellular localization of the chitinase was studied, using two types of cell fractionation methods and immunocytochemical techniques. These analyses showed that the chitinase is located in the cytoplasm in *E. coli*, whereas it is exported to the periplasm in *S. marcescens*. Analysis of chitinase isolated from periplasmic fractions of *S. marcescens* carrying the cloned gene showed that export of the enzyme is not accompanied by processing at the N-terminus. The chitinase did not show any of the characteristics that have been proposed to direct the export of other non-processed extracellular proteins such as the *E. coli* haemolysin and might therefore be secreted via a hitherto unknown mechanism.

Keywords: *Serratia marcescens*, chitinase, export, signal peptide

INTRODUCTION

Several bacteria and fungi are capable of enzymic degradation of chitin, the (1 → 4)-β-linked polymer of *N*-acetyl-β-D-glucosamine (GlcNAc), for autolytic, morphogenetic or nutritional purposes (Gooday, 1990). The chitinolytic enzyme system is composed of chitinases (EC 3.2.1.14), chitobiosidases (Tronsmo & Harman, 1993) and *N*-acetyl-β-glucosaminidases (EC 3.2.1.52). Chitinases cleave glycosidic linkages randomly along the chitin chain, eventually giving *N*,*N*′-diacetyl-β-D-chitobiose [$(GlcNAc)_2$] as endproduct (Monreal & Reese, 1969), while chitobiosidases cleave off $(GlcNAc)_2$ units from the non-reducing end of the polysaccharide chain (Tronsmo & Harman, 1993). The dimer $(GlcNAc)_2$ is further hydrolysed to GlcNAc residues by *N*-acetyl-β-glucosaminidase (Monreal & Reese, 1969).

Serratia marcescens, a Gram-negative bacterium belonging to the family *Enterobacteriaceae*, is one of the most efficient bacteria for degradation of chitin (Monreal & Reese, 1969; Schickler *et al.*, 1993). Production of chitinolytic enzymes in *S. marcescens* is induced by the presence of chitin in the culture medium (Monreal & Reese, 1969). Fuchs *et al.* (1986) showed that *S. marcescens* strain QMB1466 produced five different chitinolytic enzymes upon induction with chitin. Two chitinase genes (*chiA* and *chiB*) have been isolated from this organism and their nucleotide sequences have been determined (Jones *et al.*, 1986; Harpster & Dunsmuir, 1989). A chromosomal fragment encoding a 95 kDa *N*-acetyl-β-glucosaminidase was isolated and characterized by Kless *et al.* (1989); however, no sequence was reported for this enzyme. Sundheim *et al.* (1988) cloned two chromosomal fragments encoding chitinolytic activity from *S. marcescens* BJL200. In the present study we have analysed one of these fragments and determined the nucleotide sequence of the chitinase gene located on it.

Previous studies of *S. marcescens* chitinases have in most cases not clearly revealed the cellular location of the enzymes and possible export mechanisms. Chitinolytic enzymes are normally found in the culture medium, suggesting that they are secreted. However, the chitinolytic enzymes were isolated from the culture medium after prolonged culturing times (6–8 d) (Monreal & Reese, 1969; Roberts & Cabib, 1982; Fuchs *et al.*, 1986; Jones *et al.*, 1986). Conceivably the presence of these enzymes in the culture medium may have occurred as a result of cell lysis. Generally, the translocation of bacterial proteins across the cytoplasmic membrane is directed by an N-terminal signal peptide which is removed during or shortly after the translocation step. In Gram-negative

Abbreviation: 4-MU-$(GlcNAc)_2$, 4-methylumbelliferyl-β-D-*N*,*N*′-diacetylchitobioside.

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bacteria, an additional step is required to transport the protein across the outer membrane. Alternatively the two membranes are crossed in a single step. The latter mechanism involves proteins which lack a typical N-terminal signal peptide, and which are not processed during translocation (reviewed by Lory, 1992; Wandersman, 1992; Pugsley, 1993). In the present study we used cell fractionation and immunocytochemical methods to study the location of the cloned *S. marcescens* BJL200 chitinase in *E. coli* and *S. marcescens*. These studies provide unequivocal evidence that the chitinase is exported to the periplasm in *S. marcescens* without the help of a signal peptide and without any processing.

METHODS

Bacterial strains and culture conditions. *Escherichia coli* DH1 and DH5α (Hanahan, 1983) were grown at 37 °C in Luria Broth (LB). *Serratia marcescens* BJL200 (Sundheim *et al.*, 1988) was grown at 30 °C in LB. For plates, LB was solidified with 1·5% (w/v) agar. Ampicillin was added at the following final concentrations: 50 μg ml^{-1} for *E. coli* and 300 μg ml^{-1} for *S. marcescens*.

Enzymes. DNA-modifying enzymes were obtained from New England Biolabs and were used with buffers and protocols supplied with the enzymes.

DNA techniques. Recombinant DNA work was done by using standard protocols (Sambrook *et al.*, 1989). All fragments for cloning were separated by agarose gel electrophoresis and purified with Geneclean (BIO101). Transformation of *E. coli* and *S. marcescens* was carried out by electroporation according to the protocol of Dower *et al.* (1988), using a Bio-Rad Gene Pulser.

Deletion plasmids and subclones. pLES3-1, pLES3-2 and pLES3-3 (Fig. 1) were constructed by deleting a *Mlu*I, *Sac*I and *Hpa*I fragment, respectively, from pLES3 (Fig. 1). pMAY1 (Fig. 1) was constructed by recloning the 2·3 kb *Eco*RI–*Sac*I fragment in pGEM-7f(+)Z (Promega) digested with the same enzymes. pMAY2-10 (Fig. 1) was constructed by cutting pLES3 with *Mlu*I, filling in the 3′ recessed termini with Klenow fragment of DNA polymerase I, digesting with *Eco*RI and recloning the 1·8 kb *Eco*RI–*Mlu*I fragment in pGEM-7f(+)Z cut with *Eco*RI and *Sma*I.

DNA sequence analysis. A 1·8 kb *Xba*I–*Hin*dIII fragment from pMAY2-10 (Fig. 1) was subcloned into vector M13mp18 and M13mp19 (Yanisch-Perron *et al.*, 1985) cut with the same enzymes. The nucleotide sequence was obtained by sequencing single-stranded DNA by the dideoxy chain-termination method (Sanger *et al.*, 1977) using the Sequenase DNA sequencing kit (United States Biochemical) and [^{35}S]dATP-αS (Amersham). The DNA sequence was determined starting from the universal primer, and additional primers were designed with the newly obtained sequence information using a walking strategy. Synthetic oligonucleotide primers were synthesized on an Applied Biosystems 380/381 DNA synthesizer. Ambiguous sequences due to band compression were resolved by running formamide gels (40%, v/v) as described by the supplier of the sequencing chemicals [USB Comments 17(1), 1990]. Computer-assisted sequence analysis was performed using PCGENE microcomputer software (IntelliGenetics). Searches for nucleotide and amino acid sequence similarities were done with the FASTA program (Pearson & Lipman, 1988) in the EMBL and Swiss-Prot databases. Comparison of protein sequences was performed using the computer programs COMPARE, BESTFIT, and PILEUP of the University of Wisconsin Genetics Computer Group.

Fractionation of *E. coli* and *S. marcescens* cells. Cells were harvested in the exponential growth phase (OD_{600} ~ 0·7) and the supernatant saved as the extracellular fraction. Because some cytoplasmic proteins are released into the periplasmic fraction by an osmotic shock (reviewed by Oliver, 1987), two methods were used for the preparation of periplasmic fractions. In method A the periplasmic fraction was prepared by osmotic shock (Manoil & Beckwith, 1986). In method B the periplasmic fraction was prepared by converting the cells to spheroplasts by the action of lysozyme and EDTA (Koshland & Botstein, 1980). Cytoplasmic fractions were obtained by disrupting with glass beads the cell pellet remaining after osmotic shocking or lysozyme treatment, as described by van de Guchte *et al.* (1990).

Determination of protein concentration. Protein concentrations were determined by the method of Bradford (1976), with bovine serum albumin as a standard.

Assays for detection of chitinolytic activity

(i) Plate assay. Chitinolytic activity was initially scored by the ability of a bacterial colony to produce a halo of clearing on a chitin containing agar plate, prepared by overlaying a 15 ml M9 (minimal salts, Gibco) agar base with 10 ml 1·5% (w/v) agar and 1·5% (w/v) colloidal chitin (Vessey & Pegg, 1973).

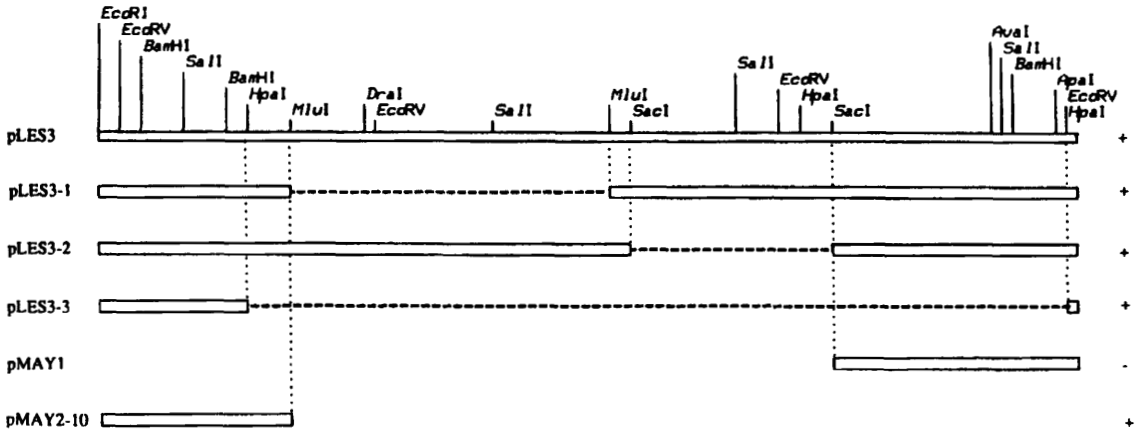

Fig. 1. Schematic representation of the 9·2 kb chitinase-coding *Eco*RI fragment isolated from *S. marcescens* BJL200, and the deletion clones constructed from this fragment. Chromosomal DNA is indicated by open boxes, and deletions are indicated by broken lines. Chitinolytic activity, as scored by the plate assay, is indicated by + on the right.

(ii) Cleavage of 4-methylumbelliferyl-β-D-*N,N'*-diacetylchitobioside [4-MU-(GlcNAc)$_2$], a chitin trimer analogue. Cell-free extracts were tested for chitinolytic activity against 4-MU-(GlcNAc)$_2$ (Sigma) (Kuranda & Robbins, 1987). The standard reaction mixture contained 70 μM 4-MU-(GlcNAc)$_2$ in 50 mM citrate phosphate buffer, pH 6·0 (total volume of 50 μl). The reaction mixture was incubated at 37 °C, and after 10 min the reaction was stopped by adding 1·95 ml 0·2 M Na_2CO_3. The 4-methylumbelliferone (4-MU) moiety is fluorescent when it becomes ionized above pH 8 and after excitation at 380 nm, it emits at a wavelength of 460 nm. The amount of 4-MU released was determined with a TKO 100 Mini Fluorimeter (Hoefer Scientific Instruments). Chitinolytic activity is given in nmol 4-MU released min^{-1} (ml culture)$^{-1}$.

(iii) Detection of chitinolytic activity after SDS-PAGE. SDS-PAGE (10%, w/v, polyacrylamide gel) was performed using a Bio-Rad Protean II minigel system essentially according to the method of Laemmli (1970), but modified with addition of 0·01% glycol chitin to the running gel (Trudel & Asselin, 1989). After electrophoresis, the gel was incubated for 3 × 20 min in 1% (v/v) Triton X-100, 25 mM Tris buffer (pH 7·5), 20 mM $MgCl_2$ at room temperature, followed by overnight incubation in 0·1% Triton X-100, 25 mM Tris buffer (pH 7·5), 20 mM $MgCl_2$, to allow renaturation of proteins. The gel was then washed in 100 mM phosphate buffer (pH 6) for 3 × 20 min, stained with Calcofluor White M2R (Sigma), destained and visualized by UV illumination, as described by Trudel & Asselin (1989). Proteins with chitinolytic activity appeared as dark bands on a fluorescent background.

β-Galactosidase assay. β-Galactosidase activity was measured in the fractions of *E. coli* DH1 by the method of Miller (1972) and expressed as nmol *o*-nitrophenol released min^{-1} (ml culture)$^{-1}$.

Aldolase assay. Aldolase activity in the fractions of *S. marcescens* was measured using the aldolase assay kit from Boehringer Mannheim according to the manufacturer's recommendations, except that the reaction was carried out at 20 °C. Activity is expressed as nmol NADH converted min^{-1} (ml culture)$^{-1}$.

Amino acid sequencing. The chitinase was purified from a periplasmic fraction of *S. marcescens* BJL200(pMAY2-10) obtained by osmotic shocking, according to the protocol of Brurberg *et al.* (1994). The amino acid sequence was determined by Edman degradation using an automatic sequenator (model 477A, Applied Biosystems).

Antibody preparation and immunocytochemical analysis. A rabbit was injected subcutaneously three times at monthly intervals with mashed SDS-polyacrylamide gel slices containing approximately 20 μg chitinase from the periplasmic fraction of *E. coli* DH1(pMAY2-10). The first injection was given with 50% (v/v) Freund's complete adjuvant, whereas the second and third injections were given with 50% Freund's incomplete adjuvant. Sera were collected after the third injection. Ultrathin sections of *S. marcescens* cells were prepared and immunogold labelled as described elsewhere (Haandrikman *et al.*, 1991).

RESULTS

Subcloning of a 9·2 kb fragment carrying a chitinase determinant

pLES3, consisting of a 9·2 kb *Eco*RI chromosomal fragment from *S. marcescens* in pBR325, was previously shown to confer chitinase activity upon *E. coli* (Sundheim *et al.*, 1988). To localize the region encoding the chitinolytic activity, several deletion derivatives of pLES3 were made (Fig. 1). The deletion derivatives were introduced into *E. coli*, and the resulting transformants were screened for chitinolytic activity by plate assay. The results indicated that the chitinolytic activity of pLES3 was located on a 1·4 kb *Eco*RI–*Hpa*I fragment (Fig. 1). A slightly larger fragment (1·8 kb *Eco*RI–*Mlu*I) was subcloned to the high-copy-number vector pGEM-7f(+)Z and the resulting plasmid was called pMAY2-10 (Fig. 1).

Table 1. **Chitinolytic activity in *S. marcescens* and *E. coli* strains**

The chitinase activity was measured in periplasmic fractions obtained by osmotic shocking. Values are the means of three independent experiments. Standard deviations were less than 10% of the means.

Strain	Activity [nmol min^{-1} (ml culture)$^{-1}$]
E. coli DH1	0
E. coli DH1(pGEM-7f(+)Z)	0
E. coli DH1(pMAY2-10)	17
S. marcescens BJL200	0·5
S. marcescens BJL200(pMAY2-10)	11

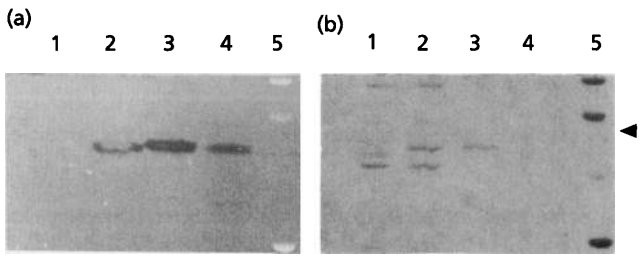

Fig. 2. **Chitinolytic activity after SDS-PAGE in a gel containing 0·01% (w/v) glycol chitin. Periplasmic fractions obtained by osmotic shocking of *E. coli* DH1(pGEM-7f(+)Z) (lane 1); *E. coli* DH1(pMAY2-10) (lane 2); *S. marcescens* BJL200(pMAY2-10) (lane 3); *S. marcescens* BJL200 (lane 4). Lane 5 contains molecular mass markers (kDa): phosphorylase *b* (94), albumin (67), ovalbumin (43) and carbonic anhydrase (30). (a) Gel stained with Calcofluor White. Chitinolytic proteins appear as dark bands (lytic zones) during UV illumination. (b) Protein staining with Coomassie Brilliant Blue G-250. The arrowhead indicates the 55 kDa chitinolytic protein.**

Overexpression of the chitinase activity in *S. marcescens*

Plasmid pMAY2-10 encoding the chitinase activity was introduced into the *S. marcescens* wild-type strain BJL200 by electroporation. The transformed *S. marcescens* showed a 20-fold increase in chitinolytic activity compared to the wild-type, as measured by hydrolysis of 4-MU-(GlcNAc)$_2$ (Table 1). Proteins produced by *E. coli* DH1(pMAY2-10),

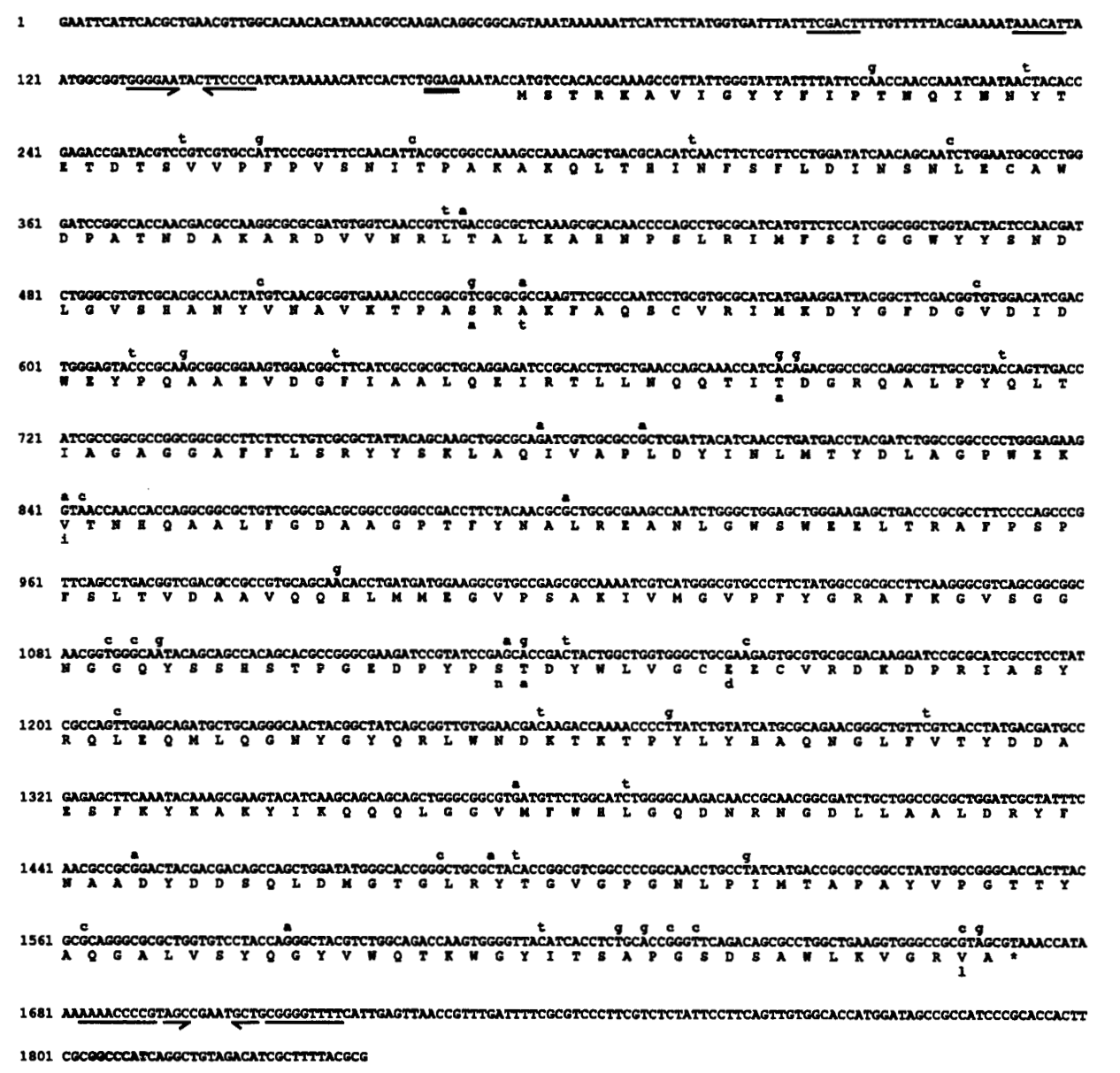

Fig. 3. Nucleotide sequence of the 1·8 kb *Eco*RI–*Mlu*I chitinase-encoding restriction fragment from *S. marcescens* BJL200. The deduced amino acid sequence of the chitinase is shown below the nucleotide sequence. Differences in nucleotide and amino acid sequence from *chiB* isolated from *S. marcescens* QMB1466 (Harpster & Dunsmuir, 1989) are indicated in lower-case type. A putative promoter −10 and −35 region is underlined. The ribosome-binding site is double underlined. The putative terminator eight bases downstream of the UAA stop codon (indicated with an asterisk) and a stem–loop structure 31 bases upstream of the ATG start codon are marked with inverted arrows.

S. marcescens BJL200 and *S. marcescens* BJL200(pMAY2-10) were analysed by SDS-PAGE, using gels containing glycol chitin as described in Methods. Fig. 2(a) shows that all three strains gave one distinct band with chitinolytic activity, which was absent in *E. coli* DH1 without pMAY2-10. Staining of the gels with Coomassie blue revealed a major 55 kDa protein band at the position of the activity band (Fig. 2b), occurring only in strains containing pMAY2-10. The observation that a Coomassie-stained chitinase band was only present in *S. marcescens* BJL200(pMAY2-10), and not in strain BJL200 lacking this plasmid, demonstrates the higher expression level of the chitinase in the transformed strain.

Nucleotide sequence analysis of the chitinase gene region

The nucleotide sequence of the 1836 bp *Eco*RI–*Mlu*I fragment encoding the chitinase gene was determined (Fig. 3). The sequence data revealed two long open reading frames (ORF1 and ORF2). ORF1 (151–1672) has an ATG start codon at position 175 which is preceded by

Table 2. Cellular location of enzymic activities in *E. coli* DH1(pMAY2-10) and *S. marcescens* BJL200(pMAY2-10)

Exponentially growing cells were fractionated by osmotic shocking (osm) or spheroplasting by lysozyme treatment (sph). Figures in the parentheses represent the percentage of the total activity in the culture. Values are the means of three independent experiments. Standard deviations were less than 10% of the means.

Cell fraction	Enzyme activity [nmol min^{-1} (ml culture)$^{-1}$]			
	Chitinase		β-Galactosidase/aldolase*	
	osm	sph	osm	sph
***E. coli* DH1(pMAY2-10)**				
Extracellular	0·6 (3)	0·9 (4)	16 (4)	4 (1)
Periplasmic	17 (78)	4·6 (20)	60 (15)	81 (19)
Cytoplasmic	4·1 (19)	17·6 (76)	322 (81)	340 (80)
***S. marcescens* BJL200(pMAY2-10)**				
Extracellular	0·03 (< 1)	0·02 (< 1)	0·3 (< 1)	0·4 (< 1)
Periplasmic	11 (77)	10·7 (81)	2.1 (2)	5 (5)
Cytoplasmic	3·3 (23)	2·5 (19)	109 (98)	98 (95)

*β-Galactosidase was used as a cytoplasmic marker for *E. coli* and aldolase was used as a cytoplasmic marker for *S. marcescens*.

a possible ribosome-binding site (Shine & Dalgarno, 1974). It could encode a protein of 499 amino acids with a calculated molecular mass of 55469 Da. ORF2 (202–1690) is located on the opposite strand of ORF1 and shares triplets with ORF1. However, in ORF2 no correctly spaced combination of a Shine–Dalgarno sequence and a start codon (ATG or GTG) could be detected, suggesting that ORF1 is the frame encoding the chitinase activity. The calculated molecular mass (55469 Da) of the protein encoded by ORF1 is in good agreement with the size of the protein with chitinolytic activity that was detected by SDS-PAGE (Fig. 2). The conclusion that ORF1 encodes the chitinase was confirmed by N-terminal sequencing of the gene product (see below).

Computer analysis of the region upstream of the coding region revealed a putative prokaryotic promoter region (Fig. 3). Between this region and the ATG start codon a stable stem–loop structure ($\Delta G = -12$ kcal mol^{-1}; -50 kJ mol^{-1}), which could be part of a regulatory mechanism for the expression of the gene, was observed (Fig. 3). A typical rho-independent transcription termination signal (Carafa *et al.*, 1990) with a free energy (ΔG) of -18 kcal mol^{-1} (-75 kJ mol^{-1}) was identified 10 nucleotides downstream from the termination codon (Fig. 3).

The deduced amino acid sequence of the chitinase was compared to other known chitinase sequences. The chitinase in this study exhibited 98·4% identity with a chitinase encoded by the *chiB* gene of *S. marcescens* strain QMB1466 (Harpster & Dunsmuir, 1989). Comparison of these proteins at the gene level revealed 96·4% identity in the coding regions and 95·8% identity when accessible upstream and downstream sequences were included in the comparison. Therefore, the chitinase gene cloned and characterized in this study will hereafter be referred to as *chiB*.

Location of chitinase B in *E. coli* cells containing *chiB*

As it took several days for *E. coli* harbouring any of the plasmids encoding chitinolytic activity (Fig. 1) to produce a clearing halo on a chitin-containing agar plate, we suspected that extracellular chitinolytic activity could be due to lysis of cells rather than to secretion of chitinolytic activity. To determine the cellular location of the chitinolytic activity, the *E. coli* DH1(pMAY2-10) cells were fractionated and the fractions were tested for chitinolytic activity against 4-MU-$(GlcNAc)_2$ (Table 2). The two methods for cell fractionation gave conflicting results with respect to the location of the chitinase activity. With the osmotic shocking method, 78% of the chitinase activity was detected in the periplasm, whereas with the spheroplast method, only 20% of the chitinase activity was detected in this fraction, most (76%) of the activity being found in the cytoplasm (Table 2). The periplasmic fractions obtained by both methods contained only 15–20% of the cytoplasmic marker enzyme (β-galactosidase). The relatively low level of β-galactosidase is consistent with its known cytoplasmic location, and indicates that there was no major contribution of cytoplasmic enzymes to the activities measured in the periplasmic fractions.

Since the two fractionation studies gave conflicting results, immunocytochemical analysis was conducted to

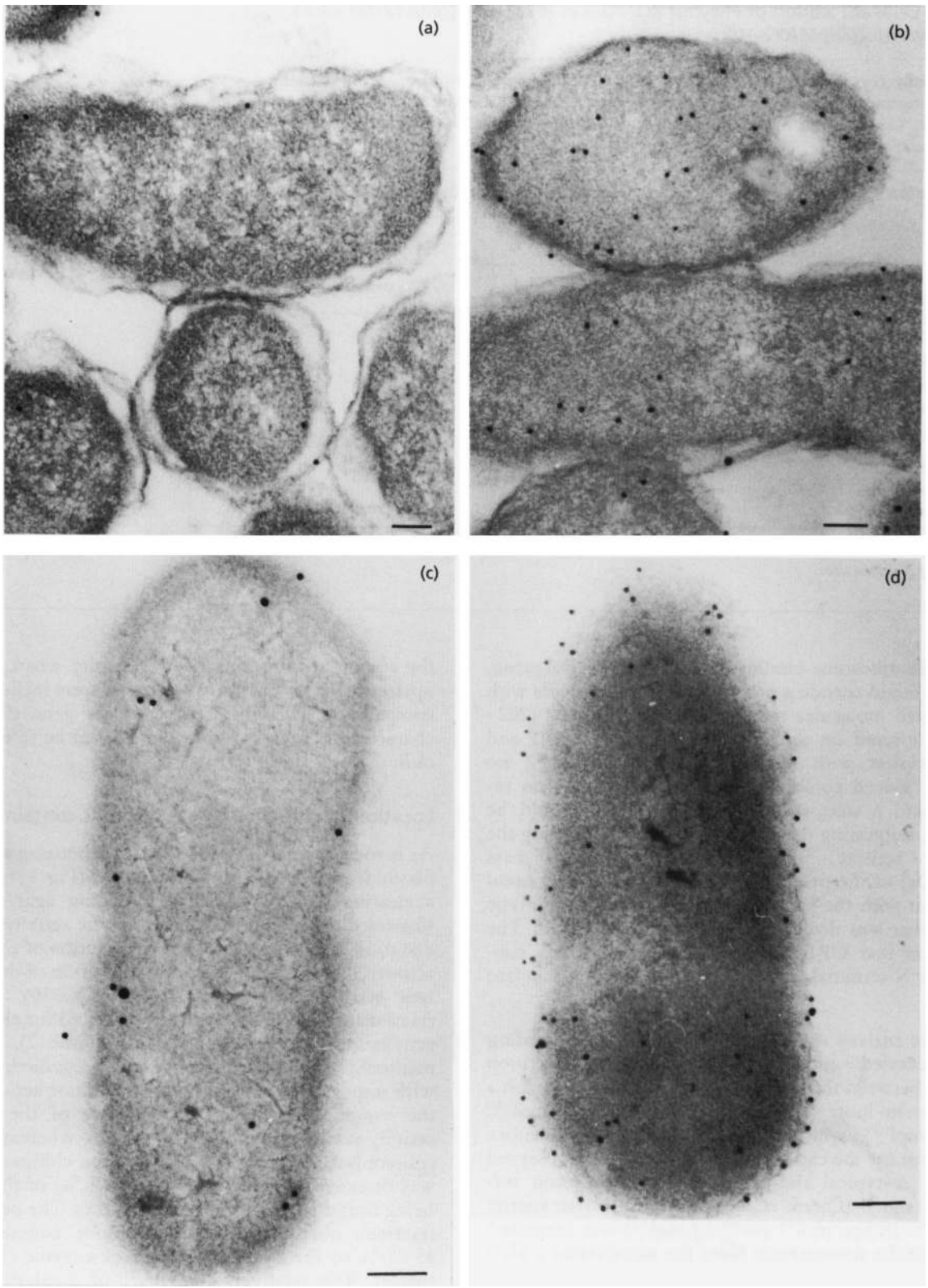

Fig. 4. Electron micrographs showing the localization of chitinase B in ultrathin sections of cells. The chitinase was visualized by immunogold labelling, using polyclonal anti-ChiB antibodies. (a) *E. coli* DH1(pGEM-7f(+)Z3), (b) *E. coli* DH1(pMAY2-10), (c) *S. marcescens* BJL200, (d) *S. marcescens* BJL200(pMAY2-10). Bars, 200 nm.

analyse the location of the chitinase in *E. coli*. Immunogold-labelled electron micrographs of *E. coli* DH1 with and without pMAY2-10 (Fig. 4) clearly showed that only *E. coli* harbouring pMAY2-10 produced the chitinase and that the enzyme was located in the cytoplasm. This result is in agreement with fractionating studies using the spheroplast method.

Location of chitinase B in *S. marcescens* cells overexpressing *chiB*

The wild-type strain of *S. marcescens*, BJL200, produced hardly any chitinolytic activity under the non-inducing conditions used in the present study. Therefore, *S. marcescens* BJL200(pMAY2-10), which overexpressed chitinase B under non-inducing conditions (Table 1), was used to determine the location of chitinase B in *S. marcescens*. The data in Table 2 show that the chitinase was mainly found in the periplasmic fraction, independent of the fractionation method that was used. The periplasmic location of chitinase B was confirmed by immunocytochemical analysis, which showed that the enzyme was almost exclusively located in the periphery of the cells (Fig. 4).

N-terminal sequence determination of chitinase B

The N-terminal part of the amino acid sequence deduced from *chiB* did not resemble signal peptides usually observed in exported prokaryotic proteins (von Heijne & Abrahamsén, 1989). To examine whether the chitinase was processed in its N-terminal region, as reported for the majority of proteins that are translocated across the cytoplasmic membrane, the N-terminus of the protein purified from the periplasmic fraction of *S. marcescens* BJL200(pMAY2-10) was sequenced. The sequence of the first 14 amino acid residues at the N-terminus was determined to be Ser-Thr-Arg-Lys-Ala-Val-Ile-Gly-Tyr-Tyr-Phe-Ile-Pro-Thr, which corresponds to amino acids 2–15 encoded by *chiB*. This shows that chitinase B is not N-terminally processed during export, except for the removal of the N-terminal methionine residue.

DISCUSSION

In the present study we identified and sequenced a gene encoding a chitinase from *S. marcescens* BJL200. The chitinase coding region identified in the cloned *S. marcescens* DNA is composed of 1497 bp coding 499 amino acids, which corresponds to a protein of 55469 Da. The data obtained from amino acid and nucleotide sequence comparison revealed that this gene is the equivalent of *chiB* isolated from strain QMB1466 of *S. marcescens* (Harpster & Dunsmuir, 1989). The nucleotide substitutions (Fig. 3) within the coding region of the *chiB* genes from the two strains are mostly synonymous. All non-synonymous substitutions occurred in residues that do not seem to be conserved among chitinases (results not shown; Butler *et al.*, 1991; Kuranda & Robbins, 1991; Watanabe *et al.*, 1992). The *chiB* gene is preceded by a typical prokaryotic promoter and followed by a strong terminator. Therefore it is most likely that *chiB* is expressed via a monocistronic transcript and not together with other chitinase genes. One might speculate about the function, if any, of ORF2 on the antisense strand. The phenomenon of a long open reading frame on the antisense strand has been shown earlier for nylon-degrading enzymes and has been suggested to be a mechanism for the evolution of new enzymes (Yomo *et al.*, 1992).

S. marcescens cells harbouring pMAY2-10 exported most of the produced chitinase to the periplasm, as shown by fractionation and immunocytochemical studies (Table 2, Fig. 4). The location of the chitinase B in the periplasm of *S. marcescens* suggests that the targets of the enzyme *in vivo* are soluble oligosaccharides capable of entering the periplasm through specific or non-specific porins in the outer membrane. Porins have solute exclusion limits around 600 Da (Weiss *et al.*, 1991), indicating that GlcNAc trimers (mol. mass 627) could be the substrate for the chitinase B. We are presently characterizing the enzymic activity of the chitinase B; preliminary results indicate that it is a chitobiosidase that releases GlcNAc dimers from the non-reducing end of chitin chains including $(GlcNAc)_{3-6}$ (Brurberg *et al.*, 1994).

The comparison of fractionation experiments with immunocytochemical analyses in the present study shows that the results of fractionation experiments can be misleading. Osmotic shocking of *E. coli* resulted in the erroneous conclusion that considerable export of the chitinase to the periplasm occurred. Our observations corroborate earlier studies that the osmotic shocking method can produce artefacts in *E. coli* (Tommassen *et al.*, 1985; Díaz *et al.*, 1989).

N-terminal amino acid sequence analysis of the purified chitinase from *S. marcescens* BJL200(pMAY2-10) showed that it is not subject to N-terminal processing during translocation to the periplasm. This contradicts an earlier report in which, on the basis of sequence analysis only, it was suggested that chitinase B possesses a 41 amino acid signal peptide (Harpster & Dunsmuir, 1989). The N-terminal methionine of the chitinase is probably removed by the action of methionyl-aminopeptidase, which has a preference for proteins with a small side chain in the penultimate position (Bem-Bassat *et al.*, 1987; Hirel *et al.*, 1989). The present results show that chitinase B of *S. marcescens* BJL200(pMAY2-10) must be exported by a secretion mechanism that differs from the general, signal-peptide-based secretion mechanism observed in prokaryotes. In *E. coli* the machinery for this secretion mechanism is either absent or non-functional for the *S. marcescens* chitinase B.

Previously, two *S. marcescens* proteins without identifiable signal peptides, a metalloprotease (Nakahama *et al.*, 1986) and a lipase (Akatsuka *et al.*, 1994), have been shown to be secreted to the culture medium without processing. As for chitinase B, these two proteins were not secreted from *E. coli* when their respective genes were introduced into this organism. However, the metalloprotease was secreted from *E. coli* in the presence of *Erwinia chrysanthemi* protease

secretion apparatus (Létoffé *et al.*, 1991) and *E. coli* α-haemolysin secretion functions (Suh & Benedik, 1992). The metalloprotease of *S. marcescens* is therefore considered to belong to a group of proteins, characterized by the α-haemolysin found in haemolytic *E. coli* strains, for which secretion appears to be independent of the general protein secretion pathway. The secretion apparatus for this group of proteins consists of two inner-membrane proteins and one outer-membrane protein, which direct the protein across both cell membranes, without stopping in the periplasm (reviewed by Lory, 1992; Wandersman, 1992; Pugsley, 1993). The presence of a similar secretion apparatus in *S. marcescens* has, so far, only been suggested (Létoffé *et al.*, 1991; Suh & Benedik, 1992).

Unlike the proteins shown to be translocated via a haemolysin-like mechanism, the chitinase of *S. marcescens* BJL200(pMAY2-10) is only exported to the periplasm and not secreted to the medium. Nevertheless, the possibility that the chitinase is exported by a haemolysin-like mechanism should be considered, since the mechanism has been shown to function for at least one secreted unprocessed *S. marcescens* protein (Létoffé *et al.*, 1991), and since the precise character of the haemolysin-like export machinery and its functional limitations are unknown. It has been shown that the secretion of a protein via a haemolysin-like mechanism is dependent on signals present in the 50 C-terminal residues (Stanley *et al.*, 1991; Létoffé *et al.*, 1991; Kenny *et al.*, 1992; Zhang *et al.*, 1993). Several authors have suggested common characteristics for these 50-residue C-terminal stretches that could direct secretion, such as the presence of an amphiphatic helix (Stanley *et al.*, 1991), a so-called aspartate-box (a stretch of small residues surrounded by a few aspartic or glutamic acid residues; Kenny *et al.*, 1992), a helix–turn–helix–strand–loop–strand motif (Zhang *et al.*, 1993), and a (Glu/Asp)-$(X)^{11}$-Asp-$(X)^{3/5}$-(Glu/Asp)-$(X)^{14}$-Asp motif (Sěbo & Ladant, 1993). Site-directed mutagenesis studies have indicated that negatively charged residues could be important (Stanley *et al.*, 1991; Kenny *et al.*, 1992). A feature seen in most proteins secreted by a haemolysin-like mechanism is the presence of repeats of the sequence GGXGXD that are involved in calcium binding (Ludwig *et al.*, 1988; Akatsuka *et al.*, 1994). The relevance of these repeats for export is, however, unknown.

The chitinase described in the present study does not contain any of the characteristics described or suggested for proteins exported via a haemolysin-like mechanism. This, together with the fact that the chitinase is directed to the periplasm instead of being secreted, suggests that export of this protein in *S. marcescens* involves a new, unknown mechanism for protein translocation.

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