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Cloning and Primary Structure of the *chiA* Gene from *Aeromonas caviae*

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The *chiA* gene from *Aeromonas caviae* encodes an extracellular chitinase, 865 amino acids long, that shows a high degree of similarity to chitinase A of *Serratia marcescens*. Expression in *Escherichia coli* yielded an enzymatically active protein from which a leader sequence was removed, presumably during transport of the enzyme across the cell membrane.

Hydrolysis of chitin to disaccharides and larger oligomeric saccharides usually takes place extracellularly by the action of chitinases. These enzymes have been shown to play an important role in biological control of soil plant pathogens (1, 6, 8). The number of chitinases isolated from a variety of organisms is rapidly expanding. Recently we compared the primary sequences of 56 known chitinases and demonstrated that many of these proteins have a central region containing several highly conserved domains (10). We have previously found processing of the secreted chitinase A (Chia_Serma) of *Serratia marcescens* in *Escherichia coli* (4, 11). Inbar and Chet isolated from soil a strain of *Aeromonas caviae* which showed a high level of chitinolytic activity (3). This bacterial strain was found to secrete chitinase into the growth medium when grown on chitin as the sole carbon source (3). In this report, we describe the cloning and sequencing of the complete *chiA* gene from *A. caviae* and comparison of the protein to closely related chitinase proteins.

The *chiA* gene of *A. caviae* was subcloned as a 4.5-kb *Hin*dIII segment in pBluescriptIISK. The strategy used is described in the legend to Fig. 1. To characterize the chitinase activity, we purified the chitinase enzyme from *E. coli*, which does not express chitin-binding activities. Strain XL1-Blue (Stratagene, La Jolla, Calif.), carrying the cloned *chiA* gene, was grown at 37°C in Luria-Bertani medium in the presence of ampicilin (100 mg/ml), and the chitinase protein was purified by standard procedures. In brief, the cells were collected, sonicated, and spun to remove debris. Colloidal chitin (0.02% [wt/vol]) was added and stirred to allow chitinase binding. The bound chitinase was collected by centrifugation, and the enzyme was dissociated from the chitin by incubation at 4°C in 10 mM HCl for 20 min. Following centrifugation, the free enzyme was dialyzed against 0.2 M sodium acetate buffer at pH 6.8. In preliminary studies, we found that in *E. coli*, part of the enzymatic activity is secreted into the medium while most of the enzymatic activity is cell associated (data not shown). We have previously found that for Chia_Serma, the enzyme accumulates in the periplasmic space unless the expression levels are very high. The enzymatic activity of the protein was assayed by the ability to degrade *p*-nitrophenyl-β-D-*N*,*N*′-diacetylchitobiose (described by Koby et al. [5]). Optimal enzymatic activity was found over a pH range of 5 to 7 in 0.1 M phosphate buffer. The highest activity was obtained at a temperature of 50°C. The purified enzyme was boiled in the presence of sodium dodecyl sulfate and β-mercaptoethanol and separated by electrophoresis on a sodium dodecyl sulfate-polyacrylamide gel. Staining with Coomassie brilliant blue revealed a single protein band with an estimated molecular mass of about 94 kDa.

The amino acid sequence at the N terminus of the protein band was determined by Edman degradation and found to be NH_2-Ala-Ala-Pro-Ala-Lys-Pro-Thr-Ile-Gly-Ser-Gly-Pro-Thr-Lys. The complete sequence of the *chiA* gene was obtained, and the deduced amino acid sequence of the chitinase protein (Chia_Aerca) yielded a large open reading frame, 2,595 nucleotides long, coding for 865 amino acids (Fig. 1). A putative ribosome-binding site (UAAGGAG) was found seven nucleotides upstream of the AUG initiation codon. However, the N terminus of the purified protein is identical to the amino acid sequence, starting at the Ala located at position 24 of the deduced amino acid sequence. This finding suggests the presence of a 23-amino-acid leader sequence, which is cleaved away, probably in the process of protein transport to the periplasmic space.

Comparison of the amino acid sequences shows that the chitinase from *A. caviae* (Chia_Aerca) is similar to chitinase A of *S. marcescens* and the chitinase of *Alteromonas* sp. strain O-7 (Fig. 1) (12). The sequence of Chia_Serma was corrected in accordance with the results reported by Perrakis et al. (9). The similarity of the chitinase of *Alteromonas* sp. strain O-7 (Chia_Altso) to chitinases A and B of *S. marcescens* has been previously noted (12). Both Chia_Aerca and Chia_Altso are larger than chitinase A of *S. marcescens* by 301 and 258 residues, respectively. This extension is restricted to the C terminus (Fig. 1).

The three-dimensional structure of the chitinase A protein of *S. marcescens* is composed of three major domains (9). The N-terminal domain (with the exception of the signal peptide) is a 134-amino-acid all-β domain similar to the fibronectin III motif. The major catalytic domain is 331 amino acids long and has an α/β barrel fold. This domain is interrupted by a 74-amino-acid domain with an α + β fold. Because of the high

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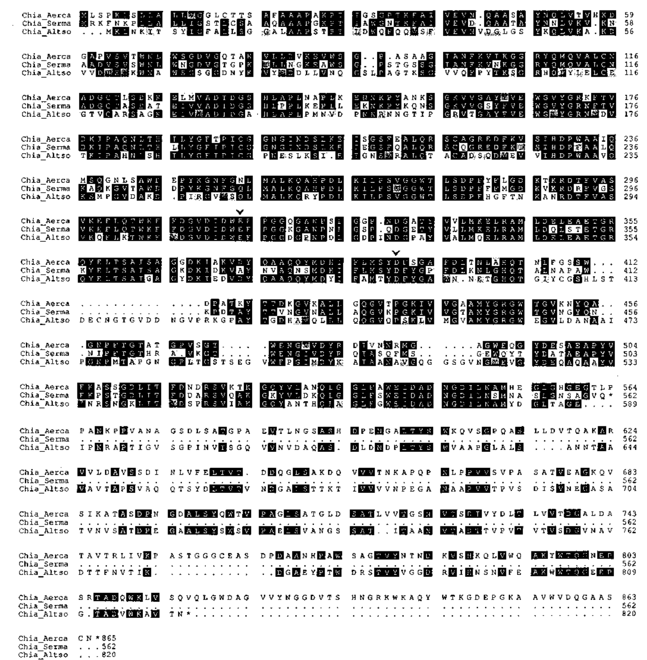

FIG. 1. Alignment of deduced amino acid sequences of closely related chitinases. The complete nucleotide sequence of the *A. caviae chiA* gene and the deduced amino acid sequence were determined. DNA of *A. caviae* was partially cleaved with restriction enzyme *Sau*3AI and ligated to vector λD69 at the unique *Bam*HI site (7). The library was amplified and screened against a DNA probe from the *chiA* gene of *S. marcescens* isolated from pLCHIA (11). Following infection of *E. coli* by selected phage clones, a clone that expressed chitinase activity was chosen. A 4.5-kb *Hin*dIII fragment was isolated and cloned. Both DNA strands were sequenced with a number of deletions and oligonucleotide primers. By using the MAP program of the Genetics Computer Group software package, the coding region of the gene was analyzed and the sequence was translated to the single-amino-acid code. The amino acid sequence alignment of the chitinases from *A. caviae* (Chia_Aerca), *S. marcescens* (Chia_Serma), and *Alteromonas* sp. strain O-7 (Chia_Altso) is shown. Residues identical to those of the *A. caviae* chitinase are shown in black, conservative changes are shaded, and dots represent gaps or shorter molecules at the N or C terminus. The alignment was generated with the PileUp application of the Genetics Computer Group package. The conserved Glu-315 and Asp-391 residues located at the active site (9) are shown by arrows.

level of similarity of Chia_Aerca and Chia_Altso to Chia_Serma, one can assume that the three enzymes have very similar three-dimensional structures, with few differences reflected by their primary structures. Asp-391 and Glu-315 of Chia_Serma, which have been suggested to be involved in the acid-base catalysis of chitin, are conserved in Chia_Aerca (9, 13, 14).

The C-terminal extension found in the chitinase of *A. caviae* is similar to that of the chitinase of *Alteromonas* sp. strain O-7 but is longer by 50 amino acid residues (Fig. 1). This part of the protein is very likely organized as a separate domain whose function is unknown. A computer search of the entire protein and of its C terminus suggested that the C-terminal domain contains two small related sequences, about 40 amino acids long. The strong similarity between these amino acid sequences suggests that they perform similar functions and arose by gene duplication (Fig. 2a). Similarly, computer searches of the available data banks revealed that the first C-terminal subdomain of Chia_Aerca (residues 766 to 804) is 51% identical to the terminal region of Chia_Altso. This subdomain shows strong similarity (48% identity) to the last 39 amino acids of the cellulase gene product (CELB1) (Fig. 2b) (2). The second C-terminal subdomain (residues 819 to 856) of

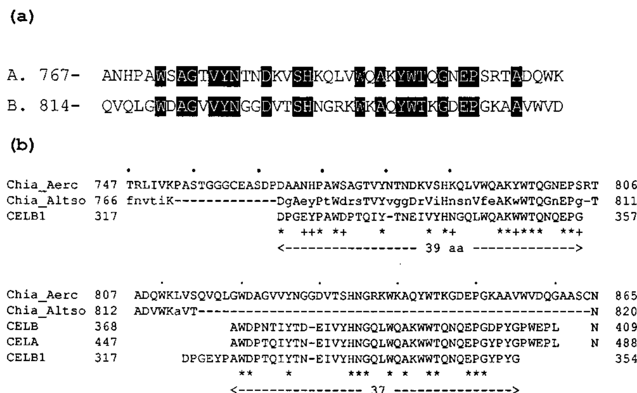

FIG. 2. Alignment of the duplication at the C terminus of Chia_Aerca and similarity to cellulase C-terminal duplication. (a) Lines A and B show the two repeating sequences at the C terminus of the *A. caviae* chitinase. The number at the left signifies the amino acid residue preceding the sequence shown. Amino acid identities between the two subdomains are shown in black (18 of 35 residues). (b) Similarity of the C-terminal repeats of the Chia_Aerca alignment between the last 119 residues of Chia_Aerca (GenBank accession no. U09139; 865 residues) and Chia_Altso (GenBank accession no. D13762; 820 residues) and the N-terminal sequences of CELA (GenBank accession no. P06565; 488 residues), CELB (GenBank accession no. P06566; 409 residues), and CELB1 (Genbank accession no. Z33876; 389 residues). Amino acid identities between all sequences are indicated by asterisks. Identities between Chia_Altso and CELB1 are indicated by plus signs. Differences between Chia_Altso and Chia_Aerca are in lowercase.

Chia_Aerca shows significant similarity (36% identity) to the same region of the cellulase gene product (CELB1). This domain also aligns with the last 40 residues of two more *Bacillus* cellulase gene products (CELA [residues 447 to 482] and CELB [residues 368 to 403]) (2). These three domains, CELA, CELB, and CELB1, are almost identical (89% identity) (Fig. 2b). These observations suggest that the C-terminal regions of the *A. caviae* chitinase and the *Bacillus* sp. strain N-4 cellulases are functionally related and may be involved in the ability of these enzymes to degrade highly hydrophobic substrates. The Trp and Tyr residues that are highly conserved between the two C termini of the *A. caviae* chitinase subdomains and the *Bacillus* cellulases possibly provide a hydrophobic protein environment, facilitating binding to chitin.

Nucleotide sequence accession number. The sequence described here has been submitted to the GenBank under accession no. U09139.

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