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Cloning and Expression of a Chitinase Gene from *Aeromonas hydrophila* in *Escherichia coli*

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An extracellular secreted chitinase gene from *Aeromonas hydrophila* was cloned in *Escherichia coli*, and the gene product was detected in the culture medium. Like the natural chitinase protein, the excreted chitinase had a molecular weight of approximately 85,000 and was subject to catabolite repression by glucose.

Various kinds of bacteria, fungi, plants, and some vertebrates (22) produce chitinase, which catalyzes the hydrolysis of the β-1,4 linkage of *N*-acetyl-D-glucosamine polymers of chitin. Because of the abundance of this compound in nature and the possibility of its conversion to useful products, increasing attention has been focused on chitinolytic microorganisms in the past few years.

The chitinolytic *Aeromonas hydrophila* JP101, a newly isolated strain from shrimp shell-enriched soil and identified by the Culture and Development Institute, Hsinchu, Taiwan, Republic of China, is able to use chitin as its carbon and nitrogen sources when grown on chitin medium and apparently synthesizes the entire enzymatic system through which degradation of chitin occurs. Although *A. hydrophila* has been reported to excrete a wide range of secretion enzymes (8, 15, 16, 27) and several genes of extracellular protein have been cloned and expressed in *Escherichia coli* (3, 4, 6, 7, 12, 23, 25), the chitin degradation system of this organism has not been studied at the gene level. Several studies on molecular characterization of chitinases (2, 5, 9–11, 14, 20, 24) suggest that this enzyme could be a useful model for fundamental studies on protein secretion, expression, and regulation as well as for applied research. Thus, we have started to clone the chitinase gene from *A. hydrophila* and to analyze the genetic information by means of molecular biology.

First, we constructed the genomic library from *A. hydrophila* JP101 as follows. *Aeromonas* chromosomal DNA was isolated by the method of Marmur (18) and partially cleaved with *Sau*3A, and fragments 3 to 20 kb in size were isolated by using sucrose density gradient centrifugation. The DNA fragments were ligated with T4 DNA ligase to *Bam*HI-digested plasmid pBR322, and the ligation mixture was used to transform *E. coli* JA221 (1). The transformants were screened on chitin-containing plates (M9 medium [17] without glucose but containing 50 μg of ampicillin per ml, 0.5% colloidal chitin, and 1.5% agar). Colloidal chitin was prepared as described by Molano et al. (21). Among the 6,000 Ap^r Tc^s transformants screened, two chitinase-positive clones were identified by the production of clear zones. The DNA inserts of the hybrid plasmids (designated pCH1 and pCH2) were characterized by digestion with restriction enzymes, and detailed physical maps showed that the two plasmid inserts were identical except that the pCH1 insert (10.7 kbp) was 1 kb smaller than that of pCH2 (Fig. 1A).

To further localize the chitinase gene, plasmid pCH1 was completely digested with *Hin*dIII, *Bam*HI, *Pst*I, or *Xho*I, and various fragments were ligated to plasmid pUC18. The results of subcloning of various restriction fragments indicated that the chitinase gene was located on the 4.5-kb *Hin*dIII fragment of pCH1. When the 4.5-kb *Hin*dIII fragment was recloned into the *Hin*dIII site of pUC18 in both orientations (pCH1001 and pCH1002), both plasmids coded for active chitinase in *E. coli* JA221. In addition, when pCH1001 and pCH1002 were transformed into *E. coli* JM101 (19), equally clear zones were observed in the presence and absence of isopropyl-β-D-thiogalactopyranoside. These data indicate that transcription of the chitinase gene was initiated at an endogenous promoter and that it is not under the control of the pUC18 β-galactosidase promoter.

To confirm that the cloned DNA fragment was derived from *A. hydrophila*, we performed Southern blot hybridization (17) with the cloned 4.5-kb *Hin*dIII fragment as a probe (Fig. 1B). The probe showed strong hybridization with the *Hin*dIII-digested plasmid pCH1001, which was used as a positive control (lane 1). An identical 4.5-kb band was also visible in the genomic *Hin*dIII digest (lane 3), and no cross-hybridization with *E. coli* DNA was detected (lanes 2 and 4). The results indicate the existence of a single chitinase gene in the *A. hydrophila* JP101 chromosome and that no deletion or DNA rearrangement in the gene had occurred during the cloning procedures.

The results from the chitin-containing plate suggest that chitinase may be secreted from *E. coli* as it is from *A. hydrophila*. To investigate this, *A. hydrophila* and *E. coli* containing the cloned chitinase gene were grown in liquid chitin medium for 3 days. Cells were removed, and the supernatants were brought to 10% trichloroacetic acid. The precipitants were rinsed with acetone and redissolved in 0.05 M Tris-HCl buffer (pH 7.2). Then the samples were examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (13). A unique protein was produced and secreted by JA221 transformed with pCH1 or pCH2, and the enzyme purified by the chitin affinity method (27) gave a single protein band with an apparent molecular size of 85 kDa (Fig. 2A, lanes 3 and 5). *A. hydrophila* secretes the 85-kDa protein, and partial purification of the *Aeromonas* chitinase activity has shown that it is associated with protein of 85 kDa (lanes 1 and 2). The inability to produce the 85-kDa protein was observed in

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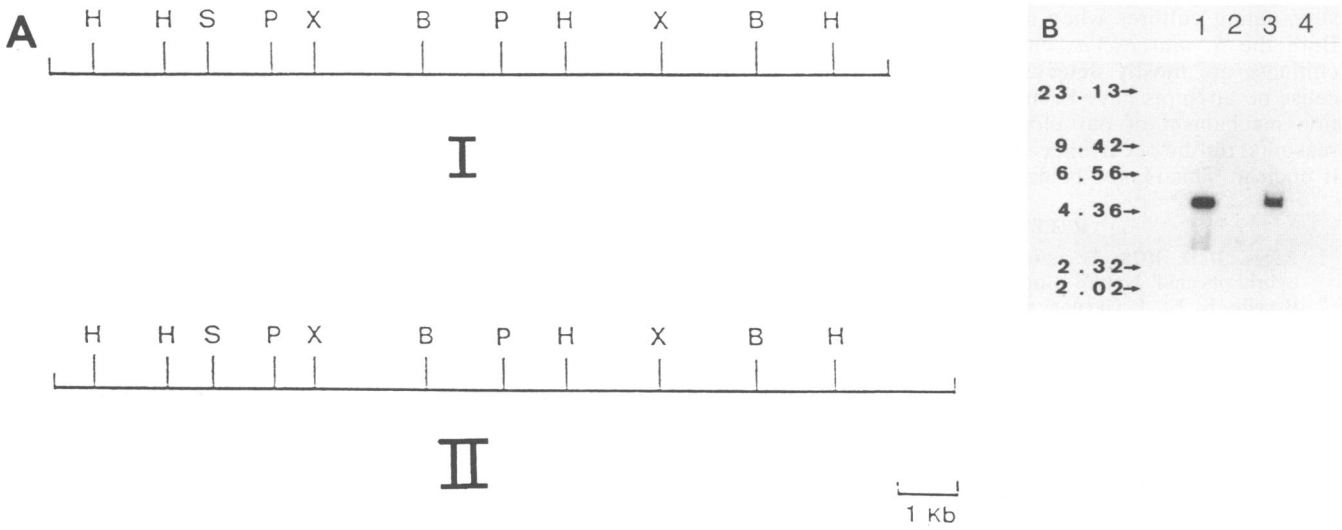

FIG. 1. (A) Restriction map of *A. hydrophila* DNA fragments encoding Chitinase: I, pCH1; II, pCH2. Restriction enzyme sites: B, *Bam*HI; H, *Hin*dIII; P, *Pst*I; S, *Sam*I; X, *Xho*I. (B) Southern hybridization analysis with the ^{32}P-labeled 4.5-kb *Hin*dIII fragment as a probe against *Hin*dIII digests of plasmid pCH1001 (lane 1), *A. hydrophila* chromosomal DNA (lane 3), and *E. coli* JA221 (lane 2) and JM101 (lane 4) chromosomal DNA. Fragment sizes (in kilobases) are indicated on the left.

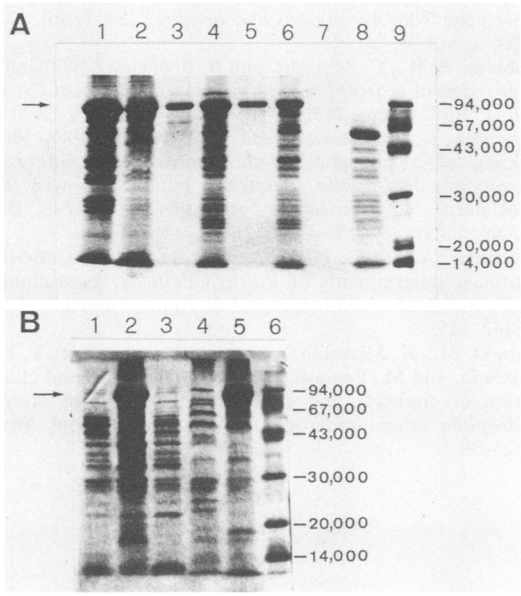

FIG. 2. SDS-PAGE analysis of chitinase proteins in culture supernatants of various bacterial strains. (A) Lanes: 1 and 2, *A. hydrophila* crude and partially purified proteins, respectively; 4 and 3, *E. coli* JA221(pCH2) crude and partially purified proteins, respectively; 6 and 5, *E. coli* JA221(pCH1) crude and partially purified proteins, respectively; 8 and 7, *E. coli* JA221(pBR322) crude and chitin affinity-purified solutions, respectively. (B) Lanes: 1 and 2, *E. coli* JM101(pCH1001) grown in chitin medium with and without 0.2% glucose, respectively; 3, *E. coli* JM101(pUC18) grown in chitin medium without 0.2% glucose; 4 and 5, *A. hydrophila* grown in chitin medium with and without 0.2% glucose, respectively. The numbers on the right indicate the molecular masses (in kilodaltons) of the protein standards. The positions of the chitinase protein is indicated by arrows.

JA221 containing pBR322 (lanes 7 and 8). We conclude that pCH1 confers the chitinase phenotype upon *E. coli* by specifying the synthesis of 85-kDa chitinase protein.

To determine whether the cloned chitinase also exhibits chitobiase activity, *E. coli* clones containing pCH1 or pCH2 were tested with 4-methylumbelliferyl-*N*-acetyl-β-D-glucosaminide as described by Wortman et al. (26). In contrast to *A. hydrophila*, which showed very strong chitobiase activity, plasmids pCH1 and pCH2 and their subclones showed no chitobiase activity.

The effects of carbon sources on chitinase production were also investigated in basal media of *A. hydrophila* and *E. coli* JM101(pCH1001). For *A. hydrophila*, chitinase production occurred only in medium containing chitin or colloidal chitin. The synthesis of chitinase was greatly suppressed when *A. hydrophila* was grown on glucose. The chitinase activity (measured by the turbidimetric method of Yabuki et al. [27]; 1 U of activity was defined as the amount of enzyme that caused a 1% decrease in A_{610} min^{-1}) in a supernatant of *E. coli*(pCH1001) was 31 U/ml, which is approximately 100-fold higher than that in *E. coli* grown on glucose. No chitinase production was observed in basal medium without chitin or colloidal chitin. That chitinase production was strongly repressed by glucose in *E. coli* and *A. hydrophila* was also shown by SDS-PAGE (Fig. 2B). These results indicate that the expression of the cloned chitinase gene in *E. coli* is inducible by chitin and is strongly repressed by glucose. The regulation profile is extremely similar to that of *A. hydrophila*.

Several extracellular *A. hydrophila* enzymes, such as amylase (6), aerolysin (4, 7), and protease (23), have been reported. Although these enzymes are extracellularly excreted by *A. hydrophila*, when they are cloned in *E. coli* the gene products are accumulated in the periplasm. Unlike the situation with the above extracellular proteins, we have shown that *A. hydrophila* chitinase is mostly found in the extracellular medium when it is cloned in *E. coli*. This is similar to the chitinases cloned from *Serratia marcescens*, which also showed that chitinases can be detected in the

supernatant cultures when they are cloned in *E. coli* (10). Both the *S. marcescens* chitinase and the *A. hydrophila* chitinase are mostly detected in the culture medium. Because no attempts have been made to investigate the secretion mechanism of our cloned chitinase by *E. coli*, the reason(s) for the occurrence of supernatant chitinase activity is unclear. This is now being investigated.

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