J. Microbiol. Biotechnol. (2004), **14**(3), 563–569

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

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Cloning and Expression of a Novel Chitosanase Gene (*choK*) from β-*Proteobacterium* KNU3 by Double Inverse PCR

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Received: September 5, 2003
Accepted: October 22, 2003

Abstract The DNA sequence of the chitosanase gene (*choK*) from β-*Proteobacterium* KNU3 showed an 1,158-bp open reading frame that encodes a protein of 386 amino acids with a novel 74 signal peptide. The degenerated primers based on the partial deduced amino acid sequences from MALDI-TOF MS analyses yielded the 820 bp of the PCR product. Based on this information, double inverse PCR cloning experiments, which use the two specific sets of PCR primers rather than single set primers, identified the unknown 1.2 kb of the *choK* gene. Subsequently, a 1.8 kb of full *choK* gene was cloned from another PCR cloning experiment and it was then subcloned into pGEM T-easy and pUC18 vectors. The recombinant *E. coli* clone harboring recombinant pUC18 vector produced a clear halo around the colony in the glycol chitosan plates. The recombinant ChoK protein was secreted into medium in a mature form while the intracellular ChoK was produced without signal peptide cleavage. The activity staining of PAGE showed that the recombinant ChoK protein was identical to the chitosanase of wild-type. The comparison of deduced amino acid sequences of *choK* revealed that there is 92% identity with that of *Sphingobacterium multivorum* chitosanase. Judging from the conserved module in other bacterial chitosanases, chitosanase of KNU3 strain (ChoK) belongs to the family 80 of glycoside hydrolases.

Key words: β-*Proteobacterium*, chitosanase, cloning, inverse PCR, MALDI-TOF

Chitosanases (EC3.2.1.132) are hydrolytic enzymes acting on chitosan, a polymer composed of β-(1-4)-linked glucosamine residues [14, 18]. Chitosanases were discovered in bacteria, fungi, and plants during the last few decades [1, 4, 10, 19]. Oligomeric chitosans obtained by enzymatic depolymerization have many potential biotechnological applications including medical and food materials, antifungal agents, and their ability to induce phytoalxin production in higher plants.

Chitosanases are distinguished from chitinases by their specificity for different degrees of acetylation and their lower apparent molecular mass. Most bacteria and fungi secrete chitosanases and chitinase separately or simultaneously. So far, both inducible and constitutive forms of chitosanases from bacteria have been purified, characterized, and cloned [5, 6, 8, 9, 23, 24].

The chitinolytic β-subdivision *Proteobacterium* KNU3 was recently isolated from soil [22]. The newly discovered strain, named as KNU3, has 99% homology with previously known chitinolytic β-*Proteobacterium* CTE108 in its 16S rRNA sequence [7]. Strain KNU3 produces 34 kDa of chitosanase in addition to two kinds of chitinases, with molecular weights of 68 kDa and 30 kDa. This strain secreted both chitinase and chitosanase when colloidal chitin or chitosan were added in a medium, respectively. So far, two kinds of chitosanases from bacteria belonging to β-*Proteobacteria* have been purified and characterized and cloned. *Matsuebacter chitosanotabidus* 3001, a new classified genus belonging to the β-subclass of *Proteobacteria*, produces 34 kDa of chitosanase (ChoA), while *Burkholderia gladioli* strain CHB101 produces 28 kDa of chitosanase and other two chitinases with molecular weights of 30 kDa and 37 kDa [13, 16, 17].

In this paper, the double inverse PCR method, which utilized the inner or nested primers, was employed to clone a novel chitosanase from a newly isolated strain. The genetic analysis of the chitosanase gene (*choK*) revealed its relationship with other β-*Proteobacterium* chitosanases. The analysis of deduced amino acid sequences of ChoK indicated that it is evolutionarily related with chitosanases from *Matsuebacter* sp. and *Sphingobacterium multivorum* that belong to the family 80 of glycoside hydrolases [20].

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MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Growth Conditions

The bacterial strain (KNU3) used in this work was initially isolated from soil and used as a donor strain of its chromosomal DNA [221]. This strain was grown aerobically in LB medium at 30°C, as previously described by Park *et al.* [13]. *E. coli* DH5α was used as a host strain for the plasmid maintenance and gene cloning. Ampicillin was added to the media at a final concentration of 50 μg/ml. Plasmid pGEM T-easy (Promega, Co., Madison, WI, U.S.A.) was used for cloning and sequencing of DNA fragments. Plasmid pUC18 (Gibco-BRL, Gaithersburg, MD, U.S.A.) was used for subcloning and expression of the *choK* gene.

DNA Preparation

Chromosomal DNA was isolated using the method of Miller *et al.* [12]. The β-*Proteobacterium* KNU3 was cultured in 400 ml of nutrient broth (Difco, Detroit, MI, U.S.A.) medium at 30°C with shaking at 180 rpm. The cells were harvested by centrifugation at 10,000 ×*g* for 15 min at 4°C. The genomic DNA was purified by CsCl/EtBr equilibrium density gradient centrifugation. The plasmid DNA was isolated by the alkaline lysis procedure of Sambrook *et al.* [15]. PCR primers were prepared from oligosynthesizing services of Bioneer (Daejeon, Korea).

Southern Hybridization

Southern hybridization was performed by DIG-labeled method as previously described [3]. β-*Proteobacterium* KNU3 total genomic DNA was digested with *Sac*I, separated by 0.8% agarose gel electrophoresis, baked at 80°C for 2 h, transferred to nylon membrane by Turboblotter System (Schleicher & Schuell, Keene, NH, U.S.A.), and hybridized to random primed DNA labeling with digoxigenin-dUTP labeled DNA probes made with the DIG DNA labeling and detection kit (Roche, Mannheim, Germany). Partial *choK* gene (820 bp) was used as probes. In order to prepare partial *choK*, sense primer, 5'-TACGGCAACGTCTTC-GACAAG-3', and antisense primer, 5'-GCTGTTGATGC-TGGTCAC-3', were synthesized and used for PCR using total genomic DNA as a template. PCR reaction was done by 28 cycles of PCR under the following reaction conditions: reaction volume, 25 μl; reaction composition, 1 μl of template, 50 pmol primer, and 0.125 μl of Ex-Taq™ DNA polymerase (Takara, Japan); cycle profile, 15 sec at 94°C, 30 sec at 56°C, 2 min at 72°C.

Inverse PCR (IPCR)

IPCR was carried out according to the method by Wang *et al.* [21] with a slight modification. Briefly, 2 μg of KNU3 genomic DNA was digested with 10 units *Sac*I for 3 h at 37°C and the enzyme was heat inactivated at 65°C for 15 min. DNA fragments were then diluted to a low concentration and incubated with a high concentration (350 U/μl) of T4 DNA ligase (Takara, Japan) at 4°C for 16 h. After the reaction mixture was purified by ethanol precipitation at -70°C for 1 h, 10 μl of circularized DNA was used as a template for the PCR amplification. The sense and antisense primer for the first inverse PCR step were synthesized on the basis of the partial *choK* gene (820 bp) from the inner PCR result. The sequences of the first IPCR primers were as follows: sense primer, 5'-GAG-ATGCCTTCGTTGTGCTCGTAA-3'; and antisense primer, 5'-CACGGGATCGATCGGCAGCATCT-3'. PCR reaction was done by 28 cycles of PCR under the following reaction conditions: reaction volume, 25 μl; reaction composition, 1 μl of template, 50 pmol primer, and 0.125 μl of Ex-Taq™ DNA polymerase (Takara, Japan); cycle profile, 15 sec at 94°C, 30 sec at 56°C, 2 min at 72°C, and a final extension at 72°C for 7 min. To confirm the inverse PCR product, 1 μl of the PCR product was used as a template for the next round of nested PCR using the second inverse primers. The sequences of second IPCR were as follows: sense primer, 5'-CTTG-TCGAAGACGTTGCCGTA-3'; and antisense primer, 5'-GTGACCAGCAT-CATCAACAGC-3'.

Gene Cloning and DNA Sequencing

The PCR products were directly inserted into the pGEM T-easy vector (Promega). Plasmid DNA was prepared by QIAprep® spin miniprep kit (QIAGEN, Hilden, Germany). The nucleotide sequence analysis was conducted at the Macrogen Co. DNA sequencing facility (Seoul, Korea) using the double-stranded dideoxy sequencing method on an ABI 377 DNA sequencer.

Expression of Recombinant Chitosanase in *E. coli*

For the expression of *choK* gene in *E. coli*, the sense primer (5'-GAGACTTGTCCACCTTCT-3') and anti-sense primer (5'-CCGTCGTCTT GCGTTCAT-3') were synthesized for PCR cloning. Purified chromosomal DNA from β-*Proteobacterium* KNU3 was used as a template. The PCR condition was 28 cycles of 94°C for 15 sec, 56°C for 30 sec, 72°C for 2 min, with a final extension at 72°C for 7 min. The amplified 1.8 kb fragment was directly inserted into the pGEM T-easy vector. The recombinant plasmid was digested with *Eco*RI and ligated into the *Eco*RI site of pUC18 (Gibco BRL) plasmid. *E. coli* DH5α cell was transformed with ligated DNA and positive clones were selected on LB agar plates containing 50 μg/ml ampicillin and 0.4% soluble chitosan.

Gel Electrophoresis and Activity Staining

SDS-PAGE was performed on a 12% SDS polyacrylamide gel containing 0.05% glycol-chitosan [2, 8]. The samples were heat-treated for 5 min at 80°C before being loaded and run at 40 mA for 40 min using a Bio-Rad Mini-Protein III Gel Kit. After the electrophoresis, the gels were washed

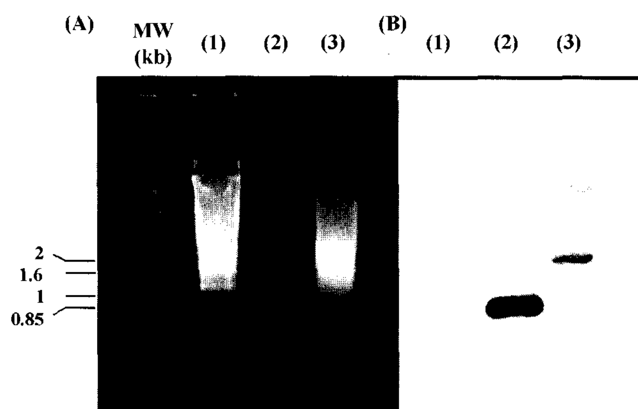

Fig. 1. Southern hybridization of enzyme-digested chromosomal DNA with PCR product probes.

(A): Agarose gel pattern with ethidium bromide. (1) *E. coli* DH5α chromosomal DNA as a negative control; (2) PCR product of partial *choK* as a positive control; (3) KNU3 chromosomal DNA digested with *Sac*I. (B): DNA pattern of Southern blot.

for 1 h at room temperature with shaking in 50 mM sodium phosphate buffer (pH 6.8) with 1% Triton X-100. The protein gels were stained with Coomassie brilliant blue G, while the gels were stained with 0.1% Congo red solution for 10 min and destained with a 1 M NaCl solution.

DNA Sequence Accession Number

The DNA sequence reported here (*choK*) was deposited in the GenBank under accession number of AY299333.

Table 1. Primers used in the inverse PCR experiments.

Primers	Sequence (5'-3')
Primer 1	TACGGCAACGTCTTCGACAAG
Primer 2	CACGGGATCGATCGGCAGCATCT
Primer 3	GTGACCAGCATCATCAACAGC
Primer 4	CTTGTCGAAGACGTTGCCGTA
Primer 5	GAGATGCCTTCGTTGTGCTCGTAA
Primer 6	GCTGTTGATGATGCTGGTCAC

RESULTS AND DISCUSSION

PCR Cloning of Partial *choK* Gene and Southern Hybridization

Previous MALDI-TOF MS analyses suggest that the amino acid sequences of *choK* gene have high homology with those of *Sphingobacter* sp. chitosanase [11, 22]. Based on identified amino acid regions, the forward and reverse degenerated primers were synthesized and used for partial *choK* gene cloning. A 820 bp PCR product was obtained and subsequently cloned into pGEM T-easy vector. The DNA sequencing analysis of partial *choK* showed that *choK* has high homology with DNA sequences of *Sphingobacter* sp. chitosanase. The existence of *choK* gene in strain KNU3 was confirmed by Southern hybridization using the partial *choK* PCR product as a probe (Fig. 1). Because a 2.0-kb-size *Sac*I-digested chromosomal band appeared in the Southern hybridization results, self-ligated library of *Sac*I-digested chromosomal

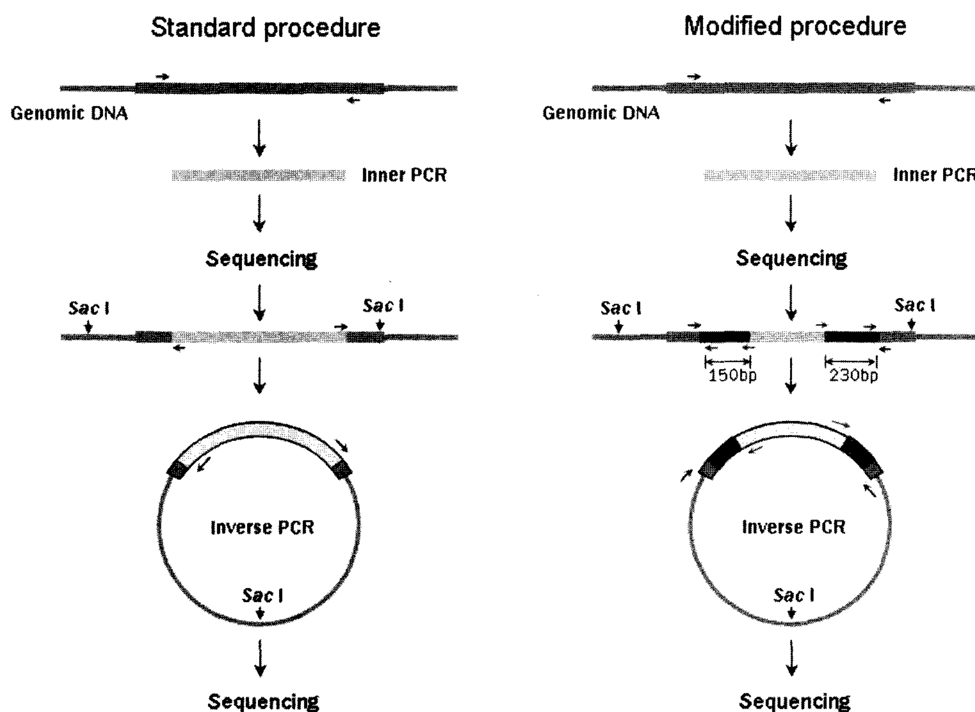

Fig. 2. The strategy for *choK* gene cloning by double inverse PCR.

DNA was used as a template DNA for further inverse PCR experiments.

Inverse PCR (IPCR) of *choK* Gene

Previous inverse PCR methods with only one set of specific primers often produce nonspecific PCR products. In fact, PCR experiments that use single set IPCR primers (primer 3, 4) alone yielded many nonspecific products. To overcome this problem and increase the sensitivity of inverse PCR, two sets specific primers of IPCR were designed and used for double inverse PCR (Table 1, Fig. 2). Firstly, the first set of IPCR primers (primer 2, 5), which were situated at the inside of the partial *choK* gene, produced a 1.6 kb inverse PCR band (Fig. 3). Secondly, to check whether this PCR product was specific or not, it was purified and used for the next round of PCR. The second set of IPCR primers (primer 3, 4) at the outside of the partial *choK* gene were used for another IPCR experiment and produced a 1.3 kb PCR product. Thirdly, the nested PCR experiments (primer 1, 5 or primer 2, 6) further proved the specific IPCR products such as 230 bp and 150 bp. The DNA sequencing of the 1.6 kb inverse PCR product revealed that the entire *choK* gene has high homology with that of previously reported *Sphingobacter* chitosanase [11]. Because another *Sac*I site existed near the termination site of translation, the remaining 38 bp of the deleted *choK* gene was obtained

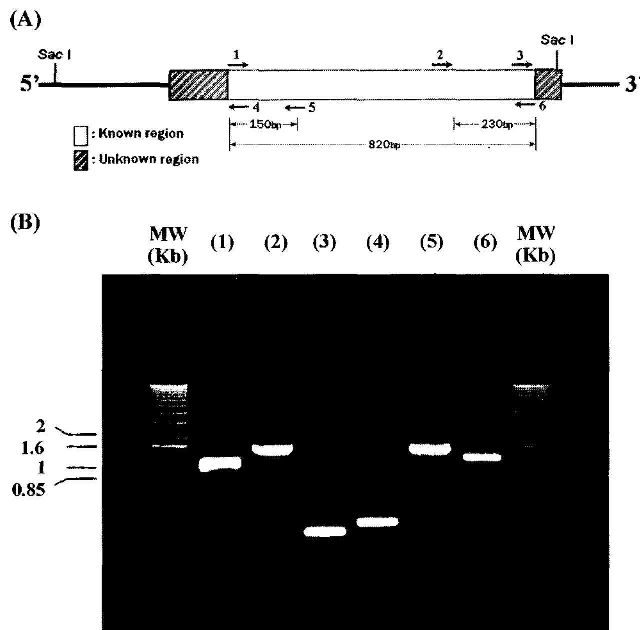

Fig. 3. Analyses of inverse PCR products of the *choK* gene.
(A) Schematic figure of overall inverse PCR. (B) Agarose gel electrophoresis of inverse PCR products. Lane MW, molecular weight marker; lane 1, PCR product by primer 3, 4; lanes 2 and 5, PCR product by primer 2, 5; lane 3, PCR product by primer 1, 5; lane 4, PCR product by primer 2, 6; lane 6, PCR product by primer 2, 5 digested with *Sac*I.

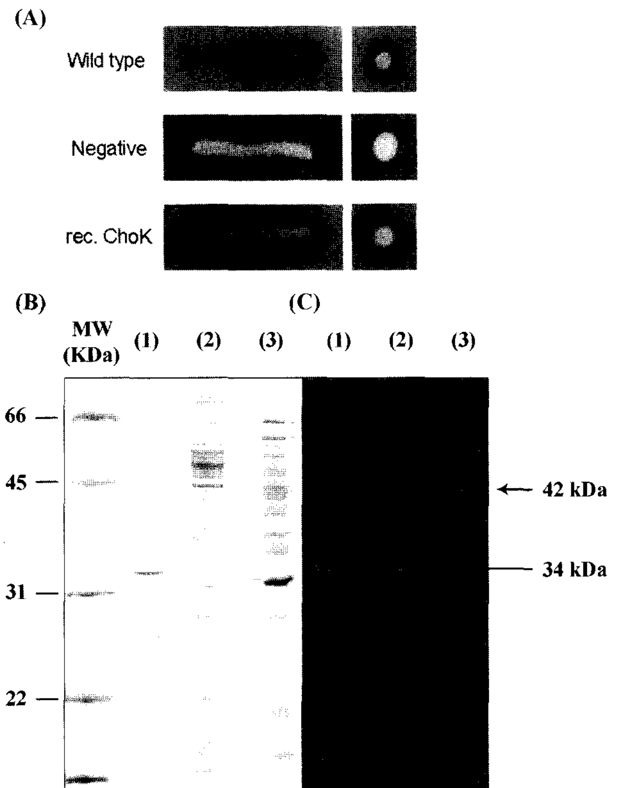

Fig. 4. Detection of chitosanase activity of recombinant ChoK by growth on chitosan plate and activity staining.
(A) Growth on soluble chitosan plates (KNU3, DH5α, recombinant, respectively). (B) Protein gel: (lane 1) purified recombinant ChoK, (lane 2) extracellular proteins, and (lane 3) intracellular proteins from recombinant *E. coli*. (C) Chitosan activity gel containing 0.05% glycol chitosan.

by another PCR experiment using the degenerated primer region of *Sphingobacter* chitosanase (data not shown). Based on the results of inverse PCR, two specific primers (explained at Materials and Methods section) were synthesized and used for PCR cloning of a full length of 1.8 kb *choK* gene. The PCR product was ligated into pGEM T-easy vector and subsequently tested for chitosanase activity.

Detection of Chitosanase Activity of Recombinant ChoK and Activity Staining

The *E. coli* transformant harboring the recombinant *choK* gene showed a clear halo around the colony in the glycol-chitosan supplemented LB plates (Fig. 4A). The expression of functional chitosanase was further confirmed by activity staining of crude extracts of recombinant *choK* clone (Figs. 4B, 4C).

The recombinant ChoK protein was extracellularly secreted with signal sequence cleavage. In contrast, the intracellular recombinant ChoK was not cleaved until the mature ChoK was secreted into the medium. The extracellular secretion

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   1 GACTTGTCCACCTTCTGCCGCCTGGACACGATCAGCCCGATCGCCTCCAGGTCGTTGGTG
  61 ATGTTGGCCACCGTCTGGGGGCTCAGCGACACCTTGTCGGCGATGTCCTTGCGCGATGCC
 121 GCGCCCTGCTGGCGGATGAAGTCCAGCACCACACGGCGGTTGTAGGGCGCGCTGGACTGT
 181 TGGGTGGCCCCTCGACTGGTGGTTCTCATGCTGGCGACTGGTCCTTCGGGATCCCCCATT
 241 CTGACGCAGCTTCACGACGGCATCACGGGGTCCGCTGACAAATCAATTTACACATCCAGT
 301 GGTTTTATTTATTGCATCGATGAAATCTTCCGCCTACATTCGCCGCCCATGGGCTGCAGC
 361 GACCCACGCCCCCGAGCGCAGGACCGGCTTGGCGAGAGTGGTGGGCGGCGGCTCTCGGGA
 421 ACCCATTCCCGTGGCGCAGGCCACGCCCCCCTACCCCCGTGTCTTTCGGAGGCAACCACC
 481 ATGCAATCTCGTACCCCCAGCGTCCGCCGCATTGGCGTGCAAGCGGCGCTCACCGTCCTG
      M  Q  S  R  T  P  S  V  R  R  I  G  V  Q  A  A  L  T  V  L
 541 GCCCTCGTCTGCGGCGCGTCCCTGACCGGCGTCGCGATCGCGGCCGGCAAGACCAAGGCG
      A  L  V  C  G  A  S  L  T  G  V  A  I  A  A  G  K  T  K  A
 601 CCGAGCGCCAACGGCCAACCCAGCGTCTACGTGCAGGACGGCTGGGTCTACACCAACACC
      P  S  A  N  G  Q  P  S  V  Y  V  Q  D  G  W  V  Y  T  N  T
 661 TTCACGGCCACCGGCCAGCCGCTGGTGACGGCCACCAAGGCCGCCGCGGCGGCCGGCGTC
      F  T  A  T  G  Q  P  L  V  T  A  T  K  A  A  A  A  A  G  V
 721 ATCCCCGTGGGCGACTCGCGCGTCTACGGCAACGTCTTCGACAAGGGTCGCAAGCTGACG
      I  P  V  G  D  S  R  V  Y  G  N  V  F  D  K  G  R  K  L  T
 781 GTCAACCAGTGGCAGGCCGTGCTCAGCATGGACGCCTATCCGGAGAACGGCACCACCAAC
      V  N  Q  W  Q  A  V  L  S  M  D  A  Y  P  E  N  G  T  T  N
 841 TATCAAGACCCGGAACCGTGGCGCTACTGCGAGGTCGATTACGAGCACAACGAAGGCATC
      Y  Q  D  P  E  P  W  R  Y  C  E  V  D  Y  E  H  N  E  G  I
 901 TCCGACTACCGCGGCGACACCTTCGGCCCGGTCGGCGTGACGACGGTCGGCGACTTCCCG
      S  D  Y  R  G  D  T  F  G  P  V  G  V  T  T  V  G  D  F  P
 961 GACTACTTCAAGAACGCCTACGCGCCCTACGTGCTGGGCAAGACCGGTGCGACCAACACC
      D  Y  F  K  N  A  Y  A  P  Y  V  L  G  K  T  G  A  T  N  T
1021 GACATGAAGAACTGGGGCGTCCAGGTCACCGGCATCGCGGCGGCGGACATGAAGGCGGAC
      D  M  K  N  W  G  V  Q  V  T  G  I  A  A  A  D  M  K  A  D
1081 GACTCGCGCCTGGACCCGTATCCGAACCTGTCCCGCACCAACTCGAAGAAGAAGGCGGCG
      D  S  R  L  D  P  Y  P  N  L  S  R  T  N  S  K  K  K  A  A
1141 CTGACCAAGATCTGCCAGGCGCTGCAGTCGGACTTCGACAACCGCCAGGCGCAGTACGTG
      L  T  K  I  C  Q  A  L  Q  S  D  F  D  N  R  Q  A  Q  Y  V
1201 ATGAGCCACTACGCCCACATCGACAGCGACAAGCTGCTGCCGGTGCTGGACGCGCTGAAG
      M  S  H  Y  A  H  I  D  S  D  K  L  L  P  V  L  D  A  L  K
1261 AAGATCGGCTTCACCAGCTTCAGCCAGTACAACCTGGTGGGACTGGCCTTCCAGGTGCAG
      K  I  G  F  T  S  F  S  Q  Y  N  L  V  G  L  A  F  Q  V  Q
1321 GTGAACACGGGATCGATCGGCAGCATCTCGGCGTTCACGCAGGTGAAGAACGCGGGCAAC
      V  N  T  G  S  I  G  S  I  S  A  F  T  Q  V  K  N  A  G  N
1381 TGCGGCAGCATGTCCAGCGAGACCTGCTTCGCCACCTACCTGACGGACCAGTACATTCGC
      C  G  S  M  S  S  E  T  C  F  A  T  Y  L  T  D  Q  Y  I  R
1441 TGGCTCTCGTCGTCCAGCCTGGGCGACGACAAGGGCAACTGCTGGCGCGCCAGCATGGCG
      W  L  S  S  S  S  L  G  D  D  K  G  N  C  W  R  A  S  M  A
1501 CTGGACATCTACAAGCAGGATCCGACGATGGGCAACGTCAGCGTGGTGAACAGCGTCATC
      L  D  I  Y  K  Q  D  P  T  M  G  N  V  S  V  V  N  S  V  I
1561 AACAGCAAGTACCCGAACAACTCCGGCAAGTGCCCGAGCTCGGGCGTGAAGTGGTCCAAG
      N  S  K  Y  P  N  N  S  G  K  C  P  S  S  G  V  K  W  S  K
1621 AACATGGCCTGGAACTGATCGCATGAAGACCGATGTCACCGCCCGCGCGGCGCAATCCC
      N  M  A  W  N  *
1681 CCCGACGCTCCGCGCTCGCGGTGGCCGCGGTCTTCGCGGTCGCGGCGGTCCTCGGGACC

1741 GCCGCCATGGCCTCGCACGCGGCAACGGATGAACGCAAGACGACGG
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Fig. 5. Open reading frame of the β-*Proteobacterium* KNU3 chitosanase gene (*choK*).

The putative signal sequence from computer analysis is represented as a shaded box. The ribosome binding sites (RBS) are marked by double underlines, while the palindromes and inverted repeated sequences at the transcriptional termination region are indicated by inverted arrows and dotted underline, respectively. The peptides underlined correspond to the results of MALDI-TOF MS analyses.

of ChoK indeed suggests that the signal sequence of *choK* gene from strain KNU3 was also functional in *E. coli* and this fact was consistent with the previously reported extracellular pattern of *Matsuebacter* sp. chitosanase [13].

DNA Sequence Analysis of β-*Proteobacterium* KNU3 Chitosanase Gene

The 1.8 kb of full-length *choK* gene was analyzed by DNA sequencing and compared with that of *Sphingobacter* chitosanase (Fig. 5). The presumed coding sequence started with an initiation codon ATG at position 481, which was preceded by a putative ribosome-binding site (GGAGG) and extended to the stop codon (TGA) located at position 1638. The putative -35 and -10 promoter sequences (ATTGCA, TACATT, respectively) were observed at 170 bp upstream of the start codon. The open reading frame of 1,158 bp would code for a 385-amino-acid polypeptide with a theoretical pI value of 5.6. The hydrophobic plot of the *choK* gene suggests that the initial N-terminal 74 amino acids peptide had typical features of signal peptides (data not shown). Previous N-terminal sequencing of the purified mature chitosanase from the KNU3 strain revealed that the signal peptide was cleaved between alanine residues 74 and 75 [22]. Cleavage at amino acid position 74 would result in a protein of 311 amino acids with a calculated molecular weight of 34,000. This is consistent with the 34-kDa-size of recombinant chitosanase estimated by SDS-PAGE. The 74-amino-acid sequence is considered to be the signal sequence responsible for the export of ChoK into the medium both in the KNU3 strain and *E. coli*.

Comparison of Deduced Amino Acid Sequences and Predictive Modeling of Catalytic Domains

The multiple comparison of deduced amino acid sequences indicated that the chitosanases from *Sphingobacter* sp. and *Matsuebacter* sp. have high homologies (92%, 74%, respectively) with that of KNU3. A phylogenetic tree was constructed for ChoK based on the clustal W method using

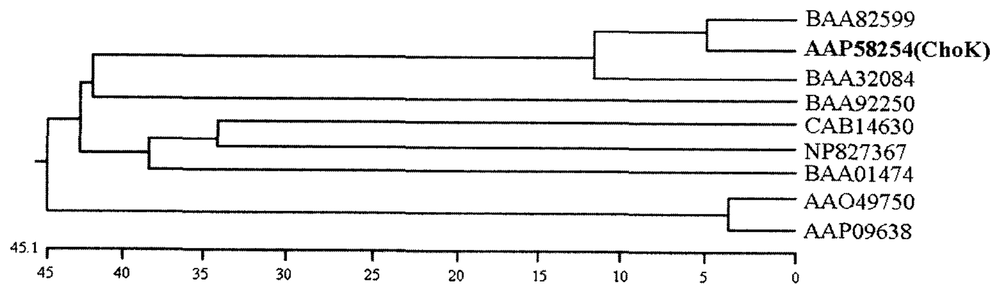

Fig. 6. Phylogenetic tree of ChoK with other known chitosanases.

The symbols represent the GenBank accession numbers for protein ID; *Sphingobacterium multivorum* (BAA82599); *Matsuebacter chitosanotabidus* (BAA32084); β-*Proteobacterium* KNU3 (AAP58254); *Aspergillus oryzae* (BAA92250); *Bacillus subtilis* 168 (CAB14630); *Streptomyces avermitilis* MA-4680 (NP827367); *Bacillus circulans* (BAA01474); *Bacillus cereus* (AAO49750); *Bacillus cereus* ATCC 14579 (AAP09638). The length of each pair of branches represents the distance between sequence pairs. The units at the bottom of the tree indicate the number of substitution events.

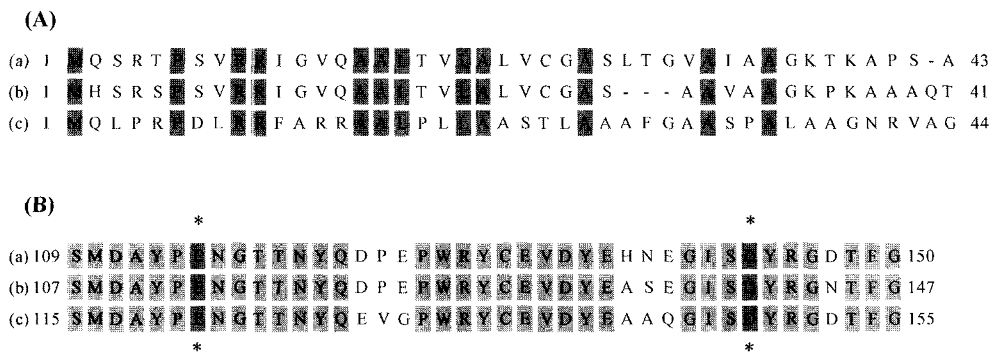

Fig. 7. Comparison of amino acid sequences in the signal peptide and catalytic domains from three bacterial chitosanases.
(A) Signal peptides comparison. (B) Catalytic domains comparison. The asterisk-labeled amino acids, Glu (E) and Asp (D), were shown to be essential for catalytic function of (a) β-*Proteobacterium* sp. KNU3 (AAP58254), (b) *S. multivorum* KST-009 (BAA82599), and (c) *Matsuebacter chitosanotabidus* 3001 (BAA32084).

lasergene software (Fig. 6). The deduced amino acid sequences were aligned with respect to the N-terminal and catalytic region of chitosanases (Fig. 7). When the three chitosanases were aligned near the putative active site of chitosanase, the two conserved residues, Glu (E) and Asp (D), were found around active sites. These facts indicated that they are closely related at the phylogenetic levels (Fig. 6).

The three-dimensional structures of active sites were compared using the predictive modelings of catalytic domains (Fig. 8). Even though there were differences in amino acid sequences, the entire pattern with closely approximating two alpha helices was not changed. This information further supports the evidence that these chitosanases originated from a common ancestor or were horizontally transferred from one organism to another during protein evolution.

In conclusion, the genetic analysis of a novel chitosanase gene (*choK*) reported here might provide a better understanding of bacterial chitosanases that belong to the family 80 of glycoside hydrolases. In addition, the double inverse PCR which is described here will provide a simple and easy method for gene cloning, as long as partial DNA sequence information is available.

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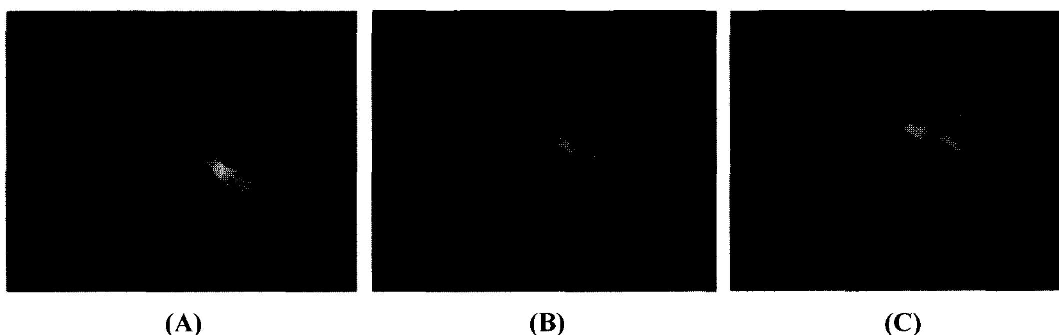

Fig. 8. Predictive modeling of catalytic domains found in three chitosanases.
(A) β-*Proteobacterium* sp. KNU3 (104-147 a.a.), (B) *S. multivorum* KST-009 (137-208 a.a.), and (C) *Matsuebacter chitosanotabidus* 3001 (108-153 a.a.). The models were created by GENO3D program (Institute de Biologie et de Chimie des Proteins, Lyon, France).

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