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RESEARCH

Cloning, DNA Sequence, and Expression of *Aeromonas caviae* WS7b Chitinase Gene

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Abstract

A chitinase-producing bacterium, designated WS7b, was isolated from a soil sample obtained from a black-pepper plantation on Bangka Island, Indonesia. Fatty-acid methyl-ester analysis indicated that the isolate was *Aeromonas caviae.* A chitinase gene from WS7b was cloned in a pUC19-based plasmid vector, but without its natural promoter. The complete nucleotide sequence of the gene was determined, and the structural gene consisted of a 2748-bp region encoding 864 amino acids. DNA sequence analysis indicated that the gene had been cloned without its promoter, and this was confirmed by chitinase-plate assay of the truncated version of the gene in *Escherichia coli.* The chitinase gene product showed amino-acid sequence similarity to chiA from *A. caviae.* Chitinase enzyme activity was determined spectrophotometrically, using colloidal chitin azure as substrate for extracellular and intracellular fractions. The ability of the chitinase cloned in *E. coli* to hydrolyze chitin was less than that of the enzyme in its indigenous host.

Index Entries: *Aeromonas caviae*; chitinase gene; DNA sequence; chitinolytic activity.

1. Introduction

Chitinase is an enzyme that catalyzes the hydrolysis of chitin, one of the world's most abundant biopolymers, which is found in nature as a major structural component of cell walls of fungi, arthropod exoskeletons, and crustacean shells. Chitinase is found in a wide variety of organisms, but its physiological role differs among them. Bacteria produce extracellular chitinase to digest chitin and utilize it primarily as a carbon and energy source *(1–4)*. In higher plants, chitinase is used for self-defense against plant pathogens and pests, i.e., to hydrolyze chitin in the cell walls of certain fungal pathogens and the cuticles of pests *(5–7)*. Chitinase has been shown to play an important role in the biological control of chitin-containing organisms, and is also important for the exploitation of natural chitinous materials *(8–11)*.

The chitinase genes of bacterial genus *Aeromonas* have, however, not been well characterized, and little information exists about the organization of the chitinase gene in this bacterium: studies of cloning and expression of the chitinase gene in *E. coli* from *A. caviae (12)*, from *Aeromonas* sp. No. 10S-24 *(13,14)*, and from *A. hydrophilia (15,16)* have been reported, in addition to studies of the utilization of chitinase produced from this bacterial genus as a mechanism of biocontrol for plant pathogenic fungi *(17)*.

In the present study, soil bacteria isolated from a black-pepper plantation on Bangka Island, Indonesia, exhibited extracellular chitinolyic activity when grown on chitin as the sole carbon source. We also describe the cloning and sequencing of the complete chitinase gene from one of those isolates, *Aeromonas caviae* WS7b. Chitinolytic activity in the indigenous host and of the *E. coli* recombinant host was compared, and enzyme production was measured semiquantitatively with spectrophotometry, utilizing colloidal chitin azure as substrate.

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Table 1
Bacterial Strains and Plasmids

Bacterial Strains and Plasmid	Relevant Characteristics	Reference
E. coli		
DH5α	F-, for α-complementation, general host for cloning	*(19)*
TOP10	F-, for α-complementation, general host for cloning	Invitrogen, Inc.
Aeromonas		
A. caviae WS7b	*chi*$^+$, soil bacterium, wildtype	This study
A. hydrophila ATCC 7965	*chi*$^+$ as positive control	*(15)*
Plasmids		
pRK415	InqP1, Tet^R	*(20)*
pUC19	*lacZ*. Ap^R	*(19)*
pAS385	MCS from pSL301(*Eco*RI–*Bal*I) cloned into pUC19 (*Sma*I–*Eco*RI), ApR	*(21)*
pWS501	4.7 kb *Hin*dIII chitinase gene from strain WS7b cloned into pRK415	This study
pWS502	4.7 kb *Hin*dIII chitinase gene from strain WS7b cloned into pUC19	This study
pWS503	pUC19 carrying 1.5 kb *Hin*dIII + *Bam*HI chitinase gene fragment from pWS502	This study
pWS504	pUC19 carrying 3.2 kb *Hin*dIII + *Bam*HI chitinase gene fragment from pWS502	This study
pWS505	pUC19 carrying 3.65 kb *Pst*I + *Hin*dIII chitinase gene fragment from pWS502	This study
pWS506	pUC19 carrying 2.9 kb XhoI + HindIII chitinase gene fragment from pWS502	This study
pWS 507	1.7 kb *Xho*I + *Hin*dIII fragment from pWS502 in pAS385	This study
pAS710	2.9 kb *Xho*I + *Hin*dIII fragment from pWS502 in pUC18NotI digested with *Sal*I + *Hin*dIII	This study
pAM1.1	pUC19 carrying 1.1 kb *Hin*dIII + *Pst*I chitinase gene fragment from pWS506	This study

2. Materials and Methods

2.1. Bacterial Strains, Plasmids, and Growth Conditions

Bacterial strains used in this study are described in **Table 1**. Soil samples from the black-pepper plantation on Bangka Island, were suspended in saline solution, and the bacteria contained within these samples were screened for extracellular chitinolytic activity on minimal medium (MM) agar supplemented with 1% (w/v) colloidal chitin *(18)*, with cycloheximide 100 µg/mL added as an antifungal agent. Bacterial isolates were identified by fatty-acid methyl-ester (FAME) analysis employing gas-liquid chromatography (Microcheck Inc., Northfield, VT). *E. coli* DH5α *(19)* and *E. coli* TOP10 (Invitrogen, Inc., Carlsbad, CA), which were used as hosts for cloning experiments throughout this study, and also their derivatives, were routinely grown in Luria–Bertani (LB) medium (broth or solidified with 1.5% agar) at 37°C for 18 h with ampicillin (100 µg/mL) added as needed. The chitinolytic WS7b strain of *A. caviae* and *A. hydrophila* ATCC 7965 as a chitinolytic positive control were grown routinely in LB agar. The chitinase plate assay was done either on LB-chitin agar or MM–chitin agar for *E. coli* derivatives and *A. caviae* WS7b, respectively. For the assessment of chitinolytic activity, *A. caviae* strain WS7b and *E. coli* transformants harboring the recombinant chitinase gene were grown overnight in LB and then diluted 10-fold in fresh MM–chitin broth at 30°C and in LB-chitin broth at 37°C, respectively, to the midexponential phase of growth. The growth media were supplemented with antibiotics as needed.

Plasmids used in this study and their relevant characteristics are described in **Table 1**. pAM1.1 is a derivative of pWS506 containing a 1.1-kb fragment of the chitinase gene upstream region, and was used in DNA sequencing as a subcloning strategy. For Southern blot hybridization, a 3.9-kb-*Eco*RI+*Sal*I fragment of the chitinase gene from *A. hydrophila*, in pJP2514 *(15)*, was used as a probe.

2.2. Recombinant DNA Techniques

Genomic DNA from all chitinolytic strains of bacteria used in the study was isolated by the cetyltrimethylammonium bromide (CTAB) method as described *(22)*. Plasmid DNA was isolated either by alkaline lysis *(19)* or with DNA purification kits (Promega, WI, or BioRad). DNA fragments were purified from agarose gels with the Gene Clean kit (Bio101 Inc., La Jolla, CA). DNA fragment ligations, recombinant DNA transformation, and other accessory techniques were done as described *(19)*. Transformants were selected either by a blue–white assay on LB agar plates containing IPTG (0.5 m*M*) and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-thiogalactopyranoside; 40 mg/mL) or by positive selection done with appropriate antibiotics.

2.3. Screening of Chitinase Gene Localization

Total genomic DNA from the chitinolytic isolates was digested with *Pst*I and screened for the chitinase gene by Southern hybridization *(19)*. A 3.9-kb *Eco*RI–*Sal*I fragment of the chitinase gene of *A. hydrophila* from pIP2514 *(15)* was used as the probe DNA. Probe DNA labeling was done by nick translation, utilizing the Bio Nick Labeling System (BRL Life Technologies), and by chemiluminescence, utilizing the Photogene Nucleic Acid Detection System (BRL Life Technologies).

2.4. DNA Sequencing and Sequence Analysis

Sequencing was done at the DNA sequencing unit of the South East Asia Center for Tropical Biology (SEAMEO BIOTROP), in Bogor, Indonesia, by dye-terminator sequencing, using the BigDye® Ready Reaction Mix and an automated DNA sequencer (ABI-Prism model 377-18; Applied Biosystems, Foster City, CA) according to the method of Sanger, as previously described *(19)*. Universal primers, m13 forward and m13 reverse, and specific internal primers were designed as required to generate double-stranded base sequences. Plasmid pAM1.1, a derivative of pWS506 carrying a 1.1-kb *Hin*dIII–*Pst*I fragment of *chi*, was used as a subcloning strategy. The database of the European Bioinformatics Institute (EBI) (URL http://www3.ebi.ac.uk) was used to obtain similarity and comparison data for chitinase gene sequence and the existing chitinase gene, as well as to analyze the deduced amino-acid sequence of the respective peptide products.

2.5. Enzyme and Protein Assays

Cultures of *A. caviae* were collected at 15, 45, and 90 h, and cultures of the other test bacteria were collected after growth to the stationary phase. Filtrates for protein and enzyme assays were collected after centrifugation of the cultures (10,000*g* at 4°C for 15 min), and were filtered through 0.45-µm pore-size filters (Millipore, Bedford, MA). Enzyme activity was measured in both extracellular and intracellular fractions. Intracellular fractions were obtained by disrupting the cell pellet four times by sonication at 15 amp for 1 min each time in cold phosphate buffer (0.2 *M*, pH 7.0). The protein concentration was measured according to the method previously described *(22)*. Chitinase activity was assayed by a modification of the method described *(24–26)*, with chitin azure as substrate *(27)*. The chitin azure pellet was suspended in 32% HCl, washed, and dialyzed in distilled water to obtain chitin in colloidal form, before sterilizing it by autoclaving. The amount of the dye released from the chromogenic chitin azure substrate—remazol brilliant blue—was measured at 590 nm. A standard curve was constructed with a chitinase standard from *Streptomyces griseus* (Sigma Chemical, St. Louis, MO) acting on colloidal chitin azure as a substrate in citrate–phosphate buffer pH 6.0 *(26)*, and with incubation at 37°C for 2 h. A series of chitinase–standard concentrations were prepared over the range of 0.0001–2 U/mL. The enzymatic activity was expressed as units of chitinase per milligram of protein. A mixture of LB and colloidal chitin azure was used as a negative control.

3. Results

3.1. Screening for the Chitinase Gene

Seven soil bacteria were isolated from soil samples of black-pepper plantation in an area that turned out to contain fewer phytopathogenic nematodes than were found in other plantation areas. These bacterial isolates exhibited extracellular chitinolytic activity after several days of incubating on chitin agar.

Isolation of the chitinase gene from the seven bacterial isolates was done by Southern hybridization. Genomic DNA from isolate WS7b yielded two hybridizing bands when probed with a 3.9-kb *Eco*RI–*Sal*I DNA fragment from *A. hydrophila* ATCC 7965 in pJP2514, whereas the remaining isolates did not show any hybridization signal (data not shown). It was possible that the chitinase gene of WS7b genomic DNA was cleaved into two separate fragments by *Pst*I, and that both fragments were hybridized to the probe. However, when WS7b genomic DNA was digested with *Hin*dIII, it showed only a single hybridization band at 4.7 kb (data not shown).

FAME analysis (Microcheck Inc.) indicated that the WS7b isolate was *A. caviae*.

3.2. Cloning and Restriction Endonuclease Mapping of Chitinase Gene from Strain WS7b

The 4.7-kb *Hin*dIII fragment of WS7b DNA was ligated into pRK415, generating a chitinolytic recombinant plasmid designated as pWS501. *E. coli* transformants harboring pWS501 were able to hydrolyze chitin, as indicated by a clear halo zone around the *E. coli* colonies after 3 d of incubation. Verification of the recombinant plasmid by agarose gel electrophoresis showed two bands, with molecular sizes of 4.7 kb and 10.5 kb, respectively, with the latter band belonging to the vector. For restriction-mapping purposes, the 4.7-kb *Hin*dIII fragment of pWS501 was subcloned in pUC19 and digested with *Hin*dIII, generating a recombinant plasmid designated pWS502 (**Fig. 1**). The restriction-endonuclease-digested fragments are shown in **Fig. 1**, and the estimated fragment sizes are presented in **Table 2**.

To locate the chitinase gene precisely, subcloning was done by digestion with several restriction endonucleases, and the shortest chitinase fragment insert that still showed chitinolytic activity was contained in the clone harboring the plasmid designated pWS506 (**Fig. 1**). *E. coli* clones harboring pWS503, pWS504, pWS505, and pWS507 did not show chitin-degrading activity until 15 d of incubation, indicating that the DNA fragments inserted into these plasmids were crucial in the expression of chitinase. Restriction analysis of pWS502 and pWS506, as well as of pAS710, showed that the chitinase gene was located adjacent to the right end of P*lac* in the plasmid vector (**Fig. 1**). The expression of this gene, in both pWS502 and pWS506 was apparently under the control of this promoter. However, in pAS710 the chitinase gene was cloned in a truncated version, which was confirmed by the chitinase plate assay of this recombinant plasmid in *E. coli*. The direction of the gene corresponded to a left-to-right direction (**Fig. 1**). Hence, it appears that the chitinase gene was cloned either without its full endogenous promoter region or with only part of this region. Furthermore, it can be suggested that the 5'-terminus of this chitinase gene was very close to the *Hin*dIII site.

3.3. Nucleotide Sequence and Structure of Chitinase Gene

DNA sequencing of the chitinase insert DNA in pWS506 yielded a 2925-bp nucleotide sequence (accession number AJ431785). An open reading frame (ORF) of 2748 bp was found to encode a putative protein of 864 amino acids. The ATG initiation codon located at nucleotide (nt) position 155 is preceded at a distance of 9 bp by a potential ribosome-binding sequence (AGGA). Three possible rho-independent transcription terminators, consisting of 6-bp, 4-bp, and 10-bp palindromic sequences, respectively, and corresponding to perfect inverted repeats in the form of potential hairpin loop structures, followed by five T residues with a 27-bp spacing, were found downstream of the TGA termination codon sequence located at nt position 2752. The UGA stop codon differed from the chitinase-gene stop codon in *Enterobacter agglomerans* (UAG) and in *Serratia marcescens* (UAA).

The nucleotide sequence data confirmed that the chitinase gene was cloned without its indigenous promoter region, and this was confirmed by a chitinase plate expression assay of the subcloned, truncated chitinase gene fragment in *E. coli*. The findings also suggested that the first

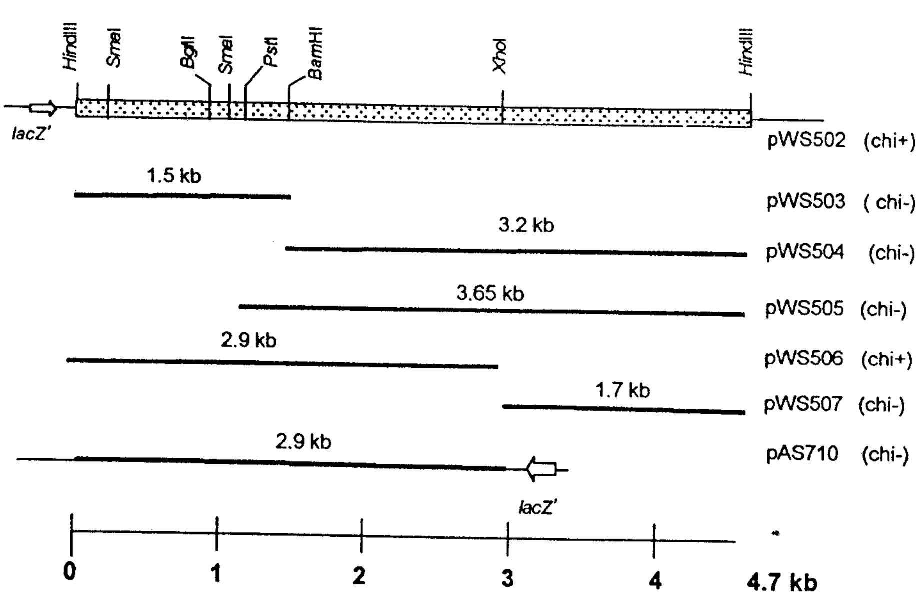

Fig. 1. Localization by restriction endonuclease cleavage and restriction mapping of the *Aeromonas caviae* WS7b chitinase gene. The size of the insert in each plasmid is shown above the line. The chitinase activity on chitin agar plates of *E. coli* transformants that harbored each recombinant plasmid is indicated in parentheses. The open bar corresponds to a 4.7-kb chitinase-gene insert.

Table 2
Estimated DNA Fragment Sizes of pWS502 Plasmid Digested with Various Restriction Endonucleases

Restriction endonuclease	Resulting DNA fragments (kb)
*Hin*dIII	4.7 ; 2.7
*Eco*RI	7.4
*Sal*I	7.4
*Bam*HI	4.2 ; 3.2
*Pst*I	3.8 ; 3.7
*Bgl*I	3.8 ; 3.4 ; 0.2
*Xho*I	7.4
*Sma*I	3.7 ; 2.9 ; 0.8
*Bam*HI + *Hin*dIII	3.2 ; 2.7 ; 1.5
*Pst*I + *Hin*dIII	3.7 ; 2.7 ; 1.1
*Xho*I + *Hin*dIII	3.0 ; 2.7 ; 1.7

nucleotide in the resulting DNA sequence is the 5'-terminal position of the insert in pWS506.

Comparison of the deduced amino acid sequence of the WS7b chitinase gene of *A. caviae* with other known prokaryotic chitinases revealed that the WS7b chitinase gene showed 97% identity with the chitinase *chiA* gene from *A. caviae* (UO9139) *(12)*, and is very closely related to the chitinase genes of *A. hydrophila* (89%) (accession number AF251793) (**Fig. 2**), *S. marcescens* (73%) (accession number Z36294), *E. agglomerans* (73%) (accession number U35121), and *Alteromonas sp.* (45%) (accession number D13762).

The level of similarity of these chitinases indicates that the amino-acid sequences of *Aeromonas* chitinases are conserved among members of this genus. The similarity of the chitinase gene from WS7b to *chiA* was noted even though they were isolated from very different locations in terms of distance and climate, but came from the same species. Therefore, the WS7b gene will hereafter be referred to as *chiA*. The N-terminal sequence of *chiA* has been previously determined and found to be NH_2-Ala-Ala-Pro-Ala-Lys-Pro-Thr-Ile-Gly-Ser-Gly-Pro-Thr-Lys *(12)*. The deduced amino-acid sequence data for our chitinase indicates the same amino-acid sequence at position 24 as in chiA. This observation therefore suggests that the N-terminus of the WS7b chitinase could be at this position, preceded by a leader sequence consisting of 23 amino acids. Alignment of the deduced amino-acid sequence indicated that the chitinases

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Chi-S.mar(Z36294)     MRKFNKPLLALLIGSTLCSAAQAAAPGKPTIAWGNTKFAIVEVDQAATAYNSLVKVKN-A 59
Chi-E.agg(U35121)     MRKFNKPLLALLIGSTLCSAAQAAAPGKPTIAWGNTKFAIVEVDQAATAYNNLVKVKN-A 59
Chi-WS7b(AJ431785)    MLSPKLSLLALLVGGLCTTSAFAAAPGKPTIGSGPTKFAIVEVNQAASAYNQLVTVHKDG 60
Chi-A.cav(UO9139)     MLSPKLSLLALLVGGLCTTSAFAAAPAKPTIGSGPTKFAIVEVNQAASAYNQLVTVHKDG 60
Chi-A.hyd(AF251793)   MFSPKHSLLALLVGGLCSTSALAAAPGKPTIGWGETKFAIIQVDQAATSYNKLVTVHKDG 60
Chi-Alt.(D13762)      ---MKLNKITSYIGFALLSGGALAAPSTPTLDWQPQQYSFVEVNVDGLGSYKQLVKAKDV 57
                         :   ::  :*     :..  ***..**:      ::::::*:  . .   . :   :

Chi-S.mar(Z36294)     ADVSVSWNLWNGDTGTTAKVLLNGKEAWSG--PSTGSSGTANFKVNKGGRYQMQVALCNA 117
Chi-E.agg(U35121)     ADVSVSWNLWNGDAGTTAKILLNGKEAWSG--PSTGSSGTANFKVNKGGRYQMQVALCNA 117
Chi-WS7b(AJ431785)    APVSVTWNLWSGDVGQTAKVLLDGKEVWSG---PAFAAGTANFKVTKGGRYQMQVALCNA 117
Chi-A.cav(UO9139)     APVSVTWNLWSGDVGQTAKVLLDVKEVWSG---PASAAGTANFKVTKGGRYQMQVALCNA 117
Chi-A.hyd(AF251793)   APVSVTWNLWSGDVGQTAKVLLDGKEVWSG---AASAAGTANFKVTKGGRYQMQVALCNA 117
Chi-Alt.(D13762)      VDISIKWNAWSGSGGDNYKVYFDDLLVNQGSLPAGTKSGVVQFPYTKSGRHQLYLELCEG 117
                      . :*:.** *.*. *  . *: ::    . .*    .     :*..:*  .*.**:*: : **:.

Chi-S.mar(Z36294)     DGCSASDATEIVVADTDGSHLAPLKEPLLEKNKPYKQNSGKVVGSYFVEWGVYGRNFTVD 177
Chi-E.agg(U35121)     DGCTASDATEIVVADTDGSHLAPLKEPLLEKNKPYKQNSGKVVGSYFVEWGVYGRNFTVD 177
Chi-WS7b(AJ431785)    HGCTLSDKKELMVADTDGSHLAPLNAPLKENNKPYANKSGKVVGAYYVEWGVYGRKFTVD 177
Chi-A.cav(UO9139)     DGCTLSDKKELMVADTDGSHLAPLNAPLKENNKPYANKSGKVVGAYYVEWGVYGRKFTVD 177
Chi-A.hyd(AF251793)   DGCTLSDKKEIVVADTDGSHLAPLNAPLQENNKPYTNKAGKVVGAYYVEWGVYGRKFTVD 177
Chi-Alt.(D13762)      TVCARSAGKEIVIADTDGAHLAPLPMNVDPNNRNNGTIPGRVTGAYFVEWGIYGRNYDVT 177
                        *: *   .*:::*****:*****    :   :*:       .*:*.*:*:****:***:: *

Chi-S.mar(Z36294)     KIPAQNLTHLLYGFIPICGGNGINDSLKEIEGSFQALQRSCQGREDFKVSIHDPFAALQK 237
Chi-E.agg(U35121)     KIPAQNLTHLLYGFIPICGGNGINDSLKEIEGSFQALQRSCQGREDFKVSIHDPFAALQK 237
Chi-WS7b(AJ43121)     KIPAQNLTHILYGFTPICGGNGINDSLKEISGSFEALQRSCAGREDFKVSIHDPWAAIQM 237
Chi-A.cav(UO9139)     KIPAQNLTHILYGFTPICGGNGINDSLKEISGSFEALQRSCAGREDFKVSIHDPWAAIQM 237
Chi-A.hyd(AF251793)   KIPAKNLTHILYGFTPICGGNGINDSLKEISGSFEALQRSCAGREDFKVSIHDPWAAVQM 237
Chi-Alt.(D13762)      KIPAHNLSHILYGFIPICGPN-ESLKSIEIGNSWRALQTACADSQDYEVVIHDPWAAVQK 236
                      ****:**:*:****  ****  *   .  .  **  .*:.***  :*  .  :*::*  ****:**:*

Chi-S.mar(Z36294)     AQKGVTAWDDPYKGNFGQLMALKQAHPDLKILPSIGGWTLSDPFFFMGDKVKRDRFVGSV 297
Chi-E.agg(U35121)     AQKGVTAWDDPYKGNFGQLMALKQARPDLKILPSIGGWTLSDPFFFMGDKVKRDRFVGSV 297
Chi-WS7b(AJ431785)    SQGNLSAWDEPYKGNFGNLMALKQAHPDLKILPSVGGWTLSDPFYFLGDKTKRDTFVASV 297
Chi-A.cav(UO9139)     SQGNLSAWDEPYKGNFGNLMALKQAHPDLKILPSVGGWTLSDPFYFLGDKTKRDTFVASV 297
Chi-A.hyd(AF251793)   GQGNLTAFDEPYKGNFGNLMALKKANPNLKILPSVGGWTLSDPFYFFSDKTKRDTFVASM 297
Chi-Alt.(D13762)      SMPGVDAKDP-IRGVYSQLMALKQRYPDLKILPSVGGWTLSDPFHGFTNKANRDTFVASV 295
                      .   .: * *     :*  :.:*****:   *:******:*********. : :*.:** **.*:

Chi-S.mar(Z36294)     KEFLQTWKFFDGVDIDWEFPGGKGANPNLGSPQ-DGETYVLLMKELRAMLDQLSAETGRK 356
Chi-E.agg(U35121)     KEFLQTWKFFDGVDIDWEFPGGKGANPNLGSPQ-DGETYVLLMKELRAMLDQLSAETGRK 356
Chi-WS7b(AJ431785)    KEFLQTWKFFDGVDIDWEFPGGQGANPSLGGPN-DGATYVVLMKELRAMLDELEAETGRQ 356
Chi-A.cav(UO9139)     KEFLQTWKFFDGVDIDWEFPGGQGANPSLGGPN-DGATYVVLMKELRAMLDELEAETGRQ 356
Chi-A.hyd(AF251793)   KEYLQTWKFFDGVDIDWEFPGGQGANPNLGGPN-DGATYVALMKELRAMLDELEAETGRQ 356
Chi-Alt. (D13762)     KQFLKTWKFYDGVDIDWEFPGGDGPNPDLGDPINDGPAYVALMQELRAMLDELEAETGRQ 355
                      *::*:****:************.*.**.**.*   **  :**  **:*******:*.*****:

Chi-S.mar(Z36294)     YELTSAISAGKDKIDKVAYNVAQNSMDHIFLMSYDFYGAFDLKN---------------- 400
Chi-E.agg(U35121)     YELTSAISAGKDKIDKVAYNVAQNSMDHIFLMSYDFYGAFDLKN---------------- 400
Chi-WS7b(AJ431785)    YELTSAISAGGDKIAKVDYQAAQQYMDHIFLMSYDFSGAFDLTN---------------- 400
Chi-A.cav(UO9139)     YELTSAISAGGDKIAKVDYQAAQQYMDHIFLMSYDFSGAFDLTN---------------- 400
Chi-A.hyd(AF251793)   YELTSAISAGGDKIAKVDYQAAQQYMDYIFLMSYDFSGAFDLKN---------------- 400
Chi-Alt.(D13762)      YELTSAIGAGYDKIEDVDYQAAQQYMDYIFAMTYDFYGAWNNETGHQTGIYCGSHLSTDE 415
                      *******.** ***  .* *:.**: **:** *:*** **::   .

Chi-S.mar(Z36294)     LGHQTALNAPAWKPDTAYTTVNGVNALLAQGVKPGKIVVGTAMYGRGWTGVNGYQNNIP- 459
Chi-E.agg(U35121)     LGHQTALNAPAWKPDTAYTTVNGVNALLAQGVKPGKIVVGTAMYGRGWTGVNGYQNNIP- 459
Chi-WS7b(AJ431785)    LPHQTNLFASSWDPATKYTADKGVKALLGQGVTPGKIVVGAAMYGRGMTGVKNYQAGNP- 459
Chi-A.cav(UO9139)     LAHQTNLFGSSWDPATKYTTDKGVKALLGQGVTPGKIVVGAAMYGRGWTGVKNYQAGNP- 459
Chi-A.hyd(AF251793)   LAHQTNLYASSWDPATKYTTDKGVKALLGQGVTPGKVVVGAAMYGRGWTGVNGYQAGNP- 459
Chi-Alt. (D13762)     CNGTGVDDNGVPRKGPAYTGDHAIQLLLQQGVQPSKLVMGVAMYGRGWEGVLDANAAIPG 475
                                          .**   :.:: ** *** *.*:*:*.******  **  . :    *
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Fig. 2. Alignment of deduced amino acid sequences of closely related chitinases. The alignment was generated with the Clustal-W program of the European Bioinformatics Institute database. Residues identical to those of the *A. caviae* chitinase are shown by asterisks, conservative changes are indicated by single and double dots, and dashes represent gaps or shorter molecules at the N or C terminus.

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Chi-S.mar(Z36294)     --FTGTATGPVKG-----TWENGIVDYRQIAGQFMS------GEWQYTYDATAEAPYVFK 506
Chi-E.agg(U35121)     --FTGTATGPVKG-----TWENGIVDYRQIASQFMS------GEWQYTYDATAEAPYVFK 506
Chi-WS7b(AJ431785)    --CTGTATGPVSG-----TWENGVVDYRDIVNNRMG------AGWEQGYDESAEAPYVFK 506
Chi-A.cav(UO9139)     --FTGTATGPVSG-----TWENGVVDYRDIVNNRMG------AGWEQGYDESAEAPYVFK 506
Chi-A.hyd(AF251793)   --FTGSATGPIKG-----TWENGVVDYRDIVNNRMG------AGWEQGYDETAEAPYVFK 506
Chi-Alt.(D13762)      NPMTAPGNGPLTGSTSEGVWEPGIMDYKAIAANAVGQGGSGVNGYEVGYDEQAQAAYVWN 535
                         *....**:.*      .** *::**: *. : :.         ::  **  *:*.**::

Chi-S.mar(Z36294)     PSTGDLITFDDARSVQAKGKYVLDKQLGGLFSWEIDADNGDILNSMNASLGNSAGVQ--- 563
Chi-E.agg(U35121)     PSTGDLITFDDARSVQAKGKYVLDKQLGGLFSWEIDADNGDILNSMNASLGNSAGVQ--- 563
Chi-WS7b(AJ431785)    ASSGDLITFDNDRSVKAKGQYVLANQLGGLFAWEIDADNGDILNAMHEGLGHGEG-TLPP 565
Chi-A.cav(UO9139)     ASSGDLITFDNDRSVKTKGQYVLANQLGGLFAWEIDADNGDILNAMHEGLGHGEG-TLPP 565
Chi-A.hyd(AF251793)   ASTGDLISFDNDRSVKAKGQYVLANQLGGLFAWEIDADNGDILNAMHEGLGNGDGGTTPP 566
Chi-Alt.(D13762)      RSNGKLITYDSPRSVIAKGQYANTHQLAGLFGWEIDADNGDLNAMYDGLTAGEIPNRAPT 595
                       *.*.**::*. ***  :**:*.   :**.***.*********:     .    .

Chi-S.mar(Z36294)     ------------------------------------------------------------
Chi-E.agg(U35121)     ------------------------------------------------------------
Chi-WS7b(AJ431785)    VNKPPVANAGSDLSATGPAEVTLKGSASHDPENGALTYSWKQVSGPQASLLDATQAKARV 625
Chi-A.cav(UO9139)     ANKPPVANAGSDLSATGPAEVTLNGSASHDPENGALTYSWKQVSGPQASLLDVTQAKARV 625
Chi-A.hyd(AF251793)   VNKPPVANAGSDLSVTGPAEVTLNGAASHDPESGVLSYSWKQVSGPQVSLLDATQAKARV 626
Chi-Alt.(D13762)      IGVSGPINVTSGQVVN------VD-AQASDLDNDPLTYSWVAAPG---LALSANNTAAVA 645

Chi-S.mar(Z36294)     ------------------------------------------------------------
Chi-E.agg(U35121)     ------------------------------------------------------------
Chi-WS7b(AJ431785)    VLDAVSSDINLVFELTVTDDQGLSAKDQVVVTNKAPQPNLPPVVSVPASATVEAGKQVSI 685
Chi-A.cav(UO9139)     VLDAVSSDINLVFELTVTDDQGLSAKDQVVVTNKAPQPNLPPVVSVPASATVEAGKQVSI 685
Chi-A.hyd(AF251793)   VLDAVSADINLVFELTVTDDHNLTAKDQVVVTNKAPQPNLPPVVTVPATASVESGKQVTI 686
Chi-Alt.(D13762)      VTAPSVAQQTSYDLTVTVNDGALSTTKTIVVVVNPEGANAAPVVTPVSDISVNEGASATV 705

Chi-S.mar(Z36294)     ------------------------------------------------------------
Chi-E.agg(U35121)     ------------------------------------------------------------
Chi-WS7b(AJ431785)    KATASDPNGDALSYQWTVPAGLSATGLDSATLVVTGSNVTSDTAYDLTLVVTDGALDATA 745
Chi-A.cav(UO9139)     KATASDPNGDALSYQWTVPAGLSATGLDSATLVVTGSNVTSDTVYDLTLVVTDGALDATA 745
Chi-A.hyd(AF251793)   KATASDPNGDALTYQWSLPAGLTATGQNSATLVVTGPSVTSDTAYDLSLVVTDGSLDASA 746
Chi-Alt.(D13762)      NVSATDPEGAALSYSWSVPAELSVANGSSATITAAN--VTADTTVPVTVTVSDGVNAVDT 763

Chi-S.mar(Z36294)     ------------------------------------------------------------
Chi-E.agg(U35121)     ------------------------------------------------------------
Chi-WS7b(AJ431785)    VTRLTVKPASTGGGCEACDPDAANHPAWSAGTVYNTNDKVSHNQLVWQAKYWTQGNEPSR 805
Chi-A.cav(UO9139)     VTRLIVKPASTGGGCEASDPDAANHPAWSAGTVYNTNDKVSHKQLVWQAKYWTQGNEPSR 805
Chi-A.hyd(AF251793)   GTRLTVKPASTGGGCEATDPDAANHPAWSASAVYNTNAKVSHKQLVWQAKYWTQGNEPSQ 806
Chi-Alt.(D13762)      TFNVTIKDG------------AEYPTWDRSTVYVGGDRVIHNSNVFEAKWWTQGEEPG- 809

Chi-S.mar(Z36294)     ------------------------------------------------------------
Chi-E.agg(U35121)     ------------------------------------------------------------
Chi-WS7b(AJ431785)    TADQWKLVSQVQLGWDAGVVYNGGDVTSHNGRKWKAQYWSKGDEPGKAAVWVDQGAASCN 865
Chi-A.cav(UO9139)     TADQWKLVSQVQLGWDAGVVYNGGDVTSHNGRKWKAQYWTKGDEPGKAAVWVDQGAASCN 865
Chi-A.hyd(AF251793)   TADQWKLLSAVQLGWNAGVAYNAGDLTNHNGRKWKAQYWTKGDEPGKAAVWVDQGAASCN 866
Chi-Alt.(D13762)      TADVWKAVTN-------------------------------------------------- 819
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Fig. 2. *continued*

from *A. caviae* strains and *Alteromonas* sp. (D13762) are larger than the chitinases from *S. marcescens* (Z36294) and *E. agglomerans* (U35121). It can also be seen that the extension is restricted to the C-terminus, as shown in **Fig. 2.** At this extended C-terminus we found two small, related sequences with strong similarity, as reported *(12)*. These two unique regions could be suggested as subdomains that perform similar functions and which arose by gene duplication.

Table 3
Chitinolytic Activity of Cloned Chitinase Gene

Strain	Activity (U/mg protein)[a]	
	Extracellular Fraction	Intracellular Fraction
E. coli DH5α	0.27	0.38
E. coli DH5α (pAS385)	0.17	0.40
E. coli DH5α(pWS506)	2.31	4.17
A. caviae WS7b	7.7[(3)]	ND[b]
A. caviae WS7b	5.92[(2)]	ND
A. caviae WS7b	0.98[(1)]	ND

[a]The chitinase activity was measured in extracellular and intracellular fractions obtained after sonication. Values are the means of three independent experiments. Standard deviations were less than 10% of the means. *E. coli* clone fractions were harvested and collected after growth to the stationary phase, whereas *A. caviae* culture filtrates 1, 2, and 3, as extracellular fractions, were collected from 15-, 45- and 90-h cultures, respectively.

[b]ND = not determined.

3.4. Enzymatic Activity of Chitinase in A. caviae and E. coli Clones

The chitinase product from *A. caviae* WS7b grown by induced culture on MM–chitin showed an increasing amount of this enzyme. However, less enzyme was produced by the noninduced culture of WS7b (data not shown). The assay for the enzyme produced in culture was performed with chitin azure as a colloid, based on our preliminary observation of very slow chitinolytic activity when chitin azure was used in its commercially available flake form. By comparison with the chitinase agar plate assay of WS7b, which showed a clearing zone after 20 h of incubation, the culture filtrate assay showed lower enzyme activity, suggesting that the chitinase expression in WS7b was inducible.

An *E. coli* clone harboring pWS506 showed higher chitinolytic activity in the intracellular than in the extracellular fraction (**Table 3**). A zone of clearing with pWS506 was observed after 3 d of incubation on chitinase agar.

4. Discussion

Expression of chitinase in *A. caviae* WS7b, either in chitin plate or in broth medium, required colloidal chitin for induction of the enzyme. Low-molecular-weight breakdown products of chitin, including D-glucosamine and *N*-acetylglucosamine have been reported to induce the synthesis of a wide spectrum of chitinolytic enzymes, i.e., *N*-acetylglucosaminidase, endochitinase, and chitobiosidase in some bacterial species such as *A. caviae*, *Ent. agglomerans*, and *Bacillus cereus*, and in the fungi *Trichoderma harzianum* and *Hirsutella* sp. *(28)*.

Although the chitinase gene in *E. coli* clone was carried by a plasmid vector with a high copy number, and was expressed under the control of the *lac* promoter, the resulting chitinase might not be well secreted in *E. coli*, as shown by the result of a assay for the extra- and intracellular fractions (**Table 3**). The extracellular chitin-hydrolytic activity exhibited by *E. coli* transformants could have resulted from cell lysis as reported *(29)*.

Chitinase WS7b was more active than in the *E. coli* clone harboring pWS506. This might have come from different expression and regulation of the chitinase gene in the *E. coli* clone as compared with that of WS7b. Ueda et al. (1998) reported that chitinase genes of *Aeromonas* sp. No. 10S-24 showed different expression in their native host than in *E. coli* *(30)*. The activity of chitinase expressed in *E. coli* was apparently due to enzyme in the whole bacterial cell or cell membrane associated enzyme *(12,16,30)*. However, the difference might also come from the possibility that WS7b carries more than one chitinase gene has been reported *(14)*.

5. Conclusion

We cloned the chitinase gene from *A. caviae* WS7b, which has been one of the well-characterized chitinase genes of the genus *Aeromonas*. The findings reported in the present study will add information about the diversity and physiological function of this gene.

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