Title

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# New chitosan-degrading strains that produce chitosanases similar to ChoA of *Mitsuaria chitosanitabida*

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**Abstract**

The beta-*Proteobacterium Mitsuaria chitosanitabida* (formerly *Matsuebacter chitosanotabidus*) 3001 produces a chitosanase (ChoA) that is classified into glycosyl hydrolase family 80. While many chitosanase genes have been isolated from various bacteria to date, they show limited homology to the *M. chitosanitabida* 3001 chitosanase gene (*choA*). To investigate the phylogenetic distribution of chitosanases analogous to ChoA in nature, we identified 67 chitosan-degrading strains by screening and investigated their physiological and biological characteristics. We then searched for similarities to ChoA by Western blotting and Southern hybridization and selected 11 strains whose chitosanases showed the most similarity to ChoA. PCR amplification and sequencing of the chitosanase genes from these strains revealed high deduced amino acid sequence similarities to ChoA ranging from 77% to 99%. Analysis of the 16S rDNA sequences of the 11 selected strains indicated that they are widely distributed in the ß- and γ- subclasses of *Proteobacterium* and the *Flavobacterium* group. These observations suggest that the ChoA-like chitosanases that belong to Family 80 occur widely in a broad variety of bacteria.

## Introduction

Chitosan, a linear polymer composed of ß-1, 4-linked glucosamine (GlcN) residues with various numbers of *N*-acetylated residues, is a deacetylated derivative of chitin. The chitooligosaccharides produced by the enzymatic hydrolysis of chitosan are widely used in the food, agricultural and pharmaceutical fields because of their various physiological activities.

Chitosanases (EC 3.2.1.132) are glycosyl hydrolases that catalyze the hydrolysis of the ß-1,4-glycosidic linkage of chitosan and thereby produce glucosamine oligosaccharides. To date, many chitosanases have been found in a variety of microorganisms, including bacteria (11, 18, 26, 29, 30, 42, 43, 44, 48), fungi (6, 8, 9, 10, 17, 38, 50), plants (27), and viruses (40). The chitosanases that have been sequenced so far have been classified into four different families in the classification system of glycosyl hydrolases, namely, Family 8, 46, 75, and 80, (12, 13, 14). This classification of the chitosanases is based on the amino acid sequence similarity of their catalytic domains. Family 8 includes five chitosanases from bacterial organisms along with cellulase, licheninase, and endo-1,4-ß-xylanase (16, 24, 45). Family 46 includes 18 chitosanases, 16 from bacterial organisms and two from *Chlorella* viruses (2, 3, 21, 22, 33, 36, 40, 46, 47). The three-dimensional structures of the Family 46 chitosanases from *Streptomyces* sp. N174 (20) and *Bacillus circulans* MH-K1 (31) and the Family 8 chitosanase from *Bacillus* sp. K17 (1) have been determined. The catalytic residues of the Family 8 and 46 chitosanases are reported to be glutamic acid (Glu) and aspartic acid (Asp) (4). Family 75 includes 17 chitosanases, 14 and three of which are from fungi and bacteria, respectively (37, 49).

Prior to this study, only two bacterial chitosanases have been classified into Family 80

(http://afmb.cnrs-mrs.fr/CAZY/). These show no significant nucleotide or amino acid sequence homology with the chitosanases in other families. We previously reported our identification of the chitosanase gene (*choA*) from *Mitsuaria chitosanitabida* (formerly *Matsuebacter chitosanotabidus*), which was then classified into Family 80 (23, 28). Furthermore, we recently reported that Glu-121 and Glu-141 are the catalytically important residues of ChoA (35). We have also succeeded in functionally expressing chitosanase in the yeast *Schizosaccharomyces pombe* (34).

In the study reported here, we identified other chitosanases that can be classified into Family 80 and investigated their phylogenetic distribution to determine how commonly this type of chitosanase occurs in nature.

## Materials and Methods

**Materials -** Restriction enzymes were purchased from TaKaRa Biomedicals (Kyoto, Japan) and New England Biolabs. Chitosan was obtained from San-in Kensetsu (Shimane, Japan). Ampicillin and 2-mercaptoethanol were purchased from Wako Pure Chemical Industries (Osaka, Japan). All other reagents were of analytical grade quality.

**Strains, plasmid, media, and culture conditions –** *M. chitosanitabida* 3001 and 67 other strains (Nos. 1-67), which were isolated from many different places in Japan, were used in this study. All were grown at 30°C with shaking in chitosan medium containing 0.5% colloidal chitosan, 0.2% $K_2HPO_4$, 0.1% $KH_2PO_4$, 0.07% $MgSO_4$, 0.05% NaCl, 0.05% KCl, 0.01% $CaCl_2$, and 0.05% yeast extract. The plasmid vector pT7 blue (TaKaRa Biomedicals) was used to clone the chitosanase genes

into the cloning host *Escherichia coli* DH5 $\alpha$. All *E. coli* strains were grown at 37°C on LB medium containing appropriate antibiotics for the selection of the transformants.

**Screening of chitosan-degrading bacteria from nature -** Samples collected from soil or water at various locations in Japan were suspended in 5ml of distilled water and particles were eliminated by stationary. The diluted supernatants were cultured in phosphate buffer with chitosan for 3 days, then plated on a chitosan plate containing 0.5% colloidal chitosan, 0.2% $K_2HPO_4$, 0.1% $KH_2PO_4$, 0.07% $MgSO_4$, 0.05% NaCl, 0.05% KCl, 0.01% $CaCl_2$, 0.05% yeast extract and 1.5% agar (pH 6.0) and incubated at 30°C to screen for chitosan-degrading bacteria, which were detected by their clear zone-forming ability.

**Physiological characteristics -** Gram-staining was performed by using the Gram-colour kit from Merck. pH and temperature tolerance were determined using LB medium. Growth at various pH values ranging from 3 to 9 and various temperatures ranging from 20 to 60°C was observed spectroscopically ($OD_{600}$) over a period of 3d. Urease activity, reduction of nitrate, indole production from tryptophan and $H_2S$ production from cysteine were determined according to Smibert and Krieg (39). Other physiological and biochemical tests were performed as described by Cowan and Steel (7).

**Analysis of isoprenoid quinones** - Quinone was extracted by using previously described methods (25). The extracted crude quinone was analyzed by normal phase thin-layer chromatography using ubiquinone 10 as a standard. Normal phase thin-layer chromatography was carried out on a Kiesel gel 60 F254 plate (Merck) with benzene-acetone (93:7, v/v). The UV-visualized band containing quinone was collected from the thin-layer chromatography plate and extracted with chloroform-methanol (1:1.

v/v). The samples were then dried and the precipitate was dissolved in ethanol. The purified quinone was further analyzed by HPLC using ethanol as the solvent phase (15).

**Western blot analysis for the detection of chitosanase -** Western blot analysis was undertaken to determine the cross-reactivity of various chitosanases with a ChoA-specific antibody (28). Cell extracts were subjected to SDS-PAGE on a 12.5% acrylamide gel performed as described by Laemmli (19) and the proteins were then transferred electrophoretically onto a PVDF membrane (Immobilon-PSQ; pore size, 0.45$\mu$m. IPVH 304FO, Millipore). To immunolabel the chitosanases, the nitrocellulose membrane was incubated at room temperature with shaking in TBS-M buffer (20mM Tris-HCl, 0.137M NaCl, 0.1M HCl, 0.25% Tween-20, and 5% dried milk) for at least 1 hr. Afterwards, the membrane was rinsed several times in TBS buffer, then incubated for 1hr with the affinity-purified rabbit antiserum against ChoA. After several rinses in TBS buffer, the membrane was incubated with a horseradish peroxidase-conjugated secondary antibody and the membrane-bound immunocomplexes were detected with an ELC$^{TM}$ system as recommended by the manufacturer (Amersham Pharmacia Biotech). The rabbit antibody specific for the chitosanases was custom-made by TaKaRa Biomedicals.

**Southern hybridization analysis –** The total genomic DNAs of various chitosan-degrading bacteria were extracted by the CTAB method as described by Sambrook et al. (32) and digested with the *Bam*HI restriction enzyme. The digested DNAs were then fractionated on a 0.7% agarose gel, denatured and neutralized, and then transferred onto a nylon membrane (Hybond-N, Amersham) by the capillary method. The Southern blot membranes were hybridized at 42°C for 10h with the *choA*

probe in buffer containing 15ml of Gold hybridization buffer, 0.07% NaCl, and 0.1% blocking agent. The membranes were then washed twice at 42°C with 20xSSC containing 0.4% SDS and 6M Urea for 20 min and twice for 20 min at room temperature with 20xSSC containing 150mmol disodium citrate and 15mmol sodium chloride. The labelled bands were visualized by using the ELC™ system according to the manufacturer`s recommendations (Amersham Pharmacia Biotech).

**PCR amplification of chitosanase genes -** PCR amplification was performed by using a DNA Thermal Cycler (Perkin-Elmer/Cetus) and the three forward primers 5`-GGAACCTCTCCTACATTC-3` (cho420-), 5`-CTGGTSACSGCSACCAAG -3` (cho748-), and 5`-ACGGTCAATCAATGGCAG-3` (cho844-) and two reverse primers 5`-CATGTTCTTSGACCACTT-3` (cho-1692), and 5`-CGCGGGTCGATGGCA-3` (cho-1773), which were designed based on the nucleotide sequence of the *choA* gene from *M. chitosanitabida* 3001. The cho748- and cho-1692 primers have a mixed base C+G (S). PCR amplification was performed with 0.5$\mu$g genomic DNA in 50$\mu$l reaction buffer supplemented with final concentrations of 1.5mM $MgCl_2$, 50$\mu$M of each dNTP, 0.1$\mu$g of each synthesized primers and 2.5 units of Ex-Taq DNA polymerase (TaKaRa). The cycle program was as follows: 1 min at 94~96°C, 2 min at 45~58°C, and 3 min at 72°C (25 cycles).

**PCR amplification of the 16S rDNA gene -** Genomic DNA was extracted from selected chitosan-degrading bacterial strains and the 16S rDNA-coding region was PCR-amplified using the two oligonucleotide primers 5`- ATCTGGTTGATCCTGCCAGT-3` (positions 2 to 21 relative to *E. coli* 16S rRNA) and 5`-GGCTACCTTGTTACGACTT-3` (positions 1510 to 1492 relative to *E. coli*

16S rRNA). The PCR program consisted of an initial denaturation step of 1 min at 95°C followed by 35 cycles of 94°C for 1 min, 48°C for 2 min, and 72°C for 3 min. The PCR products of the expected size were purified using a PCR product purification kit, then cloned into the pT7 blue plasmid vector and sequenced using the primers 5`-CCAGCAGCCGCGGTAATAC-3` (corresponding to the complementary nucleotide sequence 518-536 of *E. coli* 16S rRNA) and 5`-AAACTCAAAGGAATTGACGG-3` (corresponding to the complementary nucleotide sequence 907-926 of *E. coli* 16S rRNA). Computer-assisted analysis and comparison of DNA sequence were performed using the BLAST program in the NCBI network service.

**Phylogenetic analysis -** 16S rDNA sequences determined in this study were compared with 16S rDNA sequences of their related bacteria obtained from the GenBank using the neighbour-joining method with the CLUSTAL W program on the web (http//www.ddbj.nig.ac.jp/search/clustalw-j.html). The phylogenetic tree was drawn by the program Tree View.

**Nucleotide sequencing -** The chitosanase gene fragments in the recombinant pT7 blue plasmid were used for sequencing. Sequencing was carried out using the dideoxy-nucleotide chain termination method by using an ABI PrismTM 377 DNA sequencer (Perkin Elmer). Computer analysis of the nucleotide and deduced amino acid sequences using *choA* sequences was performed by employing DNASIS (Hitachi Software Engineering Co. Ltd., Yokohama).

**Nucleotide sequence accession numbers -** The nucleotide sequences of every 16S rDNA and partial chitosanase gene reported in this article have been assigned to Genebank. The accession numbers of the sequences are as follows: *Chryseobacterium* sp. 2, AB024308; *Herbaspellium* sp. 9,

AB024305; *Mitsuaria* sp. 12, AY856841; *Mitsuaria* sp. 13, AB024306; *Stenotrophomonas* sp. 22, AY856842; *Herbaspellium* sp. 27, AY856843; *Pseudomonas* sp. 38, AY856844; *Stenotrophomonas* sp. 45, AY856845; *Comamonas* sp. 46, AY856846; *Spingobacterium* sp. 62, AY856847; *Mitsuaria* sp. 67, AY856848 (16S rDNA sequences), *Chryseobacterium* sp. 2 AY856849; *Herbaspellium* sp. 9 AY856850; *Mitsuaria* sp. 12 AY856851; *Mitsuaria* sp. 13 AY856852; *Stenotrophomonas* sp. 22 AY856853; *Herbaspellium* sp. 27 AY856854; *Pseudomonas* sp. 38 AY856855; *Stenotrophomonas* sp. 45 AY856856; *Comamonas* sp. 46 AY856857; *Spingobacterium* sp. 62 AY856858; *Mitsuaria* sp. 67 AY856859 (partial chitosanase gene sequences).

## Results

**Physiological and biological characteristics of chitosan-degrading bacteria –** To identify additional chitosan-degrading bacteria, we screened two hundreds of samples (120 samples of the soil, 60 samples of the fresh water and 20 samples of the sea water) collected from in many places of Japan by culturing them on chitosan-containing plates and searching for clear zones generated around the bacterial colonies (see Material and Methods). This yielded 67 bacterial strains (38 from soil, 22 from fresh water and 7 from sea water) numbered No. 1 to No. 67, which were then tested for their physiological and biological properties, which are summarized in Table 1. All isolates were Gram-negative, most of which had similar physiological and biological properties to *M. chitosanitabida* 3001, but some are different, especially strain 2 and 46 produce menaquinone instead of ubiquinone. Combined with later analysis of 16S rDNA sequence (seee Fig. 3), we propose some strains belong to

in the ß- and γ- subclasses of *Proteobacterium* and the *Flavobacterium* group.

**Western blot analysis -** To determine the similarities the chitosanases of the new chitosan-degrading bacterial strains share with ChoA of *M. chitosanitabida* 3001, the isolates were subjected to Western blot analysis using a ChoA-specific antibody. Eleven isolates (Nos. 2, 9, 12, 13, 22, 27, 38, 45, 46, 62, 67) showed the same band pattern as ChoA from *M. chitosanitabida* 3001, namely, a 34 and a 42k band (Fig. 1A). The others showed four different band patterns, as summarized in Table 2. These results suggest that the 11 isolates that showed the same signal pattern as *M. chitosanitabida* 3001 may produce chitosanases that are similar to ChoA .

**Southern hybridization analysis -** To determine whether these 11 isolates have chitosanase genes that are similar to the *choA* gene from *M. chitosanitabida* 3001, Southern hybridization analysis using the *choA* gene as a probe was carried out. The probe used in this experiment contained the whole ORF region of *choA*. The detected signal patterns are shown in Figure 1B. Of the 11 isolates, only Nos. 9, 12, 46, and 67 had similar signal patterns to that of *M. chitosanitabida* 3001. Nos. 2, 22, 38, and 45 isolates showed similar signal patterns among themselves, while Nos. 13 and 62 yielded very different signals. No. 27 did not give any signals. These observations suggest that at least four of the isolates that have an anti-ChoA antibody-reactive chitosanase have a chitosanase gene that is also similar to *choA*.

**Chitosanase sequence -** We amplified the chitosanase genes from all 11 isolates using five primers (three forward and two reverse) designed on the basis of the *choA* sequence. This generated six forward:reverse primer sets. Varied denaturation and annealing temperatures were employed with

these primer sets. DNA fragments approximately 1.4kb in size were successfully amplified from isolates Nos. 2 and 67. A DNA fragment of about 0.85kb was amplified from the other isolates. These fragments were purified, ligated with the pT7 blue vector and their nucleotide sequences were determined. When the deduced amino acid sequences were aligned with the ChoA sequence (Fig.2), all the sequenced fragments showed high (over 95%) identity to ChoA, except for No. 27, which had 77% identity at the amino acid level, the one we could not detect by Southern blot analysis (Fig.1B).

**16S rDNA sequence analysis -** To determine the phylogenetic relationships between the 11 selected chitosan-degrading bacterial strains and *M. chitosanitabida* 3001, we determined the almost complete 16S rDNA sequences of these strains and subjected them to BLAST searching (http://www.ncbi.nlm.nih.gov/BLAST/). This revealed that strains Nos. 12, 13, and 67 appear to belong to the *Mitsuaria* sp., as their levels of 16S rDNA homology with *Mitsuaria chitosanitabida* 3001 16S rDNA were 99.4%, 98.4%, and 99.6%, respectively. No. 2 appears to belong to the *Chryseobacterium* sp., as its 16S rDNA homology to the 16S rDNA of *Chryseobacterium taichungense* was 97.3%. Moreover, Nos. 9 and 27 may be *Herbaspellium* sp., given their 96.7%, and 96.2% levels of homology with the 16S rDNA of *Herbaspirillum frisingense*, respectively, while Nos. 22 and 45 may be *Stenotrophomonas* sp. (98.0%, and 97.3% homology with *Stenotrophomonas maltophilia* 16S rDNA, respectively). No. 38 may be a *Pseudomonas* sp. (96.7% homology with *Pseudomonas tolaasii*), No.46 may be a *Comamonas* sp. (97.4% homology with *Comamonas testosteroni*), while No. 62 may be a *Spingobacterium* sp. (97.0% homology with *Sphingobacterium multivorum*). A phylogenetic analysis of all 11 selected isolates and their related bacteria was carried

out based on their 16S rDNA sequences (Fig. 3).

## Discussion

The chitosanases that have been sequenced to date are classified into four different families in the classification system of the glycosyl hydrolases, namely, Family 8, 46, 75, and 80 (12, 13, 14). Recently, the chitosanse from *Streptomyces griseus* HUT 6037 was found to fall into a new glycosyl hydrolase family, namely, Family 5 (41). Prior to this study, Family 80 contains only two chitosanases, those from *M. chitosanitabida* 3001 (28) and *Sphingobacterium multivorum* (23). In this study, we searched for additional bacteria that produce chitosanases resembling the Family 80-type ChoA of *M. chitosanitabida* 3001. Almost all isolates have ubiquinone-8, which is the major quinone compound of members of the ß-subclass of the *Proteobacteria* (5), but isolates Nos. 2 and 62 have menaquinone-6 and -7, respectively. Menaquinone is known to be the major quinone component of the *Flavobacterium* group. This is supported by 16S rDNA analysis of these isolates, which shows that No. 2 belongs to the *Chryseobacterium* sp. while No. 62 belongs to the *Spingobacterium* sp..

Western blot analysis with a ChoA-specific antibody revealed the intracellular chitosanase of *M. chitosanitabida* 3001 exhibited two bands, namely, a 34 and a 42k band. The 42k band is ChoA attached to its signal polypeptide, while the 34k band is the mature form of excreted. Eleven of the 67 isolates showed an identical signal pattern that suggests they produce chitosanases similar to ChoA and that bear similarly sized signal polypeptides.

Southern hybridization analysis using *choA* as the probe revealed that of the 11 isolates identified

by Western blot analysis, isolates Nos. 9, 12, 46, and 67 showed the same signal as *M. chitosanitabida* 3001. Apart from No. 27, which did not give a signal at all, the remaining isolates showed differently sized signals. Thus, it appears that the chitosanase gene from No. 27 may have low homology to *choA* compared to the others. This is supported by the deduced amino acid sequence of the No. 27 chitosanase, which showed only 77% homology to ChoA while the chitosanases of the other ten isolates showed over 95% identity with ChoA. Alignment of the deduced amino acid sequences with that of *choA* revealed the two glutamic acid residues (Glu-121 and Glu-141) reported to be putative catalytic residues for *M. chitosanitabida* 3001 ChoA (35) are conserved in all the sequenced chitosanases. Moreover, all six cysteine residues, and the Arg-150 and Asp-164 residues that are important for the catalytic activity of ChoA are all conserved (35). Phylogenetic analysis using the 16S rDNA sequences of the 11 selected isolates then showed that *choA*-like genes are widely distributed in the ß- and γ- subclasses of *Proteobacterium* and the *Flavobacterium* group in nature.

In conclusion, we characterized 11 newly isolated strains that possess a Family 80-type chitosanase, which reveals these chitosanases are widely distributed in the ß- and γ- subclasses of *Proteobacterium* and the *Flavobacterium* group in nature. This wide distribution suggests that Family 80 chitosanases occur commonly in nature.

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## LEGENDS

**Fig. 1. Western blot and Southern hybridization analysis against yielded isolates.** (A) Each isolate was grown in PYS medium overnight and then cultured five more days in chitosan liquid medium. The precipitate was separated from the culture medium by centrifugation and the intracellular chitosanase was detected by Western blot analysis using a ChoA-specific antibody and a horseradish peroxidase-conjugated secondary antibody. (B) Southern hybridization analysis was performed using total *Bam*HI-digested genomic DNA from each strain and the chitosanase gene (*choA*) from *M. chitosanitabida* 3001 as the probe.

**Figure 2. Comparison of the partial amino acid sequences of the chitosanases from the 11 selected isolates, *Spingobacterium multivorum* and *M. chitosanitabida* 3001.** The amino acid sequences of the 11 selected isolates were deduced from the nucleotide sequences of the PCR-amplified DNA fragments. Bold characters indicate different amino acid residues relative to the ChoA sequence. The two putative catalytic amino acid residues of ChoA are boxed. The asterisks (*) indicate the six cysteine residues of ChoA. The Arg-150 and Asp-164 residues, which are important for the catalytic activity of ChoA, are indicated by vertical arrows. The GenBank accession numbers are: (a) *Mitsuaria chitosanitabida* 3001, AB010493; (b) *Spingobacterium multivorum*, AB030253; (c) *Flavobacterium* sp. 2 AY856849; (d) *Herbaspellium* sp. 9 AY856850; (e) *Mitsuaria* sp. 12 AY856851; (f) *Mitsuaria* sp. 13 AY856852; (g) *Stenotrophomonas* sp. 22 AY856853; (h) *Herbaspellium* sp. 27 AY856854; (i) *Pseudomonas* sp. 38 AY856855; (j) *Stenotrophomonas* sp. 45

AY856856; (k) *Comamonas* sp. 46 AY856857; (l) *Spingobacterium* sp. 62 AY856858; (m) *Mitsuaria* sp. 67 AY856859.

**Fig. 3. Phylogenetic relationship of *M. chitosanitabida* 3001 with the 11 selected isolates and their related strains based on their 16S rDNA gene sequences.** Bar, 1 nucleotide substitution per 100 nucleotides in 16S rDNA gene sequence. The numbers at the nodes of the tree indicate bootstrap values (%) for each node of 1,000 bootstrap re-samplings. The sequences used for the comparison with the 16S rDNA genes of the isolates were obtained from GenBank. The origins and accession numbers of the sequences are as follows: *Escherichia coli*, J01859; *Pseudomonas* sp. 38, AY856844; *Pseudomonas tolaassi*, AF255336; *Xanthomonas campestris pv. camp*, AF000946; *Stenotrophomonas* sp. 45, AY856845; *Stenotrophomonas maltophilia*, AJ131903; *Stenotrophomonas* sp. 22, AY856842; *Chryseobacterium* sp. 2, AB024308; *Chryseobacterium taichungense*, AJ843132; *Sphingobacterium multivorum*, AB020205; *Spingobacterium* sp. 62, AY856847; *Rhodocyclus tenuis*, D16208; *Azoarcus denitrificians*, L33694; *Zoogloea ramigera*, D14257; *Alcaligenes faecalis*, D88008; *Herbaspellium* sp. 9, AB024305; *Herbaspellium* sp. 27, AY856843; *Herbaspirillum frisingense*, AJ238359; *Burkholderia cepacia*, X87275; *Spirillum volutans*, M34131; *Brachymonas denitrificans*, D14320; *Comamonas testosteroni,* AB064318; *Comamonas* sp. 46, AY856846; *Variovorax paradoxus*, D88006; *Rhodoferax fermentans*, D16212; *Sphaerotilus natans*, Z18534; *Leptothrix discophora*, Z18533; *Ideonella dechloratans*, X72724; *Rubrivivax gelatinosus*, AB016167; *Mitsuaria* sp. 13, AB024306; *Mitsuaria chitosanitabida* 3001, AB024307; *Mitsuaria* sp. 67, AY856848; *Mitsuaria* sp. 12, AY856841.

Table1. Comparison of physiological and biological characteristics of 11 isolated strains

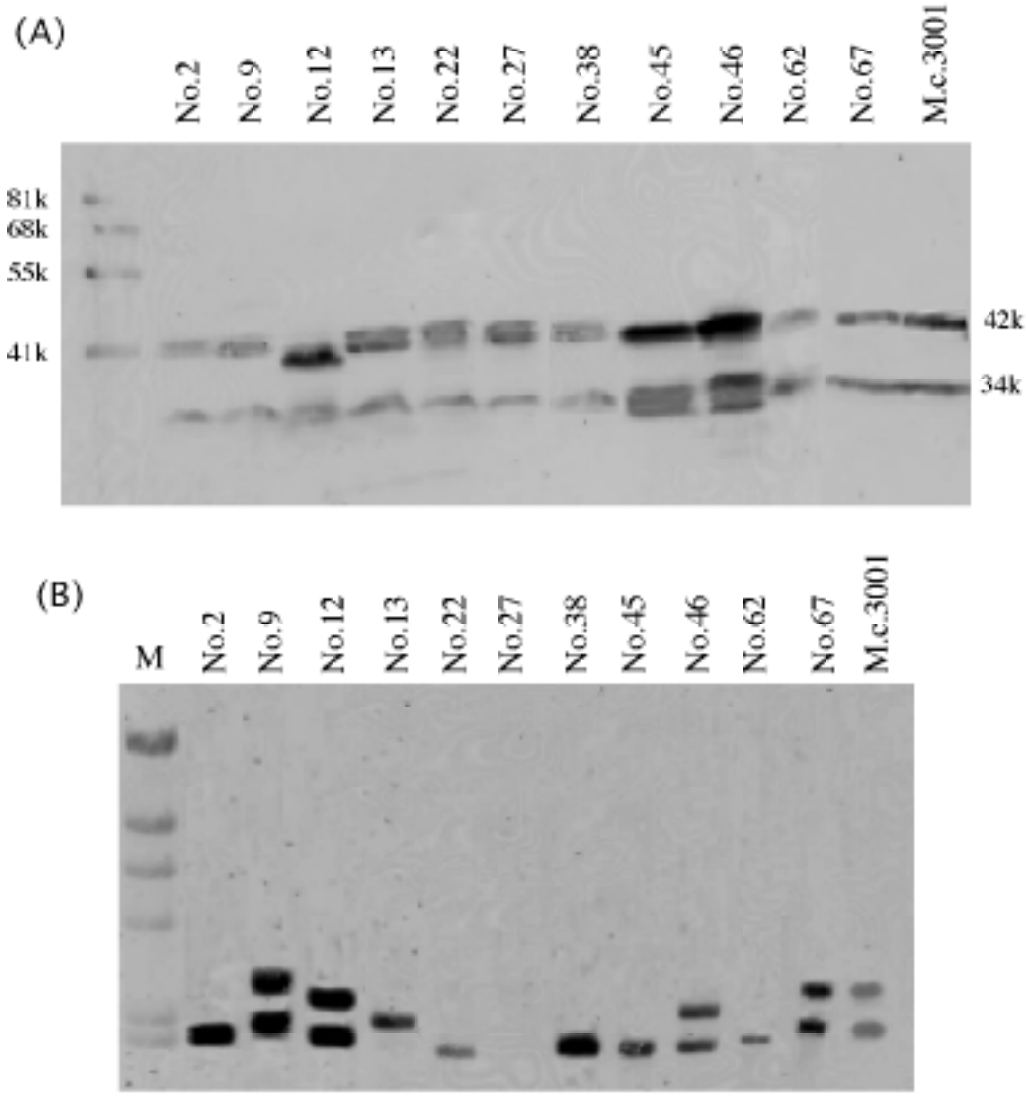
| | No.2 | No.9 | No.12 | No.13 | No.22 | No.27 | No.38 | No.45. | No.46 | No.62 | No.67 | M.c.3001 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Gram staining | - | - | - | - | - | - | - | - | - | - | - | - |
| Nitrate reduction | - | + | + | + | + | + | + | + | + | + | + | + |
| Metylred & V-P test | - | - | - | - | - | - | - | - | - | - | - | - |
| Indole production | + | - | - | - | - | - | - | - | - | - | - | - |
| O-F test | n | n | n | n | n | n | o | n | n | n | n | n |
| Oxidase production | + | + | + | + | + | + | - | + | + | + | + | + |
| Urease production | + | - | - | - | + | + | + | + | + | + | - | - |
| 2-keto-gluconate production | + | - | - | - | - | - | - | - | - | - | - | - |
| 3-keto lactose production | - | - | - | - | - | - | - | - | - | - | - | - |
| Dihydroxyacetone production | + | - | - | - | - | - | - | - | - | - | - | - |
| Catalase production | + | + | + | + | + | + | + | + | + | + | + | + |
| $H_2S$ production | - | - | - | - | - | - | + | - | - | - | - | - |
| Hydrolysis of Tween20, 40, 60 | + | + | + | + | + | + | + | + | - | + | + | + |
| Highest temperature for growth | 37°C | 34°C | 34°C | 34°C | 37°C | 40°C | 37°C | 37°C | 37°C | 37°C | 34°C | 34°C |
| pH for growth | 5-8 | 6-9 | 5-9 | 5-9 | 5-9 | 5-10 | 5-9 | 4-9 | 4-9 | 5-9 | 5-9 | 5-9 |
| Quinone type | MK-6 | UQ-8 | UQ-8 | UQ-8 | UQ-8 | UQ-8 | UQ-8 | UQ-8 | UQ-8 | MK-7 | UQ-8 | UQ-8 |

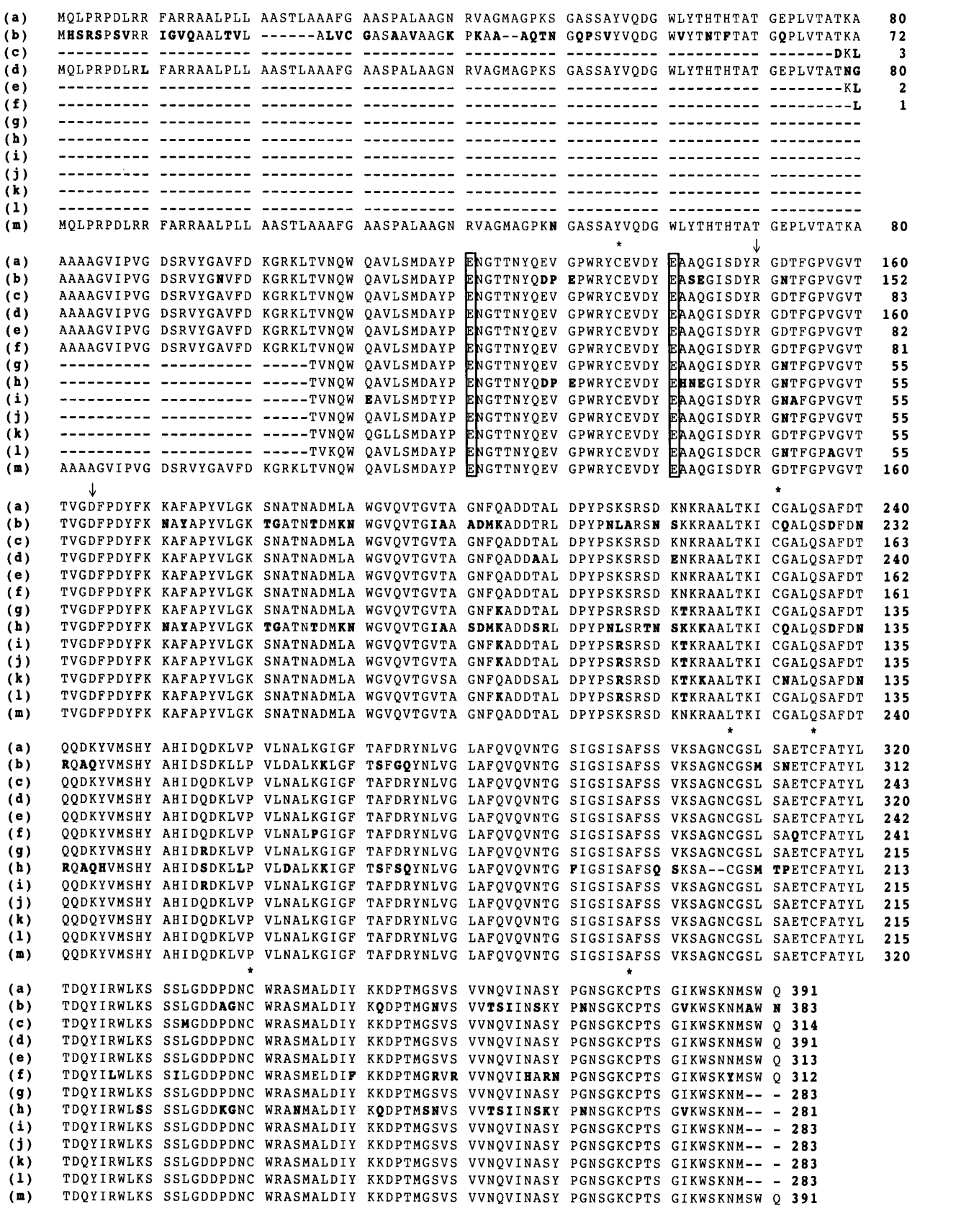
+ : positive, -: negative

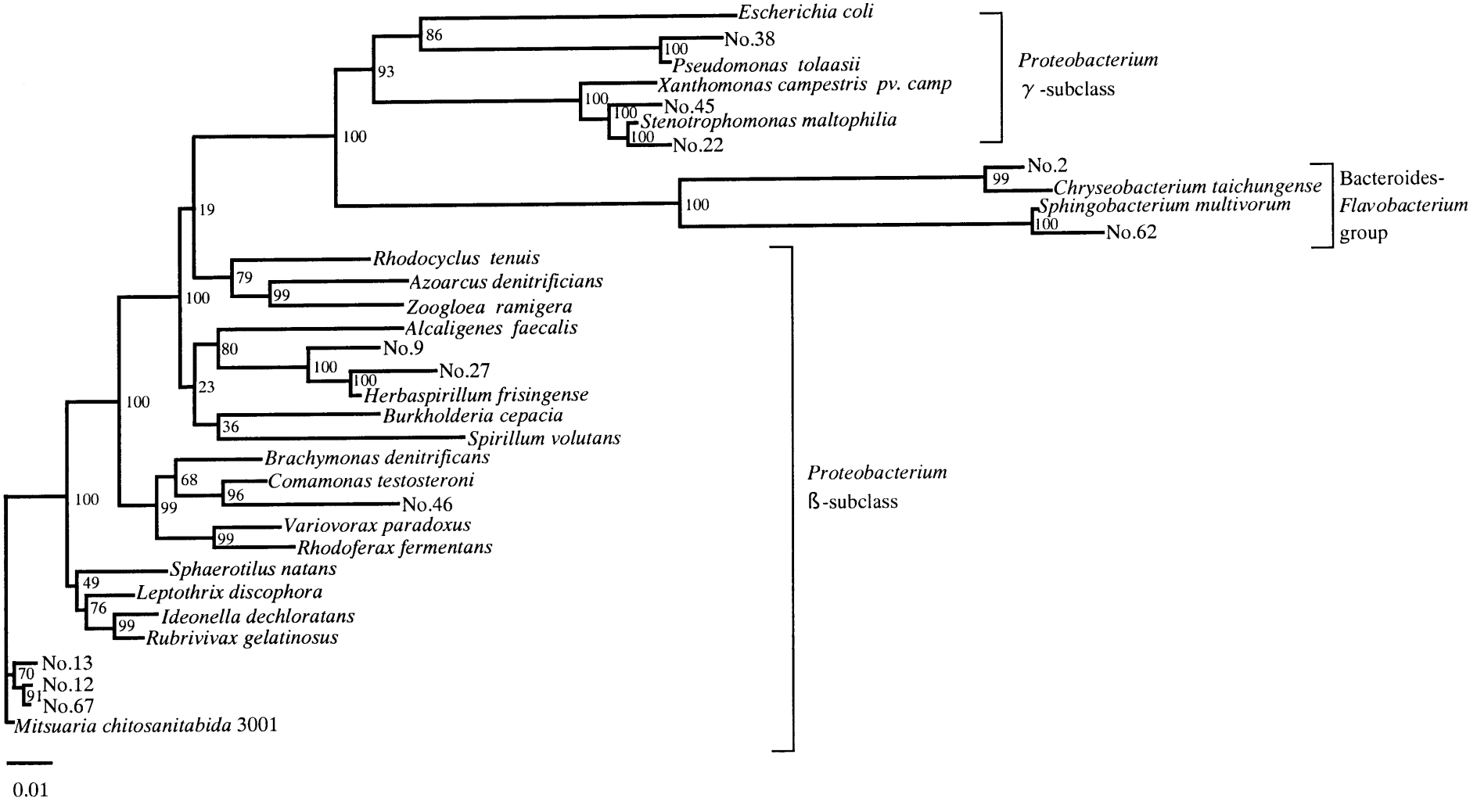
n : no action on carbohydrate, o : oxidation

Table 2. Band patterns showing the reactivity of the chitosanases from new chitosan-degrading bacteria with an anti-ChoA antibody

| Detected signal | Strains |
|---|---|
| 34k, 42k | M.c. 3001, No.2, 9, 12, 13, 22, 27, 38, 45, 46, 62, 67 |
| 34k | No. 2, 4, 7, 11, 14-17, 19-21, 26, 32, 35, 39, 59, 65 |
| 42k | No. 29, 34, 37, 41-44, 47, 50-55, 58, 60, 61, 63 |
| Signals of different size | No. 8, 23-25, 28, 30, 31, 40, 56, 57, 64, 66 |
| No signal | No. 1, 3, 5, 6, 10, 18, 33, 36, 48, 49 |


(A)
No.2
No.9
No.12
No.13
No.22
No.27
No.38
No.45
No.46
No.62
No.67
M.c.3001
81k
68k
55k
41k
42k
34k
(B)
M
No.2
No.9
No.12
No.13
No.22
No.27
No.38
No.45
No.46
No.62
No.67
M.c.3001




Escherichia coli
86
No.38
100
Pseudomonas tolaasii
93
Xanthomonas campestris pv. camp
100
No.45
100
Stenotrophomonas maltophilia
100
No.22
Proteobacterium
γ -subclass
100
No.2
99
Chryseobacterium taichungense
Sphingobacterium multivorum
100
No.62
Bacteroides-
Flavobacterium
group
19
Rhodocyclus tenuis
79
Azoarcus denitrificians
99
Zoogloea ramigera
100
Alcaligenes faecalis
80
No.9
100
No.27
100
Herbaspirillum frisingense
23
Burkholderia cepacia
36
Spirillum volutans
Brachymonas denitrificans
68
Comamonas testosteroni
96
No.46
100
99
Variovorax paradoxus
99
Rhodoferax fermentans
Proteobacterium
ß-subclass
Sphaerotilus natans
49
Leptothrix discophora
76
Ideonella dechloratans
99
Rubrivivax gelatinosus
70
No.13
91
No.12
No.67
Mitsuaria chitosanitabida 3001
0.01