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New Chitosan-Degrading Strains That Produce Chitosanases Similar to ChoA of *Mitsuaria chitosanitabida*

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The betaproteobacterium *Mitsuaria chitosanitabida* (formerly *Matsuebacter chitosanotabidus*) 3001 produces a chitosanase (ChoA) that is classified in 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 rRNA gene sequences of the 11 selected strains indicated that they are widely distributed in the β and γ subclasses of *Proteobacteria* 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.

Chitosan, a linear polymer composed of β-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 β-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, 16, 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: families 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-β-xylanase (17, 24, 45). Family 46 includes 18 chitosanases, 16 from bacterial organisms and 2 from *Chlorella* viruses (2, 3, 21, 22, 33, 36, 40, 46, 47). The 3-dimensional structures of the family-46 chitosanases from *Streptomyces* sp. strain N174 (20) and *Bacillus circulans* MH-K1 (31) and of the family-8 chitosanase from *Bacillus* sp. strain 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 3 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 to the chitosanases in other families. We previously reported our identification of the chitosanase gene (*choA*) from *Mitsuaria chitosanitabida* (formerly *Matsuebacter chitosanotabidus*) (2a), 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.

Strains, plasmid, media, and culture conditions. *M. chitosanitabida* 3001 and 67 other strains (strains 1 to 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* DH5α. 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 5 ml of distilled water, and particles were removed by sedimentation. The diluted supernatants were cultured in phosphate buffer with chitosan for 3 days, then plated onto a chitosan plate containing 0.5% colloidal chitosan, 0.2% K_2HPO_4, 0.1% KH_2PO_4,

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TABLE 1. Comparison of physiological and biological characteristics of 11 isolated strains

Characteristic	Result[a] for strain:											
	2	9	12	13	22	27	38	45	46	62	67	*M. chitosanitabida* 3001
Gram staining	−	−	−	−	−	−	−	−	−	−	−	−
Nitrate reduction	−	+	+	+	+	+	+	+	+	+	+	+
Methyl red and 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 Tween 20, 40, 60	+	+	+	+	+	+	+	+	−	+	+	+
Highest temp for growth (°C)	37	34	34	34	37	40	37	37	37	37	34	34
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

[a] +, positive; −, negative. N, no action on carbohydrate; O, oxidation.

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 color kit from Merck. pH and temperature tolerance were determined using LB medium. Growth at various pH values ranging from pH 3 to 9 and at various temperatures ranging from 20 to 60°C was observed spectroscopically (optical density at 600 nm) over a period of 3 days. 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).

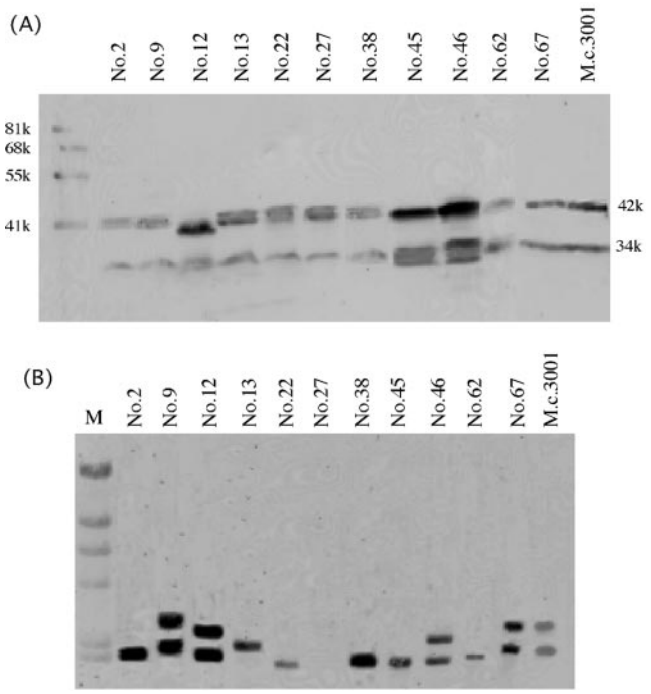

FIG. 1. Western blot and Southern hybridization analyses against isolates. (A) Each isolate was grown in PYS medium overnight and then cultured five more days in chitosan liquid medium. The precipitate was separated from the culture medium by centrifugation, and the intracellular chitosanase was detected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

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, vol/vol). The UV-visualized band containing quinone was collected from the thin-layer chromatography plate and extracted with chloroform-methanol (1:1, vol/vol). The samples were then dried, and the precipitate was dissolved in ethanol. The purified quinone was further analyzed by high-performance liquid chromatography 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 sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 12.5% acrylamide gel, performed as described by Laemmli (19), and the proteins were then transferred electrophoretically to a polyvinylidene difluoride membrane (Immobilon-PSQ; pore size, 0.45 μm; IPVH 304FO; Millipore). To immunolabel the chitosanases, the nitrocellulose membrane was incubated at room temperature with shaking in TBS-M buffer (20 mM Tris-HCl, 0.137 M NaCl, 0.1 M HCl, 0.25% Tween 20, and 5% dry milk) for at least 1 h. Afterwards, the membrane was rinsed several times in TBS buffer and then incubated for 1 h 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 ECL 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 cetyltrimethylammonium bromide method as described by Sambrook et al. (32) and digested with the BamHI restriction enzyme. The digested DNAs were then fractionated on a 0.7% agarose gel, denatured, neutralized, and transferred to a nylon membrane (Hybond-N; Amersham) by the capillary method. The Southern blot membranes were hybridized at 42°C for 10 h with the *choA* probe in a buffer containing 15 ml of Gold hybridization buffer, 0.07% NaCl, and 0.1% blocking agent. The membranes were then washed twice for 20 min at 42°C with 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) containing 0.4% sodium dodecyl sulfate and 6 M urea and twice for 20 min at room temperature with 20× SSC. The labeled bands were visualized by using the ECL 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) with the three forward prim-

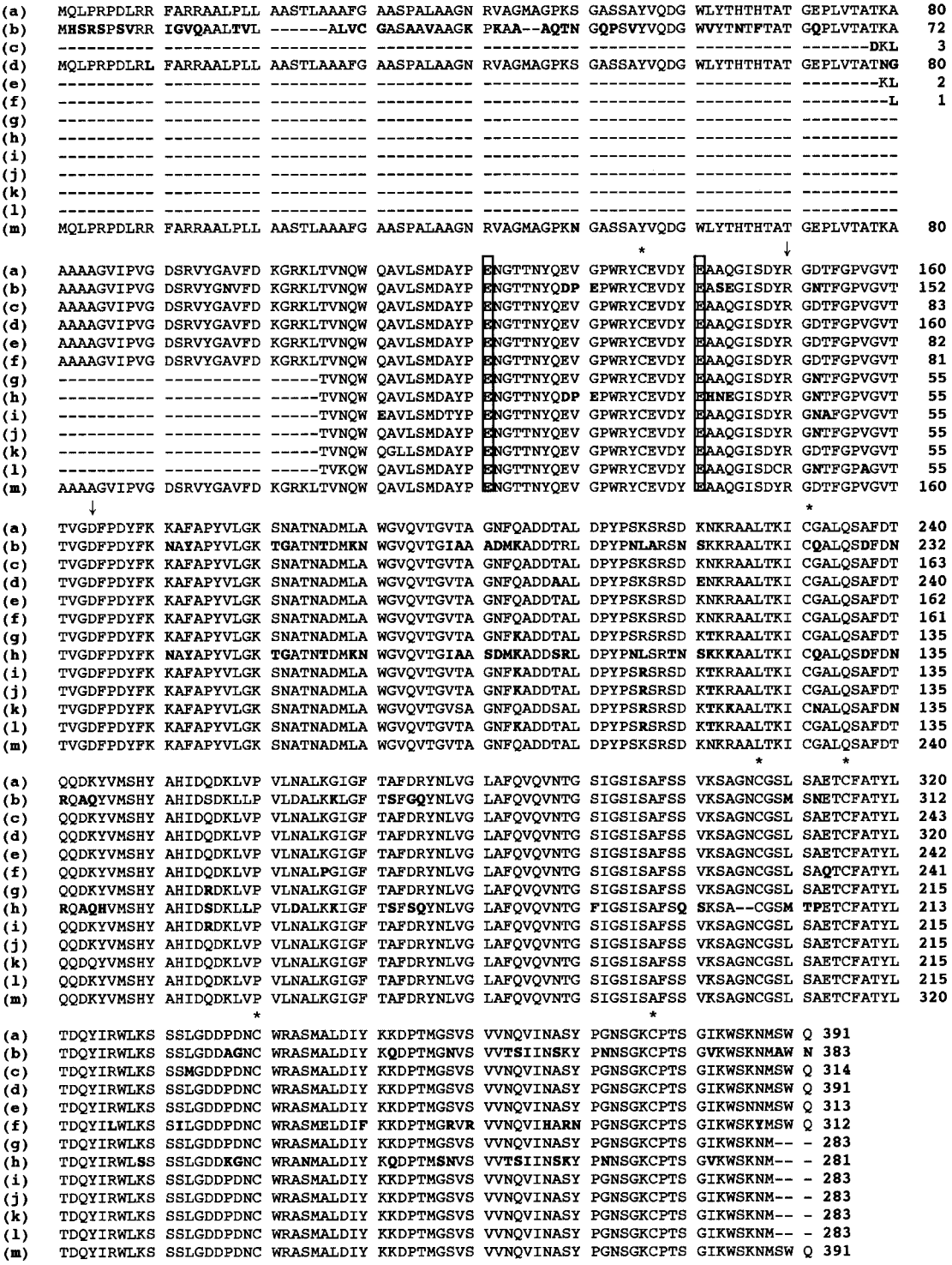

FIG. 2. Comparison of the partial amino acid sequences of the chitosanases from the 11 selected isolates, *Sphingobacterium multivorum*, and *M. chitosanitabida* 3001. The amino acid sequences of the 11 selected isolates were deduced from the nucleotide sequences of the PCR-amplified DNA fragments. Boldface indicates amino acid residues different from those of the ChoA sequence. The two putative catalytic amino acid residues of ChoA are boxed. Asterisks mark 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. Strains and GenBank accession numbers are as follows: (a) *Mitsuaria chitosanitabida* 3001, AB010493; (b) *Sphingobacterium multivorum*, AB030253; (c) *Flavobacterium* sp. strain 2, AY856849; (d) *Herbaspirillum* sp. strain 9, AY856850; (e) *Mitsuaria* sp. strain 12, AY856851; (f) *Mitsuaria* sp. strain 13, AY856852; (g) *Stenotrophomonas* sp. strain 22, AY856853; (h) *Herbaspirillum* sp. strain 27, AY856854; (i) *Pseudomonas* sp. strain 38, AY856855; (j) *Stenotrophomonas* sp. strain 45, AY856856; (k) *Comamonas* sp. strain 46, AY856857; (l) *Sphingobacterium* sp. strain 62, AY856858; (m) *Mitsuaria* sp. strain 67, AY856859.

ers 5′-GGAACCTCTCCTACATTC-3′ (cho420-), 5′-CTGGTSACSGCSACCA AG-3′ (cho748-), and 5′-ACGGTCAATCAATGGCAG-3′ (cho844-) and the two reverse primers 5′-CATGTTCTTSGACCACTT-3′ (cho-1692) and 5′-CGC GGGTCGATGGCA-3′ (cho-1773), which were designed based on the nucleotide sequence of the *choA* gene of *M. chitosanitabida* 3001. The cho748- and cho-1692 primers have a mixed base, C+G (S). PCR amplification was performed with 0.5 μg genomic DNA in 50 μl of reaction buffer supplemented with final concentrations of 1.5 mM $MgCl_2$, 50 μM each deoxynucleoside triphosphate, 0.1 μg of each synthesized primer, and 2.5 Uof Ex-Taq DNA polymerase (TaKaRa). The cycle program was as follows: 1 min at 94 to 96°C, 2 min at 45 to 58°C, and 3 min at 72°C (25 cycles).

PCR amplification of the 16S rRNA gene. Genomic DNA was extracted from selected chitosan-degrading bacterial strains, and the 16S rRNA-coding region was PCR amplified using the two oligonucleotide primers 5′-ATCTGGTTGAT CCTGCCAGT-3′ (positions 2 to 21 relative to *E. coli* 16S rRNA) and 5′-GGC TACCTTGTTACGACTT-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 primers 5′-CCAGCAGCCGCGGTAATAC-3′ (corresponding to the complementary nucleotide sequence 518 to 536 of *E. coli* 16S rRNA) and 5′-AAA CTCAAAGGAATTGACGG-3′ (corresponding to the complementary nucleotide sequence 907 to 926 of *E. coli* 16S rRNA). Computer-assisted analysis and comparison of DNA sequences were performed using the BLAST program in the National Center for Biotechnology Information network service.

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

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 Prism 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, Japan).

Nucleotide sequence accession numbers. The nucleotide sequences of all 16S rRNA and partial chitosanase genes reported in this article have been deposited in GenBank. The accession numbers of the 16S rRNA gene sequences are as follows: *Chryseobacterium* sp. strain 2, AB024308; *Herbaspirillum* sp. strain 9, AB024305; *Mitsuaria* sp. strain 12, AY856841; *Mitsuaria* sp. strain 13, AB024306; *Stenotrophomonas* sp. strain 22, AY856842; *Herbaspirillum* sp. strain 27, AY856843; *Pseudomonas* sp. strain 38, AY856844; *Stenotrophomonas* sp. strain 45, AY856845; *Comamonas* sp. strain 46, AY856846; *Sphingobacterium* sp. strain 62, AY856847; *Mitsuaria* sp. strain 67, AY856848. The accession numbers of the partial chitosanase gene sequences are as follows: *Chryseobacterium* sp. strain 2, AY856849; *Herbaspirillum* sp. strain 9, AY856850; *Mitsuaria* sp. strain 12, AY856851; *Mitsuaria* sp. strain 13, AY856852; *Stenotrophomonas* sp. strain 22, AY856853; *Herbaspirillum* sp. strain 27, AY856854; *Pseudomonas* sp. strain 38, AY856855; *Stenotrophomonas* sp. strain 45, AY856856; *Comamonas* sp. strain 46, AY856857; *Sphingobacterium* sp. strain 62, AY856858; *Mitsuaria* sp. strain 67, AY856859.

RESULTS

Physiological and biological characteristics of chitosan-degrading bacteria. To identify additional chitosan-degrading bacteria, we screened 200 samples (120 soil samples, 60 samples of fresh water, and 20 samples of seawater) collected from many places in Japan by culturing them on chitosan-containing plates and searching for clear zones generated around the bacterial colonies (see Materials and Methods). This yielded 67 bacterial strains (38 from soil, 22 from fresh water, and 7 from seawater), numbered as strains 1 to 67, which were then tested for their physiological and biological properties (summarized in Table 1). All isolates were gram negative, and most had physiological and biological properties similar to those of *M. chitosanitabida* 3001, but some were different, especially strains 2 and 46, which produce menaquinone instead of ubiquinone. Taking these results together with those of later analysis of 16S rRNA gene sequence (see Fig. 3), we propose that some strains belong to the β and γ subclasses of *Proteobacterium* and the *Flavobacterium* group.

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

Signal(s) detected	Strains
34K, 42K	*M. chitosanitabida* 3001; strains 2, 9, 12, 13, 22, 27, 38, 45, 46, 62, 67
34K	Strains 4, 7, 11, 14–17, 19–21, 26, 32, 35, 39, 59, 65
42K	Strains 29, 34, 37, 41–44, 47, 50–55, 58, 60, 61, 63
Signals of different sizes	Strains 8, 23–25, 28, 30, 31, 40, 56, 57, 64, 66
No signal	Strains 1, 3, 5, 6, 10, 18, 33, 36, 48, 49

Western blot analysis. To determine the similarities between the chitosanases of the new chitosan-degrading bacterial strains and *M. chitosanitabida* 3001 ChoA, the isolates were subjected to Western blot analysis using a ChoA-specific antibody. Eleven isolates (strains 2, 9, 12, 13, 22, 27, 38, 45, 46, 62, and 67) showed the same band pattern as *M. chitosanitabida* 3001 ChoA, namely, a 34,000-molecular-weight (34K) and a 42K band (Fig. 1A). The other isolates showed four different band patterns (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 of *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 open reading frame of *choA*. The signal patterns detected are shown in Fig. 1B. Of the 11 isolates, only strains 9, 12, 46, and 67 had signal patterns similar to that of *M. chitosanitabida* 3001. Strains 2, 22, 38, and 45 showed similar signal patterns among themselves, while strains 13 and 62 yielded very different signals. Strain 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 of approximately 1.4 kb were successfully amplified from isolates 2 and 67. A DNA fragment of about 0.85 kb was amplified from the other isolates. These fragments were purified and 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 (more than 95%) identity to ChoA, except for strain 27, the one we could

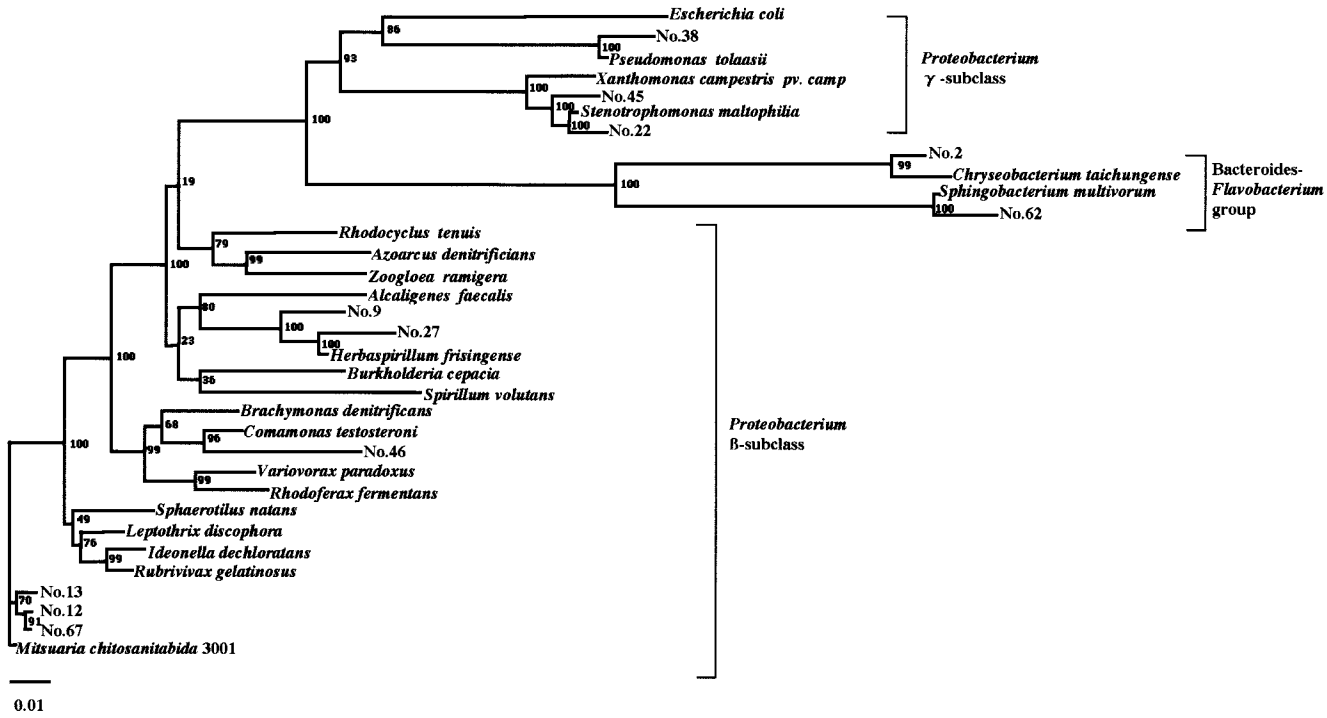

FIG. 3. Phylogenetic relationship of *M. chitosanitabida* 3001 with the 11 selected isolates and their related strains based on their 16S rRNA gene sequences. Bar, 1 nucleotide substitution per 100 nucleotides in the 16S rRNA gene sequence. The numbers at the nodes of the tree indicate bootstrap values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

not detect by Southern blot analysis (Fig. 1B), which had 77% identity at the amino acid level.

16S rRNA gene 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 rRNA gene sequences of these strains and subjected them to BLAST searching (http://www.ncbi.nlm.nih.gov/BLAST/). This revealed that strains 12, 13, and 67 appear to belong to the genus *Mitsuaria*, since their levels of 16S rRNA gene homology with *Mitsuaria chitosanitabida* 3001 were 99.4%, 98.4%, and 99.6%, respectively. Strain 2 appears to belong to the genus *Chryseobacterium*, since its 16S rRNA gene homology with *Chryseobacterium taichungense* was 97.3%. Moreover, strains 9 and 27 may be *Herbaspirillum* spp., given their 96.7% and 96.2% levels of homology, respectively, to the 16S rRNA gene of *Herbaspirillum frisingense*, while strains 22 and 45 may be *Stenotrophomonas* spp. (since they show 98.0% and 97.3% homology, respectively, to the *Stenotrophomonas maltophilia* 16S rRNA gene). Strain 38 may be a *Pseudomonas* sp. (96.7% homology with *Pseudomonas tolaasii*), strain 46 may be a *Comamonas* sp. (97.4% homology with *Comamonas testosteroni*), and strain 62 may be a *Sphingobacterium* sp. (97.0% homology with *Sphingobacterium multivorum*). Phylogenetic analysis of all 11 selected isolates and the related bacteria was carried out based on their 16S rRNA gene 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: families 8, 46, 75, and 80 (12, 13, 14). Recently, the chitosanse of *Streptomyces griseus* HUT 6037 was found to fall into a new glycosyl hydrolase family, family 5 (41). Prior to this study, family 80 contained 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 β subclass of the *Proteobacteria* (5), but isolates 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 rRNA gene

analysis of these isolates, which shows that strain 2 belongs to the genus *Chryseobacterium* while strain 62 belongs to the genus *Sphingobacterium*.

Western blot analysis with a ChoA-specific antibody revealed that the intracellular chitosanase of *M. chitosanitabida* 3001 exhibited two bands: 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 ChoA. Eleven of the 67 isolates showed identical signal patterns, suggesting that they produce chitosanases similar to ChoA and bear similarly sized signal polypeptides.

Southern hybridization analysis using *choA* as the probe revealed that of the 11 isolates identified by Western blot analysis, strains 9, 12, 46, and 67 showed the same signal as *M. chitosanitabida* 3001. Apart from strain 27, which did not give a signal at all, the remaining isolates showed signals of different sizes. Thus, it appears that the chitosanase gene of strain 27 may have low homology to *choA* compared to the others. This is supported by the deduced amino acid sequence of the strain 27 chitosanase, which showed only 77% homology to ChoA, while the chitosanases of the other 10 isolates showed more than 95% identity with ChoA. Alignment of the deduced amino acid sequences with that of *choA* revealed that 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 chitosanases sequenced. Moreover, all six cysteine residues, as well as the Arg-150 and Asp-164 residues, which are important for the catalytic activity of ChoA, are conserved (35). Phylogenetic analysis using the 16S rRNA gene sequences of the 11 selected isolates then showed that *choA*-like genes are widely distributed in the β and γ subclasses of *Proteobacteria* and in the *Flavobacterium* group in nature.

In conclusion, we characterized 11 newly isolated strains that possess family-80-type chitosanases. Our analysis reveals that these chitosanases are widely distributed in the β and γ subclasses of the *Proteobacteria* and in the *Flavobacterium* group in nature. This wide distribution suggests that family-80 chitosanases occur commonly in nature.

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