APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Feb. 2004, p. 1135–1144 Vol. 70, No. 2
0099-2240/04/$08.00+0 DOI: 10.1128/AEM.70.2.1135–1144.2004


# Distribution and Phylogenetic Analysis of Family 19 Chitinases in *Actinobacteria*

Tomokazu Kawase,[1] Akihiro Saito,[2] Toshiya Sato,[1] Ryo Kanai,[1] Takeshi Fujii,[3] Naoki Nikaidou,[1] Kiyotaka Miyashita,[2] and Takeshi Watanabe[1]*

*Department of Applied Biological Chemistry, Faculty of Agriculture, Niigata University, Niigata 950-2181,*[1] *National Institute of Agrobiological Sciences, Tsukuba, Ibaraki 305-8602,*[2] *and National Institute for Agro-Environmental Sciences, Tsukuba, Ibaraki 305-8604,*[3] *Japan*

Received 28 July 2003/Accepted 12 November 2003

**In organisms other than higher plants, family 19 chitinase was first discovered in *Streptomyces griseus* HUT6037, and later, the general occurrence of this enzyme in *Streptomyces* species was demonstrated. In the present study, the distribution of family 19 chitinases in the class *Actinobacteria* and the phylogenetic relationship of *Actinobacteria* family 19 chitinases with family 19 chitinases of other organisms were investigated. Forty-nine strains were chosen to cover almost all the suborders of the class *Actinobacteria*, and chitinase production was examined. Of the 49 strains, 22 formed cleared zones on agar plates containing colloidal chitin and thus appeared to produce chitinases. These 22 chitinase-positive strains were subjected to Southern hybridization analysis by using a labeled DNA fragment corresponding to the catalytic domain of ChiC, and the presence of genes similar to *chiC* of *S. griseus* HUT6037 in at least 13 strains was suggested by the results. PCR amplification and sequencing of the DNA fragments corresponding to the major part of the catalytic domains of the family 19 chitinase genes confirmed the presence of family 19 chitinase genes in these 13 strains. The strains possessing family 19 chitinase genes belong to 6 of the 10 suborders in the order *Actinomycetales*, which account for the greatest part of the *Actinobacteria*. Phylogenetic analysis suggested that there is a close evolutionary relationship between family 19 chitinases found in *Actinobacteria* and plant class IV chitinases. The general occurrence of family 19 chitinase genes in *Streptomycineae* and the high sequence similarity among the genes found in *Actinobacteria* suggest that the family 19 chitinase gene was first acquired by an ancestor of the *Streptomycineae* and spread among the *Actinobacteria* through horizontal gene transfer.**

Chitinase (EC 3.2.1.14) is a glycosyl hydrolase which catalyzes the degradation of chitin, an insoluble linear β-1,4-linked polymer of *N*-acetylglucosamine. This enzyme is present in a wide range of organisms, including organisms that do not contain chitin, and it plays important physiological and ecological roles. Based on amino acid sequence similarity, chitinases are classified into families 18 and 19 of glycosyl hydrolases (13, 14). The members of the two different families differ in their amino acid sequences, three-dimensional (3D) structures (9, 10, 27, 36), and molecular mechanisms of catalytic reactions and are thus considered to have different evolutionary origins.

Family 18 chitinases are widely distributed in a variety of organisms, such as bacteria, fungi, viruses, animals, and higher plants (classes III and V). On the other hand, family 19 chitinases were found only in higher plants until recently. However, since chitinase C (ChiC) of *Streptomyces griseus* HUT6037 was identified as the first family 19 chitinase in an organism other than higher plants (25), the number of family 19 chitinases found in other organisms has increased. For example, the general occurrence of family 19 chitinases in *Streptomyces* species has been demonstrated (28, 40). In addition, the recent progress of genome-sequencing projects for various organisms has revealed the presence of family 19 chitinases in some other bacteria (5, 7, 8, 11, 21, 26, 32, 35, 39, 44) and a few other organisms, including a nematode (22, 43).

Plant family 19 chitinases are thought to be part of a mechanism of defense against fungal pathogens. This role has been deduced from the following observations: (i) chitin is the major component of the cell wall of plant pathogens, (ii) chitinase is one of the pathogenesis-related proteins (12, 18, 19), and (iii) some plant chitinases exhibit antifungal activity in vitro (4, 16, 20, 31). This hypothesis has been supported by the observation that transgenic plants constructed by introducing the plant chitinase gene expressed enhanced resistance against fungal diseases (3). Since *S. griseus* ChiC exhibited significant sequence similarity to plant family 19 chitinases in the catalytic domain, the antifungal activity of ChiC was examined, and a remarkable ability of ChiC to inhibit hyphal extension of *Trichoderma reesei* was demonstrated (40). Therefore, antifungal activity may be a common characteristic of family 19 chitinases.

Although the number of organisms that have family 19 chitinases is increasing, family 19 chitinases of *Streptomyces* are of special interest because of their high levels of similarity to plant class IV chitinases and because of their antifungal activity. To clarify why the distribution of family 19 chitinases in organisms is more restricted than that of family 18 chitinases and how these types of chitinases evolved, a detailed study of *Streptomyces* family 19 chitinases is critical. In this study, to see whether organisms closely related to *Streptomyces* possess family 19 chitinases, we searched for family 19 chitinase genes in *Actinobacteria* and studied the phylogenetic relationship of the genes with those of other organisms.

* Corresponding author. Mailing address: Department of Applied Biological Chemistry, Faculty of Agriculture, Niigata University, 8050 Ikarashi-2, Niigata 950-2181, Japan. Phone: (81) 25-262-6647. Fax: (81) 25-262-6854. E-mail: wata@agr.niigata-u.ac.jp.

TABLE 1. Strains belonging to the class *Actinobacteria* used in this study

| Strain no. | Organism | Order | Suborder | Cleared zone | Signal in Southern hybridization | No. of detectable family 19 chitinase genes |
|---|---|---|---|---|---|---|
| 1 | *Actinomyces naeslundii* JCM 8349 | *Actinomycetales* | *Actinomycineae* | − | − | 0 |
| 2 | *Arcanobacterium haemolyticum* NBRC15585 | *Actinomycetales* | *Actinomycineae* | − | − | 0 |
| 3 | *Corynebacterium glutamicum* JCM 1318 | *Actinomycetales* | *Corynebacterineae* | − | − | 0 |
| 4 | *Dietzia maris* JCM 6166 | *Actinomycetales* | *Corynebacterineae* | − | − | 0 |
| 5 | *Nocardia asteroides* JCM 3384 | *Actinomycetales* | *Corynebacterineae* | − | NT[a] | NT |
| 6 | *Rhodococcus rhodochrous* JCM 3202 | *Actinomycetales* | *Corynebacterineae* | − | NT | NT |
| 7 | *Geodermatophilus obscurus* subsp. *dictyosporus* JCM 3154 | *Actinomycetales* | *Frankineae* | − | NT | NT |
| 8 | *Microsphaera multipartita* JCM 9543 | *Actinomycetales* | *Frankineae* | − | NT | NT |
| 9 | *Glycomyces harbinensis* JCM 7347 | *Actinomycetales* | *Glycomycineae* | + | + | 1 |
| 10 | *Agromyces ramosus* JCM 3108 | *Actinomycetales* | *Micrococcineae* | − | NT | NT |
| 11 | *Arthrobacter crystallopoietes* JCM 2522 | *Actinomycetales* | *Micrococcineae* | − | NT | NT |
| 12 | *Arthrobacter ramosus* JCM 1334 | *Actinomycetales* | *Micrococcineae* | − | − | 0 |
| 13 | *Cellulomonas cellulans* YCWD3 | *Actinomycetales* | *Micrococcineae* | + | + | 1 |
| 14 | *Cellulomonas fimi* JCM 1341 | *Actinomycetales* | *Micrococcineae* | + | − | 0 |
| 15 | *Curtobacterium luteum* JCM 1480 | *Actinomycetales* | *Micrococcineae* | + | − | 0 |
| 16 | *Microbacterium imperiale* JCM 1378 | *Actinomycetales* | *Micrococcineae* | − | − | 0 |
| 17 | *Dermabacter hominis* JCM 7448 | *Actinomycetales* | *Micrococcineae* | − | NT | NT |
| 18 | *Kocuria kristinae* JCM 7237 | *Actinomycetales* | *Micrococcineae* | − | − | 0 |
| 19 | *Kocuria varians* JCM 7238 | *Actinomycetales* | *Micrococcineae* | − | NT | NT |
| 20 | *Promicromonospora sukumoe* JCM 6845 | *Actinomycetales* | *Micrococcineae* | + | + | 1 |
| 21 | *Terrabacter tumescens* JCM 1365 | *Actinomycetales* | *Micrococcineae* | − | NT | NT |
| 22 | *Actinoplanes brasiliensis* JCM 3196 | *Actinomycetales* | *Micromonosporineae* | + | + | 2 |
| 23 | *Catenuloplanes japonicus* JCM 9106 | *Actinomycetales* | *Micromonosporineae* | + | + | 1 |
| 24 | *Couchioplanes caeruleus* subsp. *caeruleus* JCM 3195 | *Actinomycetales* | *Micromonosporineae* | + | − | 0 |
| 25 | *Micromonospora chalcea* JCM 3031 | *Actinomycetales* | *Micromonosporineae* | + | − | 0 |
| 26 | *Pilimelia terevasa* JCM 3091 | *Actinomycetales* | *Micromonosporineae* | − | NT | NT |
| 27 | *Luteococcus japonicus* JCM 9415 | *Actinomycetales* | *Propionibacterineae* | − | − | 0 |
| 28 | *Microlunatus phosphovorus* JCM 9379 | *Actinomycetales* | *Propionibacterineae* | − | NT | NT |
| 29 | *Actinobispora yunnanensis* JCM 9330 | *Actinomycetales* | *Pseudonocardineae* | − | NT | NT |
| 30 | *Actinokineospora riparia* JCM 7471 | *Actinomycetales* | *Pseudonocardineae* | + | + | 1 |
| 31 | *Actinopolyspora mortivallis* JCM 7550 | *Actinomycetales* | *Pseudonocardineae* | − | NT | NT |
| 32 | *Amycolatopsis orientalis* subsp. *orientalis* JCM 4600 | *Actinomycetales* | *Pseudonocardineae* | + | + | 1 |
| 33 | *Kibdelosporangium aridum* subsp. *aridum* JCM 7912 | *Actinomycetales* | *Pseudonocardineae* | + | + | 2 |
| 34 | *Saccharopolyspora erythraea* JCM 4026 | *Actinomycetales* | *Pseudonocardineae* | + | − | 0 |
| 35 | *Streptoalloteichus hindustanus* JCM 3268 | *Actinomycetales* | *Pseudonocardineae* | + | + | 1 |
| 36 | *Kitasatospora setae* JCM 3304 | *Actinomycetales* | *Streptomycineae* | + | + | 2 |
| 37 | *Actinomadura kijaniata* JCM 3306 | *Actinomycetales* | *Streptosporangineae* | + | − | 0 |
| 38 | *Microbispora rosea* subsp. *aerata* JCM 3076 | *Actinomycetales* | *Streptosporangineae* | + | − | 0 |
| 39 | *Nocardiopsis lucentensis* JCM 9420 | *Actinomycetales* | *Streptosporangineae* | + | − | 0 |
| 40 | *Nonomuraea spiralis* JCM 3286 | *Actinomycetales* | *Streptosporangineae* | + | + | 1 |
| 41 | *Planobispora rosea* JCM 3166 | *Actinomycetales* | *Streptosporangineae* | + | + | 2 |
| 42 | *Planomonospora parontospora* subsp. *antibiotica* JCM 3094 | *Actinomycetales* | *Streptosporangineae* | + | + | 2 |
| 43 | *Streptosporangium nondiastaticum* JCM 3114 | *Actinomycetales* | *Streptosporangineae* | + | − | 0 |
| 44 | *Actinocorallia herbida* JCM 9647 | *Bifidobacteriales* | | − | NT | NT |
| 45 | *Atopobium minutum* JCM 1118 | *Coriobacteriales* | | − | − | 0 |
| 46 | *Collinsella aerofaciens* JCM 10188 | *Coriobacteriales* | | − | − | 0 |
| 47 | *Eggerthella lenta* JCM 9979 | *Coriobacteriales* | | − | − | 0 |
| 48 | *Rubrobacter radiotolerans* JCM 2153 | *Rubrobacterales* | | − | − | 0 |
| 49 | *Sphaerobacter thermophilus* DSM 20745 | *Sphaerobacterales* | | − | − | 0 |

[a] NT, not tested.

## MATERIALS AND METHODS

**Bacterial strains, plasmids, media, and culture conditions.** Strains belonging to the class *Actinobacteria* used in this study are listed in Table 1. These strains were obtained from the Japan Collection of Microorganisms (http://www.jcm.riken.go.jp/), the NITE Biological Resource Center (http://www.nbrc.nite.go.jp/e-home/index-e.html/), and the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (http://www.dsmz.de/); the only exception was *Cellulomonas cellulans* YCWD3. All of the strains except YCWD3 were grown under the conditions described at the websites. *C. cellulans* YCWD3 (6) was grown at 30°C in a medium containing 1.0% mannitol, 0.2% peptone, 0.1% meat extract, and 0.1% yeast extract (pH 7.0). *Bacillus circulans* WL-12 (42) and *Serratia marcescens* 2170 (41) were grown at 30°C in Luria-Bertani medium for chromosomal DNA extraction. *Escherichia coli* JM109 was used as a general host for gene cloning, and the pT7Blue T vector (Novagen, Madison, Wis.) was used for nucleotide sequencing of amplified DNA fragments obtained by PCR. *E. coli* JM109 cells carrying a recombinant plasmid were selected on Luria-Bertani medium containing 100 μg of ampicillin per ml. The DNA fragment used as a probe for Southern hybridization was prepared from recombinant plasmid

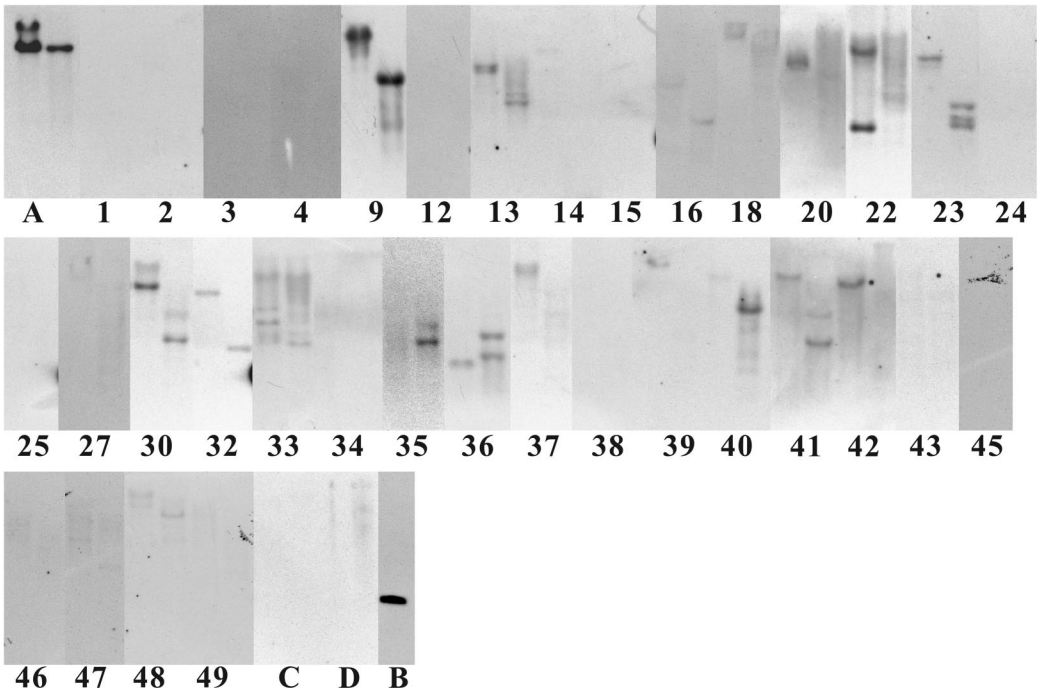


FIG. 1. Detection of family 19 chitinase genes by Southern hybridization. Chromosomal DNAs were digested with either *Pst*I (left lane of each pair) or *Sal*I (right lane of each pair). The numbers are the strain numbers in Table 1. (A) *S. griseus* HUT6037; (B) *Apa*I-*Bam*HI-digested fragment of the *chiC* gene in plasmid pGC01A; (C) *B. circulans* WL-12; (D) *S. marcescens* 2170.

pGC01A (40), which carries a 1-kb DNA fragment of the *chiC* gene of *S. griseus* HUT6037. $pSK^+$-RCC2, used in the control experiment to test amplification of a plant chitinase gene by PCR, contains a gene encoding *Oryza sativa* RCCII (24).

**Detection of chitinase activity.** *Actinobacteria* strains were streaked on agar plate medium containing 0.2% colloidal chitin, 0.05% KCl, 0.1% $K_2HPO_4$, 0.05% $MgSO_4 \cdot 7H_2O$, 0.001% $FeSO_4$, 0.05% yeast extract, and 2.0% agar (pH 7.0) and were incubated at 30°C for 3 to 14 days. Chitinase production was assessed by visual inspection of cleared zones that formed around colonies.

**Gene manipulation.** Chromosomal DNAs of various *Actinobacteria* strains were extracted from the mycelia by the method described by Hopwood et al. (15), with minor modifications. Chromosomal DNAs of *B. circulans* WL-12 and *S. marcescens* 2170 were extracted from the cells as described by Silhavy et al. (33). Other gene manipulations were performed as described by Sambrook and Russell (30).

**Southern hybridization.** Chromosomal DNAs (3 μg) of *Actinobacteria* strains were digested with restriction enzyme *Pst*I or *Sal*I, electrophoresed on a 1.0% agarose gel, and transferred onto a nylon membrane (MAGNA; OSMONICS). Probe DNA was prepared from pGC01A by digesting the plasmid with restriction enzymes *Apa*I and *Bam*HI and was labeled and detected by using an AlkPhos direct labeling detection system with CDP-Star (Amersham Biosciences, Uppsala, Sweden) according to the supplier's instructions.

**PCR amplification and determination of the nucleotide sequence of a portion of the family 19 chitinase genes of *Actinobacteria* strains.** A portion of the genes encoding family 19 chitinases of *Actinobacteria* strains was amplified by PCR by using LA *Taq* DNA polymerase (TaKaRa, Kyoto, Japan). The forward and reverse primers used for PCR were 5′-AAGCTCGCSGCSTTCCTSGC-3′ and 5′-GCACTCGAGSGCGCCGTTGAT-3′), respectively. Thirty amplification cycles of denaturation for 30 s at 98°C, primer annealing for 30 s at 50°C, and DNA synthesis for 1.0 min at 72°C were used. After the last cycle, DNA synthesis was performed for 10 min at 72°C (40). The amplified fragments were ligated with the T vector pT7Blue and were maintained in *E. coli* JM109. Nucleotide sequences of amplified fragments in the T vector were determined with an automated laser fluorescence sequencer (model 4200; LI-COR). Sequencing reactions were performed with a Thermo Sequenase primer cycle sequencing kit with 7-deaza-dGTP (Amersham Biosciences) used according to the supplier's instructions with double-stranded templates. Nucleotide sequence data were analyzed by using the GENETYX system (Software Kaihatsu Co.).

**Phylogenetic analysis.** Multiple alignments were obtained with the Clustal X (37) program. The GeneDoc (23) program was used as an editing tool for multiple alignments. Phylogenetic trees were calculated by the neighbor-joining method (29) implemented in Clustal X and were drawn by using the program TreeView. Nucleotide and amino acid sequences of family 19 chitinases were obtained from the CAZy database (http://afmb.cnrs-mrs.fr/CAZY/).

**Chemicals.** Colloidal chitin was prepared from powdered chitin purchased from Funakoshi Chemical Co. (Tokyo, Japan) by using the methods described by Jeuniaux (17). Other chemicals used in this study were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

**Nucleotide sequence accession numbers.** The nucleotide sequences obtained in this study have been deposited in the DBBJ database under accession numbers AB125373 to AB125390.

## RESULTS AND DISCUSSION

**Chitinase productivity of strains belonging to the class *Actinobacteria*.** The class *Actinobacteria* has been classified into the following five subclasses based on 16S rRNA sequences: *Actinobacteridae*, *Acidimicrobidae*, *Coriobacteridae*, *Sphaerobacteridae*, and *Rubrobacteridae* (34). Subclasses *Acidimicrobidae*, *Coriobacteridae*, *Sphaerobacteridae*, and *Rubrobacteridae* each contain only one order, and each of the orders contains only one family. On the other hand, subclass *Actinobacteridae* consists of two orders, the *Actinomycetales* and *Bifidobacteriales*. The order *Actinomycetales* comprises 10 suborders, and most of them contain many families, while the order *Bifidobacteriales* contains only one family. For example, the *Micrococcineae*, one of the suborders in the *Actinomycetales*, contains nine families. Therefore, the order *Actinomycetales* accounts for the greatest part of the *Actinobacteria*.

To initiate a study to reveal the distribution of family 19 chitinases in the *Actinobacteria*, 49 strains of various species were chosen from five orders and all suborders in the class *Actinobacteria*, as shown in Table 1. The strain in the subclass *Acidimicrobidae* was not included in this study because of the

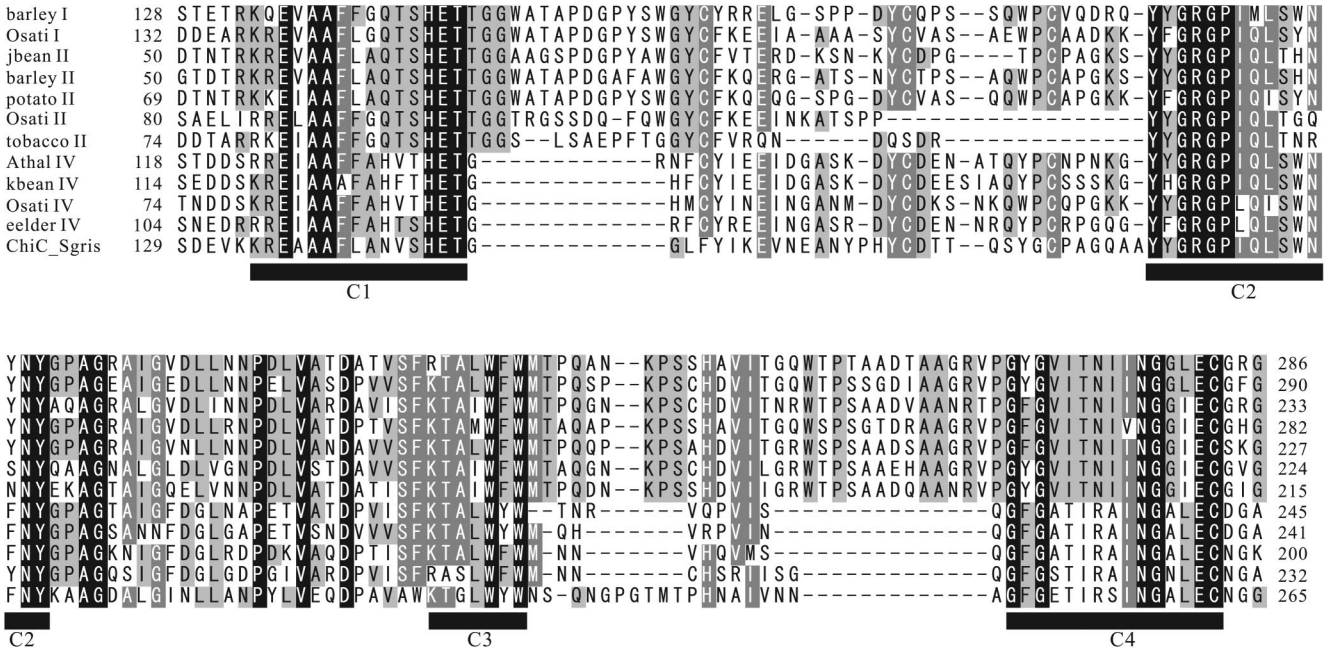


FIG. 2. Alignment of the conserved regions of family 19 chitinases used to design PCR primers. Residues conserved in all sequences are indicated by white type on a black background, while residues conserved in >80 and >60% of the proteins examined are indicated by white type on a dark gray background and by black type on a light gray background, respectively. Conserved regions of family 19 chitinases are indicated by the solid bars (C1, C2, C3, and C4). Conserved regions 1 and 2 were used to design PCR primers. barley I, barley class I chitinase (amino acid sequence accession no. Q42839); Osati I, *O. sativa* class I chitinase (Q42992); jbean II, jack bean class II chitinase (O81934); barley II, barley class II chitinase (P11955); potato II, potato class II chitinase (Q43184); Osati II, *O. sativa* class II chitinase (O80423); tobacco II, tobacco class II chitinase (P17514); Athal IV, *Arabidopsis thaliana* class IV chitinase (O23248); kbean IV, kidney bean class IV chitinase (P27054); Osati IV, *O. sativa* class IV chitinase (O04138); eelder IV, European elder class IV chitinase (Q43150); ChiC_Sgris, *S. griseus* HUT6037 ChiC (O50152).

difficulty of establishing and maintaining a culture of *Acidimicrobium ferrooxidans* DSM 10331, which is the only one strain in this subclass that is available. The chitinase production of the strains was tested by using agar plate medium containing colloidal chitin. Chitinase production was assessed by visual inspection of cleared zones that formed around colonies. As shown in Table 1, 22 of 49 strains formed cleared zones and thus appeared to produce chitinases. These 22 strains and 13 chitinase-negative strains, randomly chosen as controls, were used for further experiments.

**Detection of family 19 chitinase genes by Southern hybridization.** Southern hybridization was used to detect family 19 chitinase genes; part of the *chiC* gene of *S. griseus* HUT6037 was used as the probe. The probe used in these experiments contained the DNA region corresponding to the entire catalytic domain of ChiC and the 41-bp downstream region from the termination codon. Chromosomal DNA was extracted from the 35 strains and digested with either *Pst*I or *Sal*I. As shown in Fig. 1, clear signals were detected for 13 of the 22 strains that showed chitinase activity, suggesting that family 19 chitinase genes were present. Some strains showed only one signal, and the others showed two or three signals, indicating that multiple genes for family 19 chitinases were present. On the other hand, none of the chitinase-negative strains examined as controls showed clear signals, although very faint signals were observed with *Actinomadura kijaniata*, *Eggerthella lenta*, *Kocuria kristinae*, *Microbacterium imperiale*, *Nocardiopsis lucentensis*, and *Rubrobacter radiotolerans*.

**Detection of family 19 chitinase genes by PCR.** To confirm that the strains exhibiting hybridization signals really possess the genes for family 19 chitinases, PCR amplification of part of the family 19 chitinase genes was attempted. In order to design PCR primers, the amino acid sequences of *S. griseus* ChiC and plant class I, II, and IV chitinases were compared, and the four conserved regions were deduced from the alignment shown in Fig. 2. Forward and reverse primers were designed by using the regions corresponding to conserved regions C1 and C4, respectively, taking into account the high G+C content of *Actinobacteria*. By using this primer set, the corresponding region in the cDNA encoding class I chitinase of *O. sativa* (24) was successfully amplified in the control experiment. This fact indicates that the genes amplified with this primer set included not only genes that were very similar to the *chiC* gene of *S. griseus* HUT6037.

Chromosomal DNA was extracted from 22 chitinase-positive strains and 13 chitinase-negative control strains and then subjected to PCR amplification with the primers that we designed. All of the amplified fragments that were approximately 400 bp long were cloned and sequenced to exclude fragments of non-family 19 chitinase genes. As a result, 18 fragments of family 19 chitinase genes were obtained from 13 strains. These strains perfectly matched the strains that exhibited clear signals in the Southern hybridization experiments described above. In addition, no fragment of a family 19 chitinase was obtained from the 13 chitinase-negative strains. From *Actinoplanes brasiliensis*, *Kibdelosporangium aridum* subsp. *aridum*, *Kitasato-*

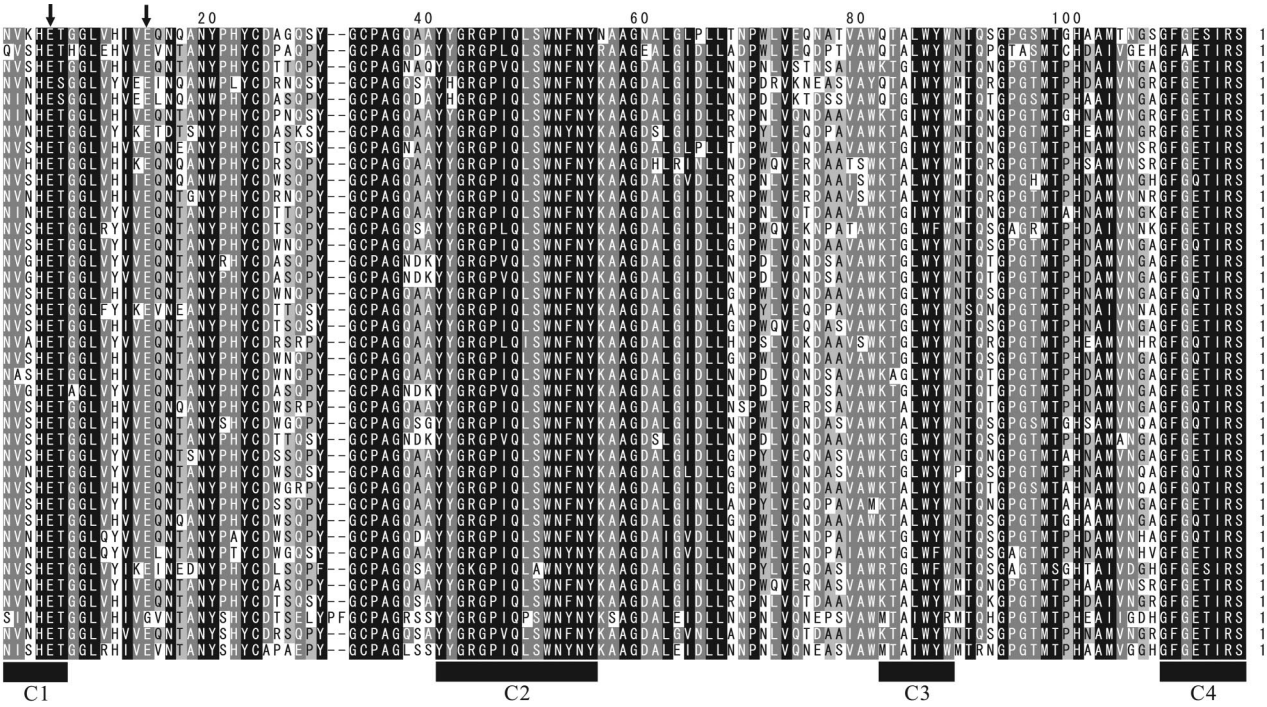


FIG. 3. Alignment of the amino acid sequences deduced from the amplified fragments of family 19 chitinases from *Actinobacteria* strains determined in this study and those reported previously. Residues conserved in all sequences are indicated by white type on a black background, while residues conserved in >80 and >60% of the proteins examined are indicated by white type on a dark gray background and by black type on a light gray background, respectively. Conserved regions of family 19 chitinases are indicated by the solid bars (C1, C2, C3, and C4). Catalytic amino acid residues of family 19 chitinases are indicated by arrows. 1, *Glycomyces harbinensis*; 2, *Cellulomonas cellulans* YCWD3; 3, *Promicromonospora sukumoe*; 4, *Actinoplanes brasiliensis* fragment 1; 5, *Actinoplanes brasiliensis* fragment 2; 6, *Catenuloplanes japonicus*; 7, *Actinokineospora riparia*; 8, *Amycolatopsis orientalis* subsp. *orientalis*; 9, *Kibdelosporangium aridum* subsp. *aridum* fragment 1; 10, *Kibdelosporangium aridum* subsp. *aridum* fragment 2; 11, *Streptoalloteichus hindustanus*; 12, *Kitasatospora setae* fragment 1; 13, *Kitasatospora setae* fragment 2; 14, *Streptomyces coelicolor* A3 (2) *chiF* (DNA accession no. AB017012); 15, *Streptomyces coelicolor* A3(2*) chiG* (AB017013); 16, *Streptomyces coelescens* ISP5421 fragment 1 (AB031749); 17, *Streptomyces coelescens* ISP5421 fragment 2 (AB031750); 18, *Streptomyces griseus* HUT6037 *chiC* (AB009289); 19, *Streptomyces ipomoeae* MAFF4023 (AB031751); 20, *Streptomyces lavendulae* (AF127374); 21, *Streptomyces lividans* 66 fragment 1 (AB031746); 22, *Streptomyces lividans* 66 fragment 2 (AB031747); 23, *Streptomyces lividans* 66 fragment 3 (AB031748); 24, *Streptomyces olivaceoviridis chi30* (AJ133186); 25, *Streptomyces prasinopilosus* fragment 1 (AB031752); 26, *Streptomyces prasinopilosus* fragment 2 (AB031753); 27, *Streptomyces* sp. strain AJ9463 *chiIS* (AB104621); 28, *Streptomyces* sp. strain S15 (AB031754); 29, *Streptomyces* sp. strain S84 (AB031755); 30, *Streptomyces* sp. strain S100 (AB031756); 31, *Streptomyces* sp. strain S159 (AB031757); 32, *Streptomyces thermoviolaceus* OPC-250 *chi25* (AB016843); 33, *Streptomyces thermoviolaceus* OPC-250 *chi35* (AB016842); 34, *Nocardiopsis prasina* OPC-131(AB086832); 35, *Nonomuraea spiralis*; 36, *Planobispora rosea* fragment 1; 37, *Planobispora rosea* fragment 2; 38, *Planomonospora parontospora* subsp. *antibiotica* fragment 1; 39, *Planomonospora parontospora* subsp. *antibiotica* fragment 2.

*spora setae*, *Planobispora rosea*, and *Planomonospora parontospora* subsp. *antibiotica*, two fragments with slightly different nucleotide sequences were amplified, indicating that two distinct family 19 genes were present. Amino acid sequences deduced from the nucleotide sequences of the amplified fragments indicated that the fragments are truly parts of the family 19 chitinase genes. Most of the amplified sequences without primer regions were 340 bp long and encoded 113 amino acid residues; the only exception was one of the two fragments amplified from *P. rosea*, fragment 2, which was 346 bp long and encoded 115 amino acids. The levels of identity of the nucleotide and deduced amino acid sequences of these amplified fragments with the sequences of the corresponding regions of *S. griseus* HUT6037 *chiC* and ChiC were 78 to 87% and 70 to 83%, respectively. Figure 3 shows an alignment of the amino acid sequences deduced from the amplified fragments and the corresponding regions of reported family 19 chitinases of *Streptomyces* species (40) and *Nocardiopsis prasina* OPC-131 (38). Four conserved regions characteristic of family 19 chitinases were found in all the deduced sequences. Except for fragment 2 from *P. rosea*, Glu residues at positions 5 and 14, which were two catalytic amino acid residues (1), were found in all sequences. In fragment 2 of *P. rosea*, a Glu residue at position 14 was replaced by Gly, and in addition, Pro and Phe were inserted at positions 31 and 32.

**Distribution and phylogenetic relationship of the genes encoding family 19 chitinases in *Actinobacteria*.** As summarized in Table 1, the strains tested in these experiments could be divided into three categories, (i) strains which did not show chitinase activity in the agar plate assay (chitinase-negative strains), (ii) strains which showed chitinase activity but for which no family 19 chitinase gene was detected (family 19-negative strains), and (iii) strains which had a family 19 chitinase gene (family 19-positive strains). Family 19-positive strains were found only in the six suborders of the order *Actinomycetales*, the *Pseudonocardineae*, *Micromonosporineae*, *Streptosporangineae*, *Glycomycineae*, *Streptomycineae*, and *Micrococcineae*. In other orders, not even a chitinase-positive

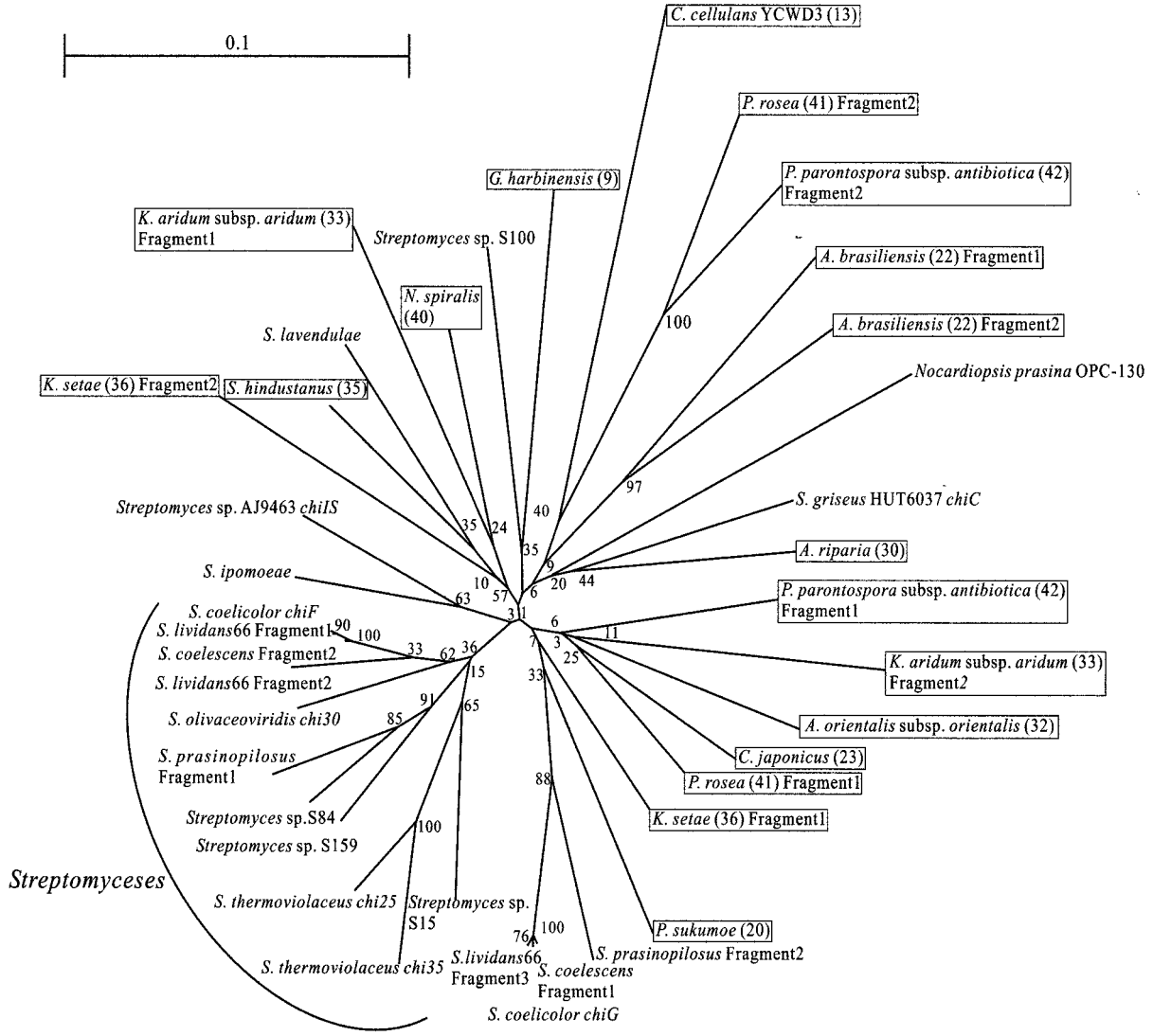


FIG. 4. Phylogenetic relationships among family 19 chitinase genes of *Actinobacteria*. An unrooted phylogenetic tree was calculated based on an alignment of conserved regions of family 19 chitinase genes from *Actinobacteria*. Organisms in which family 19 chitinase genes were found in this study are enclosed in boxes, and the numbers in parentheses are the strain numbers shown in Table 1. The numbers at the nodes are percentages which indicate the levels of bootstrap support, based on a neighbor-joining analysis of 1,000 resampled data sets.

strain was detected. All three types of strains, chitinase-negative, family 19-negative, and family 19-positive strains, were found in four suborders, the *Micrococcineae*, *Pseudonocardineae*, *Streptosporangineae*, and *Micromonosporineae*. The strains belonging to the suborders *Actinomycineae*, *Corynebacterineae*, *Propionibacterineae*, and *Frankineae* did not show chitinase activity. These results demonstrated that family 19 chitinase genes are broadly dispersed in the order *Actinomycetales*, which comprises the greatest part of the *Actinobacteria*.

To visualize the relationship among family 19 chitinases found in *Actinobacteria*, a phylogenetic tree was constructed by the neighbor-joining method (27) by using the nucleotide sequences of the amplified fragments of family 19 chitinase genes and the corresponding sequences of *Streptomyces* species determined previously (40), as shown in Fig. 4. Bootstrap values, which were an index of the accuracy of the phylogenetic tree, were higher outside the tree but lower inside the tree. Because of the low bootstrap values observed inside the tree, which were probably due to the low relative diversity of all sequences used in the analysis, it may not be appropriate to discuss the evolutionary relationships of all family 19 chitinase genes in the tree.

However, it is noteworthy that approximately one-half of the family 19 chitinase genes of *Streptomyces* species formed a stable cluster in the tree, suggesting that there is a close evolutionary relationship at least among these genes. Interestingly, the other half of the *Streptomyces* family 19 chitinase genes were dispersed in the tree with the genes found in other *Actinobacteria*.

**Comparison of amino acid sequences with the sequences of other family 19 chitinases.** Previously, the presence of family 19 chitinase genes has been reported in viruses, bacteria, nem-

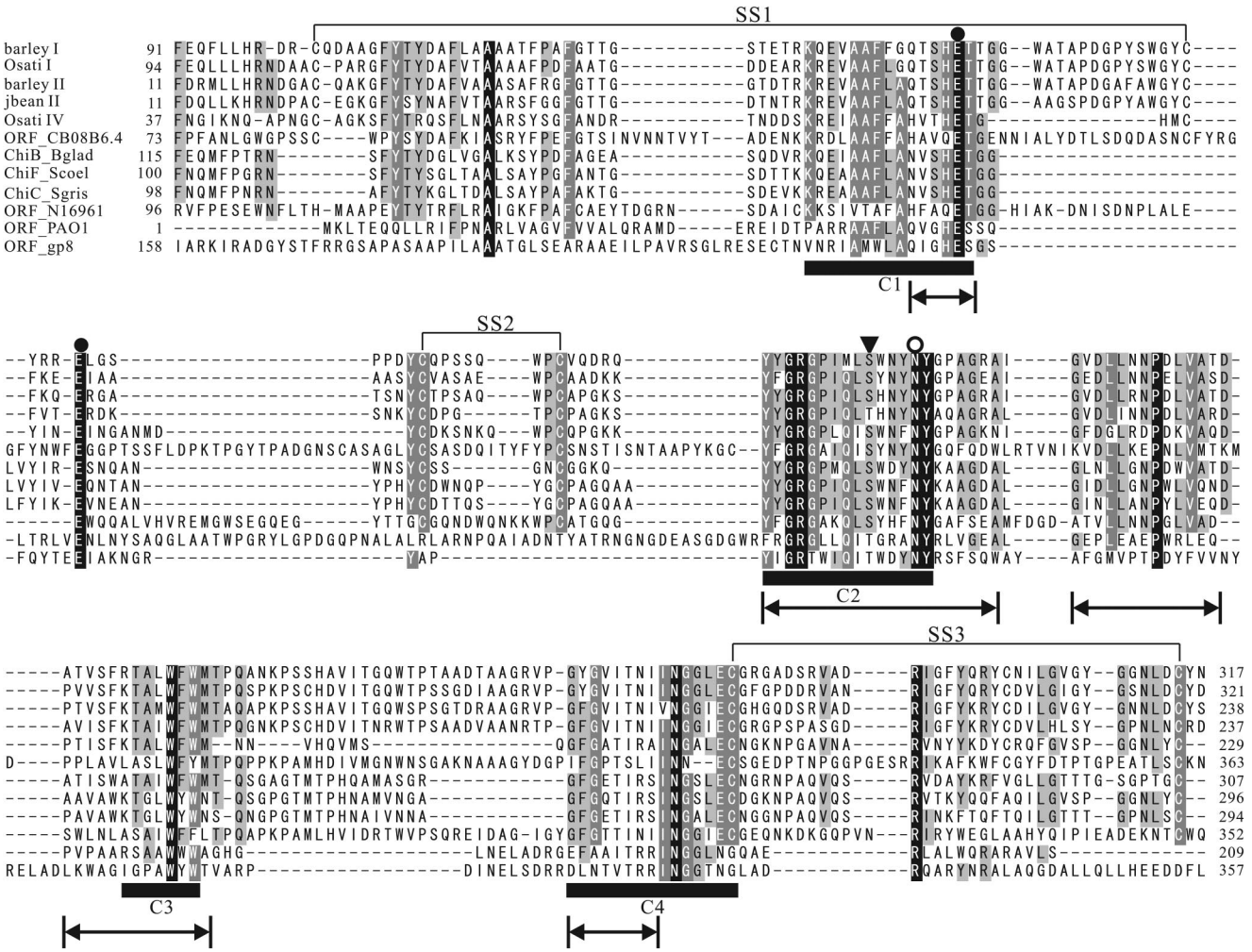


FIG. 5. Alignment of the amino acid sequences of the catalytic domains of family 19 chitinases. Residues conserved in all sequences are indicated by white type on a black background, while residues conserved in >80 and >60% of the proteins examined are indicated by white type on a dark gray background and by black type on a light gray background, respectively. The two catalytic amino acid residues of family 19 chitinases are indicated by solid circles. The open circle indicates amino acid residues related to the activity. Amino acid residues proposed to hold a water molecule are indicated by a solid triangle. SS1, SS2, and SS3 indicate the positions of disulfide bonds. Conserved regions of family 19 chitinases are indicated by the solid bars (C1, C2, C3, and C4). barley I, barley class I chitinase (accession no. Q42839); Osati I, *O. sativa* class I chitinase (Q42992); barley II, barley class II chitinase (P11955); jbean II, jack bean class II chitinase (O81934); Osati IV, *O. sativa* class IV chitinase (O04138); ORF_C08B6.4, *Caenorhabditis elegans* chitinase (Q17816); ChiB_Bglad, *B. gladioli* CHB101 ChiB (BAA92252); ChiF_Scoel, *S. coelicolor* A3 (2) ChiF (Q9Z9M6); ChiC_Sgris, *S. griseus* HUT6037 ChiC (O50152); ORF_N16961, *V. cholerae* El Tor N16961 chitinase (Q9KTW1); ORF_PAO1, *Pseudomonas aeruginosa* PAO1 chitinase (BAA83168); ORF_gp8, mycobacteriophage Bxb1 chitinase (AAG59713). The sequence regions used for the phylogenetic analysis of family 19 chitinases shown in Fig. 6 are indicated by arrows.

atodes, protozoans, and higher plants. Figure 5 shows an alignment of the amino acid sequences of some family 19 chitinases. These sequences were selected to represent sequence variations of all family 19 chitinases and categories of source organisms. The sequences of *S. griseus* ChiC and *Streptomyces coelicolor* ChiF are included as representatives of the *Actinobacteria* family 19 chitinases and are located in different clusters in Fig. 4. The alignment was first constructed with the Clustal X program and then was modified manually by referring to the 3D structures of plant class II chitinases from barley (10) and jack bean (9) and other information. Four conserved regions shown in Fig. 5, deduced in this study, were found in all sequences. Two Glu residues, which are catalytic amino acids of family 19 chitinases, were both conserved in all sequences. Asn residues corresponding to Asn124 of the barley class II chitinase, which has been suggested to participate in the catalytic reaction (1), were also well conserved. In jack bean class II chitinase, the presence of a water molecule held by hydrogen bonds to the carboxyl group of Glu90 and the hydroxyl group of Thr119 has been reported (9). This water molecule is proposed to participate as a nucleophile in the single-displacement inverting catalytic reaction (2), which is widely accepted as the catalytic mechanism of family 19 chitinases. The amino acid residues at the position corresponding to Thr119 were either Thr or Ser in all family 19 chitinases. This suggests that Ser may play the same role as Thr in the catalytic reaction as the hydroxyl amino acid. All family 19 chitinases from *Actinobacteria* have Ser residues at this position. The relative po-

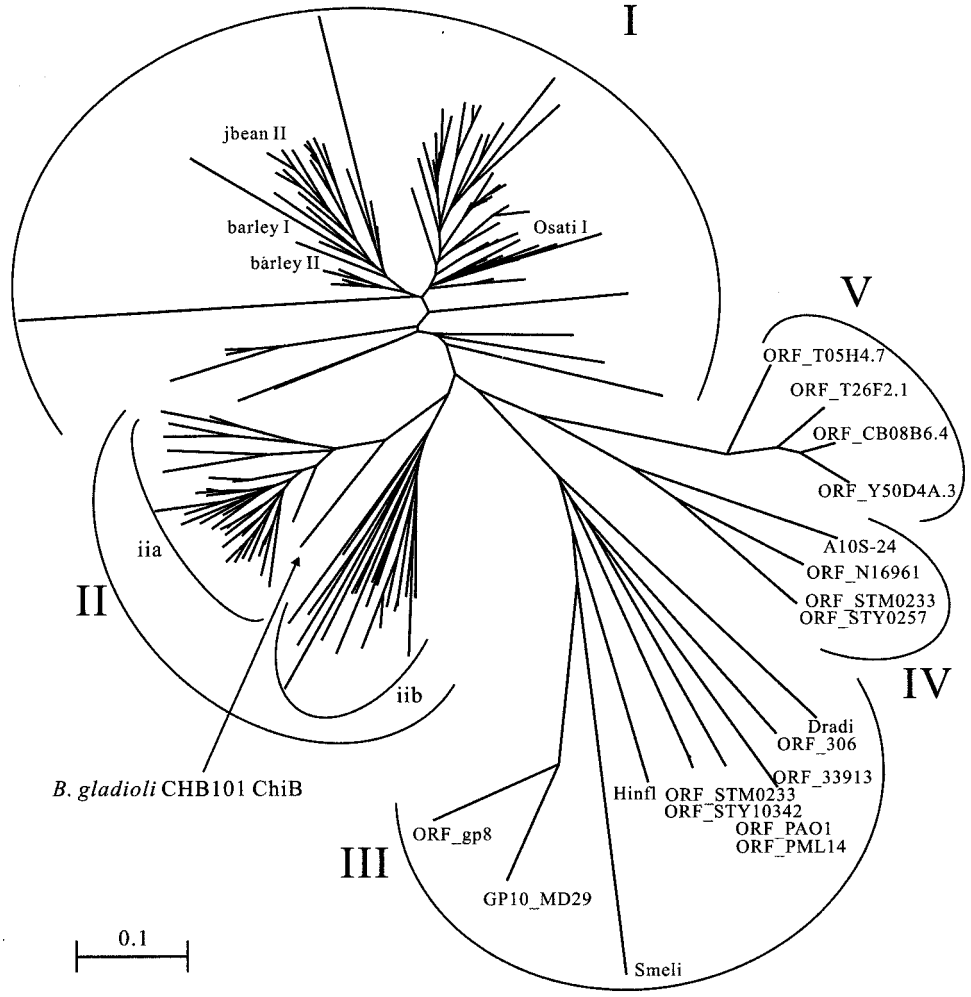


FIG. 6. Phylogenetic relationship of family 19 chitinases of *Actinobacteria* and other organisms available from a database. An unrooted phylogenetic tree was calculated based on an alignment of partial amino acid sequences of all family 19 chitinases by using the neighbor-joining method implemented in the Clustal X program. The sequence regions used for the alignment are indicated in Fig. 5. Cluster I consists of plant class I and II chitinases. Cluster II consists of *Actinobacteria* chitinases (subcluster iia) and plant class IV chitinases (subcluster iib). Cluster III mainly consists of chitinases of *Proteobacteria* and viruses. Cluster IV consists of chitinases of *Proteobacteria*. Cluster V consists of chitinases of Nematoda. barley I, barley class I chitinase (accession no. Q42839); Osati I, *O. sativa* class I chitinase (Q42992); jbean II, jack bean class II chitinase (O81934); barley II, barley class II chitinase (P11955); ORF_STM0233, *S. enterica* serovar Typhimurium LT2 chitinase (Q8ZQH4); ORF_STY10342, *S. enterica* subsp. *enterica* serovar Typhi CT18 chitinase (CAD05435); Dradi, *Deinococcus radiodurans* R1 chitinase (Q9RZ37); Smeli, *Sinorhizobium meliloti* 1021 chitinase (Q92W46); ORF_306, *Xanthomonas axonopodis* pv. citri strain 306 chitinase (AAM35357); ORF_gp8, mycobacteriophage Bxb1 chitinase (AAG59713); GP10_MD29, gene 10 protein of mycobacteriophage D29 (O64203); Hinfl, *Haemophilus influenzae* Rd chitinase (P44187); ORF_33913, *Xanthomonas campestris* pv. campestris strain ATCC 33913 chitinase (AAM42250); ORF_PAO1, *Pseudomonas aeruginosa* PAO1 chitinase (BAA83168); ORF_PML14, *Pseudomonas aeruginosa* PML14 chitinase (BAA83137); ORF_STY0257, *S. enterica* subsp. *enterica* serovar Typhi CT18 chitinase (CAD08692); ORF_STM0233, *S. enterica* serovar Typhimurium LT2 chitinase (Q8ZRN8); ORF_N16961, *V. cholerae* El Tor N16961 chitinase (Q9KTW1); A10S-24, *Aeromonas* sp. strain 10S-24 chitinase (BAA76716); ORF_Y50D4A.3, ORF_T05H4.7, ORF_T26F2.1, and ORF_C08B6.4, *Caenorhabditis elegans* chitinases (AAK68504, O16512, P92013, and Q17816).

sitions of four amino acid resides (two catalytic Glu residues, Asn, and Thr or Ser) in the 3D structures of the chitinases from jack bean and barley and *S. griseus* ChiC were very similar to each other.

Three disulfide bonds have been identified in the 3D structures of the class II chitinases from barley (10) and jack bean (9) at the corresponding positions in the two chitinases. Cys residues, which could form the three disulfide bonds (SS1, SS2, and SS3), were found to be conserved in the amino acid sequences of all family 19 chitinases from higher plants. As shown in Fig. 5, family 19 chitinases from nematodes have all six conserved Cys residues at the corresponding positions. In the sequences of *Actinobacteria* strains, *Burkholderia gladioli* CHB101, and *Vibrio cholerae* El Tor N16961, Cys residues which could form SS2 and SS3 were found, but Cys residues which could form SS1 were not found. On the other hand, family 19 chitinases from other bacteria do not have any corresponding Cys residues.

In terms of the positions of insertions or deletions, the similarity of conserved regions, and the number of the deduced

disulfide bonds, family 19 chitinases from *Actinobacteria* strains and *B. gladioli* CHB101 are more similar to plant family 19 chitinases than to family 19 chitinases from other bacteria.

**Phylogenetic analysis of family 19 chitinases.** To study the phylogenetic relationships of family 19 chitinases found in *Actinobacteria* and other organisms, a multiple alignment of the amino acid sequences corresponding to the sequence regions shown in Fig. 3 of all family 19 chitinases available from CAZy (http://afmb.cnrs-mrs.fr/CAZY/) was prepared. Then the highly variable regions containing many gaps were manually removed from the alignment, and a phylogenetic tree was constructed by the neighbor-joining method (27) based on this modified alignment. Due to the extensive diversity of the sequences, inclusion of the highly valuable regions was impractical for construction of a reliable alignment. As shown in Fig. 6, family 19 chitinases were separated into five clusters on the phylogenetic tree. Plant family 19 chitinases are located in the two clusters, clusters I and II. Cluster I consists solely of plant class I and II chitinases. On the other hand, cluster II consists of *Actinobacteria* chitinases (subcluster iia in Fig. 6) and plant class IV chitinases (subcluster iib). Cluster III mainly consists of the chitinases of *Proteobacteria* and viruses. Cluster IV consists of family 19 chitinases found in *V. cholerae* El Tor N16961, *Aeromonas* sp. strain 10S-24, *Salmonella enterica* subsp. *enterica* serovar Typhi CT18, and *Salmonella enterica* serovar Typhimurium LT2, and cluster V consists solely of Nematoda chitinases. The major group of family 19 chitinases from *Proteobacteria*, which formed cluster III, is most distantly related to the other family 19 chitinases found in various organisms. Interestingly, one of the two family 19 chitinases found in *S. enterica* subsp. *enterica* serovar Typhi CT18 and *S. enterica* serovar Typhimurium LT2 is located in cluster III for each strain, and the other is located in cluster IV.

All family 19 chitinases found in *Actinobacteria* formed the same cluster with plant class IV chitinases, and a chitinase from *B. gladioli* CHB101 exhibited a close evolutionary relationship to these chitinases. On the other hand, the family 19 chitinases of other bacteria belong to the other clusters, including cluster III, which is most distantly related to the rest of the family 19 chitinases. Therefore, the family 19 chitinases of *Actinobacteria* strains are phylogenetically unique among the family 19 chitinases of prokaryotic organisms.

We previously proposed the hypothesis that family 19 chitinases of *Streptomyces* species were acquired from plants by horizontal gene transfer (40). The results obtained in this study support the contention that this hypothesis could be expanded to the family 19 chitinases of *Actinobacteria*. The general occurrence of family 19 chitinase genes in *Streptomyces* species and the high levels of sequence similarity among the genes found in *Actinobacteria* suggest that the family 19 chitinase gene was first acquired by an ancestor of *Streptomyces* species and spread among the *Actinobacteria* through horizontal gene transfer. However, detailed analysis of the family 19 chitinases of other prokaryotic organisms from phylogenetic and biochemical viewpoints is necessary for further discussions.

## ACKNOWLEDGMENTS

We thank Yoko Nishizawa, National Institute of Agrobiological Sciences, for offering a plasmid containing the RCC2 gene. We thank Osamu Shida, Higeta Shoyu Co., Ltd., and Koji Ohnishi, Faculty of Science, Niigata University, for their valuable advice regarding phylogenetic analysis.

This work was supported in part by grant-in-aid for scientific research 14560059 from the Ministry of Education, Culture, Sports, Science, and Technology, Japan.

## REFERENCES

1. **Andersen, M. D., A. Jensen, J. D. Robertus, R. Leah, and K. Skriver.** 1997. Heterologous expression and characterization of wild-type and mutant forms of a 26 kDa endochitinase from barley (*Hordeum vulgare* L.). Biochem. J. **322:**815–822.
2. **Brameld, K. A., and W. A. Goddard III.** 1998. The role of enzyme distortion in the single displacement mechanism of family 19 chitinases. Proc. Natl. Acad. Sci. USA **95:**4276–4281.
3. **Broglie, K., I. Chet, M. Holliday, R. Cressman, P. Biddle, S. Knowlton, C. J. Mauvais, and R. Broglie.** 1991. Transgenic plants with enhanced resistance to the fungal pathogen *Rhizoctonia solani*. Science **254:**1194–1197.
4. **Collinge, D. B., K. M. Kragh, J. D. Mikkelsen, K. K. Nielsen, U. Rasmussen, and K. Vad.** 1993. Plant chitinases. Plant J. **3:**31–40.
5. **da Silva, A. C., J. A. Ferro, F. C. Reinach, C. S. Farah, L. R. Furlan, R. B. Quaggio, C. B. Monteiro-Vitorello, M. A. Van Sluys, N. F. Almeida, Jr., L. M. Alves, A. M. do Amaral, M. C. Bertolini, L. E. Camargo, G. Camarotte, F. Cannavan, J. Cardozo, F. Chambergo, L. P. Ciapina, R. M. Cicarelli, L. L. Coutinho, J. R. Cursino-Santos, H. El-Dorry, J. B. Faria, A. J. Ferreira, R. C. Ferreira, M. I. Ferro, E. F. Formighieri, M. C. Franco, C. C. Greggio, A. Gruber, A. M. Katsuyama, L. T. Kishi, R. P. Leite, Jr., E. G. Lemos, M. V. Lemos, E. C. Locali, M. A. Machado, A. M. Madeira, N. M. Martinez-Rossi, E. C. Martins, J. Meidanis, C. F. Menck, C. Y. Miyaki, D. H. Moon, L. M. Moreira, M. T. Novo, V. K. Okura, M. C. Oliveira, V. R. Oliveira, H. A. Pereira, Jr., A. Rossi, J. A. Sena, C. Silva, R. F. de Souza, L. A. Spinola, M. A. Takita, R. E. Tamura, E. C. Teixeira, R. I. Tezza, M. Trindade dos Santos, D. Truffi, S. M. Tsai, F. F. White, J. C. Setubal, and J. P. Kitajima.** 2002. Comparison of the genomes of two *Xanthomonas* pathogens with differing host specificities. Nature **417:**459–463.
6. **Doi, K., and A. Doi.** 1986. Cloning and expression in *Escherichia coli* of the gene for an *Arthrobacter* beta-(1–3)-glucanase. J. Bacteriol. **168:**1272–1276.
7. **Finan, T. M., S. Weidner, K. Wong, J. Buhrmester, P. Chain, F. J. Vorholter, I. Hernandez-Lucas, A. Becker, A. Cowie, J. Gouzy, B. Golding, and A. Puhler.** 2001. The complete sequence of the 1,683-kb pSymB megaplasmid from the $N_2$-fixing endosymbiont *Sinorhizobium meliloti*. Proc. Natl. Acad. Sci. USA **98:**9889–9894.
8. **Fleischmann, R. D., M. D. Adams, O. White, R. A. Clayton, E. F. Kirkness, A. R. Kerlavage, C. J. Bult, J. Tomb, B. A. Dougherty, J. M. Merrick, K. McKenney, G. G. Sutton, W. FitzHugh, C. A. Fields, J. D. Gocayne, J. D. Scott, R. Shirley, L. I. Liu, A. Glodek, J. M. Kelley, J. F. Weidman, C. A. Phillips, T. Spriggs, E. Hedblom, M. D. Cotton, T. Utterback, M. C. Hanna, D. T. Nguyen, D. M. Saudek, R. C. Brandon, L. D. Fine, J. L. Fritchman, J. L. Fuhrmann, N. S. Geoghagen, C. L. Gnehm, L. A. McDonald, K. V. Small, C. M. Fraser, H. O. Smith, and J. C. Venter.** 1995. Whole-genome random sequencing and assembly of *Haemophilus influenzae* Rd. Science **269:**496–512.
9. **Hahn, M., M. Hennig, B. Schlesier, and W. Höhne.** 2000. Structure of jack bean chitinase. Acta Crystallogr. D **56:**1096–1099.
10. **Hart, P. J., H. D. Pfluger, A. F. Monzingo, T. Hollis, and J. D. Robertus.** 1995. The refined crystal structure of an endochitinase from *Hordeum vulgare* L. seeds at 1.8 A resolution. J. Mol. Biol. **248:**402–413.
11. **Heidelberg, J. F., J. A. Eisen, W. C. Nelson, R. A. Clayton, M. L. Gwinn, R. J. Dodson, D. H. Haft, E. K. Hickey, J. D. Peterson, L. Umayam, S. R. Gill, K. E. Nelson, T. D. Read, H. Tettelin, D. Richardson, M. D. Ermolaeva, J. Vamathevan, S. Bass, H. Qin, I. Dragoi, P. Sellers, L. McDonald, T. Utterback, R. D. Fleishmann, W. C. Nierman, and O. White.** 2000. DNA sequence of both chromosomes of the cholera pathogen *Vibrio cholerae*. Nature **406:**477–483.
12. **Heitz, T., S. Segond, S. Kauffmann, P. Geoffroy, V. Prasad, F. Brunner, B. Fritig, and M. Legrand.** 1994. Molecular characterization of a novel tobacco pathogenesis-related (PR) protein: a new plant chitinase/lysozyme. Mol. Gen. Genet. **245:**246–254.
13. **Henrissat, B.** 1991. A classification of glycosyl hydrolases based on amino acid sequence similarities. Biochem. J. **280:**309–316.
14. **Henrissat, B., and A. Bairoch.** 1993. New families in the classification of glycosyl hydrolases based on amino acid sequence similarities. Biochem. J. **293:**781–788.
15. **Hopwood, D. A., M. J. Bibb, K. F. Chater, T. Kieser, C. J. Bruton, H. M. Kieser, D. L. Lydiate, C. P. Smith, J. M. Ward, and H. S. Schrempf.** 1985. Genetic manipulation of *Streptomyces*: a laboratory manual. John Innes Foundation, Norwich, United Kingdom.
16. **Iseli, B., T. Boller, and J. M. Neuhaus.** 1993. The N-terminal cysteine-rich domain of tobacco class I chitinase is essential for chitin binding but not for catalytic or antifungal activity. Plant Physiol. **103:**221–226.
17. **Jeuniaux, C.** 1966. Chitinase. Methods Enzymol. **8:**644–650.
18. **Jung, J. L., B. Fritig, and G. Hahne.** 1993. Sunflower (*Helianthus annuus* L.)

pathogenesis-related proteins (induction by aspirin (acetylsalicylic acid) and characterization). Plant Physiol. **101:**873–880.
19. **Kang, M. K., K. S. Park, and D. Choi.** 1998. Coordinated expression of defense-related genes by TMV infection or salicylic acid treatment in tobacco. Mol. Cell **8:**388–392.
20. **Leah, R., H. Tommerup, I. Svendsen, and J. Mundy.** 1991. Biochemical and molecular characterization of three barley seed proteins with antifungal properties. J. Biol. Chem. **266:**1564–1573.
21. **McClelland, M., K. E. Sanderson, J. Spieth, S. W. Clifton, P. Latreille, L. Courtney, S. Porwollik, J. Ali, M. Dante, F. Du, S. Hou, D. Layman, S. Leonard, C. Nguyen, K. Scott, A. Holmes, N. Grewal, E. Mulvaney, E. Ryan, H. Sun, L. Florea, W. Miller, T. Stoneking, M. Nhan, R. Waterston, and R. K. Wilson.** 2001. Complete genome sequence of *Salmonella enterica* serovar Typhimurium LT2. Nature **413:**852–856.
22. **Mediavilla, J., S. Jain, J. Kriakov, M. E. Ford, R. L. Duda, W. R. Jacobs, Jr., R. W. Hendrix, and G. F. Hatfull.** 2000. Genome organization and characterization of mycobacteriophage Bxb1. Mol. Microbiol. **38:**955–970.
23. **Nicholas, K. B., H. B. Nicholas, Jr., and D. W. Deerfield II.** 1997. Analysis and visualization of genetic variation. EMBnet News **4:**14.
24. **Nishizawa, Y., N. Kishimoto, T. Saito, and A. Hibi.** 1993. Sequence variation, differential expression and chromosomal location of rice chitinase genes. Mol. Gen. Genet. **241:**1–10.
25. **Ohno, T., S. Armand, T. Hata, N. Nikaidou, B. Henrissat, M. Mitsutomi, and T. Watanabe.** 1996. A modular family 19 chitinase found in the prokaryotic organism *Streptomyces griseus* HUT 6037. J. Bacteriol. **178:**5065–5070.
26. **Parkhill, J., G. Dougan, K. D. James, N. R. Thomson, D. Pickard, J. Wain, C. Churcher, K. L. Mungall, S. D. Bentley, M. T. G. Holden, M. Sebaihia, S. Baker, D. Basham, K. Brooks, T. Chillingworth, P. Connerton, A. Cronin, P. Davis, R. M. Davies, L. Dowd, N. White, J. Farrar, T. Feltwell, N. Hamlin, A. Haque, T. T. Hien, S. Holroyd, K. Jagels, A. Krogh, T. S. Larsen, S. Leather, S. Moule, P. O'Gaora, C. Parry, M. Quail, K. Rutherford, M. Simmonds, J. Skelton, K. Stevens, S. Whitehead, and B. G. Barrell.** 2001. Complete genome sequence of a multiple drug resistant *Salmonella enterica* serovar Typhi CT18. Nature **413:**848–852.
27. **Perrakis, A., I. Tews, Z. Dauter, A. B. Oppenheim, I. Chet, K. S. Wilson, and C. E. Vorgias.** 1994. Crystal structure of a bacterial chitinase at 2.3 Å resolution. Structure **2:**1169–1180.
28. **Saito, A., T. Fujii, T. Yoneyama, M. Redenbach, T. Ohno, T. Watanabe, and K. Miyashita.** 1999. High-multiplicity of chitinase genes in *Streptomyces coelicolor* A3(2). Biosci. Biotechnol. Biochem. **63:**710–718.
29. **Saitou, N., and M. Nei.** 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. **4:**406–425.
30. **Sambrook, J., and D. W. Russell.** 2001. Molecular cloning: a laboratory manual, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
31. **Schlumbaum, A., F. Mauch, U. Vögeli, and T. Boller.** 1986. Plant chitinases are potent inhibitors of fungal growth. Nature **324:**365–367.
32. **Shimosaka, M., Y. Fukumori, T. Narita, X. Zhang, R. Kodaira, M. Nogawa, and M. Okazaki.** 2001. The bacterium *Burkholderia gladioli* strain CHB101 produces two different kinds of chitinases belonging to families 18 and 19 of the glycosyl hydrolases. J. Biosci. Bioeng. **91:**103–105.
33. **Silhavy, T. J., M. L. Berman, and L. W. Enquist.** 1984. Experiments with gene fusions. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
34. **Stackebrandt, E., F. A. Rainey, and N. L. Ward-Rainey.** 1997. Proposal for a new hierarchic classification system, *Actinobacteria* classis nov. Int. J. Syst. Bacteriol. **47:**479–491.
35. **Stover, C. K., X. Q. Pham, A. L. Erwin, S. D. Mizoguchi, P. Warrener, M. J. Hickey, F. S. Brinkman, W. O. Hufnagle, D. J. Kowalik, M. Lagrou, R. L. Garber, L. Goltry, E. Tolentino, S. Westbrock-Wadman, Y. Yuan, L. L. Brody, S. N. Coulter, K. R. Folger, A. Kas, K. Larbig, R. Lim, K. Smith, D. Spencer, G. K. Wong, Z. Wu, I. T. Paulsen, J. Reizer, M. H. Saier, R. E. Hanchock, S. Lory, and M. V. Olson.** 2000. Complete genome sequence of *Pseudomonas aeruginosa* PA01, an opportunistic pathogen. Nature **406:**959–964.
36. **Terwisscha van Scheltinga, A. C., K. H. Kalk, J. J. Beintema, and B. W. Dijkstra.** 1994. Crystal structures of hevamine, a plant defence protein with chitinase and lysozyme activity, and its complex with an inhibitor. Structure **2:**1181–1189.
37. **Thompson, J. D., T. J. Gibson, F. Plewniak, F. Jeanmougin, and D. G. Higgins.** 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. **25:**4876–4882.
38. **Tsujibo, H., T. Kubota, M. Yamamoto, K. Miyamoto, and Y. Inamori.** 2003. Characterization of chitinase genes from an alkaliphilic actinomycete, *Nocardiopsis prasina* OPC-131. Appl. Environ. Microbiol. **69:**894–900.
39. **Ueda, M., M. Kojima, T. Yoshikawa, N. Mitsuda, K. Araki, T. Kawaguchi, K. Miyatake, M. Arai, and T. Fukamizo.** 2003. A novel type of family 19 chitinase from *Aeromonas* sp. no. 10S-24. Eur. J. Biochem. **270:**2513–2520.
40. **Watanabe, T., R. Kanai, T. Kawase, T. Tanabe, M. Mitsutomi, S. Sakuda, and K. Miyashita.** 1999. Family 19 chitinases of *Streptomyces* species: characterization and distribution. Microbiology **145:**3353–3363.
41. **Watanabe, T., K. Kimura, T. Sumiya, N. Nikaidou, K. Suzuki, M. Suzuki, M. Taiyoji, S. Ferrer, and M. Regue.** 1997. Genetic analysis of the chitinase system of *Serratia marcescens* 2170. J. Bacteriol. **179:**7111–7117.
42. **Watanabe, T., K. Suzuki, W. Oyanagi, K. Ohnishi, and H. Tanaka.** 1990. Gene cloning of chitinase A1 from *Bacillus circulans* WL-12 revealed its evolutionary relationship to *Serratia* chitinase and to the type III homology units of fibronectin. J. Biol. Chem. **265:**15659–15665.
43. **Waterston, R.** 1998. Genome sequence of the nematode *C. elegans*: a platform for investigating biology. The *C. elegans* Sequencing Consortium. Science **282:**2012–2018.
44. **White, O., J. A. Eisen, J. F. Heidelberg, E. K. Hickey, J. D. Peterson, R. J. Dodson, D. H. Haft, M. L. Gwinn, W. C. Nelson, D. L. Richardson, K. S. Moffat, H. Qin, L. Jiang, W. Pamphile, M. Crosby, M. Shen, J. J. Vamathevan, P. Lam, L. McDonald, T. Utterback, C. Zalewski, K. S. Makarova, L. Aravind, M. J. Daly, C. M. Fraser, et al.** 1999. Genome sequence of the radioresistant bacterium *Deinococcus radiodurans* R1. Science **286:**1571–1577.