【別紙２】

論文名：Screening of chitinolytic bacteria from freshwater lake and analysis of chitinase system of the isolated bacteria.

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To develop a novel type of biocontrol agent, this study was focused on bacteria that are characterized by both high chitinase activity and high biofilm development because such bacteria are thought to be secreted and concentrated chitinases in biofilms when adhered to chitin in fungal cell walls. Samples, sediments and chitin flakes immersed in the water were collected from a sand dune lake, Sakata, in Niigata, Japan. Chitin flakes are thought to be useful for collecting bacteria that have high chitinase activity and also form biofilms from freshwater environments. Chitinolytic bacteria were isolated from the sediments and immersed chitin flakes using solid media containing colloidal chitin with or without subsequent subculturing in fresh liquid medium containing chitin flakes. Thirty-one isolates from more than 5,100 isolated strains were selected to examine chitinase activity and biofilm formation. Phylogenetic analysis of these isolates based on the 16S rRNA gene sequences revealed that most isolates belonged to the family *Aeromonadaceae*, followed by the families *Paenibacillaceae*, *Enterobacteriaceae*, and *Neisseriaceae*. Based on the chitinase activity, biofilm formation, and phylogenetic analysis, four strains, one each of *Serratia* and *Andreprevotia* and two strains of *Aeromonas*, were selected for further investigation. Total chitinase activity of each strain in a medium containing chitin powder was lower than that of a reference, *Serratia marcescens* 2170. However, the specific activity of chitinases of each strain was higher than that of the reference. The molecular size of one chitinase produced by *Andreprevotia* (~121 kDa) was greater than that of typical bacterial chitinases implying a possibly new type of bacterial chitinases. In addition, the dialyzed

crude proteins containing chitinases of each isolate suppressed the hyphal growth of *Trichoderma reesei*. These results indicate that these four strains are good candidates for biocontrol agents.

A nearly full-length segment of 16S rRNA gene nucleotides of isolate, *A. salmonicida* SWSY 1.411 shows 100% identity to that of *A. salmonicida* A449 (CP000644) available in the CAZy database. In the database, *A. salmonicida* A449 (CP000644) is shown to be possessed one GH18 chitinase, two GH19 chitinases, and one AA10 protein. Based on the nucleotide sequence of each gene and the surrounding regions of that gene in *A. salmonicida* A449 genome, primers were designed for the identifying and cloning of chitinase genes in our isolate, *A. salmonicida* SWSY 1.411. Three genes involved in chitin-degradation were identified in the genomic DNA of *A. salmonicida* SWSY 1.411 by the polymerase chain reaction. Among them, one gene encodes a GH18 chitinase, one gene encodes a GH19 chitinase, and one gene encodes an AA10 protein. These genes were then cloned, sequenced, and analyzed deduced amino acid sequences. Primary structures of all deduced enzymes contain a number of functional domains; among them, one chitin-binding domain belongs to a recently classified family of carbohydrate-binding modules, CBM73. ChiA contains a CBM5, a CBM73, and belongs to subfamily A of GH18 chitinases. The catalytic domain of ChiB belongs to GH19 chitinases; ChiB contains a CBM5, a CBM73, and a PKD domain. In addition, various works have been reported that bacterial GH18 chitinases commonly play high chitinase activity toward insoluble chitins, bacterial GH19 chitinases are primary enzymes involved in the antifungal activity, and bacterial AA10 proteins in combination with chitinases play an important role on hydrolysis of chitin. These analyses indicate that the chitinase system of *A. salmonicida* SWSY 1.411 possibly plays an important role on chitin-degradation and inhibition of hyphal growth of fungi.