Characterization of *Mycobacterium* sp. isolated from yellowtail, *Seriola quinqueradiata* using *gyrB*, 16S rDNA and three types of Antigen 85 complex genes

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INTRODUCTION

Mycobacterial infection is a problem not only in mammals but also in fish, in particular *Mycobacterium* sp. infecting yellowtail (*Seriola quinqueradiata*). This microbe is an intracellular pathogen that causes greyish-white miliary granulomas in the spleen of infected fish. An efficient technique to control this bacterium has yet to be developed. Previous studies have reported that the Antigen 85 complex genes consisted of A, B and C¹⁾ genes and these genes exist in many mycobacteria. The genes are well conserved and are considered typical features in most mycobacterial species. These genes have provided the foundation for mammalian DNA vaccines against the mycobacterial infection. The *gyrB* or 16S rDNA genes have been used for bacterial classification. To characterize the *Mycobacterium* sp. found in Japanese yellowtail, we determined the complete nucleotide sequences of the Antigen 85 complex genes and partial sequence of the *gyrB* and 16S ribosomal RNA genes.

MATERIALS AND METHODS

Construction of PCR primers

To synthesize the partial fragments of each gene, universal primers (Table 1) were designed from the conserved regions of the target genes in *M. tuberculosis*, *M. avium*, *M. bovis* and *M. leprae*. All known gene sequences were obtained from Genebank.

PCR amplification

Genomic DNA of *Mycobacterium* sp. strain 9912 was used as a template for this experiment. All PCR amplifications were conducted in a Takara PCR Thermal Cycler MP (Takara shuzo Co., Ltd. Japan)

using Takara Ex Taq (Takara shuzo Co., Ltd. Japan). The PCR reaction was performed following the temperature profile: pre-heating at 94°C for 3 min; 30 cycles of denaturation [95°C, 30s], annealing [50°C, 30s], extension [72°C, 1 min]; and final extension at 72°C for 3 min.

Sequencing

To identify the sequences of each PCR product, the fragments were inserted into pGEM-T easy vector (Promega, USA). These recombinant plasmids were sequenced using Thermo sequence fluorescent labeled primer cycle sequencing kit with 7-deaza-dGTP (Amersham Pharmacia Biotech, U.S.A). The sequence reaction was carried out following the temperature profile: pre-heating at 95°C for 3 min; 30cycles of denaturation [95°C, 30s], annealing [55°C, 30s], extension [72°C, 1 min]; and lastly extension at 72°C for 5 min. Nucleotide sequences of the samples were read using DNA sequencer 4200L (Li-cor, USA). The obtained nucleotide sequences of the cloned genes were analyzed using BLAST search program.

Determination of nucleotide sequence in Antigen 85 complex genes

The analyzed partial fragments of Antigen 85 complex genes were used as probes for Southern blot hybridization. The positive signals after Southern blot hybridization provided the basis as to which part of the restriction enzyme-digested fragments of the genomic DNA that encoded the complete sequence of the Antigen 85 complex genes will be cloned for further analysis. The fragments were inserted into pUC119 vector, and the complete nucleotide sequences of the Antigen 85 complex genes were determined.

Table 1 Universal primers and target genes

primer	target genes
My85a-s 5'-ACATCAAGNTCCANTTCCA-3'	Antigen 85 A gene
My85a-a 5'-ACTTGGCNGGCANGTTGT-3'	Antigen 85 A gene
My85b-s 5'-GACGACTANAACGGCTGGGA-3'	Antigen 85 B gene
My85b-a 5'-GTTGAACACNGCGTTGTG-3'	Antigen 85 B gene
My85c-s 5'-TACAACGGNTGGGACATN-3'	Antigen 85 C gene
My85c-a 5'-GACGCAACGGNGTGTNAA-3'	Antigen 85 C gene
Gy gene F 5'-GGNGTNTCGGTGGTTAACGC-3'	gyrB gene
Gy gene R 5'-ACGATCTTGTGNANCGCAG-3'	gyrB gene
16S gene F 5'-TATCAGCTTGTGGTGNGGT-3'	16S rDNA
16S gene R 5'-TATTCACCGCAGCNTTGCTG-3'	16S rDNA

RESULTS AND DISCUSSION

Partial fragments of *gyrB* (1174bp) (Acc. No. AB074888), 16S rDNA (1090bp) (Acc. No. AB074889) and, the complete nucleotide sequence of Antigen 85 A (1014bp), B (978bp) and C (1035bp) for *Mycobacterium* sp. strain 9912 from Japanese yellowtail were analyzed. Each gene shared high homology with other reported mycobacterial genes. The *gyrB* and 16S rDNA genes shared highest homology with *M. marinum* genes (Table 2), thus suggesting the possibility that *Mycobacterium* sp. and *M. marinum* are closely related mycobacterial species. Furthermore, the amino acid sequence of Antigen 85 complex genes in *Mycobacterium* sp. showed more than 83% homology with the *M. bovis* (BCG) genes (Table 3). Previous studies have reported that the BCG derived antigen 85 complex gene provided cross protective ability following immunization against other mycobacteria.²⁾ In addition the immunoinducibility Antigen 85 complex genes have been confirmed in some mycobacterial species.³⁻⁵⁾ Therefore, the Antigen 85 complex gene cloned from the *Mycobacterium* sp. Japanese yellowtail could be utilized to establish a DNA vaccine against *Mycobacterium* sp. Moreover, if the genes are shown to possess cross protective ability against *M. marinum*, it would be useful to prevent infection of this bacteria in the other host organisms.

Table 2 The results of BLAST search for *Mycobacterium* sp. (strain 9912) *gyrB* and 16S rDNA genes partial nucleotide sequence with other mycobacteria species

	Length (nuc)	Gene with highest homology	Identity(%)
gyrB	1174bp	<i>Mycobacterium marinum</i> gyrB gene	1159/1174(98%)
16S rDNA	1090bp	<i>Mycobacterium marinum</i> 16S ribosomal RNA gene	1085/1090(99%)

Table 3 Comparison of homology(%) in *Mycobacterium* sp. (strain 9912) Antigen 85 complex genes with other mycobacteria species antigen 85 complexes

	<i>M. tuberculosis</i>		<i>M. avium</i>		<i>M. bovis</i>		<i>M. leprae</i>		<i>M. marinum</i>	
	nuc	aa	nuc	aa	nuc	aa	nuc	aa	nuc	aa
Ag85 A	82.2	83.7	84.5	87.8	79.1	83.7	79.1	79.2	97.8	97.6
Ag85 B	81.0	88.0	83.0	85.8	80.9	87.8	76.9	80.7	-	-
Ag85 C	85.3	88.6	80.9	82.7	-	-	78.2	82.1	-	-

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