***Mycobacterium pseudoshottsii* Isolated from 24 Farmed Fishes in Western Japan**

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ABSTRACT. Mycobacteria isolated from epizootics of farmed fishes in western Japan were examined for the first time using multigenotypic analysis. By analysis of the sequences of the internal transcribed spacer between the 16S and 23S rRNA genes (ITS) region and the partial 16S rRNA, *hsp65* and *rpoB* genes, *M. pseudoshottsii* was identified as the causative agent in these infections. Prior to this study, only *M. marinum* has been known as the causative agent of lethal mycobacterial disease in marine fishes in Japan.

KEY WORDS: lethal fish infection, *Mycobacterium pseudoshottsii*, mycolactone.

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Mycobacterium (M.) marinum, *M. salmoniphilum*, *M. fortuitum*, *M. chelonae* and *M. abscessus* are the most commonly identified mycobacterial fish pathogens [1, 4]. In particular, *M. marinum* is found in a wide range of saltwater species [2]. Molecular and phylogenetic analyses have facilitated the worldwide recovery of novel mycobacterial species, strains and isolates, such as *M. shottsii* [9] and *M. pseudoshottsii*, from wild marine fishes [3, 10].

M. pseudoshottsii, a slow-growing, photochromogenic mycobacterium, was initially isolated in 2005 from striped bass [10]. Its biochemical reactions, growth characteristics and mycolic acid profiles resemble those of *M. shottsii*, a nonpigmented mycobacterium that was isolated during the same epizootic outbreak [10]. However, the sequences of the 16S rRNA gene and the gene encoding the 65 kDa heat shock protein (*hsp65*) revealed that the isolate was unique [9, 10]. Initially, *M. pseudoshottsii* was found only in wild Chesapeake Bay striped bass; however, both the range of host species and the area of disease distribution have expanded to a variety of fishes and locations [13, 15]. In Japan, molecular and genotypic examinations of piscine-related nontuberculous mycobacteria (NTM) are rare. Here, we report on the genotypic analysis of mycobacteria isolated from infected fishes raised on farms in western Japan.

Twenty-four isolates were recovered from moribund yellowtails (*Seriola quinqueradiata*), greater amberjack (*Seriola dumerili*), striped jack (*Pseudocaranx dentex*), sevenband grouper (*Epinephelus septemfasciatus*), and yellowtail amberjack (*Seriola lalandi*) at fish farms in the western part of Japan from 1999 to 2008 (Table 1). The diseased fish generally showed lethargy, anorexia, emaciation

and abdominal distension with ascites. Sometimes, mass culling of the same fish group at a farm was needed because of mass mortality. In some cases, skin ulceration and eye corneal ulceration were observed. White nodules were often found in several internal organs especially in enlarged spleens and kidneys. Isolation was attempted with the affected organ, kidneys, spleen, liver and gills of each fish. These tissues were aseptically dissected, homogenized in phosphate buffered saline, inoculated on 2% Ogawa egg slant (Kyokuto Pharmaceutical Industrial, Tokyo, Japan) or homogenized with 4% NaOH for 10 min and inoculated on 1% Ogawa egg slant (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan). Incubation was performed at 23 to 25°C for 2 to 3 months. Subculture were performed for colony purification with 2% Ogawa egg slant and/or Middlebrook 7H11 agar supplemented with 10% OADC enrichment (Becton, Dickinson and Company, Fukushima, Japan).

Multigenotypic analysis was used to identify the resulting isolates. One loopful of mycobacterial colonies on Ogawa egg slant or 7H11 agar was suspended in 400 µl sterilized phosphate-buffered saline supplemented with 0.05% Tween 80 and was stored at –80°C until DNA was extracted. A frozen bacterial suspension was crushed in a bead-beating instrument (MagNalizer; Roche Diagnostics Japan, Tokyo, Japan) at 3,000 rpm for 90 sec with zirconia beads (diameter, 2 mm). Total genomic DNA was purified from the crashed suspension using a High Pure PCR Template Preparation Kit according to the manufacturer's instructions (Roche Diagnostics Japan, Tokyo, Japan) and was stored at –20°C.

An approximately 1,500-bp fragment of the 16S rRNA gene, the partial sequences of the *hsp65* and *rpoB* genes and the internal transcribed spacer between the 16S and 23S rRNA genes (ITS region) were amplified by PCR using AmpliTaq Gold polymerase (Applied Biosystems, Foster City, CA, U.S.A.) with the primers listed in Table 2. The amplicons of the isolates were sequenced using an ABI

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Table 1. Origin of the mycobacterial strains used in this study

Strain	Host fish	Isolation date	Site isolated	Location of fish farm (Prefecture)
MF01	Yellow tail (<i>Seriola quinqueradiata</i>)	Oct/19/2004	Kidney	Kagoshima
MF06	Yellow tail (<i>Seriola quinqueradiata</i>)	Sep/08/2008	Kidney	Kagoshima
MF09	Yellow tail (<i>Seriola quinqueradiata</i>)	Jul /19/2001	Kidney	Oita
MF10	Yellow tail (<i>Seriola quinqueradiata</i>)	Oct/31/2001	Kidney	Oita
MF12	Yellow tail (<i>Seriola quinqueradiata</i>)	Aug/19/2008	Kidney	Ehime
MF14	Yellow tail (<i>Seriola quinqueradiata</i>)	Aug/29/2008	Kidney	Ehime
MF31	Yellow tail (<i>Seriola quinqueradiata</i>)	Oct/18/2004	Spleen	Kagoshima
MF32	Yellow tail (<i>Seriola quinqueradiata</i>)	Oct/18/2004	Kidney	Kagoshima
MF33	Yellow tail (<i>Seriola quinqueradiata</i>)	Oct/18/2004	Kidney	Kagoshima
MF34	Yellow tail (<i>Seriola quinqueradiata</i>)	Oct/18/2004	Kidney	Kagoshima
MF35	Yellow tail (<i>Seriola quinqueradiata</i>)	Feb/02/2005	Kidney	Kagoshima
MF36	Yellow tail (<i>Seriola quinqueradiata</i>)	Feb/02/2005	Kidney	Kagoshima
MF44	Yellow tail (<i>Seriola quinqueradiata</i>)	Jul /19/2001	NC ^{a)}	Oita
MF45	Yellow tail (<i>Seriola quinqueradiata</i>)	Oct/01/2001	NC	Oita
MF46	Yellow tail (<i>Seriola quinqueradiata</i>)	NC/- /2004	NC	Ehime
MF02	Greater amberjack (<i>Seriola dumerili</i>)	Jan/06/2005	Kidney	Kagoshima
MF05	Greater amberjack (<i>Seriola dumerili</i>)	Nov/15/2005	Kidney	Kagoshima
MF07	Greater amberjack (<i>Seriola dumerili</i>)	NC/- /2006	Miliary nodule	Miyazaki
MF40	Greater amberjack (<i>Seriola dumerili</i>)	Jan/05/2004	NC	Kagoshima
MF13	Sevenband grouper (<i>Epinephelus septemfasciatus</i>)	Aug/18/2008	Kidney	Ehime
MF15	Sevenband grouper (<i>Epinephelus septemfasciatus</i>)	Oct/- /2008	Kidney	Ehime
MF04	Striped jack (<i>Pseudocaranx dentex</i>)	Nov/15/2005	Kidney	Kagoshima
MF08	Striped jack (<i>Pseudocaranx dentex</i>)	Sep/06/1999	Kidney	Oita
MF11	Yellowtail amberjack (<i>Seriola lalandi</i>)	Aug/09/2007	Spleen	Oita

a) Not clear.

Table 2. Primers used in this study

Primer	Sequence (positions)	PCR target (fragment size)	Reference
8F16S	5'-AGAGTTTGATCCTGGCTCAG- 3' (8-27)	16S rRNA gene (app. 1,500 bp)	12
1047R16S	5'-TGCACACAGGCCACAAGGGA- 3' (1,047-1,028)		
830F16S	5'-GTGTGGGTTTCCTTCCTTGG- 3' (830-849)		
1542R16S	5'-AAGGAGGTGATCCAGCCGCA- 3' (1,542-1,523)		
ITSF	5'-TTGTACACACCGCCCGTC- 3' (16S, 1,390-)	16S-23S ITS region (app. 340 bp)	11
ITSR	5'-TCTCGATGCCAAGGCATCCACC- 3' (23S, 44-)		
TB11	5'-ACCAACGATGGTGTGTCCAT- 3' (398-417)	<i>hsp65</i> (439bp)	16
TB12	5'-CTTGTCGAACCGCATACCCT- 3' (836-817)		
MF	5'-CGACCACTTCGGCAACCG- 3'	<i>rpoB</i> (342 bp)	5
MR	5'-TCGATCGGGCACATCCGG- 3'		

Prism 310 PCR Genetic Analyzer (Applied Biosystems) [6] and compared to the sequences of six strains of mycobacteria: "*M. ulcerans* subsp. *shinshuense*" ATCC33728 [6], *M. ulcerans* ATCC19423 (type strain), *M. ulcerans* Agy99 [14], *M. marinum* ATCC 927 (type strain), *M. marinum* clinical isolate strain 112509 (the preceding 5 strains originated in humans) and *M. pseudoshottsii* JCM15466 (type strain). The JCM strain was distributed by the Microbe Division of the Riken BioResource Center (BRC; Saitama, Japan). Isolate and reference sequences were deposited into the DNA Data Bank of Japan (DDBJ) under accession numbers AB548704 to AB548734 and AB642161 to AB642165.

The sequences of the 1,475-bp fragment of 16S rRNA gene from the piscine isolates showed almost complete

identity with the *M. pseudoshottsii* reference strain (99.93–100% identity). Only a single mismatch was found at nucleotide position 487 or 488 in 9 of 24 piscine isolates compared with the DNA sequence of *M. pseudoshottsii* JCM15466. However, conserved mismatches with the 5 strains that originated in humans were found at nucleotide positions 95, 969, 1,007 and 1,215 (Table 3). *M. ulcerans* Agy99 had sum upped 3-base pair insertion (TTT) at nucleotide position 1,449–1,451. Similarly, the ITS regions of the piscine isolates and the *M. pseudoshottsii* reference strain were either identical or differed at position 57, while conserved mismatches with the strains originating in humans were at nucleotide positions 30 and 62. All of the sequences of *hsp65* and *rpoB* gene fragments from the iso-

Table 3. Alignment of the 16S rRNA, ITS, *hsp65* and *rpoB* gene sequences from 24 piscine isolates and 6 reference strains^{a)}

Strain	Prefecture/ Country	Nucleotide sequence positions																	
		16S rRNA ^{b)}								ITS region				<i>hsp65</i> ^{c)}					
		95	487-8	492	969	1007	1215	1247	1288	30	57	62	83	455	571	637	639	647	797
<i>M. shinshuense</i> ATCC 33728	Nagano/Japan	T	GG	G	A	G	T	G	G	G	G	T	A	C	T	C	C	A	T
<i>M. ulcerans</i> ATCC 19423 ^T	NC ^{e)} / Australia	T	GG	A	A	G	T	G	C	G	G	T	A	T	C	C	C	A	C
<i>M. ulcerans</i> Agy99	NC / Ghana	T	GG	A	A	G	T	G	C	G	G	T	A	T	C	C	C	A	C
<i>M. marinum</i> ATCC 927 ^T	NC / USA	T	GG	A	A	G	T	A	A	G	G	T	A	C	C	C	T	G	C
<i>M. marinum</i> 112509	Tokyo/Japan	T	GG	A	A	G	T	A	A	G	G	T	G	C	C	C	T	G	C
<i>M. pseudoshottssii</i> JCM 15466 ^T	NC / USA	C	GA	A	G	T	C	A	A	A	G	C	A	C	C	T	C	G	C
MF01 (yellow tail)	Kagoshima/Japan	C	GA	A	G	T	C	A	A	A	G	C	A	C	C	T	C	G	C
MF06 (yellow tail)	Kagoshima/Japan	C	GG	A	G	T	C	A	A	A	G	C	A	C	C	T	C	G	C
MF09 (yellow tail)	Oita/Japan	C	GG	A	G	T	C	A	A	A	T	C	A	C	C	T	C	G	C
MF10 (yellow tail)	Oita/Japan	C	GA	A	G	T	C	A	A	A	G	C	A	C	C	T	C	G	C
MF12 (yellow tail)	Ehime/Japan	C	GA	A	G	T	C	A	A	A	G	C	A	C	C	T	C	G	C
MF14 (yellow tail)	Ehime/Japan	C	AA	A	G	T	C	A	A	A	G	C	A	C	C	T	C	G	C
MF31 (yellow tail)	Kagoshima/Japan	C	GA	A	G	T	C	A	A	A	G	C	A	C	C	T	C	G	C
MF32 (yellow tail)	Kagoshima/Japan	C	GA	A	G	T	C	A	A	A	G	C	A	C	C	T	C	G	C
MF33 (yellow tail)	Kagoshima/Japan	C	GA	A	G	T	C	A	A	A	G	C	A	C	C	T	C	G	C
MF34 (yellow tail)	Kagoshima/Japan	C	AA	A	G	T	C	A	A	A	T	C	A	C	C	T	C	G	C
MF35 (yellow tail)	Kagoshima/Japan	C	GA	A	G	T	C	A	A	A	G	C	A	C	C	T	C	G	C
MF36 (yellow tail)	Kagoshima/Japan	C	GA	A	G	T	C	A	A	A	G	C	A	C	C	T	C	G	C
MF44 (yellow tail)	Oita/Japan	C	AA	A	G	T	C	A	A	A	T	C	A	C	C	T	C	G	C
MF45 (yellow tail)	Oita/Japan	C	GA	A	G	T	C	A	A	A	G	C	A	C	C	T	C	G	C
MF46 (yellow tail)	Ehime/Japan	C	GA	A	G	T	C	A	A	A	G	C	A	C	C	T	C	G	C
MF02 (greater amberjack)	Kagoshima/Japan	C	GA	A	G	T	C	A	A	A	G	C	A	C	C	T	C	G	C
MF05 (greater amberjack)	Kagoshima/Japan	C	GG	A	G	T	C	A	A	A	T	C	A	C	C	T	C	G	C
MF07 (greater amberjack)	Miyazaki /Japan	C	GG	A	G	T	C	A	A	A	G	C	A	C	C	T	C	G	C
MF40 (greater amberjack)	Kagoshima/Japan	C	GA	A	G	T	C	A	A	A	G	C	A	C	C	T	C	G	C
MF13 (sevenband grouper)	Ehime/Japan	C	GA	A	G	T	C	A	A	A	G	C	A	C	C	T	C	G	C
MF15 (sevenband grouper)	Ehime/Japan	C	GA	A	G	T	C	A	A	A	G	C	A	C	C	T	C	G	C
MF04 (striped jack)	Kagoshima/Japan	C	AA	A	G	T	C	A	A	A	T	C	A	C	C	T	C	G	C
MF08 (striped jack)	Oita/Japan	C	GG	A	G	T	C	A	A	A	G	C	A	C	C	T	C	G	C
MF11 (yellowtail amberjack)	Oita/Japan	C	GA	A	G	T	C	A	A	A	G	C	A	C	C	T	C	G	C

a) Only nucleotide differences are noted. Nucleotide positions were based on the b) *E. coli* 16S rRNA gene (accession No. J01859). c) *M. tuberculosis hsp65* gene. (accession No. M15467) and d) *rpoB* gene (accession No. AF057454). e) Not clear.

lates showed complete identity with those of the *M. pseudoshottssii* sequences (Table 3). A conserved mismatch between piscine and human isolates in *hsp65* gene fragments was only found at nucleotide position 637. The results showed that the 24 piscine isolates were all identified as *M. pseudoshottssii* rather than *M. marinum*.

A lethal case of *M. marinum* in cultured yellowtails, which was identified using biological, biochemical and 16S rRNA sequence analyses, has been reported in Japan [17]. In our study, *M. pseudoshottssii* was identified as an additional source of atypical piscine mycobacteriosis and (the bacteria) had been distributed in farmed fisheries in the west part of Japan since 1999. Further studies are needed to develop an easier method to distinguish *M. pseudoshottssii* from *M. marinum* because both strains might have not been differentiated before in Japan. Their differences in susceptibility to antimicrobial agents and in capacity for human pathogenesis should be elucidated. In addition, *M. pseudoshottssii* produces a unique plasmid-encoded toxic macrolide, mycolactone F [7], suggesting that *M. pseudoshottssii* provides a reservoir in aquatic environments for the hori-

zontal transfer of the plasmid-borne genes that encode mycolactone F. Interestingly the potency of mycolactone F with regard to apoptosis in a mammalian cell line was significantly less than that of mycolactone A/B, which is produced by *M. ulcerans*, a causative agent of Buruli ulcer [17]. Further molecular, biochemical and drug susceptibility studies are needed to understand the possible role of mycolactone F in mycobacteriosis and to fully characterize piscine mycobacterial infections in Japan.

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