Molecular systematics support the revival of *Mycobacterium salmoniphilum* (ex Ross 1960) sp. nov., nom. rev., a species closely related to *Mycobacterium chelonae*

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Mycobacterial infections in fish are usually attributed to strains of *Mycobacterium marinum*, *Mycobacterium chelonae* and *Mycobacterium fortuitum*. Bacteria identified as *M. chelonae* have been isolated numerous times from salmonid fishes. Recently, this bacterium has been associated with salmon mortalities in the aquaculture industry. An *M. chelonae*-like species from salmon, '*Mycobacterium salmoniphilum*', was described in 1960. However, the species name lost standing in nomenclature when it was omitted from the 1980 Approved Lists of Bacterial Names because the species could not be distinguished with confidence from *M. fortuitum*. In the 1980s, mycobacteria isolated from salmon were characterized as a distinct subspecies, '*Mycobacterium chelonae* subsp. *piscarium*'. Again, the uncertainty of the validity of the species resulted in the subsequent withdrawal of the name. Since then, most studies have considered isolates from salmon to be *M. chelonae*. Nucleotide sequence analysis of the small-subunit rRNA, *hsp65* and *rpoB* genes was used to examine the taxonomic relatedness of type cultures and authentic isolates in our culture collection available from earlier studies. The *M. chelonae*-like strains from salmon were phylogenetically distinct from other *Mycobacterium* strains and members of the *M. chelonae* complex. Moreover, the cell-wall-bound mycolic acids were not representative of known mycolate patterns for *M. chelonae*-complex organisms. These results supported the status of the species as a separate taxon and effect the valid publication of the name '*M. salmoniphilum*' as *Mycobacterium salmoniphilum* (ex Ross 1960) sp. nov., nom. rev., with the type strain SC^T (=ATCC 13578^T =DSM 43276^T).

INTRODUCTION

Mycobacteriosis in fish is usually attributed to infections by *Mycobacterium chelonae*, *Mycobacterium fortuitum* or *Mycobacterium marinum* (Belas *et al.*, 1995; Decostere *et al.*,

2004). However, several recent studies employing DNA sequence data as well as traditional methods of characterization have identified several additional *Mycobacterium* species that infect fishes (Heckert *et al.*, 2001; Kent *et al.*, 2004; Levi *et al.*, 2003; Rhodes *et al.*, 2003, 2005; Poort *et al.*, 2006; Whipps *et al.*, 2003). Delineation of piscine *Mycobacterium* species is important not only for animal health but also for human health, as some species are potentially zoonotic. As a case in point, *M. marinum* infections in humans are often associated with exposure to fish or aquaria (Aubry *et al.*, 2002; Jernigan & Farr, 2000).

Mycobacteriosis in salmonid fishes has been reported in the literature, but the identity of the species responsible is elusive (Arakawa & Fryer, 1984; Ashburner, 1977;

Abbreviations: ITS, internal transcribed spacer; SSU, small subunit.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene, *hsp65* and *rpoB* sequences of *M. salmoniphilum* strains are DQ866764–DQ866770, DQ866777–DQ866783 and DQ866790–DQ866797 (respectively DQ866768, DQ866777 and DQ866790 for the type strain).

A tree resulting from Bayesian analysis of *rpoB* sequences and a strict consensus tree from parsimony analysis of ITS sequences are available as supplementary material with the online version of this paper.

Brocklebank *et al.*, 2003; Bruno *et al.*, 1998; Ross, 1960, 1970). In 1960, Ross described the salmon mycobacterium as a unique species and proposed the name '*Mycobacterium salmoniphilum*' (Ross, 1960). However, Gordon & Mihm (1959) identified these isolates as *M. fortuitum*. Penso *et al.* (1962) proposed that only one of the three strains was truly *M. fortuitum*, and Tsukamura *et al.* (1967) supported this finding with numerical classification analysis based upon 101 characters and suggested that they 'not be named *M. fortuitum*'. Some of this taxonomic confusion may be explained by the observation that mycobacteria from salmonid fishes exhibit biochemical characteristics of both *M. chelonae* and *M. fortuitum*. The inability to distinguish these isolates confidently from *M. fortuitum* resulted in the omission of '*M. salmoniphilum*' from the Approved Lists of Bacterial Names (Skerman *et al.*, 1980).

Using a broad panel of biochemical analyses, Arakawa & Fryer (1984) tested additional mycobacterial isolates from salmon, found that they were most like *M. chelonae* and assigned them to '*M. chelonae* subsp. *piscarium*'. The subspecies name was later withdrawn (Arakawa *et al.*, 1986), as serological analyses could not separate '*M. chelonae* subsp. *piscarium*' from *M. chelonae* subsp. *chelonae* or *M. chelonae* subsp. *abscessus*. Nonetheless, these and other studies (Ashburner, 1977; Brocklebank *et al.*, 2003; Bruno *et al.*, 1998) attributed salmon mycobacteriosis to *M. chelonae* or an *M. chelonae*-like species.

The body of evidence suggests that these mycobacteria isolated from salmon represent a distinct species that is similar to *M. chelonae* or *M. fortuitum*. Therefore, we propose that the name '*M. salmoniphilum*' suggested by Ross (1960) is valid and its revival warranted. We employed molecular and chemical methods in this study to resolve these conflicting data and to clarify the taxonomic position of mycobacteria isolated from salmon.

METHODS

Bacterial strains. Isolates maintained in our culture collection, obtained from salmonid fishes, described as '*M. salmoniphilum*' by

Ross (1960) or '*M. chelonae* subsp. *piscarium*' (Arakawa & Fryer, 1984) and *M. chelonae* of Bruno *et al.* (1998) are listed in Table 1. Cultures were grown on solid-phase media normally used for mycobacteria, including Middlebrook 7H10, Löwenstein-Jensen, blood agar or MacConkey agar, and in Middlebrook 7H9 liquid broth, at 28–30 °C. Biochemical analyses were conducted using standard methods (Kent & Kubica, 1985).

Mycolic acids. Chemical analysis of the mycolic acids was conducted with HPLC for identification of mycobacteria as reviewed by Butler & Guthertz (2001).

DNA isolation and PCR amplification. DNA was extracted from cultures using the UltraClean microbial DNA isolation kit (MoBio Laboratories). Amplification of target genes by PCR was conducted using standard methods and primers of Kent *et al.* (2004), Poort *et al.* (2006), Selvaraju *et al.* (2005) and Adékambi *et al.* (2003). DNAs extracted from uninfected fish tissues were used as negative controls and were consistently negative. Amplification products were either purified directly with the QIAquick PCR Purification kit or excised from the gel and purified using the QIAgen Gel Extraction kit (Qiagen). Sequences were obtained directly from amplification products at the Nevada Genomics Center (Reno, NV, USA).

Phylogenetic analysis. Nucleotide sequences of the small-subunit (SSU) rRNA gene from salmonid isolates were aligned to those of other rapidly growing type strains of mycobacteria. *Mycobacterium tuberculosis* H37Rv^T and *Mycobacterium leprae* TN were used as outgroups. With the sequences of the internal transcribed spacer (ITS) and heat-shock protein 65 (*hsp65*) and RNA polymerase β -subunit (*rpoB*) genes, analyses focused only on the *M. chelonae* complex, i.e. *M. chelonae*, *M. abscessus*, *Mycobacterium immunogenum*, *Mycobacterium massiliense* and *Mycobacterium bolletii*, and other relevant sequences from BLAST matches on GenBank. *M. fortuitum* strains were used as the outgroup based on recent phylogenetic analyses (Adékambi & Drancourt, 2004; Devulder *et al.*, 2005) and our preliminary analyses. Alignments were created with CLUSTAL_X (Thompson *et al.*, 1997) and edited manually. Gaps were treated as a fifth character state. Parsimony analyses were conducted in PAUP*4.01 (Swofford, 1998). Maximum-parsimony analysis employed a heuristic search with 10 repetitions of random sequence addition and tree bisection and reconnection branch swapping. Bootstrap confidence values were calculated with a heuristic search using simple sequence addition and 100 replicates. Bayesian analyses were conducted in MrBayes (Ronquist & Huelsenbeck, 2003) under a general time-reversible (GTR) model, with 10⁶ generations, tree sampling every 100 generations and a burn-

Table 1. Isolates of salmonid mycobacteria examined in this study

Isolate	Year	Location	Host
AUS	1969	Victoria, Australia	Chinook salmon (<i>Oncorhynchus tshawytscha</i>)
BAN	1964	Oregon, USA	Cutthroat trout (<i>Oncorhynchus clarkii clarkii</i>)
ELK	1981	Oregon, USA	Chinook salmon (<i>O. tshawytscha</i>)
SIL	1966	Oregon, USA	Coho salmon (<i>Oncorhynchus kisutch</i>)
TRA	1982	Oregon, USA	Coho salmon (<i>O. kisutch</i>)
MON	1982	Montana, USA	Whitefish (<i>Prosopium williamsoni</i>)
MT1890 (=NCIMB 13533)	1998	Shetland, Scotland, UK	Atlantic salmon (<i>Salmo salar</i>)
MT1900	1998	Shetland, Scotland, UK	Atlantic salmon (<i>S. salar</i>)
CAR (=ATCC 13756)	1960	Washington, USA	Chinook salmon (<i>O. tshawytscha</i>)
OR (=ATCC 13757)	1960	Washington, USA	Steelhead trout (<i>Oncorhynchus mykiss</i>)
SC ^T (=ATCC 13758 ^T =DSM 43276 ^T)	1960	Washington, USA	Chinook salmon (<i>O. tshawytscha</i>)

in of 100 trees. Distance and maximum-likelihood analyses were also conducted in PAUP*4.01; they yielded equivalent results to parsimony and Bayesian analysis and are therefore not shown.

RESULTS AND DISCUSSION

Phylogenetic analysis of the SSU rRNA gene from type strains of the rapidly growing mycobacteria and of isolates assigned to '*M. salmoniphilum*' confirmed the affiliation of this species to the *M. chelonae* complex (Fig. 1) as defined previously (Brown-Elliott & Wallace, 2002; Adékambi *et al.*, 2004, 2006; Selvaraju *et al.*, 2005). The result was consistent with previous studies that have identified these isolates as *M. chelonae* based primarily on biochemical analyses (Arakawa & Fryer, 1984; Brocklebank *et al.*, 2003; Bruno *et al.*, 1998). Some common characteristics within this group are a positive 3 day arylsulfatase test, negative nitrate reductase test and the absence of pigmentation. Differential biochemical characteristics include utilization of glucose or citrate and, highly significantly, a lack of growth at or above 37 °C (Table 2).

To verify that '*M. salmoniphilum*' isolates were not phylogenetically intermixed with other members of the *M. chelonae* complex, multiple strains of each species were

analysed using DNA sequences from *hsp65* (Fig. 2), *rpoB* (Supplementary Fig. S1 available in IJSEM Online) and the ITS (Supplementary Fig. S2). These analyses consistently revealed that '*M. salmoniphilum*' is monophyletic. Our analyses included a broad range of isolates, including those from the original description of '*M. salmoniphilum*' (ATCC 13756, ATCC 13757 and ATCC 13758), those described as '*M. chelonae* subsp. *piscarium*' by Arakawa & Fryer (1984), identical strains MT1890 and MT1900 (results for MT1890 only are shown) from Atlantic salmon incorrectly assigned to *M. chelonae* (Bruno *et al.*, 1998) and sequences obtained independently of our study from isolate SOL 803 from salmon in a hatchery in eastern Canada (GenBank accession no. AY489137) (Fig. 1). One of the original isolates of Ross (1960), ATCC 13756, was more like *M. fortuitum* by DNA sequencing, consistent with the observations of Penso *et al.* (1962) that one of Ross's isolates was *M. fortuitum*. These data also illustrated that more than one species of *Mycobacterium* may infect salmon. Indeed, *Mycobacterium neoaurum* has also been reported from salmon (Roberts, 2001).

Other species within this complex tended to form well-supported clades in all of the analyses with the exception of the *M. abscessus* clade, which was rendered paraphyletic by the addition of *M. massiliense* and *M. bolletii* (Fig. 2). Estimates of phylogeny of the *M. chelonae* complex varied

Fig. 1. Tree resulting from Bayesian phylogenetic analysis of SSU (16S) rRNA gene sequences from strains of '*M. salmoniphilum*' and other rapidly growing mycobacteria. GenBank accession numbers and strain designations are provided. Numbers at nodes are percentage clade credibility values/bootstrapped confidence values from parsimony analysis; -, less than 50 %.

Table 2. Biochemical characteristics of salmonid mycobacteria and related species

Related species are abbreviated as follows: Mch, *M. chelonae*; Mab, *M. abscessus*; Mim, *M. immunogenum*; Mma, *M. massiliense*; Mfo, *M. fortuitum*. Data were compiled from Ross (1960), Arakawa & Fryer (1984), Adékambi *et al.* (2004) and Schinsky *et al.* (2004). Growth temperature was determined on Middlebrook 7H10, Löwenstein–Jensen, Sauton or Ogawa media. ND, Not done. All mycobacteria shown are positive for growth at 24 or 25 and 30 °C and negative for growth at 42 and 45 °C.

Characteristic	ATCC 13756	ATCC 13757	ATCC 13758 ^T	BAN	ELK	SIL	TRA	MON	Mch	Mab	Mim	Mma	Mfo
Utilization of:													
Glucose	+	+	+	+	+	+	+	+	–	–	–	–	+
Citrate	+	+	+	+	+	+	+	+	+	–	–	–	+
Nitrate reductase	–	–	–	–	–	–	–	–	–	–	–	–	+
Tolerance of 5 % NaCl	ND	ND	ND	–	–	–	–	–	–	+	–	+	+
Growth at 37 °C	–	–	–	–	–	–	–	–	+	+	+	+	+

depending on the gene analysed, as illustrated by the unstable position of '*M. salmoniphilum*' in these trees. Analyses of the SSU rRNA gene and *rpoB* suggested that '*M. salmoniphilum*' is sister to *M. chelonae* (Fig. 1 and Supplementary Fig. S1), whereas *hsp65* analysis showed *M. immunogenum* to be the nearest neighbour (Fig. 2) and, with ITS analysis (Supplementary Fig. S2), *M. abscessus* was the sister species. Although a consensus cannot be established, all analyses supported '*M. salmoniphilum*' as an independent lineage, as opposed to a subset of another species.

Sequence similarity between members of the *M. chelonae* complex and '*M. salmoniphilum*' was high in the SSU rRNA gene (98.6–99.6 %) and progressively lower in the *hsp65* (92.0–96.7 %), *rpoB* (92.2–95.8 %) and ITS (86.3–95.9 %) sequences. Intrinspecifically, DNA sequences of '*M. salmoniphilum*' isolates from geographically distant locales in eastern and western North America, Australia and Europe possessed a minimum DNA sequence similarity for each gene region as follows: SSU rRNA gene (99.8 %), *hsp65* (97.7 %), *rpoB* (98.0 %) and ITS (98.7 %).

Fig. 2. Tree resulting from Bayesian phylogenetic analysis of *hsp65* gene sequences from strains of '*M. salmoniphilum*' and other members of the *M. chelonae* complex. See legend to Fig. 1 for other details.

Two-dimensional TLC of cell-wall mycolic acids of '*M. salmoniphilum*' by Arakawa & Fryer (1984) suggested similarities to *M. chelonae* and *M. abscessus*, having α and α' mycolates. Isolates lacked the epoxy mycolates found in *M. fortuitum*. HPLC patterns of mycolic acids confirmed that the study isolates shared some characteristics with the *M. chelonae* complex and with the *M. fortuitum* complex (Fig. 3). However, new HPLC analysis of cell-wall mycolic acids of '*M. salmoniphilum*' isolates visually demonstrated two separate peak groups, lacking the middle peaks found

in *M. fortuitum* (Fig. 3a; Butler & Kilburn, 1990). Although the HPLC patterns were similar to that of *M. fortuitum* (Fig. 3a), the number of mycolic acid peaks and the peak heights were different for '*M. salmoniphilum*' (Fig. 3c). Thus, the distribution of mycolic acids supported the separation of the study isolates from species within the *M. chelonae* complex and *M. fortuitum*.

We conclude that '*M. salmoniphilum*' is a pathogen of salmonid fishes that is phylogenetically and physiologically distinct from other members of the *M. chelonae* complex. The description of the salmon mycobacterium by Ross (1960) was consistent with our findings, but the name '*M. salmoniphilum*' has no current standing in nomenclature. Thus, in accordance with Rules 28a and 33a, b and c of the *International Code of Nomenclature of Bacteria* (Lapage *et al.*, 1992), we propose the revival of this name based on our analysis of Ross's original isolates and other well-identified strains and ascribe them to *Mycobacterium salmoniphilum* sp. nov., nom. rev. We designate ATCC 13758^T as the type strain based on the following criteria. Ross (1960) submitted three isolates to the ATCC; two were identified in this study as *M. salmoniphilum* (ATCC 13757 and ATCC 13758^T) by DNA sequencing, and one was confirmed as *M. fortuitum* (ATCC 13756). Strain ATCC 13758^T is also available from the DSMZ as DSM 43276^T and therefore satisfies the ICSP requirement for deposit of type strains in two collections in different countries.

Description of *Mycobacterium salmoniphilum* (ex Ross 1960) sp. nov., nom. rev.

Mycobacterium salmoniphilum (sal.mo.ni.phi'lum. L. n. *salmo* -onis a salmon; Gr. adj. *philos* loving; N.L. neut. adj. *salmoniphilum* salmon-loving).

The description is as given by Ross (1960) and also follows the descriptions of '*M. chelonae* subsp. *piscarium*' Arakawa & Fryer (1984) and '*Salmoniphilum*' strains of *M. fortuitum* (Tsukamura *et al.*, 1967). Cells are acid-fast bacilli, consistent with species of the genus *Mycobacterium*. Growth occurs on Middlebrook 7H10 agar, blood agar, MacConkey agar and Löwenstein-Jensen slants, forming cream-coloured, smooth, shiny colonies, visible after 4–6 days. Following incubation periods exceeding 10 days, colonies tend to appear waxy, with an irregular border and 'fried egg' morphology. Growth is observed at room temperature (20 °C) and with incubation at 28 to 30 °C. Weak or delayed growth may occur at 10 °C, and no growth is observed at or above 37 °C. Bacilli are generally slender and straight or slightly curved, with some short and thick. Dimensions range from 1 to 4 µm in length and 0.25 to 0.6 µm in width. Isolates have been recovered from infected salmonid fishes (although there may be other susceptible species), found throughout the viscera and predominantly in the kidney. Closely related species are members of the *M. chelonae* complex, i.e. *M. chelonae*, *M. abscessus*, *M. immunogenum*, *M. massiliense* and *M. bolletii*, sharing the

Fig. 3. Mycolic acid HPLC profiles for *M. fortuitum* ATCC 6841^T (a), *M. abscessus* ATCC 19977^T (b) and strain ATCC 13758^T (*M. salmoniphilum* sp. nov., nom. rev.) (c).

characteristics of a positive 3 day arylsulfatase test, negative nitrate reductase test and the absence of pigmentation. Strains can be distinguished from these species by a positive test for glucose utilization and by DNA sequences. Sequence analysis of the SSU rRNA gene, *hsp65*, *rpoB* and ITS regions shows that isolates are phylogenetically and consistently distinct from other members of the *M. chelonae* complex. Mycolic acid HPLC patterns are visually distinct, forming two clusters of mycolates.

The type strain is strain SC^T (=ATCC 13758^T =DSM 43276^T), isolated from Chinook salmon (*Oncorhynchus tshawytscha*) at the Spring Creek fish hatchery in Washington state, USA.

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