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Tuberculosis in seals caused by a novel member of the *Mycobacterium tuberculosis* complex: *Mycobacterium pinnipedii* sp. nov.

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A comparison of *Mycobacterium tuberculosis* complex isolates from seals (pinnipeds) in Australia, Argentina, Uruguay, Great Britain and New Zealand was undertaken to determine their relationships to each other and their taxonomic position within the complex. Isolates from 30 cases of tuberculosis in six species of pinniped and seven related isolates were compared to representative and standard strains of the *M. tuberculosis* complex. The seal isolates could be distinguished from other members of the *M. tuberculosis* complex, including the recently defined '*Mycobacterium canettii*' and '*Mycobacterium caprae*', on the basis of host preference and phenotypic and genetic tests. Pinnipeds appear to be the natural host for this 'seal bacillus', although the organism is also pathogenic in guinea pigs, rabbits, humans, Brazilian tapir (*Tapirus terrestris*) and, possibly, cattle. Infection caused by the seal bacillus is predominantly associated with granulomatous lesions in the peripheral lymph nodes, lungs, pleura, spleen and peritoneum. Cases of disseminated disease have been found. As with other members of the *M. tuberculosis* complex, aerosols are the most likely route of transmission. The name *Mycobacterium pinnipedii* sp. nov. is proposed for this novel member of the *M. tuberculosis* complex (the type strain is 6482^T = ATCC BAA-688^T = NCTC 13288^T).

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Abbreviations: BCG, Bacille Calmette–Guérin; FAFLP, fluorescent amplified fragment length polymorphism; PZA, pyrazinamide; SS, seal spoligotype; TCH, thiophen-2-carboxylic acid hydrazide.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain 6482^T is AF502574.

INTRODUCTION

The *Mycobacterium tuberculosis* complex has traditionally consisted of four members: *M. tuberculosis* (*sensu stricto*), which primarily infects human and primates; *Mycobacterium bovis*, which predominantly causes tuberculosis in cattle (Karlson & Lessel, 1970), but can also cause disease in a wide variety of other animals, including man; *M. bovis* Bacille Calmette–Guérin (BCG), an attenuated strain that is used for vaccination; *Mycobacterium africanum*, a heterogeneous group of isolates responsible for human tuberculosis in Africa, which appears to be intermediate between *M. tuberculosis* and *M. bovis* (Castets *et al.*, 1969); and *Mycobacterium microti*, a less frequently isolated pathogen that traditionally causes tuberculosis in voles (Wells & Oxen, 1937; Wells & Robb-Smith, 1946), but has been identified more recently as a cause of disease in immunocompromised humans (van Soolingen *et al.*, 1998). Each member of the *M. tuberculosis* complex is associated with a specific primary host, although infection is known to occur in various alternative hosts. Although all of these strains effectively share the same 16S rRNA gene sequence (Rogall *et al.*, 1990b) and high DNA–DNA homology (from hybridization studies), they can be separated by some phenotypic characteristics (Grange & Yates, 1994) and, as they have different primary hosts, they have been regarded as separate species (Collins *et al.*, 1982). More recently, two novel strains have been described: '*Mycobacterium canettii*', a novel smooth variant of *M. tuberculosis* that was first isolated from a Somali-born patient (van Soolingen *et al.*, 1997) and subsequently from a Swiss patient exposed in Africa (Pfyffer *et al.*, 1998); and '*Mycobacterium caprae*' (basonym: *M. tuberculosis* subsp. *caprae*), a strain that occurs primarily in Spanish goats and has recently been elevated to species level (Aranaz *et al.*, 1999; Niemann *et al.*, 2002; Aranaz *et al.*, 2003). *M. tuberculosis*, *M. africanum*, *M. microti* and *M. bovis* were accepted as separate species by using a combination of phenotypic characteristics and apparent host specificity. '*M. canettii*' and '*M. caprae*' were accepted by virtue of host preference and phenotypic and genetic characteristics.

Previous logical arguments have suggested that all members of the *M. tuberculosis* complex are sufficiently similar to belong to the species *M. tuberculosis* (Wayne, 1984; Tsukamura *et al.*, 1985; van Soolingen *et al.*, 1997). Acceptance of such an approach would cause currently accepted species (*M. bovis*, *M. microti* and *M. africanum*) to warrant classification at the subspecies level.

Between 1986 and 1995, *M. tuberculosis* complex organisms were isolated from cases of tuberculosis in captive or wild Australian sea lions (*Neophoca cinerea*), New Zealand fur seals (*Arctocephalus forsteri*), an Australian fur seal (*Arctocephalus pusillus doriferus*) and a seal trainer who worked with the affected colony in Australia (Forshaw & Phelps, 1991; Thompson *et al.*, 1993; Cousins, 1995; Woods *et al.*, 1995). Similar organisms were recovered from captive Southern sea lions (*Otaria flavescens*), wild South American fur seals (*Arctocephalus australis*) and a wild Subantarctic fur seal (*Arctocephalus tropicalis*) in Uruguay or Argentina during the period 1989–2000 (Bernardelli *et al.*, 1994, 1996; Castro Ramos *et al.*, 1998; Bastida *et al.*, 1999). Between 1996 and 1998, *M. tuberculosis* complex organisms were recovered from two South American fur seals in a zoological collection in Great Britain and a Brazilian tapir (*Tapirus terrestris*) housed in an adjacent enclosure, and from two New Zealand fur seals in New Zealand in 1997 and 1998 (Hunter *et al.*, 1998).

Many of the isolates obtained from cases of tuberculosis in Australia, Uruguay and Argentina have been well-characterized (Cousins *et al.*, 1993; Bernardelli *et al.*, 1996; Cousins, 1996; Alito *et al.*, 1999; Zumárraga *et al.*, 1999) and this information, together with preliminary tests on the seal isolates from Great Britain and New Zealand, suggested that the seal bacillus (Cousins *et al.*, 1993), isolated from pinnipeds from all four continents, may be a unique member of the *M. tuberculosis* complex. A recent study of four Australian and six Argentinian seal isolates by fluorescent amplified fragment length polymorphism (FAFLP) has further substantiated the hypothesis that the seal bacillus occupies a unique taxonomic position within the *M. tuberculosis* complex (Ahmed *et al.*, 2003). This report consolidates the results of tests performed previously and provides additional information, resulting in a comprehensive comparison of isolates available from pinniped-related cases of tuberculosis, and indicates that the seal bacillus should be considered as a novel species of the *M. tuberculosis* complex.

METHODS

Bacterial strains. *M. tuberculosis* complex isolates recovered from 30 pinnipeds and a seal trainer from 1985 to 2000 were available for study (Table 1). Isolates recovered from two guinea pigs and two rabbits after pathogenicity experiments in Australia, a bovine in New Zealand (this isolate had a restriction endonuclease analysis pattern similar to those of the Australian seal isolates) and a Brazilian tapir were also included. Where appropriate, isolates were compared to representative (and reference) strains of *M. tuberculosis* (H37Rv or Mt14323), *M. africanum* (TMC3), '*M. caprae*' (CIP 105776^T), *M. microti* (NCTC 8710^T), *M. bovis* (AN5) and *M. bovis* BCG (P3) (Table 2).

Phenotypic characteristics. Isolates were examined for growth and phenotypic characteristics according to standard procedures (Vestal, 1975). *In vitro* susceptibility patterns to isoniazid, rifampicin, streptomycin and ethambutol were determined for three isolates from Australia, three from Argentina, one from Uruguay, two from Great Britain and two from New Zealand by using the Mycobacterial Growth Indicator Tube (MGIT; Becton Dickinson) system (Bernardelli *et al.*, 1999; Morcillo *et al.*, 2000).

Pathogenicity studies in guinea pigs and rabbits. Isolates from two Australian seals (Au-1 and Au-2) were each injected into a guinea pig and a rabbit, and three isolates from Argentina (Ar-1, Ar-2 and Ar-3) and the isolate from Uruguay (U-1) were inoculated into guinea pigs, to examine the pathogenicity of the seal isolates.

Table 1. *M. tuberculosis* complex isolates recovered from pinnipeds or related cases from various countries, 1985–2000

Isolate (reference no.)	Source	Year
Australia		
Au-1 (6481)	Captive *Neophoca cinerea* (Australian sea lion)	1985
(4823F)	Guinea pig LN ex 6481	
(4524D)	Rabbit lung ex 6481	
Au-2 (6482^T)	Captive *Arctocephalus forsteri* (New Zealand fur seal)	1986
(4821D)	Guinea pig LN ex 6482	
(1676)	Rabbit lung ex 6482	
Au-3 (6510)	Captive *Neophoca cinerea*	1986
Au-4 (6954)	Captive *Neophoca cinerea*	1986
Au-5 (6866)	Captive *Arctocephalus forsteri*	1986
Au-6 (6884)	Captive *Neophoca cinerea*	1986
Au-7 (146-D)	Human (seal trainer)	1988
Au-8 (14109)	Wild *Neophoca cinerea* 1	1991
Au-9 (14126)	Wild *Neophoca cinerea* 2	1992
Au-10 (92/1161T)	Wild *Arctocephalus pusillus doriferus* (Australian fur seal)	1992
Au 11 (A95-127)	Wild *Arctocephalus forsteri*	1995
Uruguay		
U-1 (1337)	Captive *Otaria flavescens* (Southern sea lion)	1987
Argentina		
Ar-1 (1203-5)	Wild *Arctocephalus australis* (South American fur seal)	1989
Ar-2 (M-17-92, 1489-50)	Wild *Arctocephalus australis*	1992
Ar-3 (M-31-92, 1855-8)	Wild *Arctocephalus australis*	1992
Ar-4 (M-38-92, 1862-4)	Wild *Arctocephalus australis*	1992
Ar-5 (M-47-92, 1866-7)	Wild *Otaria flavescens*	1992
Ar-6 (M-11-91, 1868-9)	Wild *Arctocephalus australis*	1995
Ar-7 (M-05-95, 1920-1)	Wild *Arctocephalus australis*	1995
Ar-8 (M-35-95, 1981-2)	Wild *Arctocephalus australis*	1995
Ar-9 (M-08-96, 2003 & 5)	Wild *Arctocephalus australis*	1996
Ar-10 (M-55-95, 2007-9)	Wild *Arctocephalus tropicalis* (Subantarctic fur seal)	1996
Ar-11 (M-10-96, 2027 & 9)	Wild *Arctocephalus australis*	1996
Ar-12 (M-33-96, 2050-3)	Wild *Arctocephalus australis*	1996
Ar-13 (M-02-99, 2186)	Wild *Arctocephalus australis*	1999
Ar-14 (M-30-00, 2192)	Wild *Arctocephalus australis*	2000*
Ar-15 (M-33-00, 2225-6 & 9)	Wild *Arctocephalus australis*	2000*
United Kingdom		
UK-1 (623/971757)	Captive *Arctocephalus australis*	1996
UK-2 (624/97)	Captive *Tapirus terrestris* (Brazilian tapir)	1996*
UK-3 (2281)	Captive *Arctocephalus australis*	1998
New Zealand		
NZ-1	Bovine	1991*
NZ-1	Wild *Arctocephalus forsteri*	1997
NZ-2	Wild *Arctocephalus forsteri*	1998

*Unpublished.

Mycolic acid analysis. Mycolic acid profiles of two representative isolates (Au-1 and Au-2) were examined by HPLC, according to previously published procedures (Butler *et al.*, 1996, 1999).

Tests for MPB70. All isolates were tested for the presence of the MPB70 antigen either by using the immunoperoxidase test (Corner *et al.*, 1988; Veerman *et al.*, 1990; Liébana *et al.*, 1996) and/or by performing SDS-PAGE on antigen preparations (Cousins *et al.*, 1993; Alito *et al.*, 1999).

16S rDNA sequence determination and PCR-based tests for genetic markers. PCR-mediated amplification of 16S rDNA was performed by using procedures described previously (Edwards *et al.*, 1989; Kirschner *et al.*, 1993). The nucleotide sequences obtained were compared to all known 16S rDNA mycobacterial sequences in GenBank and the *M. bovis* sequence (available at the Sanger website, http://www.sanger.ac.uk/Projects/M_bovis/) by using the FastA application. Sequences were aligned with the program PILEUP from the Genetics Computer Group (GCG) version 9 UNIX software

Table 2. *M. tuberculosis* complex isolates that were tested by spoligotyping and used to prepare the dendrogram (Fig. 2)

AFS, Australian fur seal; ASL, Australian sea lion; Bj, Beijing strain; COB, country of birth; NZFS, New Zealand fur seal; SAFS, South American fur seal; TMC, Trudeau Mycobacterium Collection.

Species/strain	Source of isolate	Country of origin/provided by
M. africanum		
1. TMC 3	Reference strain, human origin	
2. TMC 12	Reference strain, human origin	
3. TMC 54	Reference strain, human origin	
4. 19884 (4163/69)	Clinical isolate, human origin	Australia
5. 19887 (5166/88)	Clinical isolate, human origin	Australia
6. 19890 (486/93)	Clinical isolate, human origin	Australia
7. 22054	Clinical isolate, human origin	Australia
M. bovis/*M. bovis* BCG		
8. BCG Japanese	Vaccine strain	Richard Wallace, USA
9. BCG Russian	Vaccine strain	Richard Wallace, USA
10. BCG Pasteur	Vaccine strain	RIVM, Netherlands
11. AN5	Reference strain, cattle origin	CSIRO, Australia
12. 3958	Cattle	Australia (Western Australia)
13. 6205	Cattle	Australia (Western Australia)
14. 11487	Cattle	Australia (Western Australia)
15. 14457	Cattle	Australia (Western Australia)
16. 14899	Cattle	Australia (Western Australia)
17. 15145	Cattle	Australia (Western Australia)
18. 17319	Cattle	Australia (Queensland)
19. 17898	Cattle	Australia (Queensland)
20. 20007	Red deer	Canada
21. 22950	Goat	Spain
M. microti		
22. NCTC 8710^T	Reference strain, vole origin	
23. 3377	Vole	T. Jenkins, UK
24. 3381	Vole	T. Jenkins, UK
M. tuberculosis		
25. H37Rv	Reference strain, human origin	RIVM
26. 14323	Reference strain, human origin	RIVM
27. 26079	Clinical isolate, human origin	Diagnosed in Australia, COB Indonesia
28. 26141	Clinical isolate, human origin	Diagnosed in Australia, COB Indonesia
29. 26142	Clinical isolate, human origin	Diagnosed in Australia, COB Vietnam
30. 26152	Clinical isolate, human origin	Diagnosed in Australia, COB Vietnam
31. 27204	Clinical isolate, human origin, Bj	Diagnosed in Australia, COB India
32. 27206	Clinical isolate, human origin	Diagnosed in Australia, COB Yugoslavia
33. 27214	Clinical isolate, human origin, Bj	Diagnosed in Australia, COB Vietnam
34. 27222	Clinical isolate, human origin	Diagnosed in Australia, COB Australia
35. 27230	Clinical isolate, human origin	Diagnosed in Australia, COB Afghanistan
'*M. canettii*'		
36. So93	Clinical isolate, human origin	D. van Soolingen, Netherlands
'*M. caprae*'		
37. CIP 105776^T	Goat	Spain
38. 4/21	Goat	Spain
39. CB27	Goat	Spain
Seal bacilli		
40. SS-1, 146-D	Clinical isolate, seal trainer	Australia
41. SS-1, 6482^T	Captive NZFS	Australia
42. SS-1, 92/1162/T	Wild AFS	Australia
43. SS-2	Wild SAFS	Argentina
44. SS-3, 24890	Captive SAFS	Great Britain
45. SS-4, 25878	Wild NZFS	New Zealand

package. Phylogenetic analyses of the sequence data were done with programs from the Phylogeny Inference Package (PHYLIP) as described previously (Floyd *et al.*, 1996). The pairwise comparison program GAP, also from the GCG package, was used to determine the position of consensus strand nucleotides, relative to those of *Escherichia coli* (GenBank number J01859). The 16S rDNA sequence of strain 6482^T was deposited in GenBank under accession number AF502574.

PCR-based tests for known genetic markers. All isolates were tested by PCR for the presence of mycobacterial 16S rDNA, the gene that encodes the MPB70 antigen, the IS*6110*, IS*1081* and *mtp40* sequences (Del Portillo *et al.*, 1991) and the P_{AN} promoter region that is present in pathogenic mycobacteria (Gormley *et al.*, 1997) by using previously published methods (Liébana *et al.*, 1996; Zumárraga *et al.*, 1999). Representative isolates were tested for *katG* and *gyrA* gene sequence polymorphisms at codons 463 and 95, respectively, by using methods described previously (Zumárraga *et al.*, 1999). Allele-specific polymorphisms were examined at nt 285 of the *oxyR* gene (Sreevatsan *et al.*, 1996), which differentiates *M. bovis* and '*M. caprae*' (adenine) from other members of the *M. tuberculosis* complex (guanidine), and in codon 57 (nt 169) of the *pncA* gene, which is responsible for pyrazinamide (PZA) resistance (Espinosa de los Monteros *et al.*, 1998), which is consistent with *M. bovis*.

Spoligotyping. All but two isolates were tested for known spacers between direct repeats in the DR allele by using the spoligotyping method developed by Kamerbeek *et al.* (1997) and performed as described previously (Aranaz *et al.*, 1996; Zumárraga *et al.*, 1999). Spoligotyping results were analysed by electronic scanning of images and converting and analysing them by using GelCompar version 1.3, as described previously (Romano *et al.*, 1995; Cousins *et al.*, 1998a, b). The patterns obtained from the South American isolates were compared to a database that consisted mostly of *M. bovis* isolates from Argentina. In addition, the patterns obtained from all seal isolates were compared with a large database of patterns that contained approximately 700 *M. tuberculosis* complex isolates, including approximately 500 *M. bovis* isolates from cattle, buffalo, deer, wild animals and humans from Australia and other countries (Cousins *et al.*, 1998a); they were also compared to the CDC database (Jack Crawford, personal communication). The seal isolates and representative and reference strains of the *M. tuberculosis* complex (Table 2) were included in a dendrogram of spoligotype patterns that were generated by using Dice UPGMA analysis (GelCompar, version 3.1; Applied Maths) to examine the clonal relationships between them.

FAFLP. Heat-killed cells of isolates from three Australian sea lions, one Australian fur seal and six South American fur seals were digested by using *Eco*RI/*Mse*I and analysed by FAFLP, using methods described previously (Ahmed *et al.*, 2002, 2003). Analyses were based on the differential amplification of 131 genomic loci. Standard genomic DNA from *M. tuberculosis* H37Rv, *M. bovis* AN5, *M. africanum* and *M. microti* (NCTC 8710^T) was used for comparative FAFLP analysis.

RESULTS AND DISCUSSION

Morphology, growth and phenotypic characteristics of seal isolates

A description of the physiological characteristics of the taxon can be found in the formal description. Biochemical testing clearly confirmed that the seal isolates belonged to the *M. tuberculosis* complex. The negative reactions in the nitrate reduction and niacin accumulation tests were consistent with *M. bovis* (Grange & Yates, 1994) (Table 3), a fact that led to their initial identification as such in Australia (Forshaw & Phelps, 1991), Argentina (Bernardelli *et al.*, 1996) and Great Britain. In some cases, varying amounts of niacin were produced, which is similar to results reported for *M. africanum* (Grange & Yates, 1994). In most cases, the seal isolates grew preferentially on media that contained sodium pyruvate, although some (including NZ-2 and NZ-3) also grew on Löwenstein–Jensen medium that contained glycerol. Slight differences from typical *M. bovis* isolates were noted in Australia and Argentina, in that the

Table 3. Phenotypic properties of the seal bacillus, compared to other members of the *M. tuberculosis* complex

Species: 1, *M. tuberculosis* (classic); 2, *M. tuberculosis* (Asian); 3, *M. africanum* (type I); 4, *M. africanum* (type II); 5, *M. microti*; 6, seal bacillus; 7, *M. bovis*; 8, '*M. caprae*'; 9, *M. bovis* BCG. Data were taken from references cited in the text. Abbreviations: +, positive; −, negative; V, variable; NA, not applicable.

Characteristic	1	2	3	4	5	6	7	8	9
Nitrate reduction	+	+	−	+	−	−	−	−	−
Niacin accumulation	+	+	V	V	+	−*	−	−	−
Pyruvate preference	−	−	−	−	−	+	+	+	+
Stimulated by glycerol	+		−	−	−	−	−	−	+
MPB70 antigen	−	−	−	−	−	−	+	?	+
Resistance to:									
TCH	+	−	−	+	−	−†	−	−‡	−
PZA	−	−	−	−	−	−	+	−	+
Pathogenicity in:									
Guinea pig	++		++		−	++	++	NA	−
Rabbit	−		+/−		−	+++	++	NA	−

*Occasional strains, including the isolates from New Zealand, gave weak or positive reactions in the niacin accumulation test.
†New Zealand strains were resistant to 1 μg TCH ml^{-1}, but sensitive to 10 μg TCH ml^{-1}.
‡Resistant to 1 and 2 μg TCH ml^{-1}, but sensitive to 5 and 10 μg TCH ml^{-1}.

cord formation observed after Ziehl–Neelsen staining was loose; further investigations uncovered differences between the seal isolates and *M. bovis*, including its susceptibility to PZA.

Pathogenicity and potential host range

Isolates inoculated into guinea pigs produced significant lesions or death within 6 weeks and those inoculated into rabbits caused death within 6 weeks, confirming that the isolates were fully virulent in both laboratory animals. The finding of a bovine isolate in New Zealand with characteristics indistinguishable from those of isolates from fur seals in New Zealand waters suggests that the seal bacillus is also capable of infecting cattle. This fact, combined with knowledge of its ability to cause disease in humans (Thompson *et al.*, 1993) and tapirs, suggests that the seal bacillus has the potential for a host range that extends beyond those of *M. tuberculosis*, *M. africanum* and *M. microti*.

Mycolic acid analysis

HPLC chromatograms of isolates Au-1 and Au-2 demonstrated a single cluster pattern that was consistent with species of the *M. tuberculosis* complex (data not shown) as reported previously (Butler *et al.*, 1991), providing additional evidence that these organisms belonged to the *M. tuberculosis* complex.

Tests for the MPB70 antigen

All seal isolates were negative when tested for the MPB70 antigen, despite containing the *mpb70* gene. The MPB70 antigen is considered to be characteristic of *M. bovis* (Corner *et al.*, 1988; Liébana *et al.*, 1996) and can be demonstrated by dot-blot immunoperoxidase (Liébana *et al.*, 1996) or SDS-PAGE (Cousins, 1996; Alito *et al.*, 1999). In a previous study, >97 % of *M. tuberculosis* isolates, 100 % of *M. microti* isolates and 90 % of *M. africanum* isolates were negative for the MPB70 antigen, whereas >99 % of *M. bovis* isolates were positive (Liébana *et al.*, 1996). In this regard, the seal bacillus was more like other members of the *M. tuberculosis* complex than *M. bovis*. The reported presence of the MPB70 antigen in a single isolate of *M. microti* from an alpaca (Alito *et al.*, 1999) was contrary to the findings of Liébana *et al.* (1996), who tested seven isolates of *M. microti* (including the reference strain).

16S rDNA sequence determination

16S rDNA sequencing is an accepted method of confirming the species designation of mycobacterial isolates (Böddinghaus *et al.*, 1990; Rogall *et al.*, 1990a, b). The 16S rDNA consensus strand (1400 nt) from the seal isolates demonstrated 99·9 % similarity to those of *M. tuberculosis* (GenBank number X58890) and *M. bovis* (available from the Sanger website at http://www.sanger.ac.uk/Projects/M_bovis/). A single nucleotide substitution (C→T) in the consensus strand occurred at *E. coli* position 1256 (data not shown). Phylogenetic analysis demonstrated that the consensus sequence was on the same branch as that of *M. tuberculosis*. The 16S rDNA regions of the isolates that were sequenced (1030 bp) were consistent with the sequence of the *M. tuberculosis* complex.

PCR-based testing of genetic markers

The *gyrA* and *katG* gene sequences of all seal isolates were identical (Table 4). These genetic markers are accepted methods of confirming that isolates belong to the *M. tuberculosis* complex (Thierry *et al.*, 1990, 1993;

Table 4. Genetic properties of the seal bacillus, compared to other members of the *M. tuberculosis* complex

Species: 1, *M. tuberculosis* (classic); 2, *M. tuberculosis* (Asian); 3, *M. africanum* (type I); 4, *M. africanum* (type II); 5, *M. microti*; 6, seal bacillus; 7, *M. bovis*; 8, '*M. caprae*'; 9, *M. bovis* BCG. All species contain IS*6110* and IS*1081*, although some Asian strains of *M. tuberculosis* lack IS*6110*. Present (1–5), between one and five of the 3′ spacers (39–43) are present; NIL, none of the five 3′ spacers (39–43) are present.

Locus	1	2	3	4	5	6	7	8	9
mtp40	+*	+	+*	+*	−†	+	−	−	−
pncA C57	CAC (His)	CAC	CAC	CAC	CAC	CAC	GAC (Asp)	CAC	GAC
katG C463	CTG (Leu), CGG (Arg)	CTG/CGG	CTG	CTG	CTG	CTG	CTG	CTG	CTG
oxyR nt 285	G	G	G	G	G	G	A	A	A
gyrA C95	AGC (Ser), ACC (Thr)	−	ACC	ACC	ACC	ACC	ACC	ACC	ACC
Spoligotyping: spacers 39–43	Present (1–5)		Present (1–5)		NIL	NIL	NIL	NIL	NIL

*Very occasionally, members of these species lack the *mtp40* gene (Liébana *et al.*, 1996).

†Seven of seven isolates (100 %) were negative for *mtp40* (Liébana *et al.*, 1996), whereas one isolate tested by Bernardelli *et al.* (1996) was reported as positive.

Spoligotype pattern observed with 43 spacers in DR locus	Isolate/strain/type	No. isolates				
		Australia	Argentina/ Uruguay	Great Britain	New Zealand	Total
	M. tuberculosis H37Rv					
	M. africanum TMC 03					
	M. africanum TMC 12					
	M. microti NCTC 8710^T					
	M. bovis BCG P3					
	M. bovis Sp01					
	Seal bacillus SS-1	15	13			28
	Seal bacillus SS-2		1			1
	Seal bacillus SS-3			3		3
	Seal bacillus SS-4				3	3
	Total	15	14	3	3	35

Fig. 1. Results of spoligotyping of seal-related isolates from Australia, Uruguay, Argentina, Great Britain and New Zealand, compared to reference strains of the *M. tuberculosis* complex. ■, Hybridization with spacer; □, no hybridization with spacer. NCTC, National Collection of Type Cultures; TMC, Trudeau Mycobacterium Collection.

Collins & Stephens, 1991; Cousins *et al.*, 1991; Groenen *et al.*, 1993; Liébana *et al.*, 1996; Gormley *et al.*, 1997; Sreevatsan *et al.*, 1997). Results from sequencing of the *mtp40*, *pncA* and *oxyR* genes clearly demonstrated that the seal isolates were genetically more consistent with *M. tuberculosis* and *M. africanum* than with *M. bovis*.

DNA spoligotyping

Four different spoligotypes were identified in the seal isolates; all lacked the spacers 39–43, which are known to be characteristic of *M. bovis* (Fig. 1). All of the isolates from Australia and all but one of the Argentinian isolates had a unique but identical pattern, designated seal spoligotype 1 (SS-1). The remaining Argentinian isolate was designated SS-2. The three isolates from Great Britain had identical spoligotypes (SS-3) that differed by one spacer from the other seal spoligotypes. The seal isolates from New Zealand and the isolate from a New Zealand bovine had identical spoligotypes (SS-4) that lacked six spacers that were present in all other seal isolates. When compared to reference (and representative) strains of *M. tuberculosis*, *M. africanum*, *M. microti*, *M. bovis*, '*M. canettii*' and '*M. caprae*', the seal isolates formed a distinct cluster within the *M. tuberculosis* complex (Fig. 2). Spoligotyping confirmed that the seal isolates from Australia, Argentina, Uruguay and Great Britain were closely related. The finding of three spoligotypes with only minor differences from 29 isolates that originated from these diverse geographical regions indicated a clonal relationship between these isolates, which in turn suggests that the infection may have originated from a single source as a relatively recent event. Considering that these cases were diagnosed over a period of more than 15 years and that many of these populations inhabit geographically separate territories, a more likely explanation is that the DR locus exhibits considerable genetic stability in the seal bacillus. The spoligotype identified in the New Zealand isolates clustered with those of the other seal isolates but was genetically further removed, confirming a closer relationship to the other seal isolates than to other members of the *M. tuberculosis* complex. Similar small differences in spoligotype were evident among a group of '*M. caprae*' isolates (Aranaz *et al.*, 1999). Spoligotyping has previously been used to define clonal relationships of the Beijing family of *M. tuberculosis* (van Soolingen *et al.*, 1995; Qian

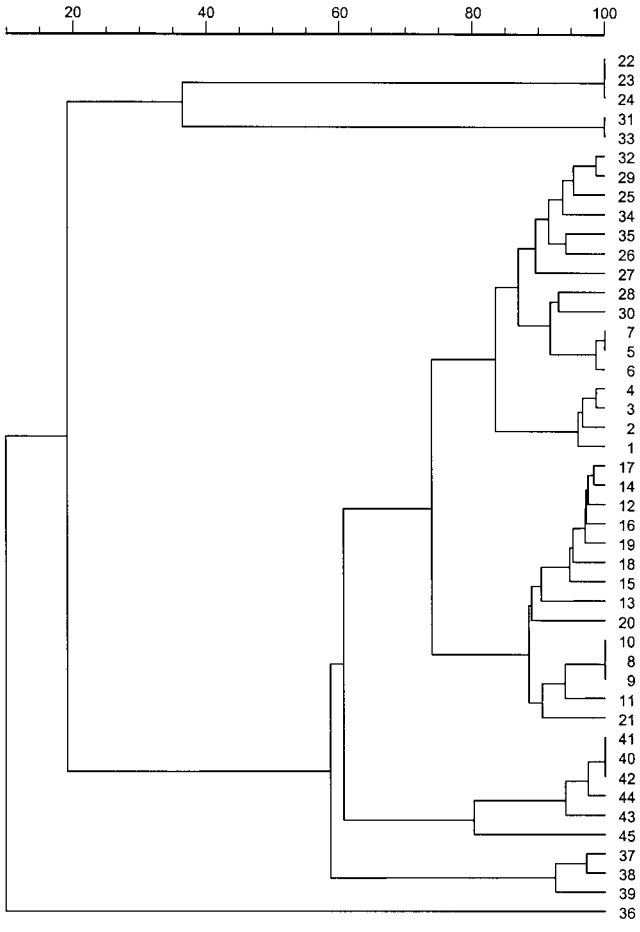

Fig. 2. Dendrogram showing the relationship of established members of the *M. tuberculosis* complex and the seal bacillus, as revealed by spoligotyping. Strain designations are given in Table 2.

et al., 1999; Anh *et al.*, 2000) and has been used to trace the global spread of this strain. It has also been proved to be useful in defining populations of *M. microti* (van Soolingen *et al.*, 1998) and, in this study, demonstrated its usefulness in defining the limited genetic diversity of the seal bacillus.

Dendrograms constructed by using GelCompar indicated there was a close relationship between all seal-related isolates. Other methods of typing these isolates, including RFLP analysis with IS*1081*, IS*6110*, DR, PGRS and pUCD and VNTR (variable number of tandem repeats) typing, confirmed these findings (data not shown). Many investigators accept that members of the *M. tuberculosis* complex may be represented along a continuum with major peaks that correspond to each of the designated species. It is probable that the seal bacillus has evolved from another *M. tuberculosis* complex organism and has found a unique niche in this marine host. A study by Behr & Small (1999) that identified deletion events in *M. bovis* BCG has elicited information on the evolution of BCG strains. Similar evolutionary insights into the origin of the seal bacillus have been gained by using comparative genomic technologies that were described previously (Brosch *et al.*, 2002; Mostowy *et al.*, 2002). In both studies, the seal strain was separated from classical *M. bovis* by at least six deletions. The seal bacillus has a similar number of deletions to *M. microti* and Brosch *et al.* (2002) suggest that, along with *M. microti* and '*M. canettii*', the seal bacillus contains a unique deletion. These deletion studies provide further evidence that the seal bacillus should be designated as a separate species within the *M. tuberculosis* complex.

FAFLP

All 10 seal isolates produced indistinguishable results. When compared to the published sequences of *M. tuberculosis* strains CDC1551 and H37Rv and *M. bovis* strain AN5, up to 18 highly polymorphic FAFLP markers for the rapid identification of the seal bacillus were identified. In these studies, three loci appeared to be unique to the seal bacillus, 12 were shared with *M. bovis* and three were shared with *M. tuberculosis*. Further studies that include some of these loci may result in the identification of species-specific markers that are potentially useful for the development of PCR-based diagnostics for the seal bacillus. The identical genotype of all seal isolates that were tested by FAFLP confirmed their close clonal relationship, which had been identified by spoligotyping. It also substantiated previous studies that used FAFLP, which suggested that this technique may play a role in discriminating between mycobacterial species, including members of the *M. tuberculosis* complex (Goulding *et al.*, 2000; Huys *et al.*, 2000).

Description of *Mycobacterium pinnipedii* sp. nov.

Mycobacterium pinnipedii (pin.ni.pe′di.i. N.L. gen. neut. n. *pinnipedii* of a pinniped, referring to the host animal from which the organism was first isolated).

Isolates can be recovered from the lung and associated lymph nodes of tuberculous pinnipeds, and occasionally from mesenteric lymph nodes and organs such as the liver. Acid/alcohol-fast, non-spore-forming, non-motile bacilli with loose cord formation. Growth is generally enhanced by sodium pyruvate and usually occurs within 3–6 weeks of incubation on egg-based media at 36–37 °C. Colonies are dysgonic, rough, flat and non-photochromogenic. Isolates are negative for nitrate reduction and generally negative for niacin accumulation; some isolates demonstrate low-to-medium reactions for niacin. Susceptible to 50 μg PZA ml^{-1} and 1 μg thiophen-2-carboxylic acid hydrazide (TCH) ml^{-1} (isolates have occasionally demonstrated resistance to 1 μg TCH ml^{-1}, but are susceptible to 10 μg ml^{-1}). Pathogenic in guinea pigs and rabbits; the apparent incidental infection of a human, bovine and tapir indicates that they may have a wide host range. All isolates contain the sequences IS*6110*, IS*1081*, *mpb70* and *mtp40*, yet fail to produce detectable MPB70 antigen. The *pncA* gene contains CAC (His) at codon 57 and the *oxyR* gene shows G at nt 285, similar to *M. tuberculosis*, *M. microti* and *M. africanum*. The seal isolate spoligotypes form a cluster that is clearly different from those of all other members of the *M. tuberculosis* complex. The isolates are susceptible to isoniazid, rifampicin, streptomycin, ethambutol and paraminosalicylic acid.

The type strain is 6482^T (=ATCC BAA-688^T=NCTC 13288^T).

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