***Actinomyces marimammalium* sp. nov., from marine mammals**

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Three strains of a previously undescribed *Actinomyces*-like bacterium were isolated from samples taken from two dead seals and a porpoise. Biochemical testing and PAGE analysis of whole-cell proteins indicated the strains were phenotypically similar to each other but different from previously described *Actinomyces* and *Arcanobacterium* species. Comparative 16S rRNA gene sequencing studies showed the organisms from marine animals were genetically closely related and represent a hitherto unknown subline within the genus *Actinomyces* (sequence divergence values > 6% with recognized species). Based on phylogenetic and phenotypic evidence it is proposed that the unknown bacterium from the seals and a porpoise should be classified as *Actinomyces marimammalium* sp. nov. The type strain is CCUG 41710^T.

Keywords: taxonomy, phylogeny, *Actinomyces marimammalium*, 16S rRNA

INTRODUCTION

In recent years, the Gram-positive asporogenous rod-shaped organisms of the genus *Actinomyces* and related taxa have attracted the attention of clinical microbiologists, mainly because of a growing awareness of their presence in clinical specimens and also because of increased taxonomic interest (Funke *et al.*, 1997a). Knowledge of the taxonomic inter-relationships of species of the genus *Actinomyces* has improved dramatically in recent years particularly with the use of 16S rRNA gene sequencing (Pascual *et al.*, 1997a; Lawson *et al.*, 1997). Indeed, the application of molecular sequence data in conjunction with improved phenotypic methods of analysis has not only resulted in better species identification but also greatly facilitated the recognition of new taxa, particularly from human clinical sources [e.g. *Actinomyces europaeus* (Funke *et al.*, 1997b); *Actinomyces graevenitzi* (Pascual *et al.*, 1997b); *Actinomyces neu*i subsp. *anitratus* and *neu*i (Funke *et al.*, 1994); *Actinomyces radingae* (Wüst *et al.*, 1995); *Actinomyces turicensis* (Wüst *et al.*, 1995); *Actinomyces urogenitalis* (Nikolaitchouk *et al.*, 2000); *Actinobaculum schaalii* (Lawson *et al.*, 1997);

Arcanobacterium bernardiae (Funke *et al.*, 1995)]. The presence of *Actinomyces*-like organisms in wild and domestic animals has received much less attention, although there are indications that the implementation of improved diagnostic methods will result in a better understanding of their host distribution and possible association with animal disease (Pascual *et al.*, 1997a, 1999). In this article, we report the polyphasic characterization (using phenotypic and molecular techniques in concert) of three unusual *Actinomyces*-like bacteria isolated from two dead seals and a porpoise. Based on the presented findings, yet another new species of the genus *Actinomyces*, *Actinomyces marimammalium* sp. nov., is described.

METHODS

Cultures and cultivation. Strain M1749/98/1^T (CCUG 41710^T) was isolated in mixed culture with *Escherichia coli*, *Streptococcus* spp. and *Actinobacillus* spp. from multiple organs (lung, spleen, liver, kidney, mesenteric lymph node) of a dead male hooded seal (*Cystophora cristata*) which had suffered from pneumonia, whereas strain M514/99/2 (CCUG 43292) was isolated from the small intestine of a dead grey seal (*Halichoerus grypus*) which had been shot. Strain M1349/94/2 (CCUG 42833) originated from the lung of a dead harbour porpoise (*Phocoena phocoena*). The unidentified *Actinomyces*-like isolates were cultured on Columbia agar (Difco) supplemented with 5% sheep blood at 37 °C, in air plus 5% CO₂. The strains were biochemically

Abbreviation: CCUG, Culture Collection of the University of Göteborg.

The GenBank accession number for the 16S rRNA gene sequence of strain CCUG 41710^T is AJ276405.

Fig. 1. Similarity dendrogram based on whole-cell protein patterns of *Actinomyces marimammalium* sp. nov. and related species. Levels of correlation are expressed as percentages of similarity for convenience.

characterized by using the API rapid ID32Strep and API coryne systems according to the manufacturer's instructions (API bioMérieux).

Protein profiling. PAGE analysis of whole-cell proteins was performed as described by Pot *et al.* (1994). For densitometric analysis, normalization and interpretation of protein patterns the GCW 3.0 software package (Applied Maths) was used. The similarity between all pairs of traces was expressed by the Pearson product moment correlation coefficient converted for convenience to a percentage similarity.

16S rRNA gene sequencing and phylogenetic analysis. The 16S rRNA genes of the isolates were amplified by PCR and directly sequenced using a *Taq* Dye-Deoxy Terminator Cycle Sequencing kit (Applied Biosystems) and an automatic DNA sequencer (model 373A, Applied Biosystems). The closest known relatives of the new isolates were determined by performing database searches. These sequences and those of other known related strains were retrieved from the GenBank or Ribosomal Database Project libraries and aligned with the newly determined sequences using the program PILEUP (Devereux *et al.*, 1984). The resulting multiple sequence alignment was corrected manually and a distance matrix was calculated using the programs PRETTY and DNADIST (using the Kimura 2-correction parameter) (Felsenstein, 1989). A phylogenetic tree was constructed according to the neighbour-joining method with the program NEIGHBOR (Felsenstein, 1989). The stability of the groupings was estimated by bootstrap analysis (500 replications) using the programs SEQBOOT, DNADIST, NEIGHBOR and CONSENSE (Felsenstein, 1989).

RESULTS AND DISCUSSION

The three isolates consisted of Gram-positive straight to slightly curved rods, some of which displayed branching. Cells were non-acid-fast and non-spore-forming. The strains grew under aerobic and anaerobic conditions and were catalase-negative. The isolates phenotypically resembled each other, producing acid from glucose, maltose, lactose and *N*-acetyl- β -glucosamine. None of the isolates produced acid from L-arabinose, D-arabitol, cyclodextrin, mannitol, melibiose, melizitose, methyl- β -D-glucopyranoside, pullulan, raffinose, ribose, sorbitol, sucrose, tagatose or D-xylose. All strains were positive for alanine phenylalanine proline arylamidase, β -galactosidase, β -galacturonidase and glycyl tryptophan arylamidase, but were negative for arginine dihydrolase, pyrazinamidase, α -galactosidase, α -glucosidase, β -glucosidase, β -glucuronidase, β -mannosidase, pyroglutamic acid arylamidase, pyrolydonyl arylamidase and urease. Variable reactions were observed for alkaline phosphatase. The three isolates failed to produce alkaline phosphatase using the API rapid ID32Strep system, but strains CCUG 41710^T and CCUG 43292 were weakly positive using the API coryne system. None of the isolates hydrolysed aesculin, gelatin or hippurate and they were Voges-Proskauer-negative and failed to reduce nitrate. The rapid ID32Strep codes for CCUG

41710^T, 42833 and 43292 were 40016601000, 40002600000 and 40016601000, respectively, and those for the coryne system were 0520162, 0420160 and 0520160, respectively. The cellular morphology and general biochemical reactions of the isolates were consistent with their preliminary assignment to the genus *Actinomyces*. An examination of the long-chain cellular fatty acids of a representative strain (CCUG 41710^T) revealed acids of the straight-chain saturated and monounsaturated types with hexadecanoic (C16:0, 39.8%), octadecanoic (C18:0, 22.8%) and *cis*-delta 9-octadecenoic (C18:1*cis* 9, 31.5%) predominating. This cellular fatty acid composition is consistent with members of the genus *Actinomyces*.

The results of a comparative analysis of whole-cell protein profiles of the unknown strains and other *Actinomyces* species by SDS-PAGE is shown in Fig. 1. The three isolates were recovered in a single group which was well separated from all other reference species examined. The phylogenetic position of the unknown organisms was determined by comparative 16S rRNA gene sequencing. The almost complete gene sequence (> 1500 nt) of the three isolates was determined and pairwise analysis showed these to be highly related (> 99.5%). Sequence database searches confirmed the unknown bacterium was most closely related to species of the genus *Actinomyces*. Highest sequence similarity (approx. 93%) was shown with *Actinomyces hordeovulneris*, other species showing lower levels of relatedness (Table 1). Treeing analysis further demonstrated the distinctiveness of the unknown *Actinomyces* species (as exemplified by strain CCUG 41710^T) (Fig. 2). The bacterium formed a relatively long subline clustering with *Actinomyces hordeovulneris*, although bootstrap resampling demonstrated the association between these taxa was not statistically significant (bootstrap value 68% for 500 tree replications) (Fig. 2).

It is apparent from both phenotypic and phylogenetic evidence that the two unidentified isolates recovered from seals and the isolate from a porpoise represent a hitherto unknown *Actinomyces* species. Both 16S rRNA sequence divergence values and treeing analysis show the bacterium represents a distinct subline within the genus *Actinomyces*. The nearest phylogenetic relative of the unknown bacterium corresponds to *Actinomyces hordeovulneris*, although a sequence divergence of 7% shows this affinity is not particularly close. The unidentified bacterium can be biochemically readily distinguished from *Actinomyces hordeovulneris* in not fermenting pullulan and D-xylose, and not producing α -galactosidase. By contrast *Actinomyces hordeovulneris* ferments these carbohydrates and is α -galactosidase-positive. The unknown bacterium also differs markedly from *Actinomyces hordeovulneris* in PAGE analysis of whole-cell proteins. Thus based on the results of the reported polyphasic taxonomic study, we consider the unknown bacterium merits classification as a new species of the genus *Actinomyces*, for

Table 1. 16S rRNA gene sequence similarity values for *Actinomyces marimammalium* sp. nov. against all currently recognized *Actinomyces* species and phylogenetically related species

Species	Accession no.	16S rRNA sequence similarity (%)
<i>Actinobaculum schaalii</i>	Y12329	90.5
<i>Actinobaculum suis</i>	S83623	89.1
<i>Actinomyces bovis</i>	X81061	91.5
<i>Actinomyces bowdenii</i>	AJ234039	90.4
<i>Actinomyces canis</i>	AJ243891	92.7
<i>Actinomyces denticolens</i>	X80412	90.2
<i>Actinomyces europaeus</i>	Y08828	91.7
<i>Actinomyces georgiae</i>	X80413	90.8
<i>Actinomyces gerencseriae</i>	X80414	88.3
<i>Actinomyces graevenitzii</i>	Y09589	90.2
<i>Actinomyces hordeovulneris</i>	X82448	92.9
<i>Actinomyces howellii</i>	X80411	90.6
<i>Actinomyces hyovaginalis</i>	X69616	91.7
<i>Actinomyces israelii</i>	X82450	88.7
<i>Actinomyces meyeri</i>	X82451	91.7
<i>Actinomyces naeslundii</i>	X81062	91.5
<i>Actinomyces odontolyticus</i>	AJ234040	92.0
<i>Actinomyces radingae</i>	X78719	88.4
<i>Actinomyces slackii</i> (clone 1)	AJ234066	90.3
<i>Actinomyces turicensis</i>	X78720	90.3
<i>Actinomyces urogenitalis</i>	AJ243791	91.7
<i>Actinomyces viscosus</i>	X82453	91.1
<i>Arcanobacterium bernardiae</i>	X79224	90.4
<i>Arcanobacterium haemolyticum</i>	AJ234059	91.3
<i>Arcanobacterium phocae</i>	X97049	91.5
<i>Arcanobacterium pyogenes</i>	X79225	91.0
<i>Mobiluncus curtisii</i>	X53186	91.0

which the name *Actinomyces marimammalium* sp. nov. is proposed. Tests which are useful in distinguishing *Actinomyces marimammalium* from some other animal *Actinomyces* species are shown in Table 2.

Description of *Actinomyces marimammalium* sp. nov.

Actinomyces marimammalium (ma.ri.mam.ma'li.um. L. neut. n. *mare* the sea; M.L. neut. gen. pl. n. *mammalium* of mammals; M.L. *marimammalium* of marine mammals).

Cells are straight to slightly curved rods, some of which exhibit branching. Cells stain Gram-positive, are non-acid-fast and non-motile. Colonies are grey, entire, circular, convex and pin-point to 0.5 mm in diameter at 48 h. Non-haemolytic. Facultatively anaerobic and catalase-negative. Acid is produced from D-glucose, maltose, lactose and N-acetyl- β -glucosamine. Acid is not produced from L-arabinose, D-arabitol, cyclodextrin, mannitol, melibiose, melezitose, methyl- β -D-glucopyranoside, pullulan, raffinose, D-ribose, sorbitol, sucrose, trehalose, tagatose or D-xylose. Acid may or may not be produced from

glycogen. Aesculin, gelatin and hippurate are not hydrolysed. Alanine phenylalanine proline arylamidase, β -galactosidase, β -galacturonidase, leucine arylamidase and glycyl tryptophan arylamidase are produced. α -Galactosidase, α -glucosidase, β -glucosidase, β -glucuronidase, arginine dihydrolase, pyroglutamic acid arylamidase, pyrazinamidase, urease, β -mannosidase and pyrolydonyl arylamidase are not produced. Activity for alkaline phosphatase may or may not be detected. Acetoin is not produced. Nitrate is not reduced to nitrite. The end products of glucose metabolism are acetic, lactic and succinic acids. The G + C content of DNA is 66 mol%. Isolated from sea mammals (hooded seal, grey seal and harbour porpoise). Habitat is not known. The type strain is CCUG 41710^T (CIP 106509^T). The type strain exhibits the characteristics of the species and produces acid from glycogen.

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Fig. 2. Unrooted tree showing the phylogenetic relationships of *Actinomyces marimammalium* sp. nov. and some other high G+C content Gram-positive bacteria. The tree, constructed using the neighbour-joining method, was based on a comparison of approximately 1327 nt. Bootstrap values, expressed as a percentage of 500 replications, are given at branching points. Scale bar, 1% sequence divergence.

Table 2. Tests useful in distinguishing *Actinomyces marimammalium* from some other animal *Actinomyces* species

+, Positive; –, negative; v, variable; +⁽⁻⁾, a few strains negative; –⁽⁺⁾, a few strains positive.

Species	Acid from* :						Production of† :							Reduction of NO ₃	Hydrolysis of aesculin
	L-Ara	Mlz	D-Rib	Suc	Tre	D-Xyl	α-Gal	α-Glu	β-Glu	Pyr	GTA	NAG	APPA		
<i>A. marimammalium</i> sp. nov.	–	–	–	–	–	–	–	–	–	–	+	+	+	–	–
<i>A. bovis</i>	–	–	–	+ ⁽⁻⁾	–	–	–	–	v	–	v	– ⁽⁺⁾	–	–	+ ⁽⁻⁾
<i>A. bowdenii</i>	–	+	v	+	+	–	+	v	+	v	–	–	+	+	+
<i>A. canis</i>	+	–	+	v	–	+	+	+	–	+	v	+	+	–	–
<i>A. denticolens</i>	–	v	v	+	v	v	+	+	+	v	–	–	v	+	+
<i>A. hordeovulneris</i>	–	–	–	v	v	+	+	v	+	v	+	+	+	–	+
<i>A. howellii</i>	–	v	–	+	v	+	+	+	+	+	–	v	+	–	+
<i>A. hyovaginalis</i>	+ ⁽⁻⁾	–	+	+	–	+	+	+	+	v	–	v	+	v	+
<i>A. slackii</i>	–	–	–	+	+	+	+	+	+	+	–	–	–	+	+

* Fermentation of: L-Ara, L-arabinose; Mlz, melezitose; D-Rib, D-ribose; Suc, sucrose; Tre, trehalose; D-Xyl, D-xylose.

† α-Gal, α-galactosidase; α-Glu, α-glucosidase; β-Glu, β-glucosidase; Pyr, pyrazinamidase; GTA, glycyl tryptophan arylamidase; NAG, N-acetyl-β-glucosaminidase; APPA, alanyl-phenylalanine proline arylamidase.

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