

## *Streptococcus halichoeri* sp. nov., isolated from grey seals (*Halichoerus grypus*)

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Phenotypic and phylogenetic studies were performed on six unidentified, Gram-positive, catalase-negative, chain-forming *Streptococcus*-like organisms recovered from grey seals. Biochemically the six strains were highly related to each other, but they did not appear to correspond to any recognized species of the genus *Streptococcus*. Comparative 16S rRNA gene sequencing studies confirmed that phylogenetically the strains were members of the genus *Streptococcus*, but sequence divergence values of greater than 3% compared with reference streptococcal species demonstrated that the organisms from seals represent a novel species. SDS-PAGE analysis of whole-cell proteins confirmed the phenotypic distinctiveness of the seal organisms. Based on biochemical criteria and molecular chemical and genetic evidence, it is proposed that the unknown organism from seals be classified as a novel species, *Streptococcus halichoeri* sp. nov., the type strain of which is CCUG 48324<sup>T</sup> (= CIP 108195<sup>T</sup>).

The genus *Streptococcus* represents the largest group of catalase-negative, Gram-positive-staining cocci. The genus has undergone considerable expansion in the past 15 years, mainly due to the increasing application of molecular-based methodologies, which has helped to delineate a plethora of new species. The genus currently comprises over 50 species, many of which are associated with human clinical and veterinary sources (Facklam, 2002). Despite the considerable increase in the number of newly described streptococcal species, only *Streptococcus phocae* and *Streptococcus iniae* have originated from marine mammals. *S. phocae* has been reported only from seals (Skaar *et al.*, 1994) and cetaceans (G. Foster, unpublished), whereas *S. iniae*, although originally recovered from freshwater dolphins (Pier & Madin, 1976), has subsequently been found in aquacultures of fish and has even been isolated from humans (Weinstein *et al.*, 1997). During the course of a study of taxonomically problematic, catalase-negative, Gram-positive cocci from marine mammals, we have characterized a novel *Streptococcus*-like organism from

grey seals (*Halichoerus grypus*). Based on the presented findings, we describe a novel species, *Streptococcus halichoeri* sp. nov.

Six bacterial isolates were recovered from different grey seals. Three strains designated M512/02/1<sup>T</sup> (= CCUG 48324<sup>T</sup>), M72/03/04 (= CCUG 48325) and M2279/96/5 were recovered from animals following post-mortem in Inverness, UK, whereas strains M188/00/1, M159/01/2 and M466/02/1 (= CCUG 48323) were isolated from grey seals sampled at a rehabilitation centre in Cornwall, UK. The unidentified strains were cultured on Columbia Blood agar base supplemented with 5% sheep blood at 37 °C, under aerobic conditions. Organisms were characterized biochemically using the API Rapid ID 32Strep and API 20Strep systems (API bioMérieux) according to the manufacturer's instructions. To assess the overall phenotypic resemblance of the novel isolates and reference species, a comparative analysis of whole-cell protein profiles by SDS-PAGE was performed. SDS-PAGE of whole-cell proteins was performed as described by Pot *et al.* (1994) and Vandamme *et al.* (1998). 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 using the Pearson product moment correlation coefficient, converted for convenience to a percentage similarity. The G + C content (mol%) of the DNA of strain CCUG 48324<sup>T</sup> was determined

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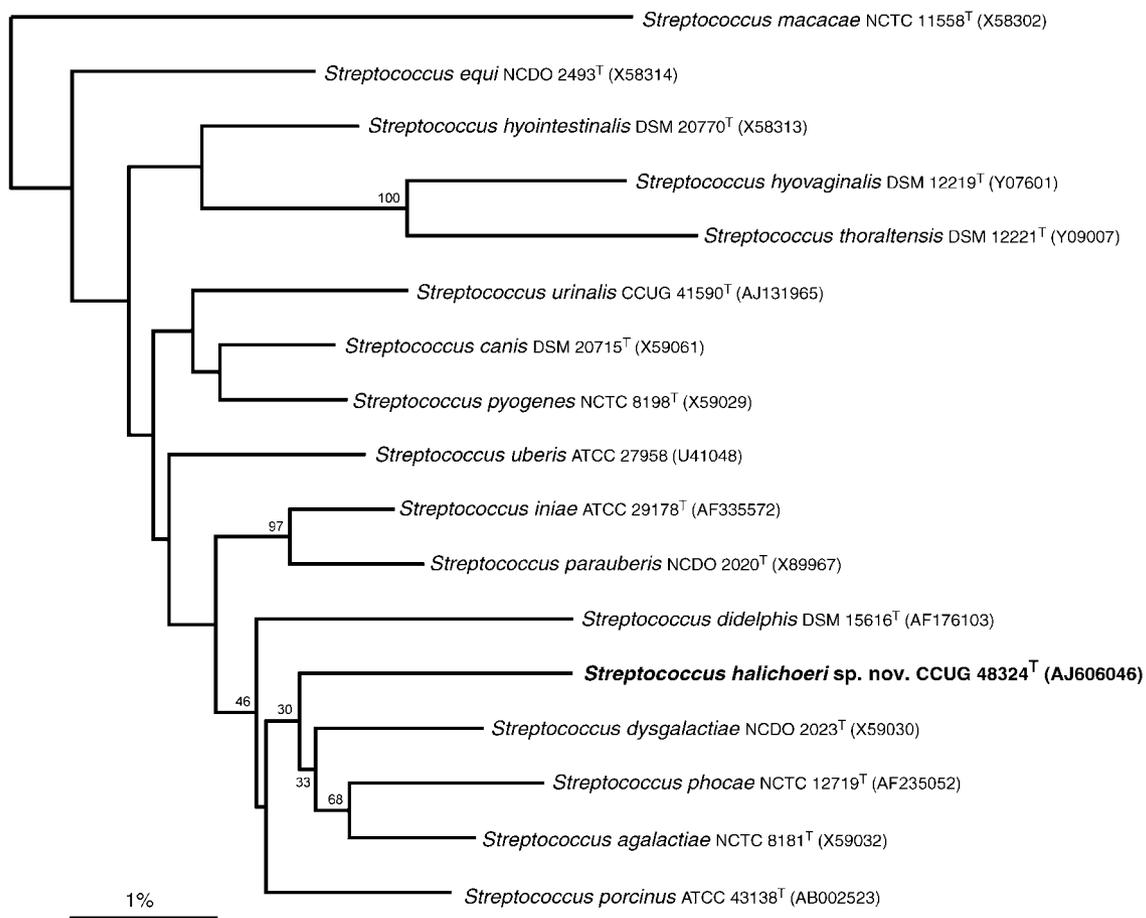
**Abbreviations:** CCUG, Culture Collection of the University of Göteborg, Sweden; CIP, Collection of Bacterial Strains of the Institute Pasteur, France.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain CCUG 48324<sup>T</sup> is AJ606046.

by HPLC as described by Mesbah *et al.* (1989). For phylogenetic analysis, 16S rRNA genes were amplified by PCR and sequenced directly 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 novel isolates were determined by performing database searches. Closely related sequences were retrieved from EMBL and aligned with the newly determined sequences using the program DNATools (Rasmussen, 1995). The resulting multiple sequence alignment had approximately 100 bases at the 5' end of the rRNA omitted from further analysis, because of alignment uncertainties due to the highly variable region V1, using the program GeneDoc (Nicholas *et al.*, 1997). A phylogenetic tree was constructed according to the neighbour-joining method (Saitou & Nei, 1987) with the programs DNATools and TREEVIEW (Page, 1996) and the stability of the groupings was estimated by bootstrap analysis (1000 replications).

The unidentified organisms recovered from grey seals were

Gram-positive cocci that occurred in pairs or short chains. The organisms were facultatively anaerobic and catalase-negative. Using the API Rapid ID 32Strep system, the unknown cocci closely resembled each other, producing acid from cyclodextrin, maltose, mannitol, ribose and pullulan. Some strains produced acid from lactose, but acid was not formed from any of the other carbohydrates in this test system. All of the isolates were Voges–Proskauer-positive and all showed activity for arginine dihydrolase, alkaline phosphatase, alanine phenylalanine proline arylamidase and pyrroglutamic acid arylamidase. All other enzyme tests gave negative results. Using the API 20Strep system, all of the strains produced acid from mannitol and ribose, but variable results were obtained with amygdalin. They were all Voges–Proskauer-positive and displayed activity for arginine dihydrolase, alkaline phosphatase, leucine arylamidase and pyrrolidonyl arylamidase. All other tests were negative with the API 20Strep system. The morphological and general biochemical characteristics of the unidentified cocci from seals were consistent with their tentative assignment to the genus *Streptococcus*,



**Fig. 1.** Unrooted tree, based on 16S rRNA gene sequences, showing the phylogenetic relationships of *S. halichoeri*. Bar, 1% sequence divergence.

but they did not correspond to any recognized species. To investigate further their phenotypic resemblance to streptococci, whole-cell protein profiling studies were performed on the novel strains. Three of the seal isolates were examined and were found to possess closely related protein patterns, forming a tight cluster (>90% intra-group similarity) that was distinct from those of all other reference streptococcal species (data not shown). The nearest neighbours to the unknown species from the PAGE analysis corresponded to *Streptococcus thoralensis* and *Streptococcus hyovaginalis*, joining the cluster formed by the seal organisms at approximately 65% similarity. 16S rRNA gene sequencing studies were performed to ascertain the phylogenetic affinities of the unidentified *Streptococcus*-like organisms. Almost-complete gene sequences (>1400 bases) of three of the seal strains (M512/02/1<sup>T</sup>, M72/03/04 and M466/02/1) were determined and pair-wise analysis revealed 100% sequence similarity, showing that the organisms were genetically highly related. Searches of the GenBank database revealed streptococci to be the nearest phylogenetic relatives of the unidentified bacterium. Phylogenetic analysis confirmed the association of the unidentified seal bacterium with the genus *Streptococcus*, with the unknown bacterium showing an affinity with a number of species within the 'pyogenic' subcluster. A tree based on a reduced dataset showing the nearest phylogenetic relatives of the unknown bacterium is depicted in Fig. 1. The G+C content of the DNA of a representative strain (M512/02/1<sup>T</sup>)

of the unknown bacterium was determined and corresponded to 39 mol%. This value is similar to those of other close phylogenetic relatives of the seal bacterium (e.g. *Streptococcus agalactiae*, 34–36 mol%; *Streptococcus dysgalactiae*, 40 mol%; *Streptococcus porcinus*, 37 mol%; *S. iniae*, 33 mol%; *Streptococcus uberis*, 37.5 mol%; *Streptococcus parauberis*, 36 mol%).

From the comparative 16S rRNA gene sequence analysis, it was evident that the unidentified, catalase-negative, coccus-shaped bacterium from seals represents a hitherto unknown streptococcal species. Phylogenetically, the unknown bacterium forms a distinct subline within the genus, but it did not display a statistically significant association with any recognized species of the genus. Highest 16S rRNA gene sequence similarity (96.9%) was shown with *S. phocae*, an organism also originating from seals (Skaar *et al.*, 1994). However, whole-cell-protein profiling showed that the organisms recovered from grey seals in this study were phenotypically quite distinct from *S. phocae*. Similarly, the unidentified seal bacterium and *S. phocae* were biochemically very different. The unidentified organisms differed from *S. phocae* in forming acid from a broader range of carbohydrates, including cyclodextrin, mannitol, pullulan and ribose, and by being arginine dihydrolase-positive. In addition, unlike *S. phocae*, the unknown organisms did not display  $\beta$ -haemolytic activity. Phylogenetically it is evident that the unidentified coccus-shaped organisms

**Table 1.** Tests useful in distinguishing *Streptococcus halichoeri* from closely related streptococci

Species: 1, *S. halichoeri*; 2, *S. agalactiae*; 3, *Streptococcus canis*; 4, *Streptococcus didelphis*; 5, *S. dysgalactiae*; 6, *S. iniae*; 7, *S. phocae*; 8, *S. porcinus*; 9, *S. uberis*; 10, *S. thoralensis*. Information on Lancefield antigens and haemolytic reactions are from Facklam (2002). Biochemical results were obtained from the API Rapid ID 32Strep test system (present study). +, Positive; -, negative; v, variable.

Characteristic	1	2	3	4	5	6	7	8	9	10
Lancefield antigen	B	B	G	None	A, C, G, L	None	C, F	E, P, U, V, none	E, none	None
$\beta$ -Haemolytic	-	+	+	+	-	+	+	+	-	-
Acid from:										
L-Arabinose	-	-	-	-	-	-	-	-	-	+
Cyclodextrin	+	-	-	-	-	v	-	-	-	-
Glycogen	-	-	-	-	v	+	v	-	v	-
Mannitol	+	-	-	-	-	+	-	+	+	+
Pullulan	+	+	+	+	+	+	-	+	v	+
Ribose	+	+	+	+	v	+	-	+	+	+
Sorbitol	-	-	-	-	v	-	-	+	+	+
Sucrose	-	+	+	+	+	+	-	+	+	+
Trehalose	-	v	v	+	+	+	-	+	+	+
Production of:										
Arginine dihydrolase	+	+	+	+	+	v	-	+	+	+
$\beta$ -Glucuronidase	-	+	-	+	+	+	-	+	+	+
Pyroglutamic acid arylamidase	+	-	-	-	-	+	v	-	v	-
Origin	Grey seals	Humans, bovine	Dogs, cats, humans	Opossums	Humans, animals	Dolphins, fish, humans	Seals, cetaceans	Swine, humans	Bovine	Swine

from seals represent a hitherto unknown species. PAGE analysis of whole-cell proteins confirmed the distinctiveness of the seal organisms and the production of distinct biochemical profiles [API Rapid ID32Strep numerical profile 1430(1)3141001 and API 20Strep profile 1163100(1)] served to distinguish the novel bacterium from all other described streptococcal species. Therefore, based on both phenotypic and phylogenetic criteria, we are of the opinion that the unknown bacterium from grey seals merits assignment to a novel species within the genus *Streptococcus*, for which the name *Streptococcus halichoeri* sp. nov. is proposed. Tests that are useful in distinguishing *S. halichoeri* from other closely related streptococcal species are shown in Table 1.

### Description of *Streptococcus halichoeri* sp. nov.

*Streptococcus halichoeri* (ha.lich.oe'ri. N.L. gen. n. *halichoeri* of a seal of the genus *Halichoerus*, systematic genus name of the grey seal).

Cells stain Gram-positive and are cocci that occur in pairs or short chains. Non-spore-forming. Colonies are white, umbonate, non-haemolytic and 0.5 mm in diameter after 24 h incubation on sheep blood agar. Lancefield serological group B. Facultatively anaerobic and catalase-negative. Using API test kits, acid is produced from cyclodextrin, maltose, mannitol, ribose and pullulan. Acid may or may not be formed from lactose. Acid is not produced from L-arabinose, D-arabitol, glycogen, inulin, mannose, melibiose, melezitose, methyl  $\beta$ -D-glucopyranoside, raffinose, sorbitol, starch, sucrose, D-tagatose or trehalose. Arginine dihydrolase, alkaline phosphatase, alanine phenylalanine proline arylamidase, leucine arylamidase and pyroglutamic acid arylamidase are produced. Activity is not detected for  $\alpha$ -galactosidase,  $\beta$ -galactosidase,  $\alpha$ -glucosidase,  $\beta$ -glucuronidase, glycyl tryptophan arylamidase,  $\beta$ -mannosidase, N-acetyl- $\beta$ -glucosaminidase or urease. Aesculin and hippurate are not hydrolysed. Acetoin is produced. Isolated from grey seals (*Halichoerus grypus*).

The type strain is CCUG 48324<sup>T</sup> (=CIP 108195<sup>T</sup>). The G+C content of its DNA is 39 mol%.

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