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Identification and Molecular Characterization of Beta-Hemolytic Streptococci Isolated from Harbor Seals (*Phoca vitulina*) and Grey Seals (*Halichoerus grypus*) of the German North and Baltic Seas

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Bacteriological investigations of seals of the German North and Baltic seas resulted in the isolation of bacteria of the genus *Streptococcus* belonging to Lancefield's serological groups C, F, and L. According to biochemical, serological, and 16S ribosomal DNA analysis, the group C and group F streptococci were identified as *Streptococcus phocae*. The group L streptococci could be classified as *Streptococcus dysgalactiae* subsp. *dysgalactiae*.

The harbor seal (*Phoca vitulina*) is the most common representative of the pinnipeds in the Wadden Sea of the German North Sea (8, 12). The grey seal (*Halichoerus grypus*) is also resident in the Wadden Sea, but in much lower numbers than the harbor seal. Both mammals are present in the Wadden Sea for the whole year (14). Harbor and grey seals are occasionally found on the German coast of the Baltic Sea. During the seal epidemic caused by phocine distemper virus in the North and Baltic seas in 1988 and 1989, 60% of the harbor seal population died. Only a few grey seals were affected in this period. Until 2001, the number of harbor seals increased again to approximately 20,000 individuals in the Wadden Sea. Grey seals could be found in numbers from 50 to 100 in the same area (3). Between May 2002 and February 2003, a new epidemic occurred in the North and Baltic seas, again associated with the occurrence of phocine distemper virus (11). A total of about 22,500 harbor seals and 824 grey seals died during this new epidemic (www.waddensea-secretariat.org).

However, during all these periods, beta-hemolytic streptococci were isolated from seal carcasses (4, 5, 6). The present study was performed to identify and further characterize these beta-hemolytic streptococci.

A total of 72 beta-hemolytic streptococci isolated from 57 organs of 39 harbor seals and 9 organs of 4 grey seals were investigated in this study. The beta-hemolytic streptococci were isolated from approximately 30% of 226 seal organs investigated between 1995 and 2000. Other bacteria isolated from these organs were *Escherichia coli* (48%), *Pseudomonas* spp. (17%), *Neisseria* spp. (13%), *Staphylococcus epidermidis* (13%), and alpha-hemolytic (1%) and gamma-hemolytic (46%) streptococci. Of the 72 beta-hemolytic streptococci, 61 were isolated from 39 harbor seals of the German North Sea, 2 were from two grey seals of the North Sea, and 9 were from two grey seals from the Baltic Sea. The animal designations, the places of discovery of the seals, and the tissues from which the beta-hemolytic streptococci were isolated are shown in Table 1.

The beta-hemolytic streptococci were cultivated under microaerobic conditions in a candle jar. For comparative purposes, *Streptococcus phocae* reference strains 8399 H1 (NCTC 12719) and 8190 R2, kindly provided by I. Skaar (Central Veterinary Laboratory, State Veterinary Laboratories of Norway, Oslo, Norway), were used.

The bacteria were investigated for serological properties by using autoclaved extracts (13) and group-specific antisera by agar gel diffusion and with a commercial grouping kit (Streptokokken-Identifizierungstest; Oxoid, Wesel, Germany) and for biochemical properties by using a commercial identification system (API 50 CH; bioMerieux, Laupheim, Germany).

The 16S rRNA gene of *S. phocae* 8399 H1 was amplified by use of the oligonucleotide primers described by Hutson et al. (10). The DNA preparation was performed as described previously (1, 2). The amplicon of the 16S rRNA gene of *S. phocae* 8399 H1 was sequenced using the facilities of the university (Institut für Medizinische Mikrobiologie, Justus-Liebig-Universität Gießen, Giessen, Germany).

A restriction fragment length polymorphism analysis of the 16S rRNA gene of the cultures was subsequently performed using the 16S ribosomal-DNA-specific oligonucleotide primer described by Bentley and Leigh (7) with the sequence 5′-GAG AGT TTG ATC TGG CTC AGC A-3′ as primer 1 and the oligonucleotide primer with the sequence 5′-CGG GTG TTA CAA ACT CTC GTG GT-3′ described previously (1, 2) as primer 2. The restriction enzymes *Ear*I and *Hin*cII (BioLabs, Schwalbach, Germany) were selected with the computer program clone manager (version 4.1; Scientific and Educational Software) and used for restriction fragment length polymorphism analysis. For this, 30 μl (*Ear*I) and 14 μl (*Hin*cII) of the

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TABLE 1. Origins of 72 beta-hemolytic streptococci isolated from 39 harbor seals and 4 grey seals of the North and Baltic seas

Animal designation[a]	Location[b]	Status of animal when found[c]	Tissue(s) of origin
P1	Amrum	K	Lung
P2	Dagebüll	K	Spleen
P3	Nordstrand	K	Liver, spleen
P4	St. Peter-Ording	S	Liver, lung, kidney, spleen
P5	Kampen on Sylt	S	Liver
P6	—	S	Intestine
P7	Pellworm	—	Lung
P8	St. Peter-Ording	K	Lung
P9	St. Peter-Ording	N	Lung
P10	Ulvesbüll	K	Lung
P11	Rantum on Sylt	K	Articulation; lung
P12	Friedrichskoog	K	Lung
P13	Hörnum on Sylt	K	Extremity
P14	Amrum	S	Articulation
P15	Amrum	S	Articulation
P16	Wenningstedt on Sylt	K	Articulation, liver, lung
P17	Amrum	K	Lung
P18	Lorenzenplate	A	Anus
P19	Hörnum on Sylt	K	Lung
P20	—	S	Liver
P21	List on Sylt	K	Lung (2)
P22	Hörnum on Sylt	K	Liver, lung, spleen
P23	Hörnum on Sylt	S	Skin
P24	Sealstation Helgoland	D	Eye
P25	Pellworm	S	Tongue
P26	Amrum	S	Lung
P27	Helgoland	K	Blubber, eye, lung, spleen
P28	Pellworm	K	Eye
P29	Friedrichskoog	K	Eye
P30	Helgoland	K	Tongue
P31	Oland	K	Eye
P32	St. Peter-Ording	K	Liver
P33	Hörnum on Sylt	S	Lung (2)
P34	Rantum on Sylt	K	Liver, lung, kidney
P35	List on Sylt	K	Liver, lung (2), skin
P36	Hörnum on Sylt	S	Intestine
P37	Helgoland	S	Lung
P38	List on Sylt	K	Eye, liver, lung (2)
P39	Sealstation Friedrichskoog	A	Eye
H1	St. Peter-Ording	K	Lung
H2	Kleiner Haft	N	Intestine, liver (2), lung (2), kidney, spleen, tongue
H3	Vitt on Rügen	S	Lung
H4	Sealstation Friedrichskoog	A	Mouth

[a] P, *P. vitulina*; H, *H. grypus*.
[b] Amrum, Dagebüll, Friedrichskoog, Lorenzenplate, Helgoland, Hörnum, Kampen, List, Nordstrand, Oland, Pellworm, Rantum, St. Peter-Ording, Ulvesbüll, and Wenningstedt are all in the North Sea. Kleiner Haft and Vitt are in the Baltic Sea. —, no information available.
[c] A, alive; D, died in a seal station; N, caught in fishing nets; K, killed because of poor condition; S, stranded; —, no information available.

amplicons were incubated with 4 μl (*Ear*I) and 3 μl (*Hin*cII) of the enzymes, respectively, for 2.5 h at 37°C. Before selecting the restriction enzymes the V2 region of the 16S rRNA gene of *S. phocae* 8399 H1 was comparatively investigated with 16S ribosomal DNA V2 regions of various streptococcal species. The latter were obtained from Bentley and Leigh (7) and Abdulmawjood and Lämmler (2).

Antibiotic susceptibilities were determined according to the recommendations of the Bundesinstitut für Gesundheitlichen Verbraucherschutz und Veterinärmedizin, Berlin, Germany.

Genomic DNA was prepared and digested with the restriction enzyme *Apa*I for macrorestriction analysis of the cultures as described previously (16, 17). The restriction patterns were analyzed according to the recommendations of Tenover et al. (18).

All 72 bacteria appeared to be gram-positive, catalase-negative cocci and were surrounded by a wide zone of complete β hemolysis.

According to the serogrouping results the 72 beta-hemolytic streptococci could be classified as serogroup C ($n = 8$), F ($n = 61$), and L ($n = 3$).

Biochemical properties of the bacteria were determined with the commercial test system API 50 CH. The 8 group C and the 61 group F streptococci displayed almost identical biochemical properties. The group C and group F streptococci were generally positive in fructose, glucose, maltose, mannose, *N*-acetyl-glucosamine, and ribose reactions and mostly negative for all the other substrates investigated. The three group L streptococcal isolates were uniformly positive in fructose, galactose, glucose, glycogen, maltose, mannose, *N*-acetyl-glucosamine, ribose, starch, sucrose, and trehalose reactions. According to cultural, serological, and biochemical properties, the 69 strep-

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1    GATCCTGGCT CAGGACGAAC GCTGGCGGCG TGCCTAATAC ATGCAAGTAG AACGCCTCTT
61   GCTTAGTGCT TGCACTAAGT AAGAGSAGTT GCAGAYACGG GTGAGTAACG CGTAGGTAAC
121  CTACCTTATA GCGGGGGATA ACTATTGGAA ACGATAGCTA ATACCGCATA AGAAGAGTTA
181  ACACATGTTA ATTCTTTAAA AGGGGCAATT GCTCCACTAT GAGATGGACC TGCGTTGTAT
241  TAGCTAGTTG GTGAGGTAAC GGCTCACCAA GGCAACGATA CATAGCCGAC CCTGAGAGGG
301  TGATCGGCCA CACTGGGACT GAGACACGGC CCAGACTCCT ACGGGATGGC AGCAGTAGGG
361  AATCTTCGGC AATGGACCGM MAGTCTGACC GAGCAACGCC GCGTGAGTGA AGMASGTTTT
421  CGGATCGTAA AGCTCTGTTG TTAGAGAAGA ATGATGGTGG GAGTGGAAAA TCCACCATGT
481  GACGGTAACT AACCAGAAAG GGACGGCTAA CTACGTGCCA GCAGCCGCGG TAATACGTAG
541  GTCTGCGAGC GTTGTCCGGA TTTATTGGGC GTAAAGCGAG CGCAGGCGGT TTGATAAGTC
601  TGAAGTTAAA GGCCGTGGCT CAACCATGGT TCGCTTTGGA AACTGTCAAA CTTGAGTGCA
661  GAAGGGGAGA GTGGAATTCC ATGTGTAGCG GTGAAATGCG TAGATATATG GAGGAACACC
721  GGTGGCGAAA GCGGCTCTCT GGTCTGTAAC TGACGCTGAG GCTCGAAAGC GTGGGGAGCA
781  AACAGGATTA GATACCCTGG TAGTCCACGC CGTAAACGAT GAGTGCTAGG TGTTAGACCC
841  TTTCCGGGGT TTAGTGCCGT AGCTAACGCA TTAAGCACTC CGCCTGGGGA GTACGACCGC
901  AAGGTTGAAA CTCAAAGGAA TTGACGGGGG CCCGCACAAG CGGTGGAGCA TGTGGTTTAA
961  TTCGAAGCAA CGCGAAGAAC CTTACCAGGT CTTGACATCC TTCTGACCCG GCCTAGAGAT
1021 AGGCTTTCTC TTCGGAGCAG AAGTGACAGG GTGGTGCATG GTTGTCGTCA GCTCGTGTCG
1081 TGAGATGTTG GGTTAAGTCC CGCAACGAGC GCAACCCCTA TTGTCTAGTT GCCATCATTA
1141 AGTTGGGCAC TCTAGCGAGA CTGCCGGTAA TAAACCGGAG GAAGGTGGGG ATGACGTCAA
1201 ATCATCATGC CCCTTATGAC CTKGGGCTAC ACACGTGCTA CAATGGTTGG TACAACGAGT
1261 TCGCAAGCCG GTGACGGCAA GCTAATCTCT TAAAGCCAAT CTCAGTTCGG ATTGTAGGCT
1321 GCAACTCGCC TACATGAAGT CGGAATCGCT AGTAATCGCG GATCAGCACG CCGCMGGTGA
1381 ATACGTTCCC GGGGCTTGTA CMACACCGCC CGTBACACCA CGAGAGTTTG TAACACCCGA
1441 AGTCGGTGAG GTAACCTTTT AGGAGCCAGC CGCCTAAGGT GGGATAGATG ATTGGGGTGA
1501 AGTCGTAACA AGGTAGCCGT ATCGGAAGGT GCGGCTGGAT CACCTCCTTT CT
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FIG. 1. Sequence of the 16S rRNA gene of the *S. phocae* reference strain 8399 H1; the V2 region (26 nucleotides) of the 16S rRNA gene is marked separately (accession number AF235052).

tococci of serogroup C and F were classified as *Streptococcus phocae* and the three group L streptococci were classified as *Streptococcus dysgalactiae* subsp. *dysgalactiae* serovar L. The biochemical and serological properties of both species corresponded to the findings given by Skaar et al. (15) and Lämmler and Hahn (13), respectively.

For molecular identification the 16S rRNA gene of the *S. phocae* reference strain 8399 H1, also including the V2 region, was sequenced (Fig. 1) and compared with 33 V2 region sequences of different streptococcal species and subspecies. The V2 region of *S. phocae* 8399 H1 appeared to be unique, showing differences of 4 to 16 nucleotides from the V2 region sequences of the other streptococcal species and subspecies investigated (data not shown). The subsequently selected restriction enzyme *Ear*I specifically digested the 16S rRNA gene of all 69 *S. phocae* isolates, yielding three characteristic DNA fragments with sizes of 170, 380, and 840 bp (Fig. 2). *Hin*cII revealed two characteristic fragments for all 69 *S. phocae* with sizes of 180 and 1,230 bp (data not shown).

The species *S. phocae* was first mentioned by Skaar et al. (15). These authors isolated *S. phocae* from different organs of harbor seals. In addition, this species was detected in infections of fur seals (9). The appearance of group C- and group F-specific group antigen among *S. phocae* isolates has been described as a common property of this species (15).

Determination of antibiotic resistances revealed that all *S. phocae* and all *S. dysgalactiae* subsp. *dysgalactiae* serovar L isolates were uniformly sensitive to amoxicillin-clavulanic acid, bacitracin (0.04 IU), bacitracin (10 IU), cephacetrile, cefotaxime, cefoxitin, clindamycin, erythromycin, minocycline, ofloxacin, oxacillin, piperacillin, penicillin G, sulfamethoxazole-trimethoprim, and tetracycline. Most of the *S. phocae* strains showed an intermediate reaction to gentamicin, and two of the *S. dysgalactiae* subsp. *dysgalactiae* serovar L strains were resistant and one strain was sensitive to gentamicin. Nearly all *S. phocae* and all *S. dysgalactiae* subsp. *dysgalactiae* serovar L isolates were resistant to kanamycin, nalidixic acid, and streptomycin. Only one *S. phocae* culture showed an in-

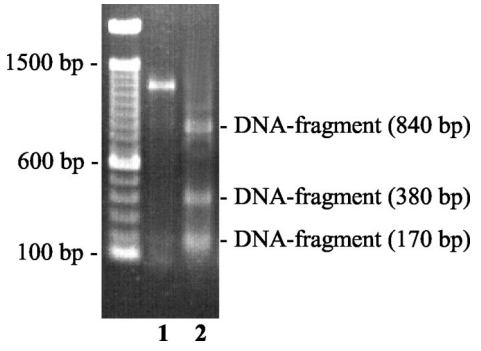

FIG. 2. Amplicon of the 16S rRNA gene of *S. phocae* 8399 H1 before (1,390 bp) (lane 1) and after (lane 2) digestion with *Ear*I.

TABLE 2. *Apa*I restriction patterns of 69 *S. phocae* isolates from 39 harbor seals and 4 grey seals from the North and Baltic seas

Animal	Tissue of origin	Restriction pattern[a]
P1	Lung	I
P2	Spleen	II
P3	Liver	III
	Spleen	III
P4	Liver	III
	Lung	III
	Kidney	III
	Spleen	III
P5	Liver	IV
P6	Intestine	III
P7	Lung	V
P8	Lung	—
P9	Lung	VI
P10	Lung	VII
P11	Articulation	VII
	Lung	VIII
P12	Lung	—
P13	Extremity	I
P14	Articulation	IX
P15	Articulation	X
P16	Articulation	II
	Liver	XI
	Lung	II
P17	Lung	XII
P18	Anus	X
P19	Lung	XIII
P20	Liver	XIV
P21	Lung (isolate 1)	IV
	Lung (isolate 2)	IV
P22	Liver	XIII
	Lung	XIII
	Spleen	XV
P23	Skin	XI
P24	Eye	VI
P25	Tongue	I
P26	Lung	XVI
P27	Blubber	XVII
	Eye	XVIII
	Lung	XVII
	Spleen	XVII
P28	Eye	XIX
P29	Eye	VIII
P30	Tongue	XX
P31	Eye	XXI
P32	Liver	X
P33	Lung (isolate 1)	VII
	Lung (isolate 2)	XXII
P34	Liver	XXIII
	Lung	VII
	Kidney	VII
P35	Lung (isolate 1)	II
	Lung (isolate 2)	XXIV
	Skin	XXV
P36	Intestine	XVI
P37	Lung	X
P38	Eye	XVII
	Liver	XVII
	Lung (isolate 1)	XVII
	Lung (isolate 2)	XX
P39	Eye	—
H1	Lung	XXVI
H2	Intestine	XXVII
	Liver (isolate 1)	XXVII
	Lung (isolate 1)	XXVII
	Lung (isolate 2)	XXVII
	Kidney	XXVIII
	Tongue	XXVIII
H3	Lung	XXVII
H4	Mouth	XXIX

[a] —, the DNA of the cultures could not be digested by *Apa*I and separated by PFGE.

termediate reaction to streptomycin. The uniform sensitivity of the strains to almost all of the antibiotics tested could possibly be explained by a lack of contact of these animals and bacteria with the various antibiotics.

To analyze possibly existing epidemiological relations, the isolates were subjected to macrorestriction analysis of their chromosomal DNA by pulsed-field gel electrophoresis (PFGE) using the rare-cutting enzyme *Apa*I. PFGE analysis of 66 *S. phocae* cultures revealed 29 different DNA patterns. There were identical as well as nonidentical DNA fragment patterns for isolates from one animal and from different animals (Table 2). However, most of the DNA fingerprints were not identical, indicating that multiple bacterial clones were distributed among the harbor seal and grey seal population of the North and Baltic seas and that cross infections between animals seem to be rare. This is in contrast to previously investigated *S. dysgalactiae* subsp. *dysgalactiae* serovar L isolates from harbor porpoises of the North and Baltic seas. In that study, single *S. dysgalactiae* subsp. *dysgalactiae* serovar L clones or at least closely related clones could be found in the various specimens (17).

Because of the occurrence of multiple *S. phocae* clones obtained from seal organs and the isolation of the *S. phocae* together with other bacterial species, the importance of this bacterial species for various health conditions remains unclear.

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