Comparative polyphasic characterization of *Streptococcus phocae* strains with different host origin and description of the subspecies *Streptococcus phocae* subsp. *salmonis* subsp. nov.

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A polyphasic study was undertaken to clarify the taxonomic position of *Streptococcus phocae* strains isolated from Atlantic salmon (*Salmo salar*) cage-farmed in Chile. Four salmon and three seal isolates showed minor differences in the SDS-PAGE protein analysis. Thus, a major protein band present in the salmon isolates, of approximately 22.4 kDa, was absent in the pinniped strains, regardless of the growth media employed. In addition, the pinniped strains showed protein bands with molecular masses of 71.5 and 14.2 kDa, when grown on trypticase soy agar supplemented with 1 % NaCl, or 25.6 kDa, when grown on Columbia blood agar, not present in the Atlantic salmon strains. A high similarity in the matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS spectra of the strains was observed, although some minor peaks were absent in the fish isolates. Fatty acid methyl esters from isolates with different host origin significantly ($P<0.05$) differed in the content of $C_{16:0}$, $C_{17:0}$, $C_{18:1}\omega9c$, $C_{20:4}\omega6,9,12,15c$ and summed features 3, 5 and 8. The salmon isolates formed a separate cluster in the phylogenetic analysis of housekeeping genes, separately or as concatenated sequences. Sequence divergences among salmon and seal strains were in the range of inter-subspecies differentiation for *groEL* (2.5 %), *gyrB* (1.8 %), *recN* (2.1 %), *rpoB* (1.7 %) and *sodA* (2.0 %) genes. DNA–DNA hybridization results confirmed those of sequencing, showing reassociation values between seal and salmon strains close to the borderline of species definition. Differences in growth at low temperatures and in the haemolytic capacities were also observed between both groups of isolates. On the basis of all these results, the salmon isolates represent a novel subspecies of *S. phocae*, for which the name *Streptococcus phocae* subsp. *salmonis* subsp. nov. is proposed. The type strain is C-4^T (=CECT 7921^T=DSM 24768^T). The subspecies *Streptococcus phocae* subsp. *phocae* subsp. nov. is automatically created. An emended description of *S. phocae* is also provided.

Abbreviations: DDH, DNA–DNA hybridization; FAME, fatty acid methyl ester; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight.

The GenBank/EMBL/DDBJ accession numbers for the *rpoB*, *sodA*, *gyrB*, *groEL* and *recN* gene sequences generated in this study are FR845994 to FR846023 (Table S1).

Three supplementary tables and four supplementary figures are available with the online version of this paper.

The genus *Streptococcus* comprises bacteria that are Gram-positive, catalase-negative and non-motile cocci. Phylogenetic analysis and identification of representatives of this genus have been based on 16S rRNA gene sequence comparison (Bentley *et al.*, 1991), but the lack of variability of the 16S rRNA gene sequences did not allow the discrimination of closely related species or subspecies. Other

molecular taxonomic methods, including DNA–DNA hybridization (DDH) and various PCR-based techniques, have been further employed for the identification and phylogenetic studies of streptococci (Bentley & Leigh, 1995; Gillespie *et al.*, 1997; Poyart *et al.*, 1998; Kawamura *et al.*, 1999; Chatellier *et al.*, 1998; Baele *et al.*, 2001; Facklam, 2002; Hassan *et al.*, 2003). On the basis of these techniques, species of the genus *Streptococcus* were taxonomically split off into six major clusters (Kawamura *et al.*, 1995). At the time of writing, 81 species and 17 subspecies with validly published names are recognized as members of the genus (Parte, 2014) some of which are commensals of the tegument or mucous membranes in both humans and animals.

Streptococcus phocae, a β-haemolytic member of the pyogenic streptococcal group (Köhler, 2007), was first isolated in Norway from clinical specimens of harbour seal (*Phoca vitulina*) affected by pneumonia or respiratory infection (Skaar *et al.*, 1994). The pathogen was further identified in other pinnipeds, including Cape fur seal (*Arctocephalus pusillus pusillus*) (Henton *et al.*, 1999), ringed seal (*Phoca hispida*) (Raverty *et al.*, 2004), grey seal (*Halichoerus grypus*) (Vossen *et al.*, 2004), Caspian seal (*Phoca caspica*) (Kuiken *et al.*, 2006), spotted seal (*Phoca largha*) (Hueffer *et al.*, 2011), California sea lions (*Zalophus californianus*) (Johnson *et al.*, 2006) and other marine mammals including southern sea otter (*Enhydra lutris nereis*) (Imai *et al.*, 2009), in several countries of north-western Europe, North America, Africa and the Caspian Sea. Since 1999, *S. phocae* has also been isolated from diseased Atlantic salmon (*Salmo salar*) causing serious economic losses in the salmon industry in Chile during the summer months (Romalde *et al.*, 2008).

The morphological, physiological and biochemical characteristics useful in the identification of *S. phocae* have been detailed by several authors (Skaar *et al.*, 1994; Gibello *et al.*, 2005; Romalde *et al.*, 2008). These phenotypic results indicated that *S. phocae* constitutes a homogeneous taxon with typical characteristics useful for the identification of this pathogen. However, serological studies demonstrated antigenic heterogeneity within this species, with the seal isolates classified into Lancefield's serogroups F and, in a much minor extent, C or untypable (Skaar *et al.*, 1994; Vandamme *et al.*, 1997), and the salmon isolates into serogroup G (Romalde *et al.*, 2008).

From a genetic point of view, recent studies employing pulsed-field gel electrophoresis (PFGE), random amplification of polymorphic DNA (RAPD), enterobacterial repetitive intergenic consensus sequence PCR (ERIC-PCR) and repetitive extragenic palindromic PCR (REP-PCR) have shown a high degree of genetic homogeneity among Atlantic salmon isolates, which displayed profiles very distinct from those of the type strain *S. phocae* ATCC 51973^T isolated from a seal in Norway (Gibello *et al.*, 2005; Valdés *et al.*, 2009). Interestingly, a remarkable heterogeneity was observed in *S. phocae* strains isolated from seals using PFGE (Vossen *et al.*, 2004).

The serological and genetic discrepancies underline the necessity of major comparative studies, in order to elucidate the taxonomic position of *S. phocae* strains isolated from salmon and pinnipeds within the genus *Streptococcus*. The aim of the present study was to compare both groups of isolates using a polyphasic approach, which included whole-cell protein profiles using one-dimensional gel electrophoresis and a proteomic approach, fatty acid composition and five housekeeping genes coding for metabolic functions.

A total of four *S. phocae* strains isolated from Atlantic salmon (2857, 696, C-4^T and LM-13-Sp) and two strains obtained from seals (*P. vitulina*; 8150 and P23) kindly provided by C. Lämmler (Institut für Tierärztliche Nahrungsmittelkunde, Justus-Liebig-Univertität Gießen, Germany) were included in this study. The identity of each isolate was confirmed as *S. phocae* by standard phenotypical procedures (Gibello *et al.*, 2005; Romalde *et al.*, 2008), and using the PCR-based analysis described by Alber *et al.* (2004). The type strain *S. phocae* ATCC 51973^T from the American Type Culture Collection was used for comparative purposes. The bacteria were grown on Columbia sheep blood agar (CBA) (AES Laboratories) and on tryptone soy agar supplemented with 1 % NaCl (TSA-1) (Difco Laboratories) under aerobic conditions at 22–25 °C for 48 h. Stock cultures were maintained frozen at −80 °C in criobille tubes (AES Laboratories) or in tryptone soy broth (TSB-1) with 15 % (v/v) glycerol.

The whole-cell proteins from *S. phocae* strains were prepared from bacterial cultures grown on TSA-1 and CBA (Pot *et al.*, 1994). Each bacterium was suspended in PBS (pH 7.4) at a concentration of 10^9 c.f.u. ml^{-1}, washed once in the same saline solution by centrifugation (8000 ***g***), and maintained at −30 °C until required. After determining their protein concentration using Pierce BCA Protein Assay (Thermo Scientific), samples (25 μg μl^{-1}) were electrophoresed by SDS-PAGE [12 and 4 % (w/v) acrylamide in the resolving and the stacking gels, respectively] (Laemmli, 1970). Protein bands were stained with Coomassie blue R-250 in 50 % (v/v) methanol and 10 % (v/v) acetic acid. Protein profiles were analysed using the TotalLab software package.

The analysis of whole-cell proteins by SDS-PAGE clearly revealed two patterns, mainly associated with the growth medium (Fig. 1). When the *S. phocae* cells were grown on TSA-1, the pinniped strains differed from the Atlantic salmon strains by the absence of a major protein band with an approximate molecular mass of 22.4 kDa and the presence of two protein bands with molecular masses of 71.5 and 14.2 kDa. Although, all *S. phocae* strains grown on CBA shared a considerable number of common bands between 116 and 14.2 kDa, a difference in two protein bands was observed in the profile for the pinniped strains, which lacked a band of 22.4 kDa and showed the presence of one protein band with molecular mass of 25.6 kDa not present in the Atlantic salmon strains.

Preparation of fatty acid methyl esters (FAMEs) from exponential cultures of each strain grown at 25 °C on TSA-1 plates was performed according to the Microbial Identification System (MIDI) as described by Sasser (1990).

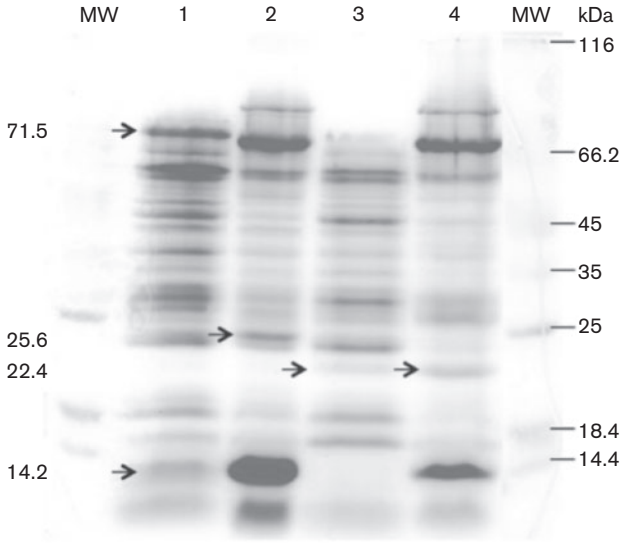

Fig. 1. Electrophoresis protein profiles of strains belonging to *Streptococcus phocae* strains. Lanes: MW, molecular size markers; 1 and 2, ATCC 51973^T; 3 and 4, C-4^T. Odd and even lanes correspond to growth in TSA-1 and CBA, respectively. Numbers on the right indicate positions in the molecular size marker (kDa). Numbers on the left indicate the molecular size of differential bands.

FAMEs were analysed by GC in an Agilent 6850 gas chromatographic system (Agilent Technologies). The operating system was Chemstation (Agilent Technologies) and Sherlock MIS (MIDI). The temperature programme ramped from 170 °C to 288 °C at 28 °C min^{-1} with a split ratio of 40 : 1 and head pressure of 144.8 kPa. FAME analysis was conducted on three sets of each isolate cultured and treated under identical conditions.

The complete fatty acid compositions of both salmon and pinniped strains are detailed in Table 1. The fatty acids that were found in trace quantities (<1 %) in all strains are not shown. FAMEs from isolates with different host origin significantly ($P<0.05$) differed in the content of $C_{16:0}$, $C_{17:0}$, $C_{18:1}\omega9c$, $C_{20:4}\omega6,9,12,15c$ and summed features 3, 5 and 8. As the species *S. phocae* is not included in MIDI databases of clinical and environmental bacteria, all seal strains tested were identified as *Streptococcus pyogenes*-GC-subgroup D, while the salmon isolates showed similarity to *Moraxella atlantae* (0.24 ± 0.07). Variability and problems in the amounts of some fatty acids have been reported for different culture conditions (medium, temperature and incubation period) (Piñeiro-Vidal *et al.*, 2008). In our experience the differences are not due to culture conditions; all *S. phocae* strains were cultured under identical conditions, therefore FAME analysis constitutes a valuable technique for epizootiological differentiation of *S. phocae* isolates.

Two *S. phocae* strains isolated from Atlantic salmon (C-4^T and 2857) and one seal strain (ATCC 51973^T), representing the main serotypes described in this pathogen (Romalde *et al.*, 2008), were selected for use in the whole-cell matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS analysis. Protein extraction was performed with

Table 1. Cellular fatty acid compositions of *Streptococcus phocae* strains

Values are percentages of total fatty acids; those amounting to <1 % of the total fatty acids in all strains studied are omitted. Identical culture conditions were used. TR, Traces (less than 1.0 %); ND, not detected.

Fatty acid	Pinniped strains		Salmon strains			
	ATCC 51973^T	P23	696	2857	LM-13-Sp	C-4^T
$C_{12:0}$	2.40	2.40	1.71	3.11	1.27	3.04
$C_{14:0}$	3.06	4.14	2.64	4.35	1.75	3.75
$C_{16:1}\omega9c$	4.85	5.49	1.74	TR	TR	TR
Summed feature 3*	8.02	9.28	2.90	1.52	1.19	1.34
$C_{16:1}\omega5c$	1.20	1.21	ND	ND	ND	ND
$C_{16:0}$	31.19	29.57	19.21	20.25	17.23	20.0
$C_{17:0}$	1.33	1.10	TR	TR	TR	TR
Summed feature 5*	13.21	14.07	33.85	33.24	38.37	34.19
$C_{18:1}\omega9c$	13.91	13.79	20.35	20.19	22.06	20.06
Summed feature 8*	8.40	8.43	3.61	2.90	3.26	3.06
$C_{18:0}$	10.71	8.43	8.48	8.59	8.43	8.27
iso-$C_{19:0}$	TR	ND	ND	ND	ND	ND
$C_{20:4}\omega6,9,12,15c$	1.23	1.22	2.99	3.13	3.40	3.06

*Summed features are groups of two or four fatty acids that cannot be separated by GLC using MIDI systems. Summed feature 3 comprised $C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$. Summed feature 5 comprised $C_{18:2}\omega6,9c$ and/or anteiso-$C_{18:0}$. Summed feature 8 comprised $C_{18:1}\omega7c$ and/or $C_{18:1}\omega6c$.

ethanol, formic acid and acetonitrile. Ethanol (70 %) was added to the pellet and mixed thoroughly before the addition of 50 µl acetonitrile. Processed samples were placed onto a spot of a steel target plate (Bruker Daltonics), air-dried at room temperature and overlaid with 1 µl matrix solution (saturated solution of α-cyano-4-hydroxy-cinnamic acid in 50 % acetonitrile supplemented with 2.5 % trifluoracetic acid). Mass spectra were acquired with an Ultraflex II MALDI-TOF MS (Bruker Daltonics). The measured mass of spectra was 2000 to 20 000 Da. The spectra were externally calibrated using the strain *Escherichia coli* FV9180 (kindly donated by Dr J. Blanco, University of Santiago de Compostela, Spain) as a standard. Manual/visual estimation of the mass spectra was performed using FlexAnalysis 2.4 software (Bruker Daltonics). For automated data analysis, raw spectra were processed using the MALDI BioTyper 1.1 software (Bruker Daltonics).

When the whole-cell-MALDI-TOF MS was used for the differentiation of *S. phocae* strains, although a high overall similarity on the fingerprint spectra from whole cells was observed, some minor peaks (6147 and 10182 Da) were absent in the fish isolates (Fig. S1, available in the online Supplementary Material). It is important to note that MALDI-TOF MS has become a standard method in many clinical laboratories (Davies *et al.*, 2012; Moon *et al.*, 2013), and has been successfully employed for identification and relationship analysis of different streptococci, including *S. pyogenes* (Wang *et al.*, 2012), *Streptococcus pneumoniae* and *Streptococcus mitis* (Ikryannikova *et al.*, 2013), or the viridans group streptococci (Kärpänoja *et al.*, 2013).

In 2002, a report of the ad hoc committee for the re-evaluation of the species definition in bacteriology encouraged the classification of novel species based on housekeeping gene analysis using comparison of at least five genes, provided that there is a sufficient degree of congruence between the technique used and the traditional method of DNA–DNA reassociation (Stackebrandt *et al.*, 2002). In order to discriminate among seal and salmon *S. phocae*, we determined the sequence of five housekeeping genes, β-subunit of RNA polymerase (*rpoB*), manganese-dependent superoxide dismutase (*sodA*), B-subunit of DNA gyrase (*gyrB*), 60 kDa heat-shock protein (*groEL*), and recombination and repair protein (*recN*) genes, for all strains. These genes were selected on the basis of previous studies indicating that they represent the best tools to differentiate members of the genus *Streptococcus* and to analyse the evolution of this bacterial group (Glazunova *et al.*, 2009, 2010).

Chromosomal DNA was extracted using InstaGene Purification Matrix (Bio-Rad) according to the manufacturer's protocols. The concentration and quality of each DNA sample was examined spectrophotometrically at 260 nm and adjusted to a concentration of 20–30 ng μl^{-1}. Extracted DNA was maintained at −20 °C until used for PCR. One microlitre of each DNA solution was used in the respective amplification reaction.

Amplification of the *rpoB*, *sodA*, *gyrB*, *groEL* and *recN* genes was performed as described by Glazunova *et al.* (2009, 2010). All PCR assays described in this work were performed in a Mastercycler personal (Eppendorf) apparatus using the commercial kit Ready-To-Go PCR beads (Amersham Pharmacia Biotech) according to the manufacturer's instructions. PCR products were purified using QIAquick PCR purification kits (Qiagen) and sequenced using a GenomeLab DTCS-Quick Start kit (Beckman Coulter) in an ABI 373A automated DNA sequencer (Applied Biosystems). Sequence corrections and analysis were performed with the DNASTAR Seqman program (Lasergene). The resulting sequences were compared with those available in GenBank and EMBL (http://www.ebi.ac.uk) using the program BLAST. Phylogenetic trees were reconstructed using the neighbour-joining and maximum-likelihood algorithms (Posada, 2008; Tamura *et al.*, 2007) (FigTree v1.3.1; http://tree.bio.ed.ac.uk/). Distance matrices were calculated using Kimura's two-parameter correction. Stability of the groupings was estimated by bootstrap analysis (1000 replicates) using the program MEGA version 4.0 (Tamura *et al.*, 2007).

Fragments of 680, 435, 531, 757 and 1215 bp were amplified and sequenced for *rpoB*, *sodA*, *gyrB*, *groEL* and *recN* genes, respectively, for all strains. The phylogenetic analysis of the concatenated sequences indicated that *S. phocae* has a monophyletic origin. Two clusters, related to host origin (salmon or seal), could be identified within the species showing bootstrap values of 100 % (Fig. 2 and Fig. S2). Similar results were obtained in the analysis of the individual genes (Fig. S3). Divergences in nucleotide sequences between the salmon and seal isolates for *groEL* (2.5 %), *gyrB* (1.8 %), *recN* (2.1 %), *rpoB* (1.7 %) and *sodA* (2.0 %) genes were in the range described for inter-subspecies divergence, with values very similar to those displayed between two (out of three) subspecies of *Streptococcus equi* (Table S2), or among the different subspecies of *Streptococcus gallolyticus* (Glazunova *et al.*, 2009, 2010).

DDH experiments were done with the hydroxyapatite method using microtitre plates (Ziemke *et al.*, 1998) with a hybridization temperature of 60 °C. The experiments were performed in triplicate using four strains, two isolated from seal (including the type strain ATCC 51973^T and strain P23) and two isolated from salmon (C-4^T and 2857).

DDH values (Table 2) were higher than 94 % between strains with the same host origin, but ranged from 63.2 to 74.5 % between isolates from different hosts, seal and salmon. A threshold value of 70 % DDH has been established for the definition of a novel bacterial species (Stackebrandt *et al.*, 2002), but these experiments can have an accuracy of about 10 % in very closely related isolates (Rosselló-Mora & Amann, 2001). In the case of the two groups of *S. phocae* isolates the values obtained are only slightly higher than the 70 % recommended threshold, supporting the other differences observed between salmon and seal isolates.

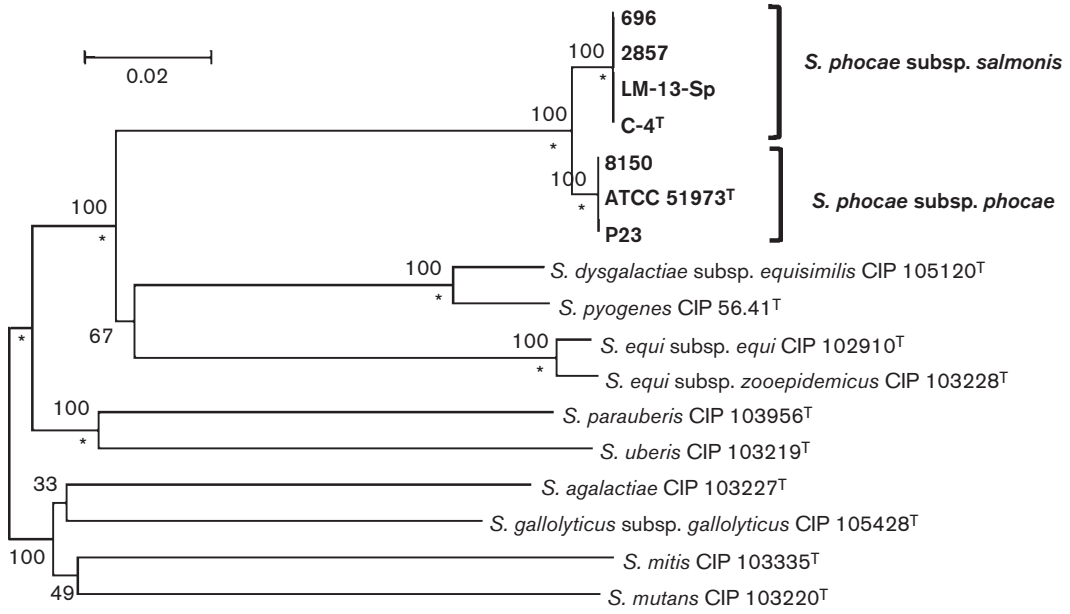

Fig. 2. Neighbour-joining tree obtained from the concatenated sequences of the housekeeping genes *rpoB*, *sodA*, *gyrB*, *groEL* and *recN* from several *S. phocae* strains isolated from Atlantic salmon and seal in comparison with related species of the genus *Streptococcus*. Significant brootstrap values of 1000 replicates appear next to the corresponding branches. Nodes coincident in the maximum-likelihood tree are indicated by an asterisk. The nucleotide sequences generated in this study (Table S1) have been deposited in the GenBank database, from where sequences from relative species were also obtained. Bar, 0.02 substitutions per position.

Taking into account the results of the present polyphasic study, we consider that the isolates from Atlantic salmon merit subspecies status within *S. phocae*. The name *Streptococcus phocae* subsp. *salmonis* subsp. nov. is proposed for the isolates from Atlantic salmon. Differential characteristics from the other subspecies, *S. phocae* subsp. *phocae* subsp. nov. (automatically created according to Rule 40b of the Bacteriological Code), are the growth at low temperatures (4 and 10 °C) (Table S3), the weak haemolytic activity of sheep and human erythrocytes (Fig. S4), and their belonging to the group G of the Lancefield serotyping scheme.

Table 2. DNA–DNA hybridization among the representative strains of *S. phocae* isolated from seal and salmon

Experiments were performed in triplicate and values shown indicate the mean ± SD.

Isolate	ATCC 51973^T	C-4^T
Seal isolates		
ATCC 51973^T	100	72.5 ± 4.3
P23	99.7 ± 3.2	73.1 ± 3.8
Salmon isolates		
C-4^T	74.5 ± 2.1	100
2857	63.2 ± 1.8	94.7 ± 1.8

Emended description of *Streptococcus phocae*

Streptococcus phocae (pho′cae. L. gen. n. *phocae* of a seal).

The description is essentially as given by Skaar *et al.* (1994). Additional features are that growth may occur between 4 and 40 °C and that according to the Lancefield serotyping scheme, isolates may belong to groups F, C or G or be nontypable.

Description of *Streptococcus phocae* subsp. *phocae* subsp. nov.

Streptococcus phocae subsp. *phocae* (pho′cae. L. gen. n. *phocae* of a seal).

Possesses the characteristics previously described by Skaar *et al.* (1994) for the species *Streptococcus phocae*.

The type strain, SVL 8399 H1^T (=CCUG 35103^T=LMG 16735^T=ATCC 51973^T), is that previously assigned to the species *S. phocae*.

Description of *Streptococcus phocae* subsp. *salmonis* subsp. nov.

Streptococcus phocae subsp. *salmonis* (sal.mo′nis. L. gen. n. *salmonis* of salmon, since it was first discovered in salmon).

Strains confirm the species description and also exhibit the following characteristics. Growth occurs at 4 and 10 °C on

blood agar and TSA-1, haloes of β-haemolysis on sheep and human blood agar are $\leqslant 1$ mm, and the isolates belong to Lancefield group G.

The type strain, C-4^T (=CECT 7921^T=DSM 24768^T), was isolated from Atlantic salmon in Chile (Romalde *et al.*, 2008).

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