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(72) Inventeurs/Inventors:
DUESUND, HENRIK, NO;
MENDOZAS, JULIO, CL
(73) Propriétaire/Owner:
CERMAQ GROUP AS, NO
(74) Agent: DEETH WILLIAMS WALL LLP

(54) Titre : NOUVEL ISOLAT DE PISCIRICKETTSIA SALMONIS
(54) Title: NOVEL PISCIRICKETTSIA SALMONIS ISOLATE

(57) **Abrégé/Abstract:**

The present invention provides a novel *Piscirickettsia salmonis* strain useful as a therapeutic agent, more particular in vaccines. The present invention furthermore provides a novel growth medium useful for the culturing of *Piscirickettsia* spp..

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(71) Applicant: **CERMAQ AS** [NO/NO]; P.O.BOX 144 Sentrum, N-0102 Oslo (NO).

(72) Inventors: **DUESUND, Henrik**; Universitetet i Bergen, Institutt for Biologi, Tormøhlensgate 53A, N-5006 Bergen (NO). **MENDOZAS, Julio**; Mainstream Chile S.A., Auda.Diego Portales 2000, 10th Floor, Puerto Montt (CL).

(74) Agent: **ONSAGERS AS**; Munkedamsvn. 35, P.O. BOX 1813 Vika, N-0123 Oslo (NO).

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(54) Title: NOVEL PISCIRICKETTSIA SALMONIS ISOLATE

(57) Abstract: The present invention provides a novel *Piscirickettsia salmonis* strain useful as a therapeutic agent, more particular in vaccines. The present invention furthermore provides a novel growth medium useful for the culturing of *Piscirickettsia spp.*



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Novel *Piscirickettsia salmonis* isolate

Field of invention

The present invention relates to a *Piscirickettsia salmonis* isolate useful as a therapeutic agent. More particularly, the present invention relates to a vaccine comprising a novel *P. salmonis* isolate that is used in a fish vaccine to protect salmonid fish against piscirickettsiosis, also referred to as salmonid rickettsial septicemia (SRS).

Background of the invention

Chile is the second largest producer of Atlantic salmon (*Salmo salar*) in the world. In 2007 Chile faced a major crisis after several outbreaks of Infectious Salmon Anemia (ISA) causing a setback in the salmon industry. The national salmon industry has since been building up and Chile produced an estimated amount of 515,000 tons in 2012, making the latter year the most productive year in the history of Chilean aquaculture (Pérez C: Consumo de antibióticos totalizó 338 toneladas el 2012. In: Salmon Xpert).

Although having successful years of salmonid production, the industry has constantly been facing problems with *Piscirickettsia salmonis*, the causative agent of salmonid rickettsial septicemia (SRS). Currently, *P. salmonis* is considered the most important species causing high mortalities at many farming sites in Chile. *P. salmonis* was responsible for 82 % of the total use of antibacterial products in Chile in 2012 (Perez et al, supra), pinpointing the importance of detailed knowledge about the bacteria in order to develop alternative and efficient treatment methods.

The family *Piscirickettsiaceae* (Fryer et al (2005), Family II. *Piscirickettsiaceae* fam. nov. In: Bergey's Manual of Systematic Bacteriology Volume 2: The Proteobacteria Part B: The Gammaproteobacteria. Edited by Garrity G, Brenner DJ, Krieg NR, Staley JR, vol. 2, 2nd edn. Web: Springer; 2005: 180-184, Validation List no. 106, Euzéby J: Validation of publication of new names and new combinations previously effectively published outside the IJSEM. International Journal of Systematic and Evolutionary Microbiology 2005, 55(6):2235-2238) contains Gram-negative, aerobic, coccoid, rod or spiral shaped and occasionally pleiomorphic bacteria. Species within the family are isolated predominantly from the marine environment (Fryer et al., supra), but isolation from fresh water environment has also been reported (Gaggero et al: First Isolation of *Piscirickettsia-Salmonis* from Coho Salmon, *Oncorhynchus-Kisutch* (Walbaum), and Rainbow-Trout, *Oncorhynchus-Mykiss* (Walbaum), during the Fresh-Water Stage of Their Life-Cycle. Journal of fish diseases 1995, 18(3):277-279). All members of the family belong to the class *Gammaproteobacteria* with 16S rRNA similarities as the main unifying factor (Fryer et al, (2005) supra).

Piscirickettsiaceae contains six genera; *Piscirickettsia*, *Cycloclasticus*, *Hydrogenovibrio*, *Methylophaga*, *Thioalkalimicrobium*, and *Thiomicrospira* (Fryer et al, (2005) supra).

Although having a phylogenetic relationship, these genera vary in phenotypic characters and share only a few common characteristics (Fryer et al. (2005), supra).

The genus *Piscirickettsia* contains one species, *Piscirickettsia salmonis* (Fryer et al.(1992), *Piscirickettsia-Salmonis* Gen-Nov, Sp-Nov, the Causative Agent of an
5 Epizootic Disease in Salmonid Fishes. Int J Syst Bacteriol, 42(1):120-126), and as of yet no other species has been described.

P. salmonis is a Gram-negative, predominantly coccoid, non-motile, non-encapsulated, aerobic, and highly fastidious facultative intracellular bacteria that is approx. 0.5-1.5 µm in diameter (Fryer and Lannan CN: Family II. Piscirickettsiaceae fam. nov. In: Bergey's
10 Manual of Systematic Bacteriology Volume 2: The Proteobacteria Part B: The Gammaproteobacteria. Edited by Garrity G, Brenner DJ, Krieg NR, Staley JR, vol. 2, 2nd edn. Web: Springer; 2005: 180-184, Gomez et al.: Additional evidence of the facultative intracellular nature of the fish bacterial pathogen *Piscirickettsia salmonis*. Arch Med Vet 2009, 41(3):261-267, Cvitanich et al.: The Isolation of a Rickettsia-Like
15 Organism Causing Disease and Mortality in Chilean Salmonids and Its Confirmation by Koch Postulate. Journal of fish diseases 1991, 14(2):121-145).

Although initially being described as non-motile it has been revealed that *P. salmonis* possess a set of flagellar genes that under certain conditions may be synthesized (Wilhelm et al.: A vaccine against the salmonid pathogen *Piscirickettsia salmonis* based
20 on recombinant proteins. Vaccine 2006, 24(23):5083-5091). The bacteria replicates *in vitro* within cytoplasmic inclusions in host cells (Fryer et al. (1990), Isolation of a Rickettsiales-Like Organism from Diseased Coho Salmon (*Oncorhynchus-Kisutch*) in Chile. Fish Pathol, 25(2):107-114), and has recently proved to induce cell death by apoptosis in trout macrophages (Rojas et al.: *Piscirickettsia salmonis* induces apoptosis
25 in macrophages and monocyte-like cells from rainbow trout. Journal of cellular biochemistry 2010, 110(2):468-476). Optimal temperatures for replication *in vitro* are 15-18 °C, and no replication occurs above 25 °C or below 10 °C (Fryer et al. (1990), supra, Fryer and Mauel: The Rickettsia: An emerging group of pathogens in fish. Emerg Infect Dis 1997, 3(2):137-144). *P. salmonis* has typically been cultured on Chinook salmon (*Oncorhynchus tshawytscha*) cell lines, CHSE-214 (Lannan CN, Winton JR, Fryer JL: Fish Cell-Lines - Establishment and Characterization of 9 Cell-Lines from Salmonids. In Vitro Cell Dev B 1984, 20(9):671-676), and was first described as an obligatory intracellular bacteria (Fryer et al., 1992, supra). However, *P. salmonis* has
30 later been found to grow on artificial cell-free medium (Mikalsen et al.: Agar culture of *Piscirickettsia salmonis*, a serious pathogen of farmed salmonid and marine fish. FEMS microbiology letters 2008, 278(1):43-47, Mauel et al.: Culture of *Piscirickettsia salmonis* on enriched blood agar. Journal of veterinary diagnostic investigation: official publication of the American Association of Veterinary Laboratory Diagnosticians, Inc 2008, 20(2):213-214, Yanez et al.: Two novel blood-free solid media for the culture of
35 the salmonid pathogen *Piscirickettsia salmonis*. Journal of Fish Diseases 2013, 36(6):587-591, WO2008/002152).

P. salmonis was first observed in 1989 and caused a novel disease of Coho salmon (*Oncorhynchus kisutch*) reared in marine net pens near Puerto Montt, Chile (Bravo and Campos: Coho salmon syndrome in Chile. Fish Health Section Newsletter, American Fisheries Society 1989, 17:3). The pathogen was initially termed unknown agent (UA) and the disease caused by the agent have been referred to as Coho salmon syndrome (Branson and Diazmunoz : Description of a New Disease Condition Occurring in Farmed Coho Salmon, *Oncorhynchus-Kisutch* (Walbaum), in South-America. Journal of fish diseases 1991, 14(2):147-156), salmonid rickettsial septicaemia (Cvitanich et al.: The Isolation of a Rickettsia-Like Organism Causing Disease and Mortality in Chilean Salmonids and Its Confirmation by Koch Postulate. Journal of fish diseases 1991, 14(2):121-145) and piscirickettsiosis (Fryer et al, 1992, supra, and Lannan and Fryer: *Piscirickettsia-Salmonis*, a Major Pathogen of Salmonid Fish in Chile. Fish Res 1993, 17(1-2):115-121). *P. salmonis* has been confirmed in various countries including Chile, Norway, Canada, USA, Ireland, Scotland and Greece.

Although promising vaccine research have been described in previous literature (Wilhelm et al.: A vaccine against the salmon pathogen *Piscirickettsia salmonis* based on recombinant proteins, Vaccine, 2006, 24 (23): 5083-5091, Kuzyk et al., An efficacious recombinant subunit vaccine against the salmonid rickettsial pathogen *Piscirickettsia salmonis*. Vaccine 2001, 19 (17-19):2337-2344), there are as of yet no commercially available vaccines that are able to handle the ongoing SRS problems Chilean fish farmers are facing today. For unknown reasons, the vaccines available have variable effect. The complexities of SRS in regards to bacterial isolates are not fully studied and this may accomplice to the varying vaccine effect observed.

As a consequence of lack in alternative treatment methods, many farmers choose to treat their fish with antibacterial products, however with variable effect as the bacterium responds poorly to the available antibiotics. According to a report published by the National Fisheries and Aquaculture, Sernapesca, the salmon industry in Chile used 337.99 tons of antibiotics in 2012, 206.8 in 2011, 143.2 in 2010, 184.5 in 2009, 325.6 in 2008 and 385.6 in 2007 (Perez, 2013, supra). As the production increased in 2012, the highest use of antibacterial products were recorded, most of which solely dedicated to the treatment of SRS (Perez, 2013, supra). To find efficient treatment methods and to enhance vaccine development against SRS, knowledge about individual strains on a phenotypic and genotypic level is important.

Genome sequences make it possible to design multilocus sequence typing (MLST) schemes by enabling identification of candidate MLST loci for the development of oligonucleotide primers for their amplification and sequencing by PCR (cf. Maiden (2006), Multilocus sequence typing of bacteria. Annu Rev Microbiol 2006, 60:561-588. MLST was proposed as an accurate tool for strain-typing and measurement of genetic variability Maiden et al. (1998). A set of individual housekeeping genes or candidate MLST loci that encode proteins under stabilizing selection for conserving a metabolic important function are commonly selected for MLST schemes. Such conservative genes

are perfect for the performance of diversity mapping and strain differentiation, as these genes should not be subject to diversifying selection. In this regard, MLST schemes function as a tool for the potential discovery of a new species. MLST schemes have been applied to a wide range of species and have proven to be a robust standard of typing

5 Larsen et al. (2012).

The main objective of the present invention is to provide an isolate of the causative agent of salmonid rickettsial septicaemia (SRS) that may be used as an agent in a vaccine useful in the preparation of *P. salmonis* infections in farmed fish.

10 It is furthermore an object of the present invention to provide a vaccine to protect against *P. salmonis* infections and SRS in farmed fish.

Summary of the invention

The present invention provides an isolated *Piscirickettsia* spp. strain that when administered to fish result in efficient immunization against further *P. salmonis* infections. The *Piscirickettsia* spp. strain according to the present invention can be used in vaccines against SRS, alone or in combination with other antigens from other fish pathogenic organisms, such as e.g. live, attenuated, killed or inactivated bacteria or virus in polyvalent vaccines.

20 The *Piscirickettsia* spp. strain of the present invention differs from *P. salmonis* strains previously described in the prior art. The present inventors have inter alia performed a phenotypic and genetic characterization of a large number of *Piscirickettsia* spp. isolates from Chile and Canada and compare them to the type-strain of *P. salmonis*, LF-89. This was done to obtain information about possible presence of heterogeneous clades that may explain the variable vaccine effect and the variable clinical expressions observed in the field.

25 According to one embodiment, the present invention provides a *P. salmonis* isolate, wherein said isolate is selected from the group consisting of:

- a) a *P. salmonis* isolate ID8079 deposited under the Budapest Treaty with the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (DSMZ) on 6th of June 2013 under deposit number DSM 27338; and
- 30 b) a *P. salmonis* isolate having substantially the same genotypic and phenotypic characteristics as the *P. salmonis* isolate of a).

35 The new *P. salmonis* according to the present invention differs from other *P. salmonis* isolates previously disclosed in the prior art as seen from the experimental data presented herein. In particular, the *P. salmonis* isolate according to the present invention, considering the genotyping and the phenotypic traits of the new isolate ID8079 is a candidate of a new sub species of *P. salmonis*. It is noted that the new

isolate provides improved characteristics compared with earlier known *P. salmonis* isolates of importance e.g. when being utilized in vaccine production. For example, the new isolate of the present invention provides:

- 5 ▪ Faster and better growth characteristic as seen on optimized agar compared to the LF-89 type strain.
- Growth on several more different growth mediums compared to the type strain.
- 10 ▪ Growth at a wider and lower temperature range is seen compared to the type strain. In particular, the *P. salmonis* isolate of the present invention grow in the area of 8 – 25 °C.
- A hemolytic activity is seen on blood agar, which is not seen on the type strain.
- The new isolate produce alpha and beta-glucosidase.

- 15 According to one embodiment, the present invention provides a *P. salmonis* strain deposited with DSMZ under deposit number DSM 27338.

The present invention furthermore provides for vaccines comprising a *P. salmonis* isolate according to the present invention.

20

The present invention furthermore provides for methods for manufacturing vaccines according to the present invention.

- 25 The present inventors have furthermore found a new culturing media, more particular an agar, that is particularly suitable for culturing of the *P. salmonis* isolate of the present invention. The present invention thus provides a growth medium, comprising animal blood, such as ovine blood, and fetal serum derived from animal blood, such as fetal bovine serum. In particular the present invention provides a growth medium comprising tryptic soy agar, ovine blood, cysteine, glucose, serum derived from animal blood, and
- 30 ferric nitrate. According to one embodiment of the present invention provides a growth medium comprising a growth medium comprising tryptic soy agar, defibrinated ovine blood, red sea salt, cysteine, glucose, serum derived from animal blood, and ferric nitrate

35 **Drawings**

Figure 1 Illustrate the scale with three growth zones used to estimate bacterial growth. Green zone indicate little growth (+). Orange zone indicate moderate growth (++). Red zone indicate vigorous growth (+++). Symbols: S, start of streak; E, end of streak.

- 40 **Figure 2** shows the unrooted phylogeny visualizing division of isolates into three clusters on the basis of the concatenated 16S-ITS genes (left) and the *tbpB* gene (right). Scale bar show the number of substitutions as a proportion of branch lengths.

Figure 3 shows the total percentage cumulative mortality in the different groups. High virulence IP: Intraperitoneal injected fish, High virulence w/ lice: Cohabitants co-infected with *L. salmonis*, High virulence: Cohabitants

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Figure 4 shows the total percentage cumulative mortality of the different groups, including shedder fish (dotted lines).

Detailed description of the invention

10 The present invention provides a new *P. salmonis* isolate that can be used in vaccines to obtain protection against further *P. salmonis* infection and thus the prevention of the development of SRS in farmed fish.

15 The *P. salmonis* of the present invention was obtained after comprehensive isolation, culturing and analysis of a large number of isolates obtained in field from Atlantic salmon or rainbow trout diagnosed with or suspected of having SRS. The closer analysis of the phenotypic characteristics of the obtained isolates surprisingly revealed that one of the isolates showed growth characteristics that are particularly advantageous for a vaccine candidate. Furthermore, the genotypic analysis showed that the novel
20 strain ID8079 could be grouped together with the isolates ID8765, ID8558, ID8465, and ID8466, differing inter alia from the type strain LF-89, which could be grouped together with the isolates ID8741, ID8670, ID817, and ID1850.

25 The novel *P. salmonis* isolate named ID8079 herein is deposited under the Budapest Treaty with the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (DSMZ) on 6th of June 2013 under deposit number DSM 27338.

30 Upon infection of a fish with an amount of the isolate according to the present invention sufficient to develop SRS, the fish will develop symptoms of said disease. Common symptoms in the acute stages are hemorrhages in the brain, eyes and skin, splenomegaly and congestion in internal organs. Common symptoms in the chronic stages are e.g. whitish nodules in the liver which progress to granulomes and the liver becomes yellowish, and the hemorrhages in the skin progress to pustuls and finally to caverns in the muscle, and in respect of splenomegaly, pericarditis and fibrin deposit is seen in the
35 abdomen.

40 The *P. salmonis* isolate according to the present invention has shown improved growth characteristics compared with the type strain LF-89, and also compared with other isolates disclosed herein. The *P. salmonis* isolate was e.g. shown to be a significantly faster growing isolate, within the broadest temperature range, compared with the type strain LF-89 and also compared with other isolates disclosed herein. The *P. salmonis* isolate according to the present invention was furthermore shown to be less fastidious as

to growth on different growth media compared with the type strain and also compared with the other isolates disclosed herein. In particular, the present *P. salmonis* isolate according to the present invention was found to grow particularly fast on the growth medium according to the present invention.

5

A further distinctive feature of the *P. salmonis* isolate according to the present invention surprisingly tested positive α -glucosidase and β -glucosidase, which are contrary to the type strain LF-89 and the further *P. salmonis* isolates disclosed herein.

10 The *P. salmonis* isolate according to the present invention is furthermore a Gram negative coccoid bacterium, with a size within the area of 0.4 – 1.8 μm , and with similar cell morphology as other *P. salmonis* isolates.

A *P. salmonis* isolate according to the present invention is to be understood to mean a *P. salmonis* strain with the above characteristics, i.e. with substantially the same phenotypic and genotypic characteristics as the isolate ID8079.

Although the present inventors have isolated and characterized a particular strain disclosed herein with *inter alia* the advantageous growth characteristics and which may be used in the preparation of a fish vaccine, i.e. ID8079, the skilled person will acknowledge that other strains being closely related to ID8079, i.e. having substantially the same genotypic and phenotypic characteristics, is meant to be covered by the present invention.

25 Thus, it is to be understood that the present invention covers *P. salmonis* strains exerting more or less equivalent genotypic and phenotypic characteristics as the isolate ID8079 (DSM 27338), i.e. having substantially the same growth characteristics and also substantially the same ability to induce protection against *P. salmonis* infection in farmed fish when used in the preparation of a fish vaccine.

30

The skilled person will acknowledge that various substitutions, deletions or additions to the genomic sequence may be introduced by commonly available gene technology methods or randomly during culturing of a bacterium strain, and that such alteration may be introduced on purpose or randomly/accidentally without substantially changing the characteristics of said strain. It is to be understood that *P. salmonis* strains differing from the strains of the present invention merely by the purposely or randomly introduction of such alteration in the genome that do not substantially effect the phenotypic characteristics are covered by the present invention. The expression “having substantially the same genotypic characteristics” is thus to be understood to mean *P. salmonis* strains where the relevant parts of the genome of the strains to be compared has a high degree of sequence identity compared with the strain ID8079 (DSM 27338).

40

It is furthermore to be understood that also further mutants originating from the *P. salmonis* isolate of the present invention, i.e. such as a functionally equivalent mutant having substantially the same genotypic and phenotypic characteristics, is covered by the present invention.

- 5 The term “mutant thereof” as used herein is thus to be understood to mean a strain derived from a *P. salmonis* isolate of the present invention, in particular derived from the strain ID8079 (DSM 27338), by means of e.g. genetic engineering, mutagenizing conditions or by culturing.

- 10 It is also to be understood that inactivated or attenuated *P. salmonis* strains originating from a *P. salmonis* strain according to the present invention is covered by the present invention.

- The *P. salmonis* isolate of the present invention may be used to prepare a vaccine useful in preventing *P. salmonis* and the development of SRS in farmed fish. The expression “vaccine” as used herein it to be understood to refer to any type of biological agent in an administrable form capable of stimulating a protective immune response in a recipient fish after being administered said vaccine. The biological agent included in a vaccine is commonly an antigen, such as a pathogen organism (live, attenuated or inactivated), or parts thereof (e.g. a suitable protein comprising a useful epitope), allowing the immune system to develop a response which is accelerated in case of later infections by said pathogen.
- 15
20

- When used as the antigen in a vaccine, the *P. salmonis* isolate according to the present invention may be used as an attenuated or inactivated form of said isolate. An attenuated form of the *P. salmonis* isolate according to the present invention maintains the ability to induce protection against SRS although it is no longer as virulent as the *P. salmonis* ID8079. An attenuated form of the *P. salmonis* isolate according to the present invention may be obtained by any method well known to the skilled person. For example, attenuated form may be obtained by serial passages or by mutagenesis techniques well known to the skilled person.
- 25

- 30 The *P. salmonis* isolate according to the present invention may also be included in polyvalent vaccines. As used herein, a polyvalent vaccine is a vaccine comprising two or more different biological agent in an administrable form capable of stimulating a protective immune response in a recipient fish after being administered said vaccine. In particular, a polyvalent vaccine stimulates the immune system of the recipient against two or more different pathogens. A non-limiting list of pathogens that may be included in a polyvalent vaccine (in live, attenuated or inactivated form) is live, attenuated or inactivated fish virus selected from the group consisting of Infectious Hematopoietic Necrosis Virus (IHNV), Infectious Pancreatic Necrosis Virus (IPNV), Infectious Salmon Anemia Virus (ISAV), Salmon Pancreatic virus (SAV), Viral Hemorrhagic Septicemia Virus (VHSC), Cardiomyopathy Syndrom Virus (CMSV) and/or live, attenuated or
- 35
40

inactivated bacteria selected from the group consisting of *Aeromonas sp.*,
Bifidobacterium sp., *Brevibacterium sp.*, *Cytophaga sp.*, *Edwardsiella sp.*,
Flavobacterium sp., *Francisella sp.*, *Lactococcus sp.*, *Leuconostoc sp.*, *Listonella sp.*
5 *Moritella viscosa*, *Mycobacterium sp.*, *Nocardia sp.*, *Pediococcus sp.*, *Photobacterium*
damsel, *Pseudomonas sp.*, *Renibacterium sp.*, *Rhodococcus sp.*, *Streptococcus sp.*,
Vibrio sp., and *Yersinia sp.*.

The *P. salmonis* isolate of the present invention may also be included in polyvalent
vaccines wherein the one or more further biological agent consists of one or more
10 recombinant protein, e.g. comprising suitable epitopes useful in immunization of a
recipient fish against a fish pathogen, e.g. a fish pathogens selected from the group
listed above.

The *P. salmonis* isolate of the present invention may also be included in polyvalent
15 vaccines wherein the one or more further biological agents are provided as a DNA
vaccine plasmid constructed in order to enable the expression of a nucleic acid sequence
encoding an antigen or epitope useful in immunization of a recipient fish against a fish
pathogen, e.g. a fish pathogens selected from the group listed above. Examples of DNA
vaccines developed for vaccination of fish is described in Heras et al., Fish Shellfish
20 Immunol., 2009, 27, pp. 120-129, EP 1 818406 A1, and WO2008/031572.

Polyvalent vaccines according to the present invention may thus be prepared from whole
cells, killed and/or attenuated virus or bacteria, protein extracts isolated from the above
mentioned pathogens, isolated or recombinant antigens, DNA vaccine plasmids, and/or
25 mixtures thereof.

The vaccine according to the present invention may be administered to fish by any of
methods for administering vaccines well known to the skilled person. For example, the
vaccine according to the present invention may be administered by intramuscular or
30 intraperitoneal injection. Vaccination may also be accomplished through oral ingestion
or by means of aqueous immersion.

The vaccine according to the present invention comprises an effective immunization
dose of *P. salmonis*. The term "effective immunization dosage" is to be understood to
35 mean the amount which will induce immunity in a farmed fish when challenged by a
virulent strain of *P. salmonis*. Immunity is considered obtained when the level of
protection of vaccinated fish is significantly higher than in a comparable population of
unvaccinated fish. Based on the general knowledge of vaccine formulation, the skilled
person will be able to identify the appropriate dose to be included in the vaccine, e.g.

based on the form of the pathogen (inactivated, attenuated, etc.), the size of the recipient fish, etc.

Vaccination of fish by injection can be performed either with or without adjuvant.

5 Adjuvants are introduced in order to enhance the efficacy of the vaccine. Mineral oil adjuvants are commonly used in fish vaccines and may be included in the vaccine according to the present invention. A non-limited example of an oil adjuvant that may be included in the vaccine of the present invention is mannide oleate in a mineral oil solution. Suitable mineral oils to be included are e.g. white mineral oil, i.e. sorbitan
10 oleate, or polyoxyethylene sorbitan monooleate. Also synthetic mineral oils, such as those commercially available under the name Montanide ISA563, Montanide USA 575, Montanide ISA 711, and Montanide 760 may be used. Other useful adjuvant that may be used in a fish vaccine according to the present invention is e.g. squalene,
15 muramuldiptides, lipopolysaccharides, and glycans or glucans, or inorganic adjuvants such as an aluminium hydroxide and calcium phosphates. Yet a further useful adjuvant is Carbopol polymers (polymers of acrylic acid cross linked with polyalkenyl ethers or divinyl glycol), such as Carbopol®.

The vaccine according to the present invention is generally administered along with a
20 pharmaceutically acceptable carrier or diluent. The particular pharmaceutically acceptable carrier or diluent used is not critical in respect of the present invention. Any well known carrier or diluent commonly used in vaccines to be administered to fish may be used. The vaccine according to the present invention may e.g. be formulated as a water-in-oil-emulsion, an oil-in-water-emulsion or a water-in-oil-in water emulsion.

25 Injection of the vaccine according to the present invention may be performed on a commercial scale using a fixed dose automatic repeating syringe or an automatic injection vaccination machine designed to deliver a fixed dose of e.g. 0.1 or 0.2 ml per fish. The vaccine may be injected through the body wall into the intra-peritoneal cavity
30 or by injecting the vaccine into the dorsal sinus. The vaccination is preferably performed following anesthetization.

SRS was first observed in salmonids. Salmonids are elongated, ray-finned bony fish, having a small adipose fin without fin rays between the dorsal fin and the tail. Many
35 salmonids live in the sea, but enter fresh water to spawn. Although SRS is most commonly a problem in the farming of salmonids, and thus may be found in e.g. rainbow trout, Atlantic salmon, coho salmon, Chinook salmon, pink salmon, masou salmon, SRS-like syndromes are also found in non-salmonids, such as seabass (e.g. white seabass (*Atractoscion nobilis*)).

40 The present invention furthermore provides a novel growth medium for the cultivation of *P. salmonis* and bacteria belonging to the *Piscirickettsia* genus. In particular, the

present growth medium is useful in cultivation of the *P. salmonis* isolate according to the present invention.

The present inventors have found that a growth medium suitable for culturing the *P. salmonis* isolate of the present invention comprising animal blood and serum derived from animal blood, in particular ovine blood and fetal bovine serum. The growth medium is further based on a mixture comprising tryptic soy agar, ovine blood, glucose, cysteine, fetal serum derived from animal blood, such as fetal bovine serum, and ferric nitrate. According to one embodiment of the invention, defibrinated ovine blood is used. According to a further embodiment, the present growth medium comprises tryptic soy agar and ovine blood, sea salt such as red sea salt, glucose, cysteine, fetal bovine serum and ferric nitrate. An amount of sea salt in the area of 15 – 25 g/L was furthermore found applicable. Furthermore, it was found that a preferable amount of L-cysteine in the growth medium according to the present invention is at least 1 g/L.

The growth medium according to the present invention may be prepared by first dissolving tryptic soy agar and sea salt, such as e.g. red sea salt, in water (preferably reverse osmosis water), and after heating (in autoclave), defibrinated blood is added and the mixture is kept in a water bath at 80°C until a brown mixture is obtained. After cooling, the remaining ingredients may be added. The pH of the growth medium is preferably adjusted if need be (to about 6.8 ± 0.2). The growth medium may then be transferred to any suitable agar plate. Upon plating of the isolates according to the present invention on agar plates comprising the growth medium according to the present invention, a translucent layer of bacteria could be seen the following day. The *P. salmonis* isolate ID8079 showed a significant faster growth on the growth medium of the present invention compared with the type strain FL-89.

The present invention may be better understood by reference to the following non-limiting examples, which are provided as exemplary of the invention. The following examples are presented in order to more fully illustrate the preferred embodiments of the invention. They should in no way be construed as limiting to the broad scope of the invention.

Examples

Example 1 Phenotypic characterization

Bacterial isolates

Bacterial isolates included in the study were initially isolated in the field from Atlantic salmon and rainbow trout diagnosed with SRS at various farming sites in Chile (Table 2). The Canadian isolate CA-9 was isolated from Atlantic salmon after suspicion of SRS. A sample of kidney tissue from the hosts Atlantic salmon and rainbow trout was streaked out on an agar plate and sent to the Fish Disease group at the University of

Bergen where sub-cultures of the isolates were made and stored for later characterization.

5 All of the respective isolates included in the present study were isolated in lab and preserved at -80 °C in a 1.8 ml Nunc tube. An overview of all of the isolates included in the study is presented in Table 1.

Isolation, propagation and preservation

10 All the isolates were initially cultured on a CHAB agar (Mikalsen J et al (2008), Agar culture of *Piscirickettsia salmonis*, a serious pathogen of farmed salmonid and marine fish. Fems Microbiology Letters, 278(1):43-47.), and later sub-cultured on an optimized novel SRS blood agar developed for the present study (see table 2 below) to do the comparative characterization. Culture of *P. salmonis* was carried out by defrosting Nunc cryo tubes containing the respective isolates from -80 °C to 4°C (30 min to 1 h). A
15 subsample of 100 µl of the bacterial solution was added and streaked to an agar plate (passage one after freeze, P1F) using an L-shaped spreader (VWR, U.S.A.), and incubated at 19 °C for growth.

20 When successfully growing a pure culture of the bacteria, DNA was extracted followed by PCR targeting the 16S rRNA gene. The gene was sequenced to confirm that the bacteria belonged to *Piscirickettsia sp.* After confirmation, a new passage (P2F) was made.

25 P2F was stored at -80 °C following the freezing protocol for *P. salmonis* isolates from agar plates; *P. salmonis* cultures with no more than 5 - 7 days of incubation were used for freezing. A volume of 3 ml of sterilized PBS 1.5% NaCl was applied to the plate. The plates were gently moved detaching the bacterial culture from the agar. Totally 900 µl of the inoculums mix and PBS 1.5% NaCl was added to a 1.8 ml Nunc tube followed by 900 µl of Biofreeze (Biochrom, Germany). The solution was gently mixed to obtain a
30 homogenous mixture. Nunc tubes were then put in a @Styrofoam box with paper, to avoid rapid cooling, at -80 °C before being put in sample box five hours later.

Table 1: Dataset for all of the *Piscirickettsia* spp. isolates included in the study.

Isolate	LF-89	CA-9	8741	8670	817	1850	8079	8765	8558	8465	8466
Country	Chile	Canada	Chile	Chile	Chile	Chile	Chile	Chile	Chile	Chile	Chile
Region	Puerto Montt (X)	British Columbia	Cabuco (X)	Chiloe Centro (X)	Cabuco (X)	Calbuco (X)	Chiloe Centro (X)	Chiloe Centro (X)	Chiloe Centro (X)	Chiloe Centro (X)	Chiloe Centro (X)
Location	-	Vancouver Island	Chidhuapi 4	Imelev	Chidhuapi 1	Chidhuapi 1	Calen 1	Calen 2	Chiloe Centro (X) Los Pinos (Lin Lin Island)	Calen 1	Calen 1
Isolation date	1989	12.2012	18.08.2012	07.08.2012	25.10.2012	03.06.2012	13.02.2012	24.08.2012	22.05.2012	19.06.2012	23.05.2012
Water source	Sea water	Sea water	Sea water	Sea water	Sea water	Sea water	Sea water	Sea water	Sea water	Sea water	Sea water
Water temp (°C) at isolation date	-	-	9,6	9,8*	7,2	6,2	9,5	7,8	10,0	7,3	7,0
Oxygen (mg/l) at isolation date	-	-	7,7	7,5*	11,1	11,1	12,7	10,0	10,3	10,1	10,3
Host	Coho salmon	Atlantic salmon	Rainbow trout	Atlantic salmon	Rainbow trout	Rainbow trout	Atlantic salmon	Atlantic salmon	Atlantic salmon	Atlantic salmon	Atlantic salmon
Wild or farmed fish	Farmed	Farmed	Farmed	Farmed	Farmed	Farmed	Farmed	Farmed	Farmed	Farmed	Farmed
Isolation tissue	-	Kidney	Lesion (Skin, Muscle)	Kidney	Lesion (Skin, Muscle)	Lesion (Skin, Muscle)	Kidney	Kidney	Kidney	Kidney	Kidney
Biomass (kgs) at isolation month	-	-	1 428 837	0	1 640 620	1 123 847	1 259 751	2 131 379	1 716 946	1 431 076	1 259 751
Lice data: juvenile	-	-	2,4	1,9	4,0	2,0	2,4	2,8	1,3	3,2	2,5
Lice data: female ov	-	-	1,4	1,5	4,5	1,0	1,0	1,6	1,7	2,0	2,6
Lice data: adults	-	-	1,5	1,5	4,0	0,0	0,3	3,4	1,6	2,0	2,7
Mortality (%)	-	0,03	36,5	15,7	34,3	34,3	33,1	23,5	19,3	33,1	33,1
Mortality caused by <i>P. salmonis</i>	-	-	13,2	5,1	6,7	6,7	14,8	5,4	3,6	14,8	14,8
Mortality caused by Sea lions	-	-	2,0	0,3	0,4	0,4	4,5	2,2	2,9	4,5	4,5
Mortality caused by other diseases	-	-	0,7	0,5	2,1	2,1	0,6	0,1	2,5	0,6	0,6
Mortality caused by other causes***	-	-	20,6	9,7	25,1	25,1	13,2	15,7	10,3	13,2	13,2
Days to reach max. mortality	-	-	92	124	108	108	38	129	243	38	38
Severity***	-	-	Sub-acute	Sub-acute	Sub-acute	Sub-acute	Acute	Sub-acute	Chronic	Acute	Acute
Diseases signs	-	-	Classic	Classic	Classic	Classic	Classic	Classic	Classic	Classic	Classic
Antibiotics (kg) used against <i>P. salmonis</i> *****	-	-	450,1	52,8	158	158	579,2	200,8	0	579,2	579,2

Symbols: *, data from 31.07.2012; **, average *Caligus* sp. pr fish; ***, includes deformed-, mature- and rejected fish and fish preyed on by birds etc.; ****, severity in days to reach the higher mortality by SRS; acute (1 to 50 days), sub-acute (51 to 150 d.), and chronic (>150 d.), *****; antibiotic use as pure drug throughout the cycle.

A new passage (P3F) was used for DNA extraction following sequencing of the housekeeping genes. P3F and later passages were used in the phenotypical characterization of the isolates. All procedures involving handling of the bacterial isolates were performed in a sterile and safe environment provided by ScanLaf Mars
5 Safety Class 2 (LaboGene™, Denmark) ventilation bench.

Colony and cell morphology

To examine CFUs and determine exact morphology, a magnifying glass was used. The colonies were given time to grow so that proper morphological studies could be
10 performed. Preferably, colonies measuring >1.0 mm in diameter was used for determining morphology.

All isolates were gram-stained. This was done as follows; a 40 µl drop of deionised water was added to a microscopy slide. 1-2 colonies of cultures were added to the
15 drop on the microscopy slide and streaked out gently. A sterile plastic bacterial loop (VWR, U.S.A.) was used when preparing slides for microscopy. The microscopy slide was left to dry at room temperature for 10 minutes. The slide was put in 100 % methanol for 3 min, before being added drops of Gram's crystal violet solution (Sigma-Aldrich, U.S.A.) for 60 sec and then rinsed carefully with tap water. Gram's
20 iodine solution (Sigma-Aldrich, Germany) was added for 60 sec following Gram's decolorizer solution (Sigma-Aldrich, U.S.A.) and counterstaining with Gram's safranin solution (Sigma-Aldrich, U.S.A.) for the same amount of time. The microscopy slide was left to dry at room temperature for 10 min. A cover glass was placed on the slide and a drop of immersion oil (Sigma-Aldrich) added before
25 examining the bacteria under light microscope at 10X100.

In respect of colony morphology, examination of sub-cultured *Piscirickettsia spp.* showed CFUs of the same characteristics as previously described by Mikalsen et al. (2008), Agar culture of *Piscirickettsia salmonis*, a serious pathogen of farmed
30 salmonid and marine fish. FEMS microbiology letters 2008, 278(1):43-47. Colonies were observed as slightly convex, grey-white, shiny, and centrally opaque with translucent, slightly undulating margins (Mikalsen et al. (2008), supra). No distinctive colony morphology was observed for the different isolates included in present study.

35 In respect of cell morphology, examination of the different *Piscirickettsia spp.* isolates included in present study showed Gram-negative coccoid bacteria with a size of 0.4-1.8 µm in diameter. No distinctive cell morphology was observed, and the varying cell-size applied to all of the isolates.

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Media for growth studies

In order to promote the growth of the fastidious *P. salmonis* isolates, an optimal standard growth medium had to be made for the phenotypic characterization.

- 5 Austral-TSFe (Yanez AJ, Silva H, Valenzuela K, Pontigo JP, Godoy M, Troncoso J, Romero A, Figueroa J, Carcamo JG, Avendano-Herrera R: Two novel blood-free solid media for the culture of the salmonid pathogen *Piscirickettsia salmonis*. Journal of fish diseases 2012) was modified three times replacing Sodium Chloride (Sigma-Aldrich) with 15 or 25 g L⁻¹ of red sea salt (Red Sea, U.S.A). Glucose
10 concentration was increased to 6 g L⁻¹ for the modifications containing 15 or 25 g L⁻¹ of red sea salt. Additionally, a glucose concentration of 12 g L⁻¹ was tested on the modification with 25 g L⁻¹ red sea salt. Tryptic Soy Agar (BD Difco™, U.S.A) was modified to follow manufacturer's recommendation of 40 g L⁻¹. Defibrinated sheep blood (Oxoid Limited, U.K) was added in a concentration of 5 % as a
15 supplement.

Table 2 Salmonid Rickettsial Syndrome Blood Agar (SRS-BA), a new growth medium for *Piscirickettsia* spp.

#	Ingredient	Formula	Product #	Supplier	Amount
1	Reverse Osmosis (RO) Water	H ₂ O	-----	-----	1000 ml
2	Tryptic Soy Agar	-----	236950	BD Difco™, U.S.A.	40 g
3	Red Sea Salt	-----	-----	Red Sea, U.S.A.	20 g
4	Defibrinated Sheep Blood	-----	SR0051	Oxoid Limited, U.K.	50 ml
5	L-cysteine*	C ₃ H ₇ NO ₂ S	C7880-100G	Sigma-Aldrich	1 g
6	D-glucose**	C ₆ H ₁₂ O ₆	49158-1KG-F	Sigma-Aldrich	6 g
7	Fetal Bovine Serum (FBS)	-----	SV30160.03	Thermo Scientific HyClone, U.S.A.	50 ml
8	Ferric Nitrate***	Fe(NO ₃) ₃	F8508-100G	Sigma-Aldrich	0.2 mM

20 Symbols: *, filter-sterilized (0.2 µm) in RO water (50 ml); **, filter-sterilized (0.2 µm) in RO water (25 ml); ***, filter-sterilized (0.2 µm) in RO water (5 ml).

- Ingredients 1-3 were autoclaved at 121 °C for 15 min. Ingredient 4 was added directly after autoclave and agar was set to water bath at 80 °C until chocolate brown in color. Subsequently the agar was cooled down to 50 °C and ingredients 5-
25 8 were added. The pH was adjusted to 6.8±0.2 before adding the agar in 22 ml portions to empty, sterile agar plates.

- The Austral-TSFe modification with 15 g L⁻¹ of red sea salt and 6 g L⁻¹ glucose was tested on all of the isolates except CA-9, 817 and 1850 which were included in
30 the study at a later time. A small, translucent layer of bacteria could be seen the following day after plating out 100 µl of the isolates 8079, 8765, 8558, 8465 and 8466 directly from frozen stock. Growth was confirmed at day two by a thicker

layer of bacteria, showing rapid growth for the latter *Piscirickettsia sp.* isolates. No growth was observed for the isolates LF-89, 8741 and 8670. However, minor growth was observed when streaking out a generous sample of the latter three bacterial isolates.

5

The addition of red sea salt to a total of 25 g L⁻¹ seemed to have neither positive nor negative effects on the less fastidious isolates (8079, 8765, 8558, 8465 and 8466), and a minor positive effect was observed on the highly fastidious isolates (LF-89, 8741, 8670, CA-9, 817 and 1850) after streaking out a generous sample of

10

The Austral-TSFe modification with 25 g L⁻¹ of red sea salt and 12 g L⁻¹ glucose was tested on all of the isolates in present study. A negative effect with regard to growth was recorded for all of the isolates. No growth was observed for the highly fastidious isolates while minor growth was observed for the less fastidious isolates. SRS-BA promoted a very good growth for all of the isolates including the highly fastidious ones, indicating that cysteine which for this specific modification was increased by 100 % to 1 g L⁻¹, may have been the limiting factor that inhibited growth in earlier modifications. Since growth was observed at both 15 and 25 g L⁻¹ for earlier modifications, it was decided to test 20 g L⁻¹ which didn't seem to have positive nor negative effect on any of the isolates, indicating that a salt range of 15-25 g L⁻¹ may be used without significant change in growth.

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Temperature range

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Three colonies measuring 1 mm in diameter were streaked out on SRS-BA growth medium and incubated at the following temperatures; 8, 11, 16, 19, 22, 25 and 37 °C. Growth was recorded using the defined scale; no growth (-), little growth (+), moderate growth (++) and vigorous growth (+++) in accordance to Figure 1. However, in cases where high densities of thriving bacteria were observed in a zone, statuses were adjusted positively (additional "+" added). Counting and measurement of the colonies was performed to provide additional data for a possible indication of optimum temperature for bacterial growth.

30

Growth was recorded at day 3-7 and 10-14 for all of the isolates included in the present study. A summary showing recorded growth at the end of the experiment (day 14) is presented in Table 4.

35

A weakly to positive growth status was recorded at the temperatures 16 °C, 19 °C and 22 °C after 3 days for all of the isolates. The strain ID8079 (DSM 27338) was recorded as the fastest growing isolate in the experiment giving it a vigorous growth status at 22 °C. Additionally, ID8079 (DSM 27338) was given vigorous growth status at 16 °C and 19 °C on day 5 and 6 respectively, making the isolate distinct

40

from the rest based solely on growth. The isolate 8558 was the only isolate recorded to grow at 25 °C, and the type strain LF-89 was the only isolate not recorded to grow on 8 °C. Overall it was apparent that the less fastidious *Piscirickettsia* sp. isolates (8079, 8765, 8558, 8465 and 8466) grew better than the highly fastidious *P. salmonis* isolates (LF-89, 8741, 8670, CA-9, 817 and 1850). The isolate 8558 produced the highest number of colonies counting 53, while the isolate 8466 produced the largest colonies measuring >3.2 mm. in diameter.

It was apparent that the optimum temperature for maximum culture growth was in the range 19 °C – 22 °C for the less fastidious isolates, and in the range 16 °C – 19 °C for the highly fastidious isolates. Overall the isolates were recorded to have a growth temperature range of 8 °C – 25 °C. No growth occurred at 37 °C.

Table 4 A summary presenting registered growth at the termination of the experiment (day 14), at different incubation temperatures.

Isolate	Growth temp	Day 14	CFU (dia. mm.)
		Growth	
LF-89	8 °C	-	
	11 °C	+	
	16 °C	++	
	19 °C	++	
	22 °C	+	
	25 °C	-	
	37 °C	-	
8741	8 °C	+	
	11 °C	+	
	16 °C	++	
	19 °C	+++	
	22 °C	+	4 (1.1)
	25 °C	-	
	37 °C	-	
EM8670	8 °C	+	
	11 °C	+	
	16 °C	++	1 (0.8)
	19 °C	++	
	22 °C	++	
	25 °C	-	
	37 °C	-	
817	8 °C	+	
	11 °C	+	
	16 °C	++	
	19 °C	++	
	22 °C	++	1 (0.9)
	25 °C	-	
	37 °C	-	
8079	8 °C	+	
	11 °C	+	
	16 °C	+++	38 (1.7)
	19 °C	+++	48 (1.9)
	22 °C	+++	30 (1.8)
	25 °C	-	
	37 °C	-	
EM8765	8 °C	+	
	11 °C	+	
	16 °C	++	21 (1.5)
	19 °C	+++	24 (2.6)
	22 °C	+++	34 (2.6)
	25 °C	-	
	37 °C	-	
8558	8 °C	+	
	11 °C	++	
	16 °C	+++	5 (1.1)
	19 °C	+++	40 (2.0)
	22 °C	+++	53 (2.2)
	25 °C	+	
	37 °C	-	
8465	8 °C	+	
	11 °C	+	
	16 °C	+++	41 (1.7)
	19 °C	+++	42 (2.1)
	22 °C	+++	23 (1.6)
	25 °C	-	
	37 °C	-	
8466	8 °C	+	
	11 °C	+	
	16 °C	+++	41 (1.7)
	19 °C	+++	42 (2.1)
	22 °C	+++	23 (1.6)
	25 °C	-	
	37 °C	-	

11 ° C	+		11 ° C	+	
16 ° C	++		16 ° C	+++	32 (2.1)
1850 19 ° C	++		8486 19 ° C	+++	35 (3.2)
22 ° C	+		22 ° C	+++	42 (3.0)
25 ° C	-		25 ° C	-	
37 ° C	-		37 ° C	-	
8 ° C	+				
11 ° C	+				
16 ° C	++	1 (0.9)			
CA-9 19 ° C	+++	16 (1.2)			
22 ° C	++	12 (1.3)			
25 ° C	-				
37 ° C	-				

Symbols: -, no growth; +, little growth; ++, moderate growth; +++, vigorous growth;

Growth media test

- Two colonies measuring 1 mm. in diameter were streaked out on SRS-BA, Austral-
 5 TSHem (Yanez AJ, Silva H, Valenzuela K, Pontigo JP, Godoy M, Troncoso J, Romero A, Figueroa J, Carcamo JG, Avendano-Herrera R: Two novel blood-free solid media for the culture of the salmonid pathogen *Piscirickettsia salmonis*.
 Journal of fish diseases 2012), cysteine heart agar w/blood (CHAB) (Mikalsen J, Skjaervik O, Wiik-Nielsen J, Wasmuth MA, Colquhoun DJ: Agar culture of
 10 *Piscirickettsia salmonis*, a serious pathogen of farmed salmonid and marine fish. FEMS microbiology letters 2008, 278(1):43-47), CHAB w/0.2 mM Fe, blood agar (BA), BA w/2 % NaCl, marine agar (MA) and tryptone-yeast extract-salts agar w/Glc (FLPA) (Cepeda C, Garcia-Marquez S, Santos Y: Improved growth of
 15 *Flavobacterium psychrophilum* using a new culture medium. Aquaculture 2004, 238(1-4):75-82. Growth mediums were incubated at a temperature of 19 °C and growth was recorded the following days using the predefined scale. However, in cases where high densities of thriving bacteria were observed in a zone, statuses were adjusted positively (additional "+" added). Counting and measurement of colonies was performed to provide additional data.
- 20 Growth was recorded at day 3-7 and 10-14 for all of the isolates included in the present study. A summary of growth at termination of the experiment (day 14) is presented in Table 5.
- 25 A weakly to positive growth status was recorded after 3 days on SRS-BA for all of the isolates. The weakly to positive growth was recorded until day 7 for all of the isolates on SRS-BA except 8079 which had a more rapidly growth giving it a moderate growth status at day 5. Of the *P. salmonis* isolates (LF-89, 8471, 8670, 817, 1850 and CA-9) positive growth on CHAB and CHAB w/Fe was only recorded
 30 for the type strain LF-89 starting at day 5-6. No growth was recorded on the other growth mediums for the fastidious isolates during the period of the experiment. Nor

was any growth recorded on MA and FLPA during the period of the experiment for the *Piscirickettsia* sp. isolates (8079, 8765, 8558, 8465 and 8466). The isolate 8558 did not grow on BA which was recorded as positive for the other less fastidious isolates. Additionally, the isolate 8465 did not grow on Austral-TSHem. Vigorous growth was recorded on SRS-BA for the isolates 8079, 8765 and 8558 while on BA w/2% NaCl only for 8079. The isolate 8765 produced the highest number of colonies (64) measuring >2.5 mm. in diameter. On the basis of how long it took to form colonies, their numbers and size, the optimal growth medium was concluded to be SRS-BA for all of the isolates.

Table 5 A summary presenting registered growth at the different growth mediums at termination of the experiment (day 14).

Isolate	Growth medium	Day 14	
		Growth	CFU (dia. mm.)
LF-89	SRS-BA	++	9 (1.0)
	CHAB	+	2 (0.9)
	CHAB w/Fe	+	1 (0.8)
	Austral-TSHem	-	
	BA	-	
	BA w/2 % NaCl*	-	
	MA	-	
	FLPA	-	
8741	SRS-BA	++	11 (1.3)
	CHAB	-	
	CHAB w/Fe	-	
	Austral-TSHem	-	
	BA	-	
	BA w/2 % NaCl	-	
	MA*	-	
	FLPA	-	
EM8670	SRS-BA	++	11 (1.4)
	CHAB	-	
	CHAB w/Fe	-	
	Austral-TSHem	-	
	BA	-	
	BA w/2 % NaCl*	-	
	MA	-	
	FLPA	-	
817	SRS-BA	++	2 (0.9)
	CHAB	-	
	CHAB w/Fe	-	
	Austral-TSHem	-	
	BA	-	
	BA w/2 % NaCl	-	
	MA	-	
	FLPA	-	
	SRS-BA	++	3 (1.0)
	CHAB	-	
	CHAB w/Fe	-	

Isolate	Growth medium	Day 14	
		Growth	CFU (dia. mm.)
8079	SRS-BA	+++	50 (2.1)
	CHAB	++	16 (1.8)
	CHAB w/Fe	++	18 (1.5)
	Austral-TSHem	++	10 (1.8)
	BA	++	12 (1.1)
	BA w/2 % NaCl*	+++	3 (0.8)
	MA	-	
	FLPA	-	
EM8765	SRS-BA	+++	64 (2.5)
	CHAB	+	16 (1.1)
	CHAB w/Fe	+	14 (1.5)
	Austral-TSHem	+ (w)	1 (1.5)
	BA	+	14 (1.0)
	BA w/2 % NaCl	+	2 (1.0)
	MA	-	
	FLPA	-	
8558	SRS-BA	+++	30 (1.8)
	CHAB	+	13 (1.1)
	CHAB w/Fe	+	2 (0.9)
	Austral-TSHem	+	13 (1.2)
	BA	-	
	BA w/2 % NaCl	+	20 (1.0)
	MA	-	
	FLPA	-	
8465	SRS-BA	++	12 (1.8)
	CHAB	+	13 (1.1)
	CHAB w/Fe	+	9 (1.2)
	Austral-TSHem	-	
	BA	+	12 (1.0)
	BA w/2 % NaCl	++	12 (1.0)
	MA	-	
	FLPA	-	
	SRS-BA	++	22 (1.7)
	CHAB	++	10 (1.1)
	CHAB w/Fe	+	11 (1.0)

1850	Austral-TSHem	-		8468	Austral-TSHem	+	4 (1.9)
	BA	-			BA	++	10 (1.0)
	BA w/2 % NaCl	-			BA w/2 % NaCl	++	19 (1.2)
	MA	-			MA*	-	
	FLPA	-			FLPA	-	
	SRS-BA	++	13 (1.1)				
	CHAB	-					
	CHAB w/Fe	-					
CA-9	Austral-TSHem	-					
	BA	-					
	BA w/2 % NaCl	-					
	MA*	-					
	FLPA	-					

Symbols: -, no growth; + (w), weak growth; +, little growth; ++, moderate growth; +++, vigorous growth; *, contaminated; SRS-BA, Salmonid Rickettsial Syndrome blood agar; CHAB, cysteine heart agar with blood; BA, blood agar; MA, marine agar; FLPA, tryptone-yeast extract-salts agar with glucose.

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API ZYM test

API ZYM kit (BioMérieux, U.S.A) for the detection of bacterial enzymes was used according to the manufacturer's protocol, except that incubation temperature was set at 19 °C and incubation time at 24 h. A 6.0 McFarland standard (Appendix 2) presented in manufacturer's protocol was made in advance for turbidity comparison.

An overview of the results from the API ZYM test performed on the isolates is presented in Table 6. To expand the overall API ZYM profile image, results of three external isolates (NVI 5692, NVI 5910 (LF-89 like) and NVI 5786 (EM-90 like)) presented by Mikalsen et al. (2008), supra were included for comparison. Identical results were observed for all of the internal isolates when performing triplicate tests.

Table 6 API ZYM profiles recorded for the isolates.

#	Enzyme	LF-89	8741	CA-9	8670	817	1850	8079	8765	8558	8465	8466	NVI 5692	NVI 5910	NVI 5786
1	Negative control	—	—	—	—	—	—	—	—	—	—	—	—	—	—
2	Alkaline phosphatase	W	W	+	W	W	W	+	+	+	+	+	—*	W	+
3	Esterase (C4)	W	W	W	W	W	W	W	W	W	W	W	+	W	+
4	Esterase Lipase (C8)	W	W	W	W	W	W	W	W	W	W	W	+	W	+
5	Lipase (C14)	—	—	—	—	—	—	—	—	—	—	—	W	W	W
6	Leucine arylamidase	+	+	+	+	+	+	+	+	+	+	+	W	W	+
7	Valine arylamidase	+	+	+	+	+	+	+	+	+	+	+	—	—	—
8	Cystine arylamidase	W	W	—	—	—	—	—	—	—	—	—	—	—	—
9	Trypsin	—	—	—	—	—	—	—	—	—	—	—	—	—	—
10	Chymotrypsin	—	—	—	—	—	—	—	—	—	—	—	—	—	—
11	Acid phosphatase	+	+	+	W	W	W	+	+	+	+	+	+	—	+
12	Naphthol-AS-BI-phosphohydrolase	+	+	+	W	+	+	+	+	+	W	+	+	—	+
13	α -Galactosidase	—	—	—	—	—	—	—	—	—	—	—	—	—	—
14	β -Galactosidase	—	—	—	—	—	—	+	—	—	—	—	—	—	—
15	β -Glucuronidase	—	—	—	—	—	—	—	—	—	—	—	—	—	—
16	α -Glucosidase	—	—	—	—	—	—	+	—	—	—	—	—	—	—
17	β -Glucosidase	—	—	—	—	—	—	—	—	—	—	—	—	—	—
18	N-acetyl- β -Glucosaminidase	—	—	—	—	—	—	—	—	—	—	—	—	—	—
19	α -mannosidase	—	—	—	—	—	—	—	—	—	—	—	—	—	—
20	α -fucosidase	—	—	—	—	—	—	—	—	—	—	—	—	—	—

Symbols: —, negative reaction (0); w, weakly positive (1-2); +, positive reaction (3-5); *, very weakly positive on one of four replicate tests.

- 5 The following enzymes were recorded as identical for all of the internal isolates; esterase (C4) (weakly positive), esterase lipase (C8) (weakly positive), lipase (C14) (negative), leucine arylamidase (positive), valine arylamidase (positive), trypsin (negative), chymotrypsin (negative), α -galactosidase (negative), β -glucuronidase (negative), β -glucosidase (negative), n-acetyl- β -glucosaminidase (negative), α -mannosidase (negative) and α -fucosidase (negative). A comparison with the
- 10 external isolates show that the isolates in present study do not vary much in profile for these specific enzymes. However, valine arylamidase was recorded as negative for the external isolates which was the opposite of what was recorded for the isolates in present study. Lipase (C14) was recorded as weakly positive for the
- 15 external isolates in comparison to a negative reaction for the internal isolates.

Alkaline phosphatase was recorded as positive (CA-9, 8079, 8765, 8558, 8465, 8466) or weakly positive (LF-89, 8741, 8670, 817, 1850) for all of the isolates except the external isolate NVI 5692 where Mikalsen et al. (2008), supra, recorded

20 a very weak reaction in one of four replicate tests. Cystine arylamidase was recorded as positive for LF-89 and 8741 whereas recorded as negative for the rest of the isolates. Acid phosphatase was recorded as positive (LF-89, 8741, CA-9, 8079, 8765, 8558, 8465, 8466) or weakly positive (8670, 817, 1850) for all of the isolates

except the external isolate NVI 5910 which is presented as negative. Additionally, the isolate NVI 5910 is presented as negative for Naphthol-AS-BI-phosphohydrolase whereas the rest of the isolates are recorded as positive (LF-89, 8741, CA-9, 817, 1850, 8079, 8765, 8558, 8466) or weakly positive (8670, 8465).

- 5 Interestingly, the isolate 8079 gave a positive (3/5) reaction for β -galactosidase and α -glucosidase while the rest of the isolates gave negative results, making 8079 stand out with a unique profile for these specific enzymes.

Agglutination test

- 10 Agglutination test was performed on the isolates LF-89 and 8079. A volume of 5 μ l of control serum and serum from experimentally *P. salmonis* (isolate ID449) challenged fish (Utne T: Challenge experiment with a putative low virulent Chilean *Piscirickettsia salmonis* field isolate and sea lice (*Lepeophtheirus salmonis*) in Atlantic salmon (*Salmo salar*). Bergen: University of Bergen; 2013) were added to a
- 15 microscopy slide. A loop of bacteria was transferred to 100 μ l PBS and vortexed for 30 sec until a homogenous bacterial suspension was obtained. A 5 μ l sample of the bacterial suspension were added to the fish serum and gently spread out. Additionally, 10 μ l of the bacterial suspension were added to a microscopy slide to be used as a PBS control. The slides were examined in light microscope.

- 20 The isolates LF-89 and 8079 agglutinated in all fish serums tested, including the control serum provided by MSD Animal Health. No agglutination was recorded when using PBS as a negative control.

Other tests

- 25 The isolate disclosed herein was also subjected to antibiotic test, experiments studying the oxidative/fermentative production of acid from glucose, an indole test, a test for oxidase, catalase, and cefinase, respectively, and finally a H_2S test. For these tests, identical results were obtained for all of the isolates disclosed herein.

30

Example 2 Genotypic characterization

DNA extraction

- 35 In order to perform an optimized DNA extraction from a pure culture of *P. salmonis*, E.Z.N.A Tissue DNA extraction kit was used with a modified kit protocol. Pure culture of *P. salmonis* was added to a 1.5 ml Eppendorf tube containing 100 μ l DEPC-water and vortexed for 30 sec to obtain a homogenous mix. First centrifugation step (5 min at 13,000 g) intended to pellet insoluble tissue debris, was not performed when working with the easily soluble colonies from pure
- 40 culture. Elution was performed twice in 50 μ l (10mM Tris, pH=8.5) portions to

increase the overall DNA yield. DNA was taken immediately for evaluation of quality using NanoDrop and then stored in freezer at -20 °C.

Polymerase Chain Reaction (PCR)

- 5 PCR and sequencing was performed using specific primers for the 12 genes included in the study (Table 7). Amplification was based on a standard reaction mixture containing 5 µl 10 X ThermoPol Buffer (BioLabs, New England), 1.5 units (0.3 µl) Taq DNA polymerase (BioLabs, New England), 2.5 mM dNTP (Sigma-Aldrich, U.S.A.) , 10 µM of forward and reverse primer (Sigma-Aldrich, U.S.A.)
- 10 and 2 µl DNA added Nuclease-Free water (Ambion, U.S.A.) or DNase, RNase free water (Sigma-Aldrich, U.K.) to a final solution of 50 µl. Amplification was performed in a GeneAmp PCR system 2700 (Applied BioSystems) at 94 °C for 3 min; 35 cycles of 94 °C for 30 sec (denaturation), 57.5 °C for 30 sec (annealing (T_m)), 72°C for 2 min (synthesis) followed by 72 °C for 8 min.
- 15 A volume of 25 µl of 1-step qPCR ROX mix (Thermo Scientific) was used for some of the isolates (8558, EM8765, EM8670, CA-9, ID817, and ID1850), replacing ThermoPol Buffer, dNTP and Taq DNA polymerase. First PCR step (polymerase activation) was performed at 95 °C for 15 min when using 1-step qPCR ROX mix. PCR product was then visualized by gel electrophoresis and purified in an Arktik
- 20 Thermal Cycler (Thermo Scientific) at 37 °C for 15 min and 80 °C for 15 min, using a mixture of 1 µl ExoSap (Affymetrix®, U.S.A.) added 2,5 µl PCR product.

Table 7 Primers used for PCR analysis in the present study:

Target gene	Primer	Direction	Sequence (5'-3')	Reference
16s rRNA	Eug B27F	Fwd	AGAGTTTGATCMTGGCTCAG	[81]
	Eug A1518R	Rev	AAGGAGGTGATCCANCCRCA	[81]
ITS	SRS-ITS/F	Fwd	GTACACACCGCCCGTCACAC	Present study
	SRS-ITS/R	Rev	CCTCACGGTACTAGTTCACTATCGG	Present study
dnaK	SRS-dnaK/F2	Fwd	CCGTGTCGTGTGGCGCTAAAA	Present study
	SRS-dnaK/R2	Rev	TTGAGATTGAGCCTGCTCCGC	Present study
	SRS-dnaK3/F1	Fwd	CCGCGTGTGATTGAGAGTGC	Present study
	SRS-dnaK3/R1	Rev	CGTCATCACCCCACCCATGG	Present study
groEL	SRS-groEL/F1	Fwd	CTTCGGTACCGGTTCCCGTC	Present study
	SRS-groEL/R1	Rev	TCTTGCACTTTCTCGCGGTCG	Present study
	SRS-groEL/F2	Fwd	GTGAAGCTCTGGCAACACTCGTC	Present study
	SRS-groEL/R2	Rev	AGGAAGCTCTGCAACCATCGC	Present study
tbpB	SRS-tbpB/F1	Fwd	AACTGGGCAGGCGTCACTGTT	Present study
	SRS-tbpB/R1	Rev	CGGCGCGTCTCTAATGTTCTG	Present study
	SRS-tbpB2/F2	Fwd	CCAAGCTGGATCACCGCCAT	Present study
	SRS-tbpB2/R2	Rev	AAAGATAGGCCAGCCACGC	Present study
mltB	SRS-mltB/F	Fwd	ACCACTCACGCGGCATCTAA	Present study
	SRS-mltB/R	Rev	ACTCAAATCATACACCGCCATTGCA	Present study
ospA	SRS-ospA/F	Fwd	AGCCGTCAAGAAGTCGGAGCT	Present study
	SRS-ospA/R	Rev	TGCCAACGACCATCCGCTTG	Present study

radA	SRS-radA/F1	Fwd	ATCAGTCGCCAGCCTGTTGG	Present study
	SRS-radA/FR1	Rev	GTCTCTCGTTGCACTGGACGA	Present study
airA	SRS-air/F1	Fwd	GGGTGCGTCCGGGGATTATG	Present study
	SRS-airA/R1	Rev	TAAGGTGCACGCAGTGGCAT	Present study
bax	SRS-bax/F1	Fwd	TCAAGGGATCTGGGAAGTGCT	Present study
	SRS-bax/R1	Rev	ACCACTGCCTATCTTGCTCAACA	Present study
tnpA	SRS-tnpA/F1	Fwd	ACCTGTTAAGTTCTCGGCCATT	Present study
	SRS-tnpA/R1	Rev	AGCCTTCACAAATGTCAACAAGTGA	Present study
elfP	SRS-elfP/F	Fwd	GCCACKGCTAATTCAGCAA	Present study
	SRS-elfP/R	Rev	STGGAATGGTCAGCCACYT	Present study

Gel electrophoresis

A gel containing 1 % agarose dissolved in 0.5 X Tris-acetate-EDTA (TAE) buffer was made. GelRed™ (Biotium, Inc., U.S.A.), a fluorescent dye used to stain the nucleic acids, was added to the agarose gel before allowing it to harden for 10 min. A volume of 1 µl of loading dye 6x was mixed with 5 µl of PCR product and added to the wells after the addition of 0.5 X TAE buffer to the electrophoresis chamber. A volume of 3 µl Smart ladder (Eurogentec) was used as a marker in the first well. The gel was run for 45 min at 90 Volts before being examined under UV-light in a Gel Logic 212 Pro (Carestream, U.S.A.) machine.

Sequencing

A BigDye® version 3.1 sequence reaction was performed in an Arktik Thermal Cycler, at 96 °C for 5 min; 30 cycles of 96 °C for 10 sec, 58 °C for 5 sec and 60 °C for 4 min, using a mixture of 6 µl deionised water, 1 µl BigDye® Terminator v3.1 Sequencing Buffer (5X) (Invitrogen), 1 µl BigDye® Terminator v3.1 Ready Reaction Premix (2.5X) (Invitrogen), 3.2 pmol (1 µl) forward or reserve primer and 1 µl purified PCR product. A volume of 10 µl of deionised water was added to each sample before delivery to the sequencing facility (Sequencing Facility, BIO at the University of Bergen, Thormøhlensgate 55, 5008 Bergen, Norway) where samples were cleaned with Agencourt CleannSeq (Beckman Coulter, Inc.) before being sequenced in a 96-capillary 3730x1 DNA Analyzer (Applied Biosystems®). Vector NTI® v9.0 Software (Invitrogen) was used following AlignX for analyzing the sequences.

Phylogeny

The phylogeny software PhyML v3.0 (Guindon S, Dufayard JF, Lefort V, Anisimova M, Hordijk W, Gascuel O: New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. Systematic biology 2010, 59(3):307-321) based on the maximum-likelihood principle was used to create an unrooted phylogenetic tree for the isolates included in present study. The default generalized time-reversible (GTR) model (Tavaré S: Some Probabilistic and Statistical Problems in the Analysis of DNA Sequences. In:

American Mathematical Society: Lectures on Mathematics in the Life Sciences. vol. 17: Amer Mathematical Society; 1986: 57-86) was selected using the nearest neighbor interchanges (NNI) and approximate likelihood-ratio test (aLRT) options.

5 *Sequence alignment and percentage identity*

The sequences obtained were aligned in AlignX to visualize and analyze genetic variations. It was apparent that the isolates could be separated into three different groups (G1, G2- and G3/CA-9) based on genetic variation. The following isolates were placed within G1; LF-89, 8741, 8670, 817, 1850 and G2; 8079, 8765, 8558, 8465, 8466. Phylogenetic branches were visualized using PhyML 3.0 (Figure 2) (Guindon et al.: New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. Systematic biology 2010, 59(3):307-321).

15 An overview of variable positions is found in Table 8. A statistics report of percentage identity (PID) was made in GeneDoc (Karl Nicholas ©2000) to determine similarities between the isolates.

Table 8 Overview of the genotypic variables.

Gene	N	No. Nucleotides	Variable positions	%	No. Amino acids	Variable positions	%
16S-ITS concatenated	11	2191	35	1.6	-	-	-
16S	11	1425	13	0.9	-	-	-
ITS	11	766	22	2.9	-	-	-
dnaK	11	1682	64	3.8	560	4	0.7
groEL	11	1429	65	4.5	476	7	1.5
tbpB	11	1879	92	4.9	626	26	4.2
mltB	11	825	16	1.9	274	4	1.5
ospA	11	347	8	2.3	115	2	1.7
radA	11	905	36	4.0	301	4	1.3
alr	11	413	20	4.8	137	5	3.6
bax	11	643	16	2.5	213	8	3.8
tnpA	11	424	1	0.2	141	1	0.7
elfP	11	473	23	4.9	157	0	0.0
Multilocus sequence typing	11	11211	376	3.4	3000	61	2.0

20 Symbols: N, the number of *Piscirickettsia salmonis* sequences.

Comparison of 10 housekeeping sequences from the *P. salmonis* isolates in present study revealed 376 variable nt. positions across 11211 nucleotides (nts). The highest number of variable positions (4.9%) was seen in the *tbpB* and *elfP* locus and lowest (0.2%) in *tnpA*. After the sequences were translated to proteins a total of 61 variable positions were revealed across a total of 3000 amino acids. The highest number of variable amino acid positions (4.2%) was seen in the *tbpB* locus and lowest (0%) in *elfP*. Although showing high genetic variation (4.9%) in nucleotides

for the elFP-gene, it was apparent that these mutations were neutral. PID was recorded to vary from 95 - 100 % when compared to the type-strain.

Table 9: An overview of all results obtained from the phenotypic and genotypic characterization.

Isolate:	LF-89	8741	CA-9	8670	817	1850	8079	8765	8558	8465	8466
Genetic group*	G1	G1	G3	G1	G1	G1	G2	G2	G2	G2	G2
Morphology	Identical 0.4-1.8 µm	Identical 0.4-1.8 µm	Identical 0.4-1.8 µm	Identical 0.4-1.8 µm	Identical 0.4-1.8 µm	Identical 0.4-1.8 µm	Identical 0.4-1.8 µm	Identical 0.4-1.8 µm	Identical 0.4-1.8 µm	Identical 0.4-1.8 µm	Identical 0.4-1.8 µm
Cell size	Slow	Slow	Slow	Slow	Slow	Slow	Fast	Fast	Fast	Fast	Fast
Growth rapidity**	-	+	+	+	+	+	+	+	+	+	+
Growth at 8 °C	+	+	+	+	+	+	+	+	+	+	+
Growth at 11-22 °C	-	-	-	-	-	-	-	-	-	-	-
Growth at 25 °C	+	+	+	+	+	+	+	+	+	+	+
Growth on; SRS-	-	-	-	-	-	-	-	-	-	-	-
BA	+	+	+	+	+	+	+	+	+	+	+
CHAB	+	-	-	-	-	-	+	+	+	+	+
CHAB w/ Fe	+	-	-	-	-	-	+	+	+	+	+
Austral-TSHem	-	-	-	-	-	-	+	+	+	+	+
BA	-	-	-	-	-	-	+	+	+	+	+
BA w/ 2% NaCl	-	-	-	-	-	-	+	+	+	+	+
MA	-	-	-	-	-	-	-	-	-	-	-
FLPA	-	-	-	-	-	-	-	-	-	-	-
API ZYM profile***	Similar NC/NC	Similar NC/NC	Similar NC/NC	Similar NC/NC	Similar NC/NC	Similar NC/NC	Unique NC/NC	Similar NC/NC	Similar NC/NC	Similar NC/NC	Similar NC/NC
TSI profile	+	+	+	+	+	+	+	+	+	+	+
Catalase	-	-	-	-	-	-	-	-	-	-	-
Cytochrome oxidase	-	-	-	-	-	-	-	-	-	-	-
Indole	-	-	-	-	-	-	-	-	-	-	-
β-Lactamase	+	+	+	+	+	+	+	+	+	+	+
H ₂ S production	+	+	+	+	+	+	+	+	+	+	+

Symbols: * genetic group based on the total dataset of housekeeping genes; **, 0-3 days (fast) or 3-< d. (slow) for visible growth to appear;***, profile unique when negative vs. positive (weak reaction vs. positive/negative give similar).

Spectroscopic DNA-DNA Hybridization

Cells were disrupted by using a Constant Systems TS 0.75 KW (IUL Instruments, Germany) and the DNA in the crude lysate was purified by chromatography on hydroxyapatite as described by Cashion et al. (1977), A rapid method for base ratio
 5 determination of bacterial DNA, Anal Biochem 81, 461-466.

DNA-DNA hybridization was carried out as described by De Ley et al. (1970), The quantitative measurement of DNA hybridization from renaturation rates. Eur J
 Biochem 12, 133-142 under consideration of the modifications described by Huss et
 al. (1983), Studies on the spectrophotometric determination of DNA hybridization
 10 from renaturation rates. Syst Appl Microbiol 4, 184-192 using a model Cary 100
 Bio UV/VIS-spectrophotometer equipped with a Peltier-thermostatted 6x6 multicell
 changer and a temperature controller with in-situ temperature probe (Varian).

15 DNA-DNA-hybridization of strain LF (ID8079) against type strain LF89 was
 performed. The following results were obtained:

% DNA-DNA similarity (in 2 X SSC at 65°C)

	strain LF89
strain ID (ID8079)	74.6 (77.2)

(Values in parentheses are results of measurements in duplicate)

20 Strain LF (ID 12-984) belongs to the same species as strain ID (ID 12-985) when
 the recommendations of a threshold value of 70 % DNA-DNA similarity for the
 definition of bacterial species by the *ad hoc* committee (Wayne et al., 1987, Report
 of the *ad hoc* committee on reconciliation of approaches to bacterial systematics. Int
 J Syst Bacteriol 37, 463-464) are considered.

25

Example 3 Challenge experiment

Pilot challenge study A collection of Piscirickettsia isolates were obtained from
 different fish farms in Chile culturing Atlantic salmon (*Salmo salar*), rainbow trout
 (*Oncorhynchus mykiss*) and Coho salmon (*O. kisutch*). All isolates were from fish
 30 showing signs of piscirickettsiosis. A selection of these isolates was used in a pilot
 challenge study of Atlantic salmon kept in fresh water at the facilities of The
 Industrial- and Aquatic Research Laboratory (ILAB) in Bergen. Fish groups kept in
 separate tanks were challenged using intraperitoneal injection (IP) of four different
 field isolates. Each isolate was injected at three different dosages; low, medium and
 35 high. The isolate chosen for this study showed a high virulence in the pilot study
 and is hereafter referred to as isolate ID8079.

The ID8079 isolate were obtained from an aquaculture facility in Chiles region X rearing Atlantic salmon. The facility experienced a disease outbreak with diagnostic signs consistent with SRS. ID8079 had been through seven before being used in the challenge experiment described below. ID8079 was originally isolated from the
 5 liver of moribund fish. The isolate is genetically different from the type strain LF-89 according to 16S gene and housekeeping gene sequences. The isolate were stored in liquid nitrogen (at -196 °C).

Study design

10 The challenge experiment using ID8079 was carried out at the facilities of ILAB, University of Bergen. The fish were kept in 150 liter (L) cubic fiberglass tanks, with lids that allowed some light to penetrate. The light was on for 12 hours a day, and automatically dimmed to simulate sunrise and sunset. Each tank had a constant water flow of approximately 375 L/hour, flowing into the tank above the water
 15 level, and draining off at the bottom through a strainer. To avoid problems with fish jumping out of the tank when opening the lid, approximately 1/3 of the water was flushed out before opening the lid. Fish that jumped out were killed and sampled. Each tank was inspected at least twice a day, and dead and moribund fish were removed. The fish were fed to appetite by the staff at ILAB.

20 Inlet water was pumped from 105 m below surface outside of Nordnes in Bergen. The water was first passed through a drum filter with 20 µm film before being treated with UV-light. Water temperature, salinity and dissolved oxygen were measured from the drain water of each tank daily by ILAB. Before bacterial
 25 challenge (day 0), the water temperature varied between 11,2°C and 12,2°C. During a four days period, at the start of the challenge, the temperature gradually increased to 15°C (±0,5°C), where it was kept throughout the experimental period. The salinity was kept between 29.2 and 32.0‰ until 7 days before the start of the challenge, when it was increased to 34,3‰ (±0,2) and it stayed at this level for the
 30 remaining period. Dissolved oxygen in drain water was measured to be between 68 and 99%, with an average of 87.5 %.

Experimental fish

35 The Atlantic salmon smolts were produced by ILAB from eggs supplied by SalmoBreed AS, and kept at full sea water during the challenge period. The mean weight at the start of the experiment was approximately 50 grams.

Screening

40 Five months before the start of the experiment, the fish were screened by Real-time RT-PCR by PatoGen® for the following pathogens; Infectious Pancreas Necrosis Virus (IPNV), Infectious Salmon Anaemia Virus (ISAV) and Piscine Myocarditis Virus (PMCV) by PatoGen Analyse AS. All fish (n = 30) were negative. At day 0,

gills from 10 fish were screened with real-time RT-PCR, for presence of the pathogens *Piscirickettsia salmonis*, Salmonid Alphavirus (SAV), *Paranucleospora theridion*, *Ichthyobodo* sp., *Epitheliocystis* (*Candidatus Branchiomonas cysticola*), Piscine Reovirus (PRV) and Infectious salmon anaemia virus (ISAV). One of the ten fish was positive for *Paranucleospora theridion*, *epitheliocystis* and PRV with CT values of 33.0, 34.1 and 35.3 respectively. After the development of PSA-assay, kidney and gill tissue from 10 fish at day 0 were tested for the presence of *P. salmonis* using this assay.

Test groups

The groups of fish were challenged in three different ways:

Intraperitoneal injection (IP),

by cohabitation (naïve test fish together with IP injected shedders), and by cohabitation and salmon lice (*Lepeophtheirus salmonis*).

In addition there were two groups acting as negative control: one group with salmon lice and one group without. The control fish were treated in the same manner as the infected groups. They were, however, always the first tanks to be checked for mortality and sampled during sampling days.

Fish in two tanks (KL and HVL) were challenged with copepodids from salmon louse 14 days before the challenge with ID8079. This was carried out by lowering the water level in the tank to a minimum, followed by adding approximately 6600 copepodids (120 per fish). The copepodids were supplied by the Sea Lice Research Center at the University of Bergen, from an in-house strain.

Table 10 Tank setup. Shedder fish were added on the day of *P. salmonis* infection while the test fish was added to the tanks three weeks before bacterial challenge.

Tank	Group	Code	Test fish	Shedders	Total
1	Control	K	55	17	72
2	Control with lice	KL	55	17	72
3	High virulence	HV	55	17	72
4	High virulence with lice	HVL	55	17	72
5	High virulence IP	HVP	55		55
Total			275	68	343

Bacterial challenge

The shedders and the IP group were injected with a 0.1 mL solution of bacteria suspended in phosphate buffered saline (PBS). Doses were estimated based on results from the pilot study and the titers were estimated by plating one dose on SRS-agar. The fish in the HVP group was given eight CFU, and the shedders in the

HV and HVL groups were given 600 CFU. The control shedders were injected with 0.1 mL PBS-solution. The shedders in the control group can be regarded as a negative control for the IP groups. The injection site was about one pelvic fin length in front of the base of the pelvic fins, which comply with the recommendations from MSD Animal Health. Prior to injection, the fish were tranquilized using tricaine mesylate (FINQUEL® vet.). Shedders were marked by clipping off the adipose fin.

Mortality

Mortalities were registered twice a day, and cumulative mortality was calculated using the formula:

$$\% \text{ Cumulative mortality} = \frac{Nd}{Nt - Nw} \times 100$$

where Nd is number of dead fish, Nt is the total number of fish at day 0 and Nw is the number of fish taken for weekly sampling.

Sampling

The original plan was to sample 5 fish at day 7, 14, 21, 28, 42, 56 and 63. However, due to high mortality sampling was not executed as planned. Conducted samplings are listed in table 3 below. The experiment was terminated between day 69 and 72, when all remaining fish were sampled.

Table 11: Overview of conducted samplings. x: sampling, -: no sampling

Sampling day	7	14	21	28	42	56	63
HV	x	x	-	-	-	-	-
HVL	-	x	x	x	-	-	-
HVP	x	x	-	-	-	-	-
K	x	x	x	x	x	x	x
KL	x	x	x	x	x	x	x

The fish were weighed and measured prior to sampling. Clinical and pathological signs were registered during necropsy. All the fish were killed by a blow to the head immediately before sampling to avoid autocatalytic degradation of the tissue. Samples were put directly on ice after extraction, and were later transferred to -30°C. Blood samples were stored in a microcentrifuge tube overnight, before centrifugation (3500 rpm, 4°C, 5 minutes), and transferring of the serum to a new tube, stored at -30°C. Tissue samples for real-time RT-PCR were taken from all fish (in chronological order): Skin/muscle, gill (2nd gill arch), spleen, liver, head kidney, heart (ventricle), CNS (cerebellum) and hindgut. Only head kidney was sampled from the shedders and dead fish. For each tissue a sample of approximately 3.0 mm³ was stored in a microcentrifuge tube at -30°C. A larger sample of the

respective tissues was stored in a Nunc container at -80°C as backup. For the groups with lice, the lice were sampled and stored at -80°C . From selected fish (at least one of the five taken out on the sampling days), samples for histology were taken from the following tissues: Gills (1st gill arch), spleen, liver, head kidney, heart and CNS. The samples were immediately placed in Karnovsky fixative and stored at 4°C .

The major clinical and pathological findings are summarized in table 12 below:

Table 12: Overview of mortality and different clinical and pathological signs observed in this trial. All numbers are presented as percentage prevalence in each group.

	Group				
	HV	HVL	HVP	K	KL
Mortality	100.0	100.0	100.0	25.8	8.5
Lower lip lesion	0.0	18.2	0.0	2.2	18.8
Exophthalmia	3.7	1.8	1.8	0.0	0.0
Skin lesions	7.4	7.3	3.6	10.9	8.3
Bleeding eye	18.5	5.5	16.4	0.0	0.0
Petechia jaw	7.4	3.6	7.3	4.3	0.0
Pale gills	9.3	5.5	5.5	0.0	0.0
Petechia skin	20.4	9.1	5.5	6.5	6.3
Fin erosion	42.6	16.4	31.0	54.3	39.6
Petechia adipose tissue	0.0	1.8	5.5	0.0	0.0
Petechia muscle	37.0	12.7	20.0	0.0	0.0
Swollen spleen	63.0	23.6	52.7	13.0	12.5
Granulated spleen	13.0	0.0	5.5	4.3	4.2
Foci in liver	51.9	27.3	56.4	0.0	0.0
Haemorrhagic foci in liver	3.7	0.0	1.8	0.0	0.0
Fecal casts	14.8	14.5	7.3	4.3	6.3
Bloody hindgut	9.3	3.6	5.5	4.3	4.2
	n = 55	n = 55	n = 55	n = 55	n = 55

The most prevalent signs observed in test groups in this experiment include white/grey circular foci in liver varying in size, swollen spleen, petechia in muscle and haemorrhage in the eye.

A high prevalence of fin erosion was observed in all groups including controls. In the two groups infected with salmon lice (HVL & KL), a higher prevalence of lower lip lesions (cf. table 12) was observed.

The mortality in all challenge groups reached 100%, see figure 3.

Both the IP-group (HVP) and the cohabitant without sea lice group (HV) had a mortality period which proceeded rapidly to >90%. The cohabitant with sea lice group (HVL) experienced a 64% cumulative mortality within the first 8 days after the addition of shedders. The cumulative mortality in control groups were 25% in the group with sea lice (KL) and 8.5% in the group without sea lice (K) at the end of the trial.

Taking in to consideration the formula used and the number of samplings in the control groups compared with the challenge groups (cf. table 1), the cumulative mortality in controls quickly reaches a high percentage as a result of a relatively high number of fish taken out for weekly samplings.

Example 4 Immunization trial

In order to investigate the Immunization abilities of the *Piscirickettsia salmonis* isolate ID 8079 the bacteria were used in a vaccine trial. The bacteria was inactivated and formulated into a vaccine by known methods and transferred to the Atlantic salmon by intraperitoneal injection, in order to produce an immune response. The test fish was subsequently challenged with the *P. salmonis* isolate ID 8079. The appropriate number non-immunized control fish was also challenged with the same isolate and worked as a positive control.

During the challenge experiment, mortality and clinical signs was monitored in all groups and samples for qPCR and bacteriology was taken of all mortalities and moribund fish. An appropriate number of fish was sampled to measure immune response by antibody response.

The challenge experiment lasted until the positive control group reached the appropriate mortality.

BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

Cermaq ASA
Grev Wedels plass 5
P.O. Box 144, Sentrum
0102 Oslo
Norway

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT
issued pursuant to Rule 7.1 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR: ID8079	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: DSM 27338
II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION	
The microorganism identified under I. above was accompanied by: () a scientific description (X) a proposed taxonomic designation (Mark with a cross where applicable).	
III. RECEIPT AND ACCEPTANCE	
This International Depositary Authority accepts the microorganism identified under I. above, which was received by it on 2013-06-06 (Date of the original deposit) ¹ .	
IV. RECEIPT OF REQUEST FOR CONVERSION	
The microorganism identified under I. above was received by this International Depositary Authority on (date of original deposit) and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on (date of receipt of request for conversion).	
V. INTERNATIONAL DEPOSITARY AUTHORITY	
Name: Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures Address: Inhoffenstr. 7 B D-38124 Braunschweig	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s): Date: 2013-06-19

¹ Where Rule 6.4 (i) applies, such date is the date on which the status of international depositary authority was acquired.

BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE



INTERNATIONAL FORM

Cermaq ASA
Grev Wedels plass 5
P.O. Box 144, Sentrum
0102 Oslo
Norway

VIABILITY STATEMENT
issued pursuant to Rule 10.2 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

I. DEPOSITOR	II. IDENTIFICATION OF THE MICROORGANISM
Name: Cermaq ASA Grev Wedels plass 5 Address: P.O. Box 144, Sentrum 0102 Oslo Norway	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: DSM 27338 Date of the deposit or the transfer: 2013-06-06
III. VIABILITY STATEMENT	
The viability of the microorganism identified under II above was tested on 2013-06-10 ¹ . On that date, the said microorganism was ☒ ² viable ☐ ³ no longer viable	
IV. CONDITIONS UNDER WHICH THE VIABILITY TEST HAS BEEN PERFORMED ⁴	
V. INTERNATIONAL DEPOSITARY AUTHORITY	
Name: Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures Address: Inhoffenstr. 7 B D-38124 Braunschweig	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s):  Date: 2013-06-19

¹ Indicate the date of original deposit or, where a new deposit or a transfer has been made, the most recent relevant date (date of the new deposit or date of the transfer).

² In the cases referred to in Rule 10.2(a) (ii) and (iii), refer to the most recent viability test.

³ Mark with a cross the applicable box.

⁴ Fill in if the information has been requested and if the results of the test were negative.

CLAIMS

1. A *Piscirickettsia salmonis* (*P. salmonis*) isolate ID8079 deposited under the Budapest Treaty with the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (DSMZ) on the 6th of June 2013 under deposit number DSM 27338.
2. A vaccine that induces protection against *P. salmonis* infection in fish comprising the *P. salmonis* isolate according to claim 1.
3. The vaccine according to claim 2 comprising an inactivated or attenuated form of the isolate according to claim 1.
4. The vaccine according to claim 2 or claim 3, further comprising one or more pharmaceutically acceptable carriers.
5. The vaccine according to any one of claims 2 to 4, further comprising an adjuvant.
6. The vaccine according to any one of claims 2 to 5, further comprising one or more biological agents useful in providing protection against other fish pathogens than *P. salmonis*, wherein the biological agent is selected from the group consisting of whole cells, killed and/or attenuated virus, protein extracts isolated from a fish pathogen other than *P. salmonis*, isolated or recombinant antigens, DNA vaccines, and mixtures thereof.
7. The vaccine according to claim 6, wherein the whole cells are killed and/or attenuated bacteria.
8. The vaccine according to claim 6 or claim 7, wherein said fish pathogens other than *P. salmonis* are
 - a) live, attenuated or inactivated fish virus selected from the group consisting of Infectious Hematopoietic Necrosis Virus (IHNV), Infectious Pancreatic Necrosis Virus (IPNV), Infectious Salmon Anemia Virus (ISAV), Salmon Pancreatic virus (SAV), Viral

Hemorrhagic Septicemia Virus (VHSC), and Cardiomyopathy Syndrome Virus (CMSV);
or

b) live, attenuated or inactivated bacteria selected from the group consisting of *Aeromonas sp.*, *Bifidobacterium sp.*, *Brevibacterium sp.*, *Cytophaga sp.*, *Edwarsiella sp.*, *Flavobacterium sp.*, *Francisella sp.*, *Lactococcus sp.*, *Leuconostoc sp.*, *Listonella sp.*, *Moritella viscosa*, *Mycobacterium sp.*, *Nocardia sp.*, *Pediococcus sp.*, *Photobacterium damsel*, *Pseudomonas sp.*, *Renibacterium sp.*, *Rhodococcus sp.*, *Streptococcus sp.*, *Vibrio sp.*, and *Yersinia sp.*.

9. The vaccine according to any one of claims 2 to 8, wherein the vaccine is formulated for administration to fish by a route selected from the group consisting of intraperitoneal injection, intramuscular injection, immersion, bath and oral administration.
10. The vaccine according to claim 9, wherein the vaccine is formulated for administration to a salmonid.
11. The *P. salmonis* isolate according to claim 1 for use in a pharmaceutical formulation.
12. The *P. salmonis* isolate according to claim 1 for use in a vaccine that induces protection against *P. salmonis* infection in fish.
13. Use of the vaccine according to any one of claims 2 to 10 for preventing *P. salmonis* infection in a fish.

Figure 1

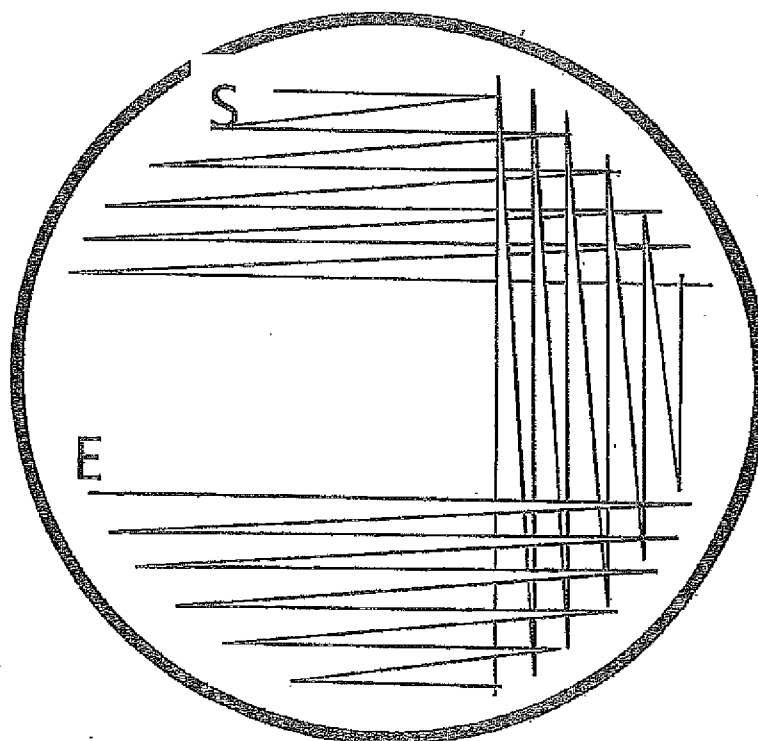


Figure 2

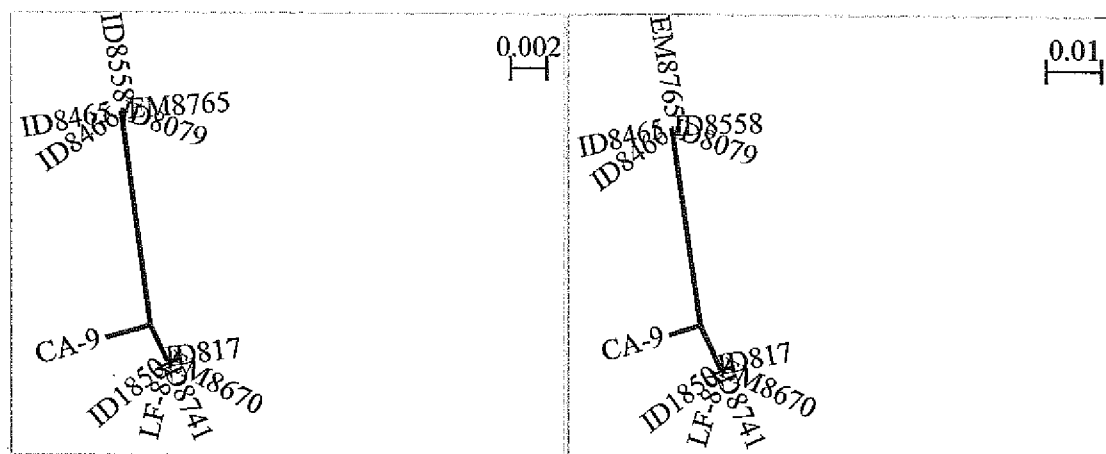


Figure 3

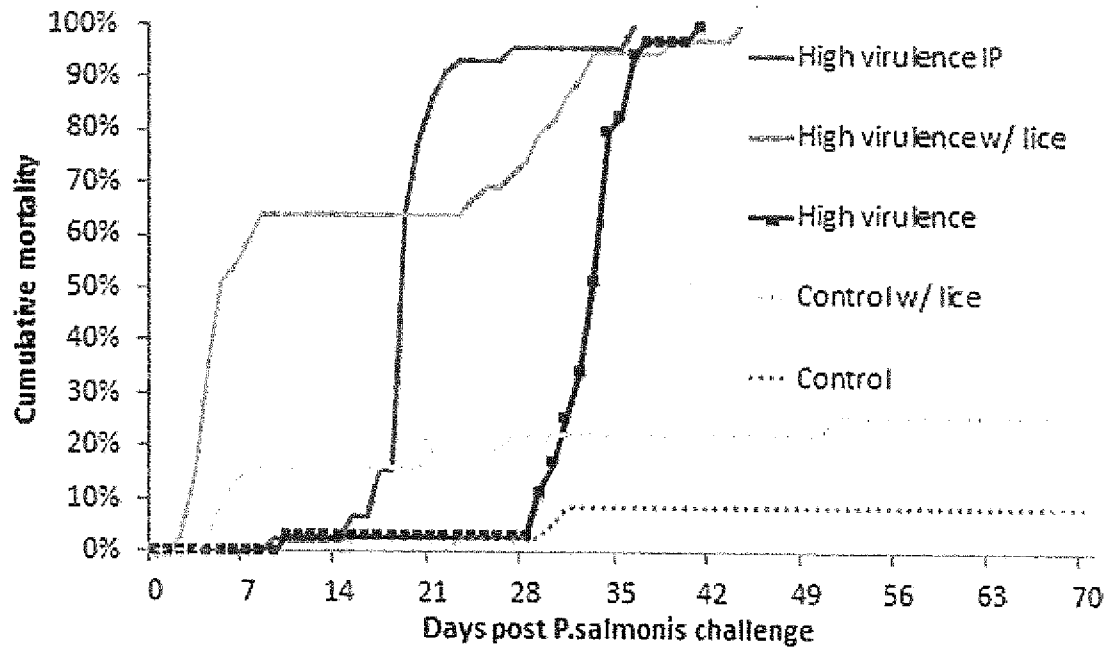


Figure 4

