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Strawberry disease lesions in rainbow trout from southern Idaho are associated with DNA from a *Rickettsia*-like organism

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ABSTRACT: Strawberry disease (SD) in the USA is a skin disorder of unknown etiology that occurs in rainbow trout *Oncorhynchus mykiss* and is characterized by bright red inflammatory lesions. To identify a candidate bacterial agent responsible for SD, we constructed 16S rDNA libraries from 7 SD lesion samples and 2 apparently healthy skin samples from SD-affected fish. A 16S rDNA sequence highly similar to members of the order *Rickettsiales* was present in 3 lesion libraries at 1%, 32% and 54% prevalence, but this sequence was not found in either healthy tissue library. Based on phylogenetic analysis, this *Rickettsia*-like organism (RLO) sequence is most closely related to 16S rDNA sequences of bacteria that may form a novel lineage within the *Rickettsiales*. We used nested PCR assays to screen 25 SD-affected fish for RLO or *Flavobacterium psychrophilum* DNA. Sixteen lesion samples were positive for the RLO sequence and 4 of the matched healthy samples were positive resulting in a significant association between SD lesions and presence of RLO DNA. While *F. psychrophilum* is reportedly associated with 'cold water strawberry disease' in the UK, we found no significant association between SD lesions and the presence of *F. psychrophilum* DNA. The statistical association between SD lesions and presence of RLO DNA is not proof of etiology, but these data suggest that RLO may play a role in SD in southern Idaho, USA.

KEY WORDS: Strawberry disease · *Oncorhynchus mykiss* · *Rickettsia* · 16S rDNA library · CWSD · WWSD · RMS

INTRODUCTION

Strawberry disease (SD) is a skin disorder of unknown etiology that occurs in rainbow trout *Oncorhynchus mykiss* in the USA. SD is characterized by bright red, raised inflammatory lesions that occur mainly in market-sized fish (Olson et al. 1985, Oman 1990) (Appendix 1, Fig. A1, available at: www.int-res.com/articles/suppl/d082p111_app.pdf). Although the disease is self-limiting within 10 wk and causes no changes in weight gain or behavior, morbidity rates can be as high as 80% (Olson et al. 1985). The unsightly lesions lead to product downgrade or rejection at rates of 50 to 75% at aquaculture facilities in southern Idaho, USA (Erickson 1969, Oman 1990). While SD was first described in Washington state, USA in the 1950s this condition has been recognized throughout the western USA (Olson et al. 1985). Very similar conditions, also with unknown etiology, have been reported in Europe including 'warm water strawberry disease' (WWSD) in the UK and France, and a recently described 'red mark syndrome' (RMS), or 'cold water

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strawberry disease' (CWSD) in the UK (Fleury et al. 1985, Ferguson et al. 2006, Verner-Jeffreys et al. 2008).

Results from Idaho producer surveys show no consistent management practices or facility, diet, or water conditions that predispose trout to SD, although stress is thought to aggravate the condition (Olson et al. 1985, Oman 1990). While previous surveys did not control for potentially confounding variables, there is evidence that SD is caused by a transmissible agent. Oman (1990) reported some success in transmitting the condition by experimental inoculation with SD lesion homogenate. Verner-Jeffreys et al. (2008) were able to demonstrate repeatable transmission of RMS/CWSD by cohabitation. Oral treatment with oxytetracycline (OTC) is used to manage the disease at some farms and is thought to reduce recovery time by as much as 50% (Erickson 1969, Olson et al. 1985, Oman 1990). Limited transmission studies and apparent response to chemotherapeutic treatment (OTC) are consistent with the hypothesis that SD results from a primary or secondary bacterial infection. Consequently, we investigated the bacterial community associated with SD lesions by constructing and comparing 16S rDNA libraries from SD lesions and matched healthy skin samples from SD-affected fish.

MATERIALS AND METHODS

Sample collection. Fish were sampled from 4 different trout farms in southern Idaho, USA from 2006 to 2007 (see Table 1). Farms A, C and D are operated by the same company. SD-affected fish were identified by farm staff and isolated 24 to 48 h prior to sampling. Fish were euthanized in tricaine methanesulfonate (MS-222, 200 mg l^{-1}, Argent Chemical Laboratories) and sections of lesion and surrounding healthy skin were removed from the skin surface down to and including underlying muscle, which appeared normal. Samples were stored in either 95% ethanol or in 10% neutral buffered formalin. Apparently healthy skin and underlying muscle were also collected from a site corresponding to the lesion on the opposite flank or distal to the lesion on the same flank.

Histology. Formalin-fixed skin samples were trimmed and dehydrated through graded ethanol processing and embedded in paraffin wax blocks for histological analysis. Paraffin wax blocks were sectioned at 4 µm and stained with hematoxylin and eosin (Washington Animal Disease Diagnostic Laboratory). Lesion severity was classified by level of inflammation: 0, no inflammation; 1, inflammation in stratum spongiosum; 2, inflammation in stratum spongiosum and stratum compactum; 3, inflammation in dermis, extension into subcutis and muscle, with or without ulceration; 3+, 3 with extensive inflammatory infiltrate and ulceration.

DNA extraction. Tissue samples (2 mm^3) were collected from the center or margin of SD lesions and total DNA was extracted using a Qiagen DNeasy Tissue kit with a modified protocol. Briefly, ethanol-stored lesion sections were washed twice in sterile 1× PBS (phosphate buffered saline) and macerated with a sterile microtube pestle (USA Scientific) in 180 µl ATL buffer. Proteinase K (40 µl of 20 mg l^{-1} solution) was added and tubes were vortexed and then incubated overnight at 60°C followed by addition of 20 µl (20 mg l^{-1}) of Proteinase K and incubation for 2 to 4 h at 65°C. DNA was eluted in 100 µl filter-sterilized water, quantified using a Nanodrop 1000 spectrophotometer (Nanodrop Technologies), and stored at –20°C.

16S rDNA library construction. We used universal 16S PCR to amplify the population of bacterial 16S rDNA sequences from extracted samples. Each PCR reaction (50 µl) included 1 U of Platinum High Fidelity *Taq* polymerase (5 U $µl^{-1}$, Invitrogen) and associated 1× PCR buffer, 2 mM $MgSO_4$, 0.2 mM of each dNTP, 0.4 µM of forward and reverse primers (eubacterial primers 20F, 5′-AGA GTT TGA TCA TGG CTC AG-3′, Weisberg et al. 1991; and 517R, 5′-ATT ACC GCG GCT GCT GG-3′, Muyzer et al. 1993), and 250 ng template DNA. Thermal cycling conditions followed a touchdown protocol as follows: initial denaturation for 2 min at 95°C; 15 cycles of 95°C for 30 s, 60.6°C for 30 s decreasing 0.5°C every cycle after the first, extension at 68°C for 45 s; 15 cycles with annealing temperature of 53.6°C for 30 s; final extension at 68°C for 10 min. To facilitate TA cloning, terminal adenines were incorporated at the 3′ ends of PCR products by addition of 1 U *Taq* polymerase (Fisher Scientific) and incubation at 72°C for 10 min. These products were then cleaned using Qiaquick PCR Purification spin columns (Qiagen) and the products were cloned into a pCR4 vector (TOPO TA Cloning Kit for Sequencing; Invitrogen) and transformed into TOP10 cells. Transformants were picked into 96-well plates containing Luria broth (LB), 100 mg l^{-1} ampicillin, and 12.5% sterile glycerol, and stored at –80°C. Three replicates of each plate were made; one without glycerol served as PCR template.

Sequencing and analysis of 16S rDNA libraries. Cloned inserts were PCR amplified from crude lysates using M13 primers. Reactions (25 µl) consisted of 0.5 U *Taq* polymerase (Fisher Scientific) and associated 1× reaction buffer, 2.5 mM $MgCl_2$, 0.2 mM each dNTP, 0.4 mM each primer, and 1 µl of lysed library clones. Lysate was prepared by repeated freeze-thaw cycles. Thermal cycling conditions included initial denaturation at 95°C for 5 min, 35 cycles of 95°C for 30 s, 55°C for 1 min, and 72°C for 1 min, with a final extension at 72°C for 10 min. PCR products were electrophoresed on a 1% agarose gel to confirm the presence of 1 band at ca. 700 bp. Plates were shipped to Functional Bio-

sciences (Madison, Wisconsin, USA) for clean-up and sequencing. Resulting trace files were imported into Sequencher (Gene Codes) for vector trimming and manual inspection of base calls. Sequences were submitted to MEGABLAST (Zhang et al. 2000) to identify the closest match to existing 16S rDNA sequences in GenBank. Sequences having matches with E-scores $>10^{-2}$ were considered unidentified. The E-score (expect score) is the probability that a sequence in a database matches the query sequence by chance. E-scores close to zero are considered significant.

Recovery of *Rickettsia*-like organism (RLO) 16S rDNA. A segment of 16S rDNA sequence was recovered from an RLO using an RLO-specific forward primer (RLO1, 5′-ATC GCT ACA AGAC GAG CCC ATG CAA-3′, this study) and a eubacterial reverse primer (1541R, 5′-AAG GAG GTG ATC CAN CCR CA-3′, Suzuki & Giovannoni 1996). The recovered sequence was aligned with the sequence obtained from the libraries to give the final 1276 bp sequence. The PCR reaction (50 µl) included 2 U of Platinum High Fidelity *Taq* polymerase (Invitrogen) and associated 1× reaction buffer, 2 mM $MgSO_4$, 0.2 mM of each dNTP, 0.4 mM of each primer, and 500 ng template. Thermal cycling conditions included initial denaturation at 94°C for 2 min, 30 cycles of 94°C for 30 s, 57°C for 30 s, 68°C for 1 min and a final extension at 68°C for 10 min. Addition of 3′ adenosine overhangs, cleaning of PCR products, cloning, and PCR amplification of cloned inserts were performed as described above. PCR products were sent to Amplicon Express (Pullman, Washington, USA) for clean-up and sequencing. Trace files were processed and submitted to MEGABLAST as described above. The recovered sequence was assigned GenBank accession number EU555284.

Phylogenetic analysis of *Rickettsiales* 16S rDNA sequences. 16S rDNA sequences for representative species of the order *Rickettsiales* were acquired from GenBank. Sequences were imported into MEGA 4.0 (Tamura et al. 2007) and aligned using ClustalW (Thompson et al. 1994). Phylogenetic trees were generated from this alignment in MEGA 4.0 using UPGMA with bootstrap values from 500 iterations. Evolutionary distances were calculated using the maximum composite likelihood method with complete gap removal; 1123 positions were used in the analysis.

Nested PCR for detection of RLO and *Flavobacterium psychrophilum* 16S rDNA sequences. The external PCR reaction for both assays included universal 16S rDNA primers 20F (5′-AGA GTT TGA TCA TGG CTC AG-3′, Weisberg et al. 1991) and U1510R (5′-GGT TAC CTT GTT ACG ACT T-3′, Lane 1991). Nested PCR reactions included two RLO-specific primers (RLO1, 5′-ATC GCT ACA AGA CGA GCC CAT GCA A-3′; RLO2, 5′-TAT TAC CGC GGC TGC TGG CA-3′) or previously published primers specific for *F. psychrophilum* 16S rDNA (Toyama et al. 1994). The external reaction mixture was the same for both nested assays. This included a 25 µl reaction volume with 0.5 U of *Taq* polymerase (Fisher Scientific) and associated 1× PCR buffer, 1.0 mM $MgCl_2$, 0.1 mM of each dNTP, and 0.4 mM of each primer. Template was 250 ng of total DNA extracted from lesions and apparently healthy skin as described above. The internal reaction for the RLO-specific assay used the same reagent concentrations as the external reaction except that 1 µl of the external reaction product was used as template. The internal reaction for *F. psychrophilum* followed previously published conditions (Wiklund et al. 2000) and included 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, and 1.5 U of *Taq* polymerase. Primer concentration was increased from 0.2 µM to 0.4 µM. Thermal cycling conditions were the same for external and internal reactions for both assays, with an initial denaturation at 95°C for 2 min followed by 35 cycles of 95°C for 30 s, 57°C for 30 s, 72°C for 90 s (external reaction) or 60 s (internal reaction) and a final extension at 72°C for 10 min. Fisher's exact probability test or chi-square test were used to test for a nonrandom association between either *F. psychrophilum* or RLO 16S rDNA products and SD lesions.

RESULTS

Gross and histological observations

Gross and histological characteristics of SD lesions from the present study were consistent with those described by Olson et al. (1985) except for 2 samples of early lesions that showed inflammation beginning in the stratum spongiosum (Table 1; Appendix 1, Figs. A1 & A2). Apparently healthy skin samples from 5 of the SD-affected fish and 5 raceway controls also had inflammation in the stratum spongiosum (data not shown). Of the lesions examined in this study, 84% (21/25) had extensive inflammation on histological examination and were scored as 3 or 3+ (Table 1). Inflammation was evident for lesions of varying severity (revealed by gross examination; data not shown). The 4 lesion samples graded as 1 or 2 had pigment and scale loss with minimal swelling, consistent with early lesions (Olson et al. 1985).

Identification of bacterial sequences from 16S rDNA libraries

Both lesion and healthy skin libraries included a similar representation of organisms at the phylum and subphylum levels (Fig. 1). In the phylum *Firmicutes*,

Table 1. *Oncorhynchus mykiss.* Rainbow trout skin samples and nested PCR results. Farms A to D are located in southern Idaho. Lesion: used as template for lesion tissue library; Healthy: used as template for healthy tissue library. Rickettsia-like organism (RLO) and *Flavobacterium psychrophilum* (*F. psych.*) detected in paired samples (lesion and healthy) from the same fish by nested PCR. Inflammation score: 0: no inflammation; 1: inflammation in stratum spongiosum; 2: inflammation in dermis; 3: inflammation in dermis, subcutis and muscle with or without ulceration; 3+: 3 with extensive infiltration of inflammatory cells and ulceration

Fish no.	Used for 16S rDNA library (no. of sequences recovered)	RLO detected lesion/healthy	*F. psych.* detected lesion/healthy	Inflammation score
Farm A				
1	Lesion (88)	+/–	–/–	3
2	Lesion (131), healthy (82)	+/–	+/–	3
3		–/–	–/–	3
4		–/–	–/–	3
9		+/–	–/–	3
10		+/+	+/–	3
Farm B				
11	Lesion (72)	+/+	–/–	3
12		+/+	+/–	3
13	Lesion (68)	+/+	–/–	3
14		+/–	–/–	3
15		+/–	–/–	1
16		–/–	–/–	3
19	Lesion (94), healthy (92)	–/–	–/–	1
Farm C				
21		+/–	+/–	3
22	Lesion (80)	–/–	–/–	3
23		–/–	–/–	3+
24		+/–	–/–	3+
25	Lesion (69)	+/–	–/–	2
26		–/–	–/–	2
Farm D				
31		+/–	–/–	3
32		–/–	–/–	3+
33		+/–	–/–	3
34		+/–	–/–	3+
35		+/–	–/–	3
36		–/–	–/–	3

there was a dominant sequence that shared 96% identity with an uncultured *Mycoplasma* species present in 2 lesion libraries and 1 healthy skin library at 2%, 89% and 4% prevalences, respectively. The subphylum *Alphaproteobacteria* was represented mostly by a *Rickettsia*-like sequence (hereafter referred to as RLO). The RLO sequence was present in 3 lesion libraries at 1%, 32% and 54% prevalences, but was not found in either healthy tissue library. No other sequences were dominant in lesion libraries and absent in healthy libraries. A few sequences belonging to the phylum *Bacteroidetes* were present in either the lesion or healthy libraries. *Flavobacterium psychrophilum* sequences were found in only 1 lesion library at 2% prevalence. Both lesion and healthy libraries included sequences belonging to various uncultured and unidentified bacteria (Fig. 1; Appendix 2, Table A1).

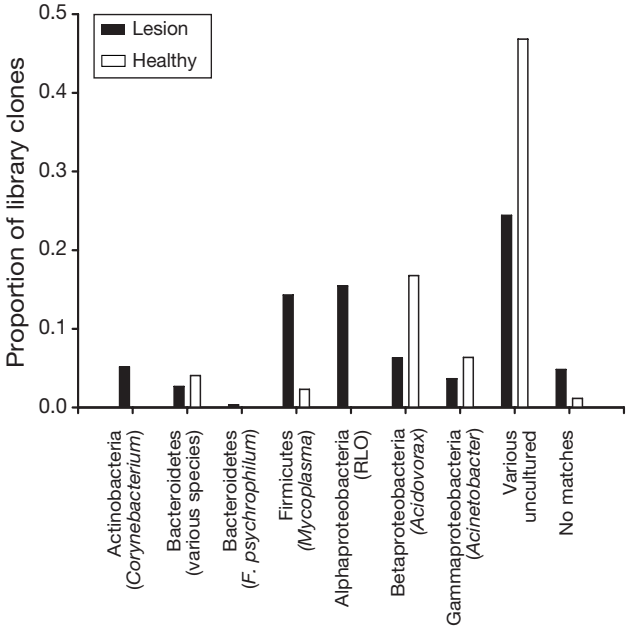

Fig. 1. Proportion of dominant bacteria recovered from 16S rDNA libraries generated from lesions (n = 7) or from healthy skin (n = 2). *F.*: *Flavobacterium*

Phylogenetic analysis of RLO 16S rDNA sequence

A near full-length 16S rDNA sequence was recovered, and phylogenetic analysis indicates a relatively close match with other members of the order *Rick-*

ettsiales (Fig. 2). The RLO sequence from the lesion libraries appears most closely related to a 16S rDNA sequence of a *Rickettsiales* bacterium recovered from ixodid ticks (Fig. 2). Other phylogenetic models and distance methods generated trees with similar topologies (data not shown).

Detection of RLO and *Flavobacterium psychrophilum* 16S rDNA

Sixteen of 25 lesion samples and 4 matched healthy samples were positive for the RLO sequence, resulting in a significant association between SD lesions and presence of RLO DNA ($p < 0.001$, chi-square test). RLO DNA was detected in apparently healthy samples only in those fish whose lesions were also RLO positive (Table 1). Only 4 lesion samples and no healthy samples were positive for *Flavobacterium psychrophilum* 16S rDNA; there was no significant association between SD lesions and the presence of *F. psychrophilum* DNA ($p = 0.06$, Fisher's exact test) although these *F. psychrophilum*-positive samples were also positive for RLO (Table 1).

DISCUSSION

We constructed 16S rDNA libraries from SD lesions and healthy fish skin to identify potential bacterial pathogen(s) associated with SD. 16S rDNA libraries are particularly useful for this purpose because sequence recovery is not dependent on culturing organisms, although the relative proportions of different sequences is not necessarily representative of the original template abundance (owing to potential bias in DNA recovery and template amplification). Thus, proportional estimates discussed herein are preliminary and require more quantitative methods for confirmation. Nevertheless, 3 sequences were dominant in lesion libraries, with a *Rickettsia*-like sequence (RLO) dominant in 2 lesion libraries and present in a third lesion library, but absent in both healthy libraries. These results were consistent with our preliminary 16S rDNA libraries made from swabs of SD lesions in which the same RLO sequence was present in 5 of 14 lesion-derived sequences and absent in the 19 sequences from apparently healthy raceway controls (data not shown).

Members of the order *Rickettsiales* have an obligate intracellular lifestyle and are generally susceptible to tetracyclines (Yao & Moellering 2003). These properties are consistent with an inability to culture the SD agent using conventional bacteriological or cell culture media, and with ancillary reports that the condition responds to OTC treatment. *Rickettsia* or RLOs have been associated with fish, although none is known to cause a specific fish disease (Fryer & Mauel 1997). Several members of *Rickettsiales* are important human

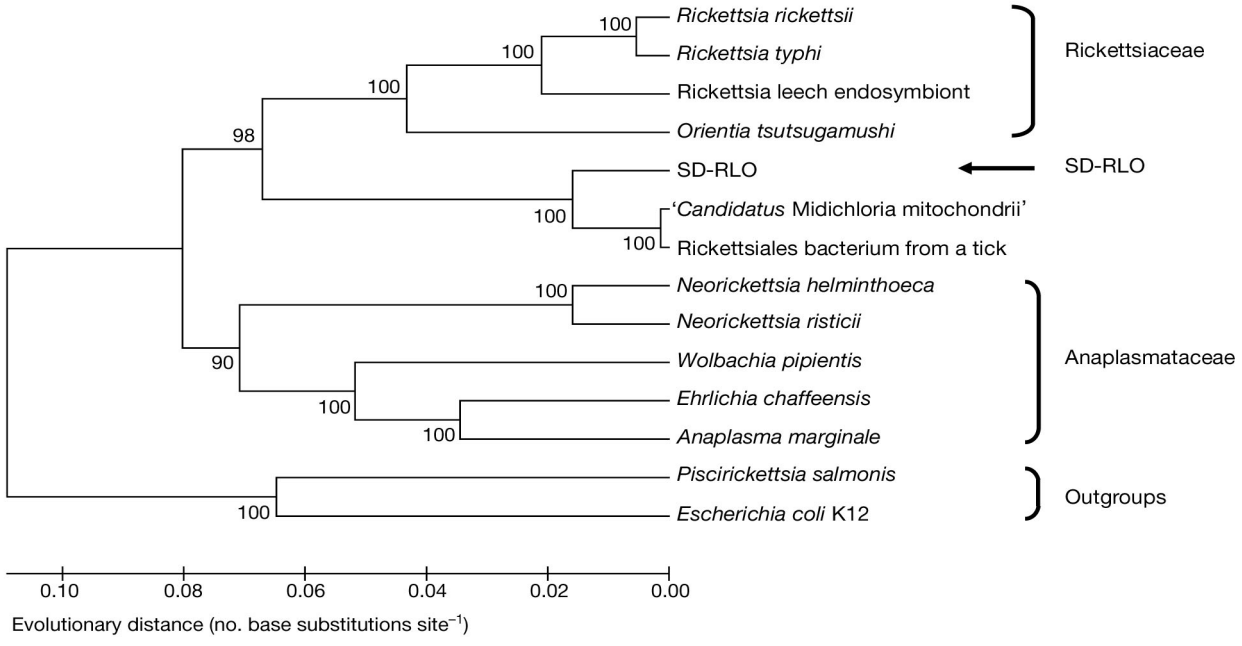

Fig. 2. Phylogenetic tree of *Rickettsia*-like organism associated with strawberry disease lesions (SD-RLO) and representative members of the order *Rickettsiales* (see 'Materials and methods' for details). GenBank accession numbers used in this analysis (from top to bottom): L36217, I36221, AB66351, D38625, EU555284 (this study), AJ566640, AF525482, NHU12457, AF037211, AF179630, AF1147752, AY077769, AY498637, and NC_00913

and animal pathogens, some of which can cause skin manifestations such as maculopapular rash and eschars, which are necrotic wounds at the site of tick bites (Parola et al. 2005). In most cases, *Rickettsia* are associated with arthropod vectors; however, members of this order have diverse and complex life cycles (Perlman et al. 2006). Rickettsial or *Rickettsia*-like organisms have been found as endosymbionts in amoebae, leeches and trematodes, and as pathogens or potential pathogens in bivalves and corals. Fryer & Mauel (1997) reported several cases of unidentified RLOs observed in, or isolated from, diseased fish or fish cell lines. These RLOs were found in marine and freshwater species throughout the world. Many examples have since been identified as strains of *Piscirickettsia salmonis*, an intracellular fish pathogen that is phenotypically similar to *Rickettsia*, but genetically unrelated. Specific examples of fish-associated RLOs include an endosymbiont of the fish-pathogenic amoeba species *Nuclearia simplex*, which infects the gills and other organs of *Rutilus rutilus* (carp family) (Perlman et al. 2006). In addition, salmon poisoning in dogs is caused by *Neorickettsia helminthoeca*; the bacterium is associated with parasitic trematodes in salmon. Wild rainbow trout have also been reported to harbor 16S rRNA sequences with 95% identity to *Neorickettsia risticii*, the causative agent of Potomac horse fever (Pusterla et al. 2000). Thus, while no RLOs are known to cause fish disease, RLOs are present in aquatic environments and are associated with fish.

Phylogenetic analysis of a 1276 bp segment of the SD-RLO 16S rDNA sequence placed this sequence within the order *Rickettsiales* and positioned closest to the family *Rickettsiaceae*. Small subunit ribosomal DNA sequences with close identity to this lineage have been detected by PCR in several ixodid tick species, amoebae, humans and microbial mats (Sassera et al. 2006). One of these sequences, '*Candidatus* Midichloria mitochondrii' (formerly Iric ES1), has been visualized within the mitochondria of ovarian cells in the tick *Ixodes ricinus* using electron microscopy and *in situ* molecular hybridization (Beninati et al. 2004, Sassera et al. 2006). It is unlikely that the RLO detected in the SD lesion libraries is associated with ticks, which are terrestrial; however, the detection of related 16S sequences in amoebae and microbial mats provides a more likely habitat for the RLO within an aquatic environment (Sassera et al. 2006).

Flavobacterium psychrophilum has been proposed as a potential etiologic agent for RMS/CWSD in Scotland (Ferguson et al. 2006). RMS/CWSD is a condition very similar to SD and was first distinguished in the UK in 2003 (Verner-Jeffreys et al. 2008). RMS/CWSD purportedly differs from SD by the presence of heterophils within established lesions in connective tissues found between the epidermis and dermis, and dermis and subcutis, in addition to involvement of organs other than the skin, including pathological changes in the liver, kidney and spleen as well as exopthalmia, myocarditis and skeletal deformities (Bruno et al. 2007, Verner-Jeffreys et al. 2008). Warm water strawberry disease (WWSD), which has long been known simply as 'strawberry disease' in the UK and France, is potentially a condition different from both SD and RMS/CWSD in that it affects fish reared at temperatures >15°C and may be responsive to vitamin C (Ferguson et al. 2006, Verner-Jeffreys et al. 2008). Interestingly, a survey of Idaho trout producers found SD outbreaks occurring at temperatures ranging from 8.8°C to 21°C (Oman 1990). Olson et al. (1985) also reported no correlation between water temperature and SD, and all the farms sampled for present study use constant temperature (14.5°C) spring water. More information is required on all 3 of these conditions to determine whether described pathological differences are due to distinct diseases or a reflection of other confounding variables such as trout lines, management or other environmental factors.

Verner-Jeffreys et al. (2008) also used a 16S rDNA library approach to identify a bacterial agent associated with RMS/CWSD; however, the authors did not detect the RLO sequence described here. Universal primers are often not truly universal; the primers used in the present study matched with 5 times more *Rickettsiales* sequences than those used by Verner-Jeffreys et al. (2008), as determined by ProbeMatch (Cole et al. 2007). In addition, the universal primers used by Verner-Jeffreys et al. (2008) had 3 mismatches at the 5′ end of the forward primer and 2 mismatches on the reverse primer compared to the RLO sequence we detected, whereas the primers in our study had only one mismatch in the forward primer. Thus, either the RLO is not present in the RMS/CWSD lesions, or the primers used in that study (op. cit.) were insufficiently matched to amplify the RLO template. The nested assay described in the present study could quickly address this question.

Only 4 SD lesion samples and none of the healthy skin samples in our study were positive for *Flavobacterium psychrophilum* DNA ($p = 0.06$, Fisher's exact test). This finding is consistent with those of Verner-Jeffreys et al. (2008) who did not find an association between *F. psychrophilum* and RMS/CWSD. In addition, use of tissue Gram stains of SD lesion sections in the present study (data not shown) and by Olson et al. (1985) revealed no filamentous bacteria consistent with *F. psychrophilum* infection. Intermittent isolation or low-level detection of *F. psychrophilum* by PCR, as well as concurrent detection of RLO in *F. psychrophilum*-positive lesions in this study (Table 1),

suggest an opportunistic role for this bacterium (rather than it acting as a primary pathogen of SD). In contrast, the RLO sequence was significantly associated with SD lesions in rainbow trout based on a nested PCR assay ($p < 0.001$, chi-square test). We note that, despite the statistical association, RLO DNA was detected in only 64% of 25 lesion samples (50 to 70% of lesions at any farm, Table 1). This may be a function of time of collection relative to lesion progression, or formation of microcolonies that could result in less than 100% detection. Four matched and apparently healthy samples from SD-affected fish were positive for RLO DNA (but only when lesion samples were also positive) and might indicate detection of very early stage lesions or the ability of this organism to become systemically distributed.

To better evaluate the association between SD and the RLO, PCR testing should be extended to other types of skin lesions. We have tested lesions and apparently healthy skin from an *Aeromonas salmonicida*-infected fish and these appeared negative for the presence of RLO (data not shown); efforts to obtain other skin lesions are underway. We recently detected RLO sequences from 2 of 6 rainbow trout that had overwintered in a lake in Washington state and were exhibiting lesions consistent with SD both grossly and by histology (data not shown). In addition, 12 apparently healthy raceway controls (1 to 4 fish from each of 4 farms) were negative for the RLO sequence (data not shown).

While a significant association between the RLO sequence and SD is not proof of causation, in lieu of any other consistent candidate organism, it is reasonable to hypothesize that the RLO is the primary or a component cause of SD. Recognizing that an RLO might be the etiologic agent points to new avenues of investigation, including efforts to recover viable RLO using arthropod cell lines (Munderloh et al. 1996) that may be more permissive to rickettsial agents. Until Koch's postulates can be satisfied (Koch 1884), efforts should determine whether there is a correlation between RLO template abundance and lesion development using methods such as quantitative PCR. This type of associative data will further support or refute the potential role of the RLO in SD pathogenesis (Fredricks & Relman 1996).

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