IDENTIFYING THE ETIOLOGIC AGENT OF

STRAWBERRY DISEASE

IN RAINBOW TROUT

By

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IDENTIFYING THE ETIOLOGIC AGENT OF

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IN RAINBOW TROUT

Abstract

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Strawberry disease (SD) is an inflammatory skin disorder of unknown etiology that leads to downgrading or rejection of farmed rainbow trout (*Oncorhynchus mykiss*) at processing and thus has an economic impact on trout producers. An infectious cause for SD is suggested by studies of limited scope that showed transmission and by the apparent response of SD to antibiotic treatment. I used culture-independent methods (16S rDNA libraries) to identify candidate bacterial pathogens from seven SD lesions and two healthy skin samples from SD-affected fish. A 16S rDNA sequence highly similar to members of the order *Rickettsiales* was present in three lesion libraries and absent in healthy tissue libraries. I developed and applied a nested-PCR assay to screen 25 SD-affected fish for 16S rDNA from this *Rickettsia*-like organism (RLO). Sixteen of 25 lesion samples and four of the 25 healthy samples were positive for the RLO sequence indicating a significant positive association between SD lesions and the presence of RLO DNA ($P < 0.001$).

Efforts to culture the RLO organism were unsuccessful. Instead, I developed a quantitative-PCR assay (qPCR) to enumerate RLO in lesions of varying severity under the working hypothesis that if RLO is the etiologic agent of SD, then there should be a positive

correlation between lesion severity and RLO copy number. The assay targeted the RLO 16S rDNA sequence and had an analytical detection sensitivity of 100 copies. I then tested 18 lesions from 13 fish representing high or low lesion severity as judged by gross examination. QPCR detected higher numbers of RLO (mean of 11,015 copies) in high severity lesions compared to lower numbers (mean of 3,160 copies) in low severity lesions ($P < 0.001$). Samples of unaffected skin from SD-affected fish were all negative except two samples (121 and 139 copies). My results demonstrate a positive correlation between copy number and lesion severity. This information combined with the association between SD lesions and the presence of RLO supports the hypothesis that the RLO is the etiologic agent of SD.

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Dedication

This dissertation is dedicated to my parents

whose love, support and encouragement

are deeply appreciated.

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GENERAL INTRODUCTION

Strawberry disease (SD) is a skin disorder of unknown etiology that occurs in rainbow trout (*Oncorhynchus mykiss*). SD is characterized by bright red or yellow raised inflammatory lesions which occur mainly in market-sized fish (Ferguson et al. 2006, Oman 1990). Although the disease is self-limiting within 5-8 weeks and it causes no changes in weight gain or behavior, incidence within a population can be as high as 80% (Olson et al, 1985) and the unsightly lesions lead to product downgrade or rejection with associated economic losses to trout producers. Harvest losses as high as 50% have been reported at aquaculture facilities in southern Idaho (Erickson 1969). A survey of Idaho producers in the late 1980's indicated that 9 out of 19 farms experienced yearly outbreaks of SD (Oman 1990). Reports of SD in wild-caught fish have been noted in California, Washington and Oregon (Oman 1990). While SD was first described in Washington State in the 1950's it has since been recognized throughout the western United States and in Canada (Olson et al. 1985).

Strawberry disease lesions occur in random locations on the trunk of rainbow trout and are described as variably hyperemic and ulcerated with a progressive thickening of the epithelium towards the center of the lesion (Oman 1990). Loss of epithelium and scales can be seen in both early-stage and severe lesions. Microscopic analysis of lesions shows extensive infiltration of lymphocytes along with some macrophages and polymorphonuclear leukocytes (Olson et al. 1985, Oman 1990); however, our preliminary analysis of presumed early-stage SD lesions shows infiltration mainly by lymphocytes and macrophages with little or no polymorphonuclear leukocytes present. Infiltration of the dermis, epidermis and occasionally the muscle layer by mononuclear inflammatory cells has been noted. These observations indicate a focal inflammatory response, rather than systemic involvement (Olson et al. 1985, Oman 1990).

While SD has only been described in rainbow trout, similar skin conditions have been observed in cutthroat trout (*O. trutta*), whitefish (*Prosopium williamsonii*), and Chinook salmon (*O. tshawytscha*). No causative agent has been identified for any of these conditions (Oman 1990).

Conditions similar to SD, also with unknown etiology, have been described in the United Kingdom. Warm water strawberry disease (formerly known as strawberry disease) occurs during May to September when water temperatures exceed 16°C and the condition is thought to respond to treatment with vitamin C or oxytetracycline (Barker & Algoet 2000, St. Hilaire & Jefferey 2004, Ferguson et al. 2006, Verner-Jeffreys et al. 2006). Red Mark Syndrome (RMS) or cold water strawberry disease (CWSD) has been described in rainbow trout from the UK since 2003 (Verner-Jeffreys et al. 2008). RMS/CWSD occurs at temperatures less than 15°C and is thought to improve when fish are moved into warmer water (Verner-Jeffreys et al. 2008). Like SD, RMS/CWSD is self-limiting, responds to oxytetracycline and is almost identical in appearance to SD in the USA except that heterophils are present later in the disease and inflammatory lesions are seen in other organs of RMS/CWSD-affected fish, especially the heart (Verner-Jeffreys et al. 2008). It is unclear if these differences are due to genetic differences in host response or differences in etiology.

Results from self-reported surveys suggest that there are no management practices or facility, diet, or water conditions that pre-dispose trout to strawberry disease, although stress is thought to aggravate or initiate the condition (Olson et al. 1985, Oman 1990). It should be noted that these surveys made no effort to control for a variety of potential confounding factors and biases. Nevertheless, there is evidence that SD is caused by a transmissible agent. In an unpublished thesis, Oman (1990) reported some success in transmitting the condition by experimental inoculation and Erickson (1969) reported that the condition might be transmitted

via co-habitation although these efforts have not been replicated by other fish health experts (S. LaPatra, personal communication). In another set of experiments, tissue homogenate from SD lesions killed cell culture monolayers whereas homogenate from normal tissue did not affect cell cultures, but it is unclear what agent caused host cell death in this assay (Oman 1990). There have been ancillary reports that oral treatment with oxytetracycline reduces recovery time as much as 50% (Erickson 1969, Olson et al. 1985, Oman 1990). Limited transmission studies and apparent response to chemotherapeutic treatment is consistent with the hypothesis that SD results from a primary or secondary infectious process. In general, fish skin disorders can be caused by bacteria, viruses, protozoa, fungi, allergies, and sunburn.

Due to the similarity between SD and goldfish ulcer disease, it has been suggested that atypical strains of *Aeromonas salmonicida* may be responsible for strawberry disease, although there is no conclusive evidence supporting this suggestion. In a survey of bacterial flora of water and fish at six hatcheries in southern Idaho, Oman (1990) recovered *A. hydrophila*, *Pseudomonas* spp., *Bacillus* spp. and *Staphylococcus* species at facilities both with or without a history of SD while *A. salmonicida* was not recovered. Fleury *et al.* (1985) recovered *Flavobacterium columnaris* and another unidentified *Flavobacterium* species from SD lesions and others have detected *F. psychrophilum* from lesions using PCR (Ferguson et al. 2006). Routine recovery of *A. hydrophila* from SD lesions led St-Hilaire and Jeffery (2004) to hypothesize a role for this agent. They suggested that SD is caused by an allergic response to *A. hydrophila* toxin, thereby explaining inconsistent recovery of the bacterium from SD lesions. An allergic response to endotoxin or exotoxin produced by gut flora was also listed by Olson et al. (1985) as a suspected cause of SD, although no supportive evidence was presented. Fleury et al. (1985) reported that an adeno-like virus was isolated from the skin lesions of two rainbow trout exhibiting SD from

France. Despite the association of these different agents with SD lesions, no one has been able to definitely demonstrate that any of these agents are responsible for SD.

There have been several unpublished reports of chlamydia-like or rickettsial intracellular organisms associated with SD lesions (reviewed in Oman 1990). If SD is caused by an obligate intracellular pathogen, it would explain the fact that the condition responds to antibiotic treatment but that no one has recovered an infectious agent using conventional microbiological methods. A member of Rickettsiales is also consistent with lack of growth in axenic culture but apparent growth in cell culture observed by Oman. Oman (1990) prepared tissue homogenate from SD lesions and showed that this material could kill cell culture monolayers whereas control homogenate had no effect. Thin sections from pelleted cell cultures revealed pleomorphic membrane-bounded bodies (PMB) with size and shape consistent with PMB's from members of the order Rickettsiales. Morphology of intracellular microorganisms within these PMB's, as seen with transmission electron microscopy (TEM) was also consistent with Rickettsiales (Oman 1990). Three of four lesions stained with Kinyoun's acid-fast stain had small, red, coccoid structures in the cytoplasm of cells and within scale pockets. The putative intracellular organisms were positive with Rickettsial-Pinkerton stain consistent with what would be seen with rickettsial organisms. In one case, Oman (1990) was able to induce lesions that were histologically consistent with SD by inoculating scarified skin tissue with lesion homogenate. Attempts to induce lesions by injection, intubation, and cohabitation failed, but samples were small and a number of factors may have prevented successful transmission. Unfortunately, Oman (1990) did not confirm that lesion homogenate from the one successful inoculation experiment could once again kill cell culture monolayers.

In summary, strawberry disease causes bright red inflammatory lesions in market-ready rainbow trout resulting in loss of profitability to trout producers. Limited transmissibility of SD to disease-free fish and reduction in healing time with oxytetracycline suggest a bacterial agent is responsible for SD. The strongest line of evidence indicates this agent may be a member of Rickettsiales: positive staining for rickettsia in lesions; visualization of rickettsia-like pleomorphic membrane-bounded bodies within cell culture infected with SD lesion homogenate; killing of cell culture monolayers with SD lesion homogenate with no cytolysis of cultures infected with healthy fish skin homogenate. Therefore, we hypothesize that a Rickettsiales-like organism (RLO) is responsible for SD in rainbow trout.

The strongest proof of causation relies on satisfaction of Koch's postulates: isolation of an organism from diseased animals; growth of the organism in pure culture; recreation of disease in naïve animals following administration of the pure organism; and re-isolation of the organism from experimentally infected animals (Koch 1884). Many pathogens, however, cannot yet be cultured or are opportunistic pathogens that are present in healthy hosts at low levels so causation cannot be shown. Koch himself experienced this difficulty when attempting to identify the etiologic agents of leprosy and cholera (Fredricks and Relman 1996). Fredricks and Relman (1996) formally proposed that detection of pathogen-associated nucleic acid could serve as as an alternative to Koch's postulates. Their molecular guidelines include detection of pathogen-associated nucleic acid sequence or higher copy numbers in most cases of disease, especially in areas of pathology; no detection of sequence in healthy hosts or tissues; decreasing copy number of pathogen-associated sequence during recovery and increasing copy number during relapse or disease onset; correlation of sequence copy number with disease severity; and visualization of sequence in affected tissues using *in situ* hybridization. There should be agreement between the

proposed pathogen and the characteristics of the disease based on comparisons with closely related organisms. Results of molecular-based studies should be reproducible over time, by different researchers and different methods (Fredricks and Relman 1996).

Our preliminary investigations into the cause of SD involved culture-independent methods to compare bacterial communities in SD lesions and healthy fish skin. Lesions from three fish were swabbed and the DNA extracted from these swabs was pooled and used to construct 16S rDNA libraries. Swabs of apparently healthy fish skin were used to make a separate library. Five of the fourteen bacterial sequences retrieved from the lesion library matched a partial 16S rDNA sequence for an uncultured Rickettsiales bacterium isolated from *Ixodes ricinus* ticks. This Rickettsiales sequence was not among the 19 bacterial sequences recovered from the healthy library. These results provided proof of concept that DNA sequence-based detection of the RLO could be used to test the hypothesis that RLO causes SD. My project sought to test the hypothesis that an RLO is the etiologic agent of SD by determining if there is an association between RLO and SD lesions (Chapter 1) and a positive correlation between RLO copy number and lesion severity (Chapter 2).

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CHAPTER ONE

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

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/d082_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 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 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 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 MgSO4, 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 MgCl2, 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 separated by electrophoresis using a 1% agarose gel to confirm the presence of one 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 MgSO4, 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; 1,123 base 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 MgCl2, 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 MgCl2, 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 two samples of early lesions that showed inflammation beginning in the stratum spongiosum (Table 1; Appendix 1, Figs. A1 &A2). Grossly normal skin samples from five of the SD-affected fish

and five 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 four 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, there was a dominant sequence that shared 96% identity with an uncultured *Mycoplasma* species present in two lesion libraries and one healthy skin library at prevalence of 2%, 89% and 4%, respectively. The subphylum Alphaproteobacteria was represented mostly by a Rickettsia-like sequence (hereafter referred to as RLO). The RLO sequence was present in three lesion libraries at 1%, 32% and 54%, 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 one 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).

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 Rickettsiales (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 four 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 four lesion samples and no healthy samples were positive for *F. 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 ribosome operon copy number, DNA recovery and template amplification). Thus, proportional estimates discussed herein are preliminary and require more quantitative methods for confirmation. Nevertheless, three sequences were dominant in lesion libraries, with a *Rickettsia*-like sequence (RLO) dominant in two 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 five 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 oxytetracycline 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 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* organisms 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*, which 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 1,276 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 identifies potential RLO hosts more likely to inhabit aquatic environments (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 three 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 herein. Universal PCR primers are often not truly universal; by pure chance the primers used in the present study matched with five 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 three mismatches at the 5'end of the forward primer and two 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 four SD lesion samples and none of the healthy skin samples in our study were positive for *F. 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 localized formation of microcolonies that could result in decreased sensitivity. Four matched and apparently healthy samples from SD-affected fish were positive for RLO DNA (but only in fish that had lesion samples that 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 were negative for the presence of RLO (data not shown); efforts to obtain other skin lesions are underway. We recently detected RLO sequences from two of six 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 (one to four fish from each of four 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 agent growth. 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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TABLES

Table 1. Rainbow trout (*Oncorhynchus mykiss*) 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.*) was 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 +/- 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

FIGURES

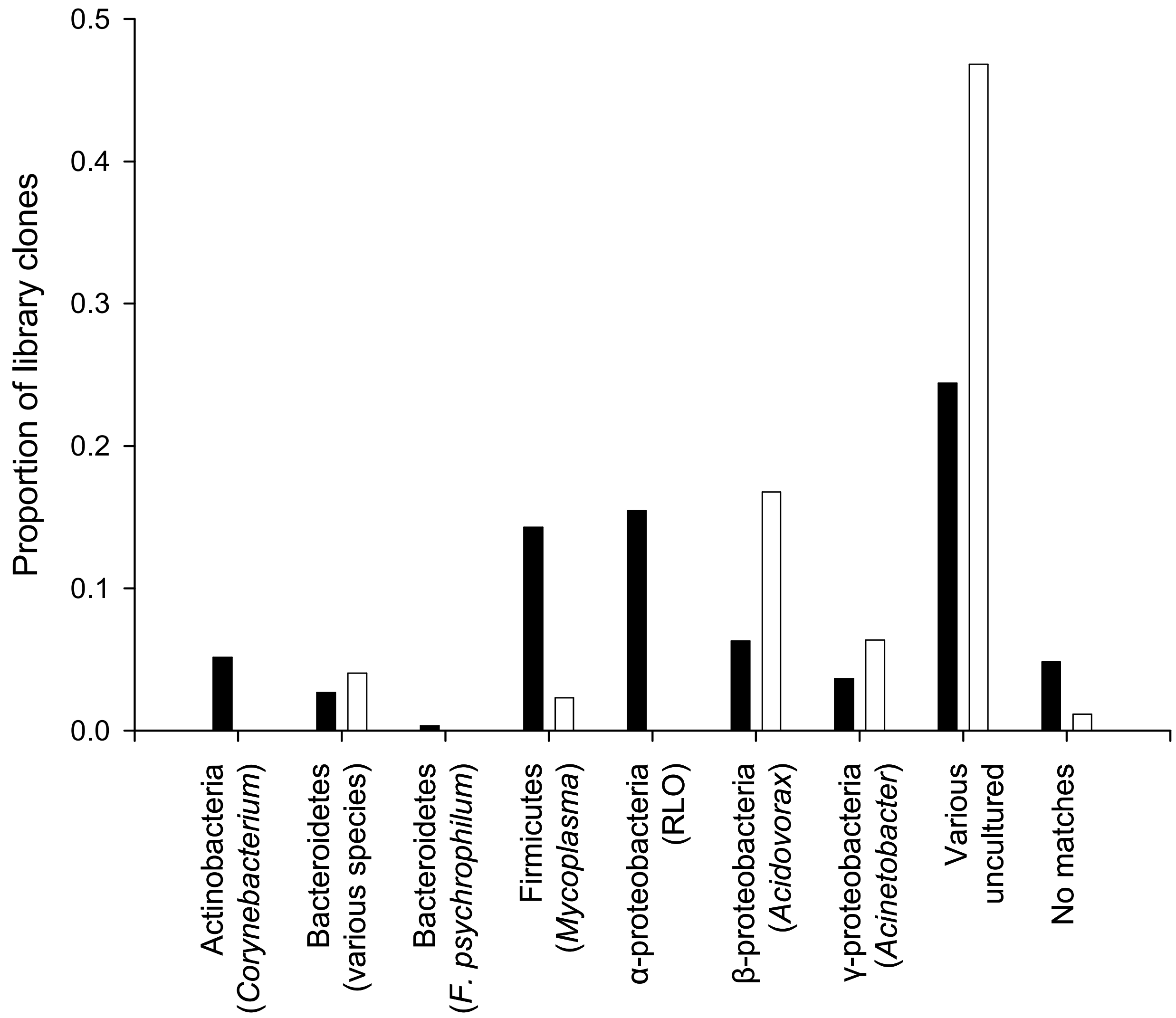

Figure 1. Proportion of dominant bacteria recovered from 16S rDNA libraries generated from lesions (n = 7; closed bar) or from healthy skin (n = 2; open bar).

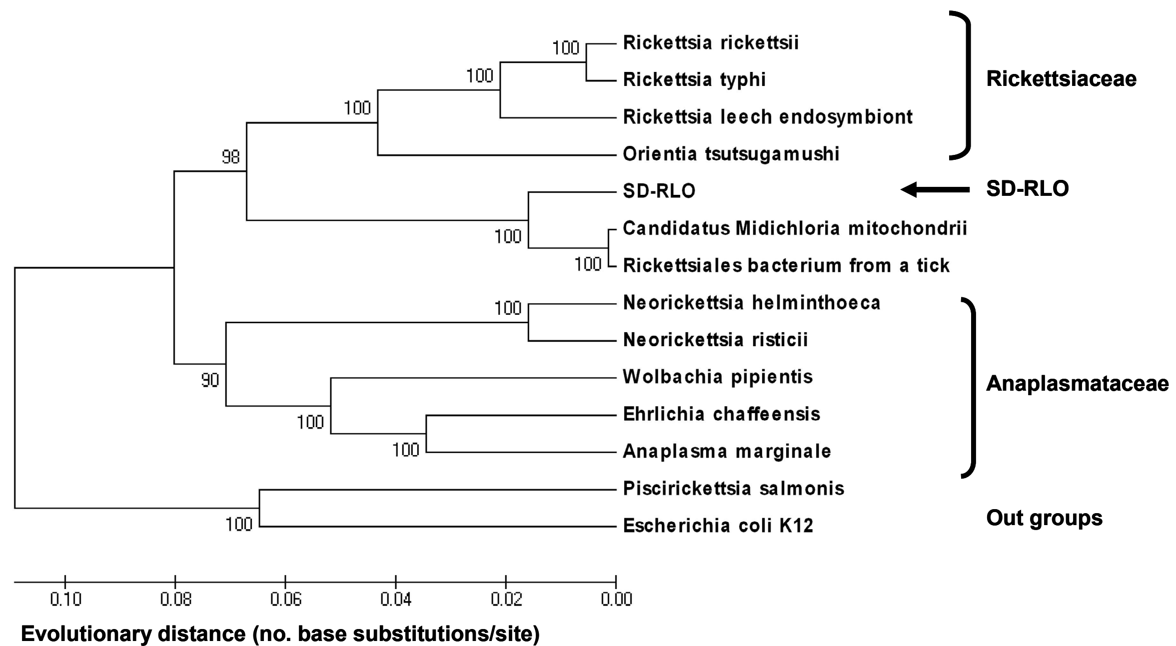

Figure 2. Phylogenetic tree of *Rickettsia*-like organism (SD – RLO) and representative members of the order Rickettsiales: *Anaplasma marginale* (AY077769), *Candidatus* Midichloria mitochondrii (AJ566640), *Ehrlichia chaffeensis* (AF1147752), *Escherichia coli* K12 (NC_00913), *Neorickettsia helminthoeca* (NHU12457), *Neorickettsia risticii* (AF037211), *Orientia tsutsugamush i*(D38625), *Piscirickettsia salmonis* (AY498637), Rickettsiales bacterium It86 (AF525482), *Rickettsia* leech endosymbiont (AB66351), *Rickettsia rickettsii* (L36217), *Rickettsia typhi* (I36221), SD-RLO sequence (EU555284, this study), *Wolbachia pipientis* (AF179630).

SUPPLEMENTAL DATA

Case definition for strawberry disease lesions. Strawberry disease (SD) lesions are found on random locations on the trunk and generally do not affect fins or the head. Early SD lesions are first evident as small round foci with a red center characterized by slight lifting of the scales, whereas established SD lesions are thickened bright red lesions that typically have scale loss and may have a central area of necrosis (Fig. A1). Microscopically, these lesions consist of a locally severe ulcerative dermatitis characterized by moderate to large numbers of lymphocytes and macrophages admixed with abundant accumulations of edema fluid within the stratum spongiosum and stratum compactum (Fig. A2). The areas of inflammation are transected by frequent small blood vessels lined with reactive endothelial cells (neovascularization) and are occasionally disrupted by minimal to locally extensive foci of necrosis that obliterate the normal architecture. The tunica media of scattered superficial dermal blood vessels is disrupted by accumulations of fibrin and edema fluid interspersed with degenerative to pyknotic inflammatory cells. Some of these vessels are partially to completely thrombosed by accumulations of fibrin and cellular debris. In the most severely affected lesions, the above inflammatory changes within the dermis extend through and obliterate the subcutis and multifocally infiltrate the skeletal muscle. In areas in which the skeletal muscle is disrupted by the inflammation, the myocytes range from degenerate with fragmentation and vacuolation of the sarcoplasm to necrotic with contracted cellular borders, hypereosinophilic sarcoplasm and pyknotic nuclei.

Fig. S1. A representative strawberry disease lesion presenting on a rainbow trout collected in Southern Idaho.

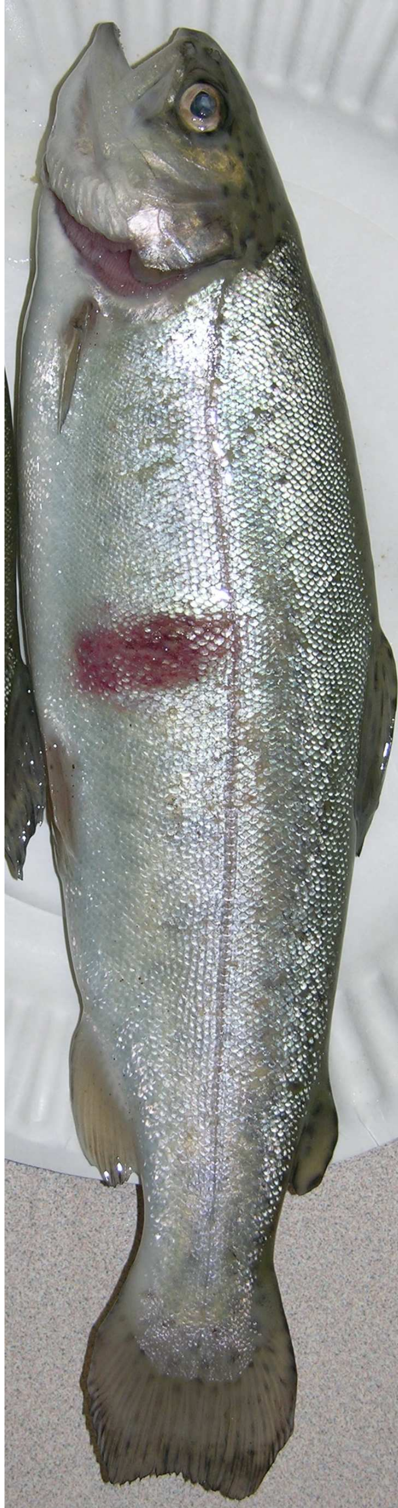

Fig. S2. Photomicrographs of (A) healthy fish skin stained with hematoxylin and eosin, 4X magnification. Note clearly demarcated architecture of the tissue; (B) strawberry disease lesion stained with hematoxylin and eosin, 4X magnification. Note loss of epidermis and scales; (C) strawberry disease lesion stained with hematoxylin and eosin, magnification 60X. Large arrow indicates occluded blood vessel, small arrow indicates edema.

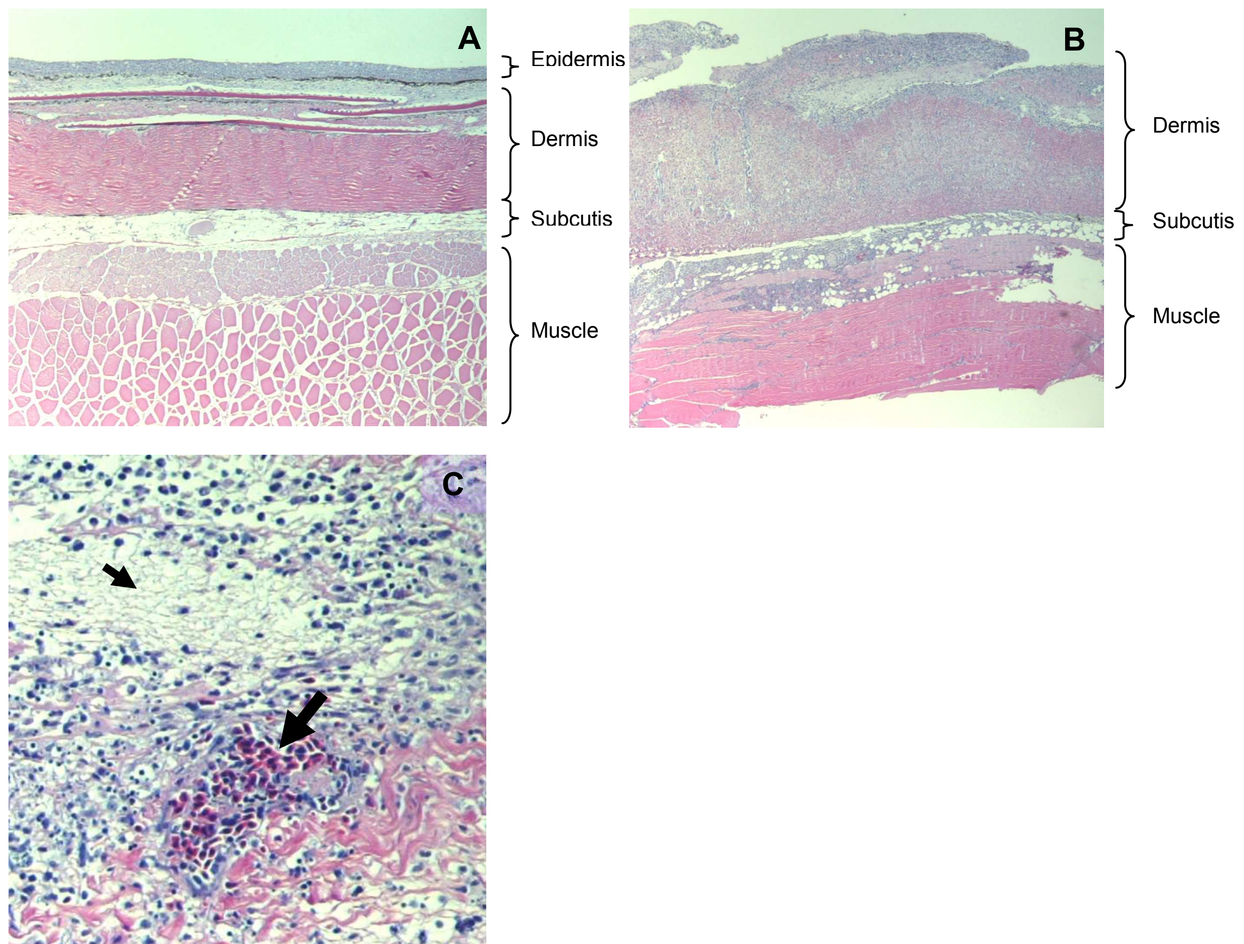

Table S1. Accession numbers, descriptions and counts of 16S rDNA sequences recovered from strawberry disease (SD) lesion and healthy skin libraries.

Accession	Description	Lesion	Healthy
AB015518.1	Unidentified proteobacterium	1[b]	0
AB039334.1	*Brevibacillus agri*	1	0
AB059480.1	*Clostridium* sp.	0	4
AB066340.1	*Brevibacterium* sp.	1	0
AB075683.1	*Enterococcus* sp.	1	0
AB111104.1	*Proteobacterium*	0	1
AB128872.1	Uncultured bacterium	14[a]	5[a]
AB166881.1	*Phenylobacterium koreense*	1	0
AB177316.1	Uncultured bacterium	2	0
AB195776.1	Aquatic bacterium	3	0
AB240505.1	Uncultured bacterium	3	0
AB255115.1	Uncultured bacterium	3	9
AB294318.1	Uncultured bacterium	2[b]	0
AF145257.1	*Corynebacterium xerosis*	1	0
AF205140.1	*Ehrlichia* sp. 'HGE agent'	1[b]	0
AF276640.1	*Corynebacterium* sp.	2	0
AF385525.1	*Streptococcus* sp.	3	0
AF408936.1	*Pseudomonas* sp.	1	0
AF427039.2	*Pseudoxanthomonas taiwanensis*	1	0

AF451251.1	*Acinetobacter* sp.	4	6
AF513962.1	Uncultured *Propionibacterineae* bacterium	15	4
AF525481.1	*Rickettsiales* bacterium It62	92	0
AF525482.1	*Rickettsiales* bacterium It86	2	0
AF539679.1	*Acinetobacter* sp.	4	1
AJ269515.1	*Moraxella osloensis*	1	0
AJ295562.1	Uncultured rape rhizosphere bacterium	0	1
AJ310412.1	*Subtercola pratensis*	2	0
AJ313027.1	*Brevibacillus* sp.	2	0
AJ489328.1	*Geobacillus thermoleovorans*	1	0
AJ548899.1	Uncultured bacterium	3	3
AJ550464	*Bacillus silvestris*	14[a]	0
AJ575540.1	Uncultured actinobacterium	1	0
AJ622907.1	*Kocuria carniphila*	2	0
AJ717350.1	*Agrococcus jenensis*	1	0
AJ786020.1	*Bacteroidetes* bacterium	1	0
AJ871304.1	*Modestobacter versicolor*	1	0
AM111056.1	*Arthrobacter* sp.	1	0
AM159183.2	*Chryseobacterium hispanicum*	0	1
AM159306.1	Uncultured *Clostridiaceae* bacterium	0	2
AM184302.1	*Comamonas* sp.	1	2
AM230493.1	*Flavobacterium succinicans*	0	1
AM237384.1	*Pedobacter cryoconitis*	1	0

AM279215.1	*Pedobacter soli*	1	0
AM285013.1	*Variovorax* sp.	2	0
AM423086.1	*Chryseobacterium hominis*	0	1
AM690925.1	Uncultured actinobacterium	7	0
AM697118.1	Uncultured bacterium	1	0
AM697234.1	Uncultured bacterium	1	0
AM697409.1	Uncultured bacterium	1	0
AM697491.1	Uncultured bacterium	0	1[b]
AM711590.1	*Sphingomonas* sp.	1	0
AY064412.2	*Rhizobium* sp.	3	0
AY095437.1	Uncultured yard-trimming-compost bacterium	3	0
AY173079.1	*Streptococcus bovis*	1	0
AY176770.1	*Acinetobacter lwoffii*	1	0
AY193101.1	Uncultured proteobacterium	1	0
AY211144.1	*Microbacterium barkeri*	9	0
AY214753.1	Uncultured candidate division OP11 bacterium	5[b]	0
AY250098.1	Uncultured bacterium	1	0
AY258065.1	*Acidovorax* sp.	2	0
AY268331.1	Uncultured bacterium	1	0
AY297809.1	Beta proteobacterium	5	0
AY332197.1	*Bacillus* sp.	1	0
AY345531.1	Bacterium W20	1	0
AY349412.1	*Sphingomonas* sp.	1	0

AY437440.1	Uncultured *Bradyrhizobium* sp.	1	0
AY444817.1	*Bacteroidetes* bacterium	2	0
AY456700.1	*Pseudomonas* sp.	1	0
AY494684.1	Uncultured *Bacteroidetes* bacterium	2	0
AY504457.1	*Paenibacillaceae* bacterium	1	0
AY527757.1	Uncultured bacterium	1	0
AY559415.1	Uncultured bacterium	1	0
AY568513.2	*Bradyrhizobium elkanii*	2	0
AY594193.1	*Tepidimonas arfidensis*	0	1
AY632569.1	*Geobacillus stearothermophilus*	2	0
AY661998.1	Uncultured bacterium	0	1
AY662494.1	*Flavobacterium psychrophilum* strain CSF 259-93	2	0
AY770721.1	Uncultured gammaproteobacterium from sea squirt	1	0
AY881680.1	Uncultured *Acinetobacter* sp.	0	1
AY898005.1	Uncultured organism	1	0
AY907742.1	Uncultured bacterium	1	0
AY947925.1	Uncultured betaproteobacterium	2	0
AY947969.1	Uncultured *Bacteroidetes* bacterium	1	0
AY948064.1	Uncultured alphaproteobacterium	1	0
AY957950.1	Uncultured bacterium	1	0
AY958993.1	Uncultured bacterium	1	0
AY959164.1	Uncultured bacterium	0	1
AY960261.1	Uncultured *Pseudomonadaceae* bacterium	1	0

AY960264.1	Uncultured *Enterobacteriaceae* bacterium	2	0
AY960266.1	Uncultured *Enterobacteriaceae* bacterium	1	0
AY960268.1	Uncultured *Enterobacteriaceae* bacterium	0	7
AY962272.1	Uncultured bacterium	1	0
AY963424.1	Uncultured bacterium	1	0
AY972175.1	*Pseudomonas putida*	0	1
AY987770.1	*Aeromonas* sp.	2	0
CP000312.1	*Clostridium perfringens*	2	0
CP000323.1	*Psychrobacter cryohalolentis*	1	0
CP000425.1	*Lactococcus lactis* subsp. *cremoris*	2	0
CP000539.1	*Acidovorax* sp.	21	15
DQ017928.1	Uncultured bacterium	3	0
DQ067012.1	Uncultured bacterium	1	0
DQ076434.1	Uncultured *Flectobacillus* sp.	1	0
DQ117534.1	Bacterium #WM-A4	0	1
DQ165180.1	Uncultured bacterium	0	3
DQ202198.1	Uncultured bacterium	2	0
DQ211400.1	Uncultured bacterium	1	0
DQ211452.2	Uncultured *Bacteroidetes*/*Chorobbi* group bacterium	0	2[b]
DQ221470.1	Uncultured bacterium	0	2
DQ228418.1	Uncultured bacterium	1[b]	0
DQ256330.1	Uncultured bacterium	1	0
DQ256357.1	Uncultured bacterium	3	1

DQ293994.1	*Pleurocapsa* sp.	1	0
DQ294626.1	*Diaphorobacter* sp.	9	3
DQ295866.1	*Pelosinus fermentans* strain	1	0
DQ316827.1	Uncultured *Bacteroidetes* bacterium	4	3
DQ336990.1	Uncultured bacterium	3	2
DQ337018.1	Uncultured bacterium	3	6
DQ337585.1	*Kaistia* sp.	1	0
DQ340193.1	Uncultured *Mycoplasma* sp.	12	4
DQ342824.1	Uncultured bacterium	1	0
DQ354708.1	Uncultured bacterium	0	1
DQ354709.1	Uncultured bacterium	5	5
DQ378249.1	Uncultured soil bacterium	0	1
DQ396035.1	Uncultured organism	1[b]	0
DQ404678.1	Uncultured bacterium	1	0
DQ409957.1	Uncultured gammaproteobacterium	0	1
DQ413165.1	*Sphingobium* sp.	2	0
DQ447856.1	Uncultured bacterium	2	0
DQ447857.1	Uncultured bacterium	0	1
DQ456408.1	Uncultured bacterium	4	0
DQ463263.1	Uncultured bacterium	2	0
DQ532127.1	Uncultured bacterium	14	4
DQ532191.1	Uncultured bacterium	0	1
DQ532278.1	Uncultured bacterium	1[b]	0

DQ538104.1	Uncultured bacterium	0	1
DQ642353.1	Uncultured bacterium	0	1[b]
DQ675503.1	Uncultured bacterium	1	0
DQ676998.1	Iron-reducing enrichment	1	0
DQ677850.1	Uncultured gammaproteobacterium	2[b]	0
DQ815250.1	Uncultured bacterium	0	1
DQ824612.1	Uncultured bacterium	1	0
DQ824738.1	Uncultured bacterium	1	0
DQ828466.1	Uncultured actinobacterium	1	0
DQ829086.1	Uncultured proteobacterium	1	0
DQ829228.1	Uncultured *Chloroflexi* bacterium	1[b]	0
DQ820310.1	Uncultured actinobacterium	0	1
DQ905990.1	Uncultured *Bacteroidetes* bacterium	1	1
DQ980905.1	Uncultured bacterium	1	0
DQ990935.1	Uncultured bacterium	1[b]	0
EF018188.1	Uncultured bacterium	0	1
EF018676.1	Uncultured *Bacteroidetes* bacterium	0	1
EF029243.1	Uncultured bacterium	1	0
EF029379.1	Uncultured bacterium	2	0
EF029394.1	Uncultured bacterium	3	0
EF029422.1	Uncultured bacterium	1	0
EF029789.2	Uncultured bacterium	1	0
EF032665.1	Uncultured alphaproteobacterium	1	0

EF033499.1	Uncultured *Acidovorax* sp.	14	10
EF033514.1	*Acidovorax* sp.	2	4
EF061133.1	*Sphingomonas* sp.	2	1
EF061965.1	Uncultured gammaproteobacterium	3	1
EF071401.1	Uncultured *Firmicutes* bacterium	2	0
EF095770.1	*Alacligenes* sp.	0	1
EF111185.1	Uncultured betaproteobacterium	0	1
EF196977.1	Uncultured proteobacterium	0	1
EF197017.1	Uncultured proteobacterium	1	0
EF208659.1	Uncultured bacterium	1	0
EF221160.1	Uncultured alphaproteobacterium	1[b]	0
EF392911.1	Uncultured bacterium	1	0
EF392912.1	Uncultured bacterium	1[b]	0
EF399436.1	Uncultured bacterium	0	1
EF409261.1	Uncultured bacterium	4	3
EF409289.1	Uncultured bacterium	1	0
EF409294.1	Uncultured bacterium	1	0
EF409299.1	Uncultured bacterium	4	0
EF409306.1	Uncultured bacterium	21	15
EF427922.1	Uncultured bacterium	1	0
EF429742.1	Uncultured bacterium	1	0
EF446186.1	Uncultured bacterium	1	0
EF488749.1	*Lysobacter* sp.	0	1

EF507945.1	Uncultured bacterium	0	1
EF511193.1	Uncultured bacterium	0	1
EF515472.1	Uncultured bacterium	1	0
EF525671.1	*Acinetobacter baumannii*	2	0
EF540427.1	Uncultured soil bacterium	1	0
EF540468.1	*Dietzia* sp.	2	0
EF540479.1	*Sphingopyxis* sp.	1	0
EF554889.1	*Ralstonia* sp.	1	0
EF555515.1	*Geobacillus* sp.	1	0
EF574595.1	Uncultured bacterium	3	0
EF574595.1	Uncultured bacterium	7	0
EF590029.1	Uncultured bacterium	0	2
EF590043.1	Uncultured bacterium	2[b]	0
EF613734.1	Uncultured *Clostridiaceae* bacterium	0	1
EF632919.1	Uncultured bacterium	0	3
EF660493.1	Uncultured bacterium	1	1
EF670442.1	*Propionibacterium acnes*	1	0
L14626.1	*Arcobacter butzlerii*	1	0
X84680.1	*Corynebacterium vitarumen*	29	0
X95305.1	*Acinetobacter* sp.	11	4
Y14146.1	*Burkholderia* sp.	0	1
Z49719.1	*Legionella bozemanii*	1	0

Totals	486	163

[a] E-score > 10^{-2}

[b] E-score < 10^{-3}

All other E-score = 0

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.

ATTRIBUTIONS

The above manuscript was published in *Diseases of Aquatic Organisms* Nov 2008 82: 111- 118 as:

Strawberry Disease lesions in rainbow trout (*Oncorhynchus mykiss*) from southern Idaho are associated with DNA from a *Rickettsia*-like organism

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Sonja J. Lloyd collected and sampled fish, trimmed lesions samples in preparation for histological sectioning and staining and evaluated the resultant histology slides with the pathologist. I extracted DNA from fish skin samples and constructed 16S rDNA libraries, prepared the libraries for sequencing, analyzed sequence data and performed phylogenetic analysis. I developed the nested-PCR assay and performed all PCR except for the recovery of full length RLO 16S rDNA sequence. This was performed by a technician, Stacey LaFrentz. Along with my major advisor, Douglas R. Call, I designed experiments, analyzed data and prepared the above manuscript.

Scott E. LaPatra is our collaborator in Southern Idaho. Fish used in this study were identified by his staff and generously donated by Clear Springs Foods, Inc. who also provided access to a laboratory at the Clear Springs Foods research facility for sampling.

Kevin R. Snekvik is a veterinary pathologist who performed the histological analysis and developed the inflammation scoring scheme.

Sophie St-Hilaire served as a liaison between our laboratory and trout producers and provided assistance and training during the first two sampling trips.

Kenneth D. Cain is co-principal investigator on this project and developed the initial proposal for this project along with Douglas R. Call.

Douglas R. Call is co-principal investigator on this project. He, along with Kenneth D. Cain, developed the initial project proposal. As my major advisor he provided significant assistance with experimental design, data analysis and manuscript preparation.

APPENDIX

DETECTION OF RLO IN ADDITIONAL SAMPLES

Additional skin lesions, apparently healthy skin and other organs have been tested for the presence of RLO. A producer in southern Idaho observed unusual skin lesions in rainbow trout and sent samples of lesion and apparently healthy skin from ten affected fish for histology and nested PCR for RLO. The fish were from a younger age group than those normally considered susceptible to SD and the producer reported that the gross appearance of lesions was not consistent with SD. The histological examination of lesions revealed dermatitis inconsistent with SD. The inflammation primarily affected the subcutis and muscle with infiltrates consisting mainly of heterophils and macrophages, whereas SD exhibits infiltrates of lymphocytes and macrophages in the dermis. RLO was not detected in any of the ten healthy skin samples or in nine of the lesion samples. One lesion sample was RLO-positive, which is not surprising because the source farm for these samples has had previous outbreaks of SD and thus we might expect RLO might be present in the environment. This could also represent a nonspecific PCR amplification or lab contamination, but sequencing was not used to confirm the product and negative control samples worked accordingly for the nested PCR assay. There was no significant association between these lesions and the presence of RLO DNA ($P = 0.5$) and the lesions were not consistent with SD histologically suggesting that the RLO is specific for SD lesions and not simply present in any skin lesion; however, further testing of other types of skin lesions is necessary to further increase confidence in the specific association between RLO for SD.

RLO has been detected in hatchery-reared rainbow trout used to stock a lake in Washington State. In May 2008 and April 2009, fish exhibiting inflammatory lesions grossly consistent with SD were sampled and submitted to the Washington Animal Disease Diagnostic

Laboratory (Pullman, WA) for histology and nested PCR for RLO. There was no evidence of bacterial, fungal or parasitic agents in the samples which were histologically consistent with SD. Two of six samples from 2008 and three of four samples from 2009 were RLO-positive. These results support the association of RLO and SD lesions and show that the RLO is present outside the trout farm environment or can be disseminated from the farm environment and maintained in other locations. Detection of RLO in environmental samples would begin to uncover the ecology of the organism and the disease.

Skin lesions, spleens and livers from rainbow trout with RMS, an SD-like condition recently described in the UK (Ferguson et al. 2006, Verner-Jeffreys et al. 2008), were received for testing by nested PCR for the presence of RLO as part of a study conducted by Stirling University, Scotland. RLO DNA was detected in eight of nine skin lesions, six of 13 spleen samples and two of ten liver samples (M. Mettselaar et al., poster presentation at Scottish Aquaculture - A Sustainable Future conference, Edinburgh, 2009). These results show that RLO is present in fish geographically distant from the Pacific Northwest and indicate that SD and RMS may share the same etiology.

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CHAPTER TWO

Quantitative PCR demonstrates a positive correlation between a *Rickettsia*-like organism and severity of strawberry disease lesions in rainbow trout (*Oncorhynchus mykiss*)

INTRODUCTION

Strawberry disease (SD) is an inflammatory skin condition of unknown etiology that affects farmed rainbow trout (*Oncorhynchus mykiss*) in the USA. Unsightly red lesions on the flanks of market-ready fish can lead to product downgrade or rejection at the time of processing; rejection rates of 50 -75% have been reported by farms in Southern Idaho (Erickson 1969, Oman 1990). Fish show no change in behavior or weight gain and recover spontaneously in approximately eight weeks although treatment with oxytetracycline is thought to reduce recovery time (Olson et al. 1985, Oman 1990). Despite the lack of mortality, morbidity rates can reach up to 80% resulting in significant losses for trout producers (Olson et al. 1985). An infectious etiology for SD has been suggested by transmission experiments that used either cohabitation with healthy fish (Erickson 1969) or inoculation of healthy fish with SD lesion homogenate (Oman 1990). Apparent improvement with antibiotic treatment suggests a bacterial agent may be responsible for SD. Previous attempts to isolate a candidate bacterial agent using conventional culture methods have been unsuccessful (Olson et al. 1985, Oman 1990). More recently, culture-independent methods (16S rDNA libraries) were used to identify potential bacteria associated with SD. A *Rickettsia*-like organism (RLO) was found in SD lesions and a nested-PCR assay was used to show a significant association between SD lesions and RLO 16S rDNA (Lloyd et al. 2008).

Conditions similar to SD, also with unknown etiology, have been described in the United Kingdom. Warm water strawberry disease (formerly known as strawberry disease) occurs during May to September when water temperatures exceed 16°C and the condition is thought to respond to treatment with vitamin C or oxytetracycline (Barker & Algoet 2000, St. Hilaire & Jefferey 2004, Ferguson et al. 2006, Verner-Jeffreys et al. 2006). Red Mark Syndrome (RMS) or cold water strawberry disease (CWSD) has been described in rainbow trout from the UK since 2003 (Verner-Jeffreys et al. 2008). RMS/CWSD occurs at temperatures less than 15°C and is thought to improve when fish are moved into warmer water (Verner-Jeffreys et al. 2008). Like SD, RMS/CWSD is self-limiting, responds to oxytetracycline and is almost identical in appearance to SD in the USA except that heterophils are present later in the disease and inflammatory lesions are seen in other organs of RMS/CWSD-affected fish, especially the heart (Lloyd et al. 2008, Verner-Jeffreys et al. 2008). It is unclear if these differences are due to genetic differences in host response or differences in etiology. Verner-Jeffreys et al. (2008) showed transmission of RMS/CWSD by cohabitation and some RMS/CWSD cases have tested positive for the same RLO 16S rDNA sequence found in SD lesions (M. Metselaar, poster at Scottish Aquaculture - A Sustainable Future conference, Edinburgh, 2009) using a previously published nested-PCR assay (Lloyd et al. 2008), which suggests that RMS and SD may have the same etiology.

Fulfillment of Koch's postulates is the traditionally accepted means for proving that a given organism is the etiologic agent of a disease; unfortunately, efforts by several groups to isolate candidate microbial pathogens, including the RLO, have been inconsistent or unsuccessful. Detection of nucleic acid sequences has been proposed by Fredricks and Relman (1996) as a proxy for the growth of an organism. We have already shown that the RLO 16S rDNA sequence is associated with SD lesions and that it is present in RMS lesions. Fredricks and

Relman (1996) also argue that showing a correlation between nucleic-acid data and severity or stage of disease supports a hypothesis of causation. In this study, we describe a quantitative real-time PCR assay to detect the RLO 16S rDNA sequence and test the hypothesis that RLO copy number is positively correlated with SD lesion severity.

MATERIALS AND METHODS

Sample collection. All fish were sampled from farms operated by the same company in Southern Idaho, USA. Fish #9 and #10 were sampled from Farm A in November of 2006 and the remaining fish were sampled from Trout Farm B in March of 2008. SD-affected fish were identified by farm staff and isolated 2 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 was grossly normal. Samples were stored in either 95% ethanol or in 10% neutral buffered formalin. Grossly normal 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. Photographs of fish taken prior to sampling were used to assess lesion severity. Lesions classified as “low severity” showed no to minimal swelling and were generally smaller in total extent compared to lesions of “high severity” (Figure 1). Lesions of high severity were characterized by an inflammatory region that was clearly raised above the proximal, uninvolved tissue and these lesions usually involved more total surface area compared with low severity lesions (Figure 1).

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 (4 µm) and stained with hematoxylin and eosin (H&E), Gomori's trichrome stain and Movat's pentachrome stain (Washington Animal Disease Diagnostic Laboratory, Pullman, WA). Sample identifiers were blinded from the reviewer of the H&E-stained slides. Inflammation was graded as follows: 0, no inflammation; 1, inflammation in stratum spongiosum; 2, inflammation in dermis; 3, inflammation in dermis, subcutis and muscle with or without ulceraton; 3+, changes listed in grade 3 with extensive infiltration of inflammatory cells and ulceration.

DNA extraction. Tissue samples (2 mm^3) were collected from the center 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 (Qiagen). 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 AE buffer (Qiagen), quantified using a Nanodrop 1000 spectrophotometer (Nanodrop Technologies), and stored at –20°C. The RLO 16S rDNA sequence was cloned as an insertion sequence in vector pCR4 (Invitrogen) from a previous study (Lloyd et al. 2008). Cultures of the clone were grown overnight at 37°C in Luria broth with 100 µg ml^{-1} ampicillin and 1.5 ml aliquots were stored at -20°C until plasmid isolation was performed. Plasmids were isolated from thawed aliquots using the Promega Mini Prep kit as per manufacturer's instructions after washing the bacterial pellet in 1X PBS. Quantity and purity of plasmid DNA was determined by both electrophoresis with a 1% agarose gel stained with

ethidium bromide and visualized by UV illumination using an Alpha Imager (Alpha Innotech Corporation) and UV spectroscopy on a Nanodrop 1000 spectrophotometer.

Nested PCR for detection of RLO sequence in tissue and in water. Nested PCR for the detection of RLO 16S rDNA in SD-affected tissue or plasmid standards was performed as previously described in Lloyd et al. (2008) with the following exceptions: the eubacterial 16S rRNA reverse primer, 517R, was used in place of universal reverse primer U1510 for the first round of PCR (Table 1); all primer concentrations were 400 nM; dNTP's were increased from 100 µM to 200 µM; extension time of the second PCR amplification (internal segment amplification) was reduced from 60 s to 20 s to eliminate non-specific amplification products. PCR products were electrophoresed in a 1% agarose gel, stained with ethidium bromide and visualized by UV illumination using an Alpha Imager. The analytic sensitivity of the nested-PCR assay was assessed using dilutions of plasmid DNA containing the RLO 16S rRNA target sequence. Dilutions were prepared in UV-irradiated Nanopure water and in the presence of 500 ng of RLO-negative fish genomic DNA. RLO-negative fish DNA was extracted from clinically normal rainbow trout from an SD-free facility as described above. Starting quantities of the plasmid ranged from 250 ng to 250 zg in 5 µl. RLO plasmid standards were also diluted in water only with starting quantities ranging from 12.5 fg to 0.8 ag. Nested PCR was performed as above and the lowest starting quantity that gave a discernible band in two experiments was considered the expected limit of detection.

Real-time quantitative PCR assay for RLO 16S rRNA sequence. A Taqman® assay was developed to detect the RLO 16S rRNA sequence and a rainbow trout reference gene, insulin growth factor I (*igf1*). *Igf1* was used to normalize RLO copy number to the amount of fish DNA in the sample, also expressed as copy number. Primers and probes to detect the RLO 16S rDNA

sequence were designed using BeaconDesigner 3.00 (Premier Biosoft International) (Table 1). *Igf1* primers and probe were previously published (Kelley et al. 2004). Probes were 5' labeled with FAM and 3' labeled with Black Hole Quencher 1. Primers and probes were synthesized by Integrated DNA Technologies. Both BLASTN 2.2.21+ (Altschul et al. 1997) and ProbeMatch (Cole et al. 2007, Cole et al. 2009) were used to assess the *in silico* specificity of the RLO primers and probe. Plasmids for RLO standard curves were generated using DNA extracted from the skin of an SD-affected fish that was RLO-positive by nested PCR as described above. The RLO 16S rRNA sequence for the Taqman assay was amplified using nested PCR with universal 16S rRNA primers 20F and 1541R for template generation and the primers designed for the RLO 16S rRNA Taqman assay to generate the fragment for cloning. Each PCR reaction (25 μl) included 0.83 U of Platinum High Fidelity Taq polymerase (Invitrogen) and associated 1X reaction buffer, 2 mM $MgSO_4$, 0.2 mM of each dNTP, 0.4 μM of each primer, and 250 ng template DNA or 1 μL of the external reaction in the case of nested PCR. Thermal cycling conditions for the RLO nested PCR included initial denaturation at 95˚C for 2 min, 35 cycles of 95˚C for 30 s, 54˚C for 30 s, 68˚C 90 s (external reaction) and 20 s (internal reaction) followed by a final extension at 68˚C for 10 min. PCR products were electrophoresed through a 1% agarose gel to confirm the presence of a single band at the expected sizes of 150 bp or 67 bp for RLO and *igf1*, respectively. To facilitate TA cloning, terminal adenines were incorporated at the 3’ termini of the PCR product by addition of 1 U *Taq* polymerase (Fisher Scientific) and incubation at 72°C for 10 min. The product was then cleaned using a Qiaquick PCR Purification spin column (Qiagen), cloned into pCR4 vector (TOPO TA Cloning Kit for Sequencing, Invitrogen) and transformed into TOP10 cells. Transformants were picked into 2 ml cryotubes with Luria broth, 100 mg mL^{-1} ampicillin, and 12.5% sterile glycerol, and stored at –80°C.

Cloned inserts were PCR amplified from transformed colonies 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. Thermal cycling conditions included initial denaturation at 95°C for 5 min, 35 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 20 s, with a final extension at 72°C for 10 min. PCR products were electrophoresed through 1% agarose gels to confirm the presence of 1 band at *ca* 415 bp. PCR products were purified (see above) and sequenced (Amplicon Express, Pullman, WA, USA). Resulting trace files were imported into Sequencher (Gene Codes) for vector trimming and manual inspection of base calls. Sequences were submitted to MEGABLAST (Altschul et al. 1997) to confirm identity of the cloned insert to the RLO 16S rRNA sequence (accession number EU555284). Positive plasmids were extracted using the Qiagen Mini Prep kit as per the manufacturer's protocol. Plasmid DNA quantities were determined by absorbance at 260 nm using the Nanodrop 1000 UV spectrophotometer.

QPCR reactions (50 μl) consisted of 1x Biorad iQ Supermix (50 mM KCl, 20 mM Tris-HCl at pH 8.4, 0.2 mM of each dNTP, 25 U/ml iTaq hotstart DNA polymerase, 3 mM $MgCl_2$, plus proprietary stabilizers), 0.4 μM of each primer and 2.5 μM of each probe. Sample template was added at a starting quantity of 250 ng DNA in 5 μl. Samples and standards were run in triplicate with each probe. The plate was placed in a Biorad iCycler and run under the following reaction conditions: 2 min at 95˚C followed by 40 cycles of 95˚C for 30 s, 55˚C for 30 s, and 72˚C for 20 s. The threshold for the PCR baseline subtracted curve fit relative fluorescence units was set at 100 for all experiments. Benchtop analytical sensitivity was assessed using decreasing starting quantities of the RLO standard plasmid (4.2ng – 4.2 ag, corresponding to 10^9 copies – 1 copy) alone as well as the RLO standard plasmid mixed with 500 ng of RLO-negative fish DNA

and run in triplicate as described above. RLO-negative fish genomic DNA was used as template to assess the analytical sensitivity of the *igf1* reaction (1315 ng – 1.315pg, corresponding to 10^9 copies – 1 copy). The lowest starting quantity with a standard deviation of ≤0.5 among triplicate wells was considered the limit of detection. Copy number was calculated from starting quantity based on the size and sequence of the plasmid DNA using the Sequence Manipulation Suite (Stothard 2000) or the size of the rainbow trout genome and copy number of *igf1* (2 copies per diploid genome). Lesion samples were tested for RLO by qPCR as described above using 250 ng of template in 5 µl. Results were expressed as RLO copy number per *igf1* copy number based on standard curves that were included with each run. RLO standard curves consisted of dilutions of plasmid standard (16 pg – 128 fg or 3.8×10^6 – 3.0×10^4 copies) in 500 ng of RLO-negative fish DNA. IGF standard curves were made from dilutions (500 ng – 0.8ng or 3.8×10^5 – 608 copies) of RLO-negative fish DNA. Absolute quantification methods were used to determine copy number: a sample's mean cycle threshold (Ct) for each probe was converted to a Log starting quantity using the linear equations from standard curves that were performed with each experiment; starting quantity was then used to determine the number of copies based on the mass of one haploid rainbow trout genome (1.315 pg) or the RLO plasmid standard (4.2 ag). All samples with RLO mean Ct values ≤37 and standard deviations < 0.50 were subjected to Kruskal-Wallis one-way ANOVA with post hoc planned comparisons (NCSS Statistical and Power Analysis Software).

RESULTS

Analytic sensitivity of PCR assays. The nested-PCR assay could detect one copy (4.6 ag) of the purified RLO plasmid standard diluted in water while the qPCR assay was less sensitive with a detection limit of 100 copies (420 ag, Table 2). When mixed with a background of 500 ng fish DNA, RLO plasmid standards for both nested PCR and qPCR had analytic sensitivities on the same order of magnitude: 544 copies (2.5 fg) were detected by nested PCR and 100 copies (420 ag) were detected by qPCR (Table 2). We have no way of determining the number of 16S rRNA operons that are present in a single genome, but if the RLO is similar to other rickettsia, which have a single 16S operon, then the RLO copy number corresponds to number of bacterial cells (Rurangirwa et al. 2002). The limit of detection for the qPCR assay was 100 RLO copies both in water and in the presence of fish DNA indicating that fish DNA does not interfere with the qPCR reaction. The nested-PCR assay, however, had reduced sensitivity in the presence of fish DNA. The relationship between cycle threshold and Log starting quantity of RLO plasmid diluted in water, or in a background of 500 ng fish DNA, was linear over the range of starting quantities detected by qPCR (100 to 1×10^9 copies; $y = -3.275 \times X + 11.683$, $r = 0.994$, and $y = -3.405 \times X + 11.361$, $r = 1.0$, respectively). The gene used to normalize RLO copy number to the amount of rainbow trout DNA present in a sample was the single copy gene, *igf1*. Detection of one copy of *igf1* represents one rainbow trout genome equivalent. The performance of the *igf1* qPCR assay was assessed using dilutions of 1.315 µg to 1.315 pg starting quantities of fish DNA (1 - 1×10^9 copies, Table 2). The relationship between the Log starting quantity and threshold cycle was linear throughout the range of detection (10 to 1×10^9 copies; $y = -2.964 \times X + 29.141$; $r = 0.981$).

Quantification of RLO in lesions of varying severity. By gross inspection the SD lesions included in this study represent two groups with low or high severity as determined by presence or absence of marked swelling. All lesions were either round or oval, although some of the low severity lesions were irregular in shape. Lesions from both groups varied in color from white to red and were mostly devoid of scales; however, half of the high severity lesions were bright red while most of the low severity lesions were white or pink indicating less hemorrhage and/or vascular congestion that was supported by histological examination (data not shown). Histological examination also showed almost no remarkable difference in these lesions despite the variation in gross appearance; all but one of the lesions were graded as 3 or 3+ indicating inflammation in the dermis and the underlying subcutis and muscle with possible erosion of the epidermis (Table 3).

Eleven low severity lesions and seven high severity lesions from 12 fish were subjected to qPCR analysis to determine RLO copy number. All of the lesions were positive for RLO 16S rDNA by nested PCR while two grossly normal skin samples from SD-affected fish also tested positive by nested PCR (Table 3). The association between presence of the RLO marker and lesions was statistically significant ($P < 0.001$ by Fisher's exact probability test). No RLO was detected by qPCR in any of the grossly normal skin samples (data not shown) except for two samples that were also RLO-positive by nested PCR. In this case, two of three replicate wells in the qPCR assay detected RLO from the normal skin sample at cycle thresholds over 35, corresponding to RLO copy numbers close to the limit of detection for this assay (121 and 139 copies of RLO for fish #41 and #47, respectively, Table 3).

The mean RLO copy number was significantly different between the high and low severity lesions as well as the grossly normal skin samples from SD-affected fish ($P < 0.001$ for

Kruskal_Wallis one-way ANOVA , Figure 2). RLO copy alone was highly correlated ($r = 0.99$ by Spearman Rank correlation test) with RLO copy number normalized to the number of rainbow trout cells as determined by *igf1* copy number; as a consequence, only RLO copy number is reported below. High severity lesions had RLO copy numbers ranging from 1,648 – 27,124 (mean 11,015 copies, Figure 2) and on average had higher RLO copy numbers compared with low severity lesions (Table 3). Two exceptions were high severity lesions that had RLO copy numbers more often associated with low severity lesions (sample #10 and #52b). Low severity lesions had RLO copy numbers ranging from 318 – 5,866 with a mean of 3,160 copies (Table 3, Figure 2).

Gomori's trichrome and Movat's pentachrome staining of histology slides. We examined representative sections from four lesions in the low severity group (#47a, #47b, #49b and #54) and three lesions in the high severity group (#49, #53 and #55). These lesions had mild to severe inflammation that extended through the stratum spongiosum of the dermis with the most severe lesions having inflammation that extended through the stratum compactum of the dermis and into the subcutis. Gomori's trichrome staining was used to evaluate the collagen fiber thickness and arrangement within the dermis in an attempt to visualize the deposition of thin, poorly organized strands of immature collagen amongst the remnant mature dermal collagen and inflammatory infiltrates. This would be most evident in the stratum compactum, which is composed of thick, densely packed collagen. Trichrome staining revealed that in the most severe inflammatory lesions only sparse deposition of immature collagen was present. Movat's pentachrome staining was initially used to evaluate the same slides for the presence of fibrin deposition, suspected to be more frequent in acute inflammatory lesions. Significant fibrin deposition was not evident in any of the evaluated sections. Movat's pentachrome staining did

reveal increasing amounts of mucin deposition within both the stratum spongiosum and stratum compactum of the dermis that was histologically correlated with severity of inflammation. The sections with the most severe inflammatory infiltrates had focally extensive accumulations of mucin within the stratum compactum that widely separated the collagen bundles with frequent loss of the thick bundles of mature collagen such that the normal yellow staining of this layer was completely lost. Similar to the Gomori's trichrome stain, significant accumulations of immature collagen were not evident in the examined sections. Staining showed that significant immature collagen deposition and evidence of granulation tissue formation were not present in any of the examined sections supporting the conclusion that these lesions range from early to possibly mid infection and have yet to reach a healing phase in the disease process.

DISCUSSION

A qPCR assay was developed to detect and quantify RLO 16S rDNA in SD lesions of varying severity. The assay could detect as few as 100 copies of the RLO plasmid standard and thus is at least as sensitive as a nested-PCR assay previously described by Lloyd et al. (2008), which can detect 544 copies. With similar to increased sensitivity, the qPCR assay could replace the nested assay leading to reduced risk of false positives due to contamination and it offers a significant advantage of requiring less time and handling to complete the assay and presumably increased diagnostic specificity given the requirement for probe hybridization coupled with PCR amplification. The level of sensitivity of the RLO qPCR assay is approximately 10 times lower than other qPCR assays used detect and quantify pathogenic bacteria in clinical samples where reported values range from one to tens of copies of either purified plasmid standard or plasmid

standard in a background of competing DNA (Pusterla et al. 2000, Kuoppa et al. 2002, Eremeeva et al. 2003). A similar qPCR assay reportedly detected *Mycobacterium leprae* in skin biopsies (Martinez et al. 2006) where sensitivity of the assay was assessed using purified genomic *M. leprae* DNA and 5 genome copies, or 25 fg, was the limit of detection. The RLO qPCR assay can detect the RLO target in 100 copies, or 0.42 fg, starting quantity within a background of fish DNA suggesting this level of detection is comparable to that of the *M. leprae* assay. It is notable that the *M. leprae* qPCR assay was sufficient to assess *M. leprae* load in clinical specimens throughout the clinical spectrum of leprosy including patients with paucibacillary tuberculoid leprosy who had few to no bacteria detectable in their skin by acid-fast staining (Martinez et al. 2006). The *M. leprae* qPCR assay was at least 20 times more sensitive than conventional bacteriological detection and was at least as sensitive as conventional PCR methods. Similarly, the qPCR assay described here is able to detect and quantify RLO in SD lesions and apparently healthy skin almost to the level indicated by benchtop sensitivity tests.

All SD lesions and only two healthy skin samples from SD-affected fish were RLO positive by nested PCR and qPCR ($P < 0.001$ by Fisher's exact probability test), consistent with a significant association between the presence of RLO DNA and SD lesions reported in a previous study (Lloyd et al. 2008). SD lesions of high severity had greater numbers of RLO DNA compared to low severity lesions. Fredricks and Relman (1996) proposed nucleic acid-based detection of an organism as an alternative to growth when a culture system or other means of obtaining purified organism and reproducing disease is lacking. Detection of higher numbers of a nucleic acid sequence corresponding to a candidate pathogen in more severe lesions or in early or mid-stage lesions would support the supposition of a causative role for the agent under investigation. Not all lesions classified as "high severity" had higher RLO copy number

compared with the low severity lesions. For example, the lesion from fish #10 was red-rimmed with a white center possibly due to large numbers of lymphocytes. Lesions similar in appearance are considered "recovering" according to trout farm managers and this lesion may indeed be recovering, although gross or histological markers corresponding to stage of SD infection, especially in healing lesions, have yet to be identified. Special stains of representative lesion sections from both groups showed little sign that lesions were healing suggesting that most lesions examined in this study were early to active lesions, although lesion #10 was not examined with staining. Low levels of RLO were also detected in the high severity lesion from fish #52. This lesion was white and less swollen than the most severe lesions which were red. It may be that this lesion was misclassified and would group as such given more refined criteria. Other possible sources of the variation in lesion RLO copy number include heterogeneity of samples because only a small portion of the lesion was sampled and the bacterium may be unequally distributed within the lesion. Variance could also be introduced by the inability to clearly distinguish disease stage using gross and/or histopathological characteristics where some lesions could be misclassified based on our gross assessment procedures. Finally, variation in host inflammatory response might contribute to variance in classification of lesion severity relative to RLO copy number. Regardless of these potential sources of variance, there is a clearly evident and statistically significant relationship between lesion severity and RLO copy number consistent with the hypothesis that RLO is the single or a component cause of SD in rainbow trout.

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TABLES

Table 1. Primer and probe sequences for nested PCR and Taqman qPCR assays.

Primers and Probes	Sequence (5' – 3')	Reference
16S rDNA, 27F	AGAGTTTGATCCTGGCTCAG	Weisberg et al. 1991
16S rDNA, 517R	ATTACCGCGGCTGCTGG	Muyzer et al. 1993
16S rDNA, 1541R	AAGGAGGTGATCCANCCRCA	Suzuki & Giovannoni 1996
RLO1, nested PCR	ATCGCTACAAGACGAGCCCATGCAA	Lloyd et al. 2008
RLO2, nested PCR	TATTACCGCGGCTGCTGGCA	Lloyd et al. 2008
RLO q-PCR, F	GGCTCAACCCAAGAACTGCTT	this study
RLO q-PCR, R	GTGCAACAGCGTCAGTGACT	this study
RLO q-PCR probe	CCCAGATAACCGCCTTCGCCTCCG	this study
IGF-I q-PCR, F	CAGTTCACGGCGGTCACAT	Kelley et al. 2004
IGF-I q-PCR, R	CCGTAGCTCGCAACTCTGG	Kelley et al. 2004
IGF-I qPCR probe	CCGTGGTATTGTGGACGAGTGCTGC	Kelley et al. 2004

Table 2. Analytical sensitivity of nested PCR and qPCR assays. Dilution series of plasmid standards were tested in water or in the presence of 500 ng fish DNA by nested PCR or qPCR. Positive detection (+) by nested PCR was defined as the presence of a ~300 bp band after electrophoresis. Positive detection (+) by qPCR was defined as a mean cycle threshold ≤ 36 with a standard deviation among triplicate wells < 0.50. *Igf1* is the fish house keeping gene used to normalize copy number calculations. Sensitivity was determined using dilutions of fish DNA.

	RLO plasmid standard in water		RLO plasmid standard in 500 ng fish DNA		*igf1* in dilutions of fish DNA
Copy number	Nested	qPCR	Nested	qPCR	qPCR
10^9	+++[a]	+++[a]	+++	+++	nd[b]
10^8	+++	+++	+++	+++	nd
10^7	+++	+++	+++	+++	nd
10^6	+++	+++	+++	+++	+++
10^5	+++	+++	+++	+++	+++
10^4	+++	+++	+++	+++	+++
10^3	+++	+++	+++	+++	+++
10^2	+++	- ++	+++	- ++	+++
10	++ -	- - -	+++	- - -	+ - -
1	++ -	- - -	- - -	- - -	- - -

no template	- - -	- - -	- - -	- - -	- - -

[a] nd = not determined

Table 3. Rainbow trout (*Oncorhynchus mykiss*) skin samples and qPCR results. Inflammation score: 0: no inflammation; 1: inflammation in stratum spongiosum; 2: inflammation in dermis; 3: inflammation in dermis, subcutis and muscle with or without ulceraton; 3+: 3 with extensive infiltration of inflammatory cells and ulceration. Lesion severity: healthy: no lesion or swelling; low: minimal swelling; high: severe swelling. Healthy samples (H) negative for RLO by qPCR are not included. Multiple lesions from the same fish are indicated by lower case letters.

Sample	Inflammation score	Severity	RLO copy no.	RLO/IGF copy no.
41H	1	healthy	121	4.47×10^{-4}
47H	2	healthy	139	8.21×10^{-4}
47a	2	low	318	1.56×10^{-3}
49b	3	low	910	5.53×10^{-3}
41b	3	low	1082	3.09×10^{-3}
10	3	high	1648	7.57×10^{-3}
54	3	low	2925	1.07×10^{-2}
52	3	low	2993	1.68×10^{-2}
48	3+	low	3309	1.09×10^{-2}
47b	3	low	3427	1.83×10^{-2}
45b	3	low	3863	1.50×10^{-2}
52b	3	high	4696	2.70×10^{-2}
51b	3	low	4696	2.65×10^{-2}

43	3	low	5367	3.88×10^{-2}
45a	3	low	5866	2.58×10^{-2}
55	3+	high	8190	2.28×10^{-2}
41a	3	high	10431	2.92×10^{-2}
53	3+	high	12457	1.02×10^{-1}
9	3	high	12556	5.99×10^{-2}
49a	3+	high	27124	6.86×10^{-2}

FIGURES

Figure 1. Representative strawberry disease lesions from (A) low, and (B) high severity groups.

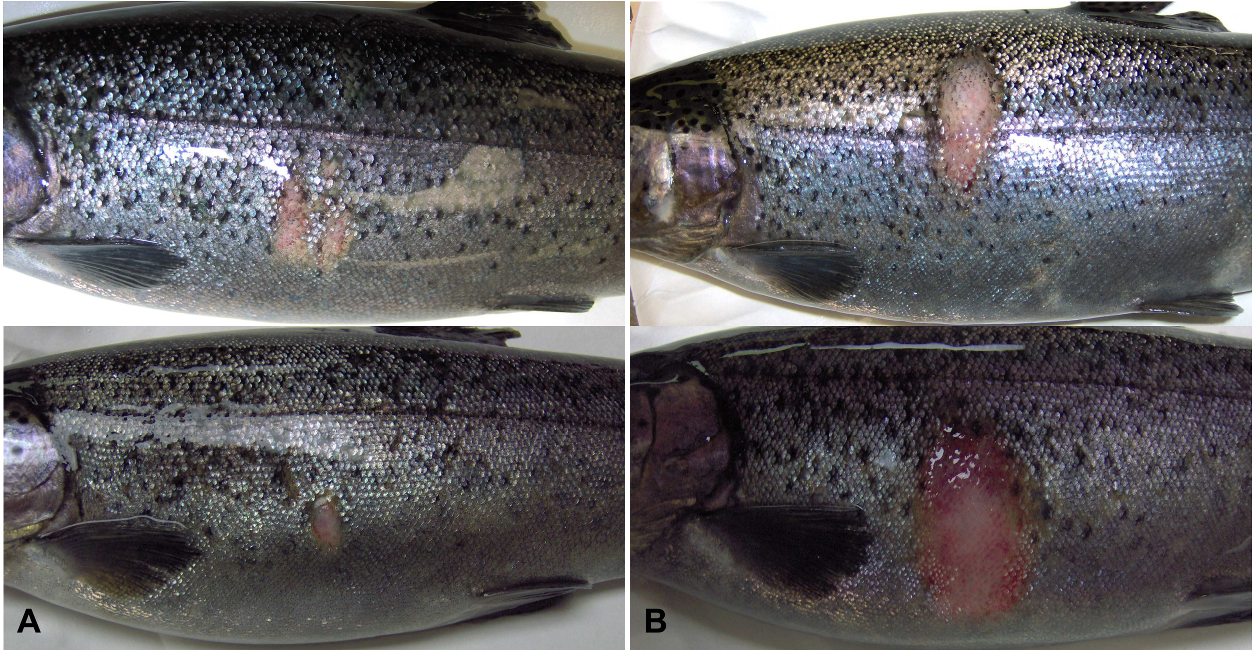
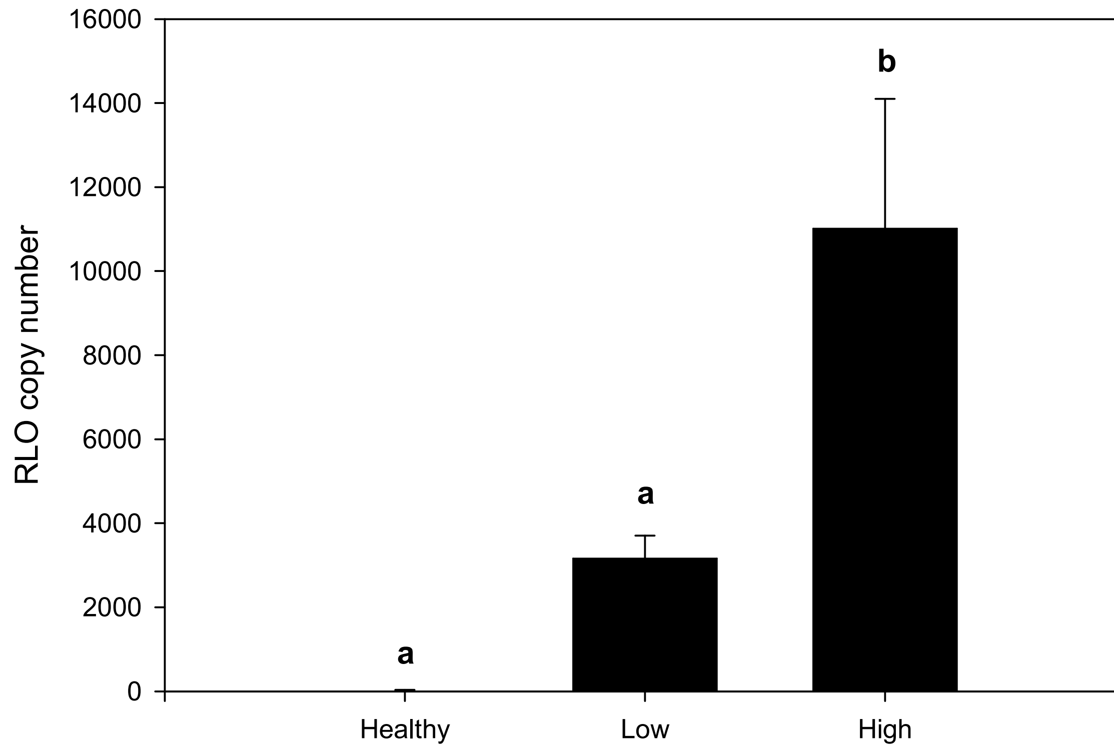


Figure 2. Mean RLO copy number in apparently healthy skin samples from SD-affected fish, low severity and high severity lesions. Error bars indicate standard error of the mean. a, b define statistically different groups (Kruskal-Wallis ANOVA followed by planned comparisons).



ATTRIBUTIONS

The above manuscript will be submitted as:

Quantitative PCR demonstrates a positive correlation between a *Rickettsia*-like organism and severity of strawberry disease lesions in rainbow trout (*Oncorhynchus mykiss*)

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Sonja J. Lloyd collected and sampled fish, trimmed lesions samples in preparation for histological sectioning and staining and read the resultant histology slides with the pathologist. I extracted DNA from fish skin samples and performed nested PCR and qPCR. Along with my major advisor, Douglas R. Call, I designed experiments, analyzed data and prepared the above manuscript.

Scott E. LaPatra is our collaborator in southern Idaho. Fish used in this study were identified by Clear Springs staff and generously donated by Dr. LaPatra and Clear Springs Foods, Inc. who also provided access to a laboratory at the Clear Springs research facility for sampling.

Kevin R. Snekvik is a veterinary pathologist who performed the histological analysis and developed the inflammation scoring scheme.

Kenneth D. Cain is co-principal investigator on this project and developed the initial proposal for this project along with Douglas R. Call.

Douglas R. Call is co-principal investigator on this project. He, along with Kenneth D. Cain, developed the initial project proposal. As my major advisor he provided significant assistance with experimental design, data analysis and manuscript preparation.

CONCLUSION

Strawberry disease (SD) is an inflammatory skin disorder of unknown etiology that affects farmed rainbow trout (*Oncorhynchus mykiss*). High morbidity rates in market-sized fish lead to downgrading or rejection at processing resulting in an economic impact on trout producers. SD is thought to respond to the antibiotic oxytetracycline and some experiments have shown the disease can be transmitted to unaffected animals through cohabititation with unaffected fish or injection of SD lesion homogenate. These observations suggest that an infectious agent is responsible for SD; however, efforts to grow a candidate pathogen from SD lesions or affected fish have proved unsuccessful. Fulfillment of Koch's postulates is generally considered sufficient evidence for causation. In the absence of a culture system or other means of obtaining purified organism with which to attempt to induce disease in unaffected fish, Fredricks and Relman (1996) propose nucleic-acid based detection of the organism as an alternative to isolation.

We hypothesize that an RLO organism is the etiologic agent of SD. This hypothesis is supported by both the statistical association found between the presence of RLO 16s rDNA and SD lesions (Chapter 1), and by the positive numerical correlation between RLO 16s rDNA copy number and disease severity (Chapter 2). Our data also found no correlation between SD lesions and the presence of *F. psychrophilum*, which has been proposed by others as a possible etiologic agent (refs). While these data are an important step to understanding the basis of SD, there are many unanswered questions and possible avenues for continued investigation. For instance, while the association between RLO with SD supports our hypothesis, the molecular evidence does not allow us to reject the alternative hypothesis that RLO is a lesion-adapted commensal

that is present but unrelated to disease etiology. Further testing of non-RLO skin lesions would help address this question because if RLO is found closely associated with any trout skin lesion, the causal link between RLO and SD would be severely weakened. While we were able to identify a positive correlation between lesion severity and RLO copy number, this analysis was based on crude classification of lesion severity that does not necessarily reflect the stage of infection. A more powerful analysis would be to correlate RLO copy number with disease progression. This can't be done (yet) at an experimental level, but our hypothesis is that RLO copy numbers will be lower in early lesions and in recovering lesions while progressively higher in mid- and late-stage lesions. This might be accomplished by isolating a cohort of SD-affected fish that exhibit a range of SD lesions (by gross examination) and destructively sampling individual fish over a period of time until all remaining fish within the isolation tanks are fully recovered. In addition, visualization of the RLO in diseased host or tissues should be attempted further. Our efforts to identify RLO organisms by electron microscopy have not been successful (data not shown), but *in situ* hybridization of DNA probes followed by confocal imaging or using family or genus-level antibodies against rickettsiae followed by immuno-gold deposition might enhance the probability of detecting the agent. Attempts to culture the RLO, not described here, have been unsuccessful; however, these attempts should continue and high-copy RLO samples could be used as template to amplify phylogenetically informative genes that might better inform *in vitro* culture efforts. The molecular evidence presented here supports our hypothesis; however, isolation of RLO and induction of disease would satisfy Koch's postulates providing definitive evidence that RLO is the causative agent of SD in rainbow trout.