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Characterization of *Renibacterium salmoninarum* with reduced susceptibility to macrolide antibiotics by a standardized antibiotic susceptibility test

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ABSTRACT: Three cohorts of juvenile and subadult Chinook salmon *Oncorhynchus tshawytscha* received multiple treatments with macrolide antibiotics for bacterial kidney disease (BKD) during rearing in a captive broodstock program. A total of 77 mortalities among the cohorts were screened for *Renibacterium salmoninarum*, the etiologic agent of BKD, by agar culture from kidney, and isolates from 7 fish were suitable for growth testing in the presence of macrolide antibiotics. The minimum inhibitory concentration (MIC) of erythromycin and azithromycin was determined by a modification of the standardized broth assay using defined medium. The American Type Culture Collection (ATCC) type strain 33209 exhibited a MIC of 0.008 µg ml^{-1} to either erythromycin or azithromycin. Isolates from 3 fish displayed MICs identical to the MICs for the ATCC type strain 33209. In contrast, isolates from 4 fish exhibited higher MICs, ranging between 0.125 and 0.250 µg ml^{-1} for erythromycin and between 0.016 and 0.031 µg ml^{-1} for azithromycin. Sequence analysis of the mutational hotspots for macrolide resistance in the 23S rDNA gene and the open reading frames of ribosomal proteins L4 and L22 found identical sequences among all isolates, indicating that the phenotype was not due to mutations associated with the drug-binding site of 23S rRNA. These results are the first report of *R. salmoninarum* with reduced susceptibility to macrolide antibiotics isolated from fish receiving multiple antibiotic treatments.

KEY WORDS: Antimicrobial agent susceptibility testing · Macrolide antibiotic · Reduced susceptibility · *Renibacterium salmoninarum* · Bacterial kidney disease

INTRODUCTION

Salmon and trout are among the most intensively cultured fish for both direct harvest and for release into the natural environment. In the northwestern part of the continental USA, Pacific salmon species are reared by governments, tribes, and private organizations for supplementation or restoration of natural runs of salmon, and there are captive broodstock programs for highly endangered populations (Flagg & Mahnaken 1995, Arkush & Siri 2001). One of the most persistent diseases observed among cultured salmon is bacterial kidney disease (BKD), which is caused by the Gram-positive microorganism *Renibacterium salmoninarum*. While acute BKD can cause high mortality among juvenile fish prior to release from rearing facilities, it is possible that most infected juveniles are asymptomatic. *R. salmoninarum* can be transmitted horizontally among cohabiting fish as well as vertically through intraovum transfer, and fish may be suscep-

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tible to infection throughout the entire life cycle. For fish culturists, the windows of opportunity to deliver therapeutics against BKD are limited, and antibiotics are the most commonly used agents.

By 1990, erythromycin was demonstrated to be potentially effective against vertical transmission of *Renibacterium salmoninarum* (Bullock & Leek 1986, Evelyn et al. 1986, Brown et al. 1990), and injection of female broodstock with erythromycin is currently a routine procedure in many US rearing facilities (e.g. Washington Department of Fish and Wildlife 2002, US Fish and Wildlife Service 2005). In the US, erythromycin may be used in food salmonids under an Investigational New Animal Drug (INAD) application, although approval is still pending (National Coordinator for Aquaculture New Animal Drug Applications 2007). In captive broodstock programs for endangered stocks of salmon, a percentage of fish are held through the entire life cycle, and the risk of BKD epidemics can be high. In those programs oral or injected macrolides may be administered anytime during growth to maturity.

Azithromycin, a semi-synthetic derivative of erythromycin, has a broader spectrum of activity against bacteria than erythromycin, and it is active against Gram-positive bacteria, *Mycobacterium avium-intracellulare,* and some Gram-negative species (Ballow & Amsden 1992, Kirst 2002). Unlike erythromycin, azithromycin may not be administered to food fish. In Chinook salmon, azithromycin accumulates to higher levels and is eliminated more slowly than erythromycin (Fairgrieve et al. 2006), a pattern that is consistent with the pharmacokinetics observed in mammalian cells for this antibiotic (Peters et al. 1992, Bosnar et al. 2005). Furthermore, azithromycin is transferred from injected female broodstock to offspring and persists up to 70 d after the start of exogenous feeding (Fairgrieve et al. 2006).

Since the Swann report in 1969 in the UK (Swann 1969), there has been concern about the contribution of agricultural use antibiotics to generating drug-resistant bacterial strains that cause disease in both man and food animals (Shea et al. 2004, Gilchrist et al. 2007). The potential for selecting *Renibacterium salmoninarum* isolates with reduced susceptibility to erythromycin was documented approximately 20 yr ago when Bell et al. (1988) generated an isolate with reduced susceptibility to erythromycin by successive growth of the isolate in increasing concentrations of erythromycin in broth culture. The authors did not determine the underlying mechanisms, but they did demonstrate that *R. salmoninarum* was capable of developing resistance to erythromycin under intense selective pressure. Because macrolides are widely administered to cultured salmonids in the US to treat BKD, it is important to determine whether *R. salmoninarum* with altered drug susceptibility can arise in treated fish. As an initial assessment, we screened a population of captive broodstock Chinook salmon that had received multiple treatments with macrolide antibiotics for BKD. In order to assess the level of susceptibility to antibiotics, a standardized susceptibility test was developed.

MATERIALS AND METHODS

Fish culture and treatments. *Oncorhynchus tshawytscha* were collected as parr from natal streams in the Grande Ronde Basin by the Oregon Department of Fish and Game in August of 2003, 2004, and 2005, and cohorts were identified as BY02, BY03, and BY04, respectively (Table 1). Fish were collected and reared under provisions of the US Endangered Species Act, and their care and welfare was monitored by a Technical Oversight Team. Fish were reared in first-passage well water until smoltification approximately 9 mo after collection, and then transferred to filtered (to 5 µm), ultraviolet light-treated, single-passage seawater. Fish were fed at an initial rate of 2.5% of body weight d^{-1} with Nutra Fry (Skretting), and the rate was tapered to a final adult rate of 1% of body weight d^{-1} with Vitalis Salmon Brood (Skretting).

Within 7 d of collection, all fish received an intraperitoneal injection of azithromycin (Zithromax; Pfizer) at a dose of 20 mg kg^{-1} body weight. Subsequently, when mortality due to clinical BKD occurred, all fish in a cohort were treated with erythromycin by injection (20 or 40 mg kg^{-1} body weight) or by feeding (100 mg kg^{-1} body weight) (Table 1). Injected erythromycin was Osborn Gallimycin 200 (Bimeda) delivered by intraperitoneal administration. Orally administered erythromycin was delivered by custom formulation of feed with Gallimycin R PFC (Bimeda) to a final concentration of 50 mg erythromycin phosphate g^{-1} feed.

Isolate collection. All spontaneous mortalities were necropsied, and kidney tissue was aseptically probed and struck onto modified selective KDM2 (SKDM2) agar plates (1% Bacto-peptone w/v, 0.05% yeast extract w/v, 0.05% L-cysteine w/v, 10% newborn calf serum v/v [Gibco/BRL], 5% nurse medium v/v, 1.2% agar w/v; adjusted to pH 7.5) (Austin et al. 1983) and modified SKDM2 agar plates supplemented with 0.0125 µg ml^{-1} azithromycin. Agar plates with azithromycin were used to enrich for bacteria with altered drug susceptibility, whereas agar plates without azithromycin were used to confirm that isolates originated from fish, not by *in vitro* selection. Isolates were transferred to basal KDM broth (1% Bacto-peptone w/v, 0.05% yeast extract w/v, 0.05% L-cysteine w/v;

Table 1. *Oncorhynchus tshawytscha*. Date (including date of transfer to seawater), mode, dose, and duration of macrolide treatment. –: no antibiotic treatment

Broodyear cohort	Treatment date (mo/yr)	Macrolide	Mode of treatment	Dose (mg kg^{-1} body weight)	Duration of treatment (d)
BY02	8/03	Azithromycin	Injection	20	1
	4/04	Azithromycin	Injection	20	1
	5/04	–	Transfer to seawater	–	–
	6/04	Erythromycin	Oral	100	28
	10/04–11/04	Erythromycin	Oral	100	21
	11/04	Erythromycin	Injection	40	1
	12/04	Erythromycin	Injection	40	1
	3/05	Erythromycin	Injection	40	1
	4/05	Erythromycin	Injection	20	1
	4/05–5/05	Erythromycin	Oral	100	29
	5/05	Erythromycin	Injection	40	1
BY03	8/04	Azithromycin	Injection	20	1
	5/05	–	Transfer to seawater	–	–
	6/05	Erythromycin	Oral	100	29
	7/05	Erythromycin	Injection	40	1
BY04	8/05	Azithromycin	Injection	20	1
	5/06	–	Transfer to seawater	–	–

adjusted to pH 7.5). If the originating plate did not contain azithromycin, no azithromycin was added to the broth. If the originating plate contained azithromycin, azithromycin was added to a final concentration 0.0125 µg ml^{-1}. Culture purity was ascertained by Gram stain morphology and by epifluorescence microscopy using an anti-*R. salmoninarum* polyclonal antibody (Kirkegaard and Perry).

Generation time determination. Broth cultures were established in basal KDM at an optical density at OD_{525} nm between 0.05 and 0.1 (which is approximately 1 to 2×10^8 bacterial cells) by the membrane filtration technique, a method to directly enumerate bacterial cells by antibody-mediated fluorescence microscopy (Elliott & Barila 1987). ODs were read at 525 nm for tube cultures and at 595 nm for microplate cultures. Measurements were taken at the beginning (time 0) and approximately every 24 h for 72 to 96 h to ensure that only log phase growth was included for generation time calculations.

Minimum inhibitory concentration (MIC) testing. MIC assays using 2 different culture volumes were tested. A larger volume test (10 ml starting volume) was performed in glass tubes, and a smaller volume test (200 µl) was performed in 96-well microplates. Both assays used basal KDM broth with incubation at 15°C and agitation at 150 rpm. For each strain, a culture containing no antibiotic was included in each assay. In addition, a *Renibacterium salmoninarum* strain with high susceptibility (American Type Culture Collection, ATCC, type strain 33209) was included for comparison within assays. Cultures for both tests were grown to an OD_{525} range of 0.8 to 1.0 immediately prior to testing. Erythromycin (Sigma) was dissolved in 30% ethyl alcohol to a stock concentration of 10 mg ml^{-1}, and azithromycin (Zithromax; Pfizer) was dissolved in sterile distilled water to a stock concentration of 125 mg ml^{-1}. Serial dilutions of erythromycin and azithromycin for testing were made from the stocks with sterile distilled water immediately prior to use (Clinical and Laboratory Standards Institute 2006). Each isolate was tested at lower passage number (Passage 2 or 3) and at higher passage number (Passage 9 or 10). The concentration of antibiotic that produced a detectable reduction in growth was defined as the MIC (Clinical and Laboratory Standards Institute 2006).

In the tube MIC assays, bacterial growth was assayed in serial 2-fold dilutions of antibiotic ranging from 0.004 to 2 µg ml^{-1} in KDM2 broth to a total volume of 10 ml. Bacterial cultures at all drug concentrations were established at an OD_{525} range of 0.05 to 0.10 (approximately 1 to 2×10^8 bacterial cells). All OD_{525} measurements were performed in polystyrene cuvettes with a 10 mm light path (Fisher Scientific) using a 2-position UV-visible recording spectrophotometer (Shimadzu). Measurements were taken at the start of the assay (time 0) and approximately every 24 h up to 4 d.

In the microplate MIC assays, culture growth was assayed in serial 2-fold dilutions of drugs ranging from 0.0125 to 0.20 µg ml^{-1} in basal KDM broth using

200 µl per well. For each drug dilution, bacterial cell concentrations were established at four 2-fold dilutions with an OD_{525} nm range of 0.0125 to 0.1. Each bacterial and drug concentration test was performed in duplicate. Cultures were established in sterile, polystyrene, flat-bottomed 96-well tissue culture plates (Costar). Prior to OD_{525} reading, the wells were mixed by gentle pipette action to thoroughly resuspend bacterial cells. Measurements were taken at the start of the assay (time 0) and approximately every 24 h up to 4 d.

Sequencing 23S rDNA and ribosomal proteins L4 and L22. Bacteria were grown in basal KDM broth and chromosomal DNA isolated as previously reported (Rhodes et al. 2000). Target sites were amplified from chromosomal DNA with the primers (Operon) specified in Table 2 using high fidelity Vent (exo-) DNA polymerase (New England Biolabs). Amplicons were purified by silica spin column (MoBio) and subjected to sequencing using BigDye terminator mix v3.1 and analyzed with an ABI3100 (Applied Biosystems). DNA sequences from fish-derived isolates were compared to those from the type strain ATCC 33209. There are 2 copies of the 23S rRNA genes in *Renibacterium salmoninarum* (Grayson et al. 2000). Because the 23S rDNA sequence is identical for at least 5 kb around the sequencing target site, one primer pair amplified both copies of the 23S rDNA, and the sequences were examined for heterozygosity.

Kidney BKD enzyme-linked immunosorbent assay (BKD ELISA) and antibiotic activity assay. Kidney samples were collected and analyzed for bacterial antigen levels with a polyclonal anti-*Renibacterium salmoninarum* antibody following the method of Pascho & Mulcahy (1987). Kidney tissue was analyzed for residual antibiotic activity by the method of Fairgrieve et al. (2006).

RESULTS

Isolate frequency, generation time of isolates, and kidney tissue assays

A total of 77 fish that were treated with macrolide antibiotics were screened by culture for *Renibacterium salmoninarum*; 10 fish were from the BY02 cohort, 56 fish were from the BY03 cohort and 11 fish from the BY04 cohort. *R. salmoninarum* isolates suitable for testing were obtained from 7 fish, and all 7 fish were from the BY02 cohort. Six isolates were derived on agar plates without azithromycin supplementation, and 2 isolates were derived on plates with azithromycin supplementation. For one fish (05-372), testable isolates were derived from both the modified SKDM2 plate (05-372 K) and on the modified SKDM2 plate supplemented with azithromycin (05-372 A).

The average generation time ranged from an average of 24.5 h (range 24.2 to 25.0 h) for isolate 06-383 to an average of 34.2 h (range 25.9 to 41.9 h) for isolate 05-372 K (Fig. 1). However, there was no significant difference in generation time among the isolates (ANOVA, $p = 0.092$). Analysis of kidney tissue for bacterial antigen levels showed that all source fish producing testable isolates had clinical BKD at death (Fig. 2). Most of the fish that did not produce testable isolates (51 of 68 fish) exhibited a low BKD ELISA value, indicating these fish had no infection or low-grade infections (Fig. 2). Kidney tissue for antibiotic activity analysis was available for 59 of the 77 fish. Detectable levels of macrolide activity were found in only 4 fish and ranged from 0.68 to 1.00 µg equivalents of azithromycin activity g^{-1} tissue (data not shown). Tissue antibiotic activity was available for 2 of the 7 fish that produced isolates for testing (05-334 and 05-882), and there was no detectable activity in either kidney sample.

Table 2. Primers used for PCR amplification from chromosomal DNA and amplicon sequencing

Primer name	Sequence (5′ to 3′)	Target gene	Application
Rsal23SrRNA1F	CATCGACGCGAGGTTCCAGCCGCCCGT	23S ribosomal DNA	Amplification, sequencing
Rsal 23SrRNA2R	GCCAAACCATGCCGTCGATATGGACTC	23S ribosomal DNA	Amplification, sequencing
Rsal 23SrRNA3F	GTAGCGAAATTCCTTGTCGG	23S ribosomal DNA	Sequencing
Rsal 23SrRNA4R	GGTATTTCAACGATGACTCCA	23S ribosomal DNA	Sequencing
RsalL4F1	GAGAACGTCACACCACGTTGAACCT	Ribosomal protein L4	Amplification
RsalL4R2	CTTGATCTCGGTCTTGTTCGAGCGC	Ribosomal protein L4	Amplification
RsalL4F3	GTCCCTGCTGCTCATCAAG	Ribosomal protein L4	Sequencing
RsalL4R4	GTCGATTAGGCCGTAGCTC	Ribosomal protein L4	Sequencing
RsalL22F1	GGTGTTCGTGACCGAGTCGATGGTC	Ribosomal protein L22	Amplification
RsalL22R2	CGATTTCAACCTTGGCAATGCCAGC	Ribosomal protein L22	Amplification
RsalL22F3	TGTGAAGGACGACCGTAAG	Ribosomal protein L22	Sequencing
RsalL22R4	TGCCCGTAGACATGAGTTG	Ribosomal protein L22	Sequencing

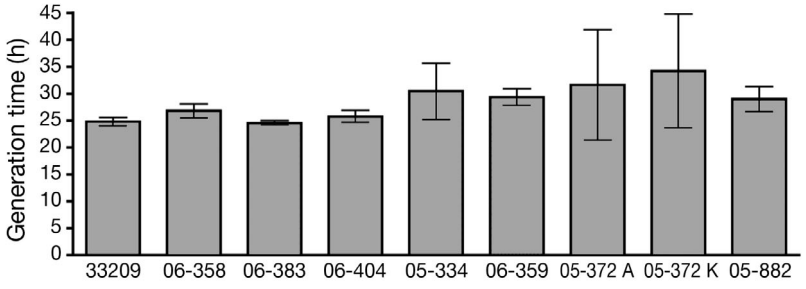

Fig. 1. *Renibacterium salmoninarum*. Average generation time (± range) for ATCC type strain 33209 and for 8 isolates from treated fish for at least 2 replicated experiments

assay (Table 3). The correlation between the MICs was not perfect (Spearman's rho = 0.750, p = 0.066), and values for 2 of the 7 tested isolates did not agree (Table 3). Generation time in the microplate assays was approximately twice as long as the corresponding generation time in the tube assays (Fig. 3), and maximum optical density readings in the microplate assays were below 0.8 (data not shown), indicating that the cultures were not under optimal *in vitro* conditions for growth.

MICs of macrolides

In the standardized tube assay, the ATCC 33209 strain exhibited high susceptibility to both erythromycin and azithromycin, with a MIC value of 0.008 µg ml^{-1} for each drug (Table 3), and isolates 06-358, 06-383, and 06-404 displayed identical MICs (Table 3). In contrast, 5 isolates (05-334, 06-359, 05-372 A, 05-372 K, and 05-882) exhibited erythromycin MICs that were 15 to 30 times higher than that for ATCC 33209, ranging between 0.125 and 0.250 µg ml^{-1} (Table 3). These same isolates also displayed higher MICs to azithromycin than ATCC 33209, ranging between 0.016 and 0.031 µg ml^{-1}. Among the isolates with reduced susceptibility, there was no correlation between erythromycin and azithromycin MICs (Spearman rho = –0.67, p = 0.233).

To assess the utility of the microplate assay, azithromycin MIC values obtained by the microplate assay were compared to those obtained by the tube

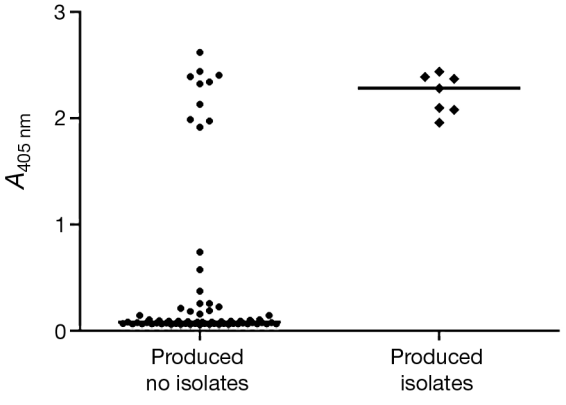

Fig. 2. *Oncorhynchus tshawytscha*. Bacterial kidney disease enzyme-linked immunosorbent assay (BKD ELISA) values of fish screened by culture, comparing fish that produced testable *Renibacterium salmoninarum* isolates (produced isolates) with those that failed to produce isolates (produced no isolates). A_{405nm}: absorbance at 405 nm. Horizontal line: median absorbance

23S rDNA sequences and ribosomal proteins L4 and L22 sequences

A 924 bp region of the 23S rDNA that encompasses the known mutational hotspot for macrolide resistance was amplified by high fidelity PCR, sequenced, and examined for heterozygosity. There was no difference in sequence throughout this region between ATCC 33209 and all of the isolates.

To determine whether there were mutations in the ribosomal proteins L4 and L22, the entire open reading frames for these genes were amplified and sequenced using primers positioned in the flanking non-coding regions. Again, sequences from all isolates were identical to those for ATCC 33209.

DISCUSSION

Erythromycin is widely used to treat BKD in hatcheries and broodstock rearing programs to attempt to reduce vertical transmission of *Renibacterium salmoninarum* to progeny. The selection of erythromycin over

Table 3. *Renibacterium salmoninarum*. Minimum inhibitory concentration (MIC) values for macrolide antibiotics based on tube and microplate assays. ND: test not done

Isolate	Tube		Microplate
	Erythromycin MIC (µg ml^{-1})	Azithromycin MIC (µg ml^{-1})	Azithromycin MIC (µg ml^{-1})
ATCC 33209	0.008	0.008	0.008
05-334	0.125	0.031	0.016
06-358	0.008	ND	0.008
06-359	0.125	0.031	0.031
05-372 A	0.250	0.016	0.016
05-372 K	0.250	0.016	0.031
06-383	0.008	0.008	0.008
06-404	0.008	ND	0.008
05-882	0.250	0.031	0.031

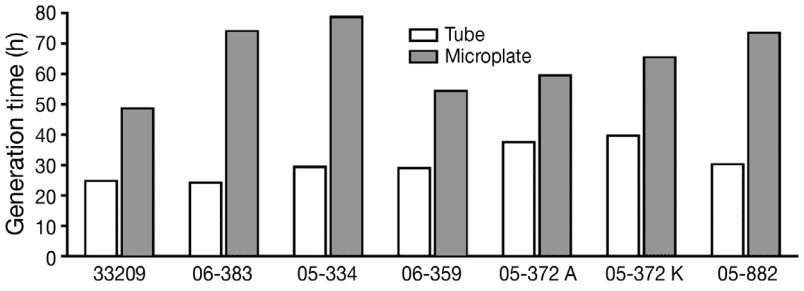

Fig. 3. *Renibacterium salmoninarum*. Comparison of generation times for ATCC type strain 33209 and for 6 isolates from treated fish obtained from the tube assay and the microplate assay for azithromycin

currently approved antibiotics for use in salmon aquaculture (oxytetracycline and sulfadimethoxine/ ormetoprim) appears to be based on longer persistence in progeny and greater pharmacological activity against Gram-positive bacteria (Evelyn et al. 1986, Brown et al. 1990). Concern about the selection of bacteria with decreased susceptibility to erythromycin and the proof-of-principle using *in vitro* selection was demonstrated nearly 20 yr ago (Bell et al. 1988). We isolated *R. salmoninarum* with reduced susceptibility to erythromycin and azithromycin from treated fish, demonstrating that reduced macrolide susceptibility can occur under conditions of repeated drug administration. These isolates are described as having reduced susceptibility, rather than resistant, because the MICs are much lower than those observed for resistant human or animal pathogens, with MICs ranging from 0.5 µg ml^{-1} to greater than 16 µg ml^{-1} (Hyde et al. 2001).

Previous antibiotic susceptibility tests of *Renibacterium salmoninarum* have reported wide variations in results. A test of 40 *R. salmoninarum* isolates, including ATCC 33209, with 5 different erythromycin formulations on KDM2 agar plates found the highest susceptibility to erythromycin stearate at 0.01 µg ml^{-1} (Austin et al. 1983). A separate study of ATCC 33209 reported an erythromycin MIC of 0.1 µg ml^{-1} and an azithromycin MIC of 0.05 µg ml^{-1}, but the media used was slightly acidic (pH 6.8) and it included 10% horse serum (Gutenberger et al. 1989). It is known that erythromycin potency declines with pH (Sabath et al. 1968, Toala et al. 1970), and ingredients with high chemical variability such as horse serum can unpredictably affect MICs (Jones & Barry 1987, Reinert et al. 2003). A third assessment of erythromycin susceptibility for 11 *R. salmoninarum* isolates (including ATCC 33209) reported inhibitory concentrations that ranged from 0.62 to 5.47 µg ml^{-1} (Bandín et al. 1991). However, that study did not test concentrations lower than 0.62 µg ml^{-1}, and the endpoint was defined as the concentration that completely inhibited growth, rather than the accepted definition of MIC. These disparate results emphasize the importance of utilizing a defined medium and a standardized protocol for performing antibiotic susceptibility testing.

The method and criteria we employed are consistent with standardized CLSI protocols (Clinical and Laboratory Standards Institute 2006), with temperature and timing modifications that are necessary for the optimal growth of *Renibacterium salmoninarum*. The protocol for the tube assay permits a culture without antibiotic to achieve stationary growth by the ending time point (72 to 96 h), with a generation time of 24 to 36 h. Although we attempted to develop a microplate assay protocol, the small volume culture conditions were not optimal for *R. salmoninarum* growth, resulting in much longer generation times (48 to 75 h). Therefore, the tube assay protocol is the recommended method for MIC testing.

Macrolide resistance can arise through several mechanisms. Many resistances are due to acquisition of genes encoding new enzymes and/or proteins, and these genes are usually associated with mobile elements that can be readily transferred between unrelated genera. Because we did not search for newly acquired genes among our isolates, this is a formal possibility for the observed phenotypes. Modification of the macrolide target site can occur through methylation of 23S ribosomal RNA (*erm* methylases), by mutation of 23S ribosomal DNA positions A2058 or A2059 (*E. coli* numbering), or by mutation of ribosomal proteins L4 or L22 that interact with the target site (Gaynor & Mankin 2003, Franceschi et al. 2004, Roberts & Sutcliffe 2005). Although no homologues to *erm* family genes have been found in the *Renibacterium salmoninarum* genome (M. Strom pers. comm.; GenBank accession CP000910), methylation might occur through unrelated methylases. Resistance due to mutations has been found in 1 to 4% of isolates that do not carry known acquired macrolide resistance genes, including a variety of Gram-positive bacteria (Roberts & Sutcliffe 2005). However, we observed no evidence for mutations in the 23S rDNA target site or in the L4 and L22 genes of any of the *R. salmoninarum* isolates. Methylation of rRNA or mutation of rRNA, L4, or L22 genes tend to confer a high level of resistance, with MICs for erythromycin often exceeding 16 µg ml^{-1} in Gram-positive bacteria such as *Streptococcus pneumoniae* (Reinert et al. 2003, Calatayud et al. 2007). However, the largest observed MIC for erythromycin in *R. salmoninarum* was 0.25 µg ml^{-1}. Taken together, these findings suggest that alterations of the macrolide target site or interacting proteins are not responsible for the observed phenotype.

The observation that only 4 of 59 fish had detectable drug activity in the kidney was not surprising. Erythromycin has a relatively short half-life in Chinook salmon, whether administered orally or by intraperitoneal injection. Orally administered erythromycin is not detectable in juvenile Chinook salmon muscle tissue by 10 d after treatment (Moffitt & Schreck 1988) or in whole body extracts of juvenile Chinook salmon by 21 d after administration (Fairgrieve et al. 2005). In subadult Chinook salmon, no erythromycin can be detected in kidney, a target tissue for accumulation, by 92 d after injection (authors' pers. obs.). Recently, risk assessment of erythromycin in trout muscle provided evidence for a reduction in withdrawal time to 255 degree-days (i.e. 25.5 d at 10°C) for fish for human consumption (Esposito et al. 2007). Because *R. salmoninarum* has a slow generation time of approximately 24 h, it is possible that this bacterium can survive high drug levels in tissues, especially for drugs with relatively rapid elimination kinetics.

In summary, we have isolated *Renibacterium salmoninarum* with reduced susceptibility to erythromycin and azithromycin from fish that received multiple macrolide treatment. A standardized protocol for drug susceptibility testing in a broth assay was developed to characterize the isolates. While the MICs were not sufficiently elevated to consider these isolates drug resistant, the reduced susceptibility phenotype did not require the continuous presence of drug and may represent an intermediate step toward drug resistance. These results demonstrate that *R. salmoninarum* with reduced susceptibility to macrolides can arise *in vivo*.

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