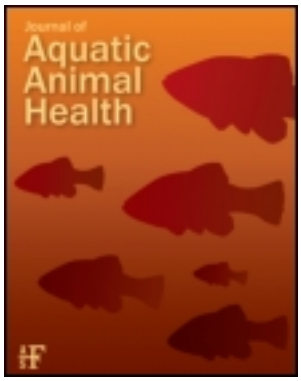

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Randall W. Oplinger [a] & Eric J. Wagner [a]
[a] Utah Division of Wildlife Resources, 1465 West 200 North, Logan, Utah, 84321, USA

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ARTICLE

Control of *Flavobacterium psychrophilum*: Tests of Erythromycin, Streptomycin, Osmotic and Thermal Shocks, and Rapid pH Change

Randall W. Oplinger* and Eric J. Wagner
Utah Division of Wildlife Resources, 1465 West 200 North, Logan, Utah 84321, USA

Abstract

***Flavobacterium psychrophilum*, the etiological agent that causes bacterial coldwater disease, has been implicated in significant reductions in the numbers of salmonids reared at fish hatcheries. In this study, we performed a series of in vitro experiments to test the effectiveness of rapid temperature, pH, and osmotic pressure changes in killing three strains of the bacterium. We also evaluated the effectiveness of antibiotics (erythromycin, streptomycin, and a penicillin–streptomycin mixture) against *F. psychrophilum*. The bacterium tolerated temperatures of 40–50°C for up to 30 min (when acclimated to 15°C). The bacterium can survive lower temperatures for >60 min. Although temperatures ≥55°C appeared to kill *F. psychrophilum* on contact, we found that eyed eggs of Rainbow Trout *Oncorhynchus mykiss* were not able to survive short (<60-s) exposures at these temperatures. We found that rapid changes in pH (15-min exposures to pH 3.0, 4.0, 5.0, 7.0, 9.0, 10.0, and 11.0) and osmotic pressure (15-min exposures to 0, 6, 8, 10, 12% sodium chloride) were not effective at killing the bacterium. Erythromycin concentrations up to 2,000 mg/L for 15 min were also ineffective. However, streptomycin concentrations ≥5,000 mg/L killed the bacterium during a 15-min exposure. The combination of penicillin and streptomycin was also effective, killing the bacterium at doses as low as 2.5×10^6 IU penicillin + 2,500 mg/L streptomycin. Our trials demonstrate that elevated temperatures and the combination of penicillin and streptomycin can kill *F. psychrophilum* under in vitro conditions. Erythromycin and rapid changes in pH and osmotic pressure are not effective at killing the bacterium.**

Disease outbreaks in fish hatcheries can significantly increase the average cost of producing fish as well as reducing the numbers of fish that are reared and ultimately stocked into the wild. Bacterial coldwater disease, caused by the long, thin gram-negative bacterium *Flavobacterium psychrophilum*, has been implicated in significant reductions in the numbers of fish stocked in western North America. For example, the State of Utah estimates that 25–30% of the Rainbow Trout *Oncorhynchus mykiss* raised in the state's hatchery system each year are lost to *F. psychrophilum* (C. Wilson, Utah Division of Wildlife Resources, personal communication). Bacterial coldwater disease has significantly influenced hatcheries worldwide, and improved methods for controlling the disease are needed.

Unfortunately, few methods of controlling *F. psychrophilum* are available to hatchery managers. Considerable emphasis has been placed on research on egg disinfection methods that can prevent the vertical transmission of the disease (e.g., Kumagai et al. 2004; Wagner et al. 2008, 2010). Vaccination also has the potential to prevent the vertical transmission of the disease. Vaccines, however, are still under development and may not be available for use for a number of years (LaFrentz et al. 2004; Plant et al. 2009). Antibiotics can be effective at preventing the horizontal transmission of *F. psychrophilum.* Some strains are susceptible to florfenicol but resistant to oxytetracycline and sulfadimethoxine (Bruun et al. 2000). Unfortunately, these three drugs are the only antibiotics approved for aquaculture use by the U.S. Food and Drug Administration (USFDA 2011).

Despite our knowledge of methods for the control of the disease, *F. psychrophilum* continues to be a major problem in fish hatcheries. This suggests that the methods that are currently used

*Corresponding author: randyoplinger@utah.gov
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to control the disease are not effective and that further research on methods for controlling the spread of *F. psychrophilum* is required. A number of studies of *F. psychrophilum* have reported on the efficacy of several antibiotics, including oxytetracycline, amoxicillin, ormetoprim-sulfadimethoxine, ampicillin, doxycycline, enrofloxacin, florfenicol, flumequine, gentamicin, oxolinic acid, sarafloxacin, and sulfamethoxazole-trimethoprim (Rangdale et al. 1997; Bruun et al. 2000; Dalsgaard and Madsen 2000; Schmidt et al. 2000; Kum et al. 2008; Hesami et al. 2010). Two other candidate antibiotics include erythromycin and streptomycin. Erythromycin is primarily used against gram-positive species, but it has been used with success against some gram-negative species (Lorian and Sabath 1970). Streptomycin is a broad-spectrum antibiotic that is effective against both gram-positive and gram-negative species (Schatz et al. 1944). Streptomycin and erythromycin are not approved for aquaculture use in the United States. Although these drugs may be beneficial for the industry, approval may take several years. Therefore, alternative control methods that rely on FDA-approved compounds or creative treatment methods should be pursued further. For example, sudden changes in temperature could be effective at killing *F. psychrophilum* that is attached to the surface of objects such as eggs or hatchery equipment (e.g., waders, nets). *Flavobacterium psychrophilum* has been reported to grow at temperatures ranging from 4°C to 23°C (Nematollahi et al. 2003). The critical thermal maximum for *F. psychrophilum* has not been reported. Sudden changes in pH or sodium chloride concentration could present other methods for killing surface-attached *F. psychrophilum.* Madetoja et al. (2003) successfully cultured *F. psychrophilum* for over 50 d at a salinity of 6%. However, the bacteria used by Madetoja et al. (2003) were not exposed to a rapid osmotic shock but instead were acclimated to the test conditions. In contrast, Suomalainen et al. (2005) found in vitro that 15-min exposures to a pH of 4.6 and 60-min exposures to sodium chloride concentrations of 4% or pH of 4.86–5.0 significantly reduced (by ~99%) the numbers of the bacterium *F. columnare*. It is possible that rapid changes in pH or sodium chloride concentration are also effective at controlling *F. psychrophilum*.

Bacterial coldwater disease is a major source of mortality for hatchery-reared salmonids. New methods for the control of *F. psychrophilum* need to be investigated. In this study, we conducted a series of in vitro tests to assess the viability of several new control methods for the disease. We determined whether rapid changes in temperature, pH, or osmotic pressure have the potential to kill *F. psychrophilum*. We also determined the concentrations of erythromycin, streptomycin, and a penicillin–streptomycin mixture required to kill the bacterium during a 15-min exposure.

METHODS

We performed a series of in vitro tests to determine the tolerance of *F. psychrophilum* to rapid changes in temperature, pH, and osmotic pressure. We also determined the toxicity of several antibiotics to the bacterium. Each of the described experiments was performed twice. The purpose of the first trial was to establish the basic tolerance limits of *F. psychrophilum* to the treatments. During this first trial, we tested the treatments on a single strain of the bacterium (ATCC 49510). The second set of trials were performed at a later time and were designed to determine whether the tolerances determined during the first trial are similar across strains. During this second trial, three strains (ATCC 49510, Clear Springs Foods 259-93 and Utah Division of Wildlife Resources strain 09-131) of *F. psychrophilum* were tested. The latter two strains were isolated from moribund Rainbow Trout. An established PCR procedure (Wiklund et al. 2000) was used to confirm that each strain was *F. psychrophilum.* Both trials used identical methodology. On occasion, the treatments tested varied between trials. The treatments selected for the second trial were based on the results from the first trial. Any differences between the two trials are discussed in more detail below.

Experiment 1: temperature.—Electric water baths (Precision Model 66802) were used to create temperatures of 25, 30, 35, 40, 45, 50, 55, 65, 75, and 85°C. As a control, a 15°C bath was also created by placing a plastic tub filled with water on a laboratory benchtop. Ice or hot water was added to maintain the temperature at 15°C. A rack containing test tubes (16 × 125 mm) filled with 15 mL of sterilized, hatchery well water (pH = 7.6; hardness and alkalinity = 220 mg/L) were added to each bath. Prior to the experiment, the tubes were allowed to sit in the water baths for at least 15 min to reach the desired treatment temperature. The culture containing *F. psychrophilum* was held at 15°C until the start of the experiment.

Ninety-six hours prior to the start of the experiment, *F. psychrophilum* was added to an Erlenmeyer flask containing 100 mL of a maltose-infused tryptone yeast extract broth (MAT; 0.4% tryptone, 0.04% yeast extract, 0.05% $CaCl_2$, 0.05% $MgSO_4$, 1% maltose, and 0.02% $C_2H_3NaO_2$; Crump et al. 2001). The bacteria were slowly stirred using a stir-plate and were cultured at 15°C. To start the experiment, 200 μL of the inoculated MAT broth was added to the test tubes within the water baths. The solution was mixed by drawing two 4-mL aliquots of the solution from each test tube into a sterilized pipette. The solution was immediately returned to the test tube. Immediately afterwards, 100 μL of solution was withdrawn and added to a petri dish (100 × 15 mm) containing tryptone yeast extract salt agar media (TYES; 0.4% tryptone, 0.04% yeast extract, 0.05% $CaCl_2$, 0.05% $MgSO_4$; Holt et al. 1993). The solution was distributed using a sterilized spreader. The petri dishes were wrapped with laboratory film and incubated at 15°C.

Every 10 min for 60 min after the addition of the bacteria, the solution in each test tube was mixed and plated as described above. Bacteria were only added to each tube once. Therefore, repeated samples were taken from each test tube for the first 60 min after the addition of *F. psychrophilum*. For every five test tubes that were inoculated with *F. psychrophilum*, a sixth tube was established as a procedural control. This tube was not inoculated with bacteria but was otherwise handled in the same manner as the other test tubes.

During the initial trial (in which only the ATCC strain of the bacterium was included), we tested temperatures of 15, 25, 35, 40, 45, 55, 65, 75, and 85°C. Samples were withdrawn from the test tubes 0, 10, 20, 30, 40, 50, and 60 min after the addition of the bacteria (the 0-min sample was actually collected 5–10 s after the addition of the bacteria). Five replicate test tubes of *F. psychrophilum* were exposed to each temperature. During a follow-up trial (ATCC, Clear Springs Foods, and Utah strains were compared), we tested temperatures of 15, 35, 40, 45, 50, and 55°C and samples were removed from the tubes 0, 5, 10, 30, and 60 min after the addition of *F. psychrophilum*. Five replicate test tubes of each strain of *F. psychrophilum* were exposed to each temperature.

In addition to testing the ability of thermal shocks to kill *F. psychrophilum*, we tested the ability of eyed Rainbow Trout eggs to tolerate a temperature of 55°C. In this trial, two water baths were created using well water (pH = 7.4; total hardness = 180 mg/L). One water bath contained water that was heated to 55°C and the other water at 13°C (control), the same temperature the eggs experienced during incubation. A mesh bag was added to each water bath, and eggs ($N = 10$, drawn haphazardly from a bucket containing eggs from 10 females) were added. The eggs were exposed to the temperature for 10, 30, or 60 s. The mesh bag was then removed and placed into a beaker containing ambient-temperature well water (~13°C) for 10 s and was then transferred to a vertical stack incubator tray. After 48 h in the incubation tray, the eggs were removed from the bag and the number of live eggs or fry was counted. Three replicate groups of 10 eggs were exposed to each combination of exposure duration (10, 30, or 60 s) and temperature (13°C or 55°C).

Experiment 2: pH.—In vitro methods were used to determine whether rapid changes in pH can be used to kill *F. psychrophilum*. For this experiment, several series of test tubes were placed into a rack, each series containing four test tubes. The first tube (12 × 75 mm) was filled with 2.0 mL of sterilized well water. Shortly (<30 min) prior to the start of the experiment, 1.0 mL of MAT broth that was inoculated with *F. psychrophilum* was added to this test tube. The second test tube (16 × 125 mm) contained 15 mL of sterilized well water. The pH of the well water in these test tubes was adjusted (using hydrochloric acid or sodium hydroxide). The pH was measured with a freshly calibrated Orion Model SA 720 pH meter. To ensure that the pH did not change during the experiment, readings were taken both before and after the experiment. The third and fourth test tubes contained 15 mL of sterilized well water and served as rinse tubes. Five test tube series (replicates) were prepared for each pH tested. The bacteria used in this experiment were acclimated to a pH of 7.2. Each series of test tubes were held at 15°C.

To conduct the experiment, a sterilized plastic strip (50 × 5 mm) was dipped into the first test tube containing *F. psychrophilum*. After 10 s, the strip was transferred by sterile forceps to the tube containing the pH-adjusted well water. After 15 min, the strip was transferred to the first rinse tube. After a 2-s rinse, the strip was rinsed in the other rinse tube for two additional seconds. After rinsing, the strip was streaked twice (once per side of the strip) across the central axis of a 100-mm-diameter petri dish containing TYES media. The petri dish was then wrapped in laboratory film and incubated at 15°C. During the preliminary trial (in which only the ATCC strain was included), five pH treatments (4.0, 5.0, 7.0, 9.0, and 10.0) were tested. During the second trial (which included all three strains of *F. psychrophilum*), we tested pH levels of 3.0, 4.0, 7.0, 10.0, and 11.0.

Experiment 3: sodium chloride.—The methods used to test the effect of rapid changes in osmotic pressure on *F. psychrophilum* were identical to the procedures used to test the effect of pH change. In the preliminary trial, we evaluated 15-min exposures to sodium chloride concentrations of 0, 2, 4, 6, and 8% (pH = 7.2). The second trial (which determined repeatability across strains) tested 15-min exposures to sodium chloride concentrations of either 0% or 12%. For both trials, we exposed five test strips (replicates) from each strain to each sodium chloride concentration.

Experiment 4: streptomycin, erythromycin, and streptomycin–penicillin mixture.—The in vitro methods were also used to test the toxicity of streptomycin (Sigma Aldrich S9137), erythromycin (Sigma Aldrich E5389), and a commercial penicillin–streptomycin mixture (Sigma Aldrich P4333; 10,000 IU penicillin and 10 mg streptomycin/mL) against *F. psychrophilum*. For the preliminary trial with streptomycin, we tested concentrations of 0, 100, 500, 1,000, 5,000, and 10,000 mg/L. For the follow-up trial (with all three strains of *F. psychrophilum*), we tested 0, 100, 500, 1,000, and 2,500 mg/L. For erythromycin we tested 0, 10, 100, 500, 1,000, and 2,000 mg/L during the preliminary trial and 0 and 2,000 mg/L during the secondary trial. For the commercially prepared penicillin–streptomycin mixture, we tested concentrations of 0, 1, 5, 10, 25, 50, 75, and 100% of the prepared product (diluted with sterilized hatchery well water) during the preliminary trial and 0, 1, 5, 10, 25, and 50% during the secondary trial. Depending on the concentration and antibiotic, either five or ten replicate test strips were exposed. All dilutions were made into sterilized well water. *F. psychrophilum* was exposed to the antibiotics for 15 min.

Data analysis.—For all experiments, the petri dishes were removed from the incubator once every 48 h over an 8-d observation period. Bacterial growth was noted as presence/absence. The morphology of all the colonies cultured was consistent with *F. psychrophilum*. Gram stains were collected from 5 to 10 randomly selected petri dishes with bacterial growth from each experiment to confirm that the cultured bacteria was consistent with *F. psychrophilum*. The gram stain results were consistent with *F. psychrophilum* in all instances. The percentage of tubes from each replicate that had *F. psychrophilum* growth was determined. In addition, a probit analysis was performed using the PROC PROBIT procedure in SAS (SAS 1998) to estimate the 50%- and 99%-lethal concentrations (LC50 and LC99

values) for each antibiotic treatment. The LC50 and LC99 values represent the antibiotic concentrations for which growth would only be expected in 1% (LC99) or 50% (LC50) of the replicates during a 15-min exposure. For the thermal shock test, the probit analysis was used to estimate the temperature required for *F. psychrophilum* growth on either 1% (LC99) or 50% (LC50) of the replicates 0, 10, 30, or 60 min after the introduction of the bacterium.

RESULTS

Experiment 1: Temperature

During the first portion of the experiment, in which the basic temperature tolerance of *F. psychrophilum* was determined using just the ATCC strain of the bacterium, no bacteria were cultured from any replicates treated at temperatures ≥55°C regardless of treatment duration (Table 1). In contrast, *F. psychrophilum*

TABLE 1. Percentages of plates of TYES media with culturable *Flavobacterium psychrophilum* ($N = 4$ or 8 plates, depending on the temperature and iteration). The bacteria were exposed to temperatures ranging from 15°C to 85°C for up to 60 min. Data for the ATCC 49510 strain were collected in two iterations. In the first iteration (ATCC 1), the basic temperature tolerance of *F. psychrophilum* was determined; in the second iteration (ATCC 2), data were also collected for the Clear Springs Foods 259-93 (CSF 259-93) and Utah Division of Wildlife Resources 09-131 (09-131) strains.

Temp (°C)	Time (min)	ATCC 1	ATCC 2	CSF 259-93	09-131	Temp (°C)	Time (min)	ATCC 1	ATCC 2	CSF 259-93	09-131
15	0	100	100	100	100	45	0	100	100	100	100
	10	100				45	5	25	0	0	0
	20	100				45	10	50	0	25	25
	30	100				45	20	100			
	40	100				45	30	25	0	0	0
	50	100				45	40	0			
	60	100	100	100	100	45	50	0			
25	0	100				45	60	0	0	0	0
	10	100				50	0	75	100	25	25
	20	100				50	5	0	0	0	0
	30	100				50	10	0	0	0	0
	40	100				50	30	0	0	0	0
	50	100				50	60	0			
	60	100				55	0	0	0	0	0
30	0	100				55	5		0	0	0
	10	100				55	10	0	0	0	0
	20	100				55	20	0			
	30	100				55	30	0	0	0	0
	40	100				55	40	0			
	50	100				55	50	0			
	60	100				55	60	0			
35	0	100	100	100	100	65	10	0			
	5	100	100	100	100	65	20	0			
	10	100	100	100	100	65	30	0			
	20	100				65	40	0			
	30	100	0	25	25	65	50	0			
	40	100				65	60	0			
	50	100				75	10	0			
	60	100	50	0	0	75	20	0			
40	0	100	100	100	100	75	30	0			
	5	0	100	100	25	75	40	0			
	10	63	75	75	25	75	50	0			
	20	0				75	60	0			
	30	6	0	50	0	85	10	0			
	40	0				85	20	0			
	50	0				85	30	0			
	60	0	0	75	0	85	40	0			
						85	50	0			
						85	60	0			

TABLE 2. Estimated temperatures (°C) required for 50% (LC50) and 99% (LC99) reductions in the number of *Flavobacterium psychrophilum* after 0, 10, 30, or 60 min of exposure at increased temperature. Estimates were derived using a probit analysis. The bacteria were acclimated to 15°C prior to the thermal shock.

Time (min)	LC50	LC99
0	51	52
10	43	54
30	38	49
60	37	38

was cultured at all temperatures ≤35°C. At temperatures of 40–50°C, the bacterium did not survive the entire 60-min exposure duration. At both 40°C and 45°C, we were able to culture *F. psychrophilum* from plastic strips that were treated for ≤30 min. At 50°C, we recovered bacteria from strips that were treated for ≤5 min.

In the second trial, in which three strains of *F. psychrophilum* were tested, the results were similar across strains. Regardless of strain, bacteria were able to survive exposure to temperatures ≤30°C for at least 60 min (Table 1). All strains were killed on contact at temperatures ≥55°C. The CSF 259-93 strain survived for at least 60 min at temperatures ≤40°C. We were only able to recover the 09-131 strain after a 10-min exposure at 40°C. This strain survived for at least 30 min at temperatures ≤35°C. Both the CSF 259-93 and the 09-131 strains survived for 10 min at 45°C.

The probit analysis (Table 2) showed that the temperature required to result in no bacterial growth decreased with exposure time. For example, in 10-min exposures, a temperature of 54°C was estimated to remove bacteria from 99% of the replicates but that only 38°C was required if the exposure period lasted 60 min (Table 2). Not surprisingly, higher temperatures were required to reduce growth to 1% of the isolates than to 50% of the isolates. In the egg treatments, no eggs that were exposed to a temperature of 55°C survived. Therefore, this temperature exceeds the short-term (10–60-s) tolerance limit of eyed Rainbow Trout eggs.

Experiments 2 and 3: pH and Sodium Chloride

Rapid changes in pH (3.0–11.0) were not effective in killing *F. psychrophilum.* Regardless of strain, we recovered bacteria from every replicate. Similarly, rapid changes in sodium chloride concentration were not effective in killing the bacterium. Since we were able to recover the bacterium from all of the strips included in these tests, we were not able to calculate LD50 and LD99 values for the effect of rapid changes in pH and osmotic pressure on the bacterium.

Experiment 4: Streptomycin, Erythromycin, and Penicillin–Streptomycin Mixture

We found that streptomycin is effective at controlling *F. psychrophilum*. During the first trial, we found that the ATCC strain of the bacterium was killed after a 15-min exposure to concentrations ≥5,000 mg/L (Table 3). The probit analysis showed that a 15-min exposure to a streptomycin concentration of 505 mg/L would kill 50% of *F. psychrophilum* and that a concentration of 567 mg/L would be required to kill 99% of the bacteria. During the second trial, we found that all three strains were able to survive treatment at concentrations of at least 2,500 mg/L. Bacteria were cultured from a lower percentage (20% versus 40%) of the replicates from the CSF 259-93 and 09-131 strains than the ATCC strain, suggesting that these strains are more susceptible to streptomycin treatment than the ATCC strain. Because we did not test any concentrations that killed 100% of the *F. psychrophilum*, we were not able to perform a probit analysis on the effects of streptomycin on the CSF 259-93 and 09-131 strains of the bacterium. We recovered *F. psychrophilum* from every strip treated with erythromycin (regardless of strain and concentration), indicating that concentrations >2,000 mg/L are required to kill *F. psychrophilum* with a 15-min exposure (Table 3).

TABLE 3. Percentages of TYES media plates with culturable *Flavobacterium psychrophilum* ($N = 5$ plates for all treatments except the 0% and 25% concentrations of the penicillin–streptomycin mixture [$N = 10$]). The bacteria were exposed to various concentrations of streptomycin, erythromycin, or a commercial penicillin–streptomycin mixture for 15 min. The concentrations listed for the penicillin–streptomycin mixture refer to the percentage concentrations in our test solution, which were obtained by dilution with sterilized well water. See Table 1 for additional information.

Antibiotic	Concentration (mg/L)	ATCC 1	ATCC 2	CSF 259-93	CSF 09-131
Streptomycin	0	100	100	100	100
	100	100	100	100	60
	500	60	90	80	50
	1,000	0	70	40	50
	2,500		40	20	20
	5,000	0			
	10,000	0			
Erythromycin	0	100	100	100	100
	10	100			
	100	100			
	500	100			
	1,000	100			
	2,000	100	100	100	100
Penicillin + streptomycin	0%	100	100	100	100
	1%	100	100	90	80
	5%	80	90	20	0
	10%	80	60	0	0
	25%	0	0	0	0
	50%	0	0	0	0
	75%	0			
	100%	0			

TABLE 4. Estimated concentrations of a penicillin–streptomycin mixture required for 50% (LC50) and 99% (LC99) reductions in the number of *Flavobacterium psychrophilum* after 15 min of exposure. Estimates were derived using a probit analysis. The listed concentrations are the percentages of a commercially prepared solution that contained 10,000 IU penicillin + 10 mg streptomycin/mL.

Strain	LC50	LC99
ATCC 49510	11.2	47.7
CSF 259-93	2.5	15.3
09-131	1.1	1.4

The commercial penicillin–streptomycin mixture was effective in killing the ATCC 49510 strain of *F. psychrophilum* during 15-min exposures to concentrations of ≥25% of that of the prepared product (i.e., 2.5×10^6 IU penicillin + 2,500 mg streptomycin / L were required; Table 3). The CSF 259-93 and 09-131 strains of the bacterium were more susceptible to treatment with the penicillin–streptomycin mixture and were killed at concentrations ≥10% of the commercial product (i.e., 1.0×10^6 IU penicillin + 1,000 mg streptomycin / L were required). The probit analysis showed that lower concentrations of the penicillin–streptomycin mixture would be required to kill the CSF 259-93 and 09-131 strains of the bacterium than to kill the ATCC strain (Table 4).

DISCUSSION

Our results demonstrate that sudden increases in temperature might be able to kill *F. psychrophilum*, as we were not able to culture bacteria that were treated at temperatures ≥55°C. The bacterium is able to survive for at least 60 min at temperatures between 15°C and 35°C. Other research has shown that *F. psychrophilum* can reproduce when incubated at temperatures between 4°C and 23°C, with the optimum growth rate being achieved at 15°C (Holt 1987; Bernardet and Kerouault 1989). Clearly, *F. psychrophilum* can tolerate short exposures to temperatures greater than optimal for incubation. The genus *Flavobacterium* contains species that can tolerate conditions that range from freezing (e.g., *F. hibernum*; McCammon et al. 1998) to ones with temperatures exceeding 70°C (e.g., *F. thermophilum*; Oshima and Yamakawa 1974). Because of its relation to these species, it is likely that *F. psychrophilum* has similar attributes and thus can tolerate suboptimal thermal conditions for short periods of time. In addition, most bacteria species possess heat shock proteins that help protect their cellular membranes and essential nucleic acids and proteins during periods of thermal stress (Oshima and Yamakawa 1974; Lindquist 1986). Our *F. psychrophilum* culture was incubated at 15°C. It is possible that bacteria acclimated to different temperatures would respond to the conditions tested in this study differently. Regardless, our results suggest that thermal shock can be used to remove surface-attached *F. psychrophilum*. These findings could be useful in the disinfection of equipment. Our findings, however, show that elevated temperature cannot be used for the disinfection of Rainbow Trout eggs. Previous research has shown that stressed bacteria can enter a "viable but nonculturable" state (Barer et al. 1993). As a result, the conditions under which we were not able to culture *F. psychrophilum* may not have killed the bacterium. Instead, the thermal shock may simply have caused it to enter a state in which it was not culturable.

We observed some differences among strains at some of the temperatures tested. In particular, the CSF 259-93 strain was the only strain that was cultured after 60 min of exposure at 40°C. This may indicate that this strain tolerates higher temperatures than do the other strains. Alternatively, it is possible that temperatures in the 40–50°C range represent a "gray area" in which *F. psychrophilum* can either grow well or be killed outright. Bacteria from the other strains may also have survived 60 min of treatment at 40°C, but we were not able to culture them. Regardless, we consistently were not able to culture bacteria from samples that were treated at temperatures >55°. We advise that this temperature be used as a minimum for disinfecting surfaces that may be contaminated with *F. psychrophilum*.

Antibiotic treatment could provide another avenue for killing *F. psychrophilum*. Previous research has established that the injection of steelhead (anadromous Rainbow Trout) brood with erythromycin helped reduce the prevalence of the disease among the progeny (Brown et al. 1997). However, in our study, a 15-min exposure to concentrations up to 2,000 mg/L was insufficient to kill *F. psychrophilum*. To our knowledge, injections with streptomycin have not been attempted. For streptomycin, we determined effective concentrations against *F. psychrophilum* and recommend concentrations ≥5,000 mg/L to kill the bacterium. Currently, erythromycin and streptomycin are not approved for aquaculture use by the FDA. Penicillin has been used for treating other species of bacteria in fish culture (DeCew 1972; Brown et al. 1990), but we are not aware of any literature on controlling *F. psychrophilum* infections with this antibiotic. Lumsden et al. (1996) noted that *F. psychrophilum* was susceptible to penicillin in a disk test, an observation that has been corroborated by our research (Oplinger, personal observation). Bustos et al. (1995), however, noted that a Chilean isolate of *F. psychrophilum* was not susceptible to penicillin, although the concentration of the sensitivity disk was not reported. In this study, the combination of penicillin and streptomycin was effective in killing *F. psychrophilum* at lower concentrations (2.5×10^6 IU penicillin + 2,500 mg/L streptomycin) than streptomycin alone. Previous research has found that penicillin and streptomycin can work synergistically against bacteria (Watanakunakorn 1971). It is possible that this synergism also operates with respect to the control of *F. psychrophilum*.

We observed variation among the three *F. psychrophilum* strains to the antibiotics tested. In general, the ATCC 49510 strain was the least susceptible to the antibiotics. Both the CSF 259-93 and Utah 09-131 strains had similar susceptibility to streptomycin. The Utah strain was the most susceptible to the penicillin–streptomycin mixture. Our findings corroborate the

work of other researchers, who have also found differences in antibiotic susceptibility among *F. psychrophilum* strains (Bruun et al. 2000). It is not known why these differences in susceptibility exist. It is likely, however, that the strains have different antibiotic exposure histories and that the more susceptible strains have historically received less antibiotic exposure than the less susceptible strains.

Osmotic shock and changes in pH have been used less frequently to kill bacteria. Working with *Flavobacterium columnare*, Suomalainen et al. (2005) demonstrated that 15-min exposures to a pH of 4.6 and 60-min exposures to sodium chloride concentrations of 4% or pH of 4.86–5.00 significantly reduced bacteria numbers. In the present study, we found that these two processes were ineffective in killing *F. psychrophilum*. Unlike those in other studies, our bacteria were not conditioned to the sodium chloride and pH conditions tested (e.g., Madetoja et al. 2003), so our data represent a true "shock." Bayer (1967) showed that *Escherichia coli* is more susceptible to sudden decreases in osmotic pressure when growing logarithmically, so culture age is a potential difference among the studies. Future research should test other *F. psychrophilum* strains and life stages to confirm the repeatability of the results observed in this study.

Using in vitro tests, we were able to establish guidelines for the elimination of *F. psychrophilum* through the use of thermal shocks and antibiotics. In the current study, streptomycin (especially when used synergistically with penicillin) was shown to be effective against *F. psychrophilum*. The treatment conditions and concentrations required to kill bacteria in vitro are not necessarily the same as those required in vivo (Gee and Sarles 1942;Bruun et al. 2003). In practice, poor water circulation (if thermal shocks are utilized) or biofilms (Stewart and Costerton 2001) reduce the effectiveness of treatments. Regardless, in vitro studies are a necessary first step in testing new treatment methods against *F. psychrophilum*. Future research should test thermal shocks, streptomycin, and the penicillin–streptomycin mixture in vivo to establish the effectiveness of these treatments in a fish culture setting. In addition, future research should determine the effects of these treatments on egg and larval development.

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