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ISOLATION AND PHENOTYPIC CHARACTERIZATION OF AN OXIDASE-NEGATIVE *AEROMONAS SALMONICIDA* CAUSING FURUNCULOSIS IN COHO SALMON (*ONCORHYNCHUS KISUTCH*)

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ABSTRACT: An oxidase-negative *Aeromonas salmonicida* was isolated from coho salmon (*Oncorhynchus kisutch*) suffering from an epizootic of furunculosis at the state hatchery near Belfair, Washington. Typical, oxidase-positive *A. salmonicida* was isolated concurrently from the same population of fish. Mortality was controlled with medicated feed treatments. Evidence supporting the identification of the two types of *A. salmonicida* is presented. Methods for the proper identification of oxidase-negative *A. salmonicida* isolates are evaluated.

Key words: *Aeromonas salmonicida*, cytochrome oxidase, furunculosis, Coomassie Brilliant Blue agar, coho salmon, *Oncorhynchus kisutch*.

INTRODUCTION

Three subspecies of *Aeromonas salmonicida* are recognized in *Bergey's Manual of Systematic Bacteriology* (Popoff, 1984). These subspecies differ from other aeromonads in being nonmotile (Popoff, 1984) and from each other in several biochemical characteristics (Elliott and Shotts, 1980; Paterson et al., 1980). All subspecies of *A. salmonicida* are pathogenic to various fish species and cause several diseases, including furunculosis, goldfish ulcer disease and carp erythrodermititis. Except for an infrequently isolated strain of motile *Aeromonas*, *A. salmonicida* is the only common fish pathogen that produces a brown, water soluble pigment on media containing 0.1% tyrosine or phenylalanine. Although this characteristic is not universal in all *A. salmonicida* isolates, it nevertheless is used in identification schemes (Amos, 1985).

Several methods exist to isolate and identify *A. salmonicida* from diseased fish (Shotts and Bullock, 1975, 1976; McFaddin, 1980) but the most commonly cited method (Amos, 1985) relies on the use of nonselective, nondifferential media for primary isolation, followed by biochemical and serological characterization of the isolated bacteria. *Aeromonas salmonicida* may be presumptively diagnosed by isolating a short (1–2 × 0.8 μm), gram negative, oxidase-positive, nonmotile rod (Amos, 1985).

We report here the isolation and phenotypic characterization of a cytochrome oxidase-negative *A. salmonicida* causing furunculosis in coho salmon (*Oncorhynchus kisutch*).

MATERIALS AND METHODS

On 18 May 1989, approximately 842,000 coho salmon fingerlings averaging 1.8 g were transported from Washington Department of Fisheries' (WDF) Skykomish Hatchery near Sultan, Washington (47°52'N, 121°49'W), to pond 1 of WDF's Coulter Creek Hatchery near Belfair, Washington (47°24'N, 121°49'W) for final rearing before transfer to net pens in winter. These fish were hatched from eggs obtained from adults that had been trapped and spawned at Skykomish Hatchery in the fall of 1988. Before transfer to Coulter Creek Hatchery, the fish were reared at Skykomish Hatchery in untreated water taken from May Creek and Wallace River. Approximately 3,143,000 fingerling coho salmon remained at Skykomish Hatchery for final rearing after the other fish had been transferred to Coulter Creek Hatchery and other stations.

On 13 June 1989, about half of the coho salmon at Coulter Creek Hatchery averaging 3.7 g were transferred from pond 1 to the adjacent pond 2, resulting in approximately equal numbers of fish in each pond. Both ponds were supplied with untreated Coulter Creek water.

Mortalities increased shortly thereafter and

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representative dead and moribund fish from both ponds were examined on June 26 using methods described by Amos (1985), except that kidney tissues were inoculated onto Coomassie Brilliant Blue (CBB) agar (Wilson and Horne, 1986), which is a modification of Tryptic Soy agar (TSA), rather than TSA. Inoculated plates were incubated at 20 C until bacterial colonies could be subcultured onto TSA (Difco Laboratories, Detroit, Michigan 48232, USA) and identified. Two isolates from different fish were subcultured and characterized biochemically and confirmed serologically by the direct fluorescent antibody test (FAT) using FITC-conjugated *A. salmonicida* antiserum supplied by the National Fish Health Research Laboratory (Kearneysville, West Virginia 25430, USA). Initial biochemical and serological tests were conducted as described by Amos (1985).

Initial antibiotic sensitivity was tested using a variation of the disc diffusion technique (Difco Laboratories, 1984). Isolates tested were cultured for 24 hr in Tryptic Soy Broth (TSB; Difco) at 22 C and inoculated onto Mueller Hinton (MH) agar plates (Difco) without adjusting the turbidity of the culture. Inoculated plates with each of three sensitivity discs (Difco and BBL Microbiology Systems, Cockeysville, Maryland 21030, USA) containing either Romet (ormetoprim/sulfadimethoxine; Hoffman-La Roche, Inc., Nutley, New Jersey 07110, USA), oxytetracycline (Abbott Laboratories, North Chicago, Illinois 60064, USA), or Sarafloxacin (compound A-56620; Abbott) were incubated for 48 hr at 22 C before zones of inhibition were measured.

To confirm our initial identification of the isolates, more extensive biochemical tests were conducted on both isolates as well as on a reference isolate of *A. salmonicida* (3.123) from the culture collection of the National Fish Health Research Laboratory, Kearneysville, West Virginia 25430. Carbohydrate utilization profiles were determined by inoculating cultures into O-F basal medium (Difco) supplemented with 1.0% of the carbohydrate. A green to yellow color change within 72 hr was recorded as positive. Other biochemical tests followed standard methodology (MacFaddin, 1980). The cytochrome oxidase reactions were determined from 24- to 48-hr cultures grown on triple sugar iron (TSI; Difco), TSA, and 5% sheep blood agar (BA). Two methods were used for the determination of cytochrome oxidase. The first involved streaking a loop full of each isolate onto a Pathotec CO strip (Organon Teknika Corporation, Durham, North Carolina 27704, USA) and the second consisted of placing a drop of oxidase reagent onto a filter paper and streaking the culture across the paper. The reagent was prepared by dissolving 0.1-g N,N,N′,N′-tetramethyl-p-phenylene diamine (Sigma Chemical Company, St. Louis, Missouri 63178, USA) in 10 ml deionized water. For both methods, the development of a blue or purple color within 30 sec indicated a positive cytochrome oxidase reaction.

Antibiotic sensitivity profiles were determined by using the Kirby-Bauer method (Bauer et al., 1966). Briefly, 24 hr cultures grown in TSB were centrifuged at 5,000 × g for 15 min, and washed in pH 7.2 phosphate buffered saline. Final cell suspensions were adjusted to approximately 10^6 CFU/ml. The final suspension was swabbed onto MH agar plates and antibiotic discs (BBL) were positioned on the agar surface. Diameters of inhibition zones were measured in millimeters after 24 hr incubation at 25 C.

Beginning 27 June fish in both ponds 1 and 2 at Coulter Creek Hatchery received oxytetracycline-medicated feed (Moore-Clark Company, La Conner, Washington 98257, USA) for 14 days at a calculated dose of 82.5 mg per kg of fish per day. On 11 August, representative dead and moribund fish from both ponds averaging 7.2 g were examined and cultures taken using the methods described previously. Bacterial isolates were subcultured, identified, and tested for antibiotic sensitivity by methods described previously.

Beginning 14 August, fish in Pond 2 were treated with Romet-medicated feed (Moore-Clark Company) for 5 days at a calculated dose of 50 mg per kg of fish per day. On 16 October, representative healthy, dead, and moribund fish averaging 15.1 g from both ponds were examined as described previously.

Representative healthy, dead, and moribund coho salmon averaging 6.5 g being reared at Skykomish Hatchery were examined on August 22 and bacterial isolates were characterized by the methods described previously. These fish originated from the same group of coho salmon shipped to Coulter Creek Hatchery. Isolation of bacteria from fish at Skykomish Hatchery that were similar to those found at Coulter Creek Hatchery would provide evidence for the source of infection at Coulter Creek Hatchery.

A subculture of the oxidase-negative isolate has been deposited in the American Type Culture Collection (Bethesda, Maryland 20852, USA; ATCC Number 49385).

RESULTS

Most moribund and dead fish examined 26 June at Coulter Creek Hatchery showed no external or internal signs of bacterial infection other than soft kidneys. A few fish had hemorrhaged fin bases and a single

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TABLE 1. Characteristics of *Aeromonas salmonicida* isolates obtained from coho salmon at Washington Department of Fisheries' Coulter Creek Hatchery in June (isolates 1 and 2), and August (isolates 3 to 9) 1989.

Characteristic	Isolate								
	1	2	3	4	5	6	7	8	9
Morphology	cb[a]	cb	cb	cb	cb	cb	cb	cb	cb
Gram stain	−	−	−	−	−	−	−	−	−
Motility	−	−	−	−	−	−	−	−	−
Oxidase	+(1)[b]	−(1)	+(3)	+(3)	−(1)	−(4)	−(1)	−(3)	−(8)
Brown pigment on TSA[c]	+	+	+	+	+	+	+	+	+
Blue colonies on CBB[d]	+	+	+	+	+	+	+	+	+
Triple Sugar Iron	K/A	A/A	K/AG	K/AG	K/AG	K/AG	K/A	K/A	K/AG
Gelatin liquefaction	+	+	+	+	+	+	+	+	+
Sucrose catabolism	ND[e]	−	−	−	−	−	−	−	−
Salicin catabolism	ND	ND	+	+	+	+	+	+	+
Glucose fermentation	ND	ND	+	+	+	+	+	+	+
Indole	ND	ND	−	−	−	−	−	−	−
0/129 sensitivity	ND	−	−	−	−	−	−	−	−
FAT[f]	+	+	+	+	+	+	+	+	+
Antibiotic									
Oxytetracycline (30 mcg)	46[g]	0	0	0	0	0	0	0	0
Sarafloxacin[h] (5 mcg)	37	43	37	39	42	38	38	40	36
Romet[i] (25 mcg)	40	39	33	34	36	33	36	37	34

[a] Coccobacillus.
[b] Number of colonies tested for oxidase shown in parentheses.
[c] Tryptic soy agar.
[d] Coomassie brilliant blue agar.
[e] No data.
[f] Fluorescent antibody test for *A. salmonicida*.
[g] Zone of inhibition (mm).
[h] Compound A-56620 (Abbott Laboratories, North Chicago, Illinois 60064, USA).
[i] Ormetoprim/sulfadimethoxine (Hoffmann-La Roche, Inc., Nutley, New Jersey 07110, USA).

fish had a small, shallow muscle lesion that was closed and necrotic. Mortality in pond 2 was approximately double that in pond 1 and losses peaked at approximately 0.08% per day. Stained kidney imprints revealed the presence of large rod-shaped bacteria in 12 of 15 fish examined. Inoculations on CBB agar from kidney tissue of fish yielded dark blue colonies that produced a brown, diffusible pigment within two days from all fish. Other colony types were not detected. Because previous experience with CBB agar indicated that these colonies were characteristic of *A. salmonicida*, only one colony from each of two fish from different ponds was subcultured onto TSA for further study. Isolate 1 was obtained from a fish in pond 2 and isolate 2 from a fish in pond 1. With the significant exception of isolate 2 being oxidase-negative, both isolates were biochemically similar in initial tests (Table 1) and were presumptively identified as *A. salmonicida*. Both isolates were strongly positive by FAT. Isolate 2 also differed from isolate 1 in that it showed no zone of sensitivity to oxytetracycline.

Extensive characterization of isolates 1 and 2 showed that both isolates were biochemically homogeneous except for the lack of cytochrome oxidase activity in isolate 2 (Table 2). This isolate was consistently negative for cytochrome oxidase, whether grown on TSI, TSA, or BA. There was no discrepancy between the two methods used for the determination of cytochrome oxidase. Except for the cytochrome oxidase reaction, both isolates were biochemically consistent with criteria described for *A. salmonicida*. Antibiotic sen-

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TABLE 2. Biochemical profiles of an oxidase-positive (#1) and an oxidase-negative (#2) isolate of *Aeromonas salmonicida* from Coulter Creek Hatchery, Washington. Isolate 3.123 from the culture collection at National Fish Health Research Laboratory, Kearneysville, West Virginia was used as the reference strain.

Biochemical test	Isolate 1	Isolate 2	Isolate 3.123
Triple Sugar Iron	K/A	K/A	K/A
Brown pigment (TSA)	+	+	+
Citrate utilization	−	−	−
Bile-esculin hydrolysis	+	+	+
Phenylalinine deaminase	−	−	−
Nitrate reduction	+	+	+
Urease	−	−	−
Motility	−	−	−
Indole	−	−	−
Malonate	−	−	−
Methyl red	+	+	+
Voges-Proskauer	−	−	−
Gelatin liquefaction	+	+	+
Glucose fermentation	+	+	+
Arginine dehydrolase	−	−	−
Lysine decarboxylase	−	−	−
Ornithine decarboxylase	−	−	−
Cytochrome oxidase	+	−	+
Catalase	+	+	+
Blue colonies on CBB	+	+	+
Acid production from:			
Adonitol	−	−	−
Arabinose	−	−	+
Arabitol	−	−	−
Cellobiose	−	−	−
Dulcitol	−	−	−
Erythritol	−	−	−
Fructose	+	+	+
Galactose	+	+	+
Glucose	+	+	+
Inositol	−	−	−
Lactose	−	−	−
Levulose	+	+	+
Maltose	+	+	+
Mannitol	+	+	+
Mannose	+	+	−
Melibiose	−	−	−
Raffinose	−	−	−
Rhamnose	−	−	−
Salicin	+	+	+
Sorbose	−	−	−
Sorbitol	−	−	−
Sucrose	−	−	−
Trehalose	−	−	−
Xylose	−	−	−

TABLE 3. Antibiotic sensitivity profiles for an oxidase-positive and an oxidase-negative isolate of *Aeromonas salmonicida* from Coulter Creek Hatchery, Washington.

Antimicrobial and concentration	Zone of inhibition (mm) Oxidase positive	Oxidase negative
Ampicillin (10 mcg)	29	34
Bacitracin (10 units)	0	0
Chloramphenicol (30 mcg)	37	36
Erythromycin (15 mcg)	24	20
Gentamicin (10 mcg)	23	20
Kanamycin (30 mcg)	22	22
Nalidixic acid (30 mcg)	39	36
Neomycin (30 mcg)	24	22
Novobiocin (30 mcg)	18	22
Oxolinic acid (2 mcg)	33	33
Oxytetracycline (30 mcg)	32	0
Polymyxin b (300 units)	17	19
Streptomycin (10 mcg)	12	14
Sulfadiazine (0.25 mg)	34	0
Sulfamethoxozole/trimethoprim (25 mcg)	33	32

sitivity profiles (Table 3) for the two isolates differed, isolate 2 being resistant to oxytetracycline and sulfadiazine.

Consistent with the antibiotic sensitivity results, treatment of affected fish with oxytetracycline was only partly effective and mortality in pond 2 remained elevated. When fish from both ponds were again examined in August, clinical signs of disease were similar to those seen in June. Typical blue bacterial colonies were isolated on CBB agar from nine of the fish examined. Multiple colonies from seven of these fish were subcultured onto TSA and purified isolates (numbered 3 to 9) were tested biochemically, serologically, and for antibiotic sensitivity (Table 1). Oxidase-negative and -positive isolates were recovered from fish from both ponds, but only one type was cultured from any particular fish. All isolates obtained in August were resistant to oxytetracycline.

After the fish were treated with Romet-medicated feed in August, mortality returned to normal. *Aeromonas salmonicida* was not isolated from fish examined on 16 October. An estimated 1.4% of the popu-

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lation died during the epizootic attributed to *A. salmonicida* at the Coulter Creek Hatchery.

Oxidase-positive *A. salmonicida* was isolated from 10 of 20 dead or moribund coho salmon at Skykomish Hatchery in August but was not isolated from healthy fish. No oxidase-negative isolates were cultured from any fish at that hatchery.

DISCUSSION

An unusual fish pathogen, a cytochrome-oxidase negative *A. salmonicida*, was isolated from coho salmon reared at the Coulter Creek Hatchery. The source of infection is unclear because only oxidase-positive *A. salmonicida* was isolated from the same stock of fish being reared at Skykomish Hatchery, from which they were transferred. Because Coulter Creek Hatchery is supplied with untreated Coulter Creek water, feral fish in the creek are a potential source of infection, but these have not been examined. It is possible that the unusual oxidase reaction of isolates in this case resulted from a spontaneous mutation of the typical *A. salmonicida*; however, this was not investigated.

During the course of the furunculosis epizootic at Coulter Creek Hatchery, selection for antibiotic resistance by *A. salmonicida* appears to have occurred there. Before any antibiotic treatment, both oxytetracycline susceptible and resistant isolates were recovered from diseased fish. In August, however, following treatment with oxytetracycline, only oxytetracycline resistant bacteria were isolated from infected fish. Selection for antibiotic resistance was most likely due to the elimination of susceptible bacteria through oxytetracycline therapy of infected fish, and may also involve the transfer of drug resistance plasmids. Such plasmids have been detected in Aeromonads (Popoff, 1984) and other fish pathogenic bacteria (Aoki and Kitao, 1985).

The importance of subculturing several colonies from different fish for identification or antibiotic sensitivity tests cannot be stressed too strongly. Our isolations revealed the presence of both oxidase-positive and -negative strains that initially showed dissimilar antibiotic sensitivity in the same population of fish. We were fortunate to have subcultured both strains in June, since only two isolates were chosen randomly for further testing. We could have just as easily selected only one type, which may have yielded different conclusions than those reported here. It is unknown whether previous furunculosis epizootics at Coulter Creek Hatchery included oxidase-negative strains of *A. salmonicida*, because extensive characterization of bacterial isolates was not performed.

Many authors have reported atypical strains of *A. salmonicida* that cause disease in fish (Elliott and Shotts, 1980; Evelyn, 1971; Paterson et al., 1980; McCarthy, 1975) but, in all cases, the divergence from atypical strains of *A. salmonicida* involves the lack or delayed production of pigment or differences in certain biochemical reactions. Oxidase-negative *A. salmonicida* isolates have not been previously reported to occur and apparently are extremely rare. Our identification of an oxidase-negative isolate of bacteria that otherwise has characteristics leading to its identification as *A. salmonicida*, therefore, is significant, yet problematic, since a positive oxidase test is among the most important characteristics of Aeromonads (Popoff, 1984).

Great care must be exercised when identifying fish pathogenic bacteria using the schemes outlined by Shotts and Bullock (1975, 1976), Amos (1985), and Department of Fisheries and Oceans (1984). All of these methods rely on results of the oxidase test early in the sequence of steps leading to identification. Considering only oxidase results at this point would have led to the identification of our isolate as a member of the Enterobacteriaceae. Other characteristics of the isolate, particularly the dark blue colony color on CBB agar, pigment production, morphology, and the fact that it was isolated concurrently with typical *A. salmonicida* from the same pop-

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ulation of fish suffering epizootic mortality, led us to question its placement in the Enterobacteriaceae.

Coomassie Brilliant Blue is a protein specific dye included in CBB agar that results in dark blue colonies of bacteria that have the A-layer protein (Udey, 1982; Wilson and Horne, 1986). Fish pathogenic bacteria other than *A. salmonicida* have not been reported to have an A-layer. Markwardt et al. (1989) cultured *A. salmonicida* and six other bacteria and found that only *A. salmonicida* yielded dark blue colonies on CBB agar. The strongest initial evidence that both our oxidase-positive and -negative isolates were *A. salmonicida* was the colony color on CBB, the lack of motility, and the brown pigment production. Further biochemical and FAT results confirmed our belief that both isolates were, in fact, *A. salmonicida*.

The inclusion of CBB agar in schemes to identify *A. salmonicida* has proved valuable in our work, is used routinely for primary isolation of fish pathogenic bacteria by the Washington Department of Fisheries, and will become even more valuable if oxidase-negative strains are found elsewhere. Caution should be exercised in identifying colonies that are not dark blue on CBB, however, since some A-layer negative *A. salmonicida* strains are pathogenic to fish (Johnson et al., 1985) and would not yield dark blue colonies.

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