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Isolation and Characterization of *Edwardsiella tarda* from Fall Chinook Salmon (*Oncorhynchus tshawytscha*)†

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A new bacterial pathogen of chinook salmon (*Oncorhynchus tshawytscha*) was isolated from fish in Oregon's Rogue River. The bacteria are biochemically and serologically related to strains of *Edwardsiella tarda*. Initially isolated from chinook salmon, the bacteria were also pathogenic for steelhead and rainbow trout (*Salmo gairdneri*), and channel catfish (*Ictalurus punctatus*). The 50% lethal doses for chinook salmon, steelhead trout, and channel catfish injected intraperitoneally and maintained in 18°C water were 4.1×10^6, 5.6×10^6, and 4.0×10^5, respectively. When chinook salmon and rainbow trout were injected intraperitoneally and held in 12°C water, the mean lethal doses were 6.4×10^7 and 1.7×10^6, respectively. The invasiveness of the organism was low in steelhead trout exposed to the bacteria by the waterborne route. The optimum growth temperature of the bacteria in brain heart infusion broth was approximately 35°C. The guanine plus cytosine content of DNA obtained from *E. tarda* isolated from salmon was 59 mol%.

During the fall of 1979 a large number of prespawning wild adult fall chinook salmon (*Oncorhynchus tshawytscha*) died in the Rogue River, Oregon. During an investigation of a similar mortality in 1980, *Edwardsiella tarda* were isolated from some of the dead salmon. In 1981, the bacteria were again observed by the direct fluorescent-antibody test (DFAT) in a larger number of dead salmon. *E. tarda*, characterized by Ewing et al. (4), have been isolated from a variety of vertebrates (3, 8, 10, 16, 23) and invertebrates (1, 18). In fish, *E. tarda* have generally been associated with warm-water species (11, 15, 21–23, 25). This report documents the first isolation of *E. tarda* from salmonid fish and from any fish species in the Pacific Northwest of the United States. The organism is pathogenic for chinook salmon, steelhead and rainbow trout (*Salmo gairdneri*), and channel catfish (*Ictalurus punctatus*).

MATERIALS AND METHODS

Isolation and detection. *E. tarda* isolates were obtained from moribund and dead prespawning wild adult fall chinook salmon in the Rogue River, Oregon. The bacteria were cultured by streaking kidney tissue on brain heart infusion agar (BHIA; Difco Laboratories, Detroit, Mich.). In addition, thioglycollate broth (THB; Difco) was inoculated with kidney tissue of certain fish. Bacterial growth from THB was subcultured onto BHIA. All preparations were incubated at 22°C for a minimum of 96 h.

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In 1981, BHIA and THB were inoculated with kidney tissue from 16 freshly killed adult fall chinook salmon. In addition, material from the intestinal tracts of the same fish was cultured on salmonella-shigella agar (Difco).

During the 1981 examinations, smears from kidney tissue and intestinal contents of freshly killed and spawned adult fall chinook salmon as well as kidney tissue smears of prespawning and postspawning fall chinook salmon carcasses in various stages of decomposition were examined by DFAT. Antisera were prepared against a chinook salmon *E. tarda* isolate and were labeled with fluorescein after purification of the immunoglobulin G component in an affigel blue column (Bio-Rad Laboratories, Richmond, Calif.).

Characterization. The *E. tarda* isolates from fall chinook salmon were characterized by morphological, biochemical, and serological comparison with known strains of *E. tarda* (ATCC 15947, ATCC 15948, FL-77-4) and *Edwardsiella ictaluri* (7; ATCC 33202, AL-79-164). The morphology and motility of the bacteria were determined microscopically by Gram stain and wet mount, respectively. Biochemical results were obtained by inoculating API20E and API50L test strips (Analytab Products, Plainview, N.Y.) and triple sugar iron agar slants (BBL Microbiology Systems, Cockeysville, Md.). Pathotec CO test strips (General Diagnostics, Morris Plains, N.J.) were used to test for the presence of cytochrome oxidase.

The chinook salmon *E. tarda* isolates were compared serologically by slide agglutination and DFAT. Specific rabbit antisera for *E. tarda* were obtained from the National Fish Health Laboratory, Leetown, W. Va. The sensitivity of the *E. tarda* type strain and each isolate from fall chinook to selected antibiotics was determined by the agar diffusion method by using Dispens-O-Discs (Difco) on BHIA.

The effect of temperature on the growth rate of *E. tarda* was determined by using a temperature gradient incubator (Scientific Products Div., McGaw Park, Ill.). Test tubes containing 7 ml of brain heart infusion broth (BHIB) were preincubated at 5°C increments from 5 to 50°C. The tubes were then inoculated with 0.1 ml of an 18-h culture of *E. tarda* grown in BHIB. Optical density was read every 20 min in a Klett-Summerson colorimeter. Doubling time of the organism was calculated from a graph of optical density versus time.

E. tarda isolated from salmon and *Escherichia coli* WP2 were grown in BHIB for 24 h at 25 and 37°C, respectively. Cells were harvested by centrifugation, and DNA was isolated by the method of Marmur (14). Sodium perchlorate was omitted, and protein was removed from the lysed culture by extraction with a mixture of Tris-saturated phenol (50 mM Tris, pH 8.0), chloroform, and isoamyl alcohol at a 25:24:1 ratio. Samples of bacterial DNA dissolved in 0.1× SSC (0.015 M NaCl, 0.0015 M trisodium citrate, pH 7.0) were adjusted to an absorbance of approximately 0.5 at 260 nm before overnight dialysis against 200 volumes of 0.1× SSC. The absorbance of each preparation at 260 nm was recorded at 0.5°C increments between 65 and 90°C with a Beckman model DU-8 computing spectrophotometer equipped with a Tm Compuset Module. Interval time was set at 2.0 min. Absorbance readings were corrected for thermal expansion, and the melting temperature was determined. The guanine plus cytosine content of *E. tarda* DNA was calculated with the equation of Mandel et al. (13). DNA extracted from *E. coli* WP2 (19; 51.0 mol% guanine plus cytosine) was used as a standard.

Infectivity. Virulence of *E. tarda* was tested in spring chinook salmon, steelhead and rainbow trout, and channel catfish. The fish were held in 68-liter aquariums supplied with pathogen-free water at 12 or 18°C. Duplicate groups of 10 fish for salmonids or 7 fish for channel catfish were used in the infectivity studies. The virulence of the bacteria was tested by either intraperitoneal (i.p.) injection or by waterborne exposure. Fish injected i.p. received 0.1 ml of a known dilution of *E. tarda* washed by centrifugation and suspended in 0.85% sterile saline. Control fish were injected i.p. with 0.1 ml of 0.85% sterile saline. *E. tarda* were injected i.p. into chinook salmon (mean weight, 56 g), steelhead trout (mean weight, 21 g), and channel catfish (mean weight, 7.6 g) held in 18°C water and into chinook salmon (mean weight, 63 g) and rainbow trout (mean weight, 2.5 g) held in 12°C water.

Steelhead trout (mean weight, 21 g) held in aquariums at 18°C were exposed to *E. tarda* by the waterborne route. Aquariums of 68-liter capacity were filled to contain 20 liters of water (including volume of fish). The bacteria were grown in BHIB at 22°C, enumerated by plate count, and added directly to the aquariums to give final concentrations which ranged from 3.8×10^4 to 3.8×10^7 or from 2.0×10^5 to 2.0×10^8 cells per ml of aquarium water. After a 15-min contact period, waterflow was resumed and the bacterial cells were diluted out (5). Sterile BHIB was added to the aquariums containing control fish.

For detection of the bacteria in exposed animals, kidney tissue of dead fish and of survivors was cultured on BHIA and Gram stained. *E. tarda* were identified by colony and cell morphology, motility, negative oxidase reaction, positive indole test, and reactions in triple sugar iron agar slants.

RESULTS

Isolation and detection. *E. tarda* were recovered more often when THB rather than BHIA was used as a primary isolation medium. The bacteria were isolated from 19% of the chinook salmon when THB was used as a culture medium but from only 2% of those cultured on BHIA. All isolates grew well on BHIA upon subculture from THB. The *E. tarda* isolates were obtained when the water temperature in the Rogue River ranged from 17 to 20°C.

E. tarda were detected in high numbers by DFAT in kidney smears of 14 of 29 prespawning and 23 of 46 postspawning fall chinook salmon carcasses examined in 1981. The bacteria were not detected in cultures of intestines or kidneys of 16 freshly killed and spawned salmon, nor was it detected by DFAT in smears of the same tissues.

Characterization. Biochemically, the bacterial isolates from chinook salmon were identical to one another and to known *E. tarda* strains but different from *E. ictaluri* (Table 1). The *E. ictaluri* strains were negative for indole and H_2S production and urease positive, whereas *E. tarda* was positive for indole and H_2S production and urease negative. Comparison of the antimicrobial sensitivity of the *E. tarda* isolates from chinook salmon and the *E. tarda* type strain is shown in Table 2. When treated with specific antisera for *E. tarda*, all of the fish isolates agglutinated or fluoresced.

E. tarda from chinook salmon grew best at approximately 35°C with a doubling time of 34 min. Doubling times at 15, 20, 25, 30, and 40°C were 178, 86, 39, and 66 min, respectively. No growth occurred below 10 or above 45°C in the 24-h incubation period.

The guanine plus cytosine content of DNA from *E. tarda* isolated from salmon was calculated to be 59 mol%. This is in the upper range of guanine plus cytosine values for *E. tarda* (6; 56 to 59 mol%) and above that reported for *E. ictaluri* (7; 53 mol%).

Infectivity. *E. tarda* isolated from chinook salmon were pathogenic for chinook salmon, steelhead and rainbow trout, and channel catfish when injected i.p. For chinook salmon, steelhead trout, and channel catfish held at 18°C, 50% lethal dose values were 4.1×10^6, 5.6×10^6, and 4.0×10^5 bacterial cells, respectively, and were 6.4×10^7 bacterial cells for chinook salmon and 1.7×10^6 bacterial cells for rainbow trout held in 12°C water. The number of steelhead trout held at 18°C that died after waterborne exposure to *E. tarda* ranged from 0 to 5% and 0 to 15% in duplicate experiments. Deaths

TABLE 1. Biochemical reactions of *E. tarda* isolates from Rogue River fall chinook salmon and known *E. tarda* and *E. ictaluri* isolates

Substance	Rogue River *E. tarda* isolates[a]	Known *E. tarda* isolates[b]	Known *E. ictaluri* isolates[c]	Substance	Rogue River *E. tarda* isolates[a]	Known *E. tarda* isolates[b]	Known *E. ictaluri* isolates[c]
Glycerol	+	+	+	D(+)trehalose	−	−	−
Erythritol	−	−	−	Inuline	−	−	−
D(−)arabinose	−	−	−	D(+)melezitose	−	−	−
L(+)arabinose	−	−	−	D(+)raffinose	−	−	−
Ribose	+	+	+	Dextrin	−	−	−
D(+)xylose	−	−	−	Amylose	−	−	−
L(−)xylose	−	−	−	Starch	−	−	−
Adonitol	−	−	−	Glycogen	−	−	−
Methyl-xyloside	−	−	−	Gas from glucose	+	+	+
Galactose	+	+	+	Teepol (0.4%)	+	+	+
D(+)glucose	+	+	+	Teepol (0.6%)	+	+	+
D(−)levulose fructose	+	+	+	Gluconate	+	+	+
				Urea	−	−	+
D(+)mannose	+	+	+	*o*-Nitrophenyl-β-D-galactopyranoside	−	−	−
L(−)sorbose	−	−	−				
Rhamnose	−	−	−	Catalase	+	+	+
Dulcitol	−	−	−	Nitrate reduction	+	+	+
Meso-inositol	−	−	−	Indole	+	+	−
Mannitol	−	−	−	Methyl red	+	+	+
Sorbitol	−	−	−	Voges-Proskauer	−	−	−
Methyl-D-mannoside	−	−	−	Citrate	−	−	−
				Arginine dihydrolase	−	−	−
Methyl-D-glucoside	−	−	−				
N-acetyl-glucosamine	+	+	+	Lysine decarboxylase	+	+	+
Amygdalin	−	−	−	Ornithine decarboxylase	+	+	+
Arbutine	−	−	−				
Esculin	−	−	−	Hydrogen sulfide	+	+	−
Salicin	−	−	−	Tryptophan deaminase	−	−	−
D(+)cellobiose	−	−	−				
Maltose	+	+	+	Gelatin liquefaction	−	−	−
Lactose	−	−	−	Oxidase	−	−	−
D(+)melibiose	−	−	−	Triple sugar iron	K/AG[d]	K/AG	K/AG
Sucrose	−	−	−				

[a] Combined results from five isolates.
[b] Combined results from ATCC 15947 (type strain), ATCC 15948, and FL-77-4 (largemouth bass, Auburn University).
[c] Combined results from ATCC 33202 (type strain) and AL-79-164 (both provided by Auburn University).
[d] Alkaline/acid with gas production.

occurred when cell concentrations were 3.8×10^5 bacteria per ml or greater.

DISCUSSION

The bacteria isolated from chinook salmon were identified as *E. tarda* after a comparison of the biochemical characteristics of the isolates with those of previously described *E. tarda* strains. Agglutination and fluorescence reactions after treatment with antisera specific for *E. tarda* confirmed the identification of these isolates and showed serological relatedness.

The improved isolation of *E. tarda* in THB compared with BHIA was significant because THB is not routinely used in fish disease diagnostics. The higher isolation rate of the organisms in THB (19%) than BHIA (2%) indicated that THB acted as an enrichment medium. On subsequent subculture to BHIA from THB these isolates grew well, again indicating that THB enhanced growth.

E. tarda has not been previously reported to be pathogenic for salmonid fish. Meyer and Bullock (15), using an *E. tarda* isolate from channel catfish, reported that no deaths occurred in brown trout (*Salmo trutta*) held in 13°C water after i.p. injection with 8×10^7 bacterial cells. However, in this study, rainbow trout and chinook salmon held in 12°C water were killed when injected i.p. with fewer than 8×10^7 bacterial cells.

The chinook salmon *E. tarda* was pathogenic for all species tested when i.p. injected. High numbers of bacteria ($>10^5$) were required to

TABLE 2. Antimicrobial sensitivity of *E. tarda* isolates from chinook salmon (*Oncorhynchus tshawytscha*) compared with the *E. tarda* type strain

Antimicrobial agent[a]	Concn. (μg)	Level of sensitivity[b]	
		E. tarda from Chinook salmon isolates[c]	*E. tarda* type strain[d]
Novobiocin	5	R	++
Triple sulfa	300	++	+++
Sulfathiazole	300	R	+++
Sulfadiazine	300	R	R
Erythromycin	5	R	R
Erythromycin	15	+	++
Oxytetracycline	30	+++	+++
Tetracycline	10	+++	+++
Kanamycin	30	++	++

[a] Dispens-O-Discs (Difco).
[b] Describes the zone of inhibition in millimeters: 0–9 (+); 10–19 (++); 20–29 (+++); R, resistant.
[c] Five isolates.
[d] ATCC 15947.

produce 50% lethal doses. Of the species tested, channel catfish were the most susceptible. Reports of the virulence of *E. tarda* in i.p. injected channel catfish have ranged from 1×10^1 cells that caused death within 10 days (12; experimental conditions not described) to 8.0×10^6 and 9.0×10^7 cells that killed two of five and four of five fish, respectively, within 10 days (15). The latter experiment was carried out in 27°C water with channel catfish (5 to 10 cm long).

The low mortality observed after waterborne exposure of steelhead to high numbers of *E. tarda* (2.0×10^8 cells per ml) suggests that the bacteria were only slightly invasive under these experimental conditions. Since channel catfish were more susceptible to *E. tarda* than were steelhead trout after i.p. injection, one might expect a lower 50% lethal dose for channel catfish as compared with steelhead trout when bacteria are added to the water. High susceptibility of channel catfish to waterborne challenge with a similar bacterium has been reported by Hawke (6), who observed a 100% mortality when channel catfish (mean length, 2 cm) were exposed to 7.5×10^3 *Edwardsiella* sp. (now named *E. ictaluri* [7]) cells per ml of water. Water temperature and contact time were not reported.

The percentage of fall chinook salmon found to have *E. tarda* by DFAT of kidney tissue was similar for prespawning (48%) and postspawning carcasses (50%) examined during 1981. However, no *E. tarda* were detected by DFAT or by culture of kidney and intestinal material from freshly killed and spawned fall chinook salmon. The absence of *E. tarda* in the kidney and intestinal tracts of freshly killed fish and the presence of bacteria in high numbers in a large percentage of dead fish suggests that *E. tarda* is an opportunistic bacterium in chinook salmon.

E. tarda has not been previously reported from any fish species in the Pacific Northwest. The organism has been isolated from a variety of mammals, birds, reptiles, and amphibians throughout the world (9, 10, 17, 20, 23, 24) and has been found in sea gulls near the mouth of the Rogue River (2) and in sea mammals along the Oregon coast (3). Thus, the potential sources of reservoir hosts that could have released this bacterium into the Rogue River system and nearby coastal waters are numerous. The difficulty in isolating *E. tarda* from chinook salmon and its absence in kidneys and intestinal tracts of freshly killed fish indicates that the bacterium was not a common pathogen of chinook salmon and may infect stressed and dying fish or both. Infected fish and carcasses may also act as a potential reservoir for the infection of humans (21) or other piscivorous animals abundant in the Rogue River system.

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