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MOLECULAR AND GENETIC CHARACTERIZATION OF CYTOCHROME OXIDASE-NEGATIVE *AEROMONAS SALMONICIDA* ISOLATED FROM COHO SALMON (*ONCORHYNCHUS KISUTCH*)

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ABSTRACT: Cytochrome oxidase variants of the bacterial fish pathogen, *Aeromonas salmonicida*, were characterized for genetic and molecular homology with cytochrome oxidase-positive isolates that typically induce furunculosis in salmonids. Protein and lipopolysaccharide moieties of the cytochrome oxidase-negative variants were similar to their typical counterparts, based on sodium-dodecyl-sulfate polyacrylamide gel electrophoresis. Pathogenicity of aberrant isolates to brook trout (*Salvelinus fontinalis*) was similar to typical cytochrome oxidase-positive isolates. Colorimetric deoxyribonucleic acid (DNA) hybridization in 96-well microplates yielded homology values greater than 82.5% for typical aberrant *A. salmonicida* isolates when photobiotinylated DNA for reference *A. salmonicida* 3.101 was used as a probe. The only variation of these isolates from typical *A. salmonicida* was a negative cytochrome oxidase reaction.

Key words: *Aeromonas salmonicida*, cytochrome oxidase, furunculosis, polyacrylamide gel electrophoresis, DNA hybridization.

INTRODUCTION

Since *Aeromonas salmonicida* originally was described as the cause of furunculosis in salmonids, it has remained a principal pathogen of cultured and feral fish. The bacterium, once believed to be restricted to salmonids, has infected and caused disease in fish of numerous families from freshwater, estuarine, and even marine environments (Austin and Austin, 1987). Systemic infections, typically induced by the microorganism, generally are characterized by severe leukopenia (Fuller et al., 1977), hemorrhage, tissue necrosis, and muscle degeneration (McCarthy, 1980; McCarthy and Roberts, 1980). Often, "furuncule-like" lesions develop within the dermis of chronically infected fish. The bacterium also induces diverse pathology in nonsalmonid hosts (Bullock et al., 1983).

Despite a wide diversity in host and geographic ranges, *A. salmonicida* has a remarkable phenotypic and genotypic homology (McCarthy, 1980; Paterson et al., 1980). Based on only subtle differences in biochemical characteristics, three subspecies currently exist (Krieg and Holt, 1984). Generally, there is little need for such specific differentiation to diagnose the bacterium in infected fish. In fact, Amos (1985) says that isolation of a Gram-negative, nonmotile bacillus that is cytochrome oxidase-positive and produces acid from glucose is sufficient to provide presumptive diagnosis. Although the production of brown pigment may assist diagnosis, this characteristic is not consistent among all isolates (Kimura, 1969; Elliot and Shotts, 1980).

A cytochrome oxidase (CO) variant of *A. salmonicida* recently was described as the etiological agent of disease in coho salmon (*Oncorhynchus kisutch*) from the Pacific Northwest (Chapman et al., 1991). Although the authors presented a biochemical characterization of the bacterium, the molecular and genetic aspects of the CO-negative phenotype were not addressed. To effectively diagnose and manage disease caused by these CO-negative isolates, it is essential to understand the genetic homology between the CO-negative and CO-positive strains, the stability of the CO-negative phenotype, and the virulence of the bacterium. Therefore, we conducted this study to compare pathogenicity, molecular aspects of protein and lipopolysaccharide constituents, and genetic relatedness between CO-positive and CO-negative isolates of *A. salmonicida*.

MATERIALS AND METHODS

Bacterial isolates

Fingerling coho salmon (*Oncorhynchus kisutch*) were held in two ponds located at the Washington Department of Fisheries' Coulter Creek Hatchery (47°24'N, 121°49'W) near Belfair, Washington (USA). In late June 1989 two isolates were cultured from kidney, a CO-positive *A. salmonicida* (3.148) and a CO-negative *A. salmonicida* (3.149). In mid-August three additional isolates were cultured from fish held in pond 2: one CO-positive (3.151), and two CO-negative (3.150, 3.152). Reference *A. salmonicida* isolates 3.10, 3.64, 3.101, 3.123, 3.125, and 3.136 were from the culture collection at the National Fish Health Research Laboratory, Leetown, West Virginia (USA).

All *A. salmonicida* isolates were biochemically characterized as described by Chapman et al. (1991). The CO reaction was determined using Pathotec CO strips (Organon Teknika Corporation, Durham, North Carolina, USA) and 1% N,N,N',N'-tetramethyl-p-phenylene diamine (Sigma Chemical Company, St. Louis, Missouri, USA) on 24- to 48-hr cultures grown on triple sugar iron agar (TSI) (Difco Laboratories, Detroit, Michigan, USA), tryptic soy agar (TSA) (Difco), and blood agar base (Difco) supplemented with 5% sheep blood (Whittaker Bioproducts, Walkersville, Maryland, USA). The appearance of a blue-purple color within 30 sec was recorded as a positive CO reaction.

Infectivity trial

Isolates 3.148 and 3.149 were grown in tryptic soy broth (TSB) (Difco) for 24 hr at 25 C. Broth cultures were centrifuged at 5,000 × g for 15 min, the supernatant discarded, and the pellet resuspended in sterile phosphate-buffered saline (PBS, pH 7.2). Final bacterial suspensions were adjusted to a transmittance (T) of 95% at a wavelength of 525 nm in a Spectronic-20D spectrophotometer (Milton Roy Company, Ivyland, Pennsylvania, USA). A 1:1,000 dilution of the 95% T suspension was used to inject fish.

Brook trout (*Salvelinus fontinalis*), obtained from White Sulphur Springs National Fish Hatchery, White Sulphur Springs, West Virginia, were stocked into two 70-gal (280 l) tanks at a density of 20 fish, (mean weight per fish = 70.9 g; SE = 6.0) per tank. Each tank was equipped with a flow-through supply of 12 C spring water. Fish from one tank received approximately 190 *A. salmonicida* in 0.1 ml of an intraperitoneal (ip) injection of the CO-positive isolate 3.148; the others received about 210 bacteria in an 0.1 ml ip injection of the CO-negative isolate 3.149.

Mortality was recorded for 21 days after challenge. Dead fish were removed daily for bacteriologic examination. Kidney tissue was analyzed from each fish for the presence and number of *A. salmonicida* on 0.01% Comassie Brilliant Blue (CBB) (Udey, 1982) plates using the methods of Miles and Mirsa (1938). Colonies from a countable dilution were inoculated onto TSI agar and identified as *A. salmonicida* based on the following: Gram-negative nonmotile fermentative rod, alkaline over acid reaction of TSI, positive for gelatinase and brown pigment production, and negative for ornithine dihydrolase and indole. Cytochrome oxidase reactions were determined from 24 hr cultures grown at 25 C on TSA.

Electrophoresis

Protein and lipopolysaccharide (LPS) components of the cell envelope were analyzed by a sodium-dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Bacteria were incubated for 24 hr on TSA slants and standardized to a 30% T (525 nm) in sterile PBS (pH 7.2) on a Spectronic-20D spectrophotometer (Milton Roy Company, Ivyland, Pennsylvania). Whole-cell sonicates for protein analysis were prepared for protein analysis (Pyle and Cipriano, 1986). Proteinase K (PK) preparations were prepared by the methods of Hitchcock and Brown (1983) for the analysis of LPS.

Electrophoresis was conducted as described by Laemmli (1970); 4% acrylamide (Bio-Rad Laboratories, Richmond, California, USA) was used in the stacking gel and 12% acrylamide in the resolving gel. Twenty microliters of each protein and LPS preparation from test isolates and reference strains were loaded in gel slots, and the conditions of electrophoresis were identical to those described by Cipriano and Pyle (1985). Protein bands in the gel were made visible by the staining method of Hitchcock and Brown (1983).

Deoxyribonucleic acid (DNA) hybridization

Bacteria were inoculated into brain-heart infusion broth (BHIB) (Difco), incubated overnight at 25 C, and brought to 30% T at 510 nm. Five ml of this suspension was inoculated into 95 ml of BHIB. Cells were collected by centrifugation after 24 hr growth at 25 C with gentle shaking.

The DNA was isolated by a method modified from Saghai-Maroof et al. (1984). Bacterial cells were suspended in 3.8 ml of 100 mM tris (pH 8.0) (Sigma), 10 mM ethylenediamine tetraacetic acid (EDTA) (Sigma), 1.4 M sodium chloride (NaCl), 2% hexadecyltrimethylammonium bromide (CTAB) (Sigma) and 0.2% 2-mercaptoethanol (Sigma). One-tenth ml of a 10 mg/ml

TABLE 1. Brood trout mortality and bacteriologic culture data for a 21-day infectivity trial using CO-negative isolate 3.149 and CO-positive isolate 3.148.

	3.148	3.149
Total fish mortality	17/20[a]	20/20
Mean CFU ($\times 10^7$/g kidney; SD $\times 10^6$)[b]	4.32 (1.14)	3.96 (1.07)
No. *A. salmonicida*/total no. colonies	75/76 (98)[c]	80/84 (95)
Cytochrome oxidase positive (%)	75/75[d]	0/80

[a] Number of fish dead/number of fish tested.
[b] Mean colony-forming units of *A. salmonicida* ($\times 10^7$/g kidney) (standard deviation $\times 10^6$/g kidney).
[c] Number of colonies identified as *A. salmonicida* ÷ total number of bacterial colonies examined (percent positive).
[d] Number of colonies with a positive CO reaction ÷ number of colonies tested.

lysozyme solution (Sigma) was added, and the suspensions were incubated 15 min at 25 C before 0.1 ml of a 10 mg/ml PK solution (Sigma) was added. After overnight incubation at 60 C, suspensions were extracted twice with equal volumes of a 24:1 ratio of chloroform-isoamyl alcohol, and nucleic acids were precipitated by the addition of two volumes of ethanol and collected by rolling the DNA strands onto a glass rod. The DNA was further purified by two precipitations in ammonium acetate. Nucleic acids were dissolved in 1 ml TE buffer (10 mM tris, pH 8.0, and 1 mM EDTA) at 60 C, and 0.5 ml 7.5 M ammonium acetate was added and mixed by inversion. After 30 min at 0 C, any precipitate was removed by a 5 min centrifugation at 12,000 × g, and DNA was precipitated by the addition of two volumes of ethanol. The DNA was dried under vacuum at 50 C, dissolved in 1 ml deionized water, and measured by spectroscopy (Sambrook et al., 1989). This method yielded 1.92 to 2.68 mg DNA per 100 ml culture with absorbance ratios (A_{260}/A_{280}) of 1.83 to 1.90. Absorbance ratios were calculated by dividing the absorbance of the sample at a wavelength of 260 nm by the absorbance of the sample at a wavelength of 280 nm.

Photobiotinylation of DNA, coating of 96 well plates, hybridization conditions, and plate-washing protocols were as described by Ezaki et al. (1989), except that hybridization was allowed to proceed at 45 C for 18 hr. The extent of hybridization was measured by the colorimetric method described here. Extravidin®-alkaline phosphatase (Sigma) at 1:1,000 dilution was used in place of the strepavidin-beta-D-galactosidase conjugate used by Ezaki et al. (1989) and incubated for 1.5 hr. After removing the conjugate solution and washing the plate twice, 100 µl substrate solution (1.0 M diethanolamine, 0.5 mM $MgCl_2$, 15 mM p-nitrophenyl phosphate, and pH 9.8) was added and the color allowed to develop at 25 C for about 2 hr until the A_{405} of the wells containing DNA homologous to the probe (strain 3.101) was ≥0.5. The A_{405} of all wells was then measured with an automatic plate reader (Molecular Devices, Menlo Park, California). Wells coated with salmon testes DNA (D-9156) (Sigma), and processed in the same manner as the sample wells, were used as blanks.

RESULTS

All cultures from Coulter Creek Hatchery and reference strains produced an alkaline over acid reaction on TSI agar, were positive for bile-esculin hydrolysis, nitrate reduction, gelatin liquefaction, glucose fermentation, and brown pigment production on TSA. All cultures and reference strains produced negative reactions for citrate, phenylalanine, urea, motility, indole, malonate, arginine, lysine, and ornithine. All reference strains and isolates 3.148 and 3.151 produced a positive CO reaction; all others were CO-negative.

Mortality of brook trout fingerlings was similar, whether injected with CO-negative isolate 3.149 or CO-positive isolate 3.148 (Table 1). Following ip challenge, each group of fish responded identically by first producing moribund fish a few days postinjection, followed by peak mortality within the first week. By day 21 only three fish from the 3.148 (CO-positive) group remained alive. Two additional infectivity trials (data not shown) had similar results.

The CO reactions from *A. salmonicida* isolated from the kidneys of dead fish remained identical to prechallenge CO reactions for the respective isolates. All cultures, regardless of CO reaction, grew as

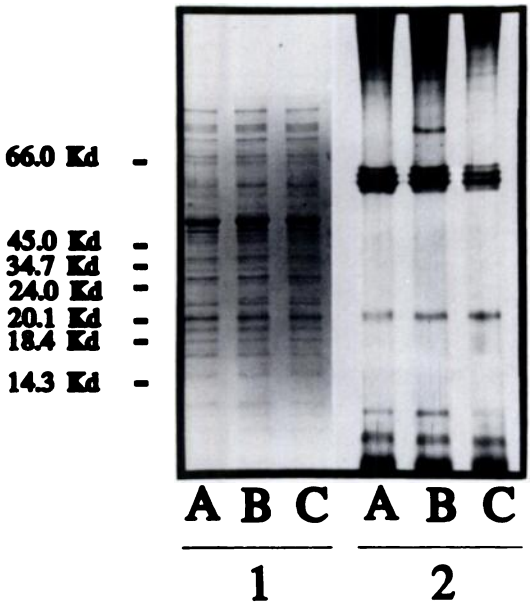

FIGURE 1. Sodium-dodecyl-sulfate polyacrylamide gel electrophoresis protein (1) and lipopolysaccharide (2) profiles of the original cytochrome oxidase-negative 3.149 (A) and cytochrome oxidase-positive 3.148 (B) isolates from coho salmon. Reference *A. salmonicida* 3.123 (C) with a phenotype of LPS^+/A^+ is shown for comparison.

blue colonies on CBB agar. The presence of an A-layer, as indicated by blue growth on CBB agar, was confirmed by SDS-PAGE. Electrophoretic banding patterns also had a high uniformity in proteins and LPS between CO-positive and CO-negative isolates. The original CO-negative (3.149) and CO-positive (3.148) isolates appeared identical to the LPS^+/A^+ phenotype of reference strain 3.123 (Fig. 1).

Using the reference *A. salmonicida* 3.101 as a probe, DNA homology for all isolates was greater than 82.5%. Homology for the CO-negative isolates ranged from 88.9 to 97.3%. For the five reference *A. salmonicida* isolates, the DNA homology was greater than 86.6%.

DISCUSSION

Oxidative phosphorylation is achieved in part by hemoproteins called cytochromes, which are the functional basis of various redox reactions involved in such processes as heterotrophic respiration (Jones, 1980). Three cytochromes (aa_3, o, and d) have oxidase activity (Jones, 1980). In bacteria these cytochromes can be present singly or in combinations of two or more, and they can be correlated to the presence or absence of a third energy-coupling site (Jones, 1980). This third site in the electron transport system generally is found in aerobic CO-positive bacteria, such as *Alcaligenes* spp. and *Pseudomonas* spp., associated with nutritionally poor environments (Jones, 1980).

Historically, the oxidase test (Kovacs, 1956) was based on the synthesis of indophenol blue from N,N-dimethyl-p-phenylenediamine and a-naphthol in the presence of oxidase as a catalyst. Today the tetramethyl salt is substituted for the dimethyl salt due to its increased stability, increased sensitivity, and decreased toxicity (Koneman et al., 1988). A blue-purple color development within 30 sec is considered a positive reaction and indicates that the microorganism contains a membrane-bound, high-potential cytochrome c coupled in close association with an active cytochrome oxidase (Jurtshuk et al., 1975). If a microorganism cannot catalyze the formation of indophenol blue, the negative reaction can be attributed to the absence of either the cytochrome oxidase or the cytochrome c; absence of the latter is most common (Jones, 1980). The molecular basis for the CO-negative phenotype in *A. salmonicida* has not been addressed in this study.

The CO reaction is a key taxonomic characteristic distinguishing members of the family Vibrionaceae (including *Aeromonas* spp.) from members of the family Enterobacteriaceae. Use of the test has been incorporated into identification schemes for bacterial fish pathogens such as *A. salmonicida* (Amos, 1985). Our results are an exception to these schemes, which discard as *A. salmonicida* any isolate that is not a nonmotile, pigment-producing, CO-positive organism.

The CO-negative 3.149 isolate used for

the infectivity study was identical to CO-positive LPS^+/A^+ *A. salmonicida* in its ability to kill fish. Mortality was first observed on day 5 postchallenge for both CO-negative 3.149 and CO-positive 3.148. Mortality for both isolates reached 50% on day 7, and for CO-negative 3.149 mortality reached 100% by day 15. Kidney cultures from all fish in both treatment groups had *A. salmonicida*. The CO-negative phenotype remained stable after passage through fish, and no CO-positive colonies were detected from cultures taken from fish injected with CO-negative 3.149 (Table 1).

The SDS-PAGE whole-cell protein profile for isolate 3.149 was similar to 3.148 and reference strain 3.123. All three isolates grew as blue colonies on 0.1% CBB, indicating the presence of A-layer (Udey, 1982). The electrophoretic profiles confirmed the presence of A-layer appearing as a 50 kd band (Figure 1). The LPS profile of 3.149 had a banding pattern identical to 3.148 and 3.123 (Fig. 1).

Quantitative DNA hybridization using plastic 96-well plates and biotin-labeled probes is a convenient technique for examining the genetic relatedness of bacteria (Ezaki et al., 1988, 1989). Homology values for all *A. salmonicida* strains and the presumptive *A. salmonicida* isolates were greater than 82.5% (Table 2), providing further evidence that all isolates described here are *A. salmonicida* (Johnson, 1984).

The CO-negative *A. salmonicida* described here were originally isolated on CBB agar (Chapman et al., 1991). The medium can routinely be used for the screening of suspect *A. salmonicida* by selecting only those colonies appearing blue on the agar surface. Blue colonies on CBB agar, even those producing a positive CO reaction and brown pigment, is not satisfactory criteria to confirm the identity of a bacterium as *A. salmonicida*. Each suspect colony must still be biochemically characterized using more than two or three tests. The medium is an effective tool for screening large numbers of colonies; however, its use and limitations must be understood.

TABLE 2. Quantitative DNA hybridization data for CO-negative and CO-positive *A. salmonicida* isolates.

Isolate	CO reaction	Mean % homology	SD
3.150	negative	97.3	17.6
3.149	negative	89.9	12.6
3.64	positive	102.9	10.4
3.152	negative	88.9	11.4
3.101	positive	100.0	0.0
3.151	positive	102.1	13.7
3.136	positive	88.0	15.6
3.148	positive	82.5	17.6
3.10	positive	86.6	17.1
3.125	positive	88.9	14.2
Escherichia coli B	negative	14.4	7.9

Our results are an exception to the practice of discarding as *A. salmonicida* any isolate that is not a nonmotile, pigment-producing, CO-positive organism. While the isolation of biochemically aberrant strains of *A. salmonicida* does not warrant such action as the redescription of the species, awareness of their existence and pathogenicity is essential for effective health management.

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