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Rodman Guy Getchell for the degree of Master of Science

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Title: COMPARISON OF *RENIBACTERIUM SALMONINARUM* ISOLATES BY ANTIGENIC ANALYSIS, ANTIMICROBIAL ASSAY AND GROWTH

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Abstract approved: _____
Dr. J. L. Fryer

The antigens of eight isolates of *Renibacterium salmoninarum* were compared serologically using immunoelectrophoretic techniques. Seven common antigens were identified by their electrophoretic mobilities in each antigenic profile. One of these common antigens, designated Fr 21-30, was partially purified by ammonium sulfate precipitation and gel filtration. Cross adsorption analysis showed Fr 21-30 to be the major surface antigen on *R. salmoninarum*. This purified antigen was heat stable and its molecular weight was estimated at 57,000 by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Growth studies and antimicrobial assay indicated cultural similarities among the selected isolates of *R. salmoninarum*.

Comparison of *Renibacterium salmoninarum* by Antigenic Analysis, Antimicrobial Assay and Growth

by

Rodman Guy Getchell

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COMPARISON OF *RENIBACTERIUM SALMONINARUM* ISOLATES BY ANTIGENIC ANALYSIS, ANTIMICROBIAL ASSAY AND GROWTH

INTRODUCTION

Bacterial kidney disease (BKD) causes losses among hatchery-reared salmonids throughout the world. Recently named *Renibacterium salmoninarum*, the kidney disease bacterium (KDB) is a slow-growing, Gram positive bacillus with an absolute requirement for cysteine (Fryer and Sanders, 1981). Control of the disease by chemotherapy is difficult because of the bacterium's intracellular occurrence (Young and Chapman, 1978). Development of a prophylactic measure such as vaccination requires knowledge of the serology and the antigenic composition of *R. salmoninarum*. The direct fluorescent antibody test (FAT) is currently an important diagnostic method for detecting KDB infected salmonids (Bullock *et al.*, 1980; Paterson *et al.*, 1981). Serological techniques form the basis for identification of *R. salmoninarum* by FAT. Early serological studies on the causative bacterium demonstrated one or more common antigens by immunodiffusion and cross agglutination (Bullock *et al.*, 1974). However, a total antigenic profile of *R. salmoninarum* has not been completed.

The purpose of this study was to obtain information on the antigenic composition of selected *R. salmoninarum* isolates. The isolates were chosen for their wide

geographical distribution and varied salmonid hosts. In addition, characterization of the major soluble antigen observed in serological tests was pursued. Another objective was to compare cultural differences among *R. salmoninarum* isolates.

Several immunoelectrophoretic techniques were used, including two-dimensional immunoelectrophoresis, to study the serological reactions between *R. salmoninarum* antigens and rabbit antiserum. Antigenic profiles were compared electrophoretically to determine common components. Cross adsorption analysis was performed to identify major surface antigens. A soluble antigen extracted from the type strain, Lea-1-74 (ATCC 33209), was partially purified and its molecular weight determined. Finally, cultural characteristics among isolates were examined by growth studies and antimicrobial assay.

LITERATURE REVIEW

The first complete review of the literature pertaining to bacterial kidney disease of salmonids was prepared by Fryer and Sanders (1981). History, etiology, epizootiology, pathology, detection, control and immunology were summarized in detail. The review presented here will survey the historical background, antibiotic sensitivity, and serology of KDB, and briefly profile the background of immunoelectrophoretic methods.

Historical Background

Bacterial kidney disease was fist described under the name Dee disease by the Furunculosis Committee (Mackie, 1933). Fourteen Atlantic salmon (Salmo salar) were found with small necrotic lesions on their spleen. Small Gram positive bacilli were demonstrated in the lesions. Splenic emulsions from the diseased fish injected into brown trout (Salmo trutta) caused mortalities in three to five weeks inferring the bacillus was the probable pathogen.

Belding and Merrill (1935) published a report on an outbreak of kidney disease at a Massachusetts State hatchery. The gross pathology, etiology and epidemiology among infected brown, rainbow (Salmo gairdneri), and brook trout (Salvelinus fontinalis) were described. Earp et al. (1953) observed kidney disease in young salmon from the Pacific Northwest. Chinook (Oncorhynchus tshawytscha), coho

(Oncorhynchus kisutch), and sockeye salmon (Oncorhynchus nerka) were diagnosed with similar pathology to that of fish from outbreaks in Massachusetts. Other species of salmonids subsequently found with BKD included pink salmon (Oncorhynchus gorbuscha)(Bell, 1961), lake trout (Salvelinus namaycush)(Ehlinger, 1963), cherry salmon (Oncorhynchus masou), kokanee salmon (Oncorhynchus nerka)(Awakura, 1978), and cutthroat trout (Salmo clarki)(Fryer and Sanders, 1981). The geographic range of BKD comprises most of Europe, Iceland, Canada, the United States and Japan (Sanders and Fryer, 1980 b).

Ordal and Earp (1956) considered the causative agent of BKD to be a species of Corynebacterium. Smith (1964) compared Dee disease of salmonids in Scotland to BKD and concluded that they were caused by the same organism. In 1978, the name Corynebacterium salmoninus was proposed for the kidney disease bacterium (KDB) (Sanders and Fryer, 1978). Citing differences between the BKD organism and the members of the genus Corynebacterium, Sanders and Fryer (1980 a; 1980 b) proposed to establish a new genus and species, Renibacterium salmoninarum, for the KDB.

Serology of Renibacterium salmoninarum

Belding and Merrill (1935) were the first to use serological methods to compare isolates of the KDB. Four

strains were found serologically identical by cross-agglutination and agglutinin adsorption with immune sera.

Further serological testing did not occur until Evelyn's (1971) study of the agglutinin response of vaccinated sockeye salmon to heat-killed R. salmoninarum. The antibodies produced were specific for strains of the KDB. Three isolates from British Columbian pink salmon all agglutinated as strongly as the homologous bacterium by five separate antisera. Evelyn also used adsorption techniques to identify the agglutinating antibody present in electrophoregrams of sera from vaccinated fish.

Bullock et al. (1974) used agglutinin and precipitin reactions to show that 10 North American strains of the KDB were serologically homogeneous. This was the first time serological relatedness of isolates from different geographical regions was examined. They also were the first to suggest serological procedures for diagnosis of epizootics, identification of isolated cultures, and possible detection of carriers. Chen et al. (1974) described an immunodiffusion test for the KDB. Specific precipitin lines were formed in agar within 24 h after rabbit antisera reacted with soluble antigens from infected tissue or from KDB cell suspensions. The procedure was offered as a more reliable method of detection than the Gram stain of smears prepared from infected tissue and faster than cultural techniques.

Lobb (1976) recommended the intestine and kidney as the best organs to sample to detect the KDB with the immunodiffusion test. Kimura et al. (1978) prepared an immunodiffusion test with a heat stable KDB antigen extracted from infected kidney. Kimura (1978) also described a new diagnostic method called the coagglutination test which used specific antibody to R. salmoninarum coated on staphylococcal protein A.

The fluorescent labelled antibody technique to detect KDB was first used by Bullock and Stuckey (1975) utilizing the indirect fluorescent antibody test. In 1980, Bullock et al. improved this diagnostic method with a faster, more sensitive direct antibody test. Banner et al. (1982) have reported a more rapid, economical, and reliable means of producing fluorescein-labelled antibody to R. salmoninarum.

The serological techniques mentioned provide greater diagnostic sensitivity. The key element in each of these tests is the presence of only one serotype of R. salmoninarum so antisera to any KDB isolate can be used. Paterson (1981) emphasized the importance of antigenic homogeneity and uniqueness in identifying R. salmoninarum with serological methods.

Recently, Austin and Rodgers (1980) studied 25 KDB isolates to determine their relatedness. They characterized the strains by cultural, biochemical, morphological, physiological and serological methods. Two distinct phena

were defined. The isolates of phenon 2 were found to be serologically homogeneous, as they all agglutinated with antisera to five phenon 2 isolates, but not at all with antisera to three European isolates outside phenon 2.

Survey of Immunoelectrophoretic Methods

Verbruggen (1975) reviewed the literature describing immunoelectrophoretic methods, listing 505 publications on the subject. The material presented here will briefly describe the history and principles of the gel-precipitation techniques used in this study.

Immunoelectrophoretic methods have combined the technique of electrophoresis established by Tiselius (Gray, 1951) with the immunochemical developments of Oudin and Ouchterlony (Williams, 1960). Tiselius analyzed and separated proteins based on their differential migration in an electric field. Oudin combined antigens and antibodies in a capillary tube filled with agar. The immunoprecipitates that formed, depended upon the rates of antigen diffusion into the antibody containing gel. Ouchterlony allowed both antigen and antibody to diffuse from small wells cut into an agar plate. This double diffusion permitted a better separation of precipitin lines and consequently was more useful than Oudin's single diffusion method (Ouchterlony, 1958).

Grabar and Williams (1953) were the first to separate

proteins by their electrophoretic mobility and specific immune reaction in a procedure appropriately named immmunoelectrophoresis. Initially, the antigens were separated by electrophoresis, then they diffused outward and met antibodies diffusing from a trough cut in the agar, and finally precipitated in a pattern of arcs (Williams, 1960).

The electrophoresis of antigen mixtures into agar gels containing antibodies was the next advance (Ressler, 1960). Laurell (1965) recognized the quantitative relation between amount of antigen and precipitate area in Ressler's work. Stressing higher resolution, as well as quantitative aspects, Laurell (1966) published his electroimmunoassay method. In this assay, electrophoresis of antigens into agarose gel containing monospecific antiserum produced rocket shaped precipitates whose height was proportional to the concentration of antigen. Laurell also developed a "crossed antigen-antibody" method which was modified by Clark and Freeman (1966) and widely used under the name two-dimensional immunoelectrophoresis. The procedure consists of two stages; electrophoretic separation of antigens in one dimension and then further electrophoresis of the antigens into an antibody containing gel placed at right angles to the movement in the first dimension. Two-dimensional immunoelectrophoresis characterizes antigens electrophoretically and quantitatively with great

sensitivity and resolving power.

Antibiotic Sensitivity of *Renibacterium salmoninarum*

Chemotherapy has been an approach to treatment of kidney disease since Rucker *et al.* (1951) reported infected sockeye fingerlings responded to sulfadiazine incorporated into the feed (6 to 12 g/100 lb fish). Following continued feeding experiments, Rucker *et al.* (1954) concluded the drug effect was bacteroistatic rather than bactericidal. Earp *et al.* (1953) showed variable results with sulfamerizine, sulfamethazine, and sulfadiazine therapy while treatment with aureomycin was ineffective.

Snieszko and Griffin (1955) studied the efficacy of sulfanilamide, sulfadiazine, Gantrisin, a mixture of sulfamerazine and sulfathiazole, chloramphenicol, terramycin, and aureomycin in the treatment of artificially infected brook trout. They found chloramphenicol (3.4 g/100 lb fish) and some of the sulfonamides (9 g/100 lb fish) delayed the progress of the infection but did not cure the fish of the disease. Allison (1958) observed the same temporary effect of sulfonamide therapy (12 g/100 lb fish) on naturally infected brook trout in Michigan.

Wolf and Dunbar (1959) broadened their approach to find a more effective treatment for BKD by testing 34 chemotherapeutics against 16 strains of *R. salmoninarum*.

Antimicrobial disc susceptibility tests indicated most of these therapeutics reduced R. salmoninarum growth. Ten of these drugs were employed in feeding trials with infected brook trout. Erythromycin (4.5 g/100 lb fish) gave the best results.

DeCew (1972) tested antibiotics for toxicity and efficacy in adult spring chinook salmon infected with R. salmoninarum and furunculosis, a disease caused by the Gram negative bacterium Aeromonas salmonicida. The combination of penicillin G procaine (15,000 to 30,000 units/kg fish), dihydrostreptomycin sulfate (19 to 38 mg/kg fish), and oxytetracycline-HCl (4 to 8 mg/kg fish) injected subcutaneously was the most effective in controlling both diseases. Banowetz (1974) indicated erythromycin (100 mg/kg fish) controlled BKD when fed to experimentally infected coho salmon, while Ampicillin (75 to 100 mg/kg fish) and potassium phenoxymethyl penicillin (Pen V-K) (75 to 100 mg/kg fish) were ineffective.

Recently, Austin and Rodgers (1980) determined the antibiotic sensitivity of 25 BKD isolates to 11 antimicrobial agents. The resistance to erythromycin (10µg/ml) by isolates clustered in phenon 2 was noted. Schneider (1980) reported minimum inhibitory concentrations for 12 antibiotics against BKD. He also noted the resistance to erythromycin (20µg/ml) by an Oregon (McKenzie Hatchery) isolate of R. salmoninarum.

MATERIALS AND METHODS

Diluent and Bacteriological Media

During antigen preparation, phosphate buffered saline (PBS) was used as a diluent. Evelyn's (1977) Kidney Disease Medium-2 (KDM-2) was modified and employed in broth form to culture *R. salmoninarum* isolates. These were prepared according to the following:

Phosphate Buffered Saline 0.01 M, pH 7.2 (PBS)

	g/1000 ml	Molarity
NaCl	7.50	0.130
KH_2PO_4	0.245	0.0018
Na_2HPO_4	0.809	0.0057
H_2O	to volume	

Modified Kidney Disease Medium-2 Broth, pH 6.5(M-KDM-2)

	g/100 ml
Peptone	1.00
Yeast Extract	0.05
L-Cysteine HCl	0.10
$Fe_2(SO_4)_3$	0.0057
H_2O	to volume

Adjust to pH 6.5

Bacterial Strains

Renibacterium salmoninarum isolates Lea-1-74 (ATCC 33209), KDV-10 pass 135, K28, K50, K70, C-384, C-37841, and

RS-21 were used as immunizing and serological test antigens. Sources of the eight isolates which were studied are listed (Table 1). A Lactobacillus species, isolated from S. clarki brood trout at Bandon Hatchery (Oregon), was cultured in Tryptic Soy Broth and used as a negative control organism.

Preparation of Immunogens

Renibacterium salmoninarum isolates Lea-1-74 and K50 were used as immunogens. Organisms were grown in a modified KDM-2 broth (M-KDM-2) which lacked serum ordinarily incorporated in KDM-2 media. Serum was not used to avoid contamination with calf serum antigens. Cultures were incubated at 18°C for 7 to 10 days in low form culture flasks under constant agitation. At the end of the incubation period, the bacteria were centrifuged at 6000 x g for 30 min (4°C), the supernatant fluid reserved for soluble antigen extraction, and the cells washed in PBS and centrifuged again under the same conditions. This was repeated three times. The pellet of cells from two 300 ml cultures was then resuspended in 10 ml PBS and disrupted by sonic treatment (four 30 s bursts, average watts = 50) by a Sonifier Cell Disruptor (Heat Systems Ultrasonics, Plainview, N.Y.). This suspension was frozen at -70°C.

Table 1. Sources of *Renibacterium salmoninarum* isolates.

Antigen Designation	Isolate Code	Country of Origin	Isolation Information	Culture[a] Obtained from
1	Lea-1-74[b] (ATCC 33209)	U.S.A.	*Oncorhynchus tshawytscha* Leaburg Hatchery	J.E. Sanders
2	KDV-10 pass 135	U.S.A.	RB-1-73 passed 135 times on Muellar-Hinton plus 0.1% cysteine	J.S. Rohovec
3	K28	France	*Oncorhynchus kisutch* Eye	B. Austin
4	K50	Norway	Salmonids reared in sea water cages	B. Austin
5	K70	England	*Salmo gairdneri* Dermal blister	B. Austin
6	C-384	Canada	*Oncorhynchus kisutch* Quinsam Hatchery	G.S. Traxler
7	C-37841	Canada	*O. nerka* reared in a sea water cage	G.S. Traxler
9	RS-21	Canada	*Salmo salar* Margaree River	W.D. Paterson

[a]Sources: J.E. Sanders and J.S. Rohovec, Department of Microbiology, Oregon State University, Corvallis, Oregon,U.S.A.; B. Austin, Fish Disease Laboratory, Weymouth, Dorset, England; G.S. Traxler, Pacific Biological Station, Nanaimo, British Columbia, Canada; W.D. Paterson, Connaught Laboratories, Willowdale, Ontario, Canada.

[b]Type Strain of *Renibacterium salmoninarum*.

Preparation of Antisera

Briefly, 2.2 ml of a mixture of equal volumes of immunogen and Freund's Complete Adjuvant (FCA) were injected subcutaneously between the scapulae (1.4 ml) and in the footpads (0.4 ml each) of a young, female New Zealand white rabbit. Preimmune sera was harvested prior to immunization. The rabbits were bled 30 days later, the first of four bleedings over the next five weeks. Eleven weeks post-immunization the rabbits were boosted with the same preparation, 2.2 ml of a 1:1 mixture of immunogen and FCA injected subcutaneously between the scapulae and into each footpad. Ten days after the booster injection the rabbits were bled again. The final three bleedings occurred over the next four weeks.

Blood was collected from the marginal ear vein of each rabbit, allowed to clot for one hour at room temperature, and centrifuged at 10,000 x g for 15 min ($4^{o}C$). After centrifugation, the serum was carefully harvested and frozen in 4 ml aliquots at $-70^{o}C$ until needed. Rabbit anti-BSA serum used to precipitate the BSA marker was obtained from Miles Laboratory (Elkhart, Ind.).

Preparation of Serological Test Antigens

Organisms were grown, harvested, washed, and sonically treated following the same procedures as used for immunogen preparation. The soluble bacterial antigens present were

cleared of cellular debris by centrifugation at 12,000 x g for 30 min (4°C). The supernatant was then concentrated by ultrafiltration to obtain a final protein concentration of 0.5 mg/ml, as measured by the method of Bradford (1976). The test reagent was obtained from Bio-Rad Laboratories (Richmond, Cal.). The ultrafiltration XM-50 membranes were purchased from Amicon (Lexington, Mass.). Cleared antigen suspensions were frozen at -70 C until needed.

Immunoelectrophoretic Methods

Immunodiffusion

The Ouchterlony immunodiffusion test to detect soluble R. salmoninarum antigens was performed on GelBond film (Marine Colloids, Inc., Rockland, Me.) adhered to glass plates. A solution containing 0.8% SeaKem Agarose (w/v) (Marine Colloids) in barbital buffer (pH 8.6, ionic strength 0.05) was poured onto the GelBond film and wells (3-mm in diam.) were cut with a Gel Punch (Gelman, Ann Arbor, Mich.). Antigens (5 µl) and antisera (10 µl) were placed in the wells and allowed to react in a moist chamber for 48 h (4°C). The gel was then soaked in 1% NaCl (w/v) for 48 h at room temperature, followed by 48 h in distilled water. After drying, the film was stained for 5 min with 250 ml of 0.5% Coomassie Brilliant Blue (w/v) dissolved in 45% ethanol, 45% distilled water, and 10% acetic acid (v/v). Decolorization was performed for 5 min in solvent

alone. The results were photographed with background lighting using a macro lens and orange (15) filter.

Immunoelectrophoresis

Immunoelectrophoresis was performed on large glass plates (12 x 12 cm) covered with GelBond film. The supporting agarose matrix used in the following three methods consisted of 0.5% agarose (w/v) in pH 8.6 barbital buffer, ionic strength 0.05, and 0.5% Triton-X-100 (v/v) detergent (Thirkill and Kenny, 1974). The electrophoresis baths contained 0.01% thimerosal and barbital buffer at ionic strength 0.1. About 25 ml of melted agarose was solidified over a plate, and wells and trough cut with a Gel Punch (Gelman). Antigens (5 µl) were placed in each 3 mm well and subjected to electrophoresis at 6 V/cm for 45 min. Antisera (200 µl) was then added to the 2 mm wide trough, and precipitation arcs were allowed to form in a moist chamber for 48 h (4°C). Photographing, washing, and staining were performed as described previously.

Rocket Immunoelectrophoresis

The plates, gels, and buffer used for rocket immunoelectrophoresis were the same as those used in the immunoelectrophoresis experiments, except only 20 ml of agarose per plate was required. The 5 mm wells containing antigen were punched out of the lower quarter of the gel.

The upper three-quarters of this gel was removed and replaced with a second-dimension gel containing 5% antisera (v/v). Antigens (20 ul) were placed in each cathodic well and electrophoresis was performed at 1.5 V/cm for 16 h. Photographing, washing, and staining were performed as described previously.

Two-Dimensional Immunoelectrophoresis

The plates, gels, and buffer used for two-dimensional immunoelectrophoresis were the same as those used in rocket and immunoelectrophoresis experiments. The 20 ml of agarose gel were about 2 mm thick when poured. The serological antigen (20 µl) mixed with 10 µl of a 1 mg/ml solution of bovine serum albumin (Sigma, St. Louis, Mo.), which acted as a marker of the migration rate of each protein, was added to a 5 mm well cut out of the cathode corner of the gel plate. First dimension electrophoresis was carried out at 7.5 V/cm for 1.5 h. The upper portion of the gel (3/4) was then removed and replaced with 15 ml of a 5% antibody containing, agarose mixture, with 20 µl of anti-BSA serum to reveal the BSA marker. The second phase was run at 1.5 V/cm for 16 h at right angles to the electrophoretic direction of the first dimension at 1.5 V/cm for 16 h. Circulating tap water (13^{o}C) was used to cool the gel plate during rocket and two-dimensional immunoelectrophoresis. Photographing, washing, and staining

were performed as described previously.

Cross Adsorption Procedures

Whole cell preparations of each isolate were grown, harvested, and washed following the same procedures as preparation of immunogens. Each isolate was then adsorbed against antiserum to isolates Lea-1-74 and K50, respectively. The procedure for adsorption was that of O'Leary et al. (1982). Briefly, a volume of antisera was added to an equal volume of wet packed cells, mixed, and allowed to incubate for 2 h at 30°C with periodic mixing. The cells were then pelleted by centrifugation at 6000 x g for 15 min (4°C) and the remaining antisera was adsorbed two more times by the same method.

The technique of rocket immunoelectrophoresis was used to screen this adsorbed antisera to determine which antigens were surface components and which antigens were common among the isolates. Wells (5 mm) were cut for each antigen in the lower quarter of the gel. The upper gel was replaced with agarose containing 5% adsorbed antiserum and the plate subjected to electrophoresis at 1.5 V/cm for 16 h. The results were recorded as previously noted with rocket immunoelectrophoresis.

Soluble Antigen Extraction

Purification

The supernatant fluid, harvested after centrifugation of cells from the culture medium, was obtained during serological antigen preparation. This soluble antigen preparation was raised to 50% saturation with ammonium sulfate (297 g/l) and stirred overnight at 4 C. The precipitate was removed by centrifugation at 6000 x g for 15 min (4^{o}C), suspended in 20 ml PBS, and dialyzed against 2 l of PBS overnight. The dialyzed protein extract was then concentrated to 4 ml by ultrafiltration over an XM-100A membrane (Amicon). The antigens present were then assayed by immunodiffusion and rocket immunoelectrophoresis.

A gel filtration column was used to separate soluble antigens from media proteins which may have been contaminants. One crude antigen preparation (Lea-1-74) was loaded onto a Ultragel AcA 44 (LKB, Gaithersburg, Md.) column (1.7 x 50 cm) in 0.01M PBS (pH 7.2). The column was eluted using the same buffer, and fractions collected. Each fraction was assayed for protein by measuring its absorbance at 280 nm with a Beckman Model 35 Spectrophotometer (Palo Alto, Cal.). Those fractions with high protein levels were assayed for soluble antigen by rocket immunoelectrophoresis and the results recorded. Fractions 21-30 were concentrated to 2 ml by

ultrafiltration over an XM-100A membrane (Amicon) and assayed by polyacrylamide-agarose gel two-dimensional immunoelectrophoresis and standard two-dimensional immunoelectrophoresis. The concentrated fractions were also tested for heat stability by placing 1 ml of the concentrate at 100°C for 30 min and then assaying by two-dimensional immunoelectrophoresis.

Polyacrylamide-Agarose Gel Two-Dimensional Immunoelectrophoresis

Polyacrylamide-agarose gel two-dimensional immunoelectrophoresis was performed to determine the molecular weight of the soluble antigens and also to confirm that the protein bands observed corresponded to actual *R. salmoninarum* antigens. The first phase was carried out using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The denaturing conditions that were used have been described in the Model 220 Vertical Slab Electrophoresis Cell Instruction Manual (Bio-Rad) (Laemmli, 1970). Ten milliliters of 3% stacking gel was overlaid on 15 ml of 9% separating gel to prepare a vertical gel slab (0.075 x 10.0 x 14.0 cm). Prior to SDS-PAGE, the antigen samples were boiled for 2 min in sample buffer containing 10% SDS (w/v) and 1.25% 2-mercaptoethanol (v/v). The molecular weight (MW) of the antigen was determined by comparison to a mixture of low MW markers (Bio-Rad) loaded between two antigen wells. Electrophoresis

was performed at a constant 10 ma in the stacking gel and 20 ma in the separating gel until the tracking dye reached the bottom of the gel. The gel was sliced vertically, and the MW marker lane and one antigen lane were stained for proteins. The other gel strip containing the second antigen lane was transferred to a GelBond film-covered plate and 15 ml of melted 5% antibody containing agarose was added to the upper portion of the plate. The gel was subjected to electrophoresis in the second dimension at 1.5 V/cm for 16 h. Washing and staining were performed as described previously. The position of the protein bands in the first dimension was compared with the position of the precipitation peak of the soluble antigen in the second dimension.

Growth of Renibacterium salmoninarum Isolates

Each isolate was grown in nephalo flasks (Bellco, Vineland, N. J.) containing 50 ml of M-KDM-2 broth incubated at 18°C on a shaker. One milliliter of a 7-day old culture in early stationary phase, diluted with M-KDM-2 to an optical density at 520 nm of 1.0, was used as the standard inoculum. The growth of isolates was monitored twice daily by recording the adsorbance at 520 nm (OD_{520}) with a Bausch and Lomb Spectronic 20.

Minimum Inhibitory Concentrations of Antimicrobics

The ability of *R. salmoninarum* to grow in different concentrations of antibiotics was measured. Sensititre plates (Gibco, Lawrence, Mass.), an *in vitro* diagnostic product, was used to determine minimum inhibitory concentrations (MIC's) of antimicrobics. This was basically a micro-version of the classical broth dilution method (Philips, *et al.*, 1978). The microtiter plate containing appropriate amounts of dried, stabilized antimicrobics was arranged to give 2-fold dilutions after addition of broth. The same standard inoculum used in the growth experiments was diluted 1:100 in Mueller-Hinton broth plus 0.1% cysteine (w/v) and used to inoculate each well (50 µl) of the Sensititre plate. After inoculation, the plate was covered with an adhesive seal and incubated at 18°C for 7 to 10 days. The MIC's for each isolate were recorded as the lowest concentration of antimicrobic that inhibited visible growth of the organism. *Staphylococcus aureus* (ATCC 25923) was included as a standard control organism with known MIC's to monitor the accuracy of the assay.

RESULTS

Comparison of *Renibacterium salmoninarum* Isolates Using Immunoelectrophoretic Methods

Immunodiffusion

The precipitin bands demonstrated by double diffusion analysis showed identity between the serological antigens of each *R. salmoninarum* isolates (Figure 1). Four distinct precipitin lines were obtained with antiserum to isolate K50, while only two bands were detectable with anti-Lea-1-74 serum. Canadian isolates 384 and 37841 showed fainter precipitin lines because of a lower total antigen concentration.

Immunoelectrophoresis

Soluble antigens of *R. salmoninarum* were analyzed by immunoelectrophoresis (Figure 2). Each isolate showed a common antigen with a well defined arc and a broad migration rate. Each isolate produced 2 to 12 precipitin lines on the anodic side of the well. Anti-Lea-1-74 serum resolved fewer distinct arcs which parallelled the immunodiffusion results. An extracted soluble antigen from isolate Lea-1-74 (Fr 21-30) was precipitated in the same position as the common antigen present in the other immunoelectrophoresis runs.

Fig. 1. Ouchterlony immunodiffusion analysis of Renibacterium salmoninarum soluble antigens. Center wells contain rabbit antisera prepared against either isolate Lea-1-74 or K50. Outer wells contain a serological test antigen or the extracted soluble antigen (Fr 21-30, well number 8). See Table 1 for serological antigen designations.

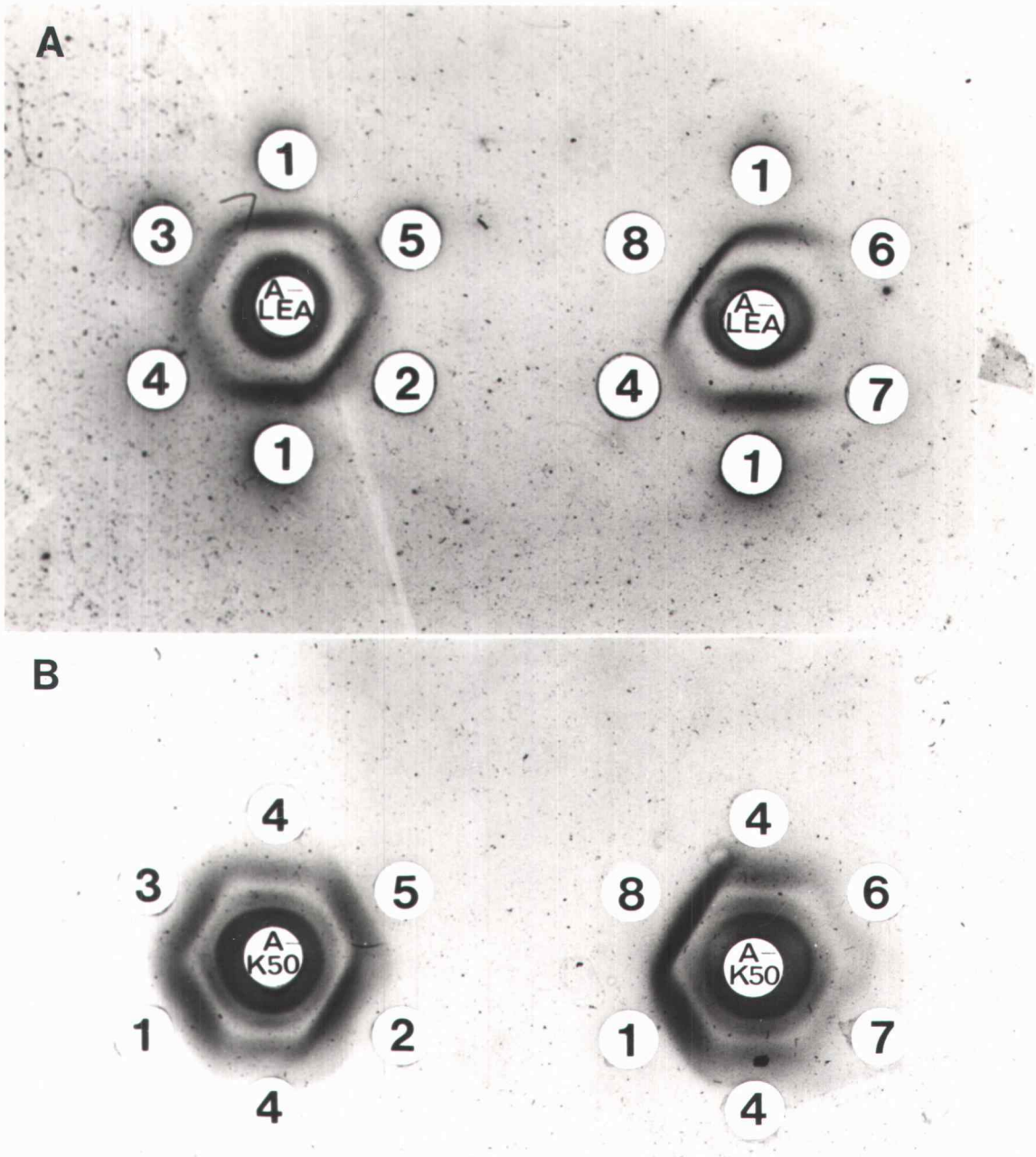

Fig. 1.

Fig. 2. Immunoelectrophoresis of Renibacterium salmoninarum soluble antigens. Center troughs contain rabbit antisera prepared against either isolate Lea-1-74 or K50. Wells contain a serological test antigen or an extracted soluble antigen (Fr 21-30, well number 8). See Table 1 for serological antigen designations. A, B, C, and D contain Lea-1-74 antiserum. E, F, G, and H contain K50 antiserum. Arrows show a common soluble antigen.

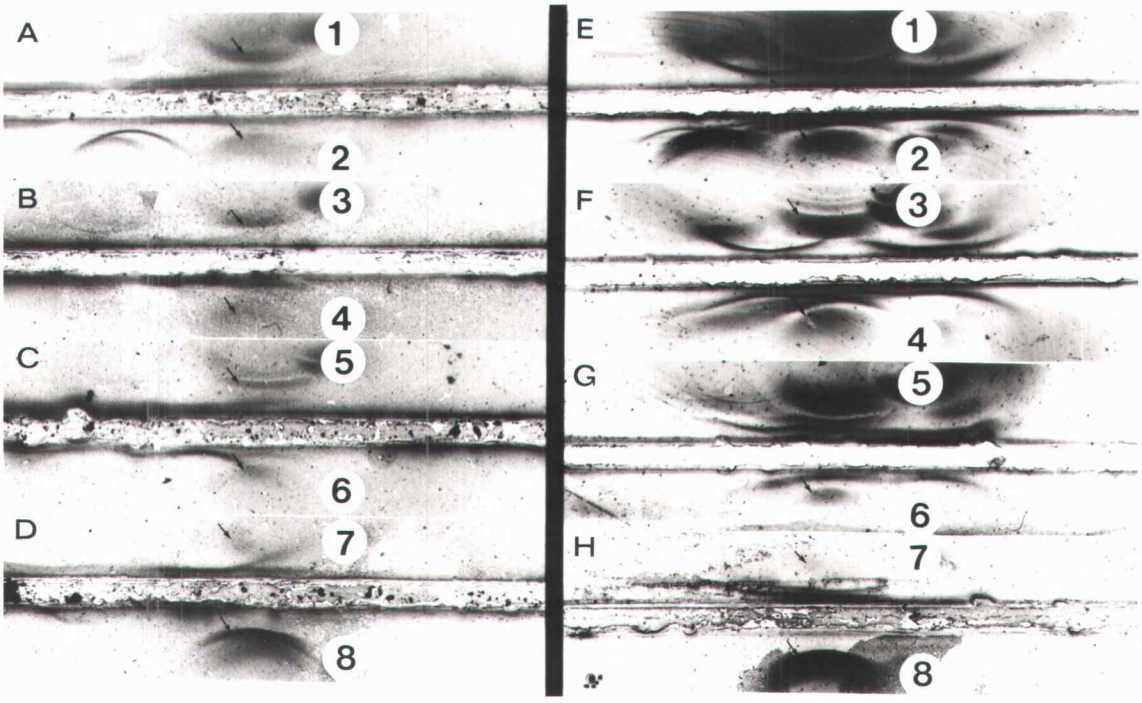

Fig. 2.

Rocket Immunoelectrophoresis

Electrophoresis of soluble antigens from KDB isolates into rabbit antiserum, prepared against isolates Lea-1-74 and K50, resulted in 7 to 15 distinct peaks (Figure 3). In this analysis anti-Lea-1-74 serum identified as many as 13 antigens, contrary to previous assays when anti-K50 serum seemed to be the stronger antiserum.

Rocket immunoelectrophoresis of Lea-1-74 or K50 antigens against preimmune sera was performed to determine whether the rabbit possessed antibodies to *R. salmoninarum* antigens prior to immunization. No precipitin lines were observed. Antigen contamination by medium components (M-KDM-2) was also tested by rocket immunoelectrophoresis against antisera to Lea-1-74 and K50. One faint peak was observed with anti-K50 serum indicating slight contamination of the K50 immunogen with a medium component. *Lactobacillus sp.*, a Gram positive fish pathogen, was used as a negative control organism to detect cross reacting antigens. Rocket immunoelectrophoresis of serological antigens from *Lactobacillus sp.* against anti-Lea-1-74 serum revealed no precipitin lines.

Two-dimensional Immunoelectrophoresis

All serological antigen preparations from *R. salmoninarum* isolates were tested by two-dimensional immunoelectrophoresis with anti-Lea-1-74 and anti-K50 sera

Fig. 3. Rocket immunoelectrophoresis of Renibacterium salmoninarum soluble antigens. The top portion of agarose contains 5% antiserum prepared against either isolate Lea-1-74 or K50 as indicated. The cathodic wells contain serological test antigens and an extracted soluble antigen (Fr 21-30, well number 8). See Table 1 for serological antigen designations. Arrows show a common soluble antigen.

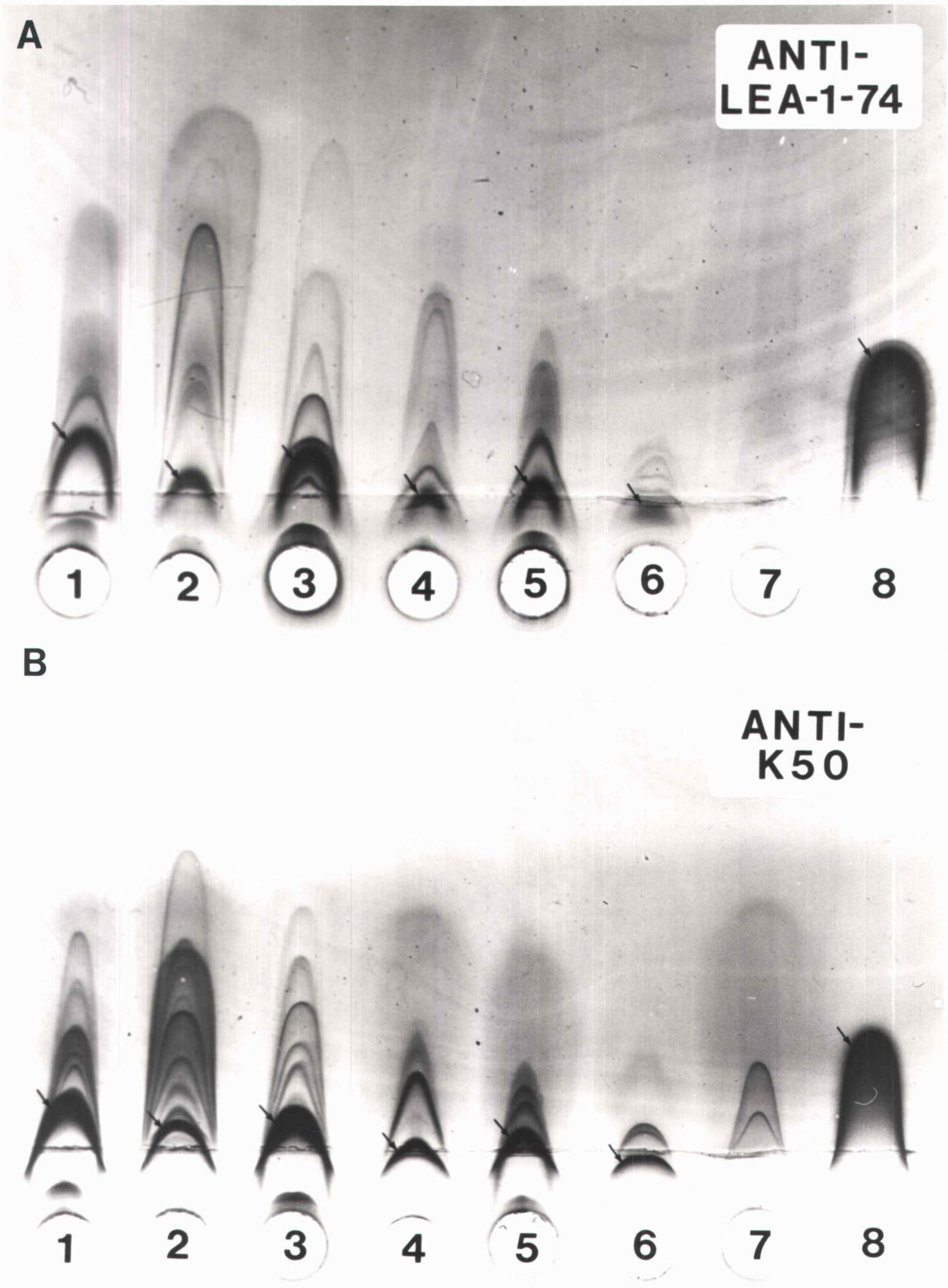

Fig. 3.

(Figures 4-7). Eight to twenty precipitin peaks were observed with each isolate, depending on relative antigen concentrations and antiserum potency. The number of precipitin peaks formed by each isolate was compared (Table 2). Anti-K50 serum produced a profile with the type strain, Lea-1-74, which closely resembled that obtained with the homologous isolate (K50). Fewer components were detected with isolates K28 and K70 against anti-K50 serum, but the profiles appeared the same. Similarly, the Canadian isolates, 384 and 37841, produced fewer peaks, but the antigens present corresponded with the major peaks of the other isolates examined in this manner.

The results with anti-Lea-1-74 serum followed the previous pattern of the K50 antiserum. Many antigens were recognized with the K50, KDV-10 and Lea-1-74 isolates, while fewer components were revealed with the K28, K70, and 384 isolates. An exception to this pattern occurred with the 37841 isolate which formed 14 peaks, one less than the homologous isolate (Lea-1-74). Profiles shown by Lea-1-74 antiserum were also similar in shape, position, and intensity of the stained peaks to anti-K50 serum profiles.

The further comparison of precipitation arcs formed by each isolate required determination of the relative electrophoretic mobilities (Table 3). Each antigen could be clearly identified by its relative migration rate versus the migration distance of the bovine serum albumin (BSA)

Fig. 4. Two-dimensional immunoelectrophoresis profiles of Renibacterium salmoninarum isolates Lea-1-74 and KDV-10. The top portion of agarose contains 5% antiserum prepared against either isolate Lea-1-74 or K50 as indicated. The cathodic wells contain the serological test antigen prepared from isolate Lea-1-74 or KDV-10. Common antigens are identified with their electrophoretic mobilities relative to bovine serum albumin (vertical bar, upper left).

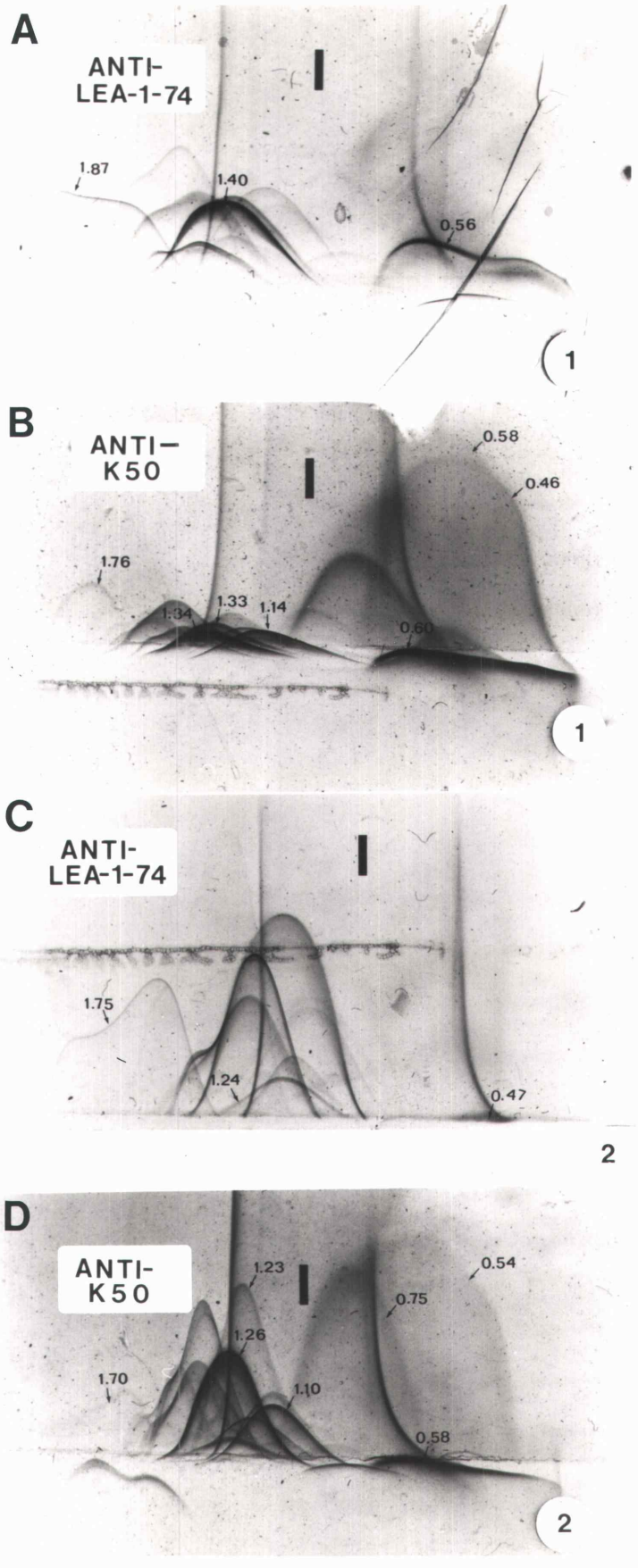
A
ANTI-
LEA-1-74
I
1.87
1.40
0.56
1
B
ANTI-
K50
I
0.58
0.46
1.76
1.33
1.14
1.34
0.60
1
C
ANTI-
LEA-1-74
I
1.75
1.24
0.47
2
D
ANTI-
K50
I
1.23
0.54
0.75
1.26
1.70
1.10
0.58
2

Fig. 4.

Fig. 5. Two-dimensional immunoelectrophoresis profiles of Renibacterium salmoninarum isolates K28 and K50. The top portion of agarose contains 5% antiserum prepared against either isolate Lea-1-74 or K50 as indicated. The cathodic well contains the serological test antigen prepared from either isolate K28 or K50. Common antigens are identified with their electrophoretic mobilities relative to bovine serum albumin (vertical bar, upper left).

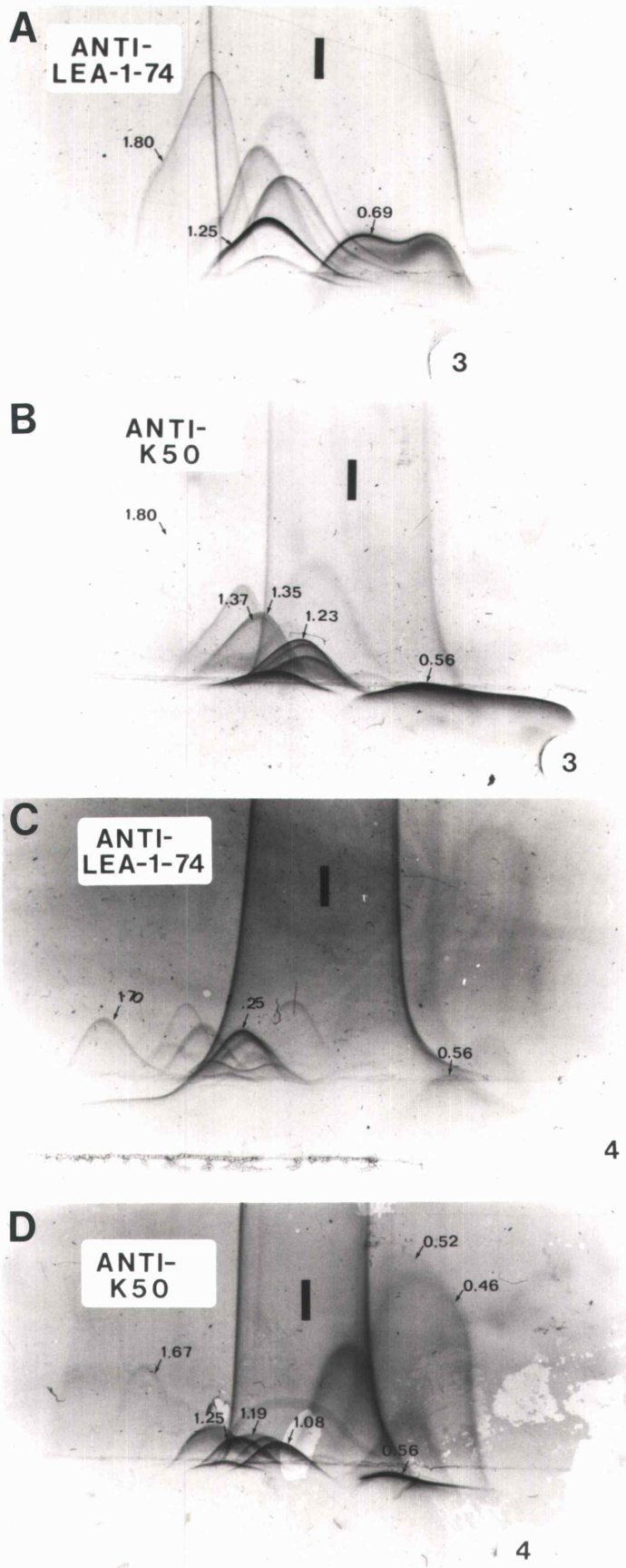
A
ANTI-
LEA-1-74
I
1.80
1.25
0.69
3
B
ANTI-
K50
I
1.80
1.37
1.35
1.23
0.56
3
C
ANTI-
LEA-1-74
I
1.70
1.25
0.56
4
D
ANTI-
K50
I
0.52
0.46
1.67
1.25
1.19
1.08
0.56
4

Fig. 5.

Fig. 6. Two-dimensional immunoelectrophoresis profiles of Renibacterium salmoninarum isolates K70 and 384. The top portion of agarose contains 5% antiserum prepared against either isolate Lea-1-74 or K50. The cathodic well contains the serological test antigen prepared from either isolate K70 or 384. Common antigens are identified with their electrophoretic mobilities relative to bovine serum albumin (vertical bar, upper left).

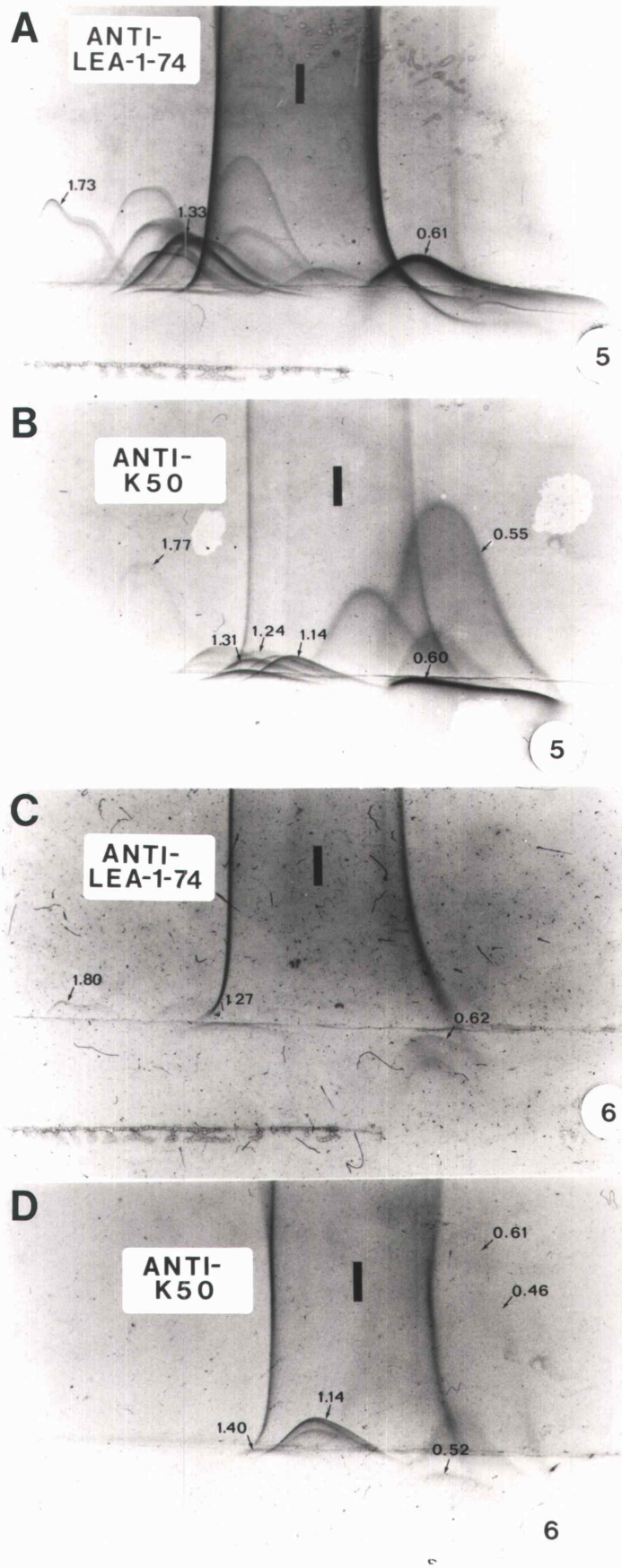
A
ANTI-
LEA-1-74
I
1.73
1.33
0.61
5
B
ANTI-
K50
I
1.77
0.55
1.31
1.24
1.14
0.60
5
C
ANTI-
LEA-1-74
I
1.80
1.27
0.62
6
D
ANTI-
K50
I
0.61
0.46
1.14
1.40
0.52
6

Fig. 6.

Fig. 7. Two-dimensional immunoelectrophoresis profiles of Renibacterium salmoninarum isolate 37841. The top portion of agarose contains 5% antiserum prepared against either isolate Lea-1-74 or K50 as indicated. The cathodic well contains the serological test antigen prepared from isolate 37841. Common antigens are identified with their electrophoretic mobilities relative to bovine serum albumin (vertical bar, upper left).

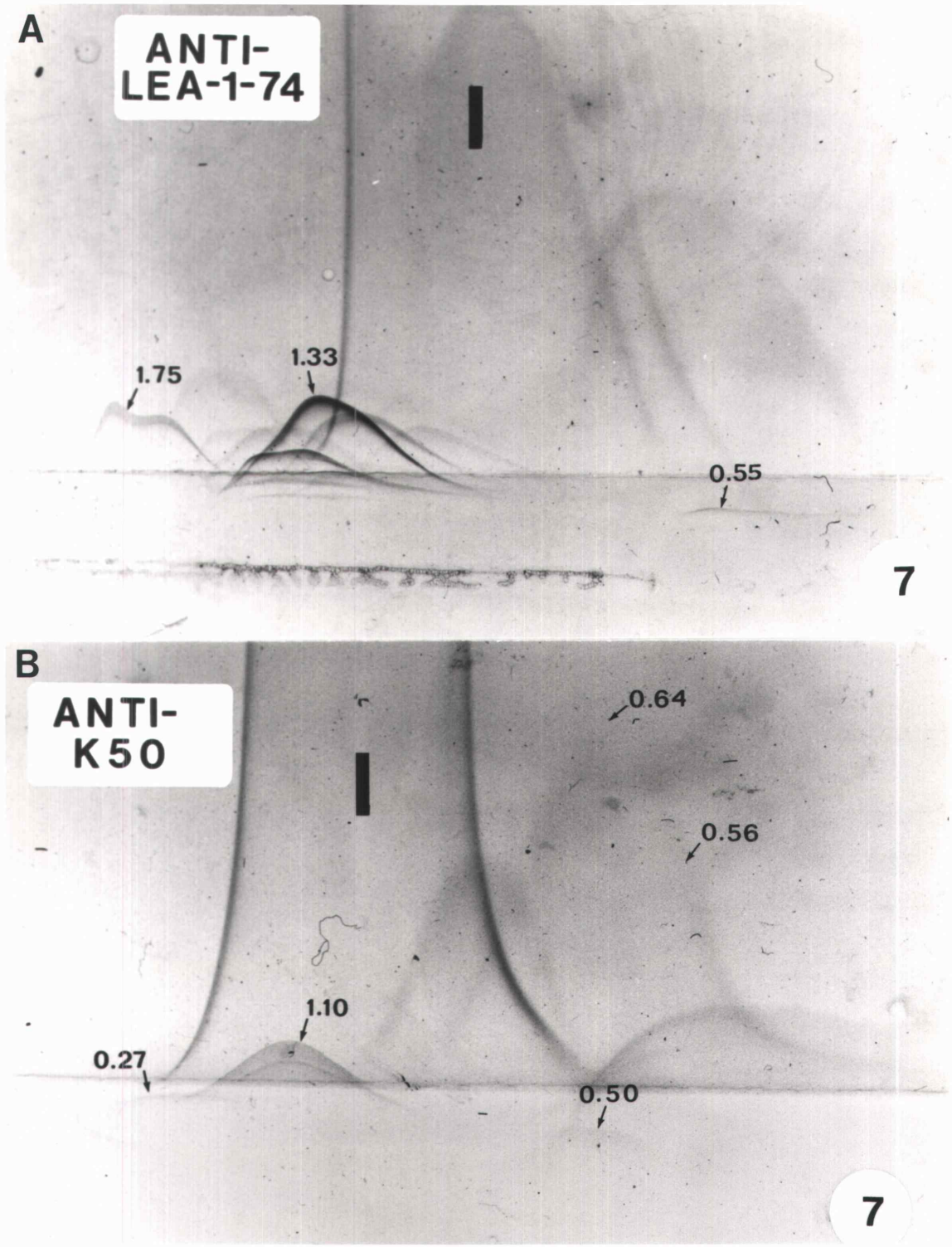

Fig. 7.

Table 2. Serological comparison of *Renibacterium salmoninarum* isolates by two-dimensional immunoelectrophoresis.

	Antigen						
	Lea-1-74	KDV-10	K28	K50	K70	384	37841
Anti-Lea-1-74 serum	15[a](1)[b]	11(0)	10(0)	15(1)	13(4)	8(2)	14(8)
Anti-K50 serum	16(2)	20(9)	13(3)	18(4)	13(4)	8(2)	8(2)
Total Antigens	17	20	13	19	17	10	16
Common Antigens	14	11	10	14	9	6	6

[a]The numbers given represent the number of precipitin lines which could be clearly distinguished on the stained films.

[b]The numbers enclosed represent unique antigens resolved by the respective antiserum.

Table 3. Relative electrophoretic mobility values detected by two-dimensional immunoelectrophoresis analysis of Renibacterium salmoninarum isolates.

Antigen versus Anti-Lea-1-74 serum						
Lea-1-74	KDV-10	K28	K50	K70	384	37841
-0.19	0.27	0.69	0.25	0.37	0.60	0.55
0.37	0.47	1.25	0.41	0.61	0.62	0.57
0.51	0.86	1.27	0.56	0.87	0.92	0.99
0.56	1.07	1.28	0.75	1.13	1.10	1.12
0.70	1.16	1.32	1.05	1.15	1.26	1.15
0.96	1.20	1.39	1.14	1.28	1.27	1.27
1.17	1.24	1.41	1.20	1.33	1.43	1.33
1.22	1.26	1.46	1.23	1.36	1.80	1.37
1.36	1.36	1.79	1.25	1.37		1.40
1.39	1.37	1.80	1.30	1.38		1.41
1.40	1.75		1.34	1.39		1.43
1.48			1.40	1.43		1.52
1.50			1.48	1.73		1.54
1.57			1.57			1.75
1.87			1.70			

Antigen versus Anti-K50 serum						
Lea-1-74	KDV-10	K28	K50	K70	384	37841
0.46	0.34	0.54	0.46	0.53	0.46	0.25
0.50	0.54	0.56	0.47	0.55	0.48	0.50
0.58	0.58	0.95	0.52	0.60	0.52	0.56
0.60	0.75	1.07	0.56	0.83	0.61	0.64
0.84	0.81	1.20	0.78	1.02	1.14	0.73
0.96	0.85	1.23	0.91	1.14	1.15	1.06
1.14	0.90	1.24	0.96	1.20	1.17	1.10
1.16	1.09	1.32	1.02	1.24	1.40	1.27
1.19	1.10	1.35	1.08	1.31		
1.27	1.11	1.37	1.13	1.37		
1.33	1.17	1.48	1.19	1.45		
1.34	1.23	1.50	1.25	1.49		
1.45	1.25	1.80	1.28	1.77		
1.51	1.26		1.35			
1.61	1.38		1.44			
1.76	1.39		1.55			
	1.40		1.67			
	1.46		1.80			
	1.65					
	1.70					

marker, which was assigned a value of 1.0. Relative mobilities measured ranged from -0.19 to +1.87. The comparative reproducibility of these mobilities between runs was good. Also, the electrophoretic order and relative size of peaks remained similar between runs. The analysis of the same isolate against anti-Lea-1-74 and anti-K50 sera resolved common components that both antisera recognized (Table 2). The unique antigens detected by one antiserum and not the other were also recorded (Table 2). For example, isolate K70 produced 13 peaks from each antiserum. Nine of these peaks were common in both profiles while each antiserum also revealed four unique components.

The comparison of the two-dimensional immunoelectrophoresis profiles produced by one antiserum against different isolates was a more difficult task. The variability in antigen concentrations between isolates resulted in tall, faint peaks when the antigen concentration was high versus shorter, more intense peaks when the amount of antigen present was low. Three common components were identified in each of the seven isolates with anti- Lea-1-74 serum. Seven common components were identified with anti-K50 serum (Table 4). Three of the components (A,D,and F) matched the common peaks resolved with antiserum to Lea-1-74. The relative mobilities of these common antigens were used to label their respective peaks (Figures 4-7). Those distinct peaks not labelled,

Table 4. Common antigens of Renibacterium salmoninarum isolates as detected by two-dimensional immunoelectrophoresis.

Antigen	Relative electrophoretic mobility of common antigens in R. salmoninarum isolates						
	Lea-1-74	KDV-10	K28	K50	K70	384	37841
A	1.76[a] (1.87)[b]	1.70 (1.75)	1.80 (1.80)	1.67 (1.70)	1.77 (1.73)	- (1.80)	- (1.75)
B	1.34	1.26	1.37	1.25	1.31	1.40	1.27
C	1.33	1.23	1.35	1.19	1.24	-	-
D	1.14 (1.40)	1.10 (1.24)	1.23 (1.25)	1.08 (1.25)	1.14 (1.33)	1.14 (1.27)	1.10 (1.33)
E	0.58	0.75	-	0.52	-	0.61	-
F	0.60 (0.56)	0.58 (0.47)	0.56 (0.69)	0.56 (0.56)	0.60 (0.61)	0.52 (0.62)	0.50 (0.55)
G	0.46	0.54	-	0.46	0.55	0.46	0.56

[a]The numbers given represent mobilities detected by anti-K50 serum.

[b]The numbers enclosed represent mobilities detected with anti-Lea-1-74 serum.

were clustered too close to each other to determine if they were common or unique components. Preliminary two-dimensional immunoelectrophoresis analysis of isolate RS-21 identified common antigen F as a major component.

Cross Adsorption Analysis

Antisera adsorbed with whole cell preparations of *R. salmoninarum* were screened by rocket immunoelectrophoresis (Figure 8). The number of antigen peaks formed above each well was recorded (Table 5). These results indicated that the soluble antigen extracted from Lea-1-74 (Fr 21-30) was a common surface component present on each of the *R. salmoninarum* isolates. This is best seen when anti-Lea-1-74 serum was adsorbed with its homologous antigens and this intense antigen peak disappeared (Figure 8A). In those cases where the antibodies to this common antigen were not completely removed by adsorption, the peak increased in height and stained less intensely (Figure 8C and 8D). The majority of peaks did not disappear or increase substantially in height, showing that these antigens were either cytoplasmic or nonexposed membrane antigens. The other peaks that did not disappear, but increased in height may have been surface antigens. The lack of any distinguishing characteristics prevented these possible surface antigens from being studied any further by this assay.

Fig. 8. Rocket immmunoelectrophoresis of anti-Lea-1-74 and anti-K50 sera following adsorption with Renibacterium salmoninarum isolates. The top portion of agarose contains 5% antiserum adsorbed with the isolate indicated (adsorbing isolate / antiserum). The cathodic wells contain serological test antigens and an extracted soluble antigen (Fr 21-30, well number 8). See Table 1 for antigen designations. See Fig. 3 for unadsorbed, control illustrations. Arrows show a common soluble antigen.

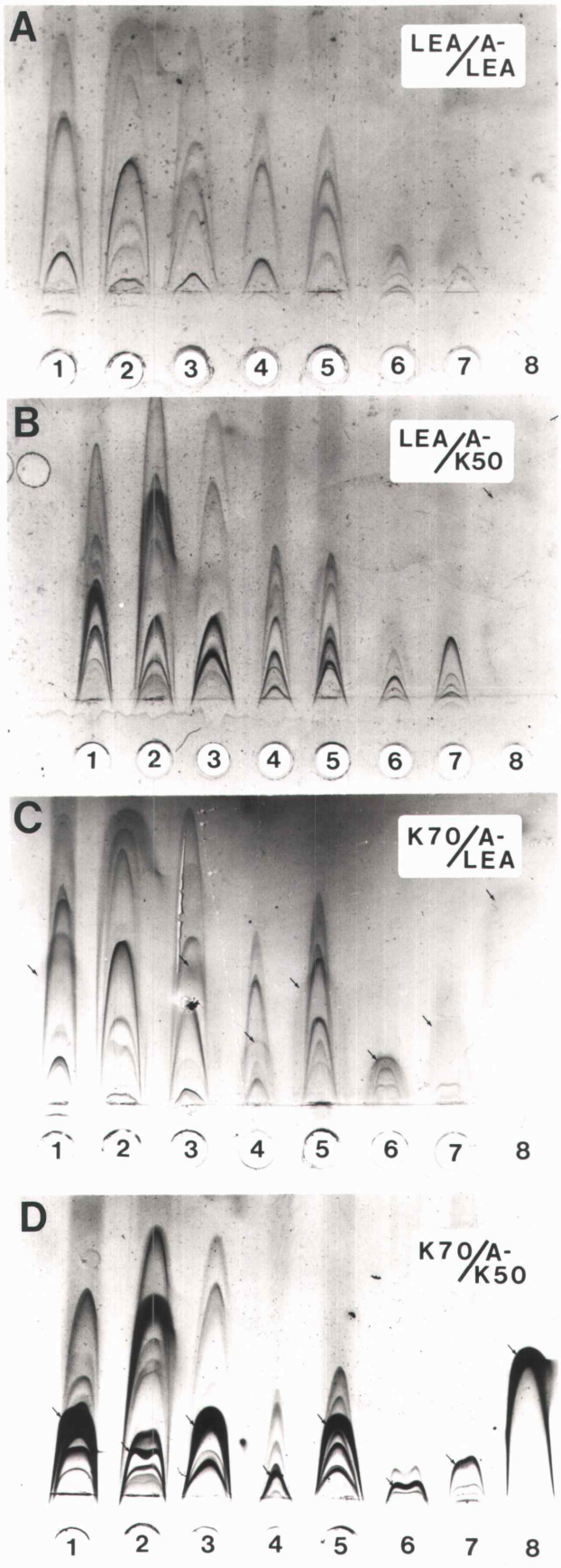
A
LEA/A-LEA
1 2 3 4 5 6 7 8
B
LEA/A-K50
1 2 3 4 5 6 7 8
C
K70/A-LEA
1 2 3 4 5 6 7 8
D
K70/A-K50
1 2 3 4 5 6 7 8

Fig. 8.

Table 5. Serological comparison of anti-Lea-1-74 and anti-K50 sera following adsorption with Renibacterium salmoninarum isolates by rocket immunoelectrophoresis.

	Antigen versus Anti-Lea-1-74 serum							
Adsorbing Isolate	Lea-1-74	KDV-10	K28	K50	K70	384	37841	21-30
Unadsorbed	14[a]	12	11	10	10	8	5	2
Lea-1-74	8	10	7	6	7	7	4	0
KDV-10	9	10	7	7	7	6	3	0
K28	7	6	4	4	5	3	2	0
K50	7	7	4	7	7	6	4	0
K70	13	11	9	8	8	8	4	1
384	10	10	10	9	9	8	4	2

	Antigen versus anti-K50 serum							
Adsorbing Isolate	Lea-1-74	KDV-10	K28	K50	K70	384	37841	21-30
Unadsorbed	14	15	13	10	10	7	7	1
Lea-1-74	12	12	8	9	9	6	6	0
KDV-10	13	15	12	9	9	7	7	0
K28	8	9	7	8	6	5	5	1
K50	14	13	11	9	9	4	7	1
K70	12	14	13	9	10	5	5	1
384	11	13	10	9	10	6	6	1

[a]The numbers given represent the number of precipitin lines which could be clearly distinguished on the stained films.

Characterization of an Extracted Soluble KDB Antigen

The immunodiffusion analysis of extracted antigens showed that KDB isolates produced a major soluble component immunologically identical and common to each isolate (Figure 9). Rocket immunoelectrophoresis of extracted antigens revealed a bullet-shaped peak with anti-K50 serum and a double peak with anti-Lea-1-74 serum (Figure 9). Minor peaks were also evident. Crude extracted antigen from one isolate (Lea-1-74) was fractionated on the gel filtration column, and fractions corresponding to high protein levels were assayed by rocket immunoelectrophoresis. Fractions 21-30 showed strong precipitin reactions and were pooled. Fractions 17-19 showed faint peaks similar to those detected when medium contamination of antigens was tested and these fractions were discarded.

Fractions 21-30 were concentrated and assayed by two-dimensional immunoelectrophoresis. Anti-K50 serum resolved a single peak which was electrophoretically identical to antigen F, while anti-Lea-1-74 serum showed a peak corresponding to antigen F and a second ill-defined arc which shadowed the other peak. This same double peak effect was seen with crude extracted Lea-1-74 (Figure 9C, well 1) and rocket immunoelectrophoresis of fraction 21-30 (Figure 3A, well 8).

Polyacrylamide-agarose gel two-dimensional

Fig. 9. Ouchterlony immunodiffusion and rocket immunoelectrophoresis of crude extracted antigens from Renibacterium salmoninarum isolates. Center wells and the top portion of agarose contain rabbit antisera prepared against either isolate Lea-1-74 or K50 as indicated. Outer and cathodic wells contain extracted soluble antigens from each isolate, including Fr 21-30 (well number 8). Extracted soluble antigens are denoted with the same numbers as those used for serological test antigens (Table 1).

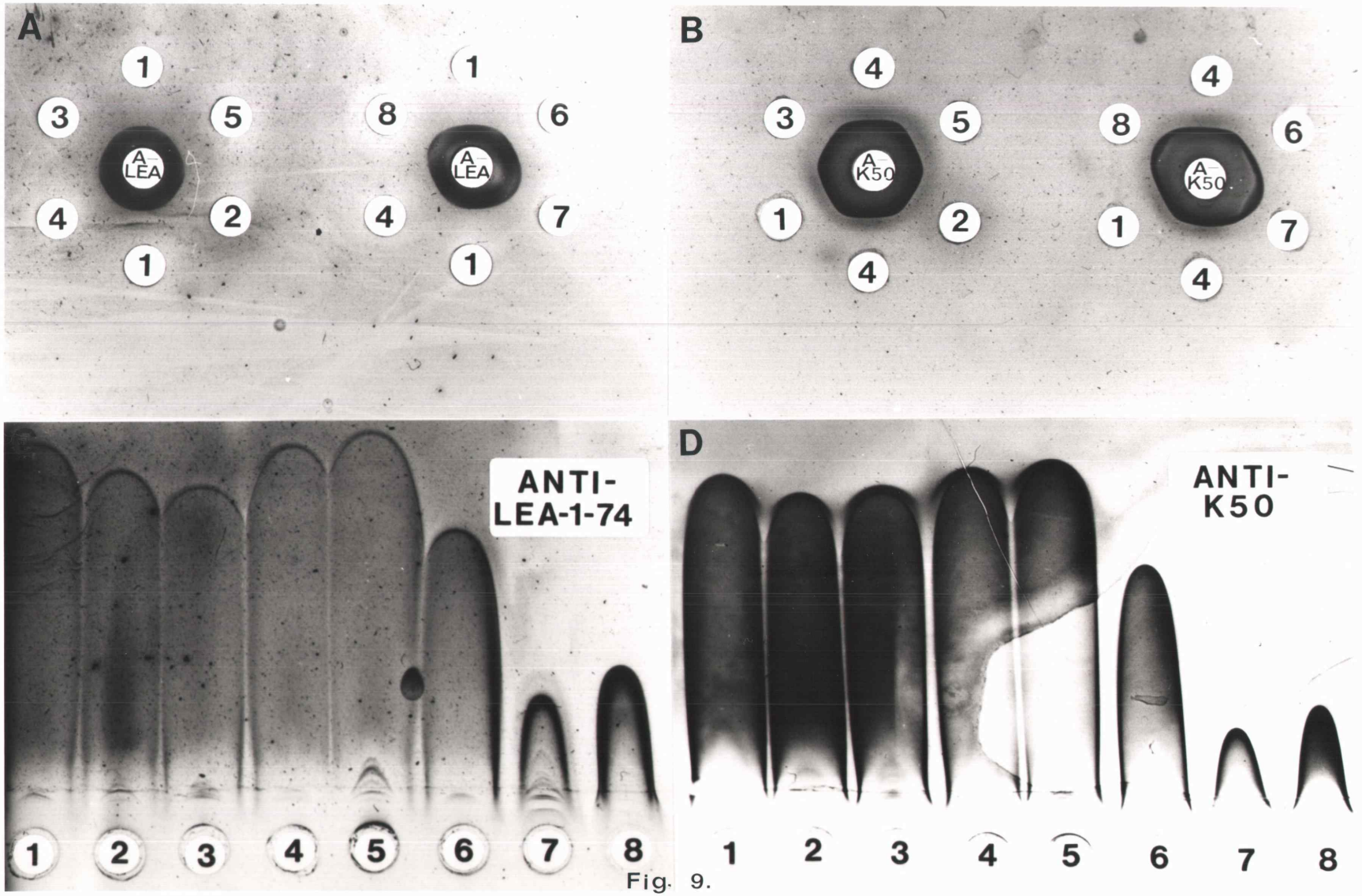
A
1
3
5
A LEA
4
2
1
1
8
6
A LEA
4
7
1
B
4
3
5
A K50
1
2
4
4
8
6
A K50
1
7
4
ANTI-LEA-1-74
1 2 3 4 5 6 7 8
D
ANTI-K50
1 2 3 4 5 6 7 8

Fig. 9.

immunoelectrophoresis of the concentrated fractions (Fr 21-30) detected a precipitin reaction in the second dimension that matched the protein bands in the first dimension (Figure 10). The estimated molecular weight of the major distinct band was 57,000 based on SDS-PAGE electrophoresis (Figure 11). An immunologically homogeneous protein mixture was present above and below this 57K band (Figure 10). Fr 21-30 was also found to be heat stable at 100°C for 30 min.

Growth Comparisons of *Renibacterium salmoninarum* Isolates

Representative growth curves follow a similar pattern, except for isolate RS-21 which grew at a much slower rate (Figure 12). Isolates Lea-1-74, KDV-10, and 37841 reached an optical density of >1.0 at 520 nm by seven days. European isolates K28 and K70 took eight and nine days, respectively, to reach this turbidity. The generation times were approximately 24 h, but results varied depending upon inoculum size, metabolic state of the inoculum, and how well the isolate was adapted to M-KDM-2 medium. The Margaree isolate (RS-21) had not been grown on M-KDM-2 before and had to adapt to this new medium. A turbidity of >1.0 $O.D._{520}$ was not obtained until twelve days after inoculation.

Minimum Inhibitory Concentration Determinations of *Renibacterium salmoninarum* Isolates

The comparison of MIC's from each isolate of *R.*

Fig. 10. Detection of partially purified, extracted antigen by polyacrylamide-agarose gel two-dimensional immunoelectrophoresis. Fractions 21-30 from the Ultragel AcA 44 column were pooled, concentrated, and subjected to electrophoresis in SDS polyacrylamide. The gel was sliced vertically and one strip subjected to immunoelectrophoresis into agarose containing anti-Lea-1-74 serum, to identify the position of the extracted antigen. The other strip, containing extracted antigen (lane B) and a mixture of low molecular weight standards (lane A) was stained with Coomassie Brilliant Blue. Zero position on the ruler is the bottom of the resolving gel.

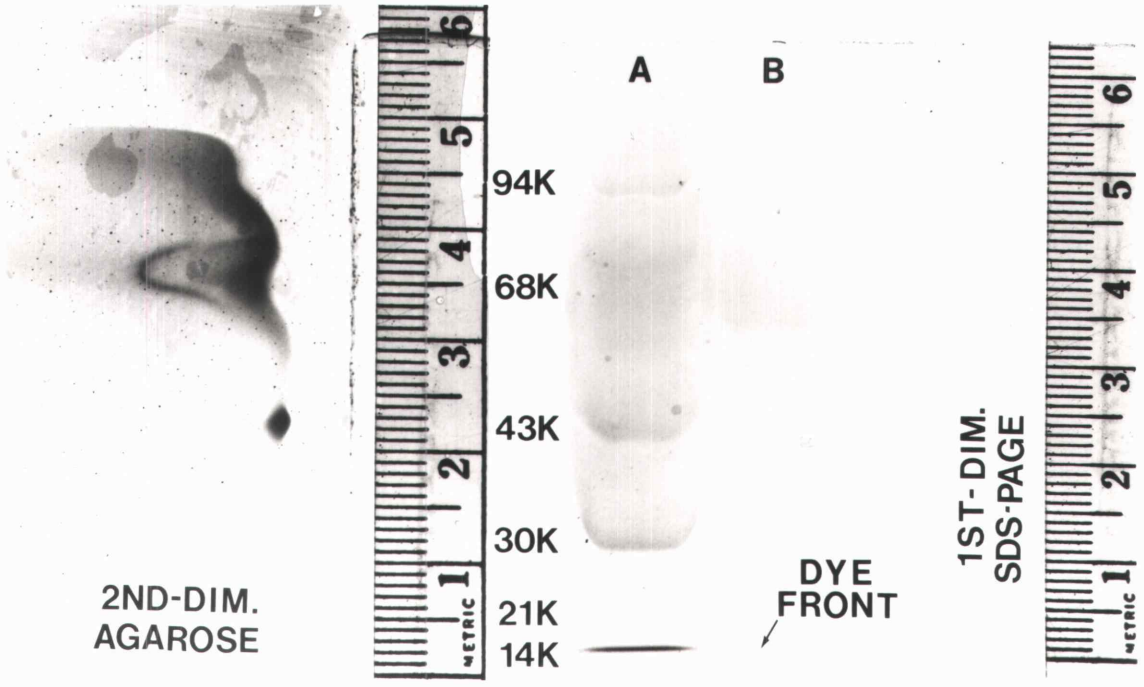
2ND-DIM.
AGAROSE
A
B
94K
68K
43K
30K
21K
14K
DYE
FRONT
1ST-DIM.
SDS-PAGE

Fig. 10.

Fig. 11. Molecular weight estimation of the extracted antigen of Renibacterium salmoninarum isolate Lea-1-74 (Fr 21-30) by SDS-polyacrylamide gel electrophoresis. Log_{10} molecular weight of the low molecular weight proteins was plotted against relative migration distance to establish a standard curve.

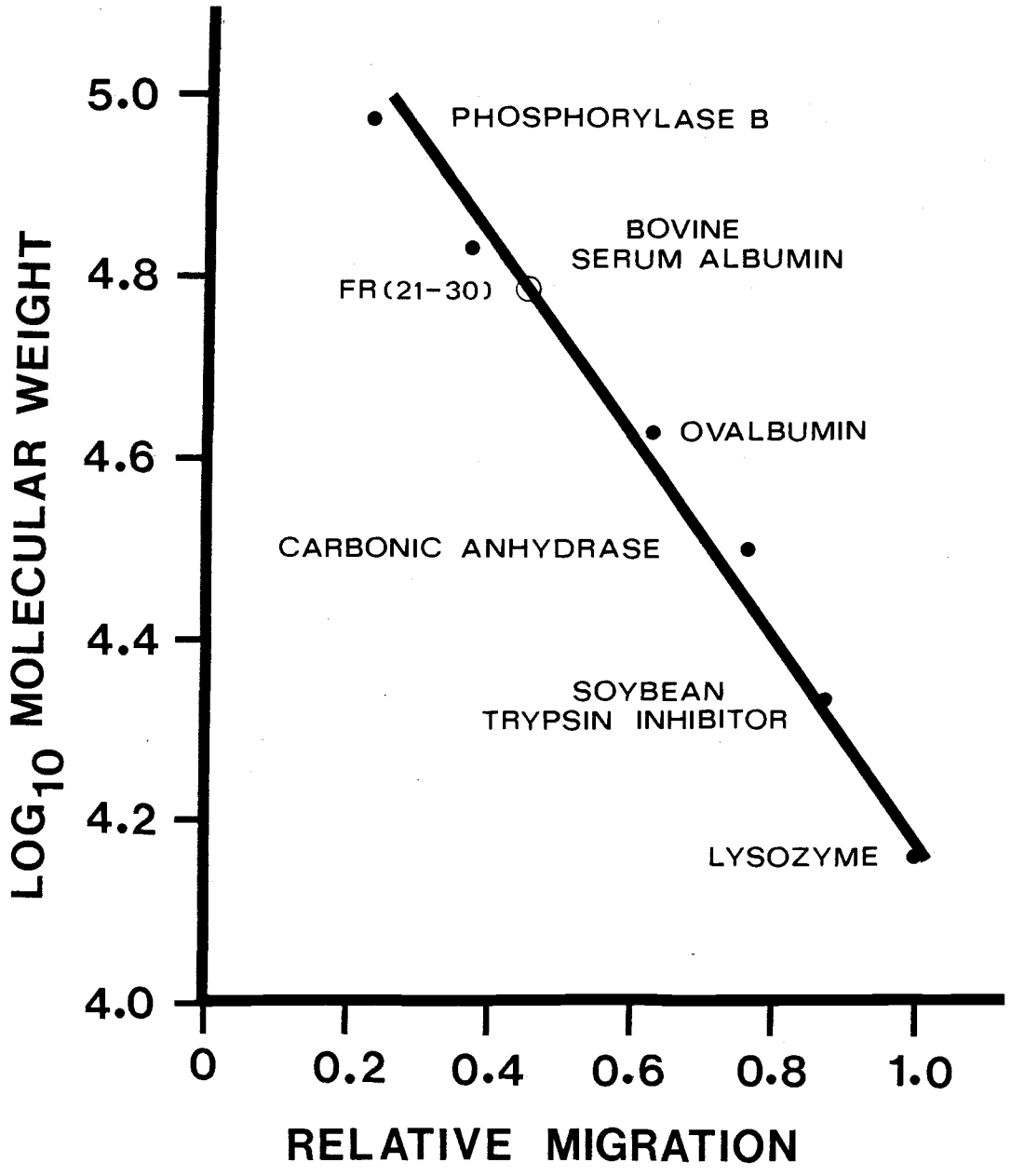
5.0
4.8
4.6
4.4
4.2
4.0
LOG10 MOLECULAR WEIGHT
PHOSPHORYLASE B
BOVINE
SERUM ALBUMIN
FR(21-30)
OVALBUMIN
CARBONIC ANHYDRASE
SOYBEAN
TRYPSIN INHIBITOR
LYSOZYME
0
0.2
0.4
0.6
0.8
1.0
RELATIVE MIGRATION

Fig. 11.

Fig. 12. Growth of Renibacterium salmoninarum in M-KDM-2 broth measured turbidimetrically. Test organisms were grown to early stationary phase in M-KDM-2 broth, diluted to 1 $O.D._{520}$ with the same medium, and 1 ml inoculated into 50 ml of M-KDM-2. Growth at 18°C was monitored by measuring absorbance at 520 nm. ●●● Lea-1-74, ○○○ KDV-10, ■■■ K28, □□□ K70, ✶✶✶ 37841, ☆☆☆ RS-21.

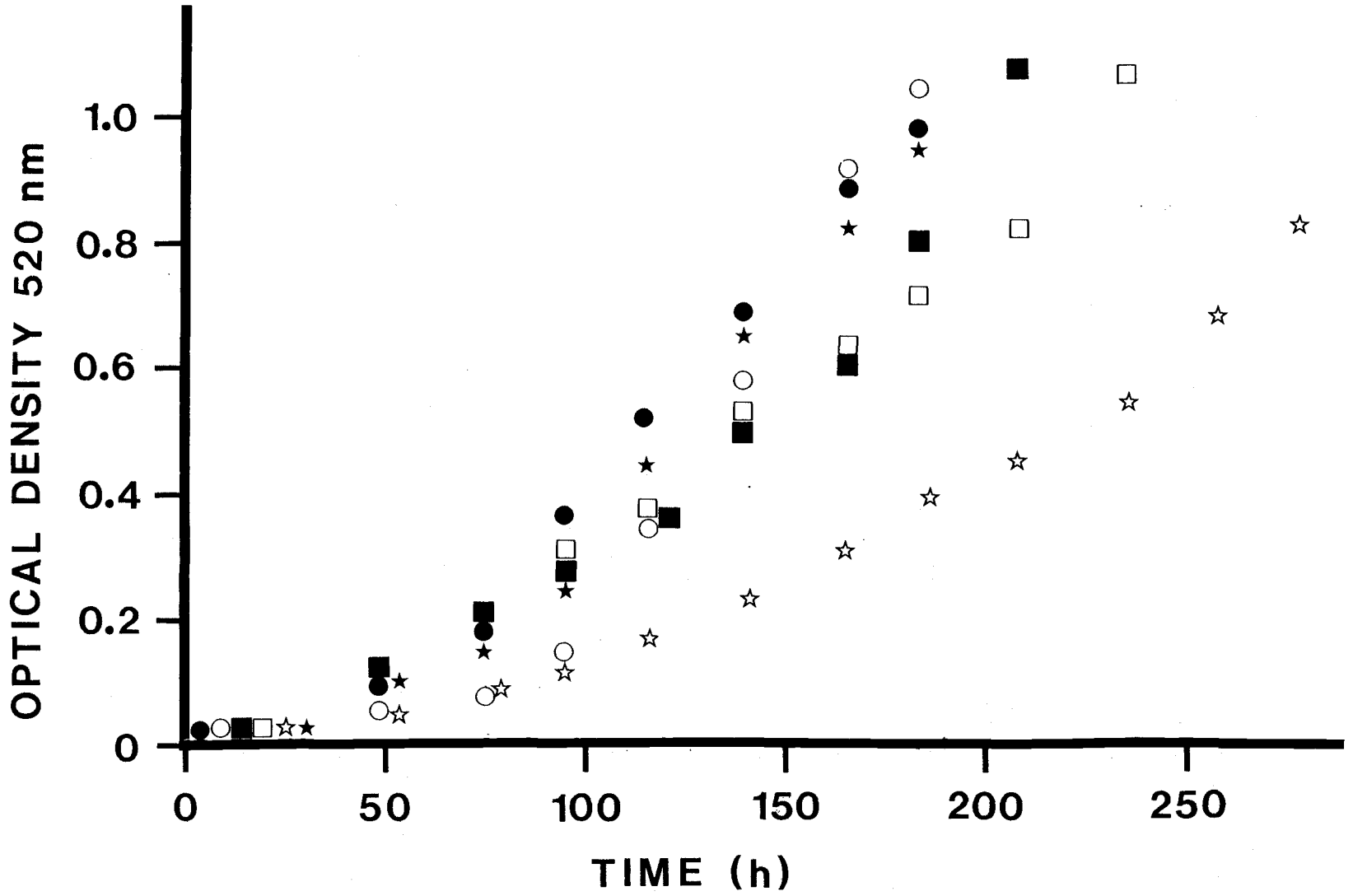
OPTICAL DENSITY 520 nm
1.0
0.8
0.6
0.4
0.2
0
0
50
100
150
200
250
TIME (h)

Fig. 12.

salmoninarum revealed similar sensitivities for those antibiotics tested (Table 6). Eight of the antimicrobics inhibited R. salmoninarum growth. Gentamicin, Kanamycin, and Methicillin had little if any effect on those isolates tested.

Table 6. Minimum inhibitory concentrations of antimicrobics for Renibacterium salmoninarum isolates.

Antimicrobics	Isolates							
	Lea-1-74	KDV-10	K28	K50	K70	384	37841	RS-21
Ampicillin (0.12-16)[b]	1.0[a]	2.0	1.0	1.0	1.0	8.0	1.0	0.5
Cephalothin (0.5-64)	2.0	2.0	2.0	2.0	2.0	4.0	2.0	1.0
Chloramphenicol (0.25-32)	1.0	4.0	2.0	4.0	1.0	4.0	4.0	1.0
Clindamycin (0.12-16)	4.0	8.0	2.0	4.0	0.5	2.0	4.0	4.0
Erythromycin (0.25-32)	1.0	2.0	1.0	1.0	0.5	1.0	1.0	0.5
Gentamicin (0.12-16)	>16	16	>16	>16	16	>16	16	16
Kanamycin (0.5-64)	>64	64	>64	>64	64	>64	>64	64
Methicillin (0.12-16)	8.0	16	8.0	16	8.0	>16	16	8.0
Penicillin G (0.06-8)	0.25	0.25	0.25	0.25	0.25	0.5	0.5	0.25
Tetracycline (0.12-16)	0.25	0.5	0.25	0.5	0.25	0.5	1.0	0.5
Vancomycin (0.25-32)	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0

[a]The numbers given represent lowest concentration (µg/ml) of antimicrobic that inhibited visible growth of the organism.

[b]The enclosed numbers represent the range of concentrations (µg/ml) present for each antimicrobic.

DISCUSSION

The comparison of eight isolates of R. salmoninarum was made by three methods. The antigenic analysis was used to examine serological relationships among the isolates. Cultures were tested for differences in growth rate. And minimum inhibitory concentrations (MIC's) assessed antibiotic susceptibilities of the isolates.

Antigenic analysis showed all R. salmoninarum isolates to be closely related serologically. This is in agreement with earlier reports indicating serological homogeneity (Bullock et al., 1974; Paterson et al., 1981). These previous works used the less sensitive methods of immunodiffusion and cross-agglutination. Using immunodiffusion, the maximum number of antigens detected in the research reported here was four, compared to twelve with immunoelectrophoresis, fifteen with rocket immunoelectrophoresis, and twenty with two-dimensional immunoelectrophoresis. The increase in sensitivity with rocket and two-dimensional immunoelectrophoresis is a result of the efficient utilization of antigen and antibody because antigens move in one direction and antibody concentration remains constant; compared to inefficient methods of immunodiffusion and immunoelectrophoresis where antigens and antibodies diffuse in all directions. The advantage of two-dimensional immunoelectrophoresis over rocket immunoelectrophoresis is the separation of the

antigen in two directions instead of one, increases the possibility of visualizing the precipitin reaction.

The eight to twenty precipitin lines observed with R. salmoninarum isolates are similar to the number of peaks found in organisms of the genera, Mycoplasma and Chlamydia (Thirkill and Kenny, 1974; Caldwell et al., 1975).

Electrophoretically common antigenic components could be identified by their similar mobilities, position among other peaks, peak shape, and staining intensity. Some components were not analyzed because their peaks were either too faint or too close to neighboring peaks to be identified consistently as common or unique. Though approximately one-half of the total antigens of each isolate were not analyzed, the common antigens that were identified formed densely staining peaks indicative of high antigen concentration. In their antigenic analysis of Mycoplasma arginini, Thirkill and Kenny (1975) studied only one-third of the total antigens (20 in all) present in each of three strains. However, the major, densely staining peaks were analyzed, which enabled antigenic relationships to be shown within the species. This study has demonstrated the presence of seven common antigens which represent a major percentage of the antigen concentration of each isolate. In addition, one of these common antigens (F) was shown to be a major surface component by cross adsorption analysis. The close antigenic relationship among

R. salmoninarum isolates was established by identifying the major serological antigens common to each profile.

The antigenic components resolved in this study showed electrophoretic mobilities ranging from near zero to well past the BSA marker. Electrophoresis moved antigens possessing a negative charge at pH 8.6. The Lea-1-74 antigen with a -0.19 mobility probably carried little charge at pH 8.6 and was transported cathodically by electroendosmosis. Anti-K50 serum consistently resolved three common antigens with low mobilities (E, F, and G), while anti-Lea-1-74 serum showed only one (F). This difference may be because the anti-K50 rabbit recognized antigens E and G, but the anti-Lea-1-74 rabbit did not. Two other common antigens identified in anti-K50 profiles (B and C), but not noted in anti-Lea-1-74 profiles, were unable to be objectively shown common to all anti-Lea-1-74 profiles. A number of variations of two-dimensional immunoelectrophoresis are available, including enhancement and suppression techniques, which would make it easier to discriminate between common and unique components (Axelson and Bock, 1972)

The nature of these resolved antigens is unknown except for the extracted antigen (Fr 21-30), which was shown to be a surface component by cross adsorption analysis. Other membrane components were perhaps solubilized by the nonionic detergent Triton-X-100, though

they may have been internally bound antigens not detected by adsorption studies. Antigen F was shown to be the same as Fr 21-30 and to have a consistent electrophoretic mobility near 0.56 in all isolates tested. The second peak or shadow resolved by anti-Lea-1-74 serum against Fr 21-30 was shown to be immunologically related to the major protein band in polyacrylamide-agarose gel two-dimensional immunoelectrophoresis of Fr 21-30. SDS-PAGE showed the molecular weight of Fr 21-30 varied, which may help explain why a shadow or double peak was seen whenever antigen F or Fr 21-30 reacted with anti-Lea-1-74 serum. Two clones of antibodies may have been present in anti-Lea-1-74 serum which reacted with different sizes of this soluble antigen. Another possibility is that antibodies were produced against two different antigenic determinants on the soluble antigen. Anti-K50 serum recognized only one size or antigenic determinant so one peak was produced against Fr 21-30 or antigen F.

Because Fr 21-30 was the only major soluble antigen extracted from spent culture medium and a major surface component, it is probably the same soluble antigen detected by previous investigators with immunodiffusion (Bullock *et al.*, 1974; Banowetz, 1974). In addition, Fr 21-30 was found stable to heating at 100^{o}C for 30 min, so Fr 21-30 is probably the same heat stable antigen extracted from KDB cells and infected kidneys by Kimura *et al.*, (1978).

Further purification and study of this extracted antigen is necessary. Serodiagnosis of R. salmoninarum infected salmonids might be possible if the soluble antigen is recognized by the fish's immune system. And if salmonids respond to this antigen, is it a protective antigen? Recently, antigens associated with protective immunity have been extracted from Vibrio anguillarum and Aeromonas salmonicida, two common, Gram negative fish pathogens (Agius et al., 1983; McCarthy et al., 1983).

Antigenic analysis of R. salmoninarum isolates showed that seven major antigens with distinct mobilities could be identified. The first of these common antigens characterized proved to be serologically identical in every isolate tested. Future research might characterize the other major components to determine their antigenic relatedness. More elaborate techniques such as tandem or intermediate gel crossed immunoelectrophoresis may be useful.

Growth experiments were conducted with a modified version of Evelyn's KDM-2 broth. Ferric sulphate was suggested as a possible supplement to the serum lacking medium used in this study (C.R. Banner, personal communication). Bovine serum was omitted because of earlier problems with contamination of antigen preparations. Despite repeated washing, serum antigens remained attached to immunogens and subsequent cross-reactions against the

bovine antigens resulted. Culture of KDB cells in M-KDM-2 broth avoided this contamination.

Growth curves recorded with M-KDM-2 broth resembled those described by Embley *et al.* (1982), where growth with cysteine supplemented Mueller-Hinton broth and KDM-2 broth reached a turbidity of >1 $O.D._{520}$ in 7 to 10 days. Fryer and Sanders (1981) reported maximum growth of the type strain by 3 to 10 days with KDM-2 broth. The growth of *R. salmoninarum* without bovine serum in M-KDM-2 may not be as rapid, but for routine culture of KDB it is more economical. A semi-defined growth medium for *R. salmoninarum* which also avoids the use of costly bovine serum and yields a maximum growth after 13 days has been reported (Embley *et al.*, 1982). This semi-defined medium may have value in nutritional investigations, but for routine cultivation M-KDM-2 is a simpler medium to prepare.

Antibiotic sensitivity testing showed that *R. salmoninarum* isolates were susceptible to eight of the eleven antimicrobics tested. Wolf and Dunbar (1959), using the disk method of sensitivity testing, also noted KDB susceptibility to chloramphenicol (30 µg/disk), erythromycin (15 µg/disk), penicillin (10 µnits/disk), and tetracycline (30 µg/disk). Austin and Rodgers (1980) found ampicillin (25 µg), chloramphenicol (10 µg), cephalophoridine (15 µg), and tetracycline (5 µg) inhibited *R. salmoninarum* growth, but did not describe their assay

method. Clindamycin and vancomycin MIC's have not been reported for R. salmoninarum.

Two studies have noted erythromycin resistance for certain KDB isolates. Schneider (1980) incorporated 20 µg/ml erythromycin in KDM-2 agar and showed heavy growth from a McKenzie Hatchery (Oregon) isolate. How much erythromycin remains active in an in vitro method like this is unknown. The other report of resistance was in the phenon 2 isolates described by Austin and Rodgers (1980). They state that some strains clustering in phenon 2 were resistant to erythromycin (10 µg), but again they fail to describe the assay method so the value of this data is questionable. R. salmoninarum isolates in this study consistently showed sensitivities to 1 µg/ml erythromycin.

In summary, R. salmoninarum isolates compared in this manuscript were all closely related serologically. Seven common antigens have been identified from two-dimensional immunoelectrophoresis profiles. One of these common antigens (F) has been extracted, purified, and shown to be a major surface antigen by cross adsorption analysis. Growth and MIC data indicate there are close cultural similarities among R. salmoninarum isolates compared herein.

SUMMARY AND CONCLUSIONS

1. Similar antigenic profiles were resolved from each isolate of *R. salmoninarum*.
2. Seven common antigens were identified by their electrophoretic mobilities in each antigenic profile.
3. A major soluble antigen (Fr 21-30) was partially purified from spent culture medium by ammonium sulfate precipitation and gel filtration.
4. Fr 21-30 was shown to be a major surface antigen by cross adsorption analysis and serologically identical in each isolate by immunodiffusion.
5. Fr 21-30 was heat stable at 100^{o}C for 30 min and its estimated molecular weight was 57,000 by SDS-PAGE.
6. *R. salmoninarum* isolates examined in this study are serologically closely related.
7. Comparisons of growth suggest cultural similarities exist among the isolates tested.
8. Minimum inhibitory concentration data agreed with published sensitivities for ampicillin, cephalothin, chloramphenicol, penicillin, and tetracycline. Previously unreported sensitivities to clindamycin and vancomycin were determined.
9. Erythromycin was found to inhibit growth at 1.0 µg/ml, in contrast to reported resistances at 10 to 20 µg/ml of this drug.

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