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Twenty-two isolates of Flexibacter columnaris were collected from different species of fish from a wide geographic range. Shieh medium was selected and modified to provide satisfactory growth of F. columnaris strains. Although there were three phenotypic variations in colony morphology among the strains observed, the environmental and biochemical characteristics as well as GC content revealed no differences among these isolates. However, when compared genetically by the method of DNA hybridization, there appeared to be two distinct groups based on the DNA homology. Group one was 81-98% homologous with Pacific Northwest strain DD3, and included isolates from Canada, Chile, Japan, Korea, Taiwan and USA (Atlantic and Pacific Coasts). Group two came from the southern portion of the USA, was less homologous (73%) with strain DD3 from the Pacific Northwest, and may be considered a different strain or species from F. columnaris. Taiwan

strains 4G and 5F belonged to the different group and were classified into new species of cytophaga as a result of low DNA homology (<29%) to the Pacific Northwest strain of F. columnaris, although all other characteristics were identical to F. columnaris.

Serological comparison of F. columnaris by crossed immunoelectrophoresis showed that all the tested strains had different antigenic profiles. Although they shared from 7 to 18 common antigens, strains varied in their composition by possessing 5 to 13 partially shared antigens analyzed by crossed immunoelectrophoresis with an intermediate gel. Strains 1S, T13, and M1 were separated into three different serotypes based on the presence of type-specific unique antigens. However, strains 82303, DD3, IC8r, and 238 failed to type because no type-specific unique antigens were found. No correlation between the serotype and geographic or host sources was observed. These common antigens were shown to be protective by cross-infection of immunized rainbow trout with formalin-inactivated whole cell bacterin.

Comparison of *Flexibacter columnaris* Strains Isolated from Fish in North America and Other Areas of the Pacific Rim

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INTRODUCTION

Flexibacter columnaris, formerly known as *Chondrococcus columnaris* or *Cytophaga columnaris*, is the etiological agent of columnaris disease. It is a recognized, widely distributed pathogen of fish, and is known to infect more than 36 species belonging to 10 families. The disease can be devastating to both juvenile and adult fish by primarily attacking the gills. Death results from respiratory failure. No vaccine against this disease is available. Prevention of columnaris disease is by avoidance of stress such as high water temperature, and crowding and handling of fish resulting in injuries and abrasions. Chemotherapy provides also an effective means of control for both external and systemic infections of *F. columnaris*.

There are three major objectives included in this research. First was selection of a growth medium for the large-scale production of vaccines based on the considerations of cell yield and growth rate. Second was a comparison of morphological, environmental, biochemical, genetic and serological characteristics among strains of *F. columnaris*. This information is essential to determine the number of strains required to produce an effective broad spectrum vaccine. Last, since serological evidence

indicates that all strains share common antigens, cross-infection experiments with immunized fish were conducted to determine if the common antigens are protective. This information is important for the development of a subunit cell vaccine instead of an inactivated whole-cell vaccine in which the protective antigens may be not dominant.

LITERATURE REVIEW

Historical Background

Columnaris disease was originally described by Davis (1923) as the cause of serious mortalities among warm-water fishes from the Mississippi River held at the U.S. Biological Station at Fairport, Iowa. The disease occurred primarily in fish which had been injured or handled at water temperatures in excess of 21.1°C. Of particular interest was the observation that when material from a lesion was placed in wet mount, the bacteria collected along bits of tissue in column-like masses. Because of this property, Davis proposed the name *Bacillus columnaris* for the organism.

Following Davis' original description, no further occurrence of columnaris disease was reported until 1943 when Fish and Rucker noted an outbreak recognized as columnaris disease among sockeye salmon being reared at the Leavenworth Hatchery of the U.S. Fish and Wildlife Service. Ordal and Rucker (1944) succeeded in isolating the etiological agent of columnaris disease from infected fish obtained during the epizootic at the Leavenworth Hatchery. Subsequently, similar organisms were isolated from lesions and organs of adult chinook and sockeye salmon, whitefish, squawfish, chubs, and suckers trapped at Rock Island Dam on the Columbia River. From 1940 to 1943 heavy mortalities occurred, particularly among

sockeye and summer chinook salmon, when they were relocated from Rock Island Dam on Columbia River to Leavenworth Hatchery where they were maintained until they spawned (Fish and Hanavan, 1948). Since 1944 numerous outbreaks of columnaris disease have been reported. The bacterium was found to be pathogenic to more than 36 species of fishes belonging to 10 families (Bullock et al., 1971). These fish represented freshwater and anadromous species, warm-water and cold-water inhabitants, adult and fingerling stocks, as well as natural and cultured populations (Nigrelli, 1943; Nigrelli and Hutner, 1945; Davis, 1949; Johnson and Brice, 1952; Rucker et al., 1953; Anacker, 1956; Anacker and Ordal, 1959; Borg, 1960; Colgrove and Wood, 1966; Bottomley and Holland, 1966; Wakabayashi and Egusa, 1966; Anderson and Conroy, 1969; Pacha and Ordal, 1970; Bootsma and Clerk, 1976; Snieszko and Bullock, 1976; Hsu and Kou, 1977; Becker and Fujihara, 1978). In addition, the etiology of "black patch necrosis" in Dover sole being reared in sea water was also ascribed to a F. columnaris-like bacterium (Campbell and Buswell, 1982). Severe economic losses have occurred as result of epizootics of this disease. A Flexibacter infection as the cause of mortality among the fry of red sea bream (Pagrus major) and black sea bream (Acanthopagrus schlegeli) reared in seawater was also found in Japan since 1976 and proposed a new species, Flexibacter maritimus (Wakabayashi et al., 1986).

Growth Media

There are six media commonly used for cultivation of F. columnaris. The ingredients of each medium are shown in Table 2.

Ordal and Rucker (1944) initially cultured the bacteria. The bacteria failed to grow on media containing the usual concentrations of nutrients and agar, but grew well when medium components were diluted. Subsequently, Cytophaga medium was found to be very satisfactory for isolation and cultivation. However, spherical and oval "microcysts" were found in culture and increased in number with the age of the culture.

An organism probably identical to F. columnaris was described by Garnjobst (1945), who reported good growth in tryptone or peptone bases and also in casein hydrolysate with appropriate mineral salts, but not in inorganic nitrogen sources. She found no apparent stimulation by vitamins, glucose, or lactose. But there was no lag period in the presence of vitamins which showed that the organism probably synthesizes any necessary vitamins.

A similar isolation was reported independently by Nigrelli and Hutner (1945). The organism grew well in a complete mixture of amino acids and also in gelatin and casein hydrolysates. Yeast extract was definitely inferior. However, the organisms were not influenced by the addition of carbohydrates to the medium, nor were they able to utilize large but non-toxic amounts of lactate,

malate, acetate, butyrate, succinate, pyruvate, L-aspartate, L-asparagin, L-glutamate, and D,L-alanine. They concluded that amino acids are used not only for energy sources, but also for structural components.

For preparation of antigens of F. columnaris, Anacker and Ordal (1959a) grew the organism in liquid medium containing tryptone and yeast infusion (TYI). Yeast infusion consists of the supernate from an autoclaved yeast suspension which has been clarified with egg albumin and sterilized by filtration (Pacha, 1961).

However, it was noted early that marked morphological aberrations occurred in cells of F. columnaris grown on synthetic media. Conversion of cells to spheroplasts was often observed. Reproducibility of growth was poor. It was thought that F. columnaris might be more sensitive to osmotic pressure changes than most eubacteria. Chase (1965) reported that F. columnaris grew on a vitamin-free, salt-free, acid hydrolyzed casein, but growth was limited unless appropriate mineral salts were provided. She developed a medium containing casein hydrolysate and Earle's balanced salt solution. The organism was found to require 12 amino acids for continuous rapid growth. Nine of these amino acids were essential (leucine, lysine, arginine, histidine, methionine, isoleucine, valine, threonine, phenylalanine). Three other amino acids (alanine, proline, serine), when deleted singly or as a

group, reduced the growth rate significantly. Phosphate, Mg^{+2}, Ca^{+2}, and Fe^{+3} were established as essential inorganic requirements. There is no indication that glucose utilization was found nor was there any stimulation of growth by this sugar. But a reduction in amino acid content restricted growth. These indicated that amino acids sufficed as both carbon and nitrogen sources, and there was an absence of a glycolytic pathway. The presence of vitamins in the form of yeast infusion in the TYI medium certainly stimulated the rate of growth. However, serial transfer on Chase medium indicated that vitamins were not required.

In 1980, Shieh examined in great detail the amounts of growth of F. columnaris in the different media by measuring the incorporation of radiolabelled amino acid into bacterial cells. Most of his investigation was in agreement with Chase's results in 1965. However, a requirement for ribonucleic acid and a stimulatory effect of $NaHCO_3$, pyruvate, and citric acid on the growth of F. columnaris were demonstrated. In addition, $BaCl_2$ and serine instead of lysine were shown to be essential for the growth of F. columnaris.

Liewes (1982) developed another culture medium, which resulted in significantly faster growth rates compared with the previous media (Bootsma and Clerk, 1976; Chase, 1965). Salts were introduced into the medium by adding 30% Earle's balanced salt solution.

Characteristics of Flexibacter columnaris

The organism is a long, slender, Gram-negative rod that measures Ø.5 to Ø.7 um wide by 4 to 8 um long. However, shape and particularly size varied with the growth phase and the composition of the medium (Pacha and Porter, 1968; Simmon and White, 1971; Glaser and Pate, 1973). Usually cells from vigorously growing cultures are longer than those from slowly growing or resting cultures. The cells are flexible and refractile and exhibit gliding motility on solid surfaces. On infected tissue, they gather in clusters resembling haystacks and then form columns, which accounts for the species designation, columnaris. Spherical and highly refractile spheroplast structures appear in older cultures (Becker and Fujihara, 1978; Pacha and Ordal, 1970).

On moist and solid medium the colonies of F. columnaris are typically yellow, spreading with a convoluted center. The spreading colonies are often relatively delicate and form a continuous sheet which ends at the edge with rhizoid-like protrusions, sometimes with a network in between. The sheet accumulated by slime may become very tough, so that it can be peeled off the agar as a pellicle or sticks tenaciously with a tendency to penetrate into the agar (Garnjobst, 1945). This characteristic colony is used as a diagnostic tool.

Liquid cultures of F. columnaris may become viscous due to the production of extracellular slime. Johnson and

Chilton (1966) purified a polysaccharide produced by F. columnaris grown in a defined amino acid medium or a synthetic medium containing casein hydrolysate. It was a high-molecular-weight homopolymer of D-galactosamine in which half of the amino groups were acetylated. This basic compound probably has a necessary but passive role in gliding motility by serving as an interface between the bacterium and its substratum (Burchard, 1980).

Flexibacter columnaris grows over a temperature range of 4 to 25°C, and some growth occurs at 37°C. The organism is strictly aerobic and nonhalophilic. Growth is inhibited by 1 % NaCl. It is actively proteolytic, produces H_2S and catalase, is unable to reduce nitrates and does not decompose tyrosine, tributyrin, nor utilize citrate. Dead bacterial cells are readily attacked and lysed, and no indole is produced from tryptophan (Pacha and Ordal, 1970; Pacha and Porter, 1968). Metabolism is presumably respiratory, with molecular oxygen as the terminal electron acceptor (Leadbetter, 1974). Sugars are not fermented but Leadbetter (1974) reports that some strains are able to utilize glucose as a carbon source.

Several investigations (Marmur et al., 1963; Mandel and Leadbetter, 1965; Colwell et al., 1966; McCurdy and Wolf, 1967) concerning the DNA base composition of myxobacteria have provided mole % GC ranges of 68 to 71 for fruiting species while non-fruiters exhibit a range of

32 to 43 mole % GC. However, the GC content in the DNA of F. columnaris strains examined varied from 29.8 to 35.9 (Wakabayashi and Egusa, 1966; Mitchell et al., 1969; Christensen, 1980) because of differences in the methods applied.

DNA homology was used to distinguish the genetic relatedness among strains of bacteria on a molecular level (Johnson and Ordal, 1968, 1969). An alternative approach is the DNA- Tm method, based on the thermal stability of heterologous DNA-DNA complexes. By using this method, Ordal (1970) showed that (a) 15 strains of columnaris isolated from the Pacific Northwest (PNW) and England were nearly 100% homologous to the reference strain isolated from the PNW; (b) two strains of columnaris from Alabama were nearly 100% homologous to a strain from Texas; (c) the reference strain from the PNW was not highly homologous (75%) with the southern strains obtained from Texas and Alabama and vice versa (74%), and (d) columnaris strains from British Columbia were genetically related to all cultures commonly found in the PNW. In summation, DNA homology indicates that two genetically related columnaris strains exist in North America, one from the PNW and one from the southern portion of the continent.

Serology of Flexibacter columnaris

Only serum agglutinin-adsorption tests have been used in attempts to differentiate the surface antigens among

strains of F. columnaris. Anacker and Ordal (1959a) indicated that antigenic differences exist within the strains collected from the PNW. There are four major serological groups and one miscellaneous group among 325 strains. All of the strains possess a common antigen which is characteristic of the species, but the strains vary in their composition of the type antigens. Each of the serological groups is composed of strains containing the identical type antigens or strains which possess at least one type antigen in common. However, the only available strain of F. columnaris from an area other than the Columbia River was a strain from Texas. The Texas strain was related to the local strain by virtue of the one common antigen and one type antigen, but its composition differed from all the other strains tested. Therefore, until a number of strains from other sources were compared with the local strains, it was impossible to know whether the Anacker's serological typing scheme was valid only for the local strains or for the majority of strains from all sources (Anacker and Ordal, 1959a).

Sanders et al. (1976) reported that two Oregon strains isolated from spring chinook and coho salmon and one Idaho strain isolated from catfish belonged to two serological groups. They shared a common antigen(s). Each group had its own unique antigen(s). In addition, the type antigen(s) influenced the agglutination titer of the

various rainbow trout antisera much more than the common antigen(s). The presence of these type-specific antigens suggests that a serologically polyvalent bacterin would produce higher levels of protection against epizootics of F. columnaris (Sanders et al., 1976).

Bootsma and Clerk (1976) also demonstrated that five of seven strains isolated from carp and goldfish in the Netherlands are antigenically related, but not identical. No correlation between serotype and geographical origin, species of host fish, or virulence was seen.

Agglutinating antisera against F. columnaris does not seem to react with other Cytophaga-like bacteria including non-pathogenic inhabitants of fish. Thus, such antisera can be a valuable diagnostic tool (Pacha and Porter, 1968; Bullock, 1972).

There have been several laboratory trials of F. columnaris bacterins. Fujihara and Nakatani (1971) described the laboratory exposure of yearling rainbow trout (Salmo gairdneri) to F. columnaris present as a natural contaminant in Columbia River water and demonstrated the development of natural immunity and immune carriers among the survivors. Active immunity to F. columnaris disease was also established in coho salmon (Oncorhynchus kisutch) by oral vaccination with heat-killed cells.

Schachte and Mora (1973) also confirmed the immunogenicity of F. columnaris to the warmwater cultured

fish, channel catfish (Ictalurus punctatus), through subcutaneous and intramuscular injection with heat-inactivated antigens.

Ransom (1975) demonstrated that juvenile coho salmon (O. kisutch) orally immunized with formalin-killed F. columnaris cells for three months were protected against columnaris disease. Bivalent bacterin consisting of F. columnaris with Yersinia ruckeri, was also protective but mixing with Aeromonas salmonicida was not protective possibly as a result of antigenic antagonism.

Liewes et al. (1982) described that the bath immunization of carp (Cyprinus carpio) with disrupted, heat-inactivated F. columnaris resulted in protection of fish from natural challenge. Although negligible agglutinatin antibody titers were observed in bath-immunized carp, specific sensitized leukocytes could be found which indicated that the immunologic memory of carp can occur without the presence of significant agglutinating antibody titers in the serum.

Survey of Immunoelectrophoresis

Crossed immunoelectrophoresis (CIEP), was initially described by Laurell (1966) and modified by Clarke and Freeman (1966). Proteins in antigen preparations were separated by electrophoresis in an agarose gel. This was followed by a second electrophoresis, at right angles to

the direction of the first, into an antibody-containing gel. Proteins which were recognized by the antibody incorporated in the top gel immunoprecipitated as rockets. This method has a greater sensitivity and resolving power than double immunodiffusion and conventional immunoelectrophoresis (Axelsen, 1971; Roberts et al., 1972; Getchell et al., 1985). The principal reason for the marked increase in sensitivity is that a large proportion if not all of the antigens are forced by the electric field into contact with the antibodies. In conventional immunoelectrophoresis, the antigens and antibodies make contact by means of simple diffusion which occurs in all directions; thus only a portion of the immunologic reagents meet and precipitate, others being lost in the surrounding agarose gel matrix. This method has been shown to be an effective tool in the investigation of complex antigen-antibody systems both qualitatively and quantitatively.

Although various species and strains of bacteria could be simply identified by the CIEP technique, experimental conditions still limited the determination of which antigens were common and which were specific for the species or strains (Roberts et al., 1972). A modified method was developed by Svendsen and Axelsen (1972) by interposing an intermediate gel between the first and the second dimensional gels of a CIEP. By this arrangement, it is possible to compare the content of an unknown antiserum

with that of a reference antiserum, by using a reference antigen. This method was originally designed for diagnostic purposes using sera from patients with infectious diseases (Axelsen and Kirkpatrick, 1973; Hoiby and Axelsen, 1973; Kaplan and Chase, 1980). Another application of crossed immunoelectrophoresis with an intermediate gel (CIEPIG) was antigenic analysis by adsorption of the common antigens in the intermediate gel in which heterologous antiserum was incorporated (Caldwell et al., 1975; Bhasin and Lapointe-Shaw, 1980; Joly and Kenny; 1982).

MATERIALS AND METHODS

Bacteria Strains

Twenty-two strains of *F. columnaris* were compared in this study. Five were isolated from fish in the Asian countries of Japan, Korea, and Taiwan. Sixteen strains came from North America, one from Canada, and the remaining fifteen from fish in the United States. One strain isolated from rainbow trout in Chile, South America, was included (Table 1).

Four mucoid strains were recognized among the twenty-two isolates. They are designated as IC8m, 244m, K4m and GA325m.

The host range from which the isolates were obtained included both warm- and cold-water fish species. Twelve strains were isolated from warm-water fish including large mouth bass (*Micropterus salmoides*), common carp (*Cyprinus carpio*), goldfish (*Carassius auratus*), channel catfish (*Ictalurus punctatus*), Japanese eel (*Anguilla japonica*), Oriental weatherfish (*Misgurnus anguillicaudatus*), large-scale sucker (*Catastomus macrocheilus*), pumpkinseed (*Lepomis gibbosus*) and Mozambique tilapia (*Tilapia mossambica*). The other ten strains were isolated from cold-water fish. They were spring chinook salmon (*Oncorhynchus tshawytscha*), coho salmon (*Oncorhynchus kisutch*) and rainbow trout and steelhead trout (*Salmo gairdneri*).

Table 1. Strains of Flexibacter isolated from fish and used in this study

Strain Designation	Date Year	Location	Host Isolated from
1S	83	Taiwan	Loach
4G	79	Taiwan	Eel
5F	80	Taiwan	Tilapia
T13	78	Japan	Goldfish
M1	83	Korea	Mirror carp
8230 3	82	Canada	Steelhead
RPTAC4	84	Chile	Rainbow
IC8r and IC8m	69	USA-Idaho	Catfish
225	57-59	USA-Washington	Salmonid fish
234	57-59	USA-Washington	Salmonid fish
235	57-59	USA-Washington	Salmonid fish
238	57-59	USA-Washington	Salmonid fish
244r and 244m	57-59	USA-Washington	Salmonid fish
DD3	69	USA-Oregon	Spring chinook
BH2	68	USA-Oregon	Coho
Rogue No. 9	80	USA-Oregon	Bass
Rogue No. 10	80	USA-Oregon	Sunfish
K4r and K4m	83	USA-Oregon	Sucker
RC	83	USA-Oregon	Coho
GA325r and GA325m	83	USA-Maryland	Goldfish
GA468	83	USA-Georgia	Catfish
GA505	84	USA-Georgia	Goldfish

Growth Media

Chase medium (Chase, 1965), Shieh medium (Shieh, 1980), and Liewes medium (Liewes, 1982) were selected for growth comparison of F. columnaris . The composition of each medium is shown in Table 2. Strain 238 was inoculated into each medium and incubated for 22 h on a shaker at 24°C. The organism was transferred at least five times under the same conditions to ensure its nutrient adaptation before testing growth responses. The growth response of F. columnaris was determined in each medium by inoculating 25 ml broth in nephelo culture flasks (Belco Glass, Inc., Vineland, NJ) with 0.36 ml of a 22 h culture grown in the each medium. Flasks were incubated on a shaker at 24°C. Absorbance at 525 nm was recorded every two hours over a 32 h period. The growth curve was plotted and generation time was calculated by comparing absorbance versus time.

Based on the growth rate and cell yield, some components in Shieh medium seemed unnecessary. The effect of glucose, pyruvate and citrate on the growth rate was determined by the same method as previously described. The Shieh medium was modified by omitting the 0.1% glucose. Further modification was made by omitting both 0.01% pyruvate and 0.001% citrate.

Table 2. The composition (% w/v) of the media used for cultivation of Flexibacter columnaris

	Cytophaga[a]	TYE[b]	TYI[c]	Chase[d]	Shieh[e]	Liewes[f]
Nitrogens						
tryptone	.05	.4	.4			
peptone					.5	
beef extract	.02					
acid hydrolyzed casein				.4		
casitone						.45
gelatin						1.0
Carbohydrates						
glucose					.1	
Na-acetate	.02			.0015	.001	.0015
Na-pyruvate					.01	
citric acid					.001	
Vitamins and Nucleic acids						
yeast extract	.05	.04			.05	.45
yeast infusion			3			
Salts						
$BaCl_2.H_2O$					.001	
NaCl				.204		.204
KCl				.012		.012
K_2HPO_4				.026127	.01	.003
KH_2PO_4				.020415	.005	
$MgSO_4.7H_2O$				.011832	.03	.006
$CaCl_2.2H_2O$				.003969	.00067	.004
Fe^{+3}				.00002		
$FeSO_4.7H_2O$					.0001	
Mn^{+2}				.0000005		
Buffer						
$NaHCO_3$					.005	

[a]Ordal and Rucker, 1944; [b]Tryptone yeast extract, Garnjobst, 1945; [c]Tryptone yeast infusion, Anacker and Ordal, 1959a; [d]Chase, 1965; [e]Shieh, 1980; [f]Liewes et al., 1982

Morphological Characteristics

Colony morphology was observed on modified Shieh agar (MSA) after 48 h incubation at 24°C. Cell morphology and size were determined by means of light microscopy. Cells grown in modified Shieh broth (MSB) for 22 h at 24°C were stained by Gram's method, were observed, and measured.

Environmental Characteristics

Aerobic or anaerobic growth was tested on MSA incubated in GasPak jars (BBL Microbiology Systems, Cockeysville, MD) under hydrogen and carbon dioxide.

The ability of the strains to grow in MSB at 4, 12, 16, 25, 37, and 42°C was observed. The incubation periods extended from 1 day to 2 wks depending on the incubation temperature used.

Tolerance to NaCl was determined by the addition of selected amounts of NaCl to MSB. Cultures were examined for growth after 3 days at 24°C. The salt concentrations used were Ø, Ø.5, and 1.Ø%.

Biochemical Characteristics

All biochemical tests were performed either in duplicate or were repeated in separate tests. Each of the test media was inoculated with young cells from MSA or broth and, unless otherwise stated, were incubated at 22-25°C. Generally each test was conducted over a three-day period with known organisms included as controls to

demonstrate either positive or negative reactions.

Protein utilization

The hydrolysis of casein was examined by inoculating the test isolates on MSA supplemented with 7.5% skim milk. After incubation, the plates were examined for clear areas in the medium near the colonies.

Modified Shieh broth was used as the basal medium for testing gelatinase production by supplementation with 12% gelatin. A positive reaction was identified by the liquefaction of gelatin surrounding the region of growth.

Carbohydrate utilization

The medium used to detect starch hydrolysis contained Ø.2% starch. A positive reaction was identified by the procedures described in Manual of Microbiological Methods (1957).

Chitin utilization was determined using a method described by Stanier (1947). Five milliliters of a mixture containing Ø.5% chitin in MSA was used. The isolates were spot inoculated on the agar and incubated for two weeks. Dissolution of the chitin around the area of growth was considered a positive test for chitinase.

A modification of the Hugh-Leifson (1953) procedure was used to test for the production of acid from glucose. The basal medium consisted of the same components as the

modified Shieh medium. Glucose was added to the basal medium at a final concentration of 1.0%.

Both Koser citrate broth (1923) and Simmons citrate agar (1926) were used for examining citrate utilization. Growth in the medium indicated ability to utilize citrate as a sole carbon source. A positive test was also indicated by the development of a deep blue color in Simmons agar after one week of incubation.

Lipid utilization

The hydrolysis of tributyrin was tested using a basal medium supplemented with 0.2% (v/v) tributyrin. Clearing around the zone of growth indicated utilization.

Miscellaneous biochemical tests

The ability of the test organisms to reduce nitrate anaerobically was tested (MacFaddin, 1980). The modified Shieh semiagar was used as a basal medium and supplemented with 0.1% potassium nitrate. Zinc dust was added to negative tubes to determine if reduction beyond the nitrite stage had occurred.

Hydrogen sulfide production was tested using lead acetate strips (Fisher Scientific, San Francisco, CA) over MSB inoculated with the test organisms. Formation of a dark color on the paper strips indicated a positive test.

The decomposition of tyrosine was examined using a method described by Lewin and Lounsbery (1969). The

isolates to be tested were inoculated on the MSA containing Ø.5% tyrosine. Disappearance of the suspended tyrosine denoted a positive test.

Ehrlich's procedure was used to test for the production of indole from tryptophan. The basal medium consisted of the same components as the modified Shieh medium. Peptone concentration in the basal medium was increased from Ø.5 to 2% (Koneman, et al., 1979).

Hydrogen peroxide (3%) was used to determine the presence of catalase. Cytochrome oxidase test strips (General Diagnostics, Morris Plains, NJ) were used to detect the presence of this enzyme.

Pacha and Porters'(1968) procedure was used to determine lytic activity. Bacterial cell agar was prepared with washed cell suspensions of Escherichia coli. Evidence of lysis was indicated by the appearance of clear zones around the areas of growth.

Genetic Characteristics

DNA isolation

For DNA preparation, Flexibacter isolates from fish were grown in one liter MSB for 22 h at 25°C. Cells were harvested by centrifugation and washed twice in sterile distilled water and frozen. Wet packed cells were suspended in 4.5 ml of distilled water and lysed with Ø.25 ml pronase b (5Ø ug/ml in Ø.15 M NaCl, Ø.Ø1 M EDTA, pH

7.0, Calbiochem, La Jolla, CA) at 50°C for 15 min. Sodium dodecyl sulfate (1.2 ml 20% w/v) was added to the mixture to facilitate lysis. The mixture was sheared by two passes through a French pressure cell at 16,000 psi. Ribonuclease (0.5 ml, 1 mg/ml in 0.15 M NaCl, pH 5.0, Sigma, St. Louis, MO) was added and the mixture incubated at 50°C for 1 h. Saline-EDTA buffer (0.15 M NaCl, 0.01 M disodium EDTA, pH 8.0) was added to a final volume of 25 ml. Protein was removed from the lysed culture by extraction with Tris-saturated redistilled phenol. DNA was purified with hydroxylapatite (Johnson, 1981). BioGel-HTP (DNA grade, Bio Rad Laboratories, Richmond, CA) was used in the procedure. DNA preparations were dialyzed against 400 volumes saline-HEPES buffer (0.02 M NaCl, 0.001 M N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid sodium salt, pH 7.0) overnight.

Determination of percent guanine plus cytosine

DNA preparations were dialyzed against 0.1 x SSC (0.015 M NaCl, 0.0015 M trisodium citrate). The absorbance at 260 nm was recorded at 1.0°C increments between 55 and 85°C with a Beckman model DU-8 computing spectrophotometer equipped with a Tm Compuset Module. The guanine plus cytosine content of *F. columnaris* DNA was calculated by the equation of Mandel et al. (1970). DNA extracted and purified from *E. coli* strain WP2 (51.0 mole% guanine plus cytosine, Seidler and Mandel, 1971) was used as a

standard.

DNA hybridization

DNA hybridization experiments were performed by the hydroxylapatite free solution method (Johnson, 1981). Unlabeled DNA preparations dissolved in saline-HEPES buffer were concentrated by lyophilization if their initial concentrations were less than 900 ug/ml. The preparations were denatured and any contaminating RNA hydrolyzed by adding 5N NaOH to a final concentration of 0.25N followed by heating for 15 min at 50°C. The mixture was allowed to cool gradually, and was neutralized with an equivalent amount of HCl. The preparation was dialyzed overnight against 600-800 volumes of saline-HEPES buffer. DNA solutions were adjusted to contain 400-600 ug DNA/ml saline-HEPES buffer.

Tritium labeled F. columnaris (strain DD3) DNA was prepared by the hydroxylapatite method and diluted with saline-HEPES buffer to contain 3.25 ug/ml (approximately 70,000 cpm/ug DNA). Labeled and competitor DNA preparations (diluted in saline-HEPES buffer to 325 ug/ml) were heated for 5 min in boiling water and cooled immediately in ice water just prior to the hybridization experiments. DNA samples and reagents were allowed to warm to room temperature to avoid the effect of temperature on sample size. Polypropylene microtubes (0.5 ml capacity,

Sarstedt, Inc., Princeton, NJ) were used as reaction vials. The reaction mixture contained the following:

50 ul denatured competitor DNA
10 ul labeled reference DNA
50 ul 0.88M NaCl-0.001M HEPES, pH 7.0

Herring sperm DNA (Sigma) was dissolved in saline-HEPES buffer, sheared by two passages at 16,000 psi in a French pressure cell and diluted to contain 325 ug/ml. This DNA preparation, having no homology to F. columnaris DNA, was used as a control to determine the amount of self renaturation of the labeled fragments. Vials were incubated in a Forma water bath (Forma Scientific, Marietta, OH) at 67.0°C (Tm-15°C) and 57.0 °C (Tm-25°C) for 23.5 h. After incubation, 100 ul were removed to determine the amount of renaturation.

Precipitated DNA was collected on glass microfibre filters (Whatman, 2.4 cm GF/C). The filters were dried, placed into scintillation vials containing 10 ml Omnifluor (New England Nuclear, Boston, MA) and counted for 10 min in a Beckman model LS 8000 liquid scintillation counter.

Serological Characteristics

A slide agglutination procedure was used to determine the serological relationship among the strains of F. columnaris. To avoid autoagglutination, serological antigens were prepared by heating cell suspensions at 50°C for 10 min. Antisera to Cytophaga psychrophila and F. columnaris strain DD3 were supplied by R. A. Holt and Dr.

J. E. Sanders of the Oregon State University. Both sera were prepared by injecting white New Zealand rabbits intramuscularly with triply washed formalin-killed cells emulsified in Freund's complete adjuvant.

Immunogen preparation

Isolates were chosen for serological comparison based on diversity of geographical and host range. Seven strains of F. columnaris: 1S, T13, M1, 82303, DD3, IC8r, and 238 were selected for immunogen preparation. Each lyophilized stock culture was inoculated into MSB and incubated for 22 h at 22°C with shaking. To ensure culture purity, Gram stains were prepared and examined. Each culture was also grown on MSA to observe homogeneity of colony morphology. The cells were harvested by centrifuging for 20 min at 6000 rpm at 4°C. Then they were washed three times with 30% Earle's balanced salt solution (EBSS, Gibco, Grand Island, NY). The packed cells were resuspended in 2 ml 30% EBSS and disrupted by sonic treatment (eight 10-sec bursts, average watts=100) by a sonifier cell disrupter (Heat Systems-Ultrasonics, Plainview, NY). The turbidity of each sonic extract was adjusted to O.D. 1.6 at 525 nm and then the extract was frozen at -70°C.

Antisera preparation

Each of seven, female, New Zealand rabbits received,

at two week intervals, four or five 2.Ø ml intramuscular injections of a particular immunogen emusified with Freund complete adjuvant. Preimmune serum was harvested prior to immunization. Rabbits were test bled from ten to eighteen days after the last injection. If agglutination titers were higher than 64, a final bleeding was made. Rabbits having immunogens 1S, M1 and DD3 received additional intraveneous injections (Ø.2, Ø.4, Ø.6, Ø.8 and 1.Ø ml respectively) at 4-day intervals. Immune sera were havested by centrifugation of the blood for 2Ø min at 3ØØØ rpm at 4°C. Each serum was sterilized by filtering through a Ø.22 um membrane filter and stored at -2Ø°C.

Antigen preparation

Strains of the bacterium were grown and harvested following the same procedures described for immunogen preparation. Cells were washed three times in Ø.ØlM phosphate buffered saline (PBS, pH 7.2). The pellet of cells was resuspended in 1Ø ml PBS containing 1% Triton X-1ØØ (Caldwell et al., 1975) and disrupted by sonic treatment for eight 1Ø sec-bursts. Then they were maintained at room temperature for 1 h to enhance the solubility of the antigens. The Triton-solubilized antigens were then cleared of cellular debris by centrifugation at 13,ØØØ g for 25 min (4°C). To prevent Triton-induced non-immunological precipitation in agar gels (Mansheim and Stenstrom, 198Ø) and to prevent hapten-

induced antibody exhaustion, the supernatant was ultrafiltered with a YM-2 membrane (MW>1,000, Amicon, Danvers, MA). After ultrafiltration, the antigen preparation was concentrated to a final protein concentration of 10-21 mg/ml, as measured by the method of Bradford (1976). The test reagent was obtained from Bio-Rad Laboratories (Richmond, CA). Cleared antigen suspensions were frozen at -70°C until used.

Crossed immunoelectrophoresis

Crossed immunoelectrophoresis and crossed immunoelectrophoresis with an intermediate gel were performed according to the procedures of Axelsen et al. (1975). The supporting matrix consisted of 0.5% agarose (electroendosmosis -Mr < 0.1, Mallinckrodt, Paris, KY) and 0.5% Triton X-100 (Sigma, St. Louis, MO) in barbital buffer, pH 8.6, 0.0375M (Laurell, 1965, Thirkill and Kenny, 1974). The electrophoresis reservoir contained barbital buffer at a concentration of 0.075M. Nine milliliters of melted agarose were solidified over a glass plate (8 x 8 cm) precovered with GelBond film (FMC Inc., Rockland, ME). One well (4mm) was cut at the cathode corner. Antigens mixed with 2 ul of a 65 ug/ul solution of bovine serum albumin (Cappel, West Chester, PA), which acted as a marker of the migration rate of each protein, were placed in each well and subjected to electrophoresis

at 7.5 v/cm for 45 min at 4°C. For CIEP the upper portion (3/4) of the gel was then removed and replaced with 5.8 ml agarose containing homologous antiserum. The second phase was run at 4 v/cm for 5 h at right angles to the electrophoretic direction of the first dimension. For CIEPIG, 1.8 ml agarose containing a heterologous antiserum was interposed between the antigen-containing gel strip and the homologous antiserum-containing gel. After the second dimensional electrophoresis, the gel was soaked in 0.3% NaCl for 12 h at 4°C, then 0.15% NaCl for 12 h at 4°C and finally for 24 h in distilled water. After drying at 68°C, the film was stained for 30 min with 0.5% Coomassie Brilliant Blue (w/v) dissolved in 45% ethanol, 45% distilled water, and 10% acetic acid. Decolorization was performed in the solvent only.

Protection Provided Fish by Common Antigens of Flexibacter columnaris

Strain 238, before challenge experiments, was passed through coho salmon (O. kisutch) for five times by water borne infection to enhance the virulence. Strain 238 was inoculated into 10 l of MSB in a microfermenter (New Brunswick Scientific, New Brunswick, NJ) for 22 h at 25°C (driving speed 100 rpm, aeration scale=1). Gram stain and colony homogeneity were examined for culture purity. The final cell concentration was 1.2 x 10^9 cells/ml, determined by plate counting with 30% EBSS containing 0.2%

gelatin as a diluent.

For artificial infections, ten healthy rainbow trout, body length about 5 cm, were placed into duplicate tanks two days before challenge at a water temperature of 18°C. Seven hundred and fifty ml of each ten-fold serial dilution of broth culture of strain 238 was poured into the bottom of each tank for 10 min. Dead fish were collected each day and cultured for F. columnaris by streaking inoculum from the kidney and gills onto MSA and tryptic soy agar plates. Percent mortality was calculated by following the formula:

$$\text{percent mortality} = \frac{\text{No. of fish which died of } F.\ columnaris}{\text{Total No. of fish} - \text{Nonspecific loss}}$$

A dosage inducing no less than 70% mortality was used in the challenge experiments.

Bacterin preparation

Strains 1S, T13, M1, 82303, DD3, IC8r, and 238 were each incubated in 10 l of MSB for 22 h at 25°C. The absence of spheroplast formation was determined by means of light microscopy. Each broth culture was inactivated by adding 0.3% formalin for 24 h at 4°C and sterility was tested in thioglycolate medium. All of the cultures were concentrated to 10^9 cells/ml using a Pellicon Cassette System (Filter HVLP 0.5 mu, Millipore, Bedford, MASS) as described by Gabler (1984).

Fish immunization

Before immunization, each bacterin was diluted to 5 x 10^8 cells/ml and the bacterin temperature was adjusted to 18°C. Each group of 15 fish was immersed in two liters of aerated bacterin for 2 min. The bacterin could be reused four times.

Challenge

Using the dosage determined previously, immunized fish were challenged by the water-borne route with strain 238 four weeks after immunization. Mortality was calculated by following the formula already indicated. Further comparisons were made by calculating the relative percent survival (R.P.S.) as follows:

$$\text{R.P.S.} = \left(1 - \frac{\text{\% mortality in immunized group}}{\text{\% mortality in control group}}\right) \times 100\%$$

RESULTS

Growth Media

The growth responses of *F. columnaris* strain 238 in three different media were compared (Figure 1). It was determined that when Chase medium was employed, a significant lag period occurred. In contrast, both Shieh and Liewes media supported excellent growth. The cells appeared typical and exhibited active gliding motility when observed in wet mount by phase microscopy. However, during logarithmic growth over a 10 h period, the generation time of strain 238 in the Shieh broth was 130 min compared to 216 min in the Liewes broth. Reproducibility of growth in Shieh broth among the other *F. columnaris* strains included in this study were similar to those of strain 238.

To determine if the glucose was metabolically necessary, a growth study was conducted with strain 238. Growth curves of strain 238 in Shieh broth with or without glucose were almost identical (Figure 2). Similar growth curves were obtained after additional modification of Shieh broth by omitting both pyruvate and citrate (Figure 3). These results indicated that glucose, pyruvate and citrate were not required for the cultivation of *F. columnaris*. This modified Shieh medium was used throughout the study.

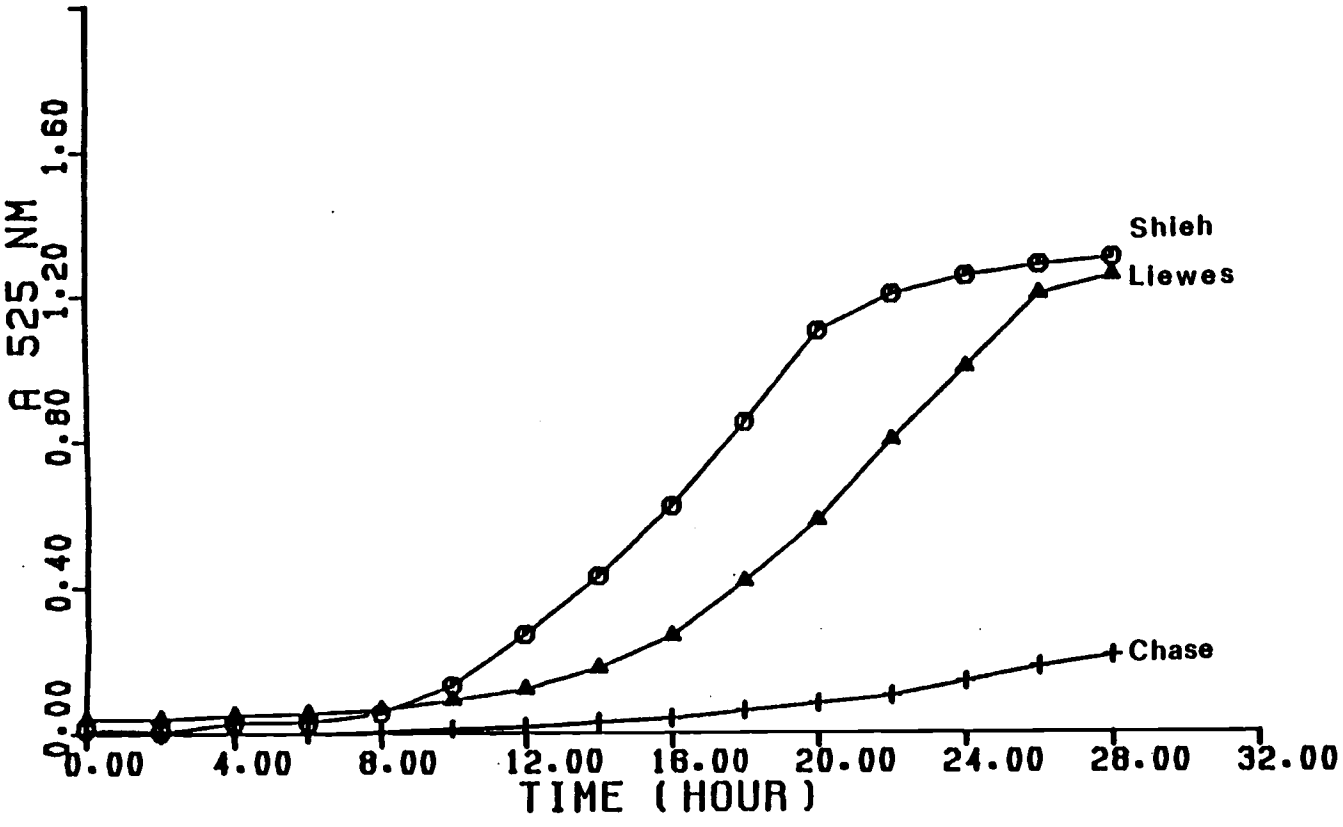

Figure 1. Growth of Flexibacter columnaris strain 238 in three different media.

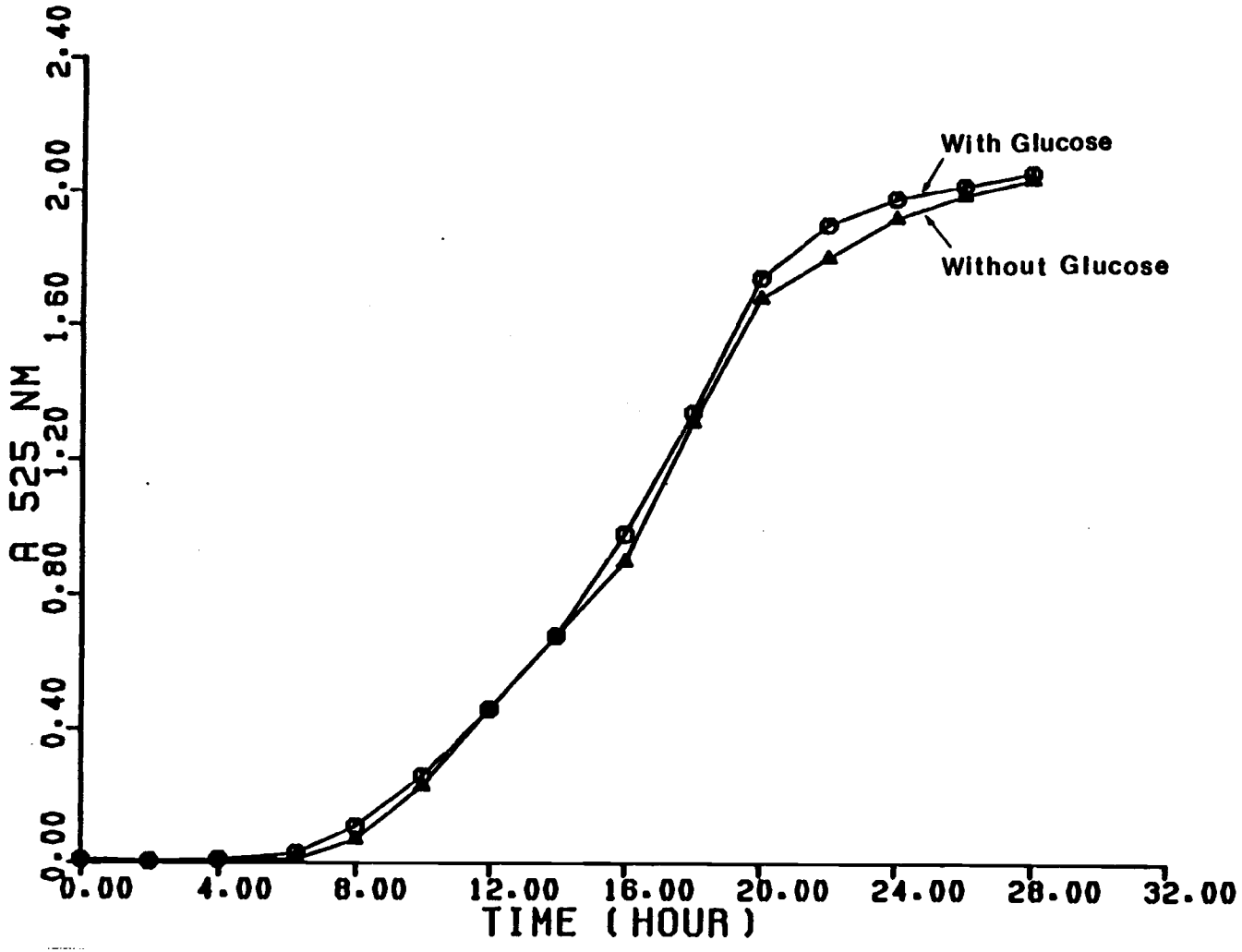

Figure 2. The effect of glucose (0.1%) on the growth of Flexibacter columnaris strain 238 in Shieh medium.

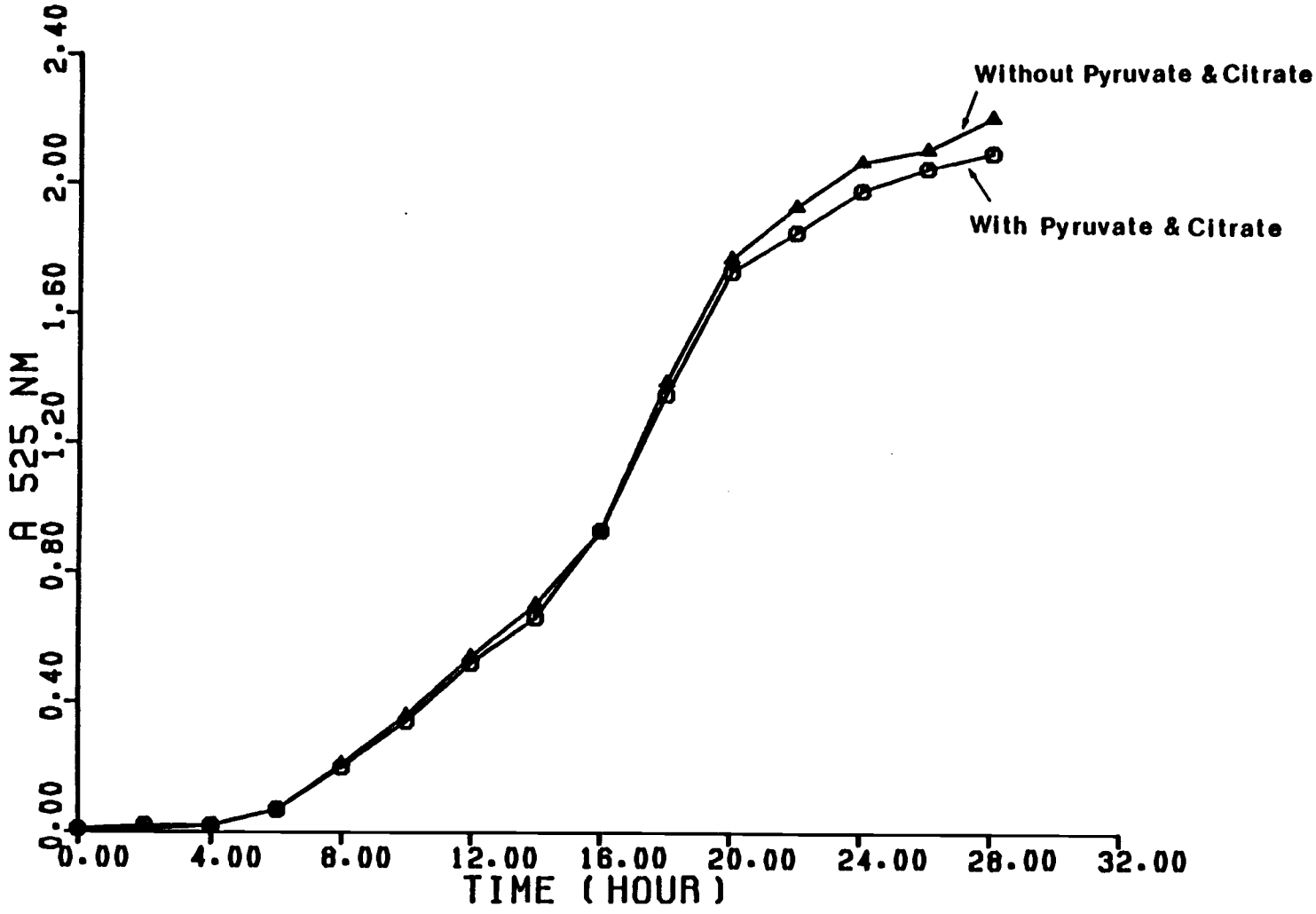

Figure 3. The effect of pyruvate (0.01%) and citrate (0.001%) on the growth of Flexibacter columnaris strain 238 in Shieh medium.

Morphological Characteristics

There were three phenotypic variations in colony morphology among the strains observed. The colonies produced by most of the columnaris isolates studied were bright yellow and had convoluted centers with rhizoid edges (Figure 4a). However, the colonies of four strains, IC8m, 244m, K4m, and GA325m, differed by not adhering to the surface of the agar and having a mucoid surface (Figure 4b). Strain GA468 formed the third colony type which was small (dia. 1.5 mm), honeycomb-like, and with short rhizoids at the edge (Figure 4c).

All strains were Gram-negative, filamentous bacteria. The bacterial cell size during the log growth phase in MSB was approximately 0.4-1.0 by 2.2-38 um. The cells were flexible and exhibited gliding motility on solid surfaces. Spherical and highly refractile spheroplasts appeared in cultures incubated for 72 hr.

Environmental Characteristics

All isolates of F. columnaris tested were aerobic. The temperature range for growth was from 12 to 37°C with the optimum at 31°C. No growth occurred at either 4 or 42°C after one week incubation. All F. columnaris cultures grew in MSB containing either no NaCl or 0.5% NaCl; however, all isolates were inhibited at a concentration of 1.0% NaCl.

Figure 4. Photomicrograph showing colony morphology of three columnaris isolates growing on modified Shieh agar (approximately 63x)

a) Strain DD3 showing the characteristic swarming nature with a convoluted center and rhizoid edges.

b) Strain IC8m showing an atypical mucoid surface.

c) Strain GA468 showing a honeycomb-like appearance.

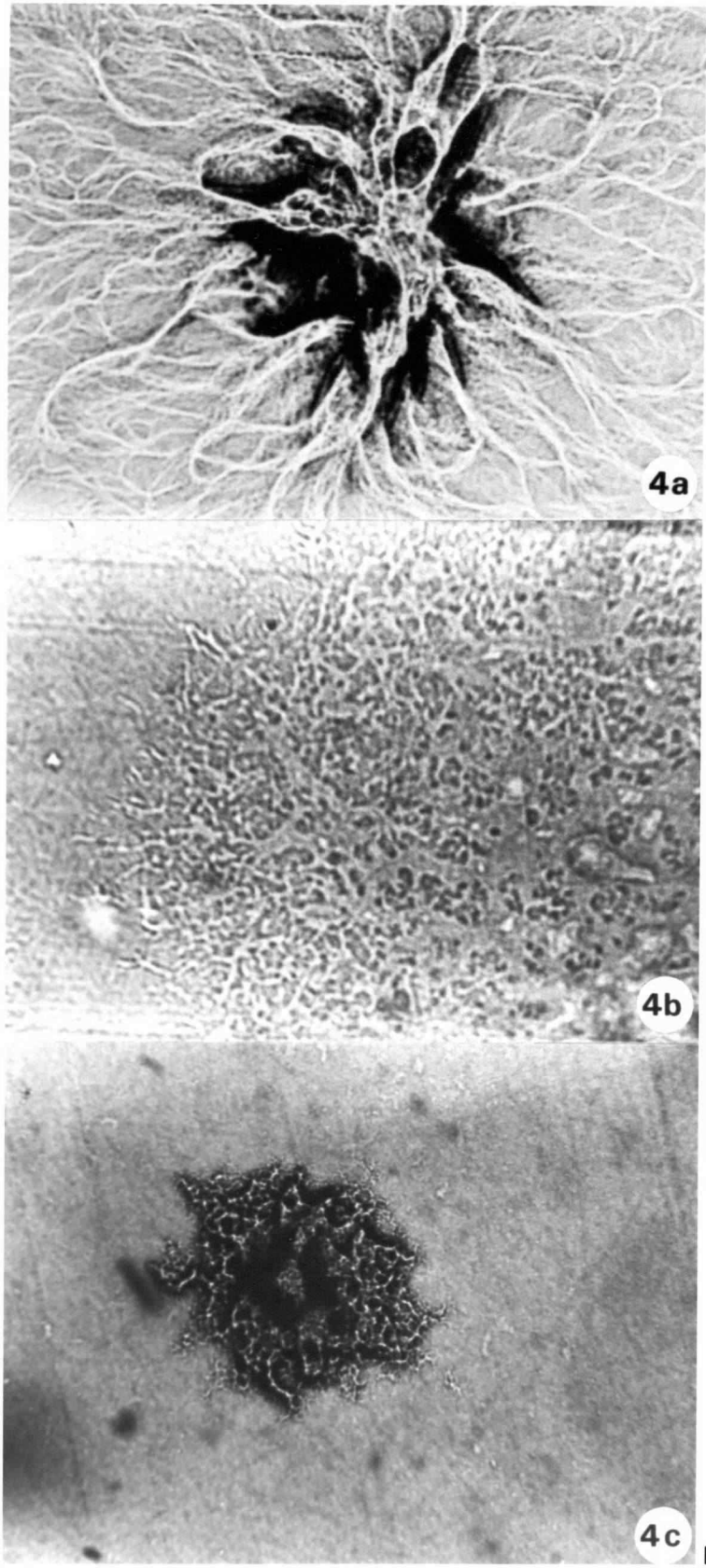

Figure 4.

Biochemical Characteristics

Biochemical test results for *Flexibacter* are found in Table 3. Except for tyrosine utilization, the reactions were identical among all the strains. All strains were proteolytic by degradation of casein and gelatin, but not lipolytic through failure to utilize tributyrin. They were unable to utilize either simple or complex carbohydrates, for example, starch and chitin were not degraded. Acid was not produced from glucose either oxidatively or fermentatively. They were also unable to utilize citrate as a sole carbon source. Nitrate was reduced to nitrogen rather than nitrite under anaerobic conditions. Hydrogen sulfide was produced. All isolates were positive for catalase and oxidase, but negative for tryptophanase through failure to produce indole. Only 6 of the 26 isolates were able to decompose tyrosine and of these six, three were mucoid strains. All isolates tested produced a swarming type of growth on the surface of *E. coli* cell agar plates and clear zones were observed around the colonies. This indicated that *Flexibacter* was able to break down *E. coli* cells and utilize the nutrients released.

Table 3. Biochemical characteristics of *Flexibacter* isolates

Test	Strain																											
	1S	4G	5F	T13	M1	82 303	RP TAC4	IC8r	IC8m	225	234	235	239	244r	244m	DD3	8H2	Rogue No. 9	Rogue No.10	K4r	K4m	RC	GA325r	GA325m	GA468	GA505
Degradation of																										
casein	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
gelatin	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
starch	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
chitin	-	-	-	-	-	-	-	-	-	-	-	-	-	-	ND	-	-	-	-	-	-	-	-	-	-	-
tributyrin	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Glucose oxidized	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Glucose fermented	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Citrate utilization	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Nitrate reduction	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
H2S production	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Tyrosine decomposition	-	+	+	-	-	-	-	-	+	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+
Indole production	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	+	-	-	+	-	-
Catalase production	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Cytochrome oxidase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Lysis of *E. coli*	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

+, positive reaction; -, negative reaction; ND, not determined

DNA Base Composition and Hybridization

The DNA of the Flexibacter strains melted at a relatively low temperature, approximately 65°C in Ø.1 x SSC. The G+C content of all strains ranged from 29.6 to 32.5 mol % (Table 4). The average %GC for the Flexibacter isolates was 31.4 ± Ø.8.

The isolates can be divided into three groups on the basis of DNA relatedness (Table 4). Reassociation values obtained with DNA from strain DD3 and other strains from group 1 ranged from 81 to 98% at Tm-25°C and from 83 to 1Ø2% at Tm-15°C. However, reassociation value of DNAs from strains DD3 and GA468 (group 2) did not exceed 73% at Tm-25°C or 72% at Tm-15°C. No more than 29% homology at Tm-25°C or 25% homology at Tm-15°C was observed with DNA from strain DD3 and DNA from Flexibacter in group 3.

Group 1 can be divided into 2 subgroups on the basis of colony morphology (Tables 4 and 5). Also 95% homology at Tm-25°C or 94% homology at Tm-15°C was observed with DNA from strain DD3 and DNA from K4m (group 1B). The morphological, environmental, biochemical and genetic comparisons of Flexibacter isolates in groups 1 through 3 were summarized in Table 5.

Table 4. DNA relatedness of isolate Flexibacter columnaris DD3 to twenty-two other strains

STRAIN	MOL % G+C	PERCENT REASSOCIATION Tm-25°C	Tm-15°C
Group 1A:			
DD3	29.6	100	100
1S	30.5	81	83
T13	32.2	88	84
M1	31.3	96	100
82303	30.3	97	99
RPTAC4	31.1	98	89
IC8r	31.5	92	84
BH2	31.1	92	93
225	31.1	93	96
234	31.9	95	94
235	31.8	93	96
238	32.0	97	98
244r	32.5	93	90
ROGUE No. 9	32.0	89	92
ROGUE No. 10	32.3	94	94
K4r	29.9	94	97
RC	31.9	92	98
GA325r	31.2	97	100
GA505	31.3	95	102
GROUP 1B:			
K4m	31.3	95	94
GROUP 2:			
GA468	31.3	73	72
GROUP 3:			
4G	31.8	29	25
5F	32.3	27	2

Table 5. Comparison of *Flexibacter* isolates in groups 1 through 3

Characteristics	Group 1A	1B	2	3
Colony morphology	rhizoid	mucoid	honeycomb -like	rhizoid
Gram stain	-	-	-	-
Bacterial morphology	F	F	F	F
Size (u)	0.4-1.0 x2.2-16.5	0.6 x2.5-13	0.6 x3-5	0.6 x3.8-37.5
Growth aerobic	+	+	+	+
anaerobic	-	-	-	-
Temp range (^{o}C)				
4	-	-	-	-
12	+	+	-	+
16	+	+	+	+
25	+	+	+	+
37	v	-	+	-
42	-	-	-	-
NaCl tolerance (%)				
0	+	+	+	+
0.5	+	+	+	+
1.0	-	-	-	-
Mole % G+C	29.6-32.5	31.3	31.3	31.8
DNA relatedness (%)	81-98	95	73	29
Tyrosine decomposition	v	v	-	+

+, positive reaction; -, negative reaction; F, filamentous; v, variable reaction

Serological Characteristics

Slide agglutination tests using antiserum against F. columnaris strain DD3 cells were conducted with each isolate to determine if they were serologically related. All isolates agglutinated in the anti-DD3 serum and no reactions occurred with normal serum, saline control, or Cytophaga psychrophila antiserum. This showed that all the Flexibacter isolates shared at least one common antigen.

Crossed immunoelectrophoresis

Preliminary CIEP experiments demonstrated that the serum from each preimmune rabbit lacked antibodies against antigens of F. columnaris. Seven antigen preparations from F. columnaris strains were subjected to CIEP against homologous antisera. Observed precipitin rockets depended on relative antigen concentration and antiserum potency. The reactions of these antigen preparations with homologous antisera disclosed the total number of antigens in each strain: 22 in strain 1S, 16 in strain T13, 20 in strain M1, 15 in strain 8230 3, 17 in strain DD3, 14 in strain IC8r, and 15 in strain 238 (Figures 5a-11a). The precipitin rockets formed by each strain were expressed by the relative electrophoretic mobilities (REM), which were determined by the relative migration rate of the precipitin rocket versus the migration distance of the bovine serum albumin marker (Table 6). The antigenic profile of each strain was different from the other

Table 6. Relative electrophoretic mobility values detected by crossed immunoelectrophoresis analysis of Flexibacter columnaris strains

No. of Antigen	Antigen versus homologous antiserum						
	1S	T13	M1	82303	DD3	IC8r	238
1	0.105	0.073	0.061	0.070	0.090	0.056	0.544
2	0.224	0.148	0.323	0.168	0.238	0.068	0.702
3	0.364	0.352	0.396	0.306	0.331	0.082	0.731
4	0.382	0.384	0.422	0.353	0.407	0.269	0.863
5	0.385	0.484	0.476	0.654	0.431	0.344	0.907
6	0.492	0.494	0.569	0.832	0.470	0.702	0.917
7	0.546	0.640	0.780	0.885	0.559	0.743	1.150
8	0.634	0.677	0.847	0.905	0.674	1.136	1.163
9	0.693	0.866	0.942	1.000	0.680	1.157	1.228
10	0.860	1.011	1.051	1.040	0.807	1.196	1.254
11	0.869	1.073	1.080	1.068	0.910	1.232	1.285
12	0.893	1.075	1.144	1.138	0.961	1.387	1.435
13	0.900	1.261	1.374	1.150	0.973	1.533	1.642
14	1.040	1.289	1.425	1.268	1.088	1.632	1.658
15	1.074	1.350	1.473	1.466	1.105		1.881
16	1.083	1.491	1.565		1.177		
17	1.119		1.601		1.314		
18	1.174		1.681				
19	1.180		1.792				
20	1.264		2.204				
21	1.339						
22	1.478						

strains not only in composition and position, but also in intensity of the stained peaks. However, the comparative reproducibility of these precipitin rockets between runs of the same isolate was good. Also, the electrophoretic order and relative size of peaks remained similar between runs.

Crossed immunoelectrophoresis with an intermediate gel

According to the Anacker and Ordal (1959a) serotyping scheme, F. columnaris strains were divided into four serogroups based on the combination of unique or type gantigens. The method of CIEPIG was used to investigate the unique antigens of strains of F. columnaris. The optimal concentrations of antigen and antisera in both intermediate and reference gels required to obtain maximum resolution of the antigen-antibody system are given in Table 7. For example, the unique antigens and common antigens of F. columnaris strain 1S were separated by the adsorption of common antigens with anti-F. columnaris T13 antiserum in the intermediate gel. Only the unique antigens that strain 1S possessed could migrate upward through the intermediate T-13 antiserum gel and precipitate in the reference gel which contained the specific anti-1S serum (Figure 5c). This method demonstrated that strain 1S possessed 8 antigens not shared by strain T13 (Figure 5c), 4 antigens not shared by strain M1 (Figure 5d), 7 antigens not shared by strain .pa

Table 7. Optimal concentrations of antigens and antisera required to obtain maximum resolution of the antigen-antibody systems in Flexibacter columnaris

Antigen	Antigen conc ug/ul	Antigen amt in well ug	Antiserum in intermediate gel ul/cm^2	Antiserum in reference gel ul/cm^2
1S	20.9	35	35	11
T13	16.0	35	35	11
M1	10.4	23	63	17
82303	14.2	20	35	23
DD3	11.0	35	35	11
IC8r	13.6	20	35	11
238	20.5	10	35	23

(Figure 5f), 9 antigens not shared by strain IC8r (Figure 5g) and 9 antigens not shared by strain 238 (Figure 5h). Among these 14 antigens (antigens f, g, h, i, j, k, m, o, p, q, r, s, t and u) which showed up in the reference gels of six plates, antigen k was not adsorbed by all heterologous antisera and therefore was type-specific to strain 1S. The rest of the 13 antigens were shared to different degrees by the other strains. Identity among these partially shared antigens was demonstrated by comparison of the REM values (Table 8).

Similarly, strain T13 had 3 antigens not shared by strain 1S, 2 antigens not shared by strain M1, 5 antigens not shared by strain 823Ø3, 4 antigens not shared by strain DD3, 5 antigens not shared by strian IC8r and 5 antigens not shared by strain 238 (Figures 6c-6h). Among these 8 antigens (antigens e, f, g, i, j, l, n and p) which showed up in the reference gels of six plates, antigens e and g were not adsorbed by all heterologous antisera and therefore were type-specific to strain T13. The rest of the 6 antigens were shared to different degrees by the other strains (Table 9).

In the same way, among 13 antigens in strain M1 analyzed by CIEPIG, antigens a and t were found not adsorbed by all heterologous antisera and, therefore, were type-specific to strain M1 (Figure 7 and Table 1Ø). However, no type-specific unique antigens were found in

strains 82303, DD3, IC8r and 238 (Figures 8 through 11 and Tables 11 through 14), although each strain possessed 5, 6, 7 and 8 antigens shared to different degrees by the other strains, respectively.

The reproducibility of CIEPIG was confirmed by the number of common antigens detected in each strain. The number of common antigens was determined by substracting the number of antigens in the reference gel from the total antigens. For example, strain DD3 had a total of 17 antigens disclosed by the reaction of antigen preparation against homologous antiserum (Table 6 and Figure 9a). After adsorption by anti-1S serum incorporated in the intermediate gel, strain DD3 showed up 1 antigen in the reference gel (Figure 9c and Table 12). This analysis indicated that strains DD3 and 1S shared 16 common antigens. The number of common antigens in the reciprocal antigen-antibody system was also determined. Strain 1S had a total 22 antigens (Table 6 and Figure 5a). After adsorption by anti-DD3 serum, strain 1S showed up 6 antigens in the reference gel (Figure 5f and Table 8). This analysis indicated that strains 1S and DD3 also shared 16 common antigens. The serological relationships between F. columnaris strains tested were summarized in Table 15. The numerator represented the total number of antigens of each strain. Each diagonal directed from the lower left to the upper right was composed of the numbers of common antigens found in heterologous antigen-antibody

systems. It showed that numbers in the lower triangle occurred in symmetrical fashion to those in the upper triangle, confirming the validity of this analysis. In addition, the number of partially shared antigens in each strain screened by the other 6 antisera was shown in Table 15. The denominator represented the number of partially shared antigens showing in the reference gels of six plates: 1S had 13, T13 had 6, M1 had 11, 823Ø3 had 5, DD3 had 6, IC8 had 7, and 238 had 8.

In summary, the seven strains tested were serologically distinct as disclosed by the different antigenic profiles analyzed by CIEP. These seven strains shared from 7 to 18 common antigens, but varied in composition by possessing 5 to 13 partially shared antigens analyzed by CIEPIG. Strains 1S, T13 and M1 were seperated into three different serotypes based on the presence of type-specific unique antigens. However, strains 823Ø3, DD3, IC8r and 238 were shown to be serologically different from each other, but failed to type because no type-specific unique antigens were found. No correlation between serotype and geographic or host origin was observed.

Figure 5. Antigenic profiles of Flexibacter columnaris strain 1S analyzed by crossed immunoelectrophoresis with an intermediate gel. The top portion of agarose contains 11 ul/cm^2 antiserum prepared against strain 1S. The intermediate portion of agarose contains 35 ul/cm^2 antiserum prepared against either strain T13, M1, 82303, DD3, IC8r, or 238 as indicated. Barbital buffer instead of antiserum incorporated into intermediate agarose was used as a control (Figure b). The cathodic well contains the serological test antigen prepared from strain 1S. Unique antigens are identified by their electrophoretic mobilities relative to bovine serum albumin (vertical bar, upper left).

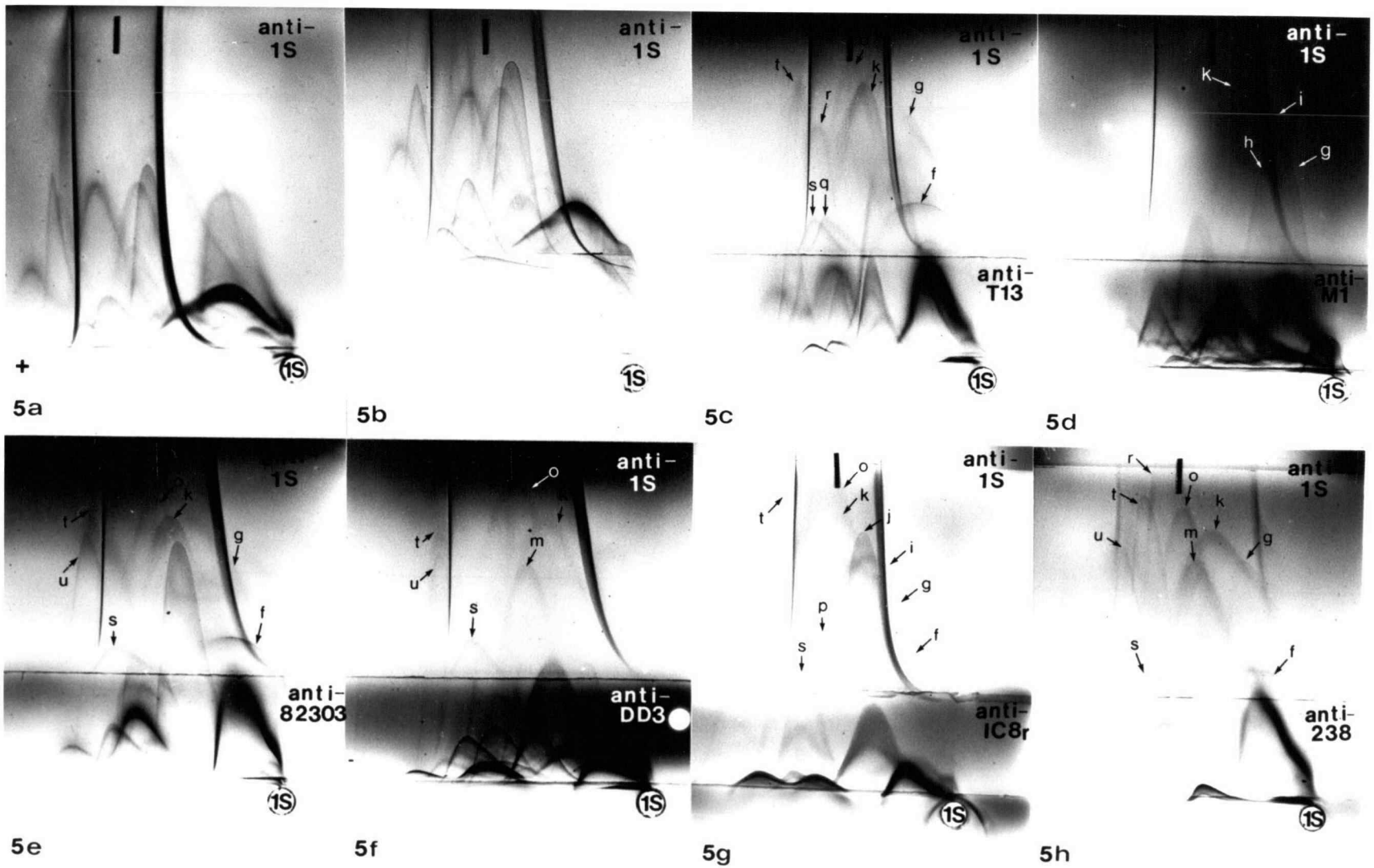
anti-
1S
+
1S
5a
anti-
1S
1S
5b
anti-
1S
t
r
k
g
s
q
f
anti-
T13
1S
5c
anti-
1S
k
i
h
g
f
anti-
M1
1S
5d
1S
t
o
k
g
u
s
f
anti-
82303
1S
5e
anti-
1S
o
t
m
u
s
anti-
DD3
1S
5f
anti-
1S
o
t
k
j
i
g
p
s
f
anti-
IC8r
1S
5g
anti-
1S
r
t
o
k
u
m
g
s
f
anti-
238
1S
5h

Figure 5.

Table 8. Relative electrophoretic mobility values of Flexibacter columnaris strain 1S analyzed by crossed immunoelectrophoresis with an intermediate gel

Antigen designation	Antigen versus anti-1S serum adsorbed by serum						
	None	anti-T13	anti-M1	anti-82303	anti-DD3	anti-IC8r	anti-238
a	0.105	-*	-	-	-	-	-
b	0.224	-	-	-	-	-	-
c	0.364	-	-	-	-	-	-
d	0.382	-	-	-	-	-	-
e	0.385	-	-	-	-	-	-
f	0.492	0.445**	-	0.444	-	0.530	0.453
g	0.546	0.555	0.525	0.448	-	0.533	0.579
h	0.634	-	0.587	-	-	-	-
i	0.693	-	0.616	-	-	0.717	-
j	0.860	-	-	-	-	0.777	-
k	0.869	0.847	0.809	0.937	0.779	0.952	0.804
l	0.893	-	-	-	-	-	-
m	0.900	-	-	-	0.915	-	0.927
n	1.040	-	-	-	-	-	-
o	1.074	0.984	-	1.063	1.015	1.046	1.068
p	1.083	-	-	-	-	1.082	-
q	1.119	1.133	-	-	-	-	-
r	1.174	1.228	-	-	-	-	1.182
s	1.180	1.243	-	1.330	1.310	1.240	1.324
t	1.264	1.337	-	1.536	1.490	1.315	1.361
u	1.339	-	-	1.580	1.568	-	1.540
v	1.478	-	-	-	-	-	-

* Dashes in each column represent the REM of antigens that were adsorbed by the heterologous antiserum.

** Values in each column represent the REM of antigens that were not adsorbed by the heterologous antiserum.

Figure 6. Antigenic profiles of Flexibacter columnaris strain T13 analyzed by crossed immunoelectrophoresis with an intermediate gel. The top portion of agarose contains 11 ul/cm^2 antiserum prepared against strain T13. The intermediate portion of agarose contains 35 ul/cm^2 antiserum prepared against either strain 1S, M1, 82303, DD3, IC8r, or 238 as indicated. Barbital buffer instead of antiserum incorporated into intermediate agarose was used as a control (Figure b). The cathodic well contains the serological test antigen prepared from strain T13. Unique antigens are identified by their electrophoretic mobilities relative to bovine serum albumin (vertical bar, upper left).

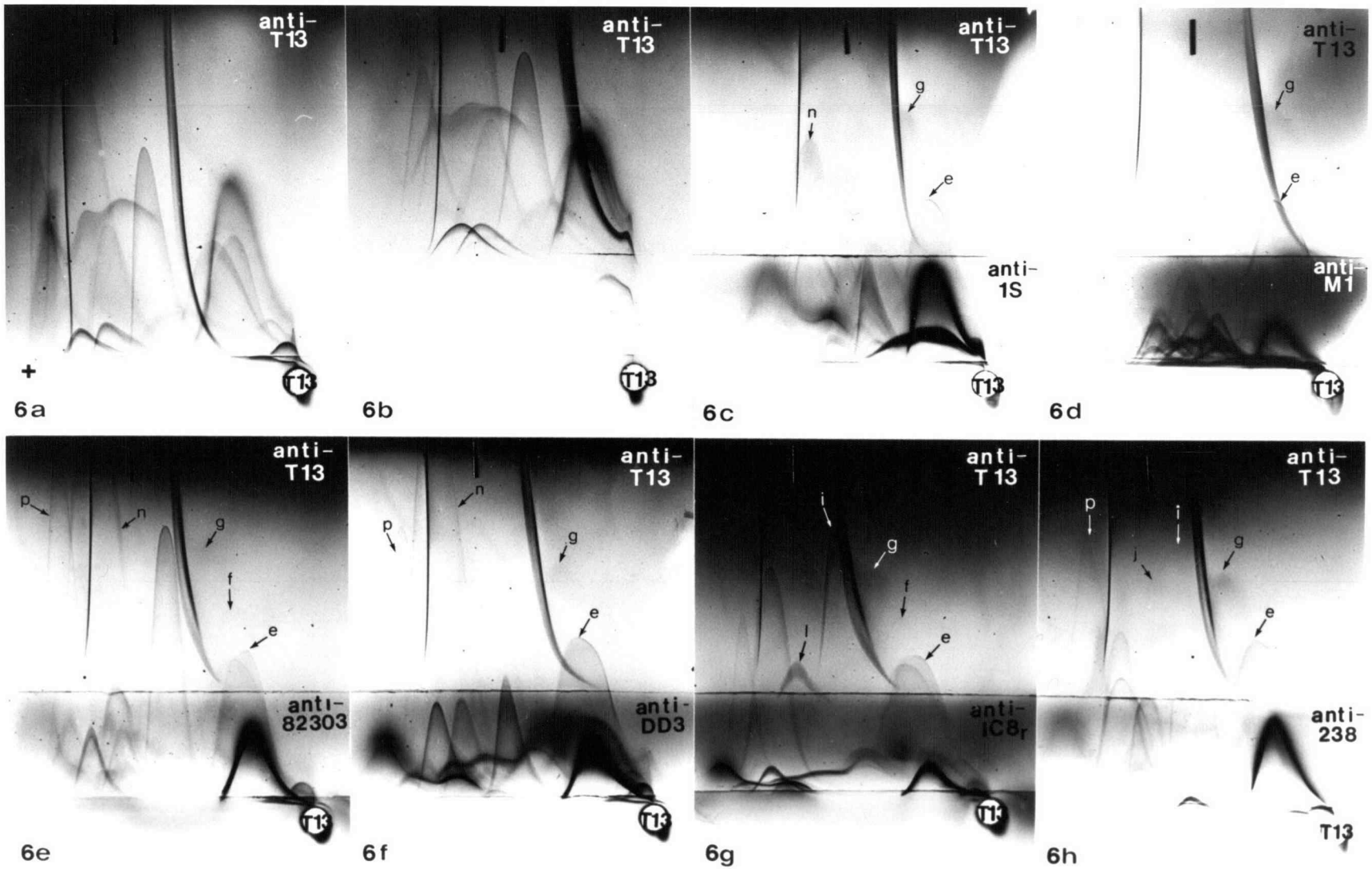
anti-
T13
+
6a
6b
n
g
e
anti-
1S
6c
anti-
M1
6d
p
f
anti-
82303
6e
anti-
DD3
6f
i
l
anti-
IC8r
6g
j
anti-
238
T13
6h

Figure 6.

Table 9. Relative electrophoretic mobility values of Flexibacter columnaris strain T13 analyzed by crossed immunoelectrophoresis with an intermediate gel

Antigen designation	Antigen versus anti-T13 serum adsorbed by serum						
	None	anti-1S	anti-M1	anti-82303	anti-DD3	anti-IC8r	anti-238
a	0.073	-*	-	-	-	-	-
b	0.148	-	-	-	-	-	-
c	0.352	-	-	-	-	-	-
d	0.384	-	-	-	-	-	-
e	0.484	0.446**	0.407	0.396	0.440	0.403	0.464
f	0.494	-	-	0.453	-	0.471	-
g	0.640	0.593	0.454	0.558	0.548	0.647	0.619
h	0.677	-	-	-	-	-	-
i	0.866	-	-	-	-	0.810	0.860
j	1.011	-	-	-	-	-	0.955
k	1.073	-	-	-	-	-	-
l	1.075	-	-	-	-	1.040	-
m	1.261	-	-	-	-	-	-
n	1.289	1.284	-	1.193	1.233	-	-
o	1.350	-	-	-	-	-	-
p	1.491	-	-	1.418	1.472	-	1.398

* Dashes in each column represent the REM of antigens that were adsorbed by the heterologous antiseurm.

** Values in each column represent the REM of antigens that were not adsorbed by the heterologous antiserum.

Figure 7. Antigenic profiles of Flexibacter columnaris strain M1 analyzed by crossed immunoelectrophoresis with an intermediate gel. The top portion of agarose contains 17 ul/cm^2 antiserum prepared against strain M1. The intermediate portion of agarose contains 63 ul/cm^2 antiserum prepared against either strain 1S, T13, 82303, DD3, IC8r, or 238 as indicated. Barbital buffer instead of antiserum incorporated into intermediate agarose was used as a control (Figure b). The cathodic well contains the serological test antigen prepared from strain M1. Unique antigens are identified by their electrophoretic mobilities relative to bovine serum albumin (vertical bar, upper left).

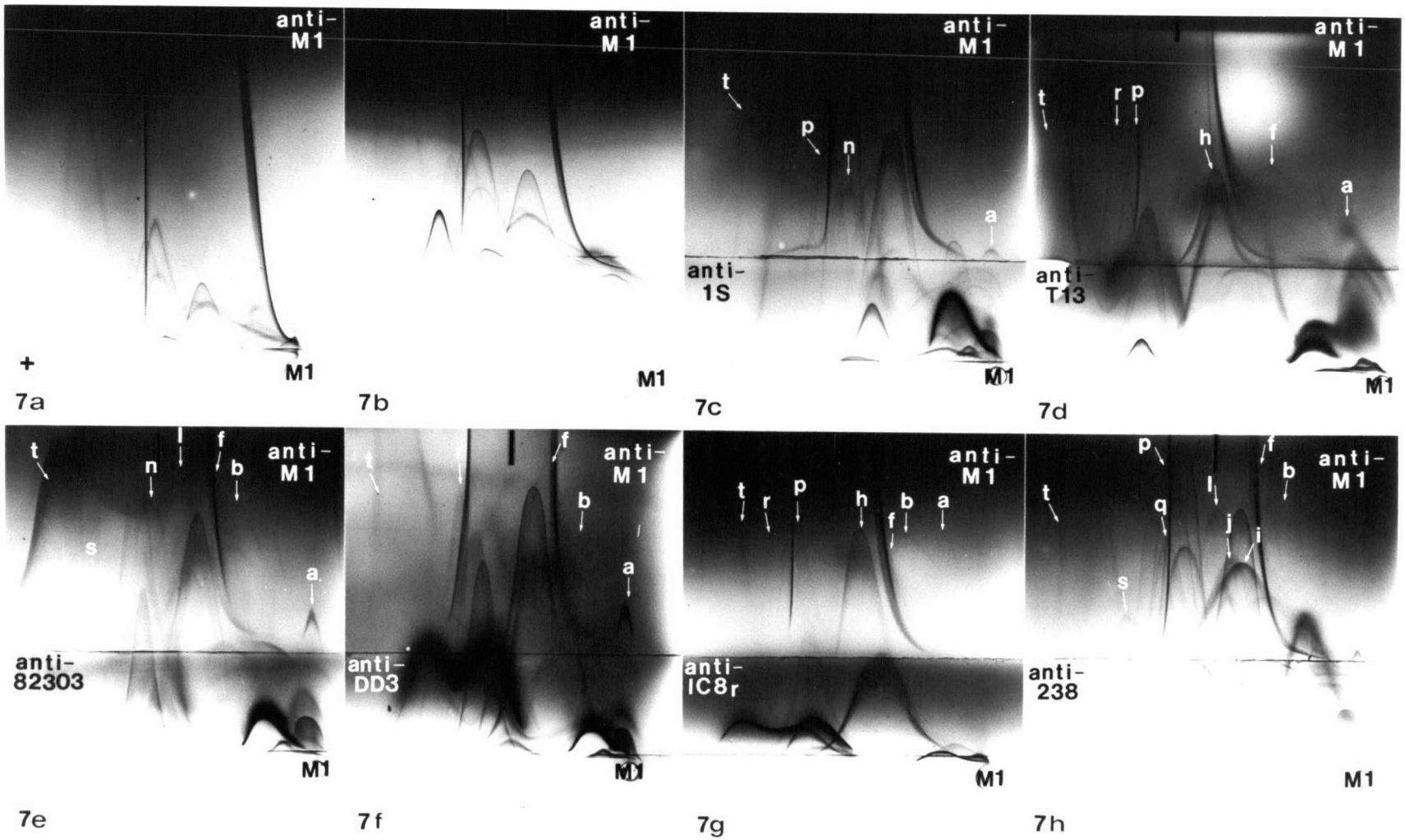
anti-
M1
+
M1
7a
anti-
M1
M1
7b
anti-
M1
t
p
n
a
anti-
1S
M1
7c
anti-
M1
t
r
p
h
f
a
anti-
T13
M1
7d
t
n
l
f
b
anti-
M1
s
a
anti-
82303
M1
7e
t
f
anti-
M1
b
a
anti-
DD3
M1
7f
anti-
M1
t
r
p
h
b
a
f
anti-
IC8r
M1
7g
p
f
anti-
M1
t
b
l
q
j
i
s
anti-
238
M1
7h

Figure 7.

Table 10. Relative electrophoretic mobility values of Flexibacter columnaris strain M1 analyzed by crossed immunoelectrophoresis with an intermediate gel

Antigen designation	Antigen versus anti-M1 serum adsorbed by serum						
	None	anti-1S	anti-T13	anti-82303	anti-DD3	anti-IC8r	anti-238
a	0.061	0.080**	0.165	0.040	0.075	0.162	0.041
b	0.323	-*	-	0.494	0.504	0.574	0.523
c	0.396	-	-	-	-	-	-
d	0.422	-	-	-	-	-	-
e	0.476	-	-	-	-	-	-
f	0.569	-	0.553	0.629	0.686	0.715	0.688
g	0.780	-	-	-	-	-	-
h	0.847	-	0.804	-	-	0.859	-
i	0.942	-	-	-	-	-	0.776
j	1.051	-	-	-	-	-	0.847
k	1.080	-	-	-	-	-	-
l	1.144	-	-	0.826	-	-	0.951
m	1.374	-	-	-	-	-	-
n	1.425	1.145	-	0.982	-	-	-
o	1.473	-	-	-	-	-	-
p	1.565	1.255	1.206	-	-	1.273	1.255
q	1.601	-	-	-	-	-	1.291
r	1.681	-	1.258	-	1.397	1.378	-
s	1.792	-	-	1.308	-	-	1.560
t	2.204	1.780	1.556	1.525	1.920	1.762	1.825

* Dashes in each column represent the REM of antigens that were adsorbed by the heterologous antiserum.

** Values in each column represent the REM of antigens that were not adsorbed by the heterologous antiserum.

Figure 8. Antigenic profiles of Flexibacter columnaris strain 82303 analyzed by crossed immunoelectrophoresis with an intermediate gel. The top portion of agarose contains 23 ul/cm^2 antiserum prepared against strain 82303. The intermediate portion of agarose contains 35 ul/cm^2 antiserum prepared against either strain 1S, T13, M1, DD3, IC8r, or 238 as indicated. Barbital buffer instead of antiserum incorporated into intermediate agarose was used as a control (Figure b). The cathodic well contains the serological test antigen prepared from strain 82303. Unique antigens are identified by their electrophoretic mobilities relative to bovine serum albumin (vertical bar, upper left).

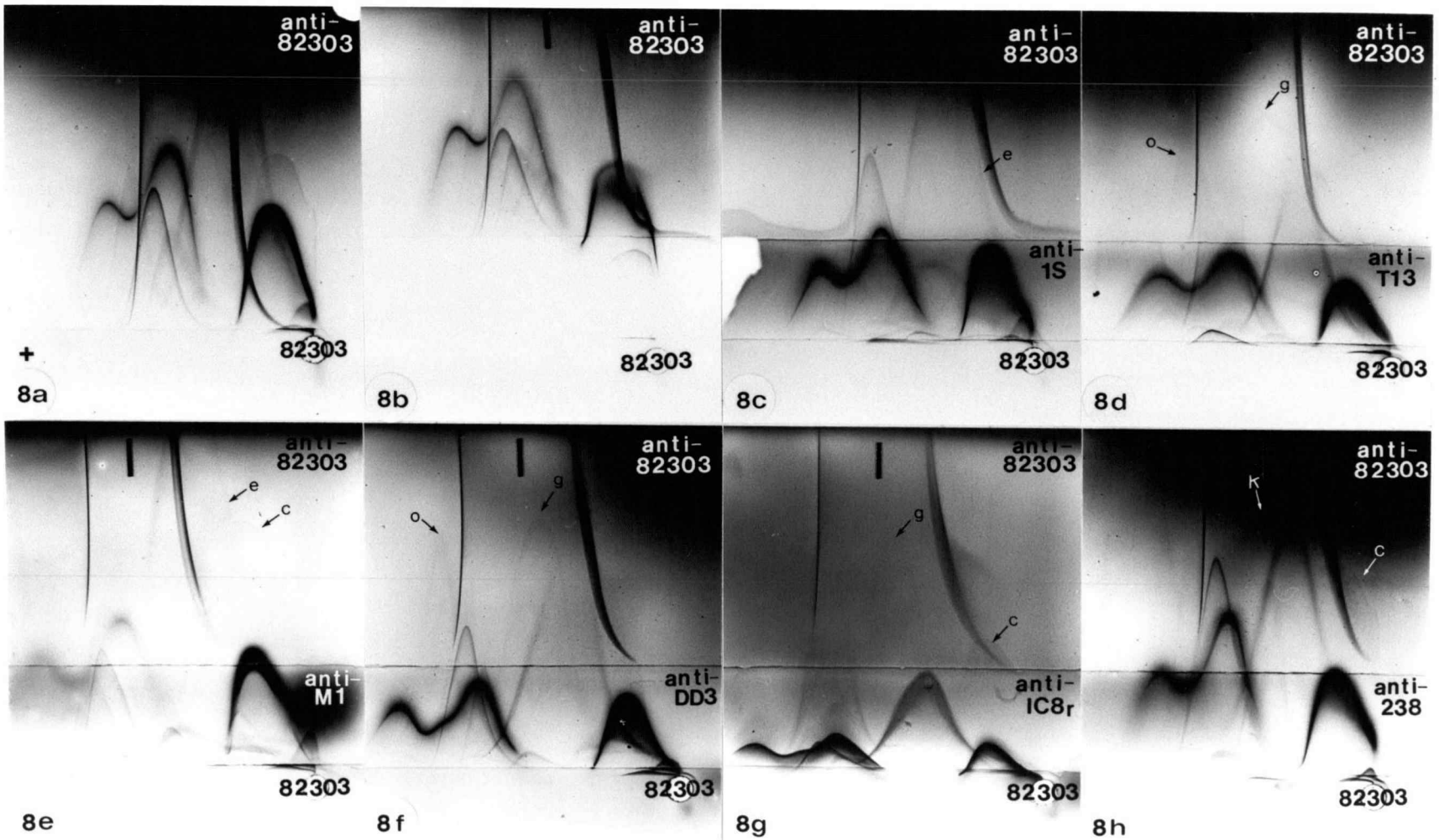
anti-
82303
82303
+
8a
anti-
82303
82303
8b
anti-
82303
e
anti-
1S
82303
8c
anti-
82303
g
o
anti-
T13
82303
8d
anti-
82303
e
c
anti-
M1
82303
8e
anti-
82303
o
g
anti-
DD3
82303
8f
anti-
82303
g
c
anti-
IC8r
82303
8g
anti-
82303
k
c
anti-
238
82303
8h

Figure 8.

Table 11. Relative electrophoretic mobility values of Flexibacter columnaris strain 82303 analyzed by crossed immunoelectrophoresis with an intermediate gel

Antigen designation	Antigen versus anti-82303 serum adsorbed by serum						
	None	anti-1S	anti-T13	anti-M1	anti-DD3	anti-IC8r	anti-238
a	0.070	–*	–	–	–	–	–
b	0.163	–	–	–	–	–	–
c	0.306	–	–	0.359	–	0.347	0.346
d	0.353	–	–	–	–	–	–
e	0.654	0.718**	–	0.673	–	–	–
f	0.832	–	–	–	–	–	–
g	0.885	–	0.872	–	0.885	0.938	–
h	0.905	–	–	–	–	–	–
i	1.000	–	–	–	–	–	–
j	1.043	–	–	–	–	–	–
k	1.063	–	–	–	–	–	1.092
l	1.136	–	–	–	–	–	–
m	1.153	–	–	–	–	–	–
n	1.263	–	–	–	–	–	–
o	1.466	–	1.411	–	1.518	–	–

* Dashes in each column represent the REM of antigens that were adsorbed by the heterologous antiserum.

** Values in each column represent the REM of antigens that were not adsorbed by the heterologous antiserum.

Figure 9. Antigenic profiles of Flexibacter columnaris strain DD3 analyzed by crossed immunoelectrophoresis with an intermediate gel. The top portion of agarose contains 11 ul/cm^2 antiserum prepared against strain DD3. The intermediate portion of agarose contains 35 ul/cm^2 antiserum prepared against either strain 1S, T13, M1, 82303, IC8r, or 238 as indicated. Barbital buffer instead of antiserum incorporated into intermediate agarose was used as a control (Figure b). The cathodic well contains the serological test antigen prepared from strain DD3. Unique antigens are identified by their electrophoretic mobilities relative to bovine serum albumin (vertical bar, upper left).

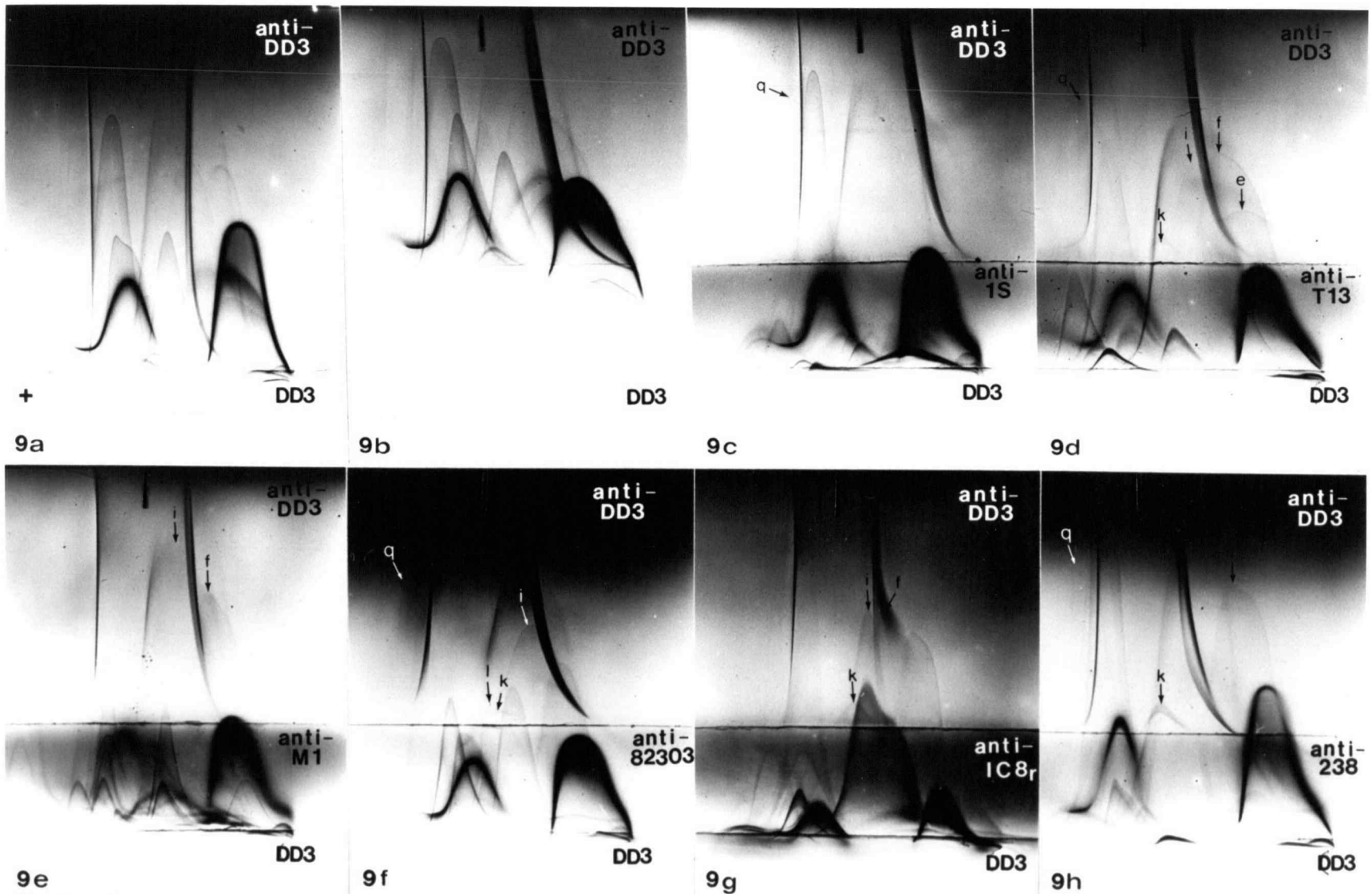
anti-
DD3
+
DD3
9a
anti-
DD3
DD3
9b
anti-
DD3
q
anti-
1S
DD3
9c
anti-
DD3
q
i
f
e
k
anti-
T13
DD3
9d
anti-
DD3
i
f
anti-
M1
DD3
9e
anti-
DD3
q
i
l
k
anti-
82303
DD3
9f
anti-
DD3
i
f
k
anti-
IC8r
DD3
9g
anti-
DD3
q
k
anti-
238
DD3
9h

Figure 9.

Table 12. Relative electrophoretic mobility values of Flexibacter columnaris strain DD3 analyzed by crossed immunoelectrophoresis with an intermediate gel

Antigen designation	Antigen versus anti-DD3 serum adsorbed by serum						
	None	anti-1S	anti-T13	anti-M1	anti-82303	anti-IC8r	anti-238
a	0.090	-*	-	-	-	-	-
b	0.238	-	-	-	-	-	-
c	0.331	-	-	-	-	-	-
d	0.407	-	-	-	-	-	-
e	0.431	-	0.454**	-	-	-	-
f	0.470	-	0.604	0.603	-	0.696	0.524
g	0.559	-	-	-	-	-	-
h	0.674	-	-	-	-	-	-
i	0.680	-	0.740	0.864	0.658	0.839	-
j	0.807	-	-	-	-	-	-
k	0.910	-	0.917	-	0.907	0.909	0.869
l	0.961	-	-	-	0.924	-	-
m	0.973	-	-	-	-	-	-
n	1.088	-	-	-	-	-	-
o	1.105	-	-	-	-	-	-
p	1.177	-	-	-	-	-	-
q	1.314	1.440	1.254	-	1.377	-	1.203

* Dashes in each column represent the REM of antigens that were adsorbed by the heterologous antiserum.

** Values in each column represent the REM of antigens that were not adsorbed by the heterologous antiserum.

Figure 10. Antigenic profiles of Flexibacter columnaris strain IC8r analyzed by crossed immunoelectrophoresis with an intermediate gel. The top portion of agarose contains 11 ul/cm^2 antiserum prepared against strain IC8r. The intermediate portion of agarose contains 35 ul/cm^2 antiserum prepared against either strain 1S, T13, M1, 82303, DD3, or 238 as indicated. Barbital buffer instead of antiserum incorporated into intermediate agarose was used as control (Figure b). The cathodic well contains the serological test antigen prepared from strain IC8r. Unique antigens are identified by their electrophoretic mobilities relative to bovine serum albumin (vertical bar, upper left).

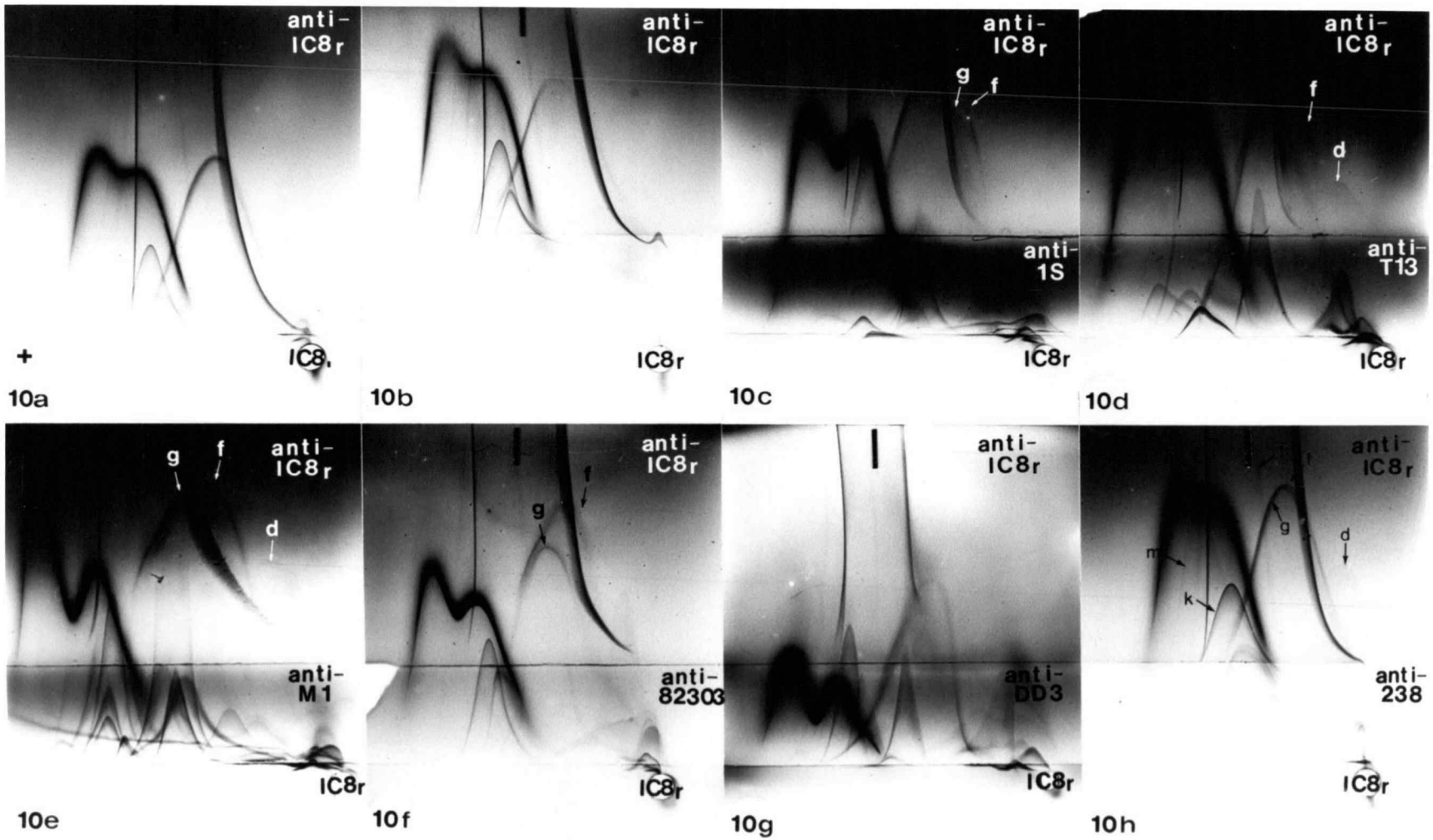
anti-
IC8r
+
IC8r
10a
anti-
IC8r
IC8r
10b
anti-
IC8r
g
f
anti-
1S
IC8r
10c
anti-
IC8r
f
d
anti-
T13
IC8r
10d
g
f
anti-
IC8r
d
anti-
M1
IC8r
10e
anti-
IC8r
g
f
anti-
82303
IC8r
10f
anti-
IC8r
anti-
DD3
IC8r
10g
anti-
IC8r
g
d
m
k
anti-
238
IC8r
10h

Figure 10.

Table 13. Relative electrophoretic mobility values of Flexibacter columnaris strain IC8r analyzed by crossed immunoelectrophoresis with an intermediate gel

Antigen designation	Antigen versus anti-IC8r serum adsorbed by serum						
	None	anti-1S	anti-T13	anti-M1	anti-82303	anti-DD3	anti-238
a	0.056	-*	-	-	-	-	-
b	0.068	-	-	-	-	-	-
c	0.082	-	-	-	-	-	-
d	0.269	-	0.287**	0.386	-	-	0.208
e	0.344	-	-	-	-	-	-
f	0.702	0.667	0.624	0.662	0.640	-	0.676
g	0.743	0.748	-	0.786	0.808	-	0.742
h	1.136	-	-	-	-	-	-
i	1.157	-	-	-	-	-	-
j	1.196	-	-	-	-	-	1.205
k	1.232	-	-	-	-	-	1.222
l	1.387	-	-	-	-	-	-
m	1.533	-	-	-	-	-	1.465
n	1.632	-	-	-	-	-	1.654

* Dashes in each column represent the REM of antigens that were adsorbed by the heterologous antiserum.

** Values in each column represent the REM of antigens that were not adsorbed by the heterologous antiserum.

Figure 11. Antigenic profiles of Flexibacter columnaris strain 238 analyzed by crossed immunoelectrophoresis with an intermediate gel. The top portion of agarose contains 23 ul/cm^2 antiserum prepared against strain 238. The intermediate portion of agarose contains 35 ul/cm^2 antiserum prepared against either strain 1S, T13, M1, 82303, DD3, or IC8r as indicated. Barbital buffer instead of antiserum incorporated into intermediate agarose was used as a control (Figure b). The cathodic well contains the serological test antigen prepared from strain 238. Unique antigens are identified by their electrophoretic mobilities relative to bovine serum albumin (vertical bar, upper left).

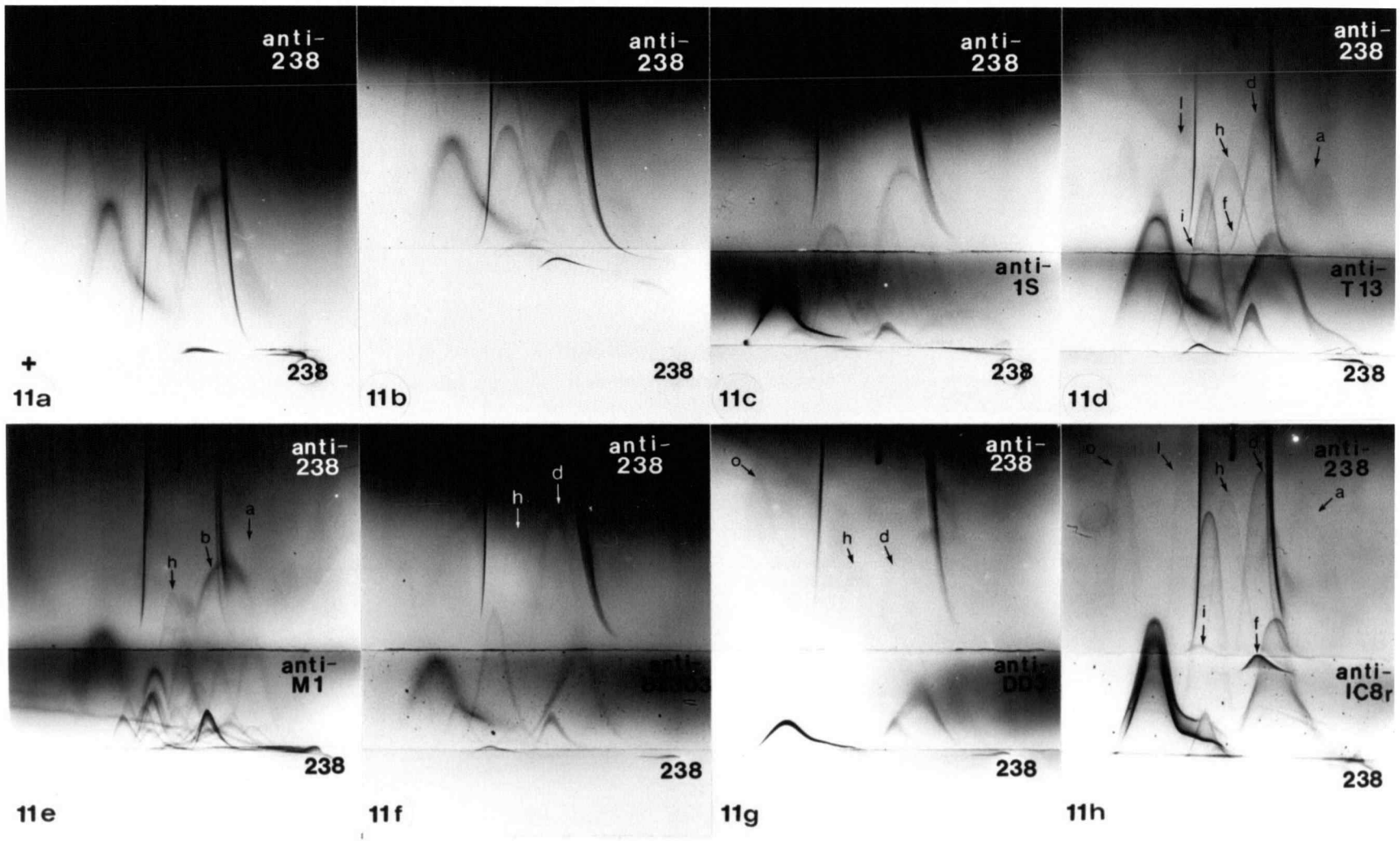
anti-
238
+
238
11a
anti-
238
238
11b
anti-
238
anti-
1S
238
11c
anti-
238
l
h
d
a
i
f
anti-
T13
238
11d
anti-
238
h
b
a
anti-
M1
238
11e
anti-
238
h
d
238
11f
anti-
238
o
h
d
anti-
DD
238
11g
anti-
238
o
l
h
d
a
i
f
anti-
IC8r
238
11h

Figure 11.

Table 14. Relative electrophoretic mobility values of Flexibacter columnaris strain 238 analyzed by crossed immunoelectrophoresis with an intermediate gel

Antigen designation	Antigen versus anti-238 serum adsorbed by serum						
	None	anti-1S	anti-T13	anti-M1	anti-82303	anti-DD3	anti-IC8r
a	0.544	-*	0.432**	0.502	-	-	0.484
b	0.702	-	-	0.680	-	-	-
c	0.731	-	-	-	-	-	-
d	0.863	-	0.794	-	0.841	0.852	0.811
e	0.907	-	-	-	-	-	-
f	0.917	-	1.038	-	-	-	0.841
g	1.150	-	-	-	-	-	-
h	1.163	-	1.054	1.076	1.113	1.171	1.055
i	1.228	-	1.257	-	-	-	1.253
j	1.254	-	-	-	-	-	-
k	1.285	-	-	-	-	-	-
l	1.435	-	1.374	-	-	-	1.346
m	1.642	-	-	-	-	-	-
n	1.658	-	-	-	-	-	-
o	1.881	-	-	-	-	1.880	1.862

* Dashes in each column represent the REM of antigens that were adsorbed by the heterologous antiserum.

** Values in each column represent the REM of antigens that were not adsorbed by the heterologous antiseru.

Table 15. Serological relationship between Flexibacter columnaris strains by crossed immunoelectrophoresis with an intermediate gel

STRAIN / ANTIGEN / SERUM	1S	T13	M1	8230 3	DD3	IC8r	238
anti-1S	22[a]/13[c]	13	16	14	16	12	15
anti-T13	14[b]	16/6	14	13	12	12	9
anti-M1	18	14	20/11	13	15	11	12
anti-8230 3	15	11	13	15/5	13	12	13
anti-DD3	16	12	15	13	17/6	14	12
anti-IC8r	13	11	13	13	14	14/7	8
anti-238	13	11	10	13	14	7	15/8

[a]Number of total antigens
[b]Number of common antigens
[c]Number of partially shared antigens

Protection Provided Fish by Common Antigens of Flexibacter columnaris

To determine whether the common antigens of F. columnaris were protective, 750 ml of broth culture of strain 238 (cell conc. 1.2 x 10^9 cells/ml) was used to challenge fish which were bath immunized with bacterins 1S, T13, M1, 82303, DD3, IC8r, or 238, respectively. Percent mortalities did not exceed 40.7% in the immunized groups compared to 98.2% in the control group. Further comparisons showed that all groups of immunized fish exhibited a relative percent survival that ranged from 58.6% to 89.6% after challenge with virulent F. columnaris (Table 16).

Table 16. Effect of common antigens of *Flexibacter columnaris* on the protection of rainbow trout (*Salmo gairdneri*) against infection with virulent strains 238 of *F. columnaris*

Bacterin	Number of fish	Number of fish that died	Percent of fish that died from *F. columnaris*	Relative percent survival
1S	59	6(6[a])	10.2[b]	89.6
T13	60	22(20)	34.5	64.9
M1	60	22(22)	36.7	62.6
82303	60	24(23)	39.0	60.3
DD3	60	23(23)	38.3	61.0
IC8r	45	17(16)	36.4	62.9
238	59	24(24)	40.7	58.6
CONTROL	60	59(54)	98.2	0

[a]Dead fish from which *F. columnaris* was isolated.

[b] $\text{Percent loss} = \frac{\text{No. of fish which died of } F.\ columnaris}{\text{Total No. of fish} - \text{Nonspecific loss}}$

DISCUSSION

There are six media commonly used for cultivation of F. columnaris. They can be divided into two categories based on the presence or absence of salts. Growth was not satisfactory in any media tested in which no salts were added. This agrees with Fijan's report (1968) that columnaris disease is less likely to occur in water of low pH. It was thought that F. columnaris might be more sensitive to osmotic pressure changes than most eubacteria (Chase, 1965).

The growth responses of F. columnaris were compared in Chase, Shieh and Liewes media with salts added. Both Shieh and Liewes media provided better growth than the Chase medium. This result was consistent with those of Shieh (1980) and Liewes (1982). It appears that yeast extract decreases the lag phase of the growth curve. Once the responsible enzymes are induced, vitamins or nucleic acids can be synthesized de novo. Therefore, it is suggested that organisms be transfered several times in the test medium to ensure nutrient adaptation before measuring growth. Comparisons of generation time were made between Shieh and Liewes media. The generation time was about 40% shorter in Shieh broth than in Liewes broth. Shieh medium may be improved by alteration of the levels of amino acids. It is also possible that a better buffer system or the availability of iron (Chase, 1965; Sword,

1966; Bullen et al., 1974; Kuo et al., 1981) may increase the growth rate.

Descriptions of the utilization of carbohydrate by F. columnaris have been confusing. The original description of F. columnaris by Ordal and Rucker (1944) reported the ability to oxidize glucose. Pacha (1961) then reported slight growth stimulation by the addition of glucose to broth cultures for two different strains of F. columnaris. Leadbetter (1974) found that some strains were able to utilize glucose as a carbon source. Shieh (1980) thought the addition of glucose to the synthetic medium may be related to the formation of extracellular polysaccharide. In contrast, no change occurred in the glucose concentration of the culture supernatant of F. columnaris after different periods of growth. Glucose exerted no effect on the growth patterns in a series of amino acid deletion mixtures (Chase, 1965). In this study, growth curves of F. columnaris in the media with and without glucose were almost identical. This indicated that glucose was not required for the cultivation of F. columnaris. It is possible that some Cytophaga-like bacteria associated with disease in fish are difficult to differentiate from F. columnaris. This may have led to the conflicting observations of carbohydrate utilization.

In this study similar growth curves were obtained after further omitting both pyruvate and citrate from Shieh medium. These results indicated that neither

pyruvate nor citrate was required for the cultivation of F. columnaris. Although the effect of amino acids on the growth rate was not determined in this study, Chase (1965) and Humphrey and Marshall (1980) showed that an increase in the casamino acid concentration increased the duration of the exponential phase and the yield of cells; however, a reduction in amino acid content restricted growth. This could indicate an absence of a glycolytic pathway with amino acids serving as a source of both carbon and nitrogen. This assumption was supported when acetate was further omitted, the growth response of F. columnaris was poor (Zaldivar, personal communication). It suggested that acetyl-CoA derived from acetate serves as a rate-limiting substrate which controls both gluconeogenesis and the tricarboxylic acid cycle.

Similarly, growth of Myxococcus xanthus, a terrestrial gliding bacterium, in minimal medium is not stimulated by a variety of carbohydrates (Bretscher and Kaiser, 1978). Radiolabeled glucose, starch, glycogen or ribose are poorly incorporated into the cell. It is unlikely that M. xanthus uses the glycolysis pathway for conversion of glucose to pyruvate. In addition, hexokinase and pyruvate kinase are apparently missing (Watson and Dworkin, 1968). The primary function of the Embden-Meyerhof enzymes are the synthesis of glucose and other carbohydrates through the gluconeogenesis pathway.

Thus MSA provides not only satisfactory growth, but also prevents overgrowth of other contaminants because of the dilute composition of the medium. Culture in MSA provides a shorter incubation time and higher cell concentrations are produced.

During this study, four mucoid strains were recognized among the twenty-two isolates examined. These colonies were yellow and had rhizoid edges; however, they differed from the typical strains of F. columnaris by not adhering to the surface of the agar and having a mucoid surface. All characteristics of cell morphology, environment and biochemistry, slide agglutination test as well as GC content revealed that they are identical to F. columnaris. Furthermore, strain K4m was selected for DNA hybridization analysis and it showed 95% homology with the Pacific Northwest strain. These results confirmed Zaldivar's observation (1985) that K4m was a spontaneous mutation of F. columnaris and not a contaminant with similar colony and cell morphology.

The strain from Georgia, GA468, had a tiny and honeycomb-like colony appearance and also demonstrated less homology (73%) to the PNW strain of F. columnaris. Kimura et al. (1978) reported that gliding bacteria similar to Georgia strain were isolated from gill of diseased salmonids. Because of its non-spreading colony type and production of yellowish, water-insoluble pigments, Kimura et al. (1978) suggested it to be a member

of Flavobacterium. However, other characterizations of Georgia strain were different from Kimura's strain. It may be considered to be a different strain or possibly a species from F. columnaris.

Taiwan strains 4G and 5F were not related to C. psychrophila on the basis of rhizoid colony morphology and nonlipolytic property. They were not F. columnaris because of a low DNA homology (≤ 29%), although the other characteristics were identical to F. columnaris. These two strains may be classified into new species of cytophage. However, unless more comparison were made among reference and Taiwan strains, it is impossible to assign specific names to strains 4G and 5F.

Although there were three phenotypic variations in colony morphology among the strains observed, the other characteristics revealed no differences among these isolates. These results are in agreement with Bullock's (1972) observations that biochemical differentiation of various aquatic myxobacteria is not practical. However, in this study, the method of DNA hybridization can classify these strains into three groups based on DNA relatedness to the reference strain isolated from fish in the PNW.

The results from the hybridizations demonstrated that strains of columnaris isolated from Oregon, Washington, Idaho and British Columbia were homologous (89-97%) to the PNW strain; the Atlantic Coast strain isolated from fish

in Maryland was also homologous (97%) to the PNW strain; strains isolated from Taiwan, Japan, Korea and Chile were genetically related (81-98%) to the PNW strain. The only isolate not homologous to the PNW strain was obtained from fish in Georgia (73%).

Combining the results obtained by Ordal (1970) and those from this study, it can be concluded that there exist two genetically related groups of F. columnaris. One group was highly homologous, and included the strains isolated from Canada, Chile, England (Ordal, 1970), Japan, Korea, Taiwan, and USA (Atlantic and Pacific Coasts). The other came from the southern portion of the USA and was not homologous with strains from the PNW. Ordal showed that two strains of columnaris from Alabama were nearly 100% homologous to a strain from Texas; the reference strain from the PNW was not highly homologous (75%) with the southern strains obtained from Texas and Alabama and vice versa (74%). Whether or not there exists any genetic relationship between Georgia strain and Texas strain or Georgia strain and Alabama strain needs further investigation.

There have been several attempts to serotype F. columnaris strains (Anacker and Ordal, 1959a; Sanders et al., 1976; Bootsma and Clerk, 1976). However, two problems were noted. First, in Anacker's scheme, although many strains were compared, all came from a limited geographic range. In Sanders' scheme, the strains came from different

areas, but limited numbers were analyzed. Second, their serotyping was based on the presence and combination of unique antigens by the agglutinin-adsorption tests. If the antibody titer was low, or the antigen was not immunogenic, these methods would not work. Therefore, a more sensitive method was required.

The method of CIEPIG used for antigenic analysis in this study was shown to be useful when the antibody titer of the serum was low. The method was used for direct comparison of antigens in a single antigenic preparation against two different antisera. Conventional adsorption procedures are confined to particulate antigens which can be separated by centrifugation. However, by using CIEPIG, soluble antigens can be adsorbed by the antiserum incorporated into the intermediate gel. In addition, serological relationships between two strains can be visualized by comparing the numbers of immunoprecipitate rockets in both intermediate and reference gels. One disadvantage of this method was the large amount of antiserum required.

Anacker and Ordal (1959a) typed 325 strains of F. columnaris from fish in the PNW and 1 strain from Texas into 4 serological groups and 1 miscellaneous group. Strain 238 was classified into group I on the basis of 1 common antigen and 2 unique antigens. About 60% of 325 strains were identical to strain 238 and were also

classified to group I. However, serological comparison of F. columnaris strains by CIEP showed that strain 238 had a different antigenic profile from any of the other 6 strains. The presence of 7 to 18 common antigens indicated these seven strains were serologically related; however, variations in the composition from 5 to 13 partially shared antigens showed that they were distinct. Strain 1S, T13 and M1 were separated into three different serotypes based on the presence of type-specific unique antigens. Strains 82303, DD3, IC8r and 238 were shown serologically different from each other analyzed by CIEPIG, but failed to type because no type-specific unique antigens were found. Results of this study indicated that a greater serological diversity may exist among F. columnaris strains than have been previously reported (Anacker and Ordal, 1959a). In Anacker and Ordals' (1959a) serotyping scheme, the Texas strain, which is the only strain from outside the PNW, is related to the PNW strains but nevertheless differs from them in antigenic composition. This agrees with the result of DNA hybridization (Ordal, 1970) that the Texas strain had less homology (74%) to the PNW strains. It seems that there is some correlation between DNA composition and antigenic structure. In this study, results of hybridization and CIEPIG showed that strains isolated from different fish from a wide geographic range were genetically related (81-97%) to the PNW strain, but they were not identical serologically.

This indicates that differences in DNA relatedness will reflect the serological heterogeneity. In summary, no correlation between the serotype and geographic or host sources was observed.

Precipitin rockets showing double peaks ("camels") were observed in the antigenic profiles of strains T13 (Figure 6), 82303 (Figure 8), and IC8r (Figure 10). Because "camel" phenomenon did not occur in the antigenic profile of every strain, the possibility that degradation of specific antigenic component through sonic treatment was expelled (Bjerrum and Bøg-Hansen, 1975). The glycosylation of antigenic components producing the similar antigenic determinants which cross-react with the antiserum may wxplain the "camel" phenomenon.

In this study, the number of common antigens in the heterologous antigen-antibody system was sometimes not equal during the reciprocal process. Two possibilities may account for this phenomenon. First, the potency of the antisera used varied, for instance, sera against strains 1S, M1 and DD3 were more powerful than other antisera and reacted with a greater number of antigens. This may be because the immune systems of the seven rabbits recognized antigens differently. Second, some components were not analyzed because their rockets were too faint to be identified. Purification and concentration of immunoglobulins against each strain from pooled antisera

are suggested. Although electrophoretically seperated antigens could be identified due to their similar mobilities, position among other rockets, rocket shape and staining intensity, it was difficult to determine the individual identities. More elaborate techniques such as tandem CIEP may be useful.

Three strategies concerning protection of fish against epizootics caused by F. columnaris were considered. It was first proposed that competition between virulent and avirulent strains for F. columnaris receptors on the gill cells would provide some protection. The mucoid strain was initially found avirulent to fish by water-borne infection. However, further investigation showed that loss of the virulence in mucoid strain results from the inability to adhere to the fish cells both in vitro and in vivo. Once the mucoid F. columnaris got into the fish body by intraperitoneal injection, fish became infected and soon died (Zaldivar, 1985). Therefore, unless a truly avirulent strain having the capacity to occupy the F. columnaris receptors on gill cells is found, this strategy is not useful.

Second, the idea that a serologically polyvalent bacterin would produce a broad spectrum of protection against epizootics of F. columnaris was considered. However, serological evidence obtained by the CIEPIG indicated that all the strains tested were heterogenous serologically. Therefore, the idea of polyvalent bacterin

seems no longer feasible.

Last, although all the strains tested were heterogeneous serologically, they all shared common antigens. In this study, cross-infection of rainbow trout immunized with the formalin-inactivated whole cell bacterin was shown to be protective. Therefore, the common antigens were proven to be protective. There have been several successful trials of F. columnaris bacterins in the laboratory. However, results of field trials have not been consistant. One possible explanation is that the common antigens became undominant by the presence of other organisms in the field trials through antigenic competition. Use of subunit cell vaccine of protective antigens may be another strategy. The nature of these common antigens needs to be investigated.

SUMMARY AND CONCLUSIONS

1. The growth responses of Flexibacter columnaris in Chase, Shieh, and Liewes media were compared. Both Shieh and Liewes media provided better growth than the Chase medium. Further comparisons on the basis of generation time were made between Shieh and Liewes media. The generation time of strain 238 in the Shieh broth was 130 min compared to 216 min in the Liewes broth. Generation time of F. columnaris was about 40% shorter in Shieh broth than in Liewes broth.

2. Glucose, pyruvate and citrate were not required in the Shieh medium for the cultivation of F. columnaris.

3. The environmental and biochemical characteristics as well as GC content revealed no differences among these numbers of isolates, although three phenotypic variations in colony morphology were observed.

4. When compared genetically by the method of DNA hybridization, there appeared to be two distinct groups. Group one was highly homologous (81-98%) to the Pacific Northwest strain, and included the strains isolated from Canada, Chile, Japan, Korea, Taiwan and USA (Atlantic and Pacific Coasts). Group two came from the southern portion of the USA and was less homologous (73%) with the strain from the

Pacific Northwest. Taiwan strains 4G and 5F were classified into new species as a result of a low DNA homology (≤29%) to the Pacific Northwest strain of F. columnaris.

5. Serological comparison of F. columnaris by the method of crossed immunoelectrophoresis showed that all the strains tested had different antigenic profiles.

6. Seven strains shared from 7 to 18 common antigens, but varied in their composition by possessing 5 to 13 partially shared antigens analyzed by the method of crossed immunoelectrophoresis with an intermediate gel.

7. Strains 1S, T13 and M1 were separated into three different serotypes based on the presence of type-specific unique antigens. However, strains 82303, DD3, IC8r and 238 were known to be serologically different from each other, but failed to type because no type-specific unique antigens were found.

8. No correlation between the serotype and geographic or host sources was observed.

9. Common antigens were shown to be protective by the cross-infection of the immunized rainbow trout with the formalin-inactivated whole cell bacterin.

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