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DNA reassociations, measured by optical and double label membrane filter techniques, were used to assess genetic relationships among Aeromonas and enterobacteria. Aeromonads from human sources, food, fresh water, fish, and pet turtles were examined. The base compositions of three A. salmonicida isolates were 58% G+C while the base compositions of 19 other aeromonads ranged from 58-61% G+C. With A. hydrophila ATCC 7966 as reference, A. salmonicida exhibited 79-85% binding under non-stringent conditions (Tm-25°C). The A. salmonicida isolates were more related to ATCC 7966 than one-half of the A. hydrophila isolates. With A. hydrophila ATCC 7966 as reference, the range in percent relative binding of aeromonads was 46-100% (Tm-25°C). Many cultures exhibited less than 70% binding with ATCC 7966 (Tm-25°C).

DNA-DNA homologies among ten environmental and human isolates of Aeromonas show that there are no correlations between

percent relative binding to the A. hydrophila reference and environmental source or human pathogenicity. The genome sizes of ten aeromonads were compared and found to vary by approximately 30%.

Percent relative bindings ranging from 20-40% were exhibited between Aeromonas reference strains and Citrobacter, Enterobacter, Klebsiella, Shigella, Arizona, and Salmonella. These studies detected the close ancestry between aeromonads and enterics and revealed the molecular heterogeneity within this group of gram-negative, fermentative, oxidase positive bacteria.

Molecular Taxonomy of Aeromonas

by

Peter Gavette Beach

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

MOLECULAR TAXONOMY OF AEROMONAS

INTRODUCTION

Aeromonads are gram negative fermentative, oxidase positive, rod-shaped bacteria. Their pathogenicity for a wide range of animals was originally described by Sanarelli (1891) after his identification of Bacillus hydrophilus fuscus (42). Aeromonads have been isolated from a wide assortment of poikilotherms and found pathogenic for frogs, fish, lizards, pigeons, guinea pigs, mice, rabbits, young dogs, cats, and humans (4). Aeromonas hydrophila from human sources have been primary agents in cases of severe gastroenteritis, septicemia, and pneumonia (21, 40, 60).

Over the past 80 years, aeromonads have been classified into a myriad of species and subspecies. This study was designed to elucidate the molecular taxonomy of the genus Aeromonas as it related to the aeromonad species and subspecies. This study also reveals the closer ancestry between aeromonads and enterics than had been previously realized. Twenty-three aeromonad isolates were compared by DNA-DNA hybridizations, genome size, G+C base composition and 23 phenotypic traits.

REVIEW OF LITERATURE

In 1891, Sanarelli named and described Bacillus hydrophilus fuscus as follows: gram-negative, motile, without brown water-soluble pigment on trypticase agar, gelatinase positive, digesting Loffler's serum, producing gas from breakdown of glycerol, and pathogenic for six animals (42). Unfortunately, none of Sanarelli's strains are extant (56). In 1900, a similar bacterium with a single polar flagellum and fermentative characteristics of Aerobacter aerogenes (now Enterobacter aerogenes) was named Aerobacter liquefaciens by Beijerinck (3). Unfortunately, Beijerinck's strain, too, is no longer extant.

In 1901, Chester (9) described a similar organism, naming it Bacillus hydrophilus. This strain has been preserved and, in 1943, Stanier renamed it Aeromonas hydrophila -- the generic name proposed by Kluyver and van Niel in 1936. Kluyver and van Niel described the genus as polarly flagellated, whose members "...ferment sugars in a way closely related to the fermentation type characteristic of the genera Aerobacter and Aerobacillus," (29).

Burgey's Manual (7th ed.) recognizes four species of Aeromonas (4). S. F. Snieszko, who prepared the chapter on Aeromonas, described them. Three of these species, Aeromonas hydrophila (Chester, 1901) Stanier, 1943, Aeromonas liquefaciens (Beijerinck, 1900)

Kluyver and van Niel, 1936, and Aeromonas punctata (Zimmerman, 1890) Snieszko, 1957, cannot be differentiated on the basis of morphological or biochemical properties, In the key to the genus, Snieszko concedes that the differentiation of these three species is based solely on source, habitat, and pathogenicity. Even this concession seems gratuitous when the sources, habitats, and pathogenicities of the three species are compared. The source of each merely reflects the source of the type species as well as the ubiquitous nature of all three. The habitat is water. A. hydrophila and A. liquefaciens have parallel pathogenicity for a number of animals and A. punctata has not been screened for its latitude of pathogenicity.

Aeromonas salmonicida, according to Snieszko, is well defined morphologically and biochemically. Unlike the other species, it is an obligate fish pathogen confined mainly to the genus Salmonidae (57, 62).

According to Eddy (14), the genus Aeromonas includes three species. He suggested that they be distinguished on the basis of the Voges-Proskauer (V-P) test, arrangement of flagella, and gas production from glucose fermentation. One species, called Aeromonas formicans, was polarly flagellated, V-P negative, and anaerogenic. This species was previously described by Scherago (44) in 1936 as a subspecies of A. punctata called A. punctata caviae. A. formicans displayed a simple mixed-acid fermentation with its major products being ethanol, acetic acid, lactic acid, succinic acid, and formic acid

(63). A. formicans was also anaerogenic, due to the absence of formic hydrogenlyase, since the gases H_2 and CO_2 could be formed only by the cleavage of formate (63).

Eddy's second species, A. liquefaciens (which included strains previously designated A. hydrophila and A. punctata), was unlike A. formicans in that it demonstrated the butanediol modification of the mixed-acid fermentation pathway. The major products of this pathway were ethanol, 2, 3-butanediol, formic acid, and the gases H_2 and CO_2 resulting from the presence of formic hydrogenlyase (63).

The third species described by Eddy was A. salmonicida. This was the non-motile, V-P negative, aerogenic species.

Ewing, Hugh, and Johnson (20) proposed in 1961 that the genus be reorganized. They took the viewpoint that since A. salmonicida appeared well definable and all other aeromonads were biochemically heterologous, all non-salmonicida aeromonads should be combined into one species. Since A. hydrophila (Chester, 1901) Stanier, 1943, held precedence among the remaining species, this omnibus grouping of aeromonads was designated A. hydrophila.

Two years later, I. W. Smith disagreed with the inclusion of A. salmonicida in the genus because it didn't vigorously generate gas during fermentation, was non-motile, and was V-P negative. She proposed that A. salmonicida be placed in a separate genus, Necromonas, in the Family Pseudomonadaceae (61). Serological

investigations have since shown a close relationship between A. salmonicida and the motile aeromonads (8, 27).

During the period 1960-1964, R. H. W. Schubert (45-52) made a thorough study of the genus. He designated three species. A. punctata and A. hydrophila differed in that A. punctata was V-P negative due to the absence of 2, 3-butanediol dehydrogenase (2, 3-BD) activity (51, 52). This enzyme dehydrogenated meso-2, 3-butanediol to acetylmethylcarbinol and diacetyl. A. salmonicida differed from A. hydrophila and A. punctata in its low limit of growth temperature (optimum 20-25°C., minimum 6°C., maximum 34.5°C.) (4). A. punctata had an optimum growth temperature of 25-30°C. and grew at 37°C. A. hydrophila had an optimum growth temperature of 30°C. with good growth at 37°C. (4).

From these three species, Smith (61) and Schubert (54) described four variants. Smith found several A. salmonicida which did not produce brown water-soluble pigment when grown on trypticase soy agar. This type was named A. salmonicida variety achromogenes. Schubert found anaerogenic variants of both A. hydrophila and A. punctata. A. hydrophila variety anaerogenes was also distinguished in that it might not be motile. A. hydrophila variety hydrophila was proposed as the non-motile variant. This variety is referred to by LeClarc and Buttiaus as A. dourgesii (31). A. punctata variety caviae was the final anaerogenic variant.

In 1968, Schubert recognized six species in the genus Aeromonas (54):

> Aeromonas punctata (Zimmerman, 1890) Snieszko, 1957.
> A. punctata subsp. caviae (Scherago, 1936) Schubert, 1964.
> A. hydrophila (Chester, 1901) Stanier, 1943.
> A. hydrophila subsp. anaerogenes. Schubert, 1964.
> A. salmonicida (Lehmann and Neumann, 1896) Griffin, Snieszko and Friddle, 1953.
> A. salmonicida subsp. achromogenes (Smith, 1963) Schubert, 1967.

In 1971, Schubert (56) published a study of two proposed neotype strains of A. hydrophila -- ATCC 7966 and ATCC 9071. The two differed only slightly on the basis of biochemical activities. It was Schubert's judgment that the characteristics of ATCC 7966 were in better agreement with the current conception of A. hydrophila.

In 1973 the Judicial Commission of the International Committee on Systematic Bacteriology decided to "conserve" the generic name Aeromonas Stanier 1943. A. hydrophila (Chester, 1901) Stanier, 1943, ATCC 7966 was designated the neotype strain (17).

One species once considered in the genus Aeromonas, A. shigelloides, has been reclassified. A. shigelloides, alternately known as C 27, remains in the Family Pseudomonadaceae as a new genus, Plesiomonas. This reclassification was based on the oxidative metabolism of carbohydrates by this organism (24).

Snieszko, in Bergey's Manual-7th ed. (4), and Schubert (55) recognize that aeromonads and certain enterics have few differences

physiologically. Using Adensonian analysis, Colwell and Mandel (11) found aeromonads to be intermediate in characteristics between enterics and pseudomonads. The chief differences between species of Aeromonas and Salmonella paratyphi A, S. typhi suis, S. typhi galarium, and Arizona are found in the arrangement of flagella (4, 21, 55) and the results of the oxidase test (21, 55). Single polar flagellation is typical of motile aeromonads (4), but mixed and peritrichous flagellation (35, 51) as well as amphitrichous and lophatrichous flagellation (36) have been observed. Motile enterics are typically peritrichous. Unlike the enterics, aeromonads are oxidase positive. The oxidase test indicates the presence of cytochrome c in the electron transport system (63). This test therefore reveals a basic difference in the respiratory electron transport system of aeromonads and enterics.

MATERIALS AND METHODS

Strains Examined

The strains used in this study are listed in Table 1.

Reference Strains

Two reference strains were selected for this study. One was the neotype of Aeromonas hydrophila (Chester) Stanier, ATCC 7966. The other was the type strain Aeromonas salmonicida (Lehmann and Neumann) Griffin, Snieszko and Friddle, ATCC 14174.

Media

The medium used for growing most cultures consisted of 1% yeast extract (Difco), 0.3% bacto peptone (Difco), and 0.1 M Tris buffer pH 7.5. Usually two liters of broth provided sufficient cells for deoxyribonucleic acid (DNA) extraction. Reference strains were propagated daily until 40-50 mg of deproteinized DNA were collected. The Vibrio species were grown in nutrient broth containing 3% NaCl.

Four variations of a glucose-basal salts medium were used for labeling cellular DNA (Table 2). Cellular DNA was labeled with tritiated thymidine at 2 μc/ml of ^{32}P at 5-6 μc/ml as $H_2{}^{32}PO_4$. Lysine and arginine doubled the yield of the A. salmonicida reference and were therefore included in the labeling medium.

Table 1. Bacterial strains examined. The following abbreviations have been used: CDC (Center for Disease Control), ATCC (American Type Culture Collection), UOMS (University of Oregon Medical School), OSU (Oregon State University), CPHL (California State Department of Public Health Laboratory).

Name	Strain	Source	Investigator Providing Strain
A. hydrophila	ATCC 7966	Canned milk	M. Mandel
"	ATCC 9071	Frog "red leg"	"
"	OSU 49	Water	R. J. Seidler
"	OSU 53	"	"
"	OSU 55	Turtle	CPHL
"	OSU 56	Human	"
"	OSU 72	Turtle	R. J. Seidler
"	OSU 86	Aquarium	"
"	OSU 94	Turtle	"
"	OSU 105	"	"
"	OSU 109	"	"
"	UOMS 2619	Human	UOMS
"	UOMS 2696	"	"
"	UOMS 8057	"	"
ssp. anaerogenes	ATCC 15467	Used oil emulsion	M. Mandel
ssp. formicans	ATCC 13137	Water	"
A. dourgesii	ATCC 23211	"	"
ssp. anaerogenes	ATCC 23212	"	"
A. liquefaciens	ATCC 14715	Salmon	"
"	OSU 3	Trout hatchery	R. H. McCoy
A. punctata	ATCC 11163	Human	M. Mandel
"	OSU 122	Carp	
ssp. caviae	ATCC 14486	Human	M. Mandel

Table 1. Bacterial strains examined. (Cont.)

Name	Strain	Source	Investigator Providing Strain
A. salmonicida	ATCC 14174	Trout	M. Mandel
"	OSU 2	Salmon	R. H. McCoy
"	OSU 19	Water	Patterson
Plesiomonas shigelloides	ATCC 14029	Human	M. Mandel
"	CDC 389-69	"	CDC
Escherichia coli	WP-2	"	J. Suit
Enterobacter hafniae	CDC 2929-70	"	CDC
E. cloacae	ATCC 13047	Spinal fluid	ATCC
Klebsiella pneuomoniae	ATCC 13883	Human	"
Citrobacter Lac$^-$	CDC 3121-70	"	CDC
" Lac$^+$	CDC 509-71	"	"
Erwinia carotovora	ATCC 15713	Potato	ATCC
Erwinia amylovora	ATCC 1269	Plant pathogen	L. Moore
Serratia marcescens	CDC 6102-65	Human	CDC
Shigella flexneri	ATCC 24570	Human feces	D. Brenner
Salmonella typhimurium	LT-2	"	J. Suit
Arizona	CDC 170-71	Human	CDC
Vibrio alginolyticus			J. Baross
Vibrio (50% G+C)		Crab	"
Plesiomonas shigelloides	ATCC 14029	Human	M. Mandel
"	CDC 389-69	"	CDC

Table 2. Variations of glucose-basal salts media used for labeling deoxyribonucleic acid of reference strains.

Defined medium for tritium labeling with thymidine:

A. hydrophila	A. salmonicida
0.3% NaCl	Same as A. hydrophila plus
0.0026% Na_2SO_4	0.005% Lysine
0.001% $MgCl_2$	0.005% Arginine
1.0% Glucose	
0.2% NH_4HPO_4	
0.6% Na_2HPO_4	
0.3% KH_2PO_4	
2.0% Casamino acids	
pH 7.5	

Defined medium for ^{32}P labeling:

A. hydrophila	A. salmonicida
0.15% NaCl	Same as A. hydrophila plus
0.0013% Na_2SO_4	0.005% Lysine
0.0005% $MgCl_2$	0.005% Arginine
2.0% Glucose	
0.1% NH_4Cl	
0.121% (0.01 M) Tris pH 7.5	
2.0% Casamino acids	

The defined media were six times more effective than yeast extract peptone medium in labeling DNA with tritium. Due to acidity of the $H_2{}^{32}PO_4$ used for labeling, adjustment of the pH was necessary even with the 0.01 M Tris buffer. All reagents were sterilized as concentrated solutions and added aseptically to sterile double distilled water. Reagents were prepared in advance and stored at room temperature. The complete glucose-basal salt medium was prepared immediately prior to use. Generally 125 ml samples in 4-6,250 ml Erlenmeyer flasks provided sufficient cells to yield 1,000 μg ^3H labeled DNA and 500 μg ^{32}P labeled DNA.

Growth Conditions

Generally strains were incubated for 16-18 hours at room temperature on a gyrotory shaker. A. salmonicida strains, however, were incubated for 18-24 hours. Should the cultures be allowed to incubate longer, autolysis and reduced DNA yields would result.

Extraction and Purification of DNA

Stationary phase cells were harvested from the growth medium by centrifugation (8, 000 X g for 5 min.). Enterobacteria and vibrios were washed once in 150 ml of saline-ethylenediaminetetraacetic acid (saline-EDTA, consisting of 0.15 M NaCl and 0.1 M EDTA, pH 8.0). The aeromonad cell pellets were not washed since saline-EDTA and freezing invariably led to premature lysis and inefficient extraction of DNA. All frozen cell pellets were suspended in saline-EDTA and lysed with 2% sodium dodecyl sulfate (SDS). E. coli and Serratia marcescens did not readily lyse when SDS was used exclusively. These two bacteria were freeze-thawed in saline-EDTA prior to lysing with 2% SDS.

DNA was extracted and purified by a modification of the Marmur procedure (33). Neutralized saline-EDTA equilibrated phenol (10% saline-EDTA, 90% liquified phenol (Mallinckrodt), pH 8.0) was used for the primary deproteinization. To increase DNA yields, the first

protein interface was re-extracted with equal volumes of phenol and saline-EDTA. DNA from the resulting aqueous layers was then deproteinized with equal volumes of 96% chloroform and 4% isoamyl alcohol (Sevag). The new aqueous layer was precipitated with two volumes of 95% ethanol, rinsed once in 70% ethanol and then dissolved in a minimal volume of chilled 0.015 M NaCl, 0.0015 M sodium citrate (0.1 X SSC) at 1-2 mg/ml.

Each DNA sample was routinely assayed for the degree of purification. Approximately 50 μg/ml of DNA was assayed in a U.V. spectrophotometer (Beckman Instruments, Inc.). Absorbance at 230, 260, 280, and 320 nm was recorded for 3-5 independent determinations. If the absorbance exceeded 0.010 at 320 nm, the sample was deproteinized again. If the 230/260 ratio was 2.2-2.4 and the 260/280 ratio 1.9 ± 0.02, the DNA was considered acceptable. The DNA was then stored at $4^{o}C$ in 0.1 X SSC over a drop of $CHCl_3$.

Determination of Tm and Guanine plus Cytosine Base Composition

Most guanine plus cytosine (G+C) determinations were made from buoyant densities (B.D.) in cesium chloride. The formula used for conversion of B.D. to G+C content was $(GC) = \frac{P - 1.660 \text{ g/cc}}{0.098}$ where P = g/cc unknown DNA (32).

The G+C base composition of the reference strain DNA preparations ATCC 7966 and ATCC 14174 were determined by Marmur and

Doty methods (34) utilizing a thermostated automatic recording spectrophotometer (Gilford Instruments, Inc.). Base compositions were determined in 0.1 X SSC using equation 5 of Mandel et al. ($G+C_x = 51 + 0.0199[Tm_x - Tm_{E.\ coli\ (WP-2)}]$) (33) (Table 4).

Tm determinations were made with sheared and dialyzed DNA (50-60 μg/ml) in the designated buffer. Samples were placed in quartz cuvettes and stoppered. The hyperchromic shift was recorded at 260 nm as the temperature was raised 0.75°C/5 min. (Table 3).

Table 3. Determination of Tm of sheared reference DNA in the renaturation buffers.

	Sheared Reference DNA					
	A. hydrophila ATCC 7966			A. salmonicida ATCC 14174		
Buffer	Tm[a]	Tm-25°C	Tm-15°C	Tm	Tm-25°C	Tm-15°C
2 X SSC 40% DMSO[b]	65.5°	40.0°	50.0°	64.0°	39.0°	49.0°
3 X SSC 20% DMSO[c]	83.8°	59.0°	69.0°	82.0°	57.0°	67.0°

[a]Tm is the mean thermal denaturation temperature of reference DNA sheared in a French press at 15,000 psi.

[b]Renaturation buffer used for double label DNA-DNA renaturations on nitrocellulose filters. In this ionic concentration, the reduction of Tm was 0.79°C/1% DMSO.

[c]Renaturation buffer used for optical renaturation technique using hyperchromic shift at 260 nm U.V. In this ionic concentration, reduction of Tm was 0.78°C/1% DMSO.

Table 4. Buoyant densities, Tm, and guanine plus cytosine base composition of 21 aeromonads.

Aeromonas isolate	Buoyant Density[a] (g/cc) of Tm	Buoyant Density[a] (°C)	G+C content (percent)
A. hydrophila ATCC 7966[b]		79.4	60[c]
" ATCC 9071	1.717	77.5	58[d]
A. hydrophila anaerogenes ATCC 15467	1.719	79.0	60[d]
A. hydrophila OSU #55	1.719		60
" OSU #56	1.719		60
" OSU #72	1.719		60
" OSU #94	1.720		61
" OSU #105	1.719		60
" OSU #2619	1.720		61
" UOMS 2696	1.718		59
" UOMS 8057	1.720		61
A. hydrophila formicans ATCC 13137	1.720		61
A. liquefaciens ATCC 14715	1.719		60
" OSU #3	1.717		58
A. dourgesii ATCC 23211	1.717		58
A. dourgesii anaerogenes ATCC 23212	1.717		58
A. punctata ATCC 11163	1.717		58
A. punctata caviae ATCC 14486	1.720		61
A. salmonicida ATCC 14174[b]	1.717	78.5	58[d]
" OSU #2	1.717		58
" OSU #19	1.717		58

Buoyant density was determined as an average of two experimental runs. The variation was ±0.001 g/cc. Tm was determined in 0.1 XSSC.

Symbols: [a]Courtesy of M. Mandel, University of Texas, M. D. Anderson Hospital and Tumor Institute, Houston, Texas.

[b]reference strains.

[c]determined by thermal denaturation only.

[d]determined by thermal denaturation and buoyant density in CsCl.

Immobilization of DNA on Filters

All materials were pre-cooled to 4°C. The filters (Schleicher and Schuell S-6, 10 cm diameter) were mounted in the filtering apparatus and washed with 200 ml of 2 X SSC. Six milligrams of tritium labeled reference DNA (500 cpm/μg) were dissolved in 100 ml of 0.1 X SSC. The DNA was denatured with 10 ml of 1 N NaOH for five minutes, then diluted with 500 ml of 6 X SSC. This mixture was then neutralized with 20 ml of 1 M NaH_2PO_4.

The neutralized, denatured DNA was poured onto the filter and allowed to gravity load for 18 to 24 hours. To hasten the filtration of the last 20-30 ml, a slight suction of approximately 5 psi was applied. Prior to removal of the filters from the filtering apparatus, the filters were marked with a pencil to indicate the confines of the area containing DNA. After loading, the filters were washed with 100 ml of 6 X SSC, drained on paper towels, and dried in a vacuum at 60-80°C for 16 hours.

Three 1ml samples were taken from (1) undenatured DNA at 50 μg/ml, (2) the denatured and neutralized DNA, and (3) the filtrate. The first group of samples were taken to confirm the specific activity of the tritiated DNA. The second and third were taken to determine the efficiency with which the denatured DNA bound to the filters. The efficiency of filter immobilization was assessed by O.D. readings

and by tritium counts before and after DNA filtration. Both methods indicated that approximately 80% of the DNA was immobilized to the filters after washing.

Once dried, the filters were punched into smaller circular filters with a paper punch (GEM). These smaller filters had a diameter of 6.5 mm. They were placed in vials and stored under vacuum at room temperature.

The activity and concentration of DNA on the respective reference filters are indicated in Table 5. The amount of single stranded DNA immobilized on the filters closely approximated the findings of Johnson and Ordal (25). The variation in concentration of DNA on the filters was $\pm$ 11%. This variation closely approximated the 12% variation reported in the literature (25).

Preparation of Renaturation Buffers

A buffer of 40% dimethylsulfoxide (DMSO) (Mallinckrodt Chemical Works, St. Louis, Mo.) and 2 X SSC was the buffer used for the membrane filter hybridizations. This buffer was prepared just prior to use. The salts tended to precipitate when added directly to concentrated DMSO, so the 10 X SSC stock solution was always added to an 80% dilution. The buffer used for optical renaturations consisted of 20% DMSO in 3 X SSC. This buffer was stable at 4°C and was prepared in advance.

Table 5. DNA-DNA renaturation results.

		Percent binding							
		Filter technique				Optical technique			
		ATCC 7966		ATCC 14174		ATCC 7966		ATCC 14174	
Aeromonas DNA	Isolate	Tm-25	Tm-15	Tm-25	Tm-15	Tm-25	Tm-15	Tm-25	Tm-15
A. hydrophila	ATCC 7966	100	100	79		100	100		77
"	UOMS 8057	100	91	61		83			
"	OSU #55	100		77					
"	OSU #94	96							
"	OSU #105	89		75					
A. liquefaciens	OSU #3	85	67	63					
A. punctata	ATCC 11163	83		78					
A. hydrophila	ATCC 9071	80		70					
"	OSU #56	80		42				50	
A. hydrophila anaerogenes	ATCC 15467	77	53	65		72			
A. punctata caviae	ATCC 14486	70	60						
A. hydrophila formicans	ATCC 13137	66	62						
A. dourgesi	ATCC 23211	74							
A. liquefaciens	ATCC 14715	65	46	82					
A. dourgesi anaerogenes	ATCC 23212	58	64				54		
A. hydrophila	OSU #109	58	59	88				85	
"	UOMS 2619	53	46						
"	OSU #72	46		53					
"	UOMS 2696	53		65					
"	OSU #49			79					
"	OSU #53			76					
"	OSU #86	86		58					
A. salmonicida	ATCC 14174	79		100	100		77	100	100
"	OSU #2	85		95	95				
"	OSU #19	84		93	93				

The genus Aeromonas is genetically heterogenous as seen by the divergence in percent binding values (46-100%) with A. hydrophila ATCC 7966 DNA as reference (Tm-25).

Preparation of Dialysis Tubing

The dialysis tubing was washed by submerging in 5% sodium bicarbonate solution and incubating at 100°C in a steam bath for one hour. The tubing was then rinsed in distilled water for approximately 30 minutes and stored in 50% ETOH at room temperature. Prior to use it was washed in 0.1 X SSC.

Preparation of DNA for Renaturation

Just before use, the DNA samples were sheared in a French press (Aminco) at 15,000 psi, yielding fragments with a double strand molecular weight of about 1×10^6 (32). Sheared DNA (600-800 μg/ml) was dialyzed to equilibrium in the preferred renaturation buffer at 4°C for 12 hours. If the DNA was not used within four days it was discarded.

Occasionally after dialysis the low molecular weight DNA became cloudy. The DNA was filtered through 0.45 μm nitro-cellulose filters in these instances and the resulting solution was comparable to the other samples.

Determination of Tm-25 and Tm-15 Incubation Temperatures

Table 3 indicates the experimentally determined mean thermal denaturation temperature (Tm) of reference strain DNA in the two

renaturation buffers. It also lists the renaturation temperatures (Tm-25 and Tm-15) used for the two hybridization techniques.

Quantitative and Qualitative Aspects of Single Stranded Tritiated DNA Immobilized on Nitrocellulose Filters

The quantity of single stranded tritiated DNA immobilized on the small filters in the filter renaturation technique varied by as much as 35%. The nitrocellulose filters with tritiated A. hydrophila ATCC 7966 DNA contained 8.2 $\pm$ 0.9 μg DNA at 352 cpm/μg. Filters with A. salmonicida ATCC 14174 DNA contained 12.5 $\pm$ 1.4 μg DNA at 500 cpm/μg.

Pilot experiments were designed to ascertain optimum conditions for the DNA membrane filter hybridization procedures. Figure 1 illustrates the binding of increasing concentrations of homologous ^{32}P labeled reference DNA to membrane bound DNA (ATCC 14174). The optimum concentration of ^{32}P DNA in solution is determined at the optimum fraction of input DNA hybridizing, whether sufficient counts appear in the homologous reaction, and whether the DNA level chosen responds well to modest concentrations of competitive homologous DNA. A level of 2 μg provided sufficient counts in the homologous reactions and also met the other criteria. Also, when blank filters were incubated with 2 μg of ^{32}P DNA, only 33 cpm of ^{32}P DNA adhered to the filters.

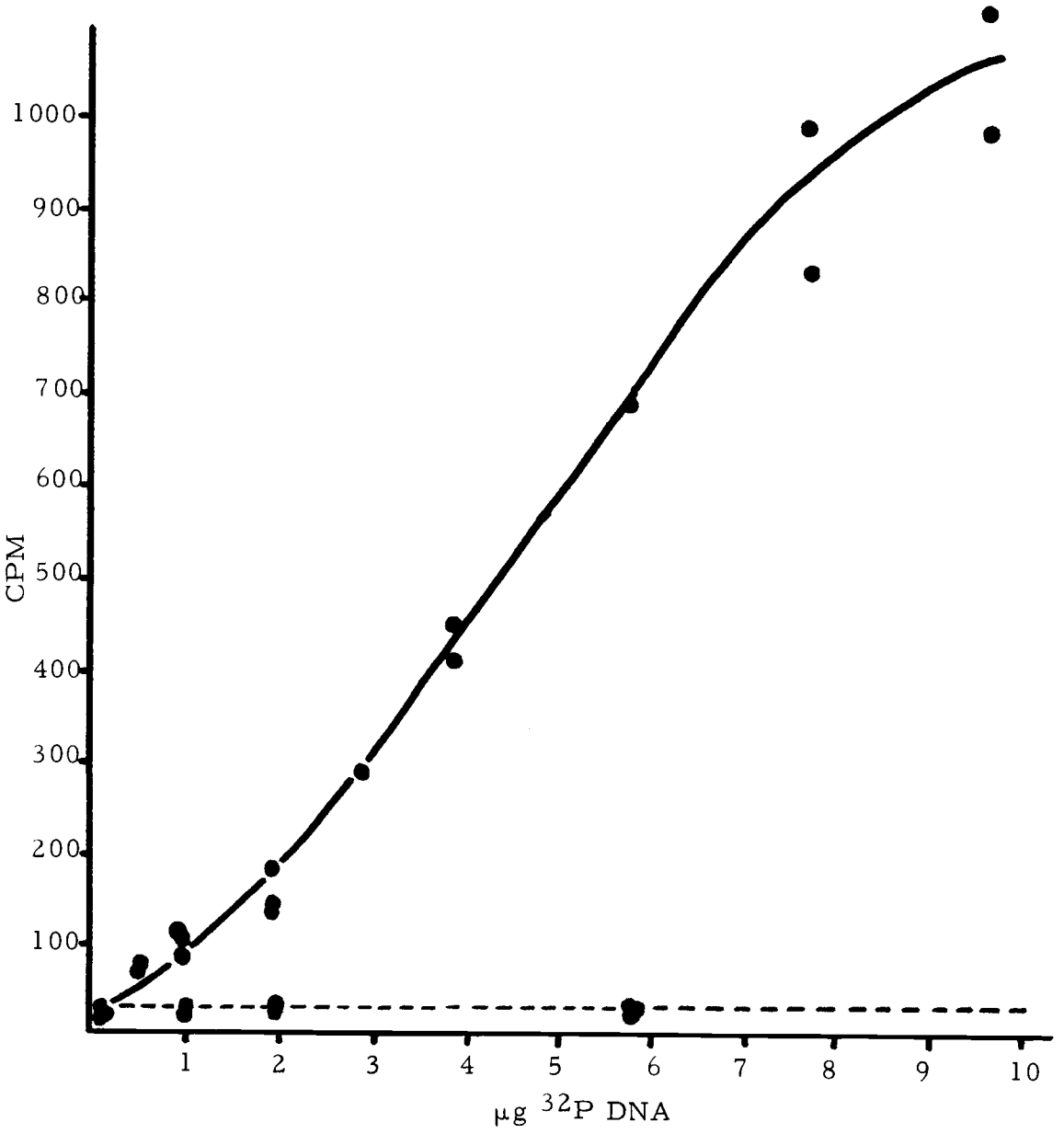

Figure 1. Renaturation kinetics of increasing concentrations of ^{32}P labeled ATCC 14174 DNA with homologous filter bound DNA. ^{32}P labl led DNA was 250 cpm/μg and sheared at 15,000 psi just prior to use. Filter bound DNA had 12.5 μg DNA/filter. Renaturations took place in 40% DMSO, 2 X SSC at 39°C (Tm-25°). Symbols: ____ renaturation profile; ----- nonspecific binding.

In a second study, conditions for optimizing DNA-DNA competition experiments were explored. These experiments are summarized in Figure 2. Increasing concentrations of unlabeled DNA were placed in competition with 2 μg ^{32}P labeled homologous DNA at 3,000 cpm/μg. The optimum concentrations for competition experiments were found to be approximately 200 μg unlabeled homologous DNA and 2 μg ^{32}P labeled homologous DNA.

Each filter with bound tritiated reference DNA was placed in a 4 dram vial with sheared, single-stranded ^{32}P reference and competitor DNA. The total volume was brought to 0.5 ml with renaturation buffer. All vials were tightly capped and incubated in a water bath at 15°C or 25°C below the reference DNA Tm as measured in the hybridization buffer. The vials were rocked continuously for 16-18 hours at 40-2 inch strokes per minute.

After incubation the filters were passed through three consecutive washes of renaturation buffer heated to renaturation temperature. This procedure was necessary to remove non-specifically adhering ^{32}P DNA. Three additional washes with 2 X SSC heated to the renaturation temperature were necessary to remove DMSO from the filters and thereby prevent quenching.

All renaturations were run in triplicate to obtain a more representative homology figure. In addition, the ^{32}P reference DNA (2 μg/0.5 ml) was incubated with a blank filter to monitor possible

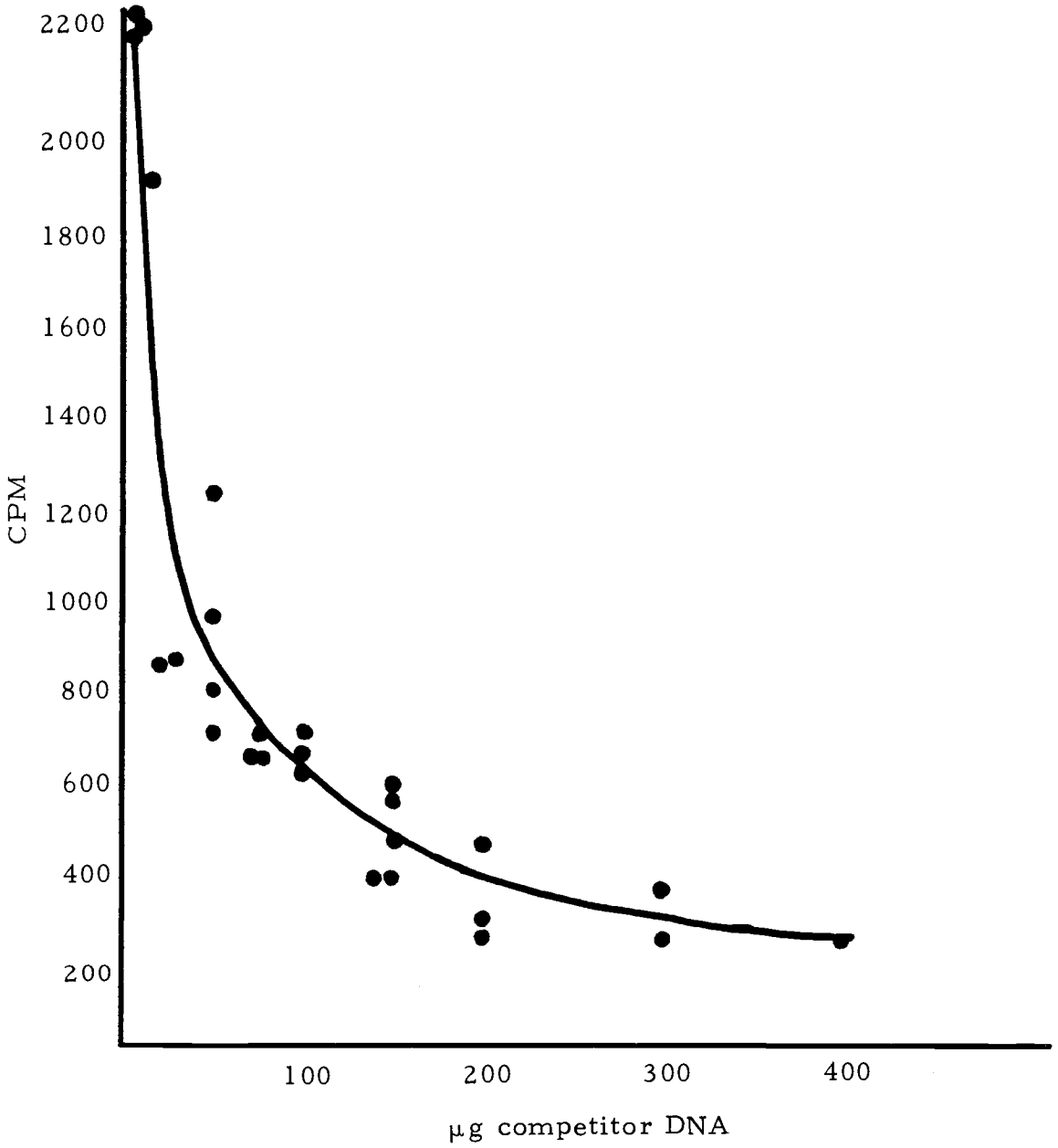

Figure 2. Renaturation kinetics of homologous DNA using the double label membrane filter technique. Competition is between 2 μg ^{32}P labeled DNA of ATCC 14174 and increasing concentrations of unlabeled homologous DNA. Both labeled and unlabeled competitor DNA was sheared at 15,000 psi just prior to use. Renaturations took place in 40% DMSO, 2 X SSC at 39°C, ^{32}P labeled DNA at 3,000 cpm/μg.

impurities influencing non-specific binding of the reference DNA to the filter. In every case the amount of non-specific ^{32}P binding to the blank filters was about 48 cpm. Since the average background radiation in the ^{32}P channel was 18 cpm, non-specific binding of the ^{32}P DNA accounted for an average of only 30 cpm for all renaturations.

Whenever the tritium count of one of three given filters varied by more than 30% of the average of the remaining two filters, it was considered an odd value and therefore discarded. This occurred in about 10% of the determinations. Figure 3 illustrates how, for a given set of two or three hybridizations from one bacterial source, percent binding was calculated once the average tritium and ^{32}P counts were determined.

In no case did the maximum homologous depression of counts fall below 350 cpm. In most experiments the depression varied from 400-1000 cpm.

Thermal Elution Profiles

Experiments were conducted in 40% DMSO and 2 X SSC. This is the same buffer used in nitrocellulose filter renaturations. Scintillation vials were filled with 5 ml of buffer, capped, and brought to the incubation temperature in a water bath heated by a thermostated heater with water circulator attached (Haake E51). An identical stoppered vial with buffer and thermometer was placed beside the vial containing

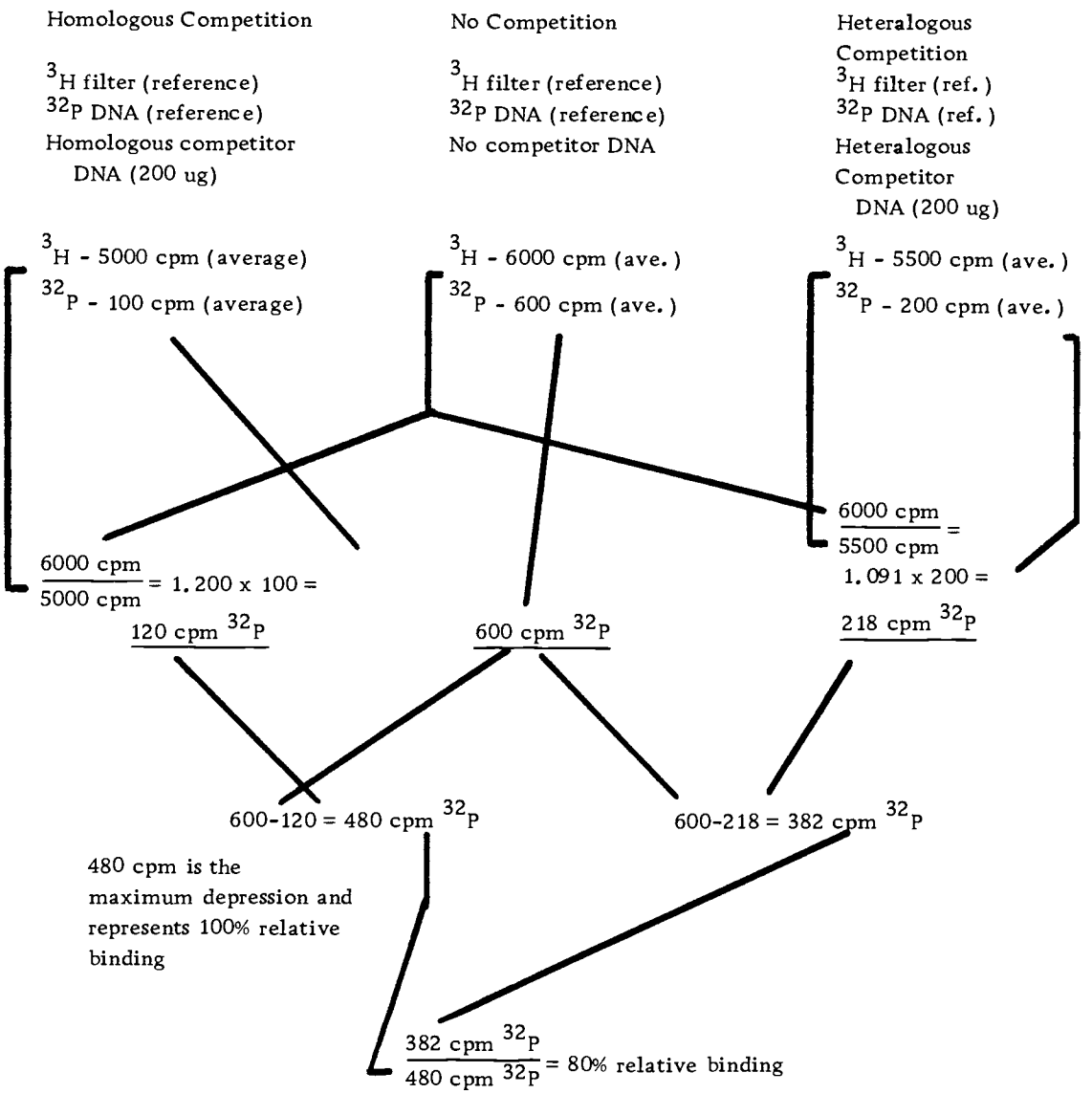

Figure 3. Flow diagram of the calculation of percent relative binding for an hypothetical example.

the filter to record the elution temperature. The filters were removed from the vials at ten minute intervals, drained by briefly touching the filter to the inside of the vial, then placed on filter paper until the next temperature equilibration was achieved. The temperature of the buffer was raised 4°C and the filter placed in another vial. The eluted ^{32}P single-stranded DNA was TCA precipitated and assayed in the liquid scintillation counter.

Optical Renaturation

Optical renaturations were conducted following the method of Seidler and Mandel (58). To make quantitative measurements of the renaturing DNA mixtures, the $Cot_{.5}$ (Moles sec./l. corresponding to time a concentration of DNA attains 50% renaturation) of the DNA preparations were first determined. The renaturation buffer used was 20% DMSO plus 3 X SSC. DNA samples of two organisms to be compared were prepared with equal amounts of recently sheared DNA (25 μg/ml). Equal amounts were determined by absorbance at 260 nm and assumed to be genome equivalents.

When genome of two test organisms had identical polynucleotide sequences, the $Cot_{.5}$ of the mixture was the same as it would have been if either DNA was renatured separately. When there were no polynucleotide sequences in common, the $Cot_{.5}$ of the mixture was the sum of the $Cot_{.5}$ values of both samples. Samples containing

intermediate polynucleotide sequences yielded proportionately intermediate $Cot_{.5}$ values.

Seidler and Mandel's (58) equation for homology (percent relative binding) from observed $Cot_{.5}$ values is:

$$\% \text{ homology} = 1 - \frac{Cot_{.5}^{mix} + (Cot_{.5}^{100} - Cot_{.5}^{0})}{Cot_{.5}^{100}} \times 100$$

The DNA was heat-denatured in a thermostated automatic recording spectrophotometer. E. coli WP-2 DNA was selected as a standard control. $Cot_{.5}$ values of 1.09 $\pm$ 0.06 at Tm-25 and 1.11 $\pm$ 0.07 at Tm-15 were considered acceptable.

Genome Size

Genome sizes were determined by using the average $Cot_{.5}$ values and the formula of Seidler and Mandel (58).

$$G = (2.2 \times 10^9 \, X/Y) - (\Delta GC_{51} \times 0.18)$$

G = genome size expressed in daltons of molecular weight

Y = $Cot_{.5}$ E. coli

X = $Cot_{.5}$ of unknown sample

GC_{51} = percent G+C difference between E. coli and the test DNA.

Biochemical Studies

Biochemical studies were generally performed following the procedures outlined by Edwards and Ewing (18). Inocula were taken from 24 hour cultures grown on nutrient agar. Cultures were incubated four days before being discarded. All cultures were incubated at 35-37°C with the exception of A. salmonicida, which was incubated at 28°C. The tests and media employed included: fermentation of 1% lactose, sucrose, cellobiose, rhamnose, sorbitol, raffinose, arabinose, and salicin; citrate utilization (Simmons Citrate Agar, Difco); indole production (Trypticase, Difco); methyl red and Voges-Proskauer (VP) tests (buffered peptone-glucose broth); growth at 37°C (nutrient agar); decarboxylation of lysine; deamination of phenylalanine (phenylalanine agar, Difco); colony appearance of EMB (EMB agar, Difco); hemolysis of sheep erythrocytes (Brain Heart Infusion, Difco with 15% blood); growth in KCN (KCN broth, Difco); the oxidase test. Indole production was tested with Kovac's reagent. A few drops of 1% α naphthol and para-aminodimethylaniline oxalate (Difco) were added to 24 hour old cultures grown on nutrient agar plates to conduct the oxidase test.

RESULTS

Guanine plus Cytosine Base Composition of Selected Aeromonads

The guanine plus cytosine base composition (% G+C) of 22 aeromonads was determined from buoyant densities in CaCl and from thermal denaturation curves. The buoyant density of three A. salmonicida strains was 1.717 g/cc with a corresponding G+C content of 58%. The buoyant densities and G+C contents of two A. dourgesii strains were identical to the A. salmonicida strains. The 17 remaining aeromonads have buoyant densities of 1.717-1.720 g/cc and a G+C content of 58-61%.

Thermal denaturation was used to determine that the A. hydrophila type species (ATCC 7966) has a base composition of 60%. It was also used to determine the base composition of three other strains and yielded results identical to those reached by ultra-centrifugation in CaCl (Table 4).

Intrageneric DNA Polynucleotide Relationships Among the Aeromonads

The results of the DNA hybridizations to two Aeromonas strains are compiled in Table 5. The results indicate that the genus Aeromonas is genetically heterogenous. The range in relative percent binding of all aeromonads to the A. hydrophila reference at the less

stringent incubation temperature (Tm-25) was 46-100%. Similarly, the range in relative percent binding to the A. salmonicida reference was 42-95%.

In most cases the drop in percent relative binding at the more restrictive incubation temperature (Tm-15) is only 10% or less. This small change would indicate that little mispairing of nucleotides occurred at the less stringent incubation temperature (Tm-25).

There is no correlation between relative percent binding and origin of the strain. Table 6 illustrates this point with ten environmental isolates. Four human isolates exhibit 53-100% relative binding, a trout isolate exhibits 85%, and five isolates from immature pet turtles (Pseudemys scripta elegans) exhibit 46-100% relative binding. Two turtle and one human isolate are indistinguishable on the basis of percent relative binding, all having greater than 96% homology to the reference strain.

DNA Polynucleotide Relationships Between Aeromonas Reference Strain DNA and DNA from Other Gram Negative Bacteria

Table 7 illustrates the percent relative binding from renaturation of various enteric and other bacterial DNA to that of the two Aeromonas references. The percent relative binding values suggest closer genetic relationships between enterics and aeromonads than does their classification. The percent relative binding values of the

Table 6. Reassociation of DNA from environmental isolates of Aeromonas with A. hydrophila ATCC 7966 DNA.

Competitor DNA	Habitat	Percent binding	
		Tm-25°C	Tm-15°C
A. hydrophila OSU #55	Turtle	100	--
" UOMS 8057	Human	100	91
" OSU #94	Turtle	96	--
" OSU #105	Turtle	89	--
A. liquefaciens OSU #3	Trout hatchery	85	67
A. hydrophila OSU #56	Human	80	--
" OSU #109	Turtle	58	59
" UOMS 2619	Human	53	46
" UOMS 2696	Human	53	--
" OSU #72	Turtle	46	--

There is no correlation between relative percent binding values of ten environmental isolates and A. hydrophila ATCC 7966. Note, for example, A. hydrophila OSU #55 and UOMS 8057. Both have greater than 96% binding values to the A. hydrophila reference yet one was isolated from a turtle while the other was isolated from a human infection.

Table 7. Relative percent binding of some gram negative bacterial DNA with Aeromonas reference DNA.

Competitor DNA	Relative percent binding		
	A. hydrophila ATCC 7966		A. salmonicida ATCC 14174
	Tm-25	Tm-15	Tm-25
A. hydrophila ATCC 7966	100	100	79
Pseiomonas shigelloides OSU #23	38	---	--
" ATCC 14029	---	---	41
Vibrio alginolyticus	37	---	--
Shigella flexneri	35	---	--
Citrobacter (lac +)	---	---	45
Citrobacter (lac -)	---	---	23
Erwinia amylovora	24	---	--
Enterobacter liquefaciens	23	---	--
Erwinia carotovora	22	0	0
Enterobacter aerogenes	21	18	44
Arizona	---	---	22
Klebsiella pneumonia	21	---	--
Escherichia coli WP-2	19	2	16
Enterobacter cloacae	15	---	--
Salmonella typhimurium (LT-2)	9	---	20
Vibrio (crab) 50% G+C	7	---	--
Enterobacter hafniae	2	---	19
S. marcescens	2	---	--

Although Aeromonas is classically classified within the family Pseudomonadaceae, the A. hydrophila reference strain has DNA which binds with DNA from Enterobacteriaceae species to a significant degree. DNA with relative percent binding of 0-20% (Tm-25) is considered low resolution or background binding. Note the drop in percent binding values for E. coli and Erwinia carotovora from Tm-25°C to Tm-15°C renaturations.

non-aeromonads to the A. hydrophila reference varies from 2-38% (Tm-25) and from 0-45% (Tm-25) when compared to the A. salmonicida reference. The many lower hybridization values of 10-40% are significant and likely indicate evolutionary affinities with the enteric bacteria.

Genome Size of Aeromonads

Table 8 illustrates the range in genome sizes exhibited by ten aeromonads. Although most of these values are based on only one or two determinations, the overall results indicate a range in genome size of about 30%.

The genome sizes of these aeromonads somewhat parallel percent relative binding values (Tm-25) to the references. For example, DNA samples from A. liquefaciens ATCC 14715 and from A. salmonicida ATCC 14174 have similar genome sizes (2,290 vs. 2,380 x 10^6 daltons) and exhibit 82% relative binding (Tm-25). However, DNA samples from A. liquefaciens ATCC 14715 and the A. hydrophila reference have genome sizes at the limits of the experimental values and exhibit only 65% relative binding. This correlation is more apparent when A. hydrophila OSU #109 is considered. Its DNA and that of the A. salmonicida reference have similar genome sizes (2,280 vs. 2,380 x 10^6 daltons) and 88% relative binding (Tm-25). However, A. hydrophila OSU #109 and the A. hydrophila reference have

Table 8. Variation in genome size of Aeromonas compared to percent binding to reference strain DNA.

	Genome size (X 10^6 daltons)	Relative percent binding Tm-25°C ATCC 7966	ATCC 14174
A. hydrophila CHL #56	1,600 (2)[a]	80	42 61
" UOMS 8057	1,600 (2)	100	61
" ATCC 7966	1,820 (4)	100	79
A. hydrophila anaerogenes ATCC 15467	2,030 (4)	77	65
A. dourgesii anaerogenes ATCC 23212	2,080 (1)	58	--
A. hydrophila UOMS 2696	2,170 (1)	53	
A. hydrophila ATCC 9071	2,210 (1)	80	70
A. hydrophila OSU #109	2,280 (1)	58	88
A. liquefaciens ATCC 14715	2,290 (1)	65	82
A. salmonicida ATCC 14174	2,380 (4)	79	100

The range in genome sizes is roughly 30%. The genome sizes of the ten aeromonads parallel DNA hybridization values of the various strains.

Symbol: [a]Standard deviation about ± 5% as determined from eight E. coli renaturation trials (Tm-25°C).

dissimilar genome sizes and only 58% relative binding (Tm-25).

In addition, aeromonads with small genome sizes similar to the A. hydrophila reference (1, 600 vs. 1, 820 x 10^6 daltons) show greater percent relative binding values to the A. hydrophila reference than to the A. salmonicida reference. DNA from A. hydrophila OSU #56 and UOMS 8057, for example, have 80 and 83-100% relative binding (Tm-25) to the DNA from the A. hydrophila reference. They have only 42-50% relative binding to the DNA from the A. salmonicida reference.

Coincidentally, the four examples in the mid-range of genome size have the lowest percent relative binding values (Tm-25) to both reference strains (53-80% binding).

Biochemical Analysis of Aeromonads

Twenty-three aeromonads were screened for 22 biochemical and physiological properties. Properties tested were chosen for presentation because, according to R. H. W. Schubert (56), they were important in differentiating the species A. hydrophila. Table 9 shows the results of these tests.

Eleven properties correlated to some degree with percent relative binding values (Tm-25) of aeromonads to A. hydrophila ATCC 7966. Based on these correlations, the aeromonads were placed into five groups (Table 10).

Table 9. Biochemical and physiological analysis of 23 Aeromonas species.

Organism	Lactose	Sucrose	Rhamnose	Raffinose	Salicin	Arabinose	Cellobiose	Sorbitol	Voges-Proskauer	Indol	Methyl Red	Citrate	Hemolysis Sheep RBC	Growth in KCN	Lysine Decarboxylase	Phenylalanine Deaminase	EMB Green Sheen	Growth at 37°C	Oxidase	24 Hour Lactose Fermentation	Brown Water-Soluble Pigment	Motility
A. hydrophila ATCC 7966	A	Ag	A	-	-	Ag	-	-	+	+	-	+	β	+	-	-	-	+	+	-	-	+
" UOMS 8057	A	Ag	A	NT	NT	NT	-	-	+	NT	NT	NT	NT	NT	NT	NT	NT	+	+	NT	-	+
" OSU #55	(+)	Ag	Ag	-	Ag	Ag	-	-	+	+	-	+	β	+	-	+	-	+	+	-	-	+
" OSU #94	(+)	Ag	A	-	Ag	Ag	A	-	+	+	-	+	β	+	-	-	-	+	+	-	-	+
" OSU #105	NT	NT	NT	NT	NT	NT	-	A	+	+	-	NT	NT	+	-	NT	NT	+	+	NT	-	+
A. liquefaciens OSU #3	NT	NT	NT	NT	NT	NT	-	-	+	NT	-	NT	NT	NT	NT	NT	NT	+	+	NT	-	+
A. punctata ATCC 11163	NT	NT	NT	NT	NT	NT	A	-	+	+	-	NT	NT	+	NT	NT	NT	+	+	NT	-	+
A. hydrophila ATCC 9071	A	Ag	A	-	-	Ag	-	-	+	+	-	-	β	-	NT	-	-	+	+	-	-	+
" OSU #56	A	A	A	-	A	A	A	A	-	+	-	+	γ	+	-	+	-	+	+	-	-	+
A. hydrophila anaerogenes ATCC 15467	A	A	-	-	-	A	A	A	-	+	-	+	γ	-	-	-	-	+	+	-	-	+
A. punctata caviae ATCC 14486	A	A	A	-	A	A	A	-	-	+	-	+	γ	+	-	+	-	+	+	-	-	+
A. hydrophila formicans ATCC 13137	A	A	A	-	-	A	A	-	-	+	-	+	γ	-	-	+	-	+	+	+	-	+
A. dourgesi ATCC 23211	Ag	Ag	A	-	A	A	-	A	-	+	-	+	γ	-	-	+	-	(+)	+	+	-	-
A. liquefaciens ATCC 14715	NT	Ag	Ag	-	Ag	Ag	-	-	-	(+)	-	+	β	-	-	-	-	(+)	+	-	-	+
A. dourgesi anaerogenes ATCC 23212	A	A	A	A	A	A	A	A	+	+	-	-	β	-	-	+	+	+	+	+	+	-
A. hydrophila OSU #109	A	Ag	A	-	Ag	A	-	A	+	+	-	+	β	+	-	+	-	+	+	+	-	+
" UOMS 2619	A	Ag	A	-	Ag	Ag	-	-	+	+	-	+	α	+	-	-	+	+	+	+	-	+
" OSU #72	A	NT	NT	NT	NT	NT	Ag	Ag	+	+	-	NT	NT	+	NT	NT	NT	+	+	+	-	+
" UOMS 2696	A	Ag	A	-	-	A	-	-	+	+	-	+	β	+	-	+	-	+	+	+	-	+
" CDC 1645-68	A	A	A	-	A	A	NT	NT	NT	+	-	+	γ	+	-	+	-	+	+	NT	-	+
A. salmonicida ATCC 14174	A	Ag	A	-	-	A	-	A	-	-	-	-	α	-	-	+	-	-	+	-	+	-
" OSU #2	A	Ag	A	-	-	A	-	A	-	-	-	-	α	-	-	+	-	-	+	-	+	-
" OSU #19	A	Ag	A	-	-	A	-	A	-	-	-	-	α	-	-	+	-	-	+	-	+	-

Symbol: (+) weak reaction or growth.
NT Not Tested

Table 10. Biochemical and physiological tests correlated with relative percent binding to A. hydrophila ATCC 7966.

Group	Organism	Relative % binding to A. hydrophila ATCC 7966	Aerogenesis	Lactose 24 hr.	Cellobiose	Sorbitol	Citrate	KCN	Voges-Proskauer	Growth 37°C	Hemolysis	Motility	Brown pigment
I	A. hydrophila ATCC 7966	100	+	-	-	-	+	+	+	+	B	+	-
	" UOMS 8057	100	+	NT	-	-	NT	NT	+	+	NT	+	-
	" OSU #55	100	+	-	-	-	+	+	+	+	B	+	-
	" OSU #94	96	+	-	A	-	+	+	+	+	B	+	-
	" OSU #105	89	+	NT	-	A	NT	+	+	+	NT	+	-
	A. liquefaciens OSU #3	85	NT	NT	-	-	NT	NT	+	+	NT	+	-
	A. punctata ATCC 11163	83	+	NT	A	-	-*	+	+	+	NT	+	-
	A. hydrophila ATCC 9071	80	+	-	-	-	-	-	+	+	B	+	-
II	A. hydrophila OSU #56	80	-	-	A	A	+	+	-	+	γ	+	-
	A. hydrophila anaerogenes ATCC 15467	77	-	-	A	A	+	-	-	+	γ	+	-
	A. punctata caviae ATCC 14486	70	-	-	A	-	+	+	-	+	γ	+	-
	A. hydrophila formicans ATCC 13137	66	-	+	A	-	+	-	-	+	γ	+	-
	A. dourgesi anaerogenes ATCC 23212	58	-	+	A	A	-	-	+	+	B	-	-
III	A. hydrophila OSU #109	58	+	+	-	A	+	+	+	+	B	+	-
	" UOMS 2696	53	+	+	-	-	+	+	+	+	B	+	-
	" UOMS 2619	53	+	+	-	-	+	+	+	+	α	+	-
	" OSU #72	46	+	+	Ag	Ag	+	+	+	+	NT	+	-
IV	A. salmonicida OSU #2	85	+	-	-	A	-	-	-	-	α	-	+
	" OSU #19	84	+	-	-	A	-	-	-	-	α	-	+
	" ATCC 14174	79	+	-	-	A	-	-	-	-	α	-	+
V	A. dourgesi ATCC 23211	74	+	+	-	A	-	-	-	(+)	γ	-	-
	A. liquefaciens ATCC 14715	65	+	-	-	-	-	-	-	(+)	B	+	+

Symbols: A = acid, g = gas in durham tube, + = positive, - = no reaction, (+) = weak reaction or growth, NT = Not Tested, * from R.H.W. Schubert's description of A. punctata (24).

The tests vary with respect to their taxonomic significance in separating the different groups. Aerogenesis, growth at 37°C, Voges-Proskauer, reaction motility, and production of a brown water-soluble pigment seem to be taxonomically useful parameters. Discounting group V, which will be discussed later, fermentation of lactose within 24 hours and hemolysis of sheep RBCs are equally reliable with two exceptions. Cellobiose and sorbitol fermentations, citrate utilization, and growth in KCN are the most variable of the selected differentiating tests.

Group I, with relative percent binding values of 80-100% (Tm-25) to the A. hydrophila reference, consists of aerogenic, Voges-Proskauer positive, 37°C positive, motile strains which do not produce a brown water-soluble pigment. Isolates of this group tend to ferment lactose slowly or not at all and usually do not ferment cellobiose and sorbitol. Isolates usually grow in KCN and are β homolytic.

Group II is best differentiated from groups I and III by its lack of gas production. Additionally, all members are cellobiose fermenters and, with one exception, are sorbitol fermenters, Voges-Proskauer positive, and γ hemolytic.

Group III ferments lactose within 24 hours. There is a unique correlation between rapid lactose fermenting strains and their range of relative percent binding values (Tm-25) to the A. hydrophila reference. All seven rapid lactose fermenters have 46-74% binding

values to the A. hydrophila reference. Only two isolates with low binding (ATCC 14486, 14717) failed to ferment lactose. Rapid lactose fermentation is the only phenotypic trait out of 22 tests which corresponds to the relative percent binding values of 46-58% which differentiates group III from group I.

Group IV, consisting of A. salmonicida, is uniquely homogeneous. All 11 traits are identical and the relative percent binding values to the A. hydrophila reference are 79-85%.

Group V, a perplexing pair of ATCC strains, has qualities suggesting a closer relationship to A. salmonicida than to any other group. These two isolates, A. liquefaciens ATCC 14715 and A. dourgesii ATCC 23211, have 65 and 74% relative binding values to the A. hydrophila reference, respectively. However, the two isolates and the three A. salmonicida strains have 7/11 traits in common with each other. The most important of these, suggesting a closer relationship between A. liquefaciens ATCC 14715 and A. salmonicida, are: elaboration of water-soluble pigment, poor growth at 37°C, 82% relative binding value (Tm-25) with the A. salmonicida reference, genome size close to the A. salmonicida reference (2,290 vs. 2,380 x 10^6 daltons), and isolation from a juvenile silver salmon -- a common host of A. salmonicida (by Beijerinck in 1900). The most important traits suggesting a close relationship between A. dourgesii ATCC 23211 and A. salmonicida are: non-motility, poor growth at 37°C, and 58% G+C

base composition.

DNA-DNA Hybridizations Among Aeromonads

In an effort to understand the intergroup and intragroup relationships better, Table 10, DNA-DNA renaturations were performed among different pairs of Aeromonas isolates. The double label membrane filter technique was used to determine percent relative binding between DNA from A. hydrophila anaerogenes ATCC 15467 and DNA from seven other aeromonads. Since ATCC 15467 and ATCC 7966 have essentially the same G+C content, renaturations were conducted at 65.5°C (Tm-25) in 2 X SSC 40% DMSO. Table 11 shows the results of these renaturations.

Table 11. Reassociation of DNA between Aeromonas hydrophila anaerogenes ATCC 15467 and other Aeromonas sp.

Group	Organism	Percent relative binding Tm-25°C A. hydrophila anaerogenes ATCC 15467
II	A. hydrophila anaerogenes ATCC 15467	100
I	A. hydrophila ATCC 7966	77[a]
I	A. hydrophila OSU #55	70
I	A. hydrophila ATCC 9071	51
II	A. hydrophila OSU #56	91
IV	A. salmonicida ATCC 14174	63, 65[b]
	A. punctata OSU #122	52
	A. hydrophila OSU #53	54, 60
	A. hydrophila OSU #86	47

All renaturations were carried out at Tm-25°C using the double label membrane filter technique. Average quantity of DNA on each ATCC 15467 filter was 8.1 ug (^{3}H DNA at 340 cpm/ug). Tm of ATCC 15467 DNA was determined experimentally to be 65.5°C in 2 X SSC 40% DMSO. All experimental details as essentially identical with those used for the other double label membrane filter techniques using ATCC 7966 and ATCC 14174 immobilized DNA filters.

Symbols: [a]Using ATCC 7966 DNA immobilized filter at Tm-25°C = 40°.

[b]Using ATCC14174 DNA immobilized filter at Tm-25°C = 39°.

The only instance where greater than 80% relative binding occurred was with DNA from OSU #56 (91% relative binding). Of the seven aeromonads used in the renaturations, only OSU #56 was a member of group II. It is reassuring that DNA from A. salmonicida ATCC 14174 gave 63% relative binding while the reciprocal test using DNA from ATCC 14714 immobilized on filters (Tm-25) showed an almost identical value of 65% (Table 5).

DNA-DNA renaturations were also performed using the optical renaturation technique (Table 12). DNA from two members of group II, ATCC 23212 and OSU #56, when renatured with DNA from ATCC 15467, demonstrated 80 and 73% relative bindings, respectively, at the more rigorous renaturation temperature (Tm-15). This indicates greater polynucleotide homogeneity between these members within group II than to members (3) outside the group.

A. liquefaciens ATCC 14715, whose DNA has more polynucleotide sequences in common with A. salmonicida than with the A. hydrophila reference (ATCC 7966), demonstrated the lowest interspecific percent relative binding (Tm-15) within the genus. DNA from A. liquefaciens ATCC 14715 renatured with DNA from A. hydrophila anaerogenes ATCC 15467 and with A. hydrophila ATCC 9071 (Tm-15) exhibited 41 and 32% relative binding, respectively. These low values are not surprising since DNA from ATCC 14715 showed only 46% relative binding (Tm-15) with the reference DNA from A. hydrophila

Table 12. Intra and intergroup reassociation of DNA among strains of Aeromonas.

		Percent relative binding Tm-15°C			
		I	I	II	III
		ATCC	ATCC	ATCC	UOMS
Group	Organism	7966	9071	15467	2696
I	A. hydrophila ATCC 7966	100		53[a]	47
I	A. hydrophila ATCC 9071		100	55	
II	A. hydrophila OSU #56			73	
II	A. dourgesii anaerogenes ATCC 23212	64[a] 54		80	
III	A. hydrophila OSU #109	59[a]			65
IV	A. salmonicida ATCC 14174	77			
V	A. liquefaciens ATCC 14715	46[a]	32	41	

Except for the high percent relative binding of A. salmonicida ATCC 14174 DNA to the A. hydrophila ATCC 7966 DNA (77%), percent relative binding values (Tm-15) within the groups have values greater than 64%. Percent relative binding values between members of the groups are equal to or less than 64%. Symbol [a] renatured via double label membrane filter technique all other values determined using optical renaturation procedure.

ATCC 7966.

Thermal Stability of DNA-DNA Duplexes

The thermal stability of interspecific DNA-DNA duplexes formed between the two reference strains are illustrated in Figure 4. The Tm_e for A. salmonicida ATCC 14174 hybridized with A. hydrophila ATCC 7966 is about 3.5C.

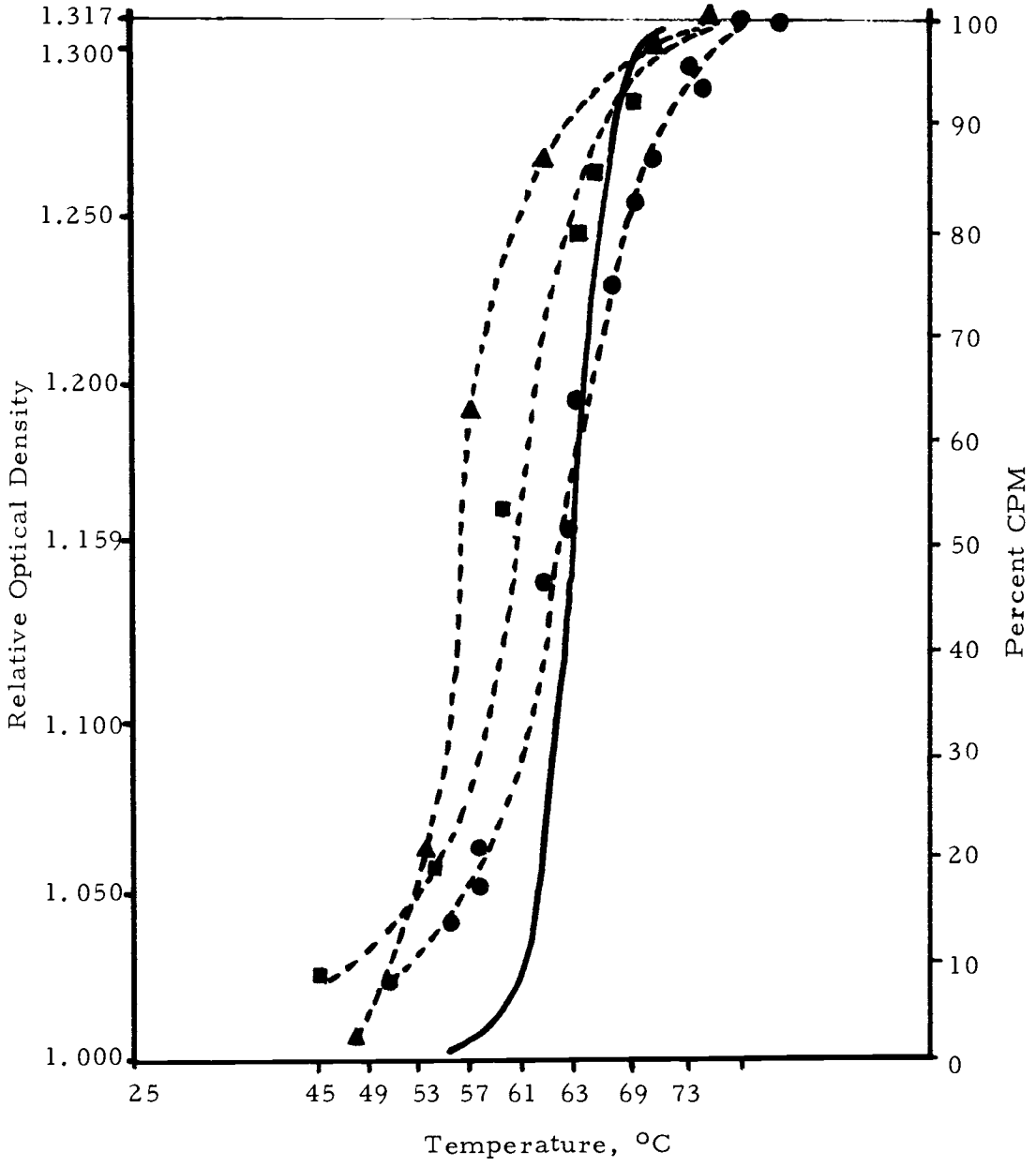

Figure 4. Thermal stability of A. hydrophila ATCC 7966 and A. salmonicida ATCC 14174 filter-bound DNA-DNA duplexes renatured on ATCC 7966 filter-bound DNA. Renatured and eluted in 2 X SSC 40% DMSO, ATCC 7966 DNA-DNA duplexes were renatured at 40°C (Tm-25) and 50°C (Tm-15) and ATCC 7966 X ATCC 14174 DNA-DNA duplexes were renatured at 39°C (Tm-25). One µg ^{32}P labeled sheared DNA was 11, 600 cpm/ug ATCC 14174 DNA and 13, 700 cpm/µg ATCC 7966 DNA. Immobilized DNA was 8.2 ug/filter ATCC 7966. Symbols: ●, A. hydrophila ATCC 7966 fragments (Tm-15); ▲ , A. salmonicida ATCC 14174 fragments (Tm-25); ■ , A. hydrophila ATCC 7966 fragments (Tm-25); solid line, optical thermal-denaturation profile of sheared ATCC 7966 DNA in same buffer.

DISCUSSION

The study of a combination of phenetic and genetic relationships among aeromonads was undertaken for two main reasons: (1) to define the diversity among isolates from various habitats, and (2) to ascertain their relatedness to the enteric bacteria.

The spectrum of guanine plus cytosine base composition for 22 aeromonad isolates studied was 58-61%. The two A. dourgesii and three A. salmonicida isolates had G+C contents of 58%. All other named species varied within the base composition spectrum.

The range in percent relative binding of Aeromonas sp. DNA was 46-100% when renatured to A. hydrophila ATCC 7966 DNA and a comparable 42-95% when renatured to A. salmonicida ATCC 14174 DNA (Tm-25). Considering the ranges of homology [percent relative binding (Tm-25)] anticipated among isolates of one species, the definition of A. salmonicida becomes uniquely important. Three isolates of A. salmonicida exhibited 79-85% homology with A. hydrophila ATCC 7966. If A. salmonicida is to continue to be acceptable as a species, then the renaturation data (Table 5) and physiological groupings (Table 10) suggest that group II is a third, yet unnamed, species.

A. salmonicida OSU #19 is typical of the three A. salmonicida strains examined. Like the other A. salmonicida strains, OSU #19 demonstrated greater than 90% homology to the A. salmonicida

reference and 79-85% homology to the A. hydrophila reference (Tm-25). The majority of the other aeromonads (11/19) show less homology to the A. hydrophila reference than do the A. salmonicida isolates.

Schubert (56) found many biochemical and physiological properties which varied from strain to strain of Aeromonas hydrophila. Table 9 is a compilation of 22 variable properties tested with 23 aeromonads. From this study, 11 properties correlated to some degree with percent relative binding values (Tm-25) of the aeromonad DNA to A. hydrophila ATCC 7966. Based upon these correlations, the aeromonads were placed into five groups (Table 10).

As illustrated in Table 10, group II includes the anaerogenic aeromonads exhibiting 58-80% homology to the A. hydrophila reference. The chief phenotypic traits which differentiate this group from group I (A. hydrophila ATCC 7966-like isolates) are anaerogenic fermentation, negative Voges-Proskauer reactions (with one exception) and no sheep RBC hemolytic enzymes. Group II is differentiable from group IV (A. salmonicida-like isolates) on the basis of cellobiose fermentation, growth at 37°C, and lack of brown water-soluble pigment.

In 1964, Adansonian analyses of aerogenic A. punctata (A. hydrophila) and anaerogenic A. formicans (A. caviae) were undertaken utilizing 134 phenotypic properties and DNA base compositions (11). Interestingly, when A. punctata ATCC 11163 (group I, Table 10) was

compared with A. hydrophila formicans ATCC 13137 (group II), the former showed only 63% interspecies similarity with the latter. Colwell and Mandel considered that a 75%+ similarity constitutes the "species level." Their analysis indicated that the genus is divisible into two or possibly three species (not including A. salmonicida). Whereas A. formicans (A. caviae) ATCC 13137 demonstrated high affinity to other strains of A. formicans, A. punctata strains did not demonstrate a similar homogeneity. They, in fact, represented a grouping more diverse than would be expected within one species.

In 1967, Schubert investigated the anaerogenic aeromonads (54). His basic assumption was that there were two species of aerogenic motile aeromonads which could be differentiated on the basis of presence or absence of the enzyme 2, 3-butanediol dehydrogenase and anaerogenic fermentation of glycerol. In his estimation the anaerogenic aeromonads were subspecies of A. punctata (subsp. caviae) or A. hydrophila (subsp. anaerogenes). Considering the earlier work by Colwell and Mandel (46), it seems reasonable in the light of the present studies that speciation should be based on the anaerogenic characteristic rather than on elaboration of 2, 3-butanediol dehydrogenase.

Aerogenicity takes precedence as a diagnostically sound criterion for speciation. The formation of gas as a result of sugar fermentation is very important for differentiating members of the enteric group. E. coli produces gas and can therefore be differentiated from

the pathogenic Shigella sp. and Salmonella typhosa, which do not produce gas. Not all strains of E. coli are of course aerogenic, however.

As a practical matter, few characteristics are infallible. The argument, therefore, over what constitutes differentiable characteristics is a philosophical rather than a biological question and must be left to the test of time and experience.

Gilardi (21), in 1967, compiled data from Eddy (14-16), Ewing (19, 20) and Schubert (50, 53-55). He reached the following conclusions: A. punctata (A. hydrophila) was motile, Voges-Proskauer positive, and aerogenic -- properties of group I isolates; A. caviae (A. formicans) was motile, V-P negative, and anaerogenic -- properties of group II isolates; A. salmonicida was non-motile, citrate negative, V-P negative, and aerogenic -- properties of group IV.

In 1970, Gilardi, Bottone, and Birnbaum (22) reviewed the genus, noting especially the work of Habs and Schubert (23) and Schubert (50, 53-55). Gilardi concluded that evidence existed for three species -- A. hydrophila, A. punctata, and A. salmonicida. In addition he, like Schubert, considered anaerogenic strains as subspecies of these three species. However, this opinion was biased by the false assumption that aeromonads were almost entirely aerogenic fermenters of glucose.

To the contrary, Ross, Rucker, and Ewing (41) found in 1966 that 100% of 14 aeromonads isolated from diseased rainbow trout were anaerogenic glucose fermenters and V-P negative. These findings

correspond to our group II. In 1970, Nygaard, Bissett, and R. M. Wood (38) found that of 48 aeromonad isolates, 47% were anaerogenic glucose fermenters.

Groups I and III (Table 10) pose a taxonomic ambiguity because they have a preponderance of traits in common. However, these two groups have the greatest divergence of polynucleotide sequences, group III demonstrating 46-58% relative binding (Tm-25) with A. hydrophila ATCC 7966 as reference. Group III is differentiable from group I only on the basis of lactose fermentation within 24 hours at 37°C. Group III is separable from group II on the basis of aerogenesis, V-P test results, and perhaps hemolysis. Group III is separable from group IV (A. salmonicida) on the basis of rapid lactose fermentation, citrate utilization, growth in KCN, V-P reaction, growth at 37°C, motility, and lack of brown water-soluble pigmentation when grown on nutrient agar medium.

The two aberrant A. salmonicida-like aeromonads, A. dourgesii ATCC 23211 and A. liquefaciens ATCC 14715 (Group V) might be classified as subspecies of A. salmonicida. They have only 6/11 traits in common with each other but, taken individually, have 8/11 traits in common with A. salmonicida (Table 10).

Group IV (A. salmonicida) and group V (A. salmonicida-like variants) are distinguishable from the other groups on the basis of their poor growth at 37°C. The value of using optimum growing

temperature as an important diagnostic test for aeromonads was considered by Gilardi (21). He stated that "...the growth at low temperatures remains the only important characteristic to separate the salmonicida group from aberrant strains of other Aeromonas species." He added, however, that "...growth at different temperatures has hitherto never been used to separate genera."

To test the validity of this approach, all three A. salmonicida strains were transferred twice at 24 hour intervals onto nutrient agar plates, incubated first at 25°C then at 34°C with heavy inocula from the 25°C plates. After incubation at 34°C, another heavy inoculum was transferred to another nutrient agar plate and reincubated at 34°C. All three A. salmonicida strains grew moderately well on the second transferred plate at 34°C. Finally, all three strains were transferred with heavy inocula to fresh nutrient agar plates and incubated at 37°C. A. salmonicida OSU #2 grew moderately after 24 hours while the other two strains did not. Two repetitions of this temperature-growth training procedure yielded the same results. "Trained" OSU #2 was successfully transferred twice at 24 hour intervals at an incubation temperature of 37°C. OSU #2 would not grow at 37°C when transferred directly from stock cultures. The "trained" strain did not elaborate the brown water-soluble pigment at 37°C but, when returned to room temperature, regained that ability. The "trained" strain was in pure culture at all times and still remained oxidase positive and

appeared morphologically identical to its precursor.

A. punctata reportedly has an optimum growing temperature of 25-30°C and A. salmonicida an optimum growing temperature of 20-25°C (11). Optimum growing temperatures were not determined for any of the strains in this study. The importance of optimum temperature as a taxonomic trait remains uncertain.

On the basis of DNA homologies, Johnson (26) proposed a taxonomic classification scheme dividing genera into varieties, species, and subspecies. His proposal took into consideration the genetic definition of Marmur, Falkow, and Mandel (36). This concept of species is grounded in the traditional phenotypic oriented taxonomy with nucleic acid renaturations accepted as one of many tools. In addition, Johnson considered the concept of genospecies proposed by A. W. Ravin (39). This concept is based on nucleic acid homologies with specific phenotypic traits used to separate the resulting groups.

Johnson proposed that genospecies should encompass 60-100% homologies with a range of 80-100% homologies constituting genovarieties. He suggested that 60-70% homologies represent genosubspecies, with 70-80% homologies of overlap. These proposals by Johnson roughly correspond with the hypothesis by Brenner that 70% or greater homologies exist between species (6).

Groups I and IV with 80-100% and 79-85% homologies, respectively, could thus be combined into one genovariety. Groups II and V

with 58-80% and 65-74% homologies could be genosubspecies. Group III would be a second genospecies with 46-58% homologies. In such cases, 24 hour lactose fermentation could be used since the two strains in group II which rapidly fermented lactose had the lowest homologies -- 58% and 66%. Thus these two strains could be added to the group III genospecies.

Interestingly, Johnson considered that 20-50% homologies designated closely related genospecies. We found eight enterics, two Erwinea, two Plesiomonas shigelloides, and a Vibrio alginolyticus isolate which fell within this range of homology. However, almost all other studies indicate that 20-50% homologies are commonly found between closely related genera and not closely related species.

Although Plesiomonas shigelloides (Aeromonas shigelloides) DNA has at least 7% lower G+C base composition than the aeromonads, two isolates demonstrated 38% and 41% homologies. Since these values are lower than the lowest value for an aeromonad (OSU #72, 46% to ATCC 7966), our data does not conflict with the placement of P. shigelloides in the genus Vibrio (24).

Vibrio alginolyticus and Shigella flexneri DNA demonstrated 37% and 35% homologies with ATCC 7966 DNA. These values suggest a closer genetic relationship between S. flexneri and Aeromonas than had previously been assumed. The finding that V. alginolyticus was genetically similar was not so surprising. Vibrio species have many

characteristics in common with aeromonads. For example, aeromonads and vibrios may produce disease in fresh water fish, ferment carbohydrates anaerogenically, and produce cytochrome oxidase. In general, there are a very few characteristics which differentiate Vibrio from Aeromonas (7). The use of cell curvature itself is of no diagnostic value since many genera have curved strains (13). Vibrio sensitivity to vibriostat 0/129 (47-59), spheroplast formation (13), tolerance to various sodium chloride concentrations (43), and amino acid decarboxylation determinations are useful tests, however, for differentiating vibrios from aeromonads (7).

Vibrio comma is so similar to anaerogenic Aeromonas that Park, in 1962, considered combining the two into a single genus (13). In 1971, H. K. Dahle and O. Sandvik (12) reported serological cross reactions between proteinase fractions produced from A. liquefaciens ATCC 14715 and two V. comma strains.

The aeromonads seem closely related to the Enterobacteriaceae. For example, the regulation of amino acid biosyntheses are similar to those operating in E. coli and dissimilar to aerobic pseudomonads. Also, anaerogenic aeromonads produce β-galactosidase which is serologically undifferentiable from that of E. coli (63). When zone electrophoresis is used, a significant number of enzyme types in Serratia marcescens, Enterobacter aerogenes, and Enterobacter cloacae are indistinguishable from those in aeromonads (J. N. Baptist,

personal communication). DNA from Enterobacter aerogenes and a lactose fermenting Citrobacter exhibit approximately 40% homologies with A. salmonicida reference DNA. Of 16 renaturations (Tm-25) between aeromonad and enteric DNA, 56% demonstrated greater than 20% homology, clearly indicating close evolutionary ties.

We believe that the detectable DNA-DNA reassociation of some members of Enterobacteriaceae is significant. Our data suggests a close association between these fermentative organisms and Enterobacteriaceae. A reassessment of Pseudomonadaceae, the taxonomic family with which the aeromonads have been classically associated, is now in order.

Based on studies with synthetic polynucleotides, thermal stability of double stranded nucleic acids was found to be sensitive to small streaches of unpaired bases (2, 30). The following formula of Ullman and McCarthy (63) is considered by Anderson and Ordal (44) to be the current best estimate of mispaired bases: 1.6 Tm_e/1% mispaired bases (Tm_e = difference in elution Tm of homologous and heterologous renaturations).

Our Tm_e of homologous DNA-DNA duplexes was comparable to that of Johnson and Ordal (25). The Tm_e of the ATCC 7966 X ATCC 14174 DNA was approximately 3.5°C (59.5°-56.0°). This value is consistent with the results of Anderson and Ordal (25) and Johnson (26) for DNA-DNA duplexes with comparable percent relative binding.

Therefore, the number of mispaired bases between DNA of Aeromonas hydrophila ATCC 7966 and A. salmonicida ATCC 14174 is approximately 2%.

The range in genome size among the aeromonads is from 1600-2400 x 10^6 daltons, roughly a 30% variation. As seen in Table 8, the genome sizes of ten selected aeromonads roughly parallels percent relative binding with the two reference strains. Group I and II isolates have the smallest genomes, about 1600-2100 x 10^6 daltons. Group III has an intermediate genome size range of approximately 2100-2300 x 10^6 daltons. Groups IV and V have the largest genome sizes with about 2300-2400 x 10^6 daltons.

Aeromonas, a diverse genus phenotypically and genetically, may be split into at least four genospecies. Our definitions of these genospecies (Table 10) are dissimilar to those of others in that they are not based upon source, habitat, optimum growth temperature, pathogenicity (4), arrangement of flagella (14), or production of 2,3-butanediol dehydrogenase (51, 53). However, the amalgamation of genetic determinations (including G+C base composition, DNA-DNA renaturations, and genome size) and phenotypic traits lends validity to our scheme.

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