DNA:DNA Reassociation Analysis of *Aeromonas salmonicida*

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DNA from 26 *Aeromonas salmonicida* strains, namely 11 'typical' and 15 so-called 'atypical' strains, was used to assess the taxonomic relatedness within the species. The genomes were characterized by determination of DNA base composition, DNA:DNA reassociation, calculation of sequence divergence following reassociation, and by genome size estimations. By comparison with DNA obtained from controls and the *Aeromonas hydrophila* group, *A. salmonicida* strains were determined to be correctly placed with respect to genus and species. *A. salmonicida* subspecies *salmonicida* (the 'typical' group) was an extremely homogeneous taxon. The 'atypical' strains were more diverse, but distinct biotypes were recognizable. The first biotype included several geographically diverse isolates from goldfish. The second recognizable biotype included strains isolated from European carp. Other 'atypical' isolates could not be grouped but showed enough internal homology to be retained within the species. The *A. salmonicida* subspecies *achromogenes* and *masoucida* were found to be closely related to the motile aeromonads. It is considered that the present classification of *A. salmonicida* is unsuitable and should be restructured to include *A. salmonicida* subspecies *salmonicida*, subspecies *achromogenes* (to include the present subspecies *masoucida*), and the reintroduced subspecies *nova*.

INTRODUCTION

The genus *Aeromonas* was originally proposed by Kluyver & van Niel (1936) to include Gram-negative bacteria associated with freshwater environments that were generally motile by means of polar flagella. Categorizing species within the genus, particularly among motile isolates, has been extremely variable and confusing. Colwell *et al.* (1986) believed that this was due, in large part, to the *a priori* phenotypic characteristics given the *Aeromonas hydrophila* group which place it midway between the Vibrionaceae and Enterobacteriaceae, i.e. sharing characteristics with each. These workers proposed removal of the genus *Aeromonas* from the family Vibrionaceae and its elevation to family status i.e. the Aeromonadaceae (Colwell *et al.*, 1986). Within the genus *Aeromonas*, as currently defined, there are two distinct groups, namely the *A. hydrophila* group which consists of the mesophilic and generally motile species *A. hydrophila*, *Aeromonas caviae* and *Aeromonas sobria* (Popoff, 1984), and the non-motile psychrophilic group referred to as *Aeromonas salmonicida*.

A. salmonicida, unlike the motile aeromonads, is rarely isolated from surface waters, and is described by Popoff (1984), in *Bergey's Manual of Systematic Bacteriology*, as an obligate pathogen of fish. The classification of this non-motile aeromonad was originally straightforward (Snieszko, 1957), but subsequently, Schubert (1967) and Kimura (1969) described two subspecies. These were listed by Schubert (1974), in the 8th edition of *Bergey's Manual of Determinative Bacteriology*, as *A. salmonicida* subsp. *achromogenes* and *A. salmonicida* subsp. *masoucida*.

Abbreviations: RBR, relative binding ratio; GU, goldfish ulcer; CE, carp erythrodermatitis.

A. salmonicida was also considered to be associated solely with the fulminant septicaemic disease, i.e. furunculosis, in salmonid fishes. More recently however, *A. salmonicida* has been isolated from chronic, ulcerative diseases in both salmonid and non-salmonid fish species including Atlantic salmon, sable fish, European carp and goldfish (Paterson *et al.*, 1980; Evelyn, 1971; Bootsma *et al.*, 1977; Elliot & Shotts, 1980). This group of isolates exhibit fastidious nutritional requirements, slow growth and distinctive biochemical properties, and because of their broader host range and the different pathogenesis so produced, have been designated 'atypical' *A. salmonicida*.

Characterization of this 'atypical' group is becoming increasingly important as the frequency of isolation continues to rise. This is due largely to the use of better primary isolation techniques, notably the presence of blood or haem in complex growth media (Ishiguro *et al.*, 1986). On the basis of numerical taxonomy McCarthy (1978) suggested that these 'atypical' strains be included in a new subspecies, i.e. *A. salmonicida* subsp. *nova*. The confusion surrounding this new group of organisms is illustrated by a number of isolates from trout which were initially classified as *Haemophilus piscium* (Snieszko *et al.*, 1950). Subsequent evidence, including DNA base composition, serology and bacteriophage susceptibilities (Kilian, 1976; Paterson *et al.*, 1980; Trust *et al.*, 1980), has indicated that *H. piscium* isolates are probably 'atypical' strains of *A. salmonicida*.

To define more accurately the classification of this new group of 'atypical' *A. salmonicida*, we have examined the DNA from a large number of isolates recovered from diverse fish species and geographical locations. The DNA has been characterized using the S1 nuclease/TCA hybridization technique (Crosa *et al.*, 1973), in conjunction with base composition and genome size estimates.

METHODS

Bacterial strains and media. The bacteria are listed in Table 1. Stock cultures were maintained at -70°C in 15% (v/v) glycerol/Tryptic Soy Broth (TSB; Gibco). Typical *Aeromonas* strains were cultured on Tryptic Soy Agar (TSA; Gibco) at 20°C for 48 h. Atypical strains were cultured on TSA supplemented with 5% (v/v) human blood for 48–72 h. *Escherichia coli* and *Vibrio anguillarum*, as control strains, were grown on TSA at 37°C and 30°C , respectively.

Isolation of DNA. Sufficient cultures to provide 1.5–2 g wet packed cells were harvested from solid media into 50 ml samples of saline/EDTA (0.15 M-NaCl, 0.1 M-EDTA, pH 8). Cells were collected by centrifugation at 17000 g for 20 min, resuspended in another 50 ml of saline/EDTA and recentrifuged. DNA was then extracted by the chloroform/isoamyl alcohol (CI) method of Marmur (1961). Following pronase, RNAase and CI extraction procedures, DNA purity was assessed using the 260/230 nm and 260/280 nm absorbance ratios. Repeated rounds of extraction were done until the absorbance ratios were greater than 2.0 and 1.8, respectively. DNA samples were then dialysed extensively against distilled water followed by dialysis against 0.42 M-NaCl. DNA samples were sonicated to an approximate M_r of 2.5×10^5 (as judged by agarose gel electrophoresis) and concentrations adjusted to $300\text{ }\mu\text{g ml}^{-1}$. Samples were divided and stored at -70°C .

DNA base composition. This was estimated using the thermal denaturation technique as described by Owen *et al.* (1969). Denaturation was measured in $0.1 \times \text{SSC}$ buffer (SSC is 0.15 M-NaCl buffered with 0.015 M-trisodium citrate at pH 7.0) in increments of $0.5^{\circ}\text{C min}^{-1}$ in a Gilford 250 spectrophotometer equipped with a Gilford 2527 thermoprogrammer, 6046 analog multiplexer and a 6051 recorder. Samples were analysed in triplicate, with a fourth cuvette containing an adenine blank to enable correction for solvent expansion. DNA base compositions were expressed as percentage guanine plus cytosine relative to the total base content (mol% G+C).

Radiolabelling of probe DNA and DNA:DNA reassociation. DNA prepared by the Marmur technique was radiolabelled *in vitro* using the 'nick-translation' procedure of Rigby *et al.* (1977) as described previously (Belland & Trust, 1982). Using tritiated dCTP and dATP specific activities of approximately 4.5×10^7 c.p.m. μg^{-1} were obtained. Reassociation experiments were done using $0.1\text{ }\mu\text{g}$ of radiolabelled probe DNA and a 1500-fold excess of cold DNA at 70°C (optimal reassociation temperature) and 85°C (stringent reassociation temperature) for 20 h in 0.42 M-NaCl. The reassociation protocol was that described previously (MacInnes *et al.*, 1979). Sequence homology was estimated by calculating the ratio of the amount of radiolabelled probe present in the S1-treated and untreated samples following subtraction of S1-resistant 'core' material ($5.3 \pm 0.8\%$ at 70°C and $7.6 \pm 1.4\%$ at 85°C). Results were then normalized to the homologous reaction value (75–83%) after correction for self-reassociation ($<3\%$) to give the relative binding ratio (RBR).

Table 1. *Bacterial strains and their mol% G + C ratios*

ATCC, American Type Culture Collection; CIP, Collection de l'Institut Pasteur, Paris, France; NCMB, National Collection of Marine Bacteria Aberdeen, UK. FL, furunculosis; GU, goldfish ulcer disease; CU, carp erythrodermatitis.

Species	Strain designation	Mol% G + C	Source
<i>Aeromonas salmonicida</i>			
'Typical'	A440 (ATCC 14174)	55.7	Brook trout, USA
	A251 (NCMB 1102)*	57.4	Atlantic salmon, Scotland, UK
	A202	57.3	Japan
	A204	57.8	Japan
	A362	56.1	Atlantic salmon, Scotland, UK
	A438	52.6	Coho salmon, BC, Canada
	A449	57.8	Brown trout, Eure, France
	A450	54.2	Brown trout, Tarn, France
	A451	55.7	Rainbow trout, Manch, France
	A470	53.6	Coho salmon, Wash., USA
	A488	54.6	Brook trout, Leetown, USA
'Atypical'	A400	56.7	Goldfish, Australia
	A406	57.3	Goldfish, Australia
	A419	56.4	Goldfish, Ark., USA
	A491	56.6	Goldfish, Md., USA
	A452	53.0	Carp, Netherlands
	A477	52.4	Carp, Denmark
	A479	54.0	Carp, Netherlands
	A480	53.3	Carp, Netherlands
	A460	55.3	Atlantic salmon, NS, Canada
	A436	57.8	Sable fish, BC, Canada
	A474	55.2	Atlantic cod, Canada
Subsp. <i>chromogenes</i>	A478 (ATCC 19261)*	59.7	Sea trout, Scotland
Subsp. <i>masoucida</i>	A206 (ATCC 27013)*	60.9	Masou, Japan
<i>Haemophilus piscium</i>	A430 (ATCC 10801)	56.1	Trout
	A442 (ATCC 15711)	56.3	Trout
<i>Aeromonas hydrophila</i>	A234 (CIP 7432)	61.9	
	A427 (ATCC 7966)*	61.9	
	A428 (ATCC 19570)	60.9	
	A516	59.8	
<i>Aeromonas sobria</i>	A235 (CIP 7433)*	57.8	
<i>Aeromonas caviae</i>	A250 (NCMB 74)*	59.9	
<i>Vibrio anguillarum</i>	V2911	44.6	
<i>Escherichia coli</i>	B (ATCC 11303)	50.9	
<i>Campylobacter fetus</i>	CIP 5396*	33.6	

* Type strain.

Sequence divergence. Homologous and heterologous double-stranded DNAs following reassociation were digested with S1 nuclease at temperature increments between 65–95 °C and the temperature at which 50% of the labelled DNA was rendered TCA-soluble was calculated ($\Delta T_{m(e)}$; Crosa *et al.*, 1973). The difference between the $T_{m(e)}$ for homologous and heterologous double-stranded DNA is defined as $\Delta T_{m(e)}$ or sequence divergence. A decrease in $\Delta T_{m(e)}$ of 1.0 °C was taken to correspond to approximately 1% base mismatching or sequence divergence (McConaughy & McCarthy, 1970). The $\Delta T_{m(e)}$ values were calculated for double-stranded DNAs reassociated at 70 °C.

Genome size estimations. Renaturation kinetics were used to estimate genome sizes (Britten *et al.*, 1974; Gillis *et al.*, 1970). Absorbance changes were monitored using sealed thermocuvettes in a Gilford model 250 spectrophotometer equipped with a thermoprogrammer (model 2527) and reference compensator. $C_0 t_{0.5}$ values were calculated from the time of 50% renaturation ($t_{0.5}$) and the initial DNA concentration (C_0), using a value of 309 for the mean M_r of a nucleotide (Park & De Ley, 1967). The genome size of a test organism (x) was calculated according to

$$C_0 t_{0.5}(x)/C_0 t_{0.5}(E. coli) = M_r(x)/M_r(E. coli)$$

E. coli K12 DNA (genome $M_r = 2.7 \times 10^9$) was used as a reference (Klotz & Zimm, 1972).

Table 2. DNA homologies (RBR; %) and sequence divergence ($\Delta T_{m(e)}$; °C) between *A. salmonicida* 'typical' reference strains [A440 (ATCC 14174) and A251 (NCMB 1102)] and 'atypical' reference strain [A419 (GU)] and representative 'typical' strains of *A. salmonicida* and selected control strains

ND, Not determined.

Source of unlabelled DNA	Reference DNA ... A440 (ATCC 14174)			A251 (NCMB 1102)			A419(GU)		
	RBR at:			RBR at:			RBR at:		
	70 °C	85 °C	$\Delta T_{m(e)}$	70 °C	85 °C	$\Delta T_{m(e)}$	70 °C	85 °C	$\Delta T_{m(e)}$
A440 (ATCC 14174)	100	100	—	97	92	0.6	83	76	1.6
A251 (NCMB 1102)	98	92	0.8	100	100	—	85	79	1.2
A362	98	89	0.5	99	92	0.2	64	49	4.2
A438	104	95	0.5	97	94	0.4	69	52	ND
A449	94	94	1.0	96	90	0.8	76	65	2.5
A450	92	90	ND	90	82	ND	74	66	ND
A451	97	93	ND	95	95	ND	80	75	ND
A470	103	99	0.2	101	96	0.2	63	50	5.1
A488	102	100	0.8	92	88	0.8	79	71	2.2
A204	88	79	2.1	82	76	2.4	84	78	1.5
A202	85	80	2.5	80	69	2.8	81	69	1.8
<i>V. anguillarum</i>	25	8		29	4		20	5	
<i>E. coli</i> B	6			10			12		
<i>C. fetus</i>	3			8			2		

RESULTS

DNA : DNA reassociation

Sequence homology results are reported in Tables 2–4 as RBR values for which standard deviations of triplicate determinations did not exceed $\pm 2.1\%$ at 70 °C and $\pm 4.2\%$ at 85 °C. Sequence divergence values ($\Delta T_{m(e)}$) were also determined in triplicate and standard deviations did not exceed ± 0.8 °C.

The 'typical' strains of *A. salmonicida* formed an extremely homogeneous group (Table 2) with only the two Japanese isolates (A202 and A204) binding at lower than 92% at the 70 °C optimal reassociation temperature (88% and 85% respectively) when probed using A440 (ATCC 14174) DNA. These data are summarized in Table 3 and show the 'typical' strains to be related at a $97 \pm 6.4\%$ level at 70 °C and to have a sequence divergence of 0.9 ± 0.8 °C. This was confirmed using a second probe DNA from another 'typical' isolate, A251 (NCMB 1102), which showed a mean RBR of $94 \pm 7.0\%$ at 70 °C with a mean $\Delta T_{m(e)}$ value of 0.9 ± 1.0 °C. Again the two Japanese isolates were the only strains which showed significant differences in genetic relatedness, i.e. $<90\%$ RBR and >1.0 °C sequence divergence. The RBR values were significantly lower when 'typical' DNAs were probed with DNA from an 'atypical' strain, A419(GU), varying from 52–85% at the optimal temperature (Table 2) and dropping substantially when the stringency of reassociation was increased. RBR values averaged $76 \pm 7.8\%$ at 70 °C and sequence divergence values averaged 2.5 ± 1.4 °C (Table 3). In several instances (e.g. A362 and A470) the values approached those recommended for separate species designation (Grimont *et al.*, 1980). Hybridization to control DNAs from *V. anguillarum* (V2911), *E. coli* B and *C. fetus* showed that, except in the case of V2911, very little genetic similarity was apparent. *V. anguillarum* is known to be much closer to *A. salmonicida* genetically than is *E. coli* (McCarthy, 1978).

The 'atypical' isolates formed a more diverse group than the 'typical' isolates. When probed using DNA from the *A. salmonicida* type strain A440 (ATCC 14174) homology values at 70 °C ranged from 60–90% with sequence divergence values ranging from 1.5 to 5.3 °C (Table 4). Mean values (Table 3) corresponded to the reciprocal experiments with an RBR value of $76 \pm 9.4\%$ at 70 °C and a sequence divergence value of 2.6 ± 1.2 °C. Two distinct clusters of

Table 3. Summary of DNA homology (RBR; %) and sequence divergence ($\Delta T_{m(e)}$; °C) among strains of *A. salmonicida* and *A. hydrophila*

Nos of isolates are in parentheses. ND, Not determined.

Reference DNA ...	<i>A. salmonicida</i>						<i>A. hydrophila</i>	
	A440		A419(GU)		A479(CE)		A428	
	RBR	$\Delta T_{m(e)}$	RBR	$\Delta T_{m(e)}$	RBR	$\Delta T_{m(e)}$	RBR	$\Delta T_{m(e)}$
<i>A. salmonicida</i>								
'Typical' (11)	97 ± 6.1	0.9 ± 0.8	76 ± 7.8	2.5 ± 1.4	ND	ND	53 ± 9.6	6.0 ± 0.8
'Atypical' (15)	76 ± 9.4	2.6 ± 1.2	76 ± 17.1	2.6 ± 1.9	76 ± 16.5	2.8 ± 2.2	55 ± 16.4	6.6 ± 1.1
GU (4)	78 ± 1.3	1.9 ± 0.1	99 ± 3.0	0.6 ± 0.5	79 ± 2.9	2.1 ± 0.3	57 ± 8.7	6.0 ± 0.2
CE (4)	74 ± 2.2	2.6 ± 0.3	76 ± 4.7	2.2 ± 0.5	96 ± 5.9	0.7 ± 0.5	53 ± 5.6	7.2 ± 0.4
Others (4)	75 ± 13.9	3.0 ± 1.7	63 ± 10.8	4.5 ± 1.3	63 ± 12.8	4.4 ± 2.2	54 ± 23.8	6.6 ± 1.5
<i>A. hydrophila</i>	49 ± 11.0	6.5 ± 0.5	53 ± 12.9	6.1 ± 0.6	ND	ND	82 ± 15.2	3.2 ± 3.5

Table 4. DNA homologies (RBR; %) and sequence divergence ($\Delta T_{m(e)}$; °C) between *A. salmonicida* 'typical' reference strains [A440 (ATCC 14174)] and 'atypical' reference strains [A419 (GU) and A479 (CE)] and representative 'atypical' strains of *A. salmonicida*

ND, Not determined.

Reference DNA ...		A440 (ATCC 14174)			A419(GU)			A479 (CE)		
Source of unlabelled DNA		RBR at:			RBR at:			RBR at:		
		70 °C	85 °C	$\Delta T_{m(e)}$	70 °C	85 °C	$\Delta T_{m(e)}$	70 °C	85 °C	$\Delta T_{m(e)}$
A400	GU	81	75	1.9	98	99	1.0	76	71	2.2
A406		80	72	1.8	96	96	1.0	79	68	2.1
A419		80	81	1.8	100	100	—	83	79	1.6
A491		78	76	2.0	103	98	0.5	78	72	2.4
A452	CE	75	65	2.5	75	70	2.4	101	95	0.5
A477		77	63	2.2	78	69	2.8	92	88	0.9
A479		72	59	3.0	81	75	1.7	100	100	—
A480		73	70	2.6	70	68	2.0	89	86	1.2
A460		86	79	1.5	54	40	5.2	61	47	3.2
A430 (ATCC 10801)		65	53	4.1	58	49	4.9	63	52	4.1
A442 (ATCC 15711)		62	59	ND	51	47	ND	61	49	4.8
A436		92	88	1.2	82	77	ND	85	79	1.7
A474		60	50	5.3	60	48	4.6	73	60	3.1
subsp. <i>achromogenes</i>										
A478 (ATCC 19261)		70	53	4.3	67	52	5.6	46	38	8.2
subsp. <i>masoucida</i>										
A206 (ATCC 27013)		90	85	1.8	71	59	2.4	53	40	6.1

genetically related organisms were apparent when probe DNAs from goldfish ulcer [A419(GU)] and carp erythrodermatitis [A479(CE)] isolates were used. The GU isolates showed a mean within group RBR value of $99 \pm 3.0\%$ while being only $79 \pm 2.9\%$ related to the CE isolate. Conversely the CE strains showed a within group relatedness of $96 \pm 5.9\%$ but were only $79 \pm 2.9\%$ related to the GU isolate used as probe. Both probe DNAs were related at significantly lower levels to the remaining 'atypical' isolates [i.e. $63 \pm 10.8\%$ for A419(GU) and $63 \pm 12.8\%$ for A479(CE)]. In particular the *A. salmonicida* subsp. *achromogenes* isolate [A478 (ATCC 19261)] appeared to be removed from both 'atypical' probe DNAs at levels exceeding those defined by Grimont *et al.* (1980) for species categorization. The *H. piscium* [A430 (ATCC 10801) and A442 (ATCC 15711)] isolates appeared to be only marginally within the species boundary and may represent a separate biotype but more strains should be examined to corroborate this. The A460 isolate appears to be much more closely related to the A479 strain used as probe than the isolate A419(GU).

Table 5. *DNA base composition and genome size of selected strains of Aeromonas*

Species	Type	Mol% G+C*	Genome size† (M _r)
<i>A. hydrophila</i> group	<i>A. hydrophila</i> , <i>A. caviae</i> and <i>A. sobria</i>	60.6 ± 1.6	3.25 ± 0.22 (100%)
<i>A. salmonicida</i>	'Typical'	55.7 ± 1.8	2.40 ± 0.20 (74%)
	'Atypical'	55.4 ± 1.6	2.05 ± 0.16 (63%)

* Calculated using a standard mol% G+C value of 50.9 for *E. coli* B.

† Values in parentheses are genome size values relative to that for the *A. hydrophila* group.

Other results summarized in Table 3 include reassociation data which illustrate the relationship between *A. hydrophila* and various groups of *A. salmonicida*. The six *A. hydrophila* isolates included representatives of each of the recognized species, i.e. *A. hydrophila*, *A. sobria* and *A. caviae* (Popoff, 1984), and thus show a large deviation from the mean RBR at 70 °C of $82 \pm 15.2\%$. The type strain of the *A. hydrophila* species (A428) was related to 'typical' isolates at a level of $53 \pm 9.6\%$ RBR, a value which is not significantly different from the level of sequence relatedness seen for the 'atypical' group of $55 \pm 16.4\%$. Although the data are not shown in Table 3, the *A. hydrophila* probe DNA bound at levels significantly higher than mean values to DNA from *A. salmonicida* subsp. *achromogenes* (71% RBR and 5.0 °C sequence divergence) and subsp. *masoucida* (82% RBR and 4.6 °C sequence divergence).

DNA base composition and genome size estimation

Mean DNA base composition data in Table 5 show that it is possible to distinguish the *A. hydrophila* group from both the 'typical' and the 'atypical' *A. salmonicida* isolates by their slightly higher mol% G+C (60.6 ± 1.6 versus 55.7 ± 1.8 and 55.4 ± 1.8 respectively). Again the two subspecies of *A. salmonicida* proved to be exceptions with mol% G+C values resembling closely those of the *A. hydrophila* group (Table 1). *A. hydrophila* was shown to have a genome size approximately 25% larger than that for 'typical' and 'atypical' isolates. The 'typical' isolates in turn appeared to have a slightly larger genome size than the 'atypical' isolates examined.

DISCUSSION

Hybridization of genome DNA is a powerful technique for defining taxonomic status, particularly among organisms related at the genus level (Brenner, 1983). Brenner *et al.* (1972), using the hydroxyapatite method, found that isolates belonging to the same species usually showed >70% homology at the optimal hybridization temperature, a slight decrease in relatedness at stringent temperatures (generally 15 °C above optimal, Wetmur & Davidson, 1968) and <5% sequence divergence. Grimont *et al.* (1980) demonstrated that values obtained using the S1 nuclease/TCA technique (Crosa *et al.*, 1973) used in this study were proportional to those obtained using the hydroxyapatite procedure, and that the aforementioned 70% homology value for species differentiation corresponded to approximately 65% at optimal temperatures and 54% at stringent temperatures. Sequence divergence values were shown to be directly comparable.

Using these criteria, we showed that *A. salmonicida* is distinct from the *A. hydrophila* group at the species level (particularly with respect to hybridization at stringent temperatures and sequence divergence) but clearly related at the genus level. This corroborates previous evidence, based on serology, physiology, phage sensitivity and DNA homology (Liu, 1961; Sebald & Veron, 1963; Popoff, 1969; McCarthy, 1978; MacInnes *et al.*, 1979), that *A. salmonicida* should remain in the genus *Aeromonas* and not be transferred to the Pseudomonadaceae as a novel genus and species, i.e. '*Necromonas salmonicida*' (see Smith, 1963). The *A. hydrophila* group, including the species *A. hydrophila*, *A. caviae* and *A. sobria*, may be distinguished from *A. salmonicida* by its slightly higher mol% G+C and larger genome size. The reduced DNA coding capacity of *A. salmonicida*, and also the lack of motility [as suggested by MacInnes *et al.* (1979)]

may reflect an evolutionary adaptation to a strictly pathogenic form of existence from a previously free-living life style.

A. salmonicida subsp. *salmonicida*, the so called 'typical' group, form an extremely homogeneous genetic group, with a mean sequence homology level of $97 \pm 6.1\%$ and a sequence divergence $< 1^\circ\text{C}$. These data are in agreement with those of MacInnes *et al.* (1979), who found $> 95\%$ sequence homology at 70°C for 11 'typical' strains, and with the data of McCarthy (1978), who reported $> 87\%$ homology at 79°C for 12 'phenon A' or 'typical' strains. A number of studies based on numerical taxonomy (Eddy & Carpenter, 1964; McCarthy, 1978) and serology (Popoff, 1969) confirm the conserved genetic nature of this group. DNA base composition in the group ranged from 52.6–57.8 mol% G + C with a mean value of 55.7 ± 1.8 , slightly lower than the mean of 56.5 ± 0.83 reported by McCarthy (1978), a result probably reflecting the different techniques used to calculate the data. The mean mol% G + C value of $55.7 \pm 1.8\%$ allows for the distinction of 'typical' strains from the *A. hydrophila* group (mean $60.6 \pm 1.6\%$) but not from the 'atypical' group (mean $55.4 \pm 1.6\%$).

'Atypical' *A. salmonicida* strains used in this study were primarily isolated from non-salmonid sources (11/15) which correspond to the 'nova' group suggested by McCarthy & Roberts (1980), but include the subspecies *achromogenes* and *masoucida* as well as two strains originally categorized as *H. piscium*. The 'atypical' organisms clearly do not belong in the homogenous group of 'typical' isolates but do not themselves form a distinct group. The type strains for the subspecies *achromogenes* and *masoucida* appear to be most closely related to the *A. hydrophila* type strain used in this study and are only distantly related to the strains from the 'nova' group used as probes. Their mol% G + C values are also closer to those of *A. hydrophila* than other *A. salmonicida* suggesting, in agreement with Popoff (1969) and Paterson *et al.* (1980), that they may represent organisms midway between the two *Aeromonas* species. We concur with the suggestion by McCarthy & Roberts (1980) that the present *A. salmonicida* subspecies *achromogenes* and *masoucida* be combined to form a single subspecies, *achromogenes*, based on numerical taxonomy (McCarthy, 1978), sequence homology and mol% G + C. Despite the fact that some strains in the subspecies (including the ATCC type strain) may in fact be chromogenic, the name *achromogenes* has historic precedence and according to rules 42 and 55 of the Bacteriological Code (Lapage *et al.*, 1976) should be retained (as suggested by McCarthy, 1978).

'Atypical' isolates, excluding the previously discussed subspecies *achromogenes* and *masoucida*, form a genetic group by reassociation analysis at least as far removed from the 'typical' strains as they are from the existing *A. salmonicida* subspecies. This group consists predominantly of isolates from non-salmonid sources and we suggest a second subspecies of *A. salmonicida* be created for these isolates, i.e. '*A. salmonicida* subsp. *nova*' [originally proposed by McCarthy (1978) and McCarthy & Roberts (1980)]. The creation of this subspecies should, however, be undertaken with the understanding that this taxon contains more than one recognizable group which may eventually warrant further subdivision. In particular, we found two distinct groups which correspond to isolates causing disease in either goldfish or carp. Although we propose including other non-salmonid isolates in the subsp. *nova* (e.g. A474, from Atlantic cod), the analysis of greater numbers of isolates than we have at this time may show up further groupings of strains which may or may not correspond to isolation source. A number of 'atypical' strains included in this study, isolated from salmonid sources such as the Kejimikujik isolate (A460) from Atlantic salmon, the A436 isolate from sable fish, and the *H. piscium* isolates from trout, did not fit in any of the taxonomic schemes we have mentioned but should be retained in the species *A. salmonicida* based on mol% G + C values and sequence homologies. These strains perhaps represent aberrant isolates and not true taxonomic groups.

'Typical' strains of *A. salmonicida* carry a large number of cryptic plasmids, which are extremely stable and persist despite decades of subculture and the use of conventional curing techniques (Toranzo *et al.*, 1983; Hackett *et al.*, 1984). Although Brenner & Falkow (1971) advise against using plasmid-carrying strains as probes in hybridization studies the accepted type strains for *A. salmonicida* subsp. *salmonicida* [A440 (ATCC 14174) and A241 (NCMB 1102)] each carry three low- M_r plasmids and a high- M_r plasmid which could not be

excluded from purified whole cell or linear DNA. Using a genome M_r of 2.40×10^9 we estimate that these plasmids represent approximately 20% of the bacterial genome. Probe strains A419 (GU) and A479 (CE) of the 'atypical' group also carried more than one plasmid, but the plasmids carried differed from each other, and from those carried by the 'typical' strains. In fact all *A. salmonicida* strains examined to date (Toranzo *et al.*, 1983; Hackett *et al.*, 1984) have more than one plasmid and analysis of plasmid DNA should enhance our ability to classify strains.

In summary, this DNA homology study has shown that the species *A. salmonicida* is related to the *A. hydrophila* group of organisms at the genus level but is distinct at the species level, indicating it should remain in the genus *Aeromonas*, or within the proposed family Aeromonadaceae (Colwell *et al.*, 1986). The species *A. salmonicida* should be divided into three subspecies which do not correspond to the groupings used by Popoff (1984), in *Bergey's Manual of Systematic Bacteriology*, but correspond to those proposed by McCarthy & Roberts (1980). *A. salmonicida* subsp. *salmonicida* is an extremely tight genetic grouping of organisms found throughout the world and should be retained as presently described. The present subspecies *achromogenes* and *masoucida* are somewhat closer to the motile aeromonads and should be combined and called *achromogenes* (by historical precedent), and we feel should be reserved for field isolates from salmonid sources that do not fit into the subsp. *salmonicida*. A new subspecies, *A. salmonicida* subsp. *nova* (McCarthy & Roberts, 1980) should be created for field isolates of *A. salmonicida* from non-salmonid sources that do not belong to the subsp. *salmonicida*. Useful phenotypic data for the identification of these groups have been provided by McCarthy (1978), Trust *et al.* (1980), Popoff (1984) and Bohm *et al.* (1986).

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