A Taxonomic Study of Bacteria Belonging to the Genus *Aeromonas* Isolated from the Water of an Aquarium Rearing Guppy *Poecilia reticulata* PETERS—Isolation and Characterization of *Aeromonas* spp.

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Thirteen strains of bacteria producing water soluble melanin-like pigment were isolated from the water of an aquarium rearing a population of guppy held at 23–25°C. These organisms were studied extensively with respect to their morphological, cultural and biochemical characteristics. The mole % G+C in their DNA was determined, as well as their sensitivity to *Aeromonas salmonicida* bacteriophages. Twelve of the thirteen strains were non motile and were similar to *Aeromonas salmonicida* in their pigment producing property, but they differed from the latter organism in certain respects. It is proposed that they should be classified as a new species of the genus *Aeromonas*.

Furunculosis is a well known bacterial disease of salmonidae. The etiological agent was originally called "*Bacterium salmonicida*" by LEHMANN and NEUMANN¹⁾ and then transferred to the genus *Aeromonas* by GRIFFIN *et al.*²⁾ Characteristic features of the organism include the following: a non-motile Gram negative rod producing a water soluble melanin-like pigment, cytochrome oxidase, and fermenting glucose. The pigment production has been regarded as one of the most important identifying characteristics.

Recently, however, several reports have revealed the presence of two subspecies of *Aeromonas salmonicida* lacking the ability to produce the pigment, *A. salmonicida* subsp. *achromogenes*^{3,4)} and subsp. *masoucida*^{5,6)}. Other species producing such pigment include *A. liquefaciens*^{7,8)} and pigment producing *Pseudomonas aeruginosa*^{9,10)}. These findings seem to have reduced the importance of pigment production in the identification of *A. salmonicida*^{11,12)}. Another well known feature of this organism is the fact that it has rarely if ever been isolated from sources other than the tissues of diseased fish^{13–16)}.

In 1975 we isolated non-motile Gram negative rods producing a water soluble melanin-like pigment from the water of an aquarium rearing a population of guppy. The characteristics of these organisms were thoroughly studied, as described in this report, in an effort to clarify the taxonomic relationship between them and *A. salmonicida*.

Materials and Methods

Bacteria were isolated from the aquarium water by inoculating plates of nutrient agar, which were incubated at 20°C for 3 days. Thirteen cultures producing a water soluble melanin-like pigment were selected and subcultured on the same medium. Seventy strains of *A. salmonicida* subsp., eight strains of motile aeromonads, two strains of *Vibrio* and one of *Escherichia*, serving as reference cultures were also cultivated on this medium, with one exception. *A. salmonicida* subsp. *achromogenes* was grown on a modified MUELLER-HINTON medium (Sensitivity Disk medium, Nissui Seiyaku Ltd., Tokyo, Japan).

For the characterization of the isolated cultures and reference strains standard methods for the study of morphological and physiological properties and biochemical reactions as outlined by COWAN¹⁰⁾ were followed.

The deoxyribonucleic acid of the 13 isolates and 7 aeromonad reference strains was prepared by the method of MARMUR¹⁷⁾ and the mole% of guanine plus cytosine of each preparation was determined according to MARMUR and DOTY¹⁸⁾.

Two of the isolated cultures and 8 reference aeromonads were tested for sensitivity to 6 *A. salmonicida* bacteriophages, using the double-agar-layer technique. The initial concentration of the phages was from 1×10^6 to 1×10^8 plaque forming units per ml.

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Table 1. Characteristics of the thirteen strains isolated

Property or reaction tested	Strain designation		Property or reaction tested	Strain designation	
	Nos. 1-12 inclusive	No. 13		Nos. 1-12 inclusive	No. 13
Gram stain	—	—	KCN (growth in)	—	—
Flagella	—	pm ^d	O/129 sensitivity	—	—
Motility	—	+	Utilization of glucose and ammonium	+	+
Growth at 5°C	+	+	Utilization of citrate (Simmons')	+	+
15°C	+	+	Hemolysis (horse blood)	+	+
25°C	+	+	Sole carbon source		
30°C	+	+	L-arginine	— ^a	—
37°C	+	+	L-histidine	—	—
Growth in 0.0% NaCl	+	+	L-glutamic acid	+	+
0.5% NaCl	+	+	DL-asparagine	+	+
3.0% NaCl	+	+	DL-serine	— ^b	—
5.0% NaCl	—	+	DL-alanine	—	—
Melanin-like pigment production at 20°C	+	+	L-glycine	—	—
37°C	+	+	L-tryptophan	—	—
Acid from glucose in aerobic	+	+	DL-threonine	—	—
anaerobic	+	+	DL-phenylalanine	—	—
Acid from arabinose	—	—	D-xylose	—	+
galactose	+	+	D-ribose	+	+
lactose	+	+	D-arabinose	—	+
mannitol	—	+	glucose	+	+
sorbitol	+	+	fructose	+	+
sucrose	+	+	galactose	+	+
Gas from glucose	—	—	maltose	+	+
glycerol	—	—	trehalose	+	+
M-R test	+	+	sorbitol	—	—
V-P reaction	—	—	starch	+	+
Butanediol dehydrogenase	—	+	pyruvate	—	—
Production of butanediol	—	—	fumarate	+	—
Oxidation of gluconate	—	—	lactate	—	—
Hydrolysis of starch	+	+	acetate	— ^c	+
esculin	—	—	malonate	—	—
tween 80	+	+	succinate	+	+
chitin	—	—	gluconate	+	+
gelatin	—	—	glycerol	+	+
casein	—	+	ethanol	—	—
Nitrate reduction	+	+	n-cetan	—	—
Indole production	—	—	benzoate	—	—
Ammonia production	+	+	phenol	—	—
Lysine decarboxylase	—	—	DL-3-hydroxy-n-butylic acid	—	—
Ornithine decarboxylase	—	—	sucrose	+	+
Arginine decarboxylase	—	+	mannitol	—	+
H ₂ S production	+	+	GC mole %	58.5-56.4	56.8
Urease	—	—	Sensitivity to		
Oxidase (Kovacs')	+	+	<i>A. salmonicida</i>	—	—
Catalase	+	+	bacteriophages		

a: No. 12 was +. b: No. 3 was +. c: No. 5 was —. d: Polar monotrichous.

Table 2. Characteristics of the isolates differentiating them from the species and subspecies of the genus *Aeromonas*

Property or reaction tested	Isolates		<i>Aeromonas salmonicida</i> subsp.					<i>Aeromonas hydrophila</i> subsp.		<i>Aeromonas punctata</i> subsp.				<i>Aeromonas</i>	
	No. 1-12	No. 13	<i>salmonicida</i> (ATCC 14174)	(NCMB 1102)	<i>aeromogenes</i> (NCMB 1110)	<i>masoucida</i> (NCMB 2020)	<i>hydrophila</i> (IAM 1018)	(NCMB 86)	<i>punctata</i> (IAM 1646)	(TUF No. 1)	(TUF No. 2)	(NUMB 74)	<i>liquefaciens</i> (EFDL)	(ATCC 11715)	
Growth at 37°C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
Brown pigment (water soluble) on TSA	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
Utilization of glucose and ammonium	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
Sole carbon source	1 ^a / 12	+	+	+	+	+	+	+	+	+	+	+	+	+	
L-arginine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
L-asparagine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
L-histidine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
L-glutamic acid	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
L-serine	1 ^a / 12	+	+	+	+	+	+	+	+	+	+	+	+	+	
L-alanine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
Growth in 5.0% NaCl	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
Butanediol dehydrogenase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
Production of	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
gas from glycerol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
gas from glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
indole in pepton	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
water containing	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
0.1% tryptophan	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
H ₂ S from 2.5%	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
pepton water	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
KCN broth (Møller's)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
Lysine decarboxylase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
V-P reaction	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
Gluconate oxidation	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
Breakdown of	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
galactose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
sucrose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
mannitol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
arabinose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
arabiose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
esculin	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
GC mole %	58.5-56.4	56.8	57.7	56.7	57.2	58.8	61.4	56.2	59.9	58.8	58.8	58.8	58.8	58.8	
Sensitivity to	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
<i>A. salmonicida</i> bacteriophages	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
a: No. of strains positive															

a: No. of strains positive

Results and Discussion

All thirteen cultures isolated proved to be Gram negative non spore forming rods measuring $0.5 \times 2-4 \mu\text{m}$, which grew well at all temperatures from 5° to 37°C , with the production of a water soluble melanin-like pigment (Fig. 1). All were non-motile except one strain, designated as No. 13. The detailed results of all the cultural and biochemical tests carried out with these cultures are presented in Table 1. The mole percentages of guanine plus cytosine fell in the range of 56.4 to 58.5. Neither isolate No. 1 or No. 13 were susceptible to any of the 6 *A. salmonicida* bacteriophages.

Table 2 outlines the cultural and biochemical characteristics of the 13 isolated strains which serve to differentiate them from the recognized species of aeromonads. The 12 reference cultures included subspecies of *A. salmonicida*, *A. hydrophila*, *A. punctata*, and *A. liquefaciens*, which were studied concurrently with the isolates.

It is apparent that the 12 non-motile isolates differed from all of the cultures of *A. salmonicida* in their ability to grow at 37°C , to utilize glucose and ammonia, to use asparagine and L-glutamic acid as sole carbon sources, and in their inability to produce butanediol dehydrogenase or to break down mannitol.

They differed from the *A. hydrophila* cultures in their production of the brown water soluble pigment, and in their inability to use histidine as a sole carbon source, or to produce butanediol dehydrogenase, indole, or gas from the fermentation of glycerol or glucose, or to grow in KCN broth.

The 12 non-motile isolates differed from the *A. punctata* subspecies in their production of the brown pigment, and their inability to use histidine as a sole carbon source, to produce indole, or gas from fermentation of glucose and their failure to grow in KCN broth, or to break down mannitol. Unlike the *A. liquefaciens* cultures they were unable to utilize histidine or alanine as sole carbon sources, or to produce butanediol dehydrogenase, indole, or gas from fermentation of glycerol and glucose. Also they failed to grow in KCN broth, to oxidize gluconate, or to break down mannitol or esculin.

From Table 1 it is evident that the cultural and biochemical characteristics of the 12 non-motile isolates were, with minor exception, quite uniform. The motile strain, No. 13, displayed a few differences in biochemical reactions. However, all 13 were Gram negative rods, oxidase positive, fermentative without gas in glucose metabolism, and non-sensitive to 0/129. Their DNAs con-

tained 56-58 mole% of G+C. These characteristics have been considered sufficient to justify classification in the genus *Aeromonas*^{16,10-12}. Furthermore they produced a water soluble melanin-like pigment, and 12 of them were non-motile. In these respects they resembled *A. salmonicida*, but differed from the latter in the properties previously cited. It is also worth noting that the isolation of this organism from waters has been very difficult, even in the presence of diseased or carrier fish¹³⁻¹⁵ whereas the cultures reported here were isolated from the aquarium water with no difficulty.

As pointed out above the isolated cultures exhibited certain characteristics that served to differentiate them from each of the recognized *Aeromonas* species, i. e. *A. salmonicida*, *A. hydrophila*, *A. punctata* and *A. liquefaciens*. However they also resembled each of these species in certain respects. Thus it was not possible to classify any of the isolates as members of any of the above species. Under these circumstances we propose to consider the 12 non-motile isolates as representatives of a new species of the genus *Aeromonas*.

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Fig. 1. Water soluble melanin-like pigment produced by isolate (strain No. 1) compared with reference strains grown on nutrient agar or modified MUELLER-HINTON agar at 20°C after 10 days.

Ar- 1: <i>Aeromonas hydrophila</i> subsp. <i>hydrophila</i>	IAM	1018
Ar- 2: <i>A. punctata</i> subsp. <i>punctata</i>	IAM	1646
Ar- 77: <i>A. liquefaciens</i>		
(chromogenic strain isolated by Paterson) ⁽¹¹⁾	ATCC	11715
Ar- 3: <i>A. salmonicida</i> subsp. <i>salmonicida</i>	ATCC	14174
Ar-133: <i>A. salmonicida</i> subsp. <i>achromogenes</i>	NCMB	1110
1-a-1 : <i>A. salmonicida</i> subsp. <i>masoucida</i>	NCMB	2020