Aeromonas media, a New Species Isolated from River Water

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During a survey for the presence of *Aeromonas salmonicida*, 45 *Aeromonas* isolates which could not be classified in the existing named species of the genus were recovered from water obtained from the River Avon in Hampshire, England. On the basis of a numerical taxonomy study, these isolates were clustered in three homogeneous phena. Phenon 1 was identified as a new species of *Aeromonas*, for which we propose the name *Aeromonas media*; strain R_M (= ATCC 33907) is the type strain of this species.

The taxonomy of *Aeromonas* is unsettled despite all of the attention which has been focused on this genus. Specifically, a satisfactory classification has to be resolved for the motile organisms (i.e., *Aeromonas hydrophila*, *Aeromonas punctata* and *Aeromonas sobria*) (12). In contrast, the nonmotile species *Aeromonas salmonicida* is regarded as relatively homogeneous (7, 29).

The motile aeromonads are ubiquitous inhabitants of freshwater and estuarine environments (9, 12, 26). In particular, *A. hydrophila* comprises a dominant component of the natural microflora of fish (27, 34). Indeed, this organism has been implicated as an opportunistic and primary pathogen of a diverse range of aquatic and terrestrial animals, including fish (4) and humans (11). In contrast, *A. salmonicida* has been recovered exclusively from fish tissues (16); in fact, the description in *Bergey's Manual of Determinative Bacteriology* mentions that this organism is not found in surface waters, but only as an obligate fish pathogen that causes furunculosis in salmonid fish (25).

The identification of fresh isolates of *A. salmonicida* relies on the absence of motility and on the production of a brown diffusible pigment on tryptone-soya agar (TSA). Thus, during a microbiological survey of a trout farm situated on the River Avon, England, a group of bacterial isolates possessing some characteristics of *A. salmonicida* were recovered from water samples plated on peptone-beef extract-glycogen agar (17), a medium selective for the isolation of *Aeromonas* spp. On the basis of overall similarity, which was computed by using numerical taxonomy methods, these organisms could not

be classified as members of previously described species of *Aeromonas*. Thus, we propose that these organisms be recognized as a new species, *Aeromonas media*.

MATERIALS AND METHODS

Collection of samples. Water samples were collected aseptically with a sampler (see below) from selected locations at a trout farm situated on the River Avon in Hampshire, England. These samples included water taken from the river approximately 100 m upstream from the fish farm, water from each of three earth fishponds, and water from a feeder channel derived from the river and located about 1 km downstream from the farm. The site was sampled at monthly intervals, beginning in October 1980 and ending in November 1981.

In addition, a survey of the River Avon was carried out, in which water samples from 11 stations on the river were collected, beginning at its source at the Kennett and Avon Canal and then at approximately 10-km intervals until the river mouth at Christchurch, Dorset, was reached. This survey was done twice in entirety, once in September 1981, and again in November 1981, and also once for the five stations downstream from the fish farm to the river mouth in June 1981.

All water samples were collected by using presterilized plastic bottles (Dippas; Sterilin Ltd., Teddington, Middlesex, England). Samples were processed within 30 min of collection at the fish farm sites. River survey water samples were processed within 2 h of collection.

Examination of samples. The water samples were diluted 10⁻⁴ with river water sterilized by passage through 0.22-µm membrane filters. Samples (0.1 ml) were pipetted in duplicate onto the surface of TSA (Oxoid Ltd., Basingstoke, Hampshire, England). In addition, 1- and 0.1-ml portions of the water samples were incorporated into peptone-beef extract-glycogen agar. All plates were incubated at 15°C for up to 14 days.

Isolation and maintenance of strains. A total of 38 bacterial isolates demonstrating several key characteristics of *A. salmonicida* (gram-negative, oxidase- and catalase-positive, nonmotile, fermentative, rod-

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TABLE 1. Strains used in this study

Phenon	Strain(s) ^a	Source of isolation	Date of isolation
1	R _L , R _M ^T , RS	Fish farm effluent	October 1980
	S1, S2	Sopley	June 1981
	Junc2, EW, I2, UP3, EN1	Road Junction, Etchilhampton water, Ibsley, Upavon, Enford	November 1981
	P43	Fish farm (pond 43), River Avon	December 1980
	Rd	Fish farm effluent, River Avon	December 1980
	P1A	Fish farm (pond 1), River Avon	February 1981
	P1E	Fish farm (pond 1), River Avon	August 1981
	CC2	Mouth of River Avon (Christchurch)	November 1981
	B	Inflow to fish farm	January 1981
	F2, B2	Effluent, inflow to fish farm	March 1981
2	B3	Inflow to fish farm	April 1981
	I1	Ibsley	June 1981
	Junc1, NET1	Road Junction	September 1981
	B4, B5	Salterton	October 1981
	S3	Inflow to fish farm	November 1981
	P12b	Fish farm (pond 1)	October 1980
	P1B	Fish farm (pond 1)	April 1981
	P1C, P1D	Fish farm (pond 1)	June 1981
	P1F	Fish farm (pond 1)	October 1981
	P43b	Fish farm (pond 43)	May 1981
	P43c	Fish farm (pond 43)	June 1981
	P43d	Fish farm (pond 43)	August 1981
	P43e	Fish farm (pond 43)	September 1981
	P43f	Fish farm (pond 43)	October 1981
	F4	Fish farm effluent	August 1981
	F5, Rd2	Fish farm effluent	September 1981
	Rd3, NET2	Fish farm effluent	October 1981
	CC1	Mouth of River Avon (Christchurch)	June 1981
	P43a	Fish farm (pond 43)	June 1981
3	I3, UP1, UP2	Ibsley, Upavon	November 1981
4	<i>A. hydrophila</i> ATCC 7966 ^T		
	<i>A. sobria</i> Popoff 310		
5	<i>A. punctata</i> subsp. <i>caviae</i> ATCC 15468 ^T		
	<i>A. hydrophila</i> NCTC 7810 ^T		
6	<i>A. salmonicida</i> NCIB 833 ^T , CCM 1307, CCM 1318		
	<i>A. salmonicida</i> 26/78	<i>Oncorhynchus</i> sp., Scotland	1978
	<i>A. salmonicida</i> 22/81	<i>Salmo trutta</i> , England	1981

^a ATCC, American Type Culture Collection, Rockville, Md.; CCM, Czechoslovak Collection of Microorganisms, Brno, Czechoslovakia; NCIB, National Collection of Industrial Bacteria, Aberdeen, Scotland; Popoff, M. Popoff, Pasteur Institute, Paris, France; NCTC, National Collection of Type Cultures, Colindale, London, England. T = type strain.

shaped organisms producing a brown diffusible pigment) and 7 closely related but motile isolates that also produced a brown pigment were recovered from the water samples (Table 1). All organisms were inoculated onto TSA slopes and maintained at room temperature. Subculturing was done every 4 weeks. In addition, stock cultures were preserved in tryptone-soya broth (Oxoid) and glycerol at -20°C.

Reference cultures. The environmental isolates were compared with 10 reference strains, including *A. punctata* subsp. *caviae* ATCC 15468^T, *A. hydrophila* ATCC 7966^T and NCTC 7810^T, *A. sobria* Popoff 310, a brown-pigmented *A. hydrophila* (strain 37/75), and *A. salmonicida* CCM 1307, CCM 1318, NCIB 833^T, 26/78, and 22/81.

Characterization of the isolates. All strains were examined for 124 characteristics as described previously (2, 8) or below. Whenever possible, TSA was

used as the basal medium, and all inoculated media were incubated at 22°C for 14 days, unless indicated otherwise, before results were recorded.

Colonial morphology and micromorphology. Colony morphology on TSA was recorded after incubation for 7 days at 22°C. Motility was determined from wet preparations. Gliding motility was assessed by the presence of swarming growth on *Cytophaga* agar (Oxoid).

Biochemical characteristics. Hydrolysis of gelatin and decarboxylation of arginine, lysine, and ornithine were tested by using the methods of Smith and Goodner (28) and Moeller (18), respectively. The production of H₂S (Kligler iron agar [Oxoid]), the hydrolysis of 5% (wt/vol) sheep blood (Oxoid) in TSA, the hydrolysis of urea (Oxoid), and the utilization of citrate (Oxoid) were recorded after incubation for 7 days at 22°C.

FIG. 1. Simplified dendrogram based on the Jaccard coefficient and unweighted average linkage.

Growth. Growth on cysteine-lactose electrolyte-deficient agar (Oxoid) and growth on thiosulfate-citrate-bile salt-sucrose agar (Oxoid) were assessed after incubation for 7 days at 22°C.

Susceptibility to antibacterial agents. Susceptibilities to antibiotics were determined by a modification of the method of Allen et al. (1). Zones of clearing around antibiotic disks placed on Mueller-Hinton agar (Oxoid) were measured as soon as growth could be detected (i.e., after incubation for 24 h at 22°C). Susceptibility to 2,4-diamino-6,7-diisopropyl pteridine (vibriostatic agent 0/129) was recorded after incubation for 24 h at 22°C.

Computer analyses. The 55 bacterial strains studied were examined for a total of 124 unit characters, which were coded in a binary format by scoring positive and negative characters as 1 and 0, respectively. Similarities between strains were calculated by using the simple matching (32) and Jaccard (30) coefficients. Sorted similarity matrices and dendrograms were obtained by using the unweighted average linkage algorithm (31).

Determination of deoxyribonucleic acid base composition. The guanine-plus-cytosine (G+C) contents of purified deoxyribonucleic acids prepared from representative organisms (14) were determined by measuring the thermal denaturation temperatures (15) with a Pye Unicam model SP 1800 recording spectrophotometer at 260 nm; this instrument was programmed for temperature increases of 1.0°C/min. G+C contents were calculated from the thermal denaturation temperatures by using the equation of De Ley (6).

Electron microscopy. Cells of representative isolates were grown and harvested by the procedure of Austin et al. (3). Cell suspensions were negatively stained with 1% (wt/vol) methylamine tungstate (EM Scope Laboratories Ltd., Ashford, Kent, England) and examined with a JEOL model CX 100 transmission electron microscope.

RESULTS

Clustering of the isolates. Six phenons and two single-member clusters were defined at the 80% similarity level by our analysis when the Jaccard

coefficient was used (Fig. 1). Thus, the 45 environmental isolates were clustered in phenon 1 through 3. However, these isolates showed little affinity to the reference cultures of *Aeromonas* included in the analysis. The latter fell into three well-separated clusters, phenon 4 through 6. Nevertheless, we determined that phenon 1 through 3 were more similar to *A. hydrophila* than to *A. salmonicida* (Fig. 1).

Characterization of the environmental isolates. All environmental isolates possessed the following properties of the genus *Aeromonas*: straight, gram-negative, fermentative, oxidase- and catalase-positive, rod-shaped organisms that produce a brown diffusible pigment on TSA, utilize a wide range of carbon compounds for energy and growth, and have a G+C content between 57 and 63 mol% (Table 2) (25). However, it was not possible to identify these isolates exactly by using the diagnostic keys in *Bergey's Manual* (5) or the diagnostic scheme of Sannohe et al. (21).

Phenon 1 contained 15 isolates, which were nonmotile rod-shaped organisms that were approximately 1 by 2 µm and had rounded ends (Fig. 2); these isolates formed round, raised, entire, shiny colonies 2 mm in diameter on TSA after incubation for 2 days at 22°C. The results of other tests are shown in Tables 2 and 3. This phenon was considered a new species of *Aeromonas*, for which we propose the name *A. media*.

Phenon 2 included 26 isolates with colonial morphologies and micromorphologies similar to those of the isolates in phenon 1. However, some phenon 2 isolates were motile by means of single polar flagella. In general, these isolates were more reactive than the organisms in phenon 1; they produced arginine dihydrolase and β-galactosidase, but not H₂S, phosphatase, or lysine or ornithine decarboxylase. Gluconate oxidation, the Voges-Proskauer reaction, and

TABLE 2. Diagnostic characters of *Aeromonas* spp. examined in this study^a

Character	Phenon					
	1	2	3	4	5	6
Motility	— ^b	V	—	+	+	—
Diffusible brown pigment	+	+	+	—	—	+
Growth on 5% (wt/vol) NaCl	—	—	—	—	+	—
Indole production	V	V	+	+	+	—
Phosphatase production	—	—	—	+	+	V
Growth at 37°C	+	+	+	+	+	—
Methyl red test	+	+	+	+	—	+
Degradation of:						
Casein	V	V	+	+	+	—
Elastin	—	—	—	+	V	+
Tyrosine	V	V	—	+	+	—
Utilization of:						
Alanine	V	+	+	+	+	—
Cellobiose	—	+	—	—	—	—
Fructose	—	+	V	+	+	+
Galactose	V	+	+	+	+	+
Lactose	V	+	+	—	—	—
Maltose	V	+	—	+	+	+
Mannose	V	+	+	+	+	—
Ribose	V	+	+	+	+	+
Sodium butyrate	V	+	—	+	+	—
Sodium citrate	V	+	—	V	+	—
Sodium formate	—	—	—	+	—	—
Sodium malate	V	+	V	+	+	+
Sucrose	—	+	—	+	+	—
Trehalose	—	+	—	+	+	+

^a In addition, the G+C contents of the phena are as follows: phenon 1, 62.3 mol%; phenon 2, 61.5 mol%; phenon 3, undetermined; phenon 4, 57.1 mol%; phenon 5, 61.6 mol%; phenon 6, 62.7 mol%.

^b +, ≥80% positive responses; —, ≤20% positive responses; V, 21 to 79% positive responses.

phenylalanine deaminase were negative. Nitrate reduction and the methyl red test were positive. Growth occurred at 4 and 37°C and in 0 to 3% sodium chloride. Esculin, blood, DNA, gelatin, ribonucleic acid starch, Tween 20, Tween 40, Tween 60, and Tween 80 were degraded, but cellulose, elastin, xanthine, and urea were not. DL-(α)-Alanine, L-(–)-arabinose, D-(+)-cellobiose, D-(–)-fructose, D-(+)-mannose, L-proline, D-(–)-ribose, L-serine, sodium acetate, sodium butyrate, sodium glutamate, sodium malate, sodium pyruvate, sodium succinate, sucrose, and D-(+)-trehalose were utilized as sole sources of carbon for energy and growth, but adonitol, meso-erythritol, ethanol, *p*-hydroxybenzoic acid, meso-inositol, inulin, L-leucine, D-(+)-melezitose, D-(+)-sorbitol, and D-(+)-xylose were not. The G+C content of a representative isolate was 61.5 ± 0.5 mol%. Although these characteristics do not match precisely the description of any *Aeromonas* species, there are a number of properties in common with *A. hydrophila* (5) (namely, motility and utilization of adonitol, alanine, arabinose, fructose, galactose, inositol,

inulin, maltose, mannitol, melezitose, serine, sodium glutamate, sucrose, trehalose, and xylose). Therefore, phenon 2 is not elevated to species status at this time; instead, further work should be carried out to elucidate more fully the taxonomic position of these organisms.

The diagnostic characteristics of phenon 3 are shown in Table 2. However, there are too few isolates for a meaningful taxonomic discussion until studies in progress are completed.

DISCUSSION

The taxonomy of *Aeromonas* has received great attention in recent years. However, some confusion about the classification of species within this genus remains. For example, at one time, *A. salmonicida* was transferred to a separate genus, *Necromonas* (29); this is in contrast to current opinion, which considers the organisms called *A. hydrophila* and *A. salmonicida* genotypically to be bona fide members of the same genus (i.e., *Aeromonas*) (13, 25). Nevertheless, atypical representatives of *Aeromonas* have been reported (33). In fact, results of some environmental studies have provided good evidence of species diversity, notably for *A. hydrophila* (10). In this case, Holder-Franklin et al. (10) described environmental isolates of *A. hydrophila* which had phenotypic traits and G+C contents that were inconsistent with the description of the taxon (25). Thus, it appears that a reappraisal of the taxonomy of *A. hydrophila* is needed, and this may clarify the position of phenon 2.

At present, the classification of the genus *Aeromonas* follows the work of Schubert (23–

FIG. 2. Negatively stained cell of strain R_m^T. Bar = 1 μm.

TABLE 3. Differential characteristics of strains in phenon 1

Character	Reaction of:	
	Phenon 1	Strain R _M ^T
Indole production	V ^a	+
Growth on:		
3% (wt/vol) NaCl	+	—
4% (wt/vol) NaCl	V	—
5% (wt/vol) NaCl	—	—
CLED ^b	+	+
MacConkey agar	+	+
TCBS ^c	V	—
Degradation of:		
Esculin	V	—
Chitin	V	+
DNA	V	—
Tyrosine	V	—
Utilization of:		
DL-(α)-Alanine	V	+
Calcium lactate	V	—
D-(+)-Galactose	V	—
Lactose	V	—
Maltose	V	—
D-(+)-Mannose	V	—
D-(—)-Ribose	V	—
Sodium butyrate	V	—
Sodium citrate	V	—
Sodium malate	V	+
Susceptibility to:		
Erythromycin	V	+
Streptomycin	—	+

^a +, Positive reaction; —, negative reaction; V, variable reaction.

^b CLED, Cysteine-lactose electrolyte-deficient agar.

^c TCBS, Thiosulfate-citrate-bile salt-sucrose agar.

25), who recognized three species, namely, *A. hydrophila* (including *A. hydrophila* subsp. *anaerogenes*, *A. hydrophila* subsp. *hydrophila*, and *A. hydrophila* subsp. *proteolytica*), *A. punctata* (including *A. punctata* subsp. *caviae* and *A. punctata* subsp. *punctata*), and *A. salmonicida* (including *A. salmonicida* subsp. *achromogenes*, *A. salmonicida* subsp. *masoucida*, and *A. salmonicida* subsp. *salmonicida*). However, the validity of *A. punctata* as a separate species has been questioned insofar as Popoff and Véron (19) consider it to be a synonym of *A. hydrophila*. Our study supports this view because the type strains of *A. punctata* subsp. *caviae* (strain ATCC 15468) and *A. hydrophila* (strain NCTC 7810) clustered together in phenon 5. These strains were distinct from *A. sobria* (phenon 4), which Popoff and Véron (19) described as a new species of *Aeromonas*.

Aeromonas spp. that produce a brown diffusible pigment have been isolated frequently from aquatic environments. Furthermore, motile rod-shaped organisms classified as *Aeromonas li-*

quefaciens (20) (an objective synonym of *A. hydrophila* [25]) have been isolated from salmon. Nonmotile organisms (H. LeClerc, Ph.D. thesis, Faculté de Médecine et de Pharmacie de Lille, Lille, France, 1962) distinct from *A. salmonicida* have been described and identified as *A. hydrophila* (22), contrary to the view that the members of *A. hydrophila* are all motile (25). Strains that possess selected characteristics of *A. salmonicida* are common in aquatic environments (10, 21), and Sannohe et al. (21) suggested that nonmotile aeromonads recovered from aquarium water in Japan belong in an as-yet-undescribed species of *Aeromonas* related to *A. salmonicida*. A comparison of phenotypic and genotypic traits shows that these organisms are very unlike the strains in phenon 1. Consequently, we decided that phenon 1 is not closely related to any of the previously described *Aeromonas* species (5). Therefore, we concluded that these strains represent a new species of the genus *Aeromonas*, for which we propose the name *Aeromonas media* (me.' di. a. f. L. adj. *medius* in the middle).

Description of *Aeromonas media* sp. nov. Gram-negative, nonmotile rod-shaped cells approximately 1 by 2 μm with rounded ends (Fig. 2).

Colonies on TSA are cream, shiny, smooth, round, raised, entire, and 2 mm in diameter after incubation for 2 days at 22°C.

A diffusible brown, nonfluorescent pigment is produced.

Cultures in peptone broth are uniformly turbid.

Chemoorganotrophic; metabolism is fermentative.

Nitrates are reduced.

Temperature range for growth: grows at 4 and 37°C but not at 42°C.

Growth occurs in 0 to 3% (wt/vol) sodium chloride.

Catalase and oxidase are produced.

Arginine dihydrolase is produced.

Lysine and ornithine are not decarboxylated.

Blood, gelatin, starch, ribonucleic acid, Tween 20, Tween 40, Tween 60, and Tween 80 are degraded, but cellulose, elastin, lecithin, xanthine, and urea, are not.

β-Galactosidase is produced, but H₂S and phosphatase are not.

The methyl red test is positive, but the Voges-Proskauer and gluconate oxidation tests are negative.

Susceptible to chloramphenicol, chlortetracycline, colistin sulfate, furazolidone, gentamicin, kanamycin, neomycin, nitrofurantoin, oxytetracycline, and tetracycline but not to ampicillin, cloxacillin, novobiocin, vibriostatic agent 0/129, penicillin G, or sulfafurazole.

Utilizes L(-)-arabinose, glycerol, mannitol, L-proline, L-serine, sodium acetate, sodium glutamate, sodium pyruvate, and sodium succinate as sole carbon sources for energy and growth, but does not utilize adonitol, D-(+)-cellobiose, *meso*-erythritol, ethanol, D(-)-fructose, *meso*-inositol, inulin, L-leucine, D-(+)-melezitose, *p*-hydroxybenzoic acid, D-(+)-raffinose, sodium benzoate, sodium formate, sodium malonate, D(-)-sorbitol, sucrose, D-(+)-trehalose, L-valine or D-(+)-xylose.

G+C content of deoxyribonucleic acid: 62.3 $\pm$ 0.2 mol%.

The type strain is strain R_m; a culture of this strain has been deposited with the American Type Culture Collection, Rockville, Md., as ATCC 33907.

Other characteristics of the type strain are shown in Table 3.

ACKNOWLEDGMENTS

D.A.A. was supported by a Sea Grant Fellowship. Assistance in obtaining computer analyses was provided by M. Goodfellow and P. West.

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