NOTE

Aeromonas culicicola* sp. nov., from the midgut of *Culex quinquefasciatus

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The taxonomic position was examined of three isolates, MTCC 3249^T, SH and SLH, from the midgut of female *Culex quinquefasciatus* and *Aedes aegyptii* mosquitoes. Numbers of cells of these isolates increased 2000-fold after a blood meal of the mosquitoes. 16S rRNA gene sequence analysis of the novel strains showed that they were highly homologous to strains of *Aeromonas*. DNA–DNA hybridization studies showed that DNA of strain MTCC 3249^T was 96 and 88 % similar to that of strains SH and SLH, respectively, and showed 54 % relatedness to *Aeromonas jandaei* and 61 % relatedness to *Aeromonas sobria*, which is below the cut-off value for species differentiation. The biochemical profiles of all three novel strains were identical. On the basis of a polyphasic approach using phenotypic analysis, 16S rRNA gene sequencing and DNA–DNA hybridization studies, a novel species is proposed for these isolates, *Aeromonas culicicola* sp. nov., with the type strain MTCC 3249^T (= NCIM 5147^T). Isolation of *A. culicicola* from the midgut of mosquitoes might help to explain the origin of *Aeromonas* infections caused without exposure to contaminated water, soil or food.

Keywords: *Aeromonas culicicola*, 16S rRNA gene, DNA–DNA hybridization

Aeromonas species are autochthonous to aquatic systems and are ubiquitous at varying temperatures, salinity and nutritional conditions (Schubert, 1987). There are numerous reports of the incidence of aeromonads in cold-blooded animals (Khardori & Fainstein, 1988). *Aeromonas* species have recently received a great deal of attention because of their role as opportunistic pathogens from environmental and food sources. Many species of *Aeromonas* are pathogens of humans and aquatic animals (Austin, 1987; Freij, 1987; Gross, 1987). Species such as *Aeromonas hydrophila*, *Aeromonas caviae*, *Aeromonas jandaei* and *Aeromonas veronii* are known to be associated with various diseases such as gastroenteritis, haemolytic uremic syndrome, septicaemia and skin and wound infections (Janda & Abbott, 1998). This demonstrates clearly that the natural habitats of these organisms are

not strictly limited to aquatic animals (Huys *et al.*, 1997).

The taxonomy of the genus *Aeromonas* is complicated and is changing continuously due to the addition of newly described species and reclassification or extended description of existing taxa. The use of terms like ‘phenospecies’ and ‘genospecies’ in *Aeromonas* classification can be highly confusing to workers outside this field (Huys *et al.*, 1997). The biochemical identification of *Aeromonas* species is time-consuming and difficult and, hence, there has been interest in finding molecular identification methods (Graf, 1999b). Recently, several methods useful in *Aeromonas* genomic species identification were proposed that include multilocus enzyme electrophoresis (Altwegg *et al.*, 1991), ribotyping (Hanninen & Siitonen, 1995), PCR (Dorsch *et al.*, 1994) and RFLP (Borrell *et al.*, 1997) of 16S rRNA genes, colorimetric DNA–DNA hybridization in microplates (Kaznowski, 1995), PCR amplification of the aerolysin gene (Khan *et al.*, 1999) and AFLP fingerprinting (Huys *et al.*, 1996).

16S rRNA gene sequencing is one of the most powerful and precise methods for determining the interrela-

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Abbreviation: HG, hybridization group.

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tionships of bacteria. Species of the genus *Aeromonas* exhibit very high levels of overall sequence similarity (98–100%) to each other. During the past few years, the taxonomy of *Aeromonas* has undergone major revisions because of the use of DNA–DNA hybridization studies. At the time of writing, the genus *Aeromonas* is represented by 14 phenospecies grouped into at least 17 hybridization groups (Martínez-Murcia, 1999; Huys *et al.*, 1997; Martínez-Murcia *et al.*, 1992). It is recommended that strains showing less than or equal to 70% DNA–DNA similarity belong to different species (Martínez-Murcia, 1999; Wayne *et al.*, 1987).

Members of *Aeromonas* are isolated not only from wound infections or septicæmic conditions but also from haematophagous animals such as the medicinal leech (*Hirudo medicinalis*). The association between leeches and *Aeromonas* strains is symbiotic, wherein the bacterium aids in the digestion of blood (Graf, 1999a). Mosquitoes serve as obligate intermediate hosts for numerous diseases that collectively represent a major cause of human mortality and morbidity worldwide. There have been attempts to generate transgenic mosquitoes refractory for the transmission of pathogens (Olson *et al.*, 1996). The midgut bacterial flora of the mosquito can be used to introduce gene products that will inhibit the development of pathogens inside the mosquitoes (Carlson *et al.*, 1995; Durvasula *et al.*, 1997). There have been few studies on the bacterial flora of laboratory-bred strains of mosquitoes and little information is available on wild specimens (Dillon *et al.*, 2000). A recent study on *Aedes triseriatus*, *Culex pipiens* and *Psorophora columbiae* indicated the presence of *Serratia marcescens*, *Klebsiella ozonae*, *Pseudomonas aeruginosa* and *Enterobacter agglomerans* (Demaio *et al.*, 1996). In this paper, we report the isolation of a novel *Aeromonas* species from the midgut of the medically important haematophagous mosquitoes *Culex quinquefasciatus* and *Aedes aegyptii*.

Wild female *Cx. quinquefasciatus* and *Ae. aegyptii* mosquitoes, either blood-fed or non-blood-fed, were collected from the surroundings of the Institute and brought live to the laboratory in cages. The endogenous bacterial flora of the mosquito midgut was isolated after surface sterilization of mosquitoes and dissection under sterile conditions. The midgut contents were suspended in physiological saline (0.85% NaCl) and serial dilutions were plated onto blood agar plates [blood agar base (Hi Media) with 5% blood]. All incubations were carried out at 30 °C for 18–24 h. Typical *Aeromonas* colonies, showing grey colour and β -haemolysis, were scored and selected for further study.

Biochemical characterization of *Aeromonas* strains was done using API 20E, API 20NE and API 50CH systems (bioMérieux) according to the manufacturer's instructions. Sensitivity to the vibriostatic agent O/129 was detected on tryptone soya agar (Oxoid) containing 10 or 150 μg O/129 ml^{-1} .

Genomic DNA was isolated from strains of *Aeromonas* by standard phenol/chloroform extraction (Sambrook *et al.*, 1989). The 16S rRNA genes of the novel isolates were amplified by PCR and sequenced directly on an ABI 377 automated DNA sequencer using the Big Dye terminator kit (Applied Biosystems). The other details of the sequencing reactions are as given in Hauben *et al.* (1997). Analysis of 16S rRNA gene sequences was done at the RDP (Ribosomal Database Project, Michigan State University, USA). *S*_{ab} values were obtained by the similarity rank analysis function at RDP I. To determine the correct extent of sequence similarity between the novel strain MTCC 3249^T and other closely related aeromonads, 16S rRNA gene sequences were retrieved from the database. Sequence alignment was done using the CLUSTAL W program at the EBI server to determine similarity values and the distance matrix. The phylogenetic tree for 16S rRNA gene sequences was drawn by using the MEGA software using 1336 nucleotides from the sequences of the type strains of all members of the genus *Aeromonas*.

Twenty reference strains of the genus *Aeromonas* representing 17 hybridization groups (HGs) were selected to study DNA–DNA similarity with *Aeromonas* strain MTCC 3249^T. The bacterial strains were grown overnight at 30 °C. Preparation and purification of DNA for this experiment was performed as described by Pitcher *et al.* (1989). DNA concentrations were measured by the diphenylamine method as described previously (Kaznowski, 1995). The DNAs were labelled with photobiotin (Vector Laboratories) according to the manufacturer's instructions. Prior to hybridization experiments, the nucleic acid was fragmented to fragments of 500–800 bp. The fragment size was checked by electrophoresis on a 1% agarose gel (Kaznowski, 1997).

Colorimetric DNA–DNA hybridizations were performed in Covalinc microplates (Nunc) (Kaznowski, 1995). Immobilization was accomplished according to the slightly modified method of Rasmussen *et al.* (1991). Calculation of T_m values was done using following formula (Grimont & Grimont, 1991):

$$T_m = (0.41 \text{ mol\% GC}) + 81.5 + (16.6 \log_{10} M) - (0.72F)$$

where mol% GC is the G+C content of the DNA (60 mol% was taken as the mean value for *Aeromonas* species), M is the sodium ion molarity and F is the percentage of formamide present in the reaction. For estimation of DNA relatedness, the absorbance of the well containing herring sperm DNA was calculated as 0% and the absorbance of the well for each reference DNA was calculated as 100%. Bacterial strains showing 70% or more relatedness to the reference strains of a given HG were assigned to that HG (Wayne *et al.*, 1987). Each cross-hybridization was performed in duplicate with four or five replicates.

Antibiotic sensitivity patterns were checked using multi-disc rings (Span Diagnostics). The concentrations of antibiotics used are given in Table 3. Depend-

Table 1. Key tests for the phenotypic differentiation of *Aeromonas culicicola* sp. nov. from other *Aeromonas* species

Species are indicated as: 1, *A. culicicola*; 2, *A. hydrophila*; 3, *Aeromonas bestiarum*; 4, *A. caviae*; 5, *Aeromonas media*; 6, *A. eucrenophila*; 7, *A. sobria*; 8, *A. veronii* biotype *sobria*; 9, *A. veronii* biotype *veronii*; 10, *A. jandaei*; 11, *A. encheleia*; 12, *A. schubertii*; 13, *A. trota*; 14, *Aeromonas allosaccharophila*; 15, *Aeromonas popoffii*. Characters are given as: +, $\geq 85\%$ strains positive; –, $\geq 85\%$ strains negative; v+, 50–84% of strains positive; v–, 50–84% of strains negative; NA, data not available. Data were adapted from Huys *et al.* (1997) and Borrell *et al.* (1998).

Character	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Motility	+	+	+	v+	–	+	+	+	v+	+	+	+	+	+	+
Production of gas from glucose	+	v+	v+	–	–	+	v+	v+	+	+	+	–	v+	+	+
Production of acid from:															
Salicin	–	v+	v+	+	+	+	–	–	+	–	+	–	–	–	–
D-Cellobiose	–	v–	v–	+	+	+	+	v+	v+	–	–	–	+	+	–
D-Mannitol	+	+	+	+	+	+	+	+	+	+	+	–	+	+	+
D-Sucrose	+	+	+	+	+	–	+	+	+	–	v–	–	v–	+	–
L-Arabinose	–	+	NA	+	+	NA	–	–	–	–	–	–	–	NA	NA
Decarboxylation of:															
Ornithine	–	–	–	–	–	–	–	–	+	–	–	–	–	–	v–
Lysine	+	+	+	–	–	–	+	+	+	+	–	+	+	+	–

ing on the diameter of the inhibition zone around the antibiotic disc, conclusions were reached according to the manufacturer's instructions.

It was found that the midgut had a mixed bacterial population representing haemolytic, late-haemolytic and non-haemolytic bacteria. The total bacterial load was found to be 1.2×10^5 per midgut, of which 75% showed haemolytic activity. The microflora of blood-fed mosquitoes (from the same geographical location but in the vicinity of human populations) was analysed by plating on blood agar; it was observed that there was a 2000-fold increase in the midgut bacterial content. Increases in the midgut bacterial content after blood feeding have been reported before (Demaio *et al.*, 1996). After a blood meal, the increase in population was due mainly to increases in the haemolytic type of bacteria. The colonies of these haemolytic bacteria were small, grey in colour with a metallic sheen and were surrounded by a zone of β -haemolysis. The isolates from *Cx. quinquefasciatus* were labelled as strain MTCC 3249^T while those from *Ae. aegyptii* were labelled as SH (small haemolytic) and SLH (small late haemolytic).

The biochemical characters of the novel strains examined in this study were identical (data not shown). Table 1 shows selected biochemical characters of strain MTCC 3249^T. The isolates were identified as belonging to the genus *Aeromonas*, since they were Gram-negative, rod-shaped, polarly flagellated, oxidase-positive, facultative aerobes. They were resistant to the vibriostatic agent O/129, could be grown at 4 °C, produced DNase and fermented glucose. It was difficult to include these *Aeromonas* strains in any known species of the genus *Aeromonas*, based only on biochemical characterization. The isolates were ornithine decarboxylase-negative, positive for the Voges–Proskauer test and did not utilize inositol or sorbitol,

thus excluding them from *A. hydrophila*, *A. veronii* biotype *sobria* (formerly *Aeromonas sobria*) or *A. caviae*. The utilization of D-sucrose distinguishes them from *Aeromonas eucrenophila*, *A. jandaei*, *Aeromonas encheleia*, *Aeromonas schubertii* and *Aeromonas trota*. Except for utilization of D-cellobiose, all the strains showed similar biochemical characters to *A. veronii* biotype *sobria* (formerly *A. sobria*).

Using universal eubacteria-specific primers, the entire 1.5 kb 16S rRNA gene was amplified by PCR. The PCR products were used directly for sequencing with universal and internal primers to obtain about 1351 bases, which represents more than 90% of the molecule. 16S rRNA gene sequences from all three novel strains of *Aeromonas* were identical (data not shown), hence only the sequence from strain MTCC 3249^T was selected for further analysis. It was compared with 16S rRNA gene sequences in the RDP II database and showed highest similarity to *A. jandaei* ATCC 49568^T, with an *S*_{ab} value of 0.999. The 16S rRNA gene sequences for all the *Aeromonas* species were retrieved from the database and aligned with the sequence of strain MTCC 3249^T in CLUSTAL W at the EMBL outstation. The 16S rRNA gene sequence of strain MTCC 3249^T was identical to all known sequences from *A. jandaei* except for one base change at position 264 with respect to other *Aeromonas* species in this region. It showed 10 nucleotide differences from *A. sobria*, with which it showed high similarity in biochemical characterization (Table 2). Species of the genus *Aeromonas* exhibit very high levels of overall 16S rRNA gene sequence similarity to each other and the number of nucleotide differences varies from 0 to 33. Two species of *Aeromonas*, *A. trota* and *A. caviae*, were found to differ only by a single nucleotide (Martinez-Murcia *et al.*, 1992). For strain MTCC 3249^T, the number of differences from reference *Aeromonas* strains in 16S rRNA gene sequences ranged from 1 to

Table 2. Comparison of *Aeromonas culicicola* sp. nov. MTCC 3249^T with reference *Aeromonas* strains based on DNA–DNA similarity and nucleotide differences in 16S rRNA

For DNA–DNA hybridization, the mean $\pm$ SD is given with the number of replicates in parentheses. ND, Not determined.

Strain	HG	DNA–DNA hybridization with strain MTCC 3249 ^T (%)	Nucleotide differences from strain MTCC 3249 ^T in 16S rRNA gene sequence
<i>A. culicicola</i> SH	–	96 $\pm$ 2 (10)	0
<i>A. culicicola</i> SLH	–	88 $\pm$ 3 (10)	0
<i>A. hydrophila</i> ATCC 7966 ^T	1	37 $\pm$ 3 (10)	15
<i>A. bestiarum</i> ATCC 51108 ^T	2	44 $\pm$ 2 (8)	14
<i>A. hydrophila</i> CDC 0434-84	3	43 $\pm$ 3 (8)	ND
<i>A. salmonicida</i> ATCC 33658 ^T	3	38 $\pm$ 4 (8)	ND
<i>A. caviae</i> ATCC 15468 ^T	4	38 $\pm$ 2 (10)	5
<i>A. media</i> CDC 0862-83	5A	43 $\pm$ 2 (6)	ND
<i>A. media</i> ATCC 33907 ^T	5B	32 $\pm$ 3 (8)	16
<i>A. eucrenophila</i> ATCC 23309 ^T	6	37 $\pm$ 2 (8)	14
<i>A. sobria</i> CIP 7433 ^T	7	61 $\pm$ 2 (10)	10
<i>A. veronii</i> biotype sobria CDC 0437-84	8/10	44 $\pm$ 3 (10)	5
<i>A. ichthiosmia</i> ATCC 49904 ^T	8/10	49 $\pm$ 2 (8)	5
<i>A. veronii</i> biotype veronii ATCC 35624 ^T	10/8	42 $\pm$ 3 (8)	5
<i>A. jandaei</i> ATCC 49568 ^T	9	54 $\pm$ 2 (10)	1
<i>Aeromonas</i> sp. ATCC 35941	11	32 $\pm$ 2 (8)	11
<i>A. schubertii</i> ATCC 43700 ^T	12	30 $\pm$ 3 (8)	18
<i>Aeromonas</i> sp. ATCC 43946	13	21 $\pm$ 4 (8)	ND
<i>A. trota</i> ATCC 49657 ^T	14	25 $\pm$ 4 (8)	6
<i>A. allosaccharophila</i> CECT 4199 ^T	15	52 $\pm$ 1 (8)	9
<i>A. encheleia</i> CECT 4342 ^T	16	35 $\pm$ 3 (8)	15
<i>A. popoffii</i> LMG 17541 ^T	17	36 $\pm$ 2 (8)	14

Fig. 1. 16S rRNA gene sequence-based phylogenetic relationships of *Aeromonas culicicola* sp. nov. MTCC 3249^T to reference strains (HGs) of the genus *Aeromonas*. The unrooted phylogenetic tree was drawn using 1336 nucleotides of 16S rRNA gene sequence using the neighbour-joining method in MEGA software. The bar represents distance values calculated in MEGA and values at nodes represent bootstrap percentages. Bootstrap values less than 50 % are not shown.

18 (Table 2). A phylogenetic tree drawn using 1336 nucleotides (Fig. 1) shows that it indeed groups very close to but separate from *A. jandaei* ATCC 49568^T.

DNA–DNA hybridization is the recommended method for defining bacterial species (Martínez-Murcia, 1999), wherein a relative binding ratio of 70 % under optimal conditions is considered to be borderline for species differentiation (Wayne *et al.*, 1987). DNA–DNA hybridization studies were performed to find out the relatedness of strain MTCC 3249^T to strains SH and SLH and to other *Aeromonas* species (Table 2).

Strain MTCC 3249^T was found to show the highest DNA–DNA similarity, 61 %, to *A. sobria* CIP 7433^T (HG7) and the lowest value, 21 %, to *Aeromonas* sp. ATCC 43946 (HG13). It showed 54 % similarity to *A. jandaei* ATCC 49568^T (HG9) and 37 % similarity to *A. hydrophila* ATCC 7966^T (HG1). Strain MTCC 3249^T showed 96 and 88 % similarity to strains SH and SLH.

Strain MTCC 3249^T showed resistance to ampicillin and amikacin, while it was sensitive to cefadroxil, ceftriaxone, cefuroxime, ciprofloxacin and norfloxacin. It showed intermediate resistance to cefoperazone,

Table 3. Antibiotic sensitivity pattern of *A. culicicola* sp. nov. MTCC 3249^T

Antibiotic	Concentration (µg)*	Cut-off zone diameter (mm)			Zone diameter observed (mm)
		Resistant	Intermediate	Sensitive	
Ampicillin	10	≤ 11	12–13	≥ 14	9
Amikacin	30	≤ 14	15–16	≥ 17	11
Cefadroxil	30	≤ 14	15–17	≥ 18	20
Cefriaxone	30	≤ 13	14–20	≥ 21	24
Cefoperazone	75	≤ 15	16–20	≥ 21	17
Cefotaxime	30	≤ 14	15–22	≥ 23	17
Cefuroxime	30	≤ 14	15–17	≥ 18	20
Ciprofloxacin	5	≤ 15	16–20	≥ 21	24
Cotrimoxazole	30	≤ 10	11–15	≥ 16	11
Erythromycin	15	≤ 13	14–22	≥ 23	18
Gentamicin	10	≤ 12	13–14	≥ 15	13
Netilmicin	30	≤ 14	15–17	≥ 18	15
Norfloxacin	10	≤ 12	13–16	≥ 17	21
Penicillin	10 U	≤ 14	15–19	≥ 20	17

* In µg except penicillin.

cefotaxime, cotrimoxazole, erythromycin, gentamicin, netilmicin and penicillin (Table 3).

Based on the results of biochemical characterization, 16S rRNA gene sequence analysis and DNA–DNA hybridization, we propose that strains MTCC 3249^T, SH and SLH represent a novel species within the genus *Aeromonas* and propose the name *Aeromonas culicicola* sp. nov.

Aeromonas is one of the most common genera found in surface water, a breeding habitat for mosquitoes (Smith *et al.*, 1998). It is therefore probable that mosquitoes acquire *Aeromonas* in their midgut from their aquatic breeding habitat. Contamination of water and the establishment of gastrointestinal mosquito carriers may be responsible for soft-tissue infections (Tapper *et al.*, 1975). Infection with *Aeromonas* is a cause of concern as most isolates of *Aeromonas* are resistant to several broad-spectrum antibiotics (Fass, 1987). In addition, *Aeromonas* strains are strongly β -haemolytic, since they secrete a potential cytotoxin called aerolysin, a membrane-interacting haemolytic enzyme (Husslein *et al.*, 1991).

To the best of our knowledge, this is the first report of the isolation of *Aeromonas* strains from the midgut of a mosquito. Reports of *Aeromonas* septicemia cases without freshwater exposure or a recognized portal of entry for the bacterium emphasize the importance of this observation (Hanninen & Siitonen, 1995). We are currently investigating the role of mosquito-borne aeromonads in such infections.

Description of *Aeromonas culicicola* sp. nov.

Aeromonas culicicola (cu.li'ci.co.la. N.L. n. *Culicidae* name of a family of mosquitoes; L. suffix *-cola* inhabitant of; N.L. n. *culicicola* inhabitant of *Culici-*

dae, to denote the family of the insect hosts from which the species was first isolated).

Cells are Gram-negative, straight, motile rods with a polar flagellum. Oxidase- and catalase-positive, reduces nitrate to nitrite and is resistant to the vibriostatic agent O/129. Shows positive reactions for DNase, Voges–Proskauer test, indole production from tryptophan, alkalization of citrate, lysine decarboxylation, arginine dihydrolase, ONPG, gelatinase and amylase production. No production of ornithine decarboxylase or urease. Optimal growth occurs after 18 h at 30 °C, but can also grow at 37 °C in LB broth. Haemolytic activity is observed after 18 h incubation at 37 °C. The type strain utilizes the following substrates as sole carbon and energy sources: starch, D-fructose, D-galactose, D-glucose, D-glycerol, glycogen, D-maltose, D-mannitol, melibiose, D-mannose, N-acetyl glucosamine, D-sucrose and D-ribose. Uniform acid production is observed with all these substrates. Does not produce acid from adonitol, amygdalin, D-arabinose, L-arabinose, D-arabitol, arbutin, methyl α -glucoside, methyl α -mannoside, α -gentiobiose, methyl β -xyloside, cellobiose, dulcitol, erythritol, aesculin, D-fucose, L-fucose, inositol, inulin, 2-ketogluconate, 5-ketogluconate, lactose, D-lyxose, melezitose, D-raffinose, rhamnose, salicin, sorbitol, L-sorbose, D-tagatose, D-turanose, xylitol or D-xylose. The type strain is resistant to ampicillin and amikacin and sensitive to cefadroxil, cefriaxone, cefuroxime, ciprofloxacin and norfloxacin. It shows intermediate resistance to cefoperazone, cefotaxime, cotrimoxazole, erythromycin, gentamicin, netilmicin and penicillin. The 16S rRNA gene sequence of the type strain is highly similar to all known sequences of *A. jandaei*, with a single base difference at position 264, while DNA–DNA hybridization studies separate it from *A. jandaei* (54 %

similarity). It shows highest similarity to *A. sobria* (61 %).

The type strain has been deposited at the Microbial Type Culture Collection at Institute of Microbial Technology, Chandigarh, India, as strain MTCC 3249^T and at the National Collection of Industrial Micro-organisms, National Chemical Laboratory, Pune, India, as strain NCIM 5147^T.

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