Aeromonas hydrophila subsp. *ranae* subsp. nov., isolated from septicæmic farmed frogs in Thailand

Geert Huys,¹ Marianne Pearson,² Peter Kämpfer,³ Rik Denys,¹ Margo Cnockaert,¹ Valerie Inglis² and Jean Swings^{1,4}

Correspondence

Geert Huys

geert.huys@rug.ac.be

^{1,4}Laboratorium voor Microbiologie¹ and BCCMTM/LMG Bacteria Collection⁴, Ghent University, K.L. Ledeganckstr. 35, B-9000 Ghent, Belgium

²Institute of Aquaculture, University of Stirling, Stirling FK9 4LA, UK

³Institut für Angewandte Mikrobiologie, Justus-Liebig-Universität Giessen, IFZ, Heinrich-Buff-Ring 26–32, D-35392 Giessen, Germany

A group of seven sucrose-negative *Aeromonas* strains (referred to as group Au) isolated from the internal organs of septicæmic farmed frogs (*Rana rugulosa*) in Thailand was subjected to a polyphasic taxonomic study including fluorescent amplified fragment length polymorphism (FAFLP) and ERIC-PCR fingerprinting, 16S rDNA sequencing, microplate DNA–DNA hybridizations and extensive phenotypic characterization. Comparison of FAFLP and ERIC-PCR fingerprints indicated that the group Au isolates belonged to the species *Aeromonas hydrophila* DNA hybridization group (HG) 1 in which they represent a genotypic subgroup closely affiliated to *A. hydrophila* subsp. *hydrophila* and subsp. *dhakensis*. One representative of the Au group exhibited $\geq 99.0\%$ 16S rDNA sequence similarity with the type strains of the two *A. hydrophila* subspecies. DNA–DNA hybridization with type and reference strains of all known *Aeromonas* taxa revealed that the Au group represented a homogeneous taxon that exhibited the highest relatedness with members of the two *A. hydrophila* subspecies, ranging from 75 to 93%. Phenotypic characterization on the basis of 152 features further revealed that the Au group isolates differed from *A. hydrophila* subsp. *hydrophila* or subsp. *dhakensis* in a total of 13 biochemical properties. Of these, assimilation of L-glycine and isobutyrate as sole carbon source, acid production from salicin and D-sucrose, and aesculin hydrolysis were of diagnostic value. From the results of this study, it can be concluded that the *Aeromonas* frog isolates of the Au group represent a new subspecies of *A. hydrophila*, for which the name *Aeromonas hydrophila* subsp. *ranae* subsp. nov. is proposed. Its type strain is Au-1D12^T (=LMG 19707^T = CCUG 46211^T).

Some members of the genus *Aeromonas* have been recognized as primary pathogens for cold-blooded (poikilothermic) or warm-blooded animal species (Gosling, 1996) and as opportunistic human pathogens (Janda & Abbott, 1996). Historically, the pathogenicity of aeromonads was first established with poikilotherms. In Southeast Asia, outbreaks of motile *Aeromonas*-associated septicæmia can reach epidemic proportions among farmed aquatic animals, leading to massive mortality rates (Joseph & Carnahan, 1994). Strains of *Aeromonas hydrophila* are also pathogenic

for amphibians, including turtles (Pasquale *et al.*, 1994) and frogs. Occasionally, motile aeromonad septicæmia in frogs has also been cited as ‘red leg’ disease, referring to haemorrhages that were occasionally observed in the leg muscles of diseased animals (Rigney *et al.*, 1978). However, identification data are only rarely reported in studies on bacterial septicæmic disease of frogs and are often based on primary phenotypic characterization. During a survey of frogs farmed in Thailand, including the species *Rana rugulosa* (Chinese bullfrog) and *Rana tigerina* (Indian bullfrog or tiger frog), Pearson *et al.* (1997) isolated a collection of motile aeromonads from the skin and intestine of both healthy and septicæmic adult frogs. Next to representatives of *A. hydrophila*, *Aeromonas veronii* and *Aeromonas caviae*, a group of unspeciated strains designated group Au was recovered exclusively from the internal organs of septicæmic animals surveyed at different farms throughout Thailand. The Au group was found to be biochemically distinct from the former species because all strains failed to

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Abbreviations: ERIC, enterobacterial repetitive intergenic consensus; FAFLP, fluorescent amplified fragment length polymorphism; HG, hybridization group.

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Supplementary tables are available in IJSEM Online.

ferment or assimilate D-sucrose, a characteristic which is also common to the species *Aeromonas eucrenophila* (Huys *et al.*, 1997a), *Aeromonas schubertii* (Hickman-Brenner *et al.*, 1988), *Aeromonas jandaei* (Carnahan *et al.*, 1991) and *Aeromonas popoffii* (Huys *et al.*, 1997b). In addition, randomly amplified polymorphic DNA (RAPD) analysis revealed a genotypic homogeneity that was unusual for a group of geographically diverse *Aeromonas* isolates (Pearson *et al.*, 1997). The current investigation was initiated to determine the taxonomic position of the unknown Au group in the genus *Aeromonas* by subjecting a group of seven representative strains to fluorescent amplified fragment length polymorphism (FAFLP) and ERIC-PCR fingerprinting, 16S rDNA sequencing, extensive phenotypic characterization and microplate DNA–DNA hybridization. The data generated indicate that the Au group genotypically belongs to *A. hydrophila* DNA hybridization group (HG) 1 but constitutes a phenotypically unique taxon in this species, for which the name *A. hydrophila* subsp. *ranae* is proposed.

Seven *Aeromonas* isolates belonging to the Au group (LMG 19707^T–LMG 19713) recovered from the liver or kidney of septicemic *Rana rugulosa* at various farm sites in Thailand were included in the present study (additional descriptive data are available as supplementary Table 1 in IJSEM Online at <http://ijs.sgmjournals.org>). Type and reference strains of all known *Aeromonas* species were obtained from the BCCM/LMG Bacteria Collection, Ghent University, Belgium. All strains were cultured aerobically on Trypticase soy agar (TSA) containing 3 % (w/v) Trypticase soy broth (BBL) and 1.5 % (w/v) bacteriological agar no. 1 (Oxoid) at 28 °C for 24 h. All Au isolates were subjected to (FAFLP) fingerprinting and the resulting band profiles were compared with the laboratory-based AEROLIB library (Huys & Swings, 1999). Microscale DNA extraction, AFLP template preparation, selective PCR amplification, electrophoresis on an automated DNA sequencer and data processing were performed as previously described (Huys & Swings, 1999). Microscale DNA extracts were also used for repetitive-sequence-based PCR fingerprinting using the ERIC1R and ERIC2 oligonucleotide primers (Versalovic *et al.*, 1991). ERIC-PCR analysis was performed twice during independent runs according to the protocol of Versalovic *et al.* (1994) with minor modifications reported by Huys *et al.* (2002). All Au frog isolates were tested for 152 physiological and biochemical properties as previously described (Huys *et al.*, 1997b). The urocanic acid assimilation test was performed according to Hänninen (1994). A total of 30 features were selected for repeated determinations. For all strains under study, both readings gave identical results, demonstrating the excellent reproducibility of the method. Antimicrobial susceptibilities were determined for six antimicrobial agents with the disc diffusion method. The following antibiotic discs (Oxoid) were applied using a ST6090 Disc Dispenser (Oxoid): nalidixic acid (30 µg), penicillin (10 µg), ampicillin (25 µg), tetracycline (30 µg), kanamycin (30 µg) and streptomycin (25 µg). Antibigrams were determined according to the conventional Kirby–Bauer method (Bauer *et al.*,

1966) with the exception that Mueller–Hinton medium was replaced by IS broth and ISA medium (Traub *et al.*, 1998). Isolates were classified into three categories based on the quantitative interpretation criteria recommended by the National Committee for Clinical Laboratory Standards (1993). Au strains LMG 19707^T, LMG 19709 and LMG 19711 were used in a DNA–DNA hybridization study that also included the type strains of all known *Aeromonas* species. Genomic DNA was prepared using a combination of the protocols of Marmur (1961) and Pitcher *et al.* (1989) as described by Goris *et al.* (1998). Hybridizations were performed using the fluorometric microplate method (Ezaki *et al.*, 1989) with modifications by Goris *et al.* (1998) at an optimal renaturation temperature of 45 °C. Fluorometric data recorded after 15 min incubation were used for calculation of the DNA–DNA hybridization values. The complete 16S rDNA sequences of strains LMG 19562^T and LMG 19707^T were determined as previously described (Huys *et al.*, 2001) using the ABI PRISM 3100 Genetic Analyser.

Previously, the use of the DNA fingerprinting technique FAFLP has proven to be a highly discriminative and relatively fast method to determine the genotypic position of unknown *Aeromonas* isolates provided that a reliable taxonomic framework is available (Huys & Swings, 1999). In the current study, the digitized FAFLP banding patterns of the Au isolates were compared with the laboratory database AEROLIB comprising well-characterized strains of all known *Aeromonas* taxa. This comparison, of which a simplified version including only the type strains of other *Aeromonas* spp. is depicted in Fig. 1, revealed that the seven Au isolates represent a homogeneous cluster delineated at a correlation level of 68 %. This cluster constitutes a subgroup within the AFLP group representing *A. hydrophila* as witnessed by its close genotypic relatedness to members of *A. hydrophila* subsp. *hydrophila* (51 % correlation) and, to a lesser extent, of *A. hydrophila* subsp. *dhakensis* (33 % correlation). The latter two taxa were recently described by Huys *et al.* (2002) to allocate two groups of *A. hydrophila* HG1 strains that were sufficiently different on a phenotypic basis to warrant their definition as separate subspecies. Visual comparison of ERIC-PCR fingerprints allowed a group of six Au isolates to be distinguished from a collection of nine reference strains encompassing the two *A. hydrophila* subspecies (Fig. 2). This differentiation was based on visual scoring of the band with an estimated size of 0.7 kb that is present in the ERIC-PCR profiles of all Au strains but absent in members of the two *A. hydrophila* subspecies. In addition, the lack of an ERIC-PCR fragment approximately 0.95 kb in size allowed the further differentiation of the Au isolates from *A. hydrophila* subsp. *hydrophila* strains that all exhibited this band in their profiles. Although the stability of these group-specific ERIC-PCR markers was demonstrated by performing duplicate experiments, it is clear that their taxonomic value should be further monitored by other workers eventually including additional Au-group-like isolates. Taken together, the AFLP and ERIC-PCR data suggest that the Au isolates are closely affiliated to

Fig. 1. Dendrogram showing the relative genotypic position of the seven Au group isolates in the genus *Aeromonas* obtained after comparison with the laboratory-based AEROLIB database (Huys & Swings, 1999). Clustering is only shown with the reference strains of *A. hydrophila* subsp. *hydrophila* and subsp. *dhakensis* and with the type strains of all other currently described *Aeromonas* species. The dendrogram was obtained following numerical analysis of digitized FAFLP band patterns using the Pearson product-moment correlation coefficient (expressed as percentage values) and the unweighted paired group method using arithmetic averages (UPGMA). LMG, BCCM/LMG Bacteria Collection, Laboratory of Microbiology, Ghent University, Ghent, Belgium; T, type strain.

A. hydrophila HG1 although they seem to constitute a separate genotypic subgroup in this taxon.

The DNA hybridization data (available as supplementary Table 2 in IJSEM Online at <http://ijs.sgmjournals.org>) clearly confirm the genotypic position of the Au group as exhibited by numerical analysis of FAFLP profiles (Fig. 1). The internal genomic relatedness of the Au group as determined from strains LMG 19707^T, LMG 19709 and LMG 19711 ranged from 93 to 103 %, indicating that the Au strains constitute a genotypically homogeneous group in the genus *Aeromonas*. In concordance with FAFLP clustering, a high

relatedness was also found between the three representatives of the Au group and the type strain and two reference strains of *A. hydrophila* subsp. *hydrophila* (80–93 %), and with the type strain of *A. hydrophila* subsp. *dhakensis* (75–87 %). On the basis of DNA–DNA hybridization values ranging from 46 to 64 %, Au strains LMG 19707^T and LMG 19711 could be clearly distinguished from the type strains of other *Aeromonas* spp. Collectively, the results from the DNA–DNA hybridization experiments clearly indicate that the Au group should be located in the species *A. hydrophila*.

Pairwise comparison of the 16S rDNA sequence of Au strain

Fig. 2. Digital photograph showing ERIC-PCR fingerprints of *Aeromonas* Au isolates and of *A. hydrophila* subsp. *hydrophila* and subsp. *dhakensis* reference strains. Lanes: 1 and 17, SmartLadder band sizing mixture (Eurogentec, Belgium); 2–7, *Aeromonas* Au isolates LMG 19707^T, LMG 19709, LMG 19710, LMG 19711, LMG 19712 and LMG 19713; 8–12, *A. hydrophila* subsp. *hydrophila* LMG 2844^T, LMG 13443, LMG 13656, LMG 13658 and LMG 13660; 13–16, *A. hydrophila* subsp. *dhakensis* LMG 19558, LMG 19559, LMG 19560 and LMG 19562^T. Arrows indicate the two ERIC-PCR markers (0.95 and 0.7 kb) that allow differentiation of Au isolates from *A. hydrophila* subsp. *hydrophila* and subsp. *dhakensis*.

Table 1. Phenotypic characteristics for differentiation of the Au group from *A. hydrophila* subsp. *hydrophila* and subsp. *dhakensis*

Characters are scored as: +, ≥85 % strains positive; –, ≥85 % strains negative.

Characteristic	Au group (n=7)	<i>A. hydrophila</i> HG1	
		subsp. <i>hydrophila</i> * (n=3)	subsp. <i>dhakensis</i> † (n=10)
Utilization of:			
Urocanic acid‡	+	–	+
L-Glycine	–	+	+
Isobutyrate	–	+	+
L-Arabinose	–	+	–
L-Fucose	–	+	–
α-Methyl D-mannoside	–	+	–
cis-Aconitate	–	–	+
DL-Aspartate	–	–	+
Caprate	–	–	+
Acid from:			
Salicin	–	+	+
Sucrose	–	+	+
L-Arabinose	–	+	–
Hydrolysis of aesculin	–	+	+

*Data from Huys *et al.* (2002) containing features previously reported by Abbott *et al.* (1992), Altwegg & Lüthy-Hottenstein (1991), Hänninen (1994) and Kämpfer & Altwegg (1992).

†Data from Huys *et al.* (2002).

‡All Au isolates displayed growth without blue colouration whereas *A. hydrophila* subsp. *dhakensis* strains exhibited growth with blue colouration on the medium described by Hänninen (1994).

LMG 19707^T (submitted under EMBL accession no. AJ508766) with those of *A. hydrophila* subsp. *hydrophila* strain LMG 2844^T=ATCC 7966^T (accession no. X60404) and *A. hydrophila* subsp. *dhakensis* LMG 19562^T (submitted under EMBL accession no. AJ508765) revealed similarity values of 99.8 and 99.0 %, respectively. Although the value of 16S rDNA sequences in differentiating between certain *Aeromonas* species has been questioned (Martinez-Murcia, 1999), these results indicate that the isolates of the Au group are phylogenetically closely affiliated to the species *A. hydrophila* HG1.

The absence of sucrose assimilation and fermentation, as displayed by all Au strains, is considered highly atypical within *A. hydrophila* HG1 (Huys *et al.*, 2002). In fact, the Au group was characterized overall by a relatively narrow spectrum of carbon sources that could be utilized or fermented. In addition to the urocanic acid test, a total of 12 negative test results allowed the differentiation of the group of Au isolates from *A. hydrophila* subsp. *hydrophila* and/or subsp. *dhakensis* because of this uniform biochemical inertness (Table 1). Among these tests, assimilation of L-glycine and isobutyrate as sole carbon source, acid production from salicin and D-sucrose, and aesculin hydrolysis were essential for the unequivocal separation of the Au group from both *A. hydrophila* HG1 subspecies. Collectively, the finding of at least 13 discriminative features

(Table 1) indicates that the Au group does not belong to *A. hydrophila* subsp. *hydrophila* or subsp. *dhakensis* and thus represents a third phenotypic subgroup in this species. All Au isolates were lysine decarboxylase- and arginine dihydrolase-positive, utilized DL-lactate, and failed to form acid from sorbitol and L-rhamnose. These characteristics are also shared by the two subspecies currently situated in *A. hydrophila* (Table 2; Huys *et al.*, 2002) and can thus be considered species-specific. As a member of the species *A. hydrophila*, the Au group can be phenotypically differentiated from *Aeromonas salmonicida* by its motility, its ability to grow at 37 °C and/or its inability to produce a brown water-soluble pigment (Pavan *et al.*, 2000). Furthermore, at least two tests are available for the separation of the Au group from each of the other species that are currently recognized in the genus *Aeromonas* (Table 2).

The DNA–DNA hybridization results and the 16S rDNA sequencing and phenotypic data reported in this study indicate that the Au group represents a new phenotypic subtaxon in the species *A. hydrophila* which deserves subspecies status in addition to the currently delineated subsp. *hydrophila* and subsp. *dhakensis* (Huys *et al.*, 2002). We propose the name *Aeromonas hydrophila* subsp. *ranae* for the seven Au strains.

Table 2. Key tests for the phenotypic differentiation of *A. hydrophila* subsp. *ranae* from other mesophilic *Aeromonas* species

1, *Aeromonas hydrophila* subsp. *ranae*; 2, *Aeromonas hydrophila* subsp. *dhakensis*; 3, *Aeromonas hydrophila* subsp. *hydrophila*; 4, *Aeromonas bestiarum*; 5, *Aeromonas caviae*; 6, *Aeromonas media*; 7, *Aeromonas eucrenophila*; 8, *Aeromonas sobria*; 9, *Aeromonas veronii* biovar *sobria*; 10, *Aeromonas veronii* biovar *veronii*; 11, *Aeromonas jandaei*; 12, *Aeromonas encheleia*; 13, *Aeromonas schubertii*; 14, *Aeromonas trota*; 15, *Aeromonas allosaccharophila*; 16, *Aeromonas popoffii*. Characters are scored as: +, ≥85 % of the strains are positive; –, ≥85 % of the strains are negative; v+, 50–85 % of the strains are positive; v–, 50–85 % of the strains are negative; ND, no data found.

	1	2*	3*	4†	5‡	6‡	7§	8‡	9‡	10‡	11‡	12§	13‡	14‡	15	16¶
Utilization of:																
DL-Lactate	+	+	+	–	v+	+	–	v+	–	–	+	–	v+	ND	ND	+
Arbutin	+	+	+	+	+	+	+	–	–	+	–	+	–	–	ND	–
D-Mannitol	+	+	+	+	+	+	+	+	+	+	+	+	–	v+	+	+
D-Melibiose	–	–	–	–	–	–	–	–	–	–	+	–	–	ND	ND	–
Acid from:																
Salicin	–	+	+	v–	+	v+	+	–	–	+	–	+	–	–	–	–
D-Cellobiose	–	–	v–	v–	v+	+	+	+	v–	v+	–	–	–	+	+	–
L-Arabinose	–	–	+	+	+	+	v+	–	–	–	–	v–	–	–	v+	v–
D-Sucrose	–	+	+	+	+	+	–	+	+	+	–	v–	–	v–	+	–
Decarboxylation of:																
Ornithine	–	–	–	–	–	–	–	–	–	+	–	–	–	–	v–	–
Lysine	+	+	+	+	–	–	–	+	+	+	+	–	v+	+	+	–
Arginine dihydrolase	+	+	+	+	+	+	+	–	+	–	+	+	+	+	v+	+

*Data from this study, Abbott *et al.* (1992), Kämpfer & Altwegg (1992), Hänninen (1994) and Huys *et al.* (2002).

†Data from Abbott *et al.* (1992), Kämpfer & Altwegg (1992) and Hänninen (1994).

‡Data from Altwegg & Lüthy-Hottenstein (1991), Abbott *et al.* (1992) and Kämpfer & Altwegg (1992).

§Data from Huys *et al.* (1997a).

||Data from Martinez-Murcia *et al.* (1992).

¶Data from Huys *et al.* (1997b).

Description of *Aeromonas hydrophila* subsp. *ranae* subsp. nov.

Aeromonas hydrophila subsp. *ranae* (ra'.nae. N.L. fem. n. *Rana* generic name of frog; N.L. gen. n. *ranae*, of a frog).

All strains of the newly proposed subspecies were isolated from farmed frogs in Thailand. All strains of *Aeromonas hydrophila* subsp. *ranae* display the following characteristics typical of the genus *Aeromonas*: Gram-negative straight motile rods, chemo-organotrophic with both oxidative and fermentative metabolism, and cytochrome oxidase and catalase are positive. Optimal growth occurs after 24 h at 28 °C on TSA medium. No brown water-soluble pigment is produced on TSA medium. Arginine dihydrolase, lysine decarboxylase and indole are positive. Three out of the seven strains, i.e. LMG 19709, LMG 19710 and LMG 19713, are Voges–Proskauer-positive. Urease, tryptophan deaminase, ornithine decarboxylase and H₂S are not produced. The following substrates are used as the sole carbon and energy sources: L-alanine (except strains LMG 19710 and LMG 19712), arbutin, L-arginine, L-aspartate (except strains LMG 19710 and LMG 19713), D-fructose, fumarate, D-galactose, D-gluconate, D-glucosamine, D-glucose, glycerol, L-histidine, DL-lactate, L-malate, D-mannitol, D-mannose, D-maltose, L-proline, pyruvate, D-ribose, L-serine, succinate and D-trehalose. None of the strains use acetamidocaproate,

N-acetyl-D-galactosamine, *cis*-aconitate, *trans*-aconitate, adipate, adonitol, 4-aminobutyrate, L-arabinose, D-arabitol, DL-aspartate (except strain LMG 19707^T), azelate, betaine, cadaverine, caprate, D-cellobiose, L-citrulline, erythritol, D-fucose, L-fucose, D-glucosaminic acid, D-glucuronate, glutarate, D-glutamate, L-glycine, 3-hydroxybenzoate, 4-hydroxybenzoate, inositol, isobutyrate, isovalerate, itaconate, lactose, lactulose, L-leucine, L-lysine, maltitol, D-melibiose, mesaconate, α-methyl D-mannoside, L-ornithine (except strain LMG 19707^T), phenylacetate, L-phenylalanine, propionate, D-raffinose, L-rhamnose, sorbate, D-sorbitol, spermidine, spermine, suberate, D-sucrose, L-tryptophan, L-valine, D-xylitol or D-xylose. Acid is uniformly produced from D-glucose, D-maltose, D-mannitol, D-mannose and D-trehalose, but not from adonitol, amygdalin, L-arabinose, D-arabitol, D-cellobiose, dulcitol (except strain LMG 19711), erythritol, inositol, α-D-melibiose, methyl-D-glucoside, lactose, D-raffinose, L-rhamnose, salicin, D-sorbitol, D-sucrose or D-xylose. All strains hydrolyse the following substrates: L-alanine-*p*NA, 2-deoxythymidine-5'-*p*NP-phosphate, gelatin, bis-*p*NP-phosphate, *ortho*-nitrophenyl-β-D-galactopyranoside, *p*NP-β-D-glucopyranoside, *p*NP-phenylphosphonate, *p*NP-phosphoryl choline and L-proline-*p*NA. None of the strains are able to hydrolyse aesculin, L-glutamate-γ-3-carboxy-*p*NA, *p*NP-α-D-glucopyranoside or *p*NP-β-D-glucuronide. All strains are

resistant to ampicillin and penicillin but sensitive to kanamycin (except strain LMG 19711), streptomycin (except strain LMG 19711) and tetracycline. Strains LMG 19707^T, LMG 19709, LMG 19710 and LMG 19713 were resistant to nalidixic acid. Isolated in 1994 from the liver and kidneys of septicaemic farmed frogs (*Rana rugulosa*) in Thailand.

The type strain has been deposited in the BCCM/LMG Bacteria Collection, Ghent University (Belgium), as strain LMG 19707^T and in the CCUG Culture Collection, University of Göteborg (Sweden), as strain CCUG 46211^T.

Significance of *Aeromonas hydrophila* subsp. *ranae* as a poikilotherm pathogen

Recently, Pearson *et al.* (2000) reported on the virulence properties of 11 *Aeromonas* isolates belonging to the Au group. All these isolates displayed high haemolytic activities conferred by *ASH1*, a haemolysin gene previously cloned from an *A. salmonicida* strain (Hirono & Aoki, 1993) that was not found in other non-Au frog *Aeromonas* isolates. Furthermore, most Au strains produced elastase and were highly cytotoxic to rainbow trout cells but did not significantly affect a mammalian cell line (Pearson *et al.*, 2000). In a supplementary study, clinically healthy *Rana rugulosa* that were challenged with isolates of the Au group reproduced the symptoms of *Aeromonas*-associated septicaemia, resulting in mortalities (M. Pearson, unpublished data). During these challenge trials, Koch's postulates were fulfilled and thus confirmed that strains of the Au group are the causal agents of septicaemia in farmed frogs. Further studies should elaborate on the role of the *ASH1* gene product in the pathogenesis of motile *Aeromonas* septicaemias of farmed frogs and other aquatic species.

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