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Characterization of *Aeromonas schubertii* Strains Recently Isolated from Traumatic Wound Infections

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Recent studies have resulted in the proposal of a new species, *Aeromonas schubertii* (mannitol, sucrose, and indole negative), formerly termed Enteric Group 501, on the basis of the study of seven strains isolated from the southeastern and southwestern United States and Puerto Rico. We have isolated two phenotypically similar *A. schubertii* strains from infected human wounds sustained in the Chesapeake Bay area. Their identification was confirmed by DNA-DNA hybridization to the Centers for Disease Control definition strain 2446-81 (ATCC 43700) for group 12. The strains were further examined for the presence of virulence-associated markers: hemolysin, hemagglutinins, cytotoxin production, agglutination in acriflavine, resistance to normal human serum, and autoagglutination phenotype. Both strains were positive for hemolysin by the plate assay, cytotoxin production at 1:10, and DNase and protease. They were resistant to human serum and negative for acriflavine agglutination, and only one of the strains was autoagglutination positive. Both strains were negative for cell-free hemolysin, hemagglutinins, pectinase, and chitinase. These isolations of *A. schubertii* further extend its previously described geographic distribution and reinforce its role as a primary causative agent of cellulitis with possible increased antimicrobial resistance.

The aeromonads are autochthonous inhabitants of aquatic environments ranging from 0 to 3% salinity (14). They are generally distinguished as being glucose-fermenting, facultatively anaerobic gram-negative rods that are oxidase positive, resistant to O/129 (Vibriostat), and motile by means of a polar flagellum. In the last 10 years, *Aeromonas* spp. have been implicated as the causative agents in a variety of human wound infections ranging from cellulitis to osteomyelitis. Additionally, a strong association between several of the *Aeromonas* spp. and gastroenteritis has been established (11). However, until recently most studies involving *Aeromonas* spp. have shown an absence of thorough phenotypic confirmation to the species level.

In fact, systematic classification of *Aeromonas* spp. has been in a state of flux since the earliest isolations by Zimmerman in 1890 and Sanarelli in 1891 (4). In 1976, Popoff and Veron (20), using the method of numerical taxonomy, proposed the species *Aeromonas hydrophila* (with biovars X_1 and X_2) and *Aeromonas sobria* sp. nov. This proposal was followed in 1981 by the study of Popoff et al. (21), who used DNA-DNA homology to show that three species were distinguishable: *A. hydrophila*, *A. sobria*, and *Aeromonas caviae* (formerly the X_2 biovar of *A. hydrophila*). However, it was also determined that each of these three species contained at least two or three distinct genotypes or hybridization groups. This observation indicated that there were additional species that could not be identified by current methods of phenotypic analysis. Fanning et al. (G. R. Fanning, F. W. Hickman-Brenner, J. J. Farmer III, and D. J. Brenner, Abstr. Annu. Meet. Am. Soc. Microbiol. 1985, C116, p. 319) confirmed and expanded Popoff's original hybridization results to include 9 to 12 DNA hybridization groups. Newly described species represent some of these groups; e.g., *Aeromonas media* (1) is associated with group 5A, *Aeromonas veronii* (6) is associated with groups 8 and 10 (6, 15), and *Aeromonas schubertii* (5) is associated with group 12.

The most recent of these proposed new species is *A. schubertii* (mannitol, sucrose, and indole negative), formerly termed Enteric Group 501. The proposal to recognize *A. schubertii* is based on the study of seven strains isolated from the southeastern and southwestern United States and Puerto Rico (5). We have isolated and characterized two phenotypically similar *A. schubertii* strains from infected human wounds sustained in the Chesapeake Bay area. Their identification was confirmed by DNA-DNA hybridization to the Centers for Disease Control definition strain for group 12 (ATCC 43700). These strains were additionally evaluated for the presence of potential virulence-associated traits.

MATERIALS AND METHODS

Case history. The two strains of *A. schubertii* examined in this study, AMC 1108-87 and AMC 4396-87, were isolated at Maryland Medical Laboratory, Inc. (Baltimore, Md.), in August 1987, from leg wounds of a 57-year-old male and a 46-year-old female, respectively, whose exposures to aquatic environments could not be determined. Strain AMC 1108-87 was isolated as the predominant organism of a mixed culture that also had a moderate growth of *Enterobacter cloacae*, while strain AMC 4396-87 was isolated in pure culture.

A detailed case history was available for only one of the patients, a 46-year-old female, who presented at a health clinic on 11 August 1987 with no preexisting conditions other than an injury to her leg sustained during a fall. Her condition was diagnosed as cellulitis, and dicloxacillin (semisynthetic penicillin) was prescribed. However, this treatment proved ineffective, and the patient subsequently returned to have the wound debrided and a specimen cultured. The laboratory results were reported as heavy growth of

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TABLE 1. Biochemical reactions of three *A. schubertii* strains

Test[a]	Reaction[b]		
	AMC 1108-87	AMC 4396-87	ATCC 43700
Oxidase	+	+	+
Sensitivity to O/129	−	−	−
String test	−	−	−
Growth in nutrient broth plus:			
0% NaCl	+	+	+
6.5% NaCl	−	−	−
Motility	+	+	+
ONPG[c]	+	+	+
Citrate (Simmons)[c]	+	+	+
Nitrate reduced to nitrite[d]	+	+	+
H_2S on triple sugar iron	−	−	−
Indole production	−	−	−
VP (O'Meara)	−	+	−
VP[c]	−	+	−
VP[d]	−	+	−
Growth in KCN	−	−	−
Esculin hydrolysis	−	−	−
Gelatin hydrolysis[c]	+	+	+
Lysine (Moeller)	+	+	+
Ornithine (Moeller)	−	−	−
D-Glucose			
Acid	+	+	+
Gas	−	−	−
Acid produced from:			
Adonitol[d]	−	−	−
L-Arabinose	−	−	−
myo-Inositol[c,d]	−	−	−
D-Mannitol	−	−	−
Raffinose[d]	−	−	−
L-Rhamnose[d]	−	−	−
Salicin	−	−	−
D-Sorbitol[d]	−	−	−
Sucrose	−	−	−

[a] ONPG, *o*-Nitrophenyl-β-D-galactopoyranoside; VP, Voges-Proskauer.
[b] −, Negative at end of appropriate incubation period; +, positive at 24 h or end of appropriate incubation period.
[c] Results obtained with API 20E.
[d] Results obtained with MicroScan Gram Negative Panel 7.

A. hydrophila, and an oral cephalosporin (500 mg two times daily) was prescribed. The patient returned 4 days later for Iodoform packing and dressing and 6 days later for a final debridement and sterile gauze redressing. Upon completion of the 15-day regimen of oral cephalosporin treatment, the patient was completely recovered.

Bacterial strains. The two strains of *A. schubertii* were quick-frozen in 2 ml of Trypticase soy broth with 10% glycerol (Remel, Lenexa, Kans.) and maintained at −70°C and subsequently subcultured to Trypticase soy agar slants (BBL Microbiology Systems, Cockeysville, Md.) and maintained at room temperature (18 to 28°C). Unless stated otherwise, all analyses were performed at 36 ± 1°C.

Phenotypic analysis. All of the standard identification tests (Table 1) were performed either by conventional methods (7, 17, 20) with media and biochemicals obtained from Remel or by rapid identification methods with API 20E (Analytab Products, Plainview, N.Y.), MicroScan Gram Negative Combo Panel 7 (Baxter Healthcare Corp., MicroScan Division, West Sacramento, Calif.), and Biolog GN Microplate (Biolog, Inc., Hayward, Calif.).

DNA relatedness analysis. DNA-DNA hybridization was conducted in duplicate by using the batch hydroxyapatite thermal elution procedure (2, 3). DNA from the definition strain of *A. schubertii* (ATCC 43700) was nick translated with ^{32}P (NEK-004; Dupont, NEN Research Products, Boston, Mass.) and reacted with unlabeled DNA from representative *Aeromonas* isolates at both the optimal reassociation temperature of 60°C and the stringent incubation temperature of 75°C. Relatedness was expressed as the relative binding ratio (RBR) and as the divergence in the melting temperature (dTm) (Table 2). RBR was calculated as a percentage to enable determination of similarity between strains. RBR values of 70% (for reactions at 60°C) and 55% (for reactions at 75°C) were considered the cutoff values for DNA relatedness.

TABLE 2. DNA relatedness of the type strain of *A. schubertii* to strains AMC 1108-87 and AMC 4396-87

Source of unlabeled DNA	Relatedness (%) to labeled DNA of *A. schubertii* ATCC 43700		dTm (°C)[a]
	60°C	75°C	
A. schubertii			
2446-81^T (ATCC 43700)	100	100	0
1108-87	97	90	2.8
4396-87	96	100	1.2
V. parahaemolyticus (ATCC 27969)	2		

[a] dTm, Difference in melting temperature between each homologous and heterologous DNA complex.

The dTm, expressed in degrees Celsius, is the difference in melting temperature between each homologous and heterologous DNA duplex. A dTm value of 5°C was considered the cutoff value for DNA relatedness.

Control reactions, in which labeled DNA was incubated in the absence of unlabeled DNA, were included, and the label-only control values were subtracted for all reactions before the RBR was calculated.

DNA from *Vibrio parahaemolyticus* (ATCC 17802) was used as a negative control for the DNA-DNA hybridizations.

Potential virulence-associated assays. (i) Autoagglutination and acriflavine agglutination. The determination as to whether these strains spontaneously pelleted in broth (SP^+) or pelleted only after boiling (PAB^+), as well as slide agglutination tests with acriflavine and with prepared O:11 antisera (16) (kindly provided by J. M. Janda, Berkeley, Calif.), were done by the method of Janda et al. (12).

(ii) Exoenzyme analysis, serum resistance, and cytotoxin assays. The strains were examined for the presence of the extracellular enzymes DNase, protease, pectinase, and chitinase by methods previously described (8, 13). Serum resistance was determined as previously described (19) by challenge of 6×10^6 to 3×10^7 CFU of each strain against 65% pooled human serum. Cytotoxin assays were performed on HEp-2 monolayers as previously described (12).

(iii) Hemolysin assay. This was performed by both the plate and tube methods. In the former, each strain was spot inoculated to Trypticase soy agar plates with 5% sheep blood and Trypticase soy agar plates with 5% horse blood (Remel) and incubated at both 37 and 22°C for 2 days. The strains were also tested for cell-free hemolysin. This involved growth in brain heart infusion broth overnight at 37°C in both static and shaken culture conditions. The shaken cultures were rotated on an orbital shaker at 100 rpm, followed by low centrifugation and filtration of the supernatants through a 0.45-μm-pore-size Acrodisc filter (Gelman Sciences, Inc., Ann Arbor, Mich.). The supernatants were then tested in serial twofold dilutions in phosphate-buffered saline against 1% (vol/vol) rabbit cells with incubation at 37°C for 1 h,

TABLE 3. In vitro susceptibility of three *A. schubertii* strains to 17 antimicrobial agents

Antimicrobial agent	MIC (μg/ml) (susceptibility)[a]			
	Range	ATCC 43700	AMC 1108-87	AMC 4396-87
Amikacin	≤2–≥16	4 (S)	8 (S)	>16 (R)
Ampicillin	≤1–≥16	>16 (R)	16 (MS)	>16 (R)
Cefazolin	≤2–≥16	16 (MS)	8 (S)	>16 (R)
Cefoxitin	≤2–≥16	<2 (S)	<2 (S)	<2 (S)
Ceftazidime	≤2–≥16	<2 (S)	2 (S)	<2 (S)
Ceftriaxone	≤4–≥6	<4 (S)	<4 (S)	<4 (S)
Cefuroxime	≤2–≥16	<2 (S)	<2 (S)	8 (S)
Cephalothin	≤8–≥16	<8 (S)	<8 (S)	<8 (S)
Ciprofloxacin	≤1–≥2	<1 (S)	<1 (S)	<1 (S)
Gentamicin	≤1–≥6	2 (S)	4 (S)	4 (S)
Imipenem	≤1–≥8	<1 (S)	<1 (S)	2 (S)
Piperacillin	≤8–≥64	<8 (S)	<8 (S)	<8 (S)
Tetracycline	≤4–≥4	<4 (S)	<4 (S)	<4 (S)
Ticarcillin-potassium clavulanate	≤1–≥6	<8 (S)	<8 (S)	<8 (S)
Ticarcillin	≤8–≥64	<8 (S)	<8 (S)	64 (MS)
Tobramycin	≤1–≥6	4 (S)	6 (I)	>6 (R)
Trimethoprim-sulfamethoxazole	<0.5/9.5–>2.0/38	<0.5/9.5 (S)	<0.5/9.5 (S)	<0.5/9.5 (S)

[a] Therapeutic guide based on National Committee for Clinical Laboratory Standards M100-2S: S, susceptible; R, resistant; MS, moderately susceptible; I, intermediate.

followed by refrigeration overnight before the final reading was made.

Hemagglutinins. Strains tested for the presence of hemagglutinins against rabbit, sheep, and rat (glutaraldehyde-fixed) erythrocytes were grown overnight on colonization factor agar at 35°C and suspended in phosphate-buffered saline to a concentration of ca. 10^{11} to 10^{12} CFU/ml. Then, 20 μl of this suspension was added to 20 μl of 3% (vol/vol) erythrocytes and mixed on a rocked-tile plate for 10 min at 60 rpm. The controls were *Escherichia coli* LY-72 and LY-91 hemagglutinin type 2 (J. Wong, M. A. Miller, and J. M. Janda, unpublished method).

Antibiotic susceptibility tests. Antibiotic susceptibility was determined by using the MIC microtube method following the standards for interpretation of the National Committee for Clinical Laboratory Standards. We determined the MICs of 17 of the most recently prescribed antimicrobial agents in a 96-well-microdilution plate, the MicroScan Gram Negative Combo Panel 7, and the results were interpreted and recorded both manually and with an AutoScan-4 reader with computer-assisted analysis by an IBM PS-60. The antimicrobial agents and concentrations are listed in Table 3.

RESULTS AND DISCUSSION

Continuing interest in the aeromonads has intensified the efforts towards classification of this group. Including the work of Hickman-Brenner et al. (6) and Schubert and Hegazi (22), there are now seven recognized or proposed species: *A. hydrophila*, *A. sobria*, *A. caviae*, *A. media*, *Aeromonas salmonicida*, *A. veronii*, *A. schubertii*, and *Aeromonas eucrenophila*.

The newest species, *A. schubertii*, is represented by DNA hybridization group 12 and to date has been isolated from abscesses, wounds, skin, pleural fluid, and blood (5). In comparison with other *Aeromonas* species, *A. schubertii* is negative for the fermentation of D-mannitol, sucrose, L-arabinose, and salicin; negative for indole production; and negative for esculin hydrolysis (5). The two clinical strains described herein further support the clinical significance of this particular species. Additionally, their isolation from the Chesapeake Bay area extends the geographic range previously described for this species.

Presumptive identification. Our strains, AMC 1108-87 and AMC 4396-87, showed the minimal characteristics for the genus *Aeromonas*, i.e., fermentative, motile gram-negative rods, oxidase positive, resistant to O/129, string test negative, not requiring NaCl for growth, not growing in 6.5% NaCl, and reducing nitrate to nitrites.

The strains were presumptively identified as *A. hydrophila* group on API 20E, but with an identification of "doubtful profile" for AMC 1108-87 and "good likelihood but low selectivity" for AMC 4396-87 by the API computer data base because of the presence of negative reactions for indole, mannitol, and sucrose. The profile numbers generated were 7206004 for both AMC 1108-87 and ATCC 43700 and 7207004 for AMC 4396-87. However, it should be noted that the API 20E data base is constructed only for *A. hydrophila*, *A. sobria*, *A. caviae*, and *A. salmonicida*.

The MicroScan Gram Negative Combo Panel 7 system gave an identification of *A. hydrophila* (probability, 99.9% correct) for only one of the strains, AMC 4396-87 (biotype no. 4000015-1), whereas it identified both AMC 1108-87 and the ATCC 43700 strain as *Vibrio mimicus* (probability, 99.9% correct) with the biotype no. 4000405-1. It should be noted that the MicroScan Data Management System did underscore the negative indole reaction for these latter two strains as being atypical. Likewise, the MicroScan Data Management System includes *A. hydrophila*, *A. sobria*, and *A. caviae* only.

Further characterization of these strains by the taxonomic schema of Popoff and Veron (20) and Janda et al. (13) and comparison with published results for the type strain *A. schubertii* (5) revealed the three strains to be essentially the same biochemically. Conversely, only AMC 4396-87 was Voges-Proskauer positive with the three different methods for detection of acetoin (Table 1). The Biolog Panel was used to analyze these strains for utilization of D-arabitol, cellobiose, *i*-erythritol, D-galactose, glycerol, *myo*-inositol, lactose, maltose, D-mannose, melibiose, and trehalose as sole carbon sources. Of these, D-galactose, glycerol, maltose, D-mannose, and trehalose were utilized by all three strains while the remainder gave negative results.

Both of these strains were weakly beta-hemolytic on Trypticase soy agar with 5% sheep blood and strongly

beta-hemolytic on Trypticase soy agar with 5% horse blood after 24 h of incubation at both 35 and 22°C. ATCC 43700 has been described as gamma-hemolytic on Trypticase soy agar with 5% sheep blood which we confirmed but further noted that it was beta-hemolytic on Trypticase soy agar with 5% horse blood after 24 h at 36°C.

Confirmed identification. The results of the DNA hybridization work showed a close relatedness between these two strains and the DNA group 12 definition strain ATCC 43700 (96 to 100%), which confirmed the identification of these strains as *A. schubertii* (Table 2).

Virulence-associated assays. Prior studies have shown a variation in virulence between species of *Aeromonas* (9, 10). For this reason, it was considered appropriate to investigate these strains for the presence of virulence-associated markers. The results for cell-free hemolysin, hemagglutinins, cytotoxin, pectinase, and chitinase were essentially negative except that both AMC 4396-87 and AMC 1108-87 were positive for cytotoxin production at a 1:10 dilution when grown under aerated conditions.

However, it is noteworthy that both strains were positive for hemolysin by the plate assay and were positive for DNase and protease. They were resistant to serum, with AMC 4396-87 showing a slightly higher serum resistance value than AMC 1108-87 (0.82 and 0.42, respectively). Also of interest is the negative acriflavine agglutination reaction which usually correlates with virulent strains (18).

Finally, the autoagglutination results revealed AMC 1108-87 to be AA^- (autoagglutination negative), ATCC 43700 to be SP^+, and AMC 4396-87 to be PAB^+. Testing of AMC 4396-87 against the O:11 somatic antisera previously described resulted in 4^+ agglutination with all three antisera and led to the conclusion that this strain is most likely a rough strain. It is therefore unlikely that this strain possesses the S layer that has been associated with invasive disease in certain populations of *A. hydrophila* and *A. sobria* (12).

Antibiotic susceptibility. The two strains had antibiograms typical of other *Aeromonas* strains, i.e., resistance to ampicillin (strain AMC 1108-87 was actually considered moderately susceptible) and susceptibility to gentamicin and tetracycline (Table 3). Additionally, AMC 4396-87 was resistant to amikacin, tobramycin, and cefazolin; therefore, it was the most resistant of the three strains tested. Otherwise, the two strains, as well as ATCC 43700, were very susceptible to the majority of the cephalosporins tested (cephalothin, cefoxitin, ceftriaxone, and cefuroxime), as well as the newer beta-lactam-inactivating drug ticarcillin-potassium clavulanate and the quinolone ciprofloxacin.

In summary, we have confirmed that *A. schubertii* should be considered when an organism resembling the aeromonads is isolated from a wound infection. Furthermore, its range of isolation has been extended up the mid-Atlantic coast region to the Chesapeake Bay.

A. schubertii will most likely be identified as *A. hydrophila* group or *A. hydrophila* by rapid identification systems such as the API 20E or MicroScan, respectively. However, the atypical reaction profile (mannitol, sucrose, and indole negative) should alert the clinical microbiologist that further identification tests are indicated.

Although many of the virulence-associated markers examined were absent in these strains, it is apparent that, at least in the case of AMC 4396-87, *A. schubertii* is capable of causing primary cellulitis. AMC 4396-87 appears to be somewhat more resistant than other *A. schubertii* strains described to date in that it is susceptible to gentamicin but resistant to tobramycin and amikacin.

A final consideration is that these strains of *A. schubertii* represented 2 of 50 *Aeromonas* species isolated in the same laboratory over a 12-month period from various clinical specimens and may suggest a higher frequency than might be suspected otherwise.

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