Multi-author Review

Research on *Aeromonas* and *Plesiomonas*

Papers presented at the 3rd International Workshop on *Aeromonas* and *Plesiomonas*, Helsingor, September 5/6, 1990*.

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I. Taxonomy, typing and isolation

Aeromonas update: New species and global distribution

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Summary. There are currently eight proposed or validated *Aeromonas* spp. of which five have been implicated in human disease: *A. hydrophila, A. sobria, A. caviae, A. veronii,* and *A. schubertii.* Recent studies have extended the geographic distribution and source of isolation of the newer species and resulted in the possibility of two new species, *A. jandaei* and *A. trota*, from diarrheal, wound, blood and environmental sources.
Key words. Aeromonas; taxonomy; biotyping; clinical.

Aeromonads are oxidase-positive, polarly flagellated, glucose-fermenting, facultatively anaerobic gram negative rods that are resistant to the vibriostatic compound 0/129 and autochthonous to aquatic environments worldwide [3, 9]. There are currently eight proposed or validated phenotypic species or phenospecies that reside among at least 12 DNA hybridization groups or genospecies [2]. The genus consists of psychrophiles and mesophiles from aquatic and soil environments encompassing a diverse disease spectrum among many warm- and cold-blooded animals [8].

The earlier literature focused on all aeromonads as *Aeromonas hydrophila,* but several large studies have shown that the majority of clinical isolates fall within three species [2, 10]: *Aeromonas hydrophila* (group 1), *A. sobria* (group 8), and *A. caviae* (group 4). However, in recent years two new species, *A. veronii* (group 10) and *A. schubertii* (group 12), were proposed as possible causes of human disease [5, 6] and one new environmental species, *A. eucrenophila* (group 6), was delineated [11].

A. veronii (group 10) was originally published as a new DNA group of ornithine decarboxylase-positive, esculin-positive, and arginine dihydrolase-negative aeromonads that may cause diarrhea [5]. However, they were later found to be genetically identical to DNA group 8 *A. sobria.* This group includes nearly all clinical *A. sobria* isolates hybridized to date. Since the type strain for *A. sobria* (CIP 7433) resides in DNA group 7, it is now considered that DNA group 8 *A. sobria* is an ornithine-negative, esculin-negative, and arginine-positive biogroup of *A. veronii* (*A. veronii* biogroup *sobria*).

A. schubertii (group 12) is also a new DNA group of mannitol-negative, indole-negative aeromonads isolated from traumatic wounds and blood [6]. *A. eucrenophila* was proposed as a new species with the type strain ATCC 23309 and is actually an aerogenic, psychrotrophic isolate from fresh water formerly identified as an *A. caviae* [11].

Research on a large number of geographically diverse *Aeromonas* strains at the University of Maryland has extended the source of isolation and geographic isolation of *A. veronii* biogroup *veronii* and *A. schubertii* with the isolation of strains from traumatic wounds incurred in and around the Chesapeake Bay area of the United States (A. Carnahan, S. Behram, A. Ali, D. Jacobs and S. W. Joseph: Systematic assessment of geographically diverse *Aeromonas* spp. as a correlate to accurate biotyping. Abstr. Annu. Meet. ASM 1990).

Further, the existence of two new possible species was determined from this numerical taxonomy study. The first was the delineation of a cluster of nine 'esculin and sucrose-negative' *A. sobria.* These were found on both the East and West Coast of the United States and Hawaii and were isolated from wounds, blood, feces, and prawn. They all hybridized with the definition strain for DNA group 9 *A. sobria* and are proposed as *Aeromonas jandaei* [4].

The second possible new species arose from a second cluster of thirteen 'esculin-hydrolysis and Voges-Proskauer-negative, and cellobiose-positive' *A. sobria* that were, in addition, sensitive to ampicillin and carbenicillin, an extremely unusual trait for clinical aeromonads. They were found on both the East and West Coast of the United States, in Bangladesh, Indonesia, and India, and all but one were isolated from human diarrheal specimens. The nomenclature strain, AH2, was hybridized against all existing DNA group definition strains and found to represent a new and distinct DNA hybridization group of aeromonads isolated from clinical specimens and are proposed as *A. trota* (unpublished data).

Previous studies have suggested that species-related disease syndromes may exist among aeromonads, such as an association between *A. sobria* and bacteremia [7] and *A. caviae* and pediatric diarrhea [1]. Therefore, it cannot be overstressed that accurate biotyping of all aeromonad isolates, regardless of source and location, should be attempted using standard methodology and media. Only then can the true significance of these newer species be determined and used to evaluate the pathogenic mechanisms and virulence potential of these engimatic, aquatic microorganisms.

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Methods for the identification of DNA hybridization groups in the genus *Aeromonas*

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Summary. Among the 269 substrates tested in assimilation tests we found some that may help in the identification of DNA hybridization groups in the genus *Aeromonas*. In addition, isoenzyme analysis and ribotyping seem to be accurate although not routine procedures that allow discrimination between genetic species.
Key words. Aeromonas; DNA hybridization groups; assimilation; ribotyping; isoenzyme analysis.

According to Bergey's Manual of Systematic Bacteriology[13] the genus *Aeromonas* contains the three mesophilic species, *A. hydrophila, A. caviae* and *A. sobria*, as well as the psychrophilic species *A. salmonicida* with the three subspecies *salmonicida, achromogenes* and *masoucida*. Since then, several new entities have been described: *A. media*[1], *A. veronii*[8], *A. eucrenophila*[15], *A. schubertii*[7], and *A. salmonicida* subsp. *smithiae*[4]. However, these species still do not reflect the genetic complexity of the genus and they do not always correlate with genetic data, e.g. DNA hybridization values[14] (and F. W. Hickman-Brenner, G. R. Fanning, M. J. Arduino, D. J. Brenner and J. J. Farmer III, Abstr. Int. Workshop on *Aeromonas* and *Plesiomonas*, p. 51, 1988). The currently known DNA hybridization groups (DHGs) are made up of strains classified into the following phenotypic species[3,10]: DHG 1, *A. hydrophila*; DHG 2, *A. hydrophila*; DHG 3, *A. hydrophila* and *A. salmonicida*; DHG 4, *A. caviae*; DHG 5A, *A. caviae*; DHG 5B, *A. caviae* and *A. media*; DHG 6, *A. eucrenophila;* DHG 7, *A. sobria*; DHG 8, *A. sobria*; DHG 9, *A. sobria;* DHG 10, *A. veronii* (DHG 8 and DHG 10 have been shown to share enough DNA homology to be included in the same DNA hybridization group, DHG 8/10); DHG 11, *A. veronii*-like strains; DHG 12, *A. schubertii*. Two strains phenotypically similar to *A. schubertii* but indole-positive and lysine-negative have been found and were referred to as '*Aeromonas* group 501'[7]. Most probably, there are still other as yet not described DNA hybridization groups.

Phenotypic characteristics allow identification of the named species[1,3–5,7,8,10,13,15] but only few tests have been described that may aid in the separation of the various DHGs within these species[3]. We have, therefore, evaluated three completely different methods for their ability to separate the various DHGs in the genus *Aeromonas*.

Assimilation tests

We have analyzed 168 *Aeromonas* strains, belonging mainly to those DHGs that occur in clinical specimens (only strains of DHG 6 and DHG 7 have not been found in clinical specimens so far), for their ability to grow on minimal medium supplemented with different substrates. For this purpose, commercialized as well as research galleries in the form of either the API 50 or the API ATB strips were kindly provided by D. Monget (API SA, La Balme-les Grottes, France). Incubation was at 29 °C for 48 h.

Variable assimilation profiles of *Aeromonas* DNA hybridization groups. Numbers are the percentage of strains positive for a given substrate. These substrates are listed in the sequence of the API 50 and API ATB strips.

Substrate	DNA hybridization group (number of strains)											
	1 (27)	2 (9)	3 (8)	4 (41)	5A (18)	5B (8)	6 (2)	7 (2)	8 (43)	9 (4)	11 (2)	12 (4)
Glucose-1-phosphate	89	66	75	0	72	87	100	100	95	100	100	75
Glucose-6-phosphate	93	100	100	5	100	100	100	100	100	100	100	100
D-Saccharic acid	0	0	0	0	0	0	50	0	0	0	0	0
Methyl-β-D-galactopyranoside	63	78	62	93	90	100	50	50	37	25	0	0
3-O-Methylglucose	44	22	0	0	6	0	0	0	16	75	0	25
Palatinose	70	44	75	2	6	0	50	50	30	25	0	0
Lactulose	26	22	25	46	44	62	50	50	21	0	0	0
Maltitol	0	0	37	2	0	0	0	0	0	0	0	0
Pullulan	96	88	100	95	83	100	100	50	88	100	100	100
Tween 20	100	100	100	93	89	100	100	50	93	100	100	100
Tween 40	100	100	100	98	89	100	100	50	93	100	100	100
Tween 80	100	100	100	98	89	88	100	50	93	100	100	100
DL-α-Glycerophosphate	89	89	100	80	89	75	100	50	58	25	50	25
Acetoacetic acid	0	0	0	0	0	0	0	0	0	25	0	0
Methylpyruvate	96	100	100	98	100	100	100	100	98	100	50	100
Oxamic acid	0	0	0	0	0	0	0	0	0	25	0	0
L-Alaninamide	0	0	0	27	56	50	0	0	12	50	0	0
L-Asparagine	100	100	100	100	100	100	100	100	100	75	100	75
S-Ethyl cysteine	0	22	0	0	6	0	0	0	2	0	0	0
L-Alanyl-glycine	93	78	62	76	72	75	100	100	86	100	100	75
Glycyl-L-proline	100	89	87	100	100	100	100	100	95	75	50	75
D-Pantothenic acid	0	0	0	0	0	0	0	0	0	25	0	0
2-Pyrrolidinone	0	0	0	0	0	0	0	0	0	25	0	0
Urocanic acid	7	89	100	100	89	100	0	0	0	25	100	0
Allantoin	0	0	12	0	0	0	0	0	0	25	0	0
Pipecolic acid	0	0	0	0	0	0	0	0	0	25	0	0
2-Pyridylacetic acid	0	0	0	0	0	0	0	0	5	25	0	0
Uric acid	0	0	0	0	0	0	0	0	0	25	0	0
Thymidine	96	100	100	93	94	87	100	100	98	75	100	75
Uridine	81	78	100	93	94	87	100	100	86	100	100	100
D-Arabinose	0	0	0	0	0	0	0	0	2	25	0	0
L-Arabinose	93	100	100	100	100	75	100	50	23	0	20	0
D-Mannose	96	100	100	27	100	100	100	100	98	100	100	100
Rhamnose	22	75	0	0	0	0	0	0	2	0	0	0
Inositol	4	0	0	0	0	0	0	0	2	0	50	0
Sorbitol	0	11	87	0	0	0	0	0	0	0	0	0
α-Methyl-D-glucoside	52	33	37	5	0	25	0	50	28	0	0	0
Arbutin	85	100	100	85	89	100	100	100	16	0	50	0
Salicin	78	100	62	85	83	87	100	100	16	0	100	0
Cellobiose	4	0	50	78	100	87	100	100	40	0	0	0
Lactose	30	22	62	54	72	100	50	50	28	0	0	0
Melibiose	0	11	0	2	0	0	0	0	7	100	0	0
Sucrose	96	100	100	100	100	100	50	100	100	0	50	0
Glycogen	96	100	100	100	100	100	50	100	93	100	100	75
β-Gentiobiose	0	0	12	54	0	0	0	0	6	0	0	0
Acetate	93	78	12	39	39	62	50	50	40	50	50	25
Butyrate	93	44	50	44	67	25	50	50	44	75	50	25
N-Valerate	48	33	12	39	56	50	50	50	37	50	0	50
N-Caproate	30	33	0	15	22	0	0	0	16	75	0	25
Heptanoate	33	22	0	10	11	0	0	0	7	0	0	25
Caprate	78	33	50	70	61	62	100	50	67	25	0	75
DL-Lactate	93	11	12	90	6	100	50	50	7	100	0	75
DL-3-Hydroxybutyrate	11	0	0	0	17	12	0	0	9	0	0	0
2-Ketoglutarate	0	0	0	2	0	0	0	0	2	25	0	0
Citrate	15	0	0	80	6	0	0	100	47	100	0	75
Tere-phtalate	0	0	0	0	0	0	0	0	0	25	0	0
Glycine	93	78	50	73	78	62	100	0	21	25	50	0
D-α-Alanine	100	100	87	83	100	75	0	50	79	75	100	50
L-α-Alanine	96	100	62	83	100	75	50	100	91	75	100	100
L-Threonine	89	100	75	90	94	87	50	0	53	75	50	50
L-Cysteine	74	44	37	22	17	0	100	100	65	50	0	25
L-Tyrosine	89	78	87	49	61	62	100	50	51	50	0	50
L-Histidine	100	100	100	95	100	100	100	50	91	75	100	75
L-Ornithine	70	100	50	76	78	37	50	0	44	50	50	0
L-Lysine	0	0	0	5	0	0	50	0	44	50	50	0
L-Citrulline	4	11	0	2	0	0	0	0	0	0	0	25
L-Arginine	96	100	75	89	100	87	50	0	70	100	100	75
L-Proline	85	78	75	90	100	87	50	100	65	50	100	50
Diaminobutane	89	78	50	73	39	25	50	50	7	0	0	100
Spermine	11	11	0	7	56	50	0	0	2	0	0	0
Glucosamine	89	100	100	100	100	100	100	100	100	100	100	100

More than two-thirds of the 269 substrates tested did not provide significant differences between DHGs. Fewer than 20% of the strains in each DHG grew on the following substrates, listed in the sequence of the API 50 and API ATB strips: D-glucuronic acid, glucuronamide, D-psicose, mucid acid, α-D-glucoheptonic acid, D-galactonic acid lactone, D-glucosaminic acid, N-methyl-D-glucamine, N-acetyl-D-galactosamine, N-acetyl-β-D-mannosamine, methyl-α-D-galactopyranoside, methyl-α-D-mannopyranoside, β-lactose, lactobionic acid, mannan, amylose, α-cyclodextrin, chondroitin sulfate, alginic acid, α-D-glucoheptonic-gamma-lactone, α-hydroxyglutaric acid-gamma-lactone, 2-furoic acid, 3-furoic acid, quinic acid, camphoric acid, L-ascorbic acid, D-isoascorbic acid, hydrocinnamic acid, trans-cinnamic acid, DL-β-phenyllactic acid, phenylpyruvic acid, tropic acid, salicylic acid, p-hydroxyphenylacetic acid, o-coumaric acid, m-coumaric acid, p-coumaric acid, acetylsalicylic acid, 2,3-dihydroxybenzoic acid, β-resorcylic acid, gentisic acid, gamma-resorcylic acid, proto-catechuic acid, α-resorcylic acid, vanillic acid, syringic acid, hippuric acid, 4-amino-hippuric acid, β-phenylethylamine, tyramine, formic acid, glyoxylic acid, 1-2-propanediol, ketomalonic acid, 2,3-butanediol, DL-α-hydroxybutyric acid, crotonic acid, 2-ketobutyric acid, pivalic acid, gamma-hydroxybutyric acid, maleic acid, glutaconic acid, α-ketovaleric acid, methylsuccinic acid, sorbic acid, tricarballylic acid, L-cysteic acid, D-threonine, succinamic acid, R(-)-leucinol, DL-carnitine, pyroglutamic acid, picolinic acid, nicotinic acid, 3-pyridylacetic acid, nicotinamide, pyridoxine, barbituric acid, oxonic acid, cytosine, thymine, uracil, adenine, guanine, hypoxanthine, xanthine, erythritol, D-xylose, L-xylose, adonitol, β-methyl-xyloside, L-sorbose, dulcitol, α-methyl-D-mannoside, amygdalin, inulin, melezitose, xylitol, D-turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, L-arabitol, 5-keto-gluconate, isobutyrate, pelargonate, oxalate, malonate, maleate, glutarate, adipate, pimelate, suberate, azelate, sebacate, glycolate, D-tartrate, L-tartrate, mesotartrate, levulinate, citraconate, itaconate, mesaconate, aconitate, phenylacetate, benzoate, o-hydroxy-benzoate, m-hydroxy-benzoate, p-hydroxybenzoate, D-mandelate, L-mandelate, phthalate, isophthalate, L-leucine, L-norleucine, DL-norvaline, DL-2-aminobutyrate, L-methionine, L-phenylalanine, D-tryptophane, L-tryptophane, trigonelline, DL-kynurenine, betaine, creatine, β-alanine, DL-3-amino-butyrate, 4-amino-butyrate, 5-amino-valerate, 2-amino-benzoate, 3-amino-benzoate, 4-amino-benzoate, urea, acetamide, sarcosine, ethylamine, butylamine, amylamine, ethanolamine, benzylamine, histamine, tryptamine, oxalacetic acid, glycyl-L-aspartic acid, glycyl-L-glutamic acid, D-raffinose, D-arabitol, 2-keto-gluconate, propionate, isovalerate, caprylate, L-isoleucine, L-valine.

More than 80% of the strains in each DHG showed growth with the following substrates: methyl-β-D-glucopyranoside, maltotriose, dextrin, glycerol, ribose, galactose, D-glucose, D-fructose, mannitol, N-acetyl glucosamine, maltose, trehalose, amidon (starch), gluconate, fumarate, DL-glycerate, pyruvate, L-serine, L-glutamate, bromosuccinic acid, inosin, succinate, L-malate, L-aspartate, glucosamine.

The percentage of strains which grew on the remaining substrates is given in the table. It should be noted that many of these test results are discrepant only for those DHGs that are represented by only a few strains (e.g. DHGs 6, 7, 9, 11, and 12) and are, therefore, statistically not significantly different. However, several substrates, some of which had been described earlier[3], seem to be useful for the separation of DHGs within the named species. For *A. hydrophila* (DHGs 1, 2 and 3) these are DL-lactate, D-sorbitol, citrate, urocanic acid, rhamnose, and acetate. For *A. caviae* (DHGs 4, 5A and 5B), DL-lactate, citrate, glucose-1-phosphate, glucose-6-phosphate, D-mannose, and β-gentobiose were most valuable. For *A. sobria* (DHGs 7, 8 and 9) DL-lactate, 3-O-methyl-glucoside, arbutin, salicin, cellobiose, melibiose, saccharose, N-caproate and L-arginine may be useful, but these data need to be confirmed using additional strains of DHG 7 and DHG 9.

Multilocus enzyme electrophoresis

Electrophoretic analysis of isoenzymes has been used in many bacterial groups (including *Aeromonas*) for taxonomic and epidemiological purposes[2,11,12,16]. Using only 4 different enzymes, we were able accurately to identify DHGs of 47 out of 48 strains analyzed blindly [M. Altwegg, A. G. Steigerwalt, J. M. Janda, and D. J. Brenner, Abstr. A. Meet. Am. Soc. Microbiol. 1989, C-235, p. 435]. The only strain not correctly identified was a strain of DHG 9 which was represented by only 2 strains in the database used. In addition, such strains are extremely rare in clinical specimens (only 1 out of 266 strains analyzed in 3 studies[3,9,10] belonged to DHG 9).

Ribotyping

The analysis of ribosomal RNA gene restriction (rDNA) patterns which was described originally as a possible taxonomic tool[6] did not show an obvious correlation to taxonomic grouping in the genus *Aeromonas*[2] [Kuijper, E. J., 1989. *Aeromonas*-associated diarrhea in The Netherlands, Ph.D. Thesis, University of Amsterdam, pp. 80–92]. However, both these studies concentrated on the high molecular-weight, intensively labeled bands. When the analysis was extended to the smaller fragments in *Sma* I digests which were clearly visible after prolonged exposure, a striking consistency of some bands within the various DHGs became apparent [G. Martinetti and M. Altwegg, manuscript in preparation].

Most of the methods that allow identification of DHGs in the genus *Aeromonas*, in particular isoenzyme analysis

and ribotyping, are usually not available in a routine diagnostic laboratory. Since at present very little is known about the virulence potential of the different DHGs, the determination of genetic groups is not recommended for these laboratories. For investigations on the pathogenicity of the various groups, however, as much information as possible on the identity of the strains should be accumulated and, in this respect, additional biochemical markers as well as enzyme analysis or ribotyping may help to circumvent DNA hybridization analysis which is even more laborious and restricted to very few laboratories.

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The taxonomy and nomenclature of the psychrotrophic aeromonads

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Summary. Following the introduction of the DNA hybridization technique several genotypes have been separated from the older phenotypically described *Aeromonas* species. Work has been undertaken on some arbutin-negative psychrotrophic *Aeromonas* strains. These were differentiated into three genotypically and phenotypically identifiable groups. One group (I) is genetically related to *A. sobria* (type 208). The two other groups (II and J) can be separated genotypically and phenotypically from the *A. sobria, A. veronii* and *A. hydrophila* genotypes. Studies on ADH-negative anaerogenic surface water aeromonads showed them to be genotypically more distant from *A. punctata* type 239 than from type 545.
Key words. Aeromonas; taxonomy; arbutin-negative *A. sobria*-like strains; *Aeromonas punctata*; '*caviae*-type'; ADH-negative anaerogenic aeromonads.

The roots of the taxonomy and systematics of the aeromonads extend into the last century. As far as systematics is concerned, a consideration of the original literature can give a useful perspective on how this 'science' has progressed up to now, and provides an incentive for work into the future. While the 'Approved Lists of Bacterial Names'[1] is the turning point of bacterial nomenclature, it is nevertheless worthwhile to look into the developments of the past in order to understand better the present-day problems of the taxonomy of *Aeromonas*.

After the theoretical conception of the genus *Aeromonas* by Kluyver and van Niel (1936)[3] and the first classification of a recognizable species of *Aeromonas*, i.e., *A. hy-*

drophila, by Stanier (1943)[9], scientific interest in this group of organisms virtually ceased. It revived in the late fifties, when some Scandinavian bacteriologists working in the field of the Enterobacteriaceae isolated strains of bacteria which possessed biochemical properties used in the taxonomy of the Enterobacteriaceae but which had the morphological characteristics of Pseudomonadaceae, i.e., they were polarly flagellated bacteria. This resulted in what may be regarded as the first phase of *Aeromonas* taxonomy.

At this time, the morphological, physiological and ecological properties of the aeromonads were analyzed and delineations were established for the Enterobacteriaceae, Pseudomonadaceae, and Vibrionaceae and, ultimately, for *Plesiomonas*. Also at this time, the species descriptions in the literature were rationalized and considered in the context of name priority. This applied especially to the *Aeromonas* species in which there existed a considerable diversity of illegitimate nomenclature. The review of old species descriptions, of newly found experimental taxonomic characteristics, a revised outline of character descriptions by former authors, and the examination of the legitimacy of names, required much detective work in order to assemble the puzzle correctly.

This period was also mainly associated with names such as Eddy, Ewing, Hugh, Snieszko and Schubert and is reflected in the eighth edition of Bergey's Manual (1974)[7]. At that time, the species concept was under active consideration. The first approach was the introduction of the technique of numerical taxonomy. When applied to psychrotrophic aeromonads there was a tendency to regard the entire psychrotrophic *Aeromonas* group as a single cluster, i.e., *A. hydrophila*.

This was achieved by the elimination of some of the specific characteristics, the amplification of general characteristics of the psychrotrophic aeromonads, and the introduction of dubious interdependent characteristics. The use of interdependent characteristics did not take into account the specific physiological properties of the different strains used in the study, e.g., the effect of different temperatures of incubation on enzyme activity within the *Aeromonas* strains tested. Although numerical taxonomy has found many areas of successful application with groups of microorganisms demonstrating phenotypical heterogeneity, it fails to discriminate species in groups possessing high phenotypical homogeneity, e.g., in the psychrotrophic aeromonads.

The next phase in the development of *Aeromonas* taxonomy was marked by the introduction of DNA-based techniques. It was De Ley et al. in 1970[2] who developed DNA hybridization techniques which provided the possibility of measuring the genetic relationship between bacterial strains. The first application of these techniques to the psychrotrophic aeromonads by Popoff et al. in 1981[6] demonstrated that the phenotypically circumscribed *Aeromonas* species, e.g. '*A. hydrophila*', comprised several genotypically different groups with genetic distances supporting species delineation.

A new basis for research was thus established but the dilemma of phenotypical identification did not find a satisfactory solution. The phenotypical description of species compiled in the seventies, which was based on large strain collections is, therefore, questionable. Today, only strains identified through DNA hybridization should be used in the classification and elaboration of species. The old phenotypical descriptions can, however, prove useful as a source of information to trace groups of strains with hitherto unknown taxonomic positions within the genus *Aeromonas*.

Studies on the phenotypical characters of species representing a distinct hybridization group show that only a few markers are required for the phenotypic differentiation of genotypically defined species. From the outset it was recognized that the pattern of breakdown of carbohydrates was of little use in the differentiation of the psychrotrophic *Aeromonas* species. However, it was the

Table 1. DNA-DNA homology studies of arbutin-negative psychrotrophic *Aeromonas* strains

Strains	Group I		II		J		G + C%
	I 100	E 138	II 115	1163	J 11	J 14	
I 100	100%	87%	58%	ND	ND	ND	59.0
E 21	80%	87%	ND	ND	44%	ND	59.0
E 138	80%	ND	63%	ND	42%	ND	59.6
II 115	ND	53%	ND	75%	ND	ND	59.6
II 124	ND	ND	ND	84%	ND	ND	ND
ATCC 11163	ND	62%	ND	100%	50%	ND	59.2
Z 32	ND	58%	ND	81%	ND	ND	59.8
J 10	62%	61%	ND	75%	51%	ND	59.8
J 11	ND	42%	ND	50%	100%	ND	60.8
J 12	ND	46%	ND	52%	86%	ND	60.2
J 14	ND	52%	ND	50%	77%	ND	60.0
J 16	ND	40%	ND	50%	77%	90%	60.0
Controls							
A. hydrophila 212	50%	46%	48%	51%	51%	50%	62.2
A. hydrophila 218	46%	51%	49%	47%	50%	58%	61.2
A. hydrophila 316	40%	40%	41%	42%	43%	40%	57.8
A. sobria 208	73%	71%	55%	62%	47%	ND	ND
A. veronii 224	58%	ND	64%	60%	49%	ND	ND

glycosides, salicin and esculin, which were found to be of crucial taxonomic importance in the separation of *A. sobria* from *A. hydrophila* by Popoff et al. in 1976[5].

It was, therefore, a heuristic concept to look into the ability to degrade naturally-occurring glycosides in order to provide an extension of existing taxonomic markers. The other glycoside, apart from esculin and salicin which was extensively used by Lee in 1987[4], was arbutin. Since arbutin cleavage appeared to be a possible criterion for taxonomic differentiation, all strains unable to break down arbutin were tested in a DNA hybridization study. Results of DNA hybridizations of these arbutin-negative strains show the presence of three groupings, I, II and J (table 1). Group I has a high genetic relatedness with type 208, i.e., *A. sobria*, whereas group II and group J can be differentiated from the *A. sobria* and *A. hydrophila* types. The more interesting phenotypical characteristics are given in table 2. This table shows that: (a) the three hybridization groups, I, II, and J do not break down arbutin; (b) compared to *A. sobria-A. veronii* hybridization types these arbutin-negative groups are least able to split the glycosides; (c) group I has a high genetic relatedness with type 208, i.e., *A. sobria*, whereas groups II and J can be differentiated from the *A. sobria* and *A. hydrophila* types; (d) the arbutin-negative groups can be further separated from *A. sobria* – *A. veronii* by their inability to ferment arabinose.

Furthermore, it can be seen that their three DNA hybridization groups can be phenotypically separated. Groups I and J can be differentiated from group II by their inability to ferment sucrose, and groups I and II from group J by the production of acetoin from glucose. Group J also shows a positive butanediol dehydrogenase reaction in addition to a negative Voges–Proskauer reaction.

The main characteristics of group II are the inability to split arbutin, prunasin, esculin and salicin, and the inability to ferment arabinose, sorbitol, lactose, 1-O-methyl-β-D-glucopyranoside, palatinose and (by most strains) l-fucose. All strains grew in KCN broth.

The main characteristics of group J are the inability to split arbutin, prunasin, esculin, salicin and the inability to ferment arabinose, sucrose, sorbitol and lactose. Organisms in this group do ferment l-fucose, cellobiose and acetylglucosamine, but show no production of acetoin (negative V-P reaction), no gluconate oxidation and a positive butanediol dehydrogenase reaction. Most strains show no growth in KCN broth.

A second area of our investigation concerned the anaerogenic species of the psychrotrophic aeromonads. In this context (taking into consideration the 'Approved Lists of Bacterial Names'[1] and the priority rules), *Aeromonas caviae* has been found to be a later and illegitimate synonym of *Aeromonas punctata*. After the validation of the species name *A. eucrenophila*[8] in the appropriate list (List no. 27)[10] in the International Journal of Systematic Bacteriology in 1988 the case was closed.

The species *A. punctata* containing the two hybridization types[6] cannot yet be phenotypically separated due to the absence of traditional biochemical markers.

Recent studies on the population of surface water aeromonads have resulted in the isolation of strains

Table 2. Biochemical characteristics of arbutin-negative psychrotrophic *Aeromonas* strains

	A. sobria-A. veronii hybrid. type 224	*A. sobria* type 208	Group I	Group II	Group J
Number of strains	6	5	3	5	4
Arbutin*	6+	4+/1−	3−	5−	4−
Prunasin*	4−/2+	4−/1+	3−	5−	4−
Esculin*	5−/1+	5−	3−	5−	4−
Salicin*	6−	5−	3−	5−	4−
Agmatin	5−/1+	5−	3−	3+/2−	4−
Sucrose	6+	5+	3−	5+	4−
Fucose	1+/5−	2+/3−	3−	1+/4−	4+
Palatinose	4+/2−	4+/1−	1−/2+	5−	4−
α-Methyl-glucopyranoside	6−	2+/3−	2+/1−	5−	4−
Cellobiose	5+/1−	3−/2+	3+	3−/2+	4+
Sorbitol	6−	5−	3−	5−	4−
Gentobiose	6−	5−	3−	5−	4−
Lactose	6−	5−	3−	5−	4−
Arabinose	6+	5+	3−	5−	4−
Pyrazinamide	4+/2−	2+/3−	3+	4+/1−	4+
Citrate	4+/2−	2+/3−	3−	3+/2−	3+/1−
Voges–Proskauer	2+/4−	3+/2−	3+	5+	4−
Gluconate oxidation	1+/5−	3+/2−	3−	4+/1−	4−
Butanediol dehydrogenase	2+/4−	3+/2−	3+	5+	4+
Lysine	4+/2−	5+	2+/1−	4+/1−	4+
Ornithine	6−	5−	3−	5−	4−
Arginine	5+/1−	5+	2+/1−	5+	4+
Acetyl-galactosamine	3+/3−	3+/2−	1+/2−	1+/4−	4+
KCN	5+/1−	4+/1−	2+/1−	5+	1+/3−

*glycosides.

Table 3. DNA-DNA homology studies of anaerogenic psychrotrophic *Aeromonas* strains

Strains	Type strains		G + C %	*A. punctata caviae*-type strains	
	83	177		239	455
83	100%	ND	61.4	64%	72%
48	95%	ND	60.2	62%	68%
64	86%	ND	61.0	54%	64%
162	80%	ND	61.8	57%	65%
51	83%	ND	60.0	45%	60%
77	88%	ND	61.0	54%	65%
101	80%	ND	60.8	63%	73%
193	93%	ND	60.6	63%	70%
29	80%	ND	61.3	57%	71%
76	84%	ND	61.3	54%	72%
158	87%	ND	60.6	57%	70%
101	80%	ND	60.8		
177	52%	100%	56.8	47%	47%

which behaved as negative in Difco arginine dehydrogenase media, but which otherwise had the properties of the anaerogenic psychrotrophic aeromonads.

The results of the DNA hybridization are given in table 3 which shows that: (a) ADH-negative strains are split into two separate groups, the one comprising 12 strains (83 group) and the other, only one strain (177 group); (b) the hybridization groups demonstrate a significant genetic distance since the G + C % of the 83 group is higher than 60% while the G + C % of the 177 strain is less than 57%; (c) hybridization of the 83 group with *A. punctata* type 239 is relatively low but for type 545 at least in some cases it is relatively high (up to 73%), so that the 83 group would seem to be genetically more distant from type 239 than from type 545; (d) the 177 strain is considerably different from type 239 and from 545.

These data and the poorly clarified position of *A. media*, indicate that the taxonomic structure and description of the group of species comprising the anaerogenic psychrotrophic aeromonads is as yet incomplete.

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Media and methods for isolation of aeromonads from fecal specimens. A multilaboratory study

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Summary. An international multilaboratory study was conducted to establish the optimal combination of culture media, incubation time and temperature for recovery of aeromonads from stools using blood ampicillin (10 mg/l) agar (BAA), starch ampicillin (10 mg/l) agar (SAA), and cefsulodin irgasan novobiocin agar (CIN) with alkaline peptone water (APW) for enrichment. Optimal recovery of aeromonads (167/186) occurred using BAA (37 °C; 24 h) and CIN (25 °C; 48 h) with APW (25 °C; 24 h) subcultured to BAA (37 °C; 24 h) and CIN (25 °C; 48 h).

Key words. Aeromonas; isolation; media; enrichment.

The isolation of *Aeromonas* spp. from human fecal specimens has become a frequent occurrence since the introduction of selective culture methods[5,8]. The most widely used selective medium for the recovery of aeromonads is 5% sheep blood agar (BAA) containing ampicillin (10 mg/l) which is usually inoculated before and after enrichment of fecal specimens in alkaline peptone water (APW), pH 8.6[7,9,12]. Cefsulodin-irgasan-novobiocin (CIN) agar, originally introduced for detection of *Yersinia enterocolitica* in feces, has also proven effective for isolation of aeromonads[1,3]. Starch-ampicillin agar (SAA), recommended for the isolation of aeromonads from foods, has not been evaluated for recovery of aeromonads from feces[13]. Many other selective media have been recommended for stool culture but no standards have emerged for media selection or incubation conditions[10,11,14,16].

In 1988, an international multilaboratory collaborative study was undertaken to determine the combination of enrichments, isolation media and incubation conditions for optimal recovery of aeromonads from fecal specimens. Five laboratories, located in Australia, France, Germany, the United Kingdom and the United States, each cultured 500 or more consecutive fecal specimens from patients with diarrhea according to the protocol presented in the figure, except that enrichments and SAA were not included in the French study and SAA was not included in the German study. Culture media evaluated were BAA (Blood Agar Base, Oxoid CM 55, containing 5% defibrinated sheep blood and 10 mg/l ampicillin), CIN (Yersinia Selective Agar, Difco 1817-17-2, plus Yersinia Antibiotic Supplement CN, Difco 3196-60-5, containing 4 mg/l cefsulodin and irgasan and 2.5 mg/l novobiocin), SAA (Phenol Red Agar Base, Difco 0098-02-1, containing 10 g/l of soluble potato starch and 10 mg/l ampicillin), and APW, pH 8.6 (Peptone Water, Oxoid CM 9). Liquid specimens were inoculated using a 10-µl calibrated loop and solid stools were diluted in a minimal amount of saline before inoculation of media. Stools submitted in Cary-Blair transport media (USA only) were inoculated using a swab. Enrichments were subcultured to plating media using a 10-µl calibrated loopful of liquid taken from the top on undisturbed APW tubes. Representative colonies were picked directly from BAA plates for spot oxidase tests. Because of acidity, oxidase tests were unreliable for colonies from CIN and SAA. Mannitol (CIN) and amylase (SAA) positive colonies were subcultured to Triple Sugar Iron Agar (TSI) and Lysine Iron Agar (LIA), and these media were incubated for 18–24 h at 37 °C. Spot oxidase tests were performed from growth on the LIA slant, and oxidase-positive colonies were definitively identified using biochemical tests shown in table 1[4,6].

Table 1. Identification of *Aeromonas* spp.

Biochemical test	% Reactivity of species		
	A. caviae n = 127	*A. hydrophila* n = 26	*A. sobria* n = 26
Glucose (gas)	0	100	100
Voges–Proskauer	0	100	100
Esculin	96	100	11
Lysine (FB)	0	100	100
Salicin	93	100	0
Arabinose	98	48	0
Hemolysin (> 1:8)	<1	100	93

Fay and Barry method[4]

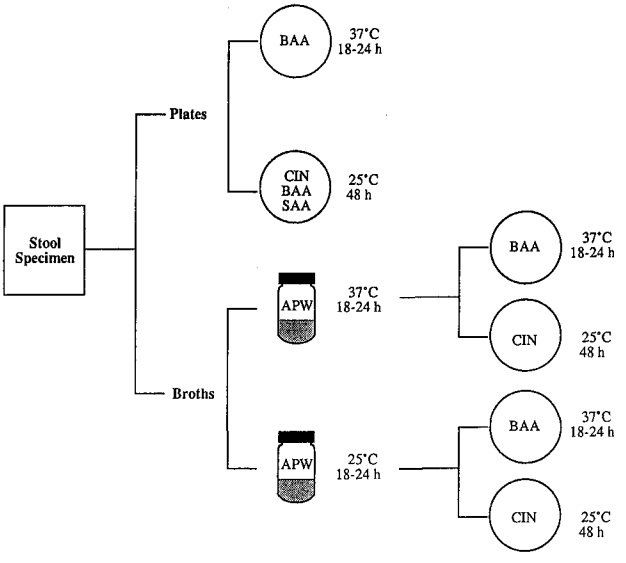
Table 2. Recovery of *Aeromonas* spp. by various culture methods. N.D., not done; [a] Abbreviation of media see the figure.

Location	Specimens	No. Positive (%)	Organisms	Primary cultures				Enrichment cultures			
				BAA[a] 25C	BAA 37C	CIN	SAA	APW25C to BAA	APW25C to CIN	APW37C to BAA	APW37C to CIN
Australia	527	61(11.6)	*A. caviae*	11	10	10	14	9	3	11	5
			A. hydrophila	7	5	1	5	9	0	5	1
			A. sobria	10	10	2	8	8	2	10	1
			A. species	0	3	0	0	2	0	2	0
France	520	5(0.96)	*A. caviae*	3	2	4	N.D.	N.D.	N.D.	N.D.	N.D.
			A. hydrophila	0	0	0	N.D.	N.D.	N.D.	N.D.	N.D.
			A. sobria	0	0	0	N.D.	N.D.	N.D.	N.D.	N.D.
			A. species	0	0	0	N.D.	N.D.	N.D.	N.D.	N.D.
Germany	500	18(3.6)	*A. caviae*	8	4	3	N.D.	15	9	9	7
			A. hydrophila	1	1	0	N.D.	2	2	2	1
			A. sobria	0	0	0	N.D.	0	0	0	0
			A. species	0	0	0	N.D.	0	0	0	0
UK	500	23(4.6)	*A. caviae*	5	5	5	8	11	12	13	12
			A. hydrophila	1	2	0	0	6	2	4	2
			A. sobria	0	0	0	0	2	0	2	0
			A. species	0	0	0	0	1	1	1	0
USA	577	79(13.7)	*A. caviae*	23	34	33	17	39	47	37	48
			A. hydrophila	3	3	1	1	4	2	2	2
			A. sobria	4	4	4	1	4	3	4	5
			A. species	0	1	1	0	0	0	1	0
Total	2624	186(7.1)		76	84	64	54	112	83	103	84

Altogether, 186 aeromonads were isolated from 2624 fecal specimens for an overall isolation rate of 7.1% (table 2). Isolation rates varied among collaborators from < 1% to 13.7%. *A. caviae* (127 strains) was most frequently isolated, followed by *A. hydrophila* and *A. sobria* (26 strains each). Seven strains of *Aeromonas* were isolated which could not be unequivocally assigned to one of these three species. BAA incubated at 37 °C for 24 h proved to be the best single medium for isolation of all three species but alone it yielded only 45.2% (84/186) of the total number of isolates. The recovery rate for *A. hydrophila* and *A. sobria* from CIN was half that of *A. caviae* which may indicate susceptibility to even 4 mg/l of cefsulodin. Cefsulodin sensitivity was particularly evident for *A. sobria* and *A. hydrophila* following APW subculture as enrichment recovery rates on CIN plates accounted for only 20–30% of the total strains for these organisms. *A. caviae* was detected equally well by all enrichment combinations. When both BAA and CIN results were used to calculate efficiency, the preenrichment recovery of aeromonads improved to 64% (119/186) of the total organisms isolated. SAA could not be adequately evaluated since two collaborators did not include it in the study, and results from the other three laboratories were contradictory. APW incubated at 25 °C and subcultured to BAA yielded 61.9% (112/186) of the total number of isolates. Incubation of APW cultures at 37 °C frequently resulted in *Pseudomonas* overgrowth when enrichments were subcultured to BAA.

The marked differences in isolation rates observed between collaborators may be related to media selection since the lowest recovery rate occurred in the laboratory which did not perform enrichment procedures. The use of Cary-Blair transport medium and swab inoculation may have contributed to the high recovery of *A. caviae* in the U.S. study as this organism is known to be sensitive to acidity. Experience in the isolation of aeromonads from stools undoubtedly contributed to the high isolation rates of two laboratories, but environmental factors (such as high summer temperatures in Iowa) may also have been important.

Geographical differences in the distribution of species was evident. *A. sobria* was common in Australia (30% of strains) but rare in other countries. *A. caviae* was commonly found in the U.S. where it accounted for 86% of all isolates. Although controversial, the pathogenic potential of *A. caviae* has been reported[2, 12]. The local distribution of strains must be considered when selecting culture methods, particularly if use of CIN is being considered. When we examined the net differences between methods, enrichment cultures contributed less that 1% of additional isolates which would have been missed on primary culture when more than one direct plating medium was used. This suggests that patients with diarrhea associated with aeromonads shed sufficient numbers of organisms to be detected on primary plating media without the need for enrichments as reported by Robinson et al.[15]. Optimal recovery of aeromonads from stools is more dependent upon the number and kinds of primary plates used for screening stools than upon the enrichment method employed. A combination of direct plating and enrichment methods is recommended until the efficiency of direct plating media is improved. BAA incubated at 37 °C and CIN, together with enrichment in APW incubated at 25 °C and subcultured to BAA and CIN, detected 89.8% (167/186) of the total isolates. This method seems appropriate for recovery of aeromonads from fecal specimens. Development of a single selective medium with 90% efficiency should be a research goal. Until that time, the specific search for aeromonads in stools will continue to be a labor- and material-intensive activity.



Optimal culture media and methods for isolation of *Aeromonas* spp. from stools. BAA, blood ampicillin agar (10 mg/l); CIN, cefsulodin irgasan novobiocin agar; SAA, starch ampicillin agar (10 mg/l); APW, alkaline peptone water.

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II. Virulence factors

Form and functions of the regular surface array (S-layer) of *Aeromonas salmonicida*

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Summary. The principal virulence factor of *Aeromonas salmonicida* is its S-layer (A-layer) which is comprised of tetragonally arrayed ~ 50 000 Mr protein subunits tethered to the cell surface via LPS. The detailed composition of its LPS is known, as is the primary sequence, and three-dimensional disposition of the A protein subunits. The A-layer physically protects the cell against bacteriophage, proteases, as well as immune and non-immune complement. The A-layer appears to be uniquely adapted towards binding biologically important molecules such as heme, and to various basement membrane proteins. In addition, the A-layer is required for macrophage infiltration and resistance. Specific mutants containing a disorganized A-layer are avirulent and provide significant protection to salmonids when applied by immersion.
Key words. Aeromonas salmonicida; A-layer; cloning; functions.

Aeromonas salmonicida is an important pathogen, causing both the systemic and ulcerative forms of the disease furunculosis in fish. The resistance of this disease to prophylaxis in the form of immersion vaccines and the persistence of infection even in 'immunized' fish prompted a detailed study of the contribution of this bacterium's cell surface to its pathogenesis.

Morphology

A. salmonicida strains differ from other typical fish pathogens with respect to colonial morphology, colonies remaining rigid and intact when grown on solid media. In addition, *A. salmonicida* cells autoaggregate readily especially in the presence of high salt concentrations, suggesting hydrophobic interactions. When individual cells are examined by thin section electron microscopy an electron dense layer immediately peripheral to the outer membrane and surrounding the cell can be seen. This structure has been called the A-layer[17].

A-layer composition

The A-layer has been shown to be principally composed of carbohydrate in the form of lipopolysaccharide (LPS) and protein (the A protein). Cellular LPS is found both as a 9 sugar containing branched core oligosaccharide linked to lipid A as well as a complete O antigen containing a linear tetrasaccharide repeat unit joined to core oligosaccharide[5,15]. Some of the O antigens project through to the surface of the S-layer[5] and cells stain uniformly with either monoclonal or polyclonal antibody[5]. This observation can be useful diagnostically since the LPS has been shown to be relatively invariant. All pathogenic strains so far isolated contain a ~ 50,000 Mr protein intimately associated with the bac-

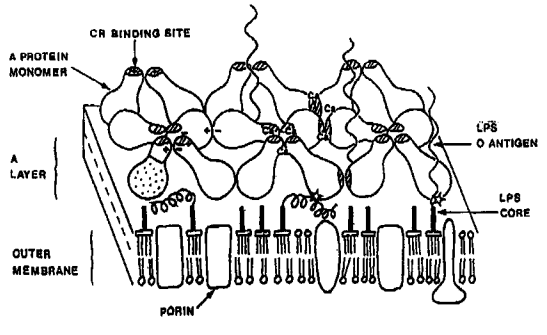
terial outer membrane[7,8]. This protein can be readily extracted with chaotropic agents or by acid depolymerization and subsequently purified by conventional chromatography[7,11]. This A protein layer has been shown by extrinsic labelling experiments to tightly cover the surface of the bacterial cell[7]. Isogenic mutants of *A. salmonicida* can be isolated devoid of either LPS, A-layer, or both, and are invariably avirulent[2,7].

Electron microscopy

Negatively stained preparations of whole bacterial cells revealed large sheets of A-layer displaced on the cell. High resolution electron microscopy of either these layers or layers released from the cell as the result of a deficiency in LPS O antigen synthesis reveal a unique tetragonal array of alternating modes of symmetry. These are indicative of S-layer proteins, two-dimensional crystalline protein arrays which have now been described for nearly 300 bacterial genera[1,15]. A computer image enhanced three-dimensional reconstruction of the *A. salmonicida* S-layer from a tilt series of electron micrographs reveals the disposition of A protein monomers as a tetragonal array of alternating heavy and light domains[6], forming an open porous structure with concave depressions formed from four subunits. The figure represents a diagrammatic form of this structure assembled on the bacterial outer membrane and anchored by its specific association with LPS O antigens. Both the three-dimensional structure as well as the association of LPS O chains with protein monomers is dependent on divalent cations[12].

Cloning and primary sequence

Restriction fragments containing the A protein gene were cloned into both λgt11 albeit unstably, and into a broad host range cosmid pLA2917, and subsequently subcloned in various vectors for DNA sequencing. The sequence revealed a single ORF of 1509 bp encoding a predicted protein of 50,856 Mr containing a typical leader sequence for protein export. Interestingly, the structure contained a direct 21 bp repeat which presumably accounts for its instability but revealed apparently less secondary structure than other S-layer proteins. Nevertheless, this appears to be the first tetragonal array S-layer protein and the only *A. salmonicida* gene to be sequenced to date (unpublished results).



Model of the cell surface and S-layer of *Aeromonas salmonicida*. The outer membrane of the bacterial cell is shown supporting the tetragonal arrayed A protein subunits tethered to the outer membrane by specific associations with LPS O antigen to form the A-layer. A protein is represented as a protein with both large and small domains and only these specific domains associate with one another. Putative binding sites for both calcium (Ca) and Congo Red (CR) are also shown.

A-layer functions

The A-layer is absolutely required for virulence since isogenic mutants are avirulent by several orders of magnitude[8]. This observation prompted an investigation into the properties of the A-layer as it pertains to pathogenesis. We were able to demonstrate that the A-layer of *A. salmonicida* is a unique multifunctional protein layer and the first S-layer to have been assigned a function.

A-protein monomers, whether assembled into an A-layer or not, readily bound the chromophoric dye Congo Red as well as a variety of other heme analogues[10]. This presumably has a function in heme or iron transport even though these cells contain both siderophore and non-siderophore iron sequestering mechanisms[4]. Indeed atypical, nutritionally fastidious strains of *A. salmonicida* can be shown to be auxotrophic for heme[7].

The A-layer can be thought of as a protective barrier against large destructive proteins such as proteases and complement[11]. In fact, both LPS core oligosaccharide as well as A protein deplete complement components from serum (unpublished results).

The A-layer has also been shown to specifically bind immunoglobulins IgG and IgM from serum[13]. This bacterium is the only gram-negative so far shown to bind immunoglobulins, presumably either as an immunoglobulin-depleting device or as masking mechanism to avoid phagocytosis.

Once more using isogenic mutants it can readily be demonstrated that the A protein or the A-layer are required for efficient entry of *A. salmonicida* into both murine and salmonid macrophages[17] (and unpublished results), where these cells somehow resist phagocytic killing mechanisms.

Finally, the A protein or A-layer appears to be a novel surface capable of binding a number of high molecular weight host basement membrane molecules such as fibronectin, laminin, and collagen-IV (unpublished results).

Attenuated strains with disorganized A-layer

Faced with a barrage of pathogenic functions associated with the A-layer, it is doubtful that humoral immunity alone would confer resistance to infection by *A. salmonicida*. Therefore, as a means to develop attenuated live cell vaccines, we isolated specific mutants which were unable

to correctly assemble the tetragonal A protein array, but still retained the ability to produce all extracellular factors. These strains were characterized as energy defective by virtue of the absence of cytochromes. However, they were still somewhat sensitive to serum killing and were avirulent. Interestingly, these strains gave significant protection, when applied by immersion, from a heavy challenge of virulent *A. salmonicida*.

Thus a fundamental knowledge of the cell surface architecture of *A. salmonicida* may lead to the development of a useful vaccine for the prevention of a furunculosis.

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New lectins and other putative adhesins in *Aeromonas hydrophila*

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Summary. The ability of strains of *Aeromonas hydrophila* to bind ^{125}I-labelled collagen types I and IV, fibronectin, laminin, lactoferrin, and immobilized mucins and orosomucoid on latex beads was found to be a property common to all the isolated strains. The binding was specific, was inhibited by homologous unlabelled glycoproteins, and was protease sensitive. The nature of the binding is discussed.
Key words. Aeromonas hydrophila; lectins; collagen; fibronectin; laminin; lactoferrin.

It may be advantageous for pathogens to produce more than one type of adhesin, especially during the early stages of the colonization process, when maximal adherence ability is needed for the organism to establish an infection successfully[12]. A number of recent studies indicate that *Aeromonas hydrophila* produces various lectins and/or adhesins which enable the pathogen to recognize different carbohydrate moieties on red blood cells and on other cells (table 1). Furthermore, the presence of lectins has been associated with disease. However, very few studies have tried to identify surface components determining an association of *A. hydrophila* cells with specific glycoconjugates on the epithelial surfaces and in the mucin layers on gut mucosal surfaces.

We have now studied the interaction between *A. hydrophila* and connective tissue proteins such as collagens, fibronectin, and laminin, iron-binding glycoproteins such as lactoferrin and transferrin, and mucins. The specificity of the binding was demonstrated by inhibition with homologous proteins, and the protein nature of the *A. hydrophila* adhesins was demonstrated by inhibition of binding of proteins by bacterial cells after various enzymatic treatments.

Materials and methods

Bacterial strains and culture conditions: Strains used in this study were isolated from diseased fish, infected hu-

Table 1. Lectins and adhesins in *Aeromonas hydrophila*

Lectin/adhesin	Target	References
Cell-associated haemagglutinins	Erythrocytes from:	
	Mammalians	1, 4, 8, 13
	Birds	13
	Fish	8
	Human	3, 4, 6, 13
Cell-free haemagglutinin	Erythrocytes from:	
	Birds	13
	Mammalians	13
Cell-associated adhesins	Buccal epithelial cells	3
	Chinook salmo embryo cells	9
	Rabbit intestinal brush borders	10
	Rainbow trout liver cells	9
	Saccharomyces cerevisiae	1, 3
	Fish mucus	9
Pili agglutinin	Hep-2 cells	5

mans, and from the environment[2]. The isolates were incubated at 32 °C for 24 h on blood agar. Bacterial colonies were suspended and washed once in 0.02 M potassium phosphate buffer pH 7.2 (PBS), and the cells resuspended in the same buffer to give approximately 10^{10} cells per ml.

^{125}I-labelled protein binding assay: Collagen types I and IV, human plasma fibronectin, laminin, lactoferrin from human milk, bovine colostrum, and bovine milk, were labelled with 0.2 mCi ^{125}I using iodo-beads[11]. The protein binding assay was quantitated as described earlier[7]. Other ^{125}I-labelled protein binding assays were performed with bacterial cells enzymatically treated as previously described[2]. For the inhibition assays, bacterial cells were pre-incubated with 100 µg of unlabelled homologous proteins or various carbohydrates and glycoproteins. Samples were incubated at 20 °C for 1 h and cells were washed with 2.0 ml of PBS and resuspended to the original volume in PBS.

Preparation of standard latex reagents and particle agglutination assay (PAA). Standard latex reagents were prepared and used as previously described[2].

Results and discussion

A preliminary screening of the isolated strains of *A. hydrophila* showed that binding of sialic acid-rich glycoproteins such as mucins and orosomucoid was a property common to all the strains (table 2). Similar findings were observed with extracellular matrix glycoproteins such as collagen types I and IV, fibronectin, and laminin (Ascencio, F., Ljungh, Å., and Wadström, T., in press). *A. hydrophila* strain A186, which showed a high binding affinity for the various glycoproteins tested, was used for further characterization of how various glycoproteins bind to bacterial cells. The binding affinity of *A. hydrophila* cells to the various glycoproteins differed among the extracellular matrix proteins, while the affinity of bacteria for the various lactoferrin preparations was almost the same (table 3). The binding of the various extracellular matrix glycoproteins and lactoferrin to *A. hydrophila* cells was specific, since it could be inhibited by homologous unlabelled glycoproteins (table 4). However, only ^{125}I-lactoferrin binding was inhibited significantly by carbohydrates and glycoproteins (table 4), and, to a lesser extent, by collagen types I or IV. Laminin binding was inhibited by asialofetuin. These findings suggest that at least lactoferrin, collagen, and laminin bind-

Table 2. Binding of ^{125}I-labelled connective tissue proteins and lactoferrin by cells of *A. hydrophila* strain A186

^{125}I-labelled protein	Percentage of binding
Connective tissue protein:	
Collagen type I	52.1
Collagen type IV	61.6
Fibronectin	36.8
Laminin	17.6
Lactoferrins:	
Human milk	35.0
Human milk (iron-saturated)	26.4
Bovine milk	37.7
Bovine colostrum	30.8

Table 3. Immobilized glycoproteins binding to *Aeromonas* strains

Latex beads coated with:	% strains with positive PAA reactivity (n = 50)
Glycoproteins:	
Asialofetuin (calf serum, type I)	63
Asialomucin (bovine submaxillary gland)	40
Bovine submaxillary mucin	53
Fetuin (calf serum, fraction V)	30
Glycoprotein (bovine, fraction VI)	37
Hog gastric mucin	59
Mucin (crude porcine stomach type II)	69
Orosomucoid (human plasma)	63
Transferrin (rabbit)	40
Ovalbumin (albumin, egg)	53
BSA	55
Without protein	55

Table 4. Inhibition of binding of ^{125}I-labelled protein to *A. hydrophila*

Inhibitor	% binding inhibition of ^{125}I-labelled					
	CnI	CnIV	Fn	Lm	LFN	LFT
Connective tissue protein:						
Collagen type I	64.8	64.1	51.0	60.0	—	—
Collagen type IV	74.0	61.0	15.0	37.0	—	—
Fibronectin	14.0	39.0	29.0	31.8	—	—
Iron-binding proteins:						
Human milk lactoferrin	—	—	—	—	62.2	65.2
Bovine milk lactoferrin	—	—	—	—	55.6	51.5
Transferrin	—	—	—	—	3.2	5.3
Ferritin	—	—	—	—	0.0	0.0
BSA (1.0 mg/ml)	5.0	17.0	0.0	28.0	16.0	2.0
Carbohydrates and glycoproteins:						
Fucose	2.0	6.6	0.0	14.0	42.8	33.1
Galactose	2.0	0.0	0.0	19.0	36.7	25.3
N-acetyl-D-galacosamine	6.5	0.0	0.0	14.0	34.3	37.5
Mannose	0.0	0.0	0.0	18.0	31.3	27.0
Asialofetuin	14.0	36.0	0.0	29.5	43.0	45.1
Bovine serum glycoproteins	0.0	0.0	0.0	18.0	10.0	0.0
Fetuin	4.8	6.6	0.0	18.0	26.0	23.1
Orosomucoid	0.0	0.0	0.0	19.0	2.0	8.3
Submaxillary asialomucin	0.0	4.8	0.0	18.0	15.0	10.0

Table 5. Effect of proteolytic enzymes on the ^{125}I-labelled protein binding to *A. hydrophila*, strain A186

Enzyme	% binding inhibition of ^{125}I-labelled				
	Cn I	Cn IV	FN	Lm	LFN
Chymotrypsin	10.3	0.0	33.0	15.0	6.0
Neuraminidase	22.0	12.5	19.0	27.0	36.4
Pepsin	28.0	20.0	18.3	17.0	37.0
Protease	14.0	12.5	50.0	12.7	16.0
Proteinase K	19.0	2.0	41.0	11.2	12.7
Trypsin	16.0	12.0	32.0	21.0	14.3

ing to *A. hydrophila* cells are mediated by a lectin-like mechanism. Since the binding of fibronectin was partially inhibited by prior treatment of bacterial cells with proteolytic enzymes (table 5), it seems most likely that fibronectin binding is mediated by a protein-protein interaction or by another specific adhesion mechanism, as has been demonstrated in other pathogens [7].

Isolation and characterization of the *A. hydrophila* surface components which recognize the various extracellular matrix glycoproteins, lactoferrin, and mucin are now in progress. Preliminary findings showed that *A. hydrophila* cell surface receptors for collagen types I and IV, fibronectin, laminin and lactoferrin can easily be removed from the cell surface by treating the bacteria with acidic glycine, urea, or distilled water, but this is not the case for the mucin receptors (data not shown). It seems that the receptor for the mucin is probably an integral membrane lectin, while the receptors for collagen, fibronectin, laminin and lactoferrin are peripheral membrane lectins.

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Iron acquisition and virulence in the motile aeromonads: Siderophore-dependent and -independent systems

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Summary. During an infection, a microbial pathogen must acquire all of its iron from the host. *Aeromonas* isolates producing the siderophore amonabactin obtain iron either from host Fe-transferrin (siderophore dependent) or from host heme-containing molecules (siderophore independent). Isolates producing the siderophore enterobactin do not utilize Fe-transferrin in serum and probably rely exclusively on host heme iron.
Key words. Siderophores; iron; virulence; transferrin; heme; amonabactin; enterobactin; *Aeromonas* species.

Iron acquisition and infection

Competition between a vertebrate host and an invading microorganism for iron is one of the points on which the outcome of an infection is balanced [5, 7, 15]. Because of the array of host iron withholding defenses, (an) efficient mechanism(s) to divert some of the metal to microbial metabolism is (are) considered essential for virulence. Part or all of the microbiostatic property of the vertebrate transferrins and lactoferrins resides in their binding and withholding of iron from some microbes. During an infection, the host's hypoferremic response also lowers

the iron present in serum transferrin, temporarily storing the metal in ferritin[5]. An inflammatory episode triggers increased production of haptoglobin, a protein that binds hemoglobin (which may be released by bacterial toxins), protecting the iron-containing heme moiety from use by some microorganisms[8]. Extracellular pathogenic bacteria (those that do not inhabit the internal compartments of host cells) have evolved various processes to override iron-withholding defenses and to access host iron depots such as Fe-transferrin (or lactoferrin) or heme (or hemoglobin). Production of siderophores is a common iron-gathering tactic of microorganisms; and some (but not all) siderophores are virulence factors which remove iron from host iron-binding proteins[5,7,15]. Microbial uptake of iron chelated by a siderophore requires a specific membrane receptor and transport process[7,15]. Acquisition of iron from Fe-transferrin without intervention of a siderophore also is accomplished by some microbes; certain of these produce a surface receptor for Fe-transferrin[9,14,18]. Iron in the host's heme compounds is used by some pathogens. Special heme receptor/transport systems, as well as lytic destruction of host cells, likely are essential to obtain this iron, although not much is known about this system. Use of heme compounds as iron sources may be siderophore independent but there are only limited data to support this assumption. Therefore, both siderophore-dependent and -independent pathogenic iron acquisition systems are evident and a single bacterial strain may be equipped with alternative iron uptake processes[5].

Iron uptake in the aeromonads

Siderophore systems. Members of the motile *Aeromonas* species produce either of the two siderophores enterobactin or amonabactin; both are phenolates containing 2,3-dihydroxybenzoic acid (2,3-DHB)[2,3]. Enterobactin has been found in various gram-negative species; and the enterobactin genetic and biosynthetic systems are being studied[4,16,17]. Amonabactin is a recently discovered siderophore (so far described only in the *Aeromonas* species) that is produced in two biologically active forms which are composed of 2,3-DHB, lysine, glycine and either tryptophan (amonabactin T) or phenylalanine (amonabactin P)[3]. Analyses of 275 isolates showed that amonabactin production was predominant (about 70%) in those classified biochemically as *A. hydrophila* and *A. caviae*; few (5%) of the *A. sobria* produced amonabactin[19]. Most of the remaining isolates produced enterobactin, although 14% of the *A. sobria* demonstrated no detectable siderophore[19].

An amonabactin biosynthetic gene (*amoA*) has been cloned[1]. It was identified in an *A. hydrophila* gene library by its complementation of an *Escherichia coli* strain carrying a mutation in the 2,3-DHB biosynthetic gene *entC*. The nucleotide sequence of *amoA* revealed a gene product with significant homology to the *entC* product (S. Barghouthi, unpublished). A sequence resembling the consensus for the 'Iron Box' iron-regulatory site was present just upstream of the *amoA* putative promoter, suggesting that *E. coli* and *A. hydrophila* have a similar iron regulon. By marker exchange mutagenesis with Tn*5* inactivated-*amoA*, an amonabactin-negative strain, *A. hydrophila* SB22, was isolated.

The capacity of amonabactin to deliver iron from Fe-transferrin in serum to the microorganism was investigated. Inhibition of bacteria by normal serum is mainly due to two components, bacteriostasis by transferrin and the bactericidal activity of complement. Heating the serum to 56 °C inactivates complement but does not destroy transferrin. To overcome iron restriction imposed by heat-inactivated serum, a microorganism must produce a siderophore functional in serum or use Fe-transferrin directly without siderophore. The siderophore enterobactin is ineffective in vertebrate sera[11]. Of 84 isolates of the *Aeromonas* species, growth in heat-inactivated serum absolutely required amonabactin production[13]. The isogenic amonabactin-negative mutant *A. hydrophila* SB22 did not grow in heat-inactivated serum unless supplemented with amonabactin. Amonabactin can function as a siderophore in serum and may be an aeromonad iron-delivery virulence factor.

Non-siderophore systems. The amonabactin-negative *A. hydrophila* SB22, as well as an enterobactin-negative strain of *A. hydrophila*, readily grew with either hematin, hemoglobin, or haptoglobin-hemoglobin complexes as the sole iron supply[13]. All of 32 other isolates (regardless of type of siderophore produced) also obtained iron from hematin or hemoglobin and 30 of these could use hemoglobin when complexed with haptoglobin. Cloned *A. hydrophila* DNA encoding the heme utilization process has been identified (L. Williamson and S. Barghouthi, unpublished). Mutating the heme utilization system will permit isolation of isogenic mutants unable to use heme. To access iron in heme, the bacteria may have to rupture host cells. The *Aeromonas* species produce various cytolytic toxins, some of which are important in virulence[6,10,12].

Conclusions

Isolates of the motile *Aeromonas* species produce either one of the siderophores enterobactin or amonabactin. Of the two, amonabactin may drive an iron-delivery system able to tap Fe-transferrin in serum. Most isolates also have a siderophore-independent mechanism that acquires iron from heme-containing host molecules. Because enterobactin is inactive in vertebrate serum, during an infection the enterobactin producing isolates may have to rely exclusively on non-siderophore heme utilization (and perhaps hemolytic activity) while the amonabactin producers have evolved both siderophore-dependent and -independent means for iron acquisition from a vertebrate host.

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Secretion and mechanism of action of the hole-forming toxin aerolysin

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Summary. *Aeromonas hydrophila* exports aerolysin as a protoxin which is activated by proteolysis after release. Aerolysin binds to the eucaryotic cell receptor glycophorin and oligomerizes, forming holes in the membrane. Important regions of the molecule have been identified by site-directed mutagenesis, and channel formation has been studied in planar lipid bilayers.
Key words. Aerolysin; toxin; oligomerization; channel formation.

Aerolysin is a major factor in the pathogenicity of *Aeromonas hydrophila*[6]. The protein crosses the inner and outer membranes of the exporting bacteria as well as the plasma membrane of eucaryotic cells, which it punctures by forming discrete channels and, as a result, it has been very useful in studies of membrane-protein interactions.

Properties of the protein

The toxin is a water-soluble protein of approximately 50 kDa which spontaneously and irreversibly aggregates at concentrations above approx. 0.5 mg/ml[2, 3, 7]. The structural genes from both *A. hydrophila* and *A. sobria* have been cloned and sequenced, and the derived amino acid sequences of the two proteins are very similar[13–15]. They predict a very hydrophilic protein which, like the porins, has little or no α-helical structure.

Export of aerolysin

Aerolysin is secreted in at least two steps. First preproaerolysin crosses the inner membrane, guided by a signal sequence which is removed co-translationally. Pulse chase experiments and studies with pleiotropic export mutants have shown that the resulting protoxin appears in the periplasm[10, 11] where it can be located by gold labelling (unpublished). Remarkably, a protonmotive force is required for proaerolysin to leave the periplasm[17], but whether there is actually a gradient

across the outer membrane, or whether transit is somehow connected to the electrochemical gradient of the inner membrane is not known.

The fact that both *Aeromonas salmonicida* and a marine *Vibrio* sp. containing the cloned structural gene export proaerolysin in the same way as *A. hydrophila* suggests that these species must produce any other proteins required for export[18,19]. In addition, since secretion of large amounts of proaerolysin by *A. salmonicida* has no effect on the export of its own proteins[18], any chaperone or carrier proteins that are needed must be very efficient or present in high concentrations.

Activation of the protoxin

The proaerolysin released by the bacteria is completely unable to lyse erythrocytes[10]. This presumably prevents damage to the cells during export, and prevents losses due to aggregation of the active toxin which might otherwise occur. The protoxin is activated by removal of about 25 amino acids from the C-terminus. *A. hydrophila* releases at least one protease which can accomplish this; and there are a variety of mammalian enzymes which are also effective[7]. Recently we have described the purification of proaerolysin from *A. salmonicida* containing the cloned structural gene[4]. In contrast to the toxin itself, proaerolysin is quite stable, since it does not aggregate even in concentrated solution.

Binding to the membrane receptor

Bernheimer and Avigad[1] were the first to show that the sensitivity of cells to aerolysin is species-specific. This is due to differences in the ability of the toxin to bind to the protein glycophorin on the cell surface[9]. Aerolysin has highest affinity for rodent glycophorin and somewhat less affinity for the human protein. Changing His^{332} in the toxin lowers affinity for the receptor, suggesting that this residue is part of the binding site[8].

Oligomerization

Binding of aerolysin effectively concentrates the protein on the cell surface. This results in aggregation to form pentameric or hexameric structures[7]. Although proaerolysin can bind equally well, it is completely unable to oligomerize, accounting for its inactivity[8]. Aggregation of aerolysin is inhibited by Zn^{2+} and histidine-reactive reagents. When His^{132} is replaced with Asn, formation of oligomers is eliminated and toxin activity is lost, evidence that this histidine participates in the aggregation process[8].

Insertion

Where in the process of hole formation membrane insertion occurs has not been convincingly established for any toxin. Since aerolysin is a hydrophilic protein with no surface activity (unpublished), it seems reasonable to assume that oligomerization precedes insertion and results in a change which exposes a hydrophobic surface. Glycophorin also appears to play a role in insertion, perhaps facilitating penetration of the surface glycocalyx[7].

Channel formation

Aerolysin forms voltage-gated channels in planar lipid bilayers which exhibit slight anion selectivity[16]. Zinc ions not only prevent channel formation by inhibiting oligomerization, but they also induce closure of preformed channels. Proaerolysin and the His^{132} mutant described above are unable to form channels in this way. Similar channels have recently been described by Chakraborty et al. using aerolysin purified from *A. sobria*[5].

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Studies on aerolysin and a serine protease from *Aeromonas trota* sp. nov.

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Summary. Hybridization of 257 mesophilic aeromonads revealed that the aerolysin gene is present in virtually all strains irrespective of origin of isolation. A probe comprising the promotor region was specific for a species now defined as *Aeromonas trota* sp. nov. Finally, isolation of a serine protease that is concomitantly expressed with aerolysin is described.

Key words. Aeromonas; aerolysin; DNA hybridization; serine protease.

In recent years, increasing attention is being focussed on the bacteria of the genus *Aeromonas* because of their association with food-borne infections and human disease[8]. *A. hydrophila* and *A. sobria* strains isolated from clinical sources are hemolytic and elaborate a potent cytotoxin. The cloning and molecular sequencing of such a hemolytic cytotoxin from an *Aeromonas* strain of clinical origin allowed its identification as aerolysin, a 54,000 dalton exoprotein that is secreted as an inactive precursor[5]. Active toxin is produced by proteolytic cleavage of the protoxin at its carboxy-terminal end[3]. We used marker-exchange mutagenesis to derive mutants that were deficient in the aerolysin gene and by virtue of the mouse infection model demonstrated that these were of reduced virulence[1]. Furthermore, purified aerolysin has been observed to exhibit lethality for mice, induce fluid accumulation in rabbit ileal segments, release inflammatory mediators from granulocytes and mast cells, and cause cytotoxicity to a variety of cell lines in vitro (cited in ref. 2). Hence the detection of this important virulence factor in *Aeromonas* strains is relevant from an epidemiological point of view.

In a study of 257 *Aeromonas* strains of both clinical and environmental origin from diverse geographical regions comprising the three main species, *A. hydrophila, A. sobria* and *A. caviae* we used DNA probes from regions proximal to and within the aerolysin gene to look for the distribution of this gene within this genus. The main findings of this study can be summarized as follows: firstly, the aerolysin gene is present in virtually all strains of the species *A. hydrophila* and *A. sobria*. The detection of the gene in about half of all *A. caviae* strains tested points to the pathogenic potential of these strains in what has by some been considered to be a non-pathogenic species. Secondly, the environment is an important reservoir of strains since the gene was present in all species regardless of the source (clinical vs environmental) of isolation. Thirdly, the DNA probe that derives from within the aerolysin gene is specific for the genus *Aeromonas* and did not hybridize to a panel of gram-negative and gram-positive strains.

A further interesting aspect of this study was that hybridization of the DNA probe encoding the promoter region of the aerolysin gene of *Aeromonas* strain AB3 was only detected in a small group of strains tested. These strains were also found to cluster biochemically and form a distinct phenetic group that we have now designated *Aeromonas trota* sp. nov. Strains belonging to the species *Aeromonas trota* have a worldwide distribution and are predominantly associated with gastrointestinal disorders.

In some cases, variable expression of hemolytic activity depending on the type of erythrocyte used was observed. Since all these strains harbor the aerolysin gene, this variability in hemolytic activity is likely to be due to differing receptor affinities of the aerolysin molecule for a particular erythrocyte species. Such differences in hemolytic activity from aerolysin molecules purified from different strains have been reported before. Our results lead us to conclude that aerolysin may be a heterogeneous family of cytolytic hemolysins present in the genus *Aeromonas*, much the same way as the family of Shiga- and Shiga-like toxins of *Shigella* and enteroinvasive *Escherichia coli*[7]. Support for this hypothesis comes from the comparison of sequences of two aerolysin genes deriving from *A. hydrophila* Ah65[4] and from the *A. trota* strain AB3[6] where the primary sequence of these genes are only 77% homologous.

Since aerolysin must be proteolytically cleaved to become active toxin, proteases involved in this activation may also be relevant virulence factors in *Aeromonas* infections. In order to isolate such a protease, we have established growth conditions under which expression of the aerolysin is maximal and looked for the concomitant presence of protease activity in these supernatant fluids. A serine protease activity was detected, and the protein responsible for this activity was purified to homogeneity after ammonium sulphate concentration of the supernatant followed by anion-exchange chromatography of the solubilized precipitate. The protein eluted as a single peak at 0.1 M NaCl in Tris-propane, HCl buffered at pH 6.9. The serine protease isolated using this procedure has a molecular mass of 70,000 daltons, has a requirement for Ca^{++} and Mg^{++} ions, a pH optimum of between 8 and 9, was inhibited by PMSF (phenyl methyl sulfonyl fluoride) and inactivated when held at 56 °C for 30 min. The molecular weight and the biochemical properties of this enzyme strongly resemble proteases that have previously been purified from *A. hydrophila*[9] and *A. salmonicida*[10]. The protease isolated from *A. salmonicida* caused

necrotic skin lesions when injected into fish, and a mutant strain lacking protease activity has been shown to be avirulent when assessed in a fish model of infection[11]. Our future studies will focus on the molecular cloning of the protease gene to resolve its role in the pathogenesis of *Aeromonas trota* infections by the construction of a site-specific protease mutant.

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Application of the polymerase chain reaction (PCR) to detection of the aerolysin gene in whole cell cultures of β-hemolytic *Aeromonas hydrophila*

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Summary. Oligonucleotide primers were used in a polymerase chain reaction protocol to detect the aerolysin gene in *Aeromonas hydrophila*. Primers detected template DNA in hemolytic, cytotoxic and enterotoxic strains of *A. hydrophila* and no amplification was detected with hemolytic *A. sobria*, non-hemolytic *A. hydrophila* and *A. caviae* strains.
Key words. Polymerase chain reaction (PCR); *Aeromonas hydrophila*; β-hemolytic; aerolysin gene.

Aeromonas species are associated with both diarrheal and extraintestinal infections in human disease[1,6]. Several extracellular products of *A. hydrophila* which have been suggested as possible contributory factors in the pathogenesis of these diseases include a β-hemolysin as well as an α-hemolysin which causes incomplete lysis of erythrocytes and which is probably of minor importance in the pathogenesis of *Aeromonas* infections[1]. Diarrhea has been associated with some strains of *Aeromonas* spp.; and both antibiotic therapy and drinking of untreated water were significant risk factors for susceptible hosts[8]. Aerolysin, first described by Bernheimer and Avigad[2], may be produced by some strains of *A. hydrophila* and is an extracellular soluble hydrophilic protein exhibiting both hemolytic and cytolytic properties. The synthetic oligonucleotide primers used in the polymerase chain reaction (PCR) described herein targeted a 209-base-pair (bp) fragment of the longest open reading frame of the published *aer* gene sequence[4].

In the present and a previous study[10], we report the development of a PCR procedure which will rapidly and specifically detect aerolysin genes in strains of hemolytic *A. hydrophila* associated with human disease. PCR results were compared with biological assays for hemolytic, cytotoxic and enterotoxic activity.

Materials and methods

Bacterial strains and culture media. A complete list of bacterial strains used in this study appears in table 1. Reference strains were defined in terms of established toxigenicity. *Aeromonas* and *Plesiomonas shigelloides* strains were fecal isolates from patients with diarrhea and were identified and speciated using the characters defined by Popoff[11]. Bacterial suspensions were prepared by inoculating 2 ml of nutrient broth followed by incubation for 2 h at 30 °C.

Determination of hemolytic, cytolytic and enterotoxic activity. Strains of *Aeromonas* spp. and *P. shigelloides* were scored for β-hemolysin activity after 24 h growth at 30 °C on Mueller-Hinton agar (Oxoid Ltd.) containing either 5% rabbit red blood cells or 5% sheep red blood cells. *Aeromonas* enterotoxin was assayed in 3-day-old suckling mice[3,7], and intestinal weight to remaining body weight ratios higher than 0.085 were considered enterotoxin positive. Cytotoxic responses were determined using Vero monkey kidney and Chinese Hamster Ovary cells (CHO).

Nucleic acid isolation and PCR. Procedures for isolation of nucleic acids (NA) and the PCR were previously described[9]. The aerolysin-specific primers (Oligonucleotide Synthesis Laboratory, Queen's University, Kingston, Ontario, Canada) are described in table 2. PCR was performed using 10 µl of a whole cell bacterial suspension with initial denaturation for 10 min at 94 °C followed by the following amplification cycles: denaturation for 2 min at 94 °C, annealing of primers for 2 min at 55 °C, and primer extension for 1 min at 72 °C with autoextension.

Results

Aerolysin oligonucleotide probes. The primer targeted a 209-bp fragment of the *aer* gene coding for aerolysin toxin in this PCR. Results of the PCR are summarized in table 1 and the figure. An amplification product of the expected size (table 2) was only observed in the PCR using DNA from β-hemolytic strains of *A. hydrophila* (figure, lane C). No similar fragments were observed in the PCR using template DNA from hemolytic *A. sobria* (lane G) or other *Aeromonas* spp. including *A. caviae* (lane F), *P. shigelloides* (lane D), *A. veronii* (lane E), and *A. schubertii* (lane H). The sensitivity limit of the PCR protocol in detecting *aer* genes in β-hemolytic strains of *A. hydrophila* was 1 ng of total NA (data not shown).

Specificity of oligonucleotide probes for detection of aerolysin gene sequences. A summary of strains, hemolytic activity, cytotoxicity to Vero and CHO cells, toxigenicity, and the PCR results appears in table 1. The PCR clearly identified all aerolysin-positive strains of *A. hydrophila* which were also cytotoxic to Vero and CHO cells and enterotoxic in suckling mice. Ten non-hemolytic isolates of *A. hydrophila* were selected for this study to

Table 1. Summary of organisms, hemolysin production, toxin profiles and PCR probe results

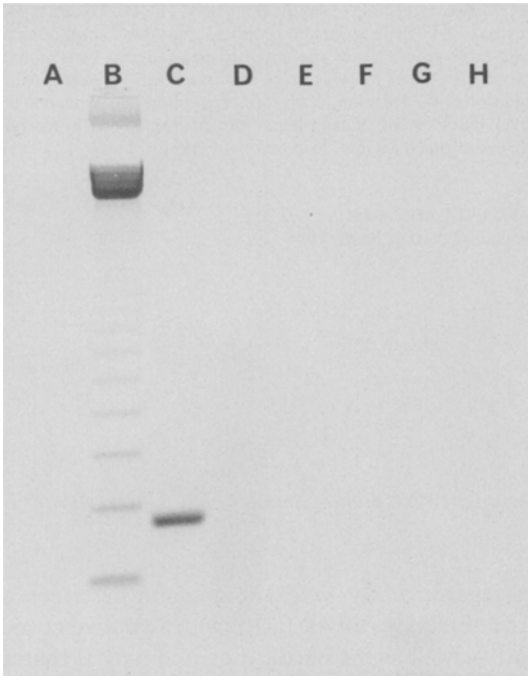
Culture	Serotype	No. of strains	Toxin(s)[a]	Hemolysin	PCR amplification product (209-bp)
Aeromonas and *Plesiomonas* strains:					
Aeromonas hydrophila		34	Cytotoxin[b], enterotoxin	+	+
A. hydrophila		4	Cytotoxin, enterotoxin	–	–
A. hydrophila		1	Cytotoxin	–	–
A. hydrophila		5	–	–	–
A. sobria		11	Cytotoxin, enterotoxin	+	–
A. sobria		1	Cytotoxin	–	–
A. caviae		6	–	–	–
A. veronii		1	–	–	–
A. schubertii		1	NT[c]	NT	–
Plesiomonas shigelloides		3	–	–	–
Other toxigenic pathogens and reference strains:					
Streptococcus pyogenes		5	Streptolysin O	+	–
Listeria monocytogenes		1	Listeriolysin	+	–
Escherichia coli	O22:H43	1	Hemolysin	+	–
Vibrio cholerae	01	1	Cholera toxin	–	–
Shigella dysenteriae	1	1	Shiga toxin	–	–
E. coli H19	O26:H11	1	VT1	–	–
E. coli E32511	O157:H-	1	VT2	–	–
E. coli 412	O139:H1	1	VTe[d]	–	–
E. coli TD427c2	O25:H-	1	LT	–	–
E. coli TD213c2	O128	1	ST	–	–
E. coli H10407	O78:H11	1	LT, ST	–	–
E. coli HB101		1	–	–	–
E. coli C600		1	–	–	–

[a] VT, verotoxin; LT, heat-labile enterotoxins; ST, heat-stable enterotoxins. [b] Cytotoxic to Vero and CHO cells. [c] NT, not tested. [d] VTe or SLT-IIv associated with edema disease in pigs – only cytotoxic to Vero cells.

Table 2. Base sequences, locations, and predicted sizes of amplified products for the aerolysin-specific oligonucleotide primers

Primer	Oligonucleotide sequence (5' –3')[a]	Location within gene[b]	Size of amplified product (base pairs)
Aero1a	CCAAGGGGTCTGTGGCGACA	645–664	
Aero1b	TTTCACCGGTAACAGGATTG	834–853	209

[a] From the published *aer* gene for aerolysin[4]. [b] In nucleotides.



Occurrence and distribution of a 209-bp amplification fragment in the PCR. Lane A, blank; lane B, 123-bp ladder (Bethesda Research Laboratories, Inc., Gaithersburg, Md.); lane C, *A. hydrophila* (aerolysin positive); lane D, *P. shigelloides* (hemolysin negative); lane E, *A. veronii* (hemolysin negative); lane F, *A. caviae* (hemolysin negative); lane G, *A. sobria* (hemolysin positive); lane H, *A. schubertii*.

establish primer specificity. No specific amplification fragments were observed in the PCR using DNA from any of the other *Aeromonas* or *Plesiomonas* strains, including several cytotoxic and enterotoxic isolates. Strains of *Streptococcus pyogenes* producing erythrogenic toxin or streptolysin O were typified by a common 200-bp amplification fragment in the aerolysin PCR (data not shown). Both the 200-bp streptococcal and 209-bp aerolysin amplification fragments were subjected to endonuclease digestion with *Nci* I. The aerolysin-specific 209-bp amplicon was cleaved into two smaller segments of 132-bp and 77-bp whereas the 200-bp streptococcal fragments remained undigested[10]. Other *Streptococcus* spp. tested were negative in the aerolysin PCR.

PCR using whole cell bacterial cultures and extracted NA. PCR studies were performed in parallel using both viable, whole cell bacterial cultures of *Aeromonas* spp. and NA extracted from the same strains. No differences were observed in the genotypic identification of *Aeromonas* strains carrying the *aer* gene and 100% concordance was observed between phenotypic assays for β-hemolysin and genotypic identification by PCR.

Discussion

It has been established that aerolysin is a virulence factor contributing to the pathogenesis of *A. hydrophila* infection[13] and very recent reports suggest that hemolytic, enterotoxic, and cytotoxic activities can be associated with a single polypeptide[12]. We have designed a PCR protocol to detect the aerolysin gene in DNA from strains of *A. hydrophila*. Although it is possible to detect the β-hemolysin biologically, these tests do not distinguish β-hemolysins from *A. hydrophila* and *A. sobria*. The PCR was species-specific in that amplification was observed only with DNA template from hemolytic *A. hydrophila* and not with template from hemolytic *A. sobria*. The PCR primers targeted the *aer* gene and clearly detected the gene sequence in DNA from representative *A. hydrophila* isolated from human sources. The PCR primers were designed to avoid regions of homology in the structural genes for *A. sobria* aerolysin[5], *E. coli* hemolysin A, or *Staphylococcus aureus* α-toxin as previously documented[4]. PCR using NA from bacterial strains known to harbor virulence-associated toxins such as cholera toxin, listeriolysin, verotoxins (VT1, VT2 and VTe), Shiga toxin and the classic LT and ST enterotoxins were consistently negative. Non-specific amplification of a similarly-sized fragment observed in some *S. pyogenes* strains is not a problem if the PCR is performed using either whole cell cultures or NA extracted from pure cultures. The presence of a *Nci* I site in the aerolysin-specific amplicon and the absence of such a site in the streptococcal fragment provides a sensitive and specific means of distinguishing the non-specific amplification product if necessary. The PCR protocol clearly identified aerolysin-producing strains of *A. hydrophila* with 100% concordance in this small group of strains and may have application as a rapid, species-specific virulence test in that other hemolytic and cytotoxic species of *Aeromonas* and other enteric pathogens tested were negative. PCR amplification results were identical in parallel tests using extracted NA and whole cell bacterial cultures. The use of whole cell bacterial cultures in the PCR has many advantages, particularly in minimizing laboratory manipulations involved in NA extraction, thereby substantially reducing the total assay time.

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Review of *Aeromonas* enterotoxins

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Summary. This report reviews the work of other investigators regarding *Aeromonas* toxins and describes work conducted in our laboratory relating to the biochemical characterization of a cytolytic factor with an antigenic moiety that cross-reacts with cholera toxin (referred to as CTC-cytolysin), as well as the purification and partial characterization of a non-CTC enterotoxin. These two toxins were produced by *Aeromonas hydrophila*, isolate SSU, and are capable of causing fluid accumulation in animal models.
Key words. Cytotoxic enterotoxin; cytotonic enterotoxin; aerolysin; immuno-cross-reactivity; neutralization; hemolysin.

Aeromonas hydrophila is a commonly occurring aquatic microorganism which has been reported to be responsible for human diarrheal disease and wound infections[7, 10, 11, 21]. Evidence has indicated that the pathogenicity of the organism may involve the action of several extracellular toxins[15, 16], including cytotoxin and enterotoxin. There has been controversy in the literature regarding whether or not the enterotoxic and cytotoxic activities of *Aeromonas* toxins reside on the same molecule[1, 8, 9, 14]. Asao et al.[1] reported the purification of a 50 kDa protein hemolysin from *A. hydrophila* culture filtrates that induced fluid accumulation in the infant mouse intestine and rabbit ileal loop assays and was cytotoxic to Vero cells. Additional studies indicated that in most cases cytotoxic activity correlated with enterotoxic activity[5, 22]. More recently, Bunning et al.[2] provided further evidence for a cytotoxic enterotoxin, demonstrating that both the hemolytic and enterotoxic activities were inhibited by antihemolysin. Chakraborty et al.[4] cloned and mapped the aerolysin gene determinant, and observed that the gene product (aerolysin) was similar to Asao's toxin in that the hemolytic and cytotoxic activities were associated with the same protein molecule. These two toxins exhibited similar molecular weights.

Evidence for a cytotonic enterotoxin also has been reported in the literature. Chakraborty et al.[3] cloned an enterotoxin gene from *Aeromonas* and demonstrated that the culture filtrates of *E. coli* containing the enterotoxin gene provoked fluid accumulation in rabbit ileal loops and in suckling mouse models. These culture filtrates, however, were negative for hemolytic and cytotoxic activities, thereby suggesting the production of a cytotonic enterotoxin. This cytotonic enterotoxin was cloned from the same isolate of *Aeromonas*, AH2, from which the aerolysin gene was cloned. These data suggested that *Aeromonas* could have either different genetic loci for the synthesis of enterotoxin, hemolysin, and cytotoxin, or the same structural gene could synthesize all three products. Earlier, Ljungh et al.[14] described a cytotonic enterotoxin produced by *A. hydrophila*, which was a relatively heat-stable (60 °C for 20 min) molecule and had a molecular weight of 15 kDa. This enterotoxin was capable of stimulating adenylate cyclase, which resulted in increased levels of intracellular cAMP in intestinal epithelial cells. These investigators also concluded that there was no antigenic cross-reactivity between the cytotonic enterotoxins of *A. hydrophila* and *Vibrio cholerae*. However, James et al.[13] reported that *A. hydrophila* produced two kinds of enterotoxin. One enterotoxin, which caused fluid accumulation in the suckling mouse assay, was heat-labile (56 °C for 30 min) and did not cross-react with cholera toxin. The second enterotoxin was heat-stable (100 °C for 30 min), caused fluid secretion in the rat perfusion system, and reacted with cholera antitoxin. In addition, Potomski et al.[17] reported on the isolation of an *Aeromonas* cytotonic enterotoxin that cross-reacted with cholera toxin and was biologically heat-stable.

This report describes the biochemical characterization of a cytolytic factor with an antigenic moiety that cross-reacts with cholera toxin (referred to as CTC-cytolysin), as

well as the purification and partial characterization of a non-CTC enterotoxin that is capable of causing fluid accumulation in animal models[6, 19, 20]. These two toxins were produced by isolate SSU of *A. hydrophila*. Our laboratory purified a CTC-cytolysin[19, 20] that is cytotoxic to CHO cells, hemolytic to rabbit red blood cells, causes fluid accumulation in rabbit ileal loops, and has lethal activity in mice.

CTC-cytolysin was purified from the culture filtrate of *A. hydrophila* SSU by ammonium sulfate precipitation, hydrophobic column chromatography using phenyl-Sepharose, and anionic exchange chromatography using DEAE Bio-Gel A, followed by gel filtration with HPLC using TSK columns 250 and 125 connected in series. The CTC-cytolysin was antigenically heat-labile (56 °C/10 min), had a molecular weight of 52 kDa and an isoelectric point of 5.1. One band at 52 kDa was detected by immunoblotting with cholera antitoxin and homologous antibodies. In addition, homologous antibodies could neutralize the tested biological activities of the molecule; however, cholera antitoxin could not neutralize any of these activities. Amino acid sequence analysis revealed that the CTC-cytolysin was a single polypeptide chain and the first 25 amino acid residues from the N-terminal end were identical to that for aerolysin as reported by Buckley's group[12].

This is the first report describing a CTC-cytolysin. In addition, this report describes the purification and partial characterization of a non-CTC enterotoxin from the same *Aeromonas* isolate SSU[6]. Non-CTC enterotoxin was isolated and purified from culture filtrate by ammonium sulfate precipitation, hydrophobic column chromatography using phenyl-Sepharose and chromatofocusing. The purified enterotoxin, unlike CTC-cytolysin, was antigenically heat-stable (100 °C/20 min), exhibited a molecular weight of 44 kDa and an isoelectric point in the range of 4.3–5.5. Western blot analysis using anti-non-CTC enterotoxin revealed a single band at 44 kDa. Cholera antitoxin failed to detect non-CTC enterotoxin antigen in Western blots. The purified non-CTC enterotoxin was biologically active as determined by in vivo and in vitro models[6]. The enterotoxic activity associated with this molecule was neutralized completely by homologous antibodies, but not by cholera antitoxin, and the molecule was free of hemolytic and cytotoxic activities. Furthermore, this toxin caused elevation of intracellular cAMP levels in CHO cells. In addition, this toxin may cause an increase in the synthesis of prostaglandin E_2 in CHO cells which ultimately may play a role in fluid accumulation in rabbit ligated intestinal loop-experiments[18].

These data suggest that some *Aeromonas* isolates can produce a cytotonic enterotoxin (non-CTC enterotoxin) as well as a cytotoxic enterotoxin (CTC-cytolysin). A comparison of these two toxins is demonstrated in the table. Presently, we are in the process of determining the

Comparison of the physicochemical, immunological and biological properties of cytotoxic and cytotonic enterotoxins of *A. hydrophila*, SSU

Properties	Cytotoxic enterotoxin	Cytotonic enterotoxin
Physicochemical		
1. Elution profile through phenyl-Sepharose (hydrophobic) column	Highly hydrophobic; eluted with 100% water	Less hydrophobic; eluted when 1 M–0.05 M $(NH_4)_2SO_4$ was used
2. Molecular size	52 kDa	44 kDa
3. Isoelectric point (pI)	5.1	4.3–5.5
Immunological		
1. Cross-reactivity to cholera toxin	Reacted with cholera antitoxin in an ELISA	No reaction with cholera antitoxin in ELISA
2. Western blot analysis	Cytotoxic enterotoxin antigen (52 kDa) was detected with both homologous and cholera toxin antibodies	Cytotonic enterotoxin antigen (44 kDa) was detected by only homologous antibodies
3. Heat stability of antigen	Heat-labile (56 °C/20 min)	Heat-stable (100 °C/20 min)
Biological		
1. Heat stability	Heat-labile (56 °C/20 min)	Heat-labile (56 °C/20 min)
2. Effect on CHO cells	Destroyed CHO cell monolayer	Elongated CHO cells
3. Effect on rabbit red blood cells	Complete lysis	No effect
4. Lethality in mice	Killed within minutes when injected intravenously	No effect
5. Enterotoxic activity	Positive in rabbit ileal loop	Positive in rabbit ileal loop
6. Skin permeability factor activity	–	Firm induration (edema)
7. Effect on cAMP	–	2.5–3-fold increase of cAMP in CHO cells
8. Effect on prostaglandin E_2 synthesis	–	2-fold increase in synthesis of PgE_2 in CHO cells

precise role of these toxins in the pathogenesis of *Aeromonas*-mediated diseases.

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Re-examination of *Rattus norvegicus* as an animal model for *Aeromonas*-associated enteritis in man

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Summary. We have developed an oral feeding model for *Aeromonas hydrophila* enteritis using *Rattus norvegicus* with clindamycin pretreatment. All animals in the clindamycin group developed a self-limited, loose stool by day four of feeding. Intestinal examination revealed evidence of enteritis. Moreover, antibiotic usage may be a predisposing risk factor to infection.
Key words. Animal model; *Aeromonas*; clindamycin; rat.

Aeromonas hydrophila is a free-living, gram-negative bacterium found in fresh water, in reptiles and amphibians, and in foods they contaminate. Recent studies have epidemiologically linked *A. hydrophila* with diarrhea in man[1,4,5,9]. Most of these diarrheal illnesses appear to be self-limited, but occasionally a cholera-like illness can result[3]. Animal models have been employed to study virulence characteristics of *Aeromonas*[2,7,8,10,12,13], and recently Pazzaglia et al. have reported on the use of a modified RITARD model as a diarrhea model[11]. However, this model is highly manipulated, labor-intensive, and expensive. In addition, some strains produce death within 24 h in this model; a rare occurrence, if ever, in man. The development of a reproducible, simple model is crucial for the evaluation of pathogenesis, prophylaxis and drug therapy. The aim of our study was to establish a rodent diarrhea model which imitates this infection in man. This oral feeding model may simulate the mode of entry and the course of illness in man. We have previously reported on preliminary observations using this model[6].

Materials and methods

A clinical isolate of *Aeromonas hydrophila* was used. The strain (DJ 188) was isolated from a child with diarrhea in Djibouti, on the eastern horn of Africa in February of 1989. The phenotype of this particular strain was hemolytic, non-autoagglutinable, failed to spontaneously pellet, and did not precipitate after boiling in brain heart infusion broth. In addition, the strain produced gas from glucose, hydrolyzed esculin, produced gas in TSI, H_2S in GCF (cysteine-iron agar), and agglutinated in the acriflavin test. The strain did not ferment arabinose nor did it agglutinate in 0:34 or 0:11 antisera. It was resistant to 10 µg of ampicillin.

Male and female Sprague-Dawley rats (avg. wt 250 g) were obtained from Charles River Breeding Labs (Wilmington, Mass., USA) and housed at the U.S. Naval Medical Research Unit No. Three, Cairo (Egypt). The rats were divided into four groups (two control groups); group A received saline placebo, and group B received clindamycin alone. Groups C and D received *A. hydrophila* with and without intramuscular clindamycin (30 mg/kg) pretreatment, respectively (as above). All groups received food and water ad libitum. *A. hydrophila* from liquid culture was fed once daily for three days at concentrations ranging from 10^2 to 5×10^8 CFU. *A. hydrophila* was grown overnight in tryptic soy broth; turbidity was adjusted to correspond to 5×10^8, after which serial dilutions and plate counts were made. By day 4 after gavaging, all the animals in group C and 3 of 8 (38%) of those in group D had loose stools which resolved within 72–96 h. The groups were monitored for a maximum of 12 days. One rat from each group was necropsied daily, starting with day four, in order to monitor the progress of the infection. Blood, liver, lung and spleen tissues were cultured to rule out break-through bacteremia.

Results

As shown in the table, intestinal colonization of *R. norvegicus* depends on the concentration of the inoculum, multiple feedings, and pretreatment with antibiotics.

Peak shedding (day 5) of organisms correlated well with diarrhea, i.e., as diarrhea resolved, colonization decreased, and by day 11 no *Aeromonas* were detected.

Histological examination of the gastrointestinal tract in ill animals revealed ileal mucosal changes consisting of congestion with shortening and broadening of the microvilli, moderate edema, and mild inflammatory reactions not observed in control animals (fig. 1). Clindamycin treatment alone caused no significant intestinal alterations. However, *Aeromonas* infection following clindamycin pretreatment resulted in major histological changes in the ileal mucosa ranging from superficial erosion to marked focal ulceration (figs 2 and 3). No evidence of infection of the underlying muscle layer or *Aeromonas*-associated bowel perforation was detected in any of the animals studied. Examination of the large intestine was unremarkable.

Extraintestinal pathology was observed in only those animals pretreated with clindamycin (group C). The pathology was suggestive of a toxin, i.e., mild cellular

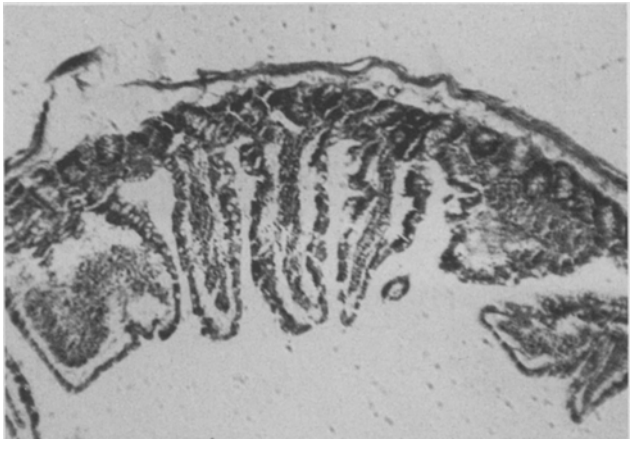

Figure 1. Photomicrograph of small intestine with moderate edema and congestion of villi, slight shortening, moderately broad with mild inflammatory reaction. This animal was fed 5×10^8 *A. hydrophila* for 3 days. H & E stain. ×30.

Intestinal colonization of infected *R. norvegicus* by time after feeding once daily for three days

		A. hydrophila feeding day											
		1	2	3	4	5	6	7	8	9	10	11	12
Clindamycin treatment (group C[a])													
Dose	No. of rats												
10^2	3	–	–	–	–	–	–	–	–	–	–	–	–
10^6	3	–	–	+[b]	+	+	–	–	–	–	–	–	–
5×10^8	3	+	+	+	+	+	+	+	+	+	+	–	–
	9												
Without clindamycin treatment (group D[a])													
Dose	No. of rats												
10^2	3	–	–	–	–	–	–	–	–	–	–	–	–
10^6	3	–	–	–	–	+	–	–	–	–	–	–	–
5×10^8	3	–	–	+	+	+	+	–	–	–	–	–	–
	9												

[a] see text; [b] +, intestinal colonization.

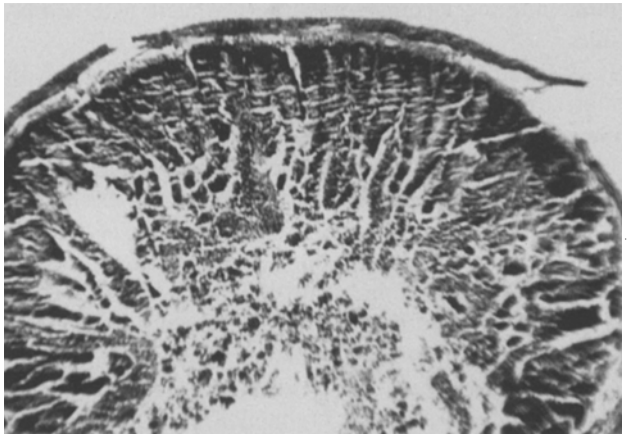

Figure 2. Photomicrograph of small intestine with superficial erosion of villi, intraluminal desquamation of epithelial cell lining and inflammatory cells, mucosa and submucosa edematous and congested. This animal was fed 5×10^8 *A. hydrophila* for 3 days after pretreatment with clindamycin. H & E stain. ×40.

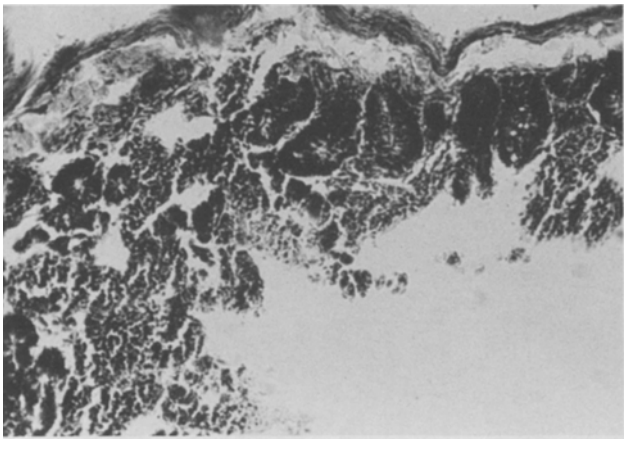

Figure 3. Photomicrograph of small intestine with prominent ulceration with short stumps of sloughed villi, congested edematous submucose with moderate inflammatory reaction. This animal was fed 5×10^8 *A. hydrophila* for 3 days after pretreatment with clindamycin. H & E stain. ×40.

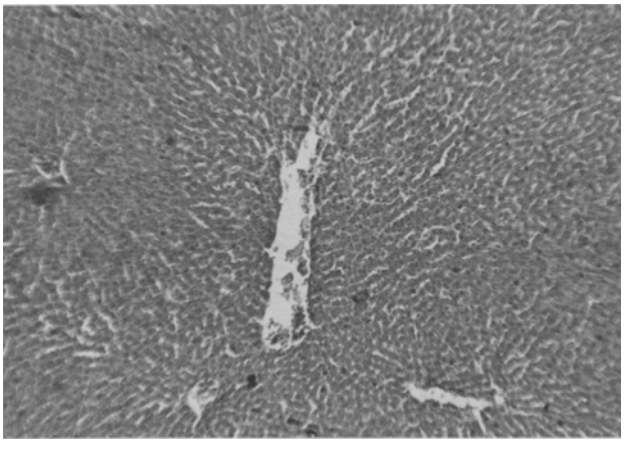

Figure 4. Photomicrograph of liver tissue with mild cellular congestion but no detectable evidence of localized infection. This animal was fed 5×10^8 *A. hydrophila* for 3 days after pretreatment with clindamycin. H & E stain. ×40.

congestion was observed in the liver and spleen but no evidence of infection was present (fig. 4). All post-mortem cultures of liver, spleen, and lung tissues were negative for bacterial growth. However, pneumonic changes characterized by an acute inflammatory cell infiltrate of the alveolar spaces, bronchial wall, and lumen were occasionally seen.

Discussion

We have described a rat model of *A. hydrophila* infection that may be relevant to human infection. The rat model had been previously employed to study *Aeromonas* toxins but was not found to be an appropriate model[8]. We modified the rat model to incorporate clindamycin pretreatment, and this modification resulted in a model for the study of *Aeromonas* colonization and disease.

Young adult (225–250 g) *R. norvegicus* appears to be a suitable animal model for studying enteritis due to *A. hydrophila*. Experimental infection produces a self-limited diarrhea in rats, and histological examination of tissues reveals mucosal damage. Thus, this model may be useful for studying the pathogenesis of *Aeromonas* enteritis and may be useful in evaluating the immune response to *Aeromonas*, and the effects of prophylactic or therapeutic interventions. Whether this model can serve in the evaluation of the relative importance of particular virulence mechanisms in *Aeromonas* spp., or as a method for determining the pathogenicity of environmental isolates remains to be seen. Further work with this model is underway to evaluate its usefulness with other enteric pathogens.

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In conducting the research described in this report, the investigators adhered to the Guide for the Care and Use of Laboratory Animals, Institute of Laboratory Animal Resources, National Research Council, DHHS Publication (NIH) 86-23 (1985).

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The phenomenon of toxin secretion by vibrios and aeromonads

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Summary. *Vibrio* and *Aeromonas* species have a remarkable ability to secrete extracellular proteins, including toxins, haemagglutinins and other virulence factors. Here we discuss the pathways and potential mechanisms of toxin secretion through the double-membraned envelopes which surround these organisms.
Key words. Protein secretion; protein folding; membranes; cholera toxin; enteric diseases.

Enteric infections caused by certain *Vibrio* and *Aeromonas* species are the result of a pathogenic process which involves the production and secretion of specific virulence factors. In the case of *V. cholerae*, cholera toxin (CT) represents the most extensively studied and characterized of these factors. CT is an exoprotein comprised of six non-covalently associated polypeptide chains, consisting of two different types; a single A subunit and five identical B subunits (for a review see Holmgren[15]). One of the most remarkable features of the production of cholera toxin is the ability of *V. cholerae* to export the individual subunits, assemble them and secrete the toxin across its bacterial outer membrane into the surrounding milieu[13]. Toxin secretion is thus an intriguing biological phenomenon involving several membrane translocation events and for which a molecular explanation has yet to be provided.

In recent years it has become apparent that cholera toxin is the prototype of a large family of related enterotoxins produced by *V. mimicus*, certain toxinogenic *Escherichia coli*, *Salmonella*, *Campylobacter*, and *Aeromonas* as well as *V. cholerae*[1-5,21,24-26,28]. The finding that cholera anti-toxin neutralizes the activity of enterotoxins produced by *Plesiomonas shigelloides* suggests that this organism may produce an extracellular cholera-like enterotoxin[6].

Studies of the biosynthesis of *E. coli* heat-labile enterotoxin (LT), which is structurally and functionally similar to cholera toxin, have revealed that the A and B subunits are synthesized as precursors with amino-terminal signal sequences[4,20,25]. Translocation of precursors across the cytoplasmic membrane is accompanied by removal of the signal sequences, followed by folding and assembly of the subunits and toxin release into the periplasmic space[8-13,22,23]. In contrast to *V. cholerae*, however, *E. coli* fails to secrete the toxin into the surrounding extracellular medium[9,10].

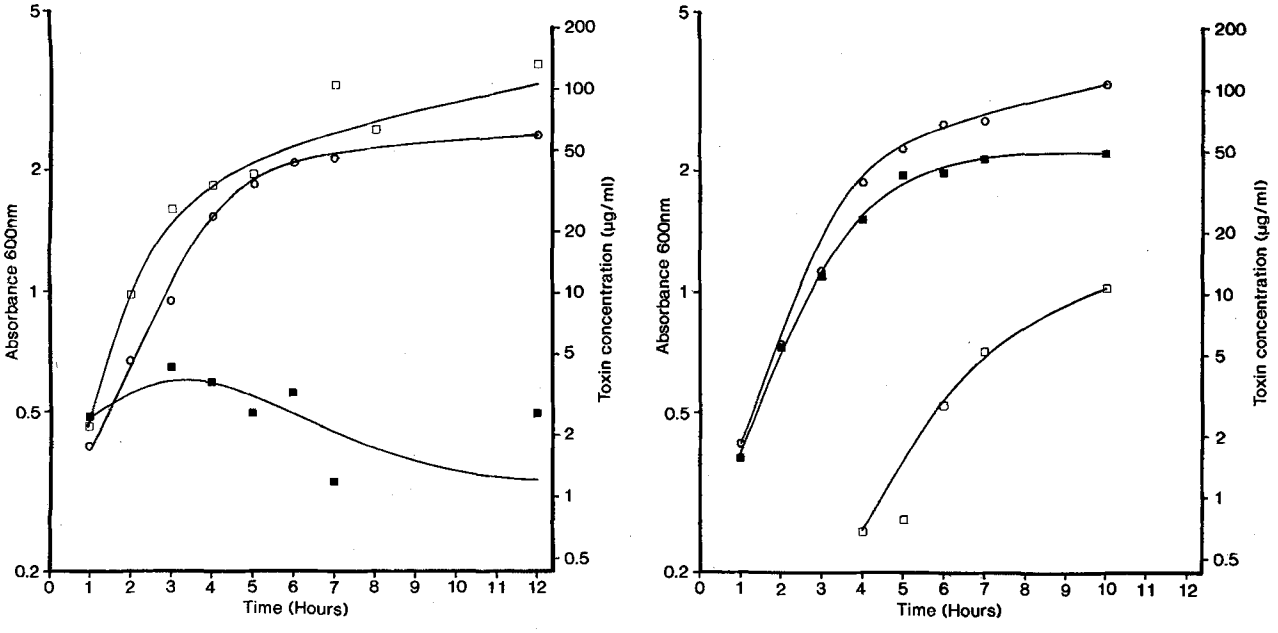
The discrepancy in the cellular location of these related enterotoxins was investigated by expressing the *E. coli* enterotoxin in *V. cholerae*[10,17]. It was found that the *E. coli* enterotoxin was efficiently secreted into the medium by *V. cholerae*. Thus the secretory event exhibited by *V. cholerae* cannot be attributable solely to the structural properties of the toxin, but must also require a specific secretory machinery present in the *V. cholerae* outer membrane but absent from *E. coli*. Genetic evidence for a secretory apparatus was implicated by Holmes and coworkers[14] when a mutant of *V. cholerae* was obtained which accumulated cholera toxin in the periplasmic space[12]. The identity of the gene(s) encoding this machinery and how they operate remains to be elucidated.

In an attempt to explore the preponderance of the toxin secretion machinery amongst enterobacteria, vibrios and aeromonads, several researchers have studied the heterologous expression of *E. coli* enterotoxin and its subunits in different gram-negative genera (table). Members of the *Enterobacteriaceae* and *Pseudomonas aeruginosa* lack the capacity to secrete oligomeric enterotoxins, whereas all *Vibrio* and *Aeromonas* species tested, including the marine *Vibrio* sp. 60 isolated by Oishi and

Studies on the heterologous expression and secretion of LT and LTB by gram-negative bacteria

Species	Plasmid vector	Toxin expressed	Toxin secretion to the medium[a]	Reference
Vibrio cholerae TRH7000	pWD600	LT	+	10
Vibrio cholerae TRH7000	pMMB68	LTB	+	22
Vibrio cholerae 569B	pCG86	LT	+	18
Vibrio alginolyticus	pMMB68	LTB	+	Hirst, unpublished
Vibrio cincinnatiensis	pMMB68	LTB	+	ibid
Vibrio fluvialis	pMMB68	LTB	+	ibid
Vibrio sp. 60 (Marine)	pMMB68	LTB	+	ibid
Aeromonas hydrophila	pMMB68	LTB	+	ibid
Aeromonas sobria	pMMB68	LTB	+	ibid
Aeromonas caviae	pMMB68	LTB	+	ibid
Aeromonas dourgesii	pMMB68	LTB	+	ibid
Escherichia coli CC118	pMMB68	LTB	–	22
Escherichia coli G6	pWD600	LT	–	10
Escherichia coli KL320	pCG86	LT	–	18
Proteus vulgaris	pMMB68	LTB	–	Hirst and Bagdasarian, unpublished
Pseudomonas aeruginosa	pMMB68	LTB	–	ibid
Shigella flexneri	pCG86	LT	–	18
Shigella sonnei	pCG86	LT	–	18
Citrobacter freundii	pCG86	LT	–	18
Enterobacter cloacae	pCG86	LT	–	18
Klebsiella pneumoniae	pCG86	LT	–	18
Salmonella typhimurium LT2	TP237Tc	LT	–	18

[a] The presence of LT or LTB antigen in the medium was determined by an immunoassay. Those species which did not secrete toxin or toxoid accumulated it within the bacterial cell.



Heterologous expression of *E. coli* heat-labile enterotoxin B subunit in wild-type marine *Vibrio* sp. 60 (left hand panel) and a mutant derivative (MVT1192) defective in exoprotein secretion (right hand panel). Bacteria harbouring plasmid pMMB68 (22) were cultured in Luria broth supplemented with 1% NaCl and 1 mM IPTG (○). At the times indicated the concentration of B subunit in the medium (□) and a fraction of sonicated cells (■) were determined using a GM1-enzyme-linked immunosorbant assay.

coworkers[19], did secrete the heterologous enterotoxoid into the medium. This poses the question of why vibrios and aeromonads maintain a capacity to secrete enterotoxins when the majority of species do not possess the genes for toxin production. One possible explanation is that the mechanism for toxin secretion is utilized for the secretion of other extracellular proteins. Indeed, vibrios and aeromonads secrete numerous extracellular proteins, including DNases, proteases, cytotoxins, hemagglutinins, neuraminidases and enterotoxins[1,7], and it may be that these or a sub-class of them are secreted via a pleiotropic secretory machinery.

Recently, several mutant derivatives of *Vibrio* sp. 60 were isolated that were pleiotropically defective in the secre-

tion of protease, amylase, DNase and haemagglutinin [16]. Expression of the B subunit of *E. coli* enterotoxin in *Vibrio* sp. 60 resulted in its secretion from the wild-type strain but its accumulation within the cells of the pleiotropic mutants (fig.; Leece and Hirst, unpublished observations). A similar observation was made when aerolysin from *A. hydrophila* was expressed in *Vibrio* sp. 60 and its mutant derivatives [27]. It is, therefore, apparent that either a major envelope defect has occurred in the mutants resulting in secondary inhibition of multiple or pleiotropic exoprotein secretory mechanisms, or that the mutations define a pleiotropic secretory apparatus utilized by both DNase, protease, aerolysin, and enterotoxins. The molecular basis for this phenomenon, as well as the reason why environmental and pathogenic vibrios and aeromonads possess an efficient toxin secretory apparatus, remain a challenging enigma for future investigation.

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III. Clinical studies

Bacteriological and clinical aspects of *Aeromonas*-associated diarrhea in The Netherlands

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Summary. Aeromonas species were isolated from 0.61% of 34,311 fecal samples during a 5-year period. Most strains belonged to DNA hybridization groups (HG) 4 (*A. caviae*), 8 (*A. sobria*), and 1 (*A. hydrophila*). Mannitol-negative *A. schubertii* (HG 12) and ornithine-positive *A. veronii* (HG 10) were not found. Multivariate analysis of the clinical data showed that *Aeromonas*-associated diarrhea cannot be attributed solely to cytotoxin production of the strains, but that it is also strongly associated with host factors.
Key words. Aeromonas-associated diarrhea; *Aeromonas* DNA hybridization group; cytotoxin-neutralizing antibodies.

In The Netherlands, interest in *Aeromonas*-associated infections was generated by the finding of *Aeromonas* in the drinking water system[5]. The drinking water is not chlorinated, and during the warmer months of the year the density of *Aeromonas* in the drinking water may increase to 1000 CFU/100 ml. Until now, outbreaks of *Aeromonas*-associated infections have not occurred. The clinical interest in *Aeromonas* was also stimulated by reports on the ability of this organism to cause diarrhea. In Tilburg, a 5-year prospective study was designed to assess the clinical and bacteriological significance of various *Aeromonas* species isolated from human fecal samples that were submitted to the Public Health Laboratory. Special attention was paid to a possible relationship between gastroenteritis and the occurrence of *Aeromonas* in surface water and drinking water.

Materials and methods

Isolation and identification of Aeromonas. Between June 1982 and May 1987 all fecal samples submitted to the Public Health Laboratory for isolation of enteropathogenic bacteria, were also investigated for the presence of *Aeromonas* by inoculation onto sheep blood agar plates with 10 mg/l of ampicillin. Oxidase- and catalase-positive gram-negative rods which reduced nitrates and did not grow in broth containing 6% NaCl were identified further using conventional biochemical methods. The strains were investigated for extracellular production of hemolysins and cytotoxins (for Vero cells) as described previously[3]. One hundred and forty-two strains were tested for DNA relatedness to the 11 DNA hybridization groups of the genus *Aeromonas* by the hydroxyapatite method[3].

Clinical and epidemiological data. For each *Aeromonas* isolate the physician was contacted and asked for clinical data and for serum of the patient from the acute and convalescent phases. With the consent of the physician, patients were asked to complete a questionnaire about the severity and duration of diarrhea, numbers of stools per day, consistency of the stools, presence of blood and mucus, occurrence of abdominal pain or cramps, and the presence of nausea, vomiting or fever. Epidemiological questions concerned exposure to surface water, recent travel and occurrence of diarrhea in the patient's immediate environment.

Cytotoxin-neutralizing assay. The cytotoxic titer of filtered supernatants of *Aeromonas* strains grown in brain heart infusion broth was defined as the highest dilution that gave distinct degeneration of 50% of the Vero cells. Toxin concentration four-fold higher than the cytotoxic titer were used in the neutralizing assay. Two-fold dilutions of sera in PBS were incubated with equal volumes of diluted cytotoxic supernatants for 2 h at 35 °C and then incubated at 35 °C on Vero cells overnight.

Results

Aeromonas species were isolated from 208 (0.61%) of 34,311 human fecal samples during the 5-year period. Aeromonas strains were isolated most frequently in summer and least frequently in winter. Other enteropathogenic bacteria isolated from fecal samples during this same 5-year period were *Campylobacter jejuni* (6.9%), *Salmonella* spp. (3.6%), *Shigella* spp. (0.33%), and *Yersinia enterocolitica* (0.67%). Of 169 *Aeromonas* strains, 32 (19%) were isolated concomitantly with 17 strains of *Salmonella*, 12 *C. jejuni*, 2 *C. difficile* and 1 enterovirus.

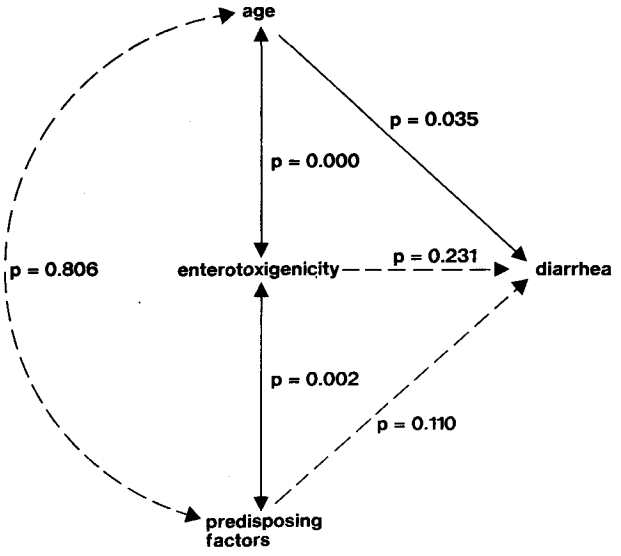
One hundred and forty-two *Aeromonas* isolates were biochemically and genetically characterized. The fecal strains were found only in DNA hybridization groups 1 to 4, 5A, and 8. Most strains were in groups 4 (57%), 8 (26%), and 1 (11%). Using the biochemical scheme of Popoff[6] for *A. hydrophila, A. sobria* and *A. caviae*, a good correlation was found between *A. caviae* and *A. sobria* with the DNA hybridization groups. However, of 26 strains biochemically identified as *A. hydrophila*, 8

(31%) were in hybridization group 8, which is *A. sobria*. Because of the poor correlation between phenotypic identification and genetic relatedness, we choose to refer to strains as 'cytotoxigenic' (cytotoxic for Vero cells; *A. hydrophila, A. sobria*, and *A. veronii*: hybridization groups 1, 2, 3, 8 and 10) and 'non-cytotoxigenic' (not cytotoxic for Vero cells; *A. caviae*: hybridization groups 4 and 5A). The distribution of *Aeromonas* by age showed a predominance of non-cytotoxigenic strains in children under the age of 5 years (46% of all strains), whereas cytotoxigenic strains were mainly cultured from patients aged 50 years or more (54% of the strains).

Of 169 *Aeromonas* strains, 19% were isolated from patients with a mixed infection, 15% from patients who used medication that could predispose the intestinal tract for colonization with *Aeromonas* (penicillin derivatives, antacids and immunotherapy), and 5% from patients with underlying diseases. The mixed infections were excluded from further evaluation. Forty-four (92%) of 48 cytotoxigenic strains were associated with gastroenteritis which showed an acute onset with watery diarrhea (57%) and persisted for more than 2 weeks in 48% of the patients. Of 10 patients (21%) who were hospitalized because of diarrhea (9 with hybridization group 8 and 1 with group 1), 7 had a severe underlying disease and 2 developed septicemia. Sixty-three (71%) of 89 non-cytotoxogenic strains were cultured from patients with mild diarrhea which was generally characterized as semisolid stools with a frequency of 1–5 stools per day. The diarrhea persisted for more than 2 weeks in 25% of the patients. Eight (8%) patients were hospitalized and none of them had an underlying disease. Seven of these patients were younger than 6 months. Ten (21%) of 48 patients with cytotoxigenic strains had been swimming, surfing, or fishing during the week before the onset of diarrhea, in contrast to 3% of patients with non-cytotoxigenic strains.

To study the relationship between diarrhea and cytotoxicity more carefully, multivariate analysis was performed with 4 variables: age (0, 1–49, and > 50 years), predisposing factors (underlying diseases, predisposing medication, travelling, contact with surface water), cytotoxicity (= enterotoxigenicity), and diarrhea. Multivariate analysis showed that the correlation between cytotoxicity and diarrhea was not significant any more, owing to two confounding factors: age and predisposing factors (fig.).

Eleven patients with acute gastroenteritis due to cytotoxigenic *Aeromonas* strains were examined for the presence of neutralizing antibodies. Five patients (46%) had developed cytotoxin-neutralizing antibodies at titers greater than 1:4. All five patients suffered from severe diarrhea and were older than 60 years. No toxin-neutralizing antibodies were found in sera of patients with chronic diarrhea (n = 5) or asymptomatic carriers (n = 1). A total of 3 (6%) of 50 healthy blood donors had cytotoxin-neutralizing serum antibodies at low titers of 1:4 or 1:8.



Multivariate analysis of clinical data from 137 patients with cytotoxigenic (enterotoxigenic) or non-cytotoxigenic *Aeromonas* strains isolated from feces.

Discussion

Aeromonas species were isolated from 0.61% of all fecal samples submitted to the Public Health Laboratory. This isolation rate would most likely increase if cultures for *Aeromonas* were only restricted to watery, bloody, or mucus-containing fecal samples. We may have missed ampicillin-sensitive *Aeromonas* strains which failed to grow on our semiselective medium.

Phenotypical identification using the scheme of Popoff was not sufficiently specific, although hybridization groups (HG) 4 and 8 could be recognized correctly [3]. Our ability to identify *A. hydrophila* (HG 1) was inadequate, since 31% belonged to another species. This incorrect identification was mainly caused by a positive reaction of esculin hydrolysis by *A. sobria* (HG 8). In our opinion, esculin hydrolysis should not be used as a major diagnostic key reaction, This is further supported by the finding that 2.5% of *A. caviae* strains hydrolyzed esculin only after 2 days and that discrepancies exist between commercial tablets and conventional methods [3]. Hydrolysis of arbutin, susceptibility to cephalotin, and fermentation of arabinose and salicin are more suitable to differentiate HG 1 from 8. We have not isolated mannitol-negative (*A. schubertii*) and ornithine-positive (*A. veronii*) strains.

Multivariate analysis of our clinical results showed that *Aeromonas*-associated diarrhea is strongly associated with host factors [2]. This is illustrated by the finding that 66 (39%) of 169 *Aeromonas* strains were from patients with mixed infections, underlying diseases, or patients who used medication that could predispose the intestinal tract to colonization with *Aeromonas*. It also indicates that non-cytotoxigenic strains (*A. caviae*) may be pathogenic for young children and individuals aged 50 years or more. In addition, it might explain why *Aeromonas* failed

to cause diarrhea after oral challenge studies of healthy marmosets and piglets[4].

Of three different serological assays (bacterial agglutination, cytotoxin neutralizing, and ELISA using cell envelopes) to measure serum antibody response against intestinal *Aeromonas* strains, only the toxin-neutralizing assay distinguished patients with acute severe diarrhea from patients with chronic diarrhea and asymptomatic carriers[1]. Cytotoxin-neutralizing antibodies were present in 46% of 11 patients who had acute severe diarrhea associated with *Aeromonas* strains predominantly from HG 8. The sensitivity of the assay may be further increased by using purified cytotoxins (or other toxins) in an ELISA.

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Aeromonas caviae: Ecologic adaptation in the intestinal tract of infants coupled to adherence and enterotoxin production as factors in enteropathogenicity

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Summary. Aeromonas caviae isolated from stools of diarrheic formula-fed infants and environmental sources produce acetic acid when grown in glucose broth, which is bactericidal (suicide phenomenon). *A. caviae* grows anaerobically in a minimal medium or under permissive conditions such as the intestinal tract of formula-fed infants. These isolates adhered to HEp-2 cells and produced a cytotoxic and a cytotonic enterotoxin which underscore their enteropathogenicity.

Key words. Adhesion; *Aeromonas caviae*; cytotoxin; ecology; enteropathogenicity; enterotoxin.

Mesophilic *Aeromonas* species are endogenous to fresh water environments and multiply therein under permissive temperature, pH and nutrient contents[17,19]. Unlike *A. hydrophila* and the majority of *A. sobria* isolates, *A. caviae* is killed by undissociated acetic acid (suicide phenomenon) produced by carbohydrate fermentation[13]. This fact accounts for its absence from acidic environments[13].

A. sobria and *A. hydrophila* are clearly recognized as gastrointestinal tract pathogens whereas *A. caviae* has been considered nonenteropathogenic[2,8,20]. Although reports by us and others have described the recovery of *A. caviae* from the stools of diarrheic patients, mostly children[1,7,11,18], its enteropathogenicity is still questioned largely because phenotypic markers and conditions for the expression of enterovirulence have been inconsistently demonstrated. Thus, while *A. hydrophila* and *A. sobria* produce an enterotoxin, cytotoxin and hemolysin to account for their enteropathogenicity[4,6,15,16,21], such parameters have not uniformly been detected in *A. caviae*[9]. In this report we further describe the requisite characteristics of *A. caviae* including unique ecologic adaptation requirements, adherence capability, and cytotoxin and enterotoxin production in support of its role as an enteropathogen.

Materials and methods

Twenty-five *Aeromonas caviae* strains were recovered from stools of symptomatic patients and four from fresh water and sewage. Stool specimens were processed for *Aeromonas* isolation by streaking 5% sheep blood agar (BBL Microbiology Systems, Cockeysville, MD) and enteric isolation media. After 24 h incubation at 35 °C, growth on blood agar was flooded with Kovacs oxidase reagent, and colonies showing a purple coloration were subcultured to blood agar and subjected to species identification[10]. Lake water and sewage specimens were pro-

cessed by membrane filtration for enumeration of *Aeromonas* (mA) method[17].

The suicidal tendency of *Aeromonas* isolates was assessed using glucose fermentation broth and the results were determined visually and by subculture[10].

To demonstrate *A. caviae* adherence to HEp-2 cells, semiconfluent HEp-2 cell monolayers grown in minimal essential medium containing 10% fetal calf serum were exposed to 2×10^6 CFU/ml *A. caviae* for 90 min. The degree of adherence was assessed by averaging the number of organisms remaining adherent subsequent to three consequent washes with phosphate buffer saline containing 0.5% minimal salt solution (PBSS)[11]. Cytotoxin and enterotoxin activity of *A. caviae* was demonstrated in the filtrates of 24 h glucose-free, double strength tryptic soy broth cultures[12]. Cytotoxicity was examined by exposing HEp-2, A549 and human fibroblast monolayers to filtrates for 5 h and was evidenced by monolayer detachment as well as rounding and loss of cell viability as assessed by trypan blue staining. Cytotonic enterotoxin production of *A. caviae* was demonstrated by feeding 0.1 ml of culture filtrates to suckling mice and was characterized by gastric distention and intestinal fluid accumulation[12].

Results and discussion

A. caviae and about 20% of *A. sobria* isolates, when grown in glucose fermentation broth, rapidly reduce the pH of the medium as a consequence of accumulating acetic acid. Growth is minimal as judged by the partial turbidity in the tubes[10,13]. *A. caviae* cells settle (autoagglutinate) to the bottom of the tube leaving the broth phase optically clear. Additionally, as viable cells cannot be recovered from these cultures, the phenomenon is termed 'suicide'[13]. The bactericidal effect of acetic acid can be negated by maintaining the pH of the culture(s) above 6.5. Also, phosphate (0.1 M) reverses the lethal effect of acetic acid[13,14].

Suicidal strains of *A. caviae* are capable of growing under anaerobic conditions in minimal medium containing 0.5% glucose[13]. In this setting glucose catabolite repression of the tricarboxylic acid cycle enzymes and stimulation of *A. caviae* pyruvate dehydrogenase result in the activation of an anaerobically energy-efficient (acetylphosphate) pathway with accumulation of acetic acid[14]. Because of the high buffering capacity and the presence of phosphate in the minimal medium, *A. caviae* survives acetic acid accumulation which, in an analogous fashion, accounts for growth of *A. caviae* in an alkaline environment but its absence in acidic waters[13].

Extrapolating these facts to the environment that may exist in the gastrointestinal tract of formula-fed infants, or those with altered gastrointestinal tract flora as a consequence of disease or antibiotic administration, allows for the construct of the events leading to the survival, colonization, and multiplication of *A. caviae*. Subsequent to acquisition by the oral route, the expression of *A. caviae* enterovirulence in the respective host, as indicated by a diarrheal illness, is within the province of the infecting *A. caviae* strain and its enteropathogenic/enteroinvasive potential or both.

In formula-fed infants, the microflora of the gastrointestinal tract consists mainly of Bacteroides species and Enterobacteriaceae which generate less acid end-products[4] as contrasted to acetic acid-producing *Bifidobacterium* in breast-fed babies[3]. In our studies, *A. caviae* was isolated from the stools of 14 symptomatic children; 10 less than 1 year of age presented with watery diarrhea (2–6 times daily) lasting from 3 to 21 days. All 10 infants were formula-fed and had a stool of > 7.5 which enhances the survival of *A. caviae*.

Once entering an environment conductive to survival, the infecting *A. caviae* strain must now colonize the gastrointestinal tract through an enteroadherent capability. We and others have shown that *A. caviae* isolates are adherent to HEp-2 cells[5,11], a feature which could also account for persistence in the permissive or modified intestinal tract. Therefore, the survival, adherence and multiplication of *A. caviae* in the gastrointestinal tract are necessary prerequisites for the initiation of gastrointestinal tract infection. The elaboration of an enterotoxic principle, or the demonstration of enteroinvasiveness, or both, would then provide prima facie evidence for enterovirulence.

In a preliminary study we reported the *A. caviae* from human and environmental sources produced a heat-stable cytotoxin active in HEp-2 cells (fig. 1), and a cytotonic enterotoxin active in the suckling mouse assay (fig. 2). The cytotoxin and enterotoxin were recoverable in filtrates of glucose-free, double strength tryptic soy broth (TSB-2x) medium[12]. Continuing with these experiments, we have further demonstrated that the cytotoxic component was also active against cultured A-549 and human fibroblast monolayers. *A. caviae* isolates regardless of source (clinical, environmental) are capable of enterotoxin production, an observation we have now extended to

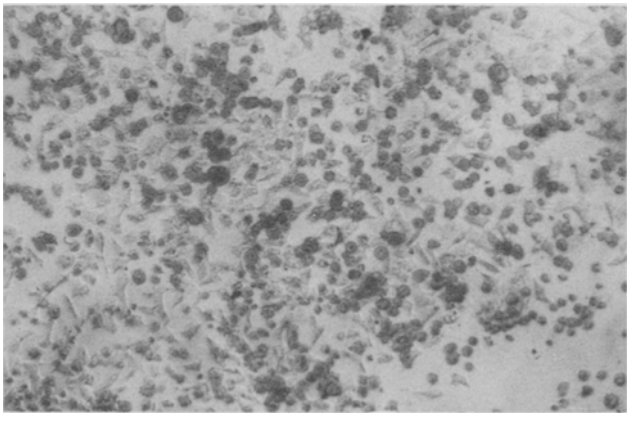

Figure 1. Cytotoxin activity of *A. caviae* in HEp-2 cells as demonstrated by monolayer detachment, cell rounding and cell death (trypan blue).

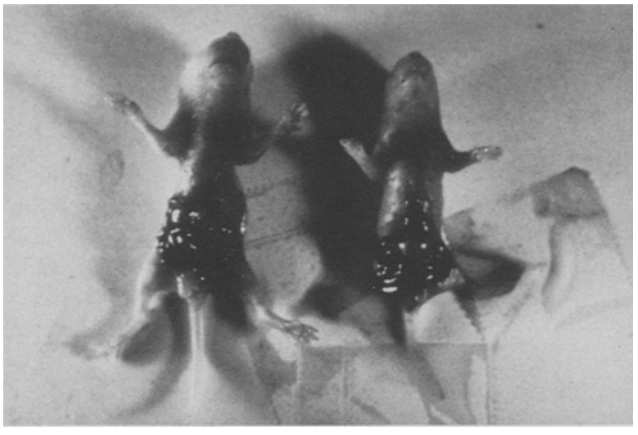

Figure 2. Enterotoxin activity of filtrate of *A. caviae* in suckling mouse assay as evidenced by gastric distention and intestinal fluid accumulation (right).

9 more *A. caviae* strains recovered from the stools of symptomatic children. These additional data further attest to its role as a bona fide enteric pathogen especially in the pediatric age group.

The provision by the host of the requisite ecologic niche for the survival and growth of *A. caviae*, in concert with its adherence capability, cytotoxin and cytotonic enterotoxin production all confer on *A. caviae* a diarrheagenic potential. The recovery, therefore, of this *Aeromonas* species from the stools of symptomatic individuals, especially infants, must be considered significant and certainly equivalent in etiology to that accorded the recovery of *A. hydrophila* and *A. sobria* under similar conditions.

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Spectrum of *Aeromonas* and *Plesiomonas* infections in patients with cancer and AIDS

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Summary. The spectrum of infection with *Aeromonas* and *Plesiomonas* included gastroenteritis, bacteremia, biliary tract infection, perirectal infection, and disseminated disease. Most patients (86%) with bacteremia were neutropenic (< 500 PMN/mm^3). Colonization of stools and sputum also occurred. Therapy with aminoglycosides, beta-lactams, trimethoprim/sulfamethoxazole, and the newer quinolones was effective in patients with AIDS and cancer.
Key words. Aeromonas; *Plesiomonas*; cancer; AIDS; neutropenia; bacteremia; gastroenteritis; quinolones.

Infections caused by *Aeromonas* species and *Plesiomonas shigelloides* are uncommon, but these organisms can cause serious, life-threatening infections particularly in patients with underlying malignancies or hepato-biliary disease[1-3,6,8]. Virtually any organ system can be involved, but the most common sites of infections are the gastrointestinal tract, the skin and soft tissues, and the bloodstream. Although there have been reports of *Aeromonas* bacteremia in cancer patients the entire spectrum of infection has not been described in detail[1,4,5,10]. Data regarding *Aeromonas* and *Plesiomonas* infections in patients with the acquired immunodeficiency syndrome are scarce[9]. In order to delineate the spectrum of infection, predisposing factors, clinical features, and therapeutic aspects of these infections, we reviewed the cases of all patients from whom these organisms were isolated and who had cancer or AIDS being treated at our institution. Our observations form the basis of this report.

Patients and methods

We reviewed the microbiological records of the University of Texas M.D. Anderson Cancer Center from July 1984 to July 1990 in order to identify patients from whom *Aeromonas* or *Plesiomonas* had been isolated from clinical specimens. By reviewing the medical records of these patients we assessed the predisposing factors leading to infection, the clinical features, and the patients' response to therapy. Infection was thought to be present if patients were febrile, had leucocytosis, or had other signs and symptoms of infection when the organisms were isolated. Colonization was thought to be present if patients were afebrile, had no other manifestations of infection, and generally stayed well without antimicrobial therapy. Antimicrobial susceptibility testing was done by the microbiology and infectious diseases laboratory of our institution using standard antibiotic impregnated discs, and standard micro-titer broth-dilution techniques[7].

Results

Twenty-eight patients with *Aeromonas* and 3 with *P. shigelloides* infection were identified. An additional 17 patients were colonized with *Aeromonas* (none with *Plesiomonas shigelloides*). Colonization of stools occurred in 14 patients and of the sputum, in 3. The underlying disorders of patients who were infected included acute leukemia (8 patients), AIDS (6 patients), lymphoma and pancreatic cancer (4 patients each), and various other solid tumors (9 patients). Most patients with lymphoma or other solid tumors had hepatic involvement. The ages ranged between 16 and 66 years, and there was a preponderance of males (24:7). Sixteen patients (52%) who developed infection were neutropenic. However, 12 of the 14 (82%) who developed *Aeromonas* bacteremia had < 500 polymorphonuclear blood cells per mm^3. Twenty patients (65%) were receiving anti-neoplastic chemotherapy when infection developed and 3 were undergoing radiation therapy (table 1).

Fourteen patients developed *Aeromonas* bacteremia. Of these 7 had acute leukemia and 12 (82%) were severely neutropenic. All were febrile to at least 101 °F (38.3 °C). Two patients developed a clinical syndrome consistent with septic shock. Three others were hypotensive but did not go on to develop shock. Only 1 patient had cutaneous lesions. These developed at the site of a crab bite which occurred prior to the development of neutropenia, and were consistent with ecthyma gangrenosum. *Aeromonas* was recovered from cutaneous lesions. Various antimicrobial agents (aminoglycosides, beta-lactams, quinolones) were used, generally in combination, for the therapy of these infections. Thirteen patients

Table 1. *Aeromonas* and *Plesiomonas* infection patient characteristics

Total number	48
Colonization	17
Infection	31
Age range	16–66 years
Sex distribution	24 male, 7 female
Acute leukemia	8 (26%)
AIDS	6
Lymphoma*	4
Pancreatic carcinoma	4
Hepatoma	3
Other solid tumors	6
Neutropenia	16 (52%)
Chemotherapy	20 (65%)
Radiation therapy	3

*Most of these patients had hepatic involvement.

(93%) responded to therapy. Eleven of the 12 neutropenic patients had neutrophil recovery. The patient who failed to respond to therapy had persistent neutropenia.

Twelve patients developed gastroenteritis with recovery of *Aeromonas* from stool specimens. Only 3 (25%) were neutropenic, and only 6 (50%) were febrile. All had > 4 bowel movements daily, and most had 7–8 loose watery stools daily. Only two patients had bloody diarrhea. Diarrhea was accompanied by abdominal pain, cramping, or tenesmus in 10 (83%) of patients. Three patients had mild to moderate dehydration. All patients had recovery of *Aeromonas* organisms in more than one stool culture. Fecal leucocytes were present in stools of all except two patients, both of whom were neutropenic. Therapy consisted of the administration of either trimethoprim/sulfamethoxazole (TMP/SMX) (7 patients) or ciprofloxacin (5 patients). Ten patients were treated with oral antibiotics whereas two were given parenteral therapy initially and changed subsequently to oral therapy. All responded to therapy with cessation of diarrhea and negative stool cultures. The average duration of therapy for *Aeromonas* bacteremia was 10 days, and for gastroenteritis, 6 days.

Two other patients with *Aeromonas* infection included one with pancreatic carcinoma and biliary obstruction who developed a biliary tract infection and another who had disseminated infection with pneumonia, bacteremia, and urinary tract infection, and a clinical picture consistent with meningitis – but normal cerebrospinal fluid values. Both responded to antibiotic therapy.

Two patients, both with AIDS, developed gastroenteritis due to *Plesiomonas shigelloides*. This illness was indistinguishable from that caused by *Aeromonas* spp. Both patients responded to therapy with oral ciprofloxacin. No relapses occurred and no maintenance therapy was given.

The third patient had acute leukemia, severe neutropenia, and developed a perirectal infection and *P. shigelloides* bacteremia. He remained neutropenic and died as a result of his infection despite appropriate antibiotic therapy.

In vitro susceptibility testing revealed that the 4-quinolones, the extended spectrum cephalosporins (cefoperazone, ceftazidime), the monobactam aztreonam, imipenem, and TMP/SMX were consistently active against these organisms (details not shown). The aminoglycosides had moderate activity, whereas the carboxy- and ureidopenicillins were not very active.

A seasonal variation in the occurrence of *Aeromonas* and *Plesiomonas* infections was noticed. Most infections (20 or 65%) occurred in the warm summer months of May through September. Also, there appeared to be an increase in the number of cases in the second half study period with 7 infections between 1984 – 1986, and 24 infections between 1987 – 1990.

Table 2. Sites of infection with *Aeromonas* spp. and *Plesiomonas shigelloides*

Site of infection	No.
Aeromonas	
Bacteremia	14
Gastroenteritis	12
Biliary tract	1
Disseminated	1
Plesiomonas	
Gastroenteritis	2
Perirectal/bacteremia	1

Comments

Our experience indicates that *Aeromonas* spp. and *P. shigelloides* can cause a variety of infections including bacteremia, gastroenteritis, and deepseated infections such as biliary tract and perirectal infections (table 2). Patients with underlying malignancies and those with acute leukemia or primary or secondary hepatic involvement appear predisposed toward these infections. Neutropenia predisposes to the development of bacteremia and other serious infections, and persistence of neutropenia is associated with an unfavorable outcome. Colonization of the gastrointestinal tract and sputum also occurs and may serve as a potential source of infection when neutropenia develops. These infections appear to be more common in the hot summer months when outdoor, water-related activities occur frequently [11]. Although we were able to obtain a history of such activity in only one patient, it might be prudent to instruct immunosuppressed patients to refrain from such activities, at least during periods of high risk such as chemotherapy-induced neutropenia.

Antimicrobial therapy is generally successful, particularly if the neutrophil count recovers. Newer antimicrobial agents, especially the 4-quinolones, appear to be potent agents, and our limited clinical experience with ciprofloxacin has been encouraging. Patients with AIDS who develop *Aeromonas* or *Plesiomonas* infections appear to respond well to therapy. In our limited experience no relapses occurred (unlike *Salmonella* infections in these patients) and it was, therefore, not necessary to use maintenance therapy once the acute infection had been treated.

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Biochemical characteristics and plasmids of clinical and environmental *Plesiomonas shigelloides*

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Summary. The biochemical characteristics of 68 clinical and 5 environmental *Plesiomonas shigelloides* isolates were determined. The reactions for ONPG, phenylalanine deaminase, motility, lactose fermentation and salicin fermentation were different from previously published findings. The reactions of the clinical and environmental isolates were very similar. The isolates were also tested for the presence of plasmids, and 40% of the strains were found to harbor between one and seven plasmids. The findings of this study will be useful for the identification and characterization of this recently recognized enteric pathogen.
Key words. Plesiomonas shigelloides; bacterial identification; bacterial plasmids; environmental pathogens.

Plesiomonas shigelloides is an oxidase-positive, fermentative, gram-negative rod currently classified as a member of the family Vibrionaceae. Several reports have implicated the organism as a cause of sporadic and epidemic diarrheal disease [1, 2, 7], and a recent study from our laboratory indicated that *P. shigelloides* is a significant cause of both locally acquired and traveller's diarrhea [3]. This study also indicated that antimicrobial therapy may be beneficial in the treatment of *P. shigelloides* diarrhea, and a subsequent study indicated that isolates of the organism are often resistant to antibiotics [4]. The purpose of the present investigation was to determine the biochemical characteristics of the *P. shigelloides* isolates from our previous studies and to determine if plasmids are present in the strains that might be associated with antibiotic resistance.

Materials and methods

Bacteria. Clinical isolates were obtained from stool cultures as previously described [3], and stored frozen in glycerol-DMSO at −80 °C until they were retrieved for biochemical testing and plasmid analysis. Environmental isolates were cultured from water by using a membrane filtration technique as previously described [6], or from oysters by using a previously described method [6].

Biochemical testing. Each isolate to be tested was subcultured on 5% sheep blood agar after removal from the freezer. Growth on blood agar plates was used to inoculate a set of 45 biochemical tests according to standard reference methods [5]. The reactions were read after 24, 48 and 72 h. Reactions noted at 24 or 48 h were considered positive. Reactions positive after 72 h of incubation were considered delayed positive. Tests not positive within 72 h were considered negative.

Plasmid analysis. Each strain was grown overnight in 10 ml brain heart infusion broth on a water bath shaker at 32 °C. Plasmids were prepared as previously described [6]. The bacteria were harvested by centrifugation, washed in 0.85% saline and lysed in 10% sodium dodecylsulfate. Chromosomal DNA was precipitated in 5 M sodium acetate. The resulting supernatants were treated with RNase and then extracted with chloroformisoamyl alcohol. Plasmid DNA was precipitated in ethanol and resuspended in distilled water. Electrophoresis was carried out in 20 cm, 0.7% agarose gels for 18 h at 20 V. Plasmid bands were visualized by staining with ethidium bromide.

Results

Water samples from three freshwater and six estuarine sites in British Columbia were cultured monthly for 18 months. Four sites in Hawaii were cultured once. Oysters from British Columbia coastal waters were also cultured monthly. Four of 69 water samples and one of 140 oyster samples yielded *P. shigelloides* (table 1).

Table 1. Environmental isolates of *P. shigelloides*

Month	Site	Water temperature (°C)	No. *P. shigelloides* per No. of sites tested	
			Water	Oysters
August	BC[a]	20	1/14	0/40
September	BC	19	1/9	0/20
October	BC	14	0/9	-
November	BC	9	1/12	1/40
February	BC	6	0/9	0/10
March	BC	8	0/12	0/30
March	Hawaii	24	1/4	-
Total	-	-	4/69	1/140

[a] British Columbia

Table 2. Biochemical characteristics of 68 clinical and 5 environmental *P. shigelloides* isolates

Test	Predicted	Percent clinical (n = 68)	Positive environmental (n = 5)
β-hemolysis	na	0	0
Oxidase	+	100	100
Nitrate	+	100	100
DNase	−	100	100
Gelatinase	−	0	0
Urease	−	0	0
Indole	+	100	100
ONPG	d	98	100
Methyl red	+	100	100
V-P	−	0	0
Citrate	−	0	0
NaCl 0%	na	100	100
3%	na	100	100
6%	−	0	0
Phenylalanine	d(w)	0	0
Lysine	+	100	100
Arginine	+	100	100
Ornithine	+	100	100
Motility	d	91	100
Glucose, gas	−	0	0
Lactose	+	81	60
Sucrose	−	6	0
Mannitol	−	0	0
Dulcitol	−	0	0
Salicin	d	3	0
Adonitol	−	0	0
Inositol	+	100	100
Sorbitol	−	0	0
Arabinose	−	0	0
Raffinose	−	0	0
Rhamnose	−	0	0
Maltose	+	100	100
Xylose	−	0	0
Esculin	−	0	0
Melibiose	d	93	80
Acetate	na	63	80
Trehalose	+	94	100
Cellobiose	−	0	0
Glycerol	d	62	80
Mucate	−	0	0

d, different reactions; w, weak reactions; na, not applicable

Biochemical characteristics. Sixty-eight clinical and five environmental *P. shigelloides* isolates were tested. The results were compared to the characterization of *P. shigelloides* published previously[8] (table 2). Characteristics of the clinical and environmental isolates were as predicted except for DNase, ONPG, phenylalanine deaminase, motility and fermentation of lactose and salicin. More than 99% of our strains were DNase and ONPG positive, motile and salicin negative but only 80% were positive for lactose fermentation. Our clinical isolates were more often positive for lactose and melibiose fermentation, but environmental isolates were more often positive for acetate and glycerol utilization. The clinical and environmental isolates were otherwise nearly identical.

Plasmid analysis. Of the 73 *P. shigelloides* strains tested, 29 had plasmids. Fifteen distinct plasmid bands were identified among the 29 strains, and 1–7 of the 15 plasmid bands were present in each strain. Although the same individual plasmid bands were found in several strains, all 29 strains had distinct plasmid banding profiles. Half of the antibiotic-resistant isolates has plasmids. However, there were no specific plasmids that were associated with resistance to individual antibiotics or groups of antibiotics. There was also no correlation of the presence of plasmids to any biochemical characteristics.

Discussion

Our recent studies and the work of others suggest that *P. shigelloides* is a recently recognized but important cause of gastroenteritis. Few studies on the biochemical characteristics of the organism have been published, and the characteristics of the organism, summarized by von Graevenitz[8], are based in some cases on only a few strains tested. Therefore, the present studies will add substantially to the body of knowledge on the identifying characteristics of *P. shigelloides*. Our results largely confirm the findings summarized by von Graevenitz[8], but they indicate that the reactions for DNase, ONPG, phenylalanine deaminase, and fermentation of lactose and salicin may be different from those previously reported. The most striking difference was for the DNase reaction. We found all of our *P. shigelloides* isolates to be DNase positive by using the toluidine blue method. This information will be helpful in the identification of *P. shigelloides* in the clinical microbiology laboratory.

The origin of *P. shigelloides* infections has not been clearly established, but previous studies have suggested a possible environmental origin[1,2,7]. Our previous studies indicated that 71% of *P. shigelloides* infections were acquired in tropical areas, but 29% were locally acquired[3]. The present findings indicate that *P. shigelloides* can be recovered both from tropical and local environments. In addition, they indicate that clinical and environmental isolates of the organism have very similar biochemical characteristics. These findings are consistent with a possible environmental origin for *P. shigelloides* infections.

Our previous studies indicate that patients with *P. shigelloides* infections might benefit from antimicrobial therapy, but most strains are resistant to ampicillin and other

penicillins, and many are resistant to tetracycline, erythromycin, and aminoglycosides other than netilmicin[3,4]. Such resistance to antimicrobial agents could be plasmid-mediated. The present studies indicate that some *P. shigelloides* isolates have plasmids, but there is no correlation between the presence of specific plasmids and resistance to antimicrobial agents. The mechanisms of antimicrobial resistance and the optimal therapy for *P. shigelloides* infections await further study.

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IV. Environmental studies

The role of motile aeromonads in the fish disease, Ulcerative Disease Syndrome (UDS)

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Summary. Ulcerative Disease Syndrome (UDS) is an epizootic fish disease characterized by the presence of severe, open dermal ulcers on the head, midbody, and dorsal regions of the fish. *Aeromonas hydrophila* and *A. sobria* were recovered more often from UDS fish than other bacteria from the genera *Vibrio, Alteromonas* and *Plesiomonas*. Representative isolates of *A. hydrophila, A. sobria, V. anguillarum, V. vulnificus, Alteromonas putrefaciens*, and *P. shigelloides* taken from UDS and healthy fish were assayed for virulence-associated factors. The aeromonads produced a wide variety of hydrolytic enzymes and expressed cell surface characteristics linked to virulence whereas the other bacterial species rarely produced the same enzymes or cell surface characteristics. The role of aeromonads in UDS is believed to be opportunistic or secondary and these bacteria are thought to play an important role in this degenerative disease.
Key words. Aeromonas; Ulcerative Disease Syndrome (UDS); virulence factors; enzymes.

Ulcerative Disease Syndrome (UDS) has been characterized as an epizootic disease of freshwater and marine fish in the lower St. Johns River system, Florida[11]. The etiological agent(s) of UDS is unknown, but it appears that motile, mesophilic aeromonads may play a role as opportunistic pathogens in UDS. The disease is characterized by severe, open dermal ulcers often extending into the surrounding musculature and located primarily on the head, dorsal, and/or tail region(s). Commercial fishermen in the lower St. Johns River system, Florida, reported that as many as 50–80% of a daily catch exhibited UDS lesions.

Several documented fish pathogens of the genera *Aeromonas, Vibrio, Alteromonas* and *Plesiomonas* were isolated from lesion tissue and blood samples. *Aeromonas hydrophila* and *Aeromonas sobria* were recovered most frequently, followed by *Alteromonas putrefaciens, Vibrio* spp., and *Plesiomonas shigelloides*[11]. Although coliform counts were usually high (350–5000 CFU/100 ml) in water samples taken from the river, coliforms were rarely recovered from lesioned or apparently healthy fish.

During UDS outbreaks, water temperature and salinity ranged from 12 to 20 °C and from 0 to 22 parts per thousand (ppt), respectively. In vitro studies on temperature and salinity growth ranges using *A. hydrophila, A. sobria, V. anguillarum, V. ordalii, V. vulnificus, P. shigelloides*, and *A. putrefaciens* recovered from lesioned fish

indicated that only the aeromonads could survive at the river's temperature and salinity extremes without loss of numbers or viability (data not shown). The vibrios all required a minimum of 10 ppt saline for growth and *P. shigelloides, V. vulnificus* and *A. putrefaciens* did not remain viable below 22 °C.

Several investigators have attempted to standardize criteria for virulence of *A. hydrophila* and *A. sobria* in both human and animal systems. Among these criteria are cell surface characteristics and agglutination reactions[6, 9, 10, 12, 14, 15, 17], production of specific enzymes[2, 5, 14, 17], binding of dyes[16, 21] and the possibility of virulence-associated plasmids[3, 8, 15]. Representative strains of *A. hydrophila* and *A. sobria* isolated from ulcerative and apparently healthy fish were evaluated in this study using several of the above criteria and additional enzyme assays, in an attempt to define the pathogenicity of these aeromonads and other UDS-associated bacteria.

Cell surface

Recently, there has been significant interest in relating virulent *Aeromonas* strains to their surface characteristics[6, 9, 10, 12, 14, 15, 17]. Highly virulent *Aeromonas* strains for mice[6, 17], humans[6, 9, 15], and fish[10, 12] have been reported to have such characteristics as autoagglutination (AA) in Beef Heart Infusion Broth (BHIB), presence of lipopolysaccharide serogroup O:11[5, 6, 9, 10, 12], presence of a surface-layer protein (S-layer)[6, 9, 10, 12, 15], and resistance to bacteriophage Aeh 1[3, 15]. In addition to autoagglutination in BHIB, the absence of agglutination in 0.2% acriflavine has also been reported to be indicative of high virulence of *A. hydrophila* in rainbow trout[10, 12]; however, other investigators have found this characteristic not to be useful for the screening of virulent strains[14, 15, 17]. It appears that the most virulent group of *A. hydrophila* and *A. sobria* has the autoagglutination characteristic of precipitation after boiling in BHIB (spontaneous precipitation negative, precipitation after boiling positive, SP−/PAB +) and shares a common O-antigen (Sakazaki O serogroup 11, O:11 +), two traits which correlate closely with the presence of the S-layer[9, 15]. The S-layer of *A. hydrophila* may enhance the bacterium's survival by increasing resistance to the bactericidal action of serum and by increasing invasiveness of the bacterium for the host.

Autoagglutination in BHIB and serogrouping assays using *A. hydrophila* and *A. sobria* isolates recovered from UDS and non-UDS fish (table 1) showed that 25% of the *A. hydrophila* and 14% of the *A. sobria* isolates from the UDS fish were characterized as SP−/PAB + and were O:11 + phenotypes, when present together, have been linked to highly virulent *A. hydrophila* and *A. sobria* strains[9, 10]. An additional 38 % of the *A. hydrophila* and 23 % of the *A. sobria* isolates exhibited either autoagglutination in BHIB or were of the O:11 serogroup. The remaining bacteria showed no autoagglutination and were O:11 negative.

Table 1. Autoagglutination pattern in Beef Heart Infusion Broth (BHIB) and Sakazaki serogroup O:11 affiliation of *Aeromonas hydrophila* and *Aeromonas sobria* isolates recovered from UDS and apparently healthy fish

Agglutination pattern[a]	*Aeromonas hydrophila* UDS	*Aeromonas hydrophila* Healthy	*Aeromonas sobria* UDS	*Aeromonas sobria* Healthy
$SP^+PAB^+/O:11^+$	3/24[b]	1/5	7/35	0/10
$SP^+PAB^+/O:11^+$	4/24	0/5	0/35	2/10
$SPPAB^+/O:11^+$	6/24	0/5	5/35	2/10
$SPPAB^+/O:11$	1/24	1/5	0/35	0/10
$SPPAB/O:11^+$	1/24	0/5	1/35	0/10
$SPPAB^-/O:11^-$	9/24	3/5	22/35	6/10

[a] SP = spontaneous precipitation in BHIB, PAB = precipitation after boiling for 1 h in BHIB, O:11 = Sakazaki somatic O-antigen group 11; + = positive affiliation, − = negative affiliation. [b] Number of strains affiliated with the agglutination pattern/number of strains tested.

Hydrolytic enzymes

The degree of pathogenicity of a bacterium is often dependent on the production of tissue damaging hydrolytic enzymes. The bacterial isolates were assayed for the production of the following enzymes by previously described techniques: hemolysin, *Staphylococcus aureus* cell lysin (staphylolysin), elastase, mucinase, fibrinolysin, hyaluronidase, casein protease, chondroitin sulfatase, DNase, and pyrazinamidase[2, 4, 5, 8, 13, 18, 20] (table 2).

Positive relationships between virulence for fish, biotyping of *Aeromonas* isolates, and the ability of *A. hydrophila* to produce elastase and staphylolysin enzymes have been demonstrated[5, 7, 17]. 77% and 73% *A. hydrophila* UDS isolates were positive for staphylolysin activity and elastase, respectively. 14% of *A. sobria* UDS isolates were positive for staphylolysin in addition to 11% that produced elastase. 60% of the *V. anguillarum* tested were positive with respect to elastase; however, none were positive for staphylolysin. Isolates of the remaining bacterial species did not produce either elastase or staphylolysin.

The majority of aeromonads tested exhibited β-hemolysis (86%) on 5% sheep blood agar, casein hydrolysis (89%), DNase production (100%) and fibrinolysin (87%) activity, whereas few produced chondroitin sulfatase (32%) or other hemolytic patterns (14%). Mucinase and hyaluronidase were produced by 37% and 39%, respectively, of the *A. sobria* isolates tested. None of the *A. hydrophila* isolates were positive for mucinase activity and only 18 % were positive for hyaluronan hydrolysis. Pyranzinamidase activity did not correlate with the presence or absence of plasmids[1, 8] for either *A. hydrophila* or *A. sobria*; it also did not seem to be a reliable phenotypic marker for *A. sobria* as previously reported[2]. Both autoagglutinating and non-autoagglutinating aeromonads were seen to exhibit similar patterns of enzyme activities. A comparison of the enzymatic activities of the aeromonads with those of other bacteria recovered from

Table 2. Detection of specific enzyme activity in bacteria isolated from UDS and healthy fish.

Bacterial isolates		Hemolysis			Specific enzymes[a]								
		β	α	γ	S	E	F	M	H	C	D	Ch	P
Aeromonas hydrophila	UDS	17/24[b]	7/24	0/24	17/22	16/22	15/22	0/22	4/22	17/22	22/22	4/22	12/22
	Healthy	4/5	0/5	1/5	2/5	1/5	4/5	0/5	1/5	4/5	5/5	3/5	1/5
Aeromonas sobria	UDS	34/36	1/36	1/36	5/36	4/36	22/28	8/28	11/28	34/36	36/36	8/28	16/28
	Healthy	8/10	2/10	0/10	1/10	0/10	7/10	4/10	4/10	10/10	10/10	4/10	6/10
Alteromonas putrefaciens[c]		4/6	2/6	0/6	0/6	0/6	0/6	6/6	0/6	0/6	4/6	0/6	0/6
Plesiomonas shigelloides[c]		0/3	3/3	0/3	0/3	0/3	nd[d]	0/3	2/3	0/3	0/3	0/3	0/3
Vibrio anguillarum[c]		0/5	0/5	5/5	1/5	3/5	1/5	0/5	0/5	1/5	0/5	0/5	0/5
Vibrio vulnificus[c]		1/3	0/3	2/3	0/3	0/3	1/3	0/3	2/3	0/3	0/3	0/3	0/3

[a] S = *Staphylococcus aureus* cell lysin; E = elastase; F = fibrinolysin; H = hyaluronidase; C = casein protease; D = DNase; Ch = Chondroitin sulfatase; P = Pyrazinamidase. [b] Number of strains positive/number of strains tested. [c] Isolated from UDS fish. [d] nd = not determined.

lesioned fish suggested that the aeromonads exhibit a greater variety of enzymatic activities than bacteria of the other species examined in this study. Bacteria that produce many of the enzymes assayed in this study have been shown previously to cause tissue damage and promote infectious diseases in humans and animals[4, 5, 13, 14, 17–20]. It is highly possible that the aeromonads participate in the development of the dermal ulcerations characteristic of UDS through the hydrolytic action of mucopolysaccharides and proteins on the dermal surface and within the dermis and surrounding tissue of affected fish.

Other assays

The bacteria were also assayed for binding of Congo Red[16] and the failure to agglutinate in 0.2% acriflavine[6, 10, 12]. The results indicated that 100% of the *Alteromonas* and *Plesiomonas* isolates tested were able to bind Congo Red, whereas only 29% of the *A. hydrophila* and 44% of the *A. sobria* were able to bind the dye (data not shown). A correlation between Congo Red binding and the presence of a 40–50 megadalton virulence plasmid in *Yersinia enterocolitica* has been reported[16]; however, a correlation between Congo Red binding and the presence of a plasmid in the bacteria used in this study was not observed. 17% of the *A. hydrophila* and 44% of the *A. sobria* isolates recovered from UDS fish were non-agglutinable in 0.2% acriflavine. Current reports question the validity of these assays to screen highly virulent *Aeromonas* species[14, 15, 17].

The lower St. Johns River system is a complex estuarine system that is subject to the activities and influences of agriculture and run-off, a large urban community and development, a United States Naval Base, heavy commercial and sport fishing, industry, as well as naturally occurring changes. It is difficult to define a single cause of UDS for such a dynamic river system. It appears that fish of all types present in the river during the months of November to March are subjected to a compromising environment. The fish then become host to opportunistic bacteria and other microorganisms. A comparison of the higher recovery of *A. hydrophila* and *A. sobria* from the blood and tissue of lesioned fish to other potential bacterial or fungal pathogens[11], as well as surface characteristics and the enzymatic activities of bacteria isolated from UDS fish, suggests that the aeromonads may play an important role in the development of UDS.

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Nutritional requirements of aeromonads and their multiplication in drinking water

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Summary. Aeromonads can utilize a wide range of low molecular-weight compounds, including amino acids, carbohydrates and long-chain fatty acids at a concentration of a few µg per liter. Utilization of biopolymers such as gelatin, casein and amylose is slow at this concentration level. The concentration of substrates available for an *A. hydrophila* strain in drinking water was usually below 10 µg of C/l. The autochthonous bacteria utilized these substrates more rapidly than the aeromonads. The multiplication of aeromonads in drinking water during distribution is therefore explained by their growth on biomass components in the biofilm and in sediments in the pipes.
Key words. Aeromonads; drinking water; growth kinetics; available substrate.

Aeromonads have been isolated from the feces of persons with diarrhea, and particularly children[1, 2, 4]. Furthermore, certain *Aeromonas* species possess enterotoxigenic properties[6, 7]. For these reasons, so-called indicative maximum values for *Aeromonas* densities have been defined by the government in The Netherlands following an incident in which a sudden and unexplained increase of the numbers of these bacteria was observed in drinking water[9]. The defined values are: 20 CFU/100 ml in drinking water leaving the production plant, and 200 CFU/100 ml in drinking water in the distribution system. Aeromonads are enumerated with the use of Ampicillin-Dextrin Agar (ADA) medium, incubated at 30 °C[5].

Aeromonas species are ubiquitous in surface water and have also frequently been observed in drinking water. Under certain conditions, the number of aeromonads in drinking water increases during distribution. Defining measures to limit the growth of aeromonads in drinking water requires information about the conditions favoring multiplication. Therefore, growth measurements with pure cultures of *A. hydrophila* have been conducted to determine (a) the nutritional versatility of aeromonads at very low substrate concentrations; (b) the growth kinetics and growth yields of *A. hydrophila* for a number of selected substrates and (c) the growth potential of drinking water for aeromonads.

The nutritional versatility of an *A. hydrophila* strain (M800) isolated from drinking water has been determined in growth measurements in drinking water (slow sand filtrate) supplemented with mixtures of organic compounds or with individual compounds, at concentrations of a few µg per liter. These experiments revealed that a wide variety of low molecular-weight compounds including amino acids, carbohydrates, and a number of long-chain fatty acids, are favorite growth substrates at this concentration level (table 1). The growth kinetics and growth constants of strain M800 were determined for a few substrates to enable calculations of substrate concentrations from growth measurements with this organism in drinking water. Yield values for acetate and

Table 1. Nutritional versatility of *A. hydrophila* strain M800 for low molecular-weight compounds at a concentration of 10 µg of C/l (adapted from van der Kooij and Hijnen[8])

Compound group	Number tested	Number with growth rate $> 0.05\ h^{-1}$
Amino acids	22	5 (23%)
Carbohydrates	15	10 (67%)
Carboxylic acids	10	1 (10%)
Long chain fatty acids	12	6 (50%)
All compounds	59	22 (37%)

glucose were 6.8×10^6 CFU/µg C and 8.0×10^6 CFU/µg C, respectively[8].

This paper reports observations on the multiplication of aeromonads on natural polymers, and the growth of the organism in drinking water.

Materials and methods

Bacterial cultures. A. hydrophila strain M800, isolated from drinking water prepared from anaerobic ground water, was used in the growth measurements. This organism is described in detail in a previous publication[8].

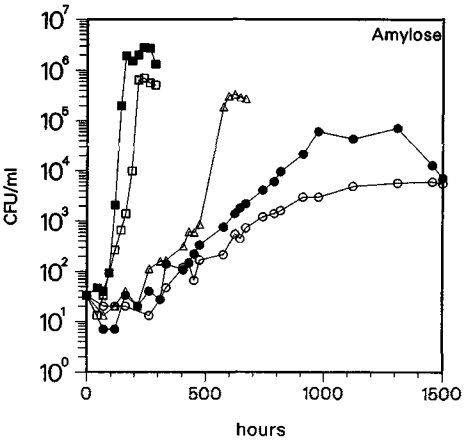
Growth measurements. Growth measurements with bacteria in drinking water were conducted in 600-ml water samples contained in thoroughly cleaned 1-l Erlenmeyer flasks. These water samples were heated at 60 °C for 30 min to inactivate the indigenous bacterial population and were subsequently inoculated with the selected organism, precultured in drinking water with a low substrate concentration. Thereafter, the flasks were incubated at 15 °C in the dark, and growth of the organism was measured by periodic colony counts. The procedures for cleaning the flasks, sample treatment, precultivation of the bacteria and determination of the colony counts have been described in detail in previous communications[8, 13].

Results and discussion

Utilization of biopolymers. A. hydrophila strain M800, like other aeromonads, can utilize a wide range of biopolymers including proteins, starch, chitin, and fats[8]. When added to drinking water at a concentration of 1 mg/l, a number of polymers promoted growth of strain M800, suggesting that hydrolysis and subsequent utilization of large molecules also occurs at relatively low concentrations (table 2). However, gelatin did not promote the growth of the organism at the concentration tested. To elucidate the effect of the concentration of the polymer, amylose was tested at a range of concentrations. A clear effect of the concentration on the growth rate and the maximum colony count was observed (fig.). From the data obtained, the substrate saturation constant (K_s), the maximum growth rate (V_{max}) and the yield factor for growth on amylose were calculated. In table 3 the results are presented in combination with values for these parameters observed in *Flavobacterium* sp. strain S12[11].

Table 2. Utilization of biopolymers by *A. hydrophila* strain M800 added to drinking water at a concentration of 1 mg/l

Compound	Maximum colony count (CFU/ml)
Blank	$< 10^4$
Gelatin	1.7×10^4
Casein	2.2×10^6
Amylose	2.3×10^6
Tween-80	5.2×10^5
DNA	9.6×10^5



Growth curves of *A. hydrophila* strain M800 at various concentrations of amylose added to drinking water. Symbols: ●, 10; △, 50; □, 100; and ■, 500 µg of amylose-C per liter; ○, blank.

The data presented in table 3 reveal that strain S12 will out-compete strain M800 at high and at low amylose concentrations. At a concentration of 10 µg of amylose-C per liter, strain S12 can multiply approximately 6 times faster than strain M800. The more favorable growth kinetics of strain S12 may be related to the smaller size of the cells of this organism, compared to the cells of strain M800. Observations on growth kinetics of another *A. hydrophila* strain and another *Flavobacterium* strain for starch[12, 13] further demonstrate that aeromonads cannot compete for these compounds with certain flavobacteria. At a concentration of 10 µg per liter, casein did not enhance the growth of strain M800. These findings reveal that at low concentrations *A. hydrophila* utilizes proteins and carbohydrates far less efficiently than low molecular-weight monomers of these compounds.

Multiplication in drinking water. Maximum colony counts of strain M800, attained in growth measurements in drinking water, were usually below 5×10^4 CFU/ml, indicating that the concentration of potentially available substrates was less than 10 µg of C per liter of water. In river water, a higher concentration was found, but here also this concentration was only a small fraction of the concentration of dissolved organic carbon (table 4). The significance of bacterial competition for available substrates in drinking water is easily demonstrated in growth measurements with strain M800 as a pure culture and in the presence of the indigenous bacterial population, respectively. In the absence of indigenous bacteria, strain M800 reached a maximum colony count (N_{max}) of 4.3×10^4 CFU/ml in drinking water prepared from ground water. No multiplication of aeromonads was observed when indigenous bacteria were present. The number of indigenous bacteria increased to a level of 1.6×10^5 CFU/ml. Obviously, substrates for aeromonads are present but under the test conditions, other aquatic bacteria can utilize these compounds much faster than the aeromonads do.

Table 3. Growth parameters for *A. hydrophila* strain M800 and *Flavobacterium* species strain S12 on amylose[a]

Organism	K_s (µg C/l)	V_{max} (doublings/h)	Yield (CFU/µg C)
A. hydrophila str. M800	93	0.26	6.4×10^6
Flavobacterium sp. str. S12	26	0.50	2.1×10^7

[a] Data for *Flavobacterium* strain S12 from Van der Kooij and Hijnen [10].

Table 4. Maximum colony counts of *A. hydrophila* strain M800 in river water and in a few types of drinking water

Water type	DOC[a] (mg/l)	N_{max} (CFU/ml)	Substrate conc. (µg ac-C eq/l)[b]
River water	3.2	9.9×10^5	145
Drinking water	1.8	1.7×10^4	2.5
Drinking water	4.2	5.4×10^4	7.9
Drinking water	8.6	2.1×10^4	3.1

[a] dissolved organic carbon; [b] $Y_{acetate} = 6.8 \times 10^6$ CFU/µg C.

Table 5. The effect of *Spirillum* sp. strain NOX, specialized in the utilization of carboxylic acids, on the growth of *A. hydrophila* strain M800 in water before and after ozonation

Organism	Maximum colony count (CFU/ml) Water before ozonation	Water after ozonation
Strain NOX	1.1×10^5	1.9×10^6
Strain M800	1.0×10^4	9.1×10^4
Strain M800 after strain NOX	1.2×10^3	6.8×10^3

An indication of the nature of compounds available for *A. hydrophila* in drinking water was obtained from growth measurements using a pure bacterial culture, *Spirillum* sp. strain NOX, an organism specialized in the utilization of carboxylic acids [11]. Table 5 shows that in ozonated water, the N_{max} value of strain M800 was clearly higher than in water prior to ozonation. This suggests that carboxylic acids, as produced by ozonation were, at least in part, utilized by strain M800. The N_{max} value of strain M800 in water in which strain NOX had reached its N_{max} value was approximately 10% of the N_{max} value attained without strain NOX. Based on the properties of strain NOX, these observations suggest that in water before and after ozonation, carboxylic acids are the major fraction (90%) of the compounds available for strain M800. Hence, the concentration of amino acids and other favorite growth substrates in drinking water is very low (< 1 µg C/l). Therefore, it is assumed that the increase of *Aeromonas* densities as observed in water supplies is especially due to the multiplication of these bacteria in biofilms and in sediments in the pipes.

Conclusion. Drinking water contains low concentrations (< 10 µg of C/l) of substrates available for aeromonads. However, autochthonous bacteria are well adapted to growth on the majority of these compounds, and consequently will outnumber the aeromonads in drinking water. In drinking water distribution systems, aeromonads probably grow on biomass components in the biofilm and in sediments.

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