mined antigenic and biological similarities between *P. shigelloides* cholera toxin-like protein and *Vibrio cholerae* enterotoxin and report these results here.

5S rRNA analysis. Results of 5S rRNA sequence analysis showed that *P. shigelloides* was much more closely related to the Enterobacteriaceae. The sequence for *P. shigelloides* is given in the figure.

Identification and conventional characterization tests. Results for most of the identification tests used in this study confirm that the strains were *P. shigelloides* and correlated well with data in the literature[5,7]. Interestingly, the majority of strains examined gave only a weak methyl red reaction, even after incubation for 7 days at 25 °C and 37 °C. Also, the *P. shigelloides* strains showed a good degree of uniformity in their reactivities. Furthermore, the results for aesculinase, amylase, gelatinase, lipase (Tween 80) and urease agreed well with the results obtained for these enzymes in other studies[5,7].

Decarboxylase activity was detected for L-arginine, L-lysine, L-ornithine, L-histidine, four of the 15 amino acid substrates examined. L-arginine, L-lysine, and L-orithine decarboxylase have been used previously in the presumptive diagnosis of *P. shigelloides*. L-histidine decarboxylase may also be useful in the diagnosis of this species. Preliminary results showed that selected strains of *Aeromonas hydrophila, A. caviae* and *A. sobria* and some *Vibrio* spp. were negative for this enzyme.

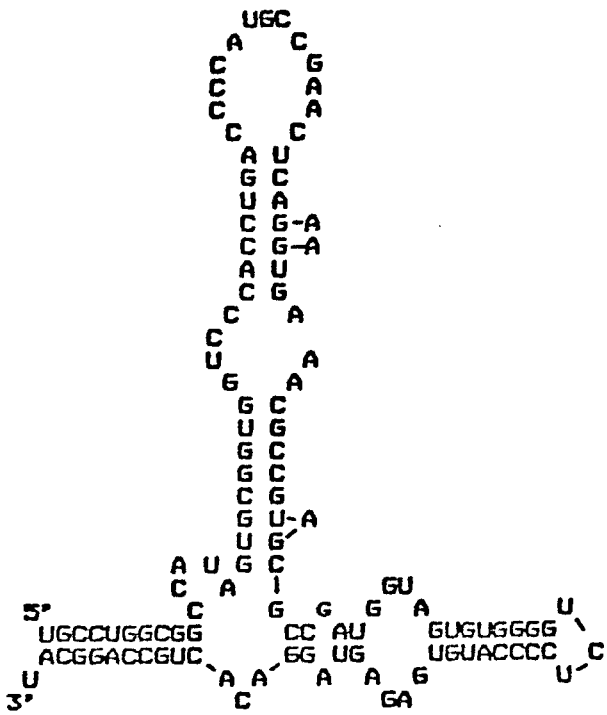
All 22 strains examined were positive for deoxyribonuclease (DNase) activity by the HCl method and most (91%) were positive using the toluidine blue 0 method described by Waller et al.[8]. Prolonged incubation (> 3 days) was usually necessary to obtain a positive reaction by either method.

Rapid characterization tests. A good correlation was observed for those enzymes tested using both 4-MUCS and API enzyme test kits. All 22 strains tested gave very strong N-acetyl-β-D-glucosaminidase activity. Similar results were obtained for this enzyme when tested using the equivalent 4-MUCS substrate and in API oxidase. This enzyme may be a potentially useful diagnostic feature for *P. shigelloides*. Most strains also gave strongly positive reactions for acid phosphatase and positive-to-strongly-positive reactions for alkaline phosphatase with API ZYM.

The overall conclusion from the results is that *P. shigelloides* is much more active enzymatically than has been reported[6,7].

CT-like and cytotoxic activity of P. shigelloides culture supernatants. The Y-1 mouse adrenal cell assay was used to measure CT-like and cytotoxic activity of culture supernatants. CT-like activity was detected in tryptic soy-inositol culture supernatants of all strains tested, with titers ranging from 4 to 16. Only ATCC 14029 and 11A produced detectable levels of CT in heart infusion broth. No activity was detected in syncase, CAYEG, or heart infusion-inositol media.

In conclusion, the data suggest a position intermediate between the Vibrionaceae and the Enterobacteriaceae for *P. shigelloides*. Furthermore, the unexpected finding of a CT-like factor produced by *P. shigelloides* suggests some interesting molecular genetic relationships of *P. shigelloides* yet to be explored.



Sequence of 5S rRNA of *P. shigelloides* ATCC 14029^T.

1 Ljungh, A., and Wadstrom, T., Infection *13* (1985) 169.
2 MacDonell, M.T., and Colwell, R.R., Syst. appl. Microbiol. *6* (1985) 171.
3 MacDonell, M.T., Swartz, D.G., Ortiz-Conde, B.A., Last, G.A., and Colwell, R.R., Microbiol. Sci. *3* (1986) 172.
4 Manorama, T.V., Taneja, V., Agarwal, R.K., and Sanyal, S.C., Toxicon Suppl. *3* (1983) 269.
5 Sakazaki, R., and Balows, A., in: The Prokaryotes: A Handbook on Habitats, Isolation and Identification of Bacteria, vol. 1, pp. 1272–1301. Eds M.P. Starr, H. Stolz, H.G. Trüper, A. Balows and H.G. Schlegel. Springer Verlag, Berlin 1981.
6 Shimada, T., and Sakazaki, R., Jap. J. med. Sci. Biol. *31* (1978) 135.
7 von Graevenitz, A., in: Manual of Clinical Microbiology, 4th edn, p. 278. Eds E.H. Lennette, A. Balows, W.J. Hausler and J.P. Truant. American Society for Microbiology, Washington, D.C. 1984.
8 Waller, J.B., Hodel, S.L., and Nuti, R.N., J. clin. Microbiol. *21* (1985) 195.

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Ecology of aeromonads and isolation from environmental samples

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Key words. Aeromonas; environmental samples.

Our knowledge of the ecology of the aeromonads has made little progress over the past ten years because systematics and procedures of isolation of these bacteria are still unsatisfactory.

General problems

The first problem is that aeromonads have until now been identified on a routine basis by phenotyping only. Ecological results,

therefore, have to be preliminary as far as the genetic level is concerned.

The present paper compromises on the problem of nomenclature insofar as the description of the aerogenic *A. hydrophila* and *A. punctata* follows Bergey's Manual 8th edition, while the description of the aerogenic *A. sobria* and of *A. caviae* follows the 9th edition. As a result of more recent findings, *A. caviae* comprises all anaerogenic aeromonads. These four are grouped together as 'psychrotrophic aeromonads' on account of their growth at 5 °C and 35 °C. The psychrophilic *A. salmonicida* is not treated in this paper.

The second problem is that of providing isolation procedures which meet the requirements of an ecological study. Of the many procedures described previously[1,3-7,9,13,15,17] only a few can be considered suitable for the isolation of aeromonads from environmental samples. Because of the close interrelationship of the different psychrotrophic *Aeromonas* species (reflected in their common sources of occurrence) culture media must also be suitable for a quantitative assessment of all species.

In order to assess such media, we plated pure cultures of three *Aeromonas* species on three media based on different selection principles: a) Dextrin-fuchsin sulfite (DFS) agar[10], b) Starch-desoxycholate (SD) agar[3] and c) Rimler-Shotts (RS) agar[15]. DFS agar uses fuchsin sulfite, the other two agars desoxycholate as selective principles. SD agar is nutritionally rich and RS agar nutritionally poor. Table 1 shows that desoxycholate reduced the numbers of colonies of 3 *Aeromonas* species significantly compared to nutrient (N) agar while DFS agar counts resembled those on N agar. In addition, desoxycholate skewed the ratios of aerogenic to anaerogenic isolates insofar as higher contents increased the percentages of colonies of anaerogenic species. Table 2 shows this for river water with a relatively high sewage content and for an oligosaprobic stream. Wahlig has indicated that *A. sobria* is even more susceptible to desoxycholate and other bile salts than is *A. hydrophila*[18]. The same studies have confirmed our opinion that DFS agar (incubated at 30 °C) is suitable for ecological studies since the psychrotrophic aeromonads grow in direct proportion to the number of each species present[18].

The use of DFS agar for the isolation of aeromonads form environmental samples

This method for the isolation of psychrotrophic aeromonads is based on papers describing various applications related to water of different qualities[10-12]. DFS agar is prepared as follows: Peptone from casein 10.0 g, Lab-Lemco powder 3.0 g, NaCl 5.0 g, Na_2HPO_4 7.5 g, dextrin 15.0 g, fuchsin 0.25 g, sodium sulfite 1.25 g, agar 20.0 g. These ingredients are dissolved in 1000 ml of distilled water (final pH circa 7.5) and heated to sterilize. After cooling to 50 °C the medium is dispensed into Petri dishes. It must be dried thoroughly before use.

Besides the advantage of not being toxic to aeromonads, DFS agar does not present the problems of false-negative oxidase reactions that may result from the direct use of oxidase reagent on colonies which ferment carbohydrates. Fuchsin sulfite indicates the formation of aldehydes and, due to the buffer capacity of Na_2HPO_4, remains unaffected by the presence of acid during fermentation.

Suitably diluted environmental samples are plated on DFS agar (0.1 ml per plate) and incubated for 24 h at 30 °C. To make a preliminary qualitative and a general quantitative assessment of bacterial growth, the plate is flooded with oxidase (NADI) reagent. The reaction is read after 1–3 min. Since this reagent is bactericidal further studies must be carried out on colonies from an untreated plate with the same dilution. Aeromonads form prominent red-colored colonies on DFS agar which, when stained with the oxidase reagent, progressively turn violet-blue, commencing mainly from the edges. Dextrin-fermenting enterobacteria (e.g., *Enterobacter, Klebsiella*) also form red colonies but these will not stain with the oxidase reagent. Pseudomonads do not form acid from dextrin and form colorless translucent colonies which stain quickly and uniformly deep blue with the oxidase reagent. Non-dextrin fermenting enterobacteria (e.g., *E. coli*) also form colorless translucent colonies which, however, are oxidase negative.

Method for the examination of contaminated waters (sewage and river water)

As a rule, surface water samples contain so many acromonads that it is feasible to plate them directly on DFS agar. The number of *Aeromonas* colonies is determined by flooding the plates after 24 h incubation at 30 °C with the oxidase reagent and counting the characteristic colonies. For species studies, 150 suspicious colonies are subcultured and identified biochemically. The first 100 of them taken at random are used for the determination of the ratio of aerogenic to anaerogenic aeromonads.

Method for the examination of uncontaminated waters (groundwater, drinking water, oligosaprobic water)

Membrane filtration. For the isolation of single colonies and subsequent quantitative assessment, a membrane filter of 0.45 µ pore size is used through which 10–100 ml of the sample are filtered[11]. The membrane is placed upside down on a DFS agar plate which is then overlayed with liquid DFS agar cooled to 42–45 °C and incubated at 30 °C for 24 h. This procedure prevents growth of pseudomonads, which are often present in such samples. When colonies suspected to be *Aeromonas* are visible the filter with the agar is cut out and placed in a sterile Petri dish. Single colonies are subcultured and identified biochemically.

Liquid enrichment. This technique is not suitable for quantitative ecological work since normally only one or two strains succeed in getting established. Analysis of populations in which the species interrelationship is being investigated thus becomes impossible. It should only be used for presence-absence tests. The primary culture is carried out in starch-bromothymol broth[10] at 10–15 °C because at higher temperatures, aeromonads will be outgrown by coliforms[11]. Plating on DFS agar follows.

The ecology of aeromonads

Psychrotrophic aeromonads originate from aquatic biotopes[7,8,10] and are present in virtually all surface waters. Their

Table 1. Comparative colony counts of *Aeromonas* strains: Nutrient agar versus selective media (30 °C). (Colony counts on nutrient agar = 100%)

Species	Strain number	DFS Agar	RS Agar	SD Agar
A. hydrophila	I/13	102.68[a]	64.37[b]	23.89[b]
A. hydrophila	917/18	104.13[a]	84.34[b]	46.11[b]
A. hydrophila	956/1	101.86[a]	34.92[b]	16.46[b]
A. punctata	III/58	106.15[a]	42.00[b]	14.50[b]
A. punctata	III/66	104.73[a]	70.64[b]	17.05[b]
A. caviae	940/29	105.16[a]	83.30[b]	37.42[b]
A. caviae	940/29	104.09[a]	75.07[b]	27.24[b]
A. caviae	III/5	97.08[a]	68.24[b]	16.08[b]

DFS, Dextrin-fuchsin-sulfite; RS, Rimler-Shotts (1% desoxycholate); SD, Starch-desoxycholate (3% desoxycholate); [a] no significant difference ($p > 0.05$); [b] significant difference ($p < 0.001$).

Table 2. Influence of the composition of selective media on the ratio of aerogenic to anaerogenic aeromonads

Sample	Medium	Aerogenic aeromonads (%)	Anaerogenic aeromonads (%)
River water M[a]	DFS	18	82
0.1 ml 10^{-2}	RS	12.5	87.5
	SD	9	91
River water E[b]	DFS	99	1
0.3 ml 10^{0}	RS	97	3
	SD	94	6

[a] High sewage content; [b] oligosaprobic stream.

Table 3. Relation of *A. hydrophila, A. punctata, A. sobria* and *A. caviae* in river water of different saprobicity (DFS agar)

River	Characteristic	*A. hydrophila*	*A. punctata*	*A. sobria*	total	%Aerogenic *Aeromonas* spp.
S.B.	Oligosaprobic	19	11	70	100	0
Sch.B.	Oligosaprobic	64	32	3	99	1
E.B.	Oligosaprobic	60	14	13	87	13
Main	Beta-mesosaprobic	39	0	30	69	31
Rhine	Beta-mesosaprobic	26	1	5	32	68

mass production occurs in waste water and in similar biotopes where intense degradation of certain high molecular weight compounds, e.g. proteins, fats, starch, etc., takes place. The ability of aeromonads to degrade simple compounds and to use them as carbon sources, however, is rather limited compared to that of other aquatic organisms like pseudomonads. Recent work has shown that the utilization of high molecular weight substances like starch by aeromonads in a poor medium, e.g., tap water, is possible only when degradable low molecular weight substances such as glucose are present[16]. The mass production of aeromonads in wastewater or in other environments where they are provided with degradable high and low molecular weight substances is thus explained. Growth competition studies have shown that temperature also plays a decisive role in the colonization of wastewater by aeromonds: at 15 °C, they were at least ten times as numerous as coliforms[12]. In contrast to *E. coli,* however, aeromonads are only very rarely present in human feces.

Aeromonads readily multiply in domestic wastewater plumbing, often reaching densities of up to 10^6–10^7/ml[13]. In sewage sludge, their concentration may be as high as 10^8/g[13]. Mass development attains a peak before reaching the sewage plant and is subsequently reduced by biological degradation. The degree of this reduction is a good measure of purification by the treatment plant. As treated waste water still contains 10^3–10^5 aeromonads/ml and many rivers and lakes still receive untreated wastewater, it must be assumed that most aeromonads found in surface waters originate from domestic and other wastewaters.

In domestic waste water, roughly 25% of the aeromonads are aerogenic ones (*A. hydrophila* and *A. sobria; A. punctata* being a rare exception) while the bulk belongs to the anaerogenic *A. caviae*[14]. In activated sludge and in percolating filters, this advantage of *A. caviae* remains unaltered. In river water, however, shifts of the ratio are observed depending on saprobicity (table 3). Polysaprobic streams show the same ratios as domestic wastewater[14] while oligosaprobic ones yield up to 100% aerogenic aeromonads. Impressive variations of these ratios between different sampling places in alpha and beta mesosaprobic rivers[14] – as compared to oligosaprobic streams – invite the interpretation that they are not due to local colonization from wastewater discharges but are rather caused by the mixing of aeromonads from surface water and from wastewater. This interpretation becomes plausible if one groups the anaerogenic *(A. caviae)* aeromonads together as 'wastewater aeromonads' and contrasts them to the aerogenic *(A. hydrophila, A. punctata, A. sobria)* species which reflect surface water ecology. The relatively small differences between the mean ratios in alpha and beta mesosaprobic waters[14] could be explained this way as well. Differences in the ratios of aerogenic to anaerogenic aeromonads are even more pronounced in groundwater. No aeromonads were found in 1000-ml samples of groundwater from undisturbed deep wells[11]. Aeromonads were, however, isolated from two shallow wells situated in a forest with only a thin layer of sand[11]; infiltration from rivers could be excluded. 50-ml samples from the wells revealed 30–40 *A. hydrophila* and no *A. caviae* colonies.

For the study of *Aeromonas* populations and ratios of a river bank infiltration zone, we chose an easily observable section of such a zone in a water production system on the lower Rhine[11]. Similar aeromonad ratios were found in river water and in water from the two wells closest to the river. Water from the well farthest from the river, however, contained only aerogenic aeromonads.

The public health importance of aeromonads in drinking water is not sufficiently well understood at present. Some guidelines regarding their occurrence may be put forward. In deep wells that are unaffected by surface conditions psychrotrophic aeromonads will not be found. In monitoring tests of drinking water, aeromonads are generally found more often than coliforms. Their evaluation must take into account local conditions which may differ even within Europe because of different standards and rules of well construction and groundwater protection.

Table 4. Maximal growth temperature of 100 *Aeromonas* isolates from water of an oligosaprobic river

	Percent	% of strains showing no growth at waterbath temp. of 37 °C	38 °C	39 °C	41 °C
A. hydrophila	61	35	12	13	1
A. punctata	14	8	4	2	0
A. sobria	15	14	1	0	0
A. caviae	10	9	1	0	0
All species	100	66	18	15	1

When surface water is used for the production of drinking water, the probability of finding aeromonads will be greater than when groundwater is used. Proper treatment and disinfection should adequately deal with these organisms.

From the ecological viewpoint, the assessment of aeromonads in water is not a closed issue. In a further study of the growth-limiting temperature in an oligosaprobic river we found densities of 250 aeromonads/ml. A considerable part of the freshly isolated *A. hydrophila* strains did not grow in a 37 °C water bath and only one strain grew at 41 °C (table 4). *A. sobria*, *A. punctata* and *A. caviae* were even more sensitive to incubation temperatures above 37 °C. On the other hand, 40 strains of *A. hydrophila* from cases of diarrhea (positive ileal loop tests) which were received by courtesy of Dr S. Sanyal from Varanasi, India, grew without exception at 41 °C, and so did two strains of *A. sobria* from clinical specimens received by courtesy of Dr E. Delbeke from the Languedoc region, France[2]. The ability of aeromonads to grow at these temperatures has not yet received proper attention in pathogenicity studies.

1 Burke, V., Robinson, J., Gracey, M., Peterson, D., and Partridge, K., Appl. envir. Microbiol. *48* (1984) 361.
2 Delbeke, E., Demarcq, M.J., Roubin, C., and Baeux, B., Presse méd. *14* (1985) 1292.
3 Hansen, J.C., and Bonde, G.J., Nord. VetMed. *25* (1973) 121.
4 Kaper, J., Seidler, R.J., Lockman, H., and Colwell, R.R., Appl. envir. Microbiol. *38* (1979) 1023.
5 McCoy, R.H., and Pilcher, K.S., J. Fish. Res. Bd Can. *31* (1974) 1553.
6 Millership, S.E., and Chattopadhyay, B., J. Hyg., Camb. *92* (1984) 145.
7 Popp, W., Münch. Beitr. Abwasserbiol. *29* (1978) 475.
8 Popp, W., Münch. Beitr. Abwasserbiol. *39* (1985) 183.
9 Rippey, S.R., and Cabelli, V.J., Appl. envir. Microbiol. *38* (1979) 108.
10 Schubert, R., Arch. Hyg. *150* (1967) 688.
11 Schubert, R., Zentbl. Bakt. Hyg., I. Abt. Orig. B *161* (1976) 482.
12 Schubert, R., Arch. Hyg. *151* (1967) 409.
13 Schubert, R., Arch. Hyg. *151* (1967) 243.
14 Schubert, R., Zentrbl. Bakt. Hyg., I. Abt. Orig. B *160* (1975) 237.
15 Shotts, E.B., and Rimler, R., Appl. envir. Microbiol. *26* (1973) 550.
16 van der Kooij, D., Visser, A., and Hijnen, W.A.M., Appl. envir. Microbiol. *39* (1980) 1198.
17 von Graevenitz, A., and Bucher, C., J. clin. Microbiol. *17* (1983) 16.
18 Wahlig, M., unpublished data (1986).

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Aeromonas and *Plesiomonas*: Isolation procedures for pathological specimens

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Key words. Aeromonas; Plesiomonas; isolation; CIN agar; *Yersinia enterocolitica.*

Aeromonas as well as *Plesiomonas* have been implicated in a wide variety of infectious diseases[6]. Isolation of these organisms from clinical specimens and from environmental and food samples which may be considered to be sources of human and animal infections is usually performed without difficulty, since both genera can easily be grown on most routinely-used common media, such as blood or MacConkey agar.

Problems may arise when quantitative recovery is required or in cases where large numbers of contaminating microflora are present. Depending on the purpose for which the culture is required, one or several of the following factors will influence the choice of medium: 1) selectivity, i.e. the ability to suppress background flora, 2) ability to differentiate between contaminating flora and the organism looked for on the basis of colonial morphology, and 3) quantitative recovery of the desired organisms.

Aeromonas hydrophila group

A variety of different media have been proposed for special applications (for a summary see von Graevenitz and Bucher[3]). Differential agents used include starch or different sugars (mannitol, trehalose, xylose). If a sugar contained in an agar is fermented, it must be realized that direct oxidase testing cannot be performed on the colonies, since oxidase reactions may become false negative at a pH ≤ 5.1[4]. To increase selectivity the use of ampicillin, novobiocin, sodium deoxycholate, sodium lauryl sulfate, Pril (a quaternary ammonium detergent) and other agents has been described. No single medium has received general acceptance. Very recently a starch-ampicillin agar has been proposed which allows quantitative recovery and ready differentiation from background flora of *Aeromonas* spp. from foods[10].

For stool specimens two systems are widely used. Blood agar containing ampicillin[7] allows direct oxidase testing but exhibits only a low selectivity. In addition, ampicillin-sensitive *Aeromonas* strains have been described[11]. Sensitivity to ampicillin seems to be especially abundant in the Philippines, where in the Kirby-Bauer-test only 57% of isolates were classified as 'resistant' (M. Kilpatrick, personal communication). A more cost-effective alternative is Cefsulodin-Irgasan-Novobiocin (CIN) agar, a medium which allows simultaneous screening for *Aeromonas* spp. as well as *Yersinia* spp.[1]. Two important points have to be considered using CIN agar[1]: 1) the medium should contain only 4 mg/l cefsulodin (e.g. Difco) instead of 15 mg/l (e.g. Oxoid), and 2) incubation should be at 25 °C, since high concentrations of cefsulodin (fig. 1) and high incubation temperatures may inhibit growth of certain *Aeromonas* strains, mainly *A. sobria* (M. Altwegg, unpublished). This medium is very selective (table), with only about 50 % of primary plates showing growth, and less then half of these with suspicious colonies, but it does not allow direct oxidase testing owing to the mannitol that is fermented. A very easy screening procedure including two MIO (motility-indole-ornithine medium, Difco Laboratories,

Selectivity of CIN agar at different Cefsulodin concentrations (% of routine stool specimens)

	Direct inoculation		APW enrichment	
	15 mg/l	4 mg/l	15 mg/l	4 mg/l
No growth	38%	31%	42%	31%
Only coliforms	40%	42%	33%	42%
Suspicious colonies	22%	27%	25%	27%