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Diversity, Persistence, and Virulence of *Aeromonas* Strains Isolated from Drinking Water Distribution Systems in Sweden

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The *Aeromonas* populations in 13 Swedish drinking water distribution systems, representing different treatments, were investigated. From each system, water samples were collected four times during the period from May to September 1994 from raw water and water after treatment and at two to five sites within the distribution system. In total, 220 water samples were collected. From samples containing presumptive *Aeromonas*, up to 32 colonies were analyzed by the PhenePlate *Aeromonas* (PhP-AE) system, which is a highly discriminating biochemical fingerprinting method. Selected isolates from different phenotypes (PhP types) were further identified by the API 20 NE system and by gas-liquid chromatography analysis of fatty acid methyl esters (FAMEs). Selected isolates were also assayed for their potential to produce hemolysin and cytotoxin and for their ability to adhere to human intestinal cells. In total, 117 water samples (53%) contained presumptive *Aeromonas* which numbered up to 10^6 CFU/100 ml in raw water and up to 750 CFU/100 ml in tap water. Among the 2,117 isolates that were subjected to typing by the PhP-AE system, more than 300 distinct PhP types were found, of which the majority occurred only sporadically. Raw (surface) water samples usually contained many different PhP types, showing high diversity indices (Di) (median Di = 0.95). The *Aeromonas* populations in samples collected from within the distribution systems were less diverse (median Di = 0.58) and were often dominated by one major PhP type that was found on several sampling occasions. Seventeen such major PhP types could be found and were represented in 1,037 isolates (49%). Identification by API 20 NE and FAME analysis revealed that most of the major PhP types were *Aeromonas hydrophila* or belonged to unidentified *Aeromonas* species. Hemolysin and cytotoxin production was observed in most major PhP types (representing 87 and 54% of the assayed isolates, respectively), and adherence was found in 89% of the isolates that produced cytotoxin. Thus, the data presented here show that although raw water may contain very diverse *Aeromonas* populations, the populations seemed to be remarkably stable within the studied water distribution systems, and that some potentially pathogenic *Aeromonas* strains could persist for several months in drinking water.

The genus *Aeromonas* comprises a group of bacteria that is widespread in natural habitats such as soil, fresh and brackish water, sewage, and wastewater (1). Over the years, the taxonomy of aeromonads has become more complicated, with the description of many new species. Currently, two different classification schemes are used for the genus *Aeromonas*: the original classification comprising the phenospecies *Aeromonas hydrophila*, *A. sobria*, *A. caviae*, and *A. salmonicida* (39), and a classification scheme of *Aeromonas* into 14 different DNA hybridization groups (HGs) or genomospecies (15). Some strains of *A. hydrophila* are capable of causing septicemia in fish and amphibians as well as extraintestinal and wound infections in humans (27), whereas *A. hydrophila*, *A. caviae* and *A. sobria* may cause enteritis in humans (9). Other species such as *A. schubertii* and *A. jandaei* are also potentially pathogenic species for humans (7, 13, 20) and may occur in water, while *A. salmonicida* is a well-known fish pathogen that is of no significance to human health (3).

The growth potential of *A. hydrophila* in drinking water, also with low concentrations of organic compounds, is well established (45). Several investigations have shown a high incidence of *Aeromonas* spp. in municipal drinking water supplies (6, 8, 24, 26). Although no verified waterborne outbreaks due to aeromonads have been reported, the possibility of waterborne *Aeromonas* infections in humans has been discussed (12, 18, 21). Aeromonads are known to produce many different putative virulence factors such as extracellular toxins, e.g., cytotoxins and enterotoxins (14). Furthermore, some strains of *Aeromonas* are able to adhere to and invade epithelial cells (22, 29). However, these properties may be found among clinical isolates as well as among isolates from other sources, including healthy humans, and are thus not clearly correlated to pathogenicity to humans (28, 38). So far, no true virulence factors have been identified among *Aeromonas* spp. that can be used to define pathogenicity in this genus. On the other hand, the pathogenicity of aeromonads is associated with certain HGs (17, 35, 41), although strain-to-strain differences may occur within the same hybridization group (23). This could mean that only a limited number of pathogenic strains exist in the genus *Aeromonas* and that expression of true virulence factors may be largely a clonal property within this genus. Hence, due to differences in the composition of *Aeromonas* populations and because most strains of a natural *Aeromonas* population are likely to be avirulent, it may prove difficult to correlate the presence of aeromonads in drinking water with the development of disease in humans. Unfortunately, studies of the pathogenicity of *Aeromonas* strains isolated from water and

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TABLE 1. Description of raw water sources and treatment processes in the 13 water distribution systems studied

Site	Raw water source	Process	Disinfection
D1	Groundwater	Rapid sand filtration, aeration, Fe/Mn filter	None
D2	Groundwater	Rapid sand filtration, aeration, Fe filter	Chlorine
D3	Groundwater	Fe/Mn filter	Chlorine
D4	Artificial groundwater[a]	Aeration	None
D5	Artificial groundwater[a]	None	None
D6	Artificial groundwater[a]	None	Sodium hypochlorite
D7	Artificial groundwater[a]	Rapid sand filtration, pH adjustment	None
D8	Surface water mixed with groundwater	Chemical flocculation, rapid and slow sand filtration	UV
D9	Surface water	Chemical flocculation, carbon filtration, pH adjustment	Sodium hypochlorite
D10	Surface water	Chemical flocculation, rapid sand filtration, pH adjustment	Sodium hypochlorite
D11	Surface water	Aeration, rapid sand filtration, carbon filtration, pH adjustment	Chlorine
D12	Surface water	Rapid sand filtration, slow sand filtration	Chlordioxide
D13	Surface water	Chemical flocculation, rapid and slow sand filtration	Chlordioxide

[a] Surface water subject to artificial infiltration.

their possible relation to isolates found in human disease have usually dealt with the characterization of only a limited number of randomly selected isolates per sample. Since we know very little about how many different *Aeromonas* strains may be present in a water sample and for how long a certain strain may persist, it is possible that potential pathogenic strains remain undetected in such studies.

To be able to obtain information on the diversity and stability of the *Aeromonas* populations in surface water and in water distribution systems, we have investigated 13 different Swedish drinking water distribution systems by sampling the raw water and at different sites in the systems on four different occasions. A large number of isolates from each sample have been subjected to typing by biochemical fingerprinting. The aims of the study have been to investigate the spread and survival potential for different *Aeromonas* strains in the water distribution systems and to investigate whether putative virulence determinants could be associated with strains that were able to persist and multiply within the systems.

MATERIALS AND METHODS

Sample collection and isolation and enumeration of strains. The *Aeromonas* populations in 13 Swedish water distribution systems (D1 to D13), with either groundwater or surface water as the raw water source (Table 1), were investigated. A total of 220 samples (in volumes of 500 ml) were collected from raw and treated water and from at least two taps at different sites within each distribution system (Table 1). From most of these sites, the samples were collected four times at approximately monthly intervals (in May, June, August, and September 1994). The samples were kept at +4°C and analyzed at the water laboratory of the Swedish Institute for Infectious Disease Control within 24 h by the membrane filtration technique. Routinely, volumes of 100 and 10 ml were filtered through membrane filters (0.45-μm-pore-size Sartorius filter), and then the filters were incubated on ampicillin-dextrin agar (ADA), a selective medium for *Aeromonas* spp. (11), at 30°C for 24 h and on m-Endo agar LES (LES [Difco Laboratories, Detroit, Mich.]) at 30°C for 44 h. Samples yielding too many bacterial colonies were diluted and analyzed again. Yellow colonies from ADA and dark red colonies with a diameter of >0.5 mm from LES were presumed to be *Aeromonas* colonies (11, 43). These colonies were picked directly from the incubated agar plates and inoculated into PhP plates (see below). When available, at least eight colonies from ADA and eight colonies from LES were assayed from each water sample. On sampling occasions 3 (in August) and 4 (in September), 16 colonies were picked from each medium. In total, 2,117 presumptive *Aeromonas* isolates were selected for phenotype (PhP) typing.

PhP typing of presumptive *Aeromonas* isolates. The PhP rapid-screening plate for *Aeromonas* (BioSys inova, Stockholm, Sweden) consists of 96-well microplates with eight rows of 11 dehydrated reagents. Eight *Aeromonas*-like colonies were inoculated into each plate, as described previously (32). The inoculated plates were incubated at 30°C, and the rate of each reaction was evaluated by measuring the absorbance value in each well after 16, 40, and 64 h of incubation with a microplate reader. After the final reading, the mean absorbance value in each well over the three readings was calculated, yielding the biochemical fingerprint, which for each isolate is based on the reactions for the 11 reagents. The biochemical fingerprints of the isolates were compared pairwise, and the similarity between each pair of strains was calculated as the correlation coefficient (r). Isolates showing correlation coefficients to each other higher than 0.975 (the identity level [30]) were assigned to the same biochemical PhP type. The diversities of bacterial populations were calculated as Simpson's diversity index (Di) (2), and clustering of correlation coefficients was performed by the unweighted pair group method with arithmetic mean (UPGMA) (42). All data processing, including optical readings, calculations of correlation coefficients and diversity indices, and clustering and printing of dendrograms, was performed with PhP software (BioSys inova).

According to the data obtained, the isolates could be subdivided into different PhP types. PhP types containing at least two isolates from the same sampling occasion in the same distribution system were named common types, and those containing only one isolate were named single types. Common PhP types occurring at several sites and/or at several occasions in the same distribution system were named major (M) types. At least one isolate representing each common type in each water sample was picked directly from one of the wells of the incubated PhP plate and stored in nutrient broth containing 20% (wt/vol) glycerol at −70°C. Some of the isolates representing single types were selected randomly and saved as well.

Phenotypic identification of selected isolates. After the last sampling occasion, all of the frozen isolates (n = 167) were cultivated on ADA and subjected to the oxidase test (BBL, Becton Dickinson Microbiology Systems, Cockeysville, Md.). They were further assayed in the PhP-48 plate, which uses biochemical fingerprints obtained from 48 selected reagents (30). Selected isolates representing common or major PhP types (n = 17), as well as some other isolates belonging to common PhP types or that, according to the data obtained from PhP typing, did not behave like normal *Aeromonas* strains (n = 30), were identified at the species level with the API 20 NE system (bioMérieux, Marcy-l'Etoile, France).

FAME analysis. Gas-liquid chromatography of cellular fatty acid methyl esters (FAME analysis) was performed on 38 isolates, most of which represented major PhP types. FAMEs were prepared and extracted by using the standardized protocol of the Microbial Identification System (MIDI; Microbial ID, Inc., Newark, Del.). Instrumentation and procedures are described elsewhere (15, 37). The selected *Aeromonas* isolates were identified by comparing the unknown FAME profiles to the AER48C database (15, 16), which contains the mean FAME profiles of all currently described phenospecies and HGs within this genus.

Determination of virulence factors. Selected isolates representing 35 common or major PhP types were assayed for hemolytic, cytotoxic, and adherence properties. Each strain was subcultured on blood agar plates (5% horse blood in brain heart infusion [Difco Laboratories]) and incubated at 22°C for 24 h.

Detection of cytotoxin and hemolysin. The cultures from blood agar plates were harvested, inoculated into two containers with 100 ml of tryptic soy broth (TSB) (Difco Laboratories), and incubated on a shaker (60 rpm) for 24 h at 22 and 37°C separately. The cultures were then harvested by centrifugation (16,000 × g) at 4°C for 30 min, and the supernatants were membrane filtered (Millipore filter; pore size, 0.22 μm). These sterile cell-free culture filtrates were tested for cytotoxic activity on Vero cells (African green monkey kidney cells [ATCC CCL 81]; Flow Laboratories, Irvine, Scotland) by exposing a monolayer

TABLE 2. Number of presumptive aeromonads found in raw water, treated water, and tap water in 13 water distribution systems

Aeromonad counts (CFU/100 ml)	No. (%) of samples from[a]:		
	Raw water (n = 50)	Treated water (n = 48)	Tap water (n = 122)
<1	19 (38)	30 (63)	54 (44)
1–10	7 (14)	9 (19)	34 (28)
11–100	1 (2)	2 (4)	27 (22)
101–1,000	9 (18)	7 (15)	7 (6)
>1,000	14 (28)	0	0

[a] n = total number of samples collected from each treatment.

of the cells to serial dilutions of the filtrates. After incubation at 37°C for 24 h in an atmosphere containing 5% CO_2, destruction of at least 50% of the Vero cells was recorded as a positive result. The production of hemolysin was assayed by recording the lysis of horse erythrocytes in agar plates (5% horse blood in brain heart infusion agar) by the sterile cell-free culture filtrates.

Adherence assay. Some of the strains showing a cytotoxic effect at dilutions of >1/4 were subject to an adherence assay. They were cultured in 100 ml of TSB at 37°C for 24 h without shaking. Human intestinal cells (Henley 407 [ATCC CCL 6]; Flow Laboratories) were grown in monolayers on glass coverslips dipped in tissue culture medium containing 10% fetal calf serum (National Veterinary Laboratory, Uppsala, Sweden), 50 U of penicillin G (National Veterinary Laboratory) and 50 μg of streptomycin (National Veterinary Laboratory) per ml. At 2 h before the adhesion experiment, the culture medium was replaced with fresh medium without antibiotics. The cell cultures were then incubated with different dilutions of bacterial suspensions (10^6 to 10^8 bacteria per ml) for 1 h at 20°C with continuous but gentle shaking. To eliminate nonattached bacteria, the coverslips were washed by being dipped several times into a series of beakers containing 0.145 M NaCl. The coverslips were fixed in methanol and stained with crystal violet for 1 to 2 min. The number of bacteria adhering to each of at least 20 cells was determined under a light microscope.

RESULTS

Occurrence of presumptive *Aeromonas* in raw water and in the distribution systems. The enumeration of presumptive *Aeromonas* from ADA and LES agar yielded similar results (Pearson product-moment correlation coefficient = 0.99). Of the 50 samples from raw water, 14 (28%) contained presumptive aeromonads in numbers exceeding 1,000 CFU/100 ml and another 9 (18%) contained between 101 and 1,000 CFU/100 ml (Table 2). All raw water samples in which *Aeromonas* counts exceeded 100 CFU/100 ml were collected from surface water, whereas 24 of 28 raw water samples collected from groundwater or from artificial ground water contained *Aeromonas* counts of <10 CFU/100 ml (Table 3). Of the 48 samples taken after the treatment stage, only 7 (15%) contained aeromonads in numbers exceeding 100 CFU/100 ml. Of the samples collected from taps within the distribution systems, 34 (28%) contained a count of presumptive aeromonads that exceeded 10 CFU/100 ml, with a maximum count of 750 CFU/100 ml in system D5 (Table 3).

The largest numbers of aeromonads in raw water were normally found in the samples collected in June, whereas the largest number in tap water were found in the samples collected in August (data not shown). Large numbers of aeromonads in surface water were normally not correlated with large numbers in treated water and/or in tap water. On the contrary, the largest numbers of aeromonads in treated water were found in water distribution systems associated with groundwater with very low *Aeromonas* counts in the raw water source (e.g., systems D2 and D5 [Table 3]).

In 5 of the 13 water distribution systems examined, *Aeromonas* counts of more than 100 CFU/100 ml were found in the tap water samples (Table 3). For two distribution systems (D8 and D10 [Table 3]), the large numbers of aeromonads in these tap water samples correlated with large numbers of aeromonads in raw water. In one distribution system (D5 [Table 3]), a high *Aeromonas* count was correlated with a high count in treated water. However, in the other two distribution systems that showed high *Aeromonas* counts in tap water samples (D1 and D4 [Table 3]), no growth of aeromonads was observed in samples from raw water or in those from treated water.

TABLE 3. Samples studied, number of presumptive *Aeromonas* organisms, and number of isolates subject to PhP typing in 13 water distribution systems

Site	Raw water			Treated water			Tap water		
	No. of samples[a]	Maximum CFU[b] (median)	No. of PhP types	No. of samples[a]	Maximum CFU (median)	No. of PhP types	No. of samples[a]	Maximum CFU (median)	No. of PhP types
Groundwater									
D1	5 (0)	0		5 (0)	0		10 (8)	200 (4)	75
D2	4 (2)	2 (1)	2	4 (4)	240 (140)	95	8 (6)	70 (8)	90
D3	4 (2)	6 (1)	20	3 (3)	110 (4)	37	13 (10)	85 (4)	145
Artificial groundwater									
D4	4 (2)	5 (1)	18	4 (2)	5 (1)	33	8 (7)	230 (16)	137
D5	3 (0)	0		4 (4)	570 (90)	104	8 (8)	750 (29)	167
D6	4 (1)	1 (0)		4 (0)	0		8 (1)	1 (0)	
D7	4 (3)	900 (790)	80	4 (0)	0		8 (0)	0	
Surface water									
D8	4 (4)	10^6 (1,400)	88	4 (0)	0		12 (6)	140 (1)	83
D9	4 (3)	250 (110)	64	4 (0)	0		8 (4)	23 (1)	27
D10	5[c] (5)	5,200 (3,200)	144	3 (0)	0		17 (11)	220 (3)	223
D11	4 (4)	5,300 (3,800)	108	4 (2)	4 (2)	9	12 (4)	45 (14)	98
D12	4 (4)	3×10^5 (3,800)	112	4 (3)	120 (2)	64	8 (3)	30 (0)	62
D13	1 (1)	940	32	1 (0)	0		2 (0)	0	
Total	50 (31)		668	48 (18)		342	122 (68)		1,107

[a] Numbers within parentheses indicate the number of samples showing any putative *Aeromonas* strains.
[b] CFU/100 ml. The value for each sample was calculated as the mean CFU/100 ml growing on ADA and LES agar.
[c] Two samples were from raw water sources outside the distribution system.

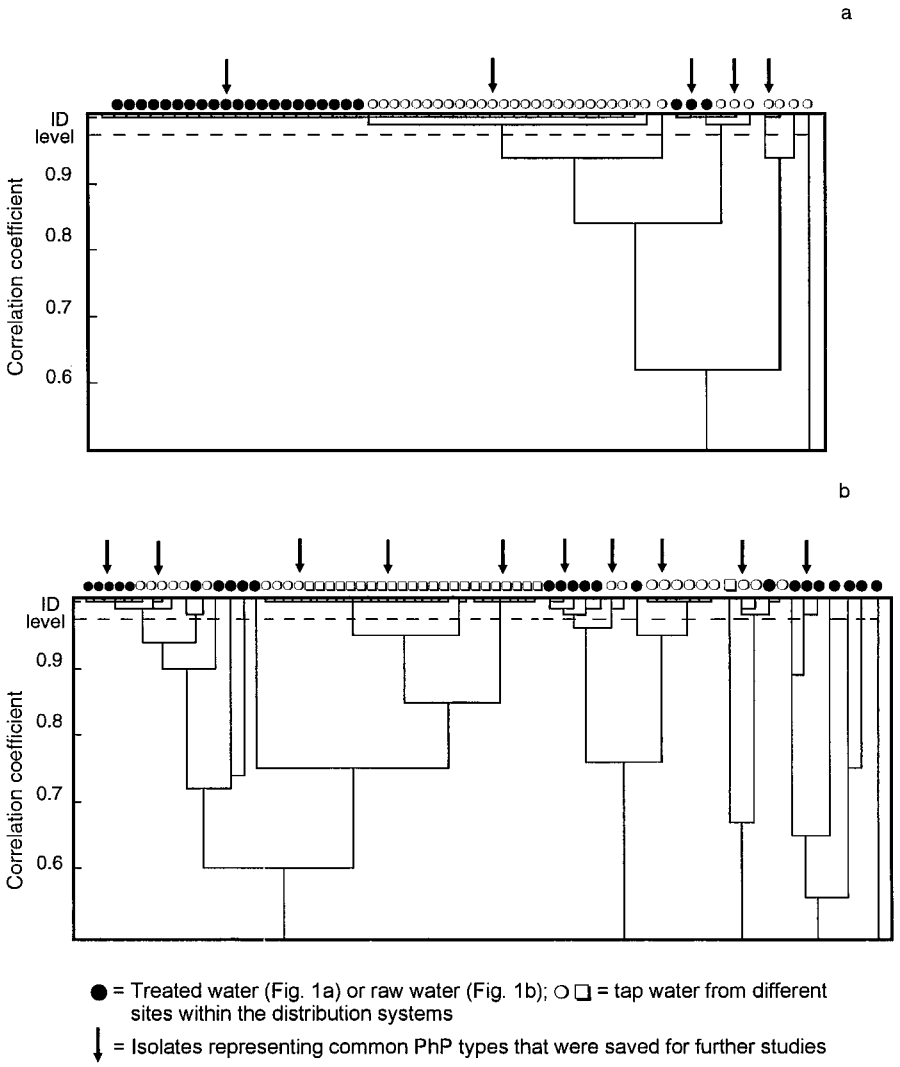

FIG. 1. Dendrogram showing the delineation of PhP types of *Aeromonas* from two water distribution systems, i.e., sample 4 from system D2 (a) and sample 3 from system D11 (b). Isolates marked with arrows were saved for further studies.

PhP types of *Aeromonas*. A total of 2,117 presumptive *Aeromonas* isolates were typed by the PhP-AE system (Table 3); 167 isolates selected among these were also assayed with the more discriminatory PhP-48 plate. The diversity obtained among these 167 isolates was 0.98 according to the PhP-48 system and 0.96 for the same isolates typed with the 11 reagents in the PhP-AE system. Thus, only 2% of the total discriminatory power was lost by reducing the number of reagents from 48 to 11. The few isolates that were identical in the PhP-AE system but differed in the PhP-48 system were usually recovered from different distribution systems. Therefore, we are quite confident that a majority of the total diversity within each sample has been accounted for by using the PhP-AE system and that the selection of one isolate per PhP type from each water sample can be regarded as representative of the entire *Aeromonas* population present in the samples. Thus, all further discussion of PhP types in the present study deals with data obtained from the PhP-AE system.

The number of PhP types found within each distribution system at any sampling occasion varied between 3 and 45, and 310 different types were found altogether. Of these, 293 PhP types occurred only sporadically, especially in samples from surface water, and were found in a total of 1,080 isolates. The remaining 1,037 isolates belonged to 17 major PhP types, which were isolated at several sites and on several occasions from 10 of the distribution systems. These major PhP types seem to be *Aeromonas* strains that have the capacity to multiply within the water distribution systems.

The diversity of the *Aeromonas* populations in different samples was highest in samples from raw water (median Di = 0.95), followed by those in treated-water samples (median Di = 0.70) and by those in samples from taps within the distribution systems (median Di = 0.58). Figure 1 displays data from one sampling occasion from two water distribution systems showing a low diversity (D2 [Fig. 1a]) and a medium to high diversity (D11 [Fig. 1b]). In system D2, only the treated-water samples and one sample from a tap contained aeromonads on this sampling occasion. Altogether, six PhP types were identified among the 57 isolates that were subjected to PhP typing. One PhP type dominated in both the treated water and the tap water and was found in 46 isolates altogether (Fig. 1a). A clear connection between the *Aeromonas* population in

TABLE 4. The putative virulence determinants hemolysin, cytotoxin, and adhesion, found in some common and major PhP types of *Aeromonas*

Virulence determinant	No. of PhP types[a]	
	Assayed	Positive
Hemolysin (20°C)	35 (1,100)	9 (605)
Hemolysin (37°C)	35 (1,100)	23 (957)
Cytotoxin[b]	35 (1,100)	11 (594)
Adhesion[c]	9 (405)	8 (369)

[a] Numbers within parentheses indicate the number of isolates from the original water samples that were represented by the actual PhP types.
[b] Titers of ≥8 were regarded as positive.
[c] Adhesion was not assayed in PhP types yielding a negative result in the cytotoxin assay.

the treated water and within the distribution system could thus be seen. The samples taken at system D11 contained 21 different PhP types, of which 16 were found in the raw water and 8 were found in the tap water. Three PhP types occurred in the raw water as well as in the tap water samples. However, the most frequently occurring PhP types in the tap waters were not recovered from the respective raw waters and thus might represent *Aeromonas* strains growing within the distribution system.

Species identification. Of the 167 isolates representing different PhP types that had been stored after the preliminary screening with the PhP-AE plate, only 10 yielded negative oxidase reaction or noncharacteristic colonies on ADA. These 10 isolates belonged to minor PhP types, representing only 27 (1.2%) of the originally studied isolates. Identification by API 20 NE was performed on 16 major as well as 31 other PhP types, representing 1,220 of the originally studied isolates. Nine PhP types (representing 7% of the original isolates) were identified as belonging to genera other than *Aeromonas* (mainly *Vibrio* or *Pseudomonas*), and four PhP types (representing 5% of the original isolates) remained unidentified. The other 34 PhP types that were characterized were identified as members of the genus *Aeromonas.*

FAME analysis was performed on 25 PhP types, representing 964 isolates. Three major PhP types that had been identified as not being *Aeromonas* spp by the API 20 NE were assigned to the genus *Aeromonas* by FAME analysis (see Table 5). The other identified PhP types were of *A. hydrophila* HG3 (PhP types representing 28% of the analyzed isolates), other *A. hydrophila* spp. (25%), *A. veronii* biogroup sobria HG8 (8%), or unknown *Aeromonas* species (38%). Thus, with FAME, PhP types representing only 1% of the original isolates were identified as being members of genera other than *Aeromonas.*

Virulence factors. Hemolytic activity at 20 and 37°C, as well as cytotoxic activity, was assayed on 35 different PhP types, representing 1,100 of the original isolates. Sometimes several isolates of the same PhP type were assayed, which resulted in some variations in the expression of the studied virulence factors. For further analysis, it has been assumed that when at least 50% of the isolates belonging to a certain PhP type show positive reactions, this PhP type possesses the capability to produce the actual virulence factor. Hemolytic activity at 20°C was observed in 9 PhP types and at 37°C in 23 PhP types (Table 4). Cytotoxic activity (titer ≥ 8) was observed in 11 types (Table 4), most of them being major types. Adhesion to human intestinal Henley 407 cells was measured only for PhP types that displayed cytotoxic activity, and a majority of those were found to show adherence (Table 4). The number of adherent bacteria per cell ranged from 3 to 20.

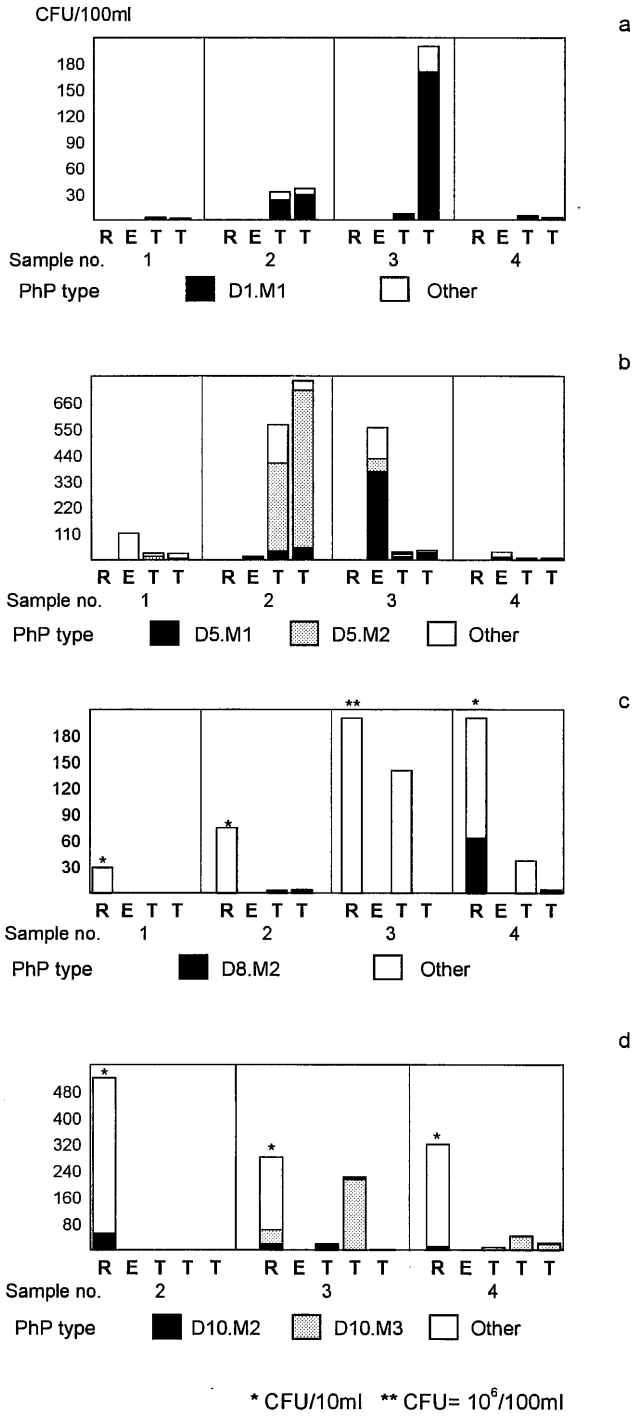

FIG. 2. Bar charts showing the total numbers of *Aeromonas* and the distribution of isolates into different PhP types in samples from four water distribution systems (D1 [a], D5 [b], D8 [c], and D10 [d]) on four different sampling occasions (indicated as sample no. 1 to 4). R denotes raw water, E denotes treated water from the exit sites of the water works, and T denotes tap water samples from different sites within the distribution systems. The major PhP types (see Table 5) are indicated as black or shaded areas on the bars. All other PhP types are indicated as white areas on the bars.

Diversity and stability of the *Aeromonas* populations in the water distribution systems. The number of aeromonads and the occurrence of major and other PhP types in four water distribution systems with different kinds of raw water are shown in Fig. 2.

TABLE 5. Properties of the 17 most common PhP types of *Aeromonas* found in 13 Swedish water distribution systems

PhP type	Persistence[a]	Source[b]	No (%) of isolates[c]	Hemolysin 37°C	Cytotoxin (titer [no. of bacteria])	Adhesion (titer [no. of bacteria])	Species according to:	
							FAME	API 20 NE
D1.M1	4	T	50 (67)	+	4	4	*A. hydrophila* HG3	*A. hydrophila*
D2.M1	3	R,E,T	40 (21)	+	10	ND[d]	*A. hydrophila* HG3	*A. hydrophila*/*A. caviae*
D2.M2	4	E,T	120 (64)	+	10	5	*A. hydrophila* HG3	*A. hydrophila*
D3.M1	2	E,T	108 (53)	+	16	3	*Aeromonas* sp.	*A. hydrophila*/*A. caviae*
D4.M1	4	T	108 (57)	+		ND	*Aeromonas* sp.	*Aeromonas* sp.
D5.M1	4	E,T	56 (21)	−		ND	*A. hydrophila* HG3	*Vibrio alginolyticus*
D5.M2	4	E,T	98 (36)	+	10	5	*A. hydrophila* HG1	*Aeromonas* sp.
D8.M2	2	R,T	37 (21)	+	10	ND	*Aeromonas* sp.	U[e]
D9.M1	2	R,T	28 (28)	−		ND	*Aeromonas* sp.	*A. sobria*
D10.M2	3	R,T	38 (10)	+	10	ND	*Aeromonas* sp.	*A. sobria*
D10.M3	3	R,T	113 (30)	+	10	ND	ND	Ah
D11.M1	4	R,T	57 (26)	−		ND	*Aeromonas* sp.	*A. sobria*
D11.M4	3	R,E,T	37 (17)	+	4	ND	ND	ND
D11.M5	3	R,E,T	25 (12)	+	100	4	*A. veronii* HG8	*A. hydrophila*/*A. caviae*
D12.M2	3	R,E,T	31 (13)	+		ND	ND	U
D12.M3	3	R,E,T	36 (15)	+	100		*Aeromonas* sp.	U
D12.M4	2	E,T	55 (23)	+			*A. veronii* HG8	*Aeromonas* sp.

[a] Indicates the number of sampling occasions on which the actual PhP type was isolated.
[b] R, raw water; E, treated water; T, tap water.
[c] Numbers in parentheses indicate the percentage of the total number of isolates studied from the actual water distribution system.
[d] ND, not done.
[e] U, unidentified.

In system D1 (Fig. 2a), which has groundwater as its raw water source, no aeromonads were found in raw water or in treated water, whereas tap water contained aeromonads in numbers up to 200 CFU/100 ml. The *Aeromonas* population in this water distribution system was clearly dominated by one single PhP type (D1.M1) belonging to *A. hydrophila* HG3, which produced hemolysin and cytotoxin and was positive in the adhesion assay (Table 5). This PhP type was found in all samples collected within the water distribution system, albeit usually in small numbers but reaching a peak in one tap water sample collected in August.

In system D5 (Fig. 2b), which has artificial groundwater as its raw water source, *Aeromonas* was also absent in raw water. On sampling occasion no. 3, the treated water contained high *Aeromonas* counts, mainly due to the presence of PhP type D5.M1. This PhP type was also recovered in minor numbers in several of the tap water samples. It belonged to genomospecies *A. hydrophila* HG3 according to FAME analysis, and it did not express any of the assayed virulence properties. On sampling occasion no. 2, both tap water samples contained high *Aeromonas* counts. The *Aeromonas* populations in both these samples were dominated by another PhP type, D5.M2, which belonged to genomospecies *A. hydrophila* HG1 and which showed hemolytic, cytotoxic, and adhesive properties (Table 5). This PhP type was also recovered in the treated water on sampling occasion no. 3, and it is possible that it had also been present in the treated water at the other sampling occasions but was not recovered in the samples collected.

Systems D8 (Fig. 2c) and D10 (Fig. 2d) both used surface water showing high *Aeromonas* counts in the raw water. However, no aeromonads were found in samples collected directly after the water treatment. In system D8, only one tap water sample contained high *Aeromonas* counts. This was due mainly to the presence of one common PhP type, which was not recovered in any other sample from system D8 (data not shown). The *Aeromonas* population in the raw water samples consisted of many different PhP types, of which one (D8.M2) was occasionally recovered in small numbers (less than 5 CFU/100 ml) from the tap water. In system D10, one PhP type (D10.M3) found in raw water was recovered in tap water samples in amounts of up to 200 CFU/100 ml. Isolates belonging to this PhP type were identified as *A. hydrophila* by API 20 NE and expressed hemolytic and cytotoxic activity.

Other water distribution systems that occasionally showed high counts of aeromonads in tap water were D2, D3, and D4 (Table 3), all of which had ground water as the raw water source. In system D2, aeromonads in tap water correlated with large numbers in treated water, and two PhP types of genomospecies *A. hydrophila* HG3 were recovered in treated water as well as in tap water (Table 5). Both of these PhP types displayed hemolytic and cytotoxic properties, and PhP type D2.M2, which was positive according to all three assays for virulence determinants, dominated the *Aeromonas* population in all the tap water samples. Also in system D3, a correlation between the number of aeromonads in treated water and in tap water was observed; this correlation was dependent mainly on the presence of one PhP type (D3.M1 [Table 4]) that remained unidentified at the HG level but reacted positively in all virulence assays. In system D4, high *Aeromonas* counts were observed only at the last sampling site within the distribution system, and this was due mainly to the presence of one PhP type (D4.M1) that could not be identified at the HG level and did not show cytotoxic activity.

DISCUSSION

For a reliable estimate of the total diversity of the bacterial populations in environmental samples, a sufficiently large number of isolates from each sample must be examined (4). Using the PhP-AE plate as a simple preliminary screening system, we were able to examine as many as 800 isolates within the same week. Based on the assignment of the isolates within each water sample into PhP types, it was possible to obtain a measure of the diversity within each sample and to select representative isolates from each sample for further PhP typing, species identification by FAME and API 20 NE analysis, and characterization of virulence factors. The numerical analysis of data in the present study has been based on the assumption

that isolates from the same water sample that are found to be identical according PhP typing results are of the same clonal origin. It can be argued that such conclusions cannot be drawn from data obtained by a simple phenotyping method alone. However, we have shown in several previous studies that the discriminatory power of the PhP rapid screening system exceeds 0.97 (measured as Simpson's diversity index [2]) for many groups of environmental bacteria (30, 32, 33, 36). Furthermore, when *Aeromonas* isolates that can be assumed to be of different clonal origins (i.e., from different water distribution systems) were compared, it was found that less than 3% of the comparisons yielded identical PhP types. This means a discriminatory power of at least 0.97 for the PhP-AE plate.

Large numbers of aeromonads as well as high phenotypic diversities of the *Aeromonas* populations were found in most samples from surface water. However, only 2 of 59 tap water samples from water distribution systems with surface water as the raw water source contained aeromonads in numbers exceeding 100 CFU/100 ml, and only 1 of these samples contained a dominating PhP type that was also found in the raw water. Thus, the aeromonads in the surface waters seemed to consist of many different clones that were not isolated repeatedly and of which very few were able to survive the water treatment process and to multiply within the distribution systems. The *Aeromonas* population in treated water and in tap water showed much lower diversities, and some of the PhP types found in such samples could also be isolated over the entire study period. We have also shown in previous investigations that *Aeromonas* strains growing within the Swedish water distribution system could persist for several months (31) and even that one single clone of *Aeromonas* could persist for at least 4 years in the water from a well in Sweden (34).

Among the more than 300 different PhP types of *Aeromonas* that were found, only a few major types were recovered in more than one water sample. Some of these PhP types were very common within the distribution systems, but they were not at all isolated from the raw water sources of these systems. They probably represent strains that are able to survive and multiply within the water distribution system. Possibly they had entered the distribution system from the raw water before the onset of the present study, or they might have been present in the raw water during the study but in such small numbers that they were not detected. Most of these major types belonged to *A. hydrophila* HG3, which has been reported to be a common genomospecies in water distribution systems (16), and *A. veronii* biogroup sobria HG8. One major type belonged to *A. hydrophila* HG1, which is one of the genomospecies most commonly associated with virulence and infections in humans (9, 10, 17, 19, 23, 25, 35). The latter PhP type, which also displayed hemolytic, cytotoxic, and adhesive properties, was found in 36% of all isolates in system D5, where it grew to large numbers in two of the tap water samples, and was sporadically isolated at all four sampling occasions.

Hemolysin production and cytotoxin production are properties that are often associated with gastrointestinal infections caused by aeromonads (41). In fact, 14 of the 17 major PhP types found in the present study consisted of isolates that were able to produce hemolysin at 37°C, and 9 of those major types contained isolates that produced cytotoxin in titers of >8. Moreover, for many bacterial enteropathogens, the ability to adhere to the intestinal mucosa is a first step in the colonization, leading to the development of disease (5), and in the present study we found that a majority of the cytotoxin-producing isolates adhered to human intestinal Henley 407 cells. Thus, it seems that many *Aeromonas* strains that were isolated in relatively large numbers from drinking water in Sweden possessed, to a great extent, the same virulence markers as we found in previous studies in isolates from humans with diarrhea (28). This raises the question whether these strains should be regarded as pathogenic clones of *Aeromonas*. Intestinal disorders that possibly could be associated with the consumption of polluted water were found in the consumers of water from only one of the studied distribution system (system D3), and the tap water from this system was in fact dominated by an *Aeromonas* strain that displayed the assayed virulence determinants.

Since drinking water is an important source of human infections and since much evidence points to the existence of pathogenic clones among the genus *Aeromonas*, it has been argued that limits on the number of aeromonads in drinking water should be specified (6, 40, 44). The data obtained in the present study have indicated that although many different *Aeromonas* strains could be present in a water distribution system, only a few of them seemed to be able to persist and multiply within the system. However, these few strains often were positive for the putative virulence factors that are normally used to determine virulence among aeromonads. If the same strains that are able to multiply within drinking water distribution systems were really true human pathogens, they obviously may cause problems to public health. Therefore, we recommend that studies on *Aeromonas* in drinking water should include not only an enumeration but also a typing of isolates and an assessment of the virulence factors of the predominant strains.

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