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Comparison of Pulsed-Field Gel Electrophoresis, Ribotyping, and Plasmid Profiling for Typing of *Vibrio anguillarum* Serovar O1

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A total of 75 *Vibrio anguillarum* serogroup O1 strains were studied with respect to their plasmid contents, ribotypes, and pulsed-field gel electrophoresis (PFGE) patterns. Eight plasmid profiles and six ribotypes were demonstrated, and one profile was dominant by both typing methods. In contrast, PFGE had very high discriminatory power, demonstrating 35 profiles. On the basis of PFGE patterns, a similarity matrix and a dendrogram were constructed. The results indicated that Scandinavian strains and southern European isolates (with some exceptions) belong to two different clonal lineages. A few strains from the United States and United Kingdom deviated considerably from each other and from Scandinavian and southern European strains.

Vibriosis caused by *Vibrio anguillarum* is one of the most important bacterial infections in fish from several countries and in several fish species (2). Primary identification of *V. anguillarum* can be performed by using biochemical criteria such as those described by Baumann and Schubert (4). Further characterization of *V. anguillarum* isolates is normally done by serotyping (34). Serogroup O1 has been shown to be the most important in salmonid fish and turbot (*Scophthalmus maximus*), while O2 is most important in cod (*Gadus morhua*) and O2 and O3 are the most important in eels (*Anguilla anguilla*) (17).

The O1 group has proved to be very homogeneous, sharing most biochemical characteristics (15). Therefore, various techniques have been used in attempts to subtype this serogroup. In previous investigations, plasmid profiling of *V. anguillarum* serovar O1 has been found to have limited value as a method for epidemiological investigation. In a study of Danish isolates (16), only two plasmid profiles were demonstrated and 62% of the strains carried only one 65- to 70-kb plasmid, equivalent to pJM1 as described by Crosa et al. (7). Restriction analysis, however, showed that restriction fragment length polymorphism of this plasmid is useful (22, 36).

Ribotyping of *V. anguillarum* can differentiate between isolates of serovar O1, with one profile dominant regardless of the fish species or geographic origin of the strain (23, 26, 27). Pulsed-field gel electrophoresis (PFGE), a technique for separation of large DNA fragments (32, 33), has proven to be a valuable tool in the differentiation of bacterial types. It has been used in both epidemiological studies of *Staphylococcus aureus* (28), *Enterococcus faecalis* (10), *Enterobacter cloacae* (11) and *Leptospira* spp. (12) and clonal studies of *Salmonella enteritidis* (25) and *Salmonella dublin* (24).

The main purpose of this investigation was to compare and evaluate the use of ribotyping, PFGE, and plasmid profiling as typing methods for strains of *V. anguillarum* serovar O1.

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MATERIALS AND METHODS

Bacterial strains. Seventy-five strains of *V. anguillarum* serovar O1 were used in this investigation. The collection included strains from 12 countries and eight fish species (Table 1). All strains were taken from the stock culture collection of our laboratory and had been stored as Luria-Bertani (LB) broth (LB broth base [Gibco BRL]) cultures with sterile glycerol (15% [vol/vol]) at −80°C. These strains had been isolated in our own laboratory or received as gifts from other laboratories.

DNA extraction and digestion for ribotyping and PFGE. Strains were grown overnight at 20°C in LB broth or on blood agar plates (blood agar base [Gibco BRL] supplemented with 5% citrate-stabilized calf blood). Three or four colonies were inoculated into LB broth and grown at 20°C until an optical density at 600 nm of approximately 0.1 was reached. Genomic DNA was prepared in chromosomal-grade agarose (Bio-Rad) as previously described (5). Digestion with restriction enzymes (for both ribotyping and PFGE) was performed by cutting a slice of one agarose plug that contained DNA and incubating it for 1 h with the relevant enzyme buffer and then for 4 h with 20 U of restriction enzyme. For ribotyping and PFGE, *Hin*dIII (Amersham or Promega) and *Not*I (Amersham) were used, respectively.

The selection of restriction enzymes for PFGE was based on testing which included the type strains for different serotypes of *V. anguillarum*. Screening was done with *Sma*I, *Bam*HI, *Cpo*I, *Sal*I, *Nae*I, *Xba*I, *Xho*I, and *Not*I (Amersham).

PFGE. For PFGE, DNA fragments were subjected to electrophoresis on 1.0% agarose (Litex LSL) gels in 0.5× Tris-borate-EDTA buffer (19) by using a modified contour-clamped homogeneous electric field (6) system (Pulsaphor Plus; Pharmacia LKB, Uppsala, Sweden). The running conditions were 12 V/cm at 14°C for 22 h. The pulse times were increased by stepping as follows: 5 s for 3 h, 9 s for 5 h, 12 s for 5 h, 20 s for 4 h, 25 s for 3 h, and 30 s for 2 h. Multimeric phage lambda (48.5-kb) DNA (Pharmacia LKB) was used as the molecular size marker.

After electrophoresis, gels were stained in ethidium bromide (Sigma) (2 μg/ml) for 15 min, destained in distilled water for 15 min, and photographed with 254-nm UV transillumination.

Ribotyping. Ribotyping was performed with DNA prepared for PFGE, a method that saved time and gave clear, sharp bands.

For ribotyping, DNA fragments were separated overnight by low-voltage (1.5 V/cm) electrophoresis on 1.2% agarose (Litex LSL) gels in 1× TAE buffer (19). Digoxigenin-labeled phage lambda DNA cut with *Hin*dIII (Boehringer, Mannheim, Germany) was used as the size marker.

*Hin*dIII-digested DNA was blotted (VacuGene XL vacuum blotting system; Pharmacia LKB) onto nylon hybridization membranes (Hybond-N; Amersham) and fixed to membranes by being baked at 80°C for 1 to 3 h. Ribotyping was performed as described by Pedersen and Larsen (27). Blotted DNA was hybridized at 56°C overnight with a digoxigenin-labeled probe made from 16S and 23S rRNAs of *Escherichia coli* (Boehringer) and incubated with alkaline phosphatase-labeled antidigoxigenin immunoglobulin (Boehringer). Probed fragments were visualized by the addition of substrates, nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate (Boehringer).

Similarities among patterns. On the basis of the band patterns obtained by PFGE, a dendrogram was created by using similarity analysis software TAXAN version 4.0 (Sea Grant College, University of Maryland). The order in which the strains were read into the similarity matrix is listed in Table 1. Clustering analysis

TABLE 1. *V. anguillarum* serovar O1 strains typed by PFGE, ribotyping, and plasmid profiling

Strains[a]	Fish species[b]	Country	Ribotype (*Hin*dIII)[c]	PFGE type (*Not*I)[d]	Plasmid content (kb)
909/88[9]	*Dicentrarchus labrax*	Italy	1	1	67
28/89	*D. labrax*	Italy	1	1	67
165/89	*D. labrax*	Italy	1	1	67
173/93	*D. labrax*	Italy	1	1	67
218/93	*D. labrax*	Italy	1	1	67
120/93	*Sparus aurata*	Italy	1	1	67
158/93	*Sparus aurata*	Italy	1	1	67
219/93	*Sparus aurata*	Italy	1	1	67
178/90[14]	*D. labrax*	Italy	1	2	89, 90
191/90	*D. labrax*	Italy	1	2	80, 90
601/91	*D. labrax*	Italy	1	2	67, 90
348/93	*D. labrax*	Italy	1	2	67, 90
90/93	*Sparus aurata*	Italy	1	2	67, 90
49/92[13]	*D. labrax*	Italy	1	3	67
493/92[12]	*Sparus aurata*	Italy	1	4	67
1159/90[7]	*Sparus aurata*	Italy	1	5	67
1/91[8]	*Mugilidae* sp.	Italy	1	6	67
76/91[10]	*Mugilidae* sp.	Italy	1	7	67
261/91[11]	*D. labrax*	Italy	1	8	67
141/93	*D. labrax*	Italy	1	8	67
143/93	*D. labrax*	Italy	1	8	67
161/93	*D. labrax*	Italy	1	8	67
274/92	*Sparus aurata*	Italy	1	8	67
53/93	*Mugilidae* sp.	Italy	1	8	67
169/93	*D. labrax*	Greece	1	8	67
170/93	*D. labrax*	Greece	1	8	67
171/93	*D. labrax*	Greece	1	8	67
89-12-199[25]	*Oncorhynchus mykiss*	Denmark	1	9	67
89-12-204	*O. mykiss*	Denmark	1	9	67
89-12-205	*O. mykiss*	Denmark	1	9	67
89-12-206	*O. mykiss*	Denmark	1	9	67
89-12-201[24]	*O. mykiss*	Denmark	1	10	67
90-11-281[26]	*O. mykiss*	Denmark	1	11	67
90-11-287	*O. mykiss*	Denmark	1	11	67
90-11-305	*O. mykiss*	Denmark	1	11	67
90-12-319	*O. mykiss*	Denmark	1	11	67
6018/1	*O. mykiss*	Denmark	1	12	67
820616-1/2	*O. mykiss*	Denmark	1	12	67
820616-1/5	*O. mykiss*	Denmark	1	12	67
820617-1/6	*O. mykiss*	Denmark	1	12	67
820721-1/11[22]	*O. mykiss*	Denmark	1	12	67
830407-1/7	*O. mykiss*	Denmark	1	12	67
830419-1/6	*O. mykiss*	Denmark	1	12	67
820617-1/8	*O. mykiss*	Denmark	1	12	67
830407-1/9[23]	*O. mykiss*	Denmark	1	13	67
830419-1/2	*O. mykiss*	Denmark	1	13	67
830422-1/1[27]	*O. mykiss*	Denmark	1	14	67
N 1793[33]	*Salmo salar*	Norway	1	15	67
NCMB 2129[34]	*O. mykiss*	Norway	1	16	67
N 1803[32]	*O. mykiss*	Norway	1	17	67
91-7-154[35]	*O. mykiss*	Norway	1	18	67
91-8-198	*O. mykiss*	Norway	1	18	67
91-8-199	*O. mykiss*	Norway	1	18	67
87-9-116	*Salmo salar*	Finland	1	19	Empty
87-9-117[31]	*O. mykiss*	Finland	1	19	67
T 246[16]	*O. mykiss*	England	1	20	67
T 265[17]	*Salmo salar*	England	1	21	50, 11.5, 5.6
51/82/2[15]	*O. mykiss*	Germany	1	22	67
R 62[1]	*Scophthalmus maximus*	Spain	1	23	67
R 73[2]	*Scophthalmus maximus*	Spain	1	24	67
PT 213[21]	*Plecoglossus altivelis*	Japan	1	25	Empty
53-507[18]	*Oncorhynchus kisutch*	United States	1	26	67
RG 75-834[10]	*O. kistuch*	United States	1	27	50, 67
90-11-286[30]	*O. mykiss*	Denmark	2	28	4.5
155/93	*D. labrax*	Italy	3	29	67
1299/92[4]	*O. mykiss*	Italy	3	29	67
152/93	*O. mykiss*	Italy	3	29	67
35/93[6]	*O. mykiss*	Italy	3	30	67

Continued on following page

TABLE 1—*Continued*

Strains[a]	Fish species[b]	Country	Ribotype (*Hin*dIII)[c]	PFGE type (*Not*I)[d]	Plasmid content (kb)
1359 A[3]	*O. mykiss*	Italy	3	31	67
1692	*O. mykiss*	Italy	3	31	67
VA 41[20]	*Salmo salar*	Canada	4	32	67
VA 12	*O. mykiss*	Canada	4	32	67
408 F[5]	*O. mykiss*	France	5	33	67
91-7-154[28]	*Scophthalmus maximus*	Denmark	6	34	50, 67
91-8-178[29]	*Scophthalmus maximus*	Denmark	6	35	54, 67

[a] Superscript numbers refer to the order in which strain data were input in the similarity matrix.
[b] *D. labrax*, sea bass; *Sparus aurata*, sea bream; *Mugilidae* sp., mullets; *O. mykiss*, rainbow trout; *Salmo salar*, Atlantic salmon; *Scophthalmus maximus*, turbot; *P. altivelis*, ayu; *O. kisutch*, coho salmon.
[c] Numbers used by Pedersen and Larsen (27).
[d] Only bands of >100 kb. were scored.

was carried out by using Jaccard's coefficient and unweighted pair group method with arithmetic averages (30).

Isolation of plasmids. Plasmid DNA was extracted for plasmid screening by the method of Kado and Liu (14) and for restriction enzyme digestion by the method of Olsen (21). Separation was done by agarose gel electrophoresis (0.8% agarose; Litex LSL) in 1× TAE buffer. Plasmid sizes were calculated by the method of Rochelle et al. (31), with *E. coli* V517 (18) and 39R861 (35) plasmids as size reference molecules.

Plasmids were examined for *Not*I restriction sites by digestion with *Not*I and evaluated by agarose gel electrophoresis.

RESULTS

Ribotyping. On the basis of ribotyping (*Hin*dIII), this collection of *V. anguillarum* strains was separated into six ribotypes (Fig. 1), with ribotype 1 including 84% (63 of 75) of these strains. Ribotype 2 included 1% (1 of 75), ribotype 3 included 8% (6 of 75), ribotype 4 included 3% (2 of 75), ribotype 5 included 1% (1 of 75), and ribotype 6 included 3% (2 of 75) of these strains (Fig. 1; Table 1).

Screening restriction enzymes for PFGE. Screening five *V. anguillarum* serovar O1 strains for suitable distribution of restriction sites showed that *Sma*I and *Nae*I gave >40 bands of <250 kb. *Bam*HI, *Sal*I, *Xba*I, and *Xho*I digestions resulted in >40 bands of <150 kb. *Cpo*I and *Not*I gave 35 to 40 bands of between 20 and 600 kb. *Cpo*I showed problems with partial digestion. On the basis of these results, *Not*I was chosen for analyses of all strains.

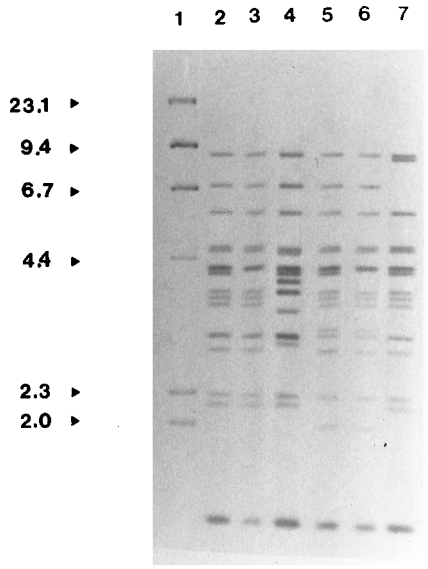

FIG. 1. Ribotypes observed among *V. anguillarum* serovar O1 strains after DNA digestion with *Hin*dIII. Lanes: 1, digoxigenin-labeled lambda DNA cut by *Hin*dIII; 2, 909/88 (ribotype 1; 63 of 75 strains); 3, VA 12 (ribotype 4; 2 strains); 4, 90-11-286 (ribotype 2; 1 strain); 5, 155/93 (ribotype 3; 6 strains); 6, 408 F (ribotype 5; 1 strain); 7, 91-7-154 (ribotype 6; 2 strains). Molecular size standards (in kilobases) are given on the left.

PFGE. Only >100-kb bands were scored. A strain was considered to be different if its PFGE pattern differed by one or more bands whose size was above this limit.

Scored this way, PFGE (*Not*I) subdivided the 75 strains of *V. anguillarum* serovar O1 into 35 PFGE types (Fig. 2), with between 1 and 12% of strains in each type (Table 1). The dominant ribotype was separated into 27 PFGE types.

During screening for the most suitable restriction enzyme (see above), 39 strains were digested with *Sma*I. *Sma*I identified a group of strains with few restriction sites for this enzyme (data not shown). This group included 1 German strain, 1 English strain, and 20 of the 21 Danish isolates tested with *Sma*I. The other group of strains yielded more than 40 bands (<250 kb) with *Sma*I, including 1 Danish strain and 16 strains from Japan, Finland, Norway, Italy, England, Spain, France, the United States, and Canada.

Similarities of PFGE patterns. Except for six Italian strains, all strains from Italy, France, and Greece clustered together when PFGE patterns were compared by similarity analysis (Fig. 3). At a lower degree of similarity, the two strains from Spain were related to the cluster of southern European strains.

All of the strains from Norway and Finland and 22 of the 23 Danish strains clustered together.

One strain from the United States, one strain from Japan, one strain from England, and six strains from Italy were included in the cluster of strains from Scandinavia.

The two strains from the United States showed a low degree of similarity to each other, which was also the case for the two strains from England.

One strain from England and one strain from the United States showed the least similarity to all of the other strains in the collection.

Plasmid profiles. Eight plasmid profiles were detected (Fig. 4 and Table 1). Most strains harbored the 67-kb virulence plasmid equivalent to pJM1. Only two strains lacked plasmids, and one strain carried only a 4.5-kb plasmid. Sixty-three strains carried only the 67-kb plasmid, and six strains contained the 67-kb plasmid and one other plasmid, with an approximate size of 50, 54, or 90 kb.

Two strains carried two large plasmids (approximately 80 and 90 kb) but not the 67-kb plasmid, and one strain carried 50-, 11.5-, and 5.6-kb plasmids without the 67-kb plasmid.

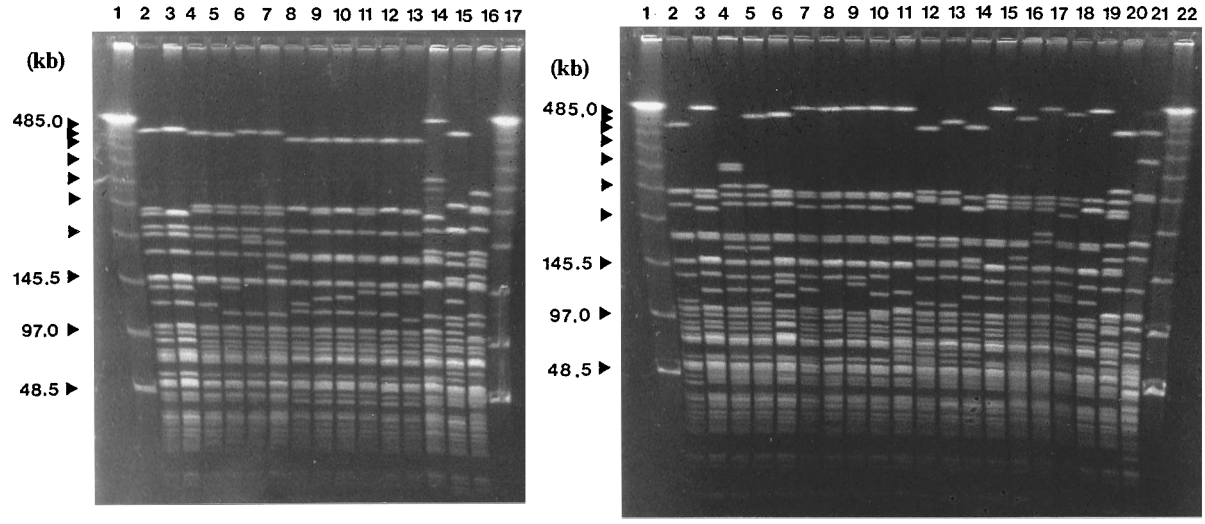

FIG. 2. PFGE types observed among *V. anguillarum* serovar O1 strains after DNA digestion with *Not*I. (A) Lanes: 1 and 17, multimers of phage lambda DNA (48.5 kb) as molecular size markers; 2, R 62 (type 23); 3, R 73 (type 24); 4, 1359 A (type 31); 5, 1299/92 (type 29); 6, 35/93 (type 30); 7, 408 F (type 33); 8, 1159/90 (type 5); 9, 1/91 (type 6); 10, 909/88 (type 1); 11, 76/91 (type 7); 12, 261/91 (type 8); 13, 493/92 (type 4); 14, 51/82/2 (type 22); 15, T 265 (type 21); 16, RG 75-834 (type 27). (B) Lanes: 1 and 22, multimers of phage lambda DNA (48.5 kb) as molecular size markers; 2, 49/92 (type 3); 3, 820721-1/11 (type 12); 4, 91-7-154 (type 34); 5, 91-8-178 (type 35); 6, 178/90 (type 2); 7, 87-9-117 (type 19); 8, N 1803 (type 17); 9, N 1793 (type 15); 10, NCMB 2129 (type 16); 11, 91-7-151 (type 18); 12, T 246 (type 20); 13, PT 213 (type 25); 14, 53-507 (type 26); 15, 830407-1/9 (type 13); 16, 89-12-201 (type 10); 17, 89-12-199 (type 9); 18, 90-11-281 (type 11); 19, 830422-1/11 (type 14); 20, VA 41 (type 32); 21, 90-11-286 (type 28).

All of the large plasmids were shown to have *Not*I restriction sites (data not shown) and were therefore unlikely to influence PFGE patterns when only >100-kb bands were scored.

DISCUSSION

In our investigation, ribotyping did not separate isolates that had originated from different countries or different fish species; therefore, it would not be suitable when a high degree of discrimination is needed. This is supported by the fact that one of the six ribotypes included 84% of the strains used in this investigation.

PFGE has been shown to be a valuable typing method for epidemiological investigations of several bacterial pathogens. In both epidemiological (10–12, 28) and clonal (24, 25) studies, PFGE proved to be highly differentiating, even when other typing methods did not provide sufficient discrimination.

In this investigation, PFGE subdivided the *V. anguillarum* collection into 35 groups and, generally, strains with origins in different countries were shown to have different PFGE types. Three Greek isolates which shared a PFGE type with a group of Italian strains (PFGE type 8) were the only exceptions. The fact that these strains also shared a ribotype suggests that they belong to the same clonal lineage.

PFGE was also found to discriminate between strains with origins in the same country. However, as with ribotyping, it was not possible to differentiate separate strains isolated from different fish species. These data suggest that individual *V. anguillarum* serovar O1 strains are pathogenic to all of the fish species described in this study and show no particular host adaptation.

The dominant ribotype (profile 1) was separated into 27 PFGE groups; thus, the PFGE method shows a degree of discrimination that may be of value in epidemiological investigations. Besides the dominant ribotype, three other ribotypes were represented by more than one isolate. Six strains from Italy with a unique ribotype (profile 3) were separated into three PFGE types, and two strains from Denmark (profile 6) were also divided into two PFGE types. Two strains from Canada (profile 4) were distinguished from each other by PFGE only when additional bands of <100 kb were scored (data not shown).

In PFGE, digested plasmids migrate according to the sizes of fragments, while uncut plasmids are reported to present aberrant mobilities (3, 20). In this study, only bands that were larger than 100 kb were scored. This size limit was chosen partly because the largest plasmid demonstrated was approximately 90 kb. Since all larger plasmids were cut at least once by *Not*I, we conclude that the presence of plasmids in DNA preparations for PFGE had no influence on the interpretation and stability of typing results.

To relate different PFGE types, a dendrogram based on the differences in band patterns was created. The conclusions drawn from the dendrogram are only general findings because the output of clustering analysis depends on the choice of similarity coefficient and linkage method and because changes could be included in clustering and levels of similarity simply by changing the order in which isolate data are input into the clustering software (data not shown). Average linkage was chosen because this method has been indicated to give the most accurate representation of data (30).

The results of the dendrogram indicate that Scandinavian strains and southern European strains belong to two different clonal lineages. In addition, although the number of isolates was limited, it appears that there is more than one clonal lineage of concern in the United States and England.

Preliminary PFGE results with *Sma*I indicated that most Danish isolates possess special properties as these strains had very few restriction sites for this enzyme. The reason for and significance of this observation are at present unknown and need further investigation.

Strains with different plasmid profiles may have identical ribotypes, but except for two strains, they always have different PFGE types, indicating that there is no correlation between

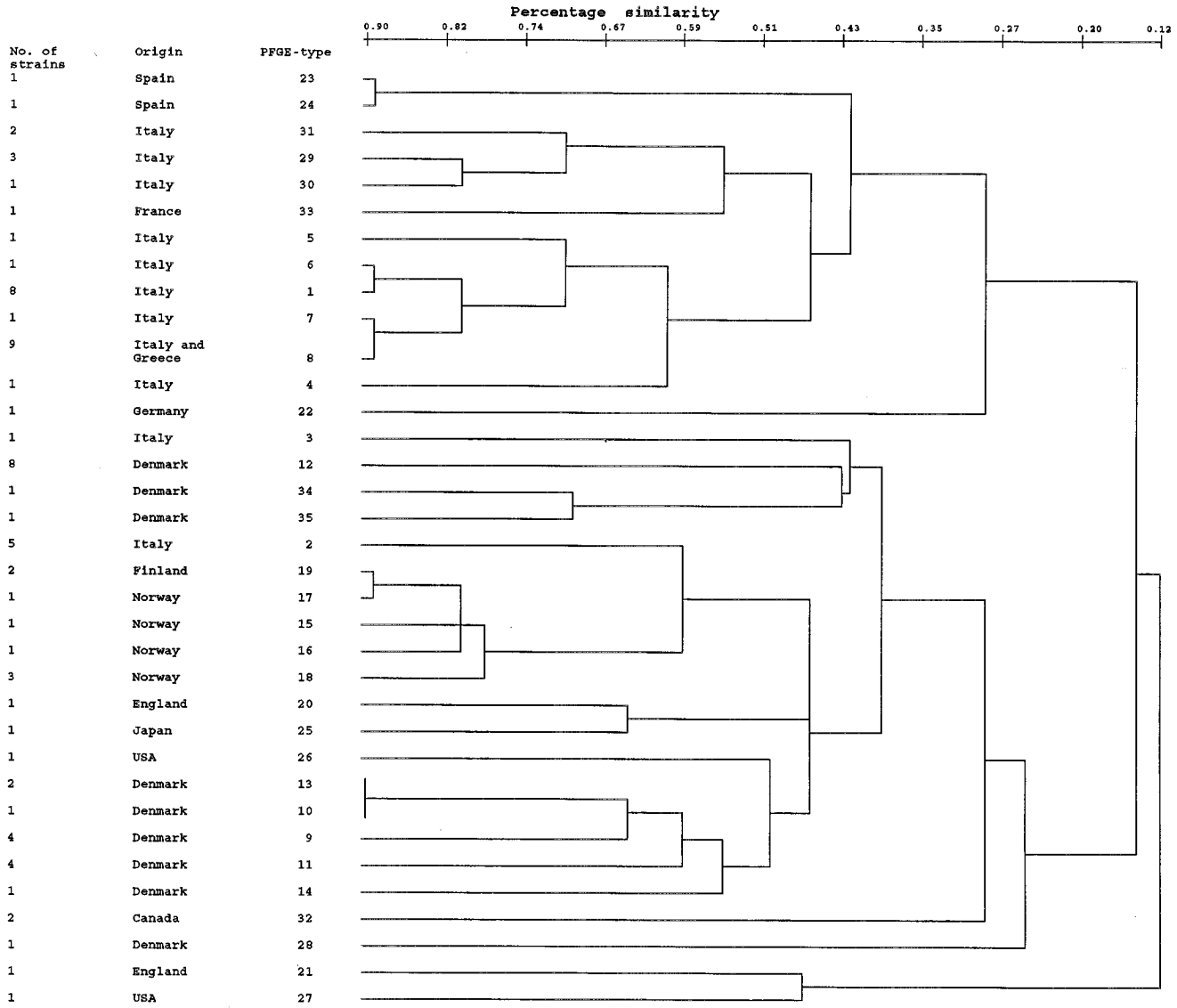

FIG. 3. Unweighted average linkage dendrogram (TAXAN version 4.0) that shows the results of cluster analysis on the basis of PFGE patterns (*Not*I) for the strains listed in Table 1. Numbers on the horizontal axis indicate percentage similarities as determined by Jaccard's coefficient.

plasmid profiling on one hand and ribotyping and PFGE on the other hand. Strains with identical plasmid profiles may have different ribotypes or PFGE types. Interestingly, the two strains with ribotype 6, both isolated from turbot in Denmark, had slightly different PFGE types and plasmid contents.

It should also be noted that the five strains that contained the approximately 90-kb plasmid contained an additional 80-kb plasmid or the 67-kb virulence plasmid. Preliminary results of work in progress in our laboratory on the genetic relationship among *V. anguillarum* plasmids indicate a close relationship between the 80-kb plasmid and the 67-kb virulence plasmid and a similarly close relationship between the 50- and 54-kb plasmids.

The two Finnish strains, 87-9-116 and 87-9-117, had identical ribotypes and PFGE profiles but different plasmid profiles. This may indicate that these two strains belong to the same clone and that one of them has lost its virulence plasmid.

The patterns obtained by PFGE have been demonstrated to be reproducible and stable for the same organism after 40 in vitro passages (1, 8, 29), repeated isolation from the same patient (13), isolation from different sites from the same patient (1), and longitudinal recovery from chronically infected patients (8).

In our investigation, we chose one test difference as the criterion for separation by considering only bands that were larger than 100 kb. The impact of this can be discussed and should be reevaluated in situations in which more detailed epidemiological data are available (9). In this study, such data were not available for all strains, so separation of these strains on the basis of a single test difference was considered to be appropriate along with clustering analysis to show similarities among strains.

In this investigation, PFGE was shown to be a typing method with a high degree of discriminative power that is superior to those of plasmid profiling and ribotyping. The diversity of PFGE types within the dominant ribotype (profile 1) suggests that PFGE is a useful supplementary tool for epidemiological analysis of *V. anguillarum* serovar O1.

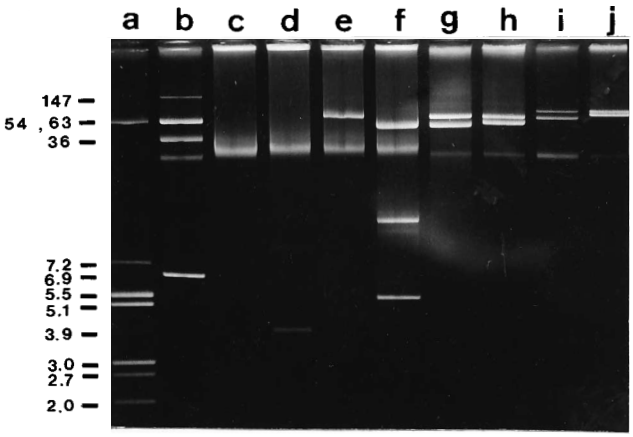

FIG. 4. Plasmid profiles of *V. anguillarum* serovar O1 strains. Lane a, *E. coli* V517 size reference plasmid molecules; lane b, *E. coli* 39R861 size reference plasmid molecules; lane c, PT 213 (empty); lane d, 90-11-280 (4.5 kb); lane e, 6018/1 (67 kb); lane f, T 265 (5.6, 11.5, and 50 kb); lane g, 91-7-154 (50 and 67 kb); lane h, 91-8-178 (54 and 67 kb); lane i, 601/91 (67 and 90 kb); lane j, 178/90 (80 and 90 kb).

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