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VIBRIO ANGUILLARUM: A COMPARATIVE STUDY OF FISH PATHOGENIC, ENVIRONMENTAL, AND REFERENCE STRAINS

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LARSEN, JENS L.: *Vibrio anguillarum: A comparative study of fish pathogenic, environmental, and reference strains.* Acta vet. scand. 1983, *24*, 456—476. — An examination of 41 fish pathogenic and 51 environmental strains of Vibrio anguillarum from Danish coastal areas, and of 8 reference strains of Vibrio anguillarum, biotype 1, showed that these strains shared rather broad-spectered activities towards many carbohydrates, amino acids, proteins and lipids.

They also exhibited rather uniform growth characteristics, including parameters as salinity (NaCl), temperature, antibiotic resistance, and growth on specific media.

A computer analysis of these strains and 3 strains of Vibrio anguillarum, biotype 2 (Vibrio ordalii), showed that the Danish strains all belonged to Vibrio anguillarum, biotype 1, and that no basis existed for setting up new biotypes.

Mean values of the G+C content in DNA of strains of the various categories of Vibrio anguillarum, biotype 1, ranged from 45.5 to 46, while the mean value for the Vibrio ordalii strains was 48.9.

The biochemically active Vibrio anguillarum seems more suitable for environmental studies than the more fastidious Vibrio ordalii.

Vibrio anguillarum; Vibrio ordalii; biochemical activities; environmental; fish pathogenic; reference strains.

Vibrio anguillarum is of etiological significance in relation to the vibriosis syndrome among cultured and will-living marine fish (*Anderson & Conroy* 1970).

Epizootics among marine fish were well-known at the beginning of this century (*Hofer* 1904), and mass deaths among eels in Italy initiated the first bacteriological examinations (*Canestrini* 1892, *Inghilleri* 1903). Bacillus anguillarum was the

name used by *Canestrini* (1892) for the responsible organism, but later *Bergman* (1909) proposed the name Vibrio anguillarum because of some resemblance with Vibrio cholerae. The taxonomy of the genus Vibrio, however, has undergone major revisions in recent years. *Baumann & Baumann* (1976) suggested Vibrio anguillarum transferred to the genus Beneckea, but in 1980 they re-evaluated their taxonomy, and the name Vibrio anguillarum was retained (*Baumann et al.* 1980).

Biochemical differences among strains of Vibrio anguillarum were observed very early by *Bergman* (1912) and *Bruun & Heiberg* (1932). *Nybelin* (1935) proposed two biotypes, A and B, with reference to the following criteria: fermentation of sucrose and mannitol, and indole production. Combination of biotypes (and also agglutination types) with the epizootological evidence about 1930 justified this separation (*Nybelin* 1935).

A third biotype named C was described by *Smith* (1961).

The species Vibrio piscium (*David* 1927), Vibrio piscium var. japonicus (*Hoshina* 1957), and Vibrio ichthyodermis (*Wells & ZoBell* 1934) were suggested transferred to Vibrio anguillarum in 1971 (*Hendrie et al.* 1971). The same year, *Evelyn* (1971) summed up the existing knowledge of the properties of Vibrio anguillarum and described a tentative archtype. During the last decennium, however, many publictaions have elucidated pronounced diversities among Vibrio anguillarum strains from various parts of the world resulting in proposals for new groups, types and phenons (*Håstein & Smith* 1977, *Baumann et al.* 1978, *Kusuda et al.* 1979, *Muroga et al.* 1979, *Ezura et al.* 1980). Vibrio anguillarum-like organisms were simultaneously described in USA and Japan (*Harell et al.* 1976, *Ohnishi & Muroga* 1976) and named Vibrio sp. 1669 and Vibrio sp. RT, respectively. Further characterization (*Strout et al.* 1978) and numerical data analyses showed that these organisms constituted a specific biotype (*Baumann et al.* 1978), which was proposed by *Schiewe* (1971) to be a new species, Vibrio ordalii. This species was described in detail by *Schiewe et al.* in 1981.

Most research has been concerned with isolates from diseased fish. Consequently, information on environmental strains is sparse, although they are of obvious epidemiological interest (*Baross & Liston* 1970, *Larsen* 1982).

This study was performed to obtain information on biochemical properties of a large set of isolates from diseased fish —

cultured and wild-living — as well as from the environment where the fish contract their infections. A number of reference strains were included for comparison.

MATERIALS AND METHODS

Strains of Vibrio anguillarum

Environmental and fish pathogenic strains were isolated according to the principles listed by *Larsen & Jensen* (1979) and *Larsen* (1982). Environmental strains originated from various Danish coastal areas.

A list of reference strains was required for comparisons. ATCC 19264 is type strain for Vibrio anguillarum according to the Approved List of Bacterial Names (*Skerman et al.* 1980). DF_3K (ATCC 33509) has been designated as type culture for Vibrio ordalii and included in the author's index of new names (*Anon.* 1982). A total of 103 strains were investigated (Table 1).

Table 1. Strains examined.

Species	Number of strains	Source	Labelled
V. anguillarum	10	Water (own isolates)	
„ „	8	Sediment (own isolates)	
„ „	7	Plankton (own isolates)	
„ „	9	Invertebrates (own isolates)	
„ „	8	Cod mucus (own isolates)	
„ „	9	Cod faeces (own isolates)	
„ „	10	Rainbow trout (Salmo gairdneri) (own isolates)	
„ „	10	Cod (Gadus morhua) (own isolates)	
„ „	11	Eel (Anguilla anguilla) (own isolates)	
„ „	10	Turbot (Scophthalmus maximus) (own isolates)	
„ „	3	Received from M. H. Schiewe	775, 1800, RG-834
„ „	1	Received from J. L. Fryer	LS 174
V. ordalii	2	Received from M. H. Schiewe	DF_3K(T), 241-S
„ „	1	Received from J. L. Fryer	MSC 275
V. anguillarum	2	Received from NCMB	NCMB 6 and 407
„ „	2	Received from ATCC	ATCC 19265 (T) and 14181
Total	103		

(T) = Type strain culture.

Morphology, motility and flagellation

Cell morphology was studied in Gram-stained preparations from blood agar plates (BA: Blood Agar base (Difco) with 5 % citrated calf blood) and broth cultures (Veal infusion broth (Difco)) incubated at 25°C aerobically for 24 h. Motility in broth was studied by dark-field microscopy. The demonstration of flagellation was made according to *Clark* (1976).

Cells for inoculation

Log-phase cells from veal infusion broth (Difco) were used routinely for inoculation, and the inoculum was most often 0.1 ml/5 ml test medium. When carry-over of nutrient might influence the results, cells from the top of colonies on BA cultured for 24 h at 25°C were used.

Biochemical tests were made according to standard microbiological methods (*Cowan* 1974, *Lautrop et al.* 1979). A few data will, however, be presented as a basis for comparison. Each test was performed once, but repeated if an inconclusive result was obtained.

Biochemical activities

Carbohydrates: The fermentative ability of the organisms was measured by the method of *Hugh & Leifson* (1953) in OF-medium (Difco) with 1 % glucose. Meat extract broth containing 0.5 % of the specific carbohydrate along with bromthymol blue as indicator was used to determine acid production. Gas productions was detected by inverted Durham tubes. To all broth culture media for Vibrio anguillarum NaCl was added to a final concentration of 2 % unless otherwise stated. These tests were read after 1, 2, 3, 4, 7, and 14 days.

Decarboxylase tests were conducted in Moeller Decarboxylase Base (Difco) with 1 % of the L-amino acids added.

Methyl-red reaction and Voges-Proskauer tests were performed in MR-VP broth (Difco); 5 ml broth was inoculated and the reactions studied daily for 4 days. 2,3-butanediol production was demonstrated after the method described by *Bullock* (1961).

Hydrogen sulphide production was detected by blackening in Triple Sugar Iron agar (Difco).

Haemolysis was tested by growth on Blood Agar Base (Difco) with 5 % citrated calf blood. Incubation aerobically at 25°C for 48 h.

Aesculin hydrolysis: The ability to hydrolyse aesculin was shown by a blackening following growth in peptone broth containing 0.1 % of aesculin and 0.1 % of ferric chloride.

Growth and reaction on selective media: The strains were all tested on MacConkey agar (Difco), SS agar (Difco), and TCBS agar (Difco). Pseudomonas selective agar (Oxoid) was used for testing cetrimide sensitivity.

Tolerance to NaCl was observed by growth in peptone water with the following concentrations of NaCl: 0, 6, and 7 per cent and on CLED (Cystein lactose electrolyte deficient medium) described by Bevis (1968).

Incubation temperature was generally 25°C except in the test at different temperatures.

Temperature range for growth: Growth at different temperature levels was determined with Veal Infusion Broth (Difco) as growth medium. Test tubes containing approx. 5 ml of the nutrient broth were inoculated and incubated at the appropriate temperatures. Observations daily for 1 week. The tubes for 37°C and 43°C tests were incubated in water bath.

Swarming was studied on Marine Agar (Difco) with 5 % citrated calf blood (*Larsen et al.* 1981).

Antibiotic sensitivity tests: Rosco Neo-sensitabs System was used for the detection of sensitivity of the strains to the antibiotics novobiocin and penicillin. The two types of discs containing 10 μg and 150 μg of the vibriostatic agent (0/129) were prepared by Rosco.

DNA base composition: The mole per cent of Guanine + Cystostine in DNA was determined by thermal denaturation at Statens Forsøgsmejeri, Hillerød, Denmark.

Numerical taxonomic analysis: The following symbols were used in the coding system applied: 0 = NC (not comparative); 1 = —; 2 = +; Blank = NT (not tested). The programme*) could analyse 300 strains with regard to a maximum of 148 features in one comparison.

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*) The programme used in this study was elaborated in Fortran V language by T. Bille and C. Jepsen, Chr. Rovsing Ltd., Herlev, Denmark.

RESULTS

Common characteristics

All V. anguillarum strains examined were gram-negative rods, catalase and oxidase positive, attacking carbohydrates fermentatively. They were motile by means of a single polar flagellum. The strains were moderately halophilic, reduced nitrate to nitrite and were sensitive to the vibriostatic agent 0/129 (10 μg).

Morphology and motility

The cell size of V. anguillarum was 0.5—1 × 1—3 μm. A varying percentage had a curved axis while others were short coli-like organisms. Most of the cells were very actively motile showing typical vibrations. Occasionally coherent cells formed 'snakes' with a 'Treponema-like motility'. In older cultures coccoid forms were predominant and motility was less pronounced.

Colony morphology and growth in broth

V. anguillarum: The colonies were circular, entire, low convex, shiny, butyrous, and semitransparent. The colony-mass was yellowish in young colonies, more or less brownish in older colonies. The diameter was ½—1 mm after 24 h, approx. 2 mm after 48 h, and 4—5 mm after 1 week of incubation. On blood agar 2 different haemolysis phenomena were observed: (i) haemolysis just beneath the colonies, (ii) a semitransparent zone surrounding the colonies, which after 3 days cleared up starting from the colonies.

After 24 h, V. ordalii was just visible under obligue light, with a diameter of approx. ½ mm after 48 h, growing to 1—2 mm during the first week of incubation. The haemolysis was of the type (i).

Growth in broth inoculated from a broth culture started in the upper part of the tube with a uniform, light turbidity which after 48 h reached the bottom; at this time a fragile pellicle developed in many strains and simultaneously a deposit appeared in the tubes. Pellicle formation was not observed among the V. ordalii strains.

Biochemical tests

Fish pathogenic, environmental and reference strains of V. anguillarum reacted rather similarly in most tests. Differences were, however, noted between V. anguillarum and V. ordalii.

Table 2. Fermentation of carbohydrates and glycosides by Vibrio anguillarum and Vibrio ordalii.

Sugars and sugar alcohols	Vibrio anguillarum							Vibrio ordalii	
	Type strain ATCC 19264	Fish pathogenic strains n = 41		Environmental strains n = 51		Reference strains biotype 1 n = 7		Type strain DF_3K*)	Reference strains biotype 2 n = 2
		+	%+	+	%+	+	%+		+
Trioses									
Glycerol	+	25	61	44	86	2	30	—	—
Glycerol gas	—	—	0	—	0	—	0	—	—
Pentoses									
Arabinose	+	21	51	19	37	4	57	—	—
Xylose	—	—	0	—	0	—	0	—	—
Adonitol	—	—	0	—	0	—	0	—	—
Hexoses									
Glucose	+	+	100	+	100	+	100	+	+
Glucose gas	—	—	0	—	0	—	0	—	—
Dulcitol	—	—	0	—	0	—	0	—	—
Fructose	+	+	100	+	100	+	100	—	+
Galactose	+	40	98	+	100	6	75	—	—
Mannitol	+	+	100	49	96	+	100	—	+
Mannose	+	+	100	+	100	+	100	—	—
Rhamnose	—	—	0	—	0	—	0	—	—
Sorbitol	+	36	88	27	53	6	75	—	—
Disaccharides									
Cellobiose	+	21	51	44	86	5	71	—	—
Inositol	—	—	0	—	0	—	0	—	—
Lactose	—	—	0	15	29	—	0	—	—
Maltose	+	+	100	+	100	+	100	+	+
Melibiose	—	—	0	9	18	—	0	—	—
Sucrose	+	39	95	47	92	+	100	+	+
Trehalose	+	40	98	+	100	+	100	—	—
Trisaccharides									
Melizitose	—	—	0	—	0	—	0	—	—
Raffinose	—	—	0	—	0	—	0	—	—
Polysaccharides									
Cellulose	+	40	98	+	100	2	25	—	—
Dextrin	+	+	100	+	100	+	100	+	1
Glycogen	+	+	100	+	100	+	100	—	—
Inulin	—	—	0	—	0	—	0	—	—
Starch	+	+	100	37	73	+	100	—	—
Glycosides									
Aesculin	—	3	7	8	16	1	13	—	—
Chitin	+	39	95	49	96	7	88	—	—
Salicin	—	3	7	3	6	1	13	—	—
ONPG	+	40	98	50	98	+	100	—	—

*) DF_3K = ATCC 33509.

Fermentation of carbohydrates and glycosides (Table 2). Glucose was attacked fermentatively without gas production. Among the V. anguillarum strains, most strains were positive for the following criteria: Glucose, fructose, galactose, mannitol, mannose, maltose, sucrose, trehalose, dextrin, glucogen, chitin and ONPG. 6 and 7 of the 8 reference strains were positive in galactose and chitin, respectively. A nagative reaction was recorded in xylose, adonitol, dulcitol, rhamnose,inositol, melizitose, raffinose, and inulin.

Except for a few environmental strains, a negative reaction was found in lactose and melibiose. In aesculin and salicin only a few strains were positive and some of these were positive in both of the two glycosides. V. ordalii only attacked glucose, fructose, mannitol, maltose, sucrose, and dextrin; DF_3K was negative in fructose and mannitol.

Activity towards amino acids, proteins and lipids (Table 3). All Vibrio anguillarum strains were positive in arginine, and except for 2 environmental strains with delayed reaction in lysine, they were negative in lysine and or-

Table 3. Activities of Vibrio anguillarum and Vibrio ordalii towards amino acids, proteins and lipids.

	Vibrio anguillarum						Vibrio ordalii	
	Type strain ATCC 19264	Fish pathogenic strains n = 41		Environmental strains n = 51		Reference strains biotype 1 n = 7		Type strain DF_3K	Reference strains biotype 2 n = 2
		+	%+	+	%+	+	%+		+
Amino acids									
Arginine dihydrolase	+	+	100	+	100	+	100	—	—
Lysine decarboxylase	—	—	0	2	4	—	0	—	—
Ornithine decarboxylase	—	—	0	—	0	—	0	—	—
Phenylalanine deaminase	—	—	0	—	0	—	0	—	—
Tryptophane-deaminase (Indole)	+	39	95	47	92	7	88	—	—
Proteins									
Gelatine liquefaction	+	+	100	50	98	+	100	—	+
Hydrolysis of casein	+	4	98	48	94	+	100	—	1
Loeffler serum digestion	+	+	100	46	90	+	100	+	1
Lipids									
Lipase (Tween 80)	+	+	100	48	94	5	71	—	—
Lecithinase	—	25	61	16	31	2	25	—	—

nithine. Generally most strains were actively proteolytic and indole positive. All fish pathogenic V. anguillarum were lipase positive, which was also the case with many environmental and reference strains. Lecithinase occurred with different frequency. Vibrio ordalii was found only to be proteolytic and negative in the other criteria.

Miscellaneous biological activities (Table 4). Besides the positive reactions with regard to catalase, oxidase, nitrate, motility and haemolysis mentioned previously, only DN-ase was a positive criterium for both V. anguillarum and V. ordalii. Negative findings were recorded for V. anguillarum as regards KCN, malonate, urease, H_2S in TSI, pigment formation and swarming. Generally most of the fish pathogens showed a high rate of positive reactions in VP, 2,3-butanediol, citrate (Sim-

Table 4. Miscellaneous biological activities of Vibrio anguillarum and Vibrio ordalii.

	Vibrio anguillarum							Vibrio ordalii	
	Type strain ATCC 19264	Fish pathogenic strains n = 41		Environmental strains n = 51		Reference strains biotype 1 n = 7		Type strain DF_3K	Reference strains biotype 2 n = 2
		+	%+	+	%+	+	%+		+
Catalase	+	+	100	+	100	+	100	+	+
Oxydase	+	+	100	+	100	+	100	+	+
Nitrate	+	+	100	+	100	+	100	+	+
DN-ase	+	+	100	49	96	+	100	+	+
KCN	—	—	0	—	0	—	0	—	—
Malonate	—	—	0	—	0	—	0	—	—
Urease	—	—	0	—	0	—	0	—	—
H_2S in TSI	—	—	0	—	0	—	0	—	—
MR	—	3	7	26	51	1	13	—	—
VP	+	40	98	36	71	6	86	—	—
2,3-butanediol	—	32	78	28	55	4	57	—	—
Citrate (Simmons)	+	39	95	32	63	6	86	—	—
NH_4/glucose medium	+	40	98	49	96	5	71	—	—
Gluconate	+	38	93	26	51	4	57	—	—
Phosphatase	+	2	5	8	16	3	38	+	+
Haemolysin	+	+	100	+	100	+	100	+	+
Pigment	—	—	0	—	0	—	0	—	—
Motility	+	+	100	+	100	+	100	+	+
Swarming	—	—	0	—	0	—	0	—	—
Pellicle formation	+	+	100	39	76	2	25	—	—

mons), NH_4/glucose medium and gluconate, while the environmental strains showed different reactions (except for NH_4/glucose medium).

The type strain and all fish pathogenic isolates produced pellicle, while 76 % of the environmental and 25 % of the reference strains of V. anguillarum showed this phenomenon. The 3 V. ordalii strains behaved very uniformly as regards these criteria. A positive phosphatase reaction was typical, while phosphatase positive isolates were rather rare among the V. anguillarum strains.

Growth conditions (Table 5). The investigated strains must be regarded as mildly halophilic because of their very sparse growth on CLED and in peptone water without NaCl. While V. anguillarum grew faintly in 7 % NaCl, V. ordalii could not grow in 6 % NaCl. Most strains grew at 5°C, but

Table 5. Growth conditions of Vibrio anguillarum and Vibrio ordalii.

	Vibrio anguillarum						Vibrio ordalii	
	Type strain ATCC 19264	Fish pathogenic strains n = 41		Environmental strains n = 51		Reference strains biotype 1 n = 7		Type strain DF_3K	Reference strains biotype 2 n = 2
		+	%+	+	%+	+	%+		+
Growth:									
on CLED	+	—	0	1	2	2	29	—	—
without NaCl	—	33*)	80	43*)	84	3*)	43	—	—
in 6 % NaCl	+	+	100	+	100	3	43	—	—
in 7 % NaCl	—	30*)	73	48*)	94	1*)	14	—	—
at 5°C	—	+	100	46	90	+	100	+	+
at 37°C	+	+	100	+	100	5	71	—	1
at 43°C	—	—	0	—	0	—	0	—	—
on McConkey	+	+	100	50	98	6	86	—	—
on SS agar	—	—	0	—	0	—	0	—	—
on TCBS agar	+	+	100	50	98	6	86	—	—
on Cetrimide agar	—	—	0	—	0	—	0	—	—
at pH 9	+	+	100	+	100	+	100	+	+
Sensitivity to:									
10 µg 0/129	+	+	100	+	100	+	100	+	+
150 µg 0/129	+	+	100	+	100	+	100	+	+
novobiocin	+	+	100	+	100	+	100	+	+
penicillin	—	—	0	—	0	—	0	+	+

*) Faint growth without NaCl and in 7 % NaCl.

none at 43°C. Growth at 37°C was common among V. anguillarum, while only 1 V. ordalii strain grew very moderately at this temperature.

Growth on MacConkey and TCBS agar was fairly typical of V. anguillarum, contrary to V. ordalii. Sensitivity to the vibriostatic agent (0/129), novobiocine, cetrimide, and growth at pH 9 was the same for the 2 species. However, V. anguillarum was resistant to penicillin and V. ordalii sensitive.

DNA base composition (Table 6). The mean values of the G+C mole percentages in DNA from all categories of V. anguillarum ranged from 45.5 to 46, and for V. ordalii the mean value was 48.9.

Table 6. DNA base composition of V. anguillarum and V. ordalii strains.

	Number of strains	Range	Mean	Standard deviation SD
V. anguillarum				
Fish pathogenic	15	45.1—47.1	46.0	0.83
Environmental	8	43.7—47.5	45.5	1.27
Reference	5	45.4—45.9	45.7	0.29
V. ordalii	3	48.3—49.3	48.9	0.57

Numerical taxonomic analysis. Based on matching coefficient analysis, the 103 strains were separated into 2 groups, recognized at the 90 % level. Group 1 comprised the V. anguillarum strains, while group 2 consisted of the 3 V. ordalii strains. Three strains, 775, 1800, and LS174, could not be included in any of these groups, but their similarity level to group 1 was approx. 80. The type strain ATCC 19264 was used with the purpose of separating the 100 V. anguillarum in different phenons. Actually, more than 70 phenons could be demonstrated, which shows a relatively great phenotypical variability among the strains.

DISCUSSION

During the last few years, clinical implications of a variety of Vibrio strains have resulted in the establishment of a rather 1981,long list of new species (*Davis et al.* 1978, *Lee et al.* 1981, *Love et al.* 1981, *Schiewe* 1981, *Hickman et al.* 1982). Among these

V. anguillarum, V. ordalii, and V. damsela represent fish pathogenic organisms (*Love et al.* 1981, *Schiewe* 1981).

It is a general conception that fish contract their infections from environmental micro-organisms (*Snieszko* 1974, *Sindermann* 1981), but of the fish pathogens mentioned above. V. anguillarum is the only one for which environmental data have been presented (*Baross & Liston* 1970, *Evelyn* 1971, *Larsen* 1982, *West & Lee* 1982). The epidemiological significance of such strains has never been stated, as this demands for a register of diagnostic methods including biochemical and serological identification procedures, followed by the demonstration of pathogenicity determinants such as colonization factors (*Mellergaard & Larsen* 1981, *Trust et al.* 1982) and other virulence factors (*Crosa et al.* 1977, *Crosa* 1980).

The available information concerns fish pathogenic strains, but data on environmental strains are equally important if environmental authorities in the future should have an opportunity to use the environmental risk ascribed to these organisms as a justification for including them in monitoring programmes of water qualty (*Larsen* 1982, *Larsen & Jensen* 1982).

For the trained bacteriologist, growth characteristics and motility of V. anguillarum give a rather good indication of its identity. The 'first stage table' of *Cowan* (1974) brings us into the genus Vibrio. This manual, as well as the Manual of Clinical Microbiology (1980), gives information only on the human pathogenic Vibrios, while Bergey's Manual (*Shewan & Véron* 1974) presents some data also on V. anguillarum.

Fermentation of carbohydrates and glycosides

Vibrio anguillarum has a very broad-spectered activity towards different carbohydrates and glucosides but it also capable of utilizing a great variety of other organic compounds as energy or carbon sources (*Baumann et al.* 1978, *Lee et al.* 1981). This broad biochemical capacity might be of ecological significance. In contrast, V. ordalii produces acid from only very few carbohydrates and not from glycosides. Comparing the type strain and the categories of V. anguillarum dealt with in this paper, there are apparently very few differences. ATCC 19264 was positive to glycerol, arabinose, sorbitol and cellobiose, while the other groups of V. anguillarum showed a different reaction. Sorbitol and cellobiose positive isolates were actually found to be pre-

valent in Japan (*Jo et al.* 1979, *Kusuda et al.* 1979). Among the environmental strains, a limited number attacked lactose and melibiose, while all other strains were negative. Lactose positive V. anguillarum has been described in Norway (*Håstein & Smith* 1977) and in Japan (*Kusuda et al.* 1979, *Ezura et al.* 1980). Only *Ransom* (1978) has mentioned melibiose-positive strains.

Apart from the data presented by *Nybelin* (1935) and *Smith* (1969) who used mannitol and sucrose, and by *Håstein & Smith* (1977) who based their biotyping on the following sugars: arabinose, cellobiose, lactose and trehalose, there exists a great deal of biochemical similarity between isolates of V. anguillarum from various parts of the world (*Evelyn* 1971, *Egidius & Andersen* 1977, *Håstein & Smith* 1977, *Larsen & Jensen* 1977, *Baumann et al.* 1978, *Jo et al.* 1979, *Kusuda et al.* 1979, *Muroga et al.* 1979, *Ezura et al.* 1980, *Lee et al.* 1981, *Schiewe* 1981).

On the basis of the present results, arabinose cannot be regarded as a valid criterium for differentiating V. anguillarum and V. fluvialis as claimed by *Blake et al.* (1980).

Activity towards amino acids, proteins and lipids

The classical human-pathogenic Vibrios are generally listed as positive to lysine and ornithine but negative to arginine (*Shewan & Véron* 1974, *Wachsmuth et al.* 1980), while the fish pathogens are frequently opposite in these criteria (*Love et al.* 1981, *Schiewe* 1981). An important step in the separation of V. ordalii is the negative reaction to arginine, which is also typical of V. hollisae, but the latter bacterium differs in other aspects (*Hickman et al.* 1982). Lysine positive V. anguillarum as found among the environmental isolates have been observed by others (*Lee et al.* 1981).

Indole production occurs frequently among the fish pathogenic and environmental strains. Many authors have recorded differences in this criterium which has also been used in different biotyping systems (*Nybelin* 1935, *Håstein & Smith* 1977) and which is of great importance for the separation of V. ordalii (*Schiewe* 1981).

The strains examined were generally proteolytic. A few strains showed some differences in their activity to gelatine, casein and serum, which might indicate some heterogenicity in enzymatic capacity. Lipase and lecithinase activity was a frequent finding among the fish pathogens. *Evelyn* (1971) described

lipase positive and *McCarthy* (1974) lipase and lecithinase positive V. anguillarum, while *Schiewe* (1981) used lipase in the differentiation of V. anguillarum and V. ordalii.

Miscellaneous biological activities

It appears from Table 4 that identical results were recorded with regard to catalase, oxidase, nitrate, DN-ase, KCN, malonate, urease, H_2S, haemolysin, pigment, motility, and swarming; results that are in accordance with most authors listed in this paper. The MR-VP reactions are rather controversial in the literature, which might be ascribed to the methods used (*Farid & Larsen* 1980). Most authors have found a low frequency of MR positive and a high frequency of VP and 2.3-butanediol positive strains. In the present study there was also relatively few MR positive fish pathogenic and reference strains, while most of these were VP positive (ATCC 19264 MR— VP+). It should also be noticed that positive reactions in 2,3-butanediol, citrate (Simmons), NH_4/glucose medium and gluconate were frequent among the fish pathogenic strains. Citrate utilization has often been reported to be negative when using Koser's citrate medium (*Smith* 1961, *Evelyn* 1971, *McCarthy et al.* 1974). *Egidius & Andersen* (1977) listed most Norwegian reference strains as positive, and also as positive in malonate. Most V. anguillarum strains can grow on Simmons citrate agar (*Jo et al.* 1979, *Kusuda et al. Muroga et al.* 1979, *Schiewe* 1981). Furthermore, *Schiewe* (1981) found that growth in Christensen's citrate medium was a common feature. The variability in citrate, VP and indole was characteristic of the isolates described by *Giorgetti & Ceschia* (1982).

Few data exist about V. anguillarum with regard to gluconate and phosphatase. *Kusuda & co-workers* (1979) found 20 of 31 strains positive in gluconate, which is the same level as for the reference strains of V. anguillarum used in the present study. The ratio among fish pathogenic and environmental isolates is respectively higher and lower (Table 4). *McCarthy et al.* (1974) stated that V. anguillarum is phosphatase positive, and *Lee et al.* (1981) found 10 of 11 strains positive for phosphatase. In the present study, the type strain was phosphatase positive, while this was the case with a relatively small percentage of the field strains. On the other hand, V. ordalii was phosphatase positive, but negative in MR, VP, 2,3-butanediol, citrate, NH_4/glucose and gluconate.

Pellicle formation was recorded for all fish pathogenic V. anguillarum and for most of the environmental isolates, but for only 2 reference strains and for no V. ordalii strains. Spontaneous loss of this property has been observed after subcultivation, and this may have contributed to the low frequency of this trait among the reference strains. *Kusuda et al.* (1979) used this property in characterization of 3 sub-groups of V. anguillarum.

Growth conditions

The present results confirm the general conception that V. anguillarum is a moderately halophilic organism. Occassionally a few strains will grow with no NaCl added to the media, or with NaCl present in trace amounts (*Nishibuchi & Muroga* 1977, *Lee et al.* 1981). This must be ascribed to a pronounced power of adaption which may also be responsible for growth at higher NaCl concentrations (*Nishibuchi & Muroga* 1977). The influence of local climatic conditions on temperature range (*Olafson et al.* 1981) may explain conflicting statements about growth at 5°C and 37°C (*Smith* 1961, *Evelyn* 1971, *Ransom* 1978, *Jo et al.* 1979, *Kusuda et al.* 1979, *Muroga et al.* 1979, *Ezura et al.* 1980, *Schiewe* 1980). The main conclusion is, however, that V. anguillarum will grow neither without NaCl nor in 7 % NaCl, but that it will grow when the concentration is 6 %. Growth occurs at 5°C and 37°C, but not at 43°C, which is also characteristic of ATCC 19264.

Information about growth on different bacteriological media is sparse (cf. *Kusuda et al.* 1979, *Lee et al.* 1981) although indicative and selective media are important for the isolation of V. anguillarum for diagnostic purposes and in ecological studies.

Both McConkey agar and TCBS are suitable media for V. anguillarum since most strains can grow on these media (Table 5). None of these media permitted growth of V. ordalii.

Both V. anguillarum and V. ordalii were found sensitive to 10 and 150 μg of 0/129. These concentrations have been considered ideal for distinguishing specied within Vibrionaceae, together with novobiocin (*Lee et al.* 1978). It should, however, be added that the use of drugs in treatment of vibriosis in fish has resulted in a development of cross-resistance to 0/129 (*Muroga et al.* 1979). V. anguillarum was resistant to penicillin, V. ordalii sensitive.

DNA base composition

The G+C mole % of the present V. anguillarum strains ranged from 45.5 to 46, while most other authors state a level of approx. 43—45 (*Cisar & Fryer* 1969, *Shewan & Véron* 1974, *Baumann et al.* 1978, *Schiewe* 1981). *Ezura & co-workers* (1980) found the same value for their phenon 2, while phenon 1 varied from 43 to 46. For V. ordalii the mean value was found to be 48.9.

Numerical taxonomic analysis

Two groups were clearly separated when the biochemical results were computerized. Group 1 consisting of most V. anguillarum strains and group 2 comprising the 3 V. ordalii. Three American V. anguillarum strains did not fall within these groups, but had a similarity to group 1 of approx. 80. These strains, however, showed such mutually different reactions that they could not be incorporated into a new specific group (species). A considerable phenotypical variability was found among V. anguillarum strains when using the criteria selected for this study. Future genotypical research may perhaps contribute to a simplification of the system.

CONCLUSION

The general conclusion is that both the environmental and the fish pathogenic Danish Vibrio anguillarum strains belong to Vibrio anguillarum, biotype 1, which shows rather broad-spectered biochemical activities, unlike the more fastidious Vibrio anguillarum, biotype 2, Vibrio ordalii.

No reason was found for establishing new biotypes within these strains, and the minimal differences between the environmental and the fish pathogenic strains make it possible that the pathogenic strains are recruited from the environment. This possibility should be considered in further investigations on serotypes, agglutination types, and in the presence of specific plasmids responsible for pathogenicity.

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SAMMENDRAG

Vibrio anguillarum: Et komparativt studium af fiskepatogene, miljø- og referencestammer.

En undersøgelse af 41 fiske-patogene og 51 miljøstammer af Vibrio anguillarum isoleret fra danske kystnære områder og 8 referencestammer af Vibrio anguillarum, biotype 1, viste, at disse stammer tilfælles havde en række biokemiske aktiviteter overfor kulhydrater, aminosyrer, proteiner og lipider.

De udviste ligeledes ret ensartede vækstegenskaber med hensyn til salinitet, temperatur, antibiotika samt vækst på specifikke substrater.

En computer-analyse af disse stammer og 3 Vibrio anguillarum, biotype 2 (Vibrio ordalii)-stammer, viste, at de danske isolater alle hørte til Vibrio anguillarum, biotype 1. Den store fænotypiske variation stammerne imellem indebar, at en etablering af nye biotyper eller phenons kun ville bibringe forvirring.

G+C indholdet i DNA for Vibrio anguillarum, biotype 1-stammerne af forskellig oprindelse varierede i middelværdierne fra 45,5 til 46, mens middelværdien for Vibrio ordalii var 48,9.

Den biokemisk aktive Vibrio anguillarum fandtes bedre egnet til miljøundersøgelser end den mere kræsne Vibrio ordalii, der ikke kunne vokse på de anvendte selektive og indikative substrater.

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