Acta vet. scand. 1981, *22*, 331—343.

From the Central Laboratory, Directorate of Fisheries, Bergen, Norway.

ISOLATION AND CHARACTERIZATION OF VIBRIO ALGINOLYTICUS AND VIBRIO PARAHAEMOLYTICUS FROM THE NORWEGIAN COASTAL ENVIRONMENT

By

Jan Gjerde and *Bjarne Böe*

GJERDE, JAN and BJARNE BÖE: *Isolation and characterization of Vibrio alginolyticus and Vibrio parahaemolyticus from the Norwegian coastal environment.* Acta vet. scand. 1981, *22*, 331—343. — Strains of Vibrio alginolyticus were regularly isolated from mussels, fish, bottom sediment and seawater from April to October. Vibrio parahaemolyticus was isolated occasionally in samples from mussels and bottom sediment in July and August. None of the species were detected in the cold season.

Isolated strains were characterized by growth requirement, morphological characteristics and biochemical tests. In addition the cellular fatty acid composition was determined and compared with standard strains from the family Vibrionaceae.

With the exception of some biochemical reactions which distinguish Vibrio alginolyticus from Vibrio parahaemolyticus, growth requirement, morphological characteristics and biochemical reactions are similar for these strains.

The close relation between Vibrio alginolyticus and Vibrio parahaemolyticus was also revealed by cluster analyses of fatty acid patterns which combined these two species into one cluster which, however, was clearly separated from the standard strains of Vibrio anguillarum.

Vibrio parahaemolyticus; Vibrio alginolyticus; fatty acid composition.

Vibrio parahaemolyticus and Vibrio alginolyticus, also called Vibrio parahaemolyticus biotype 2 (*Sakazaki* 1968), have frequently been isolated from seawater, fish, bottom sediments and mussels (*Horie et al.* 1967, *Baross & Liston* 1968, *Liston & Baross* 1973). In areas with warmer climatic conditions Vibrio parahaemolyticus has on several occasions caused food poisoning

following seafood consumption (*Molenda et al.* 1972, *Barker* 1974), and has been the cause of wound infections (*Twedt et al.* 1969).

Vibrio parahaemolyticus has also been isolated in areas with colder waters usually in the summer season (*Kristensen* 1974, *Vasconcelos et al.* 1975, *Ayres* 1978), but recently investigators have also detected the organisms in winter time at a temperature as low as 4.4°C (*van den Broek et al.* 1979).

This work is concerned with the isolation of Vibrio parahaemolyticus and Vibrio alginolyticus in the marine environment from the west coast of Norway at 60° latitude. Samples of mussels, seawater, bottom sediments and fish have regularly been investigated for the occurrence of Vibrio parahaemolyticus and Vibrio alginolyticus during a period of one year.

MATERIALS AND METHODS

Bacterial cultures

Organisms used were: Vibrio parahaemolyticus ATCC 17802, Vibrio alginolyticus NCMB 1903 and Vibrio anguillarum NCMB 6, Vibrio anguillarum V 962, V 767, V 768, V 873 and A 272 from the Culture Collection, National Veterinary Institute, Oslo, Norway.

Collection of samples

Samples of mussels, seawater, and bottom sediments from two unpolluted areas at the shore were obtained during a period of one year at least once per month with biweekly samples from July, August and September. Temperature measurements were made of the overlying water at the time the samples were obtained. A total of 48 samples of each category were examined. Samples were also obtained from newly landed cod, mackerel, and saithe, caught in the North Sea and rainbow trout from fish farms, a total of 40 fishes.

Isolation procedure

All samples were examined the same day as obtained.

The samples of mussels were washed and shucked and the content of 10 mussels were homogenized in an Ultra Turrax for 15 s. Ten grams of the homogenized mussel content were inoculated into 100 ml of Nutrient broth (Merck 5443) with 5 % NaCl

(w/v) (enrichment medium) at pH 7.5, and incubated at 37°C for 18—24 h. The samples of bottom sediments were prepared in a similar way, with 10 g mixed with 100 ml of the enrichment medium. One hundred ml of the water samples were filtered through a millipore filter 0.45 mμ pore size and the filter was placed in the enrichment medium.

From the fish 10 g of the gills, 10 g of intestinal content and 10 cm^2 of the skin were separately inoculated into 100 ml of the enrichment medium.

After the incubation period a loopful was streaked on TCBS agar (Merck 10263) and the plates were incubated at 37°C for 18—24 h. After the incubation period round green or yellow colonies 2—4 mm in diameter on the plates were respectively considered as presumptive Vibrio parahaemolyticus and Vibrio alginolyticus.

Identification procedure

All media used in the identification procedure were supplemented with NaCl (2 % w/v).

Presumptive Vibrio parahaemolyticus and Vibrio alginolyticus isolated from TCBS agar were restreaked on Nutrient agar (Merck 5450). The strains were checked for purity and reinoculated in Nutrient broth. Cultures of 18 h were used for further identification work. A total of 80 strains were examined, 15 presumptive Vibrio parahaemolyticus and 65 presumptive Vibrio alginolyticus.

Morphologcal characteristics

After 18 h of incubation at 25°C in Nutrient broth, the organisms were examined by phase-contrast microscopy for determination of cell morphology and motility. The organisms were also Gram stained.

Biochemical tests

The biochemical tests performed and media used are listed in Table 1.

Growth requirement

The requirement for salt was established by inoculating a drop of 18 h culture from Nutrient broth to tubes with Nutrient

Table 1. Description of biochemical tests employed in the identification procedure.

Test	Growth medium and test reagents supplied by Merck	Incubation period (days)
Oxidase	Nutrient agar. Test reagent: 1 % tetramethyl-p-phenylenediamine	1
Indole production	Sim medium. Test reagent: Kovacs indole reagent	2
Methyl red and Voges Proskauer	M.R.V.P. medium. Test reagent: 5 % Naphthol in abs. ethanol solution 40 % Potassium hydroxide 0.04 g Methylred to 60 ml abs. ethanol	3
Citrate utilization	Koser Citrate medium	2
Nitrate reduction	Nitrate broth. Test reagent: Griess-Ilosvays reagent	2
Casein hydrolysis	Calcium-caseinat Agar	2
Urease production	Christensens Urea Agar	2
Lysine decarboxylase	Lysine decarboxylase broth	2
Ornithine decarboxylase	Ornithine decarboxylase-Arginine dihydrolase broth	2
Arginine dihydrolase	Ornithine decarboxylase-Arginine dihydrolase broth	2
Hydrogen Sulphide production	Sim medium	2
Acid from Arabinose	OF basal medium according to Hugh and Leifson with 1 % wt/vol. concentration of the compound	2
Acid from Inositol	— „ —	2
Acid from Xylose	— „ —	2
Acid from Rhamnose	— „ —	2
Acid from Fructose	— „ —	2
Acid from Glucose	— „ —	2
Acid from Sucrose	— „ —	2
Acid from Trehalose	— „ —	2
Acid from Mannose	— „ —	2
Acid from Dulcitol	— „ —	2
Hydrolysis of starch		2

broth without NaCl, and Nutrient broth with 7 % and 10 % NaCl. The tubes were incubated at 25°C for 72 h, and the growth was determined by comparing the tubes with an uninoculated medium. The ability to grow at 42°C was established in a similar way in Nutrient broth with 2 % NaCl (w/v).

Antibiotic reactions

The reaction to antibiotics was determined by placing a disc of Bacto antibiotic sensitivity dishes on Nutrient agar plates (Merck 5450) streaked with the test organism. The strains were tested for susceptibility to chlortetracycline (10 mg), novobiocin (5 mg), Polymyxin B (50 units cons.), penicillin (2 units cons.), chloramphenicol (5 mg) and pteridine 0/129 (150 μg) after incubation for 24 h at 37°C.

Fatty acid analyses

For further identification randomly selected strains were analysed for fatty acid composition of whole cells and compared to standard strains of Vibrio parahaemolyticus, Vibrio alginolyticus and Vibrio anguillarum. A total of 11 presumptive Vibrio alginolyticus and 2 presumptive Vibrio parahaemolyticus were analysed.

After incubation in Nutrient broth cells were removed and transferred to a test tube containing 5 ml of 5 % (w/v) NaOH in 50 % (v/v) aqueous methanol. The fatty acids liberated by saponification were methylated with BCl_3/methanol, according to *Moss et al.* (1974).

Gas-liquid chromatography (g.l.c.) and g.l.c./mass spectrometry were performed as previously described (*Böe & Gjerde* 1980). The gas chromatogram of fatty acid methyl esters (FAMEs) from Vibrio parahaemolyticus ATCC 17802 is given in Fig. 1.

Numerical analyses were based on twelve FAMEs whose areas relative to 16:0 were greater than 10 %, Nos 2, 4, 15, 16, and 17 being excluded (Table 2).

The raw data were logarithmically transformed, similarity indexes calculated and cluster analysis performed as previously described, based on the unweighted pair group method (*Böe & Gjerde* 1980).

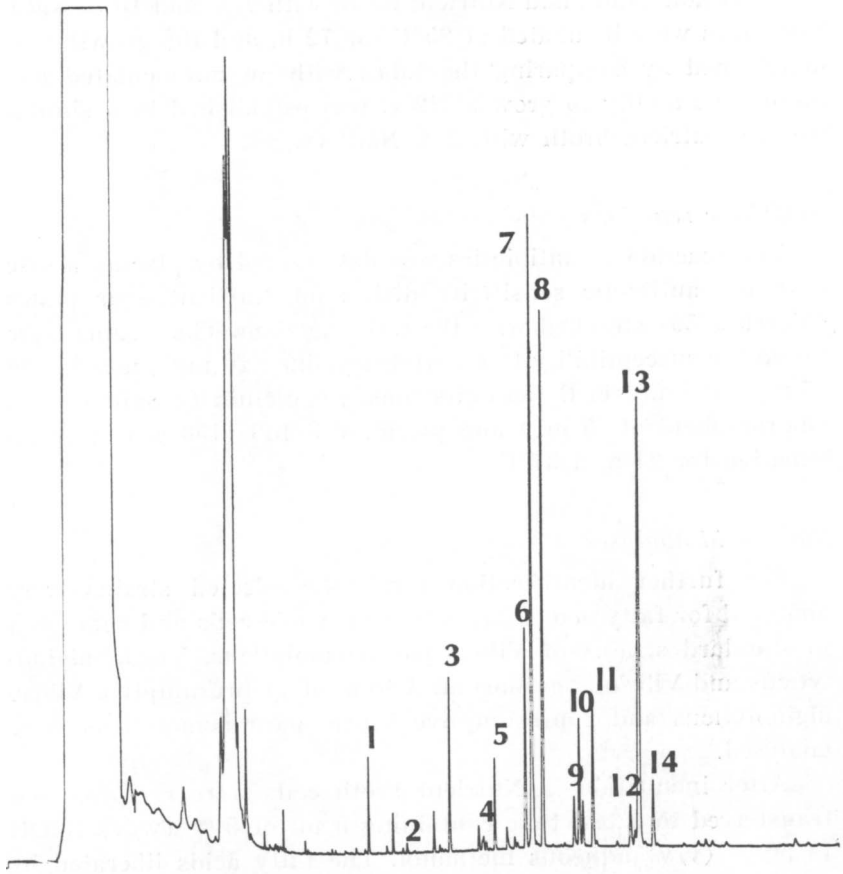

Figure 1. Gas chromatogram of fatty acid methyl esters (FAME) from Vibrio parahaemolyticus ATCC 17802. FAME numbers as in Table 2.

RESULTS AND DISCUSSION

The presumptive Vibrio alginolyticus and Vibrio parahaemolyticus from TCBS agar were found to be short to medium rods with rounded ends. Most of them had curved axis, but some examples of straight axis were also observed. All strains were actively motile and Gram negative.

Table 3 shows the results of the biochemical tests, and of the growth requirement and sensitivity to antibiotics tests.

The isolated strains of presumptive Vibrio alginolyticus reacted identically in the tests except for the methyl red reaction, where 10 strains were found to be negative. The presumptive

Table 2. Fatty acid methyl esters from Vibrionaceae.

Strain	FAME* No.	Relative peak area																
		12:0 1	13:0 2	14:0 3	a 15:0 4	15:0 5	br 16:0 6	16:1 7	16:0 8	a 17:0 9	17:0△ 10	17:0 11	br 18:0 12	18:1 13	18:0 14	19:0△ 15	19:0 16	20:0 17
1. V. anguillarum V962		14	1	23	1	2	42	183	100	4	1	3	9	91	6	1	0	0
2. V. anguillarum V767		10	1	24	1	3	38	168	100	3	1	2	3	69	6	1	0	0
3. V. anguillarum V768		23	1	29	2	4	14	210	100	8	2	5	5	114	8	1	1	0
4. V. anguillarum V873		19	1	30	1	3	34	193	100	4	1	3	2	76	7	1	0	0
5. V. anguillarum A272		24	1	31	2	3	40	183	100	4	2	3	10	67	8	0	0	0
6. V. alginolyticus 1		17	4	32	1	17	6	281	100	3	20	15	1	118	3	1	0	0
7. V. alginolyticus 2		21	3	38	1	25	0	280	100	3	28	23	1	163	4	1	0	0
8. V. alginolyticus 3		20	3	38	1	25	0	281	100	3	29	22	1	162	4	1	0	0
9. V. alginolyticus 4		22	2	57	3	19	5	269	100	4	22	18	1	109	4	1	0	0
10. V. alginolyticus 5		30	4	47	1	28	4	280	100	3	27	20	1	131	4	1	0	0
11. V. alginolyticus 6		30	2	43	3	22	6	333	100	3	25	16	1	128	0	7	0	0
12. V. alginolyticus 7		6	1	27	2	15	10	163	100	11	18	21	3	83	9	1	0	0
13. V. alginolyticus 8		6	1	25	1	12	7	170	100	9	14	17	2	74	7	0	0	0
14. V. alginolyticus 9		9	3	28	2	11	7	165	100	14	6	16	2	80	7	0	0	0
15. V. alginolyticus 10		11	1	34	2	15	5	167	100	8	10	13	1	59	5	0	0	0
16. V. alginolyticus 11		9	1	35	2	17	0	175	100	9	11	15	1	59	5	0	0	0
17. V. parahaemolyticus 1		8	1	32	2	11	14	114	100	7	11	13	0	70	12	0	0	0
18. V. parahaemolyticus 2		7	1	28	2	11	13	111	100	6	11	13	0	69	11	0	0	0
19. V. alginolyticus NCMB 1903		21	1	36	2	10	22	169	100	8	13	13	5	88	7	0	1	0
20. V. anguillarum NCMB 6		15	0	22	1	2	18	198	100	4	2	2	3	73	5	0	0	0
21. V. parahaemolyticus ATCC 17802		13	1	26	3	10	20	122	100	10	17	22	7	105	16	0	1	0

* In FAME designations, the first number gives the number of carbon atoms in the fatty acid, and the second, the number of double bonds: a designates anteiso branches; br, a branched chain, for which no standards were available (equivalent chain lengths were 15:6 for FAME no. 6 and 17:6 for FAME no. 12); △, the presence of a cyclopropane ring.

Table 3. Test response of 15 presumptive Vibrio parahaemolyticus and 65 presumptive Vibrio alginolyticus isolated from marine material.

Test	Vibrio alginolyticus	Vibrio parahaemolyticus	
		Group 1 5 strains	Group 2 10 strains
Oxidase	+	+	+
Indole	+	+	+
Methyl red	55	+	+
Voges Proskauer	+	—	+
Citrate	—	+	+
Nitrate reduction	+	+	+
Casein hydrolysis	+	+	+
Urease	—	+	+
Lysine decarboxylase	+	+	+
Ornithine decarboxylase	+	+	+
Arginine decarboxylase	—	—	—
H_2S	—	—	—
Arabinose	—	+	+
Inositol	—	—	—
Xylose	—	—	—
Rhamnose	—	—	—
Fructose	+	+	+
Glucose	+	+	+
Sucrose	+	—	—
Trehalose	+	+	+
Mannose	+	+	+
Dulcitol	+	—	—
Starch	+	+	+
Novobiocin sensitivity	+	+	+
Chlortetracycline	+	+	+
Polymyxin B	+	+	+
Penicillin	—	—	—
Chloramphenicol	+	+	+
Pteridine 0/129	+	+	+
Growth without added NaCl	—	—	—
Growth with 7 % NaCl	+	+	+
Growth with 10 % NaCl	+	—	+
Growth at 42°C	+	+	+

+: all strains positive in the test; —: all strains negative in the test; Figures: number of strains positive in the test.

Vibrio parahaemolyticus did not react identical in all biochemical tests, and were accordingly placed in two groups.

For five of the presumptive Vibrio parahaemolyticus (group 1) the results of the biochemical tests were identical to those reported earlier (*Sakazaki* 1973, *Shewan & Veron* 1974).

Ten strains of presumptive Vibrio parahaemolyticus (group 2) gave a weak positive Voges Proskauer reaction and grew in Nutrient broth with 10 % NaCl. Though it is stated that true Vibrio parahaemolyticus are negative in the Voges Proskauer reaction and do not grow in media with 10 % NaCl, the reliability of these tests have been disputed (*Kampelmacher et al.* 1972, *van den Broek et al.* 1979).

Isolation of presumptive Vibrio parahaemolyticus from seawater has shown that such strains can be misidentified as Vibrio parahaemolyticus if only a few standard bacteriological tests for identification are done (*Kaneko & Colwell* 1974). Analyses of fatty acid composition of whole cells were therefore performed as an aid in the identification besides the biochemical tests procedure (Table 2).

The phenogram based on the fatty acid analyses is given in Fig. 2. All the strains of Vibrio anguillarum form one tight cluster, while the presumptive Vibrio alginolyticus and Vibrio

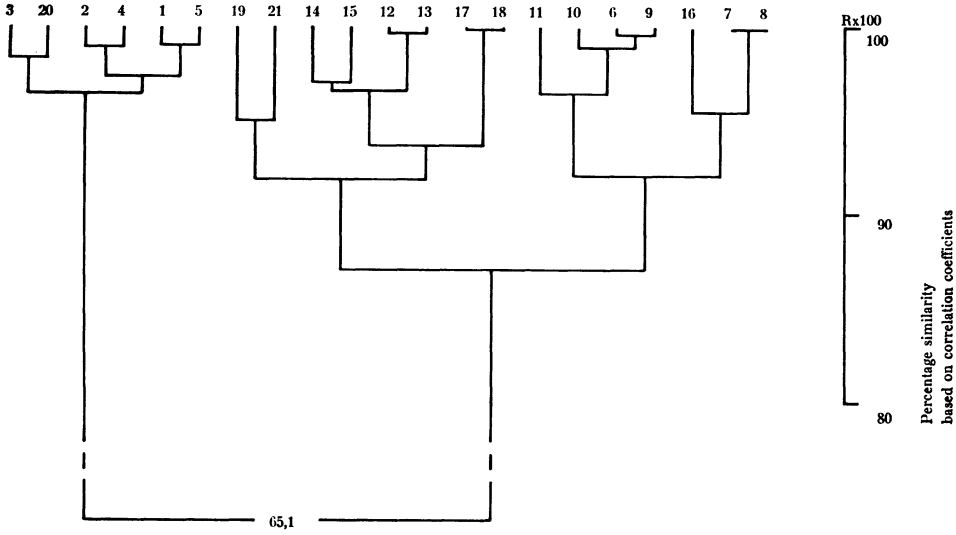

Figure 2. Phenogram of Vibrionaceae based on 12 fatty acid methyl esters. Strain numbers as in Table 2.

Table 4. Incidence of V. parahaemolyticus and V. alginolyticus in marine material.

Sampling date	Temp.	Material	Number of samples	V. alginolyticus	V. parahaemolyticus
April	6°C	Mussel	4	2	0
		Seawater	4	1	0
		Bottom sediment	4	3	0
		Skin	2	0	0
		Gills	2	2	0
		Intestine	2	2	0
May	13°C	Mussel	4	3	0
		Seawater	4	2	0
		Bottom sediment	4	4	0
		Skin	5	0	0
		Gills	5	4	0
		Intestine	5	4	0
June	13°C	Mussel	4	4	0
		Seawater	4	3	0
		Bottom sediment	4	4	0
		Skin	5	1	0
		Gills	5	4	0
		Intestine	5	4	0
July	16°C	Mussel	8	8	3
		Seawater	8	5	0
		Bottom sediment	8	8	3
		Skin	8	2	0
		Gills	8	7	0
		Intestine	8	7	0
August	15°C	Mussel	8	8	2
		Seawater	8	7	0
		Bottom sediment	8	8	1
		Skin	5	4	0
		Gills	5	5	0
		Intestine	5	5	0
September	10°C	Mussel	8	7	0
		Seawater	8	5	0
		Bottom sediment	8	7	0
		Skin	8	1	0
		Gills	8	4	0
		Intestine	8	4	0
October	8°C	Mussel	4	1	0
		Seawater	4	0	0
		Bottom sediment	4	1	0
		Skin	1	0	0
		Gills	1	0	0
		Intestine	1	1	0
November	6°C	Mussel	4	1	0
		Seawater	4	0	0
		Bottom sediment	4	1	0
		Skin	1	0	0
		Gills	1	0	0
		Intestine	1	0	0

parahaemolyticus combine with the standard strains of Vibrio alginolyticus and Vibrio parahaemolyticus into another cluster. The two clusters are connected at a rather low level of R = 0.65. A closely similar phenogram was obtained when the similarity index was based on euclidean distances (*Böe & Gjerde* 1980) instead of correlation coefficients.

Based on the morphological examination, response in the biochemical reactions and composition of fatty acids in whole cells it is concluded that the isolated organisms on TCBS agar are identified as Vibrio parahaemolyticus and Vibrio alginolyticus.

Table 4 shows the incidence of Vibrio parahaemolyticus and Vibrio alginolyticus from the marine environment and fish species. The organisms were not found in the winter from the beginning of November till the end of March.

From April to October Vibrio alginolyticus was isolated in a number of cases. Vibrio parahaemolyticus is also found to exist in the environment, but is only isolated occasionally in July and August. However, the TCBS plates were frequently overgrown with the yellow colonies of Vibrio alginolyticus which may cover the green colonies of presumptive Vibrio parahaemolyticus and reduce the frequency of detection of the latter. Occasionally small yellow colonies were observed on the TCBS plates. By microscopic examination and Gram staining, these colonies were found to be Gram+ cocci and were not further examined.

Since sea fish and mussels are normally subject to contamination with Vibrio parahaemolyticus it must be expected that the bacteria can be found in food from marine sources. However, a number of vibrio cells are needed to yield clinical symptoms in man (*Sakazaki et al.* 1979). Therefore with normal hygienic precautions taken in fish handling, these organisms do not seem to pose a pressing public health problem in cold climatic areas.

REFERENCES

Ayres, P. A.: The distribution of Vibrio parahaemolyticus in British coastal waters. Report of a collaborative study 1975—76. J. Hyg. 1978, *80*, 281—294.

Barker, W. H.: Vibrio parahaemolyticus outbreaks in the United States. Lancet 1974, *I*, 551—554.

Baross, I. & I. Liston: Isolation of Vibrio parahaemolyticus from the Northwest Pacific. Nature (Lond.) 1968, *217*, 1263—1264.

Böe, B. & J. Gjerde: Fatty acid patterns in the classification of some representatives of the families Enterobacteriaceae and Vibrionaceae. J. gen. Microbiol. 1980, *116*, 41—49.

Horie, S., K. Saheki & M. Okusumi: Quantitative enumeration of Vibrio parahaemolyticus in the sea and estuarine waters. Bull. Jap. Soc. Scient. Fish. 1967, *33*, 126—130.

Kaneko, T. & R. R. Colwell: Distribution of Vibrio parahaemolyticus and related organisms in the Atlantic Ocean off South Carolina and Georgia. Appl. Microbiol. 1974, *28*, 1009—1017.

Kampelmacher, E. H. & L. M. Van Noorle Jansen: A survey of the occurrence of Vibrio parahaemolyticus and Vibrio alginolyticus on mussels and oysters and in estuarine waters in the Netherlands. J. appl. Bact. 1972, *35*, 431—438.

Kristensen, K. K.: The occurrence of Vibrio parahaemolyticus and Vibrio alginolyticus in the sound. Nord. Vet.-Med. 1974, *22*, 188—196.

Liston, J. & J. Baross: Distribution of Vibrio parahaemolyticus in the natural environment. J. Milk Food Technol. 1973, *36*, 113—117.

Molenda, J. R., W. G. Johnsen, M. Fishbein, B. Wentz, J. Mehlman & T. A. Dadisman: Vibrio parahaemolyticus gastroenteritis in Maryland. Laboratory aspects. Appl. Microbiol. 1972, *24*, 444—448.

Moss, C. W., M. A. Lambert & W. H. Merwin: Comparison of rapid methods for analysis of bacterial fatty acids. Appl. Microbiol. 1974, *28*, 80—85.

Sakazaki, R. T.: Proposal of Vibrio alginolyticus for the biotype 2 of Vibrio parahaemolyticus. Jap. J. med. Sci. Biol. 1968, *21*, 349—362.

Sakazaki, R.: Recent trends of Vibrio parahaemolyticus as causative agent of food poisoning. *In* The Microbiological Safety of Food. B. C. Hobbs & J. H. B. Christian (eds.). Academic Press, London & New York 1973, pp. 19—30.

Sakazaki, R. T., K. Karashimada, S. Yuda, S. Sakai, Y. Asakawa, M. Yamazaki, H. Nakanishi, K. Kobayashi, T. Nishio, H. Okozaki, T. Doke, T. Shimada & K. Tamura: Enumeration of, and hygienic standard of food safety for Vibrio parahaemolyticus. Arch. Lebensmittel-Hyg. 1979, *30*, 103—106.

Shewan, J. M. & M. Veron: Vibrio. *In* Bergeys Manual of Determinative Bacteriology 8th edn. Buchanan, R. E. & N. E. Gibbons (eds.). The Williams & Wilkins Co. Baltimore 1974, pp. 340—345.

Twedt, R. M., P. L. Spaulding & H. E. Hall: Morphological cultural, biochemical and serological comparison of Japanese strains of Vibrio parahaemolyticus with related cultures isolated in the United States. J. Bact. 1969, *98*, 511—518.

Van den Broek, M. J. M., D. A. A. Mossel & A. E. Eggenkamp: Occurrence of Vibrio parahaemolyticus in Dutch mussels. Appl. environ. Microbiol. 1979, *37*, 438—442.

Vasconcelos, G. J., W. J. Stang & R. H. Laidlaw: Isolation of Vibrio parahaemolyticus and Vibrio alginolyticus from estuarine areas of Southeastern Alaska. Appl. Microbiol. 1975, *29*, 557—559.

SAMMENDRAG

Isolering og karakterisering av Vibrio alginolyticus og Vibrio parahaemolyticus fra norsk kystområde.

Vibrio alginolyticus ble regelmessig påvist i prøver fra blåskjell, fisk, bunnsediment og sjøvann i perioden fra april til oktober. Vibrio parahaemolyticus ble bare sporadisk påvist i prøver fra blåskjell og bunnsediment i juli og august. Ingen av bakteriene ble funnet i den kaldeste årstid.

Bakteriene ble identifisert på grunnlag av vekstkrav, morfologiske egenskaper og biokjemiske reaksjoner. I tillegg ble det utført undersøkelser av fettsyremønsteret som ble sammenliknet med standardbakterier fra familien Vibrionaceae.

Med unntak av enkelte biokjemiske reaksjoner som skiller Vibrio alginolyticus fra Vibrio parahaemolyticus, er vekstkrav, morfologiske egenskaper og biokjemiske reaksjoner hos disse bakteriene svært like.

Clusteranalyse av fettsyresammensetningen skilte ikke Vibrio alginolyticus fra Vibrio parahaemolyticus. Disse bakteriene dannet en gruppe som imidlertid skilte seg tydelig fra en nærbeslektet art Vibrio anguillarum.

(Received March 26, 1981).

Reprints may be requested from: Jan Gjerde, the Central Laboratory, Directorate of Fisheries, P.O. Box 185, N-5001 Bergen, Norway.