HELGOLÄNDER MEERESUNTERSUCHUNGEN
Helgoländer Meeresunters. 36, 357–373 (1983)

Taxonomic investigations of bacteriophage sensitive bacteria isolated from marine waters

K. Moebus & H. Nattkemper

Biologische Anstalt Helgoland (Meeresstation);
D-2192 Helgoland, Federal Republic of Germany

ABSTRACT: Based on 28 criteria the taxonomy of 366 phage sensitive bacterial strains isolated from marine waters (Atlantic between European continental shelf and Sargasso Sea, Bay of Biscay, North Sea near Helgoland) was investigated. Seventy-eight phage-intensity strains derived from the same Atlantic Ocean regions as the sensitive ones were tested for comparison. While in the latter considerable diversity was observed, the results obtained with the phage-sensitive bacteria are characterized by stupendous uniformity. 362 of the 366 strains are assigned to the family Vibrionaceae, some 280 of which belong to the genus *Vibrio*. As discussed, this taxonomic uniformity among the phage-sensitive bacteria is assumed to be an artifact mainly caused by the type of enrichment culture employed for the isolation of all but a few bacteriophage strains used and, to a lesser degree, by characteristics of the bacterial populations encountered.

INTRODUCTION

Although some progress has been made since about 1970, the information available on marine bacteriophages and their host bacteria is still scant. Spencer (1955, 1960) was the first who reported the isolation of 7 truly marine phages from North Sea water taken 10 miles off the Scottish coast which are active on strains of *Photobacterium phosphoreum* (1), *Pseudomonas* sp. (3), *Cytophaga* sp. (1), and *Flavobacterium* sp. (2). One of the last mentioned strains was later identified as another *Cytophaga* sp. (Colwell et al., 1966). Chaina (1965) isolated 629 bacterial strains from water and mud collected at various depths in the Indian Ocean 10 of which were sensitive to bacteriophages derived from the same samples: *Pseudomonas* sp. (5), *Flavobacterium* sp. (2), *Achromobacter* sp. (2), and *Vibrio* sp. (1). Johnson (1968) isolated another *Vibrio* sp.-phage system from an Indian Ocean mud sample collected at a depth of more than 3000 m. In the same year, Wiebe & Liston (1968) described a phage isolated from North Pacific Ocean sediment (depth 825 m) active on *Aeromonas* sp., and Espejo & Canelo (1968) reported the characteristics of a lipid-containing phage isolated from water taken 1 mile off the Chilean coast which infects bacteria tentatively classified as *Pseudomonadae*. Hidaka (1971, 1973) and Hidaka & Fujimura (1971) described phages isolated from sediment and water samples collected from the Pacific south of Kyushu, Japan, which infect strains of *Vibrio* sp. (14), *Pseudomonas* sp. (9), *Flavobacterium* sp. (4), *Achromobacter* sp. (3), and

Aeromonas sp. (2). In 1977, Hidaka reported the isolation of 7 phages from water samples collected in the southwestern Pacific at depths ranging from 1 to 200 m which are lytic for *Vibrio* sp. (3), *Pseudomonas* sp. (2), *Photobacterium* sp. (1), and *Lucibacterium* sp. (1).

Other reports are concerned with phage-host systems isolated from inshore or estuarine environments. Smith & Krüger (1955) derived a *Vibrio* sp.-phage system from a mud sample taken in San Francisco Bay. Carlucci & Pramer (1960) used *Serratia marinorubra* to enrich phages from seawater collected about 200 m offshore in New Jersey. Both the phages and their host bacteria did not require sea salts. Ahrens (1971) investigated the distribution of phages infective for the genus *Agrobacterium* in the Baltic Sea and observed a decrease in phage numbers at salinities below 8‰. In 1972, Stevenson & Albright characterized a phage attacking *Cytophaga* sp. which was isolated from water taken in the intertidal zone in British Columbia. Phages infecting luminous bacteria (*Photobacterium fischeri, Beneckea harveyi*) were isolated by Hastings et al. (1961) and Keynan et al. (1974), respectively, from samples collected at Woods Hole, Mass. (USA). Kakimoto & Nagatomi (1972) isolated 6 phages attacking *Vibrio* sp. (2), *Pseudomonas* sp. (3), and one unidentified species from water taken in Kinko Bay, Japan. Zachary (1974) described several morphologically different phages infecting *Beneckea natriegens* which were limited to brackish and marine waters collected in coastal salt marshes. According to Baumann et al. (1980), the genus *Beneckea* is abolished and its members are assigned to the genus *Vibrio*. Reports on phages lytic for *Vibrio parahaemolyticus* were published by Nakanishi et al. (1966), Sklarow et al. (1973), and Baross et al. (1978).

Moebus (1980) reported the isolation of some 250 bacteriophage strains from Atlantic Ocean water samples, and in 1981 Moebus & Nattkemper described the results of phage-host cross-reaction tests performed with 774 bacterial and 298 bacteriophage strains derived from water samples collected from the Atlantic between the European continental shelf and the Sargasso Sea as well as from the North Sea near Helgoland. As revealed by phage sensitivy patterns, the majority of the Atlantic Ocean bacteria could be grouped into 14 clusters of related strains (Moebus & Nattkemper, 1981; Moebus, 1983). To complement these findings with basic information on the taxonomy of the phage-sensitive bacteria, 444 strains were investigated for a limited number of traits as employed with the determinative scheme of Shewan et al. (1960).

MATERIALS AND METHODS

Bacteria and bacteriophages

All bacterial strains except one (H96, derived from a phage enrichment culture) were picked from seawater agar plates inoculated with freshly sampled seawater. A-series bacteria were isolated in 1979 from Atlantic water samples (Moebus, 1980). "Eastern" and "western" strains were derived from samples taken east or west, respectively, of the Azores. B-series bacteria were isolated from water samples collected in 1978 from the Bay of Biscay. H-series bacteria were derived from North Sea water samples taken near Helgoland between 1969 and 1978. The term "original host" refers to strains initially used to isolate and purify bacteriophages.

The bacteria were maintained on seawater agar slants in the refrigerator and checked for purity before use. Strains of known sensitivity to any of the bacteriophage strains available were also tested for persistence of their phage sensitivity patterns by spotting undiluted lysates. Methods and media as given by Moebus & Nattkemper (1981).

Escherichia coli B, *Staphylococcus aureus* P209, and *Pseudomonas fluorescens* (NCMB 129) were used as reference strains.

Most phages were derived from the same water sample as their original host bacteria. The method for bacteriophage isolation was described by Moebus (1980). In principle, it employs enrichment cultures set up with organic nutrients-enriched raw seawater w i t h o u t addition of prospective host bacteria. Phages infective for strains H4, H7, H17, H54, H84, H85, and H86 were isolated from enrichment cultures separately inoculated with about 5×10^7 cells ml^{-1} of these strains before incubation.

Media and methods

Seawater agar (SWA), containing 5 g peptone (Difco), 1 g yeast extract (Difco), 0.1 g $FePO_4$, and 15 g agar (Difco) per liter of "seawater mixture" (75 % aged North Sea water and 25 % distilled water). The pH was about 7.6 after autoclaving for 20 min at 121 °C.

Bacteria were grown in Petri-dishes (90 mm ∅) containing 10 ml SWA for determination of the following criteria: (a) Colony form and size (read after 2 days of incubation). (b) Pigment production: non-diffusible and diffusible pigments as well as fluorescent ones (tested under UV-light), after 1 to 3 days. (c) Luminosity, after 1 to 4 days. (d) Gram stain, according to Benson (1973). (e) Oxidase production, according to Steel (1961). (f) Flagellation, according to Mayfield & Inniss (1977) with sterile seawater mixture used to prepare cell suspensions.

Seawater bouillon (SWB) was of the same composition as SWA except for the agar content. SWB was used (in still culture) to grow bacteria for or prior to determination of the following criteria:

(a) Cell morphology: size, shape, arrangement (phase contrast microscopy, × 1250), after 1 to 2 days.

(b) Motility (phase contrast microscopy, × 800), after 1 to 2 days.

(c) Catalase production, tested with 10 % H_2O_2 after 2 days.

(d) Diffusible pigments, after up to 4 days.

(e) Gelatine liquefaction: stab culture in screw-cap tubes with 10 ml of medium (15 % gelatine in seawater mixture), incubated for 4 weeks.

(f) Starch decomposition: stab culture in Petri-dishes (60 mm ∅) containing 6 ml of starch-SWA which differs from SWA by enrichment with 0.5 % soluble starch. After 2 days the plates were flooded with Lugol's solution.

(g) Sensitivity to vibriostatic compound 0/129 (2.4-Diamino-6.7-diisopropyl pteridine): 6 ml of antibiotic-SWA (same composition as SWA except for the agar content which is 5 g Oxoid agar No. 1 per liter) were mixed with 0.15 ml of bacterial culture in Petri-dishes (60 mm ∅). After gelling of the agar, antibiotic disks were placed on the surface. Incubation for 2 days. The antibiotic disks (Schleicher & Schüll, No. 22, 9 mm ∅) were soaked with 0.1 % (w/v) solution of 0/129 in acetone and dried at 37 °C for 2 h before use (Bain & Shewan, 1968).

(h) Glucose dissimilation: MOF medium after Leifson (1963), modified according to Bölter (1977), was composed of 1 g peptone (Difco), 0.5 g ammoniumsulfate, 0.5 g TRIS-buffer, and 0.01 g phenol red per liter of seawater mixture. A 10 % solution (w/v, in distilled water) of glucose was autoclaved separately and added to a final glucose concentration of 1 % after cooling. MOF medium was used for aerobic and anaerobic cultures, the latter overlayed with paraffin (about 2.5 cm thick). Incubation for up to 14 days.

(i) Methyl red test, after Pfister & Burkholder (1965), as modified by Weyland et al. (1970): 5 g peptone (Difco) and 0.05 g K_2HPO_4 per liter of seawater mixture, with 5 g glucose added after adjusting pH 7.5. Test with methyl red after 4 days.

(j) Nitrate reduction: SWB enriched with 0.1 % KNO_3. Test for NH_3-production (with Nessler's reagent) and NO_2-production (with Lunge's reagent) after 2 and 4 days. Gas production was tested with Durham vials.

(k) Ammonia formation from peptone: SWB, tested with Nessler's reagent after 2 and 4 days.

(l) Salt requirements, tested with 3 media (incubation for up to 7 days): (1) 5 g peptone (Difco), 1 g yeast extract (Difco), and 75 g NaCl per liter of distilled water, pH 7.6; (2) 5 g peptone (Difco) and 1 g yeast extract (Difco) in mixture of 150 ml aged North Sea water and 850 ml distilled water (salinity about 5 ‰), pH 7.6; (3) SWB.

(m) Temperature sensitivity: tested in SWB incubated at 37 °C for up to 7 days. Doubtful results were controlled by streaking cultures on SWA and incubation of plates at the same temperature.

If not otherwise stated, all incubations were performed at 20 °C. Liquid media were inoculated with 1 drop of freshly grown bacterial culture in SWB.

The vibriostatic compound 0/129 was purchased from Calbiochem Company, San Diego, Cal. (USA) and all other chemicals from Merck Company, Darmstadt (FRG).

RESULTS

This investigation employed 444 bacterial strains of marine origin including 366 phage-sensitive ones. As concluded from phage sensitivity patterns, 259 of the latter strains belong to 14 clusters of related bacteria which were isolated from Atlantic Ocean water samples in 1979. Additional 29 strains of the same origin do not fit into any of these clusters. The combined 288 A-series strains are sensitive to Atlantic Ocean phages only. The remaining 78 strains are sensitive to phages isolated from the North Sea or the Bay of Biscay and originate either from these regions (31 H-series and 11 B-series strains, respectively) or from the Atlantic (36 strains). Seventy-eight A-series bacteria which are insensitive to any of the phage strains available (some 290) were investigated for comparison. They were randomly selected from our stock culture collection to include 1 to 2 strains isolated from each of 48 water samples taken between the European continental shelf and the Sargasso Sea (Moebus, 1980). The results will be presented according to this grouping of the bacteria.

Flagellation was investigated with all original hosts and some of their doubles. From the remaining bacteria, randomly selected strains were checked for flagella: 18 of the 36 A-series strains sensitive to phages from the North Sea and the Bay of Biscay, and 20 rod-shaped strains of the 78 phage-insensitive A-series bacteria.

Group I: A-series compiled in clusters 1 to 13

The 13 clusters comprise 250 Atlantic Ocean bacteria including 136 "eastern" and 114 "western" strains. (Six additional doublets belonging to cluster 1 and one of cluster 2 were excluded from this investigation.) It should be noted that formerly clusters 11 to 13 were included in cluster 1 (Moebus & Nattkemper, 1981) but had to be set apart according to recent findings (Moebus, 1983).

Traits common to (almost) all strains: Gram negative rods, motile (3 exceptions in cluster 2), oxidase positive, glucose fermented (within 1 day!), colonies unpigmented, no diffusible pigments (1 exception in cluster 2), no fluorescent pigments, non-luminous, NH_3 produced from peptone, NH_3 produced from nitrate (1 exception in cluster 5), no gas from nitrate, positive in methyl red test (2 exceptions in cluster 2 and one in cluster 4), no growth at 37 °C (1 exception in cluster 1). The results obtained for other traits are compiled in Table 1.

144 of the 150 strains tested were found to have single wavy polar flagella. In 2 moderately motile strains (clusters 2 and 3), no flagella were observed, and in 4 strains (cluster 4), unique observations were made. In 3 of these strains (2 of them highly motile), no or only few flagellated cells were found but many cells with polar stumps. In the fourth strain (highly motile), cells with up to 3 long but straight polar appendices were observed.

As for the metabolism of glucose the bacteria of group I differ from most members of the other groups by their rapid reaction in MOF medium. With no exception the change in colour from red to yellow occurred within 1 day of incubation under both aerobic and anaerobic conditions.

Diffusible pigment was observed with strain A64, one of 10 doublet strains of cluster 2 (see Moebus, 1983; Fig. 2). Its colourless colonies caused strong brown non-fluorescent colouration of the agar. Strain A734 (cluster 1) grows, though poorly, in SWB at 37 °C, producing tiny colonies an SWA at this temperature.

Seventeen strains of cluster 1 are unable to reduce nitrate to nitrite. Fifteen of them include a group of 14 doublets plus one other closely related strain (see Moebus, 1983; Fig. 1, group II + strain A699). Thirteen of these doublets also produced less NH_3 from peptone than observed in most members of cluster 1.

Regarding clusters 2, observations were made which somewhat separate its members from the bacteria compiled in clusters 1 and 3 to 13. The majority of the cluster 2 bacteria gave a comparatively weak oxidase reaction and 3 strains were found to be oxidase positive only when grown at 25 °C. Furthermore, in most members of this cluster no or weak gelatine liquefaction was observed. It should be noticed that 51 strains of cluster 2 are of western origin (see below).

Strong positive correlation was found between catalase production and starch digestion. Of the 250 strains, 192 (76.4 %) proved to be either positive (55) or negative (137) for both traits (Table 1). In some cases, correlations between traits were observed in dependence of the origin of the bacteria. As compiled in Table 2, among "eastern" strains the numbers of starch digesters (71) and non-digesters (65) are similar, however, among "western" bacteria non-digesters occurred much more often (89) than digesters (25). Origin-dependent correlations are also indicated between the traits "gelatine liquefaction" on the one hand and "starch digestion" as well as "nitrate reduced to nitrite" on the other hand (Table 2).

Table 1. Differently exhibited traits observed in bacteria of group I

Characters		Cluster 1	2	3	4	5	6	7	8	9	10	11	12	13
No. of strains tested		122	63	6	11	8	3	8	4	2	4	3	9	7
"eastern" strains		69	12		7	8	3	8	4	2	4	3	9	7
"western" strains		53	51	6	4									
Flagella:	single polar	70	30	3	3	5	3	6	4	2	3	3	7	5
	other	–	–	–	4	–	–	–	–	–	–	–	–	–
	not observed	–	1	1	–	–	–	–	–	–	–	–	–	–
	not tested	52	32	2	4	3	–	2	–	–	1	–	2	2
Sensitivity to 0/129	+	108	58	6	11	8	3	7	4	2	4	3	9	7
	–	14	5	–	–	–	–	1	–	–	–	–	–	–
Catalase versus starch digestion:	–:–	69	58	–	–	–	–	–	–	–	–	1	9	–
	+:–	4	1	2	–	–	1	4	–	–	–	–	–	5
	–:+	24	3	1	11	–	–	–	–	–	–	2	–	–
	+:+	25	1	3	–	8	2	4	4	2	4	–	–	2
Gelatine liquefaction*:	no	4	25	4	–	–	–	–	–	–	–	–	–	–
	weak	11	28	1	1	–	–	–	–	–	–	1	–	–
	moderate	61	10	1	5	5	2	8	3	–	3	–	–	1
	strong	46	–	–	5	3	1	–	1	2	1	2	9	6
Nitrite from nitrate	+	105	20	6	10	7	3	8	3	2	4	3	9	6
	–	17	43	–	1	1	–	–	1	–	–	–	–	1
Growth in salts media**: 7.5 % NaCl	5 ‰ sea salts	SWB												
+	–	+	1	–	–	–	–	–	–	–	–	–	–	–
–	–	++	6	3	2	–	–	–	–	–	–	–	–	–
+	–	++	27	3	–	3	–	–	–	–	–	2	–	–
+	+	++	1	17	–	–	–	–	–	–	–	–	–	–
++	–	++	–	–	–	1	–	–	–	–	–	–	–	–
–	–	+++	13	1	2	2	–	–	–	–	–	1	–	–
+	–	+++	47	7	2	5	2	–	2	–	–	–	6	–
–	+	+++	2	2	–	–	–	–	–	–	–	–	–	1
+	+	+++	11	28	–	–	6	2	4	3	2	4	–	1	5
++	–	+++	6	–	–	–	–	–	1	–	–	–	–	2	–
++	+	+++	4	1	–	–	–	–	1	–	–	–	–	–	–
+	++	+++	1	–	–	–	–	1	–	1	–	–	–	–	1
++	++	+++	3	1	–	–	–	–	–	–	–	–	–	–	–

* weak = up to 10 %, moderate = up to 50 %, and strong = more than 50 % of medium liquefied after 4 weeks of incubation

** + = weak growth, ++ = moderate growth, and +++ = profuse growth after 4 days of incubation

Table 2. Frequency distribution of selected traits among bacteria of group I depending on origin of bacteria. Presented are the number of strains exhibiting the respective traits

Characters		"Eastern" strains		"Western" strains	
		Gelatine liquefaction			
		−	+	−	+
		8	128	25	89
Starch digestion	−	7	58	19	70
	+	1	70	6	19
Nitrite from nitrate	−	6	9	12	37
	+	2	119	13	52

Group II: A-series bacteria of cluster 14

Cluster 14 combines 9 strains, 8 of which are "western" ones. Traits common to (almost) all strains: Gram negative rods, motile, single polar flagella (1 exception), oxidase positive, glucose fermented (within 2 to 7 days), insensitive to 0/129, colonies unpigmented, no diffusible pigments, no fluorescent pigments, non-luminous, starch not hydrolysed, gelatine liquefied (1 exception), NH_3 produced from peptone, no gas from nitrate, negative in methyl red test. Results regarding other traits are compiled in Table 3.

This group is set apart from group I mainly by the slower reaction on glucose and by the insensitivity of the bacteria to the vibriostatic pteridine 0/129. Beside this some other peculiar traits were observed.

Table 3. Differently exhibited traits observed in bacteria of group II. Salt- and temperature-dependent growth: + = weak, ++ = moderate, and +++ = profuse. F refers to flake formation

Designation of strain	A185	A335	A398	A400	A432	A897	A929	A1212	A1409
Catalase	+	+	−	+	+	−	+	−	−
NH_3 from nitrate	+	+	−	−	−	−	+	−	−
Nitrite from nitrate	+	+	−	+	+	−	+	−	−
Growth with 7.5 % NaCl	++	++	+	++ F	++ F	+	+++	−	−
Growth with 5 ‰ sea salts	+++	+++	+	+++ F	++ F	++	+++	++	++
Growth in SWB at 20 °C	+++ F	+++ F	+++	+++ F	++ F	+++	++ F	++	+++ F
Growth in SWB at 37 °C	+++ F	+++ F	−	+++ F	+++ F	−	+++ F	−	−

Five strains grow profusely in SWB at 37 °C forming large flakes which also occurred in SWB at 20 °C and, with 2 of the 5 strains, in 7.5 % NaCl- and 5 ‰ sea salts-media. Furthermore, the 8 "western" strains could be lysogenized with 1 to 6 bacteriophage strains, whereas the "eastern" one (A1409) is inhibited by 4 of the temperate phages. Details regarding interactions between these bacteria and the phages are reported by Moebus (1983).

Group III: Phage-sensitive A-series bacteria which do not fit into clusters 1 to 14

This group combines 29 bacteria which, except for 7 doublet strains, differ from each other in sensitivity to any of the phage strains tested. Nineteen are " eastern" strains and 10 are "western" ones. Traits common to (almost) all strains: Gram negative rods, motile, single polar flagella (1 strain uncertain, 1 doublet strain not tested), oxidase positive, glucose fermented (within 1 to 7 days), colonies unpigmented, no diffusible pigments, no flourescent pigments, gelatine liquefied (1 exception), NH_3 produced from peptone, no gas from nitrate. The findings for other traits are summarized in Table 4.

Table 4. Differently exhibited traits observed in bacteria of group III

"Eastern" strains	12*	1	2	1		1		2				
"Western" strains		1	1		1		1	1	1**	2	1	1
Sensitivity to O/129	+	−	+	+	−	−	+	+	−	−	−	−
Catalase	+	+	+	−	−	+	−	−	−	+	−	−
Starch digestion	+	+	−	+	+	−	+	−	+	−	+	−
NH_3 from nitrate	+	+	+	+	+	+	+	+	+	−	−	−
Nitrite from nitrate	+	+	+	+	+	+	−	−	−	−	−	−
Methyl red test	+	+	+	+	+	−	−	+	−	−	−	−

* two strains luminous
** grows at 37 °C, forms flakes under any condition tested

Significant differences between "eastern" and "western" strains were observed in regard to their activity in MOF medium. Glucose was fermented after only 1 day by 16 "eastern" and 3 "western" strains but after 2 to 7 days by 3 "eastern" and 7 "western" bacteria.

Salt requirements are as variable as observed in the members of group I (Table 1). One flake-forming strain grows well in SWB at 37 °C. Two doublet strains are luminous. Both digest starch and gelatine and grow weakly with 7.5 % NaCl.

Group IV: H-series bacteria

This group comprises 31 strains isolated from the North Sea near Helgoland between 1969 and 1978. Their phage sensitivity patterns were reported by Moebus & Nattkemper (1981; Fig. 4). Traits common to (almost) all strains: Gram negative rods,

oxidase positive, colonies unpigmented (except strain H85), no diffusible pigments, no fluorescent pigments, non-luminous, NH_3 produced from peptone, no gas from nitrate.

Table 5 summarizes the findings obtained for other traits. According to these results the members of group IV can be arranged in 3 subgroups.

Table 5. Selected traits as observed in bacteria of group IV. mt = monotrichous (single polar), pt = peritrichous, F = fermentative, O = oxidative

No. of strains	Subgroup												
	IVa			IVb							IVc		
	1	2	1	1	1	2	5	13*	1	1	1**	1^+	1^{++}
Motility	+	+	+	+	+	+	+	+	+	+	−	+	+
Flagella	mt	mt	mt	mt	mt	mt	mt	mt	mt	mt	−	mt	pt
Glucose metabolism	F	F	F	F	F	F	F	F	F	F	F	O	F
Sensitivity to 0/129	+	+	+	−	−	−	−	−	−	−	−	−	−
Catalase	+	−	−	+	+	−	−	−	−	−	+	+	−
Starch digestion	+	+	−	+	−	−	−	+	+	−	−	−	−
Gelatine liquefaction	+	+	+	+	+	+	+	+	−	−	+	−	+
NH_3 from nitrate	+	+	+	+	+	+	−	−	−	−	−	+	+
Nitrite from nitrate	+	+	+	+	+	−	−	−	−	−	−	+	+
Methyl red test	+	+	+	+	−	−	−	−	−	−	−	−	+
Growth at 37 °C	−	−	−	−	−	−	−	−	−	−	−	+	+

* see text, compare Table 6

** Strain H85, forms yellow-orange colonies on SWA; $^+$ Strain H86; $^{++}$ Strain H96

Subgroup IVa includes 4 strains which are sensitive to the pteridine 0/129, produce NH_3 and NO_2 from nitrate, and react positively in the methyl red test. Subgroup IVb combines 24 strains which are insensitive to 0/129. Most of these strains do not produce NH_3 or NO_2 from nitrate, and only one is positive in the methyl red test. In 18 bacteria of this subgroup, similarities in sensitivity to 3 H-series bacteriophages were found (Moebus & Nattkemper, 1981).

All members of subgroups IVa and IVb ferment glucose. In most strains fermentation was indicated after 2 to 8 days, in 2 strains (subgroup IVa) after only 1 day. Growth in 7.5 % NaCl medium was slight (23 strains) or moderate (3 strains). In 5 ‰ sea salts medium growth generally was enhanced: slightly in 7, moderately in 18, and profusely in 11 strains. Growth in SWB at 37 °C did not occur.

Subgroup IVc comprises 3 strains which are different from one another. Strain H85 produced yellow-orange colonies and its cells are non-motile slender rods without flagella. It ferments glucose (within 7 days) and requires sea salts for growth which was observed at 20 °C only. Strain H86 uses glucose oxidatively. Its motile cells have polar flagella and the colonies are colourless. This strain grows profusely in SWB at 20° and 37 °C and moderately in media with 7.5 % NaCl or 5 ‰ sea salts. The last strain (H96) metabolizes glucose fermentatively and grows profusely at all salt concentrations and temperatures tested. Its cells are peritrichously flagellated and spread very rapidly on SWA. The progeny of 2 or 3 cells will cover the whole agar surface in a Petri-dish of 90 mm in diameter after less than 1 day of incubation at 25 °C.

Group V: B-series bacteria

The 11 strains of this group were isolated in 1978 from water samples taken in the Bay of Biscay. With 10 strains rather uniform results were obtained. Traits common to (almost) all strains: Gram negative rods, oxidase positive, colonies unpigmented, no diffusible pigments, no fluorescent pigments, non-luminous, NH_3 produced from peptone (except strain B17), no gas from nitrate. The results for other traits are compiled in Table 6.

Table 6. Selected traits as observed in bacteria of group V. mt, F, O: see Table 5

No. of strains	4	1	1	1	1	2*	1**
Motility	+	+	+	+	+	+	−
Flagella	mt	mt	mt	mt	mt	mt	−
Glucose metabolism	F	F	F	F	F	F	O
Sensitivity to 0/129	+	+	+	−	−	−	−
Catalase	+	+	+	−	−	−	−
Starch digestion	−	+	+	−	+	+	−
Gelatine liquefaction	+	+	+	+	+	+	−
NH_3 from nitrate	+	+	+	+	+	−	−
Nitrite from nitrate	+	+	+	+	+	−	+
Methyl red test	+	+	−	+	+	−	−
Growth at 37 °C	−	−	−	−	−	−	+

* see text, compare Table 5
** Strain B17 produces no NH_3 from peptone

It should be noticed that 2 strains (marked by an asterisk in Table 6) are sensitive to the 3 H-series bacteriophage strains referred to in section "Group IV: H-series bacteria". The results obtained with these 2 strains correspond with those found with 13 H-series bacteria (marked by an asterisk in Table 5) 12 of which are sensitive to the same phages.

Strain B17 differs markedly from the 10 other bacteria of this group. Its cells are also oxidase positive, Gram negative rods but few if any were motile and no flagella were detected. Furthermore, B17 uses glucose oxidatively, does not produce NH_3 from peptone nor liquefy gelatine, and grows best in SWB at 37 °C.

Group VI: A-series bacteria sensitive to bacteriophages of H- and B-series only

This group combines 36 strains, 34 of which are sensitive to 7 phage strains of the H-series. The remaining 2 strains are infected by phage B14/2. Numerous bacterial strains included in groups already described are also sensitive to these phages. Traits common to (almost) all strains: Gram negative rods, motile (1 exception, its cells, however, are flagellated), oxidase positive (1 exception), glucose fermented (within 1 to 7 days), NH_3 produced from peptone, no gas from nitrate.

The findings regarding other traits are compiled in Table 7. Observations made with strains which belong to other groups but are sensitive to the same phages are included in Table 7 for comparison.

Table 7. Differently exhibited traits observed in bacteria of group VI. Bacteria of other groups are included for comparison

No. or designation of strains	Glucose fermented in days	Sensitivity to 0/129	Catalase	Starch digestion	Gelatine liquefaction	NH_3 from nitrate	Nitrite from nitrate	Methyl red test	Growth at 37 °C	Sensitive to phage	Remarks
2	4	−	+	−	+	−	−	−	−		
1	2	−	−	−	+	−	−	−	−		
(1)	7	−	−	+	+	−	−	−	−	H4/4	Group III
H4	4	−	−	+	+	−	−	−	−		Group IV
2	7	−	−	+	+	−	−	−	−		diffusible pigment
H17	7	−	−	−	+	+	−	−	−	H17/1	Group IV
1	4	−	−	+	+	−	−	−	−		
H27	7	−	−	+	+	−	−	−	−	H27/1	Group IV
1	1	−	+	+	+	+	+	+	−		luminous
H31	6	−	+	+	+	+	+	+	−	H31/1	Group IV
1	2	−	+	+	+	−	−	−	−		
2	4	−	+	−	+	−	−	−	−		
2	2–7	−	+	−	−	−	−	−	−		
7	2–7	−	−	+	+	−	−	−	−		
8	2–7	−	−	−	+	−	−	−	−		
(1), H7	7	−	−	−	+	−	−	−	−	H7/2 and/or H54/1 and/or H84/1	Group III, IV
H54, H84	4	−	−	−	+	−	−	−	−		Group IV
1	4	−	−	−	+	+	−	−	−		
1	7	−	−	−	−	+	−	−	−		
1	4	−	+	+	+	−	−	−	+		
1	4	−	−	+	+	−	−	−	+		
1	2	−	−	+	+	−	−	−	+++		
2	2–4	−	−	−	+	−	−	−	+++		
1	4	−	−	−	+	−	−	−	+++		oxidase negative
2	1	+	+	+	+	+	+	+	−		
(1)	1	+	+	+	+	+	+	+	−		Group I, cluster 1
(4)	1	+	−	+	+	+	+	+	−	B14/2	Group I, cluster 1
B14	1	−	−	+	+	+	+	+	−		Group V
(15)	1	+	−	−	+	+	−	+	−		Group I, cluster 1

Polar flagella were observed with 18 strains randomly selected from the members of this group. All but 2 strains (sensitive to phage B14/2) are insensitive to the pteridine 0/129. Faint pigmentation (shades of yellow and cream) was observed with 10 strains. Two other strains produced non-fluorescent diffusible pigments, and another is luminous.

Slight or moderate growth in 7.5 % NaCl medium was observed in 33 strains. With 5 ‰ sea salts growth of most strains was as profuse as in SWB at 20 °C. Only 2 strains, both growing moderately in 7.5 % NaCl medium, did not grow at all with 5 ‰ sea salts. Growth at 37 °C was observed in 6 strains.

Group VII: A-series bacteria insensitive to any of the bacteriophage strains tested

In the 78 strains of this group much greater diversity was observed than in any group of phage-sensitive bacteria. Only the most conspicuous findings will be reported shortly.

Ten strains are cocci one of which is Gram negative. It forms pink colonies on SWA and is unable to grow in SWB at 37 °C. Of the 9 Gram positive cocci, 8 strains form yellow colonies and only 1 strain does not grow at 37 °C.

Among the rod-shaped bacteria 3 Gram positive and 2 Gram variable strains were found. The Gram positive ones ferment glucose while the Gram variables are unable to metabolize it.

The remaining 63 rod-shaped bacteria are Gram negative and 52 of them are motile. Sixteen motile strains were checked for, and found to possess, flagella. In 4 non-motile strains no flagella were observed. Glucose is fermented by 58 strains, 43 of which are oxidase positive. 20 of the latter bacteria are sensitive to the vibriostatic compound 0/129. They most closely resemble the phage sensitive bacteria, which is also true in regard to other traits. The other 5 strains use glucose oxidatively. Four of them are oxidase positive and all are insensitive to 0/129.

Fourteen rod-shaped bacteria produce coloured colonies with yellow shades dominating. Two strains forming white and pink colonies, respectively, produce fluorescent pigment, and another strain is luminous. These 3 strains are motile, oxidase positive, and able to ferment glucose.

Growth at 37 °C was observed in 15 of the 63 Gram negative rods which is a much larger proportion than found with phage-sensitive bacteria. Finally, one of these 63 strains produced gas from nitrate. It is the only one observed to do so of the 444 strains tested in this investigation.

DISCUSSION

Moebus & Nattkemper (1981) reported 250 different phage sensitivity patterns observed in 326 Atlantic Ocean bacteria susceptible to phages of the same origin. On the one hand, these findings indicated considerable genetic differentiation among bacteria and phages as well. On the other hand, similarities in the phage sensitivity patterns due to which the majority of the 326 bacterial strains were placed into 2 large and 8 small clusters indicated little difference among the bacteria in terms of their taxonomic position.

Similar observations were made with bacteria and phages isolated from the North Sea. Of 31 bacterial strains 18 were found to be related to each other according to their sensitivity to 3 bacteriophage strains. Furthermore, 34 Atlantic Ocean bacteria were observed to be sensitive to the 3 North Sea phages mentioned, indicating close relationship even among bacteria isolated from far distant regions.

Taking these observations into account the chances of finding bacteria of different genera among the phage-sensitive strains investigated were expected to be limited. However, they existed, at least among some of the 14 clusters of groups I and II as well as among the members of groups III to VI.

As presented in the section "Results" stupendous uniformity was found in the 250 bacteria placed in group I. These strains are assigned to the genus *Vibrio* since for the most important traits (Gram negative rods, motile by polar flagella, oxidase positive, fermentative on glucose, and sensitive to vibriostatic pteridine 0/129) only a few exceptional strains were observed. These concern motility (3), flagellation (6), and 0/129 sensitivity (20). They can safely be included in the genus *Vibrio* due to their relationship to other strains (as indicated by phage sensitivity patterns) which on their part perfectly correspond with the majority of strains in this group. Regarding 0/129 sensitivity, contradictory results were obtained several times with doublet strains which differed only in this trait, indicating the limited stringency of this character (see also below).

A high degree of uniformity was also found in most strains combined in groups II to VI. These groups comprise 116 bacteria only 4 of which do not fit the patterns of the most important traits common to the majority of 112 strains. The latter all are Gram negative rods, motile (1 strain non-motile but flagellated), oxidase positive (1 exception in group VI, Table 7), and able to use glucose fermentatively. However, many of the 112 strains differ from the bacteria combined in group I in the following characters: insensitivity to 0/129, no production of NH_3 and nitrite from nitrate, and negative methyl red test. In 62 strains, strict correlations were found between these traits, 52 of which are sensitive to H-series bacteriophages (20 strains of group IV and 32 of group VI).

These observations complicate the decision regarding the taxonomic position of the 112 strains in question. About 30 of them can be assigned safely to the genus *Vibrio*. The remaining ones may belong to the genus *Aeromonas* since no doubt is cast on their insensitivity to 0/129 by contradictory results as observed with doublet strains among the members of group I. However, according to Bergey's Manual (Buchanan & Gibbons, 1974) members of the genus *Aeromonas* are known to produce nitrite from nitrate which was not observed in the strains in question. Furthermore, members of the genus *Vibrio* are stated to be "usually sensitive to" 0/129 (Buchanan & Gibbons, 1974, p. 340). This statement ist supported by the results obtained with bacteria of group I. Therefore, the possibility cannot be ruled out that the 0/129 insensitive strains also belong to the genus *Vibrio*.

Whether or not this is the case, there is no doubt that with the exception of 4 strains all phage-sensitive bacteria investigated belong to the family Vibrionaceae and most of them (ca. 280 of 362 strains) to the genus *Vibrio*. The 4 exceptions are strains isolated from the North Sea (3 strains) or from the Bay of Biscay (groups IV and V, respectively).

Of the H-series bacteria (Table 6), strain H85 is tentatively assigned to the genus *Flavobacterium*. It is the only truly coloured strain among the phage-sensitive bacteria investigated and belongs to the few bacteria which were not derived from the same

water sample as the infecting bacteriophage. Strain H86 is assigned to the genus Pseudomonas (group II of Shewan et al., 1960). Strain H96 may belong to the genus *Proteus*. It is the only strain ever isolated from a bacteriophage enrichment culture. Uncertain is the taxonomic position of strain B17 of group V.

The taxonomic uniformity among the phage-sensitive bacteria raises the question whether it may be an artifact caused by the methods used to isolate and maintain the bacteria and bacteriophages.

All but one (H96) of the bacterial strains were picked from plates inoculated with seawater before cultures for phage enrichment were started with the respective water sample. Since as many apparently different colonies as possible were isolated, the choice of bacteria basically should have been unbiased. However, selection certainly occurred during further handling, especially during storage, of the isolates.

Of the 1382 strains isolated from Atlantic water samples 451 did not survive until soon after return to Helgoland and about 200 additional strains were lost during the following months. Among the lost strains, coloured ones have a disproportionately large share (Moebus, 1980).

Furthermore, in bacteria placed in cluster 3 extraordinarily fast die-off was observed. When Moebus & Nattkemper (1981) investigated these bacteria, cluster 3 combined 17 strains. Only one year later their number was reduced to 10, and in spite of considerable effort made to maintain all strains of this cluster their number has shrunk meanwhile to 6. This observation, unique among the various clusters of phage-sensitive bacteria, indicates differences in the ability to survive during storage even among closely related bacteria.

Regarding the methods used to isolate bacteriophages, selection pressure on populations of bacteria and phages as well is a matter of fact. Phage enrichment requires the presence of fairly large numbers of suitable host cells. This condition is usually attained by inoculating prospective host bacteria at the beginning of the enrichment procedure to an initial titer greatly exceeding that of the natural bacterial population. The host bacteria may be isolated beforehand from the respective sample of water or sediment which is stored at low temperature until the enrichment culture is started. For technical reasons this procedure could not be employed during the expedition to the Sargasso Sea. Instead, enrichment cultures had to be started immediately after collecting the water samples using seawater supplemented with nutrients only to support growth of indigenous bacteria (Moebus, 1980). Most of the H-series and all B-series phages were derived in the same way. Under such conditions the selection pressure among bacteria is high and fast growing species will be favoured (Jannasch, 1968). Therefore, one has to expect that preferably phages will be enriched that infect bacteria able to outgrow others.

Since most of the bacteriophage strains tested by Moebus & Nattkemper (1981) are infective for bacteria all but a few of which are assigned to the family Vibrionaceae, one has to conclude that such bacteria were vastly favoured in the enrichment cultures. In theory there are two possibilities for this to happen. Vibrionaceae either outnumbered other types of bacteria in the indigenous populations from the beginning or they attained this status during the course of enrichment culture due to superior growth characteristics.

Some clues can be drawn from the following facts. Of 733 Atlantic Ocean bacteria investigated by Moebus & Nattkemper (1981), 326 are sensitive and 407 are insensitive

to the bacteriophages employed. Among the 78 randomly selected phage-insensitive strains (group VIII), 37 (= 47 %) are motile Gram negative rods, oxidase positive, and fermentative on glucose, 17 of which are also sensitive to 0/129. The 37 strains, therefore, are closely related to the phage-sensitive A-series bacteria of groups I to III and VI.

Provided that the 78 strains of group VII are a representative sample of the 407 phage-insensitive bacteria, 191 of the latter would be related to the sensitive strains. In sum, 517 of the 733 strains tested by Moebus & Nattkemper (1981) would be related ones, amounting to 70 %. Even assuming that none of the bacteria lost during storage are related to phage-sensitive strains, the 517 strains would represent 37 % of the 1382 isolates collected from Atlantic water samples.

This calculation indicates that, in the Atlantic Ocean samples, members of the genus *Vibrio* or, at least, of the family Vibrionaceae were abundant. However, to explain the fact that 100 % of the phage-sensitive A-series bacteria belong to this family one has to assume that these bacteria were favoured during enrichment culture by their growth characteristics. Therefore, the conclusion is that the taxonomic uniformity among the phage-sensitive bacteria observed in this investigation is an artifact.

Certainly the same holds true in regard to the bacteriophages. There is no reason to assume that in the Atlantic only phages exist the hosts of which belong to the taxonomic group encountered in this investigation. As can be seen from the literature cited in the "Introduction", bacteriophages active on members of 10 different genera have so far been isolated from marine and estuarine environments. However, it must be pointed out that the number of reports on phages infective for members of the Vibrionaceae (including the genera *Vibrio/Beneckea, Aeromonas, Photobacterium,* and *Lucibacterium*) is conspicuously large.

Finally, another striking result of this investigation must be considered. Among the H-series bacteria (group IV, Table 5) as well as the A-series bacteria sensitive to H-series phages (group VI, Table 7) all but 4 strains are insensitive to the pteridine 0/129, and most of them are negative in production of NH_3 and nitrite from nitrate and in the methyl red test. This combination of traits was rarely observed in the other bacteria (4 of 9 in group II, Table 3; 4 of 29 in group III, Table 4; 2 of 11 in group V, Table 6).

It is not surprising that H-series phages are active preferentially on bacteria from the Atlantic which regarding the respective traits resemble the phages' original hosts. However, it is surprising that 20 of the 28 North Sea bacteria assignable to the family Vibrionaceae exhibit exactly these traits which separate them from the vast majority of Atlantic Ocean bacteria.

These findings may be the expression of differences in the composition of bacterial and bacteriophage populations typical for the North Sea near Helgoland and the open Atlantic. It cannot be ruled out, however, that also the considerable differences in the numbers of (colony forming) bacteria per unit volume in water samples from both regions gravely affect the outcome of phage enrichment cultures started without prospective host bacteria added.

Acknowledgements. The authors are indebted to Prof. G. Rheinheimer, Dr. M. Bölter and Mrs. R. Kreibich of the Institut für Meereskunde, Kiel University, for hospitality and technical advice given to Miss H. Nattkemper during 2 weeks training in marine bacterial taxonomy.

LITERATURE CITED

Ahrens, R., 1971. Untersuchungen zur Verbreitung von Phagen der Gattung *Agrobacterium* in der Ostsee. – Kieler Meeresforsch. *27,* 102–112.

Bain, N. & Shewan, J. M., 1968. Identification of *Aeromonas, Vibrio* and related organisms. In: Identification methods for microbiologists. Part B. Ed. by M. M. Gibbs & D. A. Shapton. Acad. Pr., London, 79–84.

Baross, J. A., Liston, J. & Morita, R. Y., 1978. Incidence of *Vibrio parahaemolyticus* bacteriophages and other *Vibrio* bacteriophages in marine samples. – Appl. environ. Microbiol. *36,* 492–499.

Baumann, P., Baumann, L., Bang, S. S. & Woolkalis, M. J., 1980. Reevaluation of the taxonomy of *Vibrio, Beneckea,* and *Photobacterium:* abolition of the genus *Beneckea.* – Current Microbiol. *4,* 127–132.

Benson, H. J., 1973. Microbiological applications. Brown, Dubuque, Iowa, 345 pp.

Bölter, M., 1977. Numerical taxonomy and character analysis of saprophytic bacteria isolated from the Kiel Fjord and the Kiel Bight. In: Microbial ecology of a brackish water environment. Ed. by. G. Rheinheimer. Springer, Berlin, 148–178. (Ecological studies. *25*)

Buchanan, R. E. & Gibbons, N. E. (Eds), 1974. Bergey's manual of determinative bacteriology. Williams & Wilkins, Baltimore, 1268 pp.

Carlucci, A. F. & Pramer, D., 1960. An evaluation of factors affecting the survival of *Escherichia coli* in sea water. IV. Bacteriophages. – Appl. Microbiol. *8,* 254–256.

Chaina, P. N., 1965. Some recent studies on marine bacteriophages. – J. gen. Microbiol. *41,* Proceedings 25.

Colwell, R. R., Citarella, R. V. & Chen, P. K., 1966. DNA base composition of *Cytophaga marinoflava* n. sp. determined by buoyant density measurements in cesium chloride. – Can. J. Microbiol. *12,* 1099–1103.

Espejo, R. T. & Canelo, E. S., 1968. Properties of bacteriophage PM2: a lipid-containing bacterial virus. – Virology *34,* 738–747.

Hastings, J. W., Keynan, A. & McCloskey, K., 1961. Properties of newly isolated bacteriophage of luminescent bacteria. – Biol. Bull. mar. biol. Lab., Woods Hole *121,* 375.

Hidaka, T., 1971. Isolation of marine bacteriophages from sea water. – Bull. Jap. Soc. scient. Fish. *37,* 1199–1206.

Hidaka, T., 1973. Characterization of marine bacteriophages newly isolated. – Mem. Fac. Fish., Kagoshima Univ. *22,* 47–61.

Hidaka, T., 1977. Detection and isolation of marine bacteriophage systems in the southwestern part of the Pacific Ocean. – Mem. Fac. Fish., Kagoshima Univ. *26,* 55–62.

Hidaka, T. & Fujimura, T., 1971. A morphological study of marine bacteriophages. – Mem. Fac. Fish., Kagoshima Univ. *20,* 141–154.

Jannasch, H. W., 1968. Growth characteristics of heterotrophic bacteria in seawater. – J. Bact. *95,* 722–723.

Johnson, R. M., 1968. Characteristics of marine *Vibrio*-bacteriophage system. – J. Arizona Acad. Sci. *5,* 28–33.

Kakimoto, D. & Nagatomi, H., 1972. Study of bacteriophages in Kinko Bay. – Bull. Jap. Soc. scient. Fish. *38,* 271–278.

Keynan, A., Nealson, K., Sideropoulos, H. & Hastings, J. W., 1974. Marine transducing bacteriophage attacking a luminous bacterium. – J. Virology *14,* 333–340.

Leifson, E., 1963. Determination of carbohydrate metabolism of marine bacteria. – J. Bact. *85,* 1183–1184.

Mayfield, C. I. & Inniss, W. E., 1977. A rapid, simple method for staining bacterial flagella. – Can. J. Microbiol. *23,* 1311–1313.

Moebus, K., 1980. A method for the detection of bacteriophages from ocean water. – Helgoländer Meeresunters. *34,* 1–14.

Moebus, K., 1983. Lytic and inhibition responses to bacteriophages among marine bacteria, with special reference to the origin of phage-host systems. – Helgoländer Meeresunters. *36,* 375–391.

Moebus, K. & Nattkemper, H., 1981. Bacteriophage sensitivity patterns among bacteria isolated from marine waters. – Helgoländer Meeresunters. *34,* 375–385.

Nakanishi, H., Iida, Y., Maeshima, K., Teramoto, T., Hosaka, Y. & Ozaki, M., 1966. Isolation and properties of bacteriophages of *Vibrio parahaemolyticus.* – Biken's J. *9,* 149–157.

Pfister, R. M. & Burkholder, P. R., 1965. Numerical taxonomy of some bacteria isolated from antarctic and tropical seawaters. – J. Bact. *90,* 863–872.

Shewan, J. M., Hobbs, G. & Hodgkiss, W., 1960. A determinative scheme for the identification of certain genera of Gram-negative bacteria, with special reference to the Pseudomonadaceae. – J. appl. Bact. *23,* 379–390.

Sklarow, S. S., Colwell, R. R., Chapman, G. B. & Zane, S. F., 1973. Characteristics of a *Vibrio parahaemolyticus* bacteriophage isolated from Atlantic coast sediment. – Can. J. Microbiol. *19,* 1519–1520.

Smith, L. S. & Krueger, A. P., 1955. Characteristics of a new *Vibrio*-bacteriophage system. – J. gen. Physiol. *38,* 161–168.

Spencer, R., 1955. A marine bacteriophage. – Nature, Lond. *175,* 690.

Spencer, R., 1960. Indigenous marine bacteriophages. – J. Bact. *79,* 614.

Steel, K. J., 1961. The oxidase reaction as a taxonomic tool. – J. gen. Microbiol. *25,* 297–306.

Stevenson, J. H. & Albright, L. J., 1972. Isolation and partial characterization of a marine bacteriophage. – Z. allg. Mikrobiol. *12,* 599–603.

Weyland, H., Rüger, H.-J. & Schwarz, H., 1970. Zur Isolierung und Identifizierung mariner Bakterien. Ein Beitrag zur Standardisierung und Entwicklung adäquater Methoden. – Veröff. Inst. Meeresforsch. Bremerhaven *12,* 269–296.

Wiebe, W. J. & Liston, J., 1968. Isolation and characterization of a marine bacteriophage. – Mar. Biol. *1,* 244–249.

Zachary, A., 1974. Isolation of bacteriophages of the marine bacterium *Beneckea natriegens* from coastal salt marshes. – Appl. Microbiol. *27,* 980–982.