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TAXONOMIC STUDIES ON THE BACTERIAL STRAINS ISOLATED FROM CASES OF "SHIRASU[1]" FOOD-POISONING (*PASTEURELLA PARAHAEMOLYTICA*) AND RELATED MICROORGANISMS

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SUMMARY We studied the biological and serological properties of the strains which were first isolated in 1950 by FUJINO *et al.* from cases of "Shirasu" food-poisoning and named "*Pasteurella parahaemolytica*". We came to the following conclusions:

1) This organism should be transferred to the genus *Vibrio*;

2) The so-called "pathogenic, facultatively halophilic bacteria" isolated from various sources in recent years in Japan should be included in the same species as our isolated organisms;

3) The serotype O-1 of the pathogenic, facultatively halophilic bacteria is antigenically identical with our organism.

Thus, one of our strains should reasonably be designated as a type-strain of the *Vibrio parahaemolyticus*[2], which covers hitherto described strains of pathogenic, facultatively halophilic bacteria as well as our strains.

INTRODUCTION

On October 20 and 21, 1950 an outbreak of food-poisoning due to "Shirasu" occurred in Osaka Prefecture, in which 272 developed symptoms of acute gastroenteritis, and 20 died. Eight fatal cases were autopsied by Dr. OMURA of the Osaka University Medical School, Department of Forensic Medicine.

Using materials taken from the intestines of autopsied cases, as well as from specimens of shirasu suspected to contain the bacteria, FUJINO and his coworkers tried to obtain bacterial cultures through three successive intraperitoneal passages in mice and subsequent cultivation on blood agar plates. On October 26, they

1 Shirasu is the fry of the sardine (*Engraulis japonica* Houttuyn) which is boiled in salt water, and is sold and eaten in the half dried state.

2 We have already sent our *Vibrio parahaemolyticus* strains to the American Type Culture Collection at the request by Dr. LESSEL, and they were assigned the accession numbers 17802 an 17803.

observed two kinds of colonies on blood agar plates from which they obtained pure cultures of two different bacteria.

One of the isolated bacteria was found to be *Proteus morganii* while the other was a hitherto unknown Gram-negative, polarly monotrichous rod. According to Skerman's keys (1949), it seemed to fall into the genus *Pasteurella* and resembled *Pasteurella haemolytica*. Therefore, the organism was named *Pasteurella parahaemolytica*, FUJINO, OKUNO, NAKADA, AOYAMA, FUKAI, MUKAI and UEHO, 1951, n.sp. (FUJINO *et al.*, 1951; FUJINO *et al.*, 1953) and it was considered that mixed infection with this organism and *Proteus morganii* might have caused the severe food-poisoning.

This publication did not attract public attention until 1955 when Dr. TAKIKAWA isolated a similar organism during an outbreak of food-poisoning in his hospital in Yokohama (TAKIKAWA, 1956). Since he suspected that staphylococci were the causative agents, he used medium containing 4 per cent NaCl. However, 18 strains of Gram-negative rods with one polar flagellum were isolated from the stools of 29 cases. He was surprised by the similarities between his strains and those described in FUJINO's report. In his report (TAKIKAWA, 1958) he stated, "Among the hitherto described microorganisms only *Pasteurella parahaemolytica* isolated by Dr. FUJINO from shirasu food-poisoning cases in 1950 seems to resemble to ours, who, however, failed to notice the important halophilic properties and antigenicity of the bacteria." Since then, TAKIKAWA was able to isolate similar bacteria from sporadic cases of diarrhea which he called " food-poisoning due to the halophilic bacteria." In 1957 and 1958, many sanitary bacteriologists in Japan reported the isolation of similar bacteria from stools of cases of food-poisoning in various parts in Japan. Thus, the " halophilic bacteria " soon became a topic of interest in sanitary bacteriology in Japan, although its taxonomic position and its relation to Fujino's *Pasteurella parahaemolytica* remained unsolved.

In 1958, TAKIKAWA stated (TAKIKAWA, 1958): " *Pasteurella parahaemolytica* FUJINO has almost the same properties as my strains, not only in biological natures but also in serological characters ". In the same publication he proposed the name " *Pseudomonas enteritis* " for this organism, because he said " the data of my studies on the properties of these strains indicate they are most suitable to be grouped in *Pseudomonas* ".

YAMAZI *et al.* (1958) also found that Takikawa's *Pseudomonas enteritis* N4 strain and Fujino's *Pasteurella parahaemolytica* had the same biological properties except for a difference in acid production from arabinose. This led them to suggest that these two strains might be included in one species.

On the other hand, KOSUGE (1959) studied the thermostable as well as the thermolabile antigens of *Pseudomonas enteritis* N4 and *Pasteurella parahaemolytica* EB 102, and found that these two strains each have specific antigens as well as antigens in common.

The third attempt to clarify this organism was made by MIYAMOTO, who proposed a new subgenus *Oceanomonas* of the genus *Aeromonas* to cover all the known pathogenic, facultatively halophilic bacteria (MIYAMOTO *et al.*, 1961). The subgenus is divided into three species with respect to the actions of the organisms on chitin and alginate and their growth on desoxycholate agar:

	Chitin	Alginate	Growth on desoxycholate agar
Oceanomonas parahaemolytica	−	−	−
Oceanomonas alginolytica	+	+	+
Oceanomonas enteritidis	+	−	+

Thus, Fujino's original strains corresponded to *Oceanomonas parahaemolytica*, while Takikawa's original strains were included into *Oceanomonas enteritidis*.

This much was known when we started taxonomic studies on our strains in 1961, ten years after their first isolation. At the same time taxonomic studies on pathogenic, facultatively halophilic bacteria were undertaken by FUKUMI's group (National Institute of Health, Tokyo). In 1963, these workers concluded that the organisms should be placed in the genus *Vibrio*, and so, retaining the specific epithet "*parahaemolytica*", they proposed the name "*Vibrio parahaemolyticus*" for these organisms (SAKAZAKI *et al.*, 1963).

This paper gives results of taxonomic studies on our *Pasteurella parahaemolytica* strains. This work was performed independently to FUKUMI's group. Our reasons for accepting the specific name "*Vibrio parahaemolyticus*" proposed by SAKAZAKI, IWANAMI and FUKUMI are also given.

MATERIALS AND METHODS

1. *Strains*

1) *Pasteurella parahaemolytica*: EB 101 and EB 102 maintained in the frozen state in our Institute's Type Culture Collection and the same strains transferred to, and subcultured in nutrient agar containing 3 per cent NaCl.

2) Fifteen strains of the pathogenic, facultatively halophilic bacteria from Dr. TAKIKAWA and 12 pilot strains of the bacteria representing the serotype O–1 to O–12, from Dr. SAKAZAKI.

3) *Vibrio comma*, INABA and OGAWA, from our Type Culture Collection.

4) Four strains of vibrios, representing Gardner-Venkatraman's serotypes II (NCTC 8042), III (NCTC 30), V (NCTC 4715) and VI (NCTC 4716) from the National Collection of Type Cultures, London, and a strain NCTC 4711 representing Gardner-Venkatraman's serotype III from Dr. SAKAZAKI.

5) Six strains of vibrios representing Heiberg's types from Dr. SAKAZAKI.

6) Four strains of *Aeromonas hydrophila* (or *Aeromonas formicans*) isolated from clinical materials by Dr. LAUTROP (Statens Seruminstitut, Copenhagen). One strain each of *Aeromonas hydrophila*, *Aeromonas salmonicida* and *Aeromonas shigelloides* from Dr. SAKAZAKI.

7) *Pseudomonas aeruginosa* and *Pseudomonas fluorescens* from our Type Culture Collection.

2. *Antisera*

1) Anti-EB 101 and anti-EB 102 sera: Rabbits were immunized with heat-killed (100°C for 2 hours) cells (for anti-O sera).

Commercially available anti-O sera (O–1 to O–12) and anti-O sera prepared by Dr. OSHIRO (Hyogo Prefectural Health Laboratory) with his own pilot strains of pathogenic, facultatively halophilic bacteria were also used in these experiments.

2) Anti-*Vibrio comma* (INABA and OGAWA) sera: Anti-O sera were prepared by the same method as anti-EB 101 and EB 102.

3. *Agglutination test*

This was performed in a water-bath at 50°C for 18 hours.

4. *Biological properties*

The biological properties were tested either to the description of DAVIS and PARK (1962), or of the Guide published by the Japanese Ministry of Welfare and Health (1962), except in the following tests. The conditions for the cholera red reaction were as established previously (FUJINO *et al.*, 1964 b) with reference to BEAM's report (1959). Spheroplast formation was observed in the presence of 3 per cent glycine following the method of JAYNESS (1961). Vibriostatic agent (O/129; 2,4-diamino-6,7-diisopropyl pteridine) was kindly given through Dr. MITCHELL (Allen and Humburys Ltd.). Its action was tested according to the description in CASELITZ's report (1961). The test for haemolysis described in FUJINO's report (1951) detected only soluble haemolysin which diffused into the agar medium. Therefore, the technique for testing El Tor vibrio was employed in these experiments (WILSON and MILES, 1955).

RESULTS

1. *Biological properties of Pasteurella parahaemolytica strains and related organisms*

TABLE 1 *Generic identification of our strains EB 101 and EB 102*

	EB 101 EB 102	Vibrio[1]	Aeromonas[2]	Pseudomonas[3]
Spheroplasts regularly formed	+	+	−	−
Pleomorphic	+	+	−	−
Single polar flagellum	+	+	V	V
Fluorescence under u. v.	−	−	−	+
Growth at pH 9	+	+	V	V
Susceptible to O/129	+	+(V)	−?	−
Hugh & Leifson test	Ferment	Ferment	Ferment	Oxidize
Gas from glucose	−	−	+(V)	−
Cholera-red reaction	+	+	−	−

1 *Vibrio comma* (INABA and OGAWA); five strains representing Gardner-Venkatraman's serotypes II, III, V and VI; and six strains representing Heiberg's types.

2 Four strains of *Aeromonas hydrophila* (or *formicans*); one strain each of *Aeromonas hydrophila*, *Aeromonas salmonicida* and *Aeromonas shigelloides*.

3 *Pseudomonas aeruginosa* and *Pseudomonas fluorescens*.

V: Variable with different strains

TABLE 2 *Comparison of EB 101 EB 102 and pathogenic, facultatively halophilic bacteria*

Biological properties tested	EB 101 EB 102	Pathogenic facultatively halophilic bacteria	Biological properties tested	EB 101 EB 102	Pathogenic facultatively halophilic bacteria
Indol*	+	+	Cholera-red reaction	+	(+) V
MR	+	+	Glucose*	+	+
VP	−	d	Gas from Glucose*	−	−
Citrate (Simmonds)	+	+	Arabinose*	+	d
H_2S (TSI)*	−	−	Cellobiose	−	−
KNO_3	+	+	Lactose*	−	−
PPA	−	−	Maltose*	+	+
Urea	−	−	Rhamnose*	−	−
Malonate	−	−	Sucrose*	−	d
Liquefaction of gelatin*	+	+	Trehalose	+	+
Casein	+	+	Xylose	−	−
Hide powder	+	+	Mannitol*	+	+
Chitin	+	+	Adonitol*	−	−
Lecithin	+	+	Dulcitol*	−	−
KP-Citrate	−	−	Inositol*	−	−
KP-d-Tartarate	+	+	Sorbitol*	−	−
Catalase	+	+	Salicin*	−	−
Cytochrome oxidase	+	+	Starch	+	+
Hugh-Leifson	F	F			

Biological properties of the strains EB 101 and EB 102 marked with an asterisk (*) are from the original description by FUJINO *et al.* in 1951. We obtained the other results ourselves since 1961.

V: Variable d: Different in different subgroups F: Fermentation

1) *Comparison of generic level*

As shown in Table 1, strains EB 101 and EB 102 had all the characters required for their inclusion in the genus *Vibrio* proposed by DAVIS and PARK (1962). They were fermentative in their action on glucose in Hugh-Leifson medium, which excluded them from the genus *Pseudomonas*. Their spheroplast formation was regularly positive in 24 hours cultures in peptone medium. A higher percentage of spheroplasts were formed in medium containing 3 per cent of glycine. A definite cholera red reaction was obtained under our conditions with our strains as well as with other vibrios. Thus, our strains should be included in the genus *Vibrio*.

2) *Comparison of our strains with pathogenic, facultatively halophilic bacteria*

Table 2 gives a comparison of our strains with pilot strains of pathogenic, facultatively halophilic bacteria. The biological properties of our strains, reported in FUJINO's original publication are marked with an asterisk (*). The other results were obtained in the present experiments. The biological properties listed, cover all the keys described in the Guide published by the Japanese Ministry of Welfare and Health (1962). There was no evidence for placing pathogenic, facultatively halophilic bacteria in a different species from our strains. The cholera red reaction was always positive under our conditions. Results with Voges-Proskauer reaction, and the actions of the bacteria on arabinose and sucrose are used as criteria for distinguishing two biotypes (or subgroups) within the pathogenic, facultatively halophilic bacteria. The biological characters of our strains correspond to those of biotype 1.

2. *Antigenic structure if Pasteurella parahaemolytica strains*

The EB 101 anti-O serum reacted with Sakazaki's pilot strain O-1, to the same degree as

TABLE 3 *O-Agglutination with anti-EB 101 (heat-killed) serum*

Antigen / Antiserum	EB 101	EB 102	O-1	O-2	O-3	O-4	O-5	O-6	O-7	O-8	O-9	O-10	O-11	O-12
EB 101	800	800	800	40	40	40	40	20	20	20	20	20	20	20

TABLE 4 *O-Agglutination with anti-O sera (Oshiro)*

Antigen / Antisera	EB 101	EB 102	Homologous
O-1	3200	3200	2560<
O-2	200	200	2560<
O-3	200	200	1280
O-4	50	50	1280
O-5	50	50	640
O-6	100	200	2560
O-7	100	100	2560<
O-8	50	50	1280
O-9	200	200	1280
O-10	50	50	1280
O-11	1600	1600	2560<
O-12	50	100	1280

TABLE 5 *Absorption of anti-EB 101 serum with heat-killed cells*

	O-agglutination titer versus EB 101 antigen
Unabsorbed	1280<
Absorbed with heat-killed (100°C 2 hrs.) suspension of	
Vibrio comma (INABA)	320
Vibrio comma (OGAWA)	320
Gardner-Venkatraman	
II (NCTC 8042)	160
III (NCTC 30)	160
V (NCTC 4715)	320
VI (NCTC 4716)	320
Pathogenic, facultatively halophilic bacteria	
Pilot strain for O-1	<10
EB 101	<10

TABLE 6 *Absorption of antisera with heat-killed EB 101 strain*

Anti-O sera	O-agglutination titer versus homologous strains	
	before absorption	after absorption (repeated 4–5 times)
Vibrio comma (INABA)	1280<	640
Vibrio comma (OGAWA)	1280<	320
Gardner-Venkatraman		
II (NCTC 8042)	1280<	640
III (NCTC 30)	1280<	320
V (NCTC 4715)	1280<	1280
VI (NCTC 4716)	1280<	640
Pathogenic, facultatively halophilic bacteria		
Pilot strain for O–1	1280<	<10
EB 101	1280<	<10

with homologous strains. The reaction of other strains with EB 101 anti-O serum was less than 1 : 40 (Table 3).

Moreover, our strains EB 101 and EB 102 reacted with Oshiro's anti-O–1 serum to a titer of 1 : 3200. Cross reactions were observed between our strains and the other anti-O sera prepared by OSHIRO, and there was a reaction even up to a titer of 1 : 1600 with anti-O–11 serum (Table 4).

When EB 101 anti-O serum (titer to homologous strain: <1 : 1280) was absorbed with the pilot strain for O–1, it no longer reacted to EB 101 (Table 5). A heat-killed (100°C for 2 hours) suspension of EB 101 also completely removed anti-O–1 antibody (Table 6). Therefore, O–1 of the pathogenic, facultatively halophilic bacteria is antigenically identical with our strains.

DISCUSSION

It should be emphasized that the primary motive of our studies was not to study the taxonomic position of the pathogenic, facultatively halophilic bacteria themselves. Our first aim was rather to find which genus was the correct one for our isolated strains (which were formerly named *Pasteurella parahaemolytica*).

It should be pointed out that information on the classification of Gram-negative rods was extremely poor in the early 1950s. For example, *Vibrio* and *Pseudomonas* could be classified merely by morphological findings, and no one dared to imagine the inclusion of straight rods (without curvature of the body) in the genus *Vibrio*. Moreover, neither in Skerman's keys (1949) nor in Bergey's Manual (6th edition, 1948) was there any description of flagella in the keys to the genus *Pasteurella*. Since then, a more reasonable system for classification of Gram-negative rods has been established by introduction of Hugh-Leifson medium, the cytochrome oxidase test etc. In our Department, attempts have also been made to apply these criteria to the identification as well as to the classification of *Pseudomonas* and related Gram-negative rods recovered from clinical materials. The reports from the *Pseudomonadales* Subcommittee (1961) and extensive studies on vibrios by DAVIS and PARK (1962) were especially useful in this respect. If there is anything to be added to their schemata, it is our method for performing the cholera red reaction and also for observing spheroplast formation.

Our results leave no doubt that our strains should be included in the genus *Vibrio*, instead of *Pasteurella*. Thus, their correct name should be *Vibrio parahaemolyticus*. The same name was proposed by SAKAZAKI *et al.* for pathogenic, facultatively halophilic bacteria (SAKAZAKI, 1962 b; SAKAZAKI *et al.*, 1963). What they stated was that pathogenic, facultatively halophilic bacteria should be placed in the genus *Vibrio*, in which they might constitute an independent species. Since these authors adopted the specific epithet "*parahaemolyticus*" from the name *Pasteurella parahaemolytica* which we had proposed, it seems that they had no doubt that our strains should be included in the group formerly called pathogenic, facultatively halophilic bacteria. They compared FUJINO's original description

of *Pasteurella parahaemolytica* with their findings with pathogenic, facultatively halophilic bacteria. However, as we pointed out in the introduction, the problem of the identification of Fujino's *Pasteurella parahaemolytica* with the pathogenic, facultatively halophilic bacteria had not yet been solved. Thus, we feel it our duty to emphasize here, that it was we, not FUKUMI's group, who first presented evidence to place our organism in the genus *Vibrio*.

The second object of the present work was to see whether our strains and the pathogenic, facultatively halophilic bacteria belonged to the same species, and our results give no evidence for placing them into separate species. Recently, SAKAZAKI (1964) and ZENYOJI (1964) proposed that biotype 2 should be excluded from *Vibrio parahaemolyticus*, to form an independent species. Even so, our strains, the biological properties of which correspond to those of biotype 1, cannot be separated from the bacteria which were formerly called pathogenic, facultatively halophilic bacteria. Thus, the specific name "*Vibrio parahaemolyticus*", by which, in our opinion, our strains should be called, can be applied to most of the pathogenic, facultatively halophilic bacteria, at least, those of the biotype 1.

Takikawa's nomenclature "*Pseudomonas enteritis*" cannot be adopted since his strains were found to be the same species as ours. First, as pointed out by KAWAKITA (1960), the specific epithet "*parahaemolytica*" should be retained, even when the generic name is changed. Secondly, the inclusion of his organism in the genus *Pseudomonas* is not possible, and thirdly his usage of the Latin noun "*enteritis*" in its nominative form as a specific epithet is incorrect.

With regard to Miyamoto's nomenclature there seems to be no reason to establish a new genus (or subgenus) *Oceanomonas* to include only these organisms until the position of the genus within the Order *Pseudomonadales* is clearly defined. According to SAKAZAKI (1964 a), inclusion of this organism with *Aeromonas hydrophila* (as "subspecies *parahaemolytica*") was suggested by some members of the *Pseudomonadales* Subcommittee, whereas CASELITZ (1961) insisted on the necessity of testing its attitude to the vibriostatic agent. Although the criteria for differentiating *Vibrio* and *Aeromonas* have not yet been clearly established, it seems natural to distinguish our organism from *Aeromonas* since it is sensitive to the vibriostatic agent (FUJINO *et al.*, 1964 c) and does not produce visible gas from glucose (YASUDA *et al.*, 1962). The name *Oceanomonas* itself is a dubious one, because there is no evidence for believing that our organism had a marine origin, although this does not seem unlikely.

The third aim of this work was to see what serotype of vibrios or of pathogenic, facultatively halophilic bacteria was antigenically identical with our strains. We are of the opinion that the O antigen structure should be the ultimate criterion for defining any bacterial species, especially in the genus *Vibrio*. Our strains seemed to share common antigens with *Vibrio comma* and some vibrios representing GARDNER-VENKATRAMAN's groups, which, however, were not enough to justify their inclusion in the same species.

Among the serotypes of the pathogenic, facultatively halophilic bacteria, O–1 was found to be antigenically identical with that of our strains. SAKAZAKI (1961) once stated, "Among the 9 strains of O–1, which is corresponding to Fujino's original strains, 7 were isolated in Osaka and Hyogo Prefectures . . .", although we can find no evidence for his statement that his O–1 group corresponded to our strains in his papers. Oshiro's anti-O–11 serum also reacted with our strains at as great a dilution as 1 : 1600. Until the O antigenic classification of the bacteria formerly known as pathogenic, halophilic bacteria is definitely established, we cannot comment on this result which suggests cross reactivity.

In November 1962, SAKAZAKI, IWANAMI and FUKUMI proposed the name "*Vibrio parahaemolyticus*" (SAKAZAKI, 1962 b; SAKAZAKI *et al.*, 1963), and from our own experimental results we agreed to this name (FUJINO, 1962;

FUJINO, 1963; FUJINO and FUKUMI, 1963 FUJINO *et al.*, 1964 a). Thus, the correct name for our strains, and also for pathogenic, facultatively halophilic bacteria is *Vibrio parahaemolyticus* (FUJINO, OKUNO, NAKADA, AOYAMA, FUKAI, MUKAI, and UEHO 1951), SAKAZAKI, IWANAMI and FUKUMI 1963 ". None in the Japan Bacteriological Society has yet objected to this name and the independence of these bacteria as a species was recognised by the *Pseudomonadales* Subcommittee in 1965. However, the so-called biotype 2 should be excluded from *Vibrio parahaemolyticus*. Thus, as not all the bacteria formerly called pathogenic, facultatively halophilic bacteria have been included in the species *Vibrio parahaemolyticus*, it can be said that FUJINO and his coworkers first isolated this species and that it was not found first in any collection of " halophilic bacteria ". We hope that our strains which have been maintained in our Institute for nearly 15 years since this first isolation can be designated as type strains of the species *Vibrio parahaemolyticus*, and we consider that this report provides sufficient evidence to permit this.

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