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Concentrated culture supernatants from strains of Vibrio parahaemolyticus of gastroenteric origin, "suspected" V. parahaemolyticus isolated from cases of skin infection, nonpathogenic marine Vibrio, V. anguillarum and V. alginolyticus, were submitted to flat gel electrophoresis, followed by the examination of the polyacrylamide gels for total protein and enzyme patterns.

Variations related to the presence of single and multimolecular forms of proteolytic enzymes, lipases, esterases, phosphatases, amylases and deoxyribonucleases, permitted a differentiation among the cultures being examined. A close similarity was observed between the pathogenic cultures isolated from skin infections and the V. parahaemolyticus strains of gastroenteric origin. This group was well differentiated from the nonpathogenic Vibrio, V. anguillarum and V. alginolyticus, but under the conditions of the experiment these last

three groups could not be easily differentiated. The tests involving the detection of DNase, amylase, egg yolk lipase (mainly related to time and intensity of reaction) appeared to be the most useful for the characterization of V. parahaemolyticus and these results suggested that the application of gel electrophoresis of culture supernatants, might be of importance in the rapid identification of this bacterium. No evidence was found indicating the presence of the hemolytic factor responsible for the "Kanagawa phenomenon".

Another purpose of the present study was the determination of the heat resistance of V. parahaemolyticus, the strain ATCC 17802 being used as a test organism. The flask method was employed and peptone salt water, pH 7.2 was the suspending menstruum. Decimal reduction time (D value) was the parameter used to express the heat resistance, with values of 38.2, 2.01, and 0.51 min being observed at the temperatures of 113°, 118.4°, and 122°F, respectively.

A study to determine the influence of the composition of the recovery medium on the apparent heat resistance, showed that higher counts and survival rates were observed when Vibrio Maintenance Medium was employed for counting the heated cells, with a statistically significant difference (at five percent level) being observed when compared with Brain Heart Infusion Agar plus 2.5 percent NaCl and Trypticase Soy Agar plus 2.5 percent NaCl, without a significant difference between the last two media.

Thermal Resistance and Characterization of *Vibrio parahaemolyticus* by Gel Electrophoresis of Culture Supernatants

by

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INTRODUCTION

The presence of Vibrio parahaemolyticus in foods of marine origin is of public health significance, and in Japan during the summer, it is responsible for more outbreaks than any other type of pathogene commonly found in food and food products. This bacterium appears to have a wide distribution in the marine environment, particularly coastal waters and its occurrence has been confirmed in the United States. There is no reported outbreak of food borne infections in the U. S. due to V. parahaemolyticus, but according to Dack (1966) and Bryan (1969) this does not exclude the possibility of such occurrence, as most outbreaks are reported only for botulism, salmonellosis and staphylococcal infections and intoxications. The detection and characterization of V. parahaemolyticus involves a laborious series of isolation procedures and biochemical reactions, in order to differentiate them from other marine vibrios, such as V. anguillarum and V. alginolyticus, usually present in the same environment, but not considered to be pathogenic to man.

One purpose of this experiment was the characterization of V. parahaemolyticus and the differentiation from other nonpathogenic marine Vibrio based on in vitro tests of their extracellular enzymes,

using flat gel electrophoresis. The characteristic pattern of enzyme reactions would permit a more rapid and accurate differentiation. Another purpose of this study was to gain a better understanding of the thermal resistance of V. parahaemolyticus by the study of its destruction at different temperatures.

LITERATURE REVIEW

Experiment I

A group of facultatively halophilic organisms has been considered as the causative agent of a food borne infection that occurs in Japan during the summer months. These outbreaks are generally related to the consumption of raw sea fish and several prepared foods like "shirasuboshi" (semi-dried young sardines), and "izushi" (raw fish, vegetables, rice, salt, vinegar) which are traditional foods of the Japanese people.

These organisms were first isolated in 1951 from autopsy materials collected after an outbreak of gastroenteritis. Because of the fastidiousness of the organisms in ordinary culture media, bipolar staining characters and weak hemolytic properties, they were considered to be members of the genus Pasteurella, the name P. parahemolytica being suggested for the causal organism (cited by Miyamoto et al., 1961). According to Sakazaki (1963), the halophilism of the organism was not known, otherwise this would have excluded it from the genus Pasteurella.

Takikawa (1958) isolated several strains from outbreaks of gastroenteritis, pointing out the halophilic character and identifying the organism as being similar to the species belonging to the genus Pseudomonas, but differing serologically. He then proposed the name

P. enteritis for these organisms. Myamoto et al. (1961) studying these bacteria, proposed a new genus Oceanomonas. The authors considered that these strains showed some distinct characteristics, like low grade salt requirement, differentiating them from the genus Aeromonas (fresh water organisms), and from halophiles of higher salt requirement. According to the same authors, they were different from Vibrio since they were never found in typical curved form, which is apparent in Vibrio, and often showed pleomorphism, e.g. spherical forms. The authors did not consider the sensitivity to the vibriostatic agent 2-4 diamino 6-7 di isopropyl pteridine important from a taxonomic point of view. Three species were considered in this new genus: O. enteritidis, O. parahemolyticus and O. alginolytica.

The definitive studies on the morphological, physiological, cultural and chemical properties were reported by Sakazaki (1963). This author did not consider the halophilism an important key for the classification, by pointing out that the halophilic character changed with conditions of growth. He concluded that the organism showed a great similarity with the characteristics of the genus Vibrio, proposing the name V. parahaemolyticus (Fujino et al., 1953; Sakazaki et al., 1963) for this species. The species was divided into two subgroups on the basis of growth in peptone water containing seven and ten percent NaCl, Voges Proskauer reaction, and sucrose and

arabinose fermentation with members of subgroup 1 being noncholerageneic enteropathogenic and those of subgroup 2 with questionable pathogenicity.

In 1965, Zen Yoji et al. (cited by Sakazaki, 1968) applying computer techniques suggested that organisms in the subgroup 2 should be excluded from V. parahaemolyticus, as the similarity value between the two biotypes was approximately 80 percent. This was later confirmed by Sakazaki (1968) who proposed the name Vibrio alginolyticus for strains in subgroup 2. This bacterium is more frequently isolated from coastal sea water and sea fish than V. parahaemolyticus and sometimes it is found in the feces of human patients affected with gastroenteritis (Sakazaki, 1969, p. 123).

V. anguillarum is another marine vibrio that frequently is mistaken for V. parahaemolyticus in isolation procedures. Originally associated with red disease of eels, this organism was later described by Smith (1961) as responsible for a disease of finnock (immature Salmo trutta) in Scotland. Recently, Cisar and Fryer (1969) identified this organism as the causal agent of an epizootic of vibriosis in juvenile chinook salmon (Oncorhynchus tshawytscha) reared in a salt water impoundment on the Oregon coast. These organisms are found only in sea water and sea fish shortly after capture, and they are never found in the feces of patients suffering from gastroenteritis (Sakazaki, 1969, p. 123).

Concerning the habitat and distribution of V. parahaemolyticus, it was thought that its presence was restricted to Japanese coastal waters, but further works showed a large distribution in coastal sea waters of the United States, the Philippines, Taiwan, Hong Kong and Singapore (Sakazaki, 1969, p. 123). In the United States it was first isolated by Baross and Liston (1968) from Puget Sound and Washington Coast sediments. The organism was found in relatively large numbers during the summer from all Puget Sound water sediment and oyster samples. However, in samples collected during the early spring and early fall, the counts of V. parahaemolyticus were very low. Horie et al. (1967) observed that none of the organisms were isolated in the pelagic sea water samples; but on the other hand, it was revealed that coastal sea waters or estuarine waters contained up to 10^5 V. parahaemolyticus per 1,000 ml sample in summer season. Ward (1968) isolated organisms related to V. parahaemolyticus from frozen sediment samples from two coasts of the United States. Krantz and Colwell (1969) isolated strains of V. parahaemolyticus from lethargic and moribund blue crabs in the Chesapeake Bay (USA). These authors suggested that this bacterium is part of the marine flora and occasionally it invades marine animals where it may become a potential human health problem.

Recently, as reported by Twedt, Spaulding and Hall (1969), suspected V. parahaemolyticus were isolated from localized tissue

infections acquired by individuals living in coastal regions of the United States. According to the authors, these pathogenic vibrios are involved in an unsuspected mode of infection, via a wound or tissue injury.

The enteropathogenicity of V. parahaemolyticus has been studied by several authors. Zen Yoji et al. (1965) concluded that it was of the infectious type, with gastroenteritis developing in most cases. Symptoms usually appear 12 hours after the infected food has been eaten, although the interval may be as short as two hours or as long as 48 hours, characterized by abdominal pain and diarrhea, usually associated with nausea and vomiting; mild fever, chills and headache are also seen in most cases and a dysentery like disease with excretion of stools with mucus and blood may also occur (Sakazaki, 1969, p. 124). The incidence of this food borne infection is very high in Japan, and according to the Ministry of Welfare of Japan,73.1 percent of the outbreaks during 1963 were caused by V. parahaemolyticus (Sakazaki, 1969, p. 125).

It is not well defined if all members of the species are pathogenic. Sakazaki (1963) suggested that all members of V. parahemolyticus might be enteropathogenic for human beings. The author described that all cultures of the vibrios revealed a hemolytic zone around the colonies on ordinary blood agar plates. It was later observed that vibrios isolated from the human diarrheal stools revealed

a hemolytic activity when plated on unautoclaved Brain Heart Infusion Agar, containing five percent human blood, three percent sodium chloride and 0.001 percent Crystal Violet. However, cultures isolated from sea fish and sea water did not show such an activity in this modified medium (Sakazaki, 1968). Feeding tests with human volunteers carried out in order to clarify the enteropathogenicity of the non-hemolytic strains revealed that none of them became ill, although over 10^9 cells of the vibrios were administered.

Based on these results, it was concluded that the vibrios possess two hemolytic factors, one being common to all of them, and the other characteristic of some cultures. It was later observed that the common factor was inhibited by the addition of 0.1 percent glucose to the medium, was bound to the cell components and was heat labile, while the hemolytic factor which was found in strains from human clinical cases was free from the cell and heat stable (cited by Sakazaki, 1968b). At the 41st General Meeting of the Japan Bacteriological Society held in 1968, Fujino, the original discoverer of V. parahaemolyticus proposed that this peculiar type of hemolysis should be called "Kanagawa phenomenon" (Miyamoto et al., 1969).

The epidemiology of V. parahaemolyticus is not clear. Miyamoto et al. (1969) observed 95.3 percent positive reactions for the "Kanagawa phenomenon" in strains isolated from suspected dysentery patients, and 0.54 percent and 0.48 percent of positive

reactions from strains isolated from sea water and fish samples respectively. However, epidemiological investigations in Japan have demonstrated that sea fish and their products are the causative agents of outbreaks of gastroenteritis. Nevertheless, practically all cultures isolated from sea fish are considered apathogenic to man. According to Sakazaki (1969, p. 122), future investigations will be necessary in order to clarify this problem.

The isolation and identification of V. parahaemolyticus is a laborious procedure and there is no complete agreement among authors as to what constitutes the primary criteria for the identification.

Sakazaki, Iwanami and Tamura (1968a) developed a serological scheme of classification of V. parahaemolyticus based on 11 O groups and 41 K antigens, an antigenic scheme being established for 41 serotypes of the vibrio. It was observed that all cultures of the species possessed a common H antigen. However other authors suggested that there is no serological support for the V. parahaemolyticus isolates at the present time, and it is recommended that the chemical identification scheme be completed in order to assure the correct identification of genus and species (U.S.P.H.S., F.D.A., 1969, Sec BAM 14.03).

Sakazaki (1963; 1965; 1969) reported that V. parahaemolyticus does not ferment sucrose; however, Baross and Liston (1968)

observed that seven of the 40 strains obtained from Japan fermented this sugar. Twedt et al. (1969) observed close morphological, cultural and physiological similarities among strains isolated from different sources. However, their results did not agree completely with those reported by Sakazaki (1963), particularly concerning the pattern of salt tolerance, arabinose and cellobiose fermentation. Ward (1968) observed that many of his isolates satisfying the Japanese outline of cultural characteristics, failed to react serologically, and conversely a number of serologically reactive isolates did not conform to the scheme of classification proposed.

The use of starch gel electrophoresis in taxonomy represented an important contribution to the identification of microorganisms. Ornstein (1964) and Davis (1964) introduced the disc electrophoresis, using polycrylamide gels, that, according to the second author, offered kinds of flexibility and versatility not easily attainable with starch gels at that time. From studies of the physicochemical nature of enzymes within the tissues or cells it was discovered that single enzymes existed in multiple molecular forms. Markert and Moller (1959) proposed the term "isozyme" to characterize these forms. It was observed that similar enzymes isolated from different organisms were shown to present wide variations in their electrophoretic properties, but related organisms frequently demonstrating similar or nearly identical patterns.

Norris (1964) studying Bacillus thuringiensis divided this bacterium into ten esterase types, correlated with possession of H antigens. The author emphasized the value of gel electrophoresis as an aid to taxonomy, particularly related to bacterial esterase systems.

Lund (1965) working with gel electrophoresis of soluble protein components and esterase enzymes of some group D streptococci, demonstrated that strains of Streptococcus faecalis and its varieties "zymogenes" and "liquefaciens" possessed very similar protein patterns and contrasting strongly with the pattern shown by S. faecium and S. durans.

Robinson (1968, p. 85) studying coryneform bacteria, made a primary differentiation based on amino acid composition of the cell wall mucopeptide, followed by a further differentiation within the cell wall groups based on starch gel electrophoresis of cell free extracts and further identification of patterns for catalase, peroxidase and esterase.

Levant (1969) using polyacrylamide gel electrophoresis of concentrated supernatants of various strains of Clostridium botulinum types A, B, C, E and F, observed variations in characteristic proteolytic isozymes, lipases, lecithinases and diaphorases. The author detected a single diaphorase band in types A or B, two bands in toxic types E and F and no diaphorase in the nontoxic forms.

The taxonomy of the genus Vibrio has undergone several

revisions in the recent years, and according to Colwell (1968) certain problems remain,particularly the differentiation of species within the genus Vibrio and resolution of the relationships of Vibrio to genera of the Pseudomonadaceae and the Enterobacteriaceae. Adeyemo, Ellingsen and Colwell (1967) studying strains of Vibrio choleae, V. cholerae variety El Tor and related marine and nonmarine Vibrio strains compared data obtained by computer analysis, DNA base composition, and total protein and esterase analysis by starch gel electrophoresis. The results indicated that the strains formed a relatively homogeneous group at the species level of taxonomic relationship. The variations in esterase banding patterns which were observed did not correlate with subgroupings of V. cholerae, V. cholerae var. El Tor and "noncholera vibrios", Adansonian and DNA base composition data supporting the conclusions drawn from the electrophoretic data. The same authors concluded that the method of enzyme analysis appeared to provide valuable and taxonomically useful information.

Colwell, Adeyemo and Kirtland (1968) studied esterases and DNA base composition of strains of V. cholerae, V. parahaemolyticus, noncholera and marine vibrios. The results showed similarities when the vibrios were examined by electrophoretic methods, their soluble proteins being similar. The esterase banding patterns of the Vibrio strains, with the exception of the marine vibrios, suggested a rather

high degree of similarity, with no distinct or separate pattern for the El Tor or noncholera vibrios. V. parahaemolyticus represented a homogeneous group, with an esterase band corresponding to the slower moving band of V. cholerae. According to the authors, the DNA base composition analyses of these strains suggested a position intermediate between V. cholerae and the marine Vibrio spp. They also concluded that the information derived from esterase analyses appeared to be of considerable taxonomic value.

Experiment II

When bacterial cells or spores are exposed to heat, the proportion surviving at any time can be plotted against the heating time to give a survival curve. This curve is often exponential, and a straight line is obtained when the logarithms of survivors are plotted against time in arithmetic units. This type of curve is typical for first order chemical reactions, and it has been assumed that death of microbial cells is due to a single lethal event, occurring at random. This event could be the denaturation of an essential protein (Riemann, 1969, p. 491). However, further observations showed that the cells are destroyed by the joint action of a number of events, like the breakdown of ribosomes, loss of osmotic function of bacterial cells, and leakage of an exudate containing peptides, amino acids and nucleic acids (Riemann, 1969, p. 492).

Ordal (1970) observed in cultures of Staphylococcus aureus subjected to sublethal heat treatment, the occurrence of lesions, particularly the damage of cytoplasmic membrane with consequent leakage of cytoplasmic constituents out of the cell. There was also an alteration of the metabolic capabilities of the cell with a selective thermal inactivation of cellular enzymes and a partial denaturation of cellular proteins, and a degradation of ribosomal RNA, due to the thermal activation of enzymes which degrade the t RNA, probably a ribonuclease and a polynucleotide phosphorylase.

The heat inactivation of vegetative cells or spores are usually expressed by the use of two parameters. The "D value", also termed death rate constant or decimal reduction time, is the time required at any temperature to destroy 90 percent of the spores or vegetative cells of a given organism. Numerically, it is equal to the number of minutes required for the survivor curve to traverse one log cycle and mathematically it is equal to the reciprocal of the slope of the survivor curve (Stumbo, 1965, p. 107). The survivor curve is obtained by plotting logarithms to the base 10 of number of survivors against times of exposure.

The "Z value" represents the degrees Fahrenheit required for the thermal destruction curve to traverse one log cycle, and mathematically it is equal to the reciprocal of the slope of the thermal destruction curve (Stumbo, 1965, p. 110). The thermal destruction

curve is more conveniently constructed by plotting the logarithm of D or some multiple of D in the direction of ordinates against exposure temperatures in the direction of abscissae. In this particular case, the thermal destruction curve is called "phantom thermal death time curve", because it is supposed to have direction but no position (Schmidt, 1954, p. 730). According to Thomas, White and Longree (1966) the value of this curve is its independence from the initial inoculum concentration. The slope is assumed to be a constant value, regardless of the suspending medium and it may be extrapolated to temperatures higher than those actually tested.

According to Schmidt (1954, p. 745), there may be three general types of factors which affect the thermal resistance: (1) inherent resistance, under the same growth conditions, different strains of the same species may produce cell or spore suspensions having widely different degrees of resistance; (2) environmental influences active during the formation of cells or spores, as illustrated by the effects of age, incubation, temperature and composition of the nutrient medium; and (3) environmental influences active during the heating of the suspension, comprising all those variables such as pH, carbohydrate, protein and fat content of the substrate, colloids such as starch or soil, salt and other soluble organic or inorganic compounds present.

Stumbo (1965, p. 101) suggested that the pH of the medium, salt, sugar and fat concentration, and water content, are some of the major factors responsible for the variation in the heat resistance. The same author (1965, p. 67) emphasized the importance of the nature of the medium in which heated spores or cells suspensions are subcultured. According to the recovery medium, the counts of viable cells or spores may vary and the observed survivor curve may be altered.

Since the thermal resistance of microorganisms depends on so many factors, this would partially explain the differences in the apparent heat resistance of a same bacteria, obtained by different authors in varying conditions. Owing to the great variation in heat resistance of spores from different suspensions, even though prepared under apparently identical conditions, and also to variations in the composition of the same food products, it has been considered advisable to include in most thermal resistance determinations, a standard heating medium for reference. This medium is a phosphate buffer, prepared by mixing M/15 Na_2HPO_4 and M/15 KH_2PO_4 in order to give a final pH of 7.0 (NCA, 1968, p. 181).

Thomas et al. (1966) mention that for vegetative cells the "Z value" usually found is about $5.6 \pm 1.1^{o}C$. Riemann (1969, p. 497) states that the "Z value" for most Salmonella strains is about 4^{o} to $5.0^{o}C$, somewhat higher values (5.6^{o} to $6.4^{o}C$) being observed for S. senftenberg 775 W. Concerning Staphylococcus aureus, the

reported "Z values" observed varies from 4.7° to 7.3°C.

The resistance of Vibrio cholerae to temperature is not high, being destroyed in ten minutes at 55°C; it is particularly sensitive to drying, and does not survive long in association with the ordinary saprophytic bacilli of soil and water (Burrows et al., 1968, p. 531). Smith (1961) observed that V. anguillarum, the causal agent of vibriosis in finnock withstood heating to 40°C for ten minutes, but not to 45°C for the same time.

Fujino (1964) observed that in fish submitted to pasteurization, V. parahaemolyticus was completely destroyed by a thermal treatment of 50°C for 20 minutes or 55°C for ten minutes or 65°C for five minutes. Tenmyo (1966) observed that V. parahaemolyticus could be destroyed if heated in a peptone solution at 55°C for ten minutes, or at 60°C for five minutes. The organisms were also killed if suspended in distilled water for a short period of time. Based on these results, the author suggested that outbreaks could be prevented by washing sea fish sufficiently in fresh water or in boiling water, keeping sea fish and fish products cold, as well as cooking them before eating.

EXPERIMENT I. GEL ELECTROPHORESIS OF CULTURE SUPERNATANTS OF V. PARAHAEMOLYTICUS AND RELATED MARINE VIBRIOS

Materials and Methods

Microorganisms Used

The microorganisms were obtained from the following sources:

	Original isolation	Source
V. parahaemolyticus ATCC 17802, ATCC 17803	Isolated from cases of "shirazu" food poisoning in Japan	ATCC
V. parahaemolyticus SJ-K 4, SJ-K 20, SJ-K 32	Sea water, sea fish, patients stools in Japan	Dr. R. M Twedt, UDSHEW Public Health Service
"Suspected" V. parahaemolyticus CDC-A1334, CDC-A3454, CDC-A8198	Patient isolates in the United States	Dr. R. M. Twedt
Nonpathogenic marine Vibrio		
CB-25	Chesapeake Bay (US) mud	Dr. R. M. Twedt
CB-64	Chesapeake Bay (US) water	Dr. R M. Twedt
CB-153	Chesapeake Bay (US) oysters	Dr. R. M. Twedt
V. anguillarum LS-68-1, LS-68-2, LS-68-3	Isolated from chinook salmon disease on the Oregon Coast (US)	Dr J. L. Fryer, Dept. of Microbiology Oregon State University
V. alginolyticus ATCC 17749	Isolated from spoiled horse mackerel in Japan	ATCC

According to Twedt et al. (1969), V. parahaemolyticus strains SJ were isolated from feces of patients suffering from gastroenteritis or from food implicated in food poisoning outbreaks. The "suspected"

V. parahaemolyticus strains CDC were isolated from infections acquired by swimmers in contact with the marine environment, being cultivated from blood, sputum, discharge from eye and ear, and lesions of wrist, leg, and foot.

Culture Maintenance

Stock cultures of V. parahaemolyticus ATCC, SJ and also strains CDC, V. anguillarum and V. alginolyticus were maintained on agar slopes in screw cap tubes at room temperature using Trypticase Soy Agar (BBL) plus 2.5 percent NaCl as culture medium. Marine Vibrio strains CB were maintained using the medium with the composition proposed by Colwell et al. (1968): 2.4 percent NaCl, 0.07 percent KCl, 0.53 percent $MgCl_2 \cdot 6H_2O$, 0.70 percent $MgSO_4 \cdot 7H_2O$, 1.0 percent Proteose Peptone (Difco), 0.3 percent yeast extract (Difco), and 1.5 percent agar (Difco), with a final pH of 7.2. Throughout the experiment this medium will be referred to as Vibrio Maintenance Medium.

Before being used, the cultures were streaked on plates containing Trypticase Soy Agar or Maintenance Medium, according to the strain, being incubated for 24 hours at 28°C in the case of V. anguillarum and at 37°C for all other cultures. After observable growth, the cultures were Gram stained and examined microscopically for the presence of eventual contamination.

Preparation of Culture Supernatants

A modification of the sac culture method proposed by Donnelly et al. (1967) for the production of staphylococcal enterotoxin, was employed for the preparation of concentrated supernatant. The sac culture assembly was made from cellulose dialyzing tubing (Scientific Products), approximately 7.5 cm wide and 40 cm long. This piece was washed thoroughly in distilled water and knotted at one end, then was introduced in a 500 ml Erlenmeyer flask. Two hundred ml of double strength Brain Heart Infusion (BBL) plus 2.5 percent NaCl was placed in the sac and the open end was then knotted. The flask was sterilized in the autoclave at 121°C for 15 minutes and 100 ml of sterilized phosphate buffer (0.2 M Na_2HPO_4 and 0.2 M NaH_2PO_4, pH 7.2) plus 2.5 percent NaCl were added to the flask, outside the sac. The growth from an agar slant was washed in two ml of the buffer and added to the dilution buffer surrounding the sac.

The sac cultures were incubated for 48 hours in a rotary shaker at a temperature of 28°C for V. anguillarum and at 37°C for all the other cultures. The growth surrounding the sac was removed from the flasks and most of the cells were separated by centrifuging at 17,300 g for 20 minutes at 4°C. The supernatant was further concentrated by pouring into dialysis tubing (Scientific Products) and dialyzing against 50 percent (w/v) Polyethylene Glycol 6,000 (Fischer) for six hours at 4°C. This resulted in a reduction in volume to about

two ml--a 50 fold concentration. The concentrate was stored in small screw cap tubes and maintained at 4°C for later use. A control was prepared following the same procedure, but without inoculation with microorganisms.

Flat Gel Electrophoresis

Polyacrylamide gels were prepared according to the method of Davis (1964) using an electrophoretic unit developed for flat gel electrophoresis. Levant (1969) describes the characteristics and operation of this unit.

The concentrated supernatant (0.1 ml) was diluted with Tris-Glycine buffer, pH 8.3, in a ratio of 1:5 and gently floated on the surface of the large pore gel. The surface of the gel was divided along its length in eight parts (two cm each) using plastic tubings (Tygon) so that eight different concentrated supernatants could be submitted to electrophoresis at the same time. The current was adjusted to 50 milliamperes and the electrophoresis was continued until the tracking dye front had migrated to about 0.5 cm from the bottom of the small pore gel (approximately one hour). After completion, the protein laden gels were removed from the trough, the running gels were cut into vertical strips and submitted to the tests for enzymes.

Gel Analysis

Total Protein

After the electrophoretic fractionation, the polyacrylamide gels were suspended in 12.5 percent Trichloroacetic Acid (TCA). After 30 minutes, they were immersed in a staining solution prepared by a 1:20 dilution in 12.5 percent TCA of a one percent aqueous stock solution of Coomassie Blue (Colab). After 12 hours of staining the gels were transferred into 10 percent TCA and photographed.

Tests for Enzymes

The electrophoresed gels were cut in small strips and layered on a microscopic slide and then covered with 1.5 ml of the proper substrate embedded in agar gel. In the tests for phosphatases and esterase, the gels were immersed into a solution containing the substrate for the enzyme being studied.

Lipases

Tween 80 (polyoxyethylene sorbitan monooleate) as substrate. The method described by Sierra (1957) to detect lipolytic activity was employed, the gels being flooded with a medium containing 0.25 g NaCl, 0.005 g $CaCl_2 \cdot H_2O$, 0.5 ml of Tween 80 (one percent final concentration), 0.5 g Ionagar no. 2 (Oxoid) and distilled water up to

50 ml, and the final pH adjusted to 7.4. After incubation at 30°C, a positive reaction was indicated by formation of an opaque precipitation band, due to formation of calcium salts.

Egg yolk as substrate. Egg yolk was diluted 1:1 with physiological saline. Five percent of the above solution was added to a 1.5 percent Ionagar no. 2 solution at a temperature of 45°C, and immediately flooded on the gel. A positive reaction was indicated by the formation of an opaque precipitation band.

Proteolytic Enzymes

Casein as substrate. Five ml of sterilized reconstituted skin milk were added to five ml of 1.5 percent Ionagar no. 2 in saline. The preparation was added to the slide at a temperature of 45°C. After incubation at 30°C the gels were developed by immersion in a solution containing 1.5 g $HgCl_2$, 2 ml of concentrated HCl and 10 ml of water. A positive reaction was indicated by the formation of a clear band at the gel, made opaque by the addition of the developing reagent.

Gelatin as substrate. Gelatin (Difco) in an amount of 0.5 g and 0.5 g of Ionagar no. 2 were dissolved in 50 ml of physiological saline and the mixture heated to dissolve the agar and applied to the gel at 45°C. The same developing reagent was employed and a positive result was indicated by clear bands in the gel turned opaque by

the addition of the reagent.

Amylase

Soluble starch (Difco) in an amount of 0.1 g and 0.5 g of Ionagar no. 2 were added to 50 ml of physiological saline, being applied to the slide after the agar was dissolved and at a temperature of 45°C. After incubation at 30°C, the gel was developed by immersion in a solution containing 0.1 g of iodine, 0.2 g KI and 30 ml of distilled water. A positive result was indicated by the formation of a clear band in the gel turned blue purple by the addition of the developing reagent.

Deoxyribonuclease

DNA agar was prepared by adding 0.2 percent (w/v) DNA (calf thymus, A grade, Calbiochem) to one percent Ionagar no. 2 in saline, being the substrate placed on the gels. After incubation at 30°C, the presence of DNase was indicated by clear bands in the medium, made opaque by the addition of 1N HCl developing reagent.

Hemolysis

Ionagar no. 2 in an amount of 1.5 g was dissolved in 100 ml of physiological saline. After the agar was melted and at a temperature of 45°C, five ml of defibrinated rabbit blood was added, and the medium

flooded on the gel. A positive reaction was indicated by formation of clear bands.

"Kanagawa Phenomenon"

A modification of Wagatsuma's medium (Miyamoto et al., 1969) was employed, with the following composition: seven percent NaCl, 0.5 percent $KaHPO_4$ and 1.5 percent Ionagar no. 2 were added to 100 ml of distilled water. After dissolving by heating, mannitol to a concentration of one percent, 0.1 percent Crystal Violet (in alcohol solution to 0.1 percent) and five percent fresh defibrinated rabbit blood were added, and the medium flooded on the gel.

Phosphatases

Alkaline phosphatase. The system proposed by Burstone (1962) was utilized.

Substrate - 10 mg of sodium alpha napthyl acid phosphate (Sigma)

Diazonium salt - 20 mg of Fast Blue RR (Eastman)

Activator - 0.3 ml of 10 percent $MgCl_2$ solution

Buffer - 40 ml of 0.1 M Tris-HCl buffer, pH 8.5

The reagents were mixed immediately before use and the gels were immersed in this solution. A positive reaction was indicated by the

appearance of dark blue bands in the gel.

Acid phosphatase. The assay system consisted of the same components as those given for the alkaline phosphatase, but the pH was adjusted to 5.0

Aromatic Esterases

Substrate - 1 ml of one percent alpha naphthyl propionate (Sigma) prepared by dissolving 0.1 g in 10 ml acetone

Diazonium salt - 20 mg of Fast Blue RR

Buffer - 40 ml of 0.1 M Tris HCl buffer, pH 7.0

The reagents were mixed immediately before use and the gels were immersed in the solution, a positive reaction being indicated by formation of dark blue bands.

Due to the fact that diazonium salts also combine with functional groups of proteins, such as the free amino group of lysyl residues, negative controls were also prepared in the reactions for phosphatases and esterase. These controls were conducted by incubating the gels in solutions containing the diazonium salt but not the synthetic substrate.

Results and Discussion

Total Proteins

The results showed the presence of a large number of proteins in the culture supernatants, but differences were observed concerning the number of bands and the total protein pattern. However, a more accentuated similarity was observed between the patterns of V. parahaemolyticus (strains ATCC and SJ) and the "suspected" V. parahaemolyticus isolated from skin infection (strains CDC).

The presence of common bands with the strains of V. parahaemolyticus was also noticed concerning the nonpathogenic marine Vibrio (strains CB) but without a great similarity in the total protein pattern. All strains of V. anguillarum showed a relatively uniform pattern and different from those of V. parahaemolyticus. Figure 1 shows a comparison among strains of V. parahaemolyticus, and Figure 2 shows the total protein pattern for the different cultures examined.

The metabolic state of microorganisms may influence the number and types of proteins which can be electrophoretically separated. In order to minimize this influence, all the cultures in examination were grown under essentially the same cultural conditions. Variations in the total protein pattern, observed among different Vibrio species, probably is indicative of quantitative and qualitative differences between the enzymes present in the supernatant.

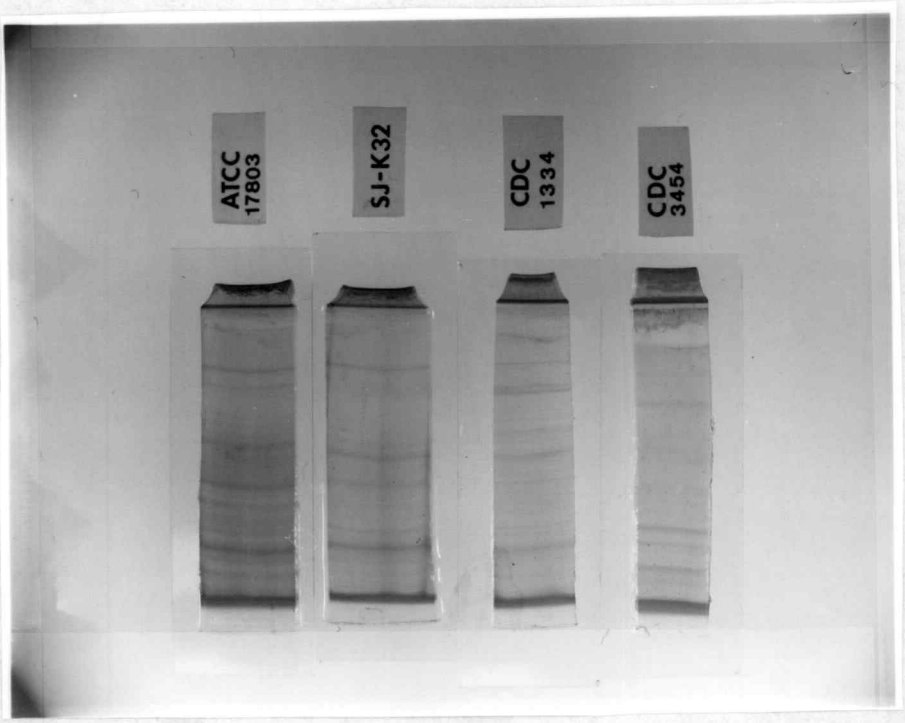

Figure 1. Total protein pattern for strains of V. parahaemolyticus and cultures isolated from skin infection.

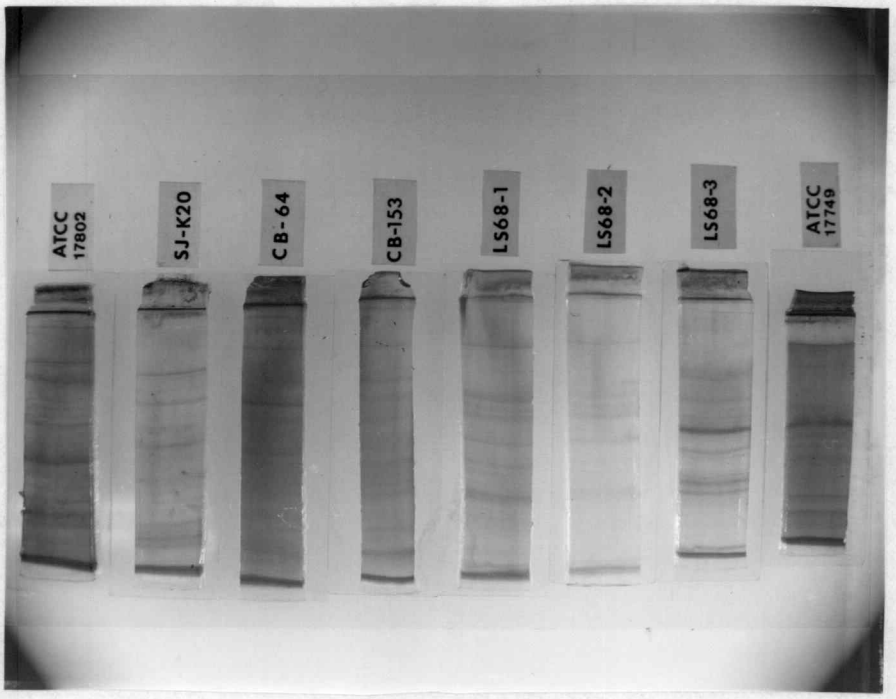

Figure 2. Total protein pattern for V. parahaemolyticus, nonpathogenic marine Vibrio (strains CB), V. anguillarum (strains LS) and V. alginolyticus (ATCC 17749).

Enzyme Tests

Table I summarizes the general results observed in the enzyme tests.

Lipases

Tween 80 as substrate. All strains and species with positive reaction showed the presence of a common band, with the same electrophoretic mobility, but with varying intensity of reaction, that was very strong in V. anguillarum and weak in V. alginolyticus. A positive result was observed after incubation of the gels for eight hours at 30°C. V. parahaemolyticus and strains CDC showed identical reactions, characterized by the presence of a second band, with lower electrophoretic mobility. Figure 3 shows the pattern for the lipase reaction.

Egg yolk as substrate. V. parahaemolyticus and the pathogenic strains CDC, showed a uniform reaction, with two bands being observed after two hours of incubation at 30°C. This reaction probably is due to a lecithinase, already shown to be present in V. parahaemolyticus (Krantz et al., 1969). The differentiation of the non-pathogenic Vibrio, V. anguillarum and V. alginolyticus, was based on the intensity of the reaction, which was visible only after 12 incubation. Figure 4 shows the observed pattern of reactions.

Table I. Characteristic activities of enzymes of V. parahaemolyticus and related marine vibrios.

Culture	Lipases		Proteolytic enzymes						Phosphatases		
	Tween 80 as substrate	Egg yolk as substrate	Casein as substrate	Gelatin as substrate	Amylase	DNase	Hemolysis	"Kanagawa phenomenon"	Alkaline	Acid	Aromatic esterase
V. parahaemolyticus											
ATCC 17802	+	++	+	+	+	+	+	-	++	++	++
ATCC 17803	+	++	+	+	+	+	+	-	++	++	++
SJ-K4	+	++	++	+	+	+	+	-	+	+	+
SJ-K20	+	++	+	+	+	+	+	-	++	++	++
SJ-K32	+	++	+	+	++	+	+	-	++	++	++
"Suspected" V. parahaemolyticus											
CDC-A1334	+	++	++	++	++	++	+	-	+	+	-
CDC-A3454	+	++	+	+	++	++	+	-	+	+	-
CDC-A8198	+	++	+	++	++	++	+	-	+	+	-
Marine Vibrio											
CB-25	++	+	-	+	-	-	-	-	-	-	++
CB-64	-	+	-	+	++	-	-	-	-	-	++
CB-153	-	+	-	+	-	-	-	-	+	+	++
V. anguillarum											
LS-68-1	++	+	++	+	-	-	-	-	-	-	++
LS-68-2	++	+	++	+	-	-	-	-	-	-	-
LS-68-3	+	+	++	+	-	-	-	-	-	-	-
V. alginolyticus											
ATCC 17749	+	+	-	++	-	+	-	-	-	-	-

++ strong reaction
+ weak reaction
- no reaction

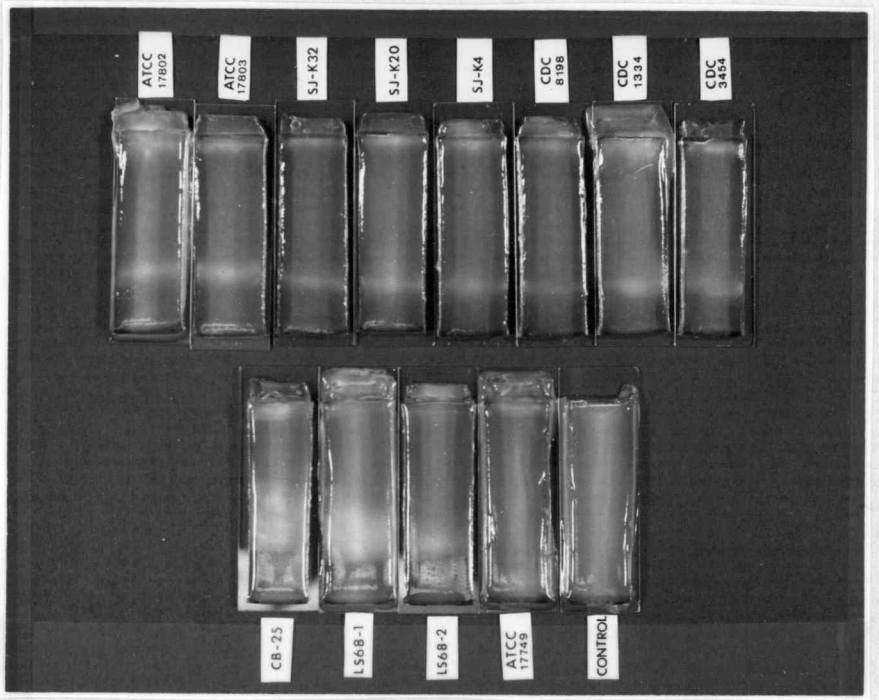

Figure 3. Pattern of reactions for lipase, using Tween 80 as substrate, and observed after eight hours incubation at 30°C.

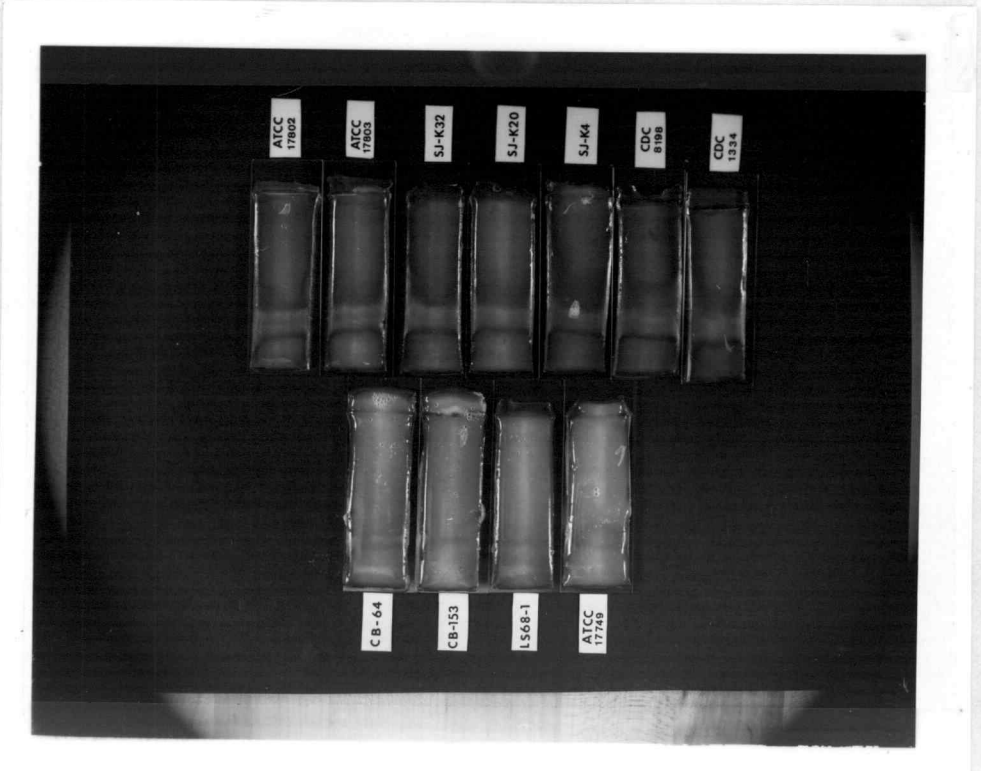

Figure 4. Pattern of reactions for egg yolk lipase, after 12 hours incubation at 30°C.

Proteolytic Enzymes

A complex pattern of multimolecular forms of enzymes (isozymes) was observed, particularly when using gelatin as substrate, some of the gels showing the presence of four bands, and at least two bands being observed in all of them. Variations were also noticed in the intensity of the reactions that were observed after eight hours incubation at 30°C.

When casein was used as substrate two bands were observed in V. parahaemolyticus and the CDC strains. However no bands were observed in the non-pathogenic or V. alginolyticus. All strains of V. anguillarum showed a uniform reaction, with the presence of two bands but with lower electrophoretic mobility than the bands in V. parahaemolyticus. Figure 5 shows the observed results.

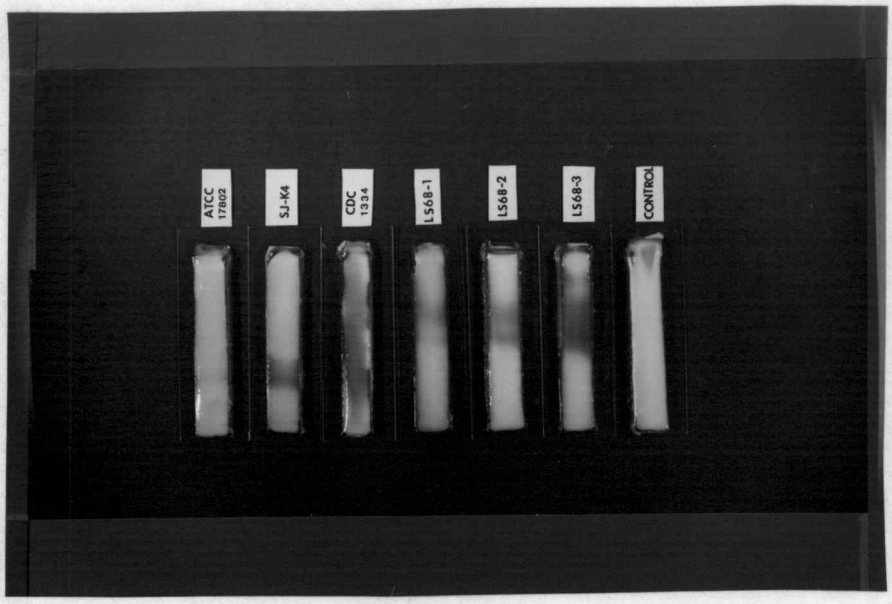

Figure 5. Pattern of reactions for casein hydrolysis, observed after incubation at 30°C for eight hours.

When gelatin was used as substrate, the reaction pattern showed variations among strains and species. The results suggested the presence of at least one common band in V. parahaemolyticus and the pathogenic strains. The nonpathogenic Vibrio (strains CB), V. anguillarum and V. alginolyticus also showed one band with the same mobility as the common band of the pathogenic strains. Figure 6 shows the observed pattern of reactions in some of the cultures being studied.

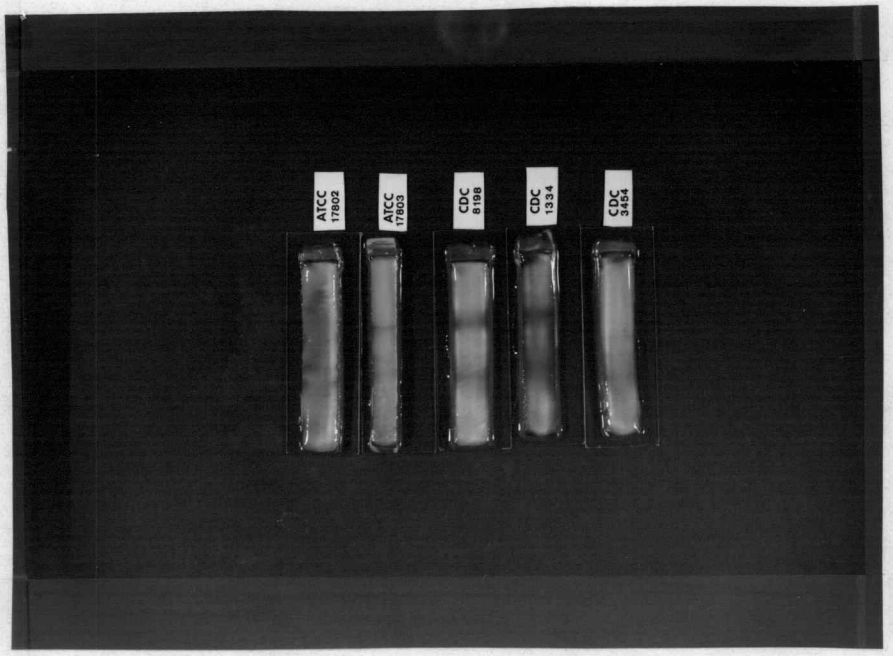

Figure 6. Pattern of reactions for gelatin hydrolysis, observed after incubation at 30°C for eight hours.

These results suggest the strong proteolytic activity presented by the Vibrio cultures under examination. Most of these marine vibrios are considered to be fish pathogens, particularly V. anguillarum, but V. parahaemolyticus has also been reported as causal

agent of a disease in blue crabs. According to Krantz et al. (1969) the presence of lipase, lecithinase, and proteolytic enzymes may contribute to the invasiveness of the bacteria.

Amylase

A positive, intense and uniform reaction was observed only in V. parahaemolyticus and the pathogenic strains (CDC) with the presence of a common band with approximately the same electrophoretic mobility in all of them. Only one nonpathogenic marine Vibrio (CB-64) showed a positive reaction. The results are shown in Figure 7.

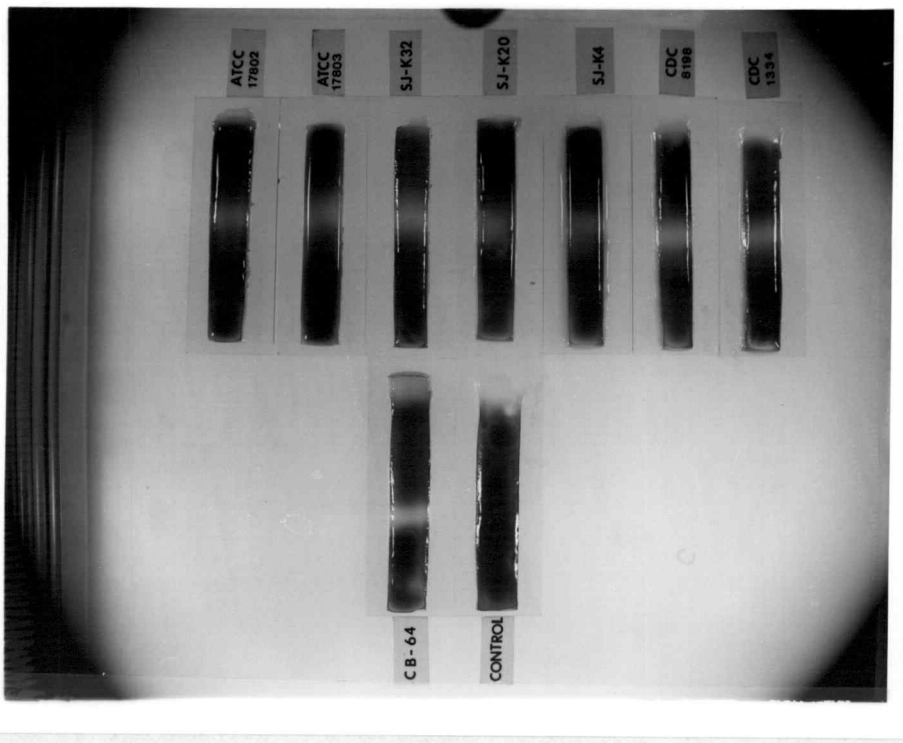

Figure 7. Pattern of reactions for starch hydrolysis observed after incubation at 30°C for eight hours.

These results agree with the observations made by Twedt et al. (1969) in which the authors noticed that practically all strains from gastroenteric origin and from skin infection, were able to hydrolyze starch, while approximately 50 percent of the nonpathogenic marine Vibrio were unable to carry out this hydrolysis. Based on this characteristic, the authors suggested that this test should be included as a criteria for separating pathogenic and nonpathogenic marine Vibrio.

Deoxyribonuclease

V. parahaemolyticus and CDC strains showed DNase activity, characterized by the presence of a common band, with the same electrophoretic mobility in all of them. The presence of a second band was apparent in some of the strains (ATCC 17802, ATCC 17803 and SJ-K32). All nonpathogenic marine Vibrio and V. anguillarum showed a negative reaction, but V. alginolyticus also presented DNase activity. Figure 8 shows the results observed.

According to Brock (1970, p. 137) there are a number of microorganisms that can utilize the nucleic acids as sources of carbon, nitrogen and energy. The nucleic acids are hydrolyzed to nucleotides by nucleases, that are extracellular enzymes. This makes it possible for the organism to utilize high molecular weight nucleic acids that cannot pass through the cell membrane, but the low molecular weight

nucleotide hydrolysis products penetrate the cell.

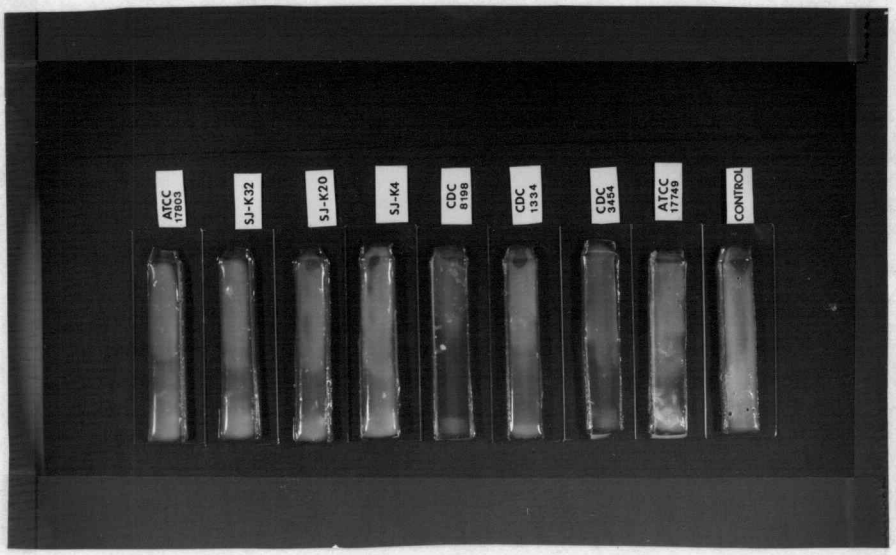

Figure 8. DNase activity, observed after incubation at 30°C for eight hours.

High DNase production is observed in the pathogenic pus-forming bacteria of the genera Streptococcus and Staphylococcus. In the pimple or boil where these organisms grow, there is a large amount of tissue destruction, and through the production of nucleases these bacteria are able to use as nutrients the nucleic acids liberated from the dead cells. Similarly, some of the "suspected" V. parahemolyticus strains (CDC) were isolated from skin and localized tissue infections, being cultivated from sputum, discharge from eye and ear, lesions of leg, foot, etc. (Twedt, et al., 1969). This fact suggests a possible explanation for the presence of DNase activity in these strains.

Hemolysis

A positive reaction was observed only in V. parahaemolyticus and the pathogenic strains with two uniform bands being formed, with relative mobilities (compared with the mobility of the tracking dye) 0.68 and 0.88. However, the intensity of the reaction was very weak, being observed only after incubation at 30°C for 12 hours.

The negative results observed in the nonpathogenic marine Vibrio (strains CB) do not mean that under different conditions these strains could not present hemolytic activity. Twedt et al. (1969) reported that 12 of the 14 nonpathogenic strains studied produced hemolysis when the cultures were streaked on Brain Heart Infusion Agar containing five percent sheep blood.

A possible explanation for the differences between pathogenic and nonpathogenic strains would be a variation in the concentration of the hemolytic factor present in larger amounts in the supernatant of the pathogenic strains. V. anguillarum is not reported as presenting hemolytic activity, according to Smith (1961) who tested on horse blood agar, and Cisar and Fryer (1969) who observed negative results when testing on sheep blood agar. According to Miyamoto et al. (1961) Vibrio alginolyticus showed a weak hemolytic activity, when growing on three percent NaCl blood agar.

"Kanagawa Phenomenon"

All the concentrated supernatants failed to show the presence of the hemolytic factor responsible for the "Kanagawa phenomenon". Sakazaki et al. (1968) and Miyamoto et al. (1969) reported that this factor is present only in the pathogenic strains, being heat stable and demonstrable in the supernatants. No relationship was found between biochemical and serological properties and the hemolytic activity of the vibrios. According to Thatcher and Clark (1968, p. 108) the hemolytic activity is reported to decrease after serial passages of hemolytic strains on artificial media, and this probably would explain the negative reaction observed.

Phosphatases

Alkaline phosphatase. A positive reaction, visible 20 minutes after immersion of the gels into the solution containing the substrate, was observed in V. parahaemolyticus and CDC strains and one of the nonpathogenic strains (CB-153). The pattern showed the presence of at least one common band among all positive strains, with a relative mobility of 0.66, while a second band with less electrophoretic mobility was observed in the strains ATCC 17803, SJ-K32, and SJ-K20.

Acid phosphatase. The pattern of reactions was similar to the alkaline phosphatase, slight variation being observed in the

electrophoretic mobilities. Again, all V. parahaemolyticus and CDC strains showed a positive reaction, and a common band with a relative mobility of 0.64 was observed in all of them, except strain ATCC 17802; this strain showed a band common with ATCC 17803 and relative mobility of 0.57. Finally, a third band with less mobility and weaker intensity was observed in four strains. Figure 9 shows the pattern for acid phosphatase.

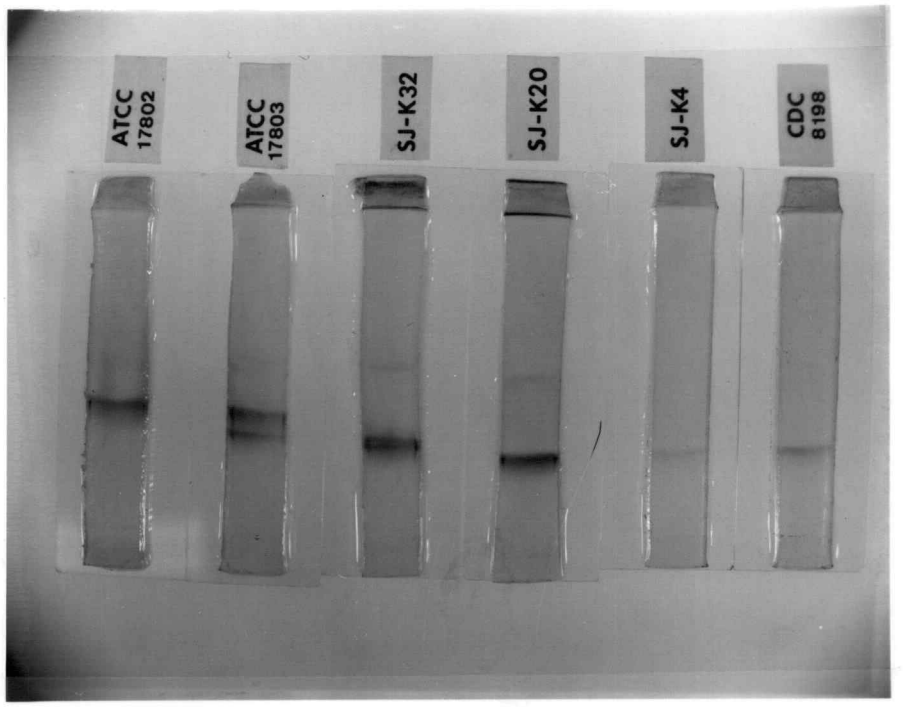

Figure 9. Pattern for acid phosphatase, observed after incubation for one hour at 30°C.

Aromatic Esterases

This was the only test in which a difference was observed between V. parahaemolyticus strains of gastroenteric origin, and the

"suspected" V. parahaemolyticus isolated from skin infections. Strains ATCC and SJ showed a pattern with the presence of three bands with approximately the same electrophoretic mobilities (0.60, 0.72, 0.78). The nonpathogenic strains (CB) and V. anguillarum LS-68-1 showed the presence of only one band, having the same mobility as the least mobile band of the V. parahaemolyticus strains (0.60). Pathogenic strains CDC, showed a negative result. Figure 10 shows the results observed.

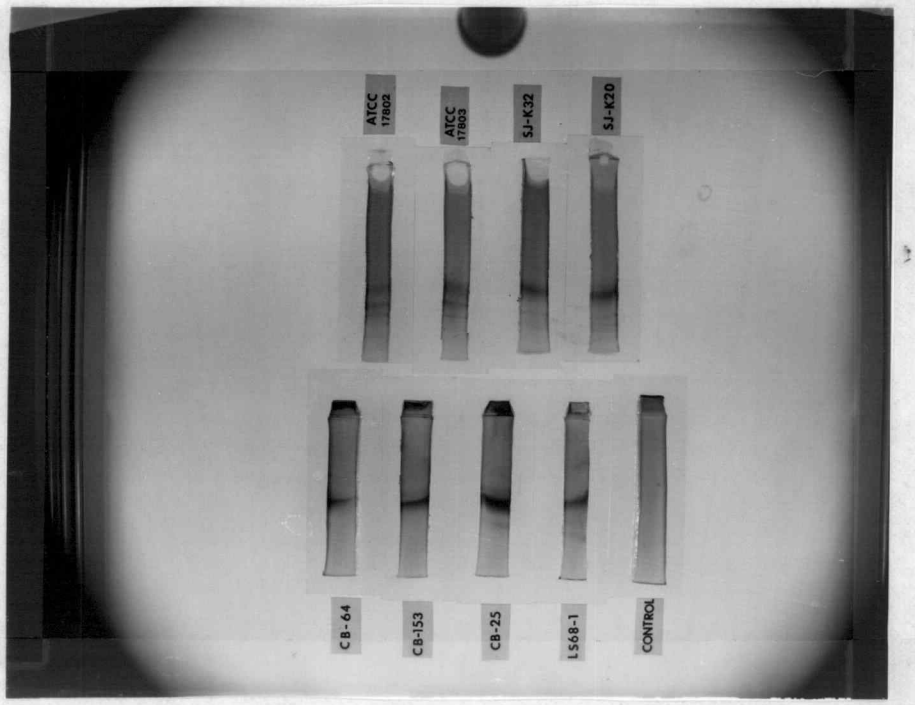

Figure 10. Pattern for aromatic esterases observed after incubation at 30°C for one hour.

The esterase pattern has been considered of value in the characterization of microorganisms (Lund, 1965; Robinson, 1968; Adeyemo et al., 1967; Colwell et al., 1968). Adeyemo et al.

(1967) observed the presence of an esterase pattern in V. cholerae and related Vibrio sp., characterized by the presence of one, two or three bands, the last one being observed in marine strains and non-cholera vibrios. Colwell et al. (1968) observed the presence of only one esterase band among 12 V. parahaemolyticus strains. However, these experiments were carried out under different conditions, using starch gel electrophoresis of extracts prepared from disrupted cells, and consequently the results are not directly comparable.

Discussion of Selected Tests

The general results obtained in this experiment suggest a great similarity between V. parahaemolyticus and the pathogenic strains CDC, independent of place and conditions of original isolation. Even considering some variations, observed particularly in the reaction for aromatic esterase, the pattern for all the other tests was identical or very similar. The nonpathogenic marine Vibrio (strains CB), V. anguillarum and V. alginolyticus, showed a great variation when compared with V. parahaemolyticus and a clear differentiation was obtained. However, the nonpathogenic cultures showed a similar pattern in most reactions.

Twedt et al. (1969) studying cultural, morphological and physiological characteristics of growing cultures of V. parahaemolyticus and nonpathogenic marine Vibrio also concluded that these two

groups could be distinguished in cultural and biochemical aspects.

The results obtained in the present experiment suggest a rapid way to differentiate potentially pathogenic strains of V. parahaemolyticus. The tests for DNase, phosphatases, amylase, and egg yolk lipase, showed the most characteristic results for the differentiation. The other tests do not appear as satisfactory, particularly due to variations in the pattern of reactions among the pathogenic strains (as in the case of aromatic esterase), identical reactions in all cultures, with slight variation in the intensity (lipase, using Tween 80 as substrate), very complex patterns, with the presence of several bands with variations in the electrophoretic mobilities (gelatin and casein hydrolysis), or very weak intensity of reaction, making difficult and inaccurate the correct observation (hemolysis).

The test for the presence of phosphatase is usually employed in the characterization of potentially pathogenic staphylococci, and it is also considered as a valuable criteria for the differentiation and separation of organisms within the genera Staphylococcus and Micrococcus. The test for DNase activity is also considered of value in order to detect pathogenic staphylococci, and differential media are available commercially, based on the assumption that there is a close correlation between staphylococcal nuclease and enterotoxin production.

These facts suggest the possibility of formulation of differential

media in order to characterize the potentially pathogenic strains of V. parahaemolyticus. Although , the observations made in this study are valid under the conditions of the experiment, it does not mean that a similar behavior would be observed in other situations. If future studies with a larger number of strains, and with growing cultures, confirm these characteristics, these tests could be employed for a more rapid identification.

The use of gel electrophoresis appears to be of value for the taxonomy of Vibrio, confirming previous observations made by other authors. The use of concentrated culture supernatants instead of extracts prepared from disrupted cells has the advantage of being less time consuming in the preparation, relatively easy to perform and with reduced possibility of contamination, a limitation being that practically only extracellular enzymes present in the supernatant can be studied.

EXPERIMENT II. THERMAL RESISTANCE OF VIBRIO PARAHAEMOLYTICUS

Materials and Methods

Culture

The bacterium studied was Vibrio parahaemolyticus ATCC 17802 (type strain). A primary culture was maintained on agar slants containing Trypticase Soy Agar (BBL) plus 2.5 percent NaCl, and covered with a layer of sterile mineral oil and incubated at room temperature.

Preparation of Inocula

From the primary culture, an inoculum was transferred to agar slants of Vibrio Maintenance Medium. After incubation at 37°C for 24 hours, the cells were washed from the surface of the slant with peptone salt water (one percent peptone, 2.5 percent NaCl and pH adjusted to 7.2), shaken thoroughly with glass beads to break up clumps, and adjusted to the desired concentration using a Spectronic 20 (Bausch and Lomb) spectrophotometer at 420 nm. A suspension containing approximately 10^8 cells/ml was employed as source of inoculum.

In every experiment, the cells employed as inoculum were obtained from a 24 hour old secondary culture, which had undergone only one transfer beyond the primary culture. This technique was used to

minimize variation and inadvertent selection of types whose heat resistance differed from that of the parent strain.

Experimental Procedure

The experiment was conducted using the flask method, according to the procedure recommended by Stumbo (1965, p. 83). A 500 ml three neck flask (Woulff bottle) was employed as substrate container. A thermometer was introduced through one neck, a small mechanical stirrer through the center one, and the third being used for introducing inoculum and withdrawing samples.

The suspending menstruum employed in the experiment was peptone salt water, with the composition mentioned above. After introduction of the menstruum, the entire assembly was sterilized at $121^{o}C$ for 15 minutes. The flask, containing 300 ml of suspending menstruum was cooled, and immersed in a thermostatically controlled water bath at the desired temperature. The assembly was located in the bath in such a way that the surface of the heating medium was well above the substrate surface in the flask, special care being taken to avoid splashing of the substrate on the walls of the flask. When equilibrium had been achieved between the temperatures of the water bath and the suspending menstruum, one ml of inoculum suspension was added to the flask (giving a final cell concentration of approximately 10^{5} cells/ml). Counts were made at zero time in order to determine

with more accuracy the initial number of cells.

At various time intervals, one ml samples were removed with a pipette to sterilized empty tubes in a cold water bath. Appropriate dilutions were made in distilled water plus 2.5 percent NaCl and plated using Vibrio Maintenance Medium as recovery medium. The plates were incubated at 37°C and counted after 24 and 48 hours, and the number of survivors being determined. The experiment was conducted at the temperatures of 113°F (45°C), 118.4°F (48°C), and 122°F (50°C), with three replications being made for each temperature.

Influence of the Nature of the Subculture Medium

In order to observe the influence of the nature of the recovery medium on the observed survivor curve of the bacteria, three recovery media were employed: Brain Heart Infusion Agar plus 2.5 percent NaCl, Trypticase Soy Agar, plus 2.5 percent NaCl, and Vibrio Maintenance Medium. The same procedure, as already described was followed, but the experiment was carried out only at the temperature of 122°F.

Treatment of Data

The "D value" was the parameter used to express the apparent heat resistance of the bacteria. At each temperature and replication,

the slope of the survivor curve was determined by the regression method. The reciprocal of the regression coefficient obtained, was used as the "D value" for that particular bacterial strain, test medium and temperature, the negative sign of the regression coefficient being ignored (Thomas, White and Longree, 1966).

Since the logarithmic order of death is assumed, the "D value" may also be calculated from the initial number of cells or spores and number surviving after some one heating time at each temperature being studied (Stumbo, 1965, p. 92; NCA, 1968, p. 190).

$$D = \frac{U}{\log a - \log b}$$

in which

U = heating time in minutes

a = initial number of microorganisms

b = number of microorganisms which survived the heating time (U).

This approach was also applied in order to determine the "D value". The variations in the survivor curve, and consequently in the "D value", according to the nature of the recovery medium, were determined by statistical analysis of variance among the "D values" obtained in different media.

Results and Discussion

Thermal Resistance at Different Temperatures

Table II summarizes the results obtained when V. parahaemolyticus ATCC 17802 was heated at different temperatures.

Table II. "D value" for V. parahaemolyticus ATCC 17802 at three temperatures.

Temperature	"D value" by formula (min)	"D value" by regression (min)	r
113°F	43.9	42.5	0.994**
	35.0	32.2	0.999**
	39.0	40.0	0.972*
Average	39.30	38.23	
118.4°F	1.92	1.83	0.994**
	2.13	2.30	0.976**
	1.82	1.92	0.972*
Average	1.95	2.01	
122°F	0.49	0.49	0.993*
	0.51	0.49	0.990**
	0.53	0.54	0.970*
Average	0.51	0.50	

*Significant at the level of 5 percent
**Significant at the level of 1 percent
r = correlation coefficient

Figures 11, 12 and 13 show the characteristics of the survivor curve obtained by plotting on semilogarithmic paper, the number of survivors related to time of exposure, the slope being determined by regression.

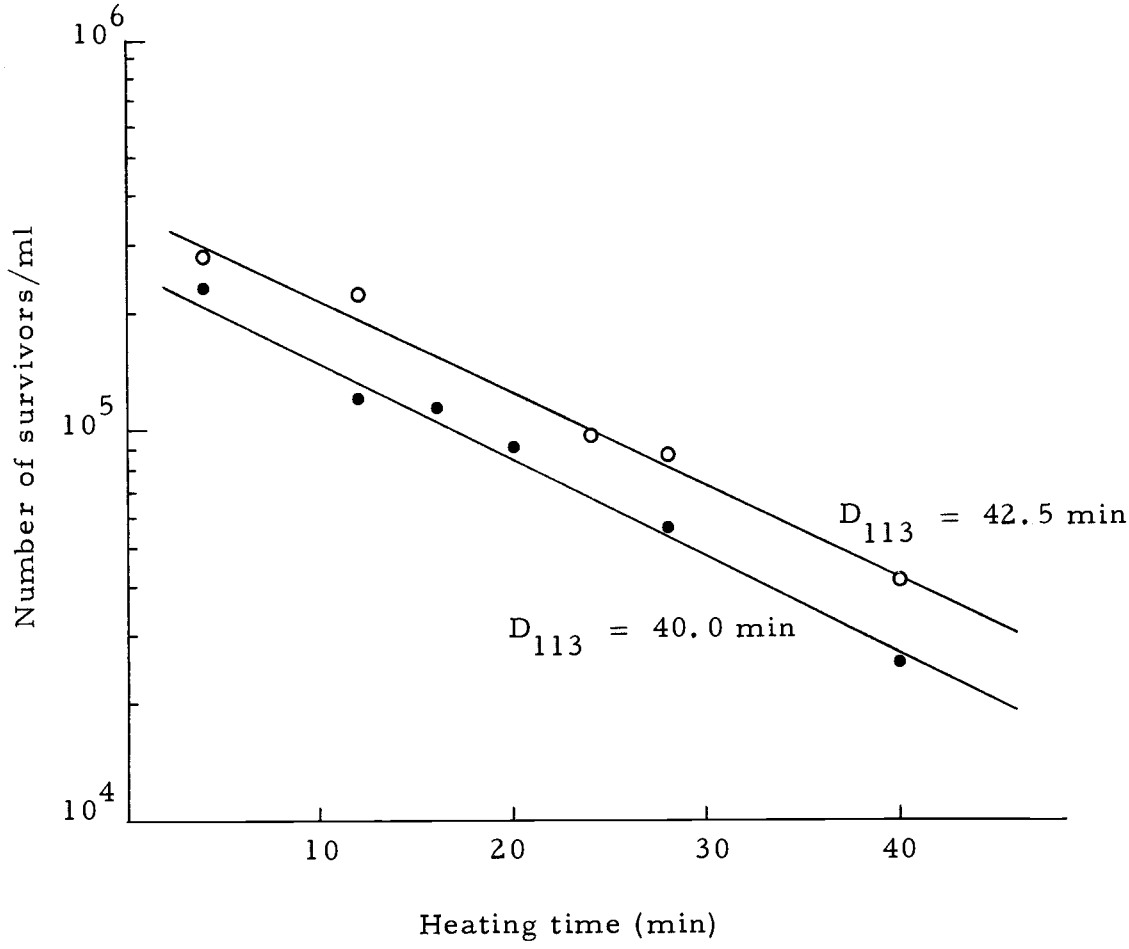

Figure 11. Logarithmic survivor curve of V. parahaemolyticus ATCC 17802, at the temperature of 113°F (45°C).

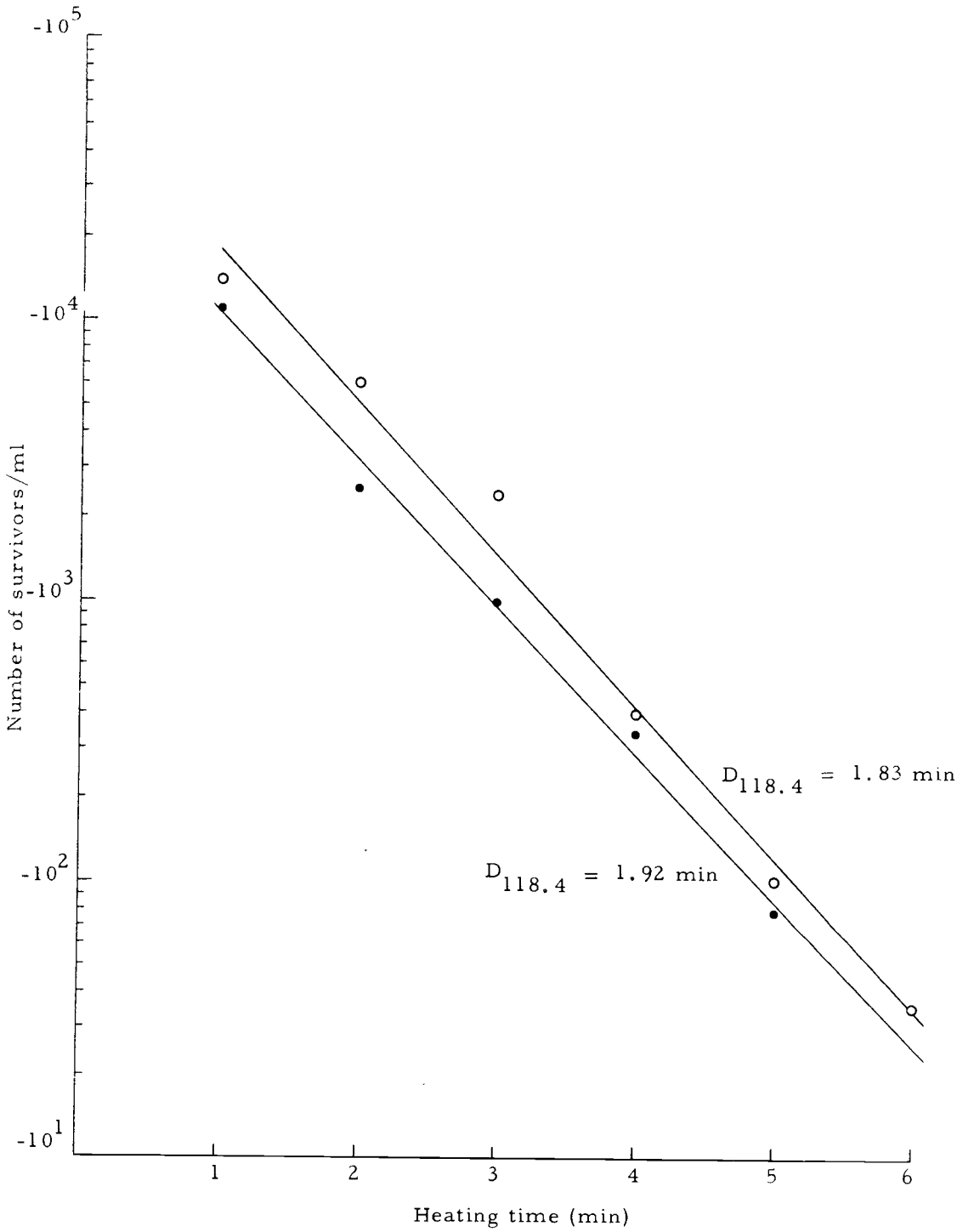

Figure 12. Logarithmic survivor curve of V. parahaemolyticus ATCC 17802, at the temperature of 118.4°F (48°C).

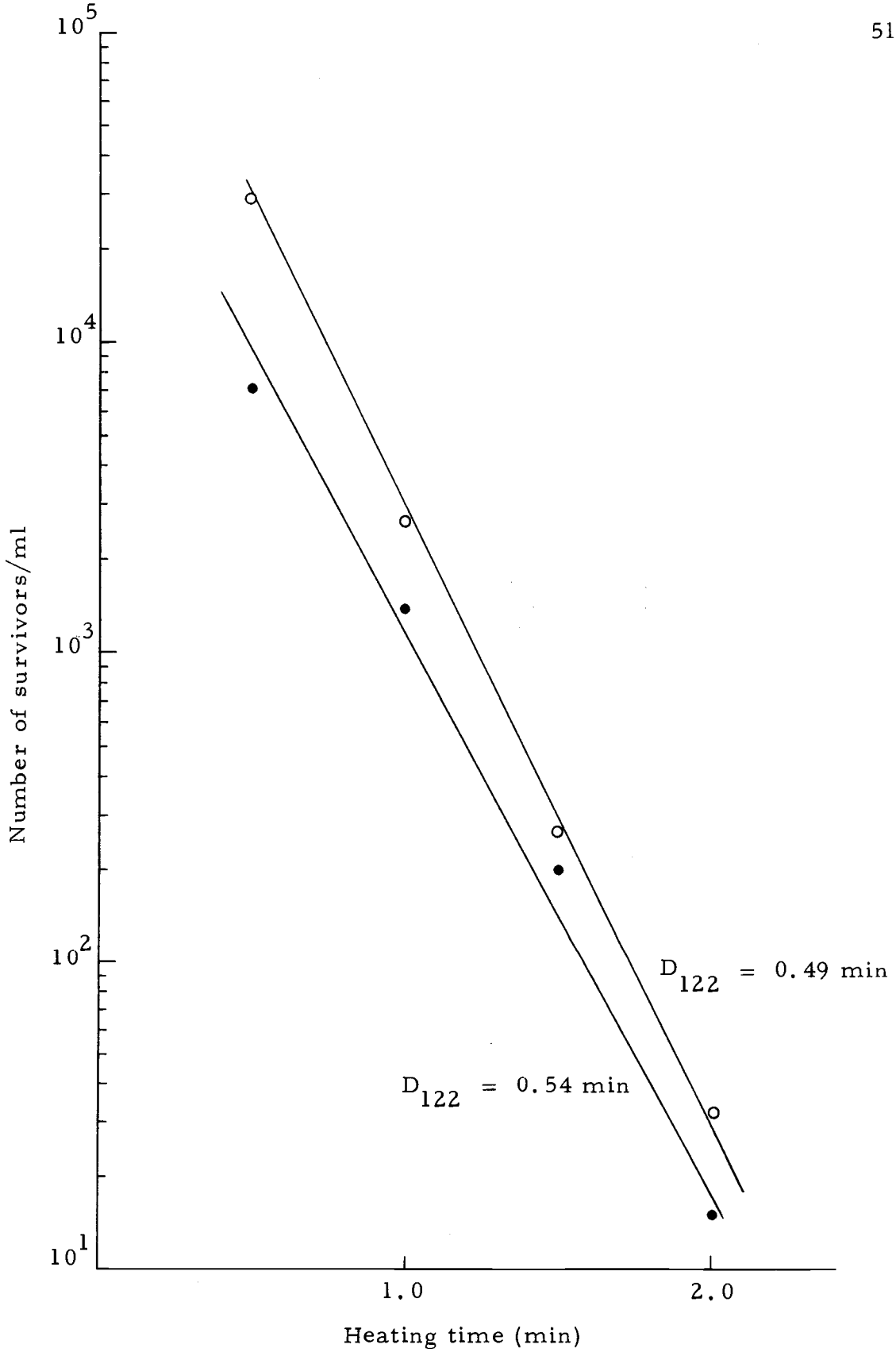

Figure 13. Logarithmic survivor curve of V. parahaemolyticus ATCC 17803, at the temperature of 122°F (50°C).

The "D values" determined by the regression method were very similar to those obtained by the formula method. According to Thomas et al. (1966) the regression method, although being much more laborious, has the advantage that it utilizes all information obtained in each experiment.

The results suggest a very low heat resistance for V. parahaemolyticus ATCC 17802. Observations by other authors under different conditions, partially confirm this characteristic in microorganisms in the genus Vibrio, and particularly V. parahaemolyticus. However, no generalizations can be made about the heat resistance of this bacterium based on the present experiment, because variations in the strain studied, and in the composition of the suspending menstruum, probably would result in a different apparent heat resistance.

The use of phosphate buffer as a standard suspending menstruum, is not indicated in the case of V. parahaemolyticus. This bacterium is reported to be destroyed when suspended in distilled water (Tenmyo, 1966) and Thatcher and Clark (1968, p. 109) recommended the use of four percent salt in peptone broth as dilution fluid instead of phosphate buffer dilution water, while other authors recommend three percent NaCl dilution water (USPHS, FDA, 1969, Sec BAM 14.06).

Influence of the Recovery Medium on the Apparent Heat Resistance

Table III shows the variations in the observed "D values" according to the composition of the recovery medium.

Table III. Variations on the apparent thermal resistance of V. parahaemolyticus ATCC 17802 at 122°F, according to the composition of the recovery medium.

Counting medium	Average D_{122} value
Brain Heart Infusion Agar + 2.5% NaCl	0.285
Trypticase Soy Agar + 2.5% NaCl	0.230
Vibrio Maintenance Medium	0.500
Variance, F	39.03*
$LSD_{.05}$	0.137
$LSD_{.01}$	0.316

*Significant at 5 percent level
LSD = Least significant difference

The results suggest that Vibrio Maintenance Medium, in which higher "D values" were observed, should be considered the most adequate recovery medium to be employed in studies of thermal resistance of V. parahaemolyticus. A significant difference at the level of five percent was observed when compared with the two other media, without a significant difference shown between the last two media.

Since a bacterial cell that fails to reproduce under conditions

considered favorable for such activity is generally considered as a dead cell (Stumbo, 1965, p. 68), it is important to select a medium for counting heated cells that will give the highest counts. This is particularly important when considering food spoilage and pathogenic bacteria, because an incorrect evaluation of the thermal resistance could result in a less severe heat processing, with higher probabilities of survival of these microorganisms. Frequently various types of media may give growth results which are equivalent for unheated cells, but different results when comparing the survivors of a heat treatment. The bacterial cells, damaged by the thermal process, generally are more fastidious in their growth and more nutritionally demanding.

These aspects were emphasized in the present experiment. Trypticase Soy Agar plus 2.5 percent NaCl and Brain Heart Infusion Agar plus 2.5 percent NaCl are media usually employed in the maintenance of V. parahaemolyticus, an intensive growth usually being observed. However, after the heat treatment, these two media were not satisfactory for the recovery of the cells.

Several possible explanations for the effect of heat in vegetative cells have been presented (Ordal, 1970), but it is difficult to relate these changes with the presence of substances in the recovery medium that will contribute to the repair of cell structures and functions. Vibrio Maintenance Medium is characterized by the presence of

mineral salts (NaCl, KCl, $MgCl_2$, $MgSO_4 \cdot 7H_2O$) besides the addition of yeast extract, and according to Twedt et al. (1969) it is the medium of choice for growing fastidious marine Vibrio. Probably, the presence of these components are related to the better recovery of heat damaged cells.

From a practical point of view, the determination of the "D value" is related to two important factors: 1) That the chances of survival in a microbial population subjected to heat are lower, the lower the density of population. It is therefore necessary from an industrial point of view to keep the number of microorganisms at the lowest possible level. 2) That it is theoretically impossible to attain complete sterility, as the survival curve is logarithmic, tending asymptotically to zero. It is, therefore, appropriate to speak of practical or "commercial sterility", without meaning that absolute sterility has been attained.

SUMMARY

Polyacrylamide gel electrophoresis of concentrated culture supernatants was carried out in order to characterize and differentiate V. parahaemolyticus from "suspected" V. parahaemolyticus cultures isolated from cases of skin infection, V. anguillarum, nonpathogenic marine Vibrio and V. alginolyticus. The differentiation was based on total protein patterns and examination of the gels for the presence of extracellular enzymes.

The results showed the presence of single and multimolecular forms of proteolytic enzymes, lipases, esterase, phosphatases, amylases and DNase, all supernatants showing the absence of the hemolytic factor responsible for the "Kanagawa phenomenon". A great similarity was observed between V. parahaemolyticus and the pathogenic cultures (strains CDC), independent of place and conditions of isolation. This group was clearly differentiated from the non-pathogenic Vibrio and from V. anguillarum and V. alginolyticus, that showed a uniform pattern in most reactions.

The tests involving the presence of DNase, amylase, egg yolk lipase (mainly related with time and intensity of reaction) appeared to be of value in the characterization of V. parahaemolyticus. The other tests, even showing some differentiation between strains and species, did not seem to provide a good characterization. The results suggested that the use of gel electrophoresis of concentrated culture

supernatants may supply evidences of possible taxonomic significance, permitting a fast identification of potentially pathogenic V. parahaemolyticus. This method is relatively easy to perform and less time consuming when compared with the use of concentrated extracts from disrupted cells.

Another purpose of the present work was to study the thermal resistance of V. parahaemolyticus ATCC 17802. The test was carried out applying the flask method, with a 24 hour old cell suspension being heated in peptone salt water, pH 7.2, as suspending menstruum. The "D value" was the parameter used to express the heat resistance, being determined at 113°, 118.4° and 122°F. The results showed a low thermal resistance, with D_{113} = 38.2 min, $D_{118.4}$ = 2.01 min and D_{122} = 0.51 min.

A comparative study related to the influence of the composition of the recovery medium on the apparent heat resistance, revealed higher survival rates, and consequently higher "D values" when Vibrio Maintenance Medium was employed. A statistically significant difference (at the level of five percent) was observed when compared with Brain Heart Infusion Agar plus 2.5 percent NaCl and Trypticase Soy Agar plus 2.5 percent NaCl. No significant difference was observed between the two other media.

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