SYSTEMATICS AND PATHOGENICITY OF VIBRIONACEAE ASSOCIATED WITH THE LARVAE OF *MACROBRACHIUM ROSENBERGII* IN HATCHERY

Thesis submitted

In partial fulfillment of the of the requirements

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By

Sarita G. Bhat

SCHOOL OF ENVIRONMENTAL STUDIES

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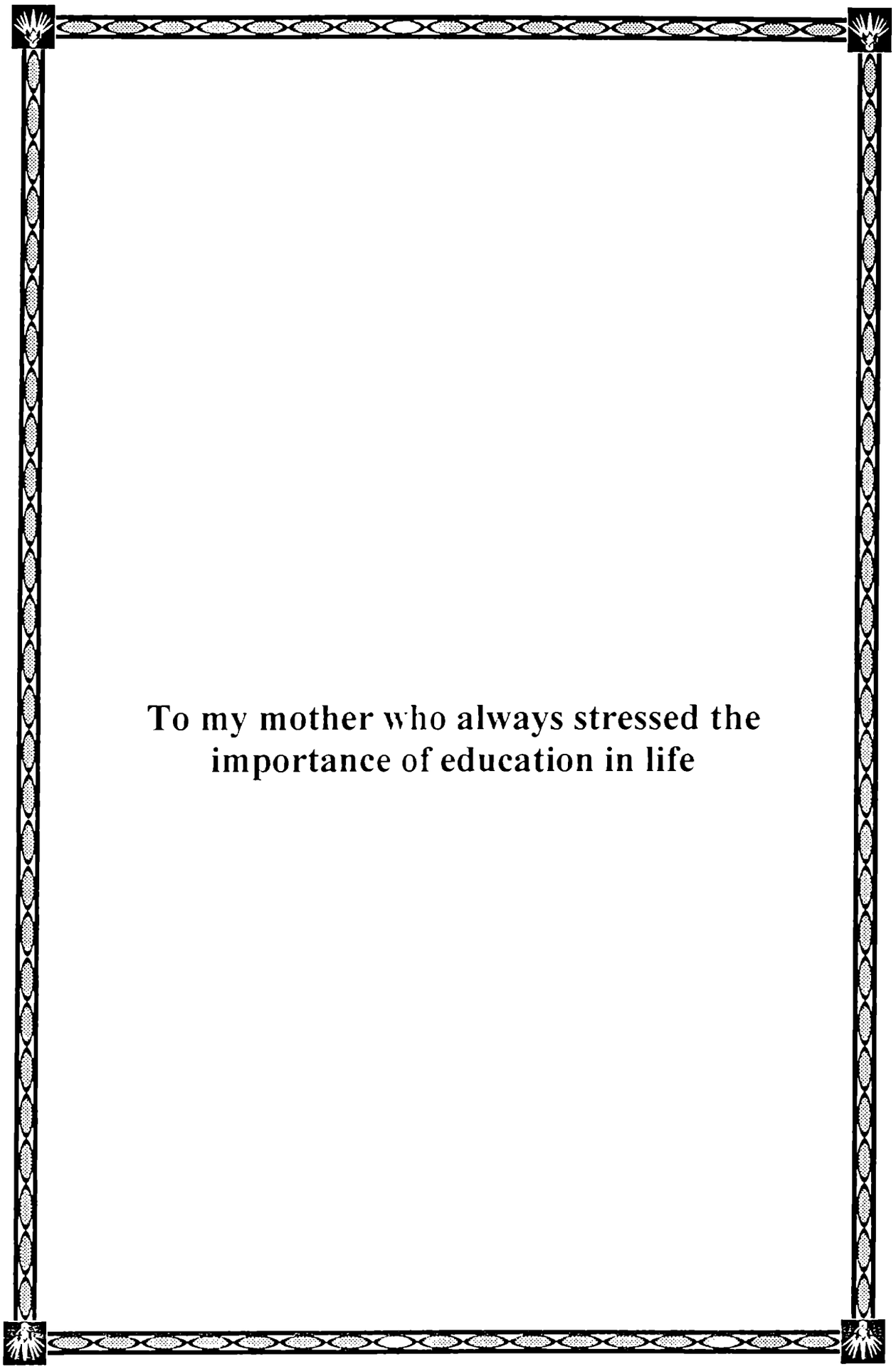
Dedicated

To the loving memory of my

Beloved father

Shri Gurupur Ganapathy Bhat



To my mother who always stressed the importance of education in life

Certificate

This is to certify that the research work presented in this thesis entitled 'Systematics and pathogenicity of Vibrionaceae associated with the larvae of *Macrobrachium rosenbergii* in hatchery', is based on the original work done by Ms. Sarita G. Bhat under my guidance, in the School of Environmental Studies, Cochin University of Science and Technology, Cochin-682016, in partial fulfillment of the requirements for the degree of Doctor of Philosophy, and that no part of this work has previously formed the basis for the award of any degree, diploma, associateship, fellowship or any other similar title or recognition.

Dr. I. S. Bright Singh
(Supervising Guide)
Reader in Microbiology
School of Environmental Studies
Cochin University of Science and Technology

Cochin-682 016
May-1998

Declaration

I hereby do declare that the work presented in this thesis entitled 'Systematics and pathogenicity of Vibrionaceae associated with the larvae of *M. rosenbergii* in hatchery' is based on the original work done, by me under the guidance of Dr. I. S. Bright Singh, Reader in Microbiology, School of Environmental Studies, Cochin University of Science and Technology, Cochin-682016, and that no part of this work has previously formed the basis for the award of any degree, diploma, associateship, fellowship or any other similar title or recognition.

Sarita G Bhat

(Sarita G. Bhat)

Cochin-682016
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CHAPTER – 1

GENERAL INTRODUCTION

1 Family Vibrionaceae - A Profile

1.1 Taxonomy : Current status

1.1.1 *Vibrio*

Members of family vibrionaceae are widely present in aquatic environment. These bacteria are primarily aquatic, associated with fish and other poikilothermic animals, where they occur as the members of the normal bacterial microbiota as well as primary or secondary pathogens (Cahill,1990; Austin and Austin , 1993). More over, they are also known to produce a wide spectrum of diseases in humans (Janda and Duffey,1988; Farmer III and Hickman-Brenner,1992).

The organisms are all Gram negative straight or curved rods, motile by means of polar flagella. They are chemo-organotrophes and facultative anaerobes capable of respiratory and fermentative metabolism. Most are oxidase positive. All members of this family utilize D-glucose as sole source or principal source of carbon and energy. Most species require 2-3 % NaCl or a sea water base for optimum growth. The mole % G + C ranges from 38- 63 (Baumann and Schubert,1984).

Various kinds of Gram negative, polarly flagellated, rod shaped bacteria were previously classified as the genus *Vibrio* in the family *Spirillaceae*. Thirty four *Vibrio* species were described by Breed (1957) in the seventh edition of *Bergey's Manual of Determinative Bacteriology*, and 207 species names were listed in the *Index Bergeyana* (Buchanan, Holt and Lessel, 1966). Since the *International Subcommittee on Taxonomy of Vibrios* (1966) reported a provisional definition for the genus *Vibrio*, however only a few species conforming to the definition have been retained in the genus. In the eighth edition of *Bergey's Manual of Determinative Bacteriology*, Shewan and Veron (1974) included five species (*V cholerae, V parahaemolyticus, V anguillarum, V fisheri and V. costicola)* in the genus. As suggested by Eddy and Carpenter (1964), the genus *Vibrio* was classified in the

family Vibrionaceae together with the genera *Aeromonas, Plesiomonas, Photobacterium* and *Lucibacterium* (Shewan and Veron, 1974). But *Lucibacterium* was found to be removed from the subsequent editions of *Bergey's Manual* especially the one on *Systematic Bacteriology*.

The first edition of *Bergey's Manual of Systematic Bacteriology* placed the genera *Vibrio, Photobacterium, Aeromonas* and *Plesiomonas* in the family Vibrionaceae (Baumann and Schubert, 1984). But at present, this family includes only the genera *Vibrio* sensu strictu, *Listonella* and *Photobacterium* (Farmer III, 1992; Farmer III and Hickman- Brenner, 1992). The genus *Aeromonas* constitutes a new family, Aeromonadaceae (*International Committee on Systematic Bacteriology, Subcommittee on the Taxonomy of Vibrionaceae,* 1992), while the status of *Plesiomonas* is uncertain (Farmer III, 1992). In addition, the number of recognized species has risen in the genus *Vibrio* from 22 (Baumann *et al.*, 1984) to 39 (Pujalte *et al.*, 1993; Holt *et al.*, 1994a; Onarheim *et al.*,1994; Ishimaru *et al.*, 1995), and in the genus *Aeromonas* from 4 (Popoff, 1984) to 14 (Schubert *et al.*, 1990 a, b; Carnahan *et al.*, 1991a,b; Martinez –Murcio *et al.*, 1992; Holt *et al.*, 1994b; Esteve *et al.*, 1995c).

Species of *Vibrio* accepted and brought under the family Vibrionaceae are given below with their corresponding mole % G + C ratios

1. *V. cholerae*	47-49 (Tm, Bd)
2. *V. metschnikovii*	44-46 (Tm)
3. *V. harveyi*	46-48 (Tm)
4. *V. campbelli*	46-48 (Tm,Bd)
5. *V. parahaemolyticus*	46-47 (Tm,Bd)
6. *V. alginolyticus*	45-47 (Tm,Bd)
7 *V. natriegens*	46-47 (Tm,Bd)
8. *V. vulnificus*	46-48 (Tm,Bd)
9. *V. nereis*	46-47 (Tm,Bd)
10.*V. fluvialis*	49-51 (Tm,Bd)
11.*V. spendidus*	45-46 (Tm,Bd)
12.*V. pelagius*	45-47 (Tm,Bd)

13 *V. nigripulchritudo*	46-47	(Tm,Bd)
14.*V. anguillarum*	44-46	(Tm,Bd)
15.*V. fisheri*	39-41	(Tm,Bd)
16.*V. logei*	40-42	(Tm,Bd)
17.*V. proteolyticus*	50.5	(Bd)
18.*V. gazogenes*	47 1	(Tm)
19.*V. marinus*	42.2	(Tm)
20.*V. costicola*	50	(Bd)

(Baumann et al.,1984)

21.Marine luminous isolates	46-48	(Chumakova *et al.*, 1973)
22.*V. aestuarianus*	43-44	(Tison and Seidler,1983)
23.*V. damsela*	43	(Love *et al.*, 1981)
24.*V. diazotrophicus*	46-47	(Guerinot *et al.*, 1982)
25.*V. hollisae*	50	(Hickman *et al.*, 1982)
26.*V. mimicus*	ND	(Davies *et al.*, 1981)
27.*V. ordalli*	44-46	(Schiewe *et al.*, 1981)
28.*V. orientalis*	45-46	(Yang *et al.*, 1983)
29.*V. tubiashii*	43-45	(Hada *et al.*, 1984)
30.*V. penaeicida*	46-47	(Ishimaru *et al.*, 1995)
31.*V. carchariae*	46	(Grimes *et al.*,1984)
32.*V. mytili*	45-46	(Pujalte *et al.*, 1993)
33.*V. navarrensis*	45-47	(Urdaci *et al.*,1991)
34.*V. salmonicida*	42	(Egidus *et al.*,1986)
35.*V. ichthyoenterii*	43-45	(Ishimaru *et al.*, 1996)
36.*V. cincinnatiensis*	45	(Brayton *et al.*, 1986)
37.*V. furnissii*	50	
38.*V. mediterranei*	42-43	(Pujalte *et al.*,1993)
39.*V. vulnificus* biovar II	46-47	(Tison *et al.*,1982)

1.1.1.1 Morphology

Vibrios grow readily on a variety of media. Most species give rise to convex smooth, creamy, white colonies with entire edges. Variants in colony morphology

may be detected in some species, particularly after repeated culture and storage on more complex media. Colonies may be rough or sometimes rugose, such colonies appear firmly attached to the medium and cannot be emulsified.

1.1.1.2 Swarming

Most strains of marine Vibrios are able to swarm on solid media. Swarming appears to be associated with the formation of long cells with many lateral flagella and is affected by a number of physical and chemical parameters, including the concentration of the agar, complexity of the medium and temperature (Baumann and Baumann,1977; de Boer *et al.*, 1975a,b; Ulitzer 1975a,b). The lateral flagella (but not the polar flagella) are essential for swarming on solid media (Shinoda and Okamoto,1977).

1.1.1.3 Growth conditions and nutritional requirements

Growth of all species of *Vibrio* is stimulated by Na^+ There is considerable variation in the ionic requirements of the various species of *Vibrio*, no single medium will allow optimum growth of all strains.

Species of *Vibrio* also vary with respect to the temperature at which growth will occur. All Vibrios grow at 20°C and most at 30°C; some grow at 4°C and 45°C and none grow at 50°C.

Many species of Vibrios will tolerate moderately alkaline conditions and will grow at pH 9 and some notably *V. cholerae* and *V. metschnikovii*, will grow at pH 10.

The species varies in their nutritional versatility; some of them grow on as few as 12 and still some others on as many as 67 of the 150 organic compounds tested as sole or principal sources of carbon and energy. These compounds include pentoses, hexoses, disaccharides, sugar acids, sugar alcohols, C_2-C_{10} monocarboxylic fatty acids and monocyclic aromatic compounds. Most *Vibrio* species also have a number of

extracellular hydrolases, which include amylase, gelatinase, lipase, chitinase, alginase, urease and deoxyribonuclease (Baumann *et al.*, 1984)

1.1.1.4 Metabolic properties

Species of *Vibrio* ferment D-glucose under anaerobic conditions by means of a mixed acid fermentation. Upon completion of fermentation, the pH of the medium ranges from 4.6-5.8. Some species produce acetoin and /or diacetyl as well as 2,3-butanediol. Two species of *Vibrio, V. fluvialis* biovar II and *V. gazogenes* produce gas, which in the latter case has been shown to be a mixture of H_2 and CO_2 (Harwood.1978)

Most species of Vibrios are oxidase positive, a property which correlates with the presence of cytochromes of the c type. *V.metschnikovii*, which is a oxidase negative species, contains cytochromes of the b, d, o and a_1 types, but lacks cytochromes of the c type(West *et al.*, 1978)

Several species of Vibrios have a constitutive arginine dihydrolase system as determined by assaying for ornithine produced from arginine under anaerobic conditions (Baumann and Baumann,1981, Baumann *et al.*, 1971a)

The descriptions of the morphological, physiological and nutritional properties of Vibrios is primarily based on the materials considered in publications (Baumann and Baumann, 1971, 1981, Baumann *et al.*,1980b; Barua and Burrows,1974; Chumakova *et al.*, 1973; Colwell,1970; Desmaschelier and Reichelt, 1981, Hendrie *et al.*, 1970; Lee *et al.*, 1978,1981a; Sakazaki, 1979; Veron, 1965,1966).

1.1.1.5 Antibiotic sensitivity

The antibiotic sensitivities of only the clinically significant species have been studied in detail (Hollis *et al.*, 1976; Huq *et al.*, 1980; Lee *et al.*, 1981a; Miwatani and Takeda, 1976; O'grady *et al* 1976). With rare exceptions they are sensitive to tetracycline which is usually the antibiotic of choice for the treatment of infections. In addition they are sensitive to a relatively wide range of antibiotics including

kanamycin, chloramphenicol, gentamycin, streptomycin and sulfonamides. *V parahaemolyticus* and *V alginolyticus* are relatively more resistant to polymyxin than other *Vibrio* species; *V.cholerae* being fully sensitive, strains of *V. parahaemolyticus* and *V. fluvialis* are relatively more resistant.

V. mimicus, a group of biochemically atypical strains of *V. cholerae*, are sensitive to 50 IU of polymyxin B (Furniss *et al.*, 1978)

1.1.1.6 Ecology and distribution of *Vibrios*

Different species of *Vibrio* vary greatly with respect to their potential to inhabit environments of different salinities and this is reflected in the wide range of Na^+ concentrations required for their optimal growth. At one extreme end of this Na^+ requirements are *V.cholerae* and *V. metschnikovii*, both of which require 5-15 mM Na^+ for optimal growth. Both these species have been isolated from freshwater as well as estuarine habitats (Desmarchelier and Reichelt, 1981, Kaper *et al.*, 1979; Lee *et al.*, 1978; Szita *et al.*, 1979). *V.splendidus* and *V. costicola* are at the other extreme, requiring 300-400 and 600-700 mM Na^+ respectively. These species would be unable to do well in environments with considerably lower salt concentrations. On the other hand, *V. fluvialis* and *V anguillarum* having an intermediate Na^+ requirement of 20-40 and 60-100 mM Na^+ could coexist with *V. cholerae* and *V. metschnikovii* in a number of estuarine and brackish water habitats, with salinities considerably lower than that of seawater (Lee *et al.*, 1978, 1981a; West *et al.*, 1980).

V. fluvialis strains are widely distributed through out the aquatic environment, particularly in brackish and estuarine waters but other sources have included shellfish and sewage. They may also be isolated from the faeces of humans suffering from diarrhoea but their enteropathogenicity has not been firmly established (Lee *et al.*, 1981)

The non-O1 cholerae have been isolated from fresh water and estuarine environments throughout the world as well as from birds, frogs and fresh water fish (Bashford *et al.*, 1979; Bisgaard *et al.*, 1978; Colwell *et al.*, 1980; Desmarchelier and

Reichelt, 1981, Kaper *et al.*, 1979; Muller, 1977; Nacescu and Ciufecu, 1978; Szeness *et al.*, 1979; Szita *et al.*,1979; West *et al.*, 1980)

Ecological studies have demonstrated that *V. parahaemolyticus* can be isolated from sea foods, as well as estuarine, neritic and brackish water (Joseph *et al.*, 1983). It is also a common inhabitant of the coastal waters and estuaries in tropical and temperate waters (Ayres and Barrow, 1978; Fujino *et al.*,1974; Kaneko and Colwell, 1978; Miwatani and Takeda, 1976; Sakazaki, 1979)

Most ecological studies of marine luminous bacteria could not distinguish between *V.spendidus* biovar I and *V harveyi;* and *V. fisheri* and *V. logei* (Ruby *et al.*, 1980). Some luminous bacteria are known to have symbiotic relationship with marine animals. *V. fisheri* has been found in the specialized luminous organs of teleost fish and squid (Fitzgerald, 1977; Herring and Morin, 1978; Nealson and Hastings, 1979; Ruby and Nealson, 1976; Tebo *et al.*, 1979). The surfaces and intestinal contents of marine animals are known to harbour *V. fisheri* and *V. harveyi* (O'Brien and Sizemore, 1979; Riechelt and Baumann, 1973; Ruby and Morin, 1979)

V. alginolyticus is very common to the coastal waters of the temperate and tropical regions(Baumann and Baumann,1981, Gotten and Scheffers, 1975). *V,. campbelli, V. natriegens, V. pelagius, V. nigripulchritudo, V. splendidus* biovar II and *V. nereis*, have been isolated in the vicinity of the Hawaiian Archipelago (Baumann *et al.*, 1971a, b); *V. neries* has been isolated from the coastal waters of England(Lee *et al* 1981*). V. fluvialis* biovar II has been isolated from the estuarine habitats of England (Lee *et al.*, 1981a) as well as from the coastal waters off the Hawaiian coast. *V costicola* has been isolated from the brines (Kushner, 1978). *V metschnikovii* has been isolated from sewage as well as from fresh water.

V. gazogenes, V. proteolyticus and *V. marinus* each represented by a single strain were isolated from a salt water marsh, wood boring crustacean and a sea water sample respectively (Colwell and Morita, 1964; Harwood, 1978; Merkel *et al.*, 1964)

V mytili (Pujalte *et al.*, 1993) has been isolated from mussels (*Mytilus edulis*) commercialized in Valencia. They are not known to be pathogenic to humans or mussels.

V tubiashii is a pathogen of bivalve mollusks (Hada *et al.*, 1984).

V ordalli, previously referred to as *V. anguillarum* biotype 2 (Schiewe *et al.*, 1981) has been routinely isolated in N. America and Japan.

V. ichthyoenterii (Ishimaru *et al.*, 1996) has been isolated from the Japanese flounder larvae in marine hatcheries in Japan. But the distribution of this organism in the environment is not known.

V penaeicida, the causative agent of vibriosis of Kuruma prawns in Japan, has also been isolated from apparently healthy prawns and water samples obtained from prawn culture ponds (Ishimaru *et al.*, 1995; de la Pena *et al.* 1993; Takahashi *et al.*, 1985)

V aestuarianus has been isolated from estuarine waters and shellfish from the coast of Oregon, U.S.A (Tison and Seidler, 1983)

Studies on bacterial populations from sea water samples, including water, sediments and marine organisms, usually focus attention on either large taxonomic groups such as genera, (Lovelace et al., 1968; Kuch and Chan, 1985) or on the isolation of certain pathogenic species (van der Broeck et al., 1979; Oliver et al., 1982; Colwell *et al*, 1984; Pujalte *et al* 1993; Ishimaru *et al.*, 1995, 1996; Ortigosa *et al.*, 1989,1994). Members of the genus *Vibrio* is ubiquitous in marine environments and some are pathogenic to humans and other animals. The occurrence and distribution of halophilic Vibrios, densities of *Vibrio* spp. , as well as isolation of several saprophytes and pathogenic species in different geographical areas have been reported(Grimes *et al.*, 1985; Dilmore and Hood, 1986; Chan *et al.*, 1986). However, the distribution and relative numbers of all species of this widespread genus are still little known.

Although the number of recognized and well characterized *Vibrio* species has dramatically increased in the recent years, their taxonomy is far from definitive. Taxonomic studies have revealed that several isolates remain unidentified (Lee *et al.*, 1981, Pujalte *et al.*, 1983; Kaper *et al.*, 1983; West *et al.*, 1986)

1.1.1.7 Pathogenicity

1.1.1.7 a Pathogenicity to humans

V cholerae serovar 01 is the causative agent of epidemic asiatic cholera; other serovars produce diarrhoea and outbreaks have been known to occur (Aldova *et al.*, 1968; Blake *et al.*, 1980; Dakin *et al.*, 1974; Kamal and Zinnaka, 1971, Mc Intyre and Feeley, 1965). The pathogenic strains of *V cholerae* 01 produce cholera toxin whose action on the mucosal cells of the small intestine is responsible for the characteristic diarrhea of the disease.

V parahaemolyticus causes gastroenteritis in humans which is usually contacted by eating contaminating sea food. (Fujino *et al.*, 1974, Miwatani and Takeda, 1976; Joseph *et al.*, 1978). More than 95 % of the strains from patients with diarrhoea are Kanagawa positive, i.e. they haemolyze human erythrocytes on Wagatsuma agar (Miyamoto *et al.*, 1969; Joseph *et al.*, 1983). Kanagawa positive (KP^+) strains are rarely isolated from environmental samples (Joseph *et al* 1983).

V. vulnificus may invade the human body from the gastrointestinal tract leading to a septicemia, particularly in patients with hepatic diseases (Blake *et al.*, 1979). *V. vulnificus, V cholerae, V. parahaemolyticus* and *V alginolyticus* have all been isolated from superficial lesions from humans, where they may simply be colonizers or opportunistic pathogens (Baumann *et al.*, 1973; Blake., *et al* 1979, 1980; Pezzlo *et al.*, 1979; Spark *et al.*, 1979).

V. fluvialis biovar I has been isolated from humans with diarrhea, suggesting that it may also be an enteropathogen, but this remains to be proved. (Huq *et al.*, 1980; Lee *et al* 1981a).

V. mimicus is a group of biochemically atypical strain of *V. cholerae*. They have been isolated from gastrointestinal infections and occasionally from various other human infections(Shandera *et al.*, 1983). They produce multiple toxins and several workers have examined the role of *V. mimicus* as a causative agent of gastroenteritis (Sanyal *et al.*, 1984; Choudhury *et al.*, 1986; Ramamurthy *et al.*, 1994).

1.1.1.7 b Vibrios pathogenic to other vertebrates and invertebrates

V anguillarum is a pathogen of marine fish and eels and a major cause of disease in fish culture (Anderson and Conroy, 1970; Sinderman,1970). Toranzo and Barja (1990),have reviewed the epizootiology of *V anguillarum* and listed the published reports on vibriosis outbreaks in fish, molluscs and crustaceans, in which *V. anguillarum* has been phenotypically identified as the causative organism. But their occurrence has been reported to be limited to temperate waters only

A number of vibrios have been isolated from diseased or dead shellfish; these include *V alginolyticus, V parahaemolyticus* and *V. logei* (Baross *et al.*, 1978; Bowser *et al.*, 1980; Vanderzant *et al.*, 1971)

Some of the strains pathogenic to larvae of bivalve molluscs and other shellfishes were found to be distinct phenotypically and genotypically from other *Vibrio spp*. These strains were assigned to the new species *V. tubiashii* (Hada et al., 1984; Bolinches et al., 1986; Lodeiros *et al.*, 1987). The genus *Vibrio* has received much attention in the recent years, resulting in an increase in the number of species recognized. Many of these species have been described as pathogens of fish and shellfish and they are as listed below

V.anguillarum and *V. ordallii* (formerly *V. anguillarum* biotype II, Harrell *et al.*, 1976; Schiewe *et al.*, 1981)
V. tubiashii (Hada *et al.*, 1984)
V damsela (Love *et al.*, 1981)
V. vulnificus biotype II (formerly *V anguillarum* type B, *V anguillicida*, Muroga *et al.*, 1976; Nishibuchi *et al.*,1980; Tison *et al* 1982)

V. carchariae (Grimes *et al.*, 1984)

V. salmonicida (Egidius *et al.*, 1986)

V. cholerae non –01 (Muroga *et al.*, 1979; Yamanoi *et al.*, 1980)

V. penaecida (Ishimaru *et al.*, 1995)

V. icthyoenteri (Ishimaru *et al.*, 1996)

A strain of *V. splendidus* was implicated in a new pathogenic syndrome of bacterial and viral aetiology that occurred in both juvenile and adult turbot (Lupiani *et al.*, 1989a). Experimental infections have confirmed the pathogenicity of *V splendidus* (Lupiani *et al* 1989b)

The public health significance of *V aestuarianus*, a new species isolated from estuarine waters and shellfish remains to be determined (Tison and Seidler.1983). However the association of this species with crabs, clams and oysters, warrants investigations into the potential of *V aestuarianus* strains as not only shellfish pathogens but also as human pathogens, since raw or improperly cooked shellfish serve as vehicles of infections by other pathogenic species.

1.1.2 *Photobacterium*

1.1.2.1 Description

Plump straight rods, 1-8 μm in length. Do not form endospores or microcysts. Gram negative, motile by one to three unsheathed polar flagella, some are non-motile, chemoorganotrophs, capable of respiratory and fermentative metabolism Grow in the presence and absence of oxygen. Do not denitrify Most strains grow in a mineral medium containing a seawater base, D-glucose and ammonium chloride. In addition to glucose, all strains utilize D-mannose, D-fructose and glycerol and grow at 20° C. Common in the marine environment, on the surfaces and in the intestinal contents of marine animals, some found as symbionts in specialized luminous organs of marine fish. The G+C content of the DNA is 40-44%.

The colonies of *Photobacterium* are not distinctive, being convex, smooth with entire edges and a white colour when grown on a complex or mineral medium such as yeast extract agar, marine agar or basal medium agar containing 0.2% (w/v) D-glucose. The colour of the colonies is somewhat whiter than that of many Gram negative, marine bacteria, probably due to a relatively lower content of cytochromes.

Species of *Photobacterium* require Na^+ for growth and are unable to grow in medium containing a seawater base in which the Na^+ has been replaced by an equimolar amount K^+ (Macleod, 1963). For optimal growth, 160-280 mM Na^+ is required, some species may also require seawater levels of Mg^+ (50mM) and Ca^+ (10mM) (Reichelt and Baumann,1974,1973). Most strains of *P leiognathi* and *P. angustum* have no organic growth factor requirement; some strains of *P. phosphoreum* require L-methionine, either alone or in combination with other amino acids (Reichelt and Baumann, 1973; Ruby *et al.*, 1980). All species grow 20°C, *P. phosphoreum* and some strains of *P. angustum* grow at 4°C while some grow at 40°C

The nutritional versatility of the species of *Photobacterium* is relatively limited, only 7-22 carbon compounds can be utilized as a sole source or principal source of carbon or energy These compounds include the hexoses and a few pentoses, diasaccharides, sugar acids, tricaboxylic acid cycle intermediates and amino acids. None of the species has an extracellular amylase or alginase; some strains may have an extracellular chitinase, lipase or gelatinase (Baumann and Baumann, 1984).

A constitutive arginine dihydrolase system has not been detected in species of *Photobacterium* (Baumann et al., 1971a,Reichelt and Baumann, 1973). Some strains form alkaline products from arginine when tested by a modification of the method of Thornley or Mϕller (Baumann and Baumann, 1981, Hendrie *et al.*, 1970).

1.1.2.2 Ecology

Strains of *P. phosphoreum* and *P. leiognathi* are widespread in the marine environment and have been isolated from sea water, the surfaces and the intestinal contents of marine animals, from the specialized luminous organs of the marine fish

(Herring and Morin,1978, Nealson and Hastings ,1979; Reichelt and Baumann. 973; Ruby and Morin,1979). *P.leiognathi* is found in fish inhabiting shallow tropical waters (0-15 meters) (Reichelt *et al.*, 1977), while *P. phosphoreum* is associated with luminous organs of fish from mid sea (200-600meters) and bathyal (600-1200 meters) habitats (Ruby and Morin, 1978).

1.1.2.3 Special features

A somewhat unusual combination of properties characteristic of *Photobacterium* is the ability to accumulate PHB as an intracellular reserve product coupled with the inability to utilize the exogenous monomer β-hydroxybutyrate as sole or principal source of carbon or energy

The genus of *Photobacterium* as presently defined does not include bioluminescence as a diagnostic trait since only two of the species are able to emit light.

1.1.2.4 Mole % G+C

1. *Photobacterium phosphoreum* 41-42
2. *Photobacterium leiognathi* 42-44
3 *Photobacterium angustum* 40-42

1.1.2.5 Species *incertae sedis*

V.psychroerythrus (D'Aoust and Kustner, 1972)

This species consists of a single, prodigiosin producing strain (D' Aoust and Kustner, 1971, D' Aoust and Gerber, 1974) which has some of the properties of the genus *Photobacterium*; it ferments D-glucose, it is motile by means of a single unsheathed polar flagellum and has a DNA base composition of 40 mol %G+C. However, DNA homology studies have indicated little or no nucleotide

complementarity (1-5%) to species of *Photobacterium* or *Vibrio* (Reichelt *et al.*, 1976; Baumann and Baumann, 1984).

1.1.2.6 Current status

At present genus *Photobacterium* (Farmer III, 1992; Farmer III and Hickman-Brenner,1992) is included in the Family Vibrionaceae, which includes *Vibrio* sensu strictu and *Listonella.*

1.1.3 *Aeromonas*

1.1.3.1 Description

The cells are straight, rod shaped with rounded ends to coccoid, 1.0-3.5 μm in length. They occur singly, in pairs or in short rods, resting stages are not known. Gram negative, generally motile by a single polar flagellum; peritrichous flagella may be formed on solid media in young cultures. One species is non-motile. Facultative anaerobes. Metabolism of glucose is both respiratory and fermentative. Carbohydrates are broken down to acid or acid and gas (CO_2 and H_2). Nitrate is reduced to nitrite. Oxidase positive. Catalase positive, optimum temperature 22-28°C, some strains do not grow at 33°C. Resistant to the vibriostatic agent 2,4-diamino-6,7-diisopropyl pteridine (O/129). Chemoorganotrophic, using a variety of sugars and organic acids as carbon sources. They occur in fresh water and sewage. Some species are pathogenic to frogs and fish. The mole % G+C of the DNA is 57-63 (Bd,Tm).

Two well-separated groups are included in the genus *Aeromonas.* Psychrophilic and non-motile aeromonads are clustered in the first group, named *Aeromonas salmonicida.* The second group consists of mesophilic, motile bacteria; this group can be divided into three species: *Aeromonas hydrophila, Aeromonas caviae* and *Aeromonas sobria* (Popoff and Veron,1976; Popoff *et al.*, 1981, Popoff, 1984). These three species will be further referred to as motile *Aeromonas* species

A. salmonicida are non motile and atrichous. Cells of motile *Aeromonas* species possess a single polar flagellum. Most of these motile species form lateral flagella in young cultures (Popoff, 1984).

Optimum growth temperatures for *A. salmonicida* is 22-25°C Most strains grow at 5°C. Maximum temperature at which growth occurs is usually 35°C. When cultured on nutrient broth at 22°C for 48 hours, colonies of *A. salmonicida* are raised, round, entire, translucent and friable. (Smith, 1963). Most strains of *A. salmonicida* produce a brown water-soluble pigment on media containing 0.1% tyrosine or phenylalanine. Pigment production does not occur anaerobically (Williamson, 1928). Some non- pigmented strains may be isolated (Smith, 1963). On blood agar haemolysis occurs rapidly, the colonies becoming greenish after 7 days (Popoff, 1984).

The optimum growth temperature for motile *Aeromonas* species is 28°C. Some strains grow at 5°C. The maximum temperature at which growth occurs is 38-41°C. on nutrient agar, colonies of motile aeromonads are round, raised with an entire edge and a smooth surface. They are translucent and white to buff in color The culture odor varies from extremely strong to absent (McCarthy, 1975). Motile *Aeromonas* species do not produce pigment. However, Ross (1962) reported the isolation of one motile *Aeromonas* strain which produced a dark red brown pigment indistinguishable from that produced by *A. salmonicida*.

The biochemical characteristics of *Aeromonas* have been reported by many workers (Eddy, 1960, 1962; Ewing *et al.*, 1961, Smith, 1963, Popoff, 1969, Mc Carthy, 1975; Popoff and Veron, 1976, Popoff, 1984, Esteve, 1995a). Acid is produced by all strains of *Aeromonas* from glucose and maltose, but not from xylose, dulcitol, m-inositol, adonitol, malonate and mucate. All strains of *Aeromonas* possess gelatinase, deoxyribonuclease, ribonuclease and Tween 80 esterase, H_2S is not produced.

The following carbohydrates are usually fermented by *A. salmonicida*: arabinose, trehalose, galactose, mannose and dextrin. The following biochemical tests

are universally negative for *A. salmonicida*: Growth in KCN broth, growth in nutrient broth containing 7.5% NaCl, urease, ornithine decarboxylase (ODC), and acidification of media containing rhamnose, sorbose, sorbitol, lactose, raffinose and cellobiose. Arginine is hydrolyzed via an Arginine dihydrolase (ADH) system.

The following physiological tests are universally positive for motile *Aeromonas:* catalase, starch hydrolysis, lecithinase, ADH, growth in nutrient broth with out NaCl, and fermentation of mannitol, trehalose, fructose, galactose and dextrin. Pectinase, ODC, phenylalanine deaminase, growth in nutrient broth containing 5% NaCl and acid production from sorbose, erythritol and raffinose (Popoff, 1984).

The following compounds serve universally as sole carbon sources for motile *Aeromonas* species: D-ribose, D-fructose, D-galactose, D-glucose, D-maltose, D-trehalose, D-gluconate, succinate, fumarate, glycerate, malate, glycerol, mannitol, aspartate and glutamate (Popoff, 1984).

1.1.3.2 Ecology

A. salmonicida is a strict parasite under natural conditions. Although these bacteria are isolated from natural waters, their existence in river water is short-lived (McGraw, 1952). *A. salmonicida* is the causative agent of fish furunculosis traditionally associated with salmon and trout (Emmerich and Weibel, 1894). Furunculosis appears to be a specific infection of fish. The pathogenic action of *A. salmonicida* may be due to its abundant growth in fish blood and tissues and production of a leucoidin (Klontz *et al.*, 1966)

Motile *Aeromonas* species occur widely in water, sludge and sewage (Leclerc and Buttiaux, 1962; Schubert *et al*, 1972; Hazen *et al*, 1978). These organisms have been isolated occasionally from apparently healthy people (Lautrop, 1961, Catsaras and Buttiaux, 1965). However fecal origin cannot explain the presence of these bacteria in the surface waters or sewage. Motile Aeromonads have long been recognized as the causal agent of 'red leg' disease in amphibians (Russel, 1898; Emmerson and Norris, 1905; Shotts *et al.*, 1972). They are also considered to be

responsible for diseases in reptiles (Camin, 1948, Page, 1962; Marcus, 1971), fishes (Haley *et al* 1967), snails (Mead, 1969), cows (Wohlegemuth *et al* 1972), and humans (Davis *et al.*, 1978). They may be a secondary invader in virus-infected fish (Heuschmann-Brunner, 1965). Mice may be infected experimentally (Schubert, 1964). Several cases of fatal human septicemia have been reported, but in all instances the patients were debilitated by some other disease (Davis *et al.*, 1978). Recently motile *Aeromonas* species were reported to be pathogenic for humans when wounds were exposed to polluted waters (Davis *et al.*, 1978). Some strains may also act as primary agents of acute diarrhoeal diseases (Bhat *et al.*, 1974, Chatterjee and Neogy, 1972, Sanyal *et al.*, 1975). A possible explanation of the potential pathogenicity of motile *Aeromonas* species has come with the finding that some strains produce a heat labile enterotoxin (Sanyal *et al.*, 1975; Wadstrom *et al.*, 1976)

Species of the genus *Aeromonas* are considered as autocthonous inhabitants of aquatic environments. *A. hydrophila* has received particular attention because of its association with human diseases. It has been isolated from both polluted and unpolluted water bodies through the world (Schubert, 1974). In addition it is considered to comprise of the portion of the normal flora of fishes, as well as other aquatic animals and plants (S imidu *et al.*, 1971, Thrust and Sparrow, 1974). It can cause infections and epizootics in a variety of animals, including alligators (Shotts *et al.*, 1972), turtles (Mc Coy and Seider, 1973), frogs (Rigney *et al* 1978), Snails (Mead, 1969), and fish (Haley *et al.*, 1967; Hazen *et al.*, 1978b).

A. hydrophila, has also been implicated in human diseases in recent years. Fatal and non fatal infections have been reported that are associated with a variety of clinical manifestations, including septicemia (Dean and Post, 1967; Von Graevenitz and Mensch, 1968; Zac-Satler, 1972; Ketover *et al.*, 1973, Tapper *et al.*, 1975; Fulghum *et al.*, 1978; Ramsay *et al.*, 1978), meningitis (Quadri *et al* 1975), endocarditis (Davis *et al.*, 1978), corneal ulcers(Feaster *et al.*, 1978), wound infections (Shackelford *et al.*, 1973, Hanson *et al.*, 1977; Fraire , 1978)

Water borne infections of *Aeromonas* have been described and Von Graevenitz and Mensch(1968) and Washington (1972), have cited a large number of

cases in which *Aeromonas* infections occurred as a result of contact with water and soil.

1.1.3.3 Mole percent G+C content

1. Aeromonas hydrophila	58-62 (Bd, Tm)
2. Aeromonas caviae	61-63 (Bd, Tm)
3. Aeromonas sobria	58-60 (Tm)
4. Aeromonas salmonicida	57-59 (Bd, Tm)

1.1.3.4 Current status

The genus *Aeromonas* constitutes a new familty, *Aeromonadaceae (International Committee on Systematic Bacteriology, Subcommittee on the Taxonomy of Vibrionaceae, 1992).* The number of recognized species have risen from 4 (Popoff, 1984) to 14 (Schubert *et al.*, 1990a, b; Carnahan *et al* 1991a, b. Martinez-Murcia *et al.*, 1992; Holt *et al.*, 1994b; Esteve *et al.*, 1995c)

1. *Aeromonas hydrophila*
2. *Aeromonas caviae*
3. *Aeromonas sobria*
4. *Aeromonas salmonicida* subsp. *salmonicida*
5. *Aeromonas jandei*
6. *Aeromonas veroni* bv. *veroni*
7. *Aeromonas encheleia*
8. *Aeromonas salmonicida* subsp. *masoucida*
9. *Aeromonas media*
10. *Aeromonas euchrenophila*
11. *Aeromonas schubertii*
12. *Aeromonas veroni* bv. *Sobria*
13. *Aeromonas allosaccharophila*
14. *Aeromonas trota*

Of the well known *Aeromonas* species the psychrophilic and non-motile *Aeromonas salmonicida* is easily identified while the identification of the mesophilic , motile *Aeromonas* species is more complex (Esteve,1995a). The motile group consists of three phenospecies genotypically heterogeneous, *A. hydrophila, A. caviae, A. sobria* and at least eight species more, in which each phenospecies correspond to one DNA homology group (Allen *et al.*, 1983, Hickmann-Brenner *et al.*, 1987; Hickmann *et al.*, 1988; Schubert and Hegazi 1988; Carnahan *et al.*, 1991a,b; Martinez-Murcia *et al* 1992; Esteve *et al.*, 1995b, c).

Methods of identification of *Aeromonas* at the genus and species levels have undergone major improvements, primarily as a result of chemo-taxonomic studies (Altwegg *et al.*, 1990, 1991, Huys *et al.*, 1994) Nevertheless, determining the assignment of motile *Aeromonas* strains to recently described species is still confusing based on biochemical characters and hence require further assessment (Esteve, 1995a).

1.1.3 Genus *Plesiomonas*

1.1.4.1 Description

The cells are round ended, straight rod-shaped, 3.0 μm in length. Resting stages not known. Gram negative, motile by means of polar flagella, generally lophotrichous. Faculatatively anaerobic, chemoorganotrophic, having both respiraory and a fermentative type of metabolism. Carbohydrates are catabolized by the production of acid but no gas. Most strains grow on mineral medium containing ammonium salts as a sole source of nitrogen and glucose as a sole source of carbon. Oxidase and catalase reactions are positive. Negative for diastase, lipase, proteinases. Positive for lysine, ornithine and arginine decarboxylases (Moller technique). Most strains are sensitive to the vibriostatic compound 0/129. Occur in fish and other aquatic animals and in a variety of mammals; probably does not belong to the normal flora of man, but can cause diarrhoea in man. The mole percent G+C of the DNA is 51 (Ch).

Cells of *P. shigelloides* typically appear as straight, sometimes long rods or even filaments. No microcysts occur No granules of intracellular poly β-hydroxybutyrate are present. The organisms are usually motile, but non-motile flagellated and non-motile atrichous strains are known to be present (Ewing *et al.*, 1961).

P. shigelloides grows in peptone broth with uniform turbidity, no sediment or pellicle is formed. On nutrient agar or blood agar at 24 hours, colonies are 1.0-1.5mm in diameter, grayish, shiny and opaque, with a slightly raised center and a smooth surface and entire edge (Sakazaki *et al.*, 1959; Habs and Schubert, 1962). No water-soluble fluorescent or brown pigment is produced

Optimum growth occurs between 37°C and 38°C, the maximum growth temperature is between 40-44°C, while the minimum temperature is 8°C. No growth occurs in nutrient broth containing 7.5% NaCl. The pH range for growth is 5.0-7 7, some strains can grow at pH 8.0. No growth occurs at pH 3.0.

Glucose, maltose, trehalose, inositol and glycerol are fermented with acid but no gas. The following carbohydrates are not fermented: starch, dexrin, glycogen, mannitol, fructose, sucrose, arabinose, aesculin, raffinose, cellobiose, salicine, sorbitol, inulin, ramnose, xylose, dulcitol and adonitol. Catabolism of lactose, galactose, mannose, salicin and chitin is variable.

The methyl red reaction is variable. The Voges-Proskauer test is negative. Other negative tests are: gluconate, malonate, citrate utilization; casein digestion, lecithinase activity, urease and phenylalanine deaminase. Indole is formed.

Antibiotic sensitivity testing of twelve *P. shigelliodes* strains indicated sensitivity to all compounds tested, ampicillin, tetracycline and chloramphenicol.

1.1.4.2 Ecology

P. shigelloides, with a few exceptions (Ellner and McCarthy, 1973) has not been isolated from wounds or inflammatory processes. The species however plays a role as a pathogen of the human intestine. Cases of diarrhoea in various degrees of severity are reported. Symptoms range from loose stools to watery choleriform excrements. Epidemic outbreaks of diarrhea attributed to *P. shigelloides* as the causative agent has been reported from Africa, India and Japan (Vandepitte et al., 1974, 1980; Bhat *et al* 1974; Sanyal *et al.*, 1975)

Apparently, *P. shigelloides* does not belong to the normal intestinal flora of man (Nakanishi *et al.*, 1969; Catsaras and Buttiaux, 1965); in only a few instances has man been found to be a symptomatic carrier of the organism. *Plesiomonas* has thus far been isolated from fish, other aquatic animals and from mammals such as swine, dogs, cats, goats, sheep and monkeys (Arai *et al.* 1980; Vandepitte *et al.*, 1980). Some animals however develop symptoms of disease (Davis *et al* 1978; Bader, 1954). The mole percent G+C of *P shigelloides* is 51 (Ch)

1.1.4.3 Current status

Plesiomonas is not included in the family Vibrionaceae or in the family Aeromonadaceae and therefore its status remains uncertain *(International Committee on Systematic Bacteriology, Subcommittee on the Taxonomy of Vibrionaceae*, 1992; Farmer III, 1992).

1.2 Vibrionaceae associated with the larvae of *Macrobrachium rosenbergii*

Macrobrachium rosenbergii, the giant fresh water prawn is one of the most ideal candidate species for aquaculture with great potential for fresh water and low saline areas of the Indian subcontinent (Kurup, 1994). As per some of the projections made recently, (Kurup, 1994) 200 million post larvae of this fast growing prawn species are required to extend their cultivation at least partially to these areas To meet this over growing demand, several hatcheries have been set up in India over the past two decades, with the prime objective to supply good quality seed at the right time. They were all based on clear water system feeding the larvae with a

combination of live feed(*Artemia* nauplii) and egg custard. In most of the cases fresh or aged seawater is diluted to 12-14 ppt with fresh water and 25% water exchange is maintained daily. However, the supply of the post larvae remained at all times far below the requirement, mainly due to the low survival rate at different stages of the larval cycle. Marked with 5-15% overall average survival and metamorphosis to post larvae, the rearing system as a whole required a through investigation to study the problem of larval mortality which had crippled fresh water farming in India for years.

Singh (1990) observed, while working out the microbiology of a typical fresh water prawn larval rearing system at the regional shrimp hatchery at Azhikode. that there existed a profound relationship between the abundance of the members of the family Vibrionaceae (Baumann *et al.*, 1984) and the mortality of the larvae, during the mid-larval cycle. The association of *Aeromonas* and *Vibrio* in sizable percentages with eggs of *M. rosenbergii* led to failure of the completion of the embryonic development and subsequent hatching. *Aeromonas* formed the major flora of the 'sick' culture system and *Pseudomonas* those of the healthy ones. Larvae with *Pseudomonas* as the major intestinal flora metamorphosed successfully, while the ones with *Aeromonas* failed to do so. In the larvae representing the 'sick' pool, though found apparently healthy at the time of sampling, with their characteristic response to light, tendency to remain at the top of the water column and zig-zag motion, progressive mortality was observed over the entire larval rearing period (Singh, 1990).

Works of a similar nature are found to be comparatively less, as more attention has been given to the penaeids than to the non-penaeids. Colorni (1985) studied bacteria associated with the larvae of *M. rosenbergii* fed with artemia nauplii and Huang *et al*, (1981) attempted to vaccinate *M. rosenbergii* with *V. anguillarum* unsuccessfully However being a decapod crustacean, the host –pathogen interactions among penaeids may very well be applicable to the non-penaeids also, especially in the larval stages as both are reared in saline waters. Vibriosis, a serious disease problem in the larval rearing systems of prawn, has been implicated as a major mortality factor (Sinderman, 1977; Lightner and Lewis. 1975; Baticados, 1986, 1988, 1990; de la Pena *et al.*, 1992; Karunasagar *et al.*, 1994; Singh *et al;* 1985; Hameed, 1994). Thus apparently for attaining better survival of larvae and

enhanced production of post larvae, an improved and appropriate managerial system to combat vibriosis has to be evolved. This includes either selective elimination of the pathogenic *Vibrios* and *Aeromonas* or suppressing their growth and multiplication in the larval rearing system. The selective removal or inhibition of the pathogenic strains of Vibrionaceae being the ultimate objective, the prime concern is how the only pathogenic strains can be segregated from the other non-pathogenic isolates from the larvae and the larval rearing system.

This sort of segregation of pathogenic strains can be achieved only by proving Koch's postulates for the isolated strains from the larvae, under defined conditions. But it is not practicable to test every culture individually for their pathogenicity *in vivo*, as their number exceeds the framework of any experimental design. Naturally, it is a fact that they have to be first identified properly applying the available system of classification based on the phenotypic, genotypic and serological characters

The classical approach to microbial identification involves the applications of keys and /or diagnostic tables. The path to an identification based on a true dichotomous key is unidirectional and a single atypical feature or error in determining a feature will result in a mis-identification. Diagnostic tables summarize the characteristics of a taxonomic group but do not indicate the hierarchial separation of the taxa. Diagnostic tables generally give the appearance of being more complicated than keys for identification of microorganisms because they contain more information. However, in cases where some features are variable for different groups, diagnostic tables are better than keys for successful identification of the an unknown microorganism.

But for identifying the members of the family Vibrionaceae which comprises of *Vibrio, Photobacterium, Aeromonas* and *Plesiomonas* (Baumann and Schubert, 1984), associated with the larvae of *M. rosenbergii*, neither the dichotomous keys nor the diagnostic tables could be employed for the following reasons:

The family Vibrionaceae is a natural group, with high information content and is composed of organisms that have in common the greatest number of characters. Such groups are termed as polythetic because this criterion does not necessarily

require that any character should be constant within a group nor does it require any one character or property to be universal for a class. This makes provisions for individuals that are exceptional in some respects. In constructing polythetic groups it is usual to give equal weight to every feature; complex characters are broken down into unit characters, each of which receive unit weight. Among all systems of taxonomic groupings, Numerical Taxonomy produces polythetic groups and thus permits the occasional exception to any character Since the heterotrophic bacteria associated with the larvae of *M. rosenbergii* is a 'natural group' and has a particular ecological niche, monothetic classification does not appear to be a feasible proposition, where all objects allotted to one class must share the character or characters under consideration. Therefore it was reasonable and scientifically sound to subject the family associated with the larvae of *M. rosenbergii* to Numerical Taxonomic analysis, which is a befitting process for such a diverse natural group

The Family Vibrionaceae alone was selected for this study because the *Vibrios* and *Aeromonas* belonged to this family (Baumann and Schubert, 1984) and were associated with, and involved in the mortality of the larvae. Under this programme the data generated based on the phenotypic characters are analyzed using Simple Matching Coefficient (Ssm) (Sokal and Michener, 1958). The isolates known as the operational taxonomic units (OTUS) clustered depending on the extent of similarity between them. The clustering was achieved by unweighted average linkage from which sorted similarity matrices and dendrograms can be constructed using the computer program TAXAN. The isolates are grouped into various clusters, each of which can be identified as a species or a subspecies. Now, for segregating the pathogenic strains, it is enough to select one culture from each cluster, which truly represents the respective cluster and using these, Koch's postulates can be proved. This approach is based on the assumption that virulence being a phenotypic character is linked with a other phenotypic characters.

Even though *Bergey's Manual* has been recognized globally as the guide to bacterial systematics, it has to be emphasized that descriptions given to a large extent are based on studies made with temperate isolates This leads one to conclude that any attempt to identify the tropical isolates with identification keys and tables

generated from this information may lead to erroneous conclusions. And there is every possibility of the existence of genotypic and phenotypic variants or even new species in this part of the aquatic ecosystem. Applications of a polythetic scheme of classification based on the principles of Numerical Taxonomy opens up exciting avenues for bringing to light, this possibility which otherwise would have been masked by the unidirectional approach as in monothetic schemes.

Another added advantage of clustering a 'natural' bacterial population by numerical taxonomy, is the ease by which genotypic characterization could be performed on the clusters by selecting a representative from each cluster This helps overcome the practical impossibility of analyzing all the isolates in a particular cluster. The genotypic characteizarion would either be mole % G+C. DNA-DNA hybridization, DNA-RNA hybridization or DNA fingerprinting.

Considering the requirement creating a broad base in the understanding of the family Vibrionaceae associated with the larvae of *M. rosenbergii*, the present work was undertaken to channelize every new information generated for developing appropriate managerial measures to protect the larvae from vibriosis during the unusually prolonged larval phase.

1.3 Objectives

The foregoing sections explained the present status of the family Vibrionaceae and its importance in the life of the larvae of *M. rosenbergii* in hatchery Based on these realizations, this work was conceptualized with the following objectives.

1. **Working out the Numerical Taxonomy of Vibrionaceae associated with the larvae of *M. rosenbergii* in hatchery.**
2. **Determination of the Guanine + Cytosine content of the DNA**
3. **Segregation of strains pathogenic to larvae**
4. **Determination of antibiotic sensitivity of the isolate, to work out a suitable chemotherapy.**

CHAPTER - II

NUMERICAL TAXONOMY AND MOLE % G+C RATIO

CHAPTER –II

NUMERICAL TAXONOMY AND MOLE % G +C RATIO

2.1 Introduction

2.1.1 Systematics based on phenotypic characters

Microbial systematics is concerned with the orderly relationship that exists among the organisms comprising the microbial world. Systematics is the study of the organisms, their diversity and inter-relationship, with the aim of arranging them in an orderly manner. Taxonomy is the process based on the established procedures and rules of describing the groups of organisms, their interrelationships and the boundaries between the microbial groups. The two functions of taxonomy are ,first to identify and describe the basic taxonomic units (species) and second to devise an appropriate way of arranging and cataloguing these units. A microbial taxonomist is concerned with classification, (ordering of organisms into groups based on their relationships), nomenclature (assigning names to the units described in a classification system), and identification (applying the system of classification and nomenclature to assign the proper names to an unknown organism and to place it into proper position within the classification system).

As mentioned above taxonomy is ordering the organisms into groups based on their relationships. In higher organisms much attention is paid to trace out the ancestry while ordering them into groups in the phylogenetic tree. But with most microorganisms, especially in the procaryotes, very little is known about their ancestry, and therefore the organisms are grouped according to their observable characters that are expressed, termed as phenetic or phenotypic, without regard to their ancestry. For all practical reasons of classifying an organism or a group, phenotypic characters are considered. The phenotypic characters observed generally

include morphological, physiological, biochemical and nutritional features of individuals of a group between which relationship has to be worked out.

In addition to phenotypic analysis, it is also possible to include genetic analysis in the development of classification schemes for microorganisms. Determination of the relative proportion of guanine (G) and cytosine (C) base pairs, compared to adenine (A) and Thymine (T) base pairs in the DNA is one of the analysis which expresses the Relative Mole % G + C. Measuring the proportion of G + C in the DNA is a crude analysis of the genome. A more practical measure of relatedness is the DNA homology between two organisms.

The classical approach to microbial identification involves the development of keys or / and diagnostic tables. An identification key consists of a series of questions that lead through a classification system to the determination of the identity of the organisms. In a dichotomous key, a series of yes/no questions is asked that lead through the branches of a flow chart to the identification of a microorganism as a member of a specified microbial group.

The path to an identification in a dichotomous key is unidirectional and a single atypical feature or error in determining a feature will result in a misidentification. Besides identification keys, diagnostic tables can also be developed to aid in microbial identification. Such tables summarize the characteristics of the taxonomic groups but do not indicate a hierarchical separation of the taxa. Diagnostic tables generally give the appearance of being more complicated than keys for the identification of microorganisms because they contain more information. However, in cases where some features are variable for different groups, diagnostic tables are better than keys for the successful identification of an unknown microorganism.

But for identifying members of the family Vibrionaceae associated with the larvae of *Macrobrachium rosenbergii*, neither dichotomous keys nor diagnostic tables could be employed for the following reasons: The above family is a 'natural' group with high information content and are composed of organisms that have in

common the greatest number of characters. Such groups are termed polythetic' because this criterion does not necessarily require that any character should be constant within a group; and also does not require any one character or property to be universal for any class, this makes provisions for individuals that are exceptional in some respects (Sneath, 1972). In constructing polythetic, groups it is usual to give equal weight to every feature; complex characters are broken down into unit characters, each of which receive unit weight. Numerical Taxonomy produces polythetic groups and thus permits the occasional exception on any character Heterotrophic bacteria associated with the larvae of *M. rosenbergii* is a natural group and has a particular ecological niche and therefore monothetic classification does not appear to be a feasible proposition, where all objects allotted to one class must share the character or characters under consideration. Therefore it was decided to study the systematics of the family Vibrionaceae associated with the larvae of *M. rosenbergii* employing the principles of Numerical Taxonomy which is a befitting taxonomic process for a natural group. The family Vibrionaceae was alone selected due to the fact that as per earlier understanding this family comprises several known species pathogenic for prawns.

Numerical Taxonomy was developed in the late 1950s, as part of the multivariate analysis in parallel with the development of computers (Sneath, 1972). As a rule when one deals with natural groups, phenetic relationships should be based on a large number of characters, so that when a percentage similarity is worked out one would not place much reliance on single value. Such tables of results are not readily analyzed by eye and therefore, the requirement for an objective method of taxonomic analysis whose first aim was to sort out individual strains of bacteria into homogenous groups (conventionally species), and which would also assist in the arrangement of the species into genera and higher groupings.

2.1.1.1 Steps in classification

The steps required in the pattern of classification are:

1 Collection of data. The bacterial strains that are to be classified have to be chosen and they have to be examined for a number of relevant properties (taxonomic characters)

2. The data must be coded and scaled in an appropriate fashion.

3. The similarity or resemblance between the strains is calculated. This yields a table of similarities (similarity matrix) based on the chosen set of characters.

4. The similarities are analyzed for taxonomic structure to yield the groups or clusters present, and the strains are arranged into phenons (phenetic groups) which are broadly equated with taxonomic groups (taxa).

5. The properties of the phenons can be tabulated for publication or for further study, and the most appropriate characters (diagnostic characters) can be chosen to set up identification systems that will allow the best identification of additional strains. These steps must be carries out in the above order

Organisms: The organisms consists of individual strains of bacteria 't' in number, called operational taxonomic units (OTUS). A good selection of strains of the group under study together with the inclusion of the type strains of the taxa under study and of related taxa are important.

Characters: A character is defined as any property that can vary between OTUS. A single character treated as independent of others is called a unit character. For Numerical Taxonomy, the characters should cover a broad range of properties: morphological, physiological and biochemical

Number of characters: The accuracy of similarity values depends critically on having a reasonably large number of characters. The number 'x' should be fifty or more. Several hundreds are required, though the taxonomic gains fall off with very large numbers.

Quality of data: The average difference in replicate tests on the same strain should be kept below 5% by rigorous standardization.

Coding of data: There are several satisfactory ways of doing this. The most familiar process of recording reactions is into positive and negative and coding 1 for positive reactions and 0 for negative reactions; this is more convenient for computation for `t ' OTUS scored for `n' characters. Naturally there should be as few gaps as possible.

The question arises as to what weight should be given to each character relative to the test. The usual practice in numerical Taxonomy is to give each character equal weight. More specifically it may be argued that unit characters should have unit weight and if character complexes are broken into a number of unit characters (each carrying one unit of taxonomic information), it is logical to accord unit weight to each unit character This philosophy is derived from the opinion of the 18^{th} century botanist Adanson, therefore Numerical Taxonomy is sometimes referred to as Adansonian (Sneath, 1972).

Example of coded n x t matrix

An example of a matrix is given below

	Reference numbers of OTUS (t)			
	1	**2**	**3**	**4**
Reference numbers of characters (n)				
1	**1**	**0**	**0**	**1**
2	**0**	**1**	**NC**	**0**
3	**1**	**1**	**1**	**0**
4	**0**	**0**	**1**	**0**
5	**1**	**0**	**0**	**1**

1= + ve

0= - ve

NC = no comparison

The estimation of resemblance

The two columns of character states (which are here taken as consisting of only 1, 0, or NC) for any given pair of OTUS can be compared over all the characters and arranged in a 2 x 2 table. For OTUS 1 and 2 in the n x t matrix given above, there is one character where both are 1, one where both are 0 and one that are 1,0 and 0,1 respectively

The 2 x 2 table is then:

	OTU-1	
	State 1	**State 0**
	(+)	**(-)**

State 1(+)	**a**	**b**
OTU-2	---	
State 0(-)	**c**	**d**

The symbol `a ' and `d ' are the number of the positive and negative matches respectively, `b ' and `c ' the number of non-matching unit characters between OTUS. The simple matching coefficient Ssm is expressed by the equation

$$\frac{a + d}{a + b + c + d} = \frac{a + d}{n}$$

In the example above the agreements over the five characters are 2, being a + d, and expressed as a percentage of the total, n i.e. 40 %.

$$\frac{2}{5} \times 100 = 40\ \%$$

The coefficient a + d /n is the simple matching coefficient Ssm. It may be given as a fraction 0.4, or as a percentage as above. If we turn to the comparison of

OTUS 1 and 3, we see that there is an NC entry in one character of the OTU-3. This only allows 4 characters to be compared, so that (a + d)/ n is

$$\frac{1 + 0}{4} \times 100 = 25\ \%$$

In this way the similarities between all the pairs of OTUS are calculated, giving the similarity or resemblance matrix of size t x t (here t=4).

This chequer board matrix is:

	Percent Ssm			
	OTUS			
	1	2	3	4
1	100			
2	40	100		
OTUS 3	25	75	100	
4	80	20	0	100

These values range from the most similar pair 1 and 4 with Ssm = 80 % to the least similar ,3 and 4 which in this example have no characters that agree. Any OTU compared with itself gives of course 100 % similarity

2.1.1.2 Taxonomic structure

A table of similarities does not by itself make evident the taxonomic structure of the OTUS. The strains will be in an arbitrary order that will not reflect the species or other groups. These similarities therefore require further manipulation to construct taxonomic groups of OTUS whish can be described at higher units and given appropriate names. There are two basic ways of revealing the taxonomic structure of the table of resemblances; the first is cluster analysis which produces discrete clusters of similar OTUS, and the other is ordination by which OTUS are arranged into some order but are not split into discrete groups. The two methods are complementary, but

for most taxonomic studies cluster analysis is more useful and here also cluster analysis is employed for revealing the taxonomic structure.

There are three different methods of cluster analysis such as

a) Single linkage
b) Average linkage
c) Total linkage

In the present work un-weighted average linkage was employed due to the reason that it permits clusters to fuse only when the average similarity between them is sufficiently high. This is probably the most satisfactory of the type of clustering methods described but it is much more difficult to write computer program for average linkage than for the other two methods and average linkage can have serious mathematical problems.

The average linkage is performed as follows. We write out the similarity values between the OTUS in the order of magnitude and the earlier example yields the list below:

%S	OTU pairs
80	**1:4**
75	**2:3**
40	**1:2**
25	**1:3**
20	**2:4**
0	**3:4**

The steps in clustering according to average linkage are as illustrated as follows:

Steps	Clusters
1. The highest values links 1 and 4 at 80 %	1, 4
2. The next highest value links 2 and 3 at 75%	2, 3

3. The next value, 40% attempts to link 1 and 2, but the computer now tests the average of the four values for 1:2, 1:3, 2:4 and 3:4. This is the average of 40 + 25 − 20 + 0 = 21.25 and this is below the current level of 40,so fusion is postponed.
4. The similarity level is reduced until it reaches 21.25 % when 1, 4 and 2, 3 joins

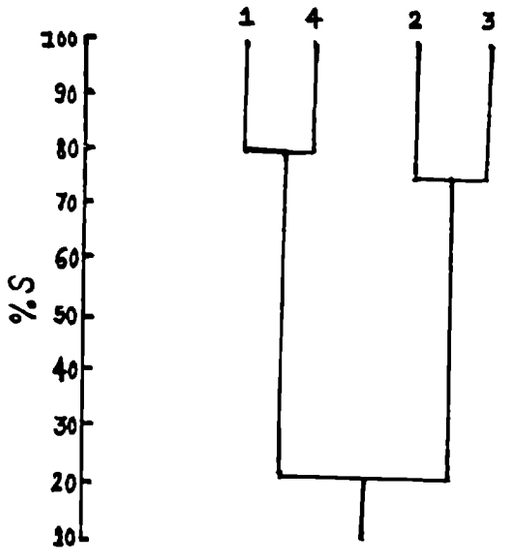
2.1.1.3 Dendrograms

A common way of displaying the results of clustering is as a tree like diagram also called a dendrogram. This is a formalized version of linkages shown above. This is more properly a phenogram as it is based on phenetic data. Dendrograms are readily drawn by following the steps given below (Fig. 1).

1. The order of OTUS as given in the final cluster is written across the top of the paper.
2. A line is dropped from each OTU until it reaches the S level at which it joins some thing else. A cross bar is drawn between the lines at this level.
3. Once a pair or cluster is formed a single line is continued down from it which in due course reaches a level where another fusion occurs and another cross bar is drawn.
4. 4. Finally, all the OTUS are joined into a cluster and a final single line can be drawn from this if desired.

The dendrogram is thus a taxonomic hierarchy, with the OTUS at the tip. The stems of the dendrograms indicate phenetic groups (phenons) and these are then taken as equivalent to natural taxa.

Fig-1



2.1.1.4 Similarity matrix (shaded diagrams)

The dendrograms may be supplemented with shaded similarity matrices after the OTUS have been arranged in the order given by the cluster analysis.

2.1.1.5 Identification of clusters

The most appropriate method of identifying a cluster is to incorporate maximum number of type strains /reference strains as OTUS along with the other OTUS by determining the taxonomic unit characters. On doing this each cluster is likely to pick up the appropriate type strain which exhibit high degree of similarity. By this way the cluster gets itself identified.

2.1.2 Systematics based on genotypic characters

2.1.2.1 Historical perspectives

Historically classification of bacteria has been based on similarities in phenotypic characters. Although this method has been quite successful, it has not

been precise enough for distinguishing superficially similar organisms or in determining phylogenetic relationships among the bacterial groups. Nucleic acid studies were first applied to such problems in bacterial classification more than 20 years ago, and has become an important component of any such study There are several advantages to be gained by basing classification on genetic relatedness (Johnson, 1984).

- A more unifying concept of a bacterial species is possible.
- Classifications based on genetic relatedness tend not to be subject to frequent or radical changes.
- Reliable identification schemes can be prepared after organisms have been classified based on their genetic relatedness.
- Information can be obtained that is useful for understanding how various bacterial groups have evolved and how they can be arranged according to their ancestral relationship.

The use of genetic characteristic in bacterial classification is relatively recent. It dates from the mid-1950s when bacterial gene transfer was discovered and Watson and Crick demonstrated the molecular basis of genetic information in the sequences of bases on the DNA molecule. Since that time the development of physicochemical techniques for the analysis of the genetic material, together with the exploitation of bacteria as genetic tools, has resulted in the accumulation of material which has proved significant for bacterial systematics (Jones, 1984).

In the last two decades it has become clear that the genetic complement of a bacterial cell lies not only in the main chromosomes but, in many cases, also in the extra-chromosomal elements such as plasmids, transposons and the lysogenic and the temperate phage. All these elements carry genetic material capable of phenotypic expression. What contribution such extra-chromosomal elements make to a particular phenotype, either by direct expression or interrelation with the chromosomal DNA of the cell, is only just beginning to be understood (Broda, 1979; Harwood, 1980; Hardy, 1981).

For the bacterial taxonomist the genetic approach to systematics has great appeal, both for its potential to reveal biologically significant, stable groupings (taxa) and for the elucidation of bacterial evolutionary relationships (phylogeny). Consequently, several of the newer taxonomic methods are being directed towards the characterization of the genetic complement of the bacteria.

2.1.2.2 DNA base compositions

The first unique feature of DNA that was recognized as having taxonomic importance was its mole percent guanine + cytosine content (mole % G+ C). Among the bacteria the mole % G +C values range from 25-75 % and the value is constant for a given organism. Some genera are homogenous in base compositions whereas others are heterogeneous, or even discontinuous (i.e. closely related bacteria have similar mole % G + C values). The latter finding is more useful taxonomically than the former, for the DNA samples showing widely different base compositions can be assumed to also have different base sequences (i.e. genetic messages). On the other hand, it cannot be assumed that base sequences are similar in two DNA samples because the overall base compositions are similar. Similar GC contents only indicate that the base sequence could be similar. Therefore it is very important to recognize that two organisms that have similar mole % G + C values are not necessarily closely related; that is because the mole % G + C values do not take into account the linear arrangement of the nucleotides in the DNA. Thus in those cases of genera showing homogeneity in DNA base composition, DNA data are not contradictory to the classification derived by other means.

In those cases where there is heterogeneity in base compositions, within a genus, the DNA data confirms the suspicion, often already aroused by the standard taxonomic methods, that unrelated bacteria are being classified together The classification of genera can be redefined so that only organisms homogenous with respect to base compositions are included in the same taxon (Hill, 1974).

The significance of guanine + cytosine content differences, from Deley's paper (1968) are summarized as follows: Differences of about less than 2 % are probably not significant with present assay methods, for, there is always some heterogeneity in the GC compositions of DNA fragments. Differences of 5% or less are usual among strains of well-defined species" Colwell and Mandel (1964), showed that the phenetic groups corresponding to common bacterial species had GC values within about this range; gene exchange between bacteria with about this degree of GC differences has been recorded (Catlin and Cunningham,1964). If however two strains differ by 20-30 %, they are distantly related taxonomically, and one would be reluctant to place them in the genus, or even in the same family However, since genera and families are poorly studied at present in this context, this last point should not be stressed or interpreted too rigidly

It should be noted that, it is only differences in GC values that are significant; two bacteria may be quite different but may have the same mole % guanine + cytosine, e.g. *Streptococcus* and *Haemophilus*. The streptococci are gram positive cocci, while the hemophilus are gram negative and fastidious organisms; both belonging to two different genera but they have the same mole % G + C ratio.

Mole percent G+C were initially determined by acid hydrolyzing the DNA, separating the nucleotide bases by paper chromatography and then eluting and quantifying the individual bases. Other methods have become popular since then such as:

a. Chemical analysis
b. Denaturation temperature
c. Buoyant density
d. Spectral analysis

2.1.2.2a Chemical analysis

DNA is hydrolyzed and subjected to paper chromatography to separate and estimate the four bases (Bendict, 1957). This method is largely superseded by the physico-chemical methods given below.

2.1.2.2b Denaturation temperature

The controlled heating of a preparation of double stranded DNA in a solution, leads to the breakdown of the hydrogen bonds between bases on the complementary strands, permitting the latter to uncoil and separate. The double stranded helix (native configuration) changes to single- stranded DNA (denatured configuration). Denatured DNA absorbs approximately 40 % more UV light at wavelength (λ) 260 nm than the native DNA. The temperature at which denaturation occurs can be determined by plotting optical density (O.D) as a function of temperature. The optical density initially remains constant, or decreases slightly, until the onset of denaturation when there is a sharp increase after which the O.D becomes constant again. The increase in O.D is called the hyperchromicity The denaturation or 'melting' temperature (Tm) is defined as that temperature corresponding to 50% hyperchromicity (Hill, 1974).

The denaturation temperature is dependent on two factors, the base composition of the DNA and the ionic strength of the solvent in which the DNA is dissolved.

a) DNA base composition

For any one solvent, when the ionic strength is constant, the greater the GC content, higher the Tm.

b) Ionic strength

The denaturation temperature is linearly related to the logarithm of the solvent

ionic strength; the more dilute the solvent the lower the Tm. Use is made of this when the DNA is of high % GC i.e. when the Tm in SSC approaches 100°C A more dilute solvent is used and the expected Tm in SSC extrapolated from the experimental one (Silvestri and Hill, 1965).

The relationship between Tm in SSC and % G+C had been derived by Marmur and Doty (1962). This relationship is satisfactory over the range of 30-70 % GC; below or above this some departure from linearity occurs.

For SSC solvent

$$GC = \frac{Tm - 69.3}{0.41}$$

Thus, higher the Tm, the higher will be the mole % G + C of the DNA.

2.1.2.2c Buoyant density method

The buoyant density of DNA increases linearly with the increase in GC content. Buoyant density is estimated by Caesium chloride density gradient ultra centrifugation (Meselson, *et al.*, 1957). The equation derived by Schildkraut *et al.*,(1962), relating buoyant density (ρ) to % GC is:

$$\rho = 1.660 + 0.098\ (GC)$$

This method has the following advantages:

1) Highly purified DNA is not required; crude nucleic acid extracts suffice.
2) Very small quantities of DNA are used, 1-2 μg.
3) The linearity of ρ to % GC is maintained below and above the 30-70 % range.
4) The presence and absence of 'satellite DNA' differing in base composition from the main populations of molecules is readily observed (Joshi *et al.*, 1963, Mandel, 1966)

2.1.2.2d Spectral analysis

The UV absorption spectrum of DNA is a summation of the individual spectra of the four bases. A simple method of calculating % GC from absorption spectra of acid-denatured DNA was given by Fredericq *et al.*, (1961). For all the methods using spectral analysis, highly purified DNA is required, as traces of proteins, whose UV absorption maximum is at λ=280 nm, lead to erroneous results. Another spectroscopic method for the determination of % GC depends on the reactivity of adenine towards brominating agents (Wang and Hashagen, 1964).

2.1.2.3 Taxonomic significance of GC content

The taxonomic significance of the mean base composition of DNA expressed as the mole percent of GC (% GC) has been discussed in several articles (DeLey, 1968; Marmur, 1961; Marmur *et al.*, 1963). General reviews of the ratios observed in different bacteria are those of Hill (1966), Rosypal and Rosypalova (1966) and DeLey (1968). These reviews and articles revealed that only differences in % GC are significant and they indicate genomic differences. Similarity in % GC does not indicate genomic similarity; as the GC ratios of *Pneumococcus, Proteus mirabilis* and man are all about the same. De Ley (1968) calculated the limits of % GC differences that can occur if two organisms share an appreciable amount of identical or almost identical cistrons. He estimates that a difference of 20-30 % in GC ratio must then mean that there is practically no nucleotide sequence in common to the two bacteria. There is therefore a firm theoretical basis for the observation that highly similar organisms must have very close GC ratios. But if the % GC difference is substantial, then the base sequence must be markedly different. Consequently, a given protein may have identical functions and the same linkage positions on the chromosome of two organisms, but if the organisms differ in % GC, the amino acid sequence of the two proteins must be notably different (Falkow, 1965).

Homogenous phenetic groups are also homogenous in % GC (Colwell and Mandel, 1964, 1965; De Ley *et al.*, 1966; Silvestri and Hill, 1966). Investigations on the molar guanine + cytosine composition (% G+C) of purified DNA have shown that strains closely resembling each other in morphological, physiological, biochemical and other properties (varieties and species)have nearly identical % GC and genera with similar properties (e.g. *Escherichia*, *Salmonella* and *Shigella*)have nearly the same range of %GC (Lee *et al.*, 1956; Belozersky and Spirin,1958; Marmur and Doty, 1962; Schildkraut *et al.*, 1962). The variations among strains of well established taxo species is quite small. It is usually reported as a range of about 2 5% (rarely greater than 4%), but would be better expressed as a standard deviation of the mean %GC of the DNA fragments (DeLey, 1968). Such standard deviation appears to be usually about 1 % for strains of a taxo species. As noted above, groups that are homogenous in %GC may yet be heterogenous phenetically. Certain genera such as *Vibrio* and *Flavobacterium*, have a very wide range of %GC, and this has been an extremely useful pointer to the need for taxonomic revision. In a number of cases, subsequent studies have led to the splitting of the genus into several, and the work of Veron and Sebald provides sufficient examples (Veron and Sebald, 1963; Veron, 1966). The discovery in a set of strains of two subgroups, different GC ratios, is a clear indication that there are at least two different phenetic groups. A difference of %GC of 5% usually implies at least a species difference, whereas a difference of less than 2% is seldom of taxonomic significance (Deley, 1968, Sneath, 1971). Sueoka (1961), pointed that two strains which differ by 10% or more GC hardly have any DNA molecules in common and therefore cannot be phylogenetically related. The reverse however is not necessarily true; two strains may have identical % GC ratios and yet be obviously different, such as the following pairs: *Escherichia* and some corynebacteria (50% GC), *P. aeroginosa* and *Mycobacterium phlei* (66% GC). Similarity in DNA base composition points to possible genetic affinity and an eventual common phylogenetic origin, but only if the organisms under comparison also display many morphological, physiological, biochemical and other similarities. Therefore DNA base composition is an important tool in bacterial taxonomy provided the strains being compared have been investigated extensively also in other ways.

2.1.2.4 Significance of %GC in this study

The study focuses attention on the Numerical Taxonomy of the family Vibrionaceae associated with the larvae of *M. rosenbergii*. The isolates obtained from both healthy and moribund larvae were divided into two based on their sensitivity to the O/129 (pteridine) compound. On comparing 233 unit characters of the operational taxonomic units along with those of the 20 standard strains based on simple matching coefficient and unweighted average linkage, by applying the computer program TAXAN, it is expected to obtain a similarity matrix based on which dendrograms could be constructed. It is expected that every phenon thus generated will pick out a standard strain and thereby get identified. Meanwhile, there would be few phena that would be left unidentified as they would not match with any of the type strains incorporated.

As explained previously % G+C ratio is an expression of the gross genotypic similarity between families /genera and species and can be considered the first step towards determining the DNA homology To complete the process of analysis, the genotypic similarity between the phena of each group and the already known species has to be worked out. Since it is not practical to examine the % G+C ratios of all the OTUS, representative strains from each cluster were selected for analysis of the mole % G+C ratio; this would represent the G+C content of the entire phena. This facilitates comparison with the already known species genotypically hereby narrowing down the affiliation of the phena to a limited number of known species of the family Once this is achieved, a phenotypic comparison of the cluster with related strains would be possible, brightening the scope for identifying the phena. Another possibility of this analysis is that in cases where the G+C ratio happens to be out of range of the accepted genera/species, new genera/species can be proposed to accommodate them also in this family This analysis further reduces the spectrum of species to which DNA homology has to be worked out, by way of DNA/DNA hybridization as the next phase of systematics

2.2 Materials and methods

2.2.1 Bacterial strains

Heterotrophic bacteria already isolated (Singh, 1990) from the larval rearing system of *Macrobrachium rosenbergii* and maintained in the Environmental Microbiology Laboratory, School of Environmental Studies, were used for the study. From this collection of cultures, isolates obtained from the moribund larvae and apparently healthy larvae were subjected to the study

Altogether 447 isolates from the moribund larval system and 300 from the apparently healthy larvae were screened for segregating the members belonging to the family Vibrionaceae. All the above cultures were purified by repeated streaking on nutrient agar plates supplemented with 2% (w/v) NaCl. Purity of the cultures were confirmed by Hucker's modification of the Gram's staining (Hucker and Conn, 1923). Working cultures were maintained on nutrient agar slopes. Unless other wise mentioned all media were supplemented with 2 % (w/v) NaCl.

2.2.1a Moribund larval system

From the moribund larvae, out of the 447 isolates screened, 204 were designated as belonging to the family Vibrionaceae. These isolates were Gram negative rods or cocci, fermentative with or without gas production in MOF medium, giving Kovac's oxidase test positive, and motile or non motile (Baumann and Schubert, 1984)

These 204 isolates were further segregated based on their sensitivity to the 0/129 compound into two groups, viz. sensitive (positive) and non sensitive (negative) (Baumann and Schubert, 1984; Bain and Shewan, 1968; Popoff, 1984). Thus, 140 isolates were found to be 0/129 negative and 64 isolates 0/129 positive.

2.2.1b Apparently healthy larval system

300 isolates were screened altogether, out of which 109 were designated as belonging to the family Vibrionaceae. These 109 isolates were Gram negative rods or cocci, fermentative with or with out gas production, gave Kovac's oxidase test positive and were either motile or non motile (Baumann and Schubert, 1984)

These 109 isolates were further segregated based on their sensitivity to the 0/129 compound into two groups viz. sensitive (positive) and non-sensitive (negative) (Baumann and Schubert, 1984, Bain and Shewan, 1968; Popoff, 1984). Thus, out of the 109 isolates, 74 were 0/129 negative and 34 isolates 0/129 positive.

2.2.1c Standard /reference strains

Twenty standard strains were subjected to the battery of tests to which the larval isolates were also subjected. The reference strains include representatives of *Vibrios* , *Aeromonas, Photobacterium* and *Plesiomonas*. The list of reference strains and the source from which they were obtained is given in Table 2-1

The four groups obtained as in 2.1.1 and 2.1.2 and the 20 reference strains procured from various sources were tested for 233 unit characters. These included biochemical, morphological and physiological characters, utilization of substances as sole sources of carbon, fermentation or production of acid or gas from sugars and carbohydrates, degradation of various substances, etc. The unit characters and the corresponding methodology of each test are given in detail in the section 2.2.

2.2.2 Unit characters

2.2.2 Biochemical tests

2.2.2.1 Methyl Red and Voges-Proskauer tests

These tests are normally carried out with cultures grown in glucose-phosphate peptone water, which has the following composition per litre:

Glucose	5g
K_2HPO_4	5g
Peptone	5g

The medium is dispensed in 5mL amounts in small culture tubes and autoclaved at 10 lbs (115° C) for 10 minutes. The inoculated tubes were then incubated for 7 days until good growth was obtained

Methyl red test

This test determines whether the production of acid from glucose has lowered and held the pH at about 4.2 or below A few drops of methyl red indicator were added to the culture and a resultant definite red color was considered positive. Shades intermediate between yellow and red were considered as doubtful positive results. The indicator was prepared by dissolving 0.1g methyl red in 300mL 95 % ethyl alcohol, which was then diluted to 500mL with distilled water,

Voges-Proskauer test

Some organisms, after producing acids from glucose, are capable of converting the acids to acetylmethyl carbinol or 2,3 butanediol which are neutral substances. Aeration in the presence of alkali then converts the products to diacetyl, which in turn reacts with the peptone constituents producing a pink colouration. Two modifications of the test viz. O'Meara's modification (O'Meara's, 1931) and Barritt's modification (Barritt, 1936) are in use and both enhance the development of color. For the present study Barritt;s modification was followed: To 1 mL of the culture was added 0.5mL 6% alcoholic solution of ∝- naphthol and 0.5mL 16% KOH solution, agitated thoroughly and maintained for 2 hours. Positive test was indicated by the development of pink coloration.

The pale pink colouration normally appears within 5 minutes but may not reach maximum red colour intensity for about an hour Negative tubes were examined and confirmed after a longer period.

2.2.2.2 3-ketolactose production

This test was originally developed by Bernearts and Deley (1963) to show the oxidation of lactose to 3-ketolactose by strains of *Agrobacterium tumefaciens.* The test was carried out by growing the organisms initially, on a nutrient agar slope having the following composition per litre:

Yeast extract	10g
Glucose	20g
$CaCO_3$	20g
Agar	20g

Two or more loopfuls of the subsequent growth are transferred to agar plates of a second medium, the growth being heaped up at one point on the plate.

The second medium has the following composition per litre

Lactose	10g
Yeast extract	1g
Agar	20g

The agar plates were incubated for 2 days and were then flooded with Benedict's qualitative reagent. The flooded plates were held at room temperature for at least 1 hour. If 3-ketolactose has been produced, a yellowish brown zone of Cu_2O develops around the colony

2.2.2.3 3-ketogluconate production

The oxidation of potassium gluconate to potassium 2-ketogluconate is a characteristic used for the identification of *Pseudomonads* and members of the family *Enterobacteriaceae*. Potassium 2-ketogluconate is a reducing substance that can be detected in a liquid medium by the development of a brown, orange or yellow precipitate of Cu_2O with Benedict's reagent.

The medium suggested by Hay es's (1951) was used. It contains per litre.

Tryptone	1.5g
Yeast extract	1g
K_2HPO_4	1g
Potassium gluconate	40g
pH 7.0	

The broth was transferred into tubes in 3mL aliquots and autoclaved at 10 lbs for 10 minutes. The tubes were inoculated and incubated at 28±0.4°C. After the development of good growth, 1ml of Benedict's qualitative reagent was added to 1mL of the liquid culture in a 15mL tube. The tube was then heated in a boiling water bath for 10 minutes and cooled rapidly The color change and the development of a precipitate were recorded after a few minutes.

2.2.2.4.The reduction of nitrate and nitrite

These reduction processes may involve

(a) assimilation, in which the nitrate is reduced to nitrite and ammonia, which is then converted to amino acids and other nitrogenous organic cell components,

(b) dissimilation, (or respiration) , in which nitrate or nitrite replaces oxygen as the terminal hydrogen acceptor, under conditions of low free oxygen availability In a

nitrate- containing medium, the nitrate may be reduced to nitrite only, or to nitrite and ammonium ions, or the nitrate may be completely assimilated. All these processes are generally referred to as nitrate reduction. When a nitrite-containing medium is used, the reduction of nitrite to ammonium ions or the complete assimilation is known as nitrite reduction. If either the nitrate or nitrite is dissimilated to a gaseous end product such as nitrogen or nitrous oxide, the process is referred to as denitrification. The reduced intermediates detected in the biochemical tests are assumed to be released mainly by dissimilatory processes.

Reduction of Nitrate to nitrite

Tests for reduction of nitrate were carried out by inoculating organisms into a suitable nutrient broth medium containing 0.1 %(w/v) KNO_3. The cultures were incubated until good growth was obtained during which time a sample was examined periodically to detect whether reduction of nitrate has occurred and to determine the stage at which it takes place.

Composition of Nutrient Broth per litre

Peptone	5g
Beef extract	5g
Yeast extract	1g
KNO_3	1g
pH 7.5	

Autoclaved at 15 lbs (121^0 C) for 15 minutes in 5mL aliquots, inoculated with the test culture and incubated at 28± 0.5 ^{0} C for 48 hours.

Preparation of reagents

Solution A.	Sulphanilic acid	1g
	5 N (glacial) acetic acid	100mL

Solution B	Dimethyl ∝-naphthylamine	0.6g
	5N (glacial) acetic acid	100mL

The presence of nitrite could be determined by adding to 5 mL of the culture 0.5ml of reagent A, followed by 0.5mL of reagent B. The development of a red color indicated that the nitrate has been reduced to nitrite.

When the nitrite test was negative, the presence of residual nitrate could be shown by adding approximately 1mg of zinc dust per mL of the culture. The development of a red color indicated that the nitrate had not been reduced by the organisms

Reduction of nitrite

The nitrite test is very sensitive and an examination of un-inoculated media free from added nitrite was always included in the study

The reduction of nitrite can be demonstrated by replacing the nitrite in the medium with 0.01% (w/v) KNO_2. A positive result is shown by a negative test for nitrite after incubation of the cultures.

The production of nitrogen gas was detected by incorporating inverted Durhams' fermentation tubes into the liquid medium.

Ammonia production could be detected by using Nessler's reagent.

2.2.2.5. Indole production

Certain bacteria produce indole by decomposition of tryptophan which is present in tryptone broth. This liberated indole reacts with the Kovacs' reagent to produce red color at the top of the medium. (Cowan and Steel, 1965)
Composition of test medium per litre:

Tryptone	1.5g
Sodium chloride	20g
pH 7.2± 0.3	

Dispensed the medium into 3mL aliquots into tubes, autoclaved at 15 lbs for 15 minutes. Inoculated and incubated for 48 hours. To each tube added about 0.5mL of Kovacs'reagent.

Preparation of Kovacs' reagent

ρ - dimethyl amino benzaldehyde	5g
Amyl alcohol	75 mL
Conc. HCl	25 mL

2.2.2.6. Hydrogen sulphide production

Many bacteria produce hydrogen sulphide from organic sulfur compounds in the medium. There are numerous tests for the detection of H_2S production and these vary widely in their sensitivity (Cowan and Steel, 1965). The relatively insensitive tests differentiate the strong H_2S producers from the others. The test organisms are grown in a medium containing a source of sulfur (e.g. peptone) and a indicator of sulphide production. (0.05% lead acetate or 0.025 % ferric ammonium citrate or 0.015% ferrous acetate); the medium turns black if H_2S is produced. A suitable medium is triple sugar iron agar (TSI) (Report, 1958), which contains per litre:

Beef extract	3g
Yeast extract	3g
Peptone	20g
Glucose	1g
Lactose	10g
Sucrose	10g
$FeSO_4.7H_2O$	0.2g

NaCl	5g
$Na_2S_2O_3$. $5H_20$	0.3g
Agar	20g
Phenol red, 0.25 solution	12mL

Heated to dissolve the solids in water, mixed and tubed in 5-6 mL aliquots. Sterilized at 15 lbs for 20 minutes and cooled to form slopes with deep butts.

Stabbed and streaked the tubes, incubated at 27°C for 24 to 48 hours. Any of the following reactions could be noticed.

a) Yellow coloration of the slope	Oxidative reaction
b) Pink or purple coloration of the slope	Alkaline reaction
c) Yellow coloration through out the tube or in the butt	Fermentative reaction
d) Blackening of the butt	H_2S production
e) Split or gas bubble in the butt	Gas production

2.2.2.7. Phenylalanine deaminase

Bacterial deamination of phenylalanine produces phenyl pyruvic acid; the keto acid turns a $FeCl_3$ indicator solution green. The reactions are typically associated with the organisms from the *Proteus* and *Providence* group, (Cowan and Steel, 1974).

Phenylalanine agar (Ewing *et al.*, 1957) slopes were made with the following ingredients per litre:

DL phenyl alanine	2g
Yeast extract	3g
Na_2HPO_4	1g
NaCl	5g
Agar	20g

Dissolved the ingredients by heating and dispensed in 3mL aliquots and sterilized at 121^0 C for 15 minutes. The tubes were solidified in a slanting position to give long slopes.

Note: When L-phenylalanine was used, only 1g was sufficient

The slopes were heavily inoculated with the test organism and incubated at 28 $\pm$ 0.5 ^{0} C for 24 to 48 hours. The area of growth was then flooded with 0.2mL of the 10 % aqueous solution of $FeCl_3$, which turned green when deamination occurred.

2.2.2.8. Amino acid decarboxylases

The amino acid decarboxylase tests demonstrates the bacterial decarboxylation of lysine, arginine, ornithine, histidine and glutamic acid, and these tests are of particular use in identifying members of *Enterobacteriaceae* (Moller, 1955). In this test the decarboxylation or the elimination, of a molecule of carbondioxide from the amino acid results in the formation of an amine with one carbon atom less. Decarboxylases are generally induced by growing the bacteria at a low pH. This is achieved, by cultivating the test organisms in a fermentable carbohydrate medium.

One percent of the L-amino acid (L (+)-Lysine dihydrochloride, L(+)-Arginine mono hydrochloride, L(+)- Ornithine monohydrochloride or L(+) – Glutamic acid) or 2 % of the DL form, was incorporated in Falkow's medium(modified from Falkow, 1958), containing per litre:

Peptone	5g
Yeast extract	3g
Glucose	1g
Bromocresol purple (0.2% solution)	10mL
Agar	3g

The solids were dissolved in distilled water and pH adjusted to 6.7; added the indicator solution. Sterilized the medium at 15 lbs for 15 minutes and made the folowing additions

1. L-lysine
2. L-ornithine
3. Histidine
4. Glutamic acid

Readjusted the pH to 6.7 if required, dispensed in 2mL aliquots and overlaid with liquid paraffin. Sterilized at 115^0 C for 20 minutes.

An inoculum from a culture of the test organisms on a solid medium was introduced with a straight inoculating wire through the liquid paraffin. The various controls included a tube containing only the basal medium that was also inoculated. Both were incubated and examined daily for 4 days. As a result of the bacterial fermentation of the glucose in the medium, the indicator turned yellow. The control tube without the aminoacid remained yellow; but a subsequent change to violet or purple in the tests indicated that alkaline degradation products were produced in the course of decarboxylation of the particular aminoacid.

2.2.2.9.Arginine dihydrolases

The ability of certain organisms to produce an alkaline reaction in arginine containing medium under relatively anaerobic conditions has been used by Thornley (1960) to differentiate between certain gram negative bacteria, especially *Pseudomonas* spp. The alkaline reaction is thought to be due to the production of ornithine , CO_2 and NH_3 from arginine.

Thornley's medium has the following composition per litre:

Peptone	1g

NaCl	5g
K_2HPO_4	0.3g
Agar	3g
Phenol red	0.01g
L (+) –arginine hydrochloride	10g
pH 7.2	

The medium was heated to dissolve the solids and dispensed in 3mL aliquots into culture tubes, overlaid with liquid paraffin to a height of 5mm and sterilized at 121^0 C for 15 minutes.

The test organisms were stab inoculated into the medium through the liquid paraffin layer Color changes are recorded after incubation at 28 ± 0.5^0 C for upto 7 days;the color change from yellowish orange to red being positive.

2.2.2.10 Terminal respiratory enzymes

2.2.2.10a Catalase test

The principle of this test is that when organisms containing catalase are mixed with hydrogen peroxide (H_2O_2), gaseous oxygen is released.

The test organisms are grown on a slope of nutrient agar A thick smear of the test organism was made from a 24 hour culture on a clean slide and a drop of hydrogen peroxide is placed on it. Immediate formation of gas bubbles indicated the liberation of oxygen and positive catalase test.

2.2.2.10b Kovacs' oxidase test (cytochrome oxidase test)

The detection of cytochrome oxidase activity is used as a differentiating test mainly for the aerobic and facultatively anaerobic groups of Gram negative bacteria.

In the organisms studied by Stanier *et al* (1966), the activity was correlated with the cytochrome of the 'c' type. Sugar free media should be used when ever posssible.

According to the methods recommended by Kovacs'(1956), the organisms were freshly grown on nutrient agar. A platinum loop was used to pick a colony and make a compact smear on a filter paper moistened with 2-3 drops of a 1% solution of tetramethyl -ρ-phenylene diamine dihydrochloride. A positive result was recorded when the smear turned violet within 10 seconds; indicating the formation of indophenol, the oxidation product of tetramethyl para phenylene diamine dihydrochloride.

2.2.2.11 Oxidation /Fermentation reactions with glucose
(The oxygen requirement for the utilization of the compound)

This characteristic is usually determined by inoculating the organisms into deep agar media in test tubes. The incorporation of a pH indicator into the medium enabled detection of changes in the pH resulting from the decomposition process to be observed in addition to visible signs of growth in different parts of the medium. Cultures are either stab inoculated with a needle after solidification or shaken cultures are prepared prior to solidification of the agar The composition of the basal medium is adjusted for the group of organisms under study/examination and should enable good growth to be obtained if the substrate is being utilized. The method is most extensively used for showing the decomposition of carbohydrates.

Acidic changes at or near the surface indicates that the substrate is being oxidized by aerobic bacteria, whereas the development of uniform acidity throughout the tube shows that facultatively anaerobic organisms are both oxidizing and fermenting the substrate. Anaerobic bacteria that ferment only the substrate, usually produce an acidic reaction in the lower part of the tube initially, but acidic materials may diffuse upwards to give an appearance of acid production through out the tube.

MOF medium (HiMedia laboratories, Bombay) was employed for the present work. Transferred 2.2 g of MOF medium to 100mL distilled water, added 1.5g agar and autoclaved at 15 lbs for 15 minutes.. Added 1% glucose or dextrose to the sterile basal medium and transferred 4mL aliquots aseptically into sterile tubes and autoclaved at 10 lbs for 10 minutes. Converted to slants with a long butt. The tubes were stabbed and streaked and incubated at 28 ±0.4°C.

The results were recorded as follows:

O –Oxidation (yellow coloration in the butt)
F -fermentation (yellow coloration through out the tube)
(F) - fermentation with gas production
Alk/N-Alkaline reaction(pink or purple coloration in the slant and no reaction in the butt)

2.2.2.12. Motility

Motility was tested by two methods, either by inoculating in soft agar medium or by hanging drop method.

A) In soft agar medium

Composition per litre:

Beef extract	3g
Peptone	10g
NaCl	5g
Agar	3gp

pH 7.2±0.1

Melted the medium and poured into tubes in 3mL aliquots and autoclaved at 15 lbs for 15 minutes. Stab inoculated the medium and incubated at 26±0.5^{0}C for 24 to 48 hours. Rhizoidal growth from the line of inoculation towards the peripheral area

was considered as the sign of motility A thick growth along the line of inoculation was considered to be negative. All negative cultures were subjected for observation by hanging drop method.

B) Hanging drop method

The organisms were grown in nutrient broth of the above composition. A loopful of the 18 to 24 hour old culture was placed in the centre of a clean coverslip. A drop of vaseline was placed on the four corners of the coverslip. Then a cavity slide was kept over the drop in such a way that the drop came within the cavity The vaseline helped to adhere the coverslip to the slide and the whole preparation was inverted quickly so that the drop of culture was seen hanging from the coverslip. The slide was placed under oil immersion objective and observed for actual displacement of the cells that could very well be differentiated from Brownian movement.

2.2.2.13 Sensitivity to the vibriostat compound,O/129 (2,4-diamino-6,7 diisopropyl pteridine)

The sensitivity of vibrios to the vibriostatic agent O/129, has long been recognized (Shewan *et al.*, 1954). This compound is very effectively used for differentiating *Vibrio* and *Photobacterium* from *Aeromonas* and *Lucibacterium*, all of them belonging to the family Vibrionaceae.

Vibrio and *Photobacterium* are sensitive to the vibriostat compound while *Aeromonas* and *Lucibacterium* are resistant.

Antibiotic assay filter paper discs of 6mm diameter (Whatman) were prepared aseptically to contain 150µg. mL^{-1} of the agent. The discs were stored in the refrigerator (4°) and used as required (Furniss *et al.*, 1978)

The nutrient agar plates were swabbed with the suspension of the test bacterial organism to get a confluent growth and the disks were placed on it with appropriate

spacing. The cultures that were sensitivity to the pteridine compound developed a clearing zone around the disc.

2.2.3. Degradation and utilization of compounds

2.2.3.1. Lecithin (Phospholipase /lecithinase production)

Bacterial phospholipases (lecithinases) decompose phospholipid complexes that occur as emulsifying agents in serum and egg yolk (Holding and Collee,1971). The enzymatic activity breaks the emulsion and liberates free fats, so that a turbidity is produced. Convenient substrates for the reaction are

1. 20% egg yolk(v/v) in saline
2. Commercially available egg yolk suspension, or preferably
3. Lecithovitellin solution

Lecithovitellin (LV) is a lipoprotein component of egg yolk and can be obtained as a clear yellow solution by mixing egg yolk with saline (Cowan and Steel, 1974).

Plate test

The test organisms were cultured on nutrient agar medium having the following composition per litre:

Peptone	5g
Beef extract	5g
Yeast extract	1g
NaCl	20g
Agar	20g
pH 7.2±0.2	

4% sterile fresh egg yolk was added to the sterile nutrient medium at 55^0 C just before the plates were poured. The test organisms were spot inoculated heavily

And incubated at 27^0 C for 24-48 hours. Phospholipase production was characterized by a zone of turbidity in the medium surrounding each colony

2.2.3.2 Degradation of lipids

2.2.3.2a Tween 20, 40, 60 and 80 (lipids) (Lipase production)

Tween compounds are generally used as substrates for detecting the hydrolysis of fats. Tweens are thermostable, water soluble,long chain fatty acid esters of a polyoxy alkylene derivative of sorbitan. Tween 80,60 and 40 are esters of oleic acid, stearic acid and palmitic acid respectively (Sierra, 1957).

Composition of the medium per litre:

Peptone	10g
NaCl	20g
$CaCl_2.H_2O$	0.1g
Tween 20,40,60,or 80	10g
Agar	20
pH 7.2±0.2	

The ingredients were dissolved and the pH adjusted. The medium was sterilized at 15 lbs for 15 minutes after dispensing in 3 mL aliquots into culture tubes. The tubes were than converted to slants. The test organisms were inoculated and the tubes incubated for 1-3 days at 27^0 C.

A positive result was seen as a opacity beneath the microbial growth in the slants, which was due to the production of calcium salts of free fatty acids (calcium soaps) and were taken as being indicative of a positive lipolytic activity.

2.2.3.2b Tributyrin (lipase production)

In general, though bacterial lipases are poorly characterized, the demonstration of lipolytic activity is sometimes of practical use. Tributyrin or glyceryl tributyrate is commonly used for studying lipolytic activity 'Tributyrin agar' medium (any nutrient agar containing tributyrin) used for the test had the following composition per litre:

Peptone	5g
Beef extract	5g
Yeast extract.	1g
NaCl	20g
Tributyrin	10g
Agar	20g

pH 7.2±0.2

Tributyrin was first mechanically blended into the nutrient broth to form a stable emulsion, agar added and sterilized at 121^0 C for 15 minutes. Plates were poured while mixing well each time.

The test organisms were spot inoculated and the inoculated plates were incubated at 27^0 C for 3-4 days.

A positive result was indicated by zones of clearing around the colonies of lipolytic organisms, where the tributyrin has been hydrolysed (Rhodes, 1959).

2.2.3.3 Gelatin (Production of gelatinases)

When proteolytic organisms are grown on a plate of nutrient medium into which gelatin (0.4%) is incorporated, zones of gelatinase activity around the colonies are demonstrated, if the plates are flooded with acid mercuric chloride solution which

reacts with gelatin in the medium to produce an opacity; where the gelatin has been hydrolysed, the medium remains clear (Frazier, 1926). The medium contains per litre:

Peptone	5g
Yeast extract	1g
Beef extract	5g
NaCl	20g
Gelatin	4g
Agar	20g

pH 7.3 ± 0.2

The prepared medium was autoclaved at 15 lbs for 15 minutes and poured into plates. The test organisms were spot inoculated and the plates incubated at 27^0 C for 24-72 hours.

Gelatinase production was tested by flooding the plates with mercuric chloride solution, of the following composition:

$HgCl_2$	5g
Conc. HCl.	20mL
Distilled water	100mL

2.2.3.4 Urease production (urea amidohydrolase)

Urease catalyses the following reaction:

$$H_2NCONH_2 + H_2O \rightarrow 2NH_3 + CO_2$$

This test was used to determine the production of the enzyme urease by microorganisms, whereby the urea is hydrolysed to form ammonia, which is highly alkaline. In a medium used for determination of urease activity, urea and a pH indicator are incorporated. A positive result is shown by a rise in the pH value

resulting from the hydrolysis of urea and a respective change in the color of the indicator used.

The medium devised by Christensen, (1946) has the following composition per litre:

Peptone	1g
NaCl	5g
Glucose	1g
KH_2PO_4	2g
Phenol red(0.2% solution)	6mL
Agar	20g
pH 6.9	

Yeast extract (0.1%) was also added for organisms requiring growth factors. The medium was prepared in bottles, sterilized and cooled to about 55^0C. A 20% solution of urea previously sterilized by filtration, was then added to give a final concentration of 2% urea in the molten medium. The completed medium was dispensed into tubes and converted to slopes. A control without urea was also included.

The tests and the controls were inoculated and incubated for 24 hours at 28 ± 0.2^0C. Urease activity caused the yellow indicator to change to red.

2.2.3.5 Aesculin hydrolysis

The ability of the microorganisms to hydrolyze this glycoside to aesculetin and glucose, can be investigated by incorporating 0.1% aesculin into a suitable nutrient agar medium that supports good growth of the organism. Ferric citrate is added to the medium at a concentration of 0.05%. A positive reaction is shown by the development of a brownish black colour that is produced by aesculetin in combination with the iron.

The method used by Gemmell and Hodgkiss, (1964) for tests with the lactobacilli which incorporated 1% aesculin into nutrient agar medium was followed. The hydrolysis was detected by the presence of coral like crystals, which are assumed to be aesculetin and a simultaneous loss of the characteristic fluorescence of aesculin in UV light.

2.2.3.6 Starch hydrolysis (Production of amylase)

Nutrient agar with the following composition per litre was used as basal medium for demonstrating amylase production

Peptone	5g
Beef extract	5g
Yeast extract	1g
NaCl	20g
Agar	20g
Soluble starch	5g

PH 7.3±0.2

The test medium was prepared according to the above composition, melted and poured into tubes in 3mL aliquots, autoclaved at 15 lbs for 15 minutes and converted into slants.

The starch agar slants were inoculated with the test organisms and the tubes were incubated until good growth was obtained at, 27°C for 24-72 hours. The tubes were flooded with Grams' iodine solution prepared in the following manner

Iodine	1g
KI	2g

Distilled water 100mL

Amylase producing or starch utilizing organisms showed a clearing zone around and beneath them. The color of the zones depended on the degree of hydrolysis of starch, when it was hydrolyzed to the stage of dextrin, then the zones were reddish brown and when the breakdown had gone further, they were colorless.

2.2.3.7 Cellulose hydrolysis (Production of cellulase)

The ability of organisms to hydrolyze naturally occurring insoluble polymers like cellulose can be routinely investigated. In the method used for the demonstration of cellulase, the polymer in the form of Whatman filter paper cut into thin strips 2-3 cms long and 1cm wide were immersed in the following medium having the composition per litre:

Solution A

NH_4Cl	5g
NH_4NO_3	1g
Na_2SO_4	2g
K_2HPO_4	3g
KH_2PO_4	1g
NaCl	10g

Solution B

$MgSO_4\ 7H_2O$	0.1g
$MgCl_2.\ 6H_2O$	4g
Yeast extract	0.01g

The pH of this mineral base medium was adjusted to 7.0 with NaOH. Solution A and Solution B were sterilized separately at 121^0 C for 15 minutes. They were then cooled to room temperature and mixed. Along with the basal medium, culture tubes containing filter paper strips were also sterlized. After mixing the two solutions, the basal medium was transferred into 3-4 mL aliquots aseptically into the tubes containing filter paper strips.

The test organisms were inoculated and incubated at 27^0C for 3-7 days or until growth appeared in the medium (seen as turbidity against an un-inoculated control). Turbidity or growth indicated that the test organisms were able to utilize cellulose as sole source of carbon and hydrolyze it by producing cellulase.

2.2.3.8 Hydrolysis of chitin (Production of chitinase)

Chitinolytic bacteria hydrolyze chitin to N-acetyl-D-glucosamine. This hydrolysis can be easily tested by incorporation of colloidal chitin in a suitable basal medium (Holding and Collee, 1971).

Purified colloidal chitin (Lingappa and Lockwood,1961) was made by treating crude chitin alternately with N-NaOH and N-HCl several times, and then with ethanol until all foreign material had been removed. The purified chitin was then dissolved in cold conc. HCl, filtered through glass wool, precipitated in distilled water and washed several times in distilled water until neutral. This colloidal chitin was then added to the nutrient basal medium having the following composition per litre:

Peptone	5g
Beef extract	5g
NaCl	20g
Colloidal chitin	5g (or 5% v/v)
Agar	20g
pH 7.5	

The medium is sterilized at 15 lbs for 15 minutes and poured into plates. The test cultures were spot inoculated and incubated for 7 days at 27^0C. Hydrolysis of chitin was represented by a halozone around the colonies.

2.2.3.9 Hydrolysis of deoxyribonucleic acid (Production of deoxyribonuclease)

A plate test for the demonstration of bacterial decomposition of nucleic acid was described by Jeffries *et al.*, (1957). DNA is readily soluble in water Fresh solution of DNA substrate (0.2%) was added to a liquid nutrient agar basal medium having the following composition per litre:

Peptone	5g
Beef extract	5g
Yeast extract	1g
NaCl	20g
DNA free acids	2g
pH 7.3±0.2	

The DNA containing medium was sterilized at 121^{0}C for 15 minutes and poured into plates as soon as the autoclaved medium had cooled to 50^{0}C. The test organisms were heavily seeded to produce a confluent growth on the plate. After incubation for 1-2 days at 27^{0}C, the plates were flooded with 2% N-cetyl, N, N, N trimethyl ammonium bromide.

Deoxyribonuclease activity resulted in the production of clear zones surrounded by a turbidity produced by the precipitation of the unaffected substrate.

2.2.3.10 Production of haemolysis

The clearly defined zones of complete hemolysis that develop around colonies of some bacteria grown on blood agar medium are referred to as zones of β-haemolysis. Blood agar was prepared as follows

Defibrinated blood	50mL
Nutrient Agar	950mL

The nutrient agar medium was sterilized by autoclaving at 15 lbs for 15 minutes and cooled to 50^0C. Added the blood aseptically, mixed and distributed into petriplates.

The plates were heavily spot inoculated and incubated at 27^0 C for 24-48 hours and observed for haemolysis

β-haemolysis: these are clearly defined zones of complete haemolysis that develops around a colony

α-haemolysis: this denotes a greenish discoloration of the blood agar medium around a colony

α'(alpha prime) haemolysis: An intermediate and rather indeterminate haemolytic effect in which there is an ill-defined zone of alteration of the blood agar without discoloration. In areas of α'-haemolysis, residual apparently unaltered red cells may be seen with the microscope.

γ-haemolysis: is used to describe the absence of effect around non-haemolytic colonies on the blood agar plate.

2.2.3.11 Hydrolysis of casein (Production of caseinase)

Casein is the milk protein and the ability of microorganisms to hydrolyze casein can be demonstrated on casein agar having the following composition per litre:

Peptone	5g
Beef extract	5g
Yeast extract	1
NaCl	20g
Agar	20g

Distilled water	750mL

pH 7.2±0.2

The medium was autoclaved at 15 lbs for 15 minutes. 30g casein in 250mL of distilled water was sterilized separately and mixed with the above medium before pouring into plates. The test organisms were heavily spot inoculated on the plates and the plates incubated at 27^0C for 24-48 hours.

Caseinase production was detected by the presence of clear zones around the test colonies.

In few instances when clearing was due to solution of the milk proteins by acid or alkaline metabolic products; it was distinguished from true proteolysis by the addition of acid mercuric chloride solution (prepared as in **2.2.3.4)**, an opaqueness in the already cleared area showed that the casein had not been hydrolyzed.

2.2.4 Utilization of sole carbon sources for nutritional screening

The basal medium, a modification of Baumann *et al.*,(1971a) given by Oliver *et al., (*1982), composed of the following per litre was used:

Solution A

NH_4Cl	5g
NH_4NO_3	1g
Na_2SO_4	2g
K_2HPO_4	3g
KH_2PO_4	1g
NaCl	10g

Solution B

$MgSO_4$ $7H_2O$	0.1g
$MgCl_2$. $6H_2O$	4g

Yeast extract	0.01g
Agar	20g

PH was adjusted to 7.0 with 1N NaOH.

Yeast extract was prepared in the form of a 0.1% stock solution in distilled water and added. Agar was used only in case of carbon sources that were insoluble; in which case plates were made and spot inoculated.

Solutions A and B were sterilized separately at 15 lbs for 15 minutes, cooled to room temperature and then mixed together (otherwise the basal medium becomes turbid).

All sugars were added to a final concentration of 0.2% (w/v). Other carbon sources were added in a final concentration of 0.1% (w/v). In case of carbon sources that were insoluble, the concentration was made to 0.1% (w/v) and plates were prepared.

In case of plates, they were spot inoculated from a suspension of the 24hour culture of the test organism in 2% saline. Control plates without the c-sources were also inoculated. All the plates were incubated at 27^0C for 3-7 days. Results were recorded on the 3^{rd} day and confirmed on the 7^{th} day

All carbon sources were sterilized at115 ^{0}C for 10 minutes. The medium containing the carbon sources were dispensed into sterile test tubes in 1mL aliquots. Control tubes without the carbon source were also maintained for each culture.

The test media were inoculated with an inoculation needle, touching the inoculum to the side of the test tube, incubated for 24-48hours at 27^0C Checked for turbidity against control. Only in case of negative results, they were incubated further for 7 days.

The compounds tested as sole sources of carbon were as follows:

1.Ribose
2.Xylose
3.Raffinose
4.Melibiose
5.Rhamnose
6.Trehalose
7.Cellobiose
8.Galactose
9.Mannose
10.Fructose
11.Sucrose
12.Arabinose
13.Inulin
14.Maltose
15.Glycogen (insoluble)
16.Sorbitol
17.Sodium pyruvate
18.Calcium lactate (insoluble)
19.Sodium glucuronate
20.Tyrosine (insoluble)
21.Adonitol
22.Lactose
23.Dulcitol
24.Glycine
25.Sodium formate
26.Proline
27.Serine
28.Cystine (insoluble)
29.Uracil (insoluble)
30.Valine
31.Sodium malonate
32.Inositol
33.Leucine
34.Tryptophan (insoluble)
35.Sodium acetate
36.Sodium gluconate
37.Mannitol
38.Sodium citrate
39.Glycerol
40.Salicine
41.Potassium-sodium tartarate
42.Succinic acid

2.2.5 Acid and gas production from compounds (Fermentation)

The fermentation of carbohydrates can be demonstrated by the production of acid or acid and gas (CO_2 and /or H_2) in liquid medium in test tubes. Hugh and Leifsons' basal medium was used for this purpose. The basal medium was supplemented with NaCl to the final concentration of 2%(w/v), because all the organisms were marine isolates.

Hugh and Leifsons' medium has the following composition per litre:

Peptone	2g
NaCl	20g
K_2HPO_4	0.3g
Phenol red (1% aq. Solution)	30mL
pH 7.2±0.3	

The carbohydrates were added to a final concentration of 0.1% (w/v). Acid production was readily observed by incorporating into the medium an appropriate pH indicator (e.g. phenol red). Gas production was observed by placing a small inverted gas (Durham's) fermentation tube in each test tube during preparation.

The basal medium was first autoclaved at 15 lbs for 15 minutes along with plugged tubes containing Durhams' tubes. All the carbohydrates were added to this sterile basal medium to the final concentration of 0.1% (w/v). The medium was dispensed into the sterile tubes aseptically and was autoclaved at 10 lbs for 10 minutes.

The tubes were inoculated with a needle and incubated at 27^0 C for 3 days and the results recorded.

The production of acid induced a change in the phenol red indicator, which changed from pink to yellow under acidic condition; and gas production was indicated by gas bubbles trapped in the Durham's tubes.

The following twenty eight carbohydrates (sugars and sugar alcohols) were used for testing the production of acid or acid and gas production.

1. Glucose
2. Fructose
3. Sucrose
4. Lactose
5. Maltose
6. Ribose
7 Xylose
8. Raffinose
9. Melibiose
10. Rhamnose
11. Trehalose
12. Cellobiose
13. Galactose
14. Mannose
15. Arabinose
16. Glycerol
17 Mannitol
18. Salicine
19 Adonitol
20. Inositol
21 Sorbitol
22. Starch
23 Glycogen
24. Dulcitol
25. Inulin
26. Butanol
27 Ethanol
28. Dextrin

2.2.6 Physiological characters (West and Colwell, 1984).

2.2.6.1 pH tolerance

The ability of the test organisms to grow at various pH conditions were demonstrated using nutrient broth supplemented with 2% (w/v) NaCl. Nutrient broth at pH 5.0, 6.0, 9.0, 10 and 12 were used for the study

The media were sterilized at 15 lbs for 15 minutes in test tubes. The test organisms were inoculated from a 24 hour old culture. Incubated at 27^0C for 24 – 48 hours. Growth was indicated by turbidity in the medium.

2.2.6.2 Temperature tolerance

The ability of the test organisms to grow at various temperatures were demonstrated. Nutrient broth with 2% (w/v) NaCl and pH 7.0± 0.3 was used. The test organisms were inoculated from a 24 hour culture and the inoculated tubes were incubated at 4, 28, 37, 45 and at 60^0C for 24-72 hours. Growth was indicated by turbidity in the medium.

2.2.6.3 NaCl tolerance

Growth at different concentrations of NaCl up to15% (w/v) was tested by observing growth in 1% tryptone broth at pH 7.0±0.3 containing varying amounts of analytical grade NaCl. The medium containing 0, 0.5, 1.0, 2.0, 5.0, 7.5, 10 and 15% NaCl were dispensed in 3mL aliquots into tubes. These broth media in culture tubes were sterilized at 15 lbs for 15 minutes and inoculated with a 24 hour culture. Growth was detected visually by observing turbidity.

The ability to grow in the absence of sodium chloride is an important preliminary diagnostic feature for the vibrionaceae members (West and Colwell, 1984).

2.2.6.4 Heavy metal tolerance

The ability of the test organisms to grow in the presence of metals such as cadmium (Cd^{+2}), chromium (Cr^{+3}), cobalt (Co^{+2}), iron (Fe^{+2}), lead (Pb^{+2}), molybdenum (MoO_4^{-2}) at 100mg/L and mercury (Hg^{+2}) at 10 mg/L, was examined in a basal medium containing 1% tryptone, pH 7.0±0.3, supplemented with 2% (w/v) NaCl (Austin *et al.*, 1977).

The tubes containing the various heavy metals were inoculated with a 24 culture and incubated at 27^0C for 24-48 hours. In all cases turbidity was considered as the manifestation of growth.

2.2.6.5 Growth in the presence of various dyes

To check the growth of the test organisms in the presence of dyes such as brilliant green, crystal violet, pyronin G and neutral red at a concentration of 0.001% (w/v), the organisms were streaked on 1% tryptone agar slants (pH 7.0±0.3), containing 2% NaCl (w/v) and incorporated with the respective dyes. The tubes were incubated at 27^{0}C for 24-48 hours and observed for growth (Austin *et al.*, 1977)..

2.2.6.6 Requirement of Mg^{+2}, K^+ and Na^{+2} for growth

In order to check the requirements of the elements such as magnesium, potassium and sodium, the test organisms were grown in a mineral medium **(2.2.4)** devoid of the respective element. Incubation was for 3-7 days at 27^{0}C.

Organisms that did not require Mg^{+2}, K^- or Na^- for growth, grew even in the medium devoid of the respective elements. Controls included the test organisms inoculated in the complete mineral medium **(2.2.4).** The organisms which had a requirement of the these elements did not grow in the mineral medium devoid of the respective element.

2.2.7 Fluorescent pigment production

The medium of King *et al.*, (1954) was used for demonstrating the ability of the test organisms to produce the fluorescent pigments, fluorescein and pyocyanin The medium of King, Ward and Raney(1954) contains per litre:

Peptone	20g
Glycerol	10g
Sodium sulphate	10g
KCl	20g
$MgCl_2.6H_2O$	1.4g
Agar	20g

pH 7.2

The medium B for fluorescein contains per .re:

Peptone	20g
Glycerol	10g
Sodium sulphate	10g
KCl	20g
NaCl	17g
$MgSO_4$. $6H_2O$	1.5g
Agar	20g
pH 7.2	

Prepared medium A and B as above. ieated to dissolve the solids and poured into tubes in 4 mL aliquots to get a generou. butt, autoclaved at 15 lbs for 15 minutes and converted to slants.

Inoculated medium A and B for fluorescein and pyocyanin production, respectively, incubated at 27^0C for 7 days d observed under UV light. Pyocyanin production was indicated by greenish fluor cence and fluorescein production was indicated by yellow fluorescence.

2.2.8 Production of levan and glucan

Glucan production typical of *Strepto. ccus sanguis* and *Streptococcus mutans* results in adherent growth on the agar which n be highly refractile, or white and dry (Starr, 1981).

Levan production typical of *Strep. coccus salinivarius* results in opaque, gummy non-adherent growth.

Levan production was tested in sucrose agar medium containing per litre:

Heart infusion	40g
Sucrose	50g
Agar	20g

Sterilized the medium at 121^0 C for 15 minutes, cooled to 50^0 C and poured into plates.

Glucan production was tested in Sucrose broth containing per litre:

Solution A

NIH thio broth	28.5g
K_2HPO_4	10g
Sodium acetate	12g
Distilled water	500mL

Solution B

Sucrose	50g
Distilled water	500mL

Solutions A and B were sterilized separately at 121^0 C for 15 minutes, cooled to 55^0C, mixed the solutions and dispensed in sterile16 x 125mm screw-capped tubes in 5mL aliquots. Inoculated and incubated for 7days at 27°C.

Glucan production was indicated when the broth was partially or completely gelled. It was also indicated when gelatinous adherent deposits formed on the bottom and walls of the culture tubes. Negative reactions were recorded when no gelling, deposit or increase in viscosity occured.

2.2.9 Antibiotic sensitivity

Sensitivity to the antibiotics were tested on nutrient agar plates supplemented with 2% (w/v) NaCl by the standard disc diffusion method. The plates were swab inoculated with the test organisms. Ready made discs from HiMedia Laboratories, Bombay, impregnated with the antibiotics were placed on the swabbed plates. Sensitivity to the antibiotics resulted in the production of clearing zones of no growth around the antibiotic discs. The plates were incubated at 28 ± 0.4^{0}C for 24 hours. The antibiotics tested were Penicillin G, Streptomycin, Oxytetracycline, Ampicillin, Tetracycline, Gentamycin, Polymyxin B, Chlortetracycline, Neomycin, Methamine mandelate, Novobiocin, Cefazolin, Amoxycillin, Nalidixic acid, Chloramphenicol, Erythromycin, Kanamycin, Bacitracin, Lincomycin and Sulfadiazine. The diameters of the zones of inhibition were also measured and the results were recorded as follows:

Diameter of restricted halo < 11mm: resistant
Diameter of restricted halo 11-20mm: moderately sensitive
Diameter of restricted halo > 20mm sensitive

2.2.10 Colonial and cellular morphology

2.2.10a Colony morphology

Colony morphology was examined after 3 days according to the criteria described by Colwell (1970). The test organisms were streaked on nutrient agar plates and incubated at 27^{0}C for 3 days. The characters recorded for each test organism included the presence of colorless, white or brown colonies; shining, opaque, transparent or translucent colonies; round, irregular and spreading colonies; entire, crenated, lobate and irregular edged colonies; flat and convex colonies and butyrous colonies.

2.2.10b Cellular dimensions (length of the organisms)

The dimensions of the test organisms were measured and recorded in micrometers using a calibrated micrometer eye piece (Erma Inc. Japan)

2.2.11 Coding of data and Numerical Taxonomical analysis

The data obtained were coded as follows: 1 for positive, 0 for negative and 9 for tests not done. The coded data were then processed by the program TAXAN, using un-weighted average linkage and Simple Matching Coefficient (Ssm) based on which sorted similarity matrices and dendrograms were constructed (Ssm; Sokal and Michener, 1958)

2.2.12 Determination of deoxyribonucleic acid base composition (mole % G + C ratio)

Bacterial isolates

From the dendrograms which were constructed, for the four groups of cultures such as **(1)** the isolates from the moribund larvae sensitive to O/129, **(2)** the isolates from the moribund larvae resistant to O/129, **(3)** the isolates from the apparently healthy larval system sensitive to O/129, **(4)** the isolates from the apparently healthy larval system resistant to O/129; one representative strain each was segregated from the base of each cluster. Wherever single cultures remained, without being clustered , they were also selected for further study. Altogether 47 representative strains were segregated for the determination of mole % G +C ratio along with 20 type strains of the family Vibrionaceae.

Method

For determining the mole percent guanine + cytosine the method using denaturation temperature (Tm) was followed (Marmur and Doty, 1961).

2.2.12a Isolation of DNA

For the isolation of DNA from the bacterial cultures, Marmur's (1961) original method modified by Hada et al., (1984) was used.

1) The cultures were grown in 500mL nutrient broth (peptone 0.5%, yeast extract 0.1%, beef extract 0.5%, pH 7.3± 0.2) containing 1.5% NaCl, for 48-72 hours to get good turbidity
2) The cultures were harvested by centrifugation (6000 x g; 15mins, 5°C)
3) The cells were washed thrice in a solution containing 0.1M NaCl, 0.05M ethylene diamine tetra acetate (pH 8.1) (TES)
4) The cells were resuspended in 25mL TES and lysis was effected by the addition of 6 mg SDS/mL (sodium dodecyl sulfate) and the mixture was placed in a 60°C water bath for 10minutes and then cooled to room temperature (R.T). Subsequently, 10mg lysozyme was added to the cell-suspension and incubated at 37° C with occasional shaking for 60 minutes.
5) 0.2 volume of TES-saturated distilled phenol was added and the mixture shaken for 30 minutes on a vortex mixer.
6) The whole mixture was shaken with an equal volume of chloroform-isoamyl alcohol mixture (24:1) in a ground glass stoppered flask for 30 minutes. The resulting emulsion was separated in 3 layers by a 15 minute centrifugation at 6000 x g.
7) The upper aqueous layer contained the nucleic acid and was carefuly pipetted out into a tube or narrow flask.
8) 2 volumes of cold (-20°C) 95% ethanol was added to the collected aqueous layer.
9) The nucleic acid was precipitated and was gently spooled onto glass rods. This is the first precipitation.
10) The spooled nucleic acid was re-dissolved in 0.1 x SSC. The mixture was shaken with an equal volume of chloroform-isoamyl alcohol mixture for 15 minutes and centrifuged at 6000 x g for 15 minutes and the aqueous layer removed. This process was repeated until no protein was seen at the interphase.

11) The supernatant obtained after the last step in the series of deproteinization, was precipitated with 2 volumes of cold (-20°C) 95% ethanol and the DNA spooled onto glass rod. This is the second precipitation. This DNA was then re-dissolved in 1-2mL 0.1 x SSC.

12) To this solution pancreatic ribonuclease (Sigma chemicals) was added to a concentration of 50 µg/ mL and incubated at 37°C for 30 minutes

13) The digest was again subjected to a series of deproteinization (at least 3) just like in the step-10.

14) The supernatant after the last such treatment was again precipitated with 2 volumes of 95% ethanol and the nucleic acid spooled off and dissolved in 9mL of 0.1 x SSC.

15) To this 1.0mL of 2.2M Na-acetate-EDTA (0.01M) mixture (pH 7.2) was added. While the solution was being stirred, 0.6 volume of isopropyl alcohol (-20°C) was added drop-wise into the vortex. The DNA was spooled with a glass rod.

16) If the yield was good, the DNA was re-dissolved in 9 mL 0.1 x SSC and re-precipitated as described in step-15.

17) The final precipitate was washed free of the acetate and salts by gently stirring the adhered precipitate in progressively increasing 70-95 % ethanol.

18) The spooled DNA was dissolved in small volume of 0.1 x SSC (2-3mL) or 1 x SSC.

2.2.12b Preparation of reagents for DNA isolation

1) TES (pH 8.1)

0.15 M NaCl	(8.766g in 1.0L D.W)
0.05 M Tris	(6.057g in 1.0L D.W)
0.05 M EDTA	(18.612g in 1.0L D.W)

Phenol (TES saturated)

Removed re-distilled phenol from the freezer and allowed to warm to room temperature and further to 60°C in a water bath to melt it. (The phenol was colourless and discarded when colored)

Added an equal volume of 1M Tris HCl (pH 8.0) to saturate the phenol (1.1), mixed and removed the aqueous phase. The phenol was extracted with tris HCl until the pH of the aqueous phase was greater than 7 Aliquoted 1.0mL of the saturated phenol into microfuge tubes. Added 0.3 mL TES to each tube. Mixed by inverting and stored in a brown bottle at –20°C until use.

2) Chloroform-isoamyl alcohol (24:1)

Added 120 mL of chloroform and 5 mL isoamyl alcohol to a brown bottle. Mixed and stored at room temperature.

3) 1 x SSC (pH 7.0)

0.15 M NaCl	(8.766g in 1.0L D.W)
0.015 M sodium citrate	(4.412g in 1.0L D.W)

4) Sodium acetate-EDTA solution (pH 7.0)

2.2 M sodium acetate	(300.696g in 1.0L D.W)
0.01 M EDTA	(37.224g in 1.0L D.W)

6) Treated pancreatic ribonuclease

0.2% w/v RNase was prepared in 0.15 M NaCl, pH5.0. The solution was heated at 80°C for 10 minutes to inactivate any contaminating DNase. From this, Rnase was added to get a final concentration of 50 μg /ml.

2.2.12c Purity of DNA

The purity of each DNA preparation was determined spectro-photometrically (Hada et al., 1984). Values of 2.0 for the ratios of optical density at 260nm to optical density at 230nm; and 1.75-1.85 for the ratio of optical density at 260nm to optical density at 280nm, were used to indicate lack of protein and ribonucleic acid contamination respectively.

$$\frac{\text{O.D at 260nm}}{\text{O.D at 230nm}} = 2.0 \text{ (lack of protein)}$$

$$\frac{\text{O.D at 260nm}}{\text{O.D at 280nm}} = 1.75\text{-}1.85 \text{ (lack of ribonucleic acid)}$$

2.2.12d Determination of mole % guanine + cytosine

The mean G + C content of the DNA was calculated by estimating the midpoint of the optical curves. The melting temperature was determined by establishing an absorbance-temperature profile (80-100°C) at 260 nm with a Shimatzu UV-visible spectrophotometer connected to a water bath. The temperature of the circulating water was increased mechanically to raise the temperature of the cuvet which held the DNA preparation at a rate of 0.5°C every five minutes, until it was thermally degraded- that is until it had completed the configurational transition from double helix to single stranded coil.

Mathematical determination by the least squares linear regression method was also used to determine the Tm value from the temperature and absorbancy values of the melting curve. (Moore, 1977). Finally the % GC ratio was determined from the thermal melting point by the equation

$$\% \text{ G + C} = \frac{\text{Tm-69.3}}{0.41}$$

The above relationship is valid when the Tm is expressed in centigrades and when the DNA is suspended in solvent containing 0.2 M Na^+ for the determination of the thermal melting profile.

The method was standardized, by running a few standard strains and E. coli ATCC 11775 as listed in Table 2-2, along with the representative strains. A comparative account of the values of % G + C obtained for the type strains in the laboratory and their accepted values are as given in the table 2-2.

2.3 Results and discussion

Halophilic bacteria already isolated from the larval rearing system of *Macrobrachium rosenbergii* and maintained in the laboratory were studied. From this collection, cultures that were associated with moribund larvae and apparently healthy larvae were subjected to numerical taxonomy

2.3.1 Moribund larvae

Out of 557 isolates obtained from the moribund larvae, 449 were found to be viable. These strains were then purified by repeated streaking on nutrient agar plates and confirmed purity by microscopic examination by Gram staining. From the 449, 204 isolates were segregated as belonging to the family Vibrionaceae based on the following characters: Gram negative rods/cocci, fermentative with or without gas production, Kovac's oxidase positive and motile/non-motile (West and Colwell, 1984). These 204 isolates were further divided into two groups based on their sensitivity to O/129 (vibriostat) compound at a concentration of 150μg/mL (Baumann and Schubert, 1984; Baumann *et al.*, 1984, Bain and Shewan, 1968). Thus out of 204 strains, 140 were O/129 negative and 64 O/129 positive.

2.3.1.a Numerical taxonomy of the strains associated with moribund larvae which were O/129 negative

The group of 140 isolates from moribund larvae grouped under the genus *Aeromonas* following Baumann *et al.*(1984), Popoff(1984) and Bain and Shewan, (1968) were tested for 233 unit characters (section 2.2; table-2-3) and were recovered after the taxonomic analysis using TAXAN in 13 clusters or phena defined at 79-83 % similarity (%S), using simple matching coefficient (Ssm) and unweighted average linkage. Phena 1-13 contained 8, 7, 3, 25, 11, 10, 25, 14, 8, 7, 7, 3 and 4 isolates respectively (Fig 2-1 and 2-2). There were 8 isolates (in this group of 140) which were found not grouping with any cluster. Interestingly, the standard strains of *Aeromonas*, such as *Aeromonas hydrophila* (ATCC 7966), *A. caviae*(ATCC 15468), *A.salmonicida* subsp. *masoucida* (ATCC 27013), incorporated with this group were

not picked up by any of the 13 phena formed, but themselves clustered together Consequently, identification of the clusters turned out to be a difficult process.

Phenon-1: Moribund larvae: O/129 negative: OTUs=8

Phenon-1 of moribund larvae contaning 8 strains was clustered at 85 %S. It produces catalase, gelatinase, lipase, caseinase, acetoin from glucose (Voges Proskauer), reduce nitrate; produce acid from glucose, mannitol, trehalose, mannose, cellobiose, glycerol and dextrin; can grow without NaCl(except one strain) and also in the presence of 7.5% NaCl; preferred a pH range of 8-10 and temperatures from 28-45°C; can tolerate heavy metals like Cd^{-2}, Fe^{+2}, Pb^{+2} and MoO_4^{-2} and can grow even in the presence of various dyes such as brilliant green, crystal violet, pyronin G and neutral red; utilize ribose, sucrose, maltose, mannitol, tyrosine and trehalose as sole source of carcbon; and are sensitive to the antibiotics such as oxytetracycline, streptomycin, tetracyclin, neomycin, polymyxin b, chlortetracycline, amoxycillin, nalidixic acid, novobiocin, chloramphenicol and kanamycin (Table 2-3).

The representative strain **AAC 1104a**, selected from this phenon has the mole % G +C content of 53.5 which is neither in the range for vibrios(39-51) nor that of the aeromonads (59-63), but some where in between. This range is quite similar to the one reported for group E-3 (Baumann *et al.*, 1971,1984) which is 54%. But phenon-1 differs from the group E-3 by being positive for chitinase, hemolysis on blood agar and utilization of xylose, galactose, salicine, and citrulline but agrees on the other characters.

Based on the resistance to O/129 alone it is quite inappropriate to group the phenon-1 under *Aeromonas* as the % G+C content is much below the accepted range for the aeromonads (59-63). Several vibrios have been found to develop spontaneous resistance to O/129 as have been reported earlier (Karunasagar et al., 1987; Ramamurthy et al., 1994; Sundaram and Murthy, 1983; Esteve, 1995). However the phenon shares several characters with both aeromonads and vibrios, but their ability to grow at higher concentrations of NaCl (7.5%) coupled with the closeness of their

%G+C to the vibrios, draws the phenon closer to the vibrios than to the aeromonads. But it has to be remembered that except one strain all the other 7 strains exhibited the ability to grow without NaCl. a property shared by the aeromonads. This phenon having mole % G+C as 53.5 and having the following as characters: resistance to O/129, motile, ability to grow at 0% and 7.5% NaCl, and inability to produce gas from glucose remains to be identified or to be given the status of a new species. Baumann et al.,(1984) suggested that the extension of the range of %G+C ratio of the vibrios was essential to include these strains, with a % G+C of 54. In this way the present phenon can very well be brought under the genus *Vibrio* and can be assigned a new species status after DNA homology studies.

Phenon-2: Moribund larvae: O/129 negative: OTUs=7

This phenon contains 7 strains clustered at 83%S. Characteristics of phenon-2 are as listed in Table 2-3. All strains are positive for catalase, oxidase, all are motile; fail to produce lysine decarboxylase and arginine dihydrolase, gelatinase, lipase, amylase, chitinase lecithinase, cellulase and none are haemolytic. They utilize ribose, mannose, fructose, sucrose, maltose, pyruvate, lactate, adonitol, tyrosine, proline, serine, tryptophan, m-inositol, mannitol, acetate and glycerol as sole carbon and energy sources; produced acid from glucose, fructose, ribose, galactose, mannitol, adonitol m-inositol and mannose; can grow in a pH range from 5-10, temperatures from 28-45°C, in medium with 0-7.5% NaCl and are resistant to penicillin G, ampicillin, novobiocin, bacitracin, lincomycin and sulphadiazine.

The mole %GC of the representative strain **(AAC1142b)** from this phenon is 39.8 which is within the range of that of *V.fischeri* (39-41) (Baumann *et al.*, 1971a 1984). However, phenon –2 differs from *V.fischeri* in the following traits: lack of bioluminescence, cell associated yellow-orange pigment and their ability to utilize gluconate, lactate, sucrose and pyruvate. But on the other hand, phenon-2 shares several other characteristics with *V fischeri* apart from the mole %G+C, such as: inability to produce arginine dihydrolase, ability to produce chitinase, utilization of cellobiose and citrate, growth in the presence of 6% NaCl, at 35°C but not at 4°C,

production of gelatinase, amylase, acid from sucrose and ornithine decarboxylase (Baumann *et al.*, 1971, 1984).

Thus the phenon-2 has been designated as *V. fischeri* in spite of a few phenotypic dissimilarities. Precisely, the strains under this phenon may be phenotypic variants of the species.

Phenon –3: Moribund larvae: O/129 negative: OTUs=3

Phenon-3 comprises 3 strains isolated from moribund larvae and which have clustered at 84 % S. They are motile, produce catalase, acetoin, arginine dihydrolase, indole. Reduce nitrate, utilize ribose, xylose, trehalose, cellobiose, galactose, fructose, sucrose, maltose, glycogen, sorbitol, pyruvate, lactate, tyrosine, proline, serine, gluconate, mannitol, glycogen and salicine as sole source of carbon; are unable to utilize melibiose, rhamnose, raffinose, glucuronate, lactose, adonitol, cystine, uracil, tryptophan, valine, malonate, m-inositol, and citrulline. Characteristics of phenon-3 are summarized in Table 2-3. Produces acid from glucose, fructose, sucrose, maltose, trehalose, mannitol, glycerol, salicine, starch, dextrin and glycogen. They also produce gelatinase, lipase, caseinase, amylase, chitinase, lecithinase, hydrolyse aesculin and do not produce DNase, or hemolysin; are able to grow between a pH range of 8-10, in the presence of 0-7.5% NaCl and temperatures between 28-45°C are preferred. Do not grow at 4 and 60°C, do not produce glucan or levan and are resistant to penicillin G, ampicillin, cefazolin and amoxycillin.

The mole % G+C of the selected representative strain (**AAC 1101b**), from phenon-3 is 41%, which is within the range of that of *Photobacterium* (40-44), *Vibrio logei* (40-42), *V. fischerii* (39-41), *V. marinus* (40-42) (Baumann and Baumann, 1984; Bauamnn *et al.*, 1971a 1984).

Phenon-3 is distinguished from *V fischerii* by a combination of properties. *V. fischerii* is negative for growth at 0% NaCl and fails to produce gelatinase and are bioluminesent. Besides *V. fischerii* has a yellow-orange cell associated pigment and

can grow only up to 6% NaCl (Baumann *et al.*, 1984). Phenon-3 on the other hand grows at 0 % and higher concentrations of NaCl (7.5%) and a few strains even up to 15%and thus does not appear to be *V. fischerii* even though they have G +C ratios within the same range.

The following combination of properties distinguishes phenon-3 from *V. logei* (40-42%). *V. logei* like *V.fischerii* are luminescent species , have a cell associated yellow orange pigment, are negative for growth at 0% NaCl, shows good growth at 4°C, but not at 30°C; and does not produce gelatinase but can utilizes lactate and pyruvate (Baumann *et al.*, 1984).

Other marine luminous organisms have been described with the DNA base compositions similar to that of phenon-3. *V. marinus*, a facultatively anaerobic organism of marine origin, which ferments glucose with the production of acid but without gas, has a mole % G+C of 40-42 % (Colwell and Mandel, 1964). However, these organisms are different from phenon-3 since they are ornithine decarboxylase and arginine dihydrolase negative, and are unable to grow at 0 and 6% NaCl and the strains show variability in their ability to produce gelatinase and are negative for gluconate utilization.

Phenon-3 disagrees with *P. phosphoreum* (% G+C ratio,41-42),also on a number of characters as the phenon is non- luminescent, fail to produce gas from glucose, do not grow at 4°C but shows good growth at 35°C; produces gelatinase, lipase, amylase and utilizes xylose, sucrose, pyruvate, proline and trehalose. Considering these dissimilarities, the phenon-3 could not be identified as *P. phosphoreum* even though the mole %G+C ratio matches very well with the latter.

However, phenon-3 shows very good resemblance to *P.angustum*(%G+C ratio,40-42) as it agrees on a number of traits for this species. Both are non-luminescent, do not produce gas from glucose, produce chitinase, lipase and gelatinase; utilize xylose, maltose, acetate, pyruvate, glucose, mannose, galactose, fructose, glycerol, ribose, gluconate, lactate, aspartate, serine, sucrose and trehalose

and fail to utilize glucuronate. Meanwhile the phenotypic differences of phenon-3 from *P.angustum* are limited to the utilization of proline, growth at 45°C and acetoin from glucose (Baumann and Baumann, 1984). Weighing the traits of phenon-3 which are in agreement and disagreement with *P.angustum* and also considering the similarity of G +C ratios, phenon-3 is designated as *P.angustum*; to be further confirmed by DNA homology studies.

Phenon-4: Moribund larvae: O/129 negative: OTUs=25

Phenon-4 comprises of 25 strains compactly clustered at 86% S. The phenotypic characters are as summarized in Table 2-3 They are oxidase positive, motile, positive for catalase, urease, lipase, caseinase, lecithinase; and negative for lysine and ornithine decarboxylase, arginine dihydrolase, amylase, chitinase, DNase and production of haemolysin; utilize sucrose, lactate, glycerol and glutamic acid as sole sources of carbon and do not utilize lactose, uracil, salicine and valine. All strains produce acid from glucose, sucrose, maltose, ribose and xylose; can grow in the pH range of 8-10, temperatures between 28-45°C, in the presence of 0-7.5% NaCl and are resistant to penicillin G, ampicillin and amoxycillin.

The mole %G+C ratio of the representative strain (**AAC639b$_1$**), from phenon-4 is 53.9, which did not fall within the range of either that of the vibrios (39-51) or the aeromonads (59-63) (Baumann and Schubert, 1984). This value is very much close to the G+C ratio of group E-3 described by Baumann *et al.* (1971), which has the mole %G+C content of 54 %. This range is intermediate between the ranges for vibrios and aeromonads, nevertheless much closer to the range for the vibrios. As suggested by Baumann *et al.*, (1984), a formal extension of the range of %G+C ratio of vibrios to 54% is essential to accommodate such species. Even though there are several characters that are in agreement with both vibrios and aeromonads (Baumann *et al.*,1984; Popoff, 1984; Sakazaki and Balows, 1981, Esteve,1995), all strains of phenon-4 are able to grow in the presence of 0 and 7.5% NaCl, a character typical of marine vibrios.

On comparing the phenotypic characters of this cluster with that of the already described species of *Vibrio*, the phenon is found to be in agreement with *V. natreigens* and *V. navarrensis* (Baumann *et al.*, 1984; Urdaci *et al.*, 1991, Ishimaru *et al.*, 1995) in several characters, but are very much distended in the %G+C ratio. Therefore it is appropriate to propose a new species status for the phenon-4 under the genera *Vibrio* taking into account their resistance to the O/129 compound, a property now widely accepted for several vibrios (Colwell, 1998, personal communication). This has to be confirmed by examining the dissimilarities of the phenon with the existing species of *Vibrio* at genomic level especially by DNA/DNA hybridization studies.

Phenon-5: Moribund larvae: O/129 negative: OTUs :11

Phenon-5 contains 11 stains clustered at 81.9 %S. Their phenotypic characters are listed in Table 2-3. Phenon –5 produces catalase, acetoin, reduces nitrate, produces indole, gelatinase, lipase, amylase, caseinase and lecithinase and are negative for DNase. The strains utilize lactate and tyrosine as sole carbon and energy sources and fail to utilize lactose, dulcitol, uracil, valine, malonate and formate. They produce acid from glucose, fructose and trehalose but fail to do so from lactose, xylose, raffinose, melibiose, rhamnose, adonitol, inositol, sorbitol, starch, inulin, butanol and ethanol; do not grow at pH 5 but show good growth from pH8-10, at temperatures ranging from 28-45°C and in the presence of 0-7.5% NaCl; but not at 4 and 60°C.

The mole %G+C content of the representative strain,(**AAC 1114b**) of this phenon, is found to be 41, which is within the range of that of *V. fischerii* (39-41), *V. logei* (40-42), *V. marinus* (40-42), *P.phosphoreum* (41-42) and *P. angustum* (40-42) (Baumann *et al.*, 1971a, 1984; Baumann and Baumann, 1984). But phenotypically phenon –5 exhibits remarkable variations with *V. fischerii*, *V. logei* and *P. phosphoreum* in several of their core characters which include the absence of biolumunescence, inability to grow at 4°C and its capability to grow in the absence of NaCl. The phenon differs from *V. marinus* also as it is ornithine decarboxylase and

arginine dihydrolase positive and do not require organic growth factors, grow at 35°C and at 6 % NaCl.

However, among the genera to which the phenon shows similarity at G+C ratio level, *P.angustum* exhibit much phenotypic agreement as both groups are non-luminescent, not pigmented, do not produce gas from glucose, produce gelatinase, lipase, chitinase and use a variety of sugars as sole carbon and energy sources.

Based on these observations the phenon-5 has been assigned specificity as *Photobacterium angustum*-like organisms which has to be further confirmed by DNA homology studies.

Phenon-6: Moribund larvae: O/129 negative: OTUs:10

This phenon has been formed by clustering 10 cultures at 83 % S which produce catalase, indole, reduce nitrate, utilize ribose, galactose and mannose as sole source of carbon, produce acid from glucose and dextrin, and grow without NaCl and can grow in the presence of 5% NaCl; prefer a temperature range of 28-45°C and a pH of 8-12; tolerate heavy metals like Cd^{+2}, Fe^{+2}, Pb^{+2} ,MoO_4^{-2} and Hg^{+2} and can grow in the presence of various dyes such as brilliant green, crystal violet, pyronine G and neutral red; and are sensitive to antibiotics such as streptomycin, tetracyclin, gentamycin, chlortetracyclin, neomycin, methamine mandelate, nalidixic acid and novobiocin (Table 2-3).

The representative strain (**AAC 1109b**), selected from the phenon has the %G+C ratio of 39.6 which is very much within the range of the G+C of *V fischeri* (39-41). However, phenon-6 differs from *V. fischeri* in several phenotypic characters as they are not bioluminescent, do not have a yellow-orange cell associated pigment and do not grow at 4°C unlike *V. fischerii*. Above all they are resistant to the vibriostat compound.

Based on these observations, phenon-6 has been differentiated from *V. fischeri* and is therefore assigned a new species status within the vibrios, which has to be further confirmed by considering the DNA homology.

Phenon-7: Moribund larvae: O/129 negative: OTUs :25

This phenon contains 25 strains which have established themselves into a compact cluster at 84%S. All strains are resistant to O/129 and produce indole, and utilize ribose, fructose, pyruvate and glutamic acid as sole source of carbon and energy. They produce acid from glucose, fructose and dextrin without gas, degrade trybutyrin do not haemolyse human blood. A pH range of 8-10 is preferred. Excect 6 strains all others require at least 0.5% NaCl in the medium for growth and can grow in the presence of up to 2% NaCl indicating slightly halophilic nature of the strains. They prefer a temperature range of 28-45°C but do not grow below 5°C. The strains in this phenon are capable of growth in the presence of brilliant green, crystal violet, pyronine G and neutral red; and are sensitive to antibiotics such as tetracyclin, gentamycin, chlortetracyclin, polymyxin B, neomycin, methamine mandelate, nalidixic acid, chloramphenicol, kanamycin and novobiocin (Table 2-3).

The mole % G+C ratio of the representative strain from this phenon **(API 684a)** , is 50.5 which is close to the range for *V costicola* (50), *V. fluvialis* (49-51), *V. furnissi* (50), *V. hollisae* (50) and *V. proteolyticus*(51) (Baumann *et al.*, 1984; Hickman *et al.*, 1982).

Phenon-7 differs from *V. costicola* by producing gelatinase, lipase, and by not utilizing sucrose and tyrosine as the sole source of carbon, and by reducing nitrate. Phenon-7 also differs by being unable to grow in the presence of more than 5% NaCl.

V fluvialis differs from phenon-7 by the following traits: It produces arginine dihydrolase but not lysine or ornithine decarboxylase, grows at 6% NaCl, and can utilize glucuronate and arabinose as sole source of carbon; besides, *V. fluvialis* produces gas from glucose.

V. furnissi also differs from phenon –7 by the same characters as for *V. fluvialis* except that *V. furnissi* does not produce gas from glucose and does not utilize glucuronate; both latter traits being in agreement with phenon –7 (Baumann *et al.*, 1984).

V. hollisae also differs from phenon-7 by being negative for the following characteristics: arginine dihydrolase, lysine and ornithine decarboxylase, gelatinase, lipase, and acid from sucrose (Hickman *et al.*, 1982).

V. proteolyticus which also has the similar %G+C also differs from phenon-7 by producing arginine dihydrolase, lysine decarboxylase and by not producing acid from sucrose (Baumann *et al.*, 1984).

Thus, phenon-7 does not fully agree phenotypically with any of the species of *Vibrio* to which it shows similarity at G+C level. Before proposing the status of a new species to the phenon, its DNA homology to the species of *Vibrio* with which it agrees at the mole %G+C level, has to be worked out by DNA-DNA hybridization

Phenon-8: Moribund larvae: O/129 negative: OTUs :14

Phenon –8 comprises 8 strains associated with moribund larvae which clustered at 80%S. The strains in this phenon are catalase and indole producers and reduce nitrate. They utilize ribose, glycogen, and puruvate as sole sources of carbon and produce acid from glucose and trehalose without gas and fail to haemolyse human blood. They prefer a pH range of 8-12, and require at least 0.5% NaCl for growth in the medium and can also grow in the presence of 5% NaCl; can grow in temperatures ranging from 28-45°C but not below 5°C. They can tolerate heavy metals like Fe^{+2}, Pb^{+2}, MoO_4^{-2} and Hg^{+2}; can grow in the presence of various dyes such as brilliant green, crystal violet, pyronine G and neutral red; and are sensitive to antibiotics such as streptomycin, tetracyclin, gentamycin, chlortetracyclin, neomycin, chloramphenicol and kanamycin. The phenon is resistant to penicillin G and ampicillin (Table 2-3).

The mole % G+C ratio of the representative of this phenon (**AAC 1128a$_2$**) has been estimated to be 47.9 which is within the G+C range of that of *V. campbelli* (46-48), *V. cholerae* (47-49), *V. harveyi* (46-48) and *V. vulnificus* (46-48).

On comparing the phenotypic characters of phenon-8 with these four species of vibrios, several characters were found to be in agreement and also in disagreement with each other and there by specificity of the phenon could not be fixed at this stage. Whether the phenon belongs to any of the four species of vibrios with which it has matching mole %GC ratio or is an entirely new species can be ascertained only after DNA homology studies.

Phenon-9: Moribund larvae: O/129 negative: OTUs :8

Phenon –9 comprises 8 strains clustered at 80 %S. The strains in this phenon are catalase producers, and utilize xylose, galactose, mannose, fructose, glycogen, glycerol and glutamic acid as sole sources of carbon and energy; they produce acid from glucose and maltose without gas, and are able to hydrolyse tween 40 and 60, prefer the pH range of 8-12, grow in the absence and in the presence of 5%NaCl, prefer temperature range of 28-45°C, but fail to grow at 4°C. They can tolerate and grow in the presence of heavy metals like Cd^{+2} , Fe^{+2}, Pb^{+2}, MoO_4^{-2} and Hg^{+2} , can grow in the presence of various dyes such as brilliant green, crystal violet, pyronine G and neutral red; and are sensitive to antibiotics such as streptomycin, tetracyclin, gentamycin, chlortetracyclin and neomycin.The phenon is resistant to oxytetracyclin, lincomycin, sulfadiazine and amoxycillin (Table 2-3).

The mole %G +C ratio of the representative strain (**AAC 1101a**), selected from this phenon has been estimated to be 40.7 This ratio falls with in the range of that of *V. fischeri* (39-41), *V. logei* (40-42), *V. marinus* (40-42) and *P. angustum* (40-42) (Baumann and Baumann, 1984; Baumann *et al.*, 1971a,1984).

However the phenon differs from *V. fischerii* and *V logei* in a few core characters such as not being bioluminescent, failing to produce a yellow –orange cell associated pigment and their inability to grow at 4°C.

Phenon –9 also differs from other marine luminescent organisms with similar DNA base compositions such as *V. marinus*. This organism differs from phenon-9 by being negative for arginine dihydrolase, ornithine decarboxylase, requires organic growth factors, cannot grow at 35°C and also in the presence of 6% NaCl, are negative for utilization of gluconate. The strains show variability in their ability to produce gelatinase which is quite agreeable with phenon-9

Meanwhile, the phenon is found to agree with most traits for *P. angustum* including its mole %G+C content and differs by being unable to grow at 4°C, failing to produce chitinase and by being able to grow at 40°C.

Based on these observations the phenon-9 has been designated as *Photobacterium angustum*-like organisms; to be confirmed by DNA homology studies.

Phenon-10: Moribund larvae: O/129 negative: OTUs: 7

Phenon-10 comprises 7 strains compactly clustered at 80 %S. The strains clustered in this phenon are non-motile, produce indole from tryptophan; utilize galactose, mannose, fructose, glycogen, sodium pyruvate, glycerol and glutamic acid as sole source of carbon and energy. They produce acid from glucose without gas, and are non haemolytic; grow between a pH range of 8-12, grow in the absence and in the presence of 5% NaCl. Prefer to grow between 28-45°C but fail to grow at 4°C. They can tolerate and grow in the presence of heavy metals like Cd^{+2} , Fe^{+2}, Pb^{+2}, MoO_4^{-2} and Hg^{+2} ; can grow in the presence of various dyes such as brilliant green, crystal violet, pyronine G and neutral red; are capable of growing in the absence of Na^{+2}, Mg^{+2} or K^{+} in broth. They are sensitive to antibiotics such as, tetracyclin,

gentamycin, chlortetracyclin and neomycin, methamine mandelate and kanamycin and are resistant to penicillin G, cefazolin, sulfadiazine and amoxycillin (Table 2-3).

The representative strain from this phenon (**AAC 880c**), has a mole %G+C ratio of 57.3% which is very well with in the range of that for *Aeromonas salmonicida* (57-59) (Popoff, 198; and is very much in agreement with the description given for *Aeromonas salmonicida* by Popoff (1984) and Sakazaki and Balows (1981) as they are non-motile, produce a water-soluble pigment, indole, hydrolyse aesculin and above all they are resistant to the O/129 compound. Based on these observations the phenon is placed under the species *Aeromonas salmonicida* to be confirmed by DNA homology

Phenon-11: Moribund larvae: O/129 negative: OTUs: 7

Phenon –11 comprises 7 strains associated with moribund larvae, clustered at 81%S. All strains produce catalase and utilize ribose, galactose, mannose, fructose, glycogen, pyruvate, lactate, glycerol, tyrosine, and glutamic acid as sole source of carbon and energy. They produce acid from glucose without gas, are unable to ferment a variety of other sugars and are not haemolytic on blood agar. They are able to grow in the pH range of 8-12, in the absence of NaCl and in the presence of 2%NaCl; fail to grow at 4°C and show good growth in the range of 28-45°C. They can tolerate and grow in the presence of heavy metals like Fe^{+2}, Pb^{+2}, MoO_4^{-2} and Hg^{+2} , can grow in the presence of various dyes such as brilliant green, crystal violet, pyronine G and neutral red and are capable of growing in the absence of Na^{+}, Mg^{+2} or K^{+}. They are all sensitive to antibiotics such as, tetracyclin, gentamycin, chlortetracyclin neomycin, methamine mandelate, nalidixic acid and kanamycin and are resistant to penicillin G, ampicillin, sulfadiazine and amoxycillin (Table 2-3).

The representative strain (**AAC 598c$_2$**) selected from phenon-11 has the mole %G+C content of 44.9%. This falls with in the range of *V. anguillarum* (44-46), *V. metschnikovii* (44-46), *V. ordalli* (44-46), *V. tubiashii* (43-45)(Baumann *et al.*, 1984, Hada *et al.*, 1984) and *V. ichthyoenterii* (43-45) (Ishimaru *et al.*, 1996)

But phenotypically there exists much disagreement between the phenon –11 and the five species of vibrios mentioned above, making it practically difficult to fix the identity of the phenon. Therefore it is proposed that the DNA homology of the phenon with the above species of vibrios have to be examined before ascribing the specificity to any of the already described species or before proposing a new species altogether.

Phenon-12: Moribund larvae: O/129 negative: OTUs: 3

Phenon-12 contains 3 strains clustered at 87 %S. They are positive for methyl red test and produce arginine dihydrolase, catalase, indole, caseinase, and cause α-hemolysis on blood agar; grow at a pH range of 8-12. Require al least 0.5% NaCl for growth and can grow only up to a concentration of 2% NaCl. Prefer a temperature range of 28-45°C but fail to grow at 4°C. They utilize galactose, mannose, glycogen, lactate, dulcitol and glutamic acid as sole sources of carbon and energy; produce acid from glucose, fructose, without gas production. They are able to tolerate and grow in the presence of heavy metals like Cr^{+3}, Fe^{+2}, and Hg^{+2} , can grow in the presence of various dyes such as brilliant green, crystal violet, pyronine G and neutral red and further they are capable of growing in the absence of Na^{+2}, Mg^{+2} or K^{+}. Except sulfadiazine they are resistant to all the antibiotics tested (Table 2-3).

The selected representative strain of phenon-12 (**ANM 718aa**) has a mole %G+C ratio of 44.4% and this falls with in the range of *V. anguillarum* (44-46), *V. metschnikovii* (44-46), *V. ordalli* (44-46), *V. tubiashii* (43-45)(Baumann *et al.*, 1984; Hada *et al.*, 1984), and *V. ichthyoenterii* (43-45) (Ishimaru *et al.*, 1996).

However on comparing the phenotypic characters of *V anguillarum*, *V. metschnikovii*, *V ordalli*, and *V. tubiashii* with that of phenon-12, profound disagreement is seen in several traits.

Meanwhile, the phenon exhibits a certain degree of agreement *with V. ichthyoenterii* in some of the phenotypic characters. The phenon-12 differs from *V. ichthyoenterii* by producing indole, arginine dihydrolase, by being positive for growth at 35°C and absence of growth in 6% NaCl, and by not producing acid from maltose and mannose. Nevertheless these characters are not sufficient enough to ascribe the phenon as *V. ichthyoenterii*.

Based on these observations it is proposed that the DNA homology of the phenon has to be worked out with the above five strains of V*ibrio* to fix the identity of this phenon.

Phenon-13: Moribund larvae: O/129 negative: OTUs :4

Phenon-13 comprises 4 strains clustered at 82 %S. They produce catalase and reduce nitrate but fail to produce indole, decarboxylate lysine and aspartic acid. They utilize ribose, xylose, raffinose, galactose, mannose, fructose, pyruvate, lactate, gluconate and glutamic acid as sole sources of carbon and energy; produce acid from glucose, maltose and trehalose without gas; prefer to grow at a pH range of 8-12, can grow in the presence of 0% and 5% NaCl. Prefer a temperature range of 28-45°C but fail to grow at 4°C. They are able to tolerate and grow in the presence of heavy metals like Fe^{+2}, Pb^{+2}, MoO_4^{-2} and Hg^{+2}, can grow in the presence of various dyes such as brilliant green, crystal violet, pyronine G and neutral red and further capable of growing in the absence of Na^{+2}, Mg^{+2} or K^{+}. All the strains are able to produce diffusible pigments (Table 2-3).

The mole %G +C ratio of the representative strain (**ANM 625**), selected from this phenon has been estimated to be 40.5. This ratio falls with in the range of that of *V. fischeri* (39-41), *V. logei* (40-42), *V. marinus* (40-42) and *P. angustum* (40-42) (Baumann and Baumann, 1984; Baumann *et al.*, 1971a 1984).

However *V. fischerii* differs from the phenon-13 by having a yellow –orange cell associated pigment, bioluminescence, inability to produce gelatinase and to utilize

D-gluconate, DL-lactate and pyruvate as sole sources of carbon and energy. In the same way *V logei* also differs from phenon-13 in all the characters mentioned above. Apart from that *V. logei* grows at 4°C and fails to grow at 30°C.

Phenon-13 shows remarkable variations in the traits reported for *V. marinus* also, as it fails to grow at 4°C, do not produce chitinase, utilize sucrose. trehalose and mannose as sole carbon and energy sources; characters which distinguish *V. marinus* from other species of vibrios (Baumann *et al.*, 1971a, 1984)

But the phenon-13 is found to show good agreement with *P. angustum* in several of the characters, except for certain traits such as the ability of the phenon to utilize proline and glucuronate as sole source of carbon and energy, being unable to grow at 4°C, but able to grow at 40°C, and to produce amylase.

Weighting the characters that are in agreement and disagreement with *P. angustum*, it is proposed to ascribe the identity of the phenon as *Photobacterium angustum* –like organisms which has to be further confirmed by DNA homology studies.

Phenon-14: Moribund larvae: O/129 negative: OTUs: 3

Phenon-14 includes 3 ATCC strains of *Aeromonas*, i.e. *A.caviae, A. hydrophila* and *A. salmonicida* subsp. *masoucida* (Fig.2-1 and 2-2), which clustered together at 83%S. These reference strains did not cluster with any of the other phena formed by the test organisms. Therefore phena 1-13 remained unidentified even after Numerical Taxonomy analysis. The characteristics tested for the reference strains are summarized in the Table 2-7

Strains from moribund larvae, O/129 negative, not clustered

Out of the 140 isolates subjected to the numerical taxonomy analysis, 7 remained without joining with any of the clusters formed. Phenotypic characters of these strains are as shown in Table 2-3.

Strain –1: AAC 672b$_1$

Strain AAC 672b$_1$ is linked to phenon-10 at 75%S. Strain AAC 672b$_1$ with the mole %G+C content of 59.4 has affiliation to *Aeromonas hydrophila* and *A. sobria* in terms of G+C content.

Among these two, the strain AAC 672b$_1$ shows great similarity to *A hydrophila* in terms of its phenotypic characters. It is motile, does not produce a brown water soluble pigment, grows at 37°C, produces indole and acetoin; acid and gas from glucose, utilizes a variety of sugars as sole source of carbon and energy, and can grow even in the absence of NaCl. But the strain differs from *A hydrophila* in its capacity to grow even in the presence of 10% NaCl, a trait universally not seen among *A. hydrophila* (Popoff, 1984).

Mean while the strain exhibits profound dissimilarities with *A. sobria* as it can utilize arabinose and salicine as sole source of carbon and energy, hydrolyse aesculin, and produce acid and gas from glucose; all characteristics to which *A. sobria* are negative.

Based on these observations the strain AAC 672b$_1$ has been placed under *A. hydrophila*, which might be another variant of this single species epithet. This has to be further confirmed by DNA homology studies.

Strain –2: AAC 669B

The strain AAC 669B is linked at 75 %S to phenon-10 and has 56.2 mole % G + C content, which is slightly lower than that of aeromonads and much higher than that of the vibrios. Therefore the strain must be subjected further to genotypic characterizations before grouping it with any of the known species /genus or elevating it to the status of a new species.

Strain –3: AAC 1108d

The strain AAC 1108d which is also linked at about 77% S to phenon-10, has a mole % G+C content of 46.5% which falls in the range of 13 species of vibrios (Table 2-7). On comparing the phenotypic characters of this strain with those of the known species, several characters were found to be in agreement and others disagreeing completely Therefore it is necessary to conduct more investigations into the genotypic relatedness with the species of vibrio, than to rely on the phenotypic characters to fix the identity of the strain.

Strain –4: API 644b

The strain API 644b is linked to phenon-10 at 72.5 % S. It has the %G+C content of 58.2, which falls within the range of *Aeromonas*, specifically that of *A. hydrophila* (58-62) and *A. sobria* (58-60). The strain API 644b agrees with motile *Aeromonas* in the following characters :oxidase positive, produce catalase, reduce nitrate to nitrite, decarboxylate lysine, hydrolyse starch, tween 80, casein and ferment trehalose, mannitol, fructose and dextrin. The strain API 644b is negative for phenyalanine deaminase, does not ferment dulcitol, rhamnose, inositol, raffinose, adonitol, cellobiose, lactose, sorbitol and glycerol like the motile aeromonads. But the strain differs from *Aeromonas* by being negative for gelatinase, DNase, lecithinase, arginine dihydrolase and being unable to grow in nutrient broth in the absence of NaCl. In addition, the strain is positive for lysine and ornithine decarboxylase and is able to grow in the presence of 7.5% NaCl, which is not observed in any of the motile aeromonads. Based on these phenotypic characteristics, the strain API 644b has been brought under the group motile aeromonads, especially to *A. hydrophila* and *A. sobria*. However the inability of the strain to grow in the absence of NaCl and its ability to grow in the presence of 7.5% NaCl opens up the possibility of expanding the base of the group of motile aeromonads, for accommodating such strains. Only DNA homology study holds the key to such differentiations.

Strain –5: AAC 654b

The strain AAC 654b is linked at 80%S with phenon-11 and has a mole % G+C ratio of 63.3 which is much closer to that of the motile aeromonads (61-63). Phenotypically this strain shows similarities in several traits and shows dissimilarity with almost the same number of characters with *A. caviae*. However, the inability of this strain to grow in the absence of NaCl, and its capability to grow in the presence of 7.5% NaCl makes the disagreements *with A. caviae* much wider. At this stage, it is inappropriate to designate the strain AAC 654b with any specificity, other than grouping it under the motile Aeromonads with close resemblance to *A.caviae*

Strain –6: AAC 536

The strain AAC 536, linked at 80 %S to phenon-12 has the mole %G+C ratio of 48.5 which falls within the range of that of *Vibrio cholerae* (47-49)(Baumann *et al.*, 1984). Phenotypically there are several traits of the strain AAC 536 which are in agreement with *V. cholerae*, and there are some others which differ Weighing all these, and by considering the closeness in the mole %GC content, the strain AAC 536 has been identified as *V cholerae*-like organism; may be a variant and which has to be confirmed by DNA homology studies.

Strain –7: ANM 1008a

The strain ANM 1008a is linked at 77 %S to phenon-11, has the mole %G+C content of 57.3 which is within the range of that of *A. salmonicida* (57-59)(Popoff, 1984). The strain is in close agreement with most of the traits characteristic of *A. salmonicida* subsp. *masoucida*. It is nonmotile, does not produce water-soluble pigment, produces indole, hydrolyses aesculin and ferments mannitol. However, the strain exhibits a gross dissimilarity with the above species by its ability to grow in the presence of 7.5% NaCl. Nevertheless, considering the %G+C content and the phenotypic characteristics, the strain ANM 1008a is designated as *A. salmonicida*-like organisms, to be confirmed by DNA homology studies

2.3.1b Numerical taxonomy of the strains associated with moribund larvae which were O/129 positive

There are 64 strains in this group isolated from the moribund larvae, all of which are O/129 sensitive. This group of 64 isolates along with the 16 standard strains of *Vibrio, Photobacterium* and *Plesiomonas* (Section 2. 2, Table2-1), were analyzed following the principles of numerical taxonomy. On completion of analysis they clustered into 3 phena and 5 isolates remained separately, unclustered. The phena 1, 2 and 3 have 17, 3 and 39 strains respectively (Fig. 2-3 and 2-4). The characteristics of all the 64 isolates are presented in Table 2-4.

In this analysis also the standard strains included in the study clustered separately into phena while some remained unclustered. This clustering together of the reference strains made the identification of the phena very difficult as none of the reference strains grouped with them. Hence identification was made based on the mole % G+C ratio of the representative strains along with the comparable phenotypic characters.

Phenon-1: Moribund larvae: O/129 positive: OTUs: 17

This phenon contains 17 strains, which formed a compact cluster at 85 %S. All the strains in this cluster produce catalase, lysine and ornithine decarboxylase, reduce nitrate, utilize galactose as the sole source of carbon, produce acid from glucose with out gas and are not haemolytic on blood agar. They prefer a pH range of 8-12, majority of them (15 out of 17 strains) can grow without NaCl, in the presence of up to 2% NaCl; prefer a temperature range of 28-45°C but fail to grow at 4°C. They tolerate and grow in the presence of heavy metals like Cd^{+2}, Fe^{+2}, Pb^{+2}, MoO_4^{-2} and Hg^{+2}; grow in the presence of various dyes like brilliant green, crystal violet, pyronine G and neutral red and further capable of growing in the absence of Na^{+}, Mg^{+2}, or K^{+} They are sensitive to streptomycin, tetracyclin, gentamycin, Chlortetrtacyclin, neomycin, methamine mandelate and nalidixic acid.

The representative strain of this phenon **(AAC 629b)** has a mole %G+C content of 43.5 which falls within the range of that of *V. aestuarianus* (43-45)(Tison and Seidler, 1983), *V. tubiashi* (43-45) (Hada et al., 1984), *V. ichthyoenterii* (43-45) (Ishimaru *et al.*, 1996) and *P. leiognathi*.(42-44)(Reichelt *et al.*, 1976).

But, phenon-1 differs from *P. leiognathi* in several traits. The strains in the phenon are non-bioluminescent, unable to grow at 4°C and are capable of growth at even 45°C. Besides, members of this phenon fail to produce H_2S, they are positive for lysine and ornithine decarboxylase and produce phenyalanine deaminase.

The phenon-1 exhibits phenotypic differences with *V. tubiashi* as well. Unlike *V. tubiashi*, the strains in this phenon are positive for lysine and ornithine decarboxylase, are unable to produce gelatinase, DNase and to utilize tyrosine as sole source of carbon and energy (Hada *et al.*, 1984).

In much the same way, members of this phenon register phenotypic differences with *V aestuarianus* by being negative for gelatinase, DNase and positive for lysine and ornithine decarboxylase. Further, unlike *V. aestuarianus* the phenon fails to produce acid from mannose, sorbitol, cellobiose and melibiose. But with regards to certain traits, phenon-1 agrees with *V. aestuarianus* by being negative for acid production from adonitol, arabinose, dulcitol, raffinose, rhamnose, salicine and xylose. Moreover, *V. aestuarianus* are easily distinguishable from pathogenic lactose-fermenting *Vibrio* spp. by its arginine dihydrolase, and lack of lysine and ornithine decarboxylases (Tison and Seidler, 1983).

Phenon-1 shows similarities and differences with *V. ichthyoenterii* also. Phenon-1 unlike *V. ichthyoenterii* is positive for acetoin production, for lysine and ornithine decarboxylase and lipase; grows at even 45°C but not all strains grow at 6% NaCl concentrations and produces acid from dexrin (16 out of 17 strains). On the other hand the phenon –1, agrees on several characters for *V. ichthyoenterii*: negative for gas from glucose, H_2S, gelatinase, growth at 4°C and acid from adonitol,

arabinose, cellobiose, dulcitol, glycerol, m-inositol, inulin, lactose, melibiose, raffinose and salicine; they are positive for growth at 0% NaCl (16 out of 17 strains).

On the basis of these it is concluded that genotypic relatedness of the phenon with the above species has to be ascertained by DNA homology studies before proposing the phenon to the status of a new species.

Phenon-2: Moribund larvae: O/129 positive: OTUs: 3

This cluster comprises of only 3 strains clustered at 85 %S. The strains in this phenon can reduce nitrate, utilize ribose, trehalose, galactose, sucrose, glycogen, calcium lactate, tyrosine, tryptophan and glutamic acid as sole source of carbon and energy; produce acid from glucose, sucrose, maltose, trehalose, cellobiose, mannose, and mannitol; hydrolyze tweens 20-80, trybutyrin and starch but fail to haemolyze on blood agar. They prefer a pH range of 8-12, and can grow even without NaCl and in the presence of 5 % NaCl; prefer a temperature range of 28-45°C but fail to grow at 4°C. They are able to tolerate and grow in the presence of heavy metals like Cd^{-2}, Fe^{+2}, $Pb^{+2}$and MoO_4^{-2} and Hg^{+2}, can grow in the presence of various dyes such as brilliant green, crystal violet, pyronine G and neutral red, capable of growth in the absence of Na^{+2}, Mg^{+2} or K^{+} They are sensitive to terracyclin, gentamycin, chlortetrtacyclin, neomycin, methamine mandelate, nalidixic acid, novobiocin, chloramphenicol, erythromycin and kanamycin.

The representative strain from this phenon (**AAC 594b**) has mole % G+C ratio of 43.2% which falls with in the range of that of *V. aestuarianus* (43-45)(Tison and Seidler, 1983), *V. tubiashi* (43-45) (Hada *et al.*, 1984), *V. ichthyoenterii* (43-45) (Ishimaru *et al.*, 1996) and *P. leiognathi*.(42-44)(Reichelt *et al.*, 1976).

The phenon exhibits profound similarity phenotypically with *V. aestuarianus* as both are able to grow in medium containing 5% NaCl but not 7.5%, fail to produce lysine and ornithine decarboxylase and H_2S. Both produce acid with out gas from glucose, sucrose, maltose, cellobiose, mannose, mannitol and trehalose; but not from

lactose, adonitol, dulcitol, raffinose, salicine or xylose. Both are sensitive to O/129 compound, chloramphenicol, gentamycin, tetracyclin, and kanamycin. Phenon-2 on the other hand differs from *V aestuarianus* in a limited number of characters such as the absence of arginine dihydrolase, DNase and failure to produce acid from lactose

With *V. tubiashi*, phenon-2 shows as much similarity as dissimilarity. Both are not luminescent, produce acid with out gas from glucose, sucrose and mannose, but not from arabinose, m-inositol, lactose, raffinose, salicine or xylose. Both produce lipase, amylase, and hydrolyze tyrosine, are unable to grow with 8% NaCl, fail to produce lysine and ornithine decarboxylase. Phenon-2 differs from *V. tubiashi* by being negative for arginine dihydrolase and DNase.

On the other hand, phenon-2 differs from *P. leiognathi* by being non-luminescent, unable to utilize succinic acid, serine and gluconate as sole source of carbon, by utilizing sucrose and trehalose and by growth at 45°C.

Phenon-2 also shares many traits with *V. ichthyoenterii*: production of acid with out gas from glucose, production of catalase, oxidase; nitrate reduction, growth at 30°C, acid from mannose, maltose, sucrose and trehalose. Besides, both are negative for lysine and ornithine decarboxylase, arginine dihydrolase, H_2S, growth at 4°C; and acid from adonitol, arabinose, dulcitol, glycerol, glycogen, m-inositol, inulin, lactose, melibiose, salicine and sorbitol. Phenon-2 differs from *V. ichthyoenterii* by growth at 35°C and lipase production.

It is very clear that phenon-2 has more affiliation to *V. aestuarianus*, *V. tubiashi* and *V. ichthyoenterii* than to *P. leignathi*. More studies on the DNA homology of the phenon with the above species are required to ascertain its identity

Phenon-3 Moribund larvae: O/129 positive: OTUs: 3

This phenon contains 39 strains clustered at 85 %S. All the strains utilize ribose and pyruvate as sole source of carbon and produce acid without gas from

glucose. They prefer a pH range of 8-12, and can grow from 0-2%NaCl and a few strains in the presence of even 5 % NaCl. Prefer a temperature range of 28-45°C, but fail to grow at 4°C; can grow in the presence of various dyes such as brilliant green, crystal violet, pyronine G and neutral red. They are sensitive to the O/129 compound, streptomycin tetracyclin, gentamycin, chlortetratacyclin, neomycin, methamine mandelate, nalidixic acid, chloramphenicol and kanamycin.

The representative strain from this phenon (**AAC 597**), has mole % G+C ratio of 43.5 % which falls within the range for *V. aestuarianus* (43-45)(Tison and Seidler, 1983), *V. tubiashi* (43-45) (Hada *et al.*, 1984), *V. ichthyoenterii* (43-45) (Ishimaru *et al.*, 1996) and *P. leiognathi* (42-44)(Reichelt *et al.*, 1976).

The members of phenon-3 show similarity with *V. aestuarianus* in certain traits, as they do not produce acid from adonitol, dulcitol, raffinose, salicine and xylose. Phenon-3 also disagrees with this species by being unable to ferment sucrose and sorbitol (except one strain).

Phenon-3 shares several phenotypical characters with *V. tubiashi*; both are not luminescent, produce acid with out gas from glucose, but not from m-inositol, lactose, raffinose, salicine or xylose. Both are unable to grow with 8% NaCl and do not utilize xylose. On the other hand unlike *V. tubiashi*, phenon-3 is negative for acid from sucrose and tyrosine hydrolysis.

Phenon-3 does not show good agreement with *P. leiognathi*, and differs from *P. leiognathi* by being nonluminescent, by growth at 35°C; and agrees by being negative for utilization of xylose and growth at 4°C. Both utilize pyruvate as sole source of carbon and energy.

Phenon-3 also shares many traits with *V. ichthyoenterii,* like production of acid with out gas from glucose, growth at 30°C but not at 4°C, negative for acid from ducitol, m-inositol, lactose, inulin, melibiose, raffinose, salicine, sorbitol and xylose; and does not produce H_2S. Phenon-3 differs from *V. ichthyoenterii* by being positive

for growth at 35°C, in the presence of 6% NaCl and negative for acid from sucrose and maltose.

The above comparisons clearly indicate that from the available information it is not possible to identify the phenon in spite of similarities shown to 4 species based on the %G+C ratio. Therefore, more studies are required, especially related to DNA homology, before this phenon can be identified.

This group also includes **phenon-4,5 and 6,** all of which comprise only of the reference strains studied along with the test organisms of this group (Fig 2-3 and 2-4).

Phenon-4:Moribund larvae: O/12 9 positive:OTUs :6

Phenon-4 comprises of ATCC strains of *Plesiomonas shigelloides, Salinivibrio costicola, P.angustum, P. leiognathi* and *P. phosphoreum* This phenon is formed at 80.7%S.

Phenon-5:Moribund larvae: O/12 9 positive:OTUs :9

Phenon-5 is linked at 76 %S to phenon-4 and contains altogether 9 strains including, *V.parahaemolyticus* ATCC, *V anguillarum* ATCC, *V alginolyticus* ATCC, *V cholerae* Ogawa, *V. harveyi, V. hollisae, V. mimicus, V, nereis* and *V metschnikovii,* clustered at 80.9 %S.

Phenon-6: Moribund larvae: O/12 9 positive:OTUs :2

Phenon-6 contains 2 reference strains , *V. vulnificus* and *V. fisherii,* clustered at 85.7%S. this phenon is linked at 66.5 % to phenon-5

The characteristics tested for the reference strains are summarized in the table 2-7

Strains from moribund larvae, O/129 positive, not clustered

There are 5 strains that do not cluster with any of the phena described earlier. They are ANM 708, ANM 610a, ANM 1020, ANM 594a and ANM 1003, and their phenotypic characteristics are summarized in Table 2-4.

Strain –I: ANM 708

The strain ANM 708 is linked at 80 %S to phenon-2. The mole %G+C ratio of this strain is 39.9 which is in the range for *V. fischeri* (39-41), and very close to that of *P. angustum* (40-42), *V logei* (40-42) and *V.marinus* (40-42) (Baumann *et al.*, 197la ,1984, Baumann and Baumann, 1984).

This strain is distinguished from *V fischerii* by a combination of properties. *V. fischerii* is negative for growth at 0% NaCl and is bioluminesent. Besides V. *fischerii* has a yellow-orange cell associated pigment and can grow up to 6% NaCl (Baumann *et al.*, 1984). On the other hand, the strain grows at 0 % but not at concentrations higher than 5% NaCl and unlike *V. fischeri* it utilizes lactate and pyruvate. Considering that they agree with each other by being negative for gelatinase, growth at 4°C and positive for growth at 30°C, the strains may be *V. fischerii* –like organism , to be further ascertained by DNA homology studies.

The strain AMN 708 is distinguished from *V. logei* (40-42) by the following combination of properties: *V. logei* like *V.fischerii*, are luminescent species, have a cell associated yellow orange pigment, are negative for growth at 0% NaCl, shows good growth at 4, but not at 30 °C. Both agree by not producing gelatinase and utilization of gluconate, and unlike *V. logei* the strain ANM 708 utilizes lactate and pyruvate (Baumann *et al.*, 1984).

V. marinus, a facultatively anaerobic organism of marine origin, which ferments glucose with the production of acid but no gas, has a mole % GC of 40-42 %). Strain ANM 708 is different from this species as it is ornithine decarboxylase

positive, doe not require organic growth factors, can grow at 0% NaCl and is positive for gluconate utilization. Both do not grow in the presence of 6%NaCl. (Colwell and **Mandel, 1964)**

However, a comparison of the traits of ANM 708 with *P. angustum*, revealed good resemblance, as it agrees on a number of traits for this species. Both are non-luminescent, do not produce gas from glucose, produce lipase, utilize xylose, pyruvate, glucose, mannose, galactose, fructose, glycerol, gluconate, lactate, aspartate, serine, and sucrose, and fail to utilize glucuronate. At the same time, the strain also shows phenotypic differences with *P.angustum* as it is positive for the utilization of proline, growth at 45°C and acetoin from the glucose (Baumann and Baumann, 1984).

However based on these dissimilarities and similarities, it is difficult to identify the strain ANM 708 with certainty For this, it is important to go for deeper studies in order to reveal their DNA relatedness.

Strain –2: ANM 610a

The strain ANM 610a is linked at 74 %S to phenon-3 This strain has a mole %GC content of 54.01, which is much closer to the %GC of group E-3 (53.6) (Baumann *et al.*, 1971). This strain agrees on all but a few characteristics for Group E-3, as the strain is negative for utilization of glycine, serine, gluconate, and positive for xylose and galactose. Unlike group E-3 bacteria this strain grows at 4°C and does not swarm on complex solid medium. Since the mole % GC ratio of this group is out of range for that of the vibrios, as suggested by Bauamnn *et al.*, (1984)an extension of the %GC range for the vibriosis required, in order to accommodate this group (Baumann *et al.*, 1971, 1984). Their DNA relatedness has to be worked out, in order to decide its taxonomic position in the family Vibrionaceae.

Strain –3: ANM 1020

The strain ANM 1020 is linked to phenon-3 at 74 %S.It has a mole %GC content of 39.9, which is in the range for *V. fischerii* (39-41), and very close to that of *P. angustum* (40-42), *V. logei* (40-42) and *V.marinus* (40-42) (Baumann et al., 1971a, 1984; Baumann and Baumann, 1984).

But the strain ANM 1020 exhibits remarkable phenotypic differences with both *V. fischerii* and *V. logei*, as the strain is not luminescent, does not have a cell associated yellow-orange pigment, negative for growth in 0%NaCl, and positive for lactate. Besides, *V. fischerii* can grow only up to 6% NaCl (Baumann *et al.*, 1984). On the other hand, the strain grows at 0 % and concentrations as high as 7.5% NaCl. Though ANM 1020 agrees with *V. fischerii* by being negative for gelatinase, growth at 4°C and positive for growth at 30°C. *V. logei* shows good growth at 4°C, but not at 30 °C, unlike the strain AMN1020. (Baumann *et al.*, 1984).

V. marinus, a facultatively anaerobic organism of marine origin, which ferments glucose with the production of acid but no gas, has a mole % GC of 40-42 %). Strain ANM 1020 is different from this species as it is negative for lysine decarboxylase,and gelatinase, does not require organic growth factors, can grow at 0% NaCl and in the presence of 6%NaCl, cannot grow at 4°C but can at 35°C(Colwell and Mandel, 1964).

However, a comparison of the traits of ANM 1020 with *P. angustum*, revealed phenotypic differences. Unlike *P. angustum*, the strain 1020 is able to grow at 45°C but not at 4°C. Besides, it also differs by being negative for gelatinase, chitinase, lysine and ornithine decarboxylase, and utilization of acetate, pyruvate, maltose, fructose, serine,gluconate and glycerol (Baumann and Baumann, 1984). Further, the strain can grow even in the presence of 7.5% NaCl. Thus only DNA homology studies of strain ANM 1020 with the above species can help fix its identity

Strain –4: ANM 594a

The strain ANM 594a is linked at 74%S to the phenon-4 (containing the reference strains). The mole %G+C ratio of this strain is 44.8% which is indistinguishable from that of This falls with in the range of *V anguillarum* (44-46), (44-46), *V. ordalli* (44-46), *V. tubiashii* (43-45)(Baumann *et al.*, 1984, Hada *et al.*, 1984) and *V. ichthyoenterii* (43-45) (Ishimaru *et al.*, 1996).

The strain ANM 594a is characterized by not having a constitutive arginine dihydrolase, which is present in *V metschnikovii, V. tubiashii* and *V anguillarum. V. metschnikovii* is different from strain ANM 594a by being Kovac's oxidase negative. It is different from *V. tubiashi* by being negative for chitinase, DNase, gelatinase, lecithinase, gelatinase, tween 80 esterase, tyrosine hydrolysis and nitrate reduction. Meanwhile, *V. anguillarum* is different by being positive for acid from arabinose, salicine and utilization of gluconate.

But the strain ANM 594a agrees with *V ordalli* in several characters apart from its %GC content. Both are negative for bioluminescence, acid without gas from glucose, arginine dihydrolase, lysine and ornithine decarboxylase, nitrate reduction, lecithinase, tyrosine hydrolysis, tween 80 esterase and acid from sorbitol; positive for Kovacs' oxidase, acid from cellobiose, sucrose, salicine, caseinase and growth at 6% NaCl. But certain degree of differences are also seen between *V. ordalli* and strain ANM 594a, where the strain under consideration is negative for chitinase and gelatinase, and positive for acetoin production and utilization of xylose and gluconate (Hada et al., 1984; Baumann et al., 1984;Ishimaru et al., 1995).

The strain exhibits good agreement *with V ichthyoenterii* in several of the phenotypic characters. Both produce acid without gas from glucose, reduce nitrate and are negative for ornithine decarboxylase, lipase, gelatinase, amylase, chitinase and growth at 4°C. Both produce acid from fructose, maltose, amnnose, sucrose, trehalose but not from xylose and m-inositol. Certain differences are also seen between *V ichthyoenterii* and the strain ANM 594a, where the latter is positive for

arginine dihydrolase, lysine decarboxylase, indole, growth at 35°C, growth in 8% NaCl; and negative for catalase, acid from amnnitol and galactose. Nevertheless these characters are not sufficient enough to identify the strain as *V. ichthyoenterii*.

Based on these observations it is difficult to identify the strain to any of the above species, though it has good similarities with both *V. ordalli* and *V. ichthyoenterii*. Therefore DNA homology of this strain has to be worked out with the above five strains of *Vibrio* to fix the identity.

Strain –5: ANM 1003

The fifth strain ANM 1003 is also linked at 74 %S to the phenon –4 comprising only of the reference strains and has a mole %G+C ratio of 54.3 which is above the range of *Vibrio* (38-51) and below the range of that of *Aeromonas* (57-63). But at the same time, very much closer to the group E-3. which has a mole %GC of 54%. However the strain ANM 1003 differs from the Group E-3 bacteria by being negative for amylase and the utilization of gluconate, cellobiose, pyruvate, glycine and serine and by being positive for xylose and galactose utilization. But strain ANM 1003 agrees on most other characters for group E-3 (Baumann *et al.*, 1971a).But the group E-3 bacteria has not yet been included in the genus *Vibrio* as an extension of the %G+C range for the vibrios would be required in order to accommodate this group (Baumann *et al.*, 1971, 1984). Therefore there is every possibility for assigning a new species status to the strain ANM 1003, after working out the DNA relatedness to the group E-3 and other *Vibrio* species.

2.3.2 Apparently healthy larvae

Out of the 442 isolates from this system 300 were found to be viable. These strains were then purified by repeated streaking on nutrient agar plates and confirmed purity by microscopic examination by Gram staining. From the 300, 109 isolates were segregated as belonging to the family Vibrionaceae following West and Colwell, (1984). These 109 isolates were further divided into two groups based on their sensitivity to O/129 (vibriostat) compound at a concentration of 150µg/mL (Baumann

and Schubert, 1984; Baumann *et al.*, 1984; Bain and Shewan, 1968). Thus out of 109 strains, 75 were O/129 negative and 34 were O/129 positive.

2.3.2a Numerical taxonomic analysis of the strains associated with apparently healthy larvae which were O/129 negative

The group of 75 isolates of vibrionaceae, O/129 negative obtained from healthy larvae of *M. rosenbergii* subjected to numerical taxonomy analysis along with the type strains of *Aeromonas* (Table 2-1), were recovered in 4 discrete clusters and 7 strains remained unclustered (Fig. 2-5 and Fig 2-6) As observed in the previous groups , the reference strains were not picked up by any of the phenons; they themselves clustered together and consequently the phena remained unidentified. This phenomenon has been previously reported (Kaper *et al.*, 1983; Lee *et al.*, 1978).

Since all the clusteres remained unidentified even after numerical taxonomy analysis, identification was done based on % G+C ratio and by comparing with the published data for the type strains. The phenotypic characteristics of the phena 1-4 are summarized in Table 2-5

Phenon-1 : Apparently healthy larvae: O/129: negative: OTUs =4

This phenon comprises 4 strains clustered at 86 %S. All the strains in this phenon are positive for methyl red, catalase, H_2S, utilization of ribose, pyruvate, and gluconate as sole source of carbon. Positive for acid from glucose, trehalose and galactose without gas, gelatinase, lecithinase, hydrolysis of tween 20-80 and tributyrin and negative for acetoin production. They prefer a pH range of 8-12, majority of them can grow without NaCl and in the presence of 0.5 to 5 % NaCl; prefer a temperature range of 28-45°C but fail to grow at 4°C. They tolerate and grow in the presence of heavy metals like Fe^{+2}, Pb^{+2}, MoO_4^{-2} and Hg^{+2}; grow in the presence of various dyes like brilliant green, crystal violet, pyronine G and neutral red and do not require K^+ in the growth medium. They are sensitive to penicillin G, oxytetracycline, streptomycin, tetracyclin, gentamycin, chlortetrtacyclin, neomycin, methamine

mandelate and nalidixic acid, novobiocin, chloramphenicol, kanamycin and sulfadiazin.

The representative strain from this phenon **(AAC730a)**, has mole %G+C content of 47 1 which is exactly the same as that of *V gazogenes* (47 1). Meanwhile it also falls within the %GC range of many species of vibrios such as *V. campbelli* (46-48), *V. harveyi* (46-48), *V. cholerae* (47-48), and *V. vulnificus* (46-48)(Baumann *et al.*, 1984). The %G+C of the isolates is also very close to that of certain other vibrios like, *V penaecida* (46-47)(Ishimaru *et al.*, 1995), *V. diazotrophicus* (46-47) (Guerinot *et al.*, 1982), *V. natriegenes* (46-47), *V. nereis* (46-47), *V. parahaemolyticus* (46-47), *V pelagius* (45-47), *V alginolyticus* (45-47), *V pelagius bv. I* (45-47), *V. nigripuchritudo* (46-47)(Baumann *et* al., 1984), *V vulnificus* bv II (46-47)(Tison *et al.*, 1982) and *V. navarrensis*(45-47) (Urdaci *et al.*, 1991).

Even though a certain degree of similarity can be seen with each species and biovars of vibrios, the phenon exhibits almost the same level of dissimilarity with them as described below.

Phenon –1 differs from *V gazogenes* by being negative for red pigmentation, gas from glucose, and positive for oxidase It is in agreement with only a few traits by being positive for utilization of salicine and sorbitol. These properties distinguishes *V. gazogenes* from all other species of vibrio.

V. campbelli agrees on the following traits of phenon-1 positive for growth at 35°C, utilization of galactose and gluconate and negative for arginine dihydrolase. With this species phenon-1 differs by being negative for lysine decarboxylase, acetoin production and positive for growth at 45°C.

Unlike *V. cholerae, V. harveyi V. parahaemolyticus, V alginolyticus and V. vulnificus* bv. I *and V. vulnificus* bv II the phenon-1 fails to produce lysine and ornithine decarboxylase, and do not grow at 6% NaCl.

V. penaecida like phenon-1 is negative for lysine and ornithine decarboxylase, arginine dihydrolase, growth in the presence of 6% NaCl. This species differs from phenon –1 by being negative for growth at 35°C and utilization of gluconate

This phenon differs from *V. diazotrophicus* by its lipolytic and gelatinolytic properties and its inability to produce arginine dihydrolase and agrees in its inability to produce lysine and ornithine decarboxylase, positive for growth at 35°C and utilization of gluconate.

V. natriegenes and *V navarrensis* is different from phenon-1 by their ability grow in 6% NaCl, produce acid from sucrose and its inability to grow at 45°C. *V. natriegenes* also differs by its ability to utilize malonate and rhamnose. Both like phenon-1 are negative for arginine dihydrolase, lysine and ornithine decarboxylase and positive for growth at 35°C, gelatinase and lipase.

V nereis is able to dihydrolyse arginine, grow in 6% NaCl, produce acid from sucrose and unable to grow at 45°C unlike the phenon-1. But both agree by being negative for gas from glucose, acetoin and utilization of cellobiose and glucuronate.

V. pelagius bv.I and II like phenon –1 are negative for arginine dihydrolase, lysine and ornithine decarboxylase, can grow at 35°C, and are lipolytic. The phenon differs from *V. pelagius* bv.I by being positive for gelatinase and from *V. pelagius* bv. II by being negative for amylase.

V. nigripuchritudo is different from this phenon by producing a black pigment.

From this it is clear that even though the phenon-1 has a mole %G+C content similar to or even close to the above mentioned species, there are gross phenotypic differences between them. Thus the final assignment of specificity to this phenon is possible only by carrying out studies on their genotypic relatedness.

Phenon-2 : Apparently healthy larvae: O/129: negative: OTUs =51

This phenon has 51 strains clustered at 86 %S. This is the largest phenon obtained in this study All strains produce indole from tryptophan, and utilizes pyruvate and gluconate as sole source of carbon. They ferment glucose without the production of gas and fail to ferment a variety of sugars such as sucrose, lactose, xylose, raffinose, arabinose, salicine, adonitol, sorbitol, dulcitol, inulin, and butanol. They fail to haemolyse and prefer a pH range of 8-12. All grow in 0.5- 2% NaCl but the majority of them fail to grow in the absence of NaCl in the medium. They prefer a temperature range of 28-45°C but are unable to grow at 4°C. They grow in the presence of various dyes like brilliant green, crystal violet, pyronine G and neutral red are inhibited by antibiotics like oxytetracycline, tetracyclin, gentamycin, polymyxin B, chlortetracyclin, neomycin, chloramphenicol, erythromycin and kanamycin.

The representative strain, (**ANM 932**) has a mole %GC content of 49.8 which falls within the range of that of *V. fluvialis*(49-51) and very much closer to that of *V. costicola* (50) and *V. furnissi*(50) (Baumann et al., 1984). A critical assessment of the phenotypic similarity and dissimilarity of the phenon with any of the above three species of vibrio listed above was not viable. However, a certain degree of similarity and dissimilarity could be worked out between them as given below.

V.fluvialis agrees with this phenon on the following traits: utilization of gluconate, growth at 35°C, negative for gas from glucose and differs by being positive for acid from sucrose, arabinose and salicine.

V. furnissi unlike the phenon-2 is positive for chitinase, for gas from glucose, and negative for growth at 45°C and agrees with this phenon by utilization of gluconate, growth at 35°C.

V. costicola on the other hand is negative for gluconate utilization, growth at 45°C quite unlike phenon-2.

This is the only possible comparison which can be made between the phenon-2 and the three species of vibrios; the inference is not strong enough to group the phenon under any of the species. A study of the DNA homology with the above species shall lead to a better understanding of their relationship.

Phenon-3 : Apparently healthy larvae: O/129: negative : OTUs =6

This phenon comprises of 6 strains clustered at 82 %S. All the strains in this phenon produce catalase, utilize ribose, pyruvate, gluconate and glutamic acid as sole source of carbon and energy; produce acid from glucose with out gas, but are unable to ferment the majority of the sugar, produce gelatinase but fail to hemolyze on blood agar. They grow between a pH range of 8-12 in the presence of 0.5-2 % NaCl; prefer a temperature range of 28-45°C but fail to grow at 4°C. They grow in the presence of various dyes such as brilliant green, crystal violet, pyronine G and neutral red. They are sensitive to oxytetracyclin, streptomycin, ampicillin, gentamycin, chlortetracyclin, neomycin, methamine mandelate, cefazolin, nalidixic acid, novobiocin, chloramphenicol, kanamycin and lincomycin.

The representative strain for this phenon ,(**ANM 723b**) has %GC ratio of 45.9 which falls in the range of *V. alginolyticus* (45-47), *V. anguillarum* (44-46), *V. metschnikovii* (44-46), *V. mytili* (45-46), *V navarrensis* (45-47), *V. ordalli* (44-46), *V. orientalis* (45-46), *V. pelagius* bv I and II (45-47), *V. splendidus* bv I and II(45-46) (Baumann *et al.*, 1984; Pujalte *et al.*, 1993; Urdaci *et al.*, 1991)

These species agree with phenon-3 in some traits while disagreeing on others. Phenon-3 like V alginolyticus is negative for arginine dihydrolase, growth at 35 °C and gelatinase and unlike it cannot utilize tyrosine or swarm on solid complex media.

The phenon like *V alginolyticus* does not produce gas from glucose nor acid from inositol. However *V. alginolyticus* differs from the phenon by utilizing arabinose, sorbitol and sucrose, and by being negative for growth at 45°C.

Phenon-3 agrees with *V. metschnikovii* by being positive for growth at 45°C gluconate utilization and negative for utilizing tyrosine. *V. metschnikovii* differs from the phenon by being oxidase positive.

V. mytili produces gas from glucose and grows in the presence of 10% NaCl quite unlike the strains in phenon-3. But both agree by growing at 35°C, not producing acid from mannose and by not producing amylase.

V. navarrensis unlike this phenon utilizes citrate as sole source of carbon and energy. Both are positive for gelatinase and growth at 35°C

V. ordalli like phenon-3 is gelatinase positive and negative for lipase, utilization of mannitol but differs by not growing even at 35°C and by utilizing gluconate.

Like *V. orientalis*, this phenon does not produce gas from glucose and negative for acid from arabinose and xylose. As with the species above, *V. orientalis* did not match fully with phenon-3, as it produces amylase and hydrolyse tween 80.

The phenon-3 agrees with *V. pelagius* bv I and II by utilizing gluconate; and by being negative for arginine dihydrolase and amylase. But the phenon differs from this species, as it can grow at 40°C and is unable to produce chitinase.

The phenon as with other species agrees only with a few traits for *V. splendidus* bv. I. Both are negative for utilization of arabinose and sorbitol and positive for growth at 40°C. But for *V. splendidus* bv I. is luminescent and arginine dihydrolase positive unlike the phenon. *V. splendidus* bv. II on the other hand agrees with this phenon by being negative for arginine dihydrolase but utilizes pyruvate. But this species cannot grow at 35 °C.

With this it is evident that the phenon-3 even though similar to many species in its %G+C content, does not have a satisfactory agreement with any of the above

species. Therefore it is obvious that identification of this phenon at this stage is not possible and that more information is necessary regarding its DNA homology with these species.

Phenon-4: Apparently healthy larvae: O/129: negative : OTUs =7

This phenon contains 7 strains which formed a compact cluster at 86 %S. All strains in this phenon gave a positive reaction to methyl red test, catalase, nitrate reduction, arginine dihydrolase and utilization of ribose, trehalose, mannose, fructose, pyruvate and gluconate as sole source of carbon and energy. The strains are all able to ferment glucose, fructose, trehalose and mannose. They are negative for amylase, chitinase, cellulase, haemolysis on blood agar and urease. They grow between a pH range of 8-12, grow well in the presence of 0.5-2 % NaCl and except one strain all are negative for growth in 0% NaCl. Prefer a temperature range of 28-45°C but fail to grow at 4°C. They grow in the presence of various dyes such as brilliant green, crystal violet, pyronine G and neutral red. They are sensitive to oxytetracyclin, ampicillin, tetracyclin, gentamycin, polymyxin B, chlortetracyclin, neomycin, methamine mandelate, cefazolin, novobiocin, chloramphenicol, kanamycin and lincomycin.

The representative strain (**API 1546**), has a mole %GC content of 38.3 which is within the range for Vibrio (38-51) and much closer to that of *V. fischerii* (39-41) (Baumann *et al.*, 1984).

There are several phenotypic characters on which the phenon and *V fisherii* both agree. Both grow at 30°C but not at 4°C, negative for amylase and chitinase.At the same time the phenon disagrees with *V fischerii* in several other characers. Unlike *V. fischerii* the members of this phenon produce arginine dihydrolase, are not luminescent, do not have a cell associated yellow-orange pigment and utilizes pyruvate as sole source of carbon and energy. Above all, the phenon-4 is resistant to O/129 and the mole %GC is slightly lower than that of *V. fischerii*

Considering all these, the phenon-4 is proposed to be a new species under the genus Vibrio. Further studies to reveal its genotypic relatedness is essential.

Phenon –5:Apparently healthy larvae: O/129: negative:OTUs: 4

Phenon-5 includes 4 strains of *Aeromonas*, i.e. *A. hydrophila* 172 (CIFT, Cochin), *A.caviae* ATCC, *A. hydrophila* ATCC and *A. salmonicida* subsp. *masoucida* ATCC(Fig.2-5 and 2-6), which clustered together at 83%S. These reference strains did not cluster with any of the other phena formed by the test organisms. Therefore phena 1-4 remianed unidentified even after Numerical Taxonomy analysis. The characteristics tested for the reference strains are summarized in the Table 2-7

Strains from apparently heathy larva, O/129 negative, not clustered

Among the group of 75 isolates from healthy larvae which were negative to the O/129 compound, 7 isolates remained unclustered with the four phena formed. The phenotypic characteristics of these isolates are summarized in table 2-5.

Strain-1: API 1561

The strain API 1561 connected to the phenon-2 at 77 %S, has a mole % GC content of 54.9% which is not within the range for *Vibrio* or *Aeromonas*, but remains close to that for the Group E-3 of Baumann *et al.*, (1971). More over this strain shares with group E-3 many characters as both are arginine dihydrolase positive, Voges-Proskauer negative, grow at 4°C, produce lipase, do not utilize xylose, salicine, glucuronate, citrulline and rhamnose, or haemolysis on blood agar bur produce chitinase. Considering all these traits, it is proposed that strain API 1561 could be a new species under genus Vibrio, closer to the group E-3 This has to be ascertained by studies on DNA homology. As suggested by Baumann et al., (1984), an extension of the mole %GC will be required in order to accommodate such groups.

Strain-2: API 1558

The strain API 1558 is linked to the phenon-2 at about 80 %S. It has a mole% G+C ratio of 60.12, which is in the range for the motile aeromonads *A. hydrophila* (58-62), *A. caviae* (61-63) and *A. sobria* (58-60)(Popoff, 1984) In general the strain API 1558 resembles motile aeromonads by the following traits which are universally positive for all motile aeromonads: catalase, DNase, gelatinase, Tween 80 esterase, lecithinase, growth in nutrient broth without NaCl and fermentation of mannose, fructose and galactose. The strain also agrees on several of the characters which are universally negative: ornithine and lysine decarboxylase and acid from raffinose. Mean while the strain differs from motile aeromonads by being negative for arginine dihydrolase and starch hydrolysis, positive for phenyalanine deaminase.

The strain agrees with *A. hydrophila* in the following characters: negative for brown water –soluble pigment, positive for esculin hydrolysis, fermentation of salicine and mannitol, but differs by its inability to produce gas from glucose.

The strain resembles *A. caviae* by being negative for brown water –soluble pigment, acetoin and gas from glucose; positive for esculin hydrolysis, fermentation of salicine and mannitol, but differs by producing acid from inositol and not utilizing succinic acid as sole source of carbon and energy.

With *A. sobria* too, the resemblance minimal, restricted to the following traits: negative for brown water –soluble pigment and fermentation of mannitol. But unlike *A. sobria*, the strain hydrolyse aesculin, and do not produce gas from glucose.(Popoff, 1984).

Weighting these similarities and dissimilarities of the traits of the strain API 1558 with these three species, it is not possible to identify the strain to any of the above previously described species other than suggesting that it belongs to motile

Aeromonas spp. DNA homology has to be worked out in order to resolve this problem.

Strain-3: AAC 727

This strain is linked to the phenon-2 at 77 %S and has a mole %GC ratio of 38.3 which is within the range for the vibrios (38-51). But it is close to that *of V. fisherii* (39-41). This strain resembles V fisherii by being negative for arginine dihydrolase, ornithine decarboxylase, acid from sucrose, pyruvate utilization and for growth at 4°C. However it differs by not being luminescent, not having a cell associated yellow-orange pigment, negative for lysine decarboxylase and producing gelatinase. More characterization at the genomic level is required for identifying the strain

Strain-4: AAC 740

The strain is linked to the strain AAC 727 at 80 %S and has a mole%GC content of 52.1% which is much higher than that of the vibrios (38-51) and much lower than that of the aeromonads (57-63)(Baumann and Schubert, 1984). This species has its GC content closer to some extent to that of *V. fluvialis* (49-51), *V. proteolyticus* (50.5), *V. costicola* (50), *V. hollisae* (50) and *V. furnissi* (50)(Baumann *et al.*, 1984). Even though this stain is linked to AAC 727 at 80 %S, its GC content is too low to have any comparison. Notably, the GC content of this strain is also lower than that of the Group E-3 (54%, Baumann *et al.*, 1971a). Therefore the first step towards identification of this strain is to assess the DNA homology with the species and groups mentioned above.

Strain-5:API 810

The strain API 810 is linked to phenon-4 at 77 %S. The mole %GC content of this strain is 36.5 %, which is much lower than that for the vibrios (38-51). However the strain has several traits in common with the vibrios. Therefore studies

characterizing its genetic relatedness with the other vibrios is required before its identity is fixed.

Strain-6: AAC 717

The strain AAC 717 joins phenon –4 at 82 %S, has a mole %G+C content of 56.7% which is out of range of that of the vibrios (38-51) and the aeromomads (57-63). More studies are required to reveal its genotypic relatedness in order to fix its identity

Strain-7:API 781

The strain API 781 is linked to the phenon-5 at 77 %S and has a % GC content of 36.1%. This value is out of range for that of the family vibrionaceae. But it complies with several of the phenotypic characters for Vibrio species. More investigations on the genotupic relatedness of this strain with vibrios which has G+C ratios much closer to that of API 781 are required before considering it a new species. (Baumann and Schubert, 1984).

2.3.2.b Numerical taxonomy of the strains associated with healthy larvae which were O/129 positive

The group of 34 isolates sensitive to the O/129 compound were subjected to numerical taxonomy analysis, along with 16 type strains. But as before the none of the reference strains were picked up by the phena formed and thus the identification of the phena became extremely difficult. From the 6 phena generated in this group, representative strains were selected along with the two strains which remained unclustered. The mole % G+C was determined and compared with that of the known species. Efforts were made to identify the clusters based on the phenotypic and genotypic similarity with the existing species under the family The morphological , physiological, biochemical and nutritional characteristics of the phena are given in Table 2-6.

Phenon –1: Apparently healthy larvae: O/129: positive: OTUs: 4

This phena contains 4 isolates which clustered compactly at 90 %S. All strains are postive for catalase, arginine dihydrolase, utilization of ribose, fructose, pyruvate and gluconate as sole source of carbon and energy They produce acid without gas from glucoseand fructose, and majority of the sugars remain unattacked. They produce gelatinase, caseinase, hydrolyse tween 20 but not 40, 60 or 80, degrade tributyrin and DNA. They prefer to grow at a pH range of 8-12 and in the presence of 0.5- 2% NaCl. They prefer a temperature range of 28-45°C, but are unable to grow at 4°C. They can tolerate and grow in the presence of heavy metals like Fe^{+2}, Pb^{+2}, MoO_4^{-2} and Hg^{+2} ,and they grow in the presence of various dyes like brilliant green, crystal violet, pyronine G and neutral red. They are inhibited by antibiotics like oxytetracycline, tetracyclin, gentamycin, polymyxin B, chlortetracyclin, neomycin, methamine mandelate, cefazolin, nalidixic acid, novobiocin, chloramphenicol, erythromycin, kanamycin, lincomycin and sufadiazine.

The representative strain (**AAC 701**), selected from this phena has a mol %GC of 48.4 % which falls within the range of *V. cholerae* (47-49) (Baumann *et al.*, 1984). The phena is in agreement with *V. cholerae* by producing catalase, utilizing gluconate, growing at 40°C and in the presence of 2% NaCl. Meanwhile, unlike *V. cholerae*, the phenon is negative for lysine and ornithine decarboxylase and does not produce acid from sucrose, mannitol and mannose (Baumann *et al.*, 1984; Sakazaki and Balows, 1981). Base on these criteria, it becomes appropriate to place this phenon under *V cholerae*.

Considering the %G+C ratio, it is only 0.4% higher than *V harveyi* (46-48), *V. campbelli*(46-48), *V. vulnificus* (46-48) (Baumann *et al.*, 1984), and marine luminous isolates of Chumakova *et al.*, 1973 (46-48). Therfore only by DNA homology studies the identity of the phenon can be fixed.

Phena-2: Apparently healthy larvae: O/129: positive: OTUs: 12

The phenon comprises 12 strains clustered at 90 %S. All the strains in this phenon are positive for catalase, acetoin from glucose, lysine and ornithine decarboxylase, indole and reduction of nitrate and nitrite. The strains utilize ribose, fructose, pyruvate, and gluconate as sole source of carbon and energy; produce acid from glucose and fructose without gas and majority of the sugars are not fermented. They produce gelatinase, caseinase. They grow in a pH range of 8-12, and in the presence of 0.5 to 5 % NaCl. They fail to grow at 4°C, and prefer a temperature range of 28-45°C. They grow in the presence of various dyes like brilliant green, crystal violet, pyronine G and neutral red. They are sensitive to the majority of the antibiotics tested.

The mole % G+C ratio of the representative strain from this phenon,**(ANM 712)**, is 45.6 which is with in the range of *V. alginolyticus* (45-47), *V. anguillarum* (44-46), *V. metschnikovii* (44-46), *V mytili* (45-46), *V navarrensis* (45-47), *V. ordalli* (44-46), *V. orientalis* (45-46), *V. pelagius* bv. I and II (45-47), *V. splendidus* bv. I and II(45-46) (Baumann *et al.*, 1984; Pujalte *et al.*, 1993; Urdaci *et al.*, 1991, Yang *et al.*, 1983).

But profound phenotypic disagreement has been observed between these species and the phenon. Unlike the phenon described above, *V. metschnikovii* is oxidase negative , *V mytili*, *V splendidus* bv I and *V. anguillarum* are negative for lysine and ornithine decarboxylase and arginine dihydrolase. *V. navarrensis* *V. ordalli* , *V. pelagius* bv. I and II and *V splendidus* bv II do not decarboxylate lysine and ornithine(Baumann *et al.*, 1984; Urdaci *et al.*, 1991, Ishimaru *et al.*, 1995). *V. orientalis* is different by being luminescent (Yang *et al.*, 1983).

But at the same time the phenon agrees with *V alginolyticus* in several of the phenotypic characters. Both are negative for arginine dihydrolase, gas from glucose and acid from arabinose, inositol and salicine and aesculin hydrolysis. Both are positive for the following characters: growth at 4°C, lysine and ornithine

decarboxylase, acetoin from glucose, nitrate reduction gelatinase and lipase. However there is also a certain degree of disgreement between the phenon and *V. alginolyticus*. Unlike the phenon, *V. alginolyticus* produce acid from sucrose, grow in 6% NaCl, swarm on solid media and utilize leucine and tyrosine (Baumann *et al.*, 1984).

Based on these characteristics it is proposed to look into the DNA homology of the phenon with all the above species of *Vibrio* mentioned above to ascertain the identity.

Phenon-3: Apparently healthy larvae: O/129 positive: OTUs: 5

This phenon contain 5 strains clustered at 90 %S. The strains in this phenon produce, catalase, , indole and reduce nitrate. They utilize ribose, fructose, pyruvate, and gluconate, mannitol and glutamic acid as sole source of carbon and energy. They produce acid without gas from glucose, mannitol and dextrin. They degrade gelatine, casein, tween 20 and 40, tributyrin and grow in a pH range of 8-12, and in the presence of 0.5 to 5 % NaCl. They fail to grow at 4°C but prefer a temperature range of 28-45°C. They grow in the presence of various dyes like brilliant green, crystal violet, pyronine G and neutral red. They are sensitive to antibiotics like oxytetracycline, streptomycin, ampicillin, tetracyclin, gentamycin, polymyxin B, chlortetracyclin, neomycin, neomycin, nalidixic acid, novobiocin, chloramphenicol, erythromycin, and kanamycin.

The representative strain from this phenon (**ANM 719**), has a mole %G+C ratio of 38.3. This is very much in the range for vibrios (38-51) and close to that of *V. fisheri* (39-41). But the phenon differs from *V. fisheri* by being non lunimescent, negative for cell-associated yellow orange pigment and positive for lysine decarboxylase and gelatinase. Besides, the phenon-3 unlike *V. fisheri* does not grow in 6%NaCl and can utilize gluconate and pyruvate. Meanwhile, the phenon like *V. fisheri* is negative for arginine dihydrolase, ornithine decarboxylase, acid from sucrose, utilization of glucuronate and growth at 4°C, but positive for growth at 30°C.

Considering all these the phenon can be designated as *V fisheri* –like even though more studies on the genotypic relatedness and similarities are required before identifying this phenon.

Phenon-4: Apparently healthy larvae: O/129 positive: OTUs: 3

The phenon-4 contains 3 strains clustered at 90 %S. The strains in this phenon are arginine dihydrolase and methyl red positive, produce, catalase, and reduce nitrate; utilize ribose, trehalose, galactose, mannose, fructose, pyruvate, and gluconate, as sole source of carbon and energy. They produce acid without gas from glucose, fructose, trehalose, galactose and mannose. They hydrolyse tween 20 and 40, produce DNase, and grow between a pH range of 8-12, and in the presence of 0.5 to 5 % NaCl. They fail to grow at 4°C but prefer a temperature range of 28-45°C. They grow in the presence of various dyes like brilliant green, crystal violet, pyronine G and neutral red. They are sensitive to most of the antibiotics tested.

The representative strain (**API 1555**), has a mole %GC content of 50.6 which falls in the range of *V. proteolyticus* (50.5) and very close to that of *V. hollisae* (50), *V. costicola* (50) and *V. furnissi* (50) (Baumann *et al.*, 1984; Hickman *et al.*, 1982).

Except for couple of traits, the phenon-4 is in good agreement *with V. proteolyticus*. Both are positive for oxidase, arginine dihydrolase, acetoin from glucose, nitrate reduction, growth at 40°C and utilization of gluconate but negative for pigmentation, growth at 4°C and utilization of sucrose and cellobiose as sole source of carbon and energy The only difference is in the utilization of sorbitol and swarming on solid medium, both traits being negative for phenon-4. Based on these traits the phenon can be designated as *V proteolyticus* –like.

However, considering the closeness of the %G+C ratio with the other species of *Vibrio* listed above, studies on the DNA relatedness to these species must be done before fixing the identity of this phenon.

Phenon-5: Apparently healthy larvae: O/129 positive: OTUs: 4

This phenon contains 4 strains clustered at 90 %S. The strains in this phenon produce catalase, indole and reduce nitrate. They utilize ribose, trehalose, galactose, fructose, pyruvate, and glycogen as sole source of carbon and energy. They produce acid without gas from glucose,fructose, ribose, trehalose, cellobiose, glycogen and dextrin. They hydrolyse gelatin, tween 60 and tributyrin; grow in a pH range of 8-12, in the presence of 0.5 to 5 % NaCl and fail to grow in the absence of NaCl. They fail to grow at 4°C but prefer a temperature range of 28-45°C. They can tolerate and grow in the presence of heavy metals like Fe^{+2}, Pb^{+2}, MoO_4^{-2} and Hg^{+2} and grow in the presence of various dyes like brilliant green, crystal violet, pyronine G and neutral red. They are sensitive to oxytetracycline, streptomycin, tetracyclin, gentamycin, chlortetracyclin, neomycin, methamine mandelate, nalidixic acid, novobiocin, chloramphenicol, erythromycin, kanamycin, lincomycin and sufadiazine.

The representative strain **(API 768)** has a mole %GC content of 60.1% which is in the range for that of *A. hydrophila* (58-62)and close to that of *A. caviae* (61-63) and *A. sobria (*58-60) (Popoff, 1984). The phenon agrees with the motile aeromonads in several traits as they produce acid from glucose, maltose, but not from xylose, adonitol, dulcitol, inositol and malonate, fail to produce H_2S, urease and ornithine decarboxylase. However the phenon differs from the aeromonads by being negative for the following tests: arginine dihydrolase, gas from glucose, Voges Proskauer, acid from arabinose, salicine, sucrose, growth in the absence of NaCl, and aesculin hydrolysis. Apart from that the phenon is sensitive to the O/129 compound whereas the aeromonads in general are resistant.

Considering all these, it is important to study the DNA homology with all the reported species of motile *Aeromonas,* before assigning the phenon a new species /genus status.

Phenon-6: Apparently healthy larvae: O/129 positive: OTUs: 4

The phenon-6 contains 4 strains clustered at 90 %S. The strains in this phenon produce catalase, acetoin from glucose, and reduce nitrate. They utilize ribose, trehalose, fructose, pyruvate, gluconate, mannitol and glutamic acid as sole source of carbon and energy. They produce acid without gas from glucose, trehalose and mannose. They hydrolyse casein and tributyrin; grow in a pH range of 8-12, in the presence of 0.5 to 5 % NaCl and fail to grow in the absence of NaCl. They fail to grow at 4°C but prefer a temperature range of 28-45°C. They can tolerate and grow in the presence of heavy metals like MoO_4^{-2} and Hg^{+2} and grow in the presence of various dyes like brilliant green, crystal violet, pyronine G and neutral red. They are inhibited by almost all the antibiotics tested.

The representative strain (**ANM 721**), has a mole %GC content of 47.3, which falls within the range of *V. cholerae* (47-49), *V. cambelli* (46-48), *V. harveyi* (46-48), *V. vunificus*(46-48), *V. tubiashi* (46-48)(Baumann *et al.*, 1984), marine luminous bacteria of Chumakova *et al.*,(1973). But phenon-6 differs from all the above species by being negative for arginine dihydrolase, lysine and ornithine decarboxylase, acid from sucrose and growth in the presence of 6% NaCl (Baumann *et al.*, 1984).

With the other species such as *V penaecida*(46-47), *V. gazogenes* (47 1), *V. natriegenes* (46-47), *V. nigripulchrituda* (46-47), *V. navarrensis* (45-47) and *V. pelagius* bv. I and II, phenon-6 is very much closer in its %GC content. But these species exhibit differences with the phenon in the following traits. *V penaecida* is negative for growth at 35°C (Ishimaru *et al.*, 1995). *V. gazogenes* produces acid from sucrose and grows in 6% NaCl (Hickman et al., 1983). *V. natriegenes* grows at 20°C and can utilize rhamnose. *V. navarrensis* is positive for citrate utilization, growth in 6% NaCl and acid from sucrose(Urdaci *et al.*, 1991). *V. nigripulchricudo* is different by producing a black pigment and is unable to grow at 35°C (Baumann *et al.*,1984). *V pelagius* biovar I and II grow at 6% NaCl and produce acid from sucrose and fail to grow at 35°C(Baumann *et al.*,1984).

The foregoing discussion suggests that the phenon-6 may be a new species under the genus *Vibrio,*which has to be ascertained by DNA homology studies.

This group also includes phenon7, 8 and 9, all of which comprise only of the reference strains studied along with the test organisms of this group (Fig 2-7 and 2-8).

Phenon-7:Apparently healthy larvae:O/129 positive: OTUs : 5

Phenon-7 comprises of single ATCC strains of *Plesiomonas shigelloides, Salinivibrio costicola, P.angustum, P. leiognathi* and *P phosphoreum* included in this study. This phenon is formed at 81.2%S. Thus phenon-7 contains 5 strains.

Phenon-8 :Apparently healthy larvae: O/129 positive: OTUs: 9

Phenon-8 is clustered at 81.2 %S and contains altogether 9 strains including, *V.parahaemolyticus* ATCC, *V. anguillarum* ATCC, *V. alginolyticus* ATCC, *V. cholerae* Ogawa, *V. harveyi, V. hollisae, V mimicus, V, nereis* and *V. metschnikovii.*

Phenon-9 :Apparently healthy larvae: O/129 positive: OTUs: 2

.Phenon-9 contains 2 reference strains , *V. vulnificus* and *V fisherii,* clustered at 85.9 %S. The characteristics tested for the reference strains are summarized in the Table 2-7

Strains from apparently healthy larvae ,O/129 positive, not clustered

The two strains under the group of isolates obtained from apparently healthy larvae and 0/129 sensitive, which remained unclustered are ANM 702 and API 1519.

Strain –1: ANM 702

ANM 702 joins with the phenon-6 at 85 % S. The mole G+C content of the strain is 63.1% which is beyond the range of *Vibrio* (39-51), *Photobacterium* (40-44) and

Aeromonas (57-63) in general although they remain closer to that of *A.caviae*(61-63). The gross phenotypic dissimilarity of the strain with *A.caviae* is its sensitivity to 0/129 to which all Aeromonads are negative. The strain agrees with *A.caviae* in the following characters: hydrolysis of aesculin, do not produce gas or acetoin from glucose or H2S,do not have brown water soluble pigment, grow at 37°C, produce indole ,ferment mannitol, do not break down mannitol, negative for ODC, growth in 5% NaCl, phenyl alanine deaminase, DNase, gelatinase, catalase. Meanwhile there is a certain degree of dissimilarity also between them. The strain does not utilize arabinose, histidine,arginine and salicine, as sole carbon sources and do not ferment salicine negative for lecithinase,starch hydrolysis,ADH,growth in 0% NaCl. Therefore only by studying the genotypic relatedness between the strain and *A.caviae*, a conclusion can be arrived at. The strain ANM 702 appears to be a new species under the family Aeromonadaceae which is resistant to 0/129 compound as 0/129 sensitivity is not a reliable diagnostic character.

Strain-2 :API 1519

The strain API 1519 joins with the phenon -6 at 81%S. The mole G+C content of the strain is 49.5% which falls in the range of *V.fluvialis* (49-51)(Baumann *et al.*,1984) and is closer to the G+C ratios of *V. hollisae*(50) (Hickmann *et al.*, 1982), *V. costicola*(50), *V. proteolyticus*(50.5) and *V. furnissii*(50) (Baumann *et al.*, 1984). The strain API 1519 exhibit phenotypic similarity with *V. fluvialis* in the following traits: reduction of nitrate negative for growth at 4°C lysine and ornithine decarboxylase, acetoin from glucose and utilization of xylose and arabinose as sole source of carbon and energy; positive for growth upto 40°C and utilize trehalose and pyruvate. They also show profound phenotypic differences as *V. fluvialis* produce lipase and chitinase, utilize mannose but not cellulose and mannitol as sole source of carbon. *V. fluvialis* do not grow at 45°C unlike the strain API 1519; hydrolyze arginine , produce acid from arabinose but grow in the presence of 6% NaCl.

This indicates that even though the %G+C is in the range of *V fluvialis*, the dissimilarities in the phenotypic chracters do not permit the inclusion of the strain API

1519 within this species. DNA homology of the strain API 1519 to *V. fluvialis* and to the other species to which closeness in G+C ratio is exhibited, should be carried out to identify the strain.

2.3.3 Numerical taxonomy of Vibrionaceae associated with the larvae of *M. rosenbergii*: A critical appraisal

Systematics of the family Vibrionaceae associated with the larvae of *M. rosenbergii* was worked out based on the principles of Numerical taxonomy. As described else where, before subjecting the OTUs to the analysis, they had been divided into O/129 sensitive and insensitive, considering the existing understanding that *Vibrio* and *Photobacterium* were sensitive and *Aeromonas* resistant to the vibriostat compound, 2,4- diamino, 6,7-diisopropyl pteridine. To match with them, the type strains were also incorporated into each of the above groups in that order as the type strains of *Vibrio* and *Photobacterium* to the OTUs sensitive to the O/129 compound; and the type strains of *Aeromonas* to the OTUs resistant to the vibriostat compound. But when the analysis was completed, it was surprisingly noticed that the discrete clusters formed at and above a percentage similarity of 80 %S, were not picking up any of the standard strains incorporated. Instead, the type strains clustered together and remained independently.

The situation of the standard strains clustering together have been previously reported by two groups of workers, Kaper *et al.*, (1983) and Lee *at al.* (1978), who observed the presence in a single cluster of all the reference strains which had been in the culture collection for several years. A possible explanation for this observation is that the strains held in a culture collection and transferred many times change in their phenotypic characters, due to loss or gain of genetic material upon repeated sub-culturing over a period of time (Kaper *et al.*, 1983).

The above situation made the identification of the phena or cluster extremely difficult as no standard strains was incorporated in to any of the phena. In order to facilitate the identification of the phena formed and also to characterize them

genotypically, one strain each, from each of the cluster formed was selected as the representative strain and the mole % G+C ratio was determined. This led to another intriguing area of bacterial systematics, where in the sensitivity to the vibriostat compound (O/129), a core character of the family Vibrionaceae was found to be of no use in segregating *Vibrio* and *Phobacterium* from *Aeromonas*. Irrespective of the response of the culture to the O/129 compound, they were with % G+C content falling with in the range for that of either *Vibrio* or *Photobacteium* or *Aeromonas* as summarized in Table 2-8 to 2-11. It was Shewan *et al.*, (1954), who originally proposed the usefulness of O/129 susceptibility for the inter-generic and inter-species differentiation with in the family. Bain and Shewan (1968), later concluded that all the O/129 sensitive Gram negative, bacilli belonged to *Vibrio* sp. The importance of this test in the systematics of Vibrionaceae, has been emphasized by several workers. In fact, Bergey's Manual of Systematic Bacteriology (Baumann and Schubert, 1984), list inhibition by 10µg and 150µg of O/129 as two of the 22 important traits that differentiated the *Vibrio* sp. and allied genera. Bur later, several workers experienced difficulty in applying this as a core diagnostic character and among them Karunasagar *et al.*, (1987), opined that for aquatic vibrios, sensitivity to the Vibriostat compound is not a valid differnetiating trait, on studying the tropical isolates. Later Ramamurthy *et al.*, (1992), observed the emergence of high frequency occurrence of O/129 resistant strains of *V. cholerae* from clinical cases of cholera in Calcutta.

On seeing the phenomenon of non integration of the type strains into the phena formed, the programme was rerun by incorporating all the 20 type stains with each of the four groups. This was done to eliminate any shortcomings, which would otherwise be brought about by grouping the type strains also into two based on their sensitivity to the O 129 compound. In spite of this reanalysis, none of the phena was found to have any of the type strains integrated. This undoubtedly indicated a significant phenotypic dissimilarity between the OTUs and the type strains. It has to be realized that when the dendrograms were constructed using unweighted average linkage, several compact clusters have been formed at high percentage similarity (at and above > 80 %S), which deserves to be called as a cohesive group. Every group of OTUs thus analysed has strains neither joined with the clusters remaining separately,

nor joining with even the type strains. Interestingly, the mole % G+C ratios of the representatives of the phena and the unclustered strains were falling within the range of that of Vibrionaceae and the newly constructed family Aeromonadaceae.

It can be postulated that the members of the family Vibrionaceae and Aeromonadaceae associated with the larvae of *M. rosenbergii* are phenotypic variants of already existing species or new species altogether By determining the mole % G+C ratio of the representative strains it is now possible to narrow down the extent of affiliations to the species of *Vibrio*, *Photobacterium* and *Aeromonas* (Table 2-8, 209, 2-10 and 2-11), and it can be hypothesized that it would be possible to fix the identity of each phena and strain based on DNA homology This is without neglecting the importance of strains and phena, whose mole % G+C ratio is outside the range of that of Vibrionaceae and Aeromonadaceae.

But at the same time, the scientific soundness of comparing a natural group with high information content, which is formed into a compact cluster, to a type strains which is isolated from an extremely different geographical area and repeatedly subcultured over years is questionable. By matching and subsequent joining of such phena to the type strains, one may be able to fix up the identity of the phena to any of the known species. But at the same time, it is a clear departure from the principles of classification of a natural group. What is required at this juncture is to examine the cohesiveness of each phena in terms of genotypic similarity and DNA homology Phenotypic characteristics of each phenon have already been described where all the strains in a particular group are either positive or negative. Likewise genotypically, if they remain cohesive, each phenon formed can be designated as an entity As the next phase the genotypic and phenotypic distance between such groups has to be measured, in order to nomenclature them in a way befitting the tropical organisms. The role of standard strains in this process is minor, just so as to know how far a natural group is away from a type strain. If at all any comparison has to be made, it is to be between the cohesive natural groups called phena of different geographical regions. This novel approach will lead to the development of a new classification scheme for tropical isolates that are characterized by high degree of diversity and information content.

Table 2-1 List of standard strains incorporated

Sl.no.	Species	Strain no.	Source
1.	*V. alginolyticus*	ATCC 17749	Dr. Yen Ling Song, Dept. of Zoology, National Taiwan University, Taipei, Taiwan
2.	*V. harveyi*	ATCC 14126	Same as above
3.	*V. nereis*	ATCC 25917	Same as above
4.	*V. anguillarum*	ATCC 19264	Same as above
5	*V. parahaemolyticus*	ATCC 17802	Same as above
6.	*V. cholerae* Ogawa	MAK 757	National Institute of Cholera and Enteric Diseases, Calcutta
7	*V. hollisae*	CDC 9041-81	Dr. Sumio Shinoda, Faculty of Pharamaceutical Sciences, Okayama University, Japan.
8.	*V. mimicus*	Q 20	Same as above
9	*V. fischeri*	MTCC 1738	Institute of Microbial Technology, Chandigarh, India
10	*V. metschnikovii*	MTCC 866	Same as above
11	*V. vulnificus*	MTCC	Same as above
12.	*A. hydrophila*	172	Dr P.K. Surendran, Central Institute of Fisheries Technology, India
13	*A. hydrophila*	ATCC 7966	From: American Type Culture collection, USA.
14.	*A. caviae*	ATCC 15468	Same as above
15.	*A. salmonicida* subsp. *masoucida*	ATCC 27013	Same as above
16.	*Plesiomonas shigelloides*	ATCC14029	Same as above
17	*Salinivibrio costicola*	ATCC 33508	Same as above
18.	*P. phosphoreum*	ATCC 11040	Same as above
19.	*P. angustum*	ATCC 25915	Same as above
20.	*P. leiognathi*	ATCC 25521	Same as above

Table 2-2 G+C ratio: comparison of the values from Bergey's Manual with the determined ones.

Serial no.	Type strain	Accepted range	Experimental value
1.	*V. mimicus* Q 20	47-49	47.07
2.	*V. fischeri* MTCC 1738	39-41	40.50
3.	*V. cholerae* Ogawa MAK 757	47-49	48.41
4.	*V. alginolyticus* ATCC 17749	45-57	47.30
5.	*A. salmonicida* subsp. *masoucida* ATCC 27013	57-59	57.80
6.	*V. vulnificus* MTCC	46-48	47.63
7.	*V. anguillarum* ATCC 19264	46-48	46.41
8.	*V. nereis* ATCC 25917	46-47	46.30
9.	*A. hydrophila* ATCC 7966	58-62	58.90
10.	*V. hollisae* CDC 9041-81	50	51.95
11.	*E. coli* ATCC 11775	48-52	50.50

Table.2-3. Characteristics of the isolates from moribund larvae (O/129 –ve)

	PHENONS													SINGLE STRAINS[a]						
	1	2	3	4	5	6	7	8	9	10	11	12	13	1	2	3	4	5	6	7
Number of strains /phenon, n =	8	7	3	25	11	10	25	14	8	7	8	3	4							
Biochemical tests																				
1. Methyl red	1	-	1	2	5	8	12	6	3	3	3	3	1	+	+	-	+	-	-	-
2. Voges Proskauer	+	4	+	7	+	9	15	5	1	4	2	-	3	+	+	-	+	-	+	-
3 Catalase	5	+	+	23	10	+	20	+	+	5	7	+	+	+	+	+	+	+	+	-
4 Nitrate reduction	+	6	+	21	+	+	24	+	5	5	3	1	+	-	+	+	+	-	+	+
5 Nitrite reduction	-	-	-	3	-	3	-	5	1	6	4	-	-	-	-	+	-	-	-	-
6 Phenyl alanine deaminase	6	3	2	21	2	1	3	-	4	4	3	-	-	-	+	-	-	-	-	-
7 2-ketolactose prod.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	N	-	N	N
8. O/129 (150μg/mL)		-	-	-		-	-		-	-	-	-			-		-	-		-
9 Lysine decarboxylase	5	-	1	-	7	7	14	13	1	2	-	-	+	-	-	+	+	-	-	-
10. Ornithine decarboxylase	1	1	1	1	9	6	9	9	1	-	-	-	1	-	+	+	+	-	-	-
11. Histidine decarboxylase	1	1	-	5	4	-	-	1	-	-	-	-	1	+	-	-	-	-	-	-
12. Aspartic acid decarboxylase	-	+	2	24	-	7	6	8	4	7	3	-	+	+	+	+	-	+	-	+
13 Arginine dihydrolase	6	-	+	1	2	4	12	6	2	3	3	3	2	-	+	-	-	+	-	+
14. 3-ketogluconate prod.	2	1	-	11	2	9	15	6	2	5	2	2	3	-	-	-	+	-	+	-
15. Nitrogen gas prod.	-	1	-	3	-	-	-	-	1	2	3	-	-	-	-	-	-	-	-	-
16. Indole	+	2	+	18	+	+	24	+	6	+	4	+	-	+	+	+	+	+	-	+
17. Ammonia prod.	+	+	+	24	+	+	23	+	+	+	5	+	+	+	+	+	-	+	-	-
18. H_2S prod. **(TSI)**	-	-	2	20	2	-	-	-	2	2	-	-	-	+	+	-	-	-	-	-
19 Yellow slant	-	1	2	+	2	4	9	2	5	6	1	+	2	+	+	-	+	-	-	+

Table 2-3. Cont'd ..

	Phenons													Single Strains[a]						
	1	2	3	4	5	6	7	8	9	10	11	12	13	1	2	3	4	5	6	7
Number of strains /phenon, n =	**8**	**7**	**3**	**25**	**11**	**10**	**25**	**14**	**8**	**7**	**7**	**3**	**4**							
20. Yellow butt	+	6	+	+	+	+	+	7	+	6	6	+	+	+	+	+	+	-	+	+
21. Gas	-	+	2	5	1	4	6	1	1	4	-	-	3	-	-	-	-	-	+	-
22. Purple slant	+	+	1	4	9	6	11	7	3	1	6	-	-	-	-	+	-	+	+	-
Utilization of sole C-source																				
23. Ribose	+	+	+	23	9	+	+	+	7	6	+	1	+	+	+	+	+	-	+	+
24. Xylose	2	2	+	23	5	7	-	10	+	6	5	2	+	+	+	+	+	+	-	+
25. Raffinose	3	3	-	6	1	1	-	1	2	1	2	-	+	-	-	-	-	-	-	-
26. Melibiose	4	3	-	3	4	4	-	5	6	2	3	2	2	-	+	+	+	-	+	+
27. Rhamnose	3	2	-	4	3	1	-	2	2	-	1	1	3	+	+	-	-	-	+	+
28. Trehalose	+	2	+	6	7	5	22	12	3	6	3	1	+	+	-	+	+	-	-	-
29. Cellobiose	3	6	+	11	6	1	3	4	-	-	3	1	1	+	+	-	-	-	-	-
30. Galactose	+	2	+	19	8	+	11	13	+	+	+	+	+	+	+	+	+	+	+	+
31. Mannose	7	+	2	16	3	+	18	13	+	+	+	+	+	+	-	+	+	+	+	+
32. Fructose	7	+	+	17	6	9	+	13	+	+	+	2	+	+	+	+	+	-	+	-
33. Sucrose	+	+	+	+	7	9	-	12	4	6	+	-	+	+	+	+	+	-	-	-
34. Arabinose	3	5	2	12	5	7	10	11	4	5	3	-	3	+	+	+	-	-	-	+
35. Inulin	4	2	2	5	2	2	-	2	6	3	2	1	-	-	-	-	-	-	+	-
36. Maltose	+	+	+	15	9	4	-	9	1	4	1	-	2	+	+	+	-	+	-	-
37. Glycogen	+	2	+	12	4	9	17	+	+	+	+	+	1	-	-	+	+	+	-	+
38. Sorbitol	7	6	+	8	5	5	-	'13	2	5	4	-	3	+	`-	+	-	-	-	+
39. Sodium pyruvate	+	+	+	24	6	6	+	+	6	+	+	1	+	+	+	+	+	+	-	+	-
40. Calcium lactate	+	+	+	+	+	9	14	13	7	6	+	+	+	+	+	+	-	+	+	+
41. Sodium glucuronate	1	-	-	2	3	3	-	8	-	2	3	-	+	-	-	-	-	+	-	+
42. Lactose	-	-	-	-	-	1	-	5	2	1	-	1	3	-	-	-	-	-	-	+

Table 2-3. Cont'd ..

	Phenons													Single Strains[a]						
	1	2	3	4	5	6	7	8	9	10	11	12	13	1	2	3	4	5	6	7
Number of strains /phenon, n =	**8**	**7**	**3**	**25**	**11**	**10**	**25**	**14**	**8**	**7**	**7**	**3**	**4**							
43. Adonitol	-	+	-	5	1	3	-	7	2		3	2	1	+	-	-	-	-	-	+
44. Tyrosine Browning	+	-	+	10	10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
45. Halozone	6	6	2	11	8	-	-	10	3	5	+	-	2	+	+	+	-	-	-	-
46. Disappearance of crystals	+	+	+	16	10	-	-	11	-	5	+	-	2	+	+	+	-	-	-	-
47. Enhanced growth	+	+	+	16	+	-	-	11	3	5	+	-	2	+	+	+	-	-	-	-
48. Dulcitol	-	1	-	3	-	1	-	5	4	1	2	+	2	-	-	+	-	-	-	+
49. Glycine	5	3	-	3	2	2	5	11	7	3	3	1	1	+	-	+	-	-	-	+
50. Proline	+	+	+	20	5	7	6	12	7	6	6	2	3	+	-	+	+	-	+	+
51. Serine	+	+	+	23	3	5	9	11	6	6	3	1	3	+	+	+	-	-	-	-
52. Cystine	-	1	-	3	1	-	-	-	1	1	-	-	-	-	-	-	-	-	-	-
53. Uracil	-	-	-	1	-	1	-	3	1	-	-	-	-	-	-	-	-	-	-	-
54. Tryptophan	-	+	-	8	1	2	-	3	3	4	1	-	-	+	-	-	-	-	-	-
55. Valine	-	-	-	1	-	-	3	2	2	2	-	-	1	-	-	-	-	-	-	-
56. Sodium malonate	-	-	-	4	-	2	1	1	1	5	3	-	3	+	-	-	-	+	-	-
57. m-inositol	-	+	-	3	1	-	1	3	3	2	6	-	3	+	-	-	-	+	-	+
58. Leucine	2	-	1	9	3	1	2	6	3	2	3	1	2	+	-	+	-	-	-	+
59. Sodium formate	-	-	-	2	-	2	4	1	1	1	2	1	1	-	-	-	-	-	-	-
60. Sodium acetate	5	+	2	22	2	-	4	2	6	3	4	1	2	+	+	-	-	-	-	-
61. Sodium gluconate	+	6	+	23	4	5	22	8	4	5	6	1	3	+	+	+	-	+	+	+
62. Mannitol	+	+	+	4	3	1	18	9	-	1	1	-	1	+	-	+	+	-	+	-
63. Sodium citrate	+	6	1	7	1	4	6	12	2	3	6	-	1	+	-	+	-	+	+	+
64. Glycerol	+	+	+	24	7	8	6	13	+	+	+	-	2	+	+	-	+	-	-	-
65. Salicine	3	2	+	-	1	1	6	-	-	-	-	-	-	+	-	-	+	-	+	-
66. K-Na-tartarate	1	1	1	3	3	1	3	1	1	-	-	-	1	-	-	-	-	-	+	-

Table 2-3. Cont'd ..

	Phenons													Single Strains[a]						
	1	2	3	4	5	6	7	8	9	10	11	12	13	1	2	3	4	5	6	7
Number of strains /phenon, n =	8	7	3	25	11	10	25	14	8	7	7	3	4							
67. Succinic acid	4	1	1	3	4	2	-	3	-	1	1	-	3	-	-	-	-	-	-	-
68. Citrulline	7	-	-	3	1	4	-	12	4	2	1	-	1	-	-	+	-	-	-	-
69. Glutamic acid	+	6	2	+	4	9	24	11	+	+	+	+	+	+	+	+	+	+	+	-
Utilization of sugars for acid/gas production																				
70. Glucose .Acid	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
71. Gas	-	5	2	13	1	2	5	-	1	-	-	-	-	+	+	-	-	-	-	+
72. Fructose .Acid	7	+	+	1	+	6	+	2	3	3	5	+	3	+	-	-	+	-	+	+
73. Gas	1	-	+	-	-	-	5	-	-	-	-	-	-	-	-	-	-	-	+	-
74. Sucrose .Acid	-	-	+	+	8	8	-	6	6	6	3	1	3	+	+	-	+	-	-	+
75. Gas	-	-	+	7	-	3	-	-	-	-	-	-	-	+	+	-	-	-	-	-
76. Lactose .Acid	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
77. Gas	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
78. Maltose .Acid	7	-	+	+	9	8	-	3	+	6	2	-	+	+	+	-	+	-	-	+
79. Gas	1	-	-	7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
80. Ribose .Acid	6	+	+	+	5	3	-	3	7	3	1	1	1	+	+	-	+	+	-	-
81. Gas	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
82. Xylose .Acid	1	-	2	+	-	1	-	-	6	1	-	-	3	+	+	-	-	+	-	-
83. Gas			-	1			-	-			-	-	-				-		-	-
84. Raffinose .Acid	-	-	-	-	-	-	-	-	-	-	-	-	2	-	-	-	-	-	-	-
85. Gas	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
86. Melibiose .Acid	-	2	-	-	-	-	-	-	-	-	-	-	2	+	-	-	-	-	+	-
87. Gas	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-

Table 2-3. Cont'd ..

	Phenon													Single Strains[a]						
	1	2	3	4	5	6	7	8	9	10	11	12	13	1	2	3	4	5	6	7
Number of strains /phenon, n =	8	7	3	25	11	10	25	14	8	7	7	3	4							
88. Rhamnose .Acid	-	-	-	-	-	-	-	-	-	-	-	-	3	-	-	-	-	-	+	-
89. Gas	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
90. Trehalose .Acid	+	2	+	17	,+	8	22	+	2	4	3	1	+	+	+	+	+	-	-	-
91. Gas	-	-	-	-	1	-	4	1	-	1	-	-	2	-	-	-	-	-	-	-
92. Cellobiose .Acid	+	1	-	-	4	-	3	1	-	-	-	-	3	+	-	-	-	-	-	+
93. Gas	-	-	-	-	-	-	-	-	-	-	-	-	2	-	-	-	-	-	-	-
94. Galactose .Acid	2	+	2	22	3	3	11	7	-	2	-	-	3	+	+	+	-	+	+	-
95. Gas	-	-	-	1	-	-	-	-	-	-	-	-	2	-	-	-	-	-	+	-
96. Mannose .Acid	+	+	1	2	2	6	17	12	-	6	3	-	2	+	-	+	-	-	+	+
97. Gas	-	-	-	1	-	1	2	-	-	1	-	-	2	-	-	-	-	-	-	-
98. Arabinose .Acid	2	4	-	9	3	1	10	11	-	1	-	-	3	-	+	+	-	-	+	-
99 Gas	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
100. Glycerol .Acid	+	6	+	18	10	4	8	5	-	5	-	-	1	-	+	-	-	-	-	-
101. Gas	-	-	-	2	-	1	1	1	-	-	-	-	-	-	-	-	-	-	-	-
102. Mannitol .Acid	+	+	+	2	7	8	23	4	-	6	3	-	3	+	-	-	+	-	+	+
103. Gas	1	1	-	-	-	1	4	1	-	-	-	-	-	-	-	-	-	-	-	-
104. Salicine .Acid	-	-	+	-	2	3	7	-	-	5	3	-	-	+	-	-	-	-	+	+
105. Gas	-	-	2	-	-	-	3	-	-	1	-	-	-	-	-	-	-	-	+	-
106. Adonitol .Acid	-	+	-	-	-	-	-	-	-	1	2	-	-	+	-	-	-	-	-	-
107 Gas	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
108. m-inositol .Acid	-	+	-	-	-	-	1	-	-	-	-	-	1	+	-	-	-	-	-	-
109. Gas																				
110. Sorbitol .Acid	5	-	-	-	-	-	-	5	-	-	-	-	3	-	-	-	-	-	-	-
111 Gas	-	-	-	-	-	-	-	-	-	-	-	-	2	-	-	-	-	-	-	-

Table 2-3. Cont'd ..

	Phenons													Single Strains[a]						
	1	2	3	4	5	6	7	8	9	10	11	12	13	1	2	3	4	5	6	7
Number of strains /phenon, n =	**8**	**7**	**3**	**25**	**11**	**10**	**25**	**14**	**8**	**7**	**7**	**3**	**4**							
112. Starch .Acid	-	-	+	-	4	9	3	13	-	2	-	-	1	-	-	+	+	-	-	+
113. Gas	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-
114. Dulcitol .Acid	-	-	-	-	1	-	-	-	-	1	-	-	1	+	-	-	-	-	-	-
115. Gas	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
116. Glycogen .Acid	+	-	+	-	9	1	17	10	-	2	-	1	-	-	-	-	+	-	-	+
117. Gas	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-
118. Inulin .Acid	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
119. Gas	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
120. Butanol .Acid	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
121. Gas	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
122. Ethanol .Acid	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
123. Gas	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
124. Dextrin .Acid	+	1	+	-	10	+	+	12	2	5	-	1	1	-	-	+	+	-	-	+
125. Gas	-	-	-	-	-	2	2	-	-	1	-	-	-	-	-	-	-	-	-	-
Degradation/utilization of compounds																				
126. Gelatin	+	-	+	12	+	1	18	1	6	1	-	-	3	+	+	-	-	+	+	-
127. Tween 20	+	-	+	17	+	6	23	12	4	4	4	-	1	+	+	+	+	-	-	-
128. Tween 40	+	-	+	23	+	6	23	13	+	4	5	-	2	+	+	+	+	-	-	+
129 Tween 60	+	-	+	24	+	6	23	12	+	4	5	-	2	+	+	+	+	-	+	+
130. Tween 80	+	-	+	24	+	6	22	11	5	4	5	-	1	+	+	+	+	-	-	-
131 Urea	-	+	2	24	1	-	-	1	6	3	-	-	1	+	+	-	-	-	-	-
132. Tributyrin	+	-	+	24	+	+	+	7	7	6	5	-	-	+	+	+	+	-	-	-
133 Starch	7	-	+	3	+	9	4	6	-	5	1	1	1	+	-	+	+	-	-	+

Table 2-3. Cont'd ..

	Phenons													Single Strains[a]						
	1	2	3	4	5	6	7	8	9	10	11	12	13	1	2	3	4	5	6	7
Number of strains /phenon, n =	**8**	**7**	**3**	**25**	**11**	**10**	**25**	**14**	**8**	**7**	**7**	**3**	**4**							
134. Casein	+	1	+	+	+	8	21	7	7	5	1	+	1	+	+	-	+	-	+	+
135. α-hemolysis	-	-	-	-	-	-	-	-	1	-		+	-		-	-	-	-	-	-
136. β-hemolysis	6	-	+	-	7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
137. γ-hemolysis	2	+		+	4	+	+	+	7	+	+	-	+	+	+	+	+	+	+	+
138. Zone color:Green	6	-	-	-	1	-	-	-	1	-	-	+	-	-	-	-	-	-	-	-
139. :Colorless	-	-	+	-	5	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-
140. Zone diameter:1mm	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
141. 2-10mm	-	-	+	-	5	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-
142. 10-20mm	3	-	-	-	2	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-
143. 20-40mm	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
144. Chitin	+	-	+	-	9	4	2	7	-	2	-	1	-	-	-	+	-	-	-	-
145. Lecithin	7	-	+	+	+	-	17	1	6	3	1	1	-	+	+	-	-	-	+	-
146. Aesculin	-	2	+	15	9	3	12	5	4	6	4	-	3	+	-	-	-	-	+	-
147. Cellulose	1	-	1	7	1	1	1	4	3	2	-	-	1	-	-	-	-	-	-	+
148. DNAase	-	2	-	-	-	1	19	-	-	1	-	-	1	-	-	-	-	-	+	+
Physiological characters																				
(a)pH tolerance																				
149. Growth at pH5	-	-	-	13	-	4	1	7	6	5	2	1	3	-	-	-	-	-	-	+
150. Growth at pH8	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
151. Growth at pH9	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
152. Growth at pH10	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
153. Growth at pH12	5	1	1	17	6	+	23	+	+	+	+	+	+	-	+	+	+	+	+	+

Table 2-3. Cont'd ..

	Phenons													Single Strains[a]						
	1	2	3	4	5	6	7	8	9	10	11	12	13	1	2	3	4	5	6	7
Number of strains /phenon, n =	8	7	3	25	11	10	25	14	8	7	7	3	4							
(b)NaCl tolerance (% w/v)																				
154. Growth without NaCl	7	+	+	+	+	+	6	4	+	+	+	2	+	+	+	-	+	-	-	+
155. Growth in 0.5% NaCl	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
156. Growth in 1.0% NaCl	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
157. Growth in 2.0% NaCl	+	+	+	+	+	+	2	+	+	+	+	+	+	+	+	+	+	+	+	+
158. Growth in 5.0% NaCl	+	+	+	+	+	9	10	+	7	6	5	-	+	+	+	+	+	+	-	+
159. Growth in 7.5% NaCl	+	+	+	+	+	7	1	10	6	4	3	-	3	+	+	+	+	+	-	+
160. Growth in 10.% NaCl	3	2	1	5	1	-	-	3	2	-	-	-	1	+	+	-	-	-	-	+
161. Growth in 15.% NaCl	2	1	1	2	-	-	-	-	2	-	-	-	-	-	-	-	-	-	-	-
(c) Temperature tolerance																				
162. Growth at 4^0C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
163. at 28^0C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
164. at 37^0C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
165. at 45^0C.	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
166. at 60^0C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
(d)Heavy metal tolerance																				
167. Cadmium (Cd^{+2})	+	+	+	24	3	+	4	13	+	+	3	-	2	+	+	+	+	-	+	-
168. Chromium (Cr^{+3})	-	-	-	7	1	5	2	12	4	5	1	+	2	-	-	+	-	-	-	+
169. Cobalt (Co^{+2})	-	-	-	5	-	2	6	3	2	2	-	1	1	-	-	-	-	-	+	+
170. Iron (Fe^{+2})	+	+	+	+	+	+	22	+	+	+	+	+	+	+	+	+	+	+	+	+
171. Lead (Pb^{+2})	+	+	+	+	+	+	22	+	+	+	+	+	+	+	+	+	+	+	+	+
172. Molybdenum (MoO_4^{-2})	+	+	+	+	+	+	22	+	+	+	+	2	+	+	+	+	+	-	+	+
173. Mercury (Hg^{+2})	1	1	-	13	1	+	22	+	+	+	+	+	+	-	-	+	+	-	+	+

Table 2-3. Cont'd ..

	Phenons													Single Strains[a]						
	1	2	3	4	5	6	7	8	9	10	11	12	13	1	2	3	4	5	6	7
Number of strains /phenon, n =	8	7	3	25	11	10	25	14	8	7	7	3	4							
(e)Growth in the presence of dyes																				
174. Brilliant green	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
175. Crystal violet	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
176. Pyronin G	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
177. Neutral red	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
(f)Requirement of Mg^{+2},K^{+} and Na^{+2} for growth																				
178. Growth in medium without Mg^{+2}	7	+	+	+	9	9	5	11	6	+	+	+	3	+	+	+	-	+	+	+
179. Growth in medium without Na^{+2}	+	+	3	24	7	+	8	+	7	+	6	+	+	+	+	+	+	+	+	+
180. Growth in medium without K^{+}	6	+	+	24	8	9	3	13	6	+	+	+	+	+	+	+	+	+	-	+
(g)Pigment production																				
181. Fluorescein	-	5	-	10	1	5	10	11	5	6	3	1	+	-	+	+	-	+	+	+
182. Pyocyanin	-	+	1	13	3	4	13	10	5	+	3	1	+	-	+	+	-	+	+	+
(h)Length of microorganisms in microns																				
183. 0.5-1.0μ	4	2	-	5	2	1	9	4	-	2	1	-	2	-	-	-	-	+	-	-
184. 1.0-1.5μ	4	4	2	11	3	5	9	4	2	4	1	-	1	+	+	+	+	-		-
185. 1.5-2.0μ		1	-	3	5	1	3	-	1	1	1	2	-		-	-	-		+	
186. 2.0-2.5μ		-	-	6	1	3	6	5	5	-	4	1	1		-	-				+
187. 2.5-3.0μ			1	-	1	-	-	1		-	-			-	-	-		-		-
(i) Colony morphology (1) Diameter																				
188. 0.5 & 0.5mm	-	2	-	-	4	2	7	-	2	-	4	2	1	-	+	-	-	+	-	-
189. 0.5-1.0mm	1	2	-	1	2	4	10	1	3	3	1	1	2	+	-	-	-	-	-	-

Table 2-3. Cont'd ..

	Phenons													Single Strains[a]						
	1	2	3	4	5	6	7	8	9	10	11	12	13	1	2	3	4	5	6	7
Number of strains /phenon, n =	8	7	3	25	11	10	25	14	8	7	7	3	4							
190. 1.0-1.5mm	-	-	1	-	-	2	5	3	-	-	-	-	-	-	-	-	-	-	-	-
191. 1.5-2.0mm	1	3	1	4	2	1	-	3	-	-	-	-	1	-	-	+	+	-	-	-
192. 2.0-2.5mm	-	-	-	-	-	1	1	2	-	-	1	-	-	-	-	-	-	-	+	-
193. 2.5-3.0mm	1	-	-	-	2	-	1	5	-	-	-	-	-	-	-	-	-	-	-	+
(2) Shape																				
194. Circular	2	+	2	5	10	+	24	10	5	4	6	+	+	+	+	-	+	+	+	+
195. Irregular	-	-	-	-	-	-	-	2	-	1	-	-	-	-	-	+	-	-	-	-
196. Spreading	6	-	1	20	1	-	1	4	3	2	1	-	-	-	-	-	-	-	-	-
(3) Margin																				
197. Entire	1	+	1	3	10	+	24	7	3	3	5	+	2	-	-	-	+	+	+	+
198. Undulate	5	-	2	13	1	-	1	2	2	4	-	-	-	-	-	+	-	-	-	-
199. Lobate	-	-	-	5	-	-	-	1	3	-	-	-	-	-	-	-	-	-	-	-
200. Crenated	2	-	-	4	-	-	-	4	-	-	2	-	2	-	-	-	-	-	-	-
(4) Elevation of growth																				
201. Convex	3	+	1	19	10	+	22	10	+	+	6	+	+	+	+	+	+	+	+	+
202. Flat	2	-	1	5	-	-	2	4	-	-	1	-	-	-	-	-	-	-	-	-
203. Low convex	3	-	1	1	1	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-
(5) Optical characters																				
204. Transparent	3	3	1	18	5	2	14	4	3	-	-	-	-	+	+	-	+	-	-	-
205. Translucent	5	4	2	8	6	8	11	9	5	+	6	+	+	-	-	+	-	+	-	+
206. Shining	-	-	-	7	-	8	6	7	6	4	6	2	3	-	-	+	+	-	-	+
207 Opaque	-	-	-	1	-	-	1	-	-	-	-	-	-	-	-	-	-	-	+	-

Table 2-3. Cont'd ..

	Phenons													Single Strains[a]						
	1	2	3	4	5	6	7	8	9	10	11	12	13	1	2	3	4	5	6	7
Number of strains /phenon, n =	**8**	**7**	**3**	**25**	**11**	**10**	**25**	**14**	**8**	**7**	**7**	**3**	**4**							
(6) Consistency																				
208. Butyrous	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
(7) Color of colony																				
209. Colorless	2	+	+	22	+	8	24	6	6	+	4	1	3	+	+	+	+	+	-	-
210. Light brown	6	-	-	3	-	2	-	8	2	-	3	2	1	-	-	-	-	-	-	+
211. White	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	+	-
Antibiotic sensitivity																				
212. Penicillin G	-	-	-	-	7	1	7	-	1	-	-	+	-	-	-	-	+	-	-	-
213. Oxytetracycline	+	1	1	1	10	+	22	9	-	2	3	+	3	-	-	-	+	+	+	-
214. Streptomycin	+	+	+	24	+	+	23	+	+	6	5	+	+	-	+	+	+	+	+	+
215. Ampicillin	-	-	-	-	2	4	8	-	1	1	-	+	-	-	-	-	+	-	-	-
216. Tetracycline	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
217. Gentamycin	+	6	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
218. Polymyxin B	+	1	+	6	9	8	+	12	1	2	3	+	3	-	-	+	+	+	+	+
219. Chlortetracycline	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
220. Neomycin	+	+	+	+	+	+	+	13	6	+	+	+	+	-	+	+	+	+	+	+
221 Methamine mandelate	7	+	+	24	+	+	+	13	6	+	+	+	+	+	+	+	+	+	+	+
222. Cefazolin	2	5	-	-	9	5	24	10	2	-	1	+	1	-	-	+	+	-	-	+
223. Amoxycillin	+	6	-	-	7	2	16	5	-	-	-	+	1	-	-	-	+	-	-	-
224. Nalidixic acid	+	+	+	+	+	+	+	8	5	6	+	+	+	+	+	+	+	+	+	+

Table 2-3. Cont'd ..

	Phenons													Single Strains[a]						
	1	2	3	4	5	6	7	8	9	10	11	12	13	1	2	3	4	5	6	7
Number of strains /phenon, n =	**8**	**7**	**3**	**25**	**11**	**10**	**25**	**14**	**8**	**7**	**7**	**3**	**4**							
225. Novobiocin	+	-	+	16	+	+	+	12	7	4	4	+	1	-	+	+	+	-	-	-
226. Chloramphenicol	+	+	+	+	+	9	+	+	7	6	5	+	+	+	+	+	+	+	+	+
227. Erythromycin	7	1	1	4	'9	7	20	9	4	2	2	+	1	-	-	+	+	+	+	+
228. Kanamycin	+	+	+	+	+	8	+	+	5	+	+	+	3	-	+	+	+	+	+	+
229. Bacitracin	3	-	2	3	+	5	12	3	2	1	1	+	-	-	-	-	+	-	-	-
230. Lincomycin	5	-	1	3	4	3	17	5	-	1	1	+	0	-	-	+	+	-	-	-
231. Sufadiazine	7	-	+	4	8	2	24	2	-	-	-	-	-	-	-	-	+	-	-	-
Production of																				
232. Levan	-	-	-	1	-	-	1	3	-	-	3	-	2	-	-	-	-	-	-	-
233 Glucan	-	-	-	4	-	-	-	1	-	-	2	-	-	-	-	-	-	-	-	-

a: strains which did not cluster (1: AAC 672b$_1$, 2:AAC 669B,3: AAC 1108d, 4:API 644b,5: AAC 654b,6: AAC 536,7: ANM 1008a)
+: all strains are positive
all strains are negative
numbers :indicate the number of strains in each phenon that are positive to a particular test
N: not done

Table.2-4 Characteristics of the isolates from moribund larvae (O/129 +ve)

	Phenon			Single Strains[a]				
	1	2	3	1	2	3	4	5
Number of strains /phenon ,n=	**17**	**3**	**39**					
Biochemical tests								
1. Methyl red	3	2	34	+	+	-	-	+
2. Voges Proskauer	16	1	34	+	+	-	+	-
3. Catalase	+	2	38	+	+	+	+	-
4. Nitrate reduction	+	+	37	+	+	+	-	+
5. Nitrite reduction	-	-	2	-	-	-	-	-
6. Phenyl alanine deaminase	-	-	2	-	-	-	-	-
7. 2-ketolactose prod.	-	-	-	N	N	N	N	N
8. O/129 (150μg/mL)	+	+	+	+	+	+	+	+
9. Lysine decarboxylase	+	-	33	+	+	-	-	+
10. Ornithine decarboxylase	+	-	31	+	+	-	-	-
11. Histidine decarboxylase	-	-	-	-	-	-	-	-
12. Aspartic acid decarboxylase	10	-	5	-	-	-	-	+
13. Arginine dihydrolase	2	-	3	-	-	-	-	+
14. 3-ketogluconate prod.	15	-	26	+	+	-	-	-
15. Nitrogen gas prod.	-	-	-	-	-	-	-	-
16. Indole	16	2	36	+	+	-	+	+
17. Ammonia prod.	+	2	36	+	-	-	+	+
18. H_2S prod. **(TSI)**	-	-	-	-	-	-	-	-
19. Yellow slant	13	2	24	+	+	+	+	-
20. Yellow butt	+	+	+	+	+	+	+	+
21. Gas	-	-	-	-	-	-	-	+
22. Purple slant	5	1	11	+	-	-	+	-
Utilization of sole C-source								
23. Ribose	15	+	+	-	+	+	+	+
24. Xylose	14	1	-	+	+	+	+	+
25. Raffinose	1	-	-	-	-	+	+	-
26. Melibiose	2	-	1	+	+	+	+	-
27. Rhamnose	3	-	5	-	-	-	+	-
28. Trehalose	14	+	16	-	+	+	+	-
29. Cellobiose	3	2	3	-	-	+	+	-
30. Galactose	+	+	4	+	+	+	+	+
31. Mannose	16	2	5	+	+	+	+	-
32. Fructose	15	2	37	+	+	-	+	+
33 Sucrose	13	+	-	+	+	+	+	+

Table 2-4. Cont'd ..

	Phenons			Single Strains[a]				
	1	**2**	**3**	**1**	**2**	**3**	**4**	**5**
Number of strains /phenon ,n=	**17**	**3**	**39**					
34. Arabinose	9	2	2	-	-	+	+	+
35. Inulin	4	1	-	+	-	-	+	-
36. Maltose	4	1	2	-	-	-	-	-
37. Glycogen	15	+	32	+	+	+	+	-
38. Sorbitol	5	2	1	+	-	-	+	-
39. Sodium pyruvate	11	2	+	+	+	-	+	-
40. Calcium lactate	12	+	10	+	-	+	+	+
41. Sodium glucuronate	7	1	-	-	-	-	+	-
42. Lactose	1	-	-	-	-	+	+	-
43. Adonitol	6	2	-	-	-	+	+	-
44. Tyrosine . Browning	-	-	-	-	-	-	-	-
45. Halozone	2	+	-	-	-	-	-	-
46. Disappearance of crystals	2	+	-	-	-	-	-	-
47. Enhanced growth	2	+	-	-	-	-	-	-
48. Dulcitol	2	-	-	-	-	+	+	-
49. Glycine	5	-	5	-	-	-	+	-
50. Proline	11	-	11	+	+	-	+	+
51. Serine	7	-	4	+	-	-	-	-
52. Cystine	-	1	-	-	-	-	-	-
53. Uracil	-	1	-	-	-	-	-	-
54. Tryptophan	2	+	-	-	-	-	-	-
55. Valine	2	2	1	-	-	-	-	-
56. Sodium malonate	2	1	-	-	-	-	-	-
57. m-inositol	-	-	-	-	-	-	+	-
58. Leucine	2	-	4	-	-	-	+	-
59. Sodium formate	2	2	5	-	-	-	+	-
60. Sodium acetate	-	1	3	-	-	-	+	-
61. Sodium gluconate	-	-	28	-	-	-	+	-
62. Mannitol	1	-	27	-	+	+	-	+
63. Sodium citrate	10	-	8	+	-	-	+	-
64. Glycerol	13	2	1	+	+	-	+	+
65. Salicine	-	1	-	-	-	-	-	-
66. K-Na-tartarate	1	1	5	-	-	-	-	-
67. Succinic acid	-	-	1	-	-	-	-	-
68. Citrulline	4	2	-	-	-	-	-	-
69. Glutamic acid	14	+	24	+	+	-	+	-
Utilization of sugars for acid/gas production								
70. Glucose .Acid	+	+	+	+	+	+	+	+
71. Gas	-	-	-	-	-	-	-	-

Table 2-4.Cont'd ..

	Phenons			Single Strains[a]				
	1	2	3	1	2	3	4	5
Number of strains /phenon ,n=	**17**	**3**	**39**					
72. Fructose .Acid	2	1	37	+	+	+	+	+
73. Gas	-	-	3	-	-	-	-	-
74. Sucrose .Acid	15	+	-	+	+	+	+	-
75. Gas	1	-	-	-	-	-	-	-
76. Lactose .Acid	-	-	-	-	-	+	-	-
77. Gas	-	-	-	-	-	-	-	-
78. Maltose .Acid	10	+	2	+	+	+	+	+
79. Gas	-	-	-	-	-	-	-	-
80. Ribose :Acid	-	-	2	-	+	+	+	+
81. Gas	-	-	-	-	-	-	-	-
82. Xylose .Acid	-	-	-	-	-	+	-	-
83. Gas	-	-	-	-	-	-	-	-
84. Raffinose .Acid	-	-	-	-	-	-	-	-
85. Gas	-	-	-	-	-	-	-	-
86. Melibiose .Acid	-	-	1	-	-	+	-	-
87. Gas	-	-	-	-	-	-	-	-
88. Rhamnose .Acid	-	1	2	-	-	-	-	-
89. Gas	-	-	-	-	-	-	-	-
90. Trehalose .Acid	15	+	15	-	+	+	+	-
91. Gas	-	-	-	-	-	-	-	-
92. Cellobiose .Acid	-	+	3	-	-	+	+	-
93. Gas	-	-	-	-	-	-	-	-
94. Galactose .Acid	3	2	4	-	-	+	+	+
95. Gas	-	-	-	-	-	-	-	-
96. Mannose .Acid	-	+	4	-	-	-	+	+
97. Gas	-	-	-	-	-	-	-	-
98. Arabinose .Acid	-	-	3	-	-	+	+	-
99. Gas	-	-	-	-	-	-	-	-
100. Glycerol .Acid	1	1	3	-	-	-	-	-
101. Gas	-	-	-	-	-	-	-	-
102. Mannitol .Acid	11	+	34	+	+	+	+	-
103. Gas	-	-	-	-	-	-	-	-
104. Salicine :Acid	-	-	-	-	-	-	+	-
105. Gas	-	-	-	-	-	-	-	-
106. Adonitol .Acid	-	-	-	-	-	-	-	-
107. Gas	-	-	-	-	-	-	-	-
108. m-inositol .Acid	-	-	-	-	-	-	-	-
109. Gas	-	-	-		-	-	-	-
110. Sorbitol .Acid	-	1	1	-	-	-	-	-
111. Gas	-	-	-	-	-	-	-	-

Table 2-4. Cont'd ..

	phenons			Single strains[a]				
	1	**2**	**3**	**1**	**2**	**3**	**4**	**5**
Number of strains /phenon ,n=	**17**	**3**	**39**					
112. Starch .Acid	16	1	9	-	+	-	+	-
113. Gas	-	-	-	-	-	-	-	-
114. Dulcitol .Acid	-	-	-	-	-	-	-	-
115. Gas	-	-	-	-	-	-	-	-
116. Glycogen .Acid	6	2	32	-	+	-	-	-
117. Gas	-	-	-	-	-	-	-	-
118. Inulin .Acid	-	-	-	-	-	-	-	-
119. Gas	-	-	-	-	-	-	-	-
120. Butanol .Acid	-	-	-	-	-	-	-	-
121. Gas	-	-	-	-	-	-	-	-
122. Ethanol .Acid	-	-	-	-	-	-	-	-
123. Gas	-	-	-	-	-	-	-	-
124. Dextrin .Acid	16	+	37	+	+	-	-	-
125. Gas	2	-	-	-	-	-	-	-
Degradation/utilization of compounds								
126. Gelatin	-	1	34	-	-	-	-	-
127. Tween 20	15	+	29	+	+	+	-	+
128. Tween 40	16	+	27	-	+	+	-	+
129. Tween 60	15	+	34	-	+	+	-	+
130. Tween 80	16	-	34	+	+	+	-	+
131. Urea	-	1	-	-	-	-	-	-
132. Tributyrin	16	+	38	+	+	+	+	+
133. Starch	16	+	8	+	+	-	-	-
134. Casein	+	2	36	+	+	-	+	-
135. α-hemolysis	-	-	-	-	-	-	-	-
136. β-hemolysis	-	-	1	-	-	-	-	-
137. γ-hemolysis	+	+	38	+	+	+	+	+
138. Zone color:Green	-	-	-	-	-	-	-	-
139. Colorless	-	-	1	-	-	-	-	-
140. Zone diameter:1mm	-	-	-	-	-	-	-	-
141. 2 -10mm	-	-	1	-	-	-	-	-
142. 10-20mm	-	-	-	-	-	-	-	-
143. 20-40mm	-	-	-	-	-	-	-	-
144. Chitin	6	1	3	-	-	-	-	-
145. Lecithin	1	-	20	-	-	-	-	-
146. Aesculin	1	-	4	-	-	-	+	-
147. Cellulose	1	-	3	+	-	-	-	+
148. DNAase	-	-	25	-	-	-	-	-

Table 2-4. Cont'd ..

	Phenons			Single Strains[a]				
	1	2	3	1	2	3	4	5
Number of strains /phenon ,n=	17	3	39					
Physiological characters								
(a) pH tolerance								
149. Growth at pH5	2	-	1	+	-	-	+	+
150. Growth at pH8	+	+	+	+	+	+	+	+
151. Growth at pH9	+	+	+	+	+	+	+	+
152. Growth at pH10	+	+	+	+	+	+	+	+
153. Growth at pH12	+	+	+	+	+	+	+	+
(b) NaCl tolerance (% w/v)								
154. Growth without NaCl	15	+	5	+	+	+	+	+
155. Growth in 0.5% NaCl	+	+	+	+	+	+	+	+
156. Growth in 1.0% NaCl	+	+	+	+	+	+	+	+
157. Growth in 2.0% NaCl	+	+	+	+	+	+	+	+
158. Growth in 5.0% NaCl	10	+	12	+	+	+	+	+
159. Growth in 7.5% NaCl	4	-	1	-	+	+	+	+
160. Growth in 10.% NaCl	1	-	-	-	-	-	+	-
161. Growth in 15.% NaCl	-	-	-	-	-	-	+	-
(c) Temperature tolerance								
162. Growth at 4^0C	-	-	-	-	+	-	-	-
163. at 28^0C	+	+	+	+	+	+	+	+
164. at 37^0C	+	+	+	+	+	+	+	+
165. at 45^0C.	+	+	+	+	+	+	+	+
166. at 60^0C	-	-	-	-	-	-	+	-
(d) Heavy metal tolerance								
167. Cadmium (Cd^{+2})	+	+	3	+	+	-	-	-
168. Chromium (Cr^{+3})	4	-	4	-	-	-	+	-
169. Cobalt (Co^{+2})	-	1	4	-	-	-	+	-
170. Iron (Fe^{+2})	+	+	36	+	+	+	+	+
171. Lead (Pb^{-2})	+	+	32	+	+	+	+	+
172. Molybdenum (MoO_4^{-2})	+	+	32	+	+	-	+	+
173. Mercury (Hg^{+2})	+	+	32	+	+	+	+	+
(e) Growth in the presence of dyes								
174. Brilliant green	+	+	+	+	+	+	+	+
175. Crystal violet	+	+	+	+	+	+	+	+
176. Pyronin G	+	+	+	+	+	+	+	+
177. Neutral red	+	+	+	+	+	+	+	+
(f) Requirement of Mg^{+2},K^+ and Na^{+2} for growth								
178. Growth in medium withoutMg^{+2}	+	+	9	+	-	+	+	+
179. Growth in medium withoutNa^{+2}	+	+	7	+	+	+	-	+
180. Growth in medium without K^+	+	2	3	-	+	+	+	+

Table 2-4. Cont'd ..

	Phenons			Single Strains[a]				
	1	2	3	1	2	3	4	5
Number of strains /phenon ,n=	**17**	**3**	**39**					
(g) Pigment production								
181. Fluorescein	3	+	11	-	-	+	+	+
182. Pyocyanin	3	+	9	-	-	+	+	+
(h) Length of microorganisms in microns								
183. 0.5-1.0μ	2	-	7	-	-	-		-
184. 1.0-1.5μ	9	1	28	-	+	-	+	
185. 1.5-2.0μ	2	1	3	-		+		-
186. 2.0-2.5μ	2	-	1	+		-		+
187. 2.5-3.0μ	-	1		-	-	-		-
(i) Colony morphology **(1) Diameter**								
188. 0.5 & 0.5mm	2	-	19	-	-	+	-	-
189 0.5-1.0mm	6	-	8	-	-	-	+	-
190. 1.0-1.5mm	7	-	7	-	-	-	-	-
191. 1.5-2.0mm	3	-	4	-	+	-	-	+
192. 2.0-2.5mm	-	-	-	-	-	-	-	-
193. 2.5-3.0mm	-	3	1	+	-	-	-	-
(2) Shape								
194. Circular	+	+	37	-	+	+	-	-
195. Irregular	-	-	2	+	-	-	+	+
196. Spreading	-	-	-	-	-	-	-	-
(3) Margin								
197. Entire	16	+	37	-	+	-	-	-
198. Undulate	1	-	-	-	-	-	-	-
199. Lobate	-	-	-	-	-	-	-	-
200. Crenated	-	-	2	+	-	+	+	+
(4) Elevation of growth								
201. Convex	+	+	38	+	+	-	-	-
202. Flat	-	-	-	-	-	+	+	+
203. Low convex	-	-	1	-	-	-	-	-
(5) Optical characters								
204. Transparent	7	-	26	-	+	+	-	-
205. Translucent	10	+	12	+	-	-	+	+
206. Shining	13	2	14	+	+	-	+	+
207. Opaque	-	-	1	-	-	-	-	-
(6) Consistency								
208. Butyrous	+	+	+	+	+	+	+	+

Table 2-4. Cont'd ..

	phenons			Single strains[a]				
	1	2	3	1	2	3	4	5
Number of strains /phenon ,n=	17	3	39					
(7) Color of colony								
209. Colorless	11	2	35	-	+	+	+	+
210. Light brown	6	1	2	+	-	-	-	-
211. White	-	-	2	-	-	-	-	-
Antibiotic sensitivity								
212. Penicillin G	12	-	35	-	+	+	-	-
213. Oxytetracycline	10	-	38	+	+	+	+	+
214. Streptomycin	+	+	+	+	+	+	+	+
215. Ampicillin	12	-	25	-	+	+	-	-
216. Tetracycline	+	+	+	+	+	+	+	+
217. Gentamycin	+	+	+	+	+	+	+	+
218. Polymyxin B	12	-	27	+	+	+	+	+
219. Chlortetracycline	+	+	+	+	+	+	+	+
220. Neomycin	+	+	+	+	+	+	+	+
221. Methamine mandelate	+	+	+	+	+	+	+	+
222. Cefazolin	12	+	38	+	+	+	-	-
223. Amoxycillin	4	-	36	-	+	-	-	-
224. Nalidixic acid	+	+	+	+	+	+	+	+
225. Novobiocin	16	+	38	-	+	+	-	-
226. Chloramphenicol	16	+	+	+	+	+	+	+
227. Erythromycin	12	+	36	-	+	+	-	-
228. Kanamycin	16	+	+	+	+	+	+	+
229. Bacitracin	13	1	33	+	+	+	-	-
230. Lincomycin	10	-	36	-	+	+	-	-
231. Sufadiazine	3	-	35	+	+	-	+	-
Production of								
232. Levan	-	-	-	+	-	-	-	-
233. Glucan	-	-	1	-	-	-	-	-

a: single strains which did not cluster (1: ANM 708; 2: ANM 610a; 3: ANM 1020; 4: ANM594a; 5: ANM 1003)

+: all strains are positive

all strains are negative

numbers :indicate the number of strains in each phenon that are positive to a particular test

N: not done

Table.2-5. Characteristics of the isolates from healthy larvae (O/129 –ve)

	Phenons				Single strains[a]						
	1	**2**	**3**	**4**	**1**	**2**	**3**	**4**	**5**	**6**	**7**
Number of strains /phenon ,n=	**4**	**51**	**6**	**7**							
Biochemical tests											
1. Methyl red	+	3	2	+	+	+	-	-	-	-	-
2. Voges Proskauer	0	41	3	5	-	-	-	+	+	-	+
3. Catalase	+	48	+	+	+	+	+	+	+	+	+
4. Nitrate reduction	2	44	4	+	+	+	-	+	-	-	-
5. Nitrite reduction	2	22	2	4	-	-	-	+	-	+	+
6. Phenyl alanine deaminase	3	8	1	1	-	+	-	-	+	-	-
7. 2-ketolactose prod.	-	-	-	-	N	N	N	N	N	N	N
8. O/129 (150μg/mL)	-	-	-	-	-	-			-	-	-
9. Lysine decarboxylase	-	8	2	2	-	-	-	-	-	+	-
10. Ornithine decarboxylase	-	5	2	1	-	-	-	-	-	+	-
11. Histidine decarboxylase	-	-	-	-	-	-	-	-	-	-	-
12. Aspartic acid decarboxylase	-	2	3	-	-	-	-	-	-	+	-
13. Arginine dihydrolase	-	39	-	+	+	-	-	-	-	-	+
14. 3-ketogluconate prod.	-	38	1	-	-	+	-	+	+	-	+
15. Nitrogen gas prod.	-	-	-	-	-	-	-	-	-	+	-
16. Indole	-	+	3	1	+	-	-	-	+	-	+
17. Ammonia prod.	+	34	1	1	+	+	+	+	+	-	+
18. H_2S prod. **(TSI)**	+	2	-	-	-	+	-	-	+	-	-
19. Yellow slant	+	7	5	2	+	-	+	-	-	-	-
20. Yellow butt	+	+	+	+	+	+	+	+	+	+	+
21. Gas	3	1	1	5	-	-	-	-	+	-	-
22. Purple slant	-	44	2	5	-	+	-	+	+	+	+
Utilization of sole C-source											
23. Ribose	+	40	+	+	+	+	+	+	+	+	+
24. Xylose	2	-	-	-	-	-	-	-	-	-	-
25. Raffinose	-	-	-	-	-	-	-	-	-	-	-
26. Melibiose	1	2	1	-	-	-	-	+	-	-	-
27. Rhamnose	2	2	1	-	-	-	-	+	-	-	-
28. Trehalose	3	9	5	+	+	-	+	-	+	-	-
29. Cellobiose	-	6	2	1	-	-	-	+	-	-	-
30. Galactose	+	7	-	5	+	+	-	-	+	-	-
31. Mannose	-	6	1	+	+	+	-	-	+	-	+
32. Fructose	-	47	2	+	+	+	+	-	+	-	+
33. Sucrose	-	-	-	-	-	-	-	-	-	-	-

Table 2-5.Cont'd ..

	Phenons				Single strains[a]						
	1	2	3	4	1	2	3	4	5	6	7
Number of strains /phenon ,n=	**4**	**51**	**6**	**7**							
34. Arabinose	-	-	-	-	-	-	-	-	-	-	-
35. Inulin	-	-	-	-	-	-	-	-	-	-	-
36. Maltose	2	5	5	2	-	-	+	+	-	-	-
37. Glycogen	-	12	1	-	-	-	-	-	-	-	-
38. Sorbitol	-	-	-	-	+	-	-	-	-	-	-
39. Sodium pyruvate	+	+	+	+	+	+	+	+	+	+	+
40. Calcium lactate	1	30	3	1	+	-	-	-	-	+	-
41. Sodium glucuronate	-	10	+	+	-	-	-	-	-	-	-
42. Lactose	-	-	1	-	-	-	-	-	-	-	-
43. Adonitol	-	-	-	-	-	+	-	-	+	-	-
44. Tyrosine . Browning	-	-	-	-	-	-	-	-	-	-	-
45. Halozone	-	3	-	-	-	-	-	-	-	+	+
46. Disappearance of crystals	-	3	-	-	-	-	-	-	-	+	+
47. Enhanced growth	-	3	-	-	-	-	-	-	-	+	+
48. Dulcitol	-	-	-	-	-	-	-	-	-	-	-
49. Glycine	1	12	1	1	-	-	-	-	-	-	-
50. Proline	2	10	1	-	+	+	-	-	-	-	-
51. Serine	2	29	1	1	-	-	-	-	-	+	-
52. Cystine	-	-	-	-	-	-	-	-	-	-	-
53. Uracil	-	-	-	-	-	-	-	-	-	-	-
54. Tryptophan	-	-	-	-	-	+	-	+	-	+	+
55. Valine	-	6	1	1	-	-	-	-	-	+	+
56. Sodium malonate	-	7	-	-	-	-	-	-	-	+	+
57. m-inositol	-	31	-	-	-	+	-	-	-	-	-
58. Leucine	1	9	1	-	-	-	-	-	-	-	+
59. Sodium formate	1	4	2	2	-	+	+	-	-	+	+
60. Sodium acetate	2	22	2	5	+	+	+	+	+	-	+
61. Sodium gluconate	+	+	+	+	+	+	+	+	+	+	+
62. Mannitol	1	44	-	1	+	+	+	-	+	+	+
63. Sodium citrate	1	22	1	-	+	-	-	-	-	+	+
64. Glycerol	1	1	-	-	-	+	-	-	+	-	-
65. Salicine	-	-	1	-	-	+	-	-	+	-	-
66. K-Na-tartarate	-	6	2	4	-	-	-	-	-	+	-
67. Succinic acid	-	2	-	-	-	-	-	-	-	-	+
68. Citrulline	-	11	-	-	-	-	-	-	-	-	+
69. Glutamic acid	1	46	+	3	+	-	+	+	-	-	+
Utilization of sugars for acid/gas production											
70. Glucose .Acid	+	+	+	+	+	+	+	+	+	+	+
71. Gas	1	-	-	-	-	-	-	-	-	-	-

Table 2-5.Cont'd ..

	Phenons				Single strains[a]						
	1	2	3	4	1	2	3	4	5	6	7
Number of strains /phenon ,n=	**4**	**51**	**6**	**7**							
72. Fructose .Acid	-	48	2	+	+	+	+	+	+	+	+
73. Gas	-	-	-	1	-	-	-	-	+	-	-
74. Sucrose .Acid	-	-	-	-	-	-	-	-	-	-	-
75. Gas	-	-	-	-	-	-	-	-	-	-	-
76. Lactose .Acid	-	-	1	-	-	-	-	-	-	-	-
77. Gas	-	-	-	-	-	-	-	-	-	-	-
78. Maltose .Acid	2	4	4	2	-	-	+	+	-	-	-
79. Gas	1	-	-	-	-	-	-	-	-	-	-
80. Ribose .Acid	2	4	2	2	-	-	-	-	-	-	-
81. Gas	-	-	-	-	-	-	-	-	-	-	-
82. Xylose .Acid	2	-	-	-	-	-	-	-	-	-	-
83. Gas	-	-	-	-	-	-	-	-	-	-	-
84. Raffinose .Acid	-	-	-	-	-	-	-	-	-	-	-
85. Gas	-	-	-	-	-	-	-	-	-	-	-
86. Melibiose .Acid	1	1	1	-	-	-	-	+	-	-	-
87. Gas	-	-	-	-	-	-	-	-	-	-	-
88. Rhamnose .Acid	2	1	1	-	-	-	-	+	-	-	-
89. Gas	-	-	-	-	-	-	-	-	-	-	-
90. Trehalose .Acid	4	9	5	+	+	-	+	+	+	-	-
91. Gas	1	-	-	3	-	-	-	-	+	-	-
92. Cellobiose .Acid	-	9	2	-	-	-	-	+	-	-	-
93. Gas	-	1	-	-	-	-	-	-	-	-	-
94. Galactose .Acid	4	7	-	5	+	+	-	-	+	-	-
95. Gas	-	-	-	-	-	+	-	-	+	-	-
96. Mannose .Acid	-	4	-	+	+	+	-	-	+	-	+
97. Gas	-	-	-	-	-	-	-	-	-	-	-
98. Arabinose .Acid	-	-	-	-	+	-	-	-	-	-	-
99. Gas	-	-	-	-	-	-	-	-	-	-	-
100. Glycerol .Acid	1	1	-	-	-	+	-	-	+	-	-
101. Gas	1	1	-	-	-	-	-	-	+	-	-
102. Mannitol .Acid	2	46	-	1	+	+	+	-	+	+	+
103. Gas	-	-	-	-	-	-	+	-	-	-	-
104. Salicine .Acid	-	-	1	-	-	+	-	+	+	-	+
105. Gas	-	-	-	-	-	-	-	-	+	-	-
106. Adonitol .Acid	-	-	-	-	-	+	-	+	+	-	-
107. Gas	-	-	-	-	-	-	-	-	-	-	-
108. m-inositol .Acid	-	29	-	-	-	+	-	-	-	-	-
109. Gas	-	-	-	-	-	-	-	-	-	-	-

Table 2-5.Cont'd

	phenons				Single strains[a]						
	1	**2**	**3**	**4**							
Number of strains /phenon ,n=	**17**	**51**	**6**	**7**							
110. Sorbitol .Acid	-	-	-	-	+	-	-	-	-	-	-
111. Gas	-	-	-	-	-	-	-	-	-	-	-
112. Starch .Acid	-	1	-	-	-	-	-	-	-	-	-
113. Gas	-	-	-	-	-	-	-	-	-	-	-
114. Dulcitol .Acid	-	-	-	-	-	-	-	-	-	-	-
115. Gas	-	-	-	-	-	-	-	-	-	-	-
116. Glycogen .Acid	-	12	1	-	-	-	-	-	-	-	-
117. Gas	-	1	-	-	-	-	-	-	-	-	-
118. Inulin .Acid	-	-	-	-	-	-	-	-	-	-	-
119. Gas	-	-	-	-	-	-	-	-	-	-	-
120. Butanol .Acid	-	-	-	-	-	-	-	-	-	-	-
121. Gas	-	-	-	-	-	-	-	-	-	-	-
122. Ethanol .Acid	-	-	-	-	-	-	-	-	-	-	-
123. Gas	-	-	-	-	-	-	-	-	-	-	-
124. Dextrin .Acid	-	20	-	1	-	-	-	-	-	-	-
125. Gas	-	-	-	-	-	-	-	-	-	-	-
Degradation/utilization of compounds											
126. Gelatin	+	43	+	5	+	+	+	+	-	+	-
127. Tween 20	+	43	1	+	+	+	-	-	+	+	-
128. Tween 40	+	41	2	+	+	+	+	+	-	+	-
129. Tween 60	3	43	1	5	-	+	+	+	+	+	+
130. Tween 80	+	44	-	5	+	+	+	+	+	-	+
131. Urea	3	3	-	-	-	+	-	-	+	-	-
132. Tributyrin	+	49	2	1	+	-	+	+	+	-	+
133. Starch	-	1	-	-	-	-	-	-	-	-	-
134. Casein	1	43	1	2	-	-	+	+	+	-	+
135. α-hemolysis			-	-	-	-	-				-
136. β-hemolysis	-	-	-	-	-	-	-	-	-	-	-
137. γ-hemolysis	+	+	+	+	+	+	+	+	+	+	+
138. Zone color:Green	-	-	-	-	-	-	-	-	-	-	-
139. Colorless	-	-	-	-	-	-	-	-	-	-	-
140. Zone diameter:1mm	-	-	-	-	-	-	-	-	-	-	-
141. 2-10mm	-	-	-	-	-	-	-	-	-	-	-
142. 10-20mm	-	-	-	-	-	-	-	-	-	-	-
143. 20-40mm	-	-	-	-	-	-	-	-	-	-	-
144. Chitin	-	2	-	-	-	-	-	-	-	-	-
145. Lecithin	+	41	2	1	-	+	+	+	+	-	+
146. Aesculin	-	1	2	2	+	+	+	+	+	-	+

Table 2-5.Cont'd ..

	Phenons				Single Strains[a]						
	1	2	3	4	1	2	3	4	5	6	7
Number of strains /phenon ,n=	17	51	6	7							
147. Cellulose	-	4	2	-	+	+	-	+	-	+	-
148. DNA	-	39	2	6	+	+	-	+	-	+	-
Physiological characters											
(a) pH tolerance											
149. Growth at pH5	-	1	-	-	-	-	-	-	-	+	-
150. Growth at pH8	+	+	+	+	+	+	+	+	+	+	+
151. Growth at pH9	+	+	+	+	+	+	+	+	+	+	+
152. Growth at pH10	+	+	+	+	+	+	+	+	+	+	+
153. Growth at pH12	+	49	4	+	+	+	-	+	+	+	+
(b) NaCl tolerance (% w/v)											
154. Growth without NaCl	3	5	2	1	-	-	+	+	+	+	+
155. Growth in 0.5% NaCl	+	+	+	+	+	+	+	+	+	+	+
156. Growth in 1.0% NaCl	+	+	+	+	+	+	+	+	+	+	+
157. Growth in 2.0% NaCl	+	+	+	+	+	+	+	+	+	+	+
158. Growth in 5.0% NaCl	+	11	1	4	+	+	-	-	+	+	+
159. Growth in 7.5% NaCl	-	2	-	1	-	-	-	-	-	+	+
160. Growth in 10.% NaCl	-	-	-	1	-	-	-	-	-	-	+
161. Growth in 15.% NaCl	-	-	-	-	-	-	-	-	-	-	-
(c) Temperature tolerance											
162. Growth at 4^0C	-	-	-	-	-	-	-	-	-	-	-
163. at 28^0C	+	+	+	+	+	+	+	+	+	+	+
164. at 37^0C	+	+	+	+	+	+	+	+	+	+	+
165. at 45^0C.	+	+	+	+	+	+	+	+	+	+	+
166. at 60^0C	-	-	-	-	-	-	-	-	-	-	-
(d) Heavy metal tolerance											
167. Cadmium (Cd^{+2})	2	9	2	2	-	+	-	-	-	+	+
168. Chromium (Cr^{+3})	-	7	-	-	-	-	-	+	-	-	+
169. Cobalt (Co^{+2})	2	10	2	2	-	+	-	+	-	+	+
170. Iron (Fe^{+2})	+	46	5	2	+	+	+	-	-	+	+
171. Lead (Pb^{+2})	+	46	5	2	+	+	+	-	-	+	+
172. Molybdenum (MoO_4^{-2})	+	46	5	2	+	+	+	-	-	+	+
173. Mercury (Hg^{+2})	+	46	5	2	+	+	+	-	-	+	+
(e) Growth in the presence of dyes											
174. Brilliant green	+	+	+	+	+	+	+	+	+	+	+
175. Crystal violet	+	+	+	+	+	+	+	+	+	+	+
176. Pyronin G	+	+	+	+	+	+	+	+	+	+	+
177. Neutral red	+	+	+	+	+	+	+	+	+	+	+

Table 2-5.Cont'd ..

	phenons				Single strains[a]						
	1	**2**	**3**	**4**	**1**	**2**	**3**	**4**	**5**	**6**	**7**
Number of strains /phenon ,n=	17	51	6	7							
(f)Requirement of Mg^{+2},K^{+} and Na^{+2} for growth											
178 Growth in medium withoutMg^{+2}	2	22	1	2	+	+	-	-	-	+	+
179. Growth in medium without Na^{+2}	1	12	1	5	-	-	-	-	-	+	-
180. Growth in medium without K^{+}	-	10	3	1	+	+	-	-	-	-	-
(g) Pigment production											
181. Fluorescein	3	41	-	5	+	+	+	-	-	-	-
182. Pyocyanin	3	33	2	3	-	+	-	-	-	-	+
(h) Length of microorganisms in microns											
183. 0.5-1.0μ	2	2	1	-	-	-	-	-	-	-	-
184. 1.0-1.5μ	1	18	4	3	+		-	-	-	+	-
185. 1.5-2.0μ	-	8	4	3	-	+			-	-	+
186. 2.0-2.5μ	1	22	1	2	-	-		-	+	-	-
187. 2.5-3.0μ	-	1	-	1	-	-	+	+	-	-	-
(I) Colony morphology (I) Diameter											
188. 0.5 & 0.5mm	-	5	3	5	-	N	-	-	N	-	N
189. 0.5-1.0mm	1	4	2	2	-	N	+	+	N	+	N
190. 1.0-1.5mm	-	9	-	-	-	N	-	-	N	-	N
191. 1.5-2.0mm	-	11	-	-	+	N	-	-	N	-	N
192. 2.0-2.5mm	-	8	1	-	-	N	-	-	N	-	N
193. 2.5-3.0mm	-	15	-	-	-	N	-	-	N	-	N
(2) Shape											
194. Circular	1	+	4	6	-	-	-	-	-	+	-
195. Irregular	-	-	2	1	+	-	+	+	-	-	-
196. Spreading	+	-	-	-	-	+	-	-	+	-	+
(3) Margin											
197. Entire	1	+	3	6	-	-	-	-	-	+	-
198. Undulate	-	-	1	1	+	-	-	-	-	-	-
199. Lobate	-	-	-	-	-	-	-	-	+	-	-
200. Crenated	3	-	2	-	-	+	+	+	-	-	+
(4) Elevation of growth											
201. Convex	+	49	5	+	+	+	+	+	+	+	+
202. Flat	-	1	1	-	-	-	-	-	-	-	-
203. Low convex	-	1	-	-	-	-	-	-	-	-	-
(5) Optical characters											
204. Transparent	+	14	5	6	+	+	-	-	-	+	-
205. Translucent	-	12	1	1	-	-	+	-	+	-	+

Table 2-5.Cont'd ..

	Phenons				Single Strains[a]						
	1	2	3	4	1	2	3	4	5	6	7
Number of strains /phenon ,n=	17	51	6	7							
206. Shining	-	6	1	2	-	-	-	-	-	-	-
207. Opaque	-	24	-	-	-	-	-	+	-	-	-
(6) Consistency											
208. Butyrous	+	+	+	+	+	+	+	+	+	+	+
(7) Color of colony											
209. Colorless	+	20	5	+	-	+	+	-	+	-	+
210. Light brown	-	5	1	-	+	-	-	-	-	-	-
211. White	-	26	-	-	-	-	-	+	-	+	-
Antibiotic sensitivity											
212. Penicillin G	+	22	5	2	+	-	-	+	-	-	+
213. Oxytetracycline	+	+	+	+	-	-	+	+	+	-	+
214. Streptomycin	+	47	+	5	+	+	+	+	-	+	+
215. Ampicillin	2	35	6	+	+	-	-	-	-	-	+
216. Tetracycline	+	+	5	+	+	+	+	+	+	+	+
217. Gentamycin	+	+	+	+	+	+	+	+	-	+	+
218. Polymyxin B	-	+	+	+	+	-	-	+	-	+	+
219. Chlortetracycline	+	+	+	+	+	+	+	+	+	+	+
220. Neomycin	+	+	+	+	+	+	+	+	+	+	+
221. Methamine mandelate	+	+	+	+	+	+	-	+	+	+	+
222. Cefazolin	2	48	+	+	-	+	-	+	-	+	+
223. Amoxycillin	-	22	5	-	-	-	-	-	-	+	-
224. Nalidixic acid	+	+	+	+	+	+	-	+	+	-	+
225. Novobiocin	+	+	+	+	+	+	+	+	+	+	+
226. Chloramphenicol	+	+	+	+	+	+	+	+	+	+	+
227. Erythromycin	2	+	+	+	+	-	+	+	+	+	+
228. Kanamycin	+	+	+	+	+	+	-	+	-	+	+
229. Bacitracin	-	23	4	-	-	-	+	-	-	-	-
230. Lincomycin	1	41	+	+	+	-	+	+	-	-	+
231. Sufadiazine	+	40	4	3	-	+	-	-	-	+	+
Production of											
232. Levan	-	3	-	-	-	-	+	-	-	-	-
233. Glucan	-	-	-	-	-	-	-	-	-	-	-

a: single strains which did not cluster (1: API 1561; 2:API 1558; 3:AAC 727; 4: AAC 740; 5: API 810; 6: AAC 717; 7: API 781)

+: all strains are positive

all strains are negative

numbers :indicate the number of strains in each phenon that are positive to a particular test

N: not done

Table.2-6 Characteristics of the isolates from healthy larvae (O/129 +ve)

	Phenons						Single Strains[a]	
	1	2	3	4	5	6	1	2
Number of strains /phenon ,n=	4	12	5	3	4	4		
Biochemical tests								
1. Methyl red	-	11	-	+	2	1	+	-
2. Voges Proskauer	2	+	4	+	-	+	-	-
3. Catalase	+	+	+	+	+	+	+	+
4. Nitrate reduction	3	+	+	+	+	+	+	+
5. Nitrite reduction	3	+	3	1	2	3	+	+
6. Phenyl alanine deaminase	1	-	-	-	-	2	-	-
7. 2-ketolactose prod.	-	-	-	-	-	-	N	N
8. O/129 (150µg/mL)	+	+	+	+	+	+	+	+
9. Lysine decarboxylase	-	+	4	+	+	+	-	-
10. Ornithine decarboxylase	-	+	-	2	-	-	-	-
11. Histidine decarboxylase	-	-	-	-	-	-	-	-
12. Aspartic acid decarboxylase	-	-	-	-	-	-	-	-
13. Arginine dihydrolase	+	-	-	+	-	-	-	-
14. 3-ketogluconate prod.	3	5	-	-	-	1	-	-
15. Nitrogen gas prod.	-	-	-	-	-	-	-	-
16. Indole	2	+	+	-	+	1	+	+
17. Ammonia prod.	+	4	2	2	-	1	-	-
18. H_2S prod. **(TSI)**	-	-	-	-	-	-	-	-
19. Yellow slant	1	3	+	1	+	+	+	+
20. Yellow butt	3	+	+	+	+	+	+	+
21. Gas	-	-	-	-	-	-	-	-
22. Purple slant	3	8	-	2	-	-	-	-
Utilization of sole C-source								
23. Ribose	+	+	+	+	+	+	+	+
24. Xylose	-	2	-	-	-	-	-	-
25. Raffinose	-	-	-	-	-	-	-	-
26. Melibiose	-	-	-	-	3	1	-	-
27. Rhamnose	-	-	-	1	3	2	-	-
28. Trehalose	1	1	4	+	+	+	+	+
29. Cellobiose	-	2	2	-	3	-	+	-
30. Galactose	2	-	3	+	+	+	+	-
31. Mannose	-	9	3	+	2	2	-	-
32. Fructose	+	+	+	+	+	+	+	+

Table 2-6. Cont'd..

	Phenons						Single Strains[a]	
	1	2	3	4	5	6	1	2
Number of strains /phenon ,n=	**4**	**12**	**5**	**3**	**4**	**4**		
33. Sucrose	-	-	-	-	-	-	-	-
34. Arabinose	-	-	-	-	-	-	-	-
35. Inulin	-	-	-	-	-	-	-	-
36. Maltose	1	2	2	1	3	3	+	+
37. Glycogen	1	11	2	-	+	3	-	-
38. Sorbitol	-	-	-	-	-	-	+	-
39. Sodium pyruvate	+	+	+	+	+	+	+	+
40. Calcium lactate	2	4	3	2	-	2	-	-
41. Sodium glucuronate	-	-	-	1	-	1	-	-
42. Lactose	-	-	-	-	-	-	-	-
43. Adonitol	-	-	-	-	-	-	-	-
44. Tyrosine . Browning	-	-	-	-	-	-	-	-
45. Halozone	-	-	-	-	-	-	-	-
46. Disappearance of crystals	-	-	-	-	-	-	-	-
47. Enhanced growth	-	-	-	-	-	-	-	-
48. Dulcitol	-	-	-	-	-	-	-	-
49. Glycine	-	-	-	-	1	1	-	-
50. Proline	-	1	2	-	2	2	+	-
51. Serine	3	2	1	-	-	-	-	-
52. Cystine	-	-	-	-	-	-	-	-
53. Uracil	-	-	-	-	-	-	-	-
54. Tryptophan	1	1	-	-	-	-	-	-
55. Valine	2	-	1	-	-	-	-	-
56. Sodium malonate	-	-	-	-	-	-	-	-
57. m-inositol	1	-	-	-	-	-	-	-
58. Leucine	1	2	-	-	-	1	-	-
59. Sodium formate	1	-	-	-	1	-	-	-
60. Sodium acetate	1	3	-	2	3	1	-	+
61. Sodium gluconate	+	+	+	+	3	+	+	+
62. Mannitol	-	9	+5	-	3	+	+	-
63. Sodium citrate	2	1	-	-	-	-	-	-
64. Glycerol	-	-	-	-	-	-	-	-
65. Salicine	-	1	-	-	-	-	-	-
66. K-Na-tartarate	1	2	-	2	-	-	-	+
67. Succinic acid	2	1	-	-	-	-	-	-
68. Citrulline	2	1	-	-	-	-	+	-
69. Glutamic acid	3	7	+	-	1	+	+	-
Utilization of sugars for acid/gas production								
70. Glucose .Acid	+	+	+	+	+	+	+	+

Table 2-6. Cont'd..

	Phenons						Single Strains[a]	
	1	2	3	4	5	6	1	2
Number of strains /phenon ,n=	**4**	**12**	**5**	**3**	**4**	**4**		
71. Gas	-	-	-	1	-	-	-	-
72. Fructose .Acid	+	+	4	+	+	3	-	+
73. Gas	-	-	-	-	-	-	-	-
74. Sucrose :Acid	-	-	-	-	-	-	-	-
75. Gas	-	-	-	-	-	-	-	-
76. Lactose .Acid	-	-	-	-	-	-	-	-
77. Gas	-	-	-	-	-	-	-	-
78. Maltose .Acid	1	2	2	1	3	3	+	+
79. Gas	-	-	-	-	-	-	-	-
80. Ribose .Acid	-	-	3	1	+	2	-	-
81. Gas	-	-	-	-	-	-	-	-
82. Xylose .Acid	-	-	-	-	-	-	-	-
83. Gas	-	-	-	-	-	-	-	-
84. Raffinose .Acid	-	-	-	-	-	-	-	-
85. Gas	-	-	-	-	-	-	-	-
86. Melibiose .Acid	-	-	-	-	3	1	-	-
87. Gas	-	-	-	-	-	-	-	-
88. Rhamnose .Acid	-	-	-	-	3	2	+	-
89. Gas	-	-	-	-	-	-	-	-
90. Trehalose .Acid	2	1	4	+	+	+	+	+
91. Gas	-	-	-	+	-	-	-	-
92. Cellobiose .Acid	-	2	2	1	+	-	+	-
93. Gas	-	-	-	-	1	-	-	-
94. Galactose .Acid	2	-	3	+	-	-	+	-
95. Gas	2	-	3	+	-	-	+	-
96. Mannose .Acid	-	9	3	+	1	2	-	-
97. Gas	-	-	-	-	-	-	-	-
98. Arabinose .Acid	-	-	-	-	-	-	-	-
99. Gas	-	-	-	-	-	-	-	-
100. Glycerol .Acid	-	-	-	-	-	-	-	-
101. Gas	-	-	-	-	-	-	-	-
102. Mannitol .Acid	2	9	+	-	3	+	+	-
103. Gas	-	-	-	-	-	-	-	-
104. Salicine .Acid	-	-	-	-	-	-	-	-
105. Gas	-	-	-	-	-	-	-	-
106. Adonitol .Acid	-	-	-	-	-	-	-	-
107. Gas	-	-	-	-	-	-	-	-
108. m-inositol .Acid	1	-	-	-	-	-	-	-
109. Gas	-	-	-	-	-	-	-	-
110. Sorbitol .Acid	-	-	-	-	-	-	+	-

Table 2-6. Cont'd..

	Phenons						Single Strains[a]	
	1	2	3	4	5	6	1	2
Number of strains /phenon ,n=	**4**	**12**	**5**	**3**	**4**	**4**		
111. Gas	-	-	-	-	-	-	-	-
112. Starch .Acid	-	3	1	-	1	-	-	-
113. Gas	-	-	-	-	-	-	-	-
114. Dulcitol :Acid	-	-	-	-	-	-	-	-
115. Gas	-	-	-	-	-	-	-	-
116. Glycogen .Acid	1	11	2	-	+	3	-	-
117. Gas	-	-	-	-	-	-	-	-
118. Inulin .Acid	-	-	-	-	-	-	-	-
119. Gas	-	-	-	-	-	-	-	-
120. Butanol :Acid	-	-	-	-	-	-	-	-
121. Gas	-	-	-	-	-	-	-	-
122. Ethanol .Acid	-	-	-	-	-	-	-	-
123. Gas	-	-	-	-	-	-	-	-
124. Dextrin .Acid	3	11	+	1	+	3	+	-
125. Gas	-	-	-	-	-	-	-	-
Degradation/utilization of compounds								
126. Gelatin	+	+	+	1	+	3	+	+
127. Tween 20	+	9	+	+	3	2	+	-
128. Tween 40	3	2	+	+	1	2	+	-
129. Tween 60	+	9	4	2	+	3	+	-
130. Tween 80	+	10	2	2	-	2	-	-
131. Urea	-	-	-	-	-	-	-	-
132. Tributyrin	+	11	+	-	+	+	+	+
133. Starch	-	1	1	-	1	-	-	-
134. Casein	+	+	+	-	3	+	+	-
135. α-hemolysis	-	-	-	-	-	-	-	-
136. β-hemolysis	-	-	-	-	-	-	-	-
137. γ-hemolysis	+	+	+	+	+	+	+	+
138. Zone color:Green	-	-	-	-	-	-	-	-
139. Colorless	-	-	-	-	-	-	-	-
140. Zone diameter:1mm	-	-	-	-	-	-	-	-
141. 2-10mm	-	-	-	-	-	-	-	-
142. 10-20mm	-	-	-	-	-	-	-	-
143. 20-40mm	-	-	-	-	-	-	-	-
144. Chitin	1	1	-	-	1	-	-	-
145. Lecithin	3	4	2	1	3	1	-	-
146. Aesculin	-	-	1	-	-	-	+	+
147. Cellulose	-	-	-	-	-	-	-	-
148. DNAase	+	6	4	+	3	2	+	-

Table 2-6. Cont'd..

	Phenons						Single Strains[a]	
	1	2	3	4	5	6	1	2
Number of strains /phenon ,n=	**4**	**12**	**5**	**3**	**4**	**4**		
Physiological characters								
(a) pH tolerance								
149. Growth at pH5	-	-	-	-	-	-	-	-
150. Growth at pH8	+	+	+	+	+	+	+	+
151. Growth at pH9	+	+	+	+	+	+	+	+
152. Growth at pH10	+	+	+	+	+	+	+	+
153. Growth at pH12	+	11	+	+	+	+	+	-
(b) NaCl tolerance (% w/v)								
154. Growth without NaCl	1	1	2	-	-	-	-	-
155. Growth in 0.5% NaCl	+	+	+	+	+	+	+	+
156. Growth in 1.0% NaCl	+	+	+	+	+	+	+	+
157. Growth in 2.0% NaCl	+	+	+	+	+	+	+	+
158. Growth in 5.0% NaCl	2	1	-	1	-	-	-	-
159. Growth in 7.5% NaCl	-	-	-	-	-	-	-	-
160. Growth in 10.% NaCl	-	-	-	-	-	-	-	-
161. Growth in 15.% NaCl	-	-	-	-	-	-	-	-
(c) Temperature tolerance								
162. Growth at 4^0C	-	-	-	-	-	-	-	-
163. at 28^0C	+	+	+	+	+	+	+	+
164. at 37^0C	+	+	+	+	+	+	+	+
165. at 45^0C.	+	+	+	+	+	+	+	+
166. at 60^0C	-	-	-	-	-	-	-	-
(d) Heavy metal tolerance								
167. Cadmium (Cd^{+2})	2	4	1	1	-	-	-	-
168. Chromium (Cr^{+3})	-	-	-	-	-	1	-	-
169. Cobalt (Co^{+2})	2	4	1	1	-	1	-	-
170. Iron (Fe^{+2})	+	11	+	2	+	3	-	-
171. Lead (Pb^{+2})	+	11	+	2	+	3	-	-
172. Molybdenum (MoO_4^{-2})	+	11	+	2	+	3	-	-
173. Mercury (Hg^{+2})	+	11	+	2	+	3	-	-
(e) Growth in the presence of dyes								
174. Brilliant green	+	+	+	+	+	+	+	+
175. Crystal violet	+	+	+	+	+	+	+	+
176. Pyronin G	+	+	+	+	+	+	+	+
177 Neutral red	+	+	+	+	+	+	+	+

Table 2-6. Cont'd..

	Phenons						Single Strains[a]	
	1	2	3	4	5	6	1	2
Number of strains /phenon ,n=	4	12	5	3	4	4		
(f) Requirement of Mg^{+2},K^{+} and Na^{+2} for growth								
178. Growth in medium without Mg^{+2}	-	3	-	2	-	1	-	-
179. Growth in medium without Na^{+2}	1	3	-	+	-	-	-	-
180. Growth in medium without K^{+}	-	2	-	-	-	-	-	-
(g) Pigment production								
181. Fluorescein	+	2	3	2	2	3	+	+
182. Pyocyanin	2	4	4	+	2	+	+	+
(h) Length of microorganisms in microns								
183. 0.5-1.0μ	1	-	-	-		-	-	-
184. 1.0-1.5μ	2	5	4	-	-	+	+	-
185. 1.5-2.0μ	-	4	1	+	-	-	-	
186. 2.0-2.5μ	1	3	-	-	+	-	-	+
187. 2.5-3.0μ		-		-	-	-	-	-
(i) Colony morphology (1) Diameter								
188. 0.5 & 0.5mm	1	3	2	+	1	-	-	+
189. 0.5-1.0mm	-	3	-	-	1	-	+	-
190. 1.0-1.5mm	-	3	2	-	2	1	-	-
191. 1.5-2.0mm	1	3	-	-	-	2	-	-
192. 2.0-2.5mm	1	-	1	-	-	1	-	-
193. 2.5-3.0mm	1	-	-	-	-	-	-	-
(2) Shape								
194. Circular	+	11	+	+	+	+	+	-
195. Irregular	-	1	-	-	-	-	-	+
196. Spreading	-	-	-	-	-	-	-	-
(3) Margin								
197. Entire	+	11	+	+	3	+	+	-
198. Undulate	-	-	-	-	-	-	-	-
199. Lobate	-	1	-	-	-	-	-	-
200. Crenated	-	-	-	-	1	-	-	+
(4) Elevation of growth								
201. Convex	3	11	+	+	+	+	+	+
202. Flat	1	1	-	-	-	-	-	-
203. Low convex	-	-	-	-	-	-	-	-
(5) Optical characters								
204. Transparent	1	10	1	2	3	2	-	+
205. Translucent	2	2	2	1	-	1	-	-

Table 2-6. Cont'd..

	Phenons						Single Strains[a]	
	1	**2**	**3**	**4**	**5**	**6**	**1**	**2**
Number of strains /phenon ,n=	**4**	**12**	**5**	**3**	**4**	**4**		
206. Shining	-	5	-	-	1	-	+	-
207. Opaque	1	-	2	-	1	1	-	-
(6) Consistency								
208. Butyrous	+	+	+	+	+	+	+	+
(7) Color of colony								
209 Colorless	2	+	3	+	+	+	+	+
210. Light brown	-	-	1	1	-	-	-	-
211 White	2	-	1	-	-	-	-	-
Antibiotic sensitivity								
212. Penicillin G	3	+	3	1	2	+	+	+
213. Oxytetracycline	+	+	+	+	+	+	+	+
214. Streptomycin	+	+	+	+	+	+	+	+
215. Ampicillin	2	+	+	2	1	+	+	-
216. Tetracycline	+	+	+	+	+	+	+	+
217. Gentamycin	+	+	+	+	+	+	+	+
218. Polymyxin B	+	11	+	+	2	+	+	+
219. Chlortetracycline	+	+	+	+	+	+	+	+
220. Neomycin	+	+	+	+	+	+	+	+
221. Methamine mandelate	+	+	+	+	+	+	+	+
222. Cefazolin	+	+	-	+	1	+	+	+
223. Amoxycillin	2	3	1	-	-	1	-	-
224. Nalidixic acid	+	+	+	+	+	+	+	+
225. Novobiocin	+	+	+	+	+	+	+	+
226. Chloramphenicol	+	+	+	+	+	+	+	+
227. Erythromycin	+	+	+	+	+	+	+	+
228. Kanamycin	+	11	+	+	+	+	+	+
229. Bacitracin	2	6	1	-	1	3	+	+
230. Lincomycin	+	10	4	+	+	+	+	+
231. Sufadiazine	+	+	2	2	+	+	+	+
Production of								
232. Levan	1	-	-	-	-	-	-	+
233. Glucan	-	-	-	-	-	-	-	-

a: single strains which did not cluster (1: ANM 702; 2: API 1519)
+: all strains are positive
all strains are negative
numbers :indicate the number of strains in each phenon that are positive to a particular test

N: not done

Table.2-7 Characteristics of the reference strains included in this study.

	Reference Strains*																			
Strain numbers	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**
Biochemical tests																				
1. Methyl red	+	+	+	+	-	+	-	+	-	+	-	+	-	-	-	-	-	-	-	-
2. Voges Proskauer	-	+	+	-	-	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-
3. Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
4. Nitrate reduction	+	+	-	+	-	+	+	-	+	+	+	+	+	+	-	+	-	-	-	+
5. Nitrite reduction	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
6. Phenyl alanine deaminase	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+	-	-	-	-
7. 2-ketolactose prod.	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
8. O/129 (150µg/mL)	+	+	+	+	+	+	+	+	+	+	+		-	-		+	+	+	+	+
9. Lysine decarboxylase	+	+	+	-	+	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
10. Ornithine decarboxylase	+	-	+	-	+	-	+	+	-	+	-	-	-	-	-	-	-	-	-	-
11. Histidine decarboxylase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
12. Aspartic acid decarboxylase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
13. Arginine dihydrolase	-	-	-	-	-	+	+	-	+	+	+	+	+	+	+	-	-	-	-	-
14. 3-ketogluconate prod.	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
15. Nitrogen gas prod.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
16. Indole	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
17. Ammonia prod.	+	+	-	-	-	+	+	-	+	+	+	+	+	+	-	-	+	-	+	+
18. H_2S prod. **(TSI)**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
19. Yellow slant	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 2-7.Cont'd..

	Reference strains*																			
Strain numbers	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**
20. Yellow butt	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
21. Gas	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
22. Purple slant	-	-	-	-	-	-	-	-	-	+	-	-	+	-	+	-	-	-	-	-
Utilization of sole C-source																				
23. Ribose	+	+	-	+	-	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-
24. Xylose	-	-	-	-	+	+	+	+	-	+	+	-	+	+	+	-	-	-	-	-
25. Raffinose	+	-	-	+	+	+	+	+	-	+	-	-	+	-	-	-	-	-	-	-
26. Melibiose	+	-	-	+	+	+	+	+	-	+	-	-	+	-	-	-	-	-	-	-
27. Rhamnose	-	-	-	-	-	+	-	-	-	+	-	-	+	-	-	-	-	-	-	-
28. Trehalose	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-
29. Cellobiose	+	+	-	-	+	+	+	-	+	+	+	-	-	-	-	-	-	-	-	-
30. Galactose	+	-	+	-	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-
31. Mannose	+	-	-	+	+	+	+	-	+	+	+	+	-	-	+	-	-	-	-	+
32. Fructose	-	+	+	-	+	-	+	+	+	+	+	+	-	-	+	-	-	-	-	+
33. Sucrose	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-
34. Arabinose	-	-	-	-	-	-	+	-	-	+	-	+	+	+	-	-	-	-	-	-
35. Inulin	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
36. Maltose	-	-	-	+	+	+	+	+	-	+	+	+	+	-	+	-	-	-	+	-
37. Glycogen	+	-	+	-	-	+	+	-	+	-	-	+	+	+	+	+	+	-	+	+
38. Sorbitol	-	-	-	-	-	-	-	+	+	+	-	+	+	+	-	-	-	-	-	-
39. Sodium pyruvate	-	-	+	+	+	+	+	+	-	+	+	-	+	+	+	-	+	-	-	+
40. Calcium lactate	-	-	+	+	+	+	+	+	-	+	+	-	+	+	+	-	-	-	-	-
41. Sodium glucuronate	-	-	+	-	-	-	+	+	-	+	+	+	-	-	-	-	-	-	-	-
42. Lactose	-	-	-	-	-	-	+	-	-	+	-	-	+	+	-	-	-	-	-	-

Table 2-7.Cont'd..

	Reference Strains*																			
Strain numbers	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
43. Adonitol	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
44. Tyrosine . Browning	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
45. Halozone	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
46. Disappearance of crystals	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
47. Enhanced growth	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
48. Dulcitol	-	-	-	+	-	+	+	+	-	+	-	+	-	-	-	-	-	-	-	-
49. Glycine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
50. Proline	+	-	+	+	-	+	+	+	+	+	-	+	-	-	-	-	-	-	-	-
51. Serine	-	-	-	-	-	-	+	-	-	+	+	-	+	-	-	-	-	-	-	-
52. Cystine	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-	-	-
53. Uracil	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
54. Tryptophan	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
55. Valine	-	-	-	-	-	-	+	+	-	+	-	-	-	-	-	-	-	-	-	-
56. Sodium malonate	-	-	-	+	-	-	+	+	-	-	-	-	+	+	-	-	-	-	-	-
57. m-inositol	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-	-	-
58. Leucine	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-
59. Sodium formate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
60. Sodium acetate	-	-	-	-	-	-	+	+	-	+	-	-	-	-	-	-	-	-	-	-
61. Sodium gluconate	-	+	-	-	-	-	+	-	-	+	+	+	+	+	-	-	-	-	-	-
62. Mannitol	-	+	-	-	+	+	-	-	-	-	+	+	+	-	+	-	-	-	-	-
63. Sodium citrate	-	-	-	-	-	-	+	-	-	+	-	+	+	+	-	-	-	-	-	-
64. Glycerol	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-
65. Salicine	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-
66. K-Na-tartarate	-	-	-	-	-	-	-	-	-	+	+	-	+	-	-	-	-	-	-	-

Table 2-7.Cont'd..

	Reference strains*																			
Strain numbers	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
67. Succinic acid	+	+	-	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-
68. Citrulline	-	-	-	-	-	-	-	+	-	+	-	-	+	-	-	-	-	-	-	-
69. Glutamic acid			-	-				+		+			+	-		-			-	
Utilization of sugars for acid/gas production																				
70. Glucose .Acid	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
71. Gas	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
72. Fructose .Acid	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+
73. Gas	-	-	-	-	-	+	+	-	-	-	-	-	+	-	-	-	-	-	-	-
74. Sucrose .Acid	-	+	+	-	+	+	+	-	+	+	+	+	+	+	+	-	+	+	+	-
75. Gas	-	-	-	-	-	+	+	-	+	+	-	-	+	-	-	-	-	-	-	-
76. Lactose .Acid	-	-	-	-	-	-	+	-	-	+	-	+	-	-	-	-	-	-	-	-
77 Gas	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
78. Maltose .Acid	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	-	+	-	+	-
79. Gas	-	-	-	-	-	+	+	-	-	+	-	-	+	-	-	-	-	-	-	-
80. Ribose .Acid	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
81. Gas	-	-	-	-	-	+	+	-	-	+	-	-	-	-	-	-	-	-	-	-
82. Xylose :Acid	-	-	-	-	-	-	+	-	-	+	-	-	-	-	-	+	-	+	+	-
83. Gas	-	-	-	-	-	-	+	-	-	+	-		-	-			-	-		-
84. Raffinose .Acid	-	-	-	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-
85 Gas	-	-	-	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-
86. Melibiose :Acid	-	-	-	-	+	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-
87. Gas	-	-	-	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-
88. Rhamnose .Acid	-	-	-	-	-	-	+	-	-	+	-	+	-	-	-	-	-	-	-	-
89. Gas	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-

Table 2-7.Cont'd..

Strain numbers	Reference strains*																			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
90. Trehalose .Acid	+	+	+	-	+	+	-	-	-	-	+	+	+	+	+	-	+	+	-	-
91. Gas	-	-	-	-	-	+	-	-	-	-	-	-	+	-	+	-	-	-	-	-
92. Cellobiose .Acid	-	-	-	-	+	-	-	-	-	-	+	-	-	-	+	-	-	-	-	-
93 Gas	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
94. Galactose .Acid	+	-	-	+	+	+	-	-	-	-	+	-	-	-	-	-	-	-	+	-
95. Gas	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
96. Mannose .Acid	+	+	-	+	+	-	-	+	-	-	+	+	+	-	+	-	-	-	+	+
97. Gas	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
98. Arabinose .Acid	+	-	-	+	-	+	-	-	+	-	-	+	-	+	-	-	-	-	+	-
99. Gas	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
100. Glycerol .Acid	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	-	+
101. Gas	-	-	-	-	-	+	-	-	-	-	-	-	+	-	-	-	-	-	-	-
102. Mannitol .Acid	-	+	-	-	+	+	-	-	-	-	+	+	+	+	+	-	-	-	-	-
103. Gas	-	-	-	-	-	+	-	-	-	-	-	-	+	-	-	-	-	-	-	-
104. Salicine .Acid	-	-	-	-	-	+	+	-	-	-	-	+	-	+	-	-	-	-	-	-
105. Gas	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
106. Adonitol .Acid	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
107. Gas	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
108. m-inositol .Acid	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
109. Gas	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
110. Sorbitol .Acid	-	-	-	-	-	-	+	-	+	+	-	+	-	-	-	-	-	-	-	-
111. Gas	-	-	-	-	-	-	+	-	+	+	-	-	-	-	-	-	-	-	-	-
112. Starch .Acid	-	-	-	-	+	-	-	-	-	-	-	-	+	+	+	-	-	-	-	-
113. Gas	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-

Table 2-7.Cont'd..

	Reference strains*																			
Strain numbers	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**
114. Dulcitol .Acid	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
115. Gas	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
116. Glycogen .Acid	-	+	-	-	+	-	+	+	-	-	-	+	+	+	-	-	-	-	-	-
117. Gas	-	+	-	-	+	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-
118. Inulin .Acid	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
119 Gas	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
120. Butanol .Acid	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
121. Gas	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
122. Ethanol .Acid	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
123. Gas	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
124. Dextrin .Acid	+	+	+	-	+	-	-	-	-	-	+	+	+	+	+	-	-	-	-	-
125. Gas	-	+	+	+	+	-	-	-	-	-	-	-	+	-	+	-	-	-	-	
Degradation/utilization of compounds																				
126. Gelatin	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-
127. Tween 20	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	-	+	-	-	-
128. Tween 40	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	-	+	-	-	-
129. Tween 60	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	-	+	-	-	-
130. Tween 80	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	-	-	-	-	-
131. Urea	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	-	-
132. Tributyrin	+	+	+	+	+	+	+	+	+	-	-	+	-	-	-	-	-	-	-	-
133. Starch	+	+	+	-	+	+	+	-	+	-	-	+	+	-	+	-	-	-	-	-
134. Casein	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 2-7.Cont'd..

	Reference strains*																			
Strain numbers	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**
135. α-hemolysis	-	-	-	-	-	-	-	-	-	-	-	-	N	N	N	N	N	N	N	N
136. β-hemolysis	+	+	+	-	+	+	-	+	+	-	-	+	N	N	N	N	N	N	N	N
137. γ-hemolysis	-	-		+	-	-	+	-	-	+	+	-	N	N	N	N	N	N	N	N
138. Zone color: Green	+	+	+	-	+	+	-	+	+	-	-	+	N	N	N	N	N	N	N	N
139. Colorless	-	-	-	-	-	-	-	-	-	-	-	-	N	N	N	N	N	N	N	N
140. Zone diameter:1mm	-	-	-	-	-	-	-	-	-	-	-	-	N	N	N	N	N	N	N	N
141. 2-10mm	+	+	+	-	+	+	-	+	+	-	-	+	N	N	N	N	N	N	N	N
142. 10-20mm	-	-	-	-	-	-	-	-	-	-	-	-	N	N	N	N	N	N	N	N
143. 20-40mm	-	-	-	-	-	-	-	-	-	-	-	-	N	N	N	N	N	N	N	N
144. Chitin	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
145. Lecithin	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
146. Aesculin	-	-	-	-	+	+	+	-	+	+	+	+	+	+	+	-	-	-	-	-
147. Cellulose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
148. DNAase	-	-	-	-	+	+	-	+	+	+	+	-	-	-	-	-	-	-	-	-
Physiological characters																				
(a) pH tolerance																				
149. Growth at pH5	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	+	-	+	-	-
150. Growth at pH8	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
151. Growth at pH9	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+
152. Growth at pH10	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+
153. Growth at pH12	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+

Table 2-7.Cont'd..

	Reference strains*																			
Strain numbers	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
(b) NaCl tolerance (% w/v)																				
154. Growth without NaCl	+	-	+	-	-	+	+	-	-	+	+	-	+	+	+	+	-	+	+	+
155. Growth in 0.5% NaCl	+	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+
156. Growth in 1.0% NaCl	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
157 Growth in 2.0% NaCl	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
158. Growth in 5.0% NaCl	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+
159. Growth in 7.5% NaCl	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	-	+	+	-	-
160. Growth in 10.% NaCl	+	+	-	-	-	-	+	-	+	+	+	+	-	-	-	-	+	+	-	-
161. Growth in 15.% NaCl	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+	+	-	-
(c) Temperature tolerance																				
162. Growth at 4^0C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
163. at 28^0C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+
164. at 37^0C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
165. at 45^0C.	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	-	-
166. at 60^0C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
(d) Heavy metal tolerance																				
167. Cadmium (Cd^{+2})	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
168. Chromium (Cr^{+3})	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
169. Cobalt (Co^{+2})	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
170. Iron (Fe^{+2})	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-	+	+	-	+
171. Lead (Pb^{+2})	+	-	-	-	-	+	+	-	-	+	-	+	+	+	+	+	+	+	+	+
172. Molybdenum (MoO_4^{-2})	+	+	+	+	-	+	+	-	+	+	-	+	+	+	+	+	+	+	+	+
173. Mercury (Hg^{+2})	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 2-7.Cont'd..

	Reference strains*																			
Strain numbers	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
(e) Growth in the presence of dyes																				
174.Brilliant green	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	+	+
175.Crystal violet	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
176.Pyronin G	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-	-	-
177.Neutral red	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-	-	+	+	+
(f) Requirement of Mg^{+2},K^{+} and Na^{+2} for growth																				
178.Growth in medium without Mg^{+2}	+	+	-	-	-	-	+	-	+	+	+	+	+	+	+	-	-	+	-	-
179.Growth in medium without Na^{+2}	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+	-	-	+	-	-
180.Growth in medium without K^{+}	+		+				+	+		+	+	+	+	+	+			+		
(g) Pigment production																				
181.Fluorescein	+	+	-	+	-	-	-	+	-	+	+	+	+	+	+	-	-	+	+	+
182.Pyocyanin	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	+
(h) Length of microorganisms in microns																				
183. 0.5-1.0μ	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
184. 1.0-1.5μn	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
185. 1.5-2.0μ	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
186. 2.0-2.5μ	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
187 2.5-3.0μ	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N

Table 2-7. Cont'd..

	Reference strains*																			
Strain numbers	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
(i) Colony morphology (1)Diameter																				
188. 0.5 & 0.5mm	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
189. 0.5-1.0mm	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	+	+	-	-
190. 1.0-1.5mm	-	-	-	+	-	-	-	+	-	+	+	-	+	-	-	-	-	-	+	+
191. 1.5-2.0mm	+	+	+	-	+	-	+	-	+	-	-	+	-	-	-	-	-	-	-	-
192. 2.0-2.5mm	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
193. 2.5-3.0mm	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
(2) Shape																				
194. Circular	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
195. Irregular	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
196. Spreading	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
(3) Margin																				
197. Entire	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
198. Undulate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
199. Lobate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
200. Crenated	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
(4) Elevation of growth																				
201. Convex	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
202. Flat	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
203. Low convex	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
(5) Optical characters																				
204. Transparent	-	-	+	+	-	-	-	+	-	+	-	-	-	+	+	+	+	-	-	-

Table 2-7.Cont'd..

	Reference strains*																			
Strain numbers	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**
205. Translucent	+	+	-	-	+	+	+	-	+	-	-	+	+	-	-	-	-	+	+	+
206. Shining	+	+	+	+	+	+	-	-	+	+	-	-	-	+	+	+	+	+	+	+
207. Opaque	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
(6) Consistency																				
208. Butyrous	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
(7) Color of colony																				
209. Colorless	-	+	+	+	-	-	-	+	-	+	-	-	-	+	-	+	-	-	+	-
210. Light brown	+	-	-	-	+	+	-	-	+	-	-	+	+	-	+	-	+	-	-	-
211. White	-	-	-	-	-	-	+	-	-	-	+	-	-	-	-	-	-	+	-	-
Antibiotic sensitivity																				
212. Penicillin G	-	-	-	-	-	-	-	+	-	-	+	-	-	-	-	+	-	-	-	-
213. Oxytetracycline	+	+	+	+	+	+	+	+	+	-	+	+	-	-	+	+	+	-	-	+
214. Streptomycin	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+
215. Ampicillin	-	-	+	+	-	+	-	+	+	-	-	-	-	-	-	+	+	+	+	-
216. Tetracycline	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
217. Gentamycin	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
218. Polymyxin B	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+
219. Chlortetracycline	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
220. Neomycin	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+
221 Methamine mandelate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Table 2-7.Cont'd..

	Reference strains*																			
Strain numbers	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
222. Cefazolin	+	+	+	+	+	-	-	+	+	-	+	+	-	-	-	+	+	+	+	+
223. Amoxycillin	-	-	-	+	-	-	-	+	+	-	-	-	-	-	-	-	+	-	-	-
224. Nalidixic acid	+	+	+	+	+	-	+	+	+	+	-	+	+	+	+	+	+	-	+	+
225 Novobiocin	+	+	+	+	+	+	-	+	+	-	+	+	+	+	-	+	+	-	+	+
226. Chloramphenicol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-	+	+
227. Erythromycin	+	+	+	+	+	+	-	+	+	-	+	+	+	+	+	+	+	-	+	-
228. Kanamycin	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+
229. Bacitracin	-	-	+	+	+	+	+	+	+	-	+	+	-	-	-	+	+	+	+	+
230. Lincomycin	+	+	+	+	+	-	+	+	+	-	+	+	-	-	-	+	+	+	+	+
231. Sufadiazine	+	-	+	-	+	-	+	+	+	-	-	-	-	+	-	+	+	-	+	+
Production of																				
232. Levan	-	-	-	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-
233. Glucan	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-

N: not done
+: Positive test
- : negative test

*1. *V. parahaemolyticus* ATCC 17802
2. *V. alginolyticus* ATCC 17749
3. *V. cholerae* Ogawa MAK 757
4. *V. hollisae* CDC 9041-81
5. *V. harveyi* ATCC 14126
6. *V. nereis* ATCC 25917
7. *V. vulnificus* MTCC
8. *V. anguillarum* ATCC 19264
9. *V. mimicus* Q 20
10 *V. fisheri* MTCC 1738
11. *V. metschnikovii* MTCC 866
12. *A. hydrophila* 172 (CIFT)
13. *A. hydrophila* ATCC 7966
14. *A. caviae* ATCC 15468
15. *A. salmonicida* subsp. *masoucida* ATCC 2701
16. *Plesiomonas shigelloides* ATCC 14029
17. *Salinivibrio costicola* ATCC 33508
18. *P. phosphoreum* ATCC 11040
19. *P.angustum* ATCC 25915
20. *P.leignathi* ATCC 25521

Table 2-8 Mole % G+C ratio , affiliation and/or identity of the phena and unclustered strains which are O/129 negative, from moribund larvae

Serial no.	Phenon/single strains	Representative cultures	Mole % G+C ratio	Species to which affiliation is seen in terms of Mole % G+C ratio
1.	Phenon-1	AAC 1104a	53.5	Group E-3
2.	Phenon-2	AAC 1142b	39.8	*V. fischeri*
3.	Phenon-3	AAC 1101b	41	*P. angustum , P. phosphoreum, V. logei, V. marinus, V. fischeri*
4.	Phenon-4	AAC $639b_1$	53.9	Group E-3
5.	Phenon-5	AAC 1114b	41	*P. angustum , P. phosphoreum, V. logei, V. marinus, V. fischeri*
6.	Phenon-6	AAC 1109b	39.6	*V. fischeri*
7.	Phenon-7	API 684a	50.5	*V. costicola, V. furnissi, V fluvialis, V. hollisae, V. proteolyticus*
8.	Phenon-8	AAC $1128a_2$	47.9	*V campbelli, V. cholerae, V. harveyi, V. vulnificus*
9.	Phenon-9	AAC 1101a	40.7	*P angustum , V. logei, V marinus, V fischeri*
10.	Phenon-10	AAC 880c	57.3	*Aeromonas samonicida*
11.	Phenon-11	ANM $598c_2$	44.9	*V anguillarum, V metschnikovii, V ordalli, V tubiashi, V ichthyoenterii*
12.	Phenon-12	ANM 718aa	44.4	*V anguillarum, V metschnikovii, V ordalli, V tubiashi, V ichthyoenterii*
13.	Phenon-13	ANM 625	40.5	*P. angustum , V. logei, V. marinus, V. fischeri*
14.	Single strain-1	AAC $672b_1$	59.4	*Aeromonas sobria, A. hydrophila*
15.	Single strain-2	AAC 669B	56.2	Out of range for Vibrionaceae
16.	Single strain-3	AAC 1108d	46.5	13 species of Vibrios
17.	Single strain-4	API 644b	58.2	*A. hydrophila, A. sobria*
18.	Single strain-5	AAC 654b	63.3	*A. caviae-like*
19.	Single strain-6	AAC 536	48.5	*V. cholerae*
20	Single strain-7	ANM 1008a	57.3	*A. salmonicida*

Table 2-9 Mole % G+C ratio , affiliation and/or identity of the phena and the unclustered strains which are O/129 positive obtained from moribund larvae

Serial no.	Phenons/single strains	Culture no.	Mole % G+C ratio	Species to which affiliation is seen in terms of Mole % G+C ratio
1.	Phenon-1	AAC 629b	43.4	*V aestuarianus, V tubiashi, V ichthyoenterii, P. leiognathi*
2.	Phenon-2	ANM 594b	43.2	*V aestuarianus, V. tubiashi, V ichthyoenterii, P. leiognathi*
3.	Phenon-3	ANM 597	43.5	*V aestuarianus, V tubiashi, V ichthyoenterii, P. leiognathi*
4	Single strain-1	ANM 708	39.9	*V. fischeri, V logei, V marinus, P. angustum*
5.	Single strain-2	ANM 610a	53.6	Group E-3
6.	Single strain-3	ANM 1020	39.9	*V. fischeri, V logei, V. marinus, P. angustum*
7	Single strain-4	ANM 594a	44.8	*V tubiashi, V. anguillarum, V ichthyoenterii, , V.ordalli*
8.	Single strain-5	ANM 1003	54.3	Group E-3

Table 2-10 Mole % G+C ratio, affiliation and/or identity of the phena and unclustered strains which are O/129 negative from apparently healthy larvae

Serial no.	Phenons/single strains	Representative cultures	Mole % G+C ratio	Species to which affiliation is seen in terms of Mole % G+C ratio
1	Phenon-1	AAC 730a	47 1	*V gazogenes, V, cholerae, V. cambelli, V. penaecida, V. diazotrophicus, V. nereis, V. parahaemolyticus, V. pelagius bv. I and II, V vulnificus bv. I, V. nigripulchritudo, V. harveyi V natreigenes, V navarrensis, V. alginolyticus*
2.	Phenon-2	ANM932	49.8	*V fluvialis, V. costicola, V, furnissi*
3.	Phenon-3	ANM 723	54.9	Group E-3
4.	Phenon-4	API 1546	38.3	*V. fischerii*
5.	Single strain-1	API 1561	54.9	Group E-3
6.	Single strain-2	API 1558	60.12	*A. hydrophila, A. caviae, A. sobria*
7.	Single strain-3	AAC 727	38.3	*V. fischerii*
8.	Single strain-4	AAC 740	52.1	*V. fluvialis, V. costicola, V, furnissi, V proteolyticus, V. hollisae*
9.	Single strain-5	API 810	36.5	Out of range of Vibrionaceae
10.	Single strain-6	AAC 717	56.7	Out of range of Vibrionaceae
11	Single strain-7	AAC 781	36.1	Out of range of Vibrionaceae

Table 2-11 Mole % G+C ratio, affiliation and/or identity of the phena and unclustered strains which are O/129 positive obtained from apparently healthy larvae

Serial no.	Phenons/ Single strains	Representative cultures	Mole % G+C ratio	Species to which affiliation is seen in terms of Mole % G+C ratio
1.	Phenon-1	AAC 701	48.4	*V cholerae, V. harveyi , V. campbelli, V. vulnificus*
2.	Phenon-2	ANM 712	45.6	*V. anguillarum, V metschnikovii, V. mytili, V. navarrensis, V. ordalli, V. orientalis, V. pelagius* bv. I and II, *V splemdidus* bv. I and II, *V. alginolyticus*
3.	Phenon-3	ANM 719	38.3	*V. fischeri*-like
4.	Phenon-4	API 1555	50.6	*V. proteolyticus, V hollisae, V costicola, V furnissi*
5.	Phenon-5	API 768	60.1	*A. hydrophila, A. sobria, A. caviae*
6.	Phenon-6	ANM 721	47.8	*V. vulnificus, V. campbelli, V. cholerae, V. harveyi, V. tubiashi,* marine luminous bacteria
7.	Single strain-1	ANM 702	63.1	*Aeromonas*
8.	Single strain-2	ANM 1519	49.5	*V. proteolyticus, V hollisae, V costicola, V. furnissi, V. fluvialis*

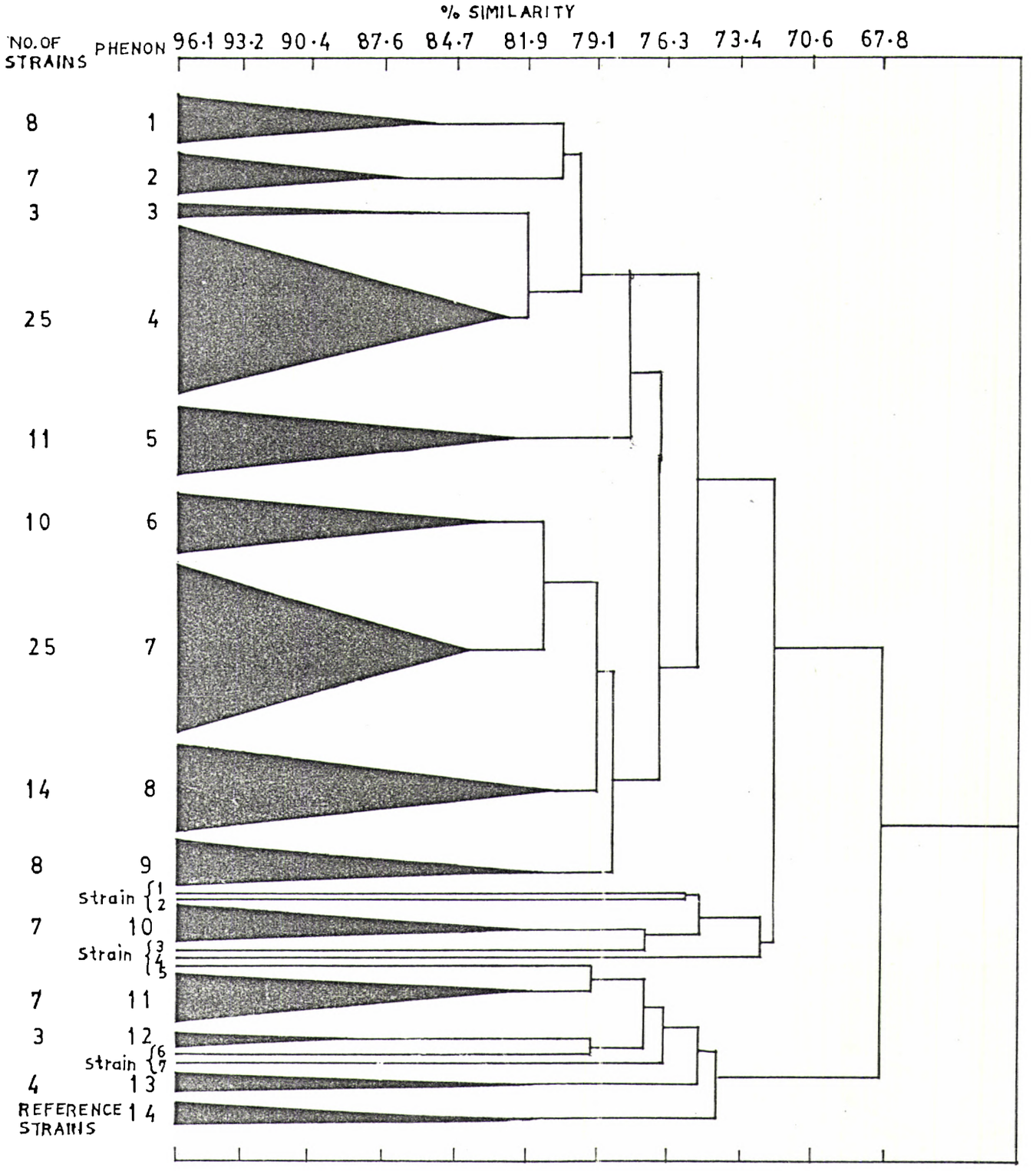


Fig. 2.2 Dendrogram of the isolates from moribund larvae, O/129 negative

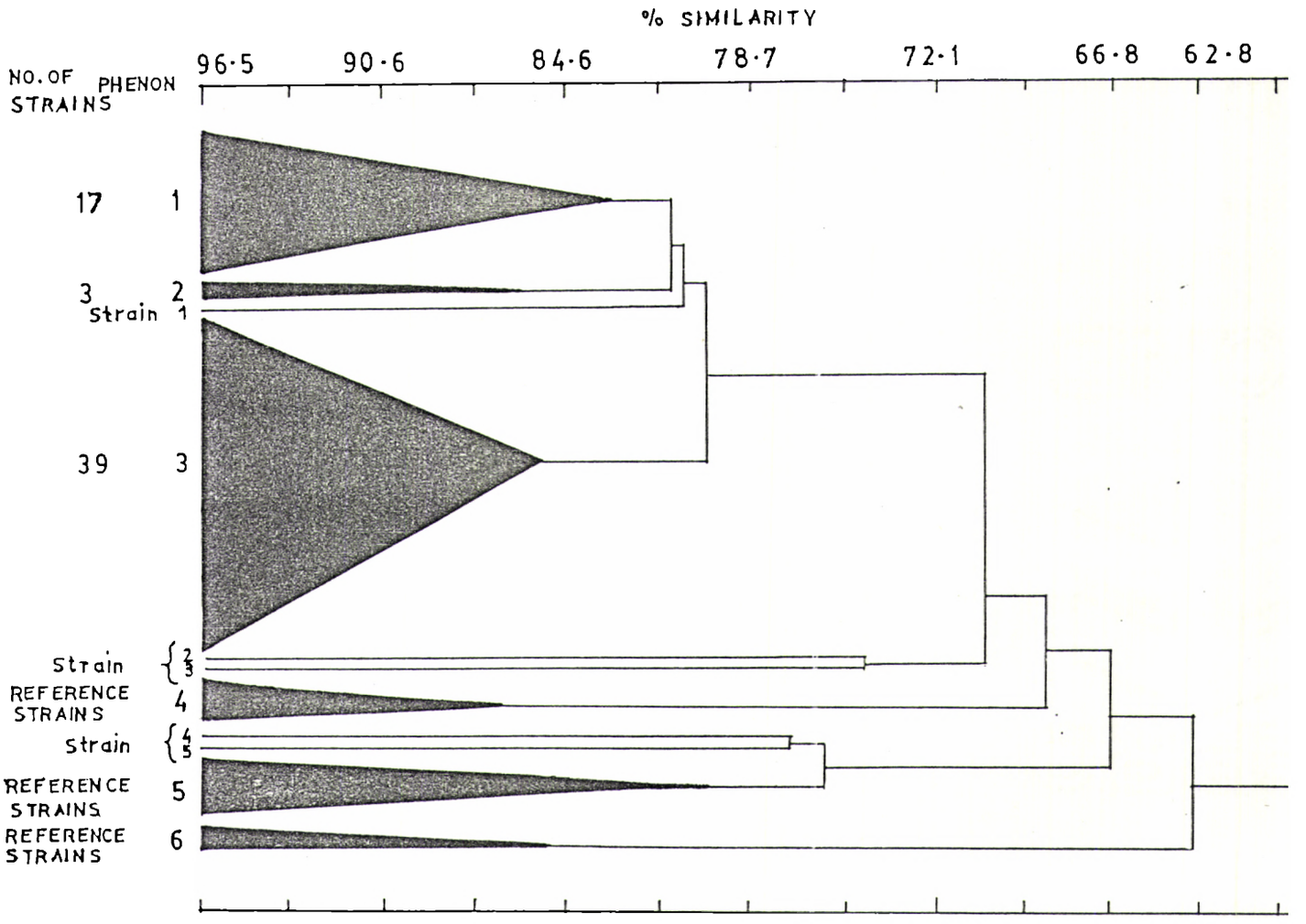


Fig. 2.4 Dendrogram of the isolates from moribund larvae, O/129 positive

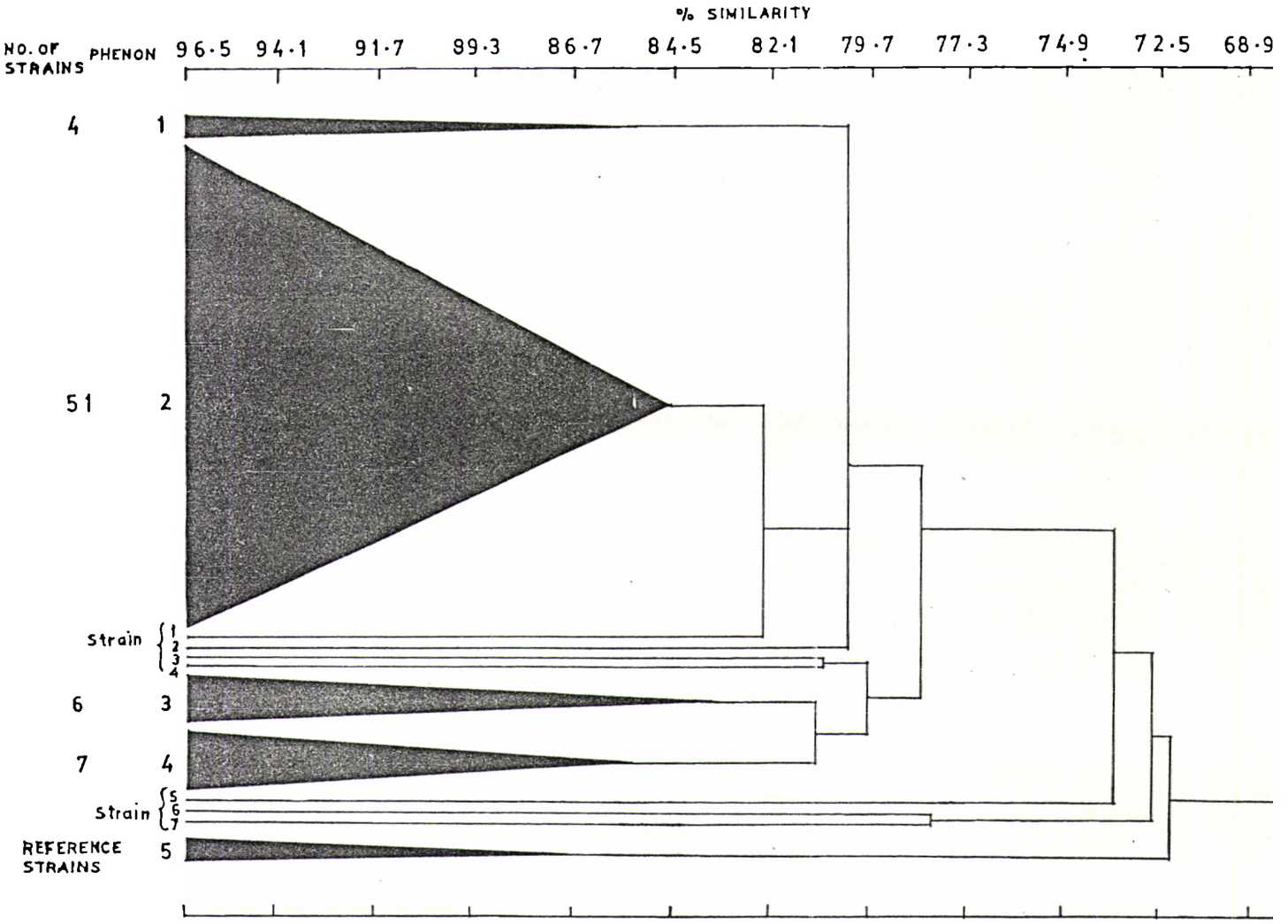


Fig. 2.6 Dendrogram of the isolates from apparently healthy larvae, O/129 negative

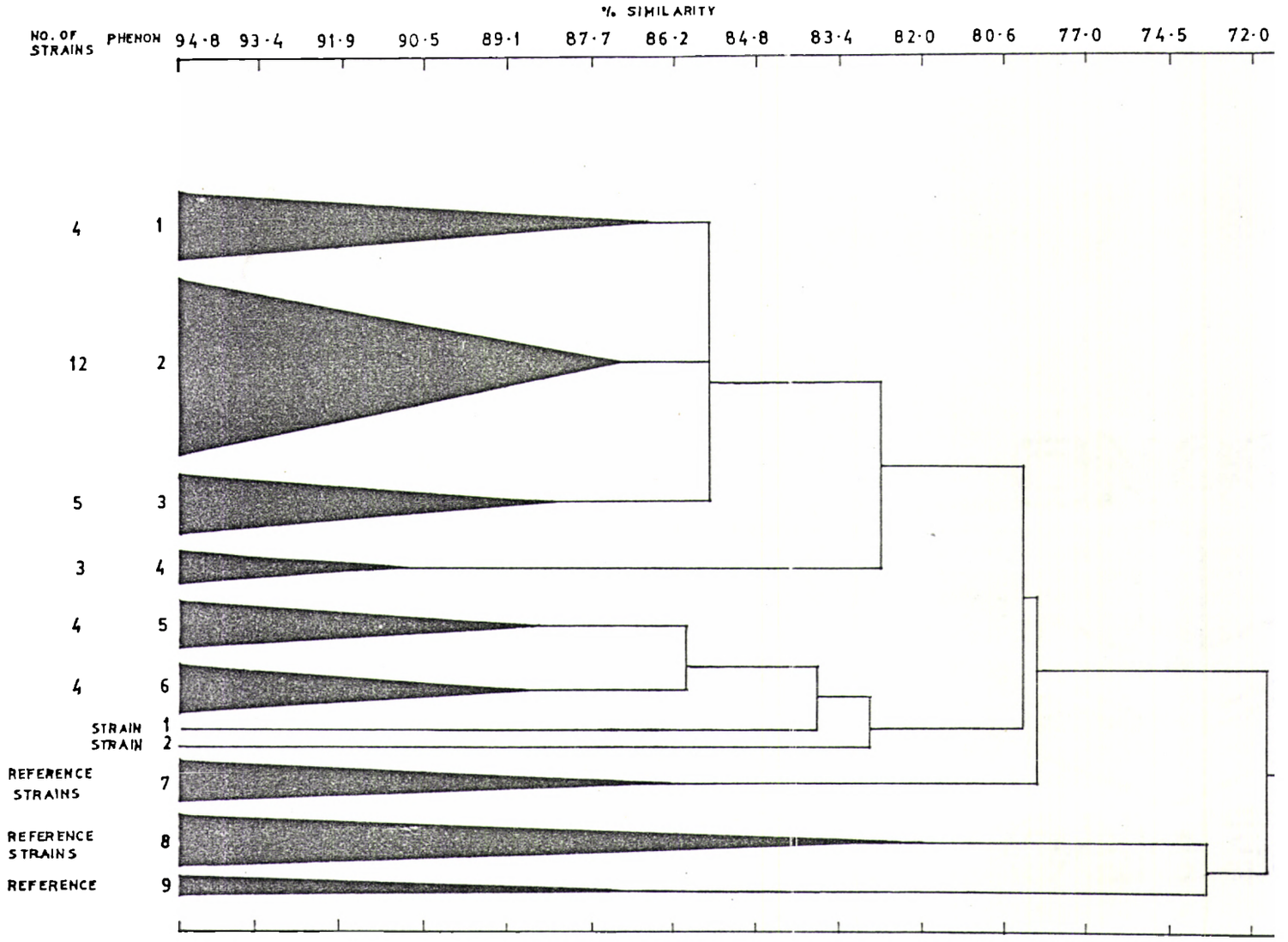


Fig. 2.8 Dendrogram of the isolates from apparently healthy larvae, O/129positive

CHAPTER - III

SEGREGATION OF PATHOGENIC STRAINS

CHAPTER-III

SEGREGATION OF PATHOGENIC STRAINS

3.1.Introduction

Development of commercial culture of shrimp has always been accompanied by the occurrence of the infectious and non-infectious diseases. Several species of bacteria have been implicated as causes of such diseases which in most of the occasions break out of several predisposing conditions resulting in mortality in the larval, post larval and juvenile stages (Lightner, 1988;Lightner *et al.*, 1992). The farming of crustaceans, particularly marine and brackish water shrimps has gained tremendous popularity in the Asia-Pacific region, South America and Central America over the past decade. Present estimates indicate that over two million hectares are dedicated to shrimp culture in these regions and regular health screening procedures should be established and strictly adhered to, as a complement of good husbandry practices to prevent calamities due to epidemics.

Walne (1956) suggested that among the cultured aquatic animals, too little attention was given to the effect of bacteria on shellfish larvae. His experiments showed, that bacterial populations in shell fish larval culture systems might be hundred times more than those in the sea. Three years later the first laboratory experiments proving the pathogenicity of specific bacteria were reported by Guillard (1959).

Now the diseases due to bacterial infections in captive- wild and cultured shrimp are very well known (Lewis, 1973 a, b; Cook and Lofton 1973; Delves-Broughton and Poupard,1973; Lightner and Lewis, 1975; Aquacop,1977;Lightner,1977). These infections may take two forms: localized pits in the cuticle (shell diseases) or generalized

septicemia; most seems to be a secondary nature (Cook and Lofton, 1973 and Lightner, 1977). Nevertheless in every reported bacterial infections in prawns reviewed up to 1983, motile, Gram negative, oxidase positive, fermentative rods have been isolated from lesions or haemolymph. Most isolates have been *Vibrio* species usually *V. alginolyticus, V. parahaemolyticus or V. anguillarum.* Certain other Gram negative rods including *Pseudomonas* spp and *Aeromonas* spp. may occasionally be involved in Bacterial Disease Syndrome in prawns (Lightner, 1983). However, all these genera and species have been reported to be among the normal microflora of these animals (Vanderzant *et al.*, 1970,1971,. Aquacop , 1977; Yasuda and Kitao, 1980; Lewis *et al.*, 1982 and Singh *et al.*, 1998) Hence, it would appear that shrimp have only opportunistic bacterial pathogens that are part of their normal microflora even though there are potentially pathogenic forms

In the most extensive study conducted by Miyamoto *et al.*, (1983), 13 genera of bacteria were identified from *Macrobrachium rosenbergii* larvae, including *Vibrio, Aeromonas* and *Pseudomonas.* Colorni (1985) described the predominant flora in healthy larvae as *Aeromonas liquifaciens, Vibrio anguillarum* and various other species of *Vibrio, Aeromonas* and *Pseudomonas* spp. Fujioko and Greso (1984) recorded the isolation of many different species of *Vibrio* including *V. alginolyticus, V. fluvialis* and *V .cholerae* non 01 from the *Macrobrachium* sp. larval culture waters. Bacteria isolated from the brown spot necrotic tissue were predominantly chitinoclastic comprising several species of the genera *Vibrio, Aeromonas* and *Pseudomonas* (Brock, 1983; El-Gamal *et al.*, 1986). Studies carried out in Malaysia showed that mass mortalities in larval culture cycles could not always be attributed to environmental and/or management faults (Anderson *et al* 1989). In many situations, pathological lesions similar to those described by other workers (Aquacop, 1977) and mid-cycle larval diseases (Brock, 1983) were seen in which bacteria or bacteria derived etiology were postulated. Anderson *et al.*, (1988) identified *Alkaligenes* sp and *Enterobacter* sp. from these diseased larvae, without demonstrating any clear role for these isolates in relation to the disease condition itself, as

they were seen as part of the normal flora of the larvae. However, systemic bacterial diseases necrosis, due to *Aeromonas* or *Vibrio* is common in hatcheries (New, 1995).

One of the most common larval problems is mid cycle disease (MCD). MCD peaks about 10 days into a 35- day larval period (Angell, 1992). Sometimes heavy mortality is also observed during the first few days after hatching. The exact etiology of this disease is not yet known even though it is speculated that there may be a link between MCD and nutritional deficiency, or the introduction of a toxic or disease carrying larval feed. Ten days post hatching is the time when the feeding of *Artemia* nauplii are significantly reduced or ceased (New, 1995).

Bacterial diseases caused by chitinolytic bacteria, *Pseudomonas, Vibrio, Beneckea, Aeromonas* and *Leucothrix* are common in prawn hatcheries(Lombardi and Labao, 1991a, b), and result in 'black' spot bacterial necrosis and gill obstruction. Studies on the involvement of bacteria, in the larval development of *M. rosenbergii* in Indian hatcheries are very limited. However, the involvement of *Vibrio* and *Aeromonas* during hatching and larval development, has been reported by Singh (1990). According to him, eggs fail to hatch out and mass mortality of larvae are common when *Vibrio* and *Aeromonas* dominate in the populations associated with the embryonated eggs and larvae; and when *Pseudomonas* happens to be the dominant flora, the larvae complete the development successfully

Even though vibrios and aeromonads were repeatedly isolated from the diseased animals, conformation of their pathogenicity by satisfying Koch's postulates, have always been difficult. In several such attempts a relatively massive dosages were administered in order to overcome the natural defense of the host and produce disease and death in the experimental animals (Vanderzant *et al.*, 1970; Lewis 1973b; Lightner and Lewis, 1975, Corliss *et al.*, 1977; Huang *et al.*, 1981). One study showed that cell free solutions of crude extracts of endotoxins and exotoxins of *V. parahaemolyticus* and *V. alginolyticus* injected in *P. satiferus*, produced significant mortalities with gross signs,

similar to those observed in actual bacterial infections (Leong and Han rahan, 1980). One of the reasons for the phenomenon is the loss of virulence due to repeated sub-culturing, inevitable for any such study Another reason may be the lack of growth factors in the medium essential for the expression of pathogenicity.

In the present study, one of the objectives was segregation of pathogens from the associated Vibrionaceae and Aeromonadaceae of the larvae. The representative strains from each phenon along with the lone isolates that did not join with any of the clusters were subjected to experiments in order to segregate the potential pathogens. Considering the possibility of the loss of virulence that may have occurred during storage and subculture, an additional step of passaging the test organisms through prawn infusion was incorporated in the protocol.

3.2. Materials and Methods

3.2.1. Selection of representative strains

The group of 204 isolates from moribund larvae and 109 isolates from healthy larvae, designated as Vibrionaceae were further segregated based on their sensitivity to the O/129 compound into two groups, viz. sensitive and non-sensitive. Each bacterial isolate was examined for a total of 233 unit characters and are analyzed using simple matching coefficient following the methods of numerical taxonomy. Clustering was by unweighted average linkage from which sorted dendrograms and similarity matrices were constructed (Chapter-2)

From the clusters obtained after the computer analysis of the data, 47 strains were selected as representatives for determining their mole % G+C ratios (Chapter-2) and for testing their pathogenicity This included cultures selected from the base of each cluster and also the isolates which did not cluster with any group and which stood out independently

3.2.2. Strain virulence improvement.

The 204 isolates from moribund larvae and 109 from the apparently healthy ones, were subjected to the continuous process of characterization and analysis over a period of two and a half years. During this period, the cultures were constantly sub-cultured and because of this, it was suspected that all the 47 representative strains might have become avirulent or less virulent.

In order to enhance the virulence of the selected isolates, they were passaged through prawn muscle (*Macrobrachium rosenbergii)* extract or infusion. This infusion acted as a substitute for a live animal passage. Such a method was adopted because re-isolation of the organism from the whole animal after inoculation with out a

contamination would be too difficult. As the infusion was filter sterilized, all its proteinic components and other unknown growth factors are expected to be protected without being denatured or degraded, and this would naturally stimulate the revival of the invasiveness, an expression which can be attributed to the pathogenicity

3.2.3. Preparation of prawn infusion

20g of prawn (*Macrobrachium rosenbergii*) muscle was macerated in 200 mL PBS (1X) (phosphate buffered saline) and centrifuged at 10,000 rpm for 30 minutes, to remove the debris. The supernatant was inactivated in a water bath at 56^0C for 30 minutes. This heating helped to coagulate the proteins and the solution was again centrifuged at 10,000 rpm for 30 minutes. The supernatant thus obtained was sterilized by passing through Seitz filter (0.45 μ pore size). The filtrate thus obtained was once again sterilized by passing through Whatman filter (0.22 μ pore size), and was stored at 4^0C.

3.2.4.Sterility testing for the prawn infusion.

2mL of the filter sterilized prawn infusion was inoculated into nutrient broth (peptone 0.5%, beef extract 0.5%, yeast extract 0.1%, NaCl 2%, pH 7.3±0.3), incubated for 24 hours at 28±0.4^{0}C and observed for growth, as indicated by turbidity

3.2.5.Preparation of phosphate buffered saline (PBS) (1X)

Dulbecco's PBS medium

1.PBS (A)	NaCl	8g	
	KCl	0.2g	
	Na_2HPO_4	1.15g	
	KH_2PO_4	0.2g	Dissolved in 800mL of distilled water

2.PBS (B)	$CaCl_2$	0.1g	Dissolved in 100mL of distilled water
3.PBS (C)	$MgCl_2\ 6H_2O$	0.1g	Dissolved in 100mL of distilled water

Solutions (A), (B) and (C) were autoclaved separately at121^{0}C for 15 minutes and stored at room temperature. When complete PBS was required, the solutions (A),(B) and (C) were added together aseptically.

3.2.6 Passages through prawn infusion

The sterile prawn infusion that was prepared as mentioned in section 3.2.3., was dispensed under aseptic conditions into sterile culture tubes, in 1mL aliquots. The cultures that were to be passaged through this infusion were grown on nutrient agar slants and 24 hours old cultures were than used as the inoculum. These inoculated prawn infusion tubes were incubated at 28±0.5^{0}C for 24-48 hours. Growth was indicated by turbidity after 24-48 hours. It was passaged successively for two more times.

From the third passage, the test organisms enriched in the prawn infusion, were used for testing the pathogenicity in the larvae of *Macrobrachium rosenbergii*.

3.2.7 Test of pathogenicity

Pathogenicity of the selected cultures passaged through prawn infusion to revive and enhance their virulence, were tested on 10-12 day old larvae of *Macrobrachium rosenbergii*. Fifteen larvae were maintained in 500mL water in 3 litre plastic basins. These larvae were maintained on a diet composed of freshly hatched *artemia* nauplii. Salinity of the water was maintained at 13ppt and for avoiding drastic fluctuations in the water temperature, the basins were maintained in another large tub filled with tap water. The temperature was 26-28^{0}C through out the experiment. Ammonia content was recorded below 0.2 ppm in the experimental tanks though out the experiment and pH was 7.0±0.3. The larvae thus maintained were challenged with the cultures from the third

passaged tubes to a final number of 10^7 cells/mL of the larval rearing water Progressive mortality over a period of 72 hours was recorded against three sets of positive and negative controls. The positive set of experiment was done by inoculating a strain of *Pseudomonas* sp. isolated from the larval rearing system, to the final number of 10^7 cells/mL. The experiment was repeated thrice with fresh batches of larvae and the average in percentage mortality was recorded. The test of significance ('t') was worked out.

3.2.8 Re-isolation of the pathogenic strains

To complete Koch's postulates, moribund larvae from both the test and negative control groups were used for re-isolating the pathogen. The larvae were washed in sterile 15 ppt seawater and disinfected with sodium hypochlorite containing 100ppm chlorine. Altogether 25 sets of larvae were macerated separately in a tissue homogenizer, with PBS and plated on nutrient agar plates by the standard pour plate method. From the test group larvae,125 colonies and from the control group 35 colonies were isolated and identified to family The χ^2 analysis was carried out to find the significance of the results obtained.

3.3 Results and discussion

Pathogenicity of the representative strains on the larvae of *M. rosenbergii* was determined. This was based on the extent of mortality that the larvae suffered due to the inoculation of the revitalized cultures as compared to the uninoculated controls. Behavioural changes such as sluggish movement, sinking to the bottom of the rearing trough, lack of sensitivity to light were also observed in the experimental sets where mortality was registered. Altogether 47 representative strains were tested. From the healthy larvae, 11 isolates were O/129 negative and 8 O/129 positive; from the moribund larvae 20 were O/129 negative while 8 were O/129 positive

Percentage mortality of the larvae from *M. rosenbergii* challenged with the test organisms from moribund larvae (O/129 negative) are given in Tables 3-1. With all test organisms uniformly higher mortality of the larvae was observed ranging from 23.06 to 89.56 %, compared to both the positive and the negative control which is significant at <0.01 level. In Table 3-2 the percentage mortality suffered by the larvae on challenging with the test organisms which are O/129 positive are summarized. It can be seen that compared to the controls, higher percentage of mortality ranging from 62.32 to 83.90 was observed, significant at <0.01 level. In Table 3-3,percentage mortality of the larvae of *M.rosenbergii* challenged with the test organisms (O/129 negative) obtained from apparently healthy larvae are summarized. Uniformly for all test organisms at significantly(at < 0.01 level), higher percentage mortality ranging from 54.64 to 82.58 compared to control groups were observed. On inoculating the test organisms (O/129 positive) obtained from apparently healthy larvae, higher percentage mortality ranging from 54.07 to 71 95 significant at <0.01 level compared to that observed in the control groups. In Table 3-5, the percentages of vibrios re-isolated from both experimental and control sets of larvae are summarized.

The data clearly indicated that all strains obtained as representatives of the phena from both apparently healthy and moribund larvae are virulent which in general is a

manifestation of the family Vibrionaceae and Aeromonadaceae. Egidius(1987) proposed that the term vibriosis is applicable in all cases of infections caused by a bacterial species of the genus *Vibrio*. But it appears that the definition has further to be modified to accommodate the infection caused by aeromonads also as they were once grouped under the family Vibrionaceae (Baumann and Schubert, 1984)even though now segregated in to a separate family Aeromonadaceae. Therefore, the definition of vibriosis may be modified as the infectious diseases caused by species of *Vibrio, Aeromonas* and *Photobacterium* in finfish and shellfishes leading to septicemia and death. On this basis the mortality caused by the strains of bacteria inoculated in to the larvae of *M. rosenbergii* shall be designated as vibriosis.

On assessing the pathogenicity of the strains against the positive and negative controls, it is postulated that the pathogenicity is widespread in the family Vibrionaceae and a general protection for the larvae against the invasive death has to be accorded as part of the husbandry practices. Interestingly, there was no profound variation between the isolates from moribund and apparently healthy larvae as far as the pathogenicity is concerned.. It has to be mentioned that an apparently healthy larval rearing system has been defined based on the comparatively better survival rate of the larvae obtained in these pools over a period of 15 to 20 days indicating precisely that the pathogens exist in both the systems and it is the introduction and existence of certain stress factors which lead to the invasion of these organisms in to the larvae. In the systems which are designated as 'apparently healthy' too, the percentage survival is only 20 against 100% mortality seen in the 'sick' pools indicating precisely that there is no difference in the distribution of the pathogenic strains.. This observation deepens the understanding that the members of the family Vibrionaceae as a whole in the larval rearing system of *M.rosenbergii* has to be considered a flora to be avoided , as they turn out to pathogenic at any moment of time.

During the time of experiment the larvae were fed with *Artemia* nauplii. Huq *et al.*, (1983) observed that *Vibrio* colonize copepod surface and it turns out to be a micro-

ecosystem for them to survive. In the present experiment, the strains inoculated in to the rearing water may have entered in to the larval alimentary canal through *Artemia* nauplii when the larvae feed on them. On studying the microbial flora of the penaeid prawns Singh *et al.*, (1990) and Singh *et al* (in press) observed that the alimentary canal of prawns formed a suitable micro-environment for *Vibrio* and *Coryneform* group to undergo no more than a few cycles of division. Lee, (1980) on studying the bacterial variability among individual penaeid shrimp digestive tracts observed that the hind gut lining has many bacteria living adhered to the gut epithelium. Attached or adhering bacteria would have a selective advantage in the flow through environment of the digestive tract(Liston,1963; Brezak and Pankratz,1977; Lee, 1980; Costerton and Cheng, 1988). Yasuda and Kitao (1980) showed that zoea of *P. japonicus* harboured more *Vibrio* in the intestine than in the rearing water. Like wise, in the present study also the pathogenic strains which enter the gut of *M. rosenbergii* larvae might be adhering to the gut epithelium and undergo multiplication. Lio (1985) reported that *Vibrio* infected post larvae of prawns by invading the haemolymph and mid gut gland.. The mortality which was observed in the larvae of *M. rosenbergii* in the present study is directly linked to the strains of the family Vibrionaceae which with all probability might have invaded the haemolymph and caused septicemic death. This was further demonstrated by re-isolating the strains from the moribund larvae at significant percentage(Table 3-5), even though it was not possible to distinguish it from those that originated from the gut or the haemolymph.

It is very much notable that none of the strains of the 47 tested, caused 100% mortality of larvae during a 72 hour experimental period. Meanwhile, Karunasagar *et al* ., (1994) on studying the mass mortality of *P. monodon* larvae due to antibiotic resistant *V. harveyi* which colonized on larval tanks, could demonstrate 100% mortality with a dosage of 10^4 cells per mL But the *V. harveyi* from seawater gave only around 50% mortality Meanwhile, *Pseudomonas* gave around 20-24 % mortality during this period. A comparison of their observation with that of the present study, even though they are

two entirely different systems suggest that the strains of Vibrionaceae isolated and studied here not may have potential pathogenicity but on the other hand they can be classified as opportunistic pathogens as they are capable of causing mass mortality during unfavorable conditions.

Table 3-1 Percentage mortality of the larvae of *M. rosenbergii* when challenged with the test organisms obtained from moribund larvae which are O/129 negative

Serial no.	Culture no.	Mole % G+C ratio	% mortality	Compared to –ve control		Compared to + ve control		Species to which affiliation is seen in terms of Mole % G+C ratio
				t value	p value	t value	p value	
1	AAC 1104a	53.5	62.70	37.1028	0.01	11.4762	0.01	Group E-3
2	AAC 1142b	39.8	63.68	40.3896	<0.01	11.5778	<0.01	*V. fisheri*
3	AAC 1101b	41	56.82	50.1575	<0.01	10.3961	<0.01	*P. angustum ,, P. phosphoreum, V. logei, V. marinus, V. fischeri*
4.	AAC 639b_1	53.9	42.51	30.1126	<0.01	6.1520	<0.01	Group E-3
5	AAC 1114b	41	58.59	32.1945	<0.01	10.2198	<0.01	*P. angustum , P. phosphoreum, V. logei, V. marinus, V. fischeri*
6	AAC 1109b	39.6	55.8	23.5530	<0.01	8.8822	<0.01	*V fisheri*
7	API 684a	50.5	23.06	8.8987	<0.01	0.5921	>0.2	*V costicola, V. furnissi, V. fluvialis, V. hollisae, V. proteolyticus*
8	AAC 1128a_2	47.9	58.3	40.1411	<0.01	10.5287	<0.01	*V campbelli, V. cholerae, V. harveyi, V. vulnificus*
9	AAC 1101a	40.7	43.54	20.3294	<0.01	5.9798	<0.01	*P angustum , V. logei, V. marinus, V. fischeri*
10	AAC 880c	57.3	59.33	67.2089	<0.01	11.3188	<0.01	*Aeromonas samonicida*
11	ANM 598c_2	44.9	86.72	53.6791	<0.01	18.1098	<0.01	*V anguillarum, V metschnikovii, V ordalli, V tubiashi, V ichthyoenterii*
12.	ANM 718aa	44.4	89.56	47.0704	<0.01	18.2791	<0.01	*V anguillarum, V metschnikovii, V ordalli, V tubiashi, V ichthyoenterii*
13	ANM 625	40.5	87.93	67.1695	<0.01	19.0196	<0.01	*P angustum , V logei, V marinus, V. fischeri*
14.	AAC 672b_1	59.4	77.68	41.4831	<0.01	15.2079	<0.01	*Aeromonas sobria, A. hydrophila*
15	AAC 669B	56.2	66.38	33.5978	<0.01	12.0737	<0.01	Out of range of Vibrionacae
16.	AAC 1108d	46.5	65.86	35.0368	<0.01	12.0731	<0.01	13 species of Vibrios
17.	API 644b	58.2	70.35	42.5931	<0.01	13.5998	<0.01	*A. hydrophila, A. sobria*
18.	AAC 654b	63.3	73.26	38.3086	<0.01	13.9757	<0.01	*A. caviae-like*
19.	AAC 536	48.5	88.40	41.3932	<0.01	17.4690	<0.01	*V cholerae*
20	ANM 1008a	57.3	72.27	46.9523	<0.01	14.2858	<0.01	*A. salmonicida*

Table 3-2 Percentage mortality of the larvae of *M. rosenbergii* when challenged with the test organisms obtained from moribund larvae which are O/129 negative

Serial no.	Culture no.	Mole % G+C ratio	% mortality	Compared to –ve control		Compared to + ve control		Species to which affiliation is seen in terms of Mole % G+C ratio
				t value	p value	t value	p value	
1	AAC 629b	43.4	83.90	60.67	<0.01	17 7584	<0.01	*V. aestuarianus, V. tubiashi, V. ichthyoenterii, P. leiognathi*
2.	ANM 594b	43.2	65.56	35 03	<0.01	1.0019	>0.2	*V aestuarianus, V tubiashi, V ichthyoenterii, P. leiognathi*
3.	ANM 597	43.5	65.28	22.4611	<0.01	10.4656	<0.01	*V. aestuarianus, V. tubiashi, V. ichthyoenterii, P. leiognathi*
4.	ANM 708	39.9	67.59	41.8255	<0.01	12.8984	<0.01	*V. fisheri, V. logei, V. marinus, P angustum*
5	ANM 610a	53.6	83.43	46.9105	<0.01	16.5828	<0.01	Group E-3
6.	ANM 1020	39 9	82.7	49 9304	<0.01	16,9423	<0.01	*V. fisheri, V logei, V marinus, P angustum*
7	ANM 594a	44.8	62.32	37 9457	<0.01	11.4294	<0.01	*V tubiashi, V anguillarum, V ichthyoenterii, , V.ordalli*
8.	ANM 1003	54.3	62.67	27 4485	<0 01	10.7029	0.01	Group E-3

Table 3-3 Percentage mortality of the larvae of *M. rosenbergii* when challenged with the test organisms obtained from apparently healthy larvae which are O/129 negative

Serial No.	Culture no.*	Mole % G+C ratio	% mortality	Compared to –ve control		Compared to + ve control		Species to which affiliation is seen in terms of Mole % G+C ratio
				t value	p value	t value	p value	
1	AAC 730a	47 1	72.1	32.1190	< 0.01	13 1299	< 0.01	*V gazogenes, V, cholerae, V. cambelli, V. penaecida, V. diazotrophicus, V. nereis, V. parahaemolyticus, V. pelagius* bv. I and II, *V. vulnificus bv. I, V nigripulchritudo, V. harveyi V. natreigenes, V. navarrensis, V. alginolyticus*
2.	ANM932	49.8	65.48	36.6361	< 0.01	12.0973	< 0.01	*V. fluvialis, V. costicola, V, furnissi*
3	ANM 723	54.9	69.55	39.0398	< 0.01	13.1952	< 0.01	Group E-3
4	API 1546	38.3	68.45	33.0666	< 0.01	12.4760	< 0.01	*V. fisherii*
5.	API 1561	54.9	54.64	34.1822	< 0.01	9.3790	< 0.01	Group E-3
6.	API 1558	60.12	69.67	53.1997	< 0.01	13.8721	< 0.01	*A. hydrophila, A. caviae, A. sobria*
7.	AAC 727	38.3	82.58	48.1869	< 0.01	16.8021	< 0.01	*V. fisherii*
8.	AAC 740	52.1	78.03	48.0304	< 0.01	15.7289	< 0.01	*V. fluvialis, V. costicola, V, furnissi, V. proteolyticus, V. hollisae*
9	API 810	36.5	73.03	41 9614	< 0.01	14.1900	< 0.01	Out of range of Vibrionaceae
10.	AAC 717	56.7	66.61	24.0699	< 0.01	10.9755	< 0.01	Out of range of Vibrionaceae
11.	AAC 781	36.1	61.3	31.9249	< 0.01	10.8211	< 0.01	Out of range of Vibrionaceae

Table 3-4 Percentage mortality of the larvae of *M. rosenbergii* when challenged with the test organisms obtained from apparently healthy larvae which are O/129 positive

Serial no.	Culture no.	Mole % G+C ratio	% mortality	Compared to –ve control		Compared to + ve control		Species to which affiliation is seen in terms of Mole % G+C ratio
				t value	p value	t value	p value	
1	AAC 701	48.4	71 95	25.8333	<0 01	12.1955	<0.01	*V cholerae, V harveyi, V campbelli, V vulnificus*
2.	ANM 712	45.6	71.87	37.0840	<0.01	13.5687	<0.01	*V anguillarum, V. metschnikovii, V mytili, V navarrensis, V. ordalli, V orientalis, V pelagius* bv. I and II, *V. splemdidus* bv. I and II, *V. alginolyticus*
3.	ANM 719	38.3	59.82	31 4686	<0.01	10.4521	<0.01	*V. fischeri* –like
4.	API 1555	50.6	54.07	26.8013	<0.01	8.8153	<0.01	*V. proteolyticus, V. hollisae, V. costicola, V. furnissi,*
5.	API 768	60.1	73.21	32.9417	<0.01	13.4484	<0.01	*A. hydrophila, A. sobria, A. caviae*
6	ANM 721	47.8	74.47	63 6724	<0.01	15 4107	<0 01	*V vulnificus, V campbelli, V cholerae, V harveyi, V. tubiashi* , marine luminous vibrios
7	ANM 702	63.1	64.41	69.5656	<0.01	12.7556	<0.01	*Aeromonas species*
8.	ANM 1519	49.5	54 76	45 5941	<0.01	9.7556	<0.01	*V proteolyticus, V hollisae, V costicola, V. furnissi, V. fluvialis*

Table 3-5. Percentage of vibrios reisolated from the experimental and control groups of larvae after pathogenicity studies

	Experimental larvae		Control larvae	
	% vibrios	% non vibrios	% vibrios	% non vibrios
	76	24	14.29	85.71
Total no of isolates	95	30	5	30
	Highly significant at p <o.o1			

CHAPTER - IV
SELECTION OF ANTIBIOTICS

CHAPTER-IV

SELECTION OF APPROPRIATE ANTIBIOTICS FOR USE IN THE LARVAL REARING SYSTEM OF *M. ROSENBERGII*

4.1. Introduction

The earliest record of aqua-culture was in China, where artificial hatching and rearing of carp *Cyprinus carpio* was carried out, dating back to 2000 B C. Aqua-culture with oysters was first carried out by the Romans. Now the culture of shell fish like clams, oysters, shrimps and prawns are common. And all the culturable species are subjected to infectious diseases and their impact on the economic viability of the aqua-culture industry is immense.

Aqua-culture is gaining importance in the world arena of fish production. The water bodies, either fresh, brackish or sea, are of considerable value for fish production through aqua-culture. The world aqua-culture production has reached 10 million tonnes and is expected to reach 20 million tonnes by the turn of the century

Disease is one of the most important factors limiting the survival, growth and production of farmed fish and shellfish. It has been estimated that about 10% of all cultured aquatic animals are lost as a result of mortalities due to infectious diseases. Highly pathogenic bacteria and viruses cause mortality of over more than 90% of the hatchery populations.

Poor hatchery management, overcrowding, unsuitable water quality, dietary imbalances, stress etc are some of the major factors contributing to the occurrence of disease in shrimp and prawn culture systems. Diseases can be of infectious or of non-

infectious aetiologies. Infectious diseases are caused by bacteria, viruses, fungi and other parasitic organisms.

Bacterial diseases in shrimp and prawn culture systems are caused by several species of bacteria such as *Vibrio, Beneckea, Aeromonas,, Pseudomonas* and *Photobacterium.* The fungal diseases in larvae are caused by *Lagenidium* and *Fusarium* spp (Sindermann, 1990).

Several species of fresh water prawns particularly the giant prawns *Macrobrachium rosenbergii* have been the subjects of intensive culture efforts in the United states, India and South East Asia during the past decade. Because spawning and larval development occurs in saline waters, these animals logically become part of marine aqua-culture. So far, approximately 15 disease conditions have been described of which at least three are of infectious origin. Disease problems with *Macrobrachium* culture have been relatively minor, compared with penaeid shrimp culture and seem to result largely from poor water quality or other stresses characteristic of the artificial environments (Sindermann, 1990). Though a large volume of literature is available on the microbiology, pathogenicity and chemo-prophylaxis against pathogens of penaeid shrimps, not much seems to have been done on chemo-prophylaxis and therapy as well as on potential pathogens of the Malaysian fresh water prawn *M. rosenbergii.*

Like many other pathogens, bacteria are always present in water and are opportunistic organisms. They may effect prawns both as primary and secondary invaders, and infection may usually develop due to adverse changes in the environment which can easily stress the prawn and thus lower its resistance A number of bacterial diseases have been reported to effect the various stages of penaeid prawns. Necrosis of the appendages may affect both larvae and post larvae (Aquacop, 1977, 1979;Vincente *et al.*, 1979; Gacutan, 1979). But in these cases the mortalities were usually due to secondary bacterial infection after cuticular injuries or due to the inability of the larvae to molt (Gacutan, 1979).

The bacterial species *Vibrio* has also been observed to effect the protozoeal stages and cause mortalities up to 80% in hatcheries (Ruangpan, 1982, PCARRD,

1985). Delves-Broughton and Poupard (1973) and Aquacop (1977) suggested that the *Vibrio* disease in *P. monodon* may be due to prevailing environmental conditions.

Luminous bacteria isolated from larval and post larval sediments were found to be present in dominant proportion in weak and dead prawns as also in rearing water samples, sediments and seawater indicating its occurrence in the natural environment. Mass mortalities are often encountered during heavy infection.

Several luminous bacteria were tested and found resistant to most commonly used antibiotics such as erythromycin, penicillin, streptomycin and sulfadiazine (Baticados, 1990, Karunasagar et al.,1994).

4.1.1 Vibrios as pathogens

As a consequence of the dominance of vibrios in the normal shrimp and prawn microflora, opportunistic *Vibrio* spp. have been the most common bacterial pathogens of cultured shrimp *Vibrio* spp. established lethal following primary infections with other pathogens, environmental stress, nutritional imbalance and /or predisposing lesions (Lightner, 1988). Diseases caused by vibrios have already assumed great importance among cultivated marine fish and shellfish populations. In fact, the emerging role of halophilic vibrios is one of the most exciting present aspects of disease studies in marine aqua-culture. The past few years have seen the elucidation of the identification of the pathogen involved but the picture is very murky and confusing. Some of the most recently occurring disease syndromes of shrimps were caused by *Vibrio* spp. Which were behaving like true pathogen rather than opportunistic invaders (Lightner *et al.*, 1992). The etiological agent of the ulcer disease in Japanese fish was recently described as having characteristics of *V. parahaemolyticus* (which causes human summer sickness) and *V. anguillarum* Kranz *et al.*, (1969) found *V. parahaemolyticus* in Chesapeake Bay crabs and postulated that the *Vibrio* is a pathogen of crab.

Vibriosis caused by species of the genus *Vibrio* has been described as the most serious disease of penaeid shrimps (Egidius, 1987, Bowser *et al.*, 1981). Increased

Vibrio and *Aeromonas* populations in the larval rearing tanks was one of the factors that reduced the survival rate of larvae and post larvae of penaeid shrimp (Hameed, 1994; Singh, 1985). Yasuda and Kitao (1980) observed poor growth of shrimps when the *Vibrio* populations were dominant in the gut. Of the *Vibrio* species described *V. parahaemolyticus*, *V. alginolyticus*, *V anguillarum* and *V. harveyi* have been described as pathogenic to penaeid shrimps (Vanderzant *et al.*, 1970; Lightner and Lewis, 1975; Lightner, 1983, 1988; Takahashi *et al.*, 1985). Hameed (1989) isolated a *Vibrio campbelli*- like bacterium from diseased hatchery reared larvae of *P.indicus*, which were pathogenic to larvae, post-larvae and adult animals. According to Lightner (1983) and Lightner *et al.*, (1992), the important aspects of vibriosis in penaeids are: *Vibrio* spp. which infect shrimp are ubiquitous and are reported from all shrimp culture region. They differ markedly in their virulence for penaeids as they do for other hosts.

Most outbreaks are a consequence of extreme stress and opportunistic pathogens. *Vibrio* spp. may not always produce experimental infection, except when massive doses are injected. Although disease may affect cultivated shell fish at any stage of its life history, a number of recent reports suggest that larval and post larval stages are particularly vulnerable.

Chitinolytic forms of *Vibrio* and *Aeromonas* species have been isolated from shell lesions of pond grown prawns. Most of the species were *V. alginolyticus* (Chong and Chao, 1986). *Vibrio* and *Aeromonas* are very common in seawater and may also be part of the normal flora of the prawn so that the disease could be a result of secondary infections after mechanical injury or trauma of the shell and underlying membranes. Shell disease was also reported to affect wild adult prawns caught off the Cochin backwaters of India (Gopalan *et al.*, 1980).

4.1.2 Chemotherapy

Chemotherapeutics are drugs that are capable of affecting or killing microorganisms, especially bacteria, in the lymphatics of organisms or selectively damaging tumor cells.

Antibiotic is the collective term for the chemically highly heterogenous substances formed from fungi, bacteria or by synthesis. In great dilution, they harm, inhibit or kill other microbes without significant harm to the microorganisms including the host.

The antibiotics act principally via the genetic apparatus of the microorganisms, on their division and therefore act mainly as bacteriostatics. The bacteriostatics is reversible and the multiplication can be carried out on afresh if the action of the antibiotics ceases.

However, a lytic effect is possible, whereby the enzymatic and oxidation processes may be affected, so that the end result obtained is a bactericidal action. This action is irreversible and is a function of the dosage.

In actual practice, it is very important that the practically attainable therapeutic levels of antibiotics lead only to bacteriostatics. It is also important that under such conditions, all normal defense functions of the microorganism should be intact, as no effects can be obtained even with the best of antibiotics, if the organism is so weakened due to infection or any other effects, that the normal defense functions are retained.

The failure of antibiotic therapy is mainly due to the following reasons:

1. Failure of the defense mechanism of the host
2. The absence of transfer media, for instance albumins or
3 The resistance of the pathogen against the antibiotics.

Chemotherapeuthic drugs interfere at a number of vulnerable sites in the cell. They interfere with the following mechanisms:

1. Cell wall synthesis
2. Membrane function

3. Protein synthesis
4. Nucleic acid metabolism and
5. Intermediary metabolism.

It is important to note that there may be a number of stages between the initial or primary effect of the drug and the eventual death of the cell that results. Also, some agents may have more than one primary site of attack or mechanism of action.

The number of chemo-therapeutics available to the crustacean and fish pathologist for the treatment of bacterial and fungal diseases is limited. Most of the drugs currently used are introduced in the animal by feeding during the treatment of systemic infections. This may result only in preventive treatment because the animals that eat it don't need it and the animals that need it don't eat it (Wood, 1986). This phenomenon is likely to be pronounced with the crustacea than with the fish. Chemo-therapeutics added to the water if unabsorbed in an active form or do not achieve effective tissue levels, only treat the surface tissues of the animals. Introduction of drugs by means of injection is not practical on any large scale. What is required for effective therapy of disease of prawns is a chemo-therapeutic that is soluble in water, non toxic to the animals at treatment concentrations, is absorbed in the active form by the animals in order to reach effective tissue levels, is rapidly excreted from the animals tissu after treatment, has a wide anti-microbial activity over a wide range of bacterial and fungal pathogens, does not interfere too greatly with the *Nitrosomonas-Nitrobacter* flora of biological gravel filters and is degradable (Broughton,1974).

Facultative pathogens, particularly but not exclusively of the *Vibrio-Pseudomonas- Aeromonas* group may under stresses of low dissolved oxygen, high temperature, dietary imbalances or high population densities, occur in epizootic proportions in cultivated populations of shell fish. Mortalities caused by non-optimum environments are thus attributed to disease. The species composition of microorganism in the vicinity of aqua-culture facilities may shift drastically, due to eutrophication or shedding of pathogens from infected stock. Disease outbreaks are clearly aspects of ecosystem dynamics (Sindermann, 1990).

The use of chemo-prophylaxis and chemotherapy is feasible in intensive culture systems but not as a substitute for good facility and population management practices. Chemotherapy should be considered a last resort method in disease control, if methods of prevention have failed as emphasized by Herman (1970) and Snieszko (1974).

The best approach to disease control emphasizes stress reduction in aqua-culture facilities. Though this is true, there are occasions when chemotherapy can be a control method of choice and facility, managers are content when they have an armament of chemical remedies readily available on the shelf. Chemo-therapeutic methods have been developed to treat some of the diseases encountered. Some of the microbial diseases can be treated successfully, especially if the treatment was instituted early, before mortalities begin.

Chemotherapy has been successfully employed in the treatment of some larval diseases of lobsters and other crustaceans (Fisher and Nelson, 1977; Fisher *et al.*, 1978). Gaffkemia in lobsters caused by the bacterium *A. viridans* var *homari* have been shown to be responsive to injections of antibiotics vanomycin and penicillin (Stewart and Arnie, 1974; Fisher *et al.*, 1978). Control of epizootic fouling of lobster larvae can be affected by chemotherapy even though improved facility management and hygiene and stress reductions are methods of choice. Fisher et al., (1978) showed that continuous addition of low concentrations of streptomycin and penicillin increased egg and larval survival.

Disease control is complicated by the continuing interplay of host susceptibility, pathogen virulence and environmental influences. Frank pathogens and their effects can be dealt with reasonably well, but much of the damage to cultured populations is caused by facultative pathogens which exerts effects when water quality is not maintained or other stresses like abnormal temperature, oxygen deficiency, inadequate diet, over crowding exist in culture facilities. Disease is always the overt symptom of marginal culture conditions, control frequently consists of improving these conditions (Sindermann, 1990).

The use of antibiotics to control bacterial infections have been instituted against necrosis of larvae and heavy mortalities in penaeids (Aquacop, 1983). Aquacop (1977), tested and used prophylactic/therapeutic drugs mainly erythromycin phosphate at 1ppm active product (AP), streptomycin-bipenicillin at 2ppm AP(2UI/mL), tetracyclin chlorhydrate at 1ppm AP, sulfametazin at 3ppm AP and furanace at 0.1 ppm AP The drug is applied every other day until the post larvae stage is reached. Treated larvae resume feeding, recover quickly and all traces of necrosis are lost after regeneration of the new appendages at the next molt (Aquacop, 1977).

Chloramphenicol has been used in hatcheries at 3ppm every day for prophylactic treatment (Sunaryanto, 1986) and found to be the most effective at prophylactic levels of 2-6 ppm every second day or therapeutic levels of 2-10 ppm with variation adjusted according to the larval stage (Aquacop, 1983).

Chemotherapy of bacterial infections may be done through direct additions of antibiotics to culture tank water during hatching, larval or post larval rearing while for older stages, incorporating the antibiotics directly into the diet could prove to be a better method (Lightner, 1983).

Chemicals added to intensive culture systems for disease controls may perform two important positive functions: They may reduce or eliminate pathogens and they may reduce and control populations of heterotrophic microorganisms which may act as facultative pathogens of animals under stress. Such chemicals can on the other hand cause problems in the culture systems.

1. They may have negative effects on biological filters in controlled recirculated systems, particularly nitrifying bacteria
2. They may have negative side effects on algal food or algae present in fish larval rearing systems
3. They may leave undesirable or harmful residues in cultured animals.

With all their limitations chemo-prophylaxis and chemotherapy still occupy significant niches for response to crisis or otherwise intractable problems, and profit oriented aqua-culture producers are most comfortable when remedies are at hand.

4.1.3 Testing resistance to antibiotics (antibiograms)

Determining the resistance of bacteria constitutes an important prerequisite for a planned therapy of bacteriosis in fish, shrimps and prawns by chemotherapeutics. Several methods are in practice for testing the resistance, of which the most important ones are: the tube test, the plate test, the hole or pit or cylinder test and the paper disc or tablet test.

The tube test or the paper disc test, which are most often used in bacteriological laboratories are described in the following. The tube test is employed for scientific investigations and enables an accurate determination of the sensitivity of fish, prawn/shrimp pathogenic bacteria to antimicrobiotics. The antimicrobial preparations are diluted and introduced into a series of test tubes containing a liquid nutrient medium (nutrient broth, peptone water), hence also called serial dilution tests (SDT). After inoculation, the strain of bacteria will not grow beyond a certain concentration. This dilution of the antibiotic is then taken as the sensitivity value (Minimum Inhibiting concentration, MIC).

According to Schaperclaus (1986) the sensitivity of the bacteria determined in liquid nutrient medium, approach the conditions in vivo closer than testing on solid culture medium. The tube test has the disadvantage that different resistant bacteria flourish and make the broth turbid, whereas only one or few colonies develop on the plate. This disadvantage can be avoided in the plate test.

The paper disc test is the most practiced and enables merely to know whether the bacteria are sensitive, moderately sensitive or resistant. Even this finding is considered quite adequate in most cases.

4.2 Materials and Methods

4.2.1.Bacterial isolates

The isolates from moribund larvae and from the apparently healthy larvae, designated as belonging to family Vibrionaceae (Chapter-2), were used in the preliminary screening studies using the 20 antibiotics and the disk diffusion method. Thus 204 strains from moribund larvae and 109 from the apparently healthy larvae were studied.

Representative strains selected from the clusters obtained after numerical taxonomy analysis were used for determining the MICs of the selected antibiotics after the preliminary screening. Thus 47 strains were tested for their antibiotic sensitivity and resistance by using the Minimum inhibitory concentrations (MICs) of the selected antibiotics.

4.2.2 Media used

4.2.2.a Nutrient agar

The nutrient agar used for the study was prepared in the laboratory using ingredients manufactured by HiMedia. Unless other wise mentioned all media used were supplemented with 2% (w/v) NaCl. The composition of the medium per litre of distilled water:

Peptone	5g
Beef extract	5g
Yeast extract	1g
Sodium chloride	20g
Agar	20g

pH at 28^0 C 7.3±0.2

4.2.2.b Nutrient broth

The composition of nutrient broth is the same as in 4.2.2a, without the agar

4.2.3 Experiment on antibiotic sensitivity using the disc diffusion method (Preliminary screening)

Antibiotic sensitivity of the 204 isolates from moribund larvae and 109 from the apparently healthy larvae, were examined using the ready to use antibiotic sensitivity disks (HiMedia). Details about the antibiotic disks used and their concentration are presented in Table-4-1 In all 20 antibiotics were screened for this study.

4.2.3.a Preparation of nutrient agar plates

Nutrient agar having the composition mentioned in 4.2.2a was prepared and sterilized by autoclaving at 15 psi for 15 minutes. In 90mm sterile petriplates, approximately 15 mL of medium was poured aseptically and the plates were kept undisturbed for solidification. The plates were dried at 37^0C overnight.

These plates were swabbed with the bacterial suspension to be tested. The bacterial suspension was prepared with 24 hour cultures. One or two loopfuls of the 24 hour old culture was suspended in 1.5% (w/v) saline. It was vortexed to obtain a homogenous suspension. Then, with sterile swabs, these bacterial suspensions were spread uniformly on the nutrient agar plates, so as to get lawn cultures. The plates were marked to divide them in to four quadrants. One antibiotic disk was then placed in each quadrant.

Thus, one bacterial culture could be screened for 4 antibiotics per plate. Each strain was screened for sensitivity to 20 antibiotics. The inoculated plates with the antibiotics were incubated for 24 hours at 28 ± 0.5^0C. The diameters of the zones of

inhibition were measured at 24 hours. The sensitivity of the isolates were recorded as follows based on their zones of inhibition (Schauperclaus, 19) as:

Diameter of halozone < 11mm: resistant
Diameter of halozone 11-20 mm: moderately sensitive
Diameter of halozone > 20mm: sensitive

4.2.4 Experiments on the Minimum Inhibitory Concentration (MIC) of the antibiotics selected after the preliminary screening.

After the preliminary sensitivity screening, 10 antibiotics were selected out of the 20 screened, for finding out their minimum inhibitory concentration (MIC). The 47 representative strains selected after the numerical taxonomy analysis, as representatives of the clusters formed , were used to test the MIC of the antibiotics. The list of 10 antibiotics ant their effective concentration are presented in table 4-6. The strains selected as representatives are the same as those selected from each phenon in chapter-2.

Working stock solutions of the antibiotics

The working standard solution of each antibiotic was prepared as 100μg/mL of effective concentration of the antibiotics in sterile nutrient broth and distributed as 1, 5, 10, 20, 40, 60, 80, and 100 μg/mL. The total volume of the broth used for each concentration was 3mL. Three controls were included while testing the MIC of each antibiotic. Control-1 contained nutrient broth (3mL) inoculated with the test culture (but with out the antibiotic) (positive control). Control-2 consisted of nutrient broth (3ml) un-inoculated.

24 hour old cultures grown on nutrient agar slants were used. From this a suspension was prepared in nutrient broth and adjusted the O.D to 0.1. A loopful from this suspension was added to the tubes containing varying concentrations of antibiotics in nutrient broth. The inoculated tubes were vortexed and incubated at

28±0.5^{0}C. The tubes were read for turbidity at 24hours, 48 hours and at 72 hours and MIC was recorded at each time.

4.2.5. Toxicity testing of the antibiotics

The antibiotics chloramphenicol, tetracyclin, streptomycin, chlortetracyclin, kanamycin, oxytetracyclin, gentamycin, neomycin, nalidixic acid and novobiocin were used to study toxicity to the larvae of *Macrobrachium rosenbergii*. Toxicity were studied at 50ppm and 100ppm of the antibiotics used. 10 day old larvae were used in this study One litre of 15 ppt sea water were kept in 3 litre plastic basins and 15 larvae were maintained per basin. The antibiotics were weighted to give concentrations of 50ppm and 100ppm and were dissolved in the water The larvae were fed once every day with *Artemia* nauplii and pellet feed. The temperature was 28-29^{0}C and the pH of the water was 7±0.5. The toxicity study was carried out on triplicates. Controls included three basins containing 15 larvae. The study was monitored for 72 hours. The results were recorded at 24 hours, 48 hours and 72 hours.

4.2.6 Protective effect of antibiotics on the larvae being challenged with the pathogenic strains

As described under chapter-3, pathogenicity of the 47 strains were tested on 10-day M. rosenbergii larvae. From this 18 were selected at random and the larvae were challenged again, but in the presence of antibiotics which had the lowest inhibitory concentration for these strains. (Table 4-7).

For the experiment,1 litre seawater (15ppt) with 10 larvae, kept in 3 litre plastic basins were used. The antibiotics such as tetracyclin, chloramphenicol and gentamycin were weighted out as shown in Table 4-12, were added to the water and inoculated with the 18 pathogenic strains to a final number of 10^7 cells/mL. Both positive with the pathogen and without the antibiotic and negative control without the pathogen and the antibiotics, were incorporated. The larvae were fed with artemia nauplii once in a day Temperature, pH, and ammonia levels were monitored and

found to be 28-29°C, 7± 0.5 and < 0.2 ppm respectively The experiment was conducted in triplicate and relative percentage survival in the presence of the antibiotics was worked out for each pathogenic strain.

4.3 Results and discussion

In order to select an appropriate chemotherapy to protest the larvae from vibriosis, 20 commercially available antibiotics were first screened, by incorporating it as part of the unit characters studied. On analyzing the data based on the principles of Numerical Taxonomy, several phena could be obtained along with strains which did not cluster with any group. These phenas and the individual strains exibited a particular pattern of sensitivity and resistance to the antibiotics tested (Table 4-2 to 4-5)

Among the 20 antibiotics tested, the 13 phena resolved from the group of isolates obtained from moribund larvae that were O/129 negative, were sensitive to only four of the antibiotics such as, tetracyclin, gentamycin, chlortetracyclin and neomycin. The pattern of sensitivity of each of the individual strains vary from each other and as a general agreement all were sensitive to tetracycline, gentamycin, chlortetracycline, methamine mandelate, nalidixic acid and chloramphenicol (Table4-2)

The three phena derived from the group of moribund larvae which are positive to O/129 compound uniformly, were inhibited by antibiotics such as streptomycin, gentamycine, teracycline, chlortetracycline, neomycin, methamine mandelate and nalidixic acid. The strains which are not clustered in this group were sensitive uniformly to the following antibiotics: oxytetracycline, streptomycin, tetracycline, gentamycin, polymyxin B, chlortetracycline, neomycin, methamine mandelate, nalidixic acid, chloramphenicol and kanamycin (Table 4-3).

The four discrete clusters generated from the group of isolates that were o/129 negative and obtained from the apparently healthy larvae are sensitive uniformly to antibiotics such as, oxytetracycline, gentamycin, chlortetracycline, neomycin, methamine mandelate, nalidixic acid, novobiocin, chloramphenicol and kanamycin. The strains from this group that remained unclustered were sensitive altogether to tetracycline, chlortetracycline, neomycin, novobiocin and chloramphenicol.(Table4-4)

The six phena derived from the group of isolates obtained from the healthy larvae, that were O/129 positive exhibited sensitivity uniformly for 11 antibiotics, such as oxytetracycline, streptomycin, tetracycline, gentamycin, chlortetracycline, neomycin, methamine mandelate, nalidixic acid, novobiocin, chloramphenicol and erythromycin. Meanwhile, the strains which remained unclustered in this group were uniformly sensitive to penicillin G, oxytetracycline, streptomycin, tetracycline, gentamycin, polymyxin B, chlortetracycline, neomycin, methamine mandelate, cefazolin, nalidixic acid, novobiocin, chloramphenicol, erythromycin, kanamycin, bacitracin, lincomycin and sufadiazine (Table 4-5).

By the general screening procedure, 11 antibiotics could be segregated as active on each of the phena and strains of all the groups. However, 4 antibiotics among them such as tetracyclin, gentamycin, chlortetracyclin and neomycin were found to inhibit all the 313 strains tested and are designated as most useful, considering the wide spectrum of activity among the family Vibrionaceae.

For determining the Minimum Inhibitory Concentration (MIC), 10 antibiotics such as chloramphenicol, streptomycin,tetracycline, gentamycin, chlortetracyclin, kanamycin, oxytetracyclin, neomycin, nalidixic acid and novobiocin were chosen.. Methamine mandelate was not tested for its MIC as it was not available commercially

Minimum Inhibitory Concentrations of the ten antibiotics against the 47 representative strains, as a cumulative effect over a period of 72 hours is summarized in Table 4-6. MIC_{50} (i.e. the lowest concentration of an antibiotic required to inhibit the growth of 50% isolates tested. has been demarcated in Table 4-6. Within a range of 1 to 100 ppm the antibiotics tested against the 47 strains gave a MIC as follows: chloramphenicol 20ppm, streptomycin 40ppm, tetracyclin 5ppm, gentamycin 20ppm, chlortetracycline 40ppm, oxytetracyclin 80ppm, neomycin 40ppm, nalidixic acid 40ppm. However even at 100ppm concentration, 100% inhibition could not be achieved and the maximum obtained was 95.95-95.74% inhibition with nalidixic acid, gentamycin and chloramphenicol. Performance of the

antibiotics such as kanamycin and novobiocin was very poor as even at 100ppm the percent inhibition attained was only 46.81%.

On testing the MIC with 18 randomly selected pathogenic strains tetracycline ranked first (inhibited 11strains), followed by gentamycin (inhibited 5 strains) and chloramphenicol (inhibited 2 stains) (Table 4-7)

Toxicity of the 10 antibiotics, at 50 and 100ppm levels on the larvae over a period of 72 hours is summarized in Table 4-8. At 50 and 100 ppm no toxicity was recorded with any of the antibiotics as the differences in the percentage survival between the test and control was not significant. Except that at 100ppm nalidixic acid exhibited toxicity significant at $P < 0.01$ levels.

The strains proved to be pathogenic to the larvae, randomly selected from the 47 representative strains were used for testing the protective effect of 3 antibiotics, such as tetracyclin, chloramphenicol and gentamycin at the MIC levels with respect to the strain tested. The relative percentage survival ranged from 50- 80 percent indicating that the antibiotics can accord protection to the larvae against a pathogenic invasion. It has to be highlighted that the three antibiotics were noticed to have a wider spectrum of activity when tested against all the 313 isolates. On testing with the 47 representative strains also, these three antibiotics were found to have a MIC at the lower level such as tetracycline(5ppm), chloramphenicol (20ppm) and gentamycin (20ppm) at 72 hours Based on these observations, these three antibiotics are recommended for applications at the above levels during times of emergency

An overall assessment of the antibiotic susceptibility of Vibrionaceae associated with the larvae of M. rosenbergii reveal the fact that the pathogens in general are sensitive to a wide spectrum of antibiotics. The system studied was not subjected to chemotherapy at any stage, and this may be cited as one of the reasons for the wide spectrum of activity observed. Meanwhile, there were resistant strains too. But one of the considerations given here for the selection of tetracycline, chloramphenicol, and gentamycin for application in the larval rearing system is the wide spectrum of activity they have. But a constant and continuous application of

these antibiotics may as usual lead to the development of resistance through transferable R-plasmids (Aoki, 1992). Therefore to avert such calamities, an appropriate schedule of antibiotic therapy has to be evolved. Generally mass mortality of the larvae happens during the 8-10th day of hatching and also during the molting stage. In a hatchery system if there is any such recurrence of mortality, a schedule of application shall be evolved beginning from 48 hours to the critical period ending 24 hours after the same. Such an approach shall considerably avoid drug abuse which generally happens in several hatchery systems. Instead of applying it in water it is always better to incorporate the antibiotic in the egg custard and give once in a day during this period. The spent water generated during and after the antibiotic treatment should be disinfected with chlorine at 300ppm and disposed off. This will help in containing the problem of developing drug resistance among the pathogenic strains in the larval rearing system.

Table –4.1 List of antibiotics tested by the disc plate method and their concentrations

Serial no	Antibiotics	conc./disk
1.	Penicillin G	10 units
2.	Streptomycin	30 mg
3.	Ampicillin	10 µg
4.	Oxytetracyclin	10 µg
5.	Tetracyclin	10 µg
6.	Gentamycin	30 µg
7	Polymyxin B	10 µg
8.	Chlortetracyclin	300 units
9.	Neomycin	30 µg
10.	Methamine mandelate	30 µg
11.	Cefazolin	3 mg
12.	Amoxycillin	10 µg
13.	Novobiocin	30 µg
14.	Nalidixic acid	30 µg
15.	Chloramphenicol	30 µg
16.	Erythromycin	15 µg
17	Kanamycin	30 µg
18.	Bacitracin	10 units
19	Lincomycin	2 µ g
20.	Sulphadiazine	300 µg

Table 4-2 Antibiotic sensitivity of the isolates from moribund larvae that are O/129 negative

		Phenons													Single strains[a]						
Serial no		1	2	3	4	5	6	7	8	9	10	11	12	13	1	2	3	4	5	6	7
	Number of strains	8	7	3	25	11	10	25	14	8	7	7	3	4							
	Antibiotics	-	-	-	-	7	1	7	-	1	-	-	+	-	-	-	-	+	-	-	-
1.	Penicillin G	+	1	1	1	10	+	22	9	-	2	3	+	3	-	-	-	+	+	+	-
2.	Oxytetracycline	+	+	+	24	+	+	23	+	+	6	5	+	+	-	+	+	+	+	+	+
3.	Streptomycin	-	-	-	-	2	4	8	-	1	1	-	+	-	-	-	-	+	-	-	-
4.	Ampicillin	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
5.	Tetracycline	+	6	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
6.	Gentamycin	+	1	+	6	9	8	+	12	1	2	3	+	3	-	-	+	+	+	+	+
7.	Polymyxin B	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
8.	Chlortetracycline	+	+	+	+	+	+	+	13	6	+	+	+	+	-	+	+	+	+	+	+
9.	Neomycin	7	+	+	24	+	+	+	13	6	+	+	+	+	+	+	+	+	+	+	+
10.	Methamine mandelate	2	5	-	-	9	5	24	10	2	-	1	+	1	-	-	+	+	-	-	+
11.	Cefazolin	+	6	-	-	7	2	16	5	-	-	-	+	1	-	-	-	+	-	-	-
12.	Amoxycillin	+	+	+	+	+	+	+	8	5	6	+	+	+	+	+	+	+	+	+	+
13.	Nalidixic acid	+	-	+	16	+	+	+	12	7	4	4	+	1	-	+	+	+	-	-	-
14.	Novobiocin	+	+	+	+	+	9	+	+	7	6	5	+	+	+	+	+	+	+	+	+
15.	Chloramphenicol	7	1	1	4	9	7	20	9	4	2	2	+	1	-	-	+	+	+	+	+
16.	Erythromycin	+	+	+	+	+	8	+	+	5	+	+	+	3	-	+	+	+	+	+	+
17.	Kanamycin	3	-	2	3	+	5	12	3	2	1	1	+	-	-	-	-	+	-	-	-
18.	Bacitracin	5	-	1	3	4	3	17	5	-	1	1	+	0	-	-	+	+	-	-	-
19	Lincomycin	7	-	+	4	8	2	24	2	-	-	-	-	-	-	-	-	+	-	-	-
20	Sufadiazine																				

a: strains which did not cluster (1: AAC 672b$_1$, 2:AAC 669B,3: AAC 1108d, 4:API 644b,5: AAC 654b,6: AAC 536,7: ANM 1008a

+: all strains are positive

all strains are negative;

numbers :indicate the number of strains in each phenon that are positive to a particular tes

Table 4-3 Antibiotic sensitivity of isolates from moribund larvae that are O/129 positive

Serial no.	Antibiotics	Phenons			Single strains[a]				
		1	2	3	1	2	3	4	5
	Number of strains, n=	17	3	39					
1.	Penicillin G	12	-	35	-	+	+	-	-
2.	Oxytetracycline	10	-	38	+	+	+	+	–
3.	Streptomycin	+	+	+	+	+	+	+	–
4.	Ampicillin	12	-	25	-	+	+	-	-
5.	Tetracycline	+	+	+	+	+	+	+	–
6.	Gentamycin	+	+	+	+	+	+	+	–
7.	Polymyxin B	12	-	27	+	+	+	+	–
8.	Chlortetracycline	+	+	+	+	+	+	+	–
9.	Neomycin	+	+	+	+	+	+	+	–
10.	Methamine mandelate	+	+	+	+	+	+	+	–
11.	Cefazolin	12	+	38	+	+	+	-	-
12.	Amoxycillin	4	-	36	-	+	-	-	-
13.	Nalidixic acid	+	+	+	+	+	+	+	–
14.	Novobiocin	16	+	38	-	+	+	-	-
15.	Chloramphenicol	16	+	+	+	+	+	+	–
16.	Erythromycin	12	+	36	-	+	+	-	-
17.	Kanamycin	16	+	+	+	+	+	+	–
18.	Bacitracin	13	1	33	+	+	+	-	-
19.	Lincomycin	10	-	36	-	+	+	-	-
20	Sufadiazine	3	-	35	+	+	-	+	-

a: single strains which did not cluster (1: ANM 708; 2: ANM 610a; 3: ANM 1020; 4: ANM594a; 5: ANM 1003)
+: all strains are positive
all strains are negative
numbers :indicate the number of strains in each phenon that are positive to a particular test

Table. 4-4. Antibiotic sensitivity of the isolates from apparently healthy larvae (O/129 –ve) which clustered into 4 phena and the 7 unclustered isolates

Serial no.	Antibiotics	Phenons				Single Strains[a]						
		1	2	3	4	1	2	3	4	5	6	7
		17	51	6	7							
1.	Penicillin G	+	22	5	2	+	-	-	+	-	-	+
2.	Oxytetracycline	+	+	+	+	-	-	+	+	+	-	+
3.	Streptomycin	+	47	+	5	+	+	+	+	-	+	+
4.	Ampicillin	2	35	6	+	+	-	-	-	-	-	+
5.	Tetracycline	+	+	5	+	+	+	+	+	+	+	+
6.	Gentamycin	+	+	+	+	+	+	+	+	-	+	+
7.	Polymyxin B	-	+	+	+	+	-	-	+	-	+	+
8.	Chlortetracycline	+	+	+	+	+	+	+	+	+	+	+
9.	Neomycin	+	+	+	+	+	+	+	+	+	+	+
10.	Methamine mandelate	+	+	+	+	+	+	-	+	+	+	+
11.	Cefazolin	2	48	+	+	-	+	-	+	-	+	+
12.	Amoxycillin	-	22	5	-	-	-	-	-	-	+	-
13.	Nalidixic acid	+	+	+	+	+	+	-	+	+	-	+
14.	Novobiocin	+	+	+	+	+	+	+	+	+	+	+
15.	Chloramphenicol	+	+	+	+	+	+	+	+	+	+	+
16.	Erythromycin	2	+	+	+	+	-	+	+	+	+	+
17	Kanamycin	+	+	+	+	+	+	-	+	-	+	+
18.	Bacitracin	-	23	4	-	-	-	+	-	-	-	-
19.	Lincomycin	1	41	+	+	+	-	+	+	-	-	+
20.	Sufadiazine	+	40	4	3	-	+	-	-	-	+	+

a: single strains which did not cluster (1: API 1561; 2:API 1558; 3:AAC 727; 4: AAC 740; 5: API 810; 6: AAC 717; 7: API 781)

+: all strains are positive

all strains are negative

numbers :indicate the number of strains in each phenon that are positive to a particular test

Table.4-5. Antibiotic sensitivity of the isolates from apparently healthy larvae (O/129 +ve) which clustered into 6 phena and 2 unclustered strains

Serial no	Antibiotics	Phenons						Single Strains[a]	
		1	2	3	4	5	6	1	2
	Number of strains, n=	4	12	5	3	4	4		
1.	Penicillin G	3	+	3	1	2	+	+	+
2.	Oxytetracycline	+	+	+	+	+	+	+	+
3.	Streptomycin	+	+	+	+	+	+	+	+
4.	Ampicillin	2	+	+	2	1	+	+	-
5.	Tetracycline	+	+	+	+	+	+	+	+
6.	Gentamycin	+	+	+	+	+	+	+	+
7.	Polymyxin B	+	11	+	+	2	+	+	+
8.	Chlortetracycline	+	+	+	+	+	+	+	+
9.	Neomycin	+	+	+	+	+	+	+	+
10.	Methamine mandelate	+	+	+	+	+	+	+	+
11.	Cefazolin	+	+	-	+	1	+	+	+
12.	Amoxycillin	2	3	1	-	-	1	-	-
13	Nalidixic acid	+	+	+	+	+	+	+	+
14.	Novobiocin	+	+	+	+	+	+	+	+
15	Chloramphenicol	+	+	+	+	+	+	+	+
16.	Erythromycin	+	+	+	+	+	+	+	+
17.	Kanamycin	+	11	+	+	+	+	+	+
18.	Bacitracin	2	6	1	-	1	3	+	+
19.	2Lincomycin	+	10	4	+	+	+	+	+
20.	Sufadiazine	+	+	2	2	+	+	+	+

a: single strains which did not cluster (1: ANM 702; 2: API 1519
+: all strains are positive
all strains are negative
numbers :indicate the number of strains in each phenon that are positive to a particular test

Table 4-6 MIC of the selected antibiotics on the 47 representative strains as percentage cumulative effect over 72 hours

Serial no.	Antibiotics	Concentration of antibiotics in ppm							
		1	5	10	20	40	60	80	100
1.	Chloramphenicol	4.25	36.17	40.42	53.19*	89.36	89.36	89.36	95.74
2.	Tetracycline	38.3	60*	62.13	66.38	68 51	68.51	77.02	83.4
3.	Streptomycin	0	2.13	4.26	10.64	57 45*	74.47	87.27	87.27
4.	Chlorteracycline	0	6.38	42.55	48.93	53.18*	53.18	55.31	59.56
5.	Kanamycin	0	0	0	40.43	40.43	40.43	40.43	46.81
6.	Oxytetracycline	0	0	8.5	14.88	36.15	46.78	51.03*	51.03
7.	Gentamycin	2.13	14.9	38.3	70.22*	87.22	93.6	95.73	95 73
8.	Neomycin	0	8.5	12.75	29 75	55.28*	74.43	80.81	89.31
9.	Novobiocin	12.77	14.9	27.67	38.3	40.43	42.56	46.81	46.81
10.	Nalidixic acid	0	0	6.38	28.15	66.44*	83.44	92.82	94.95

The numbers with * are MIC_{50}

Table 4-7 MIC (in ppm, at 72 hours) of the antibiotics against the 18 pathogenic isolates from *M. rosenbergii*

		Antibiotics									
Serial no.	Culture no.	Chloram-phenicol	Tetracy-cline	Strepto-mycin	Chlortet-racycline	Kanam-ycin	Oxytetr-acycline	Gentam-ycin	Neomy-cin	Nalidix-ic acid	Novobi-ocin
1.	ANM 932	20	5*	40	10	20	20	20	40	60	20
2.	API 1561	40	20*	40	100	20	>100	20	20	40	80
3.	AAC727	1	1*	40	10	20	>100	10	20	40	10
4.	API 810	20	5*	>100	40	>100	60	>100	>100	60	100
5.	API 781	20	5*	40	10	>100	>100	60	60	20	>100
6.	ANM 721	5	1*	80	10	20	40	20	40	20	20
7.	ANM 1008_a	40*	100	40	>100	>100	>100	80	>100	40	>100
8.	ANM 708	40*	100	40	>100	>100	>100	40	80	40	>100
9.	AAC 740	5	5*	20	20	20	40	5	5	20	1
10	API 1546	40	1*	40	10	>100	40	10	20	20	1
11.	AAC 701	20	1*	20	10	>100	40	10	20	20	1
12.	ANM $598c_2$	20	>100	80	>100	>100	>100	20*	40	40	>100
13.	ANM 718aa	>100	80	80	>100	>100	>100	40*	60	40	>100
14.	ANM 536	20	5*	40	40	>100	80	20	40	80	>100
15.	ANM 625	40	>100	40	>100	20	>100	20*	60	40	>100
16	AAC 629b	5	1*	40	10	>100	40	10	20	40	10
17	ANM 610a	>100	>100	60	>100	>100	>100	60*	100	>100	>100
18.	ANM 1020	40	>100	>100	>100	>100	>100	5*	>100	80	>100

*

Table 4-8 Toxicity testing of the antibiotics at 50ppm and 100ppm

Serial no.	Antibiotics used	Concentrations in ppm	% Average survival	p values
1.	Chloramphenicol	100	88 888	0.2-0 1
		50	97 777	0.2-0.1
2.	Tetracycline	100	37 777	0.05-0 02
		50	84 444	0.2-0 1
3.	Streptomycin	100	86.777	>0.2
		50	95 555	>0.2
4.	Chlortetracycline	100	91 111	>0.2
		50	97.777	0.2-0.1
5.	Kanamycin	100	91 111	>0.2
		50	95 555	>0.2
6.	Oxytetracycline	100	91 111	>0.2
		50	100	>0.2
7	Gentamycin	100	80.00	0.05-0.02
		50	95.555	0.2-0.1
8.	Neomycin	100	73.333	0.2-0.1
		50	86 666	>0.2
9.	Nalidixic acid	100	17 777	<0 01
		50	77 777	0.1-0 05
10.	Novobiocin	100	62.222	>0.2
		50	93.333	>0.2
11	Control without antibiotic	-	93.333	-

Table 4-9 Relative percentage survival of the larvae of *M. rosenbergii* treated with antibiotic and challenged simultaneously with the bacterial pathogen

Serial no.	Culture no.	Antibiotic used	Conc. (ppm)	Relative percentage survival (RPS)
1.	ANM 932	Tetracycline	5	66.666
2.	API 1561	Tetracycline	20	71.428
3.	AAC727	Tetracycline	1	71.428
4.	API 810	Tetracycline	5	60
5.	API 781	Tetracycline	5	75
6.	ANM 721	Tetracycline	1	50
7	ANM 1008_a	Chloramphenicol	40	75
8.	ANM 708	Chloramphenicol	40	60
9.	AAC 740	Tetracycline	5	80
10.	API 1546	Tetracycline	1	60
11.	AAC 701	Tetracycline	1	60
12.	ANM $598c_2$	Gentamycin	20	66.66
13.	ANM 718aa	Gentamycin	40	66.66
14.	ANM 536	Tetracycline	5	50
15.	ANM 625	Gentamycin	20	62.5
16.	AAC 629b	Tetracycline	1	71.428
17.	ANM 610a	Gentamycin	60	71.428
18.	ANM 1020	Gentamycin	5	71.428

CHAPTER - V
SUMMARY

CHAPTER-V

SUMMARY

The great potential for the culture of non-penaeid prawns, especially *Macrobrachium rosenbergii* in brackish and low saline areas of Indian coastal zone has not yet been fully exploited due to the non availability of healthy seed in adequate numbers and that too in the appropriate period. In spite of setting up several prawn hatcheries around the country to satiate the ever growing demands for the seed of the giant fresh water prawn, the supply still remains fear below the requirement mainly due to the mortality of the larvae at different stages of the larval cycle. In a larval rearing system of *Macrobrachium rosenbergii,* members of the family Vibrionaceae were found to be dominant flora and this was especially pronounced during the times of mortality However, to develop any sort of prophylactic and therapeutic measures, the pathogenic strains have to be segregated from the lot. This would never be possible unless they were clustered based on the principles of numerical taxonomy It is with these objectives and requirements that the present work involving phenotypic characterization of the isolates belonging to the family Vibrionaceae and working out the numerical taxonomy, determination of mole % G+C ratio, segregation of the pathogenic strains and screening antibiotics as therapeutics at times of emergency, was carried out. Results obtained and the conclusions made are summarized as follows:

- On constructing the dendrograms based on unweighted average linkage, discrete clusters or phena were formed at > 80% similarity with a few strains keeping aloof without being clustered.

- None of the phena formed were found to pick up any of the 20 type strains incorporated, instead the type strains remained clustered separately or independently

- This indicated significant phenotypic dissimilarity between the members of the family Vibrionaceae associated with the larvae of *Macrobrachium rosenbergii* and the type strains.

- It is hypothesized that members of the family Vibrionaceae associated with the larvae of *Macrobrachium rosenbergii* are phenotypic variants of the already existing species or new species altogether.

- Sensitivity to 2,4-diamino 6,7-diisopropyl pteridine (0/129), a differentiating core character of the family used to segregate *Vibrio* and *Photobacterium* from *Aeromonas* was found to be of no use in this group of isolates.

- Irrespective of the response of the cultures to 0/129,compound, they had a %G+C content falling within the range of *Vibrio, Photobacterium* or *Aeromonas.*

- There were a couple of phena and several strains within the group of Vibrionaceae defined as Gram negative rods, motile/non-motile, Kovac's oxidase positive and fermentative with mole %G+C ratio out of the range of that of Vibrionaceae, indicating strongly the existence of unrelated groups requiring segregation and a new nomenclature.

- Based on the mole % G+C ratio the phena and strains unclustered were found to show affiliation to *Vibrio aestuarianus, V. tubiashi, V. ichthyoenterii, V fischeri, V logei, V. marinus, V. anguillarum, V metschnikovii, V. ordalli, V. costicola, V. furnissi, V. fluvialis, V. vulnificus, V hollisae, V. proteolyticus, V. campbelli, V cholerae, V. harveyi V. gazogenes, V. penaecida, V. diazotrophicus, V nereis, V. pelagius V. nigripulchritudo, V. natreigenes, V. navarrensis, V. alginolyticus, V.*

mytili, V. orientalis, V splendidus, P. angustum, P. phosphoreum, P. leiognathi, A. salmonicida, A. sobria, A. hydrophilla and *A. caviae.*

- For confirming the identity of the phena and the strains un-clustered, more studies to reveal their genotypic relatedness to the existing species is required.

- Each cluster or phenon can now be called a cohesive group with much of the information content agreeing at higher level of significance (> 80% similarity) phenotypically which can be called a " natural group "

- The scientific validity of comparing and matching such natural groups to the type strains of known species is questioned as it is a clear departure from the principles of classification of natural groups.

- Since the phenotypic characters of each phenon has already been described, what is required next is to examine the cohesiveness of each phenon in terms of genotypic similarity and DNA homology

- If they remain as cohesive groups genotypically also, each phenon can then be designated as an entity

- As the next phase, the distance between such entities has to be measured genotypically and phenotypically to nomenclature them in a way befitting the tropical organism.

- In this process of nomenclature, the role of standard strains is only minor as to know how far a natural group is away from a type strain. If at all any comparison has to be made, that should be between the cohesive natural groups of different geographic regions.

- This novel approach will lead to the development of a new classification scheme for tropical isolates characterized by high degree of diversity and information content.

- The 47 representative strains used for the determination of mole % G+C ratio were also used for determining the pathogenicity of the whole family of Vibrionaceae associated with the larvae.

- On challenging the larvae with every representative strain, significantly higher percentage mortality was observed as compared to a positive and negative control.

- The death of larvae resulted in this study is designated due to Vibriosis as it is believed that the organisms invaded the haemolymph through intestine and caused septicaemia.

- It is recommended to modify the definition of Vibriosis as the infectious diseases caused by species of the family Vibrionaceae and Aeromonadaceae (*Vibrio*, *Aeromonas* and *Photobacterium*) in shellfish and finfishes leading to septicemia and death. As per the existing definition, the term Vibriosis is applicable in all cases of infections caused by bacterial species of the genus *Vibrio* alone.

- It is observed that the pathogenicity is widespread among the members of the family Vibrionaceae associated with the larvae of *Macrobrachium rosenbergii* and a general protection for the larvae against the invasive death has to be accorded as part of husbandry practices.

- There is no difference in the distribution of pathogenic strains between the moribund and apparently healthy larvae suggesting that they are opportunistic pathogens, but deserve concern as they invade the larvae at times of stress.

- As the satisfactory completion of Koch's postulates, the *Vibrio* strains inoculated could be re-isolated from the intestine and haemolymph of the larvae in significantly higher proportions compared to un-inoculated controls suggesting that the pathogens have invaded the larvae and caused mortality.

- As none of the 47 strains tested caused 100% mortality in larvae, it suggests that the strains of Vibrionaceae associated with the larvae may not be potential pathogens altogether but can be classed as opportunistic.

- By screening 20 antibiotics against 313 OTUs, 11 could be segregated as active on each phenon and strains of all the groups. Out of this, 10 antibiotics such as Chloramphenicol, Tetracycline, Gentamycin, Chlorotetracycline, Kanamycin, Oxytetracycline, Neomycin, Nalidixic acid Novobiocin and Streptomycin were selected for further study Four antibiotics among them, such as Tetracycline, Gentamycin, Chlorotetracycline and Neomycin were found to inhibit all the 313 strains tested.

- M.I.C $_{50}$ of the above antibiotics were as follows: Chloramphenicol 20ppm, Tetracycline 5ppm, Streptomycin 40ppm, Chlorotetracycline 40ppm, Oxytetracycline 80ppm,Gentamycin 20ppm, Neomycin 40ppm , Nalidixic acid 40ppm

- At 50 and 100 ppm level, the above antibiotics were found to be significantly non-toxic.

- Based on the M.I.C required, three antibiotics were selected which are Tetracycline, Gentamycin, and Chloramphenicol and they were proved to accord protection to the larvae at a significant level in the presence of the pathogenic strains.

- These antibiotics at the following concentrations are recommended for application to protect the larvae from Vibriosis: Tetracycline 5ppm, Chloramphenicol 20ppm, and Gentamycin 20ppm.

  To avoid any drug abuse and to avert the development of drug resistance in the pathogenic strains, it is recommended to apply these antibiotics orally through egg custard, beginning from 48 hours prior to onset of the critical phase in the life cycle of the larvae as observed in each larval rearing system; and 24 hours after its ending, once daily The spent water after the antibiotic treatment should be disinfected with a final concentration of 300ppm chlorine and disposed.

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