CHAPTER THREE

MATERIALS AND METHODS

SECTION ONE

3.1 Bacterial isolates

The *Aeromonas hydrophila* isolates used in this study were collected from clinical and environmental sources. The environmental isolates were obtained from several fresh water fish ponds in the University of Malaya campus. Nineteen clinical strains (SL strains) were originally recovered from diarrheal samples from the General Hospital of Colombo, Sri Lanka (courtesy of Dr. R. P. Subasinghe) and 4 clinical isolates (A isolates) were obtained from Institute for Medical Research, Malaysia. These isolates were confirmed as *A. hydrophila* with API 20E system (API System GA, La Balme Les Grottes, Montalieu-Vercieu, France) and the Biolog identification system (Biolog Microstation, USA). The latter system provides a standardized micromethod using 95 biochemical tests to identify a broad range of enteric, nonfermenting, Gram-negative bacteria. The Biolog Microlog™ computer software was used to identify the bacteria from its metabolic pattern of utilizing a panel of different biochemical sources in the Biolog microplate.

The *Vibrio cholerae* O1 isolates (clinical isolates) were obtained from the Institute for Medical Research (IMR), Malaysia (courtesy of Dr. Rohani). Twelve isolates were isolated from fecal samples of patients from the recent cholera epidemic outbreak (May, 1996) in Penang, Malaysia. The other strains were isolated from

previous local cholera outbreaks. These *Vibrio cholerae* isolates were confirmed as biovar El Tor by hemagglutination and Voges-Proskauer tests (Cheeseborough, 1984; Finkelstein and Mukerjee, 1963).

Stock cultures of these *Aeromonas hydrophila* and *Vibrio cholerae* strains were maintained in 25% glycerol in Trypticase Soy Broth (TSB) at -20°C. Routine cultures were grown on slants of nutrient agar and were kept at 4°C. The *Aeromonas hydrophila* and the *Vibrio cholerae* strains used in this study are listed in Table 3.1 and Table 3.2 , respectively.

Table 3.1 List of *Aeromonas hydrophila* isolates.

Isolates	Category	Source of Isolation
SL2	clinical	diarrheal stool
SL3	clinical	diarrheal stool
SL4	clinical	diarrheal stool
SL6	clinical	diarrheal stool
SL8	clinical	diarrheal stool
SL9	clinical	diarrheal stool
SL10	clinical	diarrheal stool
SL11	clinical	diarrheal stool
SL12	clinical	diarrheal stool
SL13	clinical	diarrheal stool
SL14	clinical	diarrheal stool
SL15	clinical	diarrheal stool
SL16	clinical	diarrheal stool
SL17	clinical	diarrheal stool
SL18	clinical	diarrheal stool
SL19	clinical	diarrheal stool
SL20	clinical	diarrheal stool
SL21	clinical	diarrheal stool
SL22	clinical	diarrheal stool
A12	clinical	diarrheal stool
A13	clinical	diarrheal stool
A14	clinical	diarrheal stool
A24	clinical	diarrheal stool
X2	environmental	fish farm, U. of Malaya
X8	environmental	fish farm, U. of Malaya
X9	environmental	fish farm, U. of Malaya
X13	environmental	fish farm, U. of Malaya
X14	environmental	fish farm, U. of Malaya
X18	environmental	fish farm, U. of Malaya
X36	environmental	fish farm, U. of Malaya
X38	environmental	fish farm, U. of Malaya
X52	environmental	fish farm, U. of Malaya
X53	environmental	fish farm, U. of Malaya
X54	environmental	fish farm, U. of Malaya
E29	environmental	fish farm, U. of Malaya
H10	environmental	fish farm, U. of Malaya
FC3	environmental	fish farm, U. of Malaya
FC4	environmental	fish farm, U. of Malaya
FP2	environmental	fish farm, U. of Malaya
FP3	environmental	fish farm, U. of Malaya
XF4	environmental	fish farm, U. of Malaya
XF5	environmental	fish farm, U. of Malaya
XF8	environmental	fish farm, U. of Malaya

Table 3.2 List of *Vibrio cholerae* isolates.

Isolates	Category	Subtypes	Source of Isolation
V38	Clinical	Inaba	Diarrheal stool
V57	Clinical	Inaba	Diarrheal stool
V113	Clinical	Ogawa	Diarrheal stool
V114	Clinical	Ogawa	Diarrheal stool
V115	Clinical	Ogawa	Diarrheal stool
V123	Clinical	Ogawa	Diarrheal stool
V124	Clinical	Ogawa	Diarrheal stool
V125	Clinical	Ogawa	Diarrheal stool
V127	Clinical	Ogawa	Diarrheal stool
V129	Clinical	Ogawa	Diarrheal stool
V130	Clinical	Ogawa	Diarrheal stool
V131	Clinical	Ogawa	Diarrheal stool
V132	Clinical	Ogawa	Diarrheal stool
V133	Clinical	Ogawa	Diarrheal stool
V135	Clinical	Ogawa	Diarrheal stool
V136	Clinical	Ogawa	Diarrheal stool
V137	Clinical	Ogawa	Diarrheal stool
V138	Clinical	Ogawa	Diarrheal stool
V140	Clinical	Ogawa	Diarrheal stool
V141	Clinical	Ogawa	Diarrheal stool
V142	Clinical	Ogawa	Diarrheal stool
V143	Clinical	Ogawa	Diarrheal stool
V144	Clinical	Ogawa	Diarrheal stool
V145	Clinical	Ogawa	Diarrheal stool
V146	Clinical	Ogawa	Diarrheal stool
V147	Clinical	Ogawa	Diarrheal stool
V1041	Clinical	Ogawa	Diarrheal stool
V1094	Clinical	Ogawa	Diarrheal stool
V1100	Clinical	Ogawa	Diarrheal stool
V1264	Clinical	Ogawa	Diarrheal stool
V1386	Clinical	Ogawa	Diarrheal stool
V1387	Clinical	Ogawa	Diarrheal stool
V1392	Clinical	Ogawa	Diarrheal stool
V1394	Clinical	Ogawa	Diarrheal stool
V1398	Clinical	Ogawa	Diarrheal stool
V1400	Clinical	Ogawa	Diarrheal stool
V1403	Clinical	Ogawa	Diarrheal stool
V1482	Clinical	Ogawa	Diarrheal stool

V1041, V1094, V1100, V1264, V1286, V1387, V1392, V1394, V1398, V1400, V1403, and V1482 are stool cultures from the recent cholera outbreak (May, 1996) in Penang.

3.2 Plasmid Profile

3.2.1 Small scale plasmid extraction

Plasmid DNA was extracted by the alkaline lysis method of Sambrook *et al.* (1989) with several modifications. A loopful of the bacteria were inoculated in 2 ml of Luria-Bertani (LB) broth and the culture was incubated overnight at 37°C with shaking. The culture was transferred to a 1.5 ml Eppendorf tube and centrifuged at 5000 rpm for 5 min. The supernatant was discarded and the bacterial pellet was resuspended in 150 µl of ice-cold Solution I and placed on ice for 10 min. 350 µl of Solution II was then added, mixed and placed on ice for 5 min before 250µl of ice-cold Solution III was added and the solution placed on ice for 10 min. The solution was then centrifuged at 4°C at 12,000 rpm for 15 min. The supernatant was removed to another Eppendorf tube and mixed with an equal volume of isopropanol for 5 min and centrifuged again for 15 min. The supernatant was removed and the DNA pellet was rinsed with 70% ethanol and left to dry before being dissolved in TE buffer and kept at -20°C. The preparation of Solutions I, II and III is explained in Section 2.

3.2.2 Agarose Gel Electrophoresis

Plasmid DNA samples were resolved by electrophoresis in submerged horizontal agarose gels in TBE (Tris-borate) buffer at 5V/cm for 1.5 hours. 0.7g of agarose was dissolved in 100ml of TBE buffer by boiling. The molten agar was cooled to 50°C before casting. The agarose gel was then placed horizontally in the buffer tank of the electrophoresis system and the gel was covered with TBE buffer. An aliquot of

DNA (10 µl) was mixed with 6× bromophenol blue tracking dye (2 µl) and the mixture was loaded into the sample wells.

After completion of a run, the gels were stained in ethidium bromide solution (0.5µg/ml) for 30 min. The DNA in the gel was visualized, photographed and analyzed with the Vilber Lourmat Bio-1D computer imaging system. The molecular weight of the plasmids were determined by including marker plasmids from *Escherichia coli* V517 (35.7 - 1.4 Mda) during electrophoresis.

3.2 Curing Experiments

Acridine orange was used as the curing agent (Winkler *et al.*, 1976) with the modification suggested by Borrego *et al.* (1991). The bacteria were inoculated in 10 ml of Brain-Heart Infusion Broth (BHIB) in a baffled flask. The flask was incubated at 37°C for 24h with shaking at 220 rpm. 2 ml culture samples were then added to 1 ml of fresh BHIB and incubated for 3h at 37°C. Then 1 ml of a solution of 20µg/ml acridine orange was added to the culture and incubated further for 24h. The cells were spun down at 3000 rpm in a centrifuge and resuspended in 2 ml of fresh BHIB. After further growth for 6h at 37°C the cells were streaked on TSA (Tryptone Soya Agar) plates and tested for their plasmid content.

3.4 Siderophore detection on agar plates

3.4.1 Growth under iron-limiting conditions and siderophore production

The strains were cultured in M9 minimal agar supplemented with Casamino Acids (Difco) 0.2% w/v. The iron chelator ethylenediamine-di-(o-hydroxyphenyl acetic acid) (EDDA) was added to M9 minimal agar at a final concentration of 10 µM to

achieve the iron-limited conditions. After overnight growth, the colonies from these plates were transferred to the chrome azurol S (CAS) siderophore detection agar of Schwyn and Neilands (1987) prepared as described by Barghouthi *et al.* (1989) which was used to detect siderophore production. The method is based on the fact that the dye chrome azurol S (Sigma, USA), incorporated in the medium, can form stable complexes with iron. Therefore, when a strain produces a diffusible siderophore (which removes the iron from the complex) the color turns to yellow-orange around the colony after 24h of growth. Isolates without a halo were classified as siderophore negative.

3.5 Siderophore assay

3.5.1 Growth of culture for assay

The cultures were grown in the MM9 liquid media of Schwyn and Neilands (1987) which consisted of MM9 salts, Pipes (Piperazine-N,N'-bis[2-ethanesulfonic acid]) buffer (Sigma, USA), and casamino acids (0.3%). Thiamine-HCl (2ppm) and nicotinic acid (2ppm) were also added as suggested by Barghouthi *et al.* (1989). The media was treated with Chelex-100 (Bio-Rad, USA) by the batch method according to the manufacturer's instructions to lower the amount of trace metal contamination and then filter sterilized. A detailed description of the preparation of the MM9 media is given in Section 2.

Siderophores were obtained by the method of Schwyn and Neilands (1987). A 10ml volume of MM9 liquid media in a baffled flask was inoculated with a loopful of bacteria and incubated in a shaker at 220 rpm at the required temperature (either 28°C or 37°C). After sufficient growth (18 - 20 hours), 100µl of this iron-starved culture

was transferred to 10 ml of the same media in a baffled flask and incubated further for 24 hours. Iron-rich conditions were obtained by addition of 2μM $FeSO_4.7H_2O$ to the MM9 medium. At the end of this period, the cells were centrifuged and the supernatant was assayed for the presence of siderophores.

3.5.2 Chrome azurol S assay

The chrome azurol S (CAS) assay solution of Schwyn and Neilands (1987) was used. 0.5 ml of the culture supernatant was mixed with 0.5 ml of CAS assay solution and after equilibrium was reached the absorbance was measured at 630nm with air as the blank. A low absorbance indicates a high production of siderophores and vice-versa. A color change from blue to orange was obtained within 5 min when catecholate siderophores are present.

3.6 Hemolysin assay

3.6.1 Growth of culture for assay

A loopful of bacteria were inoculated in 10 ml of TSB (Trypticase Soy Broth) in a baffled flask and incubated at either 28°C or 37°C for 24 hours with shaking at 220 rpm. Iron-limiting conditions were achieved by adding the iron chelator ethylenediamine-di(o-hydroxyphenylacetic) acid (EDDA) (Sigma, USA) to a final concentration of 100μM. Iron-rich conditions were obtained by using TSB without the addition of EDDA and also by adding $FeSO_4.7H_2O$ to TSB at a final concentration of 100μM.

3.6.2 Hemolytic microtitre plate assay

The hemolysin assay was performed according to the method of Asao *et al.* (1986) with several modifications. The cultures were spun down at 5000 rpm in a centrifuge and the supernatant was used immediately for the hemolysin assay. The bacterial cell-free supernatant (100µl) was added to PBS (100µl) in a V-bottomed microtitre plate, and two-fold dilutions of the supernatant were carried out. Washed 2% erythrocytes (100µl) were added to each well, and the plate was incubated for 1 hour at 37°C. The positive control was 10% saponin and the negative control, sterile TSB broth.

A red button-like formation at the bottom of the plate coupled with a clear suspension is indicative of non-hemolysis. A totally homogenous red suspension would reflect total hemolysis of the erythrocytes (Figure 3.1). Intermediate hemolysis would show up as a red button-like formation with a reddish suspension. The hemolytic activity was defined as the reciprocal of the dilution of the sample needed to produce 50% hemolysis of red blood cells.

3.6.3 Hemolysin assay using spectrophotometer

Hemolytic activity was determined by the previous procedures with slight modifications (Asao *et al.*, 1984). The supernatant was serially diluted in 0.01M Tris-hydrochloride buffer, pH 7.2, containing 0.9% NaCl. The diluted samples were dispensed in 0.75 ml amounts into Eppendorf tubes, to which was added equal amounts of washed human blood cell suspension in the same buffer. After centrifugation at 5000 rpm for 5 min at 4°C, the absorbance of supernatant was read at 540nm. The concentration of human blood cell suspension was adjusted to give an

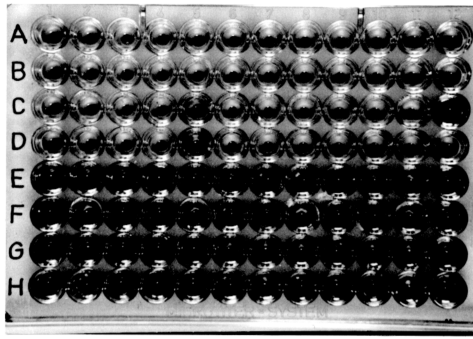

Figure 3.1

Hemolysis pattern on V-bottomed microtiter plates.

Rows A, B, C and D: Negative control (PBS + 2% erythrocytes suspension).

Rows E, F, G and H: Positive control (10% w/v saponin + 2% erythrocytes suspension)

absorbance of 0.8 at 540nm when complete hemolysis occurs under the specified conditions. One hemolytic unit (HU) was defined as the minimum dose of the sample required to produce 50% hemolysis. The positive control was 10% saponin and the negative control was sterile TSB broth.

3.7 Protease assay

3.7.1 Growth of culture for assay

The bacteria were inoculated in 10ml of TSB in a baffled flask. 1.5% w/v skimmed milk (Oxoid) was added to the broth before inoculation of the bacteria. Iron-limiting conditions were achieved by the adding the iron chelator EDDA to a final concentration of 100μM while iron-rich conditions were obtained by the addition of $FeSO_4.7H_2O$ to TSB at a final concentration of 100μM or by using TSB alone without the addition of EDDA.

3.7.2 Azocasein assay

The method of Allan and Stevenson (1981) was used with modifications. In the reaction mixture, sodium phosphate buffer (2.3 ml), 10% azocasein (0.1 ml) and bacterial cell-free supernatant (0.1 ml) were added together to make up a total of 2.5 ml. The reaction was incubated at 37°C with gentle shaking, for a duration of one hour. The reaction was stopped by the addition of 0.75 ml of the reaction mixture to 0.75 ml of 10% TCA, in an Eppendorf tube. The precipitation was left at room temperature for 30 min. The mixture was then spun at 10,000 rpm for 10 minutes to pellet the protein precipitate. A volume of 0.75 ml of the supernatant was added to 0.75 ml of 1 M NaOH and the absorbance was read at 450 nm using the Beckman

spectrophotometer relative to a blank made with an inactivated sample of bacterial supernatant.

3.8 Statistical test

The Student's t-test was applied to observe any significant difference between the siderophore, proteolytic and hemolytic activities under iron-limiting and iron-rich conditions at the different growth temperatures of 28°C and 37°C. The levels of significance used throughout this study was 5% ($p < 0.05$) and 1% ($p < 0.01$).

SECTION TWO

MEDIA & SOLUTIONS

3.9 Media

3.9.1 Nutrient agar (NA) slants

NA powder (14g) was dissolved in 500 ml of dH_2O and autoclaved at 121°C for 15 min. The sterile nutrient agar was distributed into MacCartney bottles and placed at an angle to create agar slants for the maintenance of the bacterial isolates at 4°C.

3.9.2 Brain-Heart Infusion Broth (BHIB)

The media was prepared by dissolving 37 g of Brain Heart Infusion powder in 1 liter of sterile water. The medium was then autoclaved at 121°C, 15 p.s.i. for 20 min.

3.9.3 M9 + EDDA Minimal Agar

Per liter:

To 750 ml of sterile deionized H_2O, add:

5 x M9 salts	200 ml
1M $MgSO_4$	2 ml
20% glucose	20 ml
1 M $CaCl_2$	0.1 ml
Casamino acids (Difco)	2.0 g
EDDA	1.8 mg

Bacto-agar	7.5 g

5 x M9 salts is made by dissolving the following salts in deionized H_2O to a final volume of 1 liter:

$Na_2HPO_4.7H_2O$	64 g
KH_2PO_4	15 g
NaCl	2.5 g
NH_4Cl	5.0 g

The salt solution is divided into 200 ml aliquots and sterilized by autoclaving at 121°C for 15 min.

The $MgSO_4$ and $CaCl_2$ solutions should be prepared separately, sterilized by autoclaving and added after diluting the 5 x M9 salts to 1 liter with sterile deionized water. Glucose should be sterilized by filtration before it is added to the diluted M9 salts. Casamino acids were deferrated by extraction with 3% (w/w) 8-hydroxyquinoline in chloroform solution and filter sterilized. EDDA was added to 750 ml deionized water and sterilized by autoclaving.

3.9.4 Chrome Azurol S (CAS) agar

To prepare 1 liter CAS agar medium:

To 750 ml deionized water, add:

NaOH	5.3 g
Pipes	30.24 g
Agar	15 g
10 x stock salt solution	100 ml

3.9.5 MM9 Minimal Medium

Per 1 liter:

The following components were added:

NaOH	5.3 g
Pipes	30.24 g
KH_2PO_4	0.30 g
NaCl	0.50 g
NH_4Cl	1.0 g
Casamino acids	3.0 g
Glucose	2.0 g
Thiamine-HCl (1 mg/ml)	2 ml
Nicotinic acid (1 mg/ml)	2 ml

50 g of Chelex-100 was added to the above solution and stirred for 2 hours to remove trace element contamination including iron. The Chelex-100 beads were removed by filtration and the following solutions were added:

$MgSO_4$	(1 M)	0.5 ml
$CaCl_2$ (1 M)		0.5 ml

The media was sterilized by filtration and aseptically dispensed into baffled flasks (10 ml each). To obtain iron-rich conditions, 100 µl of 200 µM $FeSO_4.7H_2O$ solution was added into each flasks to a final concentration of 2 µM $FeSO_4.7H_2O$.

3.9.6 Tryptone Soya Broth (TSB)

TSB powder (15g) was dissolved in 500 ml dH_2O and sterilised by autoclaving at 121°C for 15 min.

3.9.7 TSB-skim milk broth

A volume of 1.5 ml from a 10% skim milk solution was added to 8.5 ml of TSB broth in a 250 ml baffled flask.

3.9.8 Luria-Bertani (LB) broth

Tryptone	10.0 g
Yeast Extract	5.0 g
Sodium chloride	5.0 g
dH_2O	1 liter

The broth was autoclaved at 121°C for 15 minutes.

3.10 Solutions for siderophore assay

3.10.1 CAS assay solution

To prepare 100 ml of CAS assay solution:

A 6-ml volume of 10 mM HDTMA was placed in 100-ml volumetric flask and diluted with water. A mixture of 1.5 ml iron (III) solution (1 mM $FeCl_3.6H_2O$ in 10 mM HCl) and 7.5 ml 2 mM aqueous CAS solution was slowly added under stirring. A 4.307 g quantity of anhydrous piperazine was dissolved in water and 6.25 ml of 12 M HCl was carefully added. This buffer solution (pH 5.6) was rinsed into the volumetric flask which was then filled with water to afford 100 ml of CAS assay solution. The solution was kept in polyethylene bottles and stored in the dark.

3.11 Solutions for protease assay

3.11.1 Skim milk (10% w/v)

Skim milk (Oxoid)	10 g
dH_2O	100 ml

The solution was autoclaved for 5 min at 121°C.

3.11.2 TCA (10% w/v)

TCA	100 g
dH_2O	1 liter

3.11.3 Azocasein (10% w/v)

Azocasein (Sigma)	10 g
dH_2O	100 ml

The solution was stored at 4°C in 10 ml aliquots.

3.11.4 1M NaOH

NaOH	4 g
dH_2O	100 ml

3.11.5 0.1 M sodium phosphate buffer

The following solutions were first prepared:

1M Na_2HPO_4: 14.2g Na_2HPO_4 in 100 ml dH_2O

1M NaH_2PO_4: 15.6g NaH_2PO_4 in 100 ml dH_2O

To make 0.1 M sodium phosphate buffer:

1 M Na_2HPO_4	34.2 ml
1 M NaH_2PO_4	15.8 ml

The solution was made to a volume of 440 ml with dH_2O. The pH was adjusted to 7.2 before topping up the solution to 500 ml. The solution was autoclaved at 121°C for 20 min.

3.12 Solutions for hemolysin assay

3.12.1 PBS (pH 7.2)

KH_2PO_4	0.29 g
Na_2HPO_4	0.76 g
NaCl	4. 25 g

The chemicals were dissolved in 500 ml of dH_2O and the pH of the solution was adjusted to 7.2. The solution was autoclaved at 121°C for 15 min and kept at 4°C.

3.12.2 Tris-HCl buffer (pH 7.2)

Per 1 liter:

Tris-HCl	0.01 M
NaCl	9.0 g

The pH of the solution was adjusted to 7.2 and autoclaved at 121°C for 15 minutes.

3.12.3 2% human red blood cells (HRBC) suspension

Human red blood cells (6-7 ml blood) were pelleted in a centrifuge tube at 2000 rpm for 5 min and the supernatant discarded. The cells were resuspended in 10 ml PBS (pH 7.2) and centrifuged at 2000 rpm for 5 min. The washing of HRBC was repeated twice, and the cells were resuspended in PBS to give a concentration of 2% HRBC.

3.12.4 Saponin (10% w/v)

1 g of saponin was dissolved in 10 ml of deionized water. The solution was filter sterilized and kept at room temperature.

3.13 Solutions for plasmid curing

3.13.1 Acridine orange

2 mg of acridine orange (Sigma, USA) was dissolved in 100 ml deionized water to give a 20μg/ml solution.

3.14 Solutions for plasmid DNA extraction

3.14.1 Solution I

50 mM glucose

25 mM Tris.Cl (pH 8.0)

10 mM EDTA (pH 8.0)

This solution was autoclaved for 15 minutes at 10 lb/sq. in. and stored at 4°C.

3.14.2 Solution II (freshly prepared)

0.2 N NaOH

1% SDS

3.14.3 Solution III

5 M potassium acetate	60 ml
glacial acetic acid	11.5 ml
dH_2O	28.5 ml

3.14.4 TE Buffer (pH 8.0)

TE buffer was used for dissolving DNA.

10 mM Tris.Cl (pH 8.0)

1 mM EDTA (pH 8.0)

3.15 Solutions for agarose gel electrophoresis

3.15.1 0.5 × TBE buffer

Per 1 liter:

Tris base	5.4 g
Boric acid	2.75 g
0.5 M EDTA (pH 8.0)	2.0 ml

3.15.2 Bromophenol blue tracking dye (6× strength)

Tracking dye was added to the DNA-containing solution prior to electrophoresis to weigh down the DNA in the sample wells and to allow monitoring of DNA migration during electrophoresis.

Glycerol	30% v/v
Bromophenol blue	0.25% w/v

The solution was stored at 4°C.

3.15.3 Ethidium bromide solution (10 mg/ml)

A stock solution was prepared by the addition of 1 g ethidium bromide to 100 ml sterile dH_2O and stirred until the powder has entirely dissolved. The solution was kept in a tightly closed bottle wrapped in aluminium foil and stored at room temperature. Gloves and mask was worn during the weighing and preparation of ethidium bromide as this is a very powerful mutagen.