ISOLATION AND CHARACTERIZATION OF SYMBIOTIC BACTERIA FROM THE HERMIT CRAB

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INTRODUCTION

In terrestrial ecosystems symbiotic relationships, between bacteria and vertebrate or invertebrate animals, have been well studied. Examples are the gut symbionts of ruminants and termites. The presence of symbionts in the guts of some aquatic invertebrates has been known of for a long time, for example the presence of *Cristispira* in the crystalline style of *Saxidames gigantes* (Berkeley 1959) and the cellulose-degrading bacteria in the gut of *Teredo* or shipworm (Hidaka 1954). Endosymbiotic relationships in corals and in deep sea invertebrates have also been well studied (Conway *et al* 1989, Herry *et al* 1989).

However, very little attention has been paid to the possible symbiotic relationships between aquatic invertebrates and the bacteria present in the gut. Harris (1993) suggests that gut bacteria may contribute significantly to nutrient gain by aquatic hosts. Some organisms have been shown to maintain a permanent and consistent microbiota in the gut, which is significantly different from that of the surroundings. These organisms include the giant prawn *Macrobrachium rosenbergi* (Colorni 1985), the polychaete *Thlepus setosus* (Duchene *et al* 1988), prawns *Upogenbia africana* and *Callinessa kraussi* (Harris *et al* 1991), halothuroid *Psychropoles sp.* (Denning *et al* 1981), deep sea amphipod *Lyssianssidae hirondella* (Schwartz *et al* 1976) and sea urchin *Echinus exilentus* (Unkles 1977). In contrast another study showed an absence of bacteria in the gut of marine, wood-boring crustacea (Boyle & Mitchell 1978). Two students on the Microbial diversity Cousre in 1993 attempted to isolate bacteria from spider crabs and marine isopods but were unsuccessful. In many other studies there is no indication to suggest the bacteria isolated from the gut are anything other than ingested, transient bacteria (Harris 1993). Nagasawa & Nemoto (1988) suggest that bacteria may be an important food source for some marine invertebrates . The gut microbiota of Crustacea has not been studied in detail, but the most commonly isolated bacterial genera are *Vibrio, Pseudomonas, Flavobacterium, Aeromonas, Micrococcus,* and *Staphlycoccus* (Harris 1993).

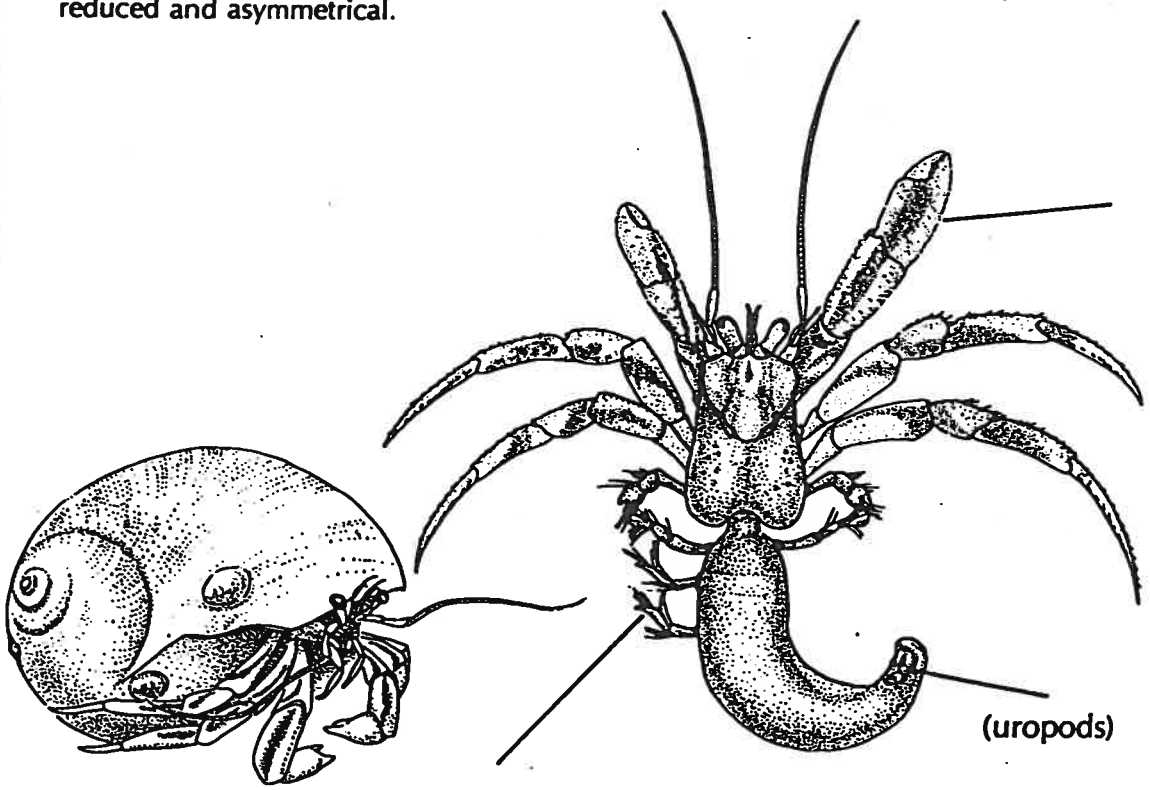
Hermit Crabs (Family: Paguridae) are a large group of Crustacea. They are a quite separate group from the true crabs or Brachyura. Hermit Crabs have a hard cephalothorax and a soft twisted, abdomen which is hidden within a discarded, snail shell (See Fig. 1) They are omnivorous detrivores and scavengers (Bliss 1982). The gut of a hermit crab is very different from that of a true crab or lobster. Typically a crab or lobster has a very short anterior gut leading into the stomach. From the stomach the food particles then pass into the hepato-pancreas. Undigested food is compacted in a short intestine and then passed out of the anus (Berrick 1986). However in the Hermit crab the anterior gut leading to the stomach and hepatopancreas is proportionally much longer. (Bullis pers. comm.). This may suggest that some pre-digestion of ingested food involving

Fig. 1.

Hermit Crabs

GENUS *Pagurus* (G. "a crab")

In snail shells. Carapace short, widest in rear; anterior portion (shield) well calcified. Claws unequal in size, right much larger than left. Fourth and fifth pair of walking legs reduced; fifth pair turned upwards. Abdomen elongated, soft, cylindrical, and spiraled. Abdominal appendages reduced to two on the left side. Uropods present but reduced and asymmetrical.



LONG-CLAWED HERMIT CRAB
Pagurus longicarpus Say 1817 (G. "long wrist")

a resident bacterial population could be occurring. To our knowledge there has been no previous study of the microbiota of the gut of the Hermit Crab.

The aims of this project are isolate and characterize bacteria from the different regions of the Hermit Crab gut. Bacteria will also be isolated from the water and sediment in the tank, where the hermit crabs are maintained, in an attempt to ascertain whether bacteria in the Hermit crab gut are resident or merely ingested from the environment.

MATERIAL AND METHODS

1. Isolation of Bacteria

A Hermit Crab was obtained from the Marine Resurces Centre, MBL. The crab was anesthetised for 2 hours at -20 °C prior to dissection. The hermit crab digestive tract was removed and divided into five part (Fig 2). Each part was transferred into sterile seawater (1 ml) and homogenized. Homogenate was examined using phase contrast microscopy, DAPI was then added to samples and examined using flouresence microscopy. F_{420} and chlorophyll flouresence was also looked for. 100 ul of homogenate was spread onto plates of three different media (SWC, CAA SW, BHI SW -Appendix 1). Further more sediment and sand from the bottom of the aquarium and scrapings from the Hermit Crab shell suspended in sterile sea water were spread directly onto plates (SWC, BHI SW, CAA SW, YEG SW).10 ml aliquots of sea water from the Hermit Crab Aquarium were filtered on to 0.2um filters. The filters were then placed onto plates of SWC and YEG-SWC Incubation conditions were aerobic and anaerobic at 20 °C.

After growth the different colonies were picked and transferred on fresh plates of the same media. To get pure cultures the isolates were streaked several times. The morphology of the bacteria and purity of the cultures were examined with the microscope.

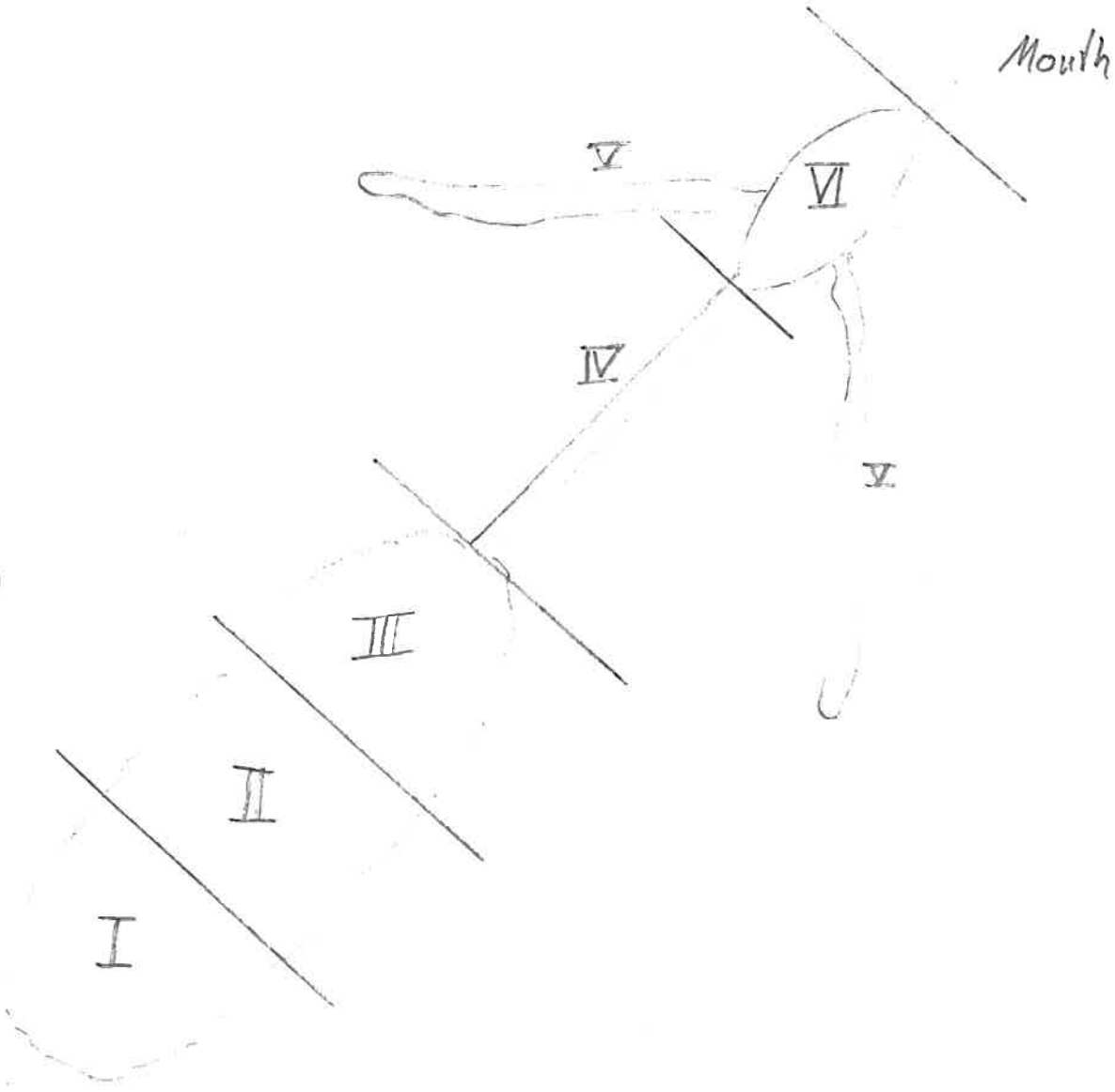


Fig. 1: different parts [illegible] digestive organs
I–III hypopharynx
II stomach
V [illegible]
VI [illegible]

2. Phenotypic Tests

Oxidase test

One colony of each isolate was picked and streaked on a filter paper. To show the oxidase activity one drop of tetramethyl p-phenylenediamine (Difco laboratories) was dropped on the colony. Development of a blue colouration indicates oxidase activity.

Luminescence

All isolates of the SWC plates were stored in the dark and examined for luminescence.

Fluorescence

All isolates were stored in the dark and irradiated with short wave UV light to look for flouresence.

Growth on Different Media

Pure cultures were spread on to plates of the different media descibed in Appendix1.

Growth Temperature

Growth at 4, 15, 20, 30, 37 and 42°C was investigated.

Requirement of Salt for Growth

The requirement for salt was tested by comparing growth of isolates on BHI made with sea water to growth on media made with distelled water.

Hydrolysis of Starch and Milk Protein

This was investigated by spreading isolates on Skim Milk plates and BHI + Starch plates (Appendix 1) and looking for zones of clearing around the colonies.

Aerobic/Anaerobic Growth

Isolates obtained aerobically were tested for their ability to grow anerobically, and likewise those isolated anaerobically were tested for their ability to grow aerobically.

Production of Acid or Alkali Under Anaerobic Growth

Production of acid or alkali under anaerobic growth was tested by placing pH paper next to the colonies on plates.

Characterisation of Pigments

Pigments were extrated from cells by mixing a small amount of cells with ethanol: methanol (70:30 V:V). A spectra was then made of the pigment.

3. Genotypic Tests

16S rRNA in situ hybridization

1 or 2 colonies were suspended in 1 ml of PBS. After centrifugation the pellets were washed two times with ice cold PBS and resuspended in 1 ml PBS. 15 ul from a dilution (1 : 100) of the cells was applied to subbed slides (0,1 % gelantine, 0,01 % $CrK(SO_4)_2$ 12 H_2O) and dried in 37 °C incubator.

The slides were treaded with ethanol and formaldehyde (90/10 v/v) for 5 min. to fix the cells and than washed two 2 times for 2 min. with H_2O in a coplin jar. After drying at 37 °C 40 ul of the probe (340 ng/ul) in hybridization mix was dropped on the cells and hybridized overnight at 37 ° C. At the next morning the slides were washed three times in 1 X SET at 37 ° C and dried vertically in a dark place. On each spot a tiny drop of mounting fluid was added and the coversliped was placed on the slide.

<u>Solutions</u>

10X SET : 1.5 M NaCl
200 mM Tris Cl pH 7.8
10 mM EDTA

hybridization mix: 400 ul 2 % BSA - 1 g/ 50 ml
400 ul 0.1 % PAA - 0.1 g/ 100 sterile water
2.0 ml 10 X SET
1.2 ml Dextran sulfate

probes: Proteobacteria (Enteric, Alphas, Betas, Gammas),
Flavobacteria
Universal probe.

PCR AND SEQUENCING OF 16S rRNA GENE

Several different approaches were investigated for lysis of cells to release DNA:

(1) 1 or 2 colonies were suspended in 50 ul of TE buffer and 17.5 ul of GeneRel aser was added. Cells were vortexed for 20 sec followed by centrifugation for 20 sec.and then 20 ul of mineral oil was overlaid in the tubes. Tubes were heated in the microwave for 5 min

(2) As above except tubes were heated in the thermocycler as described in the GeneReleaser protocol.

(3) Cells were suspended in TE buffer as above. Proteinase K was added to the tubes to give a concentration of 200ug/ml. Tubes were heated at 55°C for 2 hours, followed by heating to 95°C for 10 minutes.

(4) Cells were suspended in TE buffer as above and Lysozyme was added to a concentration of 0.5 mg/ml. Tubes were heated for 1 minute at 95°C.

(5) Cells were added directly to the PCR master-mix (see below). Tubes were then heated at 94°C for 10 minutes directly prior to addition of Taq. polymerase.

(6) Cells were suspended in 10ul of TE buffer and 25 ul of GeneReleaser. Tubes were then heated in the thermocycler as described in the GeneReleaser protocol.

PCR was then carried out as follows:

Eppendorf tubes with 25 ul of master mix 1 were placed in the Temp cycler and Ampliwax beads (2-3) were added. After the Temp cycler has headed to 80 ° C and than cooled to 35°C, 54ul of master mix 2 and 3 ul of DNA was added. A negative control with 3 ul H_2O was also carried out.

The following program of the Thermo-cycler was used. 35 cycles were carried out:

95 ° C for 45 sec (denaturing step)

50 ° C for 50 sec (annealing step)

72 ° C for 2 min. (elongation step)

After the end of the 35 cycles the temperaturec was held at 4 ° C. 5 ul of the sample was run on 0.8% agarose ethidiumbromide gel for approximately 1 h at 100 mV to check the PCR product.

Solutions

master mix 1: 2.5 ul 10X PCR buffer
10 ul $MgCl_2$
2.5 ul H_2O
2.0 ul dATP, dCTP, dTTP, dGTP
1.0 ul forward primer (universal)
1.0 ul reverse primer (universal)

master mix 2: 5.7 ul 10X PCR buffer
0.5 ul Taq enzyme
47.8 ul H_20

The PCR products were cleaned using Wizard kit. The amount of DNA was measured using the DNA Dipstick kit. DNA was then diluted if neccessary to a concentration of 50 ng/ul. This was then used for sequencing.

Results

Table 1:Growth on Different Media :Aerobic
Percentage of Plate Covered in Growth

Part of Digestive Tract	CAA-SW	BHI-SW	SWC pH 6	SWC pH 7	SWC pH 8
I	70%	100%	100%	100%	100%
II	70 %	90 %	70 %	70 %	70 %
III	30%	60%	50%	30%	80%
IV	few colonies only	10%	10%	20%	10%
V	70 %	70 %	few colonies only	few colonies only	few colonies only
VI		70 %	80 %	90 %	70 %

Table 2 :Growth on Different Media :Anaerobic

Percentage of Plate Covered in Growth

Part of Digestive Tract	CAA-SW	BHI-SW	SWC pH 6	SWC pH 7	SWC pH 8
I	80 %	50 %	50 %	80 %	50 %
II	90 %	50 %	10 %	5 %	30 %
III	10 %	50 %	70 %	90 %	70 %
IV	70 %	50 %	70 %	90 %	70 %
V	few colonies only	50 %	50 %	70	few colonies only
VI	70 %	70 %	50 %	50 %	50 %

Table 3:Growth on Plates Aerobic Sediment, Seawater and Crab Shell

SAMPLE	YEG-SW	SWC
SEDIMENT		
Plate I	> 100 colonies	60 % of plate covered
Plate II	> 100 colonies	60 % of plate covered
SEAWATER		
Plate I	> 100 colonies	> 100 colonies
Plate II	> 100 colonies	> 100 colonies
CRAB SHELL	approx. 20 colonies	approx. 20 colonies

Tab. 4 and 5: Isolates part I

isolate	pigment	colony shape	T ° C 15	20	30	37	42	bacteria shape	Motility	An-aerobic	O_2	Oxidase	acid production
BHI AN 2	N	small circular	?	+	?	?	?	rods spores?	no	+	no	negative	pH 5
BHI A	B	circular raised	+	+	+	+	-	short rods	+	?	+	positive	
BHI B	Y	circular raised	-	+	+	+	-	short rods	+	?	+	positive	
CAA 1.1	PY	flat	-	+	-	-	-	long rods	+	+	+	positive	
CAA 1.2	YO	flat	?	+	?	?	?	?		+	+	positive	
CAA 2a	YO	flat	-	+	-	-	-	small rods		?	+	positive	
CAA 2b	N	flat	-	+	-	-	-	small rods		?	+	positive	
ow6a	N	circular raised	?	+	+	+	-	rods		no	+	negative	
r8an	N	flat	?	+	+	?	?	rods		+	+	positive	no
t7an	N	flat	?	+	+	?	?	rods		+	+	positive	no
o6a	YO	circular raised	?	+	+	+	-	rods		no	+	positive	
w8a	N	circular raised	?	+	+	+	-	small rods		no	+	positive	

isolate	Colony color	CAA SW	YEG SW	SWC	BHI SW	BHI	skim-milk
BHI AN2	N	+	?	?	+	no	no
BHI A	WB	no	?	no	+	no	no
BHI B	CP	no	?	+	+	no	no
CAA 1.1	CS		?	no	no	no	no
CAA 1.2	CY	+	?	no	no	no	no
CAA 2a	T	+	?	?	+	no	no
CAA 2b	TD	+	?	+	no	no	no
ow6a	W	+	+	+	+	+	+
r8an	W	?	?	+	+	?	?
t7an	WT	?	?	+	+	?	?
o6a	O	+	+	+	+	no	no
w8a	W	+	+	+	+	+	no

Tab. 6: Isolates part II

isolate	pigment	colony shape	T ° C 15	20	30	37	42	bacteria shape	Motility	An-aerobic	O_2	Oxidase	acid production
BHI C	B	regular flat	+	+	-	-	-	short rods	no	?	+	positive	
CAA 4.0	YO	flat	-	+	-	-	-	small rods	no	no	+	positv	
CAA 4.1	PY	rand slights raised	-	+	-	-	-	small rods	no	no	+	positive	
BHI An3	N		?	+	?	?	?	small rods	+	+	no	positive	no
o8a	N	circular raised	?	+	+	+	-	rods	?	no	+	positive	
g7a	N	flat	?	+	+	+	-	rods	?	no	+	positive	
wt6a	N	circular	?	+	+	-	-	rods	?	no	+	positive	
w8a		circular	?	+	+	+	-	rods	?	no	+	positive	
t6a	N	circular	?	+	+	+	-	rods	?	no	+	positive	
w7a	N	circular	?	+	+	+	-	rods	?	no	+	positive	
t8a	N	flat	?	+	+	+	-	rods	?	no	+	positive	
w6a	N	circular raised	?	+	+	+	-	rods	?	no	+	negative	
w8an	N	flat	?	+	+	?	?	rods	?	+	+	positive	no
t7an	N	flat	?	+	+	?	?	cocci	?	+	+	positive	no
t6an	N	flat	?	+	+	?	?	rods	?	+o	+	positive	no
wg6an	N	circular	?	+	+	?	?	rods	?	+	+	positive	no
w6an	N	circular raised	?	+	+	?	?	rods	?	+	+	positive	no

Tab. 7: Isolates part II

isolate	Colony color	CAA SW	YEG SW	SWC	BHI SW	BHI	skim-milk
BHI C	CP	no	?	no	+	+	no
CAA 4.0	YC	+	?	no	no	no	no
CAA 4.1	C	+	?	no	no	no	no
BHI An3	N	+	?	?	+	no	no
o8a	WD	+	+	+	+	+	+
g7a	W	+	+	+	+	+	+
wt6a	W	no	+	+	+	+	no
w8a	W	+	+	+	+	no	no
t6a	W	+	+	+	+	+	no
w7a	W	+	+	+	+	no	no
t8a	WT	+	+	+	+	+	no
w6a	W	+	+	+	+	+	+
w8an	W	?	?	+	+	?	?
t7an	W	?	?	+	+	?	?
t6an	W	?	?	+	+	?	?
wg6an	W	?	?	+	+	?	?
w6an	W	?	?	+	+	?	?

Tab. 8 and 9: Isolates part III

isolate	pigment	colony shape	T ° C 15	20	30	37	42	bacteria shape	Motility	An-aerobic	O_2	Oxidase	acid production
BHI F	B	round raised	+	+		-	-		?	?	+	positive	
BHI G	B	raised circular	+	+	-	-	-	short rods	+	little	+	positve	
CAA 5.0	YO	flat	-	+	-	-	-	small rods	no	no	+	positive	
CAA An2	N	flat	?	+	?	?	?	small rods	+	+	no	positive	no
wt7a	N	circular	?	+	+	+	-	rods	?	no	+	positive	
t7a	N	circular	?	+	+	+	-	rods	?	no	+	positive	
wg7a	N	circular	?	+	+	+	-	rods	?	no	+	positive	
w8a	N	circular	?	+	+	+	-	rods	?	no	+	positive	
wt8a	N	circular	?	+	+	-	-	rods	?	no	+	positive	
w6an	N	circular	?	+	+	?	?	rods	?	+	+	positive	pH 4
t8an	N	flat	?	+	+	?	?	rods	?	+o	+	positive	no
t6an	N	circular	?	+	+	?	?	rods	?	+	+	positive	no
t7an	N	flat	?	+	+	?	?	cocci	?	+	+	positive	no

isolate	Colony color	CAA	YEG SW	SWC	BHI SW	BHI	Skim-milk
BHI F		no	?	+	+	?	no
BHI G	CB	no	?	no	+	no	no
CAA 5.0	YC	+	?	+	+	no	no
CAA An2	Y	+	?	?	no	no	no
wt7a	WD	+	+	+	+	+	+
t7a	W	+	+	+	+	+	no
wg7a	W	+	+	+	+	+	no
w8a	W	+	+	+	+	+	no
wt8a	W	+	+	+	+	no	no
w6an	W	?	?	+	+	?	?
t8an	W	?	?	+	+	?	?
t6an	W	?	?	+	+	?	?
t7an	W	?	?	+	+	?	?

Tab. 10 and 11: Isolates part IV

isolate	pigment	colony shape	T ° C 15	20	30	37	42	bacteria shape	Motility	An-	O_2	Oxidase	acid production
BHI H	B	round raised	+	+	+	-	-		no	no	+	positive	
CAA An3	N	circular raised	?	+	?	?	?	rods	no	+	+	positive	no
CAA An5	N	circular	?	+	?	?	?	long thin rods	no	+	no	positive	no
w8a	N	circular	?	+	+	-	-	rods	?	no	+	positive	
t8a	N	circular	?	+	+	+	-	rods	?	no	+	positive	
wg7a	N	circular	?	+	+	+	-	rods	?	no	+	positive	
wg6a	Y	circular	?	+	+	+	-	rods	+	no	+	positive	
w8an	N	circular	?	+	+	?	?	rods	?	+	+	positive	no
t8an	N	flat	?	+	+	?	?	cocci	?	+	+	positive	no
w7an	N	circular	?	+	+	?	?	cocci	?	+o	+	positive	no
t6an	N	circular	?	+	+	?	?	short rods	?	+	+	?	no

isolate	Colony color	CAA SW	YEG SW	SWC	BHI SW	BHI	skim-milk
BHI H	BrB	no	?	+	+	+	+
CAA An3	PB	+	?	?	+	+	+ no
CAA An5	T	+	?	?	no	no	+ no
w8a	W	+	+	+	+	+	no
t8a	W	+	+	+	+	+	+
wg7a	W	+	+	+	+	+	+
wg6a	Y	+	+	+	+	+	no
w8an	W	?	?	+	+	?	?
t8an	W	?	?	+	+	?	?
w7an	W	?	?	+	+	?	?
t6an	W	?	?	+	+	?	?

Tab. 12 and 13: Isolates part V

isolate	pigment	colony shape	T ° C 15	20	30	37	42	bacteria shape	Motility	An-aerobic	O_2	Oxidase	acid production
CAA 10	PY	round raised	-	+	-	-	-	small rods	no	no	+	positive	
BHI An8	N	circular raised	?	+	?	?	?	rods	+	+	+	positive	no
BHI An9.1	N		?	+	?	?	?		?	+	+	?	?
CAA An6	N	circular	?	+	?	?	?		?	+	+	positive	no
s7a	N	pyramid hard	?	+	+	+	+	rods	?	no	+		
y8a	Y	circular	?	+	+	-	-	rods	?	no	+	negative	
w8a	N	circular	?	+	+	+	-	rods	?	no	+	positive	
t6a	N	circular	?	+	+	+	-	rods	?	no	+	negative	
w6a	N	circular	?	+	+	+	-	rods	?	+	+	positive	

isolate	Colony color	CAA SW	YEG SW	SWC	BHI SW	BHI	skim-milk
CAA 10	C	+	?	+	+	+	no
BHI An8	WP	+	?	?	+	+	no
BHI An9.1	W	+	?	?	+	no	no
CAA An6	W	+	?	?	+	+	+ no
s7a	WB	+	+	+	+	+	no
y8a	Y	+	+	+	+	no	no
w8a	W	+	+	+	+	+	+
t6a	W	no	+	+	+	no	+
w6a	W	+	+	+	+	+	no

Tab. 14 and 15: Isolates part VI

isolate	pigment	colony shape	T ° C 15	20	30	37	42	bacteria shape	Motility	An-aerobic	O_2	Oxidase	acid production
BHI P	Y	round raised	-	+	+	+	+	rods	no	+	+	positive	no
BHI L	Y	round raised	-	+	-	+	-	rods	+	+	+	positive	no
CAA 11	Y	flat	-	+	+	-	-			no	+	negative	
CAA 12	N	circular	-	+	-	-	-	small rods	no	+	+	positive	
CAA An7	N	flat	?	+	?	?	?	rods	no	+	no	positive	no
w6a	N	circular	?	+	+	+	-	rods	?	no	+	positive	
t6a	N	circular	?	+	+	+	-	rods	?	no	+	positive	
t8an	N	circular	?	+	+	?	?	rods	?	+	+	positive	no
y8an	N	circular	?	+	+	?	?	rods	?	+	+	positive	no

isolate	Colony color	CAA SW	YEG SW	SWC	BHI SW	BHI	skim-milk
BHI P	WY	no	?	no	+	no	no
BHI L	W	no	?	no	+	no	no
CAA 11	CY	+	?	?	no	no	no
CAA 12	WS	+	?	no	no	no	no
CAA An7	CS	+	?	?	+	no	no
w6a	W	+	+	+	+	+	+
t6a	W	+	+	+	+	+	+
t8an	W	?	?	+	+	?	?
y8an	WC	?	?	+	+	?	?

Tab. 16 and 17: Isolates sand, biofilm and sea

isolate	pigment	colony shape	T ° C 15	20	30	37	42	bacteria shape	Motility	An-aerobic	O_2	Oxidase	acid production
sand pink	P	round	?	+	?	?	?	thin rods	+	no	+	positive	
sand 2	N	round	?	+	?	?	?	rods		+	+	positive	
sand 4	N	flat	?	+	?	?	?	long rods	+	+	+	positive	
sand 5	P		?	+	?	?	?			+	+	positive	
sand 6	N	mucoid sticky	?	+	?	?	?	cocci		no	+	positive	
sea 1	Y	flat glider	?	+	?	?	?	long thin rods	glider	no	+	?	
sea 2	N	thick	?	+	?	?	?	rods		no	+	negative	
sea 3	N		?	+	?	?	?	small rods		+	+	negative	
biofilm C	sY	circular	?	+	?	?	?	short rods		no	+	positive	
biofilm SO	O	circular	?	+	?	?	?	rods		+	+	positive	

isolate	Colony color	CAA SW	YEG SW	SWC	BHI SW	BHI
sand pink	PO	?	+	+	?	?
sand 2	C	?	?	+	?	no
sand 4	W	+	no	+	+	+
sand 5	PO	no	+	?	+	no
sand 6	W	no	+	+	+	+
sea Y1	Y	?	?	+	?	no
sea 2	BS	no	+	+	+	no
sea 3	BS	?	?	+	+	no
biofilm C	W	+	+	+	+	no
biofilm SO	BO	no	no	+	+	no

B : beige, W : white, O : orange, S : shiny, N : no pigment, Y : yellow, P : pink,
s : soluble, C : cream, Br : brown, D : dark, T: transparent
? : not determined

Tab. 18: In situ hybridisation

Isolate	Universal	Enteric	Prote α	Proteo β	Proteo γ	Flavo
BHI H	yes	no	no	no	n. d.	n. d.
sand 5	yes	no	no	no	n. d.	n. d.
CAA An3	yes	no	no	no	n. d.	n. d.
BHI An2	yes	yes	no	no	n. d.	n. d.
w3a, III	yes	no	n. d.	n. d.	no	no
o6a, I	yes	no	n. d.	n. d.	no	no
s7a, V	yes	no	n. d.	n. d.	no	no
BHI G	yes	no	n. d.	n. d.	no	no
BHI L	yes	no	n. d.	n. d.	no	no
CAA 4.1	yes	no	n. d.	n. d.	no	no
CAA 2b	yes	no	n. d.	n. d.	no	no
BHI SO	yes	no	n. d.	n. d.	no	no

Fig. 3: PCR-product, line 7, isolate w8a,III lysozyme

Fig. 4: PCR-product, line 8, isolate BHI M gene releaser

Fig. 5: PCR-product, line 15,29,30
heating

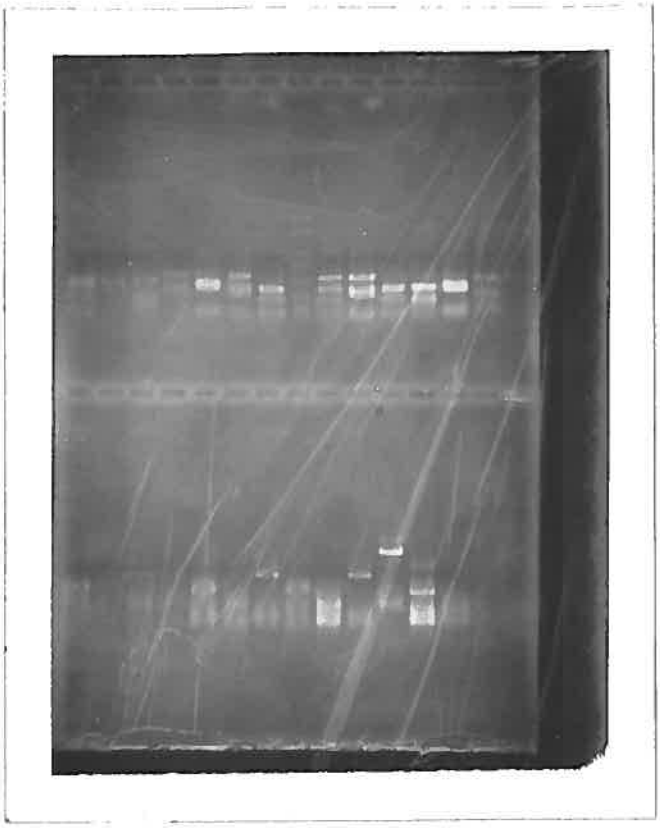
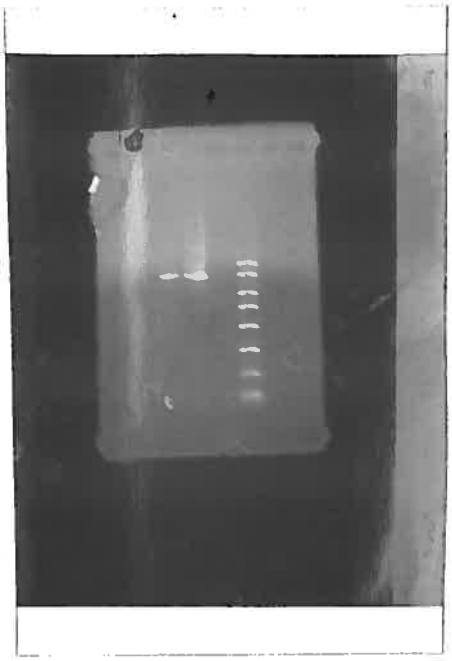


Fig. 6: PCR-product, line 3, isolate o6a,I
line 4, isolate t8a, IV



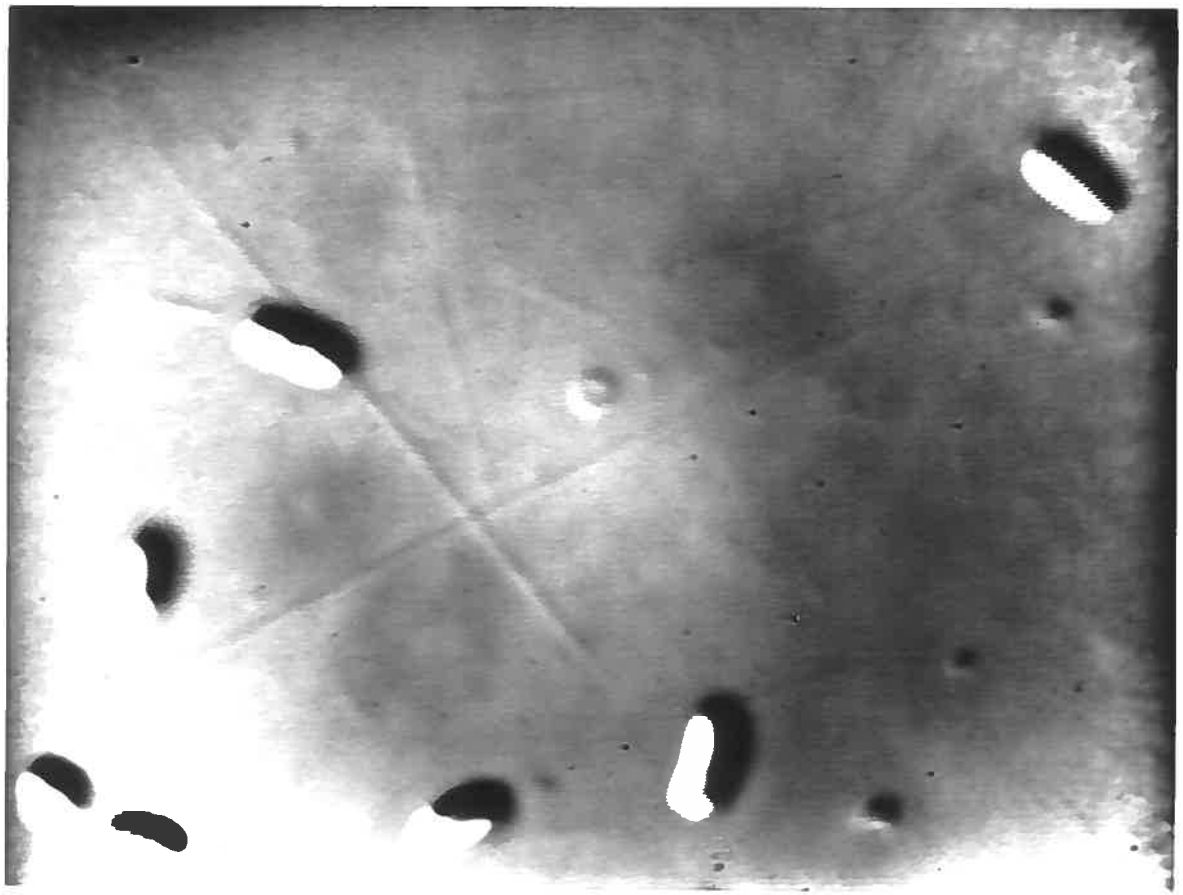


Fig. 7: Bacteria cells and tissue fibers of the hermit crab

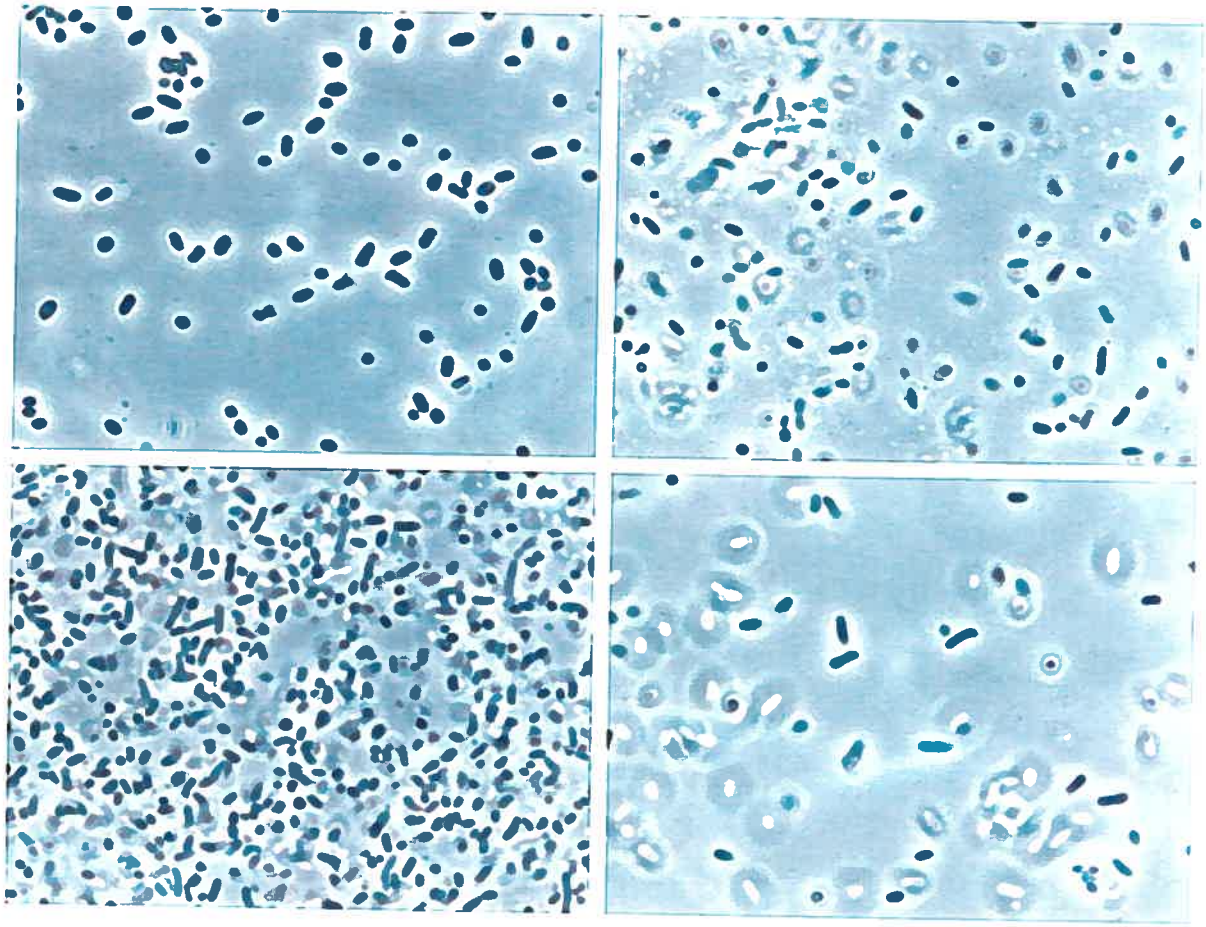


Fig. 8: top left isolate *BHI B*, top right isolate *BHI H*, bottom left isolate *BHI 2b*, bottom right *CAA 1.1*

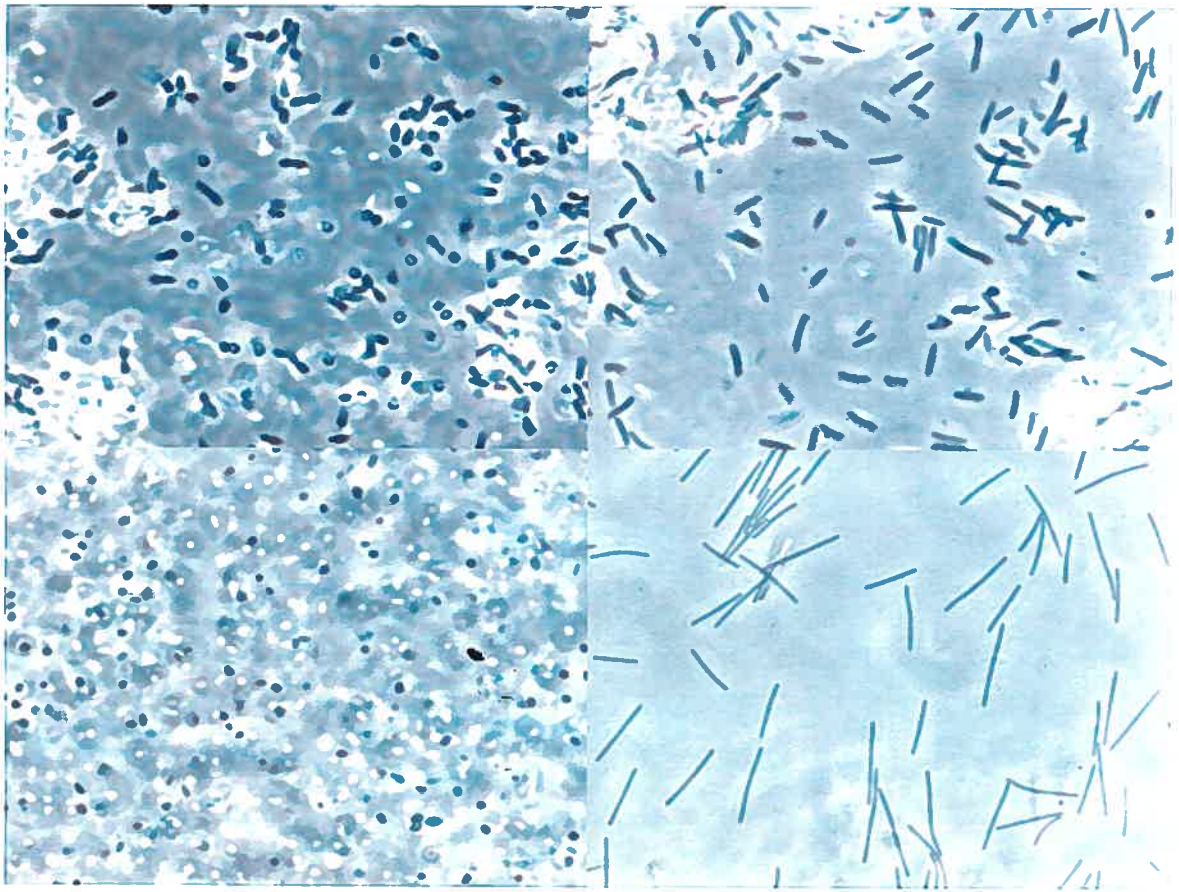


Fig. 9: top left isolate *sand 5*, top right isolate *biofilm SO*, bottom left isolate *sand 6*, bottom right *sea Y*

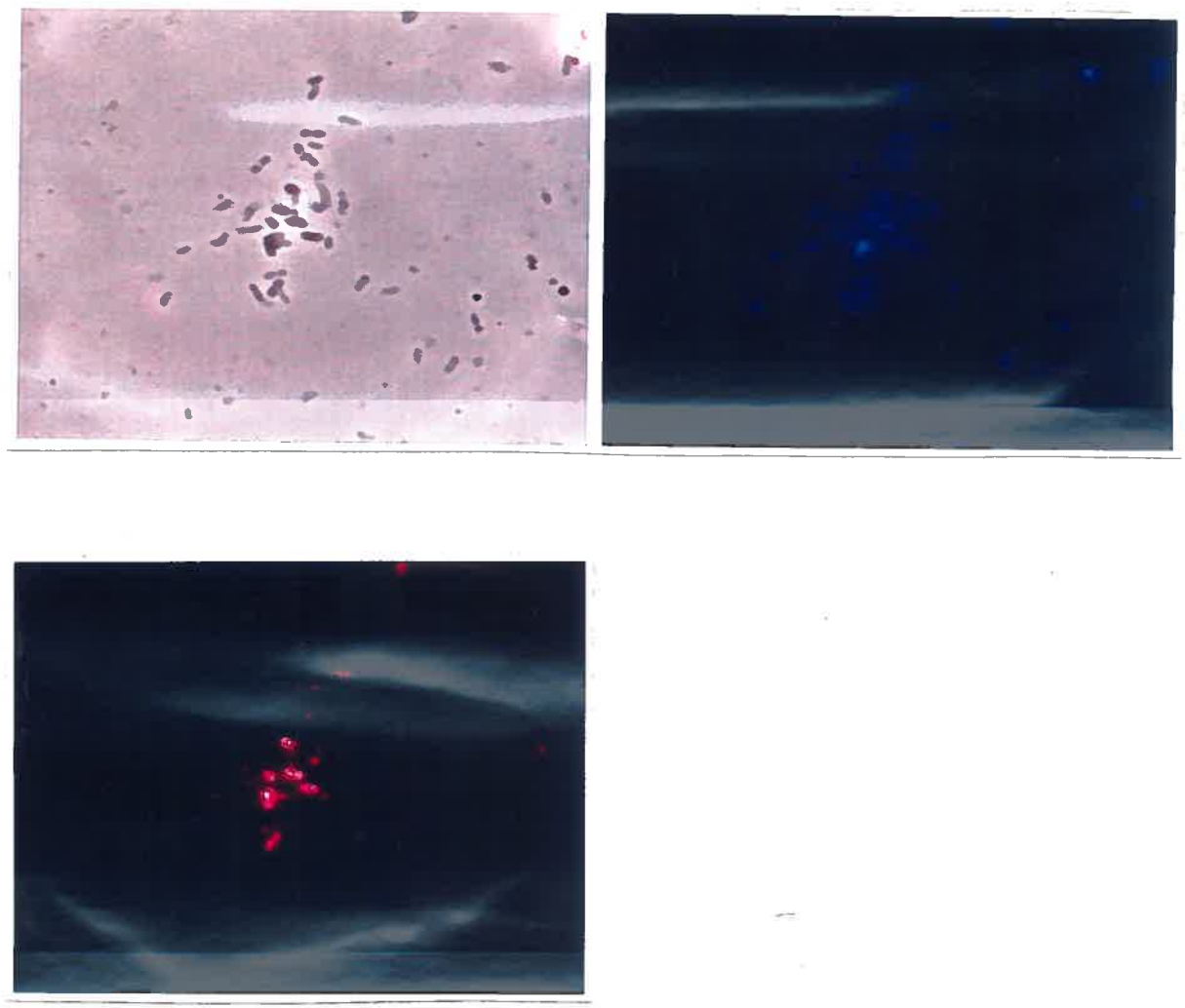


Fig. 10: in situ hybridisation, isolate o6a, I, universal probe, top left phase contrast, top right cumarin, bottom left fluroscence

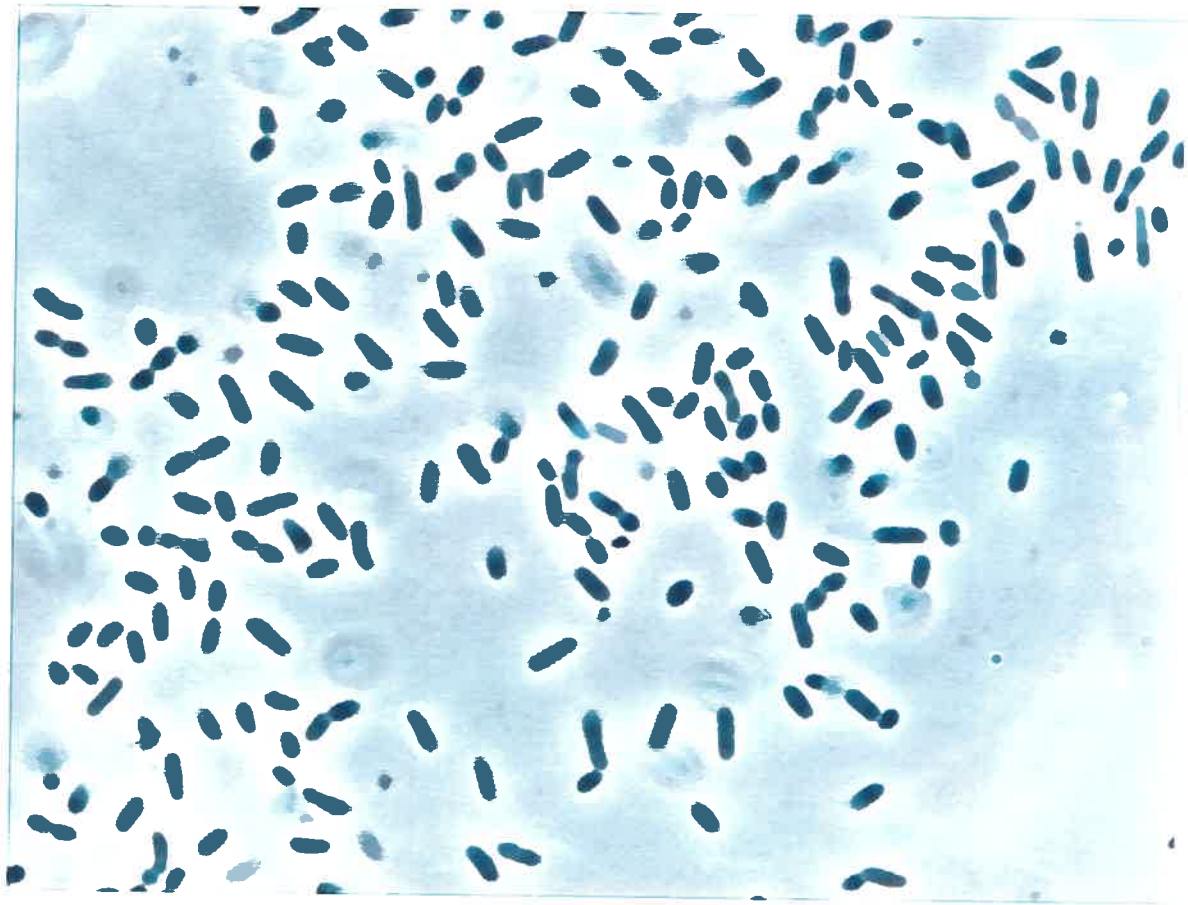


Fig. 11: isolate 06a, I

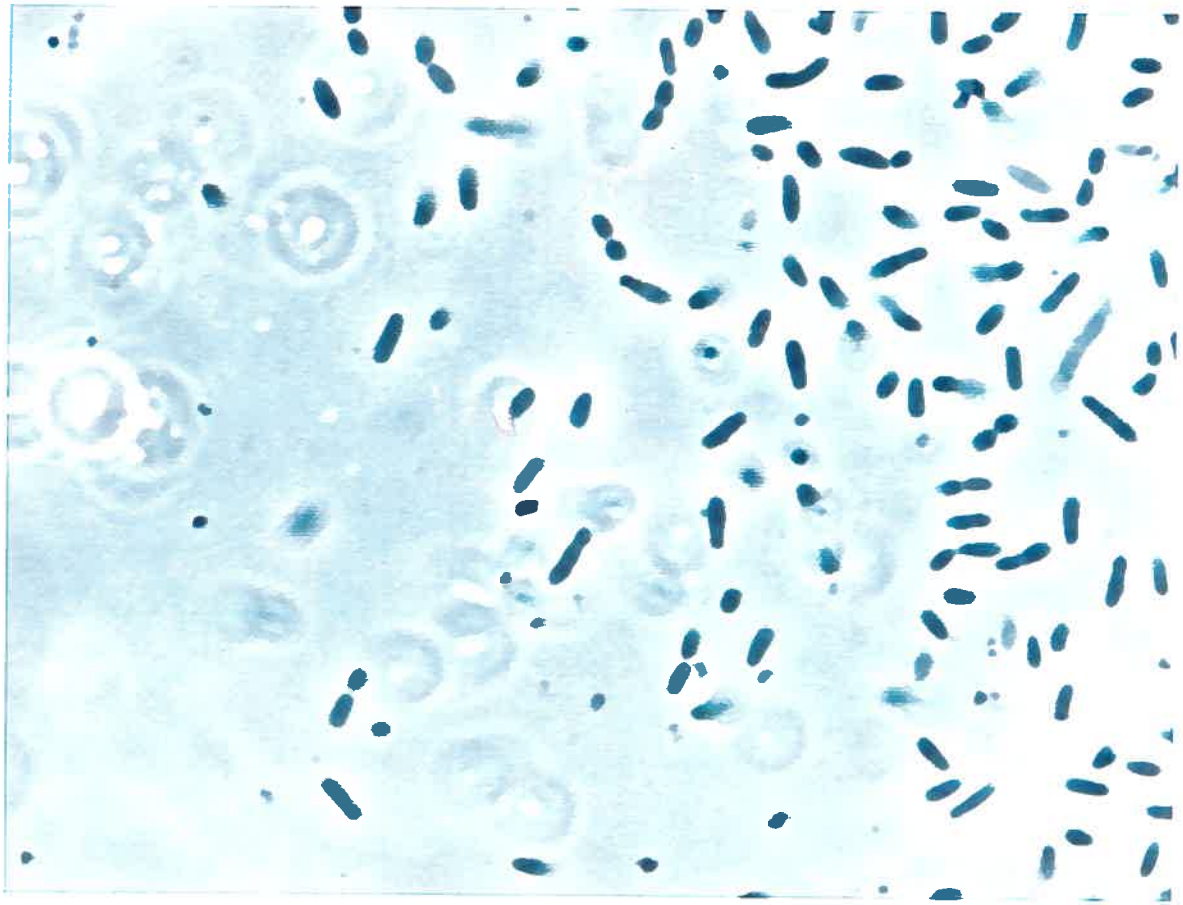


Fig. 12: Isolate t8a, IV

Fig. 13

Oba I

Ace : Met 9 : 1

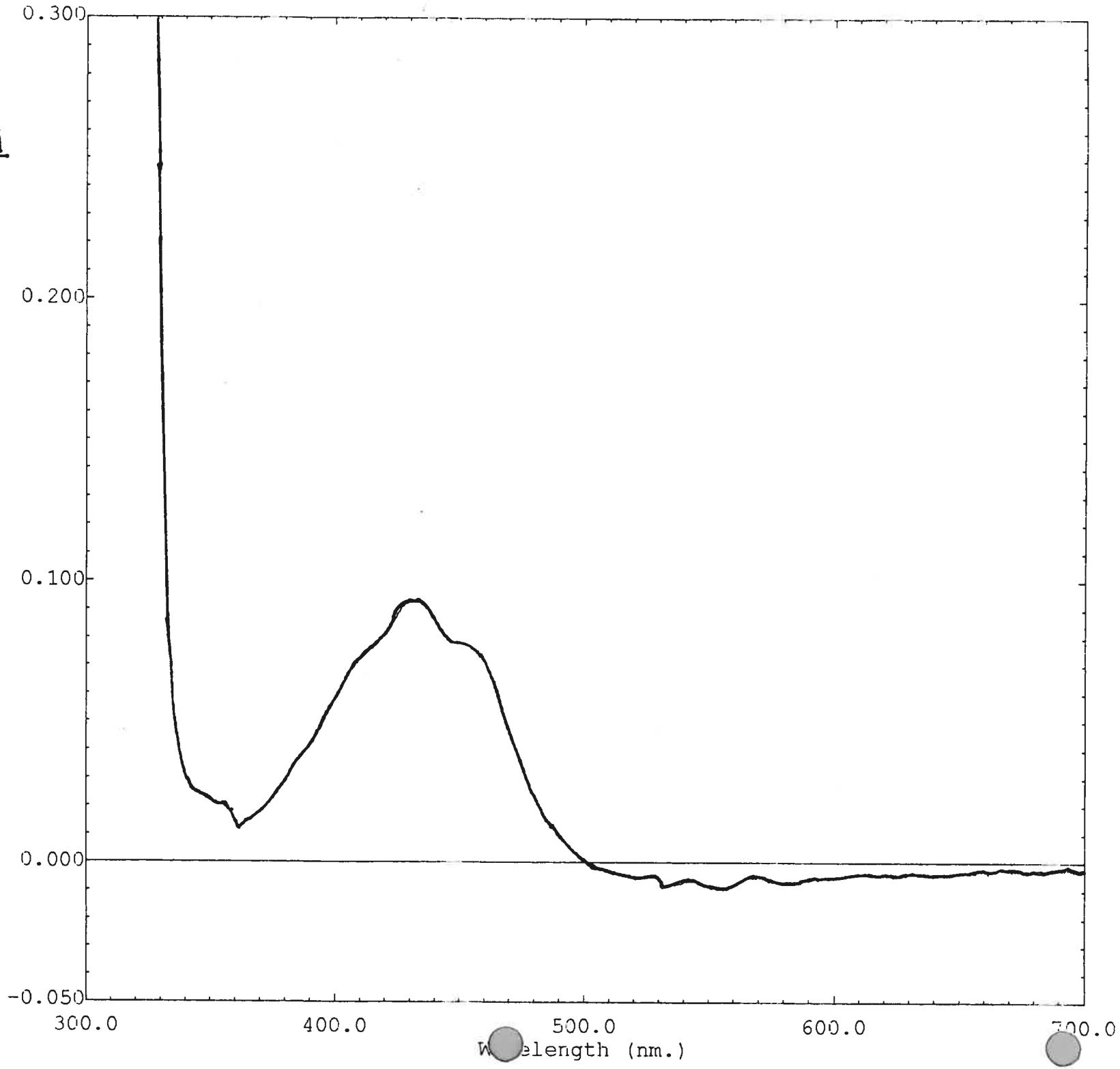


Fig 14
Sea YI

Ace : Met 7:3

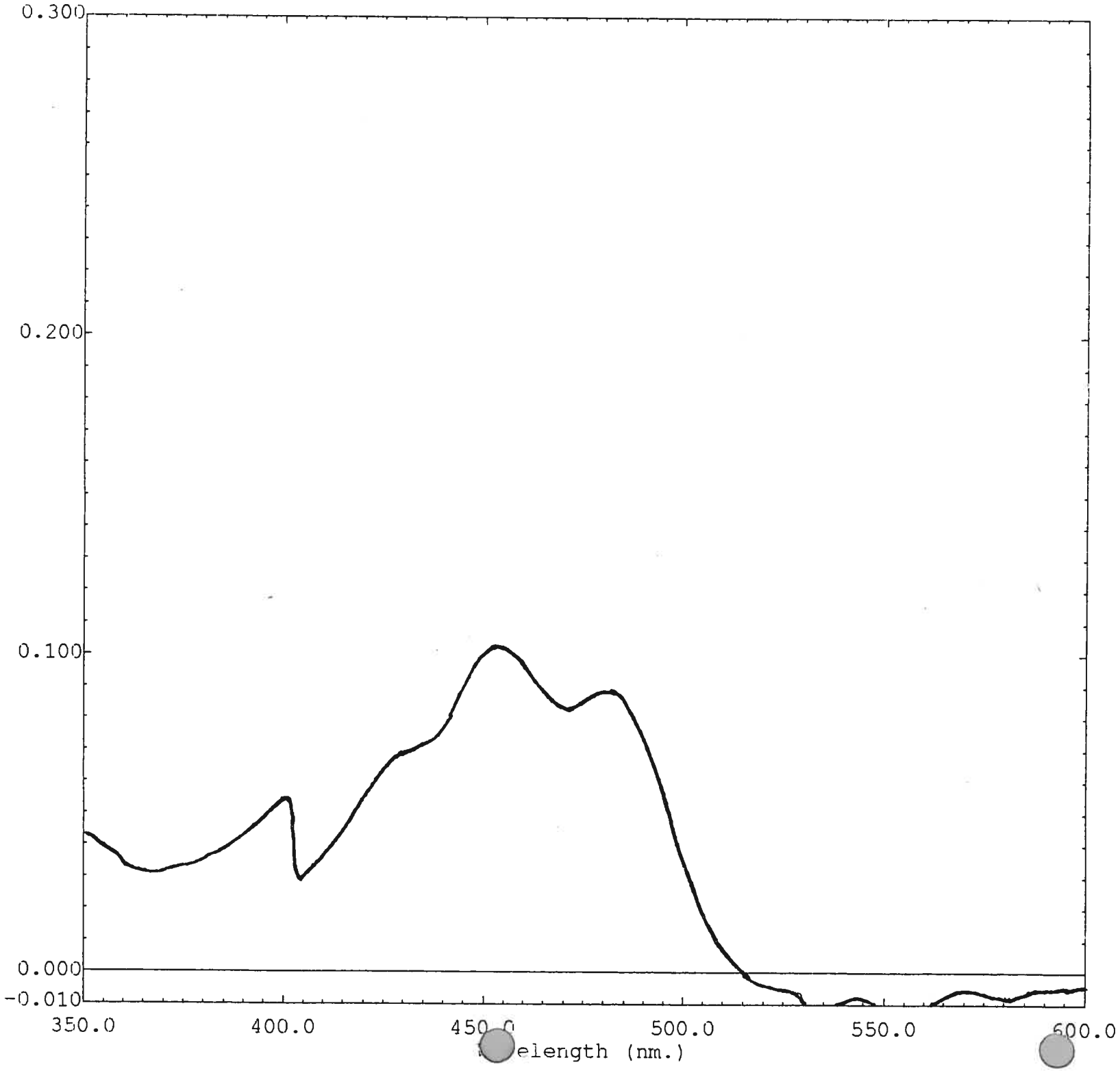


Fig. 15

(% Difference)

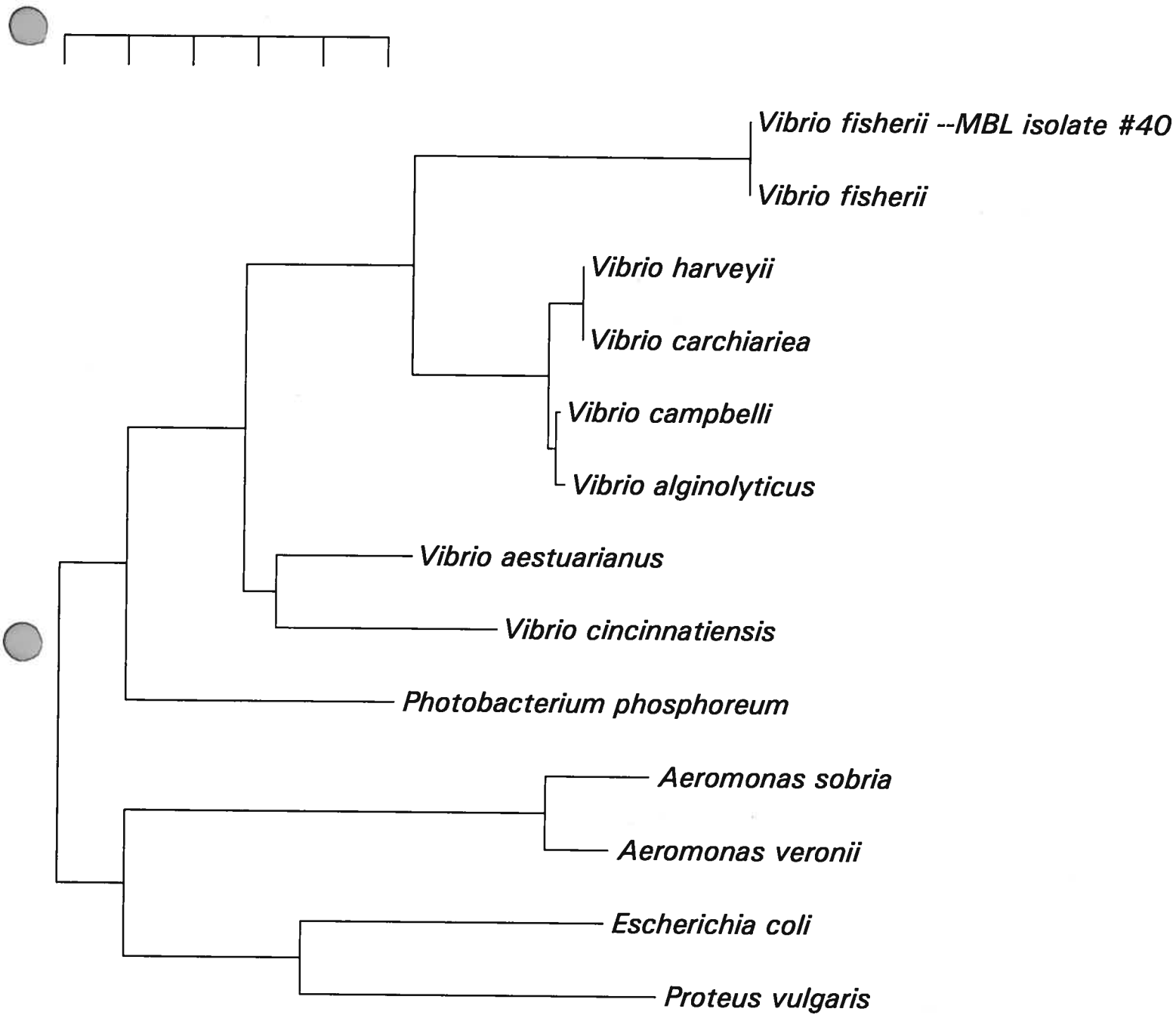


Fig 16

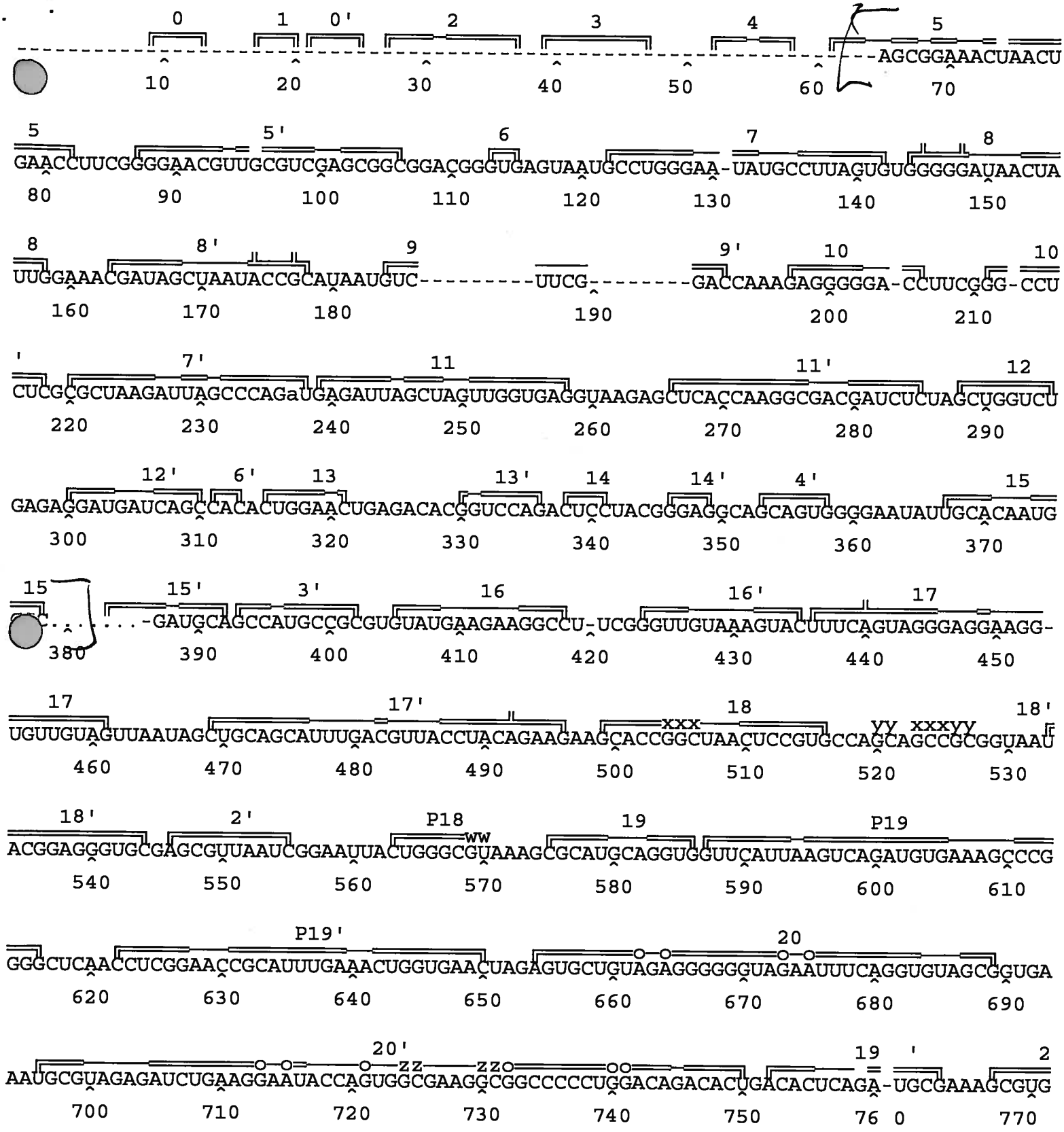
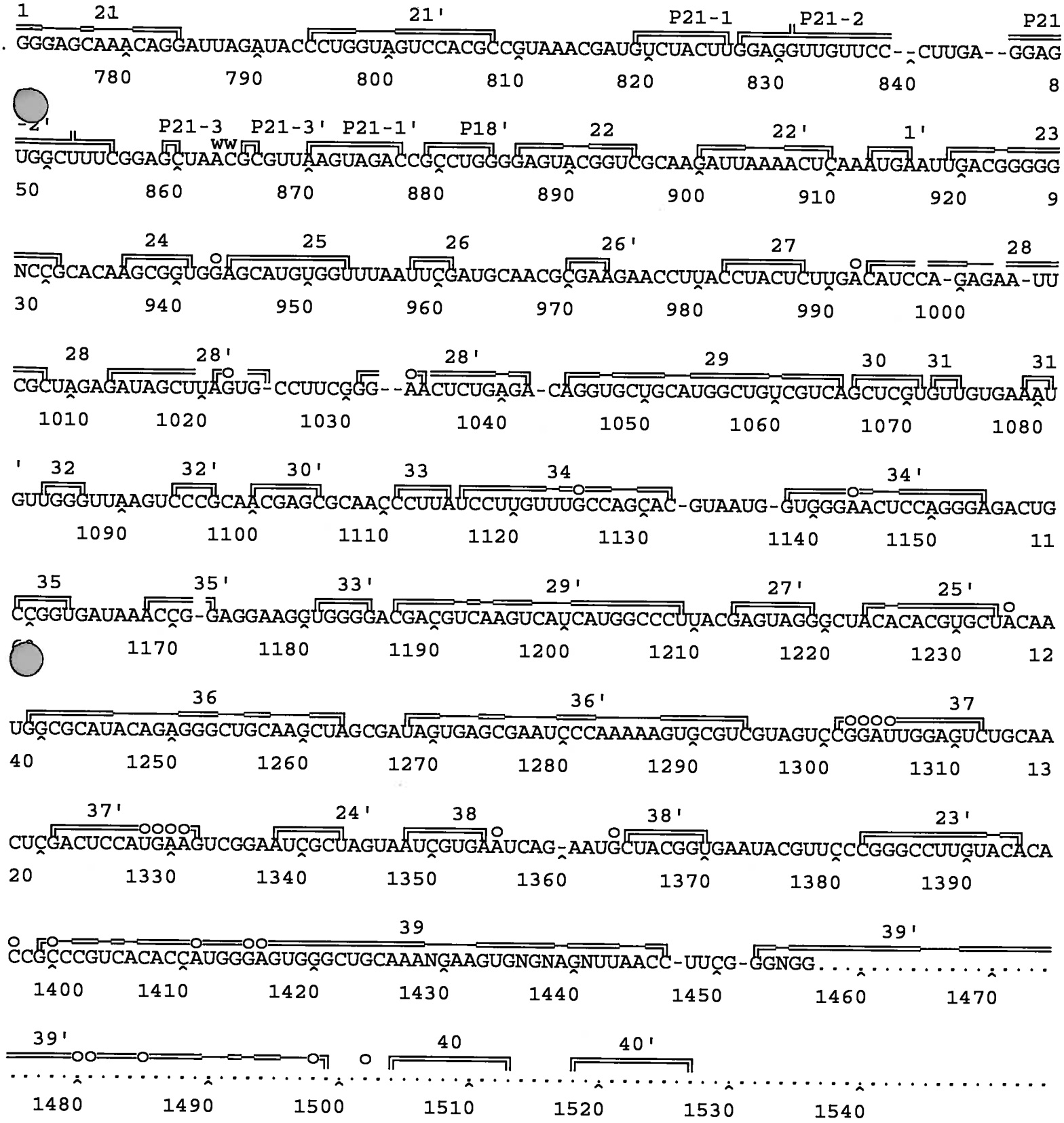

0 1 0' 2 3 4 5
AGCGGAAACUAACU
10 20 30 40 50 60 70
5 5' 6 7 8
GAACCUUCGGGGAACGUUGCGUCGAGCGGCGGACGGGUGAGUAAUGCCUGGGAA-UAUGCCUUAGUGUGGGGGAUAACUA
80 90 100 110 120 130 140 150
8 8' 9 9' 10 10
UUGGAAACGAUAGCUAAUACCGCAUAAUGUC---------UUCG--------GACCAAAGAGGGGGA-CCUUCGGG-CCU
160 170 180 190 200 210
7' 11 11' 12
CUCGCGCUAAGAUUAGCCCAGaUGAGAUUAGCUAGUUGGUGAGGUAAGAGCUCACCAAGGCGACGAUCUCUAGCUGGUCU
220 230 240 250 260 270 280 290
12' 6' 13 13' 14 14' 4' 15
GAGAGGAUGAUCAGCCACACUGGAACUGAGACACGGUCCAGACUCCUACGGGAGGCAGCAGUGGGGAAUAUUGCACAAUG
300 310 320 330 340 350 360 370
15 15' 3' 16 16' 17
C.....-GAUGCAGCCAUGCCGCGUGUAUGAAGAAGGCCU-UCGGGUUGUAAAGUACUUUCAGUAGGGAGGAAGG-
380 390 400 410 420 430 440 450
17 17' 18 18'
UGUUGUAGUUAAUAGCUGCAGCAUUUGACGUUACCUACAGAAGAAGCACCGGCUAACUCCGUGCCAGCAGCCGCGGUAAU
460 470 480 490 500 510 520 530
18' 2' P18 19 P19
ACGGAGGGUGCGAGCGUUAAUCGGAAUUACUGGGCGUAAAGCGCAUGCAGGUGGUUCAUUAAGUCAGAUGUGAAAGCCCG
540 550 560 570 580 590 600 610
P19' 20
GGGCUCAACCUCGGAACCGCAUUUGAAACUGGUGAACUAGAGUGCUGUAGAGGGGGGUAGAAUUUCAGGUGUAGCGGUGA
620 630 640 650 660 670 680 690
20' 19 ' 2
AAUGCGUAGAGAUCUGAAGGAAUACCAGUGGCGAAGGCGGCCCCCUGGACAGACACUGACACUCAGA-UGCGAAAGCGUG
700 710 720 730 740 750 76 0 770

Fig 1b (Cont.)


1 21 21' P21-1 P21-2 P21
GGGAGCAAACAGGAUUAGAUACCCUGGUAGUCCACGCCGUAAACGAUGUCUACUUGGAGGUUGUUCC--CUUGA--GGAG
780 790 800 810 820 830 840 8
-2' P21-3 P21-3' P21-1' P18' 22 22' 1' 23
UGGCUUUCGGAGCUAACGCGUUAAGUAGACCGCCUGGGGAGUACGGUCGCAAGAUUAAAACUCAAAUGAAUUGACGGGGG
50 860 870 880 890 900 910 920 9
24 25 26 26' 27 28
NCCGCACAAGCGGUGGAGCAUGUGGUUUAAUUCGAUGCAACGCGAAGAACCUUACCUACUCUUGACAUCCA-GAGAA-UU
30 940 950 960 970 980 990 1000
28 28' 28' 29 30 31 31
CGCUAGAGAUAGCUUAGUG-CCUUCGGG--AACUCUGAGA-CAGGUGCUGCAUGGCUGUCGUCAGCUCGUGUUGUGAAAU
1010 1020 1030 1040 1050 1060 1070 1080
32 32' 30' 33 34 34'
GUUGGGUUAAGUCCCGCAACGAGCGCAACCCUUAUCCUUGUUUGCCAGCAC-GUAAUG-GUGGGAACUCCAGGGAGACUG
1090 1100 1110 1120 1130 1140 1150 11
35 35' 33' 29' 27' 25'
CCGGUGAUAAACCG-GAGGAAGGUGGGGACGACGUCAAGUCAUCAUGGCCCUUACGAGUAGGGCUACACACGUGCUACAA
1170 1180 1190 1200 1210 1220 1230 12
36 36' 37
UGGCGCAUACAGAGGGCUGCAAGCUAGCGAUAGUGAGCGAAUCCCAAAAAGUGCGUCGUAGUCCGGAUUGGAGUCUGCAA
40 1250 1260 1270 1280 1290 1300 1310 13
37' 24' 38 38' 23'
CUCGACUCCAUGAAGUCGGAAUCGCUAGUAAUCGUGAAUCAG-AAUGCUACGGUGAAUACGUUCCCGGGCCUUGUACACA
20 1330 1340 1350 1360 1370 1380 1390
39 39'
CCGCCCGUCACACCAUGGGAGUGGGCUGCAAANGAAGUGNGNAGNUUAACC-UUCG-GGNGG
1400 1410 1420 1430 1440 1450 1460 1470
39' 40 40'
1480 1490 1500 1510 1520 1530 1540

Discussion

The aims of this project were to isolate and characterise bacteria from the Hermit crab gut. Previous investigators have described the crustacean gut as sterile. (Boyle & Mitchell 1978). Boyle and Mitchell examined the digestive tract of two marine and one terrestrial wood- boring isopods and one wood inhabiting amphipod using SEM and saw no evidence of any microbial life. Hughes (Microbial Diversity 1993) examined the hind gut of two species of marine isopods microscopically and revealed no visible microbiota. We also observed very little microbial life microscopically.

However we have isolated a total of 83 strains of bacteria from the Hermit Crab gut. Ten strains were also isolated either from the sea water of the tank, the sediment at the bottom of the tank and the Hermit Crab shell. Each part of the crab gut was homogenized in 1 ml of sterile sea water and then 100 ul of this was spread directly onto the various plates. Some plates were estimated to be covered with up to 2000 colonies giving a figure of 20 000 bacteria per ml of homogenate. This figure is low compared to numbers of bacteria found in the ruminant gut (10^{11} per ml) or the termite hind gut but it may explain why we saw very little microscopically, especially considering the large amounts of tissue and other gut contents present. Bacteria may also be attached to the wall of the gut also making then more difficult to observe. In some microscopic examinations of colonies we observed very narrow long fibres of what we presumed to be crab tissue. (Fig. 7) Often the bacteria were attached to these fibres. Other plates had much lower numbers of colonies on them.

We were interested to compare the numbers of bacteria isolated from each region of the crab gut. The highest numbers were isolated from Parts I and II (Hepato-pancreas) and VI (anterior gut). The highest diversity was also isolated from parts I and II. Lowest numbers of bacteria were from Parts III (Anterior end of the Hepato-Pancreas) and IV (Stomach). Interestingly although there

was very little growth of bacteria from Part IV aerobically there was much higher numbers anaerobically. Unfortunately because we did not carry out dilutions, accurate counts of numbers present in each part could not be carried out.

Using a variety of phenotypic tests we tried to characterize the isolates. It was very diificult to group the organisms with the tests we used. We found that the majority of isolates were different from each other. Significantly we found the isolates from the tank and from the surface of the Hermit Crab shell were phenotypically very different from any strains isolated from the gut of the Hermit Crab. This could support the idea that the Hermit Crab has a symbiotic population of bacteria resident in the gut.

Using 16S rRNA sequencing, presumptive identitiesand relationships between bacteria isolated can be made more easily. However we found that not one single method of cell lysis was suitable for all our isolates. PCR products were obtained from only three of our isolates and only one of these isolates was successfully sequenced. There has been much debate recently about the advantages of genotypic versus phenotypic typing of organisms. It is agreed that the culture methods used to isolate bacteria introduce a large amount of bias into the study, selecting only for bacteria which can be cultured under a particular set of conditions. This study illustrates that the protocol used for PCR also introduces bias into the study selecting for only the bacteria which can be lysed using a particular protocol or whose DNA can be amplified using a particular primer. The 16S rRNA sequence was found to be identicle to that of *Vibrio fisherii* . This is a commonly isolated from coastal and open ocean sea water, from the surfaces of squid and fish, from luminous organs and from seafood. Many strains are bioluminescent and do not grow well at 37°C. However our isolate was not bioluminescent and it also grew at 37°C.

The Hermit Crab is a detrivore and scavenger so the diet would include protein, polysaccharide and other complex carbohydrates. We have shown that bacteria isolated from the Hermit Crab gut can

hydrolyse protein and that they grow on complex media. This could suggest that they play a role in the digestion of the Hermit Crab. Most isolates grew best between 15°C to 30°C and many required seawater media. These isolates are therefore well adapted to the marine environment. Before any conclusions can be drawn about whether any of these bacteria are indeed symbionts of the Hermit Crab or are merely ingested with the food of the Hermit Crab many further investigations need to be carried out. Further physiological studies of the bacteria need to be carried out to look for enzyme activities which might be of benefit to the crab for example cellulose and polysaccharide degradation. A more complete survey of the environment may reveal more similarities between bacteria isolated from the Hermit Crab gut and those in the environment.

Appendix 1
Media

1. **Seawater complete (SWC)**

different pH: 6, 7, 8
5 g Bactopeptone
3 ml Glycerol
15 g agar
300 ml freshwater
700 ml seawater

2. **Casamino acid seawater (CAA SW)**

20 g casamino acids
1 g $KHPO_4$
0.5 g yeast extract
18 g agar
1000 ml seawater

3. **Brain heart infusion seawater (BHI SW)**

37 g brain heart infusion
0,05 % Cystein
1 drop Heme (10 %)
18 g agar
1000 ml seawater

4. **Yeast extract seawater (YEG SW)**

10 g yeast extract
10 g glucose
15 g agar
300 ml freshwater
700 ml seawater

5.**Skim Milk Seawater**

40g skim milk powder
18g agar
750 ml sea water
250 ml distelled water

6 **Starch Media**

As BHI with addition of 2g soluble starch per litre.

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