A study of the diversity of microbiota in the accessory nidamental gland of squid *Loligo pealei*

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Abstract

One of the intriguing symbiotic topics in Microbiology is squid and their bacterial symbiont. Sepiolid squid *Euprymna scolopes* and its light organ bacterial symbiont *Vibrio fisheri* has been well described in the previous reports. However, the association of non-luminescent bacteria wit ı the accessory nidamental gland of loliginid squid *Loligo pealei* has not been described in details before. In this project, I used microscopy as well as 16S rRNA-directed probes with fluorescently tagged oligonucleotide and extraction of DNA directly from the homogenized gland to study the diversity of microbiota that present in this squid's accessory nidamental gland. Using microelectrodes, I am able to detect the oxygen content and pH of the interior of the gland. I also tried to cultivate different bacteria from the gland and characterized these pure culture. Overall, this report showed that unlike *Euprymna scolopes'* light organ, the bacteria residing in the accessory nidamental gland of *Loligo pealei* have a quite diverse characteristics.

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Introduction

An intriguing topic of microbiology recently is the association of bacteria with "higher" plant and animal life. this includes "pathogen-host" relationship as well as symbiosis between bacteria and their host. One example of the latter is a small sepiolid squid, *Euprymna scolopes*, and its light organ bacterial symbiont *Vibrio fischeri*. This luminous bacterium colonizes the light organ and makes *E. scolopes'* bioluminescent. The light organ maintains a pure culture as many as 10^8 *V. fischeri*.[1]

Another squid *Loligo pealei* also has bacterial symbiont. Unlike *Euprymna*, however, *Loligo pealei* does not have a light organ and luminous bacterial symbionts. *L. pealei* is abundant in the sea around Cape Cod and Cape Hatteras, along the East Coast of United States. These animals are typically restricted to continental shelves and coastal margins and do not migrate through deep water.[2] Although *L. pealei* does not have a light organ containing bacterial symbionts, the accessory nidamental glands of female squid are heavily colonized by bacteria.[3] Unlike the bacterial population in light organ of *E. scolopes*, the bacteria present in the accessory nidamental gland are of quite diverse characteristics. But detailed examination of the microbiota of this gland has not been reported.

Using microeletrodes, I will first determine the oxygen content and pH of the interior of the accessory nidamental gland. A rapid method for the identification of bacterial cells using 16 s rRNA- directed, fluorescently tagged oligonucleotide probes, will also be employed. Direct extraction of DNA from gland samples and characterization by comparative rRNA sequence analysis will also be used. Some isolations of pure cultures in different media such as sea water complete (SWC) medium, casamino acid-sea water (CAA-Sea) medium, yeast extract-glucose seawater (YEG-Sea) medium and VB2 are obtained during this study. With this information, we may begin to understand the diversity of the microbiota present in this squid's accessory nidamental glands.

Materials and Methods

Microscopy of accessory nidamental gland of *Loligo pealei*

Loligo pealei were obtained from Marine Resources Center, Marine Biological Laboratory, Woods Hole, Massachusetts. The accessory nidamental glands were removed from the squid. Each gland was homogenized and then observed under a phase contrast microscope. Examination of F_{420} autofluorescence, chlorophyll autofluorescence and green autofluorescence were also empolyed.

Emumeration and cultivation of bacteria of interior of the accessory nidamental gland

The total number of bacteria in accessory nidamental gland was estimated by direct microscopic count. Series of dilutions of homogenized gland were made and a proper dilution was counted using a Petroff-Hausser counting chamber.

Several different media were used to isolate bacteria from the gland. The media used included: Yeast extract glucose-sea water [YEG-sea, sea water, 70% (v/v); yeast extract, 1% (w/v) and glucose, 1% (w/v)], casamino acid-sea water medium [CAA-sea, sea water, 70% (v/v); casamino acid, 2% (w/v); K_2HPO_4, 0.1% (w/v) and yeast extract, 0.05% (w/v)], sea water complete medium [SWC, sea water, 70%(v/v); Bacto-peptone, 0.5% (w/v); yeast extract, 0.3% (w/v) and glycerol, 0.3% (v/v)]. Solid media were the same composition as above except 1.5% (w/v) agar was added in YEG-sea and CAA-sea media and 2% (w/v) agar was included in SWC medium. Another solid medium used was VB2 [NaCl , 1.3% (w/v); $MgCl_2$, 0.2% (w/v); KCl , 0.07% (w/v); nutrient agar, 2.3% (w/v); yeast extract, 0.5% (w/v); Difco agar, 0.5% (w/v) and 0.1% (v/v) of ferrous ammonium sulfate solution (3% w/v)]. The accessory nidamental gland was sterilized by dipping in ethanol before homogenization to prevent other bacterial contamination from non-gland's source. Homogenized gland after dilution was either spread or streaked on the media and incubated aerobically and anaerobically in a Gas Pak jar at room temperature. After

growth, a single isolated colony was picked and restreaked on appropriate medium, then incubated at room temperature aerobically and anaerobically. Pure cultures were obtained after several transfer and then been characterized. The tests for characterization included: Gram stain, morphology, transmission electron microscopy, motility, oxidase, catalase bioluminescense, nitrate reduction and growth in dufferent media and conditions.

Determination of oxygen content and pH of the interior of the accessory nidamental gland by microelectrodes

Oxygen content of the interior of the accessory nidamental gland was determined by a Clark microelectrodes with a guard cathod. pH inside the gland was measurde by a pH microelectrode contructed from pH-sensitive glass and lead glass.[4] The oxygen was measured using intact gland while measuring pH, the gland was cut open and the interior of the gland was measured due to the gragility of the pH microelectrode and its inability to penetrate the intact gland. As a control, other parts of the squid such as gills and nidamental gland were also tested for oxygen content.

Direct extraction DNA, amplified by polymerase chain reaction, cloning and sequencing rDNA and identity

Bacterial DNA was extracted directly from homogenized accessory nidamental gland using "DNA extraction from environmental samples" method (J. Döre, unpublished data) followed by "hot start" polymerase chain reaction procedure to amplify rDNA of the bacteria in the environmental sample (the gland). Purified PCR product (rDNA) was then subjected for ligation into a commercial vector pCR II^{R} and transformed into a compentent cells (*E. coli*) using a commercial kit, Original TA cloning ^{R} kit (invitrogen Co.) Due to time restriction, Sequencing and identity were not employed.

Testing for autoinducer produced by bacteria in accessory nidamental gland

Homogenized accessory nidamental glandothe pure cultures from the gland) was streaked in the center of SWC agar. *Vibrio fischeri* MJ211 (Δ*lux*I strain) was obtained from Dr. Paul Dunlap (WHOI, Woods Hole, MA).[5] This mutant can still luminesce if stimulated by exogenous autoinducer but can not produce its own autoinducer. MJ211 was streaked alongside a streaked line of homogenized gland (or pure culture isolated from the gland), either at the same time or after the growth of bacteria from gland occureed. (Fig A). The plates then were incubated at room temperature (23°C- 25°C). The appearance of luminescense was observed after *Vibrio fischeri* MJ211 grew. If the bacteria from the accessory nidamental gland produced autoinducer, MJ211 should exhibit luminescense.

In situ hybridization using 16S rRNA-directed, fluorescently tagged oligonucleotide probes

Pure cultures isolated from accessory nidamental gland were grown in SWC liquid media overnight and 1 ml of culture was centrifuged and washed with PBS (pH 7.4) twice and resuspend in 1 ml PBS. Cells were then diluted 1 : 100 with PBS and 15 ul were spotted onto subbed, baked, 10-well slides (Cell-Line Associates, Newfield, NJ). Environmental samples (homogenized gland) were also employed by the similar method. The cells were then fixed with ethanol/formaldehyde (90/10, v/v) and hybridized with16S rRNA-directed, fluorescently tagged oligonucleotide (Rhodamine labeled) probes overnight in appropriate temperature (37°C or 45°C). The probes used here included universal probe and probes detecting the following group of bacteria: High G+C bacteria; Low G+C bacteria; Proteobacteria: Alphas group; Betas group, Deltas group and Enteric (Gammas Group); Pseudomonades and Flavobacteria. The results were examined under a microscope with fluorescent filter. The detail pricedure was described in reference 6.

Results and Comments

Accessory nidamental glands are paired organs founded in female *Loligo*, have been assumed to play a secretory role associated with the reproductive system (Fig B), and their color can be used as a marker for sexual maturity of the squid.[2] *Loligo pealei* house symbiotic bacteria within their accessory nidamental gland has been reported but not in details.[3] The results here show that some characteristics of the bacteria residing in the gland.

Microscopy of accessory nidamental gland

The results of microscopy of the homogenized gland are shown in Fig C-1 (differential interference contrast microscope) and Fig C-2 (phase contrast microscope). The results shown a mixed population of bacteria residing in the gland with different shape (rod, short rod, cocci, etc.) and motility (both motile and nonmotile). All the results of autofluorescent examination were negative (no autofluorescence beed observed).

Emumeration and cultivation of bacteria of the interior of accessory nidamental gland

The total number of bacteria in the accessory nidamental gland is about 2.3×10^{11} bacteria/ml (the data is from the average of three different counts of one squid). The results of isolation of bacteria and some of their characteristics are summarized in Table 1. There were six isolates studied in this report: strains SQ1, SQ2, SQ3, SQ4, SQ5 and SQ6. The microscopy results are shown in Fig C-3 to Fig C-8. Results of transmission electron microscope of strains SQ, SQ2, SQ3, SQ4 and SQ5 are shown in Fig D-1 to Fig D-5.

Oxygen content and pH of the interior of the accessory nidamental gland

Oxygen content shown immediately drop to totally anoxic inside the gland of a fresh sacrificed squid. The control parts of the squid (e.g. gills and nidamental gland) also shown anoxic. However, squids are highly metabolic animals and after sacrificed their circulation and exchanging of oxygen stopped. Also the oxygen in the accessory nidamental gland could be depleted by those bacteria residing in the gland since all the isolates in this study could grow aerobically. pH of the interior of the gland was about neutral (pH about 6.8) determined by pH microelectrode.

Direct extraction DNA, PCR, cloning, sequencing rDNA and identity

Bacterial DNA extracted directly from accessory nidamental gland was successfully obtained. The DNA was amplified by "hot start" PCR procedure and purified and then inserted into a vector pCR II. Unfortunately, I did not get any transformed cells in the transformation step. I had repeated three times, two of them I did not get any cell grew and the last time I got a confluent growth. The confluent growth might due to the improper preparation of plates with kanamycin and so kanamycin did not select the transformed cells. From the confluent growth plates, I washed the cells off and then spread on another kanamycin-LB plate. I got some presumbly transformed colony from this plate. In order to confirm if these are transformed cells I will have to do the plasmid preparation. However, since time is limited here I was not allow to do any further experiments for this rDNA extraction and comparative.

Autoinducer production by bacteria in accessory nidamental gland

The results shown none of the isolates as well as homogenized gland produce autoinducer complement to that of MJ211. However, this experiment only used *V. fischeri* as reference bacteria. Since autoinducer is species-specific, I do not know if they produce autoinducer that complement to other luminescent bacteria such as *Vibrio harveyi* or *Photobacterium sp.*.

In situ hybridization with 16S r-RNA directed, fluorescently tagged oligonucleotide probes

All the samples reacted with universal probes but the environmental sample (homogenized gland) reacted much weaker compared with those of pure culture. Strains SQ1, SQ2 and SQ6 reacted with Enteric (Gamma) group probes. Since environmental sample reacted much weaker with universal probe, the reason that no Enteric probe reacted with environmental sample may due to the reaction was too weak to be detected. All the rest of the probes shown no reaction with any of the samples. (Table 1) However, the reaults of the control for the probes for low G+C bacteria, Alphas and Deltas group did not shown positive, either.

Summary: The results above shown a quite diverse characteristics of the microbiota residing in the accessory nidamental gland of *Loligo pealei*. From 16S rRNA probes and the characteristics of the isolates, I could conclude at least some of the bacteria are belong to the Family Vibrionaceae (e.g. SQ1, SQ2 and SQ6).

In this study, I did not try to isolate any of the strict anaerobic bacteria from the gland. Since the interior of the gland of a fresh sacrificed squid shown totally anoxic, it would be worthwhile try to isolate some anaerobic bacteria as well as facultative ones from the gland in the future.

The direct extraction of DNA from environment and comparative rDNA sequences will help us to understand the diversity of the microbiota in the gland and can compared to those of isolates' rDNA sequences to see if there are any agreements. Since the restriction of the time here, I did not have chance to complete these experiments.

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Table 1: Some characterics of the isolates from squid accessory nidamental gland

isolate	SQ1	SQ2	SQ3	SQ4	SQ5	SQ6
initial isolate media and condition	aerobic, CAA-sea	aerobic, SWC	aerobic, SWC	aerobic, YEG-sea	aerobic, YEG-sea	anaerobic, CAA-sea
morphology	rod(curve) cocci	rod-cocci	rod	rod-cocci	big rod	big rod
flagellated (motility)	yes	yes	no	no	no	yes
Gram stain	negative	negative	negative	negative	negative	negative
oxidase	positive	positive	positive	positive	positive	positive
catalase	positive	positive	positive	positive	positive	positive
media that can grown with aerobically	CAA-sea, YEG-sea, VB2, SWC	CAA-sea, YEG-sea, VB2, SWC	YEG-sea, VB2, SWC	YEG-sea, VB2, SWC	YEG-sea, VB2, SWC	CAA-sea, YEG-sea, VB2, SWC
growth at 37 C	no	no	no	no	no	no
reacted with 16S rRNA probes	universal, Enteric probes	universal, Enteric probes	universal probe only	universal probe only	universal probe only	universal, Enteric probes
growth** in YEG-sea anaerobicall y	+/-	-	-	-	-	+/-
growth** in SWC anaerobicall y	+	+/-	-	+/-	-	+
growth **in SWC + 1% nitrate anaerobically	+	+/-	+/-	+/-	+/-	+

* All of the isolates do not grow well (or not grow at all) on YEG and CAA without sea water base.
** When growth occured, the pH of the media dropped to about pH 5.
*** All strains shown only background growth on VB2 plates anaerobically.
Figure A: Streaking for testing autoinducer

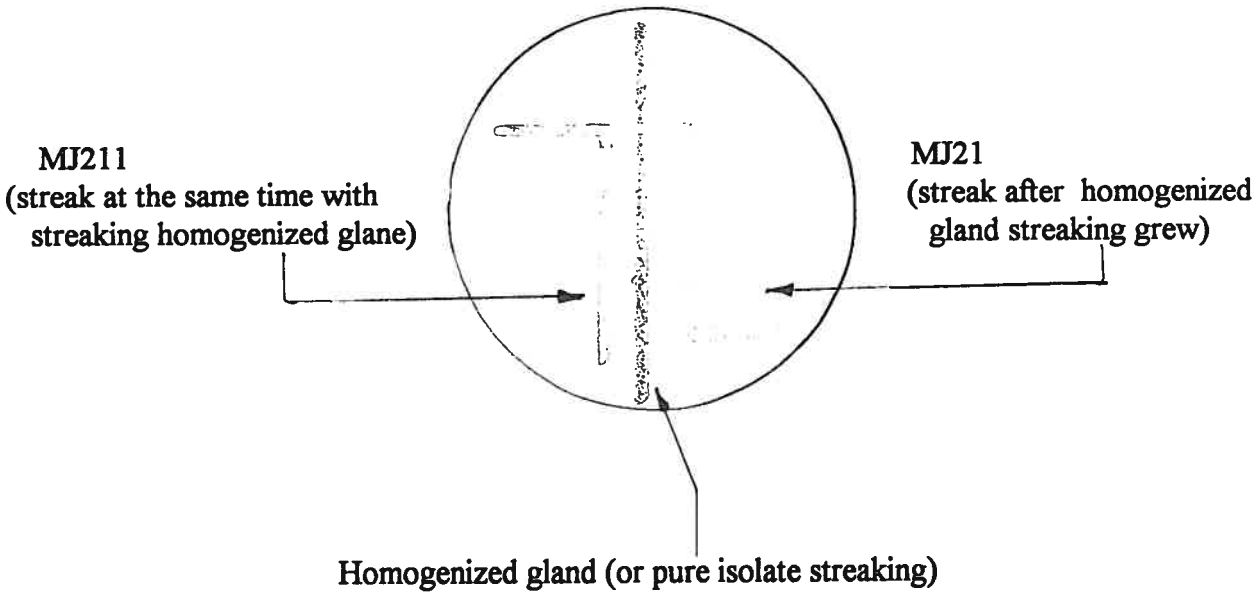

Figure B: The squid *Loligo pealei* and accessory nidamental gland

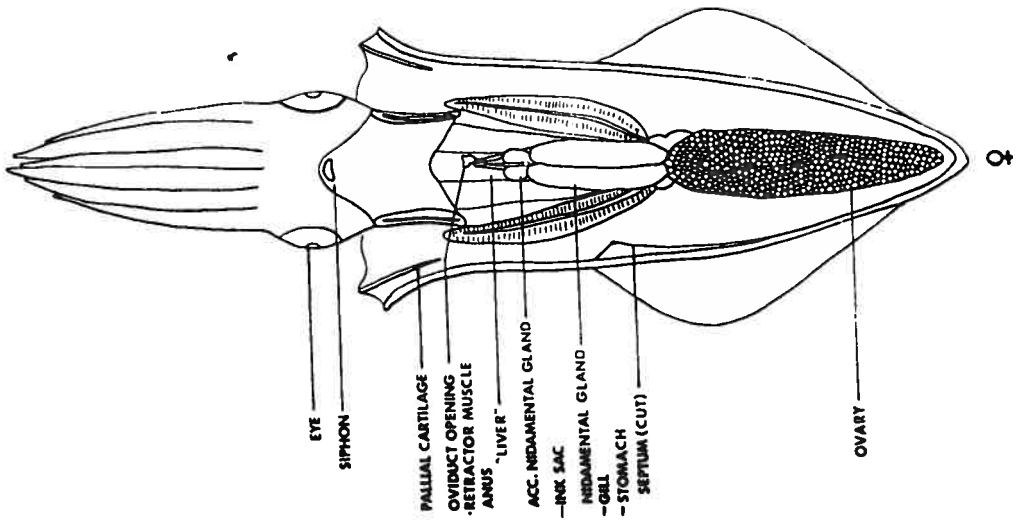

Figure legends:

Fig C:

Fig C-1: homogenized accessory nidamental gland under DIC microscope

Fig C-2: homogenized accessory nidamental gland under phase contrast microscope

Fig C-3 to Fig C-8: Strains SQ1, SQ2, SQ3, SQ4, SQ5 and SQ6 under phase contrast microscope, respectively. Strain SQ1, SQ2 and SQ6 shown highly motility while others did not.

Fig D: photograph of bacteria isolated from accessory nidamental gland under transmission electron microscope using negative staining

Fig D-1 and Fig D-2: Strain SQ1, Shown vibrio shap with flagella

Fig D-3: Strain SQ2, rod shape

Fig D-4: Strain SQ3, without flagella

Fig D-5: Strain SQ4, short rod without flagella

Fig D-6: Strain SQ5, the long rod in the center of the picture might be a contamination of the preparation.

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Figure C:Figure C-1 to Figure C-4

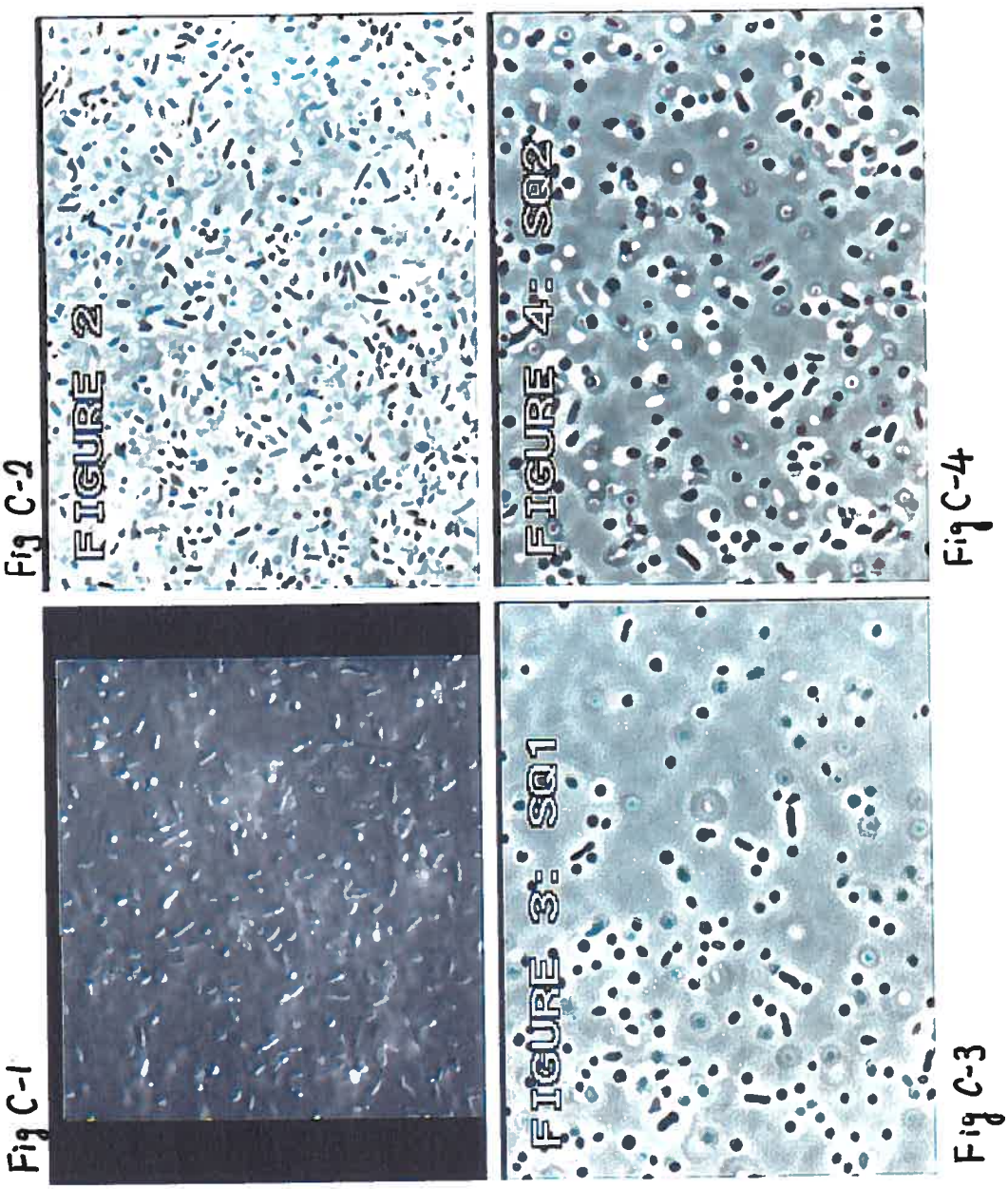

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Figure C: Figure C-5 to Figure C-8

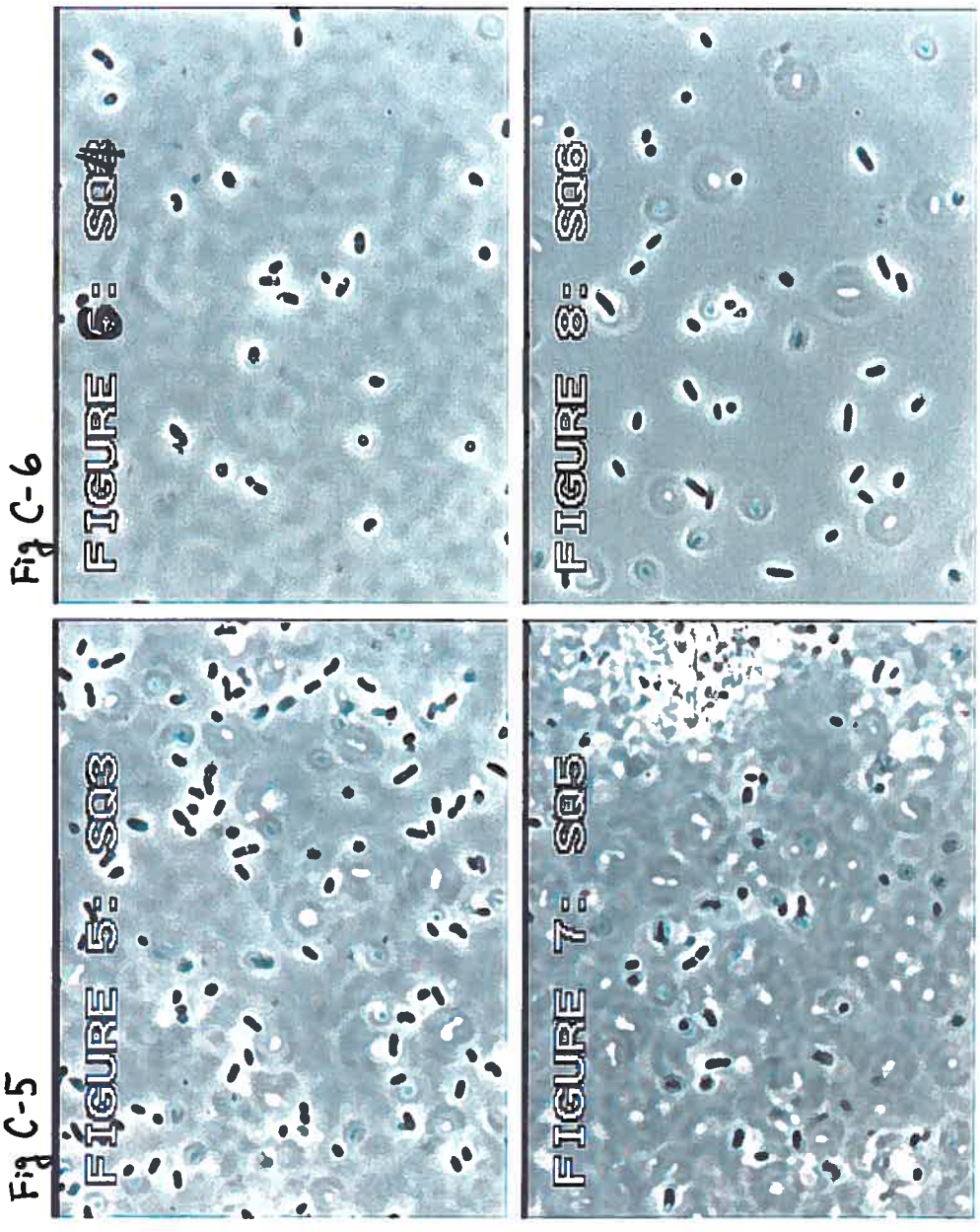

Fig C-5 Fig C-6 Fig C-7 Fig C-8

Figure D: Figure D-1 to Figure D-3

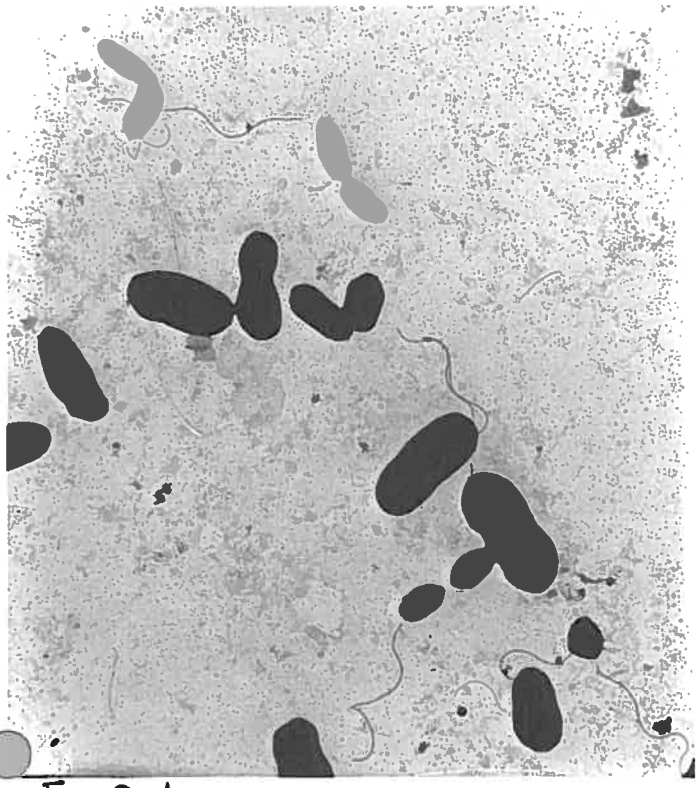

Fig D-1

Fig D-1

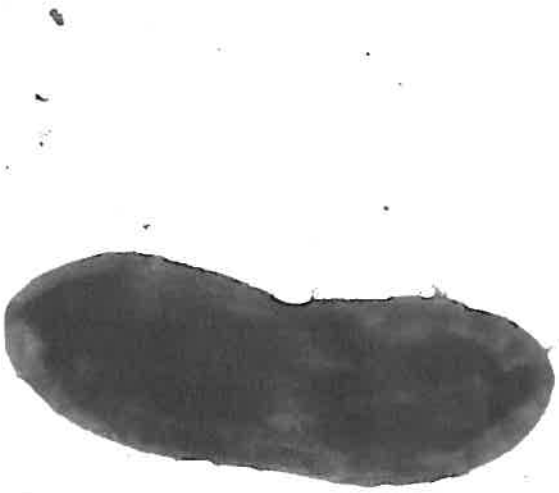

Fig D-2

Fig D-2

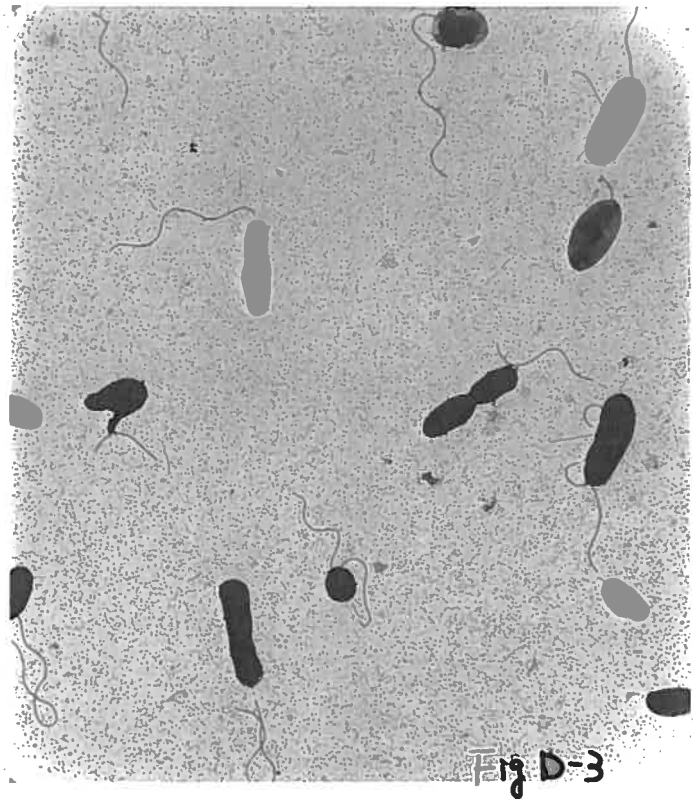

Fig D-3

Fig D-3

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Figure D: Figure D-4 to Figure D-6

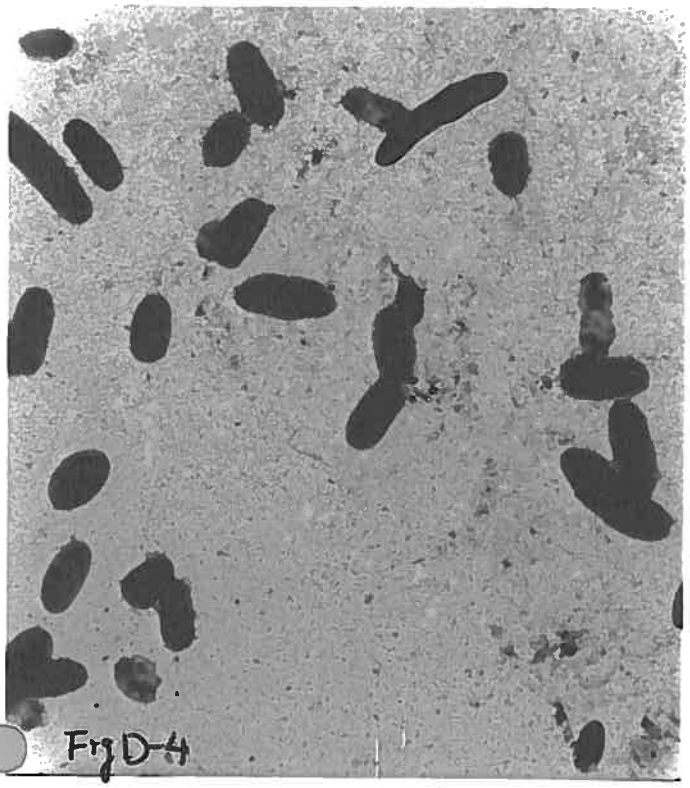

Fig D-4

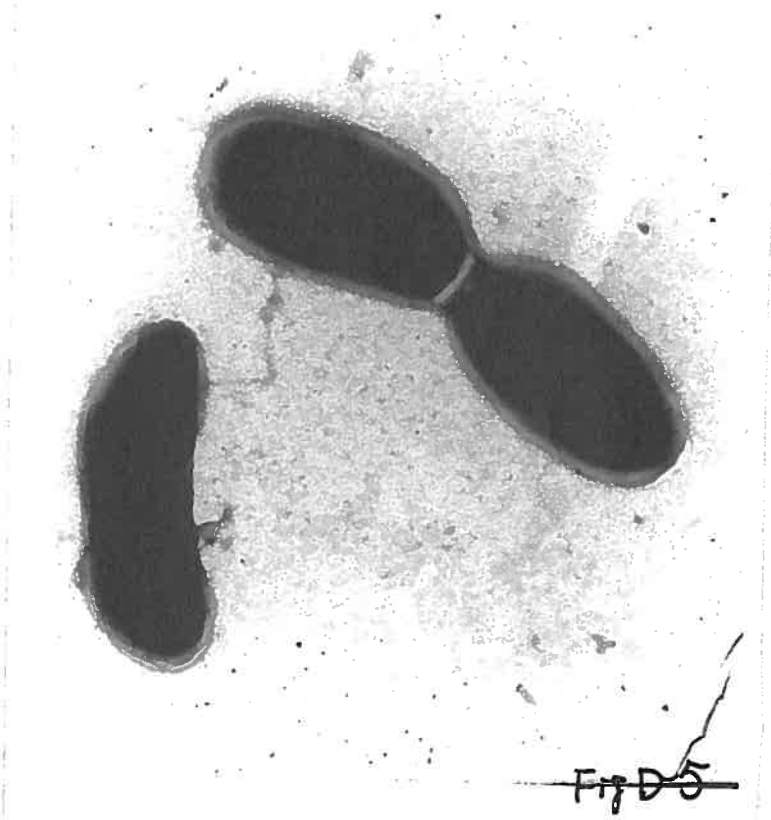

Fig D-5

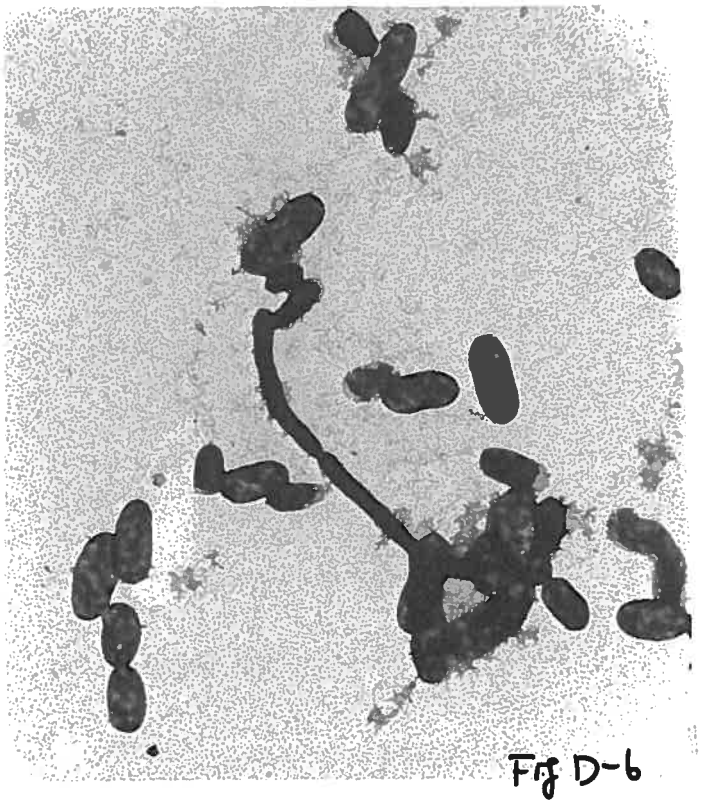

Fig D-6

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