

The Accessory Nidamental Glands of *Loligo pealei*: Diversity of Symbiotic Bacteria

Elena Barbieri¹ and Jay Gulledge²

¹Istituto di Scienze Tossicologiche, Igienistiche e Ambientali, University of Urbino—Italy
and ²Institute of Arctic Biology, University of Alaska Fairbanks.

INTRODUCTION

It is becoming obvious that bacterial symbionts play an important role in the physiology and development of many marine animals, but relatively little work has been done so far in this area. Accessory nidamental glands (ANG) present in sexually-mature female cephalopods harbor a dense bacterial community (Getzel, 1934). It has been shown that there is a cycle in the epithelial cells and in the presence of bacteria in the tubules of the gland (Bloodgood, 1977). The glands reflect the maturity state of the host, as they change from colorless in immature animals to red-orange in reproductively mature animals (Costa & Fernandez 1993). The color has been attributed to the bacteria, but no role for the coloration in the biology of squid has been determined (Richard et al. 1979). Only a few bacteria have been isolated from the gland and these have been characterized only very superficially. Traits used for characterization included: morphology (Getzel, 1934), pigmentation (Van Den Branden, 1980; Dunlap, 1990), and rudimentary biochemical characteristics (Lum-Kong & Hastings 1992). The identities of these organisms remain unresolved. A recent attempt to examine the genetic composition of the microbial community by molecular techniques revealed a rRNA sequence that suggested the presence of a phototrophic nonsulfur bacterium in the ANG (C. C. Chien, pers. comm.).

Our objective was to describe in greater detail the bacterial community of the ANG in squid (*Loligo pealei*), and to better understand the environment in which this community develops. Results of this study should lead to hypotheses regarding the symbiotic role(s) of the bacterial community in the accessory nidamental glands of cephalopods.

MATERIALS AND METHODS

We have taken a holistic approach to exploring the bacterial community of the ANG, and the environment in which this community occurs by combining traditional enrichment and isolation techniques with modern molecular and analytical techniques. Media and environmental conditions not examined in previous studies, including anaerobic conditions and those intended to select for nonsulfur phototrophic bacteria, have been employed. PCR amplification of 16S rDNA from representative isolates, as well as homogenized samples of the whole accessory nidamental glands, and subsequent sequencing of selected DNA fragments was used to characterize the composition and phylogenetic diversity of the bacterial community. No such analysis has been performed previously. In situ hybridization of specific DNA probes was used to directly detect the presence of specific bacterial groups in the gland. The O₂, CH₄, and pH of the intact gland were investigated using microelectrode probes. Additionally, the absorption spectrum of the ANG pigment was compared to pigments extracted from individual isolates exhibiting a range of coloration.

Culture media and isolations. Live, mature female ($n = 4$) *Loligo pealei* were obtained from Marine Resources Centre, Marine Biological Laboratory, Woods Hole, Massachusetts. Each squid was decapitated and a longitudinal mid-ventral incision made along the mantle to reveal the ANGs which were removed under aseptic conditions and dissected free according to Lum-Kong and Hastings (1992), and washed in filtered, autoclaved seawater. Using a dissecting microscope it was possible to distinguish between different-colored (white, yellow, and orange-red) areas of the gland. Sterile capillary pipettes were used to collect fluid from the different-colored areas. This fluid was inoculated into several media that were incubated under a variety of conditions, including aerobic, anaerobic and microaerophilic; and under full-spectrum light and in darkness. Both selective and non-selective media were chosen for phototrophic, pigment-producing, luminescent, and other marine bacteria. All cultures were incubated at 15°C, except the specific enrichments used to select presumptive *Vibrio* spp., which were incubated at 37°C

for 7 and 18 h as suggested by Buonadonna (1995), and the phototrophic enrichments, which were incubated at room temperature.

Two enrichment media were used: 1) seawater complete supplemented with L-aspartate: sea water 75% (w/v), bacto-peptone 0.5% (w/v), glycerol 0.3% v/v; L-aspartate 20 mM; 2) purple nonsulfur phototroph medium as described by Gauden and Armitage (1995). Five agar-solidified media were used for direct isolations: 1) seawater-complete supplemented with L-aspartate, which is commonly found in the nervous and reproductive systems of cephalopods (D'Aniello et al. 1995): seawater 75% (v/v), Bacto-peptone 0.5% (w/v), Glycerol 0.3% (w/v) L-aspartate 20 mM, agar 1.5%(w/v); 2) seawater-minimal, seawater, agar 1.5%(w/v); 3) squid medium: whole squid finely chopped and boiled for 1 h, agar 1.5%(w/v); 4) Sulfur Reduction Agar (SRA; Moser and Nealson, 1996); 5) LB agar supplemented with milk. One isolate was grown on a S-containing agar medium described by Moser and Nealson (1996).

Whole ANGs were homogenized in 2 ml sterile seawater and 100 μ l homogenate were inoculated into liquid enrichment media in 20 ml screw-cap culture tubes. Enrichment cultures were inoculated by serial dilution so that inocula were diluted to 10^{-2} , 10^{-4} , 10^{-6} , 10^{-8} , and 10^{-10} . Fresh homogenate was also streaked onto the solid media for direct isolations.

The total number of bacteria in the ANGs was estimated by direct microscopic count. Series of homogenized glands were made and appropriate dilutions (10^{-3} , 10^{-4} , 10^{-5} , 10^{-6}) were counted using the DNA-specific fluorochrome DAPI and epifluorescence microscopy (Porter and Feig 1982).

Phenotypic characterization of isolates. Phenotypic analyses included: Gram stain, colonial and cellular morphology, motility, oxidase, catalase, pigment absorption spectra, and F_{420} , bacteriochlorophyll *a*, and phycobilin autofluorescence analysis. Pigments were extracted by sonication of cell suspensions and extraction of hydrophilic and hydrophobic pigments in water and a 7:2 acetone: methanol mixture, respectively. Extracts were centrifuged to remove particulate material and absorption spectra were obtained using a

Shimadzu model UV-3101PC continuously-scanning spectrophotometer. F_{420} autofluorescence for methanogens, and autofluorescence for bacteriochlorophyll *a* and phycobilins were examined microscopically.

Flagellar staining was performed using the Flagella Stain kit from Carr-Scarborough (Decatur, GA) which contains 0.6% crystal violet, 2% tannic acid, 2.5% phenol, and 5.7% aluminum potassium sulfate. The protocol was modified as follows: 3 μ l of liquid (with or without cells) was placed on a microscope slide. If no cells were present in the original liquid, a colony was picked and gently resuspended in the liquid. A cover slip was placed over the sample and 3 μ l of flagella stain was placed next to the coverslip and allowed to move under the glass by capillary action. The cells were stained for 5 minutes and then viewed at 2000X magnification.

Molecular characterization of isolates and ANG bacterial community. Bacterial DNA was extracted directly from homogenized ANGs using DNA extraction from environmental samples followed by amplification of 16S rDNA by PCR. Purified PCR product (rDNA) was then inserted into a commercial plasmid vector (pCNTR) and transformed into competent cells of *Escherichia coli* using a commercial Kit (5,3Prime, Inc. CO-USA). All molecular procedures followed Paster et al. (1996). DNA was extracted from ANG samples using Gene Releaser using the protocol provided by the manufacturer. Successful extraction of DNA from individual isolates required the thermocycle protocol, also provided by the manufacturer. A universal prokaryote primer was used to amplify rDNA genes for all samples and the PCR products were checked by electrophoresis in agarose 1% gel. PCR products from the ANG samples were separated by cloning. Purified Wizard PCR prep (Promega) or cloned DNA amplify using the Sequi Therm Long Reader Cycle sequencing Kit for Li-cor fluorescent sequencing (Epicenter Technologies). Between 100 and 400 ng of template DNA was used for sequencing using the 536 reverse 16S RNA primer. The 16S rRNA gene sequences of the clones and the rDNA of the isolates were compared with sequences from known organisms by electronic retrieval from the EMBL, Gen Bank sequences used for phylogenetic analyses (Paster et al. 1996).

In situ hybridization of group-specific gene probes was performed as described by Nierzwicki-Bauer (1996). An overnight culture of isolate 219 (Table 1) was grown in SWC liquid medium. The native bacterial community of one ANG was suspended in 1 ml sterile seawater. Each suspension was diluted to approximately 10^5 cells ml^{-1} to yield a final microscopic field count of 30–300 cells. The probes are listed in Table 2.

Chemical environment of the ANG. Chemical gradients were measured within the ANG using microelectrode probes for O_2 , CH_4 , and pH, using the protocols of L. Damgaard (Pers. Comm.). In order to maintain the flow of O_2 to living tissues, live, anesthetized squid were examined. A continuously-flowing bath was constructed to deliver O_2 and anaesthetic (1.5% ethanol in seawater) to the gills. The source water was continuously sparged with air to maintain the O_2 level. Once the squid was anesthetized, a longitudinal, mid-ventral incision was made along the mantle to reveal the ANGs and the squid was secured using dissecting needles. The flow of aerated seawater was immediately directed to the gills. It was assumed that normal delivery of oxygenated blood was occurring as long as the branchial arteries remained blue in color (indicative of oxygenated blood) and regular contractions of the branchial hearts could be observed.

RESULTS

The ANG bacterial community. Microscopic examination of the intact ANG revealed a patchwork mosaic of distinct white, yellow, and orange-red areas on the gland surface. Fluid samples collected using a capillary tube was of the same color as the gland area from which it was obtained. Microscopic observation of the different-colored fluids revealed phenotypically distinct bacteria. The predominant organisms in the white fluid were thin, nonmotile rods, whereas the predominant phenotype in the the orange-red fluid was motile rods arranged in pairs end-to-end. The white fluid also contained cocci that were not observed in the orange-red fluid. Obtaining fluid from the yellow area was difficult and the phenotypes from this fluid appeared to be a mixture of those from the white and orange-red areas. The variety of morphologies present in fresh ANG homogenate are

displayed in Fig. 6. No autofluorescence was observed for F_{420} , bacteriochlorophyll *a*, or phycobilins. Direct bacterial counts yielded an estimate of 1.40×10^9 cells/gland. The gland volume was not determined.

Seven rDNA sequences obtained by PCR and cloning from a freshly homogenized ANG were sequenced and analyzed phylogenetically (Fig. 1). Five of the clones were identical and were most closely related to the genus *Roseobacter*, with the nearest known relative being *Roseobacter denitrificans* (400–450 bp; 93% similar). The other two clones were identical to one another and were most closely related to the purple nonsulfur phototrophic bacterium *Rhodovulum euryhalinum* (88% similar). These two clones were also identical to one obtained in a previous study (clone 1995, Fig. 1; C. C. Chien, pers. comm.).

In situ hybridization indicated that the community contained a wide variety of bacterial groups, including alpha and delta proteobacteria, SO_4^- reducers, flavobacteria, and two groups of archaea (Table 2; Fig. 2). Although the probe for gamma proteobacteria yielded negative results, the probe was believed to be defective.

Bacterial Isolates. Several bacterial strains were obtained from the various enrichment cultures and by direct isolation on solid media (Table 1). The largest number of isolates was obtained by direct isolation on seawater complete agar plates under aerobic conditions. Although several isolates were obtained under anoxic conditions, none were obligate anaerobes. Several of the isolates in Table 1 are suspected to be redundant.

Three of the isolates were putatively identified by rRNA sequence analysis. A 1350 bp rDNA sequence placed isolate 203 (Table 1) within the genus *Alteromonas*, its nearest known relative being *Alteromonas citrea* (96% similarity; Fig. 3). This isolate was pink-pigmented, grew both aerobically and anaerobically, and grew on a variety of media, including marine minimal agar. The flagellar stain revealed a single polar flagellum (Fig. 4), which is characteristic of *Alteromonas* (Gautier & Breittmayer 1992). Isolate 222 yielded a 450 bp DNA sequence identical to *Aeromonas allosaccharophilus* (Fig. 5). This isolate also grew on a variety of media under aerobic conditions and was apparently

capable of reducing S during anaerobic growth (Moser 1996). When grown on S medium, this isolate exhibited filamentous growth, forming chains of rods joined end-to-end. A similar morphology was also observed for an unidentified organism in fresh ANG homogenate (Fig. 6a). Finally, isolate 218 was identified as a member of the genus *Vibrio* (Fig. 7).

A yellow-pigmented isolate (219, Table 1) was examined by in situ hybridization of group-specific gene probes. Results were positive for both the alpha proteobacteria and the sulfate reducer probes (Table 2, Fig. 8).

ANG and bacterial isolate pigments. The ANGs were predominantly orange-red in color. The pigment was easily extracted in a 7:2 mixture of acetone and methanol. After repeated extraction, the ANG tissue appeared nearly white, indicating that most of the pigment was extracted. Spectral analysis indicated that the pigment was predominantly carotenoid in nature, absorbing light at wavelengths between 400 and 600 nm (Fig. 9).

In contrast to the gland pigment, extraction of pigments from individual isolates proved difficult. Yellow pigments were extracted from three isolates (214, 215, 219). The absorption spectra, which did not resemble that of the ANG pigment, were similar for all three isolates (Fig. 9). Pigments from two pink-pigmented isolates (203, 204) were not successfully extracted. As a result, the absorption spectra of mid-log-phase cell suspensions (10^5 – 10^6 ml⁻¹) of these isolates were examined, but the suspensions were too turbid to allow detection of absorption due to pigments.

Chemical gradients in the ANG. The steady-state O₂ concentration in the ANG was very low throughout (Fig. 10), pH was very close to neutral throughout, and no CH₄ was detected. Measurements in other tissues yielded similar results. Note that although we attempted to ensure normal blood flow during the measurement, it was impossible to be certain of this condition.

DISCUSSION

Using traditional culture techniques, we isolated a range of organisms from the accessory nidamental gland of *Loligo pealei* that was similar to prior studies (Getzel 1934; Van den Branden et al. 1980; Lum-Kong and Hastings 1992). Through molecular approaches, however, we have detected a much more diverse range of bacteria within the ANG than previously reported. Van den Branden et al. (1980) reported 6 unidentified, pigmented isolates from the ANG of the cuttlefish *Sepia officinalis*. Twelve isolates from the ANG of *Loligo forbesi* were only tentatively identified as members of the genera *Vibrio* and *Pseudomonas* (Lum-Kong & Hastings 1992). The 19 isolates listed in Table 1 probably represent between 8 and 12 distinct strains based on rudimentary phenotypic characteristics. Most of these isolates closely resemble isolates described in previous studies, despite the fact that we employed a greater range of culture conditions than used previously. These results suggest that the range of bacteria from the ANG that are culturable using traditional culture techniques is limited.

One of the bacterial isolates (203; Table 1) was identified by rRNA sequence analysis as a member of the genus *Alteromonas*. This strain was isolated from each ANG examined, and grew in a wide variety of conditions. Microscopic examination of ANG homogenate revealed a large number of cells morphologically similar to this isolate. Together, these observations suggest that the *Alteromonas* strain is a prevalent member of the ANG community. Fisher (1983) implicated *Alteromonas* species in the protection of shrimp (*Palaemon macrodactylus*) eggs from fungal infection. Because the ANG is a reproductive organ and is located immediately adjacent to the egg-producing nidamental glands, the antifungal activity of *Alteromonas* may suggest a mutualistic role for ANG bacteria in the cephalopod life cycle.

Another isolate (222; Table 1) was identified by rRNA sequence analysis as *Aeromonas allosaccharophila*. This organism reduced S when grown anaerobically, and grew as single rods when grown aerobically on SWC agar, but was filamentous when grown anaerobically with S (Moser 1996). The presence of this organism in the ANG was in agreement with results of in situ hybridization of the ANG bacterial community with

the SRB probe (Table 2), which is diagnostic of SO_4^- reducers, many of which can also reduce S. Whether or not this *Aeromonas* species reduces SO_4^- was not determined, but its ability to do so would provide a basis for its existence in the marine environment because seawater is SO_4^- -rich. Neither SO_4^- nor S concentrations were examined in the ANG. S reducing bacteria have not been observed in the ANG previously and were not expected. *Aeromonas* species, including *A. allosaccharophila*, are often found as pathogens in marine animals, suggesting that this organism could simply be parasitic (Austin & Gibb 1993).

Evidence was found for the presence of second unexpected bacterial group in the ANG, the anoxygenic nonsulfur phototrophic bacteria. Multiple rRNA sequences matching the genus *Roseobacter* were extracted from the ANG bacterial community. The nearest known relative was *Roseobacter denitrificans*. This organism is unusual among phototrophic bacteria in that it lives phototrophically only in the presence of O_2 or NO_3^- . Additionally, it apparently synthesizes chlorophyll in the dark, suggesting that it requires periodic light-dark cycles in order to sustain a phototrophic lifestyle (Shiba 1992). Other phototroph-like rRNA sequences were obtained from the ANG bacterial community, including sequences similar to *Rhodovulum euryhalinum* and a *Rhodobacter*-like sequence. A sequence 96% similar to *Rhodobacter sphaeroides* was obtained in a previous study (C. C. Chien, pers. comm.). This genus belongs to the alpha proteobacteria, a probe for which produced positive results when exposed to the bacterial community of the ANG.

Despite obtaining multiple phototroph-like rDNA sequences from the ANG bacterial community, no chlorophylls were apparent in the absorption spectrum of the ANG pigment. Whether the ANG actually harbors phototrophic bacteria requires confirmation through further investigation, including cultivation of such organisms from ANG inocula. The molecular data provide strong evidence, however, that organisms closely related to phototrophs exist in the ANG, and raise the possibility of a symbiotic relationship between phototrophic bacteria and cephalopods.

Results of in situ hybridization experiments with a variety of group-specific gene probes suggested that the bacterial community within the ANG is very diverse. In addition to those organisms discussed above, the ANG appeared to contain archaea, delta

proteobacteria, and flavobacteria, none of which have been reported in other studies of the ANG bacterial community. A complete knowledge of the composition of this community will require much further investigation using both molecular and traditional microbiological techniques.

The chemical environment of the ANG was difficult to analyze. We attempted to keep the ANG intact on a live animal during the microelectrode analyses to ensure uninterrupted supply of oxygenated blood to the body tissues. Although the blood in the gill arteries remained oxygenated and the branchial hearts continued to contract during the analyses, we have no way of confirming that unrestricted blood flow was provided to all tissues. Hence, it is possible that the anoxia of the ANG and other tissues was an artifact of the procedure. The ability of most of the isolates to grow anaerobically suggests that anaerobiosis is feasible within the gland. Conversely, the lack of obligate anaerobes suggests that conditions within the gland are not persistently anoxic. In contrast to O_2 concentration, the CH_4 concentration within the ANG probably would be enhanced in the event that oxygen delivery was interrupted, yet no CH_4 was found. This result corroborates the lack of F_{420} fluorescence observed in fresh ANG homogenate.

As in previous studies, we were unable to isolate bacteria that expressed pigments that could account for the orange-red color of the mature ANG. A more comprehensive survey of rRNA from the ANG bacterial community might be helpful in detecting bacterial taxa capable of producing such pigments, and thus assist in future isolation attempts.

CONCLUSIONS

By combining traditional culture techniques with modern molecular approaches, we were able to find evidence of a much more diverse bacterial community within the accessory nidamental gland of *Loligo pealei* than had been observed previously. It is important to stress that the molecular data presented here are not conclusive, but rather provide sound hypotheses for further investigation. It is also important to realize that traditional microbiological and physiological techniques are essential to develop a

reasonable understanding of the composition of the ANG bacterial community and its role in the biology of cephalopods.

A symbiotic role for bacteria in the ANG has not been determined, but there is some indication that protection of eggs from fungal attack may be involved. Unlike previous studies that have found mostly vibrios and pseudomonads using traditional culture techniques, our molecular approach suggests the presence of several unexpected bacterial types in the ANG of *Loligo*. Particularly novel is the possibility that phototrophic bacteria may have established a symbiosis with cephalopods. Further work will focus on culturing, identifying, and characterizing the organisms responsible for the DNA sequences that we obtained from the bacterial community in the ANG of *Loligo pealei*, and also further investigation of other unidentified members of the community.

ACKNOWLEDGEMENTS

We wish to thank all members of the MBL Microbial Diversity staff and faculty. In particular we thank the course directors, E. Leadbetter and A. Salyers for logistical support and valuable discussions on the project, B. Paster for assistance with molecular analyses, J. Armitage for discussions on phototrophic bacteria, P. Dunlap for discussions on ANG research, L. Damgaard for assistance with microelectrode analyses, T. Pitta for assistance with microscopy, D. Graham for photography, D. Moser for discussion and collaboration regarding S-reducing bacteria, C.-C. Chien for discussions on previous research, and R. Bulliss and P. Turk for assistance with squid handling. We are also grateful to our 1996 Microbial Diversity Course classmates for rewarding scientific and social interactions.

REFERENCES

- Austin B. and Gibb A. (1993) Emerging bacterial fish pathogens and their significance for aquiculture. Proc. First Intl. Symp. on Aquaculture Technol. Investment Opportunities, Riyadh Saudi Arabia, 11-14 April, pp. 410-425.
- Bloodgood A.R. (1977) The squid accessory nidamental gland: ultrastructure and association with bacteria. *Tissue & Cell*. 9(2): 197-208.
- Buonadonna L., (1995) Giornata studio *Vibrio cholerae*. Istituto Superiore di Sanita', 24 May Rome, Italy,
- Costa P.A.S., Fernandes F.C. (1993) Reproductive cycle of *Loligo sanpaulensis* (Cephalopoda: Lolignidae): in the Cabo frio region, Brazil, *Mar. Ecol. Prog. Ser.* 101: 91-97.
- D'Aniello A., Di Cosimo A., Di Cristo C., and Fisher G. (1995) D-Aspartate in the Male and Female Reproductive System of *Octopus vulgaris* Lam, *Gen. Comp. Endoc.* 100: 69-72.
- Dunlap P. (1990) Cultivation of symbiotic pigment-producing bacteria from the accessory nidamental glands of the squid *Loligo pealei*. *Amer. Malac. Soc. Abst.*
- Fisher W.S. (1983) Eggs of *Palaemon macrodactylus*: III. Infection by the fungus, *Lagenidium callinectes*. *Biol. Bull.* 164:214-226.
- Gauden D. E. And Armitage J. P. (1995) Electron transport-dependent taxis in *Rhodobacter sphaeroides*, *J. Bacteriol.* 177: 5853-5859.

- Gauthier, M.J. and Breittmayer, V.A. (1992). The General *Alteromonas* and *Marinomonas*. In: (A. Balows, et al., Eds.) *The Prokaryotes*, 2nd ed., pp. 3046-3070.
- Getzel D.(1934) I microbi della ghiandola nidamentale accessoria in *Sepia officinalis*, *Arch.Zool. Ital.* 20: 33-43.
- Lum-Kong A. and Hasting T. S. (1992) The accessory nidamental glands of *Loligo forbesi* (Cephalopoda: Lolignidae): characterisation of symbiotic bacteria and preliminary experiments to investigate factor controlling sexual maturation, *J. Zool. Lond.* 228: 395-403.
- Moser, D.P. 1996 MBL Microbial Diversity Course Report.
- Moser D.P. and Neelson K.H. (1996) Growth of the facultative anaerobe *Shewanella putrefaciens* by elemental sulfur reduction. *Appl. Env. Microb.* 62: 2100-2105.
- Niezwicki-Bauer, S.A. (1996). Nucleotide probes workshop, 1996 MBL Microbial Diversity Coures Exercise.
- Paster, B.J., Dewhirst, F.E, Cooke, S.M., Fusing, V., Poulsen, L.K. and Breznak, J.A. (1996) Phylogeny of not-yet-cultured spirochetes from termite guts. *Appl. Environ. Microbiol.* 62:347-352.
- Porter, K. G., Feig, Y. S. (1980). The use of DAPI for identifying and counting aquatic microflora. *Limnol. Oceanogr.* 25: 943-948.
- Richard, A., Van Den Branden, C. and Declair, W. (1979) The cycle of activity in the accessory nidamental glands from cephalopods. In: *Cyclic Phenomena in Marine Plants and Animals*, pp. 173-180. Pergamon Press, Oxford.

Shiba, T. The Genus *Roseobacter*. In: (A. Balows, et al., Eds.) *The Prokaryotes*, 2nd ed., pp. 2156-2159.

Van Den Branden C. (1980) Carotenoid producing bacteria in the accessory nidamental glands of *Sepia officinalis* L., *Comp. Biochem. Physiol.* 66B: 331-334.

Table 1. Phenotypic Characteristics of Bacterial Isolates from the Accessory Nidamental Gland of *Loligo Pealei*.

isolate	oxygen metabolism			colony characteristics		motile	cells	oxidase/	
	20%	5%	0%	color	shape			catalase	oxidase/
201	+	+	-	white	round convex	+	fat rod		+/+
202	+	+	+	white	round convex	+	rod		+/-
203	+	+	+	pink	round convex	+	rod		+/+
204	+	+	+	pink	round convex	+	rod		+/+
205	+	+	-	white	irregular	+	thin rod		+/+
206	+	-	-	white	microcolony	-	thin rod		+/+
207	+	+	+	beige	oval convex	+	thin rod		(-)?/(-)?
208	+	+	+	white	spreading	-	thin rod		+/-
209	+	+	-	beige	spreading	+	thin rod		+/+
210	+	+	+	white	spreading	-	thin rod		+/+
214	+	+	-	beige	oval convex	+	rod		+/-
215	+	+	+	beige	irregular	+	fat rod		-/-
216	+	+	-	white	microcolony	-	large coccus		-/+
217	+	+	-	white	microcolony	-	large coccus		-/+
218	+	+	+	white	round convex	+	fat rod		+/-
219	+	+	-	white	microcolony	-	large coccus		-/+
220	+	-	-	white	microcolony	-	large coccus		-/+
221	+	+	+	white	microcolony	-	large coccus		-/+
222	+	nd	+	beige	oval convex		rod		+/-

Table 2. In Situ Hybridization of Group-Specific RNA Probes

probe	isolate 219	ANG community
alpha proteobacteria	+	+
beta proteobacteria	-	-
gamma proteobacteria*	-	-
delta proteobacteria	-	+
archaea 291	-	+
archaea 635	-	+
SRB	+	+
Low G+C	-	-
High G+C	-	-
flavobacteria	-	+

*gamma probe believed to be defective

FIGURE LEGENDS

Figure 1. Phylogenetic position of 7 clones obtained from fresh ANG homogenate. The dendrogram was constructed from 400–450 base comparisons. The scale represents a 10% difference in nucleotide sequence as determined by measuring the lengths of horizontal lines connecting two species.

Figure 2. Fluorescence in situ hybridization of group-specific gene probes with cells in fresh ANG homogenate. a) universal; b) flavobacteria; c) archae 635; d) sulfate reducing bacteria; e) alpha proteobacteria; f) general coumarin stain corresponding to Fig. 2e.

Figure 3. Phylogenetic position of an rDNA sequence extracted from isolate 204 (*Alteromonas* MBL-51). The dendrogram was constructed from ~1350 base comparisons. The scale represents a 5% difference in nucleotide sequence as determined by measuring the lengths of horizontal lines connecting two species.

Figure 4. Flagellar stain of isolate 204 showing a single polar flagellum.

Figure 5. Phylogenetic position of an rDNA sequence extracted from isolate 222 (*Aeromonas* clone25). The dendrogram was constructed from ~450 base comparisons. The scale represents a 5% difference in nucleotide sequence as determined by measuring the lengths of horizontal lines connecting two species.

Figure 6. Phase contrast micrographs showing morphological diversity of organisms in fresh ANG homogenate.

Figure 7. Phylogenetic position of an rDNA sequence extracted from isolate 218 (*Vibrio* MBL218). The scale represents a 5% difference in nucleotide sequence as determined by measuring the lengths of horizontal lines connecting two species.

Figure 8. Fluorescence in situ hybridization of group-specific gene probes with cells of isolate 219. a) universal; b) archae 635; c) sulfate reducing bacteria.

Figure 9. Absorption spectrum of ANG pigment extract showing carotenoid signature and lack of chlorophyll signatures.

Figure 10. O₂ concentration gradient in the ANG of an anaesthetized squid.

Figure 1

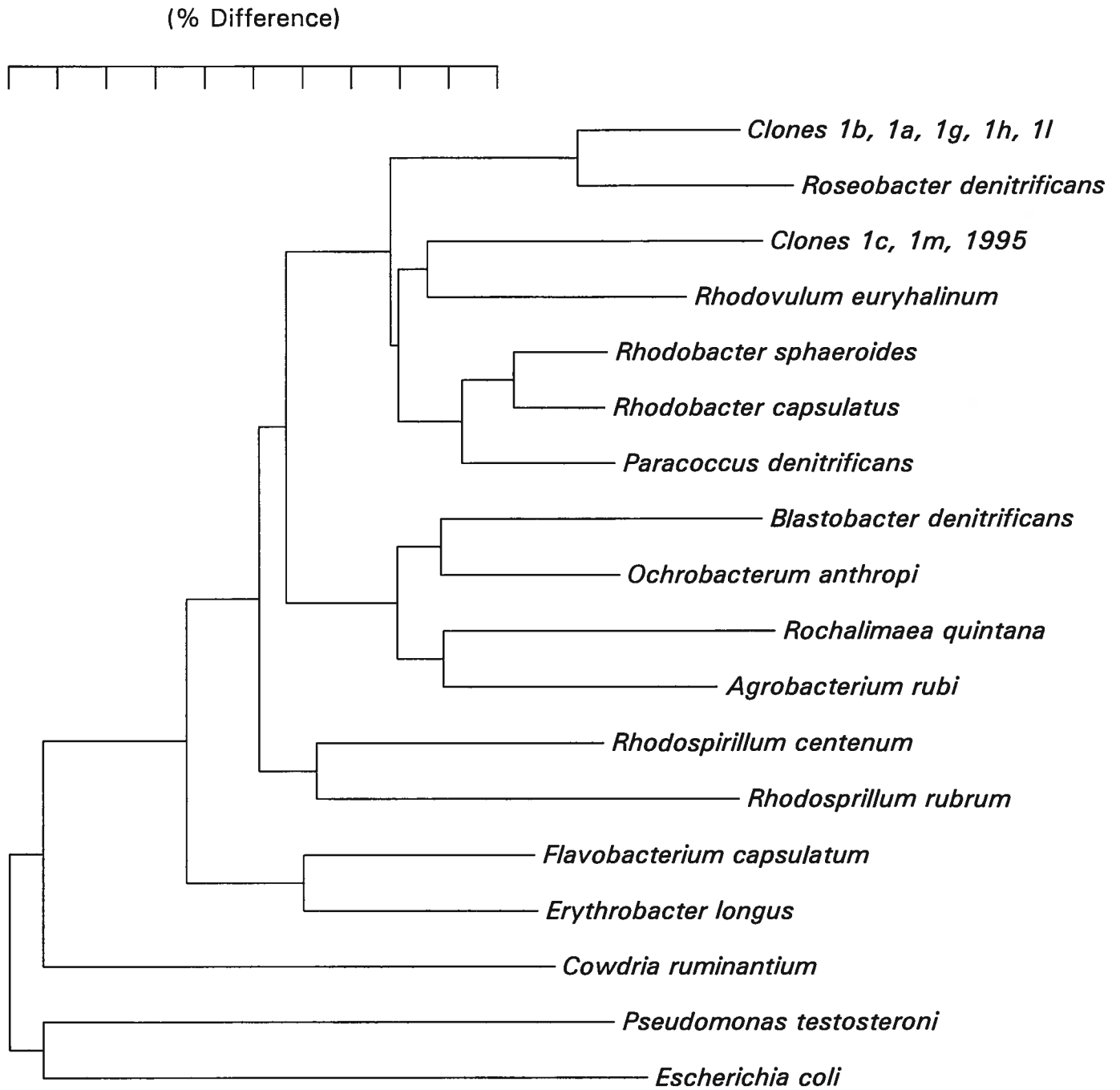


Figure 2

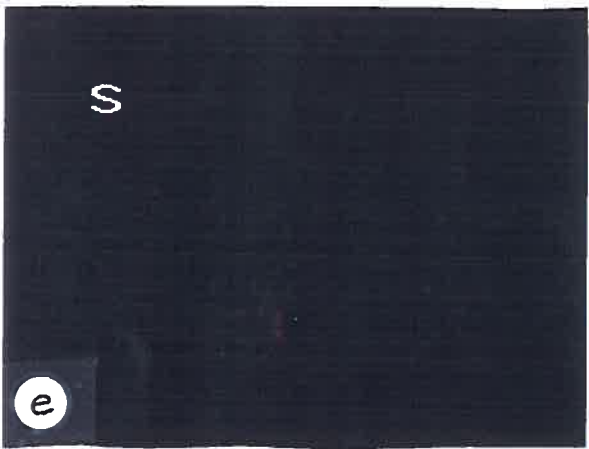
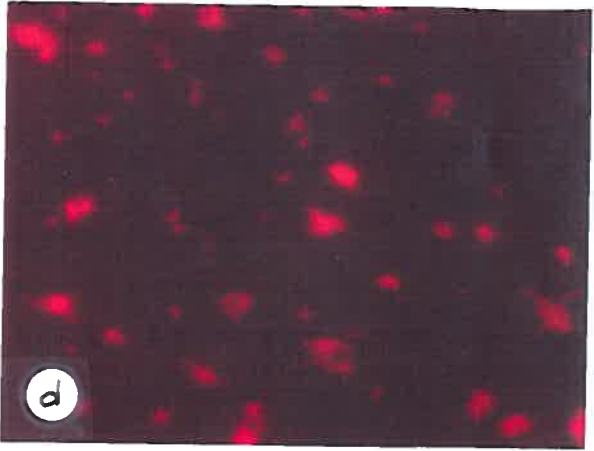
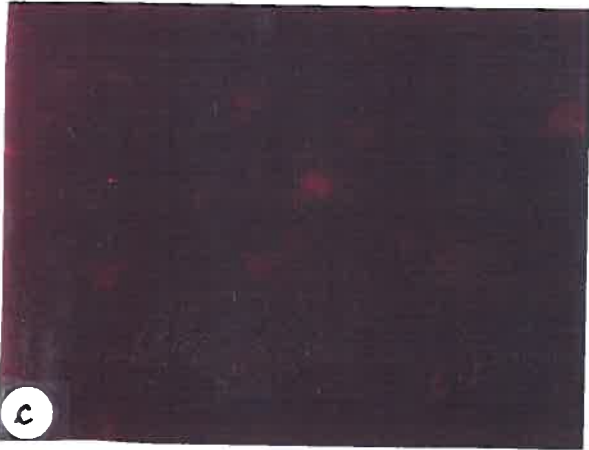
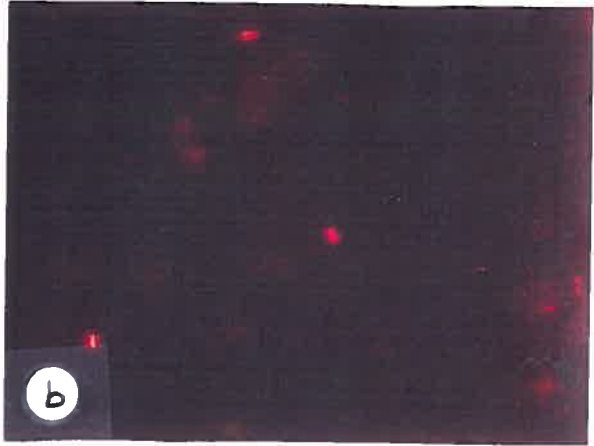
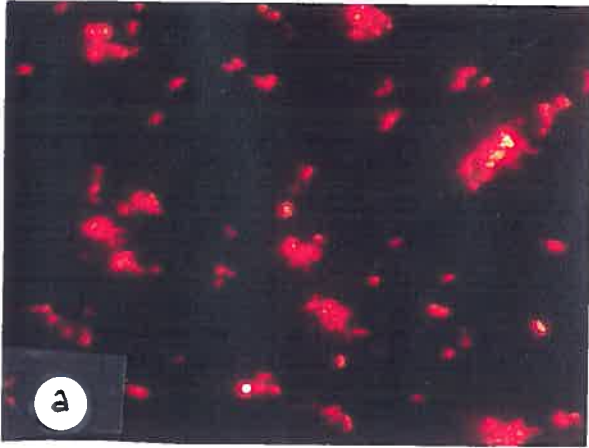


Figure 3

(% Difference)

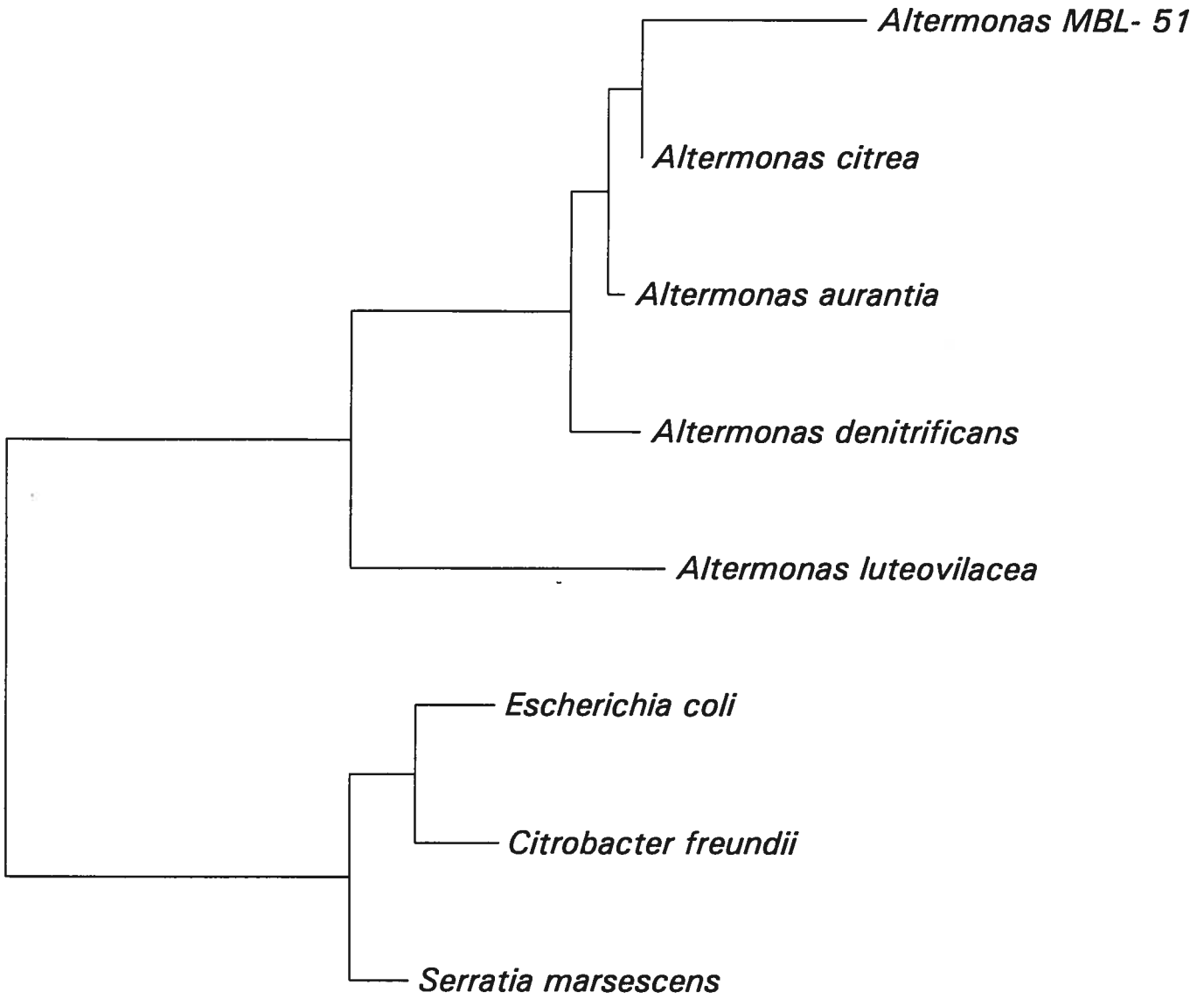
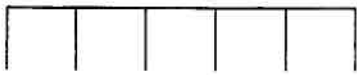
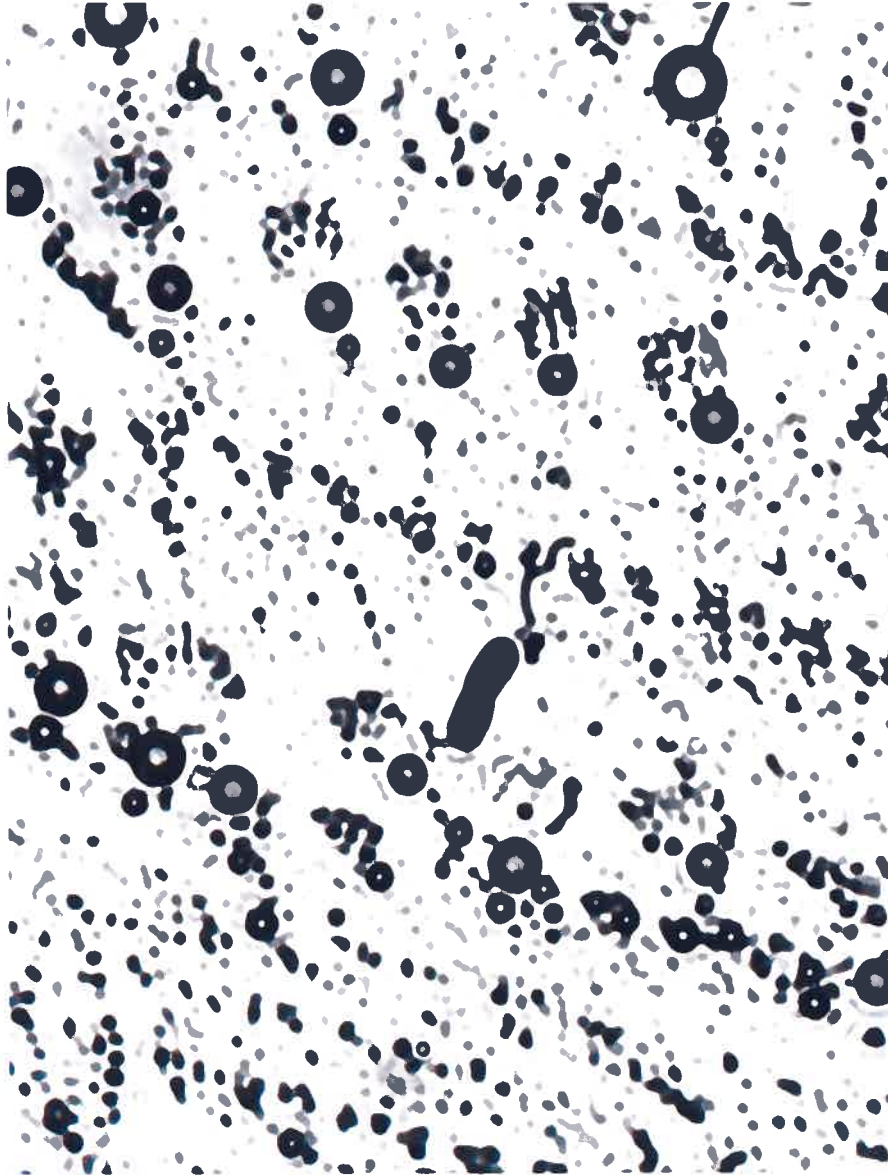


Figure 4



Handwritten text, possibly a signature or date, located below the micrograph.

Figure 5

(% Difference)

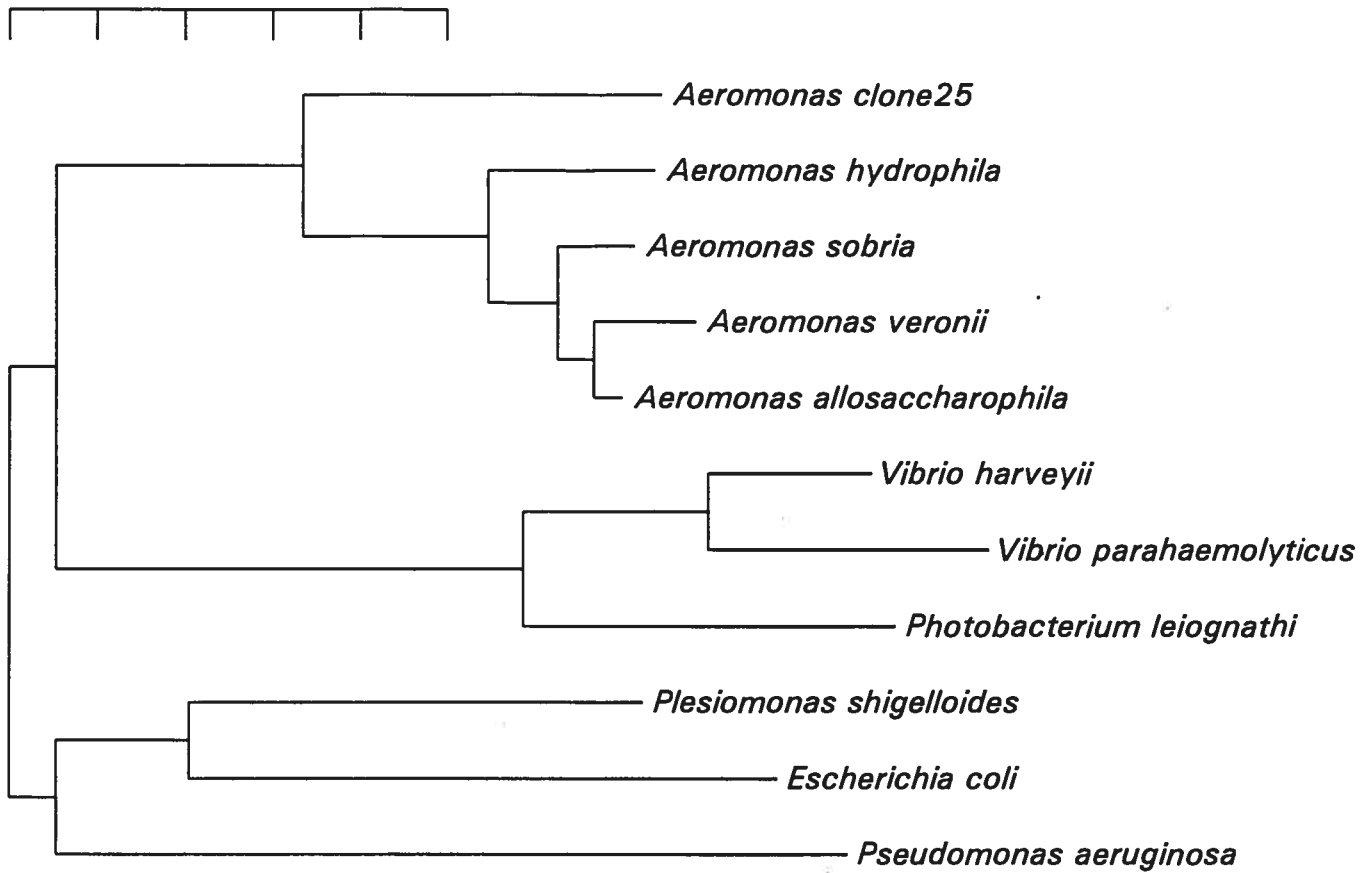


Figure 6

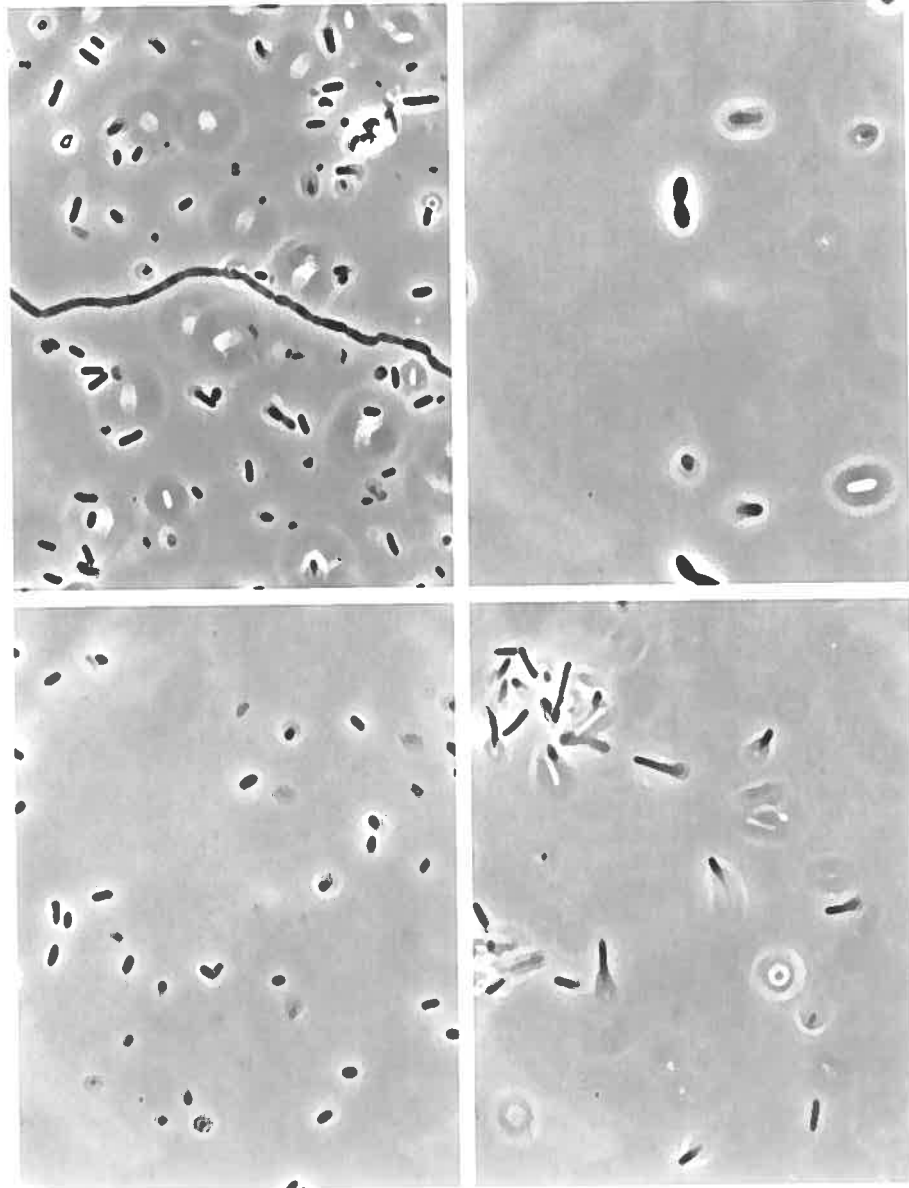


Figure 7

(% Difference)

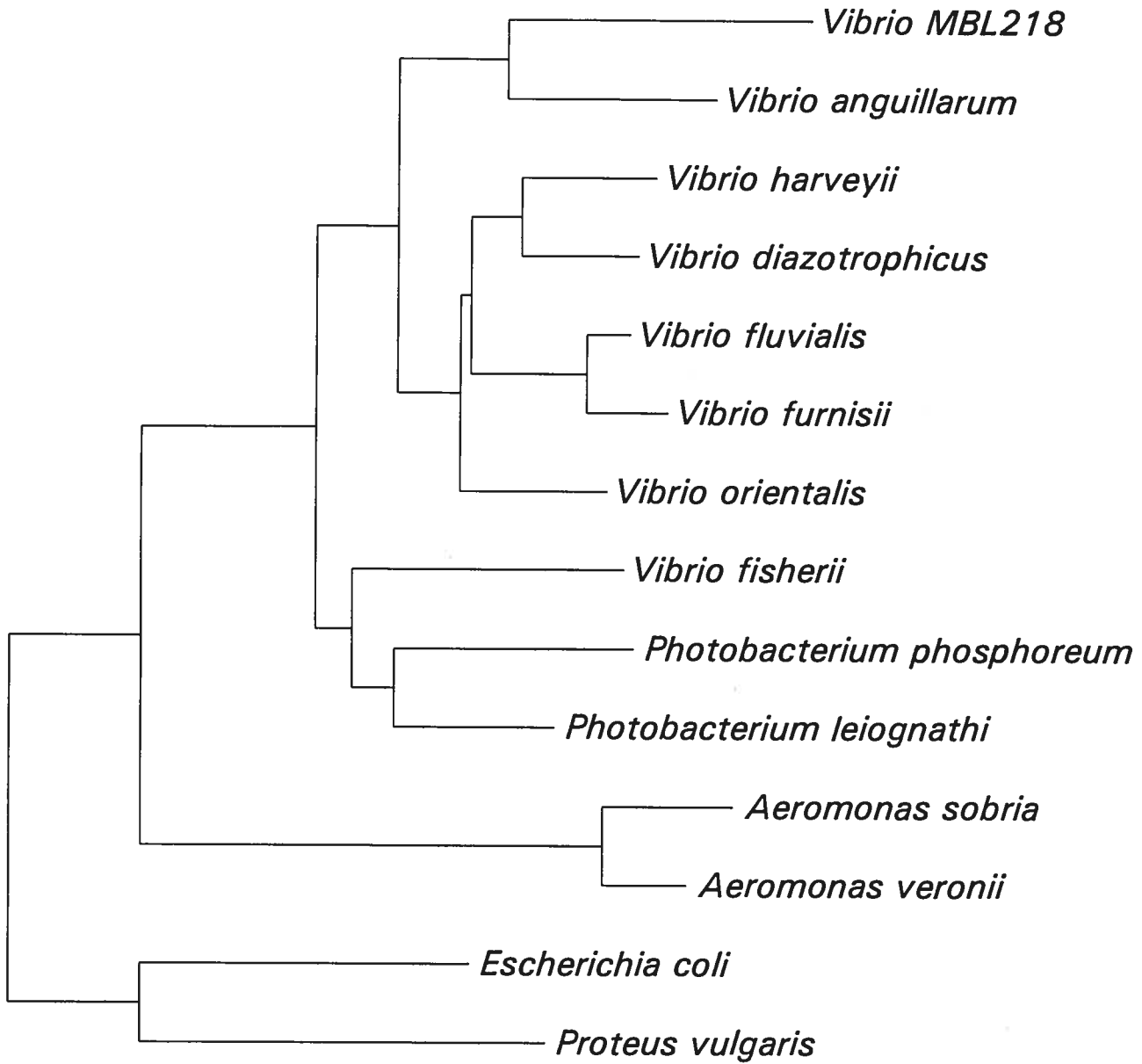
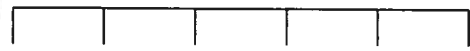


Figure 8

Yellow isolate from
Acc. Mid. Island

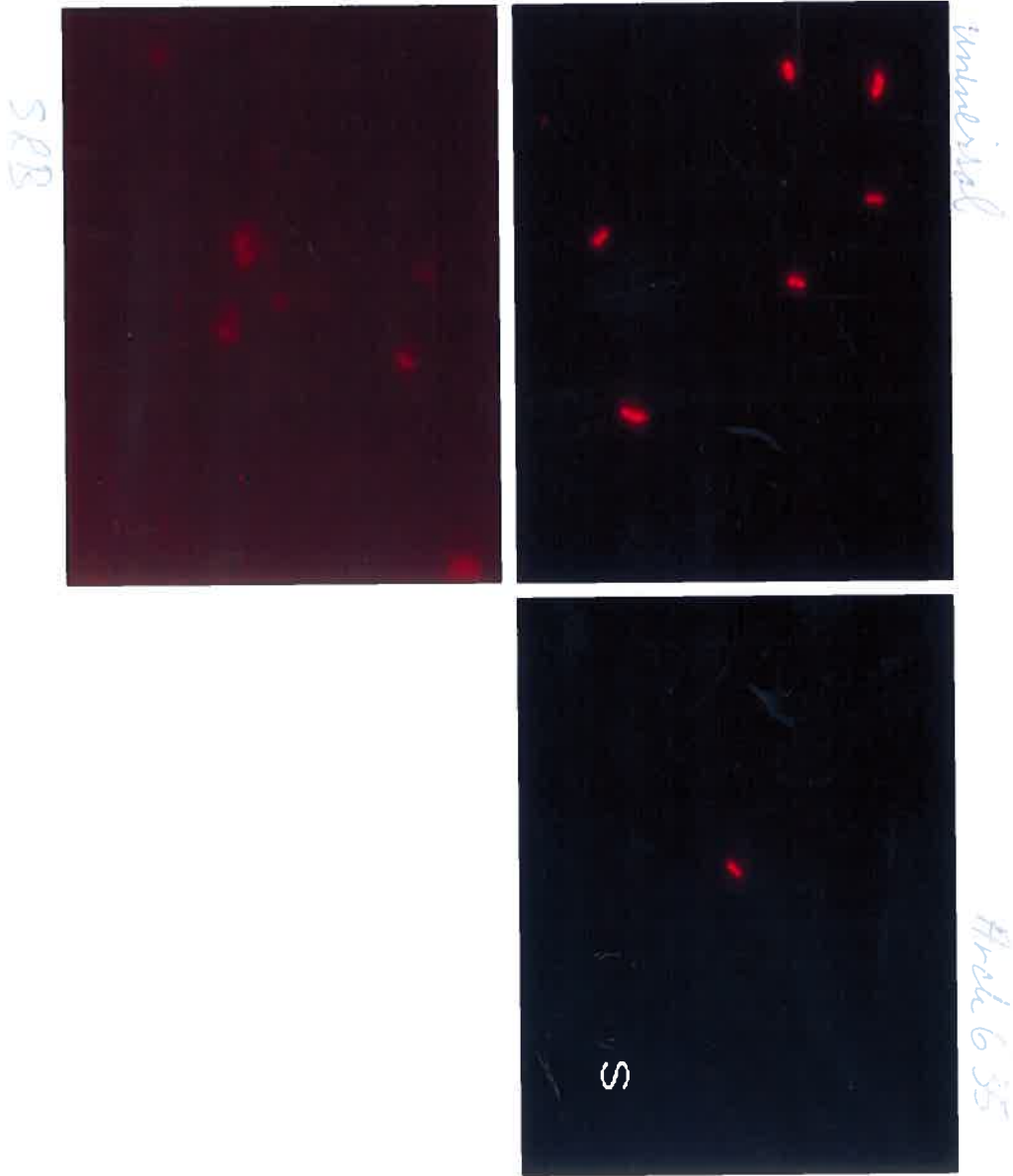


Figure 9

