Further Characterization of the Microbial Community Present in the Accessory Nidamental Gland of *Loligo pealei*

Microbial Diversity 1998 - Group IV
Cynthia Carr, Dmitri Sobolev, John Spear, Silvana Tarlera

Abstract

The purpose of this research was to further characterize the microbial community present in the accessory nidamental gland (ANG) in females of the squid *Loligo pealei*. Microscopic evaluation of the ANG homogenate (phase contract, 1000 X) revealed that the gland contained a morphologically diverse group of microorganisms. Bacteria in the shape of rosettes were also observed, and were thought to be prosthecated bacteria. Cells viewed using transmission electron microscopy (TEM) were rod-like, or T-shaped in appearance. Anaerobic liquid enrichment cultures inoculated with the ANG homogenate were constructed for sulfate reducing bacteria and methanogens. Methanogenic growth, as indicated by the production of methane, was not observed with hydrogen as the growth substrate. An enrichment culture amended with sulfate (10 mM) and hydrogen became turbid, and was subsequently transferred into a second enrichment culture containing the same substrate concentrations. HPLC analysis of the medium in the secondary enrichment revealed that acetate was being formed. Three subsequent agar shake dilution series were made using this secondary enrichment culture as the innoculum. The agar shakes were amended with lactate and sulfate, ethylene glycol and sulfate, or ethylene glycol. Growth was observed in all three series, a presumptive indication for the presence of sulfate reducing bacteria and homoacetogens. Phototrophic bacteria could not be enriched from the ANG homogenate. Molecular techniques were employed to characterize both the ANG homogenate from a young and adult squid. A clone sequenced from the young squid was found to be < 93% identical to the organism Erythrobacter BAL28. No similar clone sequence has

been retrieved before from either egg capsules or from the ANG of the female squid. Bacteria that belong to this genus are aerobic, orange-pigmented containing bacteriochlorophyll *a*. Phylogenetically, this genus represents a fourth subgroup of the alpha subclass of the proteobacteria. A second clone was recovered from the adult squid, and was < 99% identical to alpha proteobacteria egg clone D47 that was sequenced from a *Loligo* egg sac in 1997 (Microbial Diversity Course, Woods Hole, MA). Ribosomal (16S) RNA from the adult squid was shown to hybridize to flavobacteria and methylotroph probes in a checkerboard hybridization experiment.

Materials and Methods

Dissection

Fresh *Loligo pealei* were obtained from the Marine Resource Center, Marine Biological Laboratory, from stock tanks. Female squid were identified from males by looking at the ventral side of the mantle for the orange-salmon colored ANG; an internal organ visible through the fascia. The squid were placed in seawater containing buckets and brought back to the lab. Sterile materials were assembled for organ collection (petri dishes, forceps, scissors, razor blades and seawater) and a place to collect the organ arranged (glass Pyrex dish). Decapitation was done with the scissors in an expeditious manner. Basic dissection of the mantle was accomplished with the razor blade and forceps, though a scalpel would be more useful. The ANG lies ventral to the ink sac and care must be taken to not cut the ink sac while excising the gland; if this happens, the ink cannot be removed from the gland. The ANG is cut and torn out one lobe at a time, and placed into a sterile petri dish. With the addition of approximately 100-200 µl of sterile seawater the gland was chopped with a razor blade, up and down, against the floor of the dish, approximately two dozen times. Forceps were then used to lift off the ANG sac

from the orange colored bacterial contents and discarded. The dish was tipped up on side, and a pipette used to remove the fluid into a sterile Eppendorf tube. Final volume of the gland contents varied depending on initial size and how much sterile seawater was used to wash excess off of the dish. Tubes containing the ANG homogenate were then stored at 4 °C.

Microscope

For phase contrast microscopy, wet mount preparations of freshly obtained cells were made and examined under oil on Zeiss microscopes. Images were recorded digitally on a Zeiss Axioplan 2 microscope with a 3-chip CCD camera attached.

Transmission Electron Microscopy (TEM) was done at the Marine Biological Laboratory's Zeiss TEM facility. Copper grids were Formavar coated, followed by a carbon coat, then freshly glow discharged. Samples of squid cells were placed on the grid for 3-5 minutes, blotted off with a Kimwipe, then 50 µl of either 1% uranyl acetate, pH 7.0, or 1% phospho tungstic acid, pH 7.0, was placed on the grid for the same amount of time and blotted. The TEM grids were then air dried in a dessicator until image analysis. Images were made on 7 x 10 cm film negatives, and if interesting, blown up to a larger size.

Liquid Enrichments

Enrichments were prepared for sulfate reducing and methanogenic bacteria using the ANG homogenate. Reduced, freshwater medium was used for the enrichments, and each was amended with hydrogen, hydrogen and sulfate (10 mM, as sodium salt), or ethanol and sulfate (each 10 mM). Growth was indicated by an increase in turbidity or production of gas. Aliquots of medium were transferred into secondary liquid enrichments (prepared as outlined above) when growth was detected. All cultures were incubated in the dark at 30 °C, and were not

shaken. Hydrogen was replaced as needed to avoid a buildup of vacuum in hydrogen fed cultures.

Series of agar shake dilutions were prepared from secondary enrichment cultures that exhibited microbial growth. Agar shake dilutions were prepared as outlined in the course handout, "Preparation of Pure Cultures by the Agar Dilution Method", B. Schink. Three series were prepared, each to a 10^{-6} dilution, and the substrates were lactate and sulfate, ethylene glycol and sulfate, and ethylene glycol (10 mM each). When the agar solidified, each tube was purged with N_2/CO_2 gas and incubated at 30 °C.

Phototrophic enrichments were prepared using the procedure outlined in the course handout, "Enrichment for phototrophic 'non-sulfur' and 'sulfur bacteria'", Kurt Hanselmann. The enrichments were characterized as 1) high H_2S, low pH (liquid and agar); 2) low H_2S, high pH (agar only); 3) no H_2S (agar). All cultures were incubated next to a light source, at room temperature.

Gradient Cultures

An attempt to isolate microaerophillic, sulfide-oxidizing bacteria, capable of taking advantage of the oxic-anoxic transition **(1)** near the walls of the gland was made by means of a gradient culture. This culture was prepared by aseptically pouring 2 mL of sterile 20 mM sodium sulfide into a screw-top tube, allowing it to solidify under nitrogen gas, and then adding 10 mL of non-reduced bicarbonate-buffered medium (Na_2CO_3, 2.5 g/L; K_2HPO_3, 0.06 g/L; NH_4Cl, 0.01 g/L, vitamins and trace elements supplemented as to the liquid cultures, final pH set to 7.0 by bubbling with N_2/CO_2 mix and aseptically adding sterile acid or alkali) stabilized with 0.25% agar. The headspace of the tubes was then filled with air, and the tubes were then inoculated with approximately 0.05 mL of the fresh ANG homogenate and incubated at 25°C. One tube

received exactly the same treatment as the others, but no inoculum, and one was used as a negative control.

HPLC Analysis

Fatty acids were detected and quantified in liquid medium using a Waters HPLC equipped with an UV detector (wavelength of 210 nm). The oven was operated isothermally at 60 °C. A Shodex column, Ionpak KC-811, was used for separation, and the eluent was 0.1% H_3PO_4 (flow rate of 1 mL/min). Samples were prepared by transferring 400 µL of medium into sterile 1.5 mL eppendorf tubes. The tubes were centrifuged for 10 minutes at 13,000 g. The supernatant was transferred to clean HPLC vial inserts for analysis. Standards (5 mM) were prepared and analyzed with the samples.

Molecular Techniques

A DNA extraction procedure was performed on the homogenate of one entire ANG following a DNA lysis procedure prepared for the Microbial Diversity course. Phenyl:chloroform:isoamyl alcohol mix / extraction buffer was added to the homogenate together with glass beads in a bead beater tube and bead beat on a mini bead beater for one minute. Final suspension of extracted DNA was placed in 20 µl filter sterilized deionized water and frozen at -20°C.

A polymerase chain reaction (PCR) was done using an established protocol for the Microbial Diversity course. Amplification of the 16s rRNA gene was done using the primers 8-forward and 1492-reverse. Taq polymerase was added in wax bead form (Promega). Gels were run in 0.8% agarose prepared in 0.5X TBE buffer. If a strong band was visible, indicating the presence of the16s amplicon , cloning was done with either an Invitrogen Topo cloning kit, or an Invitrogen Blunt cloning kit. Approximately 24 clones were then examined for the 16s rRNA

gene insert by a second PCR amplification. All of these PCR products were then restriction enzyme digested at 37°C for restriction fragment length polymorphism (RFLP) analysis following an established protocol developed for the Microbial Diversity course. Twenty microliter samples were loaded on a 1.5% low melt agarose gel, and band pattern differences were determined visually. Based on this analysis, it was decided which samples would be DNA sequenced.

Molecular checkerboard hybridization was performed with squid homogenate DNA which had been PCR amplified using a D.I.G. forward primer and a universal reverse primer. A series of designed DNA probes were attached to a nylon filter and allowed to react with a wide variety of source DNA samples, including the squid. Probes specific for the 16S rRNA gene included *Streptococcus sanguis, Staphylococcus oralis,* all streptococci, *Actinomyces,* all cyanobacteria, all methylotrophs, all sulfate reducing bacteria, and some universal probes. A developed protocol for the checkerboard hybridization procedure was followed closely.

Results and Discussion

Collection and Dissection.

ANG's were dissected from adult and young female squid. It should be noted that during collection, squid were stressed and would expel ink in almost every case. Although probably not as significant as the long-term stress associated with catching and keeping in captivity, this stress may have altered the composition of the bacterial community. Another possible problem associated with dissection is that squid are actively moving animals in nature, and this results in constant ventilation of the mantle cavity and a relatively high partial pressure of oxygen in the blood. When the animal is not moving (e.g., during dissection), blood oxygen is consumed.

Such a change alters the partial pressure of oxygen within the ANG, and positioning of the microaerobic zone, which may also have an effect upon the bacterial population.

Although removal of the ANG was done as aseptically as possible, a certain degree of contamination occurred (presumably from the outer surface of the gland, which is in a direct contact with the environment). We assumed that such contamination was relatively minor compared to the number of bacteria originating from the gland.

Light and Electron Microscopy.

Among the variety of common bacterial shapes (cocci, motile and non-motile rods), phase-contrast microscopy revealed rod-shaped cells assembled in star-like structures of six cells (Fig. 1) in the adult ANG. "T"-shaped cells (Fig. 2), assumed to be prosthecating bacteria, were observed by transmission electron microscopy. Similar morphologies have not been observed in previous studies, which might be attributed to the gentler homogenization technique used in this report. No literature references have been found that report the presence of such bacteria in other organisms, and attempt to enrich for, and isolate them may be a direction of further studies.

Enrichments using Adult ANG Homogenate

Enrichments for methanogens and sulfate reducers on hydrogen and carbon dioxide (H_2/CO_2) and ethanol and sulfate, respectively, did not yield any growth. A primary enrichment culture was obtained with 10 mM sulfate and H_2/CO_2. Secondary enrichments were made using the primary enrichment as inoculum. HPLC analysis of the medium in the secondary enrichment showed that acetate was being formed from H_2 metabolism. Subculturing the secondary enrichment into agar shakes amended with lactate and sulfate, ethylene glycol and sulfate, or ethylene glycol was conducted to determine whether sulfate reducing bacteria and

homoacetogenic bacteria were present. Growth was observed on all electron donor/terminal electron acceptor combinations.

Growth in agar shakes with several electron donors and sulfate as terminal electron acceptor may have been indicative of the presence of sulfate-reducing bacteria in the ANG, which is consistent with the results reported by Chien (1995) and Zurek and Hughes (1997), but contradicts the checkerboard hybridization results of this study. Production of acetate with hydrogen and carbon dioxide is indicative of the presence of homoacetogenic bacteria. However, due to the lack of time, none of these bacteria were isolated to pure culture. Further study might focus on performing such isolations, and characterizing the function of these bacteria within the ANG. Analysis of fatty acids in the ANG may identify possible substrates for anaerobic bacteria, which might prove to be extremely helpful in further research. One should also note that to determine the importance of anaerobic bacteia in the ANG (i.e., percentage of all bacteria present), a quantitative serial dilution must be made.

Attempts to isolate phototrophic bacteria, the presence of which was previously suggested by molecular techniques **(3)**, were unsuccessful. Although anatomy and behavior of the squid is not inconsistent with the possibility of such organisms present within the ANG, no growth was observed after 3 weeks on any of the media used. Failure to cultivate such organisms might be due to their inability to grow on the media provided, stress on bacterial population during capture and dissection of the squid, or their absence from the ANG. In the last case, observed hybridization of the probes designed for phototrophic bacteria with organisms from ANG requires explanation.

Cultivation of the sulfide-oxidizing organisms by means of a gradient culture were also unsuccessful. No differences were observed between inoculated and control tubes. The lack of

growth may have been due to the lack of specific growth requirements, or problems associated with disruptions in oxygen gradients within the ANG during capture and dissection. A question that should be asked is how do squid avoid sulfide poisoning? Is it due to the fact that only small amounts of sulfide are produced and then oxidized abiotically, or is there a population of still uncultured microaerophilic bacteria taking advantage of the oxygen gradient? A similar symbiosis between a mollusk and sulfide-oxidizing bacteria was described for a clam living in sulfide-rich sediments **(4)**. So far there is no insufficient evidence to speculate whether this phenomenon takes place within the squid ANG, but this possibility might be investigated in further studies.

Molecular Techniques

A variety of approaches have been tried to obtain the 16S rRNA gene clones from the ANG bacteria. To date, direct cloning of the PCR products with a Topo kit on ampicillin plates worked best. Sequencing results indicated the presence of bacteria phylogenetically related to *Erythrobacter* (approximately 93% similarity) in the young squid ANG. In the adult squid, an alpha-proteobacterial clone was found, which was approximately 99% identical to the clone sequenced from a squid egg sack in 1997 **(3)**. This may be another piece of indirect evidence suggesting that squid uses the ANG content to inoculate the egg capsules. However, at this stage it requires further demonstration. Another interesting detail is how the ANG bacterial community changes as squid matures, and how (if at all) it might be controlled by the squid. This opens perspectives for the interdisciplinary studies on squid/bacteria symbiosis. Presence of an *Erythrobacter*-like clone in the young squid is consistent with the previous observations of the signal from phototrophic bacteria, however it contradicts the results of the attempt to culture phototrophs. It should be noted, however, that the phototrophic enrichment was initiated from an

adult squid, from which no such clone has been obtained so far. It is possible to propose that the population of the phototrophic bacteria is high in numbers in the young squid, but declines as the animal matures. A phototrophic enrichment procedure similar to the one used on adult ANG homegenate in this study should be attempted for a younger squid sample. At the moment, there is insufficient data to demonstrate this hypothesis, although this may be developed further in a more detailed study. A phylogenetic tree was constructed to delineate the relationships between *Erythrobacter* and the ANG and egg clones sequenced to date (see Fig. 3).

Checkerboard hybridization (see Fig. 4) yielded a signal from *Flavobacteria* and methylotrophic bacteria, the presence of which have been reported before **(3)**. No signal was detected from the sulfate-reducing bacteria, which contradicted culture observations and still requires an explanation. It is believed that there may have been problems associated with the checkerboard hybridization procedure reported in this report, since bands corresponding to the squid ANG DNA are dark and smeared (see Fig. 4). Methylotrophic bacteria, if present, might provide indirect evidence for methanogenic processes in the anoxic portion of the gland. However, the presence of these organisms suggests aerobic (or at least microaerophilic) processes within the gland. Further study of the oxygen penetration depth in the ANG (under conditions as close to natural as possible) and gradient processes may be of interest.

References

1. Kraus D, Doeller J, Powell C (1996) J. Exp Biol;199(6):1343-1352 Sulfide may directly modify cytoplasmic hemoglobin deoxygenation in Solemya reidi gills

2. Chien, Chih-Ching (1995) A study of microbiota in the accessory nidamental gland of squid *Loligo pealei*. Microbial Diversity Summer Course, Woods Hole, MA.

3. Zurek, Ludek and Debbie Hughes (1997) Microbial community of the egg capsule and accessory nidamental gland (ANG) of the squid *Loligo pealei*.

4. Felbeck, H. (1983) J. of Comp. Physiol. 152: 3-11. Sulfide Oxidation and Carbon Fixation of the Gutless Clam *Solemya reidi*.

Figure Captions

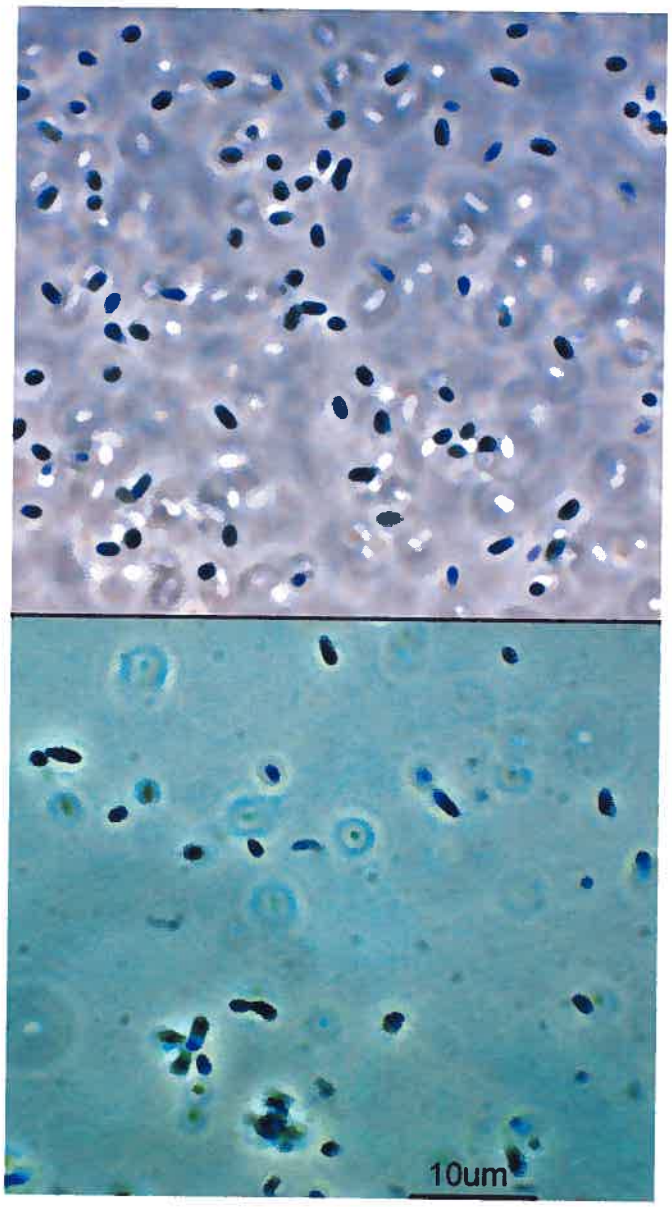
Figure 1: Digital image of a phase contrast image at 1000x under oil. The majority of cells in the field are small motile rods. In the lower left center portion of the field is what appears to be a small rosette pattern, indicating possible connectivity between cells. Such rosettes are common among prosthecating bacteria.

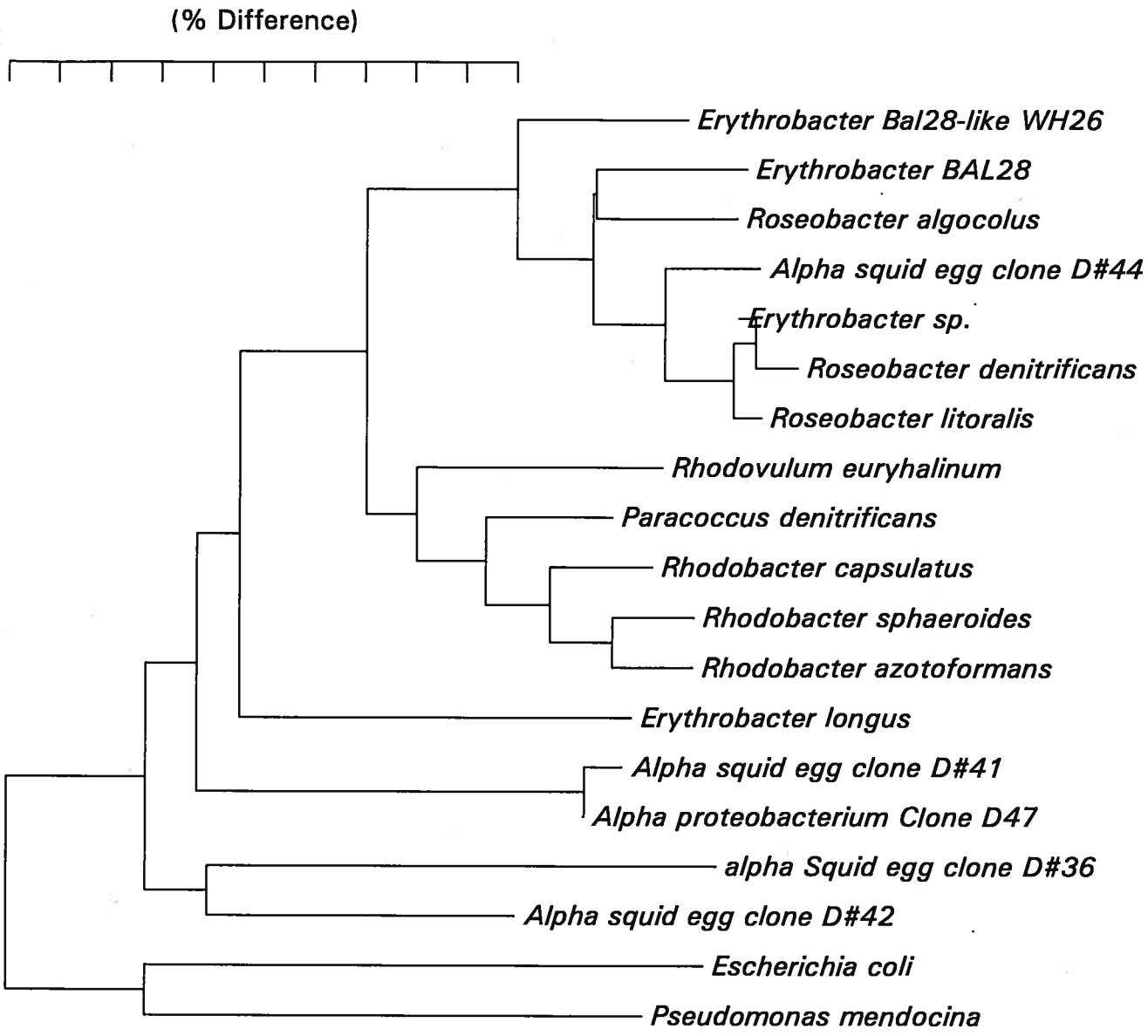
* Figure 2: A digitally encoded transmission electron microscope image obtained at 14,000x of adult ANG homegenate. Cells are "T" shape in appearance, but it is inconclusive whether or not this is actually an example of a prosthecated bacterium.

Figure 3: A phylogenetic tree indicating where this year's clones from adult ANG homegenate relate to previous studies. WH-26 is an *Erythrobacter* which is loosely related to bacteria cloned from the squid egg sac.

Figure 4: A copy of the checkerboard hybridization membrane where several bacterial probes were tested against a number of samples, one of which was D.I.G. tagged adult squid ANG homogenate DNA. Positive intersections of sample with probe are seen by small rectangles.

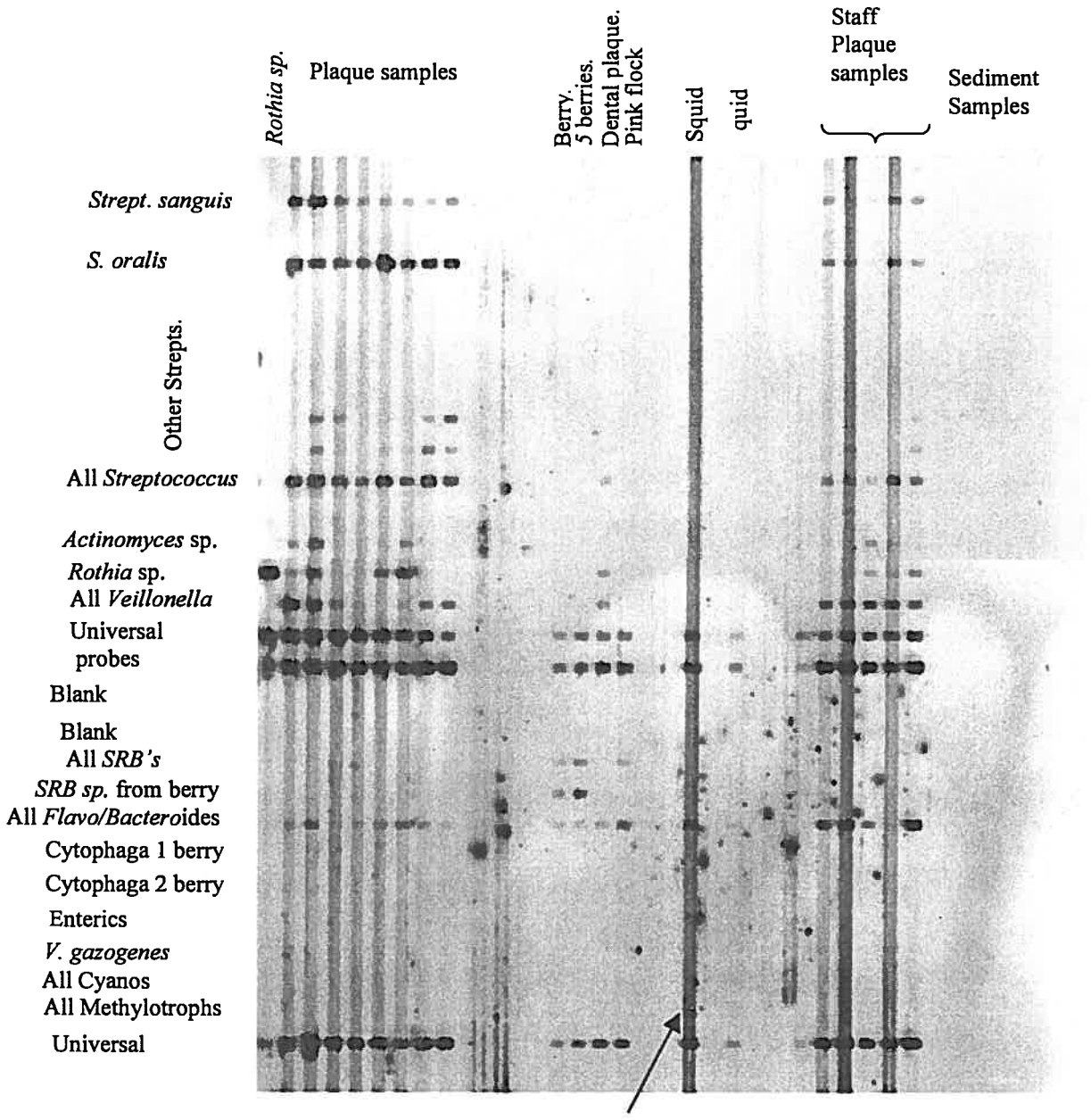
* DUE TO TECHNICAL DIFFICULTY, PHOTOS ARE NOT ENCLOSED. TOM PITTA OK'd THIS. J. SACK


10um


(% Difference)
Erythrobacter Bal28-like WH26
Erythrobacter BAL28
Roseobacter algocolus
Alpha squid egg clone D#44
Erythrobacter sp.
Roseobacter denitrificans
Roseobacter litoralis
Rhodovulum euryhalinum
Paracoccus denitrificans
Rhodobacter capsulatus
Rhodobacter sphaeroides
Rhodobacter azotoformans
Erythrobacter longus
Alpha squid egg clone D#41
Alpha proteobacterium Clone D47
alpha Squid egg clone D#36
Alpha squid egg clone D#42
Escherichia coli
Pseudomonas mendocina

June 29, 1998

Checkerboard hybridization of environmental samples



Notes: (see Bruce for explanation)

- Berry samples have specific SRB identified last year and flavobacteria, but not the specific species of *Cytophaga* identified last year (last year not all berries had both Cytophaga species)
- Pink flock has SRB's (but not the specific species found in the berry) and flavobacteria
- Squid samples have flavobacteria and 1 sample may have methylotrophs (arrow-- hard to see)
- Dental plaque samples have different distributions of streptococci and other bacteria, including flavobacteria (*Bacteroides* and related bacteria)
- Sediment samples gave no signal—probably DIG primers were not used.