Isolation and Identification of a Probable Bacterial Pathogen Affecting the Accessory Nidamental Gland of Female Cuttlefish, *Sepia officinalis*

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Introduction

Mass mortalities of female cuttlefish (*Sepia officinalis*) have been occurring in recent months at the Marine Biological Laboratory's (MBL) Marine Resources Center (MRC). The deaths have been attributed to a probable bacterial pathogen. The affection, which occurs in the accessory nidamental gland of the female cuttlefish, manifests shortly after spawning.

Most female cephalopods possess the accessory nidamental gland. This gland is pigmented yellow, orange, or red in sexually mature females (Van den Branden *et al.*, 1980). The accessory nidamental gland in adult *Sepia officinalis* is orange-red in color, and this color is attributed to carotenoid pigments found in symbiotic bacteria that inhabit the accessory nidamental gland (Van den Branden *et al.*, 1980). The accessory nidamental glands of diseased female cuttlefish at the MRC lose their characteristic orange-red color and turn a mottled brown. Diseased cuttlefish die shortly after onset of infection.

Materials and Methods

The accessory nidamental gland of a recently dead diseased cuttlefish was swabbed at necropsy and plated on marine blood heart infusion agar (MBHIA). Cultures were incubated at room temperature for three weeks at the MRC before a secondary transfer to MBHIA was conducted at the MBL's Microbial Diversity laboratory. The secondary transfer was incubated

at room temperature and examined after 24 hours and 48 hours of incubation. The probable pathogen, isolated on MBHIA, was examined utilizing light microscopy and transmission electron microscopy (TEM). Isolated bacterial colonies, diluted in sterile seawater (80%), were prepared for TEM on copper grids coated with plastic and carbon. TEM samples were heavy metal stained with 1% phosphotungstic acid (PTA; pH 7).

DNA was extracted from bacterial colonies purified on MBHIA using standard techniques (Microbial Diversity course guide, 1998). Polymerase chain reaction (PCR) was used to amplify 16S ribosomal RNA (rRNA) gene sequences. Universal forward (position 8) and universal reverse (position 1492) primers were used for sequencing. Nucleotide sequence analysis of the 16S rRNA gene was conducted with PCR products. Sequences obtained for the probable pathogen isolate were compared to 16S rRNA gene sequences present in the Genbank database. Phylogenic analysis of the sequences was conducted using the RNA1 computer program (Bruce Paster, personal communication).

The probable pathogen isolated on MBHIA plates was plated on seawater complete (SWC) agar medium in order to determine if the isolate was bioluminescent. SWC plates were aerobically incubated for four days at 13°C, 19°C, and 30°C. Cultures were examined utilizing light microscopy and observed in a dark room in order to assess bioluminescent activity

Results

Small, round, mucoid colonies with yellow pigmentation were detected on the MBHIA plates within 24 hours. After 24 hours incubation, light microscopy analysis revealed that the isolate was predominantly composed of small rapidly moving rods with characteristic gaps in the

cytoplasm (Figures 1a and 1b). Microscopy conducted after 48 hours incubation showed mixed bacterial morphologies (Figures 1c and 1d). The additional morphological types observed included non-motile rods lacking gaps in the cytoplasm, long thin "string-like" non-motile rods, and rods that exhibited spiral-like motility. The appearance of colony cultures on MBHIA did not change after 24 hours and 48 hours of incubation.

TEM showed the predominance of rod-shaped bacteria with single polar flagella (Figure 2). Curved rod-shaped bacteria lacking flagella (Figure 3) were also observed with TEM but were present in fewer numbers.

Nucleotide sequence analysis of the 16S rRNA gene (500 base pair sequence) showed that the cuttlefish isolate is more similar to *Vibrio salmonicida* and *Vibrio logeii* than to any other species sequenced (Figure 4). However, the sequence for the probable pathogen showed only 95% and 96% similarity to *V. logeii* and *V. salmonicida*, respectively.

The probable pathogen isolate grew on SWC agar medium at 13°C and 30°C. No growth was observed at 19°C. The lack of growth at 19°C was unexpected, considering that the isolate grew well at room temperature, and may be attributed to inaccurate temperature control by the incubator. Cultures grown at 13°C were pigmented yellow and resembled the mixed bacterial morphologies observed on MBHIA incubated at room temperature. Cultures grown at 30°C were white on SWC agar medium and were cocci, some of which were in chains. Bacterial cultures grown on SWC plates incubated at 13°C and 30°C did not exhibit bioluminescence.

Discussion

Nucleotide sequence analysis of the 16S rRNA gene suggests that the probable pathogen isolated from the diseased cuttlefish is of the genus *Vibrio* and most probably is a new species. *Vibrio* spp. are common inhabitants of marine environments (Benediktsdottir *et al.*, 1998), and they are known pathogens affecting marine organisms. Pathogenic *Vibrio* spp. have been isolated from skin lesions and kidneys of fish (Egidius *et al.*, 1986; Benediktsdottir *et al.*, 1998). *V. salmonicida* and *V. logeii* are two of eight species of *Vibrio* shown to cause disease in marine fish and shellfish. The other pathogens include *V. alginolyticus, V. anguillarum, V. damsela, V. harveyii, V. ordali,* and *V. vulnificus* (Benediktsdottir *et al.*, 1998). According to 16S rRNA sequence analysis, *V. salmonicida, V. logeii,* and the cuttlefish pathogen are more closely related to eachother than to the other *Vibrio* spp. known to be pathogenic to marine organisms (Figure 4).

Vibrio salmonicida causes a serious disease of salmonid fishes called cold-water vibriosis or Hitra Disease (Egidius *et al.*, 1986; Enger *et al.*, 1989; Farmer and Hickman-Brenner, 1992). *V. salmonicida* are motile curved rods with at least nine polar flagella (Egidius *et al.*, 1986). However, pleomorphism has been observed in strains of *V. salmonicida* isolated from kidneys of recently dead fish (Egidius *et al.*, 1986). Like *V. salmonicida*, the probable pathogen isolated from the cuttlefish appears to be pleomorphic.

Vibrio logeii are bioluminescent bacteria (Bang *et al.*, 1978) and were recently found to be light organ symbionts of Sepiolid squid (Fidopiastis *et al.*, 1998). *V. logeii* are motile rods with tufts of two to five polar flagella (Bang *et al.*, 1986). The probable pathogen isolated from the diseased cuttlefish is similar to *V. logeii* in that both *Vibrio* spp. exhibit yellow pigmentation

(Bang *et al.*, 1978). However, unlike *V. logeii*, the probable pathogen appears not to be a bioluminescent organism.

V. logeii has been shown to be pathogenic in tanner crabs, causing necrotic lesions of the exoskeleton (Farmer and Hickman-Brenner, 1992; Benediktsdottir *et al.*, 1998). This genus has also been isolated from scallops, fish intestines, and marine sediments (Bang *et al.*, 1978; Enger *et al.*, 1989; Farmer and Hickman-Brenner, 1992).

Suggestions for Future Research

Further studies should be conducted to confirm the results obtained by 16S rRNA gene sequence analysis of the probable pathogen isolated from the accessory nidamental gland of the diseased cuttlefish. Biochemical analysis of the isolate and further microscopic examination is needed to confirm that the isolate is indeed a new species of *Vibrio*. The different morphological types of bacteria observed from the MBHIA plates should be studied to determine if they represent different forms of the same species and hence indicate that the probable pathogen is pleomorphic, like its closest relative, *Vibrio salmonicida*.

The bacterial contents of accessory nidamental glands of apparently healthy cuttlefish should be compared to isolates from diseased cuttlefish. A useful analysis would be to examine bacteria isolated from an apparently healthy cuttlefish utilizing fluorescent *in situ* hybridization (FISH). *Vibrio* spp. are gamma-proteobacteria, and FISH could be conducted utilizing a gamma probe in order to determine if the probable pathogen is a normal inhabitant of the accessory nidamental gland of *Sepia officinalis*.

Further studies should be carried out to definitively show whether the probable pathogen isolate is bioluminescent. Although the isolates incubated on SWC did not demonstrate bioluminescence, this does not prove that this new *Vibrio* sp. is incapable of bioluminescent activity.

Finally, the isolate obtained from the diseased cuttlefish must be shown to be the primary cause of the accessory nidamental gland infection and not the result of a secondary infection. Koch's postulates should be employed to definitively prove that the isolate is indeed the causal agent of the disease causing the mass mortalities of cuttlefish at the MRC.

Figure 1

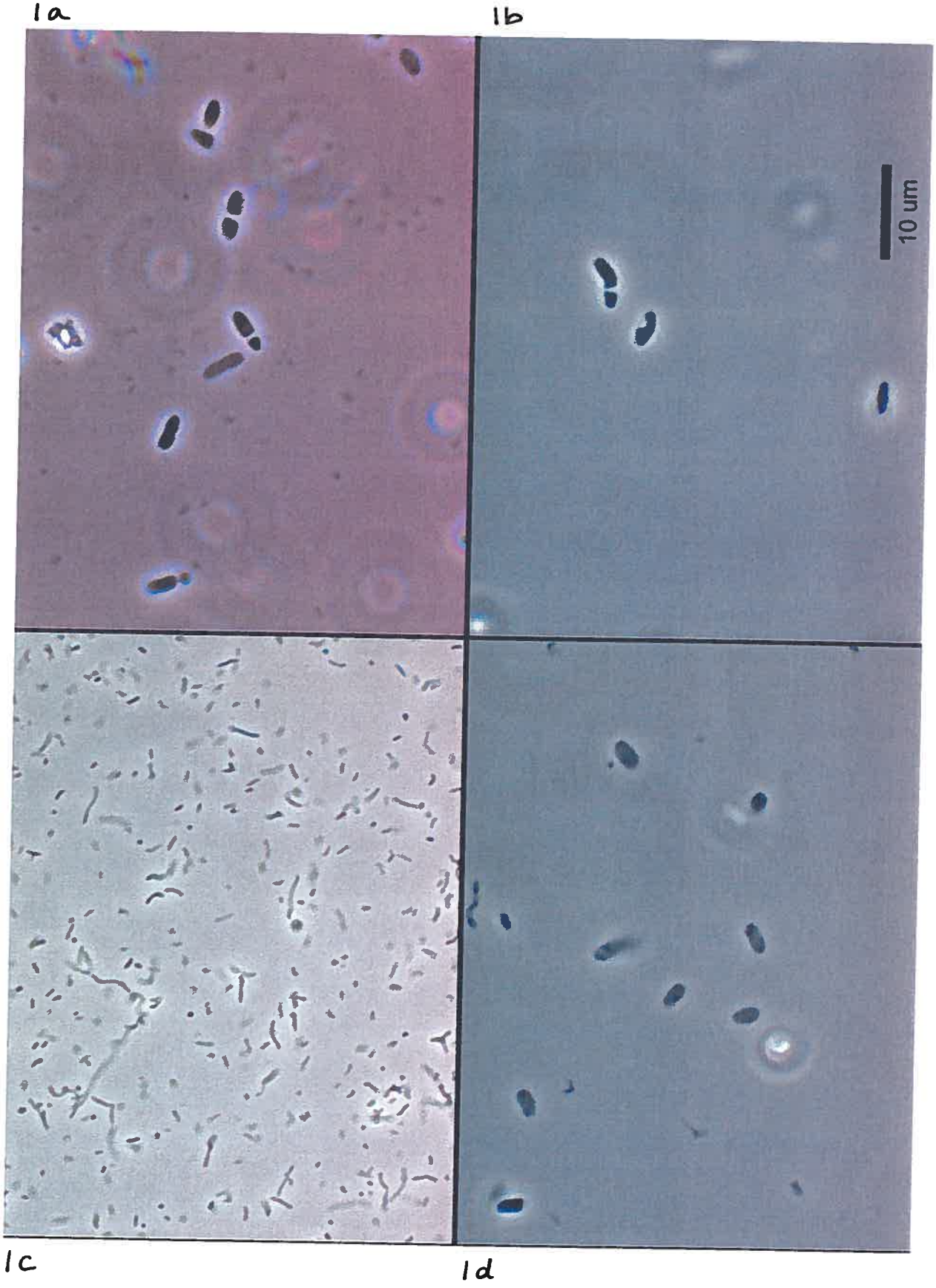


Figure 2

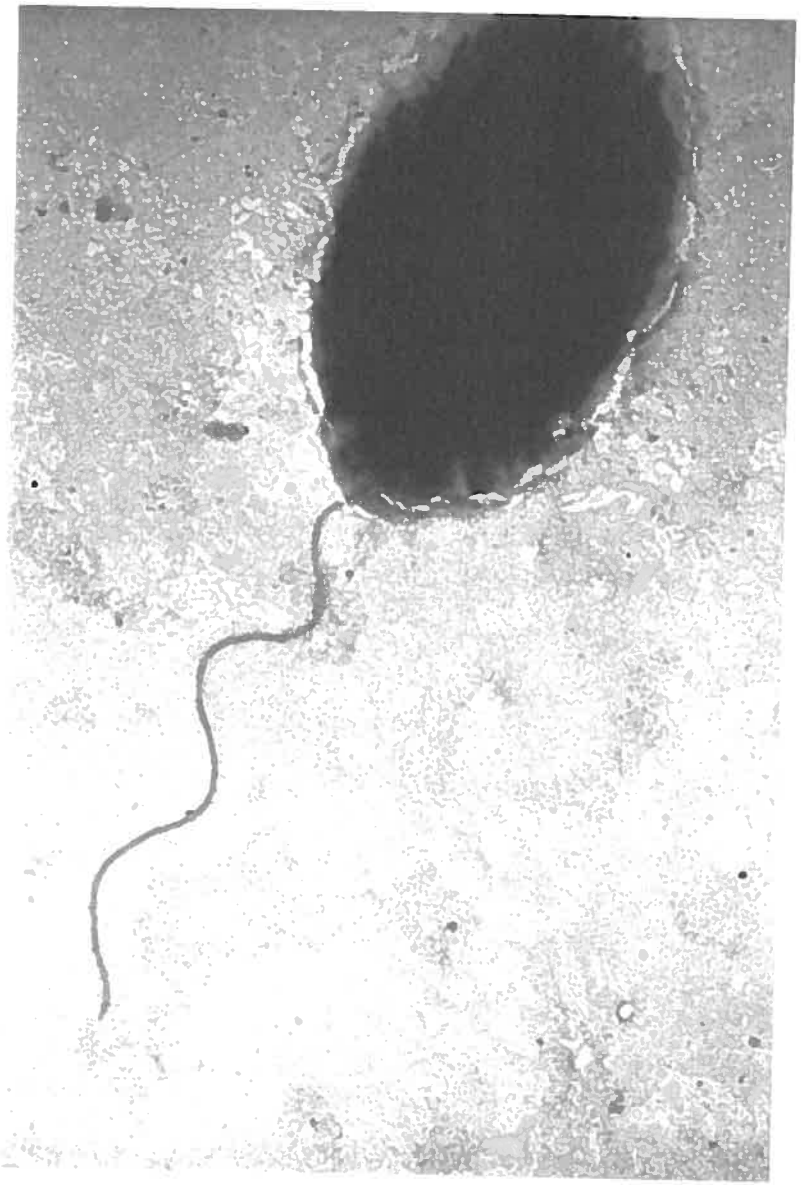


Figure 3

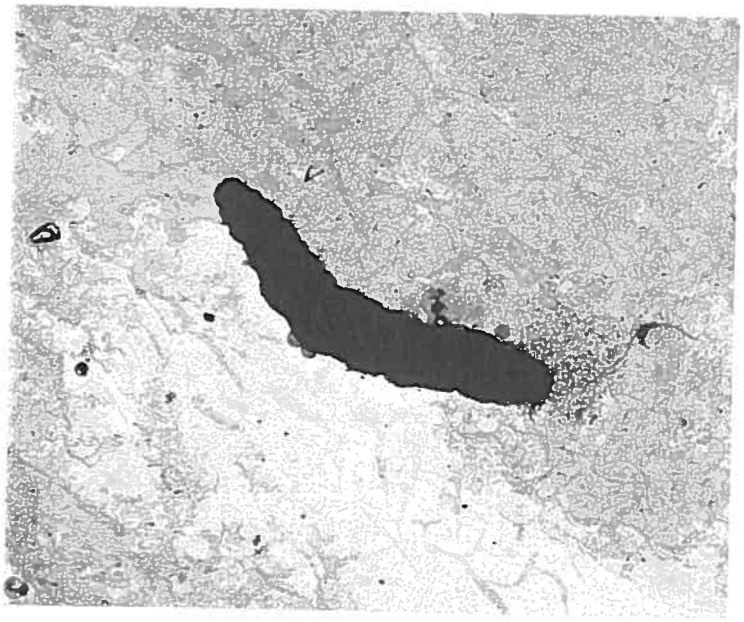
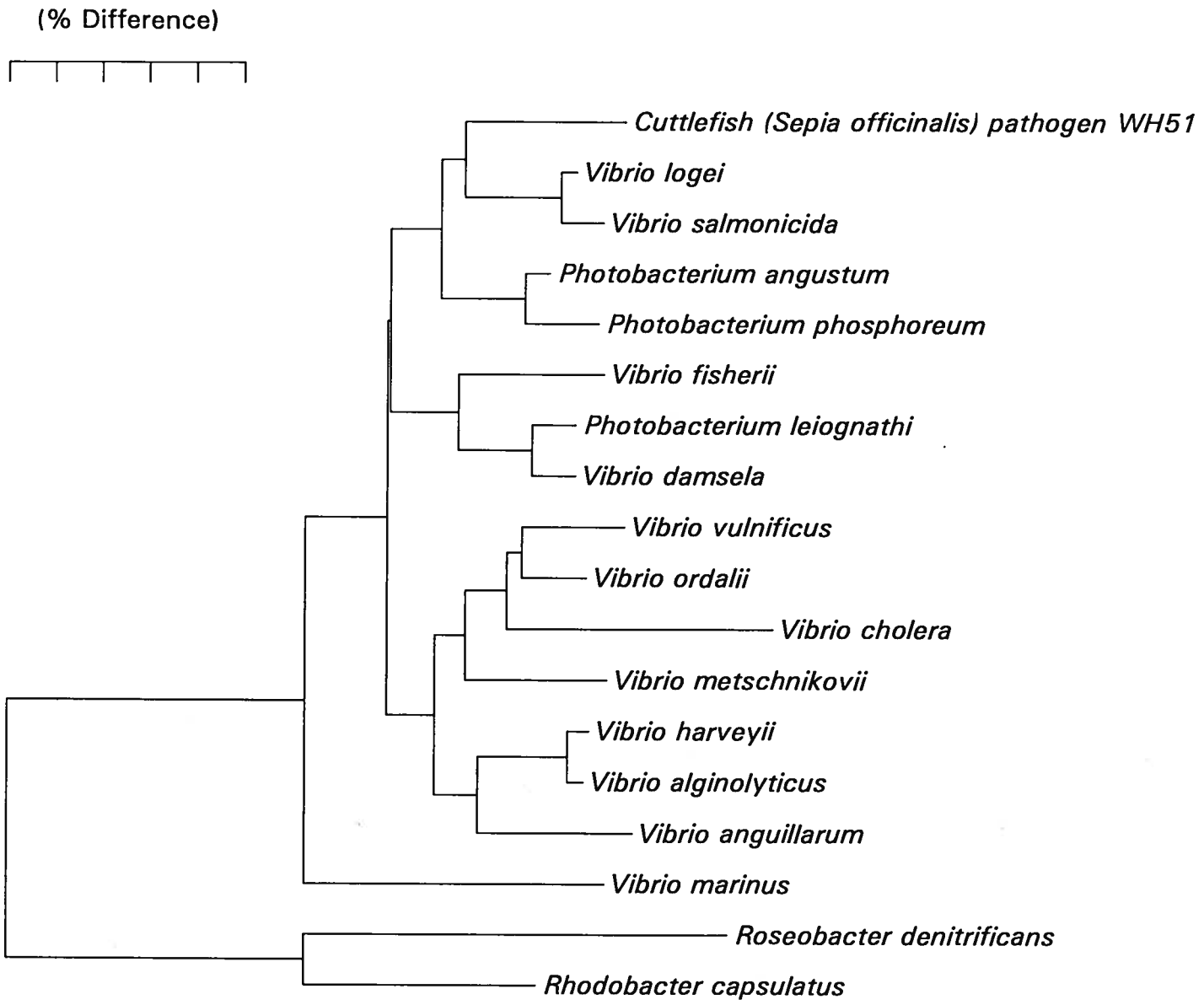


Figure 4


(% Difference)
Cuttlefish (Sepia officinalis) pathogen WH51
Vibrio logei
Vibrio salmonicida
Photobacterium angustum
Photobacterium phosphoreum
Vibrio fisherii
Photobacterium leiognathi
Vibrio damsela
Vibrio vulnificus
Vibrio ordalii
Vibrio cholera
Vibrio metschnikovii
Vibrio harveyii
Vibrio alginolyticus
Vibrio anguillarum
Vibrio marinus
Roseobacter denitrificans
Rhodobacter capsulatus

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