

## Capillaries: a useful tool for microaerophile isolation?

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### Abstract

The number and diversity of microaerophilic bacteria in nature has been underappreciated in part because of the challenges of isolation. Capillary assays for aerotaxis in microaerophiles have played a central part of chemotaxis research. This study evaluates the efficacy of capillaries as a tool for microaerophile enrichment and isolation using cultivation dependent and independent methods. Direct plating of capillary samples versus underlying microbial mat liquid with which capillaries were loaded (control) showed no difference in the percentage of total cells able to form colonies under 2% O<sub>2</sub> on SWC rich media plates after 5 days. Colonies were also tested for their ability to grow under normal atmospheric O<sub>2</sub>. Colonies picked on day 2 from both capillary and control plates grew up overnight under 21% O<sub>2</sub> but day 5 colonies took at least 3 days to become visible. 16S rDNA analysis of colonies showed that most colonies from control plates were *Marinobacterium* species whereas most of the colonies from capillary plates were *Roseobacter*-like. The closest pure cultured relative in 16S rDNA databases shared only 96% sequence identity (by BLASTn analysis) with this study's *Roseobacter* isolates, suggesting that new species of *Roseobacter* have been isolated using capillary techniques that select for microaerophilic behavior and that aerobic anoxygenic phototrophs may prefer microaerophilic conditions. Cultivation independent 16S rDNA analysis revealed an enrichment of phylotypes closely associated with known microaerophiles such as *Thiomicrospira*, *Hydrogenovibrio*, and *Arcobacter* species in capillary tubes whereas phylotypes from control samples grouped within the *Cytophaga-Flavobacterium-Bacteroidetes* group characterized by ability to degrade complex polysaccharides.

### Introduction

Life evolved in an anaerobic environment and aerobic microbes have had to invent ways to deal with the toxicity of O<sub>2</sub>. Some microbes have dealt with this by only thriving at low concentrations of O<sub>2</sub>. These are termed microaerophiles. Microaerophiles require O<sub>2</sub> as their terminal electron acceptor for respiration but are also poisoned by O<sub>2</sub> and grow only under atmospheric O<sub>2</sub> levels (21%). The

main difference between microaerophiles and those bacteria that can tolerate low O<sub>2</sub> conditions is growth rate. True microaerophiles will grow best under low O<sub>2</sub> tensions when using O<sub>2</sub> as an electron acceptor. Gradient agar tubes have been useful as screens for microaerophilic behavior and thereafter for isolation (Madigan and Martinko, 2006). Microaerophiles form discrete bands beneath zones of visible O<sub>2</sub> penetration as seen with a redox indicator dye such as resazurin. Another screen for microaerophilicity takes advantage of the aerotactic behavior of some microaerophiles. Engelmann in 1881 observed spirilla accumulating in a narrow band near but not on the air water interface in capillaries (Engelmann TW 1881 Zur Biologie der Schizomyceten Pfluegers Arch Gesamte Physiol Menschen Tier 26:537-45, Krieg and Hoffmann 1986). Beijerinck established that these spirilla were repelled by high O<sub>2</sub> and preferred low O<sub>2</sub> conditions in experiments in semi-solid media (Beijerinck, 1893; Krieg and Hoffman 1986). Despite the common observation of microaerophilic bands forming in capillaries, most of these capillary assays have been used in pure culture studies and there has been few applications to the environment to enrich for microaerophiles (Wells and Krieg, 1965). The study investigates the use of capillaries as a tool for microaerophile enrichment using direct plate counts of colonies forming under microaerobic conditions and 16S rDNA analysis of the chemotactic communities formed within capillaries.

## **Methods**

### *Sampling*

A sample of a *Beggiatoa* rich microbial mat was collected from Little Sippewissett salt marsh on June 18, 2008 and maintained in lab in a plastic box open to the atmosphere submerged in seawater until use on July 18, 2008 (Fig 1). Microscopy revealed a diversity of motile morphotypes including many *Beggiatoa*.

### *Capillary assays*

A 50 ml sample of seawater surrounding the mat was taken for capillary assays. Flat 0.4 x 4 mm Vitrotube microslide capillaries were used for microscopy of microaerophile bands. 50 round glass capillaries of dimensions 75 mm x 1.35 mm (Biological Research Inc) were used for capillary assays intended for direct plating and 16S analysis because of the ease of sampling. For these assays,  $\frac{3}{4}$  of a sterile round glass capillary tube was filled with mat seawater liquid from the 50 ml sample and sealed at one end with plasticine. The other end was exposed to the atmosphere. Techniques such as this have been described in Overmann (2005). Bands were allowed to develop over 5 hours at room temperature. Thereafter visible bands were removed from capillaries using pulled glass pipets (100ul) and pooled into a sterile 1.5 ml Eppendorf tube. A similar volume of the mat liquid taken directly from the 50 ml sample was used for analysis as a comparison to capillary samples.

### *Direct Plating*

50 ul of  $10^0$ ,  $10^{-1}$ ,  $10^{-2}$ ,  $10^{-3}$  and  $10^{-4}$  dilutions of capillary liquid and control mat liquid in 1X sterile synthetic seawater was spread on SWC media plates (per liter: 750 ml synthetic seawater, 250 ml MilliQ water, 5 g tryptone, 3 g yeast extract, 3 ml glycerol). 4 dilution series were performed for capillary and control samples (Fig 2). Plates were incubated under 2%  $O_2$  (0.5%  $C O_2$ , remainder  $N_2$ ) in a hypoxic glove box. CFUs were counted on day 5 and normalized to total cell count (DAPI) for capillary and control plates. Approximately 100 visible colonies were picked under 2%  $O_2$  from capillary and control plates on day 2 of incubation and moved to grow under 21%  $O_2$  to look obligate microaerophiles. This was performed again on day 5.

### *16S rDNA analysis*

The 16S rDNA genes were partially sequenced from 19 capillary plate colonies and 13 control plate colonies using 8F and 1492R primers using colony PCR techniques. Briefly, colonies were picked into 40 ul NP-40 and subjected to 5 min boiling at 102 C followed by freezing at -80 C and thawing at room temperature. 2 ul of the cell containing NP-40 was then used as template for 20 ul PCR reactions running at 36 cycles at an annealing temperature of 45 C. PCR products from reactions yielding the correct sized PCR product as visualized by gel electrophoresis were sequenced. Cultivation independent 16S analysis was also performed on capillary and control mat liquid samples using the 8F and 1492R primers. 5 x 10 ul spots of each sample were made onto 0.2 um GTTP filters in preparation for community 16S analysis by filter PCR. The

filter spots (~ 3mm diameter) were cut out from the filter and placed into 5 ul PCR grade water and subjected 5 cycles of freeze (-80 C) and boiling (102 C) to lyse cells on the filter. The entire filter spot with cells and PCR water was used as template for 16S PCR amplification using 8F and 1492R primers in a 50 ul reaction with 40 cycles of amplification. The correct sized products from each filter PCR for capillary and control samples were pooled for TOPO TA cloning. 48 clones from each library (capillary and control) were picked for sequencing.

#### *Cbb3-type cytochrome oxidase (ccoN) functional gene screening in isolates*

Broad specificity primers for the O<sub>2</sub> binding subunit of high affinity cbb3-type cytochrome oxidase enzymes (ccoN) were designed by Wertz and Breznak (2007) and utilized in this study to screen for ccoN genes in 16 capillary isolates and 16 plate isolates using colony PCR techniques described above. Promega 2X master mix was used in 25 ul reactions and primers were added at a final concentration of 0.2 uM. Thermocycling conditions were performed according to those proscribed by Wertz and Breznak (2007) with an annealing temperature of 53 C. PCR products were visualized on 1.5% agarose gel (0.5X TBE) stained with ethidium bromide.

## **Results and Discussion**

Direct plating at 10<sup>-1</sup> and 10<sup>-2</sup> dilutions yielded a countable number of colonies for both capillary and control plates on rich SWC media (Fig 3). Fast growing colonies that grew within a day under 2% O<sub>2</sub> produced a lot of extracellular polysacharrides and were generally light beige to white in color. The smaller colonies (Fig 3b) were generally more pigmented in color and when streaked for isolation they were rose to brown in color. Many small colonies began to appear in on day 4 and they took much longer (at least 3 days) to show visible growth when picked to grow under 21% O<sub>2</sub>. This suggests that the colonies appearing later during hypoxic incubations would be true microaerophiles with more stringent requirements for low O<sub>2</sub> and that the fast growing colonies may be microaerobic weed microbes tolerant of a wide variety of O<sub>2</sub> conditions. These “weeds” may well have been fermenting instead of respiring O<sub>2</sub> under hypoxic conditions. An improvement in experimental design would involve using acetate,

a non fermentable substrate, on defined media plates and to let incubations run longer than 5 days. The importance of a longer running experiment is borne out the results shown in figure 4. No difference was seen in the number of cultured microaerobic organisms on plates spread with capillary or control mat liquid. This may be because incubation time did not allow for the appearance of “true” microaerophiles, hypothesized to dominate on capillary plates over control plates. Another caveat is that the appearance of a colony under 2% O<sub>2</sub> does not necessarily indicate microaerophily. Growth rates at 21% and 2% O<sub>2</sub> would need to be compared to establish whether a particular bacterium were a true microaerophile. It could be that the capillary plates do indeed harbor a greater number of microaerophiles relative to the control plates despite having similar numbers of microaerobic organisms on them.

Indeed the 16S phylogeny of randomly picked capillary isolates (*Roseobacter*-like) suggests that they may be microaerophilic whereas the phylogeny of control plates isolates does not suggest this (Figure 5, 6, 7). *Roseobacter*-like organisms constitute an important component of the aerobic anoxygenic phototroph population in the world’s oceans (Yurkov and Beatty, 1998; Koblizek et al 2003, Allgaier et al, 2003; Oz et al 2005). Many of these organisms remained uncultured and are related to the uncultured SAR 83 cluster, a highly abundant group of marine picoplankton. The closest pure cultured relative in 16S databases share only 96% sequence identity (by BLASTn analysis) with this study’s *Roseobacter* isolates, suggesting that new species of *Roseobacter* have been isolated using capillary techniques that select for microaerophilic behavior

and that aerobic anoxygenic phototrophs may prefer microaerophilic conditions. Control plate isolates were primarily *Marinobacterium* species (Fig 6) which are generally chemoheterotrophic facultative anaerobes. These may have been fermenting during low O<sub>2</sub> growth on SWC rich media plates. More studies are required to determine if such bacteria are truly microaerophiles.

Capillary clone library sequences support the hypothesis that capillary assay provide a useful tool for microaerophile enrichment and isolation. About half of capillary clone sequences grouped with known gamma-proteobacteria microaerophiles such as sulfide oxidizing *Thiomicrospira* and H<sub>2</sub> oxidizing *Hydrogenovibrios* (Fig 7). The other half of the clones belonged to epsilon proteobacteria and are most likely *Arcobacters* (Fig 8). Chemotaxis in opposing sulfide and oxygen gradient tubes has been observed in the motile sulfur oxidizing bacterium *Candidatus Arcobacter sulfidicus* and suggest it prefers microaerophilic conditions (Seivert et al 2006). 16S clone sequences retrieved from control samples originating from mat liquid represented a wider range of bacterial diversity (Fig 5) with many falling the *Cytophaga-Flavobacterium-Bacteroidetes* phylum. This is not unexpected given that the mat was most likely enriched in polysaccharides. Clones sequences grouping within uncultured clades TM7, OD1, and OP11 were also observed in control samples.

A screen for the gene encoding high affinity O<sub>2</sub> binding cbb3 cytochrome oxidases, mainly found in microaerophiles, was performed on pure culture

isolates. However, PCR screens resulted in non-specific PCR products in all 32 colonies tested (16 from capillary plates, 16 from control plates). This suggests that either (1) the colonies growing microaerobically have a different type of *cbb3* that is not targeted by the primer set, (2) the PCR conditions were not optimized for the templates of interest or (3) the colonies were fermenters and would not need a *cbb3* type gene for growth at under low  $O_2$ . All reasons are likely since little is known about the diversity of high  $O_2$  affinity cytochrome oxidases in nature, PCR conditions were based directly on those recommended by Wertz and Breznak (2007) without sample optimization, and media contained many fermentable substrates. *Cbb3* PCR screens would be more useful once use of  $O_2$  for respiration under low  $O_2$  conditions was determined.

Future work to investigate the capillary based isolation of microaerophiles would involve growth curves to distinguish between organisms capable of surviving at low  $O_2$  from those capable of thriving, especially for those *Roseobacter*-like isolates. Direct plating experiments should also run longer than 5 days to make sure that isolates represent both microaerobic fast and slow growers. Defined media would also aid in selecting for microaerophiles with a specific metabolism of interest.

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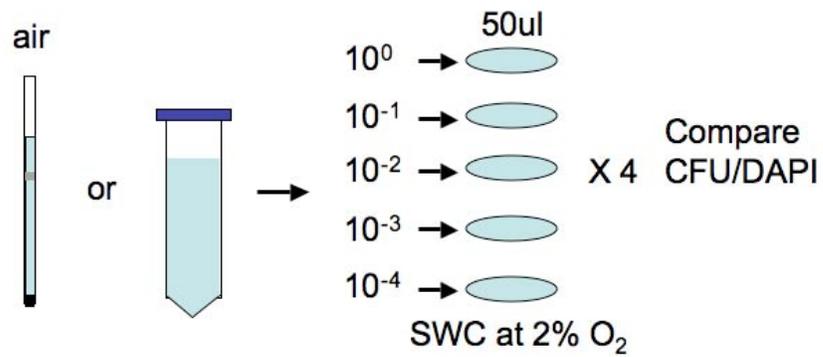
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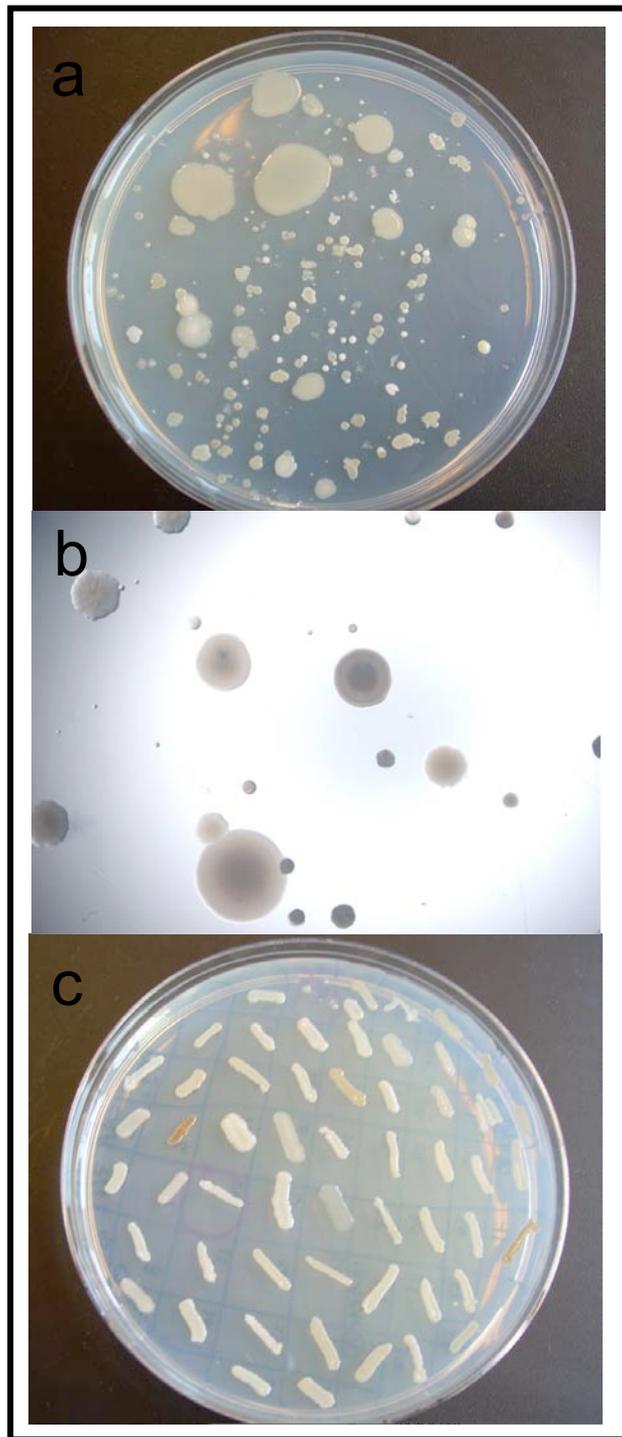
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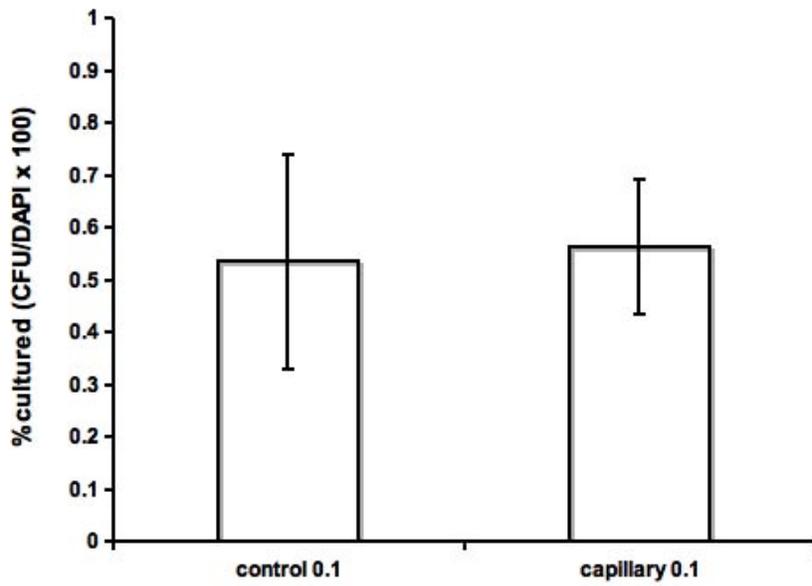
**Fig 1.** *Beggiatoa* rich microbial mat from Little Sippewissett salt marsh. The white sheen results from the predominance of filamentous microaerophilic *Beggiatoa*.



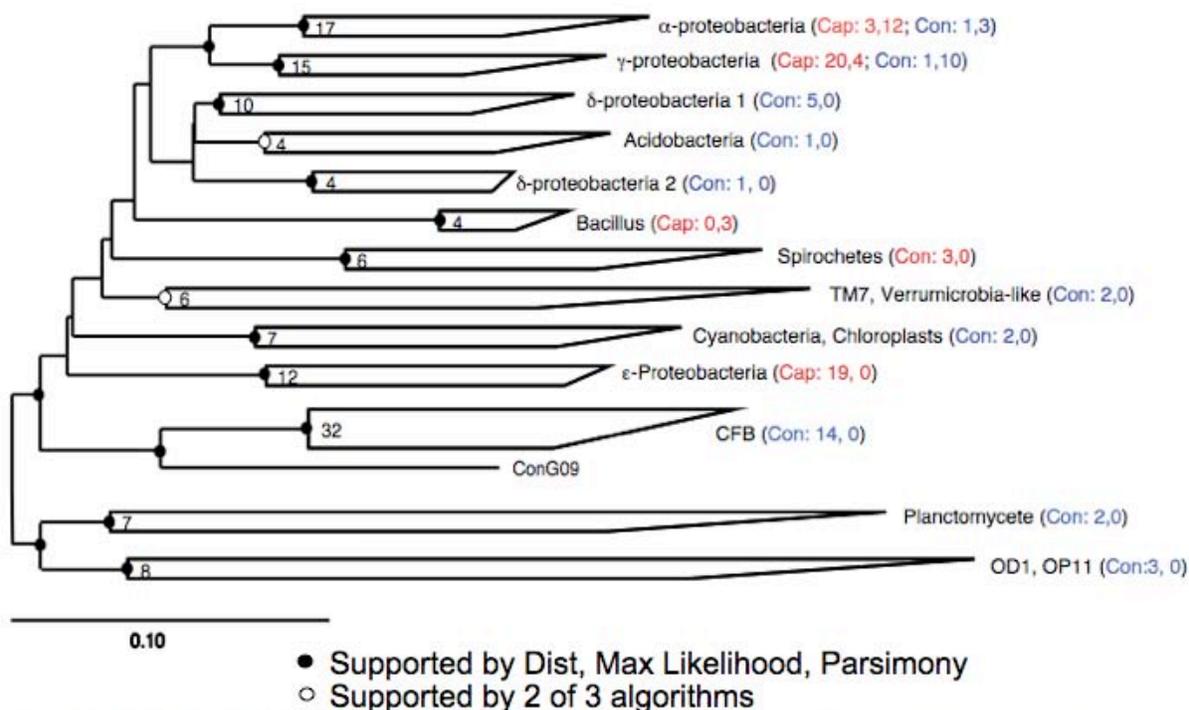
**Fig 2.** Direct plating from capillary or control mat liquid samples onto SWC plates. 4 replicate dilution series were performed for capillary and control samples. After 5 days the number of CFUs normalized to DAPI cell counts were compared for capillary and control plates.



**Fig 3.** SWC plate after 5 day incubation at 2% O<sub>2</sub> for (a) 10<sup>-2</sup> dilution of capillary samples (b) same 10<sup>-2</sup> capillary plate at close range (c) day 2 colony picks for isolation.

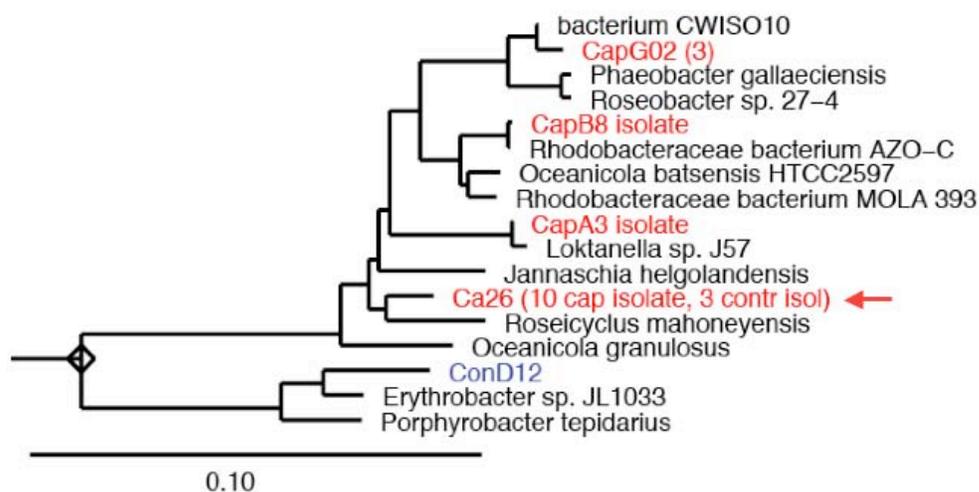


**Fig 4.** Comparison of cultured microaerobic bacteria (CFUs normalized to DAPI) appearing on  $10^{-1}$  dilution SWC plates of capillary and control sample after 5 days of incubation at 2%  $O_2$ .

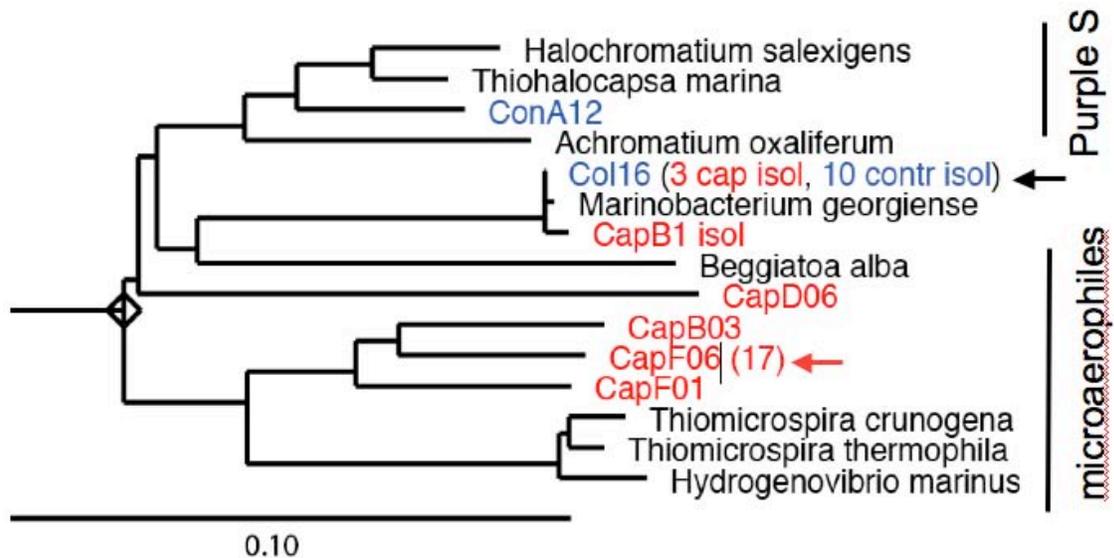


Capillary: Cap ( # clones, # isolates) Control Mat water: Con ( # clones, # isolates)

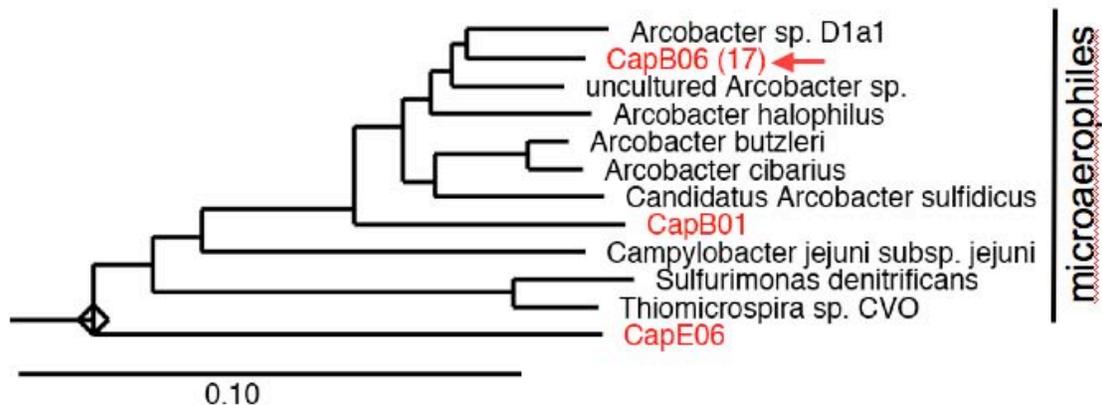
**Fig 5.** 16S rDNA phylogeny of capillary and control mat liquid isolates and clones. Phylogenetic analysis was based on 685 bp.



**Fig 6.** 16S rDNA phylogeny of capillary and control mat liquid isolates and clones falling in the  $\alpha$ -proteobacteria subclass. Most capillary isolates were *Roseobacter*-like and shared only 96% sequence identity with closest pure culture *Roseobacter* isolates.



**Fig 7.** 16S rDNA phylogeny of capillary and control mat liquid isolates and clones falling in the  $\gamma$ -proteobacteria subclass. Most control isolates were *Marinobacterium* species. Capillary clone sequences fell within a clade defined by known microaerophiles. *Thiomicrosira* and *Hydrogenovibrio*.



**Fig 8.** 16S rDNA phylogeny of capillary clones falling in the  $\epsilon$ -proteobacteria subclass. Capillary clone sequences fell within a clade defined by *Arcobacter* species, some of which have been shown to be microaerophiles. *Campylobacter jejuni* is one of the best studied microaerophiles.