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Taxonomy, Biogeography, and Evolution of Polar Gas Vacuolate Bacteria

by

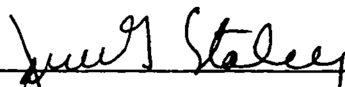
John J. Gosink

A dissertation submitted in partial fulfillment
of the requirements for the degree of

Doctor of Philosophy

University of Washington

1996

Approved by 
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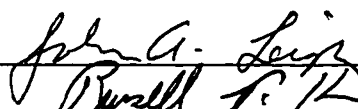

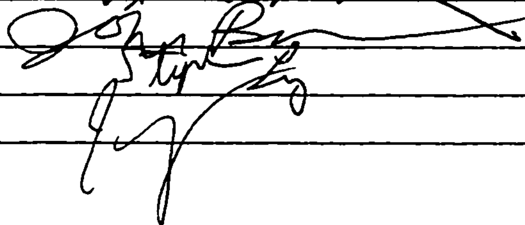

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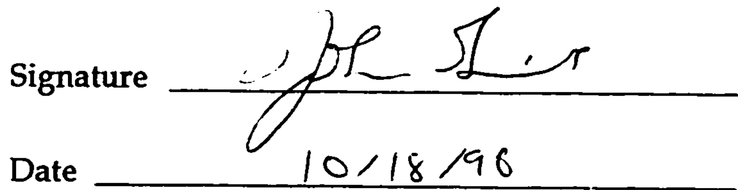

Date 10/18/96

University of Washington

Abstract

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by

John J. Gosink

Chairperson of the Supervisory Committee: Professor James T. Staley
Department of Microbiology

Over 250 gas vesicle producing (gas vacuolate) bacteria have been isolated from arctic and antarctic sea ice and water (Irgens et al., 1989; Staley et al., 1989; Gosink et al., 1993; Gosink and Staley, 1995). These strains are the first reported examples of heterotrophic gas vacuolate bacteria from a marine environment and the first reported examples of gas vacuolate bacteria from the beta Proteobacteria and Cytophaga/Flavobacteria/ Bacteroides (CFB) phylogenetic groups (Gosink and Staley, 1995). Investigation of these isolates tested three hypothesis: (1) that there are several distinct groups of gas vacuolate bacteria associated with the sea ice (2) that the species of gas vacuolate bacteria from the Arctic and Antarctic are biogeographically distinct; and (3) that the gas vesicle phenotype in polar isolates, particularly among CFB members, is the result of a recent horizontal gene transfer event. The results of this study show that several distinct groups of gas vacuolate bacteria can be cultivated from the north and south polar regions, each representing a unique species. Because none of the isolates were of the same species, there is no evidence for cosmopolitan species of polar gas vacuolate bacteria. Due to an unexpectedly high level of species diversity, however, insufficient numbers of any one group were sampled to demonstrate exclusive species in either the Arctic or Antarctic. Analysis of the CFB gas vesicle structural gene (*gvpA*) sequences showed that they formed a monophyletic clade that was not associated with any other *gvpA* sequences. This suggests that *gvpA* was not acquired by a recent horizontal gene transfer event in these species.

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LIST OF ABBREVIATIONS

AODC	Acridine Orange Direct Counts
CFB	Cytophaga/Flavobacteria/Bacteroides
PCR	Polymerase Chain Reaction
R	transition to transversion Ratio
RDP	Ribosomal Database Project
SIMCO	Sea Ice Microbial Communities

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Chapter I. Background

A. Gas Vesicles

Molecular structure and regulation. Gas vesicles are rigid, hollow, biconical, intracellular structures composed entirely of protein and they are found in certain species of Bacteria and Archaea (Walsby and Buckland, 1969). They reduce the overall density of cells which produce them (Figure 1.1). The more gas vesicles present in a bacterium, the less dense it is. This buoyancy control allows a bacterium to position itself in a vertically stratified aqueous environment at a location favorable for growth (Clark and Walsby, 1978).

In all species of gas vacuolate bacteria examined, the major structural subunit protein of gas vesicles is GVPa, a small (approx. 70 kDa) protein encoded by the *gvpA* gene (Marsac et al., 1985). GVPa is one of the most hydrophobic proteins known (Walsby and Hayes, 1989). X-ray diffraction data suggest that GVPa polypeptides assemble in a way to present most of their hydrophobic faces on the inside of the gas vesicle, and their hydrophilic faces on the outside. This makes the interior of the gas vesicle extremely hydrophobic and hence free of water (Walsby, 1994).

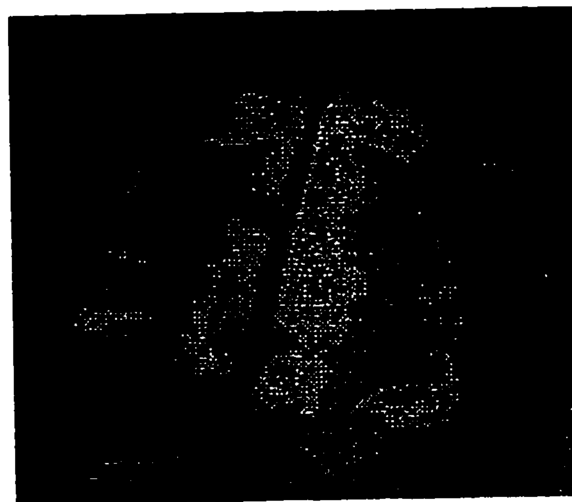


Figure 1.1 Electron micrograph of gas vesicles. Scale bar is 1 μm.

In some species , gas vesicles are also composed of a small amount of a second protein, GVPc encoded by the *gvpC* gene (Damerval et al., 1987). In the only study of the distribution of *gvpC* across species, it was found in 9 of 27 *gvpA*-containing cyanobacterial species examined (Damerval et al., 1989). It has also been found in some species of *Halobacteria*. (Walsby, 1994) GVPc helps to stabilize and strengthen the gas vesicle structure (Hayes et al., 1992). Immunolabelling studies show that GVPc binds to the outside of the gas vesicle but that removing it from the vesicle surface leaves an intact and functional gas vesicle (Walsby and Hayes, 1988; Hayes et al., 1992). Gas vesicles stripped of GVPc, however, are much more susceptible to collapse by external hydrostatic pressure (Hayes et al., 1992). In the Archaean species, *Halobacterium halobium*, *gvpC* is necessary for regulating gas vesicle shape and size (Offner et al., 1996). Offner et al. suggest that *gvpC* serves as a molecular scaffold during gas vesicle assembly (Offner et al., 1996).

Some procaryotes posses additional gas vesicle associated genes. *Halobacterium halobium* has a cluster of 14 genes called *gvpACNODEFGHIJKLM* (Englert et al., 1992). *GvpN* has a nucleotide binding site and the *gvpDE* genes regulate expression of gas vesicle proteins. The functions of the other *gvp* genes have not been examined closely and their roles remain unclear (Walsby, 1994). Some species have more than one copy of the *gvpA* gene, these extra copies have been referred to as *gvpB*, or *gvpA1*, A2 etc. The nucleotide sequence of these different copies are either identical or very similar to each other within a species (Marsac et al., 1985; Damerval et al., 1987; Damerval et al., 1989; Hayes, 1992).

Gas vesicle formation is regulated by the bacteria as it responds to its environment, although little information exists regarding the mechanisms or inducers of *gvp* expression. Because a variety of gas vacuolate bacterial species can accumulate at different levels in the same water column (Clark and Walsby, 1978), it is hypothesized that genetic regulation of gas vesicle formation varies from species to species. In *Halobacterium halobium*, gas vesicle formation is regulated by a high rate of chromosomal rearrangements or deletions; thus, gas vesicle-producing colonies spontaneously give rise to colonies lacking gas vesicles and vice versa (DasSarma, 1989). Some cyanobacteria appear to regulate their gas vesicle formation in response to light levels (Damerval et al., 1991). Konopka et al. demonstrated that

Ancylobacter aquaticus gas vesicle formation is inhibited by the presence of lysine (Konopka, 1977).

Gas vesicle function and ecology. Gas vesicles function by forming extremely hydrophobic water exclusion spaces within the cell. While gas vesicles have intermolecular gaps of at least 0.63 nm, large enough to permit the passage of water molecules, the extreme hydrophobicity of the proteins keeps water out. Gas molecules, however, are free to diffuse to the interior of gas vesicles; thus, the inside of a vesicle is filled with those gases that are dissolved in the cytoplasm, each at its respective partial pressure (Walsby, 1969). A gas molecule has a typical residence time within the gas vesicle of just 0.4 μsec (Walsby, 1994).

Gas vesicles only function at relatively shallow depths in the water column. As shown in Figure 1.2, the forces acting to collapse a gas vesicle inside a cell include the atmospheric pressure above the water column (P_a), the hydrostatic pressure of the water column (P_h), and the cell turgor pressure (P_t). Counteracting these forces are the structural rigidity of the gas vesicle, and the partial pressure of the gases within the vesicle (P_g) (Walsby, 1971). The structural rigidity of the gas vesicle is determined by the molecular nature of the bonds holding the structural proteins together and by the gas vesicle diameter. Gas vesicle diameters range from 45 nm in a species of *Trichodesmium* to 117 nm in a species of *Aphanothece* (Walsby, 1994). In general, gas vesicles with thin diameters are stronger than those with wide diameters (Walsby, 1971). Typical pressures required to crush gas vesicles vary from 0.1 to 1.3 Mpa (Walsby and Bleything, 1988) with some *Trichodesmium* species reaching 3.7 Mpa. These values correspond to depths of 10 to 370 meters in a water column (Walsby, 1994). Therefore, gas vacuolate bacteria would not be expected as indigenous inhabitants of deep oceanic waters.

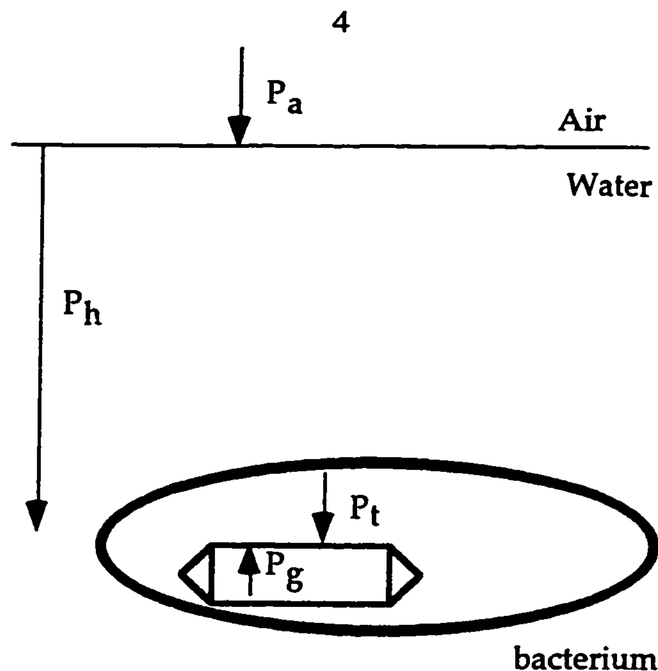


Figure 1.2. Forces acting on a gas vesicle within a cell. If $P_a+P_h+P_t-P_g >$ structural rigidity of the the gas vesicle, then it will collapse. Adapted from Walsby (Walsby, 1971).

The role played by gas vesicles in the life cycle of a bacterium depends on its ecology. For some groups of bacteria, such as oxygenic cyanobacteria, the ecological goal is to reach the upper layers of the water column to take maximum advantage of sunlight for photosynthesis (Walsby, 1994). For others such as the purple and green sulfur bacteria, the optimal depth in the water column may be a several meters below the surface in the anaerobic hypolimnion, where there is still sunlight filtering from above and hydrogen sulfide available from below (Pfennig, 1967; Clark and Walsby, 1978). One species of gas vacuolate *Clostridium* found in anaerobic sediments is thought to produce gas vesicles as a dispersal mechanism for its spores (Oren, 1983). In each case the role of the gas vesicles are the same, buoyancy regulation. However, the factors regulating the formation of gas vesicles, and effect of gas vesicle formation on the cell *in situ*, may be very different.

Evolution of Gas Vesicles. Gas vesicles are found in a wide variety of Archaea and Bacteria. Curiously, their appearance in specific bacterial groups appears sporadic. Table 1.1 is a list of known gas vacuolate bacteria.

Unfortunately, the phylogenetic identity of a number of these organisms remains to be defined, thus leaving the true extent of the gas vesicle phenotype across procaryotic phylogeny unknown.

Table 1.1. Gas Vacuolate Genera (from Krieg and Holt, 1984; Balows et al., 1992; Walsby, 1994)

Nonmotile curved rods
Ancylobacter (Microcyclus)[1]
Meniscus
Brachyarcus
Enhydrobacter

Aerobic Rods and Cocci
Aquabacter[1]
Lampropedia

Gram Positive Rods
Clostridium[1]
Desulfotomaculum[1]

Chromatiaceae
Lamprobacter
Lamprocystis
Thiodictyon
Amoebobacter
Thiopedia

Ectothiorhodospiraceae
Ectothiorhodospira[1]

Cyanobacteria
Dactylococcopsis
Microcystis[1]
Coelosphaerium
Spirulina[1]
Oscillatoria[1]
Trichodesmium
Pseudanabaena[1]
Anabaena[1]
Anabaenopsis
Nodularia[1]
Cylindropspermum
Nostoc[1]
Calothrix[1]
Gloeotrichia
Prochlorothrix[1]

Fe depositing bacteria
Siderocapsa
Ochrobium

Budding/Appendaged
Ancalomicrobium
Prosthecomicrobium
Stella[1]
Isosphera[1]

Sheathed
Leptothrix[1]

Nonphotosynthetic gliders
Pelonema
Peloploca
Achroonema

Green Bacteria
Pelodictyon
Ancalochloris
Choroherpeton
Clathrochloris[1]
Chlorochomatium
Chloroplana
Cylindrogloea
Chloronema[1?]

Others
Desulfobacterium
Desulfovibrio

Archaea (Euryacheaota)
Methanogens
Methanosarcina[1]
Methanothrix[1]

Extreme halophiles
Halobacterium[1]
Haloferax[1]
Natronobacterium[1]

[1]Species of these genera have been classified by 16S rDNA sequencing and phylogenetic analysis.

Two conditions must first be met to trace the evolution of gas vesicles via phylogenetic analysis (details of the phylogenetic methods are discussed

later in this chapter). First, the gas vesicles must be the result of homologous gene(s) in each of the test organisms. Second, these genes must show the characteristics of a good molecular chronometer, namely: (a) mutating at a stochastic rate; (b) performing a universal function that is independent of selective pressures; and (c) being appropriately large for meaningful comparison. In the case of gas vesicles, it is known that the first condition is met because the main structural component of all gas vesicles examined so far is GVPa. Both GVPa and the gene that codes for it, *gvpA*, are homologous and highly conserved in all organisms examined (Figure 1.3) (Griffiths et al., 1992). The second condition is met in that *gvpA* has the same function in all organisms examined (Walsby, 1972). Furthermore, although *gvpA* is small (approximately 210 bp), a simple preliminary test demonstrates that it has sufficient length for low resolution phylogenetic analysis.

```
                 ------->                         <------
Aph. AVEKTNSSSSLAEVIDRILDKGIVIDAWVRVSLVGIXLLAIXARIVIASV...
Ana. AVEKTNSSSSLAEVIDRILDKGIVIDAWVRVSLVGIELLAIEARIVIASVETYLKYAEAVGLTQSAAVPA
Cal. AVEKTNSSSSLAEVIDRILDKGIVVDAWVRVSLVGIELLAIEARIVIASVETYLKYAEAVGLTQSAAVPA
pse. AVEKVNSSSSLAEVIDRILDKGIVIDAWVRVSLVGIELLSIEARVVIASVETYLKYAEAVGLTASAAVPAA
Osc. AVEKVNSSSSLAEVIDRILDKGIVIDAWVRVSLVGIELLSIEARIVIASVETYLKYADAVGL...
Spi. AVEKVNSSSSLAEVIDRILDKGIVIDAWVRVSLVGIELLSVEARXVIA...
Mic. AVEKTNSSSSLAEVIDRILDKGIVIDAWARVSLVGIELLAIEARVVIASVETYLKYAEAVGLTQ...
Dac. AVEKTNSSSSLGEVVDRILDKGVVVDLWVRVSLVGIELLALEARVVI...
Amo.AKVANSTDSSSLAEVVDRILDKGIVIDAWVRVSLVGIELLAIEARVVVASVETYLKYAEAVGLTASAAVPAA
Hal.     AQPDSSSLAEVLDRVLDKGVVVDVWARISLVGIEILTVEARVVAASVDTFLHYAEEIAKIEQAELTAGAEAPEPAPEA
```

Figure 1.3. Homology of GVPa in Various Species. Adapted from Griffiths (Griffiths et al., 1992). Arrows reprent the DRILDKG and TYLKAE PCR primers that can be used to amplify an internal region of *gvpA*. Abbreviations are as follows: Aph., *Aphanizomenon flos-aquae;* Ana., *Anabaena flos-aquae;* Cal., *Calothrix* sp.; Pse., *Pseudanabaena* sp.; Osc., *Oscilliatoria agardhii;* Spi., *Spirulina* sp.; Mic., *Microcystis* sp.; Dac., *Dactlyococcopsis salina;* Amo., *Amoebobacter pedens;* Hal., *Halobacterium halobium.*

The phylogenetic relationship based upon 16S rRNA genes of all the major forms of life is shown in Figure 1.4. Phyla within boxes have members which produce gas vesicles. As can be seen from this diagram, 6 of the 11 major radiations of Bacteria, and 1 of the 2 major radiations of Archaea contain members which produce gas vesicles (Walsby, 1994).

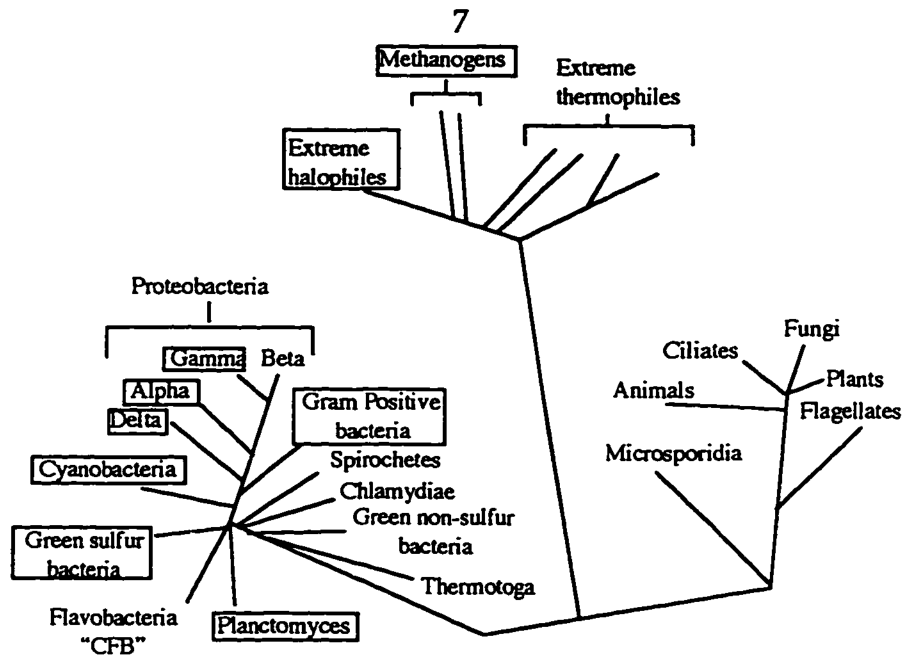
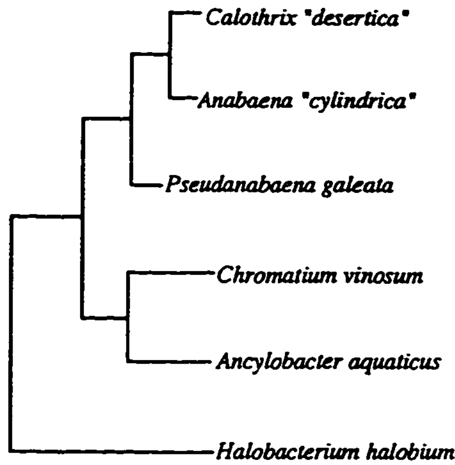


Figure 1.4 Universal phylogenetic tree determined from 16S rRNA sequence comparisons. Groups which have gas vacuolate members are in boxes. Adapted from Woese (Woese, 1987) and Wainright (Wainright et al., 1993).

Two lines of evidence support the view that gas vesicles originated early in evolution, before the divergence of Bacteria and Archaea. First, phylogenetic analysis of GVPa from several species indicates that this gene evolved with these species. The trees in Figure 1.5 are simple phylogenetic reconstructions (unpublished data) based upon 16S rRNA sequences on the left, and GVPa proteins on the right. The corresponding clades in each tree were produced from the sequences of the nearest taxonomic neighbors for which sequence data were available. For example, *Chromatium vinosum* is the nearest taxonomic neighbor of *Amoebobacter pedens* for which 16S rRNA sequence data is available. Unfortunately, protein sequence data provides only low resolution information for phylogenetic inferences. Also, it is not known if the GVPa proteins being compared are orthologs or paralogs of each other.



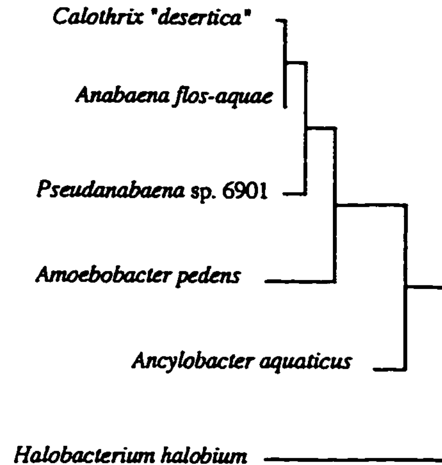


Figure 1.5 A comparison of phylogenetic trees based on 16S rRNA nucleotide sequences and GVPa protein sequences for some species of cyanobacteria, Proteobacteria, and *Halobacterium*. Both analyses were performed using unweighted generalized parsimony. Scale bars represent an average of 0.1 nucleotide or amino acid change per position.

The second line of evidence for the ancient origin of gas vesicles is based on the relative degree of conservation of GVPa and GVPc. GVPc is not required in all gas vacuolate organisms and has no known function other than helping with gas vesicle stability. Since it shows a greater degree of divergence among species than GVPa, it suggests that GVPa arose early but has evolved more slowly (Griffiths et al., 1992)

Inspection of Table 1.1 and Figure 1.4 reveals that although the gas vacuolate phenotype is evolutionarily widespread, relatively few genera actually posses it. The fact that no member of the well-characterized CFB group - or any marine heterotrophic species - has ever been described as gas vacuolate suggests that the phenotype may be the result of horizontal gene transfer. One of the aims of this investigation was to evaluate this hypothesis by examining the nucleotide sequences of *gvpA* from various polar marine gas vacuolate bacteria we have in the laboratory.

B. Polar Sea Ice

The gas vacuolate bacteria in this study were isolated from within and around the annual sea ice of the north and south polar regions (Irgens et al., 1989; Staley et al., 1989; Gosink et al., 1993). Annual sea ice provides a habitat for microbial growth, with sunlight and sea water providing energy for primary productivity and nutrients, respectively (Legendre et al., 1992). Furthermore, sea ice creates a highly stratified marine environment - both at the macroscopic scale (i.e., a solid ceiling at the sea water/ice interface), and at the microscopic scale (brine channels within the ice) (Maykut, 1985). Understanding this environment from a microbial perspective is important in understanding the biogeography and evolution of polar gas vacuolate bacteria. Our studies show that a large number of novel gas vacuolate bacteria thrive in the sea ice of the North and South Pole. Because a large variety of different gas vacuolate bacteria were isolated from the sea ice of the North and South Pole, it is possible that this unique environment provides some selection for gas vesicle production.

Physical structure of sea ice. Twenty three million square kilometers, approximately 6% of the world's surface, is covered with sea ice which melts and refreezes on a yearly basis (Maykut, 1985). Sea ice formation is a multistep process: in early fall, air and surface waters drop below the freezing point of seawater and ice slush, called "frazil ice", forms in thick sheets on the surface of the water. Eventually the frazil ice sinters into a solid layer. As the temperature continues to drop, the ice layer increases in thickness with frazil ice freezing onto the solid ice from below. On the microscopic scale, salt in the form of concentrated brine is excluded from the growing ice crystals. This forms a lattice of brine channels and pockets (Maykut, 1985). In spring, the brine pocket temperatures range from 0° C to -2°C, salinities from 1 to 5 times normal sea water concentration, and light intensities from 0.02% to 0.8% surface irradiance (Horner, 1985).

It is important to note that Earth did not always possess extensive ice caps. As recently as 39 million years ago, the earth's oceans were relatively warm and there was no extensive sea level glaciation (Knox, 1994). By 22

million years ago, global climate changes led to cooling of the earth's surface and the subsequent formation of the polar ice caps (Knox, 1994). Thus, any species or ecological feature specifically adapted to polar sea ice could not be more than 22 to 39 million years old. Since polar ice cap formation, the timescale for water circulation between the Arctic and Antarctic environments is on the order of 1000 years (Broecker and Peng, 1982; Gordon, 1986). Thus, if bacteria were carried from one pole to the other by ocean currents, they would be subjected to centuries in a radically different environment.

Biological structure. Brine channels in the polar sea ice support an extensive population of algae, diatoms, protozoa and heterotrophic bacteria. This Sea Ice Microbial Community (SIMCO) contributes a significant amount to the annual productivity of polar waters (Horner, 1982; Horner, 1985; Legendre et al., 1992). The density of the SIMCO depends on the amount of light penetration through the sea ice. Ice areas covered with snow may have little light penetration and hence a full SIMCO layer will not develop, whereas a patch of ice a few meters away may be free of snow and support an extensive algal bloom and SIMCO layer (Bunt and Wood, 1963; Grossi et al., 1984). When the ice melts in spring, the SIMCO is released into the water where it may account for massive polar phytoplankton blooms (Horner, 1982; Horner, 1985; Legendre et al., 1992).

The identity of the types of bacteria associated with the SIMCO is largely unknown. There have been no systematic studies reported prior to this work. This is surprising because annual sea ice and the SIMCO create ecosystems as large as North America, ecosystems that disappear and reappear yearly. Most microbial sea ice studies have focused either on the taxonomy of eucaryotes such as diatoms and protozoa (Horner, 1976; Grossi et al., 1984; Horner, 1985; Knox, 1994), or upon measurements of microbial activities such as total autotrophic CO_2 fixation in bulk water samples (Holm-Hansen et al., 1977; Hodson et al., 1981; Miller et al., 1984; Kogure et al., 1986; Upton and Nedwell, 1989). Recently, environmental population cloning was carried out to detect archaeal constituents of the SIMCO and polar water (DeLong, et al., 1994). Surprisingly, 16S rRNA sequences similar to those of known

hyperthermophilic Crenarchaeota showed up in large numbers. Other types of sequences detected were similar to moderately thermophilic Euryarcheota.

Previous studies of polar gas vacuolate bacteria. Studies of polar sea ice gas vacuolate bacteria began as a noteworthy side observation to studies of chitinolytic bacteria at Palmer Station, Antarctica in 1986 (Herwig et al., 1988; Irgens et al., 1989). Large numbers of gas vacuolate heterotropic bacteria were cultivated from sea ice and water. This was remarkable in that no heterotrophic gas vacuolate bacteria had been reported from marine environments. Further studies showed that gas vacuolate bacteria were also readily thriving on the other side of the Antarctic continent at McMurdo Station (Table 1.2) (Staley et al., 1989). Most of these bacteria were found in conjunction with the highest levels of chlorophyll *a* in the ice. Furthermore, most (92-100%) of the culturable bacteria from the ice column, including the gas vacuolate strains, were pigmented.

Table 1.2 Distribution of Gas Vacuolate Bacteria from McMurdo Sound in December of 1987[a]. The total number of culturable bacteria per ml of sea water or melted sea ice/percent of culturable bacteria which were gas vacuolate are indicated.

Source and depth	Site 1	Site 2	Site 4	Site 5	Site 6	Site 7
Ice 10 -40 cm	7 / 0	104/15	360 / 83	466 / 91	650 / 0	121 / 0.5
Ice 0-20 cm	87 / 0.6	1520 / 0	6280 / 0.3	11.5 / 92	5170 / 0.06	6000 / 5.4
Platelet ice or water (0-1m)	41 / 0	7065 / 0.3	120 / 16.7	30 / 4.2	10120 / 0	2300 / 9.6
Water 3-6 m	145 / 0.2	64 / 0	58 / 2.8	14 / 3.6	144 / 0	64 / 0
Water 18 - 19 m	187 / 0	26 / 1.9	47 / 0	22 / 0	30 / 0.18	47 / 1.1

a Data from (Staley et al., 1989)

Unpublished, preliminary phenotypic and phylogenetic analyses indicated that some of these isolates belonged to the beta Proteobacteria and CFB groups (Davis, 1990; Woese, 1991), but definitive taxonomic studies were not undertaken. One aim of this project was to taxonomically classify a number of the polar marine gas vacuolate strains. If some of these strains were definitively shown to belong to the beta Proteobacteria and CFB, it

would be the first example of gas vacuolate members of these groups. As discussed previously, this would also raise questions about the evolutionary origin of the gas vesicle phenotype in these strains.

C. Horizontal gene transfer

The discovery that a variety of marine bacteria, particularly members of the CFB phylogenetic group, appear to harbor gas vesicles is novel. This observation suggests two possibilities: (a) the gas vesicle genotype was inherent to ancestral CFB but that it has been lost from most members during the evolution of this group; or (b) the gas vacuolate sea ice isolates acquired the gas vesicle genes from other bacteria in the relatively recent past via horizontal gene transfer.

Horizontal gene transfer has been demonstrated for a large number of different genes across a wide variety of microorganisms (Mazodier and Davies, 1991; Smith et al., 1992). In the laboratory it is possible to transfer genes across species, genera, phyla, and domains of procaryotes. In fact, recombinant genetic techniques between radically different types of bacteria are now increasingly commonplace (Ausubel et al., 1989), with chimeric gene splicing and recombinant DNA technology forming a major industry.

Mechanisms. Horizontal (or lateral) gene transfer is the act of moving genetic material from one species to another by a process other than normal cell division. Horizontal gene transfer occurs by one of three ways: transduction, transformation, or conjugation (Brock and Madigan, 1991; Mazodier and Davies, 1991).

In transduction, DNA is transferred via a bacteriophage intermediate. Transduction is either specialized or generalized. With specialized transduction, chromosomal DNA from a specific region, typically less than a few thousand bases flanking the viral integration site in the donor cell, is transferred. In generalized transduction, DNA from anywhere on the donor cell chromosome is transferred to the recipient, due to spurious viral packaging or nonspecific integration. For transformation, naked DNA, typically from a lysed donor cell, is transferred to the recipient. In

conjugation, DNA is transferred via a specific sex pilus. This requires an appropriate donor cell, the genes of which are often encoded on a conjugative plasmid such as RSF1010, or the F-plasmid (Brock and Madigan, 1991; Mazodier and Davies, 1991).

Obstacles exist to transferring and successfully expressing genes in foreign hosts. DNA in the environment is subject to degradation by nucleases and/or binding to humic compounds. Potential recipient strains must be competent (in the case of transformation) or express the appropriate receptors (in the case of transduction) (Saunders et al., 1990; Schmieger, 1990). After donor DNA has entered the recipient, there are other barriers to its integration and expression including: DNA methylation and restriction systems, incompatible host recombination machinery, incompatible transcriptional and translational signals, differing codon preferences, post-translational protein modification systems, and lack of accessory genes relevant to the processing or functioning pathway of the transferred gene product (Saunders et al., 1990; Neilson et al., 1994).

Ecological considerations can also block the successful propagation of transferred genes. In general, high selection pressures and extensive bacterial turnover from grazing and viral attack vastly reduce the chance of a single bacterium fixing its genotype in the population (Saunders et al., 1990). Also, most bacteria in natural environments are non-dividing and, thus, transferred genes would not be propagated (Gauthier and Breittmayer, 1990; Saunders et al., 1990). Finally, if the recipient cell is dividing, bacterial microspecialization may cause the transferred genes to be a metabolic liability to the physiology and ecology of the recipient (Fry and Day, 1990). Nevertheless, the natural environment is vast (23 X 10^6 km^2 for the annual sea ice) (Maykut, 1985) and it can be estimated that the total bacterial population in the world's oceans is greater than 10^{30} individuals (Weast and Astle, 1982; Fuhrman et al., 1988). It is not surprising, then, that there are known examples of horizontal gene transfer in existing species of procaryotes.

Observations. Demonstration of horizontal gene transfer in the environment can be divided into two classes: *in situ* experiments and "natural" phylogenetic reconstructions (Saunders et al., 1990). With *in situ* experiments, genetically engineered microorganisms (GEMs) are added to

microcosms. Transfer of GEM marker genes to other organisms is monitored by periodic sampling of natural bacterial cohabitants (Saunders et al., 1990). Such experiments have demonstrated that gene transfer *in situ* is possible. For example, Neilson and coworkers (Neilson et al., 1994) transferred a large catabolic plasmid between two genera of bacteria in soils. Similar results have been generated among aquatic organisms, as reviewed in Fry (Fry and Day, 1990).

Natural phylogenetic techniques are used to trace the evolutionary history of genes in existing strains of microorganisms. Briefly, a phylogenetic tree is constructed using the gene in question from a large number of bacterial isolates. Horizontal gene transfer is indicated if this tree is substantially different from a phylogenetic tree constructed from standard genes such as 16S rRNA. Such reconstructions effectively illustrate that antibiotic resistance determinants have been transferred between different bacterial species (Mazodier and Davies, 1991) and kingdoms (Brisson-Noël et al., 1988). On a larger evolutionary scale, horizontal gene transfer has occurred even between procaryotes and eucaryotes with the genes that encode for glyceraldehyde-3-phosphate dehydrogenase, glucose-6-phosphate isomerase, Fe-superoxide dismutase, and fructose-1,6-bisphosphate aldolase (Smith et al., 1992).

D. Phylogenetic analysis

In addition to defining the history of genes in existing bacteria, phylogenetic analysis also serves to determine the evolutionary relationship between test organisms (taxa). Phylogenetic analysis is based on a group of philosophically different methods which are used to infer evolutionary relationships. All methods are based on examining features (characters) shared by the set of taxa being studied. By closely and objectively examining homologous features for each study taxon, one can infer a hypothesis about the order of divergence of the taxa (Swofford and Olsen, 1990). Other phylogenetic methods are designed to test hypothetical phylogenetic "trees" and determine how probable each is given the dataset (Felsenstein, 1985; Kishino and Hasegawa, 1989). At first, phylogenetic methods may seem

obscure to many experimental biologists because analyses do not involve physical manipulation of the test organisms. However, they are scientific methods of inquiry because they propose alternate hypotheses (trees) and then compare them with objective criteria for failure (Penny et al., 1991).

Nucleotide (and indirectly, amino acid) sequence data are the most common features examined in phylogenetic analyses today (Swofford and Olsen, 1990). Each nucleotide position of a given gene can be considered a different feature (Maddison and Maddison, 1992; Olsen and Woese, 1993). By comparing the same gene from different organisms, one is actually comparing hundreds or thousands of separate features (characters). The choice of a homologous molecule for phylogenetic analysis is determined by three basic criteria: that they (a) perform the same function in compared organisms; (b) mutate stochastically so as to demonstrate some variability between organisms; and (c) are large enough to generate meaningful comparisons. The most widely used gene to define bacterial phylogeny is the small subunit, or 16S, ribosomal RNA (rRNA). 16S rRNA, found in all bacteria, performs the same function in translational machinery. At approximately 1500 bases, the 16S gene is of reasonable size, having both variable and conserved domains to facilitate comparison between closely related and divergent bacteria. The interspersed, conserved regions are also valuable for aligning multiple sequences. A number of other genes and protein sequences also fulfill these criteria.

Additionally, it is important to conduct analyses on *orthologous* and not *paralogous* sequences (Miyamoto and Cracraft, 1991). Paralogous genes are the result of a gene duplication event that occured *before* a speciation event. As shown in Figure 1.6, phylogenetic trees drawn from paralogous sequence pairs will give topologies reflecting the gene duplication event, not the speciation event. Sequence paralogy cannot be discerned directly from sequence data; multiple copies of the gene are indicated by Southern hybridization, long range PCR, or sequencing multiple gene clones from the same organism. For 16S rRNA sequences, it has been shown that different copies within the same species have identical, or very similar sequences (Cillia, 1996; Clayton, 1995;Maidak, 1996). The work of Clayton et al. suggests that strain mishandling and mixup, interoperon differences, and between strain variation in 16S rRNA sequences may lead to most of the differences in

reported 16S rRNA sequences (Clayton, 1995). These differences are usually not sufficient to lead to phylogenetic misplacement of bacterial genera. Thus, paralogy is not a significant concern in 16S rRNA phylogenetics when considering cross genus and cross division horizontal gene transfer.

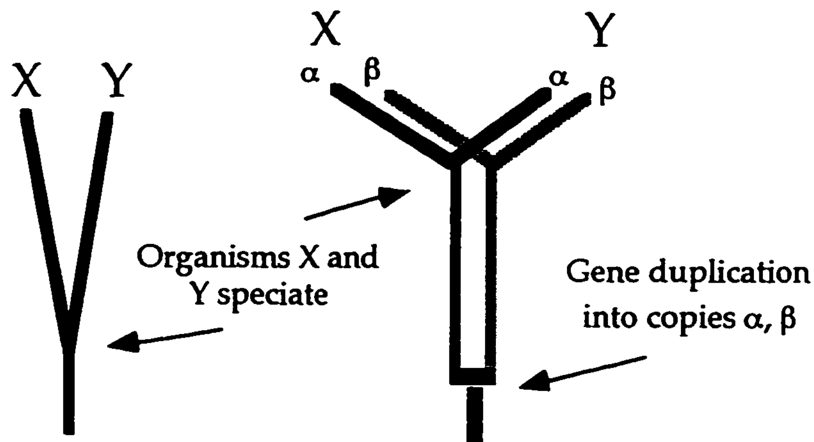


Figure 1.6 Orthology vs. paralogy in phylogenetic reconstructions. In the left hand figure there is only one copy of the gene in organisms X and Y. In the right hand figure the gene underwent a duplication event long before the organisms speciated into X and Y. An evolutionary tree based on gene copy α in organism X, and gene copy β in organism Y would not reflect the species evolution of X and Y as it would be based on paralogous gene copies

Data requirements for analysis. The first step in any phylogenetic analysis is to align the nucleotide (or amino acid) sequences of each specific gene with one other. While seemingly a trivial matter, it is quite difficult to obtain a high quality alignment of sequences from moderately different organisms (Olsen and Woese, 1993). Incorrect alignments may introduce small but false assumptions into the analysis; comparing even one incorrectly aligned nucleotide position would be as meaningless as comparing an oxidase reaction in one isolate to the amylase activity in another. A detailed, mathematical discussion of all the of the methods used to assure a good alignment is outside the scope of this dissertation (specific methods used in this work are detailed in Chapter 2). Some basic considerations will be summarized below.

In phylogenetic analysis, it is only meaningful to compare homologous features. Alignment becomes difficult when one sequence has a stretch of

nucleotides with no corresponding homologs in the other sequences in the dataset. Similarly, regions in the dataset may exist for which all of the taxa are evolving at a high rate and it is impossible to determine which nucleotides correspond to each other. Thus, nucleotide positions that have no homolog or cannot be unambiguously aligned with each other, should not be used in the analysis (Olsen and Woese, 1993).

Another confounding factor in the phylogenetic analysis of diverse species of bacteria is the effect of mole percent G+C bias in the species being compared (Woese et al., 1991). Taxa which have a significantly higher mole % G+C than other taxa in the dataset will be "unnaturally" driven to appear on the same branches of a phylogenetic tree. This propensity can be negated by "weighting" A or T ⇔ G or C substitutions compared with A ⇔ T, or G ⇔ C substitutions (Swofford and Olsen, 1990; Woese et al., 1991). In the case of protein coding regions, DNA sequence data can be converted to protein sequences and then phylogenetic methods applied. Alternatively, the contribution of second and third codon position nucleotides can be reduced or eliminated from the analysis as these positions are most likely to reflect the mole % G+C composition of the species (Swofford and Olsen, 1990).

A final data requirement is the size of the dataset. Efficiency analyses show that increasing the number of nucleotides in the dataset increases the probability of finding the correct tree (Hillis, Huelsenbeck et al., 1994; Hillis, Huelsenbeck et al., 1994). Generally, there must be more informative character positions than there are taxa (Stewart, 1993; Stewart, 1994). Informative character positions are those in which at least two of the species being analyzed share a unique nucleotide relative to the other taxa. For example, in Figure 1.7 positions 1, 2, and 3 are informative sites. Site 4 will always require a single step on all trees and therefore does not help to distinguish the most parsimonius tree. At the other extreme, incorporating too few taxa in the analysis makes the problem too simple. As shown in Table 1.3, unrooted trees for one, two, and three taxa each only have one shape. Four taxa can be joined in only one of 3 different ways. Phylogenetic analysis applied to such small datasets does not strongly fulfill the scientific goal of discarding alternate hypotheses (Platt, 1964).

Table 1.3. Number of unrooted trees by number of taxa

# of taxa	# of unrooted trees
1	1
2	1
3	1
4	3
5	15
6	105
7	945
8	10,395
9	135,135
10	2,027,025
15	$\sim 8 \times 10^{15}$
20	$\sim 2 \times 10^{20}$

From Felsenstein class notes (Felsenstein, 1996).

Phylogenetic methods. Once sequences are properly aligned, they can be evaluated by phylogenetic methods. There are three major classes of phylogenetic analysis commonly used today: parsimony, distance, and maximum likelihood methods. Each will be discussed in full below.

Parsimony methods. The first class of phylogenetic methods, parsimony methods, work by calculating the total number of expected mutational events required to fit the dataset to a given tree (Camin and Sokal, 1965). In effect, each hypothetical ancestral mutation needed to account for the sequences in the dataset is mapped to the tree. This is depicted in Figure 1.7. Different tree topologies will require mapping more or fewer ancestral mutations to the tree to take into account the dataset. As can be seen in Table 1.3, many different shapes of trees can be constructed which join taxa in a given dataset. The key to parsimony is to search through all of these alternate topologies, measuring the number of hypothesized steps for each tree, and then choose the shortest overall tree. As with distance methods, "weighting schemes" can be applied to the analysis to account for different rates of the various nucleotide substitutions (Maddison and Maddison, 1992).

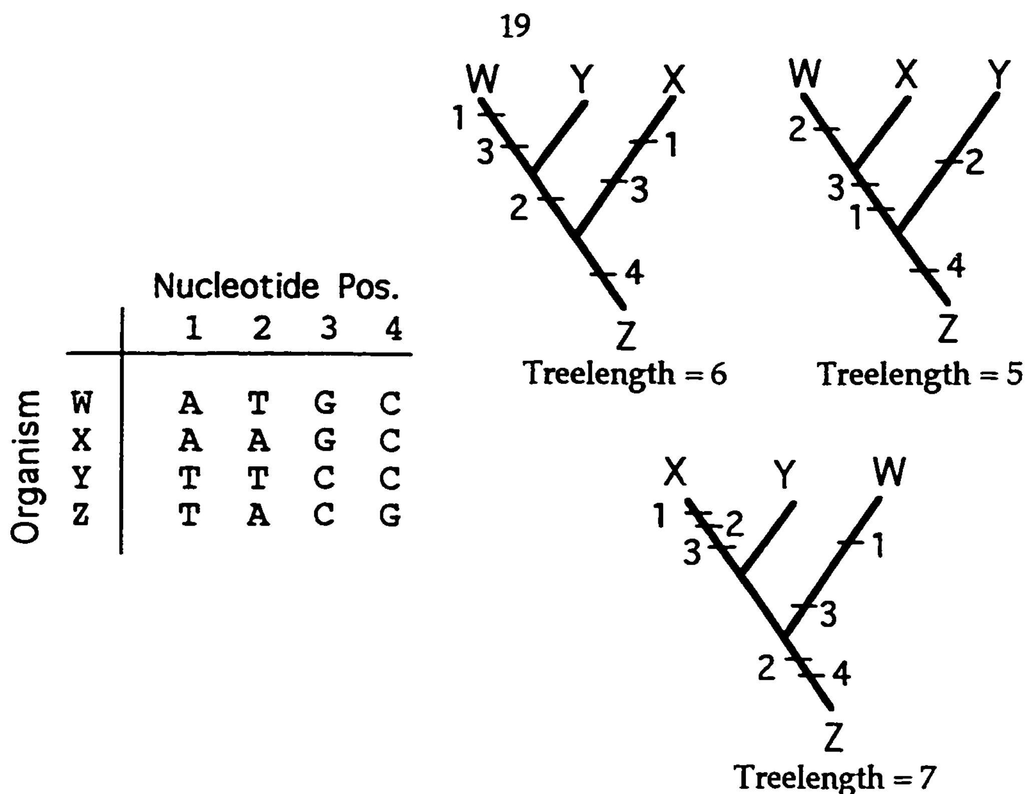


Figure 1.7 Parsimony analysis is based on the concept that the tree which requires the fewest number of assumptions is the tree which is most likely correct (also Ockham's Razor). In this case, the assumptions are the changes needed to convert one sequence into another. In the example below, 4 different organisms (W, X, Y, and Z) have the indicated nucleotides at postions 1 through 4. Hypothetical past mutations are indicated by horizontal tick marks numbered with the nucleotide position which has mutated. The tree on the upper right would be the most parsimonious tree as it requires only 5 hypothetical past mutations as compared to 6 on the left, or 7 below.

Distance methods. The second class, distance analysis, uses a two-step process (Cavalli-Sforza and Edwards, 1967; Fitch and Margoliash, 1967). In the first step, the number of differences between each possible pair of sequences of the aligned dataset are determined, yielding a distance matrix of pairwise scores. In the second step, a tree is generated linking together pairs (or groups) of taxa with branch lengths proportional to the distance (or, average distance for groups) between them.

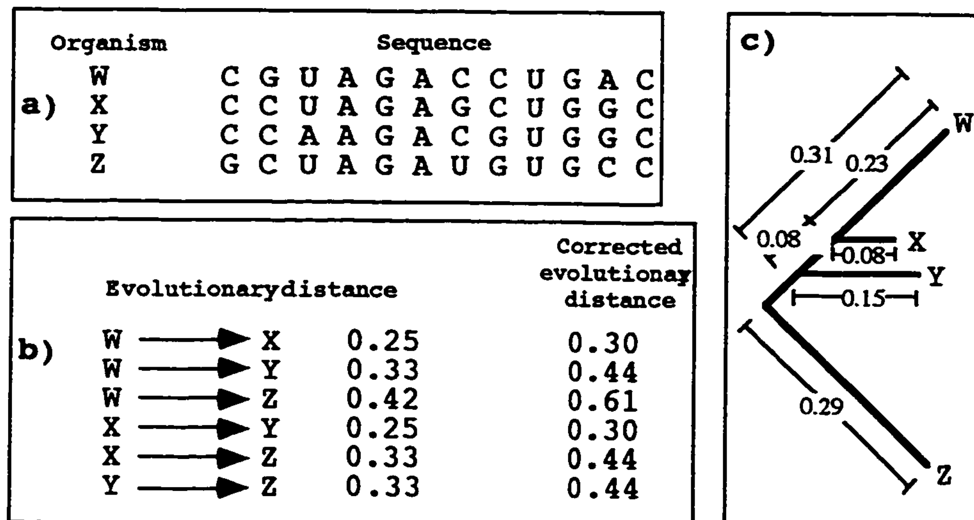


Figure 1.8 Example of a distance matrix analysis of a gene in four different species. Only short sequences are used, for illustrative purposes. The evolutionary distance shown in (b) is the percent of nonhomologous nucleotide positions between the two species. The corrected distance is a statistical correction necessary to account for either back mutations to the original genotype or additional forward mutations at the same site. A tree (c) is ultimately generated by linking together the most closely related pairs of species. Groups of linked species can be joined to other species by considering the distance between the group and the species to be added as the average distance of the individual members of the group and the added species. (Reprinted with permission of the author from The Biology of Microorganisms (Brock and Madigan, 1991)).

Notice in Figure 1.8 the column of corrected pairwise distances. This is a correction for the possibility that a nucleotide position in one of the sequences having mutated twice, the second time having mutated back to the original nucleotide. Because of this, the raw pairwise scores will always be a slight *underestimate* of the true number of mutations that actually occured. Corrections are also needed to take into account the uneven rate of transitions to transversions or any of the 12 types of nucleotide substitutions possible. Models that take these factors into account to produce corrected pairwise distances tables include the Jukes Cantor model (Jukes and Cantor, 1969), the Kimura 2-parameter model (Kimura, 1980), and the F84 and HKY models (Kishino and Hasegawa, 1989; Felsenstein and Churchill, 1996).

In a separate process, trees are generated by linking species into groups. The most closely related pair of species are linked first. The distance matrix is then recalculated with the linked species considered as a single group. The distance between this group and each of the other species in the distance matrix is given by the average of the distances to each of the members in the group. This process is repeated until all the species and groups of species have been joined together. Careful examination of Figure 1.8 will reveal that no tree topology will exactly match the corrected pairwise distances. Several methods exist to find trees that minimize this discrepancy: the least squares method (Fitch and Margoliash, 1967), UPGMA clustering (Michener and Sokal, 1957), and the Neighbor-Joining method (Kishino and Hasegawa, 1989; Felsenstein and Churchill, 1996).

Maximum likelihood methods. The third major class of phylogenetic analyses is the maximum likelihood methods (Felsenstein, 1981). Maximum likelihood is rooted in the field of statistical mathematics. Operationally, maximum likelihood methods are very complex. First, a tree topology and model of DNA evolution (e.g. transitions are 2.3 times more frequent than transversions) are chosen. Next, the observed nulceotides at a specific position in the aligned dataset (e.g. nucleotide position 547) are mapped to the tree (Figure 1.9). The hypothetical nucleotides at that position for each of the hypothetical ancestral sequences joining the observed taxa are then determined. The likelihood of the observed nucleotides (at position 547) are then calculated as the product of each of the individual likelihoods at each of the interior nodes. The overall likelihood for a given topology is scored as the product of the likelihoods for each of the nucleotide positions in the dataset. By repeating this process for all possible tree topologies one can find the tree which has the highest likelihood of having "given rise to the data" under the starting model of evolution. Because so many products must be taken, this method is computationally expensive (Felsenstein, 1981). It does, however, lend itself to a logically justifiable and statistically rigorous evaluation (see the Kishino Hasegawa test, below).

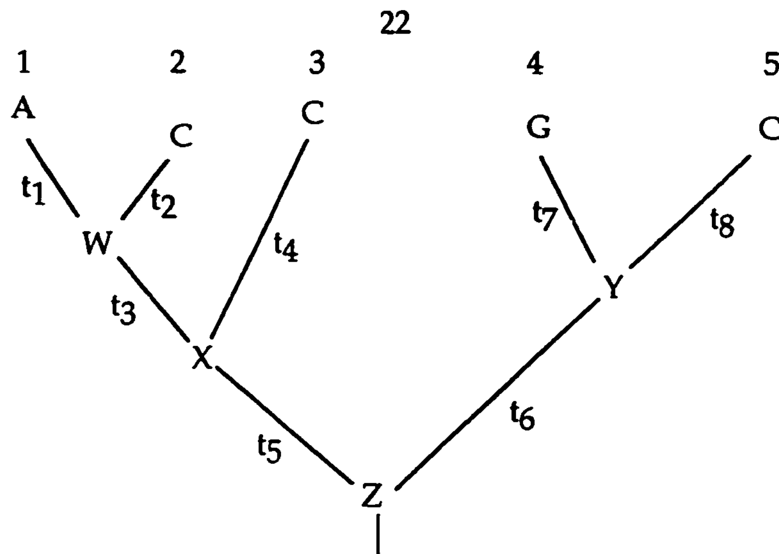


Figure 1.9 A maximum likelihood analysis for five sequences. In this tree the organisms numbered 1 through 5 each have the nucleotide shown at the tips of the branches at some particular site (e.g. position 547) in a gene. The likelihood of this tree topology is calculated by computing the probability that the A and C of organisms 1 and 2 would give rise to each of the four nucleotides at node W (t_1 times t_2). Given that organism 3 has a C, and node W has the four possibilities just calculated, the distribution of possible nucleotides at node X is calculated as t_3 times t_4. The same process is used to determine the probability of each of the four nucleotides at Y and Z. The overall likelihood of the tree at this position (e.g. 547) is the product of t_1, t_2, t_3, t_4, t_5, t_6, t_7, and t_8.

Tree validation. Trees produced from each of the three methods described above (distance, parsimony, and likelihood) are subject to testing by reanalysis using "bootstrap" methods (Felsenstein, 1985) and/or the Kishino-Hasegawa test (Kishino and Hasegawa, 1989). These tests check the "robustness" of the tree hypothesis made by the phylogenetic analysis.

Bootstrap reanalysis creates a series of datasets from the original dataset (typically 100 or 1000), each with the same overall phylogenetic "signal" and "noise" (Felsenstein, 1985). This is done by randomly copying columns from the original dataset, each column representing an alignment at a single nucleotide position. In the process of random sampling, some columns of the original dataset may be missed, and others maybe drawn repeatedly. After constructing a number of bootstrap datasets (e.g. 100), a computer program is directed to analyze each one in the same manner that the original dataset was analyzed. If a portion of the original tree is frequently reproduced from the

bootstrap datasets, then that portion is said to have strong support. The underlying premise is that if the dataset has a strong phylogenetic "signal" for a certain tree topology throughout its length, then the same tree topology should be derived even if some of the data columns are missing or others are duplicated.

The second type of test for tree robustness, the Kishino-Hasegawa test, is designed for use with trees generated by the maximum likelihood method (Kishino and Hasegawa, 1989). This test examines alternate trees produced by the same dataset and measures the probability that one tree is statistically more likely than another. Often in large datasets, or datasets with a number of very similar taxa, several trees can be produced which have nearly the same likelihood scores. The Kishino-Hasegawa test indicates whether there is any statistical justification for choosing one of these slightly different topologies over the other.

E. Biogeography

Obligate pathogens and symbionts are restricted to the range of their hosts, however it has long been held that free-living bacterial species have an unlimited biogeographic distribution. Baas-Becking stated over 60 years ago the hypothesis that "everything is everywhere: but the milieu selects — in nature and in the laboratory." (Bas-Becking, 1934). According to this hypothesis a specific bacterial habitat anywhere in the world, under the same conditions, will be filled with the same species of bacteria. This is possible because bacteria are small, hardy, and easily disseminated. Only a small particle of matter needs to be moved from one location to another to bring along an inoculum for the new area. The short generation times of most procaryotes allow them to rapidly colonize any new habitat. Surprisingly, there have been few attempts to confirm or deny this hypothesis. However, several recent studies have started to shed light on this topic.

Castenholz reported that a species of thermophilic cyanobacteria was isolated from several North American hotsprings but that it was not isolated from hotsprings in northern Alaska and Iceland (Castenholz, 1995). Although the habitats were the same, Castenholz speculated that the

cyanobacteria had not yet colonized the northern hotsprings because it is difficult for thermophilic species to travel from one hotspring to the next, and the relatively short time since the last ice age may not have been sufficient to allow colonization of these springs. Others have suggested that small differences in the habitat may lead to the presence of different types of bacteria, most of which have not yet been cultured and characterized (Ruff-Roberts et al., 1994). Indeed, 16S rRNA molecular probe-based studies have indicated that bacterial microspecialization may lead to different species of bacteria occupying similar niches in very close proximity to each other (McArthur. et al., 1990; Wood and Townsend, 1990; Britschgi and Giovannoni, 1991; DeLong, 1992; Ruff-Roberts et al., 1994).

Recent studies have also revealed the presence of similar 16S rDNA sequences in oil fields in widely disparate locations (L'Haridon et al., 1995). Margot (Margot, 1996) found 16S rDNA sequences in oil fields in the Paris Basin, France that were 98.2% similar to sequences from oil fields in the Cameroons, Africa. Others have shown, however, that bacterial species which are 99.5% identical in 16S rDNA sequence can have as low as 25 to 50% DNA/DNA reassociation (Fox et al., 1992; Stackebrandt and Goebel, 1994). Since 70% DNA/DNA reassociation is considered the standard for strains of the same species (Wayne et al., 1987), the sequences obtained from the oil fields may well represent different species.

In a more systematic study, it was found that different types of 3-chlorobenzoate degraders were isolated from from five sites on each of 4 different continents (Tiedje et ai., 1994). Approximately 600 strains were grouped on the basis of restriction fragment length polymorphisms and other molecular techniques. Similar or identical types of strains were isolated from different areas on the same continent, however different continents had only different types of strain. This suggested that microbial dispersal is not uniform and that endemic species may exist in some areas.

In contrast, later studies in Tiedje's lab indicated that strains of denitrifying, toluene degrading *Azoarcus* which were very similar to each other could be isolated from different continental areas including Brazil, Michigan, California, and Washington (Fries et al., 1995). Eight strains were examined in detail and their 16S rRNA sequences showed 97.9 to 99.9% similarity. Again, however, DNA/DNA hybridization studies were not

conducted so true species level affiliation of all of these strains can not be assessed.

A number of studies have been carried out on the taxonomy of diatoms and dinoflagellates in relation to the north and south polar sea ice. Horner (Horner, 1985) lists some of these organisms and where they were found. The majority were found at only one pole or the other, however species of some genera, most notably, *Amphiprora, Chaetoceros, Navicula, Nitzschia, Thalassiosira, Distephanus,* and *Phaeocystis* were found at both polar regions. It was also noted, however, that *Chaetoceros, Nitzschia,* and *Thalassiosira* are all planktonic and have a wide geographic distribution. Also, most of these classifications are based on gross morphological characteristics and there is some question about the validity of some species level identifications (Horner, 1985; Horner, 1996).

F. Hypotheses and experiments

Prior to this work, a large number of gas vacuolate, heterotrophic bacteria were isolated from the marine environment of Antarctica and the Arctic (Irgens et al., 1989; Staley et al., 1989). This contrasts with repeated attempts that have failed to isolate gas vacuolate heterotrophs from midlatitude, marine waters. Some of the Arctic isolates appeared to share morphological and metabolic similarities with isolates from Antarctica which was surprising given that environmental and physiological factors may prevent mixing of the populations from the two poles. Of particular interest, previous studies (Davis, 1990) suggested that some of the polar gas vacuolate isolates are related to the CFB phylum of bacteria, no members of which are known to produce gas vesicles.

Given these preliminary observations, three hypotheses were proposed to investigate the identity, distribution and origin of polar gas vacuolate populations:

(1) Several different phylogenetic groups of heterotrophic gas vacuolate bacteria are found in polar marine environments.

(2) Some gas vacuolate heterotrophic bacteria exhibit a cosmopolitan bipolar distribution.

(3) Horizontal transfer of gas vesicle genes into existing species of non-gas vacuolate bacteria gave rise to some species of polar gas vacuolate bacteria.

To address these hypotheses, a large number of polar marine gas vacuolate heterotrophs were isolated, characterized and grouped by phenotypic and chemotaxonomic methods. Representative samplings of the populations at both poles were obtained by isolating a large number of gas vacuolate bacteria from different locations over several field seasons. Identical culturing conditions were used for all the samples to ensure that the same types of bacteria could be isolated if they were present. Several rapid techniques were investigated for systematically grouping the isolates. Clustering based on fatty acid composition was chosen as the standard method.

Next, the nucleotide sequences of the 16S rRNA genes and *gvpA* genes of a representative member from each groups identified above was determined. Sequence data from the 16S rRNA genes were used to phylogenetically classify the different groups. Phylogenetic analyses based on these data were used to taxonomically identify species and to indicate their phylogeny in relationship to other polar and nonpolar bacteria. These data, in turn, suggested further tests to physiologically, ecologically, and taxonomically describe the sea ice gas vacuolate strains.

Third, the phylogenetic tree of the *gvpA* genes was compared with the phylogenetic tree of the 16S rRNA genes. Topological comparison of the two trees was used to reveal the evolutionary relationships between these organisms and their gas vesicle genes. In particular, we were interested in whether the *gvpA* genes of the CFB type isolates showed specific phyletic affiliation with any other group of *gvpA* sequences and if the *gvpA* sequences of the north and south polar isolates are closely related to each other. This was done to provide evidence on the possibility of a horizontal gene transfer event that gave rise to these strains of gas vacuolate bacteria.

Based on these studies, this dissertation describes and classifies some of the heterotrophic gas vacuolate bacteria found in polar marine environments. New taxa have been declared for some organisms that were significantly different from previously defined groups of bacteria. The total number and size of the taxonomic groups identified places a lower limit on the species diversity of gas vacuolate heterotrophs at the two poles. The

geographic distribution of these strains (biogeography) was also assessed. None of the isolates from the Arctic and Antarctic were identical, although several organism pairs closely resembled each other. Finally, the origin of the gas vesicle phenotype in these isolates was investigated by comparing the inferred evolutionary lineage of each isolate with the inferred evolutionary lineage of its *gvpA* gene. These analyses did not support the hypothesis of horizontal gene transfer, however.

This dissertation is divided according to articles I have published or manuscripts that are in preparation or submitted for publication. The structure roughly reflects the order in which the subprojects were completed. Chapter 2 contains laboratory and data analysis methods that were used throughout this work. Some methods, pertinent to only a specific part of the work may be detailed in the specific chapter where they occur. Chapters 3 to 8 correspond to published material or material in preparation from this work. Chapter 3 is significant in that it demonstrates that gas vacuolate bacteria could be found in large numbers in and around the sea ice of the arctic. Since not other marine heterotrophic gas vacuolate species had been previously reported from temperate waters it suggests that the gas vacuolate phenotype is common and (at least somewhat) specific for polar procaryotes. Chapters 4 is significant in that it shows that the polar sea ice bacteria are phylogenetically diverse on the basis of 16S rRNA phylogenetics. Chapters 5, 6, and 7 focus on classifying some of these groups of bacteria. This serves to demonstrate that these strains are not closely related to previously describe species and genera of gas vacuolate bacteria. Chapters 5 and 6 also demonstrate the diversity and biogeography of these isolates by pointing out that each of them is a different species and hence none of them are cosmopolitan with bipolar distribution. Chapter 8 addresses the question of whether the gas vacuolate phenotype was acquired by any of these isolates, particularly the CFB isolates, by horizontal gene transfer. The answer to this question sheds light on evolutionary and ecological origin of these species. Finally, chapter 9 provides an overview of the major results from this work, their significance, and future studies that would appropriately continue this project.

Chapter 2

Material and Methods

A. Collection and *in situ* measurements.

Ice cores. Isolates were obtained over five field seasons from multiple, widely separated sites of Antarctica and the Arctic (Table 2.1 and Figures 2.1 and 2.2). Gas vacuolate bacteria were recovered from antarctic sea ice and water as previously described (Staley et al., 1989) in 1986 from the U.S. Palmer Station (64°S, 64°W) (strains 23-P and 34-P) and in 1987 from McMurdo Station (78°S, 167°E) (strains S36-W(gv)1, S51-W(gv)1, 84-W(gv)1 and 90-P(gv)1). Similar techniques were used at McMurdo Station in the austral summer (November and December) of 1992 (strains 301 and 307). Arctic sea ice and water was also sampled in May of 1991 at Pt. Barrow, Alaska (strains 37 and 174) and 350 km offshore of Deadhorse, Alaska (73° 01' N, 148° 31' W) (strains 206, 214.6, 215, and 238) in April of 1992. Multiple sites were sampled for each timepoint and several ice cores and/or water samples were taken for each site. The specific sampling locations were chosen distant from areas of human activity.

Table 2.1. Isolate Number, Classification, Source Location, and Isolation Date of Polar Gas Vacuolate Strains Analyzed by 16S rDNA Sequencing.

Isolate #	Classification	Chapter	Source Location	Isolation Date
34-P	*Polaromonas vacuolata* str. 34-P	5	Penola Strait, Antarctica, 25 m[a]	November,1986
215	*Polarobacter filamentus* str. 215	7	350 km north of Deadhorse, Alaska, 0 m[a]	April, 1992
23-P	*P. irgensii* str. 23-P	7	Penola Strait, 10-25 m[a]	November, 1986
301	*P. franzmannii* str. 301	7	Site 5, McMurdo Sound, Antarctica, 25-50 cm[b]	November, 1992
238	*Octadecobacter arcticensis* str. 238	6	350 km north of Deadhorse, Alaska, 38-48 cm[b]	April, 1992
307	*O. antarcticensis* str. 307	6	Site 3, McMurdo Sound, Antarctica, 25-50 cm[b]	November, 1992
37	unclassified gamma Proteobacteria	8	Site 2, Pt. Barrow, Alaska, 120-140 cm[b]	May, 1991
174	unclassified gamma Proteobacteria	8	Site 3, Pt. Barrow, Alaska, 40-60 cm[b]	May, 1991
90-P(gv)1	unclassified gamma Proteobacteria	8	Site 6, McMurdo Sound, Antarctica, 18 m[a]	December 1987
206	unclassified gamma Proteobacteria	8	350 km north of Deadhorse, Alaska, 0-10 cm[b]	April, 1992
214.6	unclassified gamma Proteobacteria	8	350 km north of Deadhorse, Alaska, frazil[c]	April, 1992
S51-W(gv)1	unclassified gamma Proteobacteria	8	Site 7, McMurdo Sound, Antarctica, frazil[c]	December 1987
S36-W(gv)1	unclassified gamma Proteobacteria	9	Site 4, McMurdo Sound, Antarctica, 10-40 cm[b]	December 1987
84-W(gv)1	unclassified CFB	7	Site 7, McMurdo Sound, Antarctica, 18 m[a]	December 1987

[a] Depth in water column

[b] Level in ice core. 0 cm is the sea ice/water interface, "100" cm is 100 cm from the interface etc.

[c] Melted frazil ice

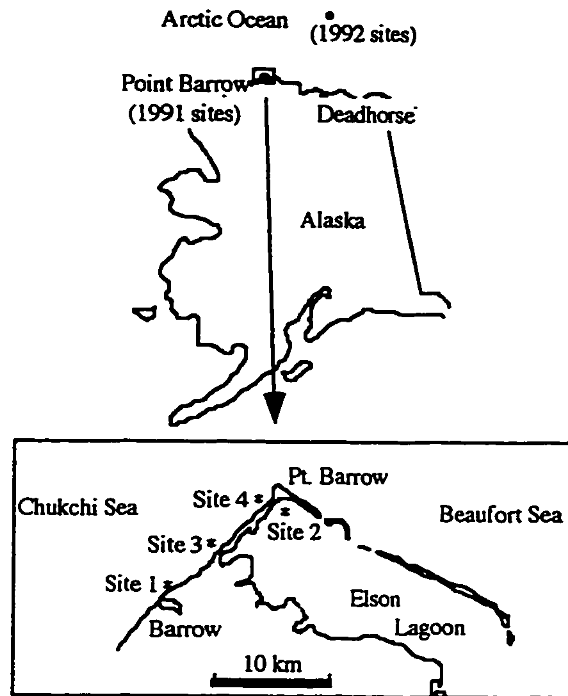


Figure 2.1. Map of Sampling Sites Off the Coast of Alaska. Lower panel shows details of the of Point Barrow vicinity showing the three open water sites (sites 1,3 and 4) in the Chukchi Sea and the Elson Lagoon site (site 2).

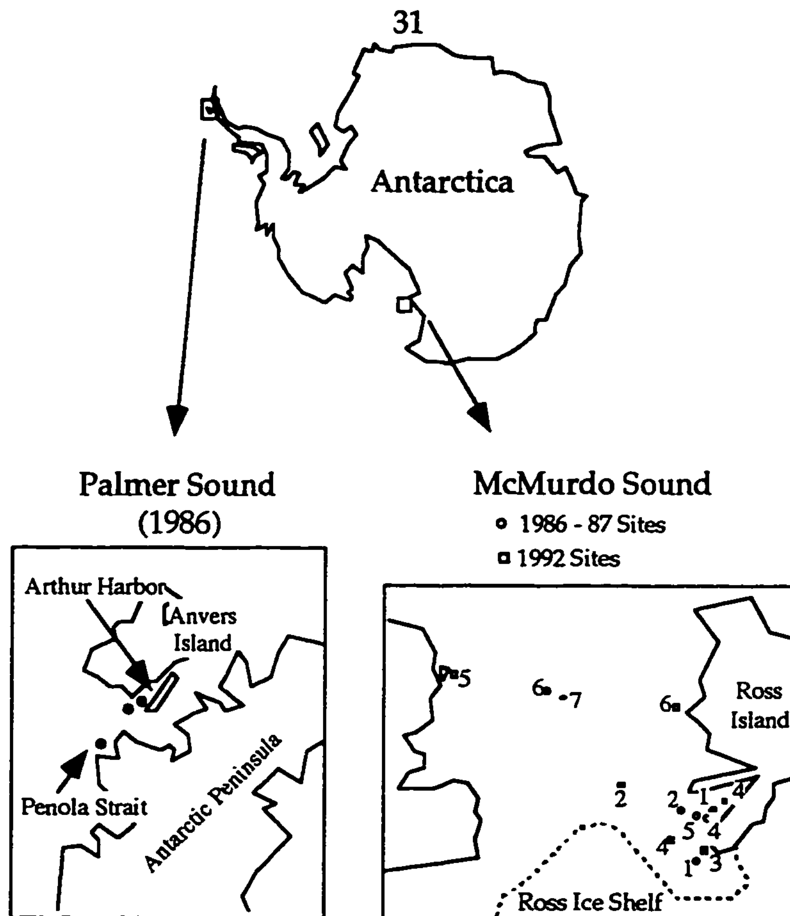


Figure 2.2. Map of Sampling Sites in Antarctica. Lower panels show details of the the McMurdo Sound and Palmer Penninsula sampling sites.

Ice cores were collected using a modified 14 cm (5 1/2") diameter Snow Ice and Permafrost Research Establishment (SIPRE) Corer (USA-Cold Regions Research and Engineering Laboratory, Hanover, N.H.). Ice cores were sectioned with a clean, ethanol-sterilized ice saw and thawed at room temperature in sterile plastic bags. A 10" power ice drill was used to make holes in the ice for water sampling.

Water samples. Water samples were taken at 0, 2, 4, or 20 m (or at the sea floor) beneath the ice with a van Dorn or Niskin water bottle rinsed with 95% ethanol between samples. Several features of sea water were described: salinity was measured with a Beckman Industrial Solu Bridge. For chlorophyll *a* measurements, 50 to 500 ml aliquots were filtered through 47 mm Whatman GF/F glass fiber filters (Whatman, Inc., Clifton NJ) and stored at -20°C in polypropylene vials in the dark. They were extracted with 90%

acetone and analyzed with a Turner Model 112 Fluorometer within five weeks of collection (Parsons et al., 1984). Acridine orange direct counts (AODCs) were determined by the method of Herwig et al. (Herwig et al., 1986). Viable counts and gas vacuolate strains were obtained by plating aliquots on SWCm medium (Irgens et al., 1989) (see below) at 4 to 6°C.

B. General microbiological methods.

Bacterial strains and cultures. Each sample was spread on sea-water cytophaga medium (SWCm) agar (Irgens et al., 1989) (and see below) plates and incubated at 4 to 6°C for 3 to 10 weeks in the dark. SWCm is a dilute medium containing beef extract, yeast extract, tryptone, and succinate in half strength artificial sea water (Irgens et al., 1989). SWCm contained (per liter of distilled or deionized water) 12 g of NaCl, 3.5 g of $MgSO_4 \cdot 7H_2O$, 2.6 g of $MgCl_2 \cdot 6H_2O$, 0.73 g of $CaCl_2 \cdot 2H_2O$, 0.5 g of ferric citrate, 0.35 g of KCl, 78 mg of Tris-HCl, 5 mg of KH_2PO_4, 0.5 ml Tris-Cl (pH 7.5), 0.5 g of tryptone (Difco Laboratories, Detroit MI), 0.4 g of beef extract (Difco), 0.4 g of yeast extract (Difco), and (for solid media) 15 g of Bacto Agar (Difco). The medium was adjusted to pH 7.6 with concentrated NaOH before autoclaving. A filter sterilized solution consisting of 10 ml of vitamin solution, 1 ml of trace element solution, and 0.2 g of sodium succinate·$6H_20$ was added to the medium after autoclaving. The vitamin solution (Staley, 1968) contained (per liter of distilled or deionized water) 10 mg of pyridoxine·HCl, 5 mg of calcium pantothenate, 5 mg of nicotinamide, 5 mg of *p*-aminobenzoic acid, 5 mg of riboflavin, 5 mg of thiamine·HCl, 2 mg of biotin, 2 mg of folic acid, and 0.1 mg of cyanocobalamine (B_{12}). Trace elements solution (modified from (Irgens, 1977)) contained (per liter of distilled or deionized water) 0.3 g of H_3BO_3, 0.2 g of $CaCl_2 \cdot 2H_2O$, 0.1 g of $ZnSO_4 \cdot 7H_2O$, 0.03 g of $MnCl \cdot 4H_2O$, 0.03 g of $Na_2MoO_4 \cdot 2H_2O$, 0.02 g of $NiCl_2 \cdot 6H_2O$, and 0.01 g of $CuCl_2 \cdot 2H_2O$ at pH 3 to 4.

Colonies of gas vacuolate bacteria often have a chalky translucent appearance that served as an initial phenotypic screen for gas vesicle producing strains (Staley, 1990). Ultimately, pure cultures were frozen with 10% glycerol or 3.5% DMSO in SWCm broth and stored at -70°C. Working cultures were maintained on SWCm agar plates and in SWCm broth tubes.

Microscopy. After three to ten weeks incubation time, wet mounts of presumptive gas vacuolate colonies were prepared for phase contrast microscopy. Cells were observed for bright, refractile regions indicative of gas vacuoles. Colonies of cells thought to contain gas vacuoles were restreaked on SWCm agar. Final confirmation of gas vesicles in presumptive gas vacuolate strains was made by electron microscopy. Electron micrographs were obtained of unstained whole cells using a JEOL-100B transmission electron microscope at 60 kV.

Fatty acid methyl ester analysis. Strains were grown on SWCm agar plates for 2 to 4 weeks at 4 or 10°C, scraped from the plates, and frozen at -80°C in 13 × 100 mm teflon lined screw cap tubes. When enough samples were collected, cells were lysed, and the whole cell fatty acids were saponified with methanolic base (MIDI, 1993). Saponified fatty acids were converted to fatty acid methyl esters by the addition of HCl. Fatty acid methyl esters were then identified and quantitated with a Hewlett Packard model 5890 Series II gas chromatograph according to recommended procedure (MIDI, 1993).

Nutritional requirements. The ability of strains to grow on a variety of carbon sources was tested using SWCm without tryptone or beef extract, and only 0.2 g/l yeast extract. Aliquots (200 μl) of this base broth along with specific carbon sources were pipeted into each well of Falcon Microtest III™ 96-well tissue culture plates. The specific carbons sources tested are indicated in the tables in chapters 6 and 7. The plates were chilled to 4°C. Cells were scraped from petri plates and suspended to slight turbidity in cold SWCm without added nutrients. 50 μl of each cell suspension was inoculated in sets of 4 into the wells of the microtiter plates to so that the final concentration of the test carbon sources was 1.0 g/l. Plates were incubated at 10°C and growth was scored 0, 24, and 35 days post inoculation with a BioTek EL311 microtiter plate reader at 600 nm.

Vitamin requirements were assayed in a similar manner. In this case the basal medium consisted of SWCm without tryptone, beef extract, yeast extract, vitamins, or trace elements solution, but to which vitamin free Casamino Acids (Difco, Detroit, MI) had been added to a final concentration of 2 g/l. Four different preparations of this basal medium were made, each of

which had all but one of the following vitamins: 0.01 μg/ml biotin, 1 μg/ml nicotinic acid, 1 μg/ml thiamine HCl, or 1 μg/ml pantothenic acid. Another preparation was made without any vitamins and a final preparation had all of the vitamins present. Cells were scraped from petri plates and washed twice, and resuspended to faint turbidity in cold SWCm without any vitamins, trace elements, or nutrients. As in the carbon source experiments, 50 μl of these cell suspensions were inoculated in quadruplicate into the wells of the microtiter plates. Cells were passed through 2 serial transfers to fresh wells of the corresponding vitamin type after 34 and 19 days growth, respectively. Growth was scored 16 days after the last transfer using a microtiter plate reader as described above.

Mole % G + C. DNA for mole % G+C determination and DNA/DNA hybridization was obtained by a hexadecyltrimethylammonium bromide miniprep method previously described (Ausubel et al., 1989). Multiple rounds of phenol/chloroform extractions and chloroform extractions with ethanol precipitation using the spooling technique were employed until high quality, pure DNA was obtained. High Pressure Liquid Chromatography was used to determine the mole % G + C from the DNA of the polar gas vacuolate strains unless otherwise indicated (Mesbah et al., 1989; Gerhardt et al., 1994).

DNA hybridization. A thermal renaturation method (Gerhardt et al., 1994) was used to determine the percent DNA-DNA reassociation between various strains and type species. For strains 238, 307, and *Roseobacter denitrificans,* the initial denaturation temperature was set at 97°C and the reannealing temperature was set at 76°C. For strains 23-P, 215, 301, and *Flectobacillus glomeratus,* the intial denaturation temperature was set at 97°C and the reannealing temperature was set at 63°C. All pairwise reassociations were run six to ten times.

C. General Methods employed for only certain identified strains:

Polaromonas **specific methods.** Strains 13-P, 41-P, 54-P, JA, and JB were characterized as previously described (Irgens et al., 1989). Features examined by R. L. Irgens include temperature optimum, generation time, pH optimum,

salinity optimum, catalase, oxidase, deaminase, lipase, nitrate reductase, amylase, protease, cysteine desulfurase, and tryptophanase. Urease was tested by standard methods (Gerhardt et al., 1981) by R. L. Irgens. Likewise, he tested resistance to various antibiotics was tested by placing a paper disk with the antibiotic onto an SWCm agar plate which had been spread with the test strain. The plates were incubated at 4°C and susceptibility was scored by a zone of clearing greater than 40 mm.

Also, for strain 34-P, the utilization of carbon sources was tested on SWCm medium from which the tryptone was eliminated, the beef extract was reduced to 0.1 g per liter and the carbon source was added to a final concentration of 0.2%. This same medium without the added carbon source was used as a control.

***Octadecobacter* specific methods.** Strains 238, 307, 308, and 309 were tested for the ability to grow at various pHs using SWCm buffered with 20 mM MES (2-[N-Morpholino]ethanesulfonic acid), ACES (2[92-Amino-2-oxoethyl)amino]ethanesulfonic acid), TAPSO (3-[N-tris(Hydroxymethyl)methylamino]-2-hydroxy-propanesulfonic acid), TAPS (N-tris[Hydroxymethyl]methyl-3-aminopropanesulfonic acid), or CHES (2-[N-Cyclohexylamino]ethanesulfonic acid) (pHs 5.5, 6.5, 7.6, 8.5, and 9.5 respectively). Growth was scored after 17 to 28 days incubation at 10°C. Salinity ranges required for growth were examined using SWCm media formulations having increasing or decreasing amounts of NaCl, $MgCl_2 \cdot 6H_20$, $MgSO_4 \cdot 7H_2O$ and KCl while keeping the other components constant. Growth was scored after incubation at 10°C for 10 days. Growth temperatures were tested by streaking onto SWCm agar plates and incubating in the dark for 19 days at 4, 10, 15, 19, and 37 °C. Oxidase, catalase, and Gram stain reactions were determined by standard methods (Gerhardt et al., 1994). Antibiotic resistances were determined by plate disk method using SWCm agar plates incubated at 4°C for 4 weeks. Resistance was scored as a zone of clearing greater than 30 mm. Nitrate reduction was tested by method 25.1.54.2 of Smibert and Krieg (Gerhardt et al., 1994). The growth medium was SWCm with 0.1% KNO_3 and 0.17% agar. Hydrolysis of gelatin and starch was assayed using 0.4% gelatin or 0.2% soluble starch in SWCm agar plates. Growth was scored after 6 to 7 weeks of incubation at 10°C by the methods 25.1.72 and

25.1.29.2 of Smibert and Krieg (Gerhardt et al., 1994). Glucose oxidation and fermentation was tested using the modified O/F (MOF) medium of Leifson (25.3.2) (Gerhardt et al., 1994). Motility was scored for cultures at different ages grown on solid and liquid media. The absorption spectra of whole cells (Shiba, 1991) and methanol extracts of ground cells (Ledyard et al., 1993) was determined with a Hitachi U-2000 spectrophotometer as previously described. Bacteriophaeophytin spectra were determined after the addition of 1/10 × volume of 1 M HCl.

Polarobacter specific methods. Strains were tested for the ability to grow on various solid and liquid media. Solid media included Nutrient agar (NA) (Difco), NA + 2.5% w/vol NaCl, trypticase soy agar (TSA) (BBL), TSA + 3% w/vol sucrose (TSAS), TSA + 3% w/vol glucose (TSAG), TSA + 2.5% w/vol NaCl, Marine agar 2216 (Difco), CLED agar (Difco), MacConkey agar (Difco), Levine EMB agar (Difco), SWCm agar (see above), Mueller Hinton medium (Difco), peptonized milk agar (PMA) (Difco), MSB agar (Larkin and Borrall, 1984), and MSB agar + 3.0% w/vol NaCl. Liquid media included MSB, MSB + 1.5% w/vol NaCl, MSB + 3.0% w/vol NaCl, Marine medium 2216 (Difco), and SWCm (see above).

Oxidation and fermenation of sugars, alcohols, and related carbohydrates were tested using a SWCm to which had been added 0.5 g/l tris-base, 0.01 g/l phenol red, and 2.5 g/l agar (Difco). The medium was adjusted to pH 7.5 with HCl, steamed for 15 minutes and, and aliquoted into flasks. The media was then autoclaved and 1/10 volume of a 10% filter sterilized test carbon source solution was added to each flask. 2.5 to 3.0 ml were dispensed into 75 × 100 mm tubes. The tubes were chilled overnight to 4°C. Turbid suspensions of each strain were stab inoculated into each of the tubes in groups of 6. One half of the tubes were then overlaid with mineral oil. Tubes were incubated at 10°C (23-P, 215, 301, and *Flectobacillus glomeratus*), or 22°C (*Flc. marinus*). Turbidity and pH changes were scored 3, 7, 14, 21, and 28 days post inoculation.

The mole percent G+C of genomic DNA from the *Polarobacter* strains was determined by thermal denaturation (Gerhardt et al., 1994).

Ethanol extracts of the cells were examined by spectroscopy. Briefly, 1 liter cultures of each strain were grown in SWCm. Cells were harvested by

centrifugation, resuspended in 10 ml of cold 95% ethanol, and sonicated for 10 seconds. The sonicated cell material was then filtered through a 0.22 μm GSWP filter and the absoption spectrum between 250 and 700 nm was determined with a Hitatchi U-2000 scanning spectrophotometer. The samples were then alkalinized with 1/10 x volume of 0.1 M NaOH. The absorption spectra were then reobtained and the peaks were examined for a bathychromatic shift, a characteristic of flexirubins (Weeks, 1981).

Biochemical tests including urease, lysine and ornithine decarboxylase, oxidase, catalase, indole formation, Voges-Proskauer reaction, NO_3^- reduction, and H_2S formation were conducted by the methods of Smibert and Krieg (Smibert and Kreig, 1994). In these tests, distilled water was replaced with the basal salt solution of SWCm. In the case of the NO_3^- reduction assay, SWCm basal salts were used with the omission of NH_4Cl. β-galactosidase activity was determined by streaking cultures onto SWCm agar plates ammended with 0.1 mM isopropyl-β-D-thio-galactopyranoside (IPTG) and 20 μg/ml 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-gal).

Macromolecule hydrolysis by the *Polarobacter* strains was similarly tested using SWCm basal salts instead of distilled water. Esculin, starch, casein, and gelatin hydrolysis were tested by the methods of Smibert and Kreig (Smibert and Kreig, 1994). Chitin hydrolysis was tested using chitin overlay plates (Herwig et al., 1988). Cellulose hydrolysis was tested both by cellulose overlay plates (Smibert and Kreig, 1994) and by examining strips of filter paper in liquid cell cultures for dissolution (Herwig, 1994).

D. Molecular Methods.

PCR amplification and cloning of 16S rRNA genes. Template genomic DNA was purified from bacteria by a hexadecyltrimethylammonium bromide miniprep method described previously (Ausubel et al., 1989). 16S rDNA genes were amplified by PCR (33 cycles of 1.5 min at 94°C, 1 min at 42°C, and 4 min at 72°C with the last step of the last cycle continuing for 10 min) from genomic DNA using the "universal" primers 8 FPL and 1492 RPL (Reysenbach et al., 1994). Reactions were carried out with PCR primers at 500 nM each, genomic DNA at 4 ng/μl, $MgCl_2$ at 1.5 mM, KCl at 50 mM, dNTPs at

150 µM, Tris-HCl (pH 8.3) at 10 mM, gelatin at 0.01% w/v, and *Taq* DNA polymerase (Pharmacia, Uppsala, Sweden) at 0.04 U/µl.

Following amplification, PCR product was subjected to end-filling with DNA pol I or Klenow (large fragment of DNA polymerase I), thus ensuring full length PCR products. After heat-inactivating the reaction, this DNA was digested with *Not* I, separated via gel electrophoresis, and isolated from 1% low-melting-point agarose (Sigma). This DNA was ligated into the dephosphorylated *Not* I site of pBluescript II KS+ (Stratagene, La Jolla, CA), and electrotransformed into competent *E. coli* DH5α.

Nucleotide sequence analysis of 16S rRNA genes. Multiple clones (except in the case of 90-P(gv)1 and 34-P) of the same orientation were pooled and sequenced using the *Taq* DyeDeoxy™ Terminator cycle sequencing kit (Applied Biosystems, Foster City, CA) with 16S rDNA specific forward and reverse primers (Dyksterhouse et al., 1995) on an ABI 373A automatic sequencer (Applied Biosystems). Additional primers were needed to sequence part of the 16S rRNA gene for some of the strains: sp3Rgamma (positions 358 to 340 relative to the *E. coli* rDNA sequence numbering (Brosius et al., 1978); ACTGCTGCCTCCCGTAGGA) for strain 90-P(gv)1, sp7Rgamma (positions 802 to 785; TACCGGGGTATCTAATCC) for strain 90-P(gv)1, sp11rhod (positions 1096 to 1114; CCGGCAACGAGCGCAACCC) for strains 307 and S51-W(gv)1, sp11flav (positions 1096 to 1114; CCTATAACGAGCGCAACCC) for strain 301, and sp13Rgamma (positions 1406 to 1390; ACGGGCGGTGTGTACAA) for strain S51-W(gv)1. Sequence contigs were assembled using the program SeqApp (Gilbert, 1992).

Sequences were submitted to the Ribosomal Database Project (RDP, version 4.0 or 5.0) (Larsen et al., 1993) for alignment by the ALIGN_SEQUENCE program (Larsen et al., 1993). Small adjustments were made by manual comparison to secondary structures provided by the RDP (Gutell, 1994). Additional, prealigned, 16S rRNA sequences were also obtained from the RDP. Sequences identified by "str. clone..." or "env. clone" indicate 16S rRNA population clones of bacteria that have not been isolated in pure culture. BLAST (Altschul et al., 1990) searches were additionally performed against GenBank to determine if other closely related sequences existed in this database. The aligned datasets were trimmed to leave only

sequence positions between, but not including, the regions defined by the 8 FPL and 1492 RPL primers. Phylogenetic trees were generated using PAUP 3.0s (Swofford, 1991) for parsimony analysis, SEQBOOT, DNADIST, NEIGHBOR, and FITCH (Felsenstein, 1989) for distance analysis, fastDNAml (Felsenstein, 1981; Olsen et al., 1994) for likelihood analysis, and DNAML (Felsenstein, 1989) for Kishino-Hasegawa tests. MacClade 3.05 (Maddison and Maddison, 1992) was used to determine transition and transversion frequencies.

In the case of one strain, 34-P, our result was also compared with the sequence as determined independently by the lab of C. R. Woese (personal communication). The sequences of 23-P and 84-W(gv)1 were determined entirely by the lab of C. R. Woese (Davis, 1990; Woese, 1991).

PCR amplification and cloning of *gvpA* genes. PCR was used to amplify *gvpA* genes from genomic DNA of polar sea ice bacteria, the same template used for previous 16S amplification. *GvpA* genes were amplified by PCR (35 cycles of 1 min at 94 °C, 1 min at 37 °C, and 1 min at 72 °C) using the following highly degenerate primer "TYLKYAE" 5'-ccggaattcNGCRTAYTTNARRTANGT-3' and primer "DRILDKG" 5'-gttctaagaTMGNATYYTNGAYAARGG-3' (nucleotides in small case correspond to *XbaI*, and *EcoRI* restriction sites. As per standard IUPAC nomenclature: N is an equimolar mixture of G,A,T, and C, R is an equimolar mix of A and G, Y is an equimolar mix of C and T, and M is an equimolar mix of A and C. The sequence for these primers is a modification of those kindly suggested by Paul Hayes (University of Bristol). Reactions were carried out using Perkin Elmer's Buffer II with 2.5 mM MgCl2, 0.2 mM dNTPs, 0.02 U/μl AmpliTaq DNA polymerase (Perkin Elmer), 1 pmol/μl of each primer, and 2 ng/μl genomic DNA. These conditions were intentionally permissive as the PCR primer TYLKYAE has 1024 fold degeneracy, and the DRILDKG primer has 512 fold degeneracy. This resulted in a total of 524,288 fold degeneracy for the reaction. It was expected, however, that some degree of mismatch would still allow priming of the template DNA.

Following amplification, PCR product was end-filled with DNA pol I or Klenow (large fragment of DNA polymerase I) and separated via gel electrophoresis. DNA fragments of approximately 150 bp were isolated from

1% low-melting-point agarose (Sigma). Strains 37 and 174 required a second round of PCR, employing the same reaction conditions and primers, to obtain adequate DNA for subsequent cloning.

PCR product was cloned into a pBluescript II KS+ cloning vector (Stratagene Inc., LaJolla CA) using one of two strategies. The first was a "double sticky end" ligation approach. PCR product and pBluescript II KS+ were each digested in separate reactions with *Eco*RI and *Xba*I prior to ligation. The second cloning strategy was a "sticky-blunt" ligation approach. PCR product was digested with *Eco*RI, which cuts in one of the primer termini of the product (and thus leaves the other terminus blunt). pBluescript II KS+ was digested with *Eco*RI and *Sma*I, the latter of which is a blunt cutter. Finally, compatible vector and insert were ligated. Each ligation was electrotransformed into competent *E. coli* DH5α.

Nucleotide sequence analysis of *gpvA* genes. Clones containing the appropriate sized insert were subjected to nucleotide sequence analysis using the *Taq* DyeDeoxy® Terminator cycle sequencing method (Applied Biosystems, Foster City, CA) and an ABI 373 or 377 automatic sequencer. In some cases, DNAs from multiple clones were pooled before sequencing to abrogate the affect of PCR-induced mutations and to obtain a consensus sequence if multiple *gvpA* alleles existed (see discussion, Chapter 8).

Sequence read editing and multiple sequence alignments were conducted using SeqApp (Gilbert, 1992). BLAST (Altschul et al., 1990) searches were performed against GenBank to find the following homologous genes: the *gvpA* sequences of *Anabaena flos-aquae* (GenBank # M32060, gene starting at position 188), *Calothrix* PCC 7601 (# M16733, X06085 genes starting at positions 441 and 761), *Pseudoanabaena* sp (# 57731), and *Halobacterium halobium* (# Y00534). Phylogenetic analysis of the *gvpA* sequences was conducted in a manner similar to that of the 16S rRNA genes. Phylogenetic trees were generated using PAUP 3.0s (Swofford, 1991) for parsimony analysis, SEQBOOT, DNADIST, and NEIGHBOR (Felsenstein, 1989) for distance analysis, fastDNAml (Felsenstein, 1981; Olsen et al., 1994) for likelihood analysis, and DNAML (Felsenstein, 1989) for Kashino-Hasegawa tests. MacClade 3.05 (Maddison and Maddison, 1992) was used to determine transition and transversion frequencies.

Nucleotide sequence accession number. Nucleotide sequences for the polar gas vacuolate 16S rDNA sequences have been deposited in the GenBank database under accession numbers U14581 to U14586, and U73721 to U73726.

Chapter 3.

Gas Vacuolate Bacteria from Arctic Sea Ice.

reprinted from (Gosink et al., 1993)

Abstract. Heterotrophic gas vacuolate bacteria have been found in north polar sea ice samples obtained near Point Barrow, Alaska, a finding consistent with their recent discovery in antarctic sea ice microbial communities. These bacteria comprised 0.2% or less of the viable bacteria isolated from sea ice cores, lower than concentrations reported for most antarctic samples. Most gas vacuolate isolates from the sea ice cores were pigmented pink, orange or yellow. An ice core from nearby saline Elson Lagoon contained an inverted sea ice microbial community with highest chlorophyll *a* concentrations and bacterial counts found in the top 0-20 cm of the ice. This surface layer also contained high concentrations (up to 186 bacteria/ml) of an nonpigmented, gas vacuolate, elongated rod-shaped bacterium.

Introduction. Gas vacuolate prokaryotes, including representatives of the eubacteria, cyanobacteria, and archaebacteria, are usually found in the water column or sediments of aquatic environments. Gas vesicles, the intracellular subunits of a gas vacuole, are hollow, gas-containing proteinaceous structures. They reduce cell density and thereby increase the buoyancy of the organism allowing it to float at a favorable depth in the water column. Therefore, gas vesicles are especially useful to organisms that live in freshwater or saline habitats which have vertical photic, thermal, or chemical stratification (Walsby, 1994). Marine environments have not been known to

contain gas vacuolate prokaryotes with the exception of cyanobacteria in the genus, *Trichodesmium* (Krieg and Holt, 1984).

Recently, however, extremely psychrophilic, pigmented, gas vacuolate bacteria of unknown classification were discovered in the sea ice of Antarctica (Irgens et al., 1989; Staley et al., 1989) living in close association with the dense bottom type sea ice microbial community (SIMCO). This SIMCO is a community of algae, protozoa, and prokaryotes that grows in microscopic brine pockets in the lower 20 cm of the ice. In the brine pockets temperatures typically range from 0°C to -2°C, salinities from 1 to 5 times normal sea water concentration, and light intensities are 0.02% to 0.8% of surface irradiance (Sullivan and Palmisano, 1984). It has been proposed that algae are the primary producers, and heterotrophic bacteria the secondary producers in this community (Sullivan and Palmisano, 1984).

The discovery of psychrophilic gas vacuolate bacteria in the SIMCO of antarctic sea ice led us to examine north polar sea ice for gas vacuolate species. This paper reports similar gas vacuolate bacteria in the sea ice off Point Barrow, Alaska.

Results and Discussion. Ice cores and underlying water samples were collected from near Point Barrow, Alaska in mid May of 1991 (Figure 2.1) by J. T. Staley. All sites were relatively free of snow cover (less than 2 cm), and all ice cores were 1.7 to 1.85 m thick. Sites 1, 3, and 4 (open water sites) were taken from the Chukchi Sea ice several hundred meters offshore in approximately 4 m of underlying water. The lower layer of ice containing the SIMCO is often soft and frazzled and can be partially lost in retreiving the ice cores which may explain some of the variability in the measurements. The core from Elson Lagoon had zones with sand and grit presumably derived from either wind-blown debris from land or from sediments of dislodged anchor ice. This site was shallow and frozen to within 15 cm of the bottom of the lagoon.

The highest concentrations of chlorophyll *a* in the three open water sites were found in the bottom 4 cm layer of ice (Figure 3.1a). This corresponded to the visually apparent reddish-brown band of primary producers in the SIMCO layer of these ice cores. The highest acridine orange counts, averaging 4×10^5 per ml, and the greatest viable counts, averaging 1.2

$\times 10^4$ per ml, were also found in this layer. For these open water sites the viable count ranged from 0.05% to 5% of the AODC, typical of results obtained from the antarctic sea ice samples (Staley et al., 1989).

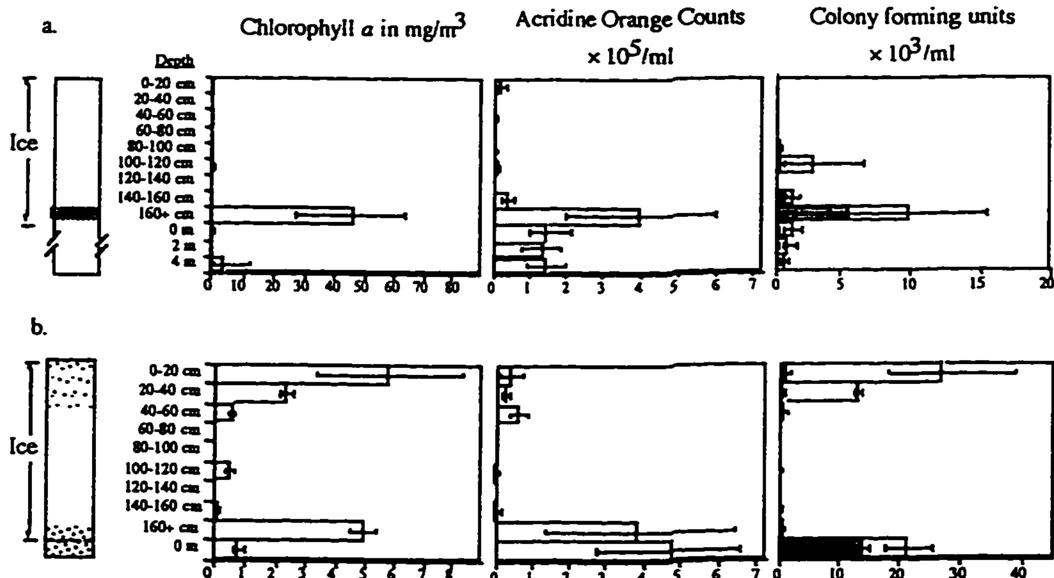


Figure 3.1. Analyses of chlorophyll *a*, acridine orange direct counts, and viable counts of bacteria from the ice core and water column samples from the Arctic. (a) Open water sites (sites 1,3, and 4) with a diagram showing a cross section of the ice and water column, stippling indicates the sea ice microbial community (SIMCO) layer. Shaded areas in viable count column indicate concentrations of pigmented bacteria. (b) The Elson Lagoon (site 2) has same legend except that stippled areas in the cross section indicate grit. Error bars indicate 1 standard deviation.

The Elson Lagoon site differed from the open water sites in several ways. First, the underlying water had a higher salinity (50‰) than the open water sites (35‰). Second, the ice extended to within 15 cm of the lagoon floor, and underlying water samples that were collected were brownish in color due to suspended particulate material. Finally, areas at the top, within, and at the bottom of the ice core as well as in the underlying water column were discolored with wind-blown grit or sediments. Not surprisingly this site showed different profiles for salinity, chlorophyll *a*, total acridine orange direct counts (AODC) and viable counts of bacteria compared to the three open water sites.

In contrast to the open water samples, the highest concentrations of chlorophyll *a* were found in the surface layers of the ice (6 mg/m^3 in the 0-20 cm layer) of Elson Lagoon (Figure 3.1 b). These values, however, were lower than that of the SIMCO values for the open water sites (46 mg/m^3). High total and viable bacterial counts were also found in the surface layer of the ice. But, unlike the open water samples, culturable counts from the surface layer of the lagoon (2.8 $\pm$ 1.0 $\times$ 10^4 bacteria/ml) were more than double that of the SIMCO from the open water samples (1.0 $\pm$ 0.7 $\times$ 10^4 bacteria/ml) and comprised about 50% of the AODC (Figure 3.1b). The high viable counts coupled with their high proportion to total counts suggests enriched organic conditions in the surface ice (Staley et al., 1982) perhaps due to the large amounts of grit blown in from the nearby land or to sediment-loaded anchor ice that is found in these layers. The high viable bacterial count in the upper 160 cm of the ice core also correlates ($r^2 = 0.98$) with the relatively high levels of chlorophyll *a* at this site. However, it seems unlikely that algal excretion alone could account for the increased numbers of bacteria because the concentration of chlorophyll *a* in the surface layers (4.0 $\pm$ 2.5 mg/m^3 in the top 40 cm) of the Elson Lagoon core was only one tenth that of the SIMCO layer of the open water cores (46 $\pm$ 18 mg/m^3).

High concentrations of chlorophyll *a* were also found in the water column below the lagoon sea ice. However, the proportion of phaeophytin to chlorophyll *a* was very high in this sample (Table 3.1). This suggests that many of the algae beneath the lagoon ice were physiologically inactive or dead (Parsons et al., 1977), perhaps due to the low levels of light that were available during the winter.

Table 3.1. Comparison of phaeophytin versus chlorophyll *a* concentrations (mg/m3) in the ice core and water column samples.

	Open Water Sites[1]			Elson Lagoon		
Depth	Phaeophytin	Chlorophyl *a*	Ratio[2]	Phaeophytin	Chlorophyl *a*	Ratio
0-20 cm	0.03	0.1	0.3	3	6	0.5
20-40 cm	-	-	-	1	2	0.5
40-60 cm	0.02	0.08	0.3	0.3	0.6	0.5
60-80 cm	-	-	-	-	-	-
80-100 cm	0.04	0.1	0.4	-	-	-
100-120 cm	0.2	0.7	0.3	0.1	0.6	0.2
120-140 cm	-	-	-	-	-	-
140-160 cm	0.1	0.2	0.5	0.08	0.2	0.4
160+ cm	10	50	0.2	5	5	1
0 m	0.2[3]	0.2	1	5	0.8	6
2 m	0.07	0.05	1	-	-	-
4 m	1	4	0.3	-	-	-

[1] The values for phaeophytin and chlorophyll *a* for the three open water sites (1,3,4) are given by the average of the results from these three sites.
[2] Ratio is the ratio of phaeophytin to chlorophyll *a*.
[3] Depth below the bottom of the ice.

Of the approximately 100,000 colonies that were visually screened for gas vacuolate bacteria, 177 were confirmed as positive by phase and electron microscopy (Figure 3.2 a,b). It was not possible to discern gas vacuolate bacteria directly from AODC. Table 3.2 shows that most (165 out of 177) of the gas vacuolate bacteria were found in the Elson Lagoon ice sample. Furthermore, most (160 of 165) of the Elson Lagoon isolates were similar looking long (5-15 μm x 1-2 μm), highly gas vacuolate rods which form white, raised colonies on SWCm . The open water isolates, in contrast, accounted for only a small proportion (12 of 177) of the gas vacuolate isolates, and most were pigmented pink, orange, or yellow. Finally, only one gas vacuolate bacterium was found in the SIMCO layer. The others were found mostly in the ice above the SIMCO layer, or in the water column below the SIMCO layer.

Figure 3.2 (a) Phase photomicrograph of a non-pigmented rod-shaped gas vacuolate bacterium isolated from Elson Lagoon. Bright areas within cells are gas vacuoles. Bar = 5 μm. (b) Electron micrograph of a non-pigmented rod-shaped gas vacuolate bacterium from Elson Lagoon showing the gas vesicles in the cell. Bar = 1 μm.

The arctic sites contained low populations of viable gas vacuolate bacteria. Gas vacuolate bacteria accounted for no more than 1% (Table 3.2) of the viable colony forming units (cfus). In contrast, some of the antarctic samples contained 91% gas vacuolate cfus (Staley et al., 1989). Most of our arctic isolates were obtained from the surface layers of Elson Lagoon, and unlike most antarctic isolates, they were not pigmented.

Table 3.2. Distribution of gas vacuolate bacteria isolated from ice core and water samples from the Arctic

Depth	Open Water Sites		Elson Lagoon	
	Total G.V.	% of Viable[1]	Total G.V.	% of Viable
0-20 cm	0	0%	32	0.4%
20-40 cm	-	-	112	1%
40-60 cm	5	1%	12	0.3%
60-80 cm	-	-	-	-
80-100 cm	3	1%	-	-
100-120 cm	2	0.03%	1	0.09%
120-140 cm	-	-	-	-
140-160 cm	0	0%	7	0.7%
160+ cm[2]	0	0%	1	0.06%
0 m[3]	1	0.08%	0	0%
2 m	0	0%	-	-
4 m	1	0.1%	-	-

[1] The values for the % of viable cfus from the open water sites (1, 3, 4) are given by the average of the results from these three sites.

[2] Corresponds to the SIMCO layer in the open water sites

[3] Depth below the bottom of the ice.

As in Antarctica (Staley et al., 1989), pigmented prokaryotes comprised the most numerous viable species in the lower ice layers (Figure 3.1). Orange, yellow, and red pigmented colonies made up approximately 60% of the the total cfus in the lowest layers of the open water site ice cores. In contrast, an average of only 9% of the colony forming units from the open water water column were pigmented. It is not understood why there is a higher concentration of pigmented bacteria in the SIMCO, but it has been suggested that pigmentation may play a role in protecting bacteria from ultraviolet radiation (Atlas and Bartha, 1987).

An interesting result of this study was that gas vacuolate bacteria were rarely found in or near the SIMCO layer (160 cm+) of the arctic sea ice (Table 3.2). In Antarctica most gas vacuolate bacteria were found either in or near the SIMCO layer although some were found in the underlying water column (Irgens et al., 1989). Additional research is needed to assess what niche the gas vacuolate bacteria occupy and to determine the role of gas vacoules in polar environments.

One of the long term objectives of our work is to determine whether any species of these sea ice bacteria are endemic in their geographic distribution. We are not aware of free-living prokaryotes endemic to specific locales on earth. There are, however, many examples of pathogens or symbionts whose distribution is dependent upon their hosts or symbiotic partners. Sea ice bacteria may be one group of prokaryotes ideally suited to the study of the biogeography of free-living prokaryotes. Many gas vacuolate sea ice bacteria isolated from Antarctica are extreme psychrophiles (Irgens et al., 1989). It seems likely that those from the north polar SIMCO are also psychrophilic. If so, it would seem that the tropical equator and the 11,000 km span of open ocean comprises a formidable barrier to dispersal or population exchange of these psychrophiles. By examining bacteria from the two polar regions we propose to address the question of whether these polar species are endemic or cosmopolitan.

Chapter 4

Biodiversity of Gas Vacuolate Bacteria from Antarctic Sea Ice and Water

Reprinted from (Gosink and Staley, 1995)

Abstract. Psychrophilic, gas vacuolate bacteria indigenous to sea ice communities have been isolated from Antarctica. Representatives of six different phenotypic groups were analyzed by 16S rDNA sequencing to determine their phylogeny. These bacteria belong to four different Eubacterial phyla including the alpha, beta, and gamma Proteobacteria and the CFB groups. This is the first report of gas vacuolate bacteria from the beta Proteobacteria and the CFB groups. All strains have unique 16S rDNA sequences that are of recent phylogenetic origin, consistent with the idea that extreme psychrophiles have evolved recently. The results of this study emphasize the importance of examining this extreme habitat more closely because it contains many unique and specially adapted bacteria.

Introduction. Approximately 23 million km^2 of the ocean surface freezes to produce sea ice on a annual basis (Maykut, 1985). In the spring and summer months an extensive community of microorganisms develops in the sea ice at the ice—sea water interface (Horner, 1985). This sea ice microbial community is dominated by diatoms whose pigments impart a reddish brown color to the layer. The community also contains bacteria and protozoa and serves as a major food source for krill (Hamner et al., 1983) and other zooplankton (Garrison et al., 1983). Polar sea ice represents an extreme environment for life on earth. Temperatures are lower than -1.9°C (freezing point of sea water) and salinities in small brine pockets and channels which harbor microbial life may reach five times that of normal sea water (Sullivan and Palmisano, 1984).

One of the principal types of cultivable bacteria associated with the sea ice community is a group of heterotrophic gas vacuolate organisms (Figure

4.1) which occurs at both the North and South Poles (Staley et al., 1989; Gosink et al., 1993). These polar gas vacuolate bacteria comprise one of the most psychrophilic bacterial groups known: most strains do not grow above 15°C, and some do not grow at temperatures above 7-10°C (Irgens et al., 1989). Heterotrophic gas vacuolate bacteria have not been reported from other marine habitats. Thus, a major goal of this investigation was to determine the phylogenetic relationship of the antarctic gas vacuolate strains to each other and to other known bacteria.

Specific materials and methods. Gas vacuolate bacteria were recovered from antarctic sea ice and water as previously described (Staley et al., 1989) in 1986 from the U.S. Palmer Station (64°S, 64°W) (strain 34-P) and in 1987 from McMurdo Station (78°S, 167°E) (strains S36-W(gv)1, S51-W(gv)1, and 90-P(gv)1). Similar techniques were used again at McMurdo Station in the austral summer (November and December) of 1992 (strains 301 and 307) (see Figure 2.1). Thus, isolates were obtained over three field seasons from multiple sites at widely separated areas of Antarctica.

The 16S rDNA sequences of the polar gas vacuolate strains were obtained as described above. Additional, prealigned, 16S rRNA sequences were also obtained from the RDP (Genbank nucleotide accession numbers M64629, M58775, M59063, M63810, D16211, L20811, X67022, M63811, D21224, X67024, L10939, and L10950). Trees were generated with a heuristic generalized unweighted parsimony search using PAUP 3.0s (Swofford, 1991). The search parameters included a simple addition sequence with one tree held at each step using the TBR swapping algorithm. The total tree length is 1811 and the Consistency Index is 0.63. Similar branching order was obtained using PAUP 3.0s for transversion parsimony (Swofford, 1991), FITCH (Felsenstein, 1989) for distance, and fastDNAml (Larsen et al., 1993) for maximum likelihood analyses (data not shown).

Results and Discussion. Gas vacuolate bacteria were recovered as previously described over 3 field seasons in Antarctica from sites in the vicinity of the U.S. Palmer and McMurdo Stations. Both areas have been the site of previous studies on sea ice and water microbial communities (Bunt and Wood, 1963; Sullivan and Palmisano, 1984; Herwig et al., 1988; Irgens et

al., 1989; Staley et al., 1989). By sampling different locations during separate seasons we were able confirm that gas vacuolate sea ice bacteria are members of the normal microbiota of this habitat (Irgens et al., 1989; Staley et al., 1989; Gosink et al., 1993). The gas vacuolate phenotype in these colonies was confirmed by phase contrast microscopy and transmission electron microscopy (Figure 4.1.).

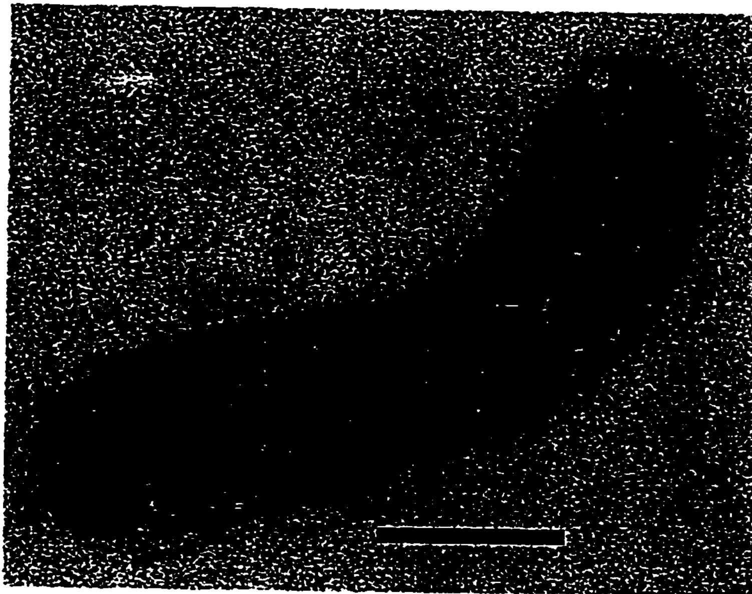


Figure 4.1. Transmission electron micrograph showing two unstained cells of gas vacuolate sea ice bacteria. The bright intracellular areas are gas vesicles. Bar represents 1.0 μm.

Our collection of South polar gas vacuolate bacteria contains over 25 strains. In order to assess the diversity of these isolates, they were first characterized by fatty acid composition (Table 4.1). The strains were initially grouped into operational taxonomic units based on whole cell fatty acid composition using the MIDI (Microbial Identification, Inc., Newark, DE) cluster analysis program (Figure 4.2). Under recommended conditions, isolates of the same strain, species, or genus have been shown to cluster at 6, 10, or 25 Euclidian distances, respectively (MIDI, 1993). Unfortunately our isolates were incapable of growth using the standard conditions employed in this commercial database. Thus it was necessary to use an artificial sea water medium (SWC) and grow them at low temperatures (Staley et al., 1989). Despite the fact that the fatty acid composition of these isolates could not be

compared to known bacteria in the MIDI database, the cluster analysis program proved useful in grouping the strains. The fatty acid compositions of isolates were reproducible even though many strains grew slowly (data not shown).

Table 4.1. Predominant fatty acids and percent compositions of type strains[a]

	Strain					
	301	307	34-P	S51-W(gv)1	S36-W(gv)1	90-P(gv)1
# of isolates in fatty acid group	8	3	5	2	1	1
13:0 iso	5					
12:0 3OH					11	
14:0				5		
15:1 isoG	11					
15:1 ω8cis						6
15:1 ω6cis	9					
16:1 ω9cis		14			13	
16:1 ω7cis	9		75	43	22	38
16:0		5	15	27	12	9
15:0 iso 3OH	17					
15:0 3OH	7					
17:1 ω9cis						9
18:1 ω9cis					16	
18:1 ω9cis/ ω12trans/ ω7cis		76	7		7	14

[a] Only fatty acids representing 5% or more of the total fatty acid composition of each strain are listed

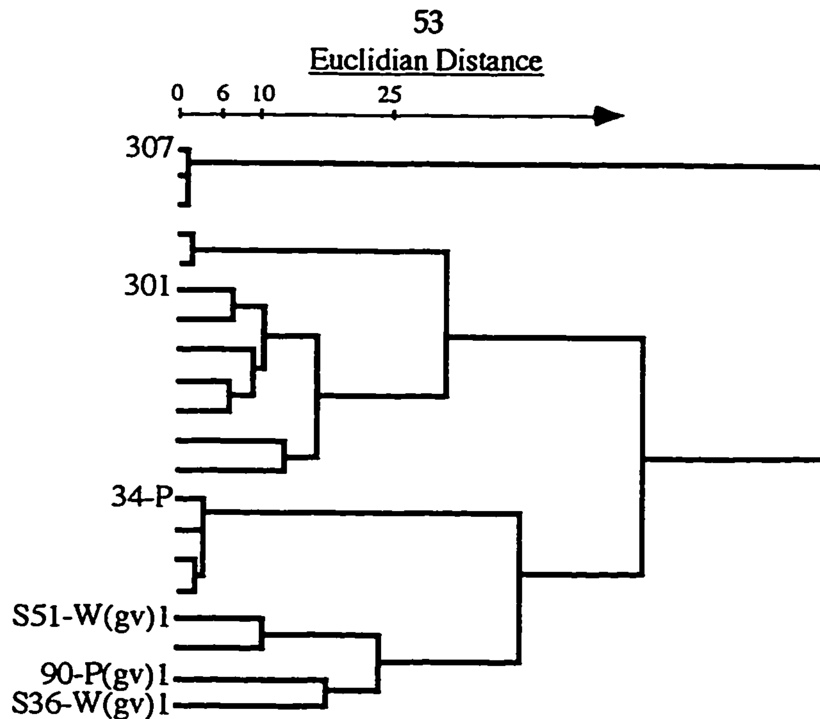


Figure 4.2. A cluster analysis dendrogram of Antarctic gas vacuolate sea ice bacteria based on whole cell fatty acid composition obtained using the MIDI system. Strains that were further characterized by 16S rDNA sequencing and phylogenetic analysis (Figure. 4.3) are indicated by their strain number designation. The designations of the other strains have been omitted for clarity.

To determine the relationship of these operational taxonomic units to each other and to other known bacteria, a representative from each of these groups was subjected to 16S rDNA sequencing and phylogenetic analysis. Due to small differences in the 16S rRNA sequences, slightly different sequencing primers had to be used for analysis of parts of the 301, 307, S51-W(gv)1, and 90-P(gv)1 16S rRNA genes. Closely related sequences were obtained from the RDP (Larsen et al., 1993) and carefully aligned with sequences from the antarctic isolates. Phylogenetic analysis of this alignment set using distance, parsimony, and maximum likelihood methods all generated similar tree topologies. One of the surprising results from these analyses is that the polar gas vacuolate bacteria do not comprise a single species or genus, but are instead phylogenetically diverse (Figure 4.3). They fall into four distinct Eubacterial phyla including the alpha, beta, and gamma Proteobacteria and the CFB group. None of these organisms is identical in 16S rDNA sequence

to that of any known prokaryotic species, and only two strains appear to belong to known genera: strain 301, appears to belong the marine genus, *Flectobacillus,* which contains a described Antarctic species, *Flectobacillus glomeratus* (Franzmann and Dobson, 1993); and strain S51-W(gv)1 is closely related to *Colwellia psychroerythrus,* a psychrophilic marine bacterium (Deming et al., 1988; DeLong et al., 1993).

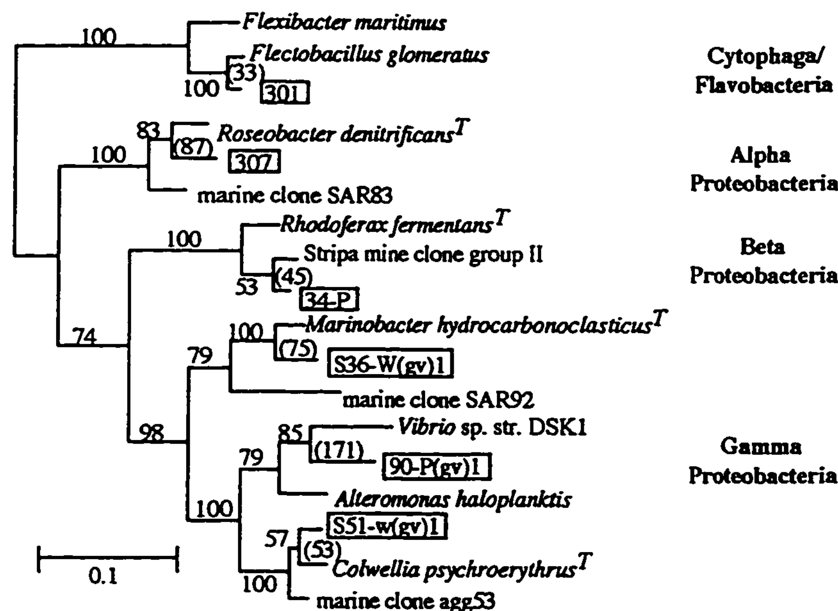


Figure 4.3. Phylogenetic relationship based on 16S rRNA sequences of representative gas vacuolate sea ice bacteria (indicated by their strain numbers in boxes) and closely related species or environmental sequences from uncultivated bacteria. This tree was the most parsimonious tree generated using PAUP 3.0s (Swofford, 1991) in a generalized, unweighted, heuristic search. Numbers above the nodes represent the percent bootstrap values for that node (500 bootstrap resamplings). Scale bar represents 0.1 change per average nucleotide position.

None of these bacteria has been previously described, indicating that many unknown species exist in this habitat. Several reasons may account for the discovery of new taxa in sea ice communities. First, few bacterial phylogenetic studies of Antarctic bacteria have been undertaken (Franzmann and Dobson, 1992; Dobson et al., 1993; Franzmann and Dobson, 1993; DeLong, Wu et al., 1994). This may be due in part to the fact that this particular environment, though important in terms of the area of earth it occupies and

unusual for its extreme conditions, is not readily accessible for study. Second, most polar research efforts have been focused on ecological, not organismal, studies (Olson et al., 1978; Sullivan and Palmisano, 1984; Horner, 1985; Friedmann and Thistle, 1993). Finally, because this is an extreme habitat, natural selection would favor the evolution of organisms adapted to extreme cold and high salinity (Sullivan and Palmisano, 1984). Therefore, the discovery of new, previously unreported species of sea ice bacteria is not entirely unexpected.

This is also the first report of gas vacuolate bacteria from the beta Proteobacteria and the CFB phyla of Eubacteria. Thus, now six of the 11 Eubacterial phyla, as well as some Archaean methanogens and extreme halophiles, are known to contain gas vesicles (Walsby, 1994).

That the gas vacuolate phenotype occurs in several disparate phylogenetic groups in the sea ice community suggests that this phenotype provides an important selective advantage to the sea ice bacteria that produce them. Gas vesicles are produced by many aquatic prokaryotes that use them to provide buoyancy to regulate their position in vertically stratified water columns (Walsby, 1994). Clearly, this habitat has a strong vertical stratification due to the phase discontinuity between the sea ice and water. Several hypotheses could be proposed for the significance of gas vesicles to sea ice bacteria, but the specific function(s) in buoyancy regulation in this habitat is not yet apparent. It is surprising that there have been no previous reports of gas vacuolate heterotrophic marine bacteria considering that many marine habitats exhibit stratification.

A phylogenetic tree of these extreme psychrophilic, gas vacuolate strains in comparison to previously reported trees of the Procaryotes (Larsen et al., 1993) indicates that they are of recent origin (Figure 4.3). This is in marked contrast to extreme thermophilic Eubacteria (such as *Aquifex* (Burggraf et al., 1992)) and Archaea (such as *Pyrococcus* (Woese, 1987)) which branch deeply in phylogenetic trees. Hence, this study is consistent both with a recent climatic origin of frozen polar ice caps (Crowley, 1986) as well as the hypothesis that thermophilic prokaryotes evolved early when Earth was much warmer (Achenbach-Richter et al., 1987).

Finally, it should be noted that some researchers have employed a novel approach to characterize prokaryotic species diversity in natural

communities that does not require the isolation of pure cultures. This approach, which analyzes the sequence of 16S rRNA genes from DNA or RNA extracted directly from the whole community, has resulted in the discovery of many new forms of microbial life in many habitats including marine habitats (for example see references (Britschgi and Giovannoni, 1991; Schmidt et al., 1991; DeLong et al., 1994)) . The results of our study indicate that, at least in this environment, not only do unique bacteria exist, but many can be isolated and grown in pure culture. Thus, cultivation studies would appear to offer a worthwhile supplemental approach to investigate the phylogenetic diversity of at least some marine communities.

Chapter 5

Polaromonas vacuolata, gen. et sp. nov., a Psychrophilic, Marine Gas Vacuolate Bacterium from Antarctica

Reprinted from IJSB (Irgens et al., 1996)

Abstract. Several previously undescribed strains of gas vacuolate heterotrophic bacteria were isolated from marine waters in the vicinity of sea ice microbial communities near the U. S. Palmer Station on the Palmer Peninsula, Antarctica. Phylogenetic analyses using 16S rDNA sequencing coupled with nutritional, metabolic, and physiological studies indicate that these strains comprise a new genus and species of Eubacteria which are herein named *Polaromonas vacuolata*. *P. vacuolata* are heterotrophic, obligately aerobic bacteria that are sometimes motile by polar flagella. All known strains are psychrophilic and do not grow at temperatures above 15°C. Growth occurs in a medium containing amino acids as sole carbon sources or in complex media containing yeast extract, beef extract, and peptone. Vitamins are not required for growth. This is the first report of gas vacuolate members of the beta Proteobacteria as determined by 16S rDNA sequence analysis. Phylogenetically, the most closely related bacteria by 16S rDNA sequence analysis is the photosynthetic non-sulfur purple bacterium *Rhodoferax fermentans*, and a hydrogen autotroph, *Variovorax paradoxus*^{T}.

Introduction. Although gas vacuolate heterotrophic bacteria are well known inhabitants of aquatic ecosystems, until recently none had been observed or isolated from marine habitats. In 1989 several types were reported from Antarctica where they were found growing in association with the sea ice community of bacteria (Irgens et al., 1989; Staley et al., 1989). Sequencing and analyses of 16S rDNA from a variety of these Antarctic gas

vacuolate bacteria revealed that they were members of either the Proteobacteria or the CFB phylogenetic groups (Gosink and Staley, 1995). Within the Proteobacteria, the alpha, beta, and gamma subgroups are represented.

This paper includes a description of gas vacuolate members of the beta Proteobacteria that were isolated from the Palmer Peninsula area in Antarctica. Evidence is presented that indicates this group of strains comprises a new bacterial genus. A new species, *Polaromonas vacuolata*, is described.

Specific materials and methods. All strains were isolated from Antarctic waters off the Palmer Peninsula near the U. S. Palmer Station, Anvers Island, Antarctica (Table 5.1). Several strains including 41-P, 54-P, J-A, and J-B were isolated from samples collected beneath sea ice. All samples were transported on ice from the sampling areas to the Palmer Station Laboratory.

TABLE 5.1. Source of strains of *Polaromonas vacuolata* from Antarctica.

Strain No.	Location	Depth (m)	Date of Collection
34-P	Penola Strait, North of French Passage	25	23 November 1986
J-A, J-B	Arthur Harbor between Torgersen and Litchfield Islands	50	30 November 1986
54-P	Arthur Harbor[1]	2	8 December 1986
41-P	Arthur Harbor	10	8 December 1986

[1]The 8 December samples were collected northwest of Palmer Station beneath sea ice

All phenotypic tests, except fatty acid analysis, were performed by R. L. Irgens.

The 16S rDNA sequence of 34-P was aligned to the most similar sequences in the RDP as described above. The following prealigned, 16S

rRNA sequences (with their GenBank accession numbers) were also obtained from the RDP and incorporated into the dataset: *Thiobacillus perometabolis* M79421-M79423, *Sphaerotilus natans* Z18534, *Rubrivivax gelatinosus*^{T} D16213, *Brachymonas denitrificans*^{T} D14320, *Comamonas testosteroni*^{T} M11224, Stripa-derived bacterium L20811, *Variovorax paradoxus*^{T} D30793, str. PAD44 D26231, *Rhodoferax fermentans*^{T} D16211, *Alcaligenes faecalis*^{T} M22508, and *Bordetella parapertussis*^{T} U04949. Finally, a BLAST search was also performed against the October 1 1995 release of GenBank to determine if there were any sequences closely related to the sequence of 34-P but which were not included in release 5.0 of the RDP. Phylogenetic trees were generated as described in Chapter 2.

Results and discussion. These bacteria are short, unicellular, gram negative rods (0.8 × 2 - 3 μm) that typically produce gas vacuoles which appear as bright refractile areas within the cells (Figure 5.1 a). Although cells are non-motile under usual culture conditions, they produce polar flagella in addition to gas vacuoles (Figure 5.1 b). Cells may be encapsulated.

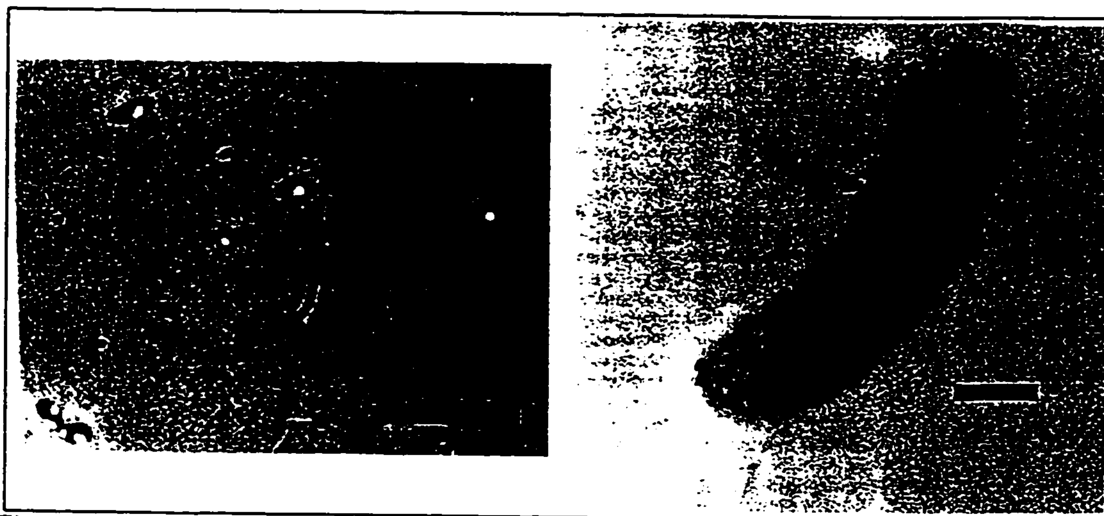


Figure 5.1. A phase photomicrograph (a) showing several cells of strain 34-P. Note the bright, irregular gas vacuoles in the cells. Bar equals 5 μm. Also, an electron micrograph (b) of 34-P showing a cell containing several gas vesicles. Flagella are not attached. Bar equals 0.5 μm.

On agar plates these bacteria produce circular, convex colonies with a smooth, glistening surface and an entire edge. The colonies are chalky white

in pigmentation. In stationary phase broth cultures containing 0.1% agar, the cells accumulate in the upper half of the tube due to gas vacuole production. No growth occurs on SWCm plates containing glucose (0.1%) or L-arginine (0.1%) as carbon sources when incubated in anaerobic jars (BBL GasPak, Baltimore, MD).

Strain 34-P grew well in the 0 to 12°C temperature range. It did not grow at 15°C. The generation time calculated from absorbance readings at 4°C was 40 hours. This strain grows when the initial pH of the medium ranges from 6.0 to 9.5. The pH of the spent medium was in all cases about 7.0, except when sugars were present in which case the pH fell to between 6.0 and 7.0. Good growth occurred at 0 to 6.0% NaCl. No growth occurred at 7.0% NaCl.

Polaromonas vacuolata, strain 34-P, tested positive for catalase, oxidase, urease, deaminase, and lipase (0.2 ml tributyrin per 100 ml SWCm). It was negative for nitrate reductase, amylase, protease (gelatin), cysteine desulfurase, and tryptophanase (indole production). The mol % G + C is 52.0 as determined by thermal melting.

Strain 34-P is sensitive to novobiocin (30 mcg), tetracycline (30 mcg), neomycin (30 mcg), and resistant to streptomycin (10 mcg) and gentamycin (10 mcg).

The organism grows well on SWCm medium, but does not grow when tryptone, yeast extract and beef extract are replaced with a B-vitamin solution (17) and NH_4Cl. Strain 34-P did grow when when these complex additives were replaced with vitamin-free casamino acids (Difco, Detroit, MI) indicating that vitamins are not required for growth. Good growth occurred in nutrient broth at 4.0 g per liter but there was no growth at 8.0 g per liter.

The following carbon sources are utilized by 34-P: lactate, malate, fumarate, citrate, succinate, 2-oxoglutarate, D-glucose, oxaloacetate, butyrate, DL-alanine, pyuvate, DL-glutamate, glycerol, DL-proline, propionate, DL-aspartate, DL-asparagine, acetate and sorbitol.

The following carbon sources are not utilized: maltose, D-fructose, sucrose, lactose, xylose, D-ribose, L-fucose, formate, L-arginine, glycine, DL-serine, malonate, DL-isoleucine, DL-lysine, DL-histidine, DL-ornithine, DL-methionine, DL-valine, cellobiose, mannose, melibiose, melezitose, rhamnose, sorbose, trehalose, methanol, ethanol, propanol, benzoate, erythritol, DL-threonine, and DL-tryptophan.

Whole cell fatty acid analyses were performed on all strains (Table 5.2). All strains contained large amounts (74 - 78%) of 16:1 ω7cis and smaller amounts of 16:0 (14 - 17%). In addition, a third fatty acid was present in lower amounts (7 - 9%). It was identified as 18:1 ω7cis or 18:1 ω9trans or 18:1 ω12trans, or possibly a combination of more than one of these. Its actual identity could not be resolved by the procedure and instrumentation used.

TABLE 5.2. Percent fatty acid compostion of strains of *Polaromonas vacuolata*[a].

Fatty Acids	strain 34-P	strain 41-P	strain J-A	strain J-B
16:1 ω7cis	75	79	74	75
16:0	17	14	15	15
18:1 ω7cis, ω9trans, or ω12trans	8	7	9	8

[a] Results for strains 34-P and 41-P are the average of two runs, the results for strains J-A and J-B are from only one run.

The 16S rDNA sequence of strain 34-P was compared with other bacteria included in the RDP (Table 5.3). By simple sequence homology, the most closely related described organisms are *Rhodoferax fermentans*, a non-sulfur purple bacterium (Hiraishi et al., 1991), and *Variovorax paradoxus*, a chemoorganotroph and facultative lithoautotroph (Davis et al., 1969; Willems et al., 1991). By this same measure, however, strain 34-P is most closely related to the environmental 16S rDNA sequences "str. Stripa" and "str. PAD44". The Stripa-derived bacterium sequence was obtained from deep groundwater in the Stripa mine in Sweden (Ekendahl et al., 1994). The "str. PAD44" or "env. PAD 44" is a sequence that was obtained from a paddy field (Ueda, 1993). However, both of these are simply environmental sequences without organisms available for comparison, so it is impossible to determine how similar the actual organisms might be to one another in other respects. Additionally, these are only partial 16S rDNA sequences so the actual relatedness might be different if the complete sequences were available.

Table 5.3. 16S rRNA Sequences Used in Phylogenetic Analysis

Species or environmental clone name	GenBank Number	16S rRNA sequence differences from 34-P: Total differences	Percent differences
str. Strip	L20811	44	5
Variovorax paradoxus[T]	D30793	75	5.2
env. PAD44	D26231	16	5.9
Rhodoferax fermentans[T]	D16211	94	6.5
Sphaerotilus natans	Z18534	100	7.4
Brachymonas denitrificans[T]	D14320	120	8.3
Rubrivivax gelatinosus[T]	D16213	122	8.4
Comamonas testosteroni[T]	M11224	123	8.5
Thiobacillus perometabolis	M79421 - M79423	97	11.3
Bordetella parapertussis[T]	U04949	173	12.1
Alcaligenes faecalis[T]	M22508	176	12.4

Phylogenetic analysis of these sequences reveals an uncertain relationship between these organisms. A preliminary phylogenetic set of the four most parsimonius trees using PAUP 3.0s (Swofford, 1991) was analyzed by MaClade 3.05 (Maddison and Maddison, 1992) to produce a substitution matrix to correct for varying rates of the 12 different types of nucleotide substitutions (e.g. A⇔C or G⇔U etc.) (data not shown). The overall rate of transitions to transversions was similarly determined to be 1.3. A rescaled consistency index weighting mask was also constructed from these trees using MacClade 3.05 (Maddison and Maddison, 1992).

The substitution matrix was reapplied to the aligned dataset in PAUP 3.0s (Swofford, 1991) and the most parsimonius trees were determined using the branch and bound option on both the original data set and 100 bootstrap resampled data sets. The orignal data set (with the applied substitution matrix) yielded four equally parsimonius trees of length 638. These trees varied in the relationship between "env. PAD44", *Rhodoferax fermentans, Variovorax paradoxus,* "str. Stripa", and strain 34-P. The consistency index for these trees is 0.386. The bootstrap values show only weak support for any particular branching structure near 34-P (Figure 5.2 a). Three similar trees were obtained when using corrections for the substitution matrix and the rescaled consistency index weighting mask simultaneously (data not shown).

The aligned data set used in the parsimony analysis was also resampled to make 100 bootstrap replicates using SEQBOOT (Felsenstein, 1989). Distance

matrix tables for both the original and the bootstrap resampled datasets were constructed using DNADIST (Felsenstein, 1989) with a Kimura 2 parameter correction, a jumbled input order, and the transition to transversion ratio set at 1.3. The original and bootstrap distance matrices were analyzed by NEIGHBOR (Felsenstein, 1989) (Figure 5.2 b). Again, the exact relationship between strain 34-P and its nearest phylogenetic neighbors is not certain. While there is clear support for 34-P in this group of beta Proteobacteria, the bootstrap values for any given branch order are quite low.

Finally, the aligned dataset was analyzed using fastDNAml (Felsenstein, 1981; Olsen et al., 1994) with the base frequencies determined empirically and the transition to transversion ratio set at 1.3 (Figure 5.2 c). Again, the more distantly related taxa show a similar branching topology to that determined in the parsimony and distance trees. As in the neighbor joining analysis, strain 34-P is between the Stripa mine clone and a clade containing *Rhodoferax fermentans* and *Variovorax paradoxus*. This is in contrast to the parsimony analysis which placed strain 34-P (with weak support) as a sister taxon to the Stripa mine clone.

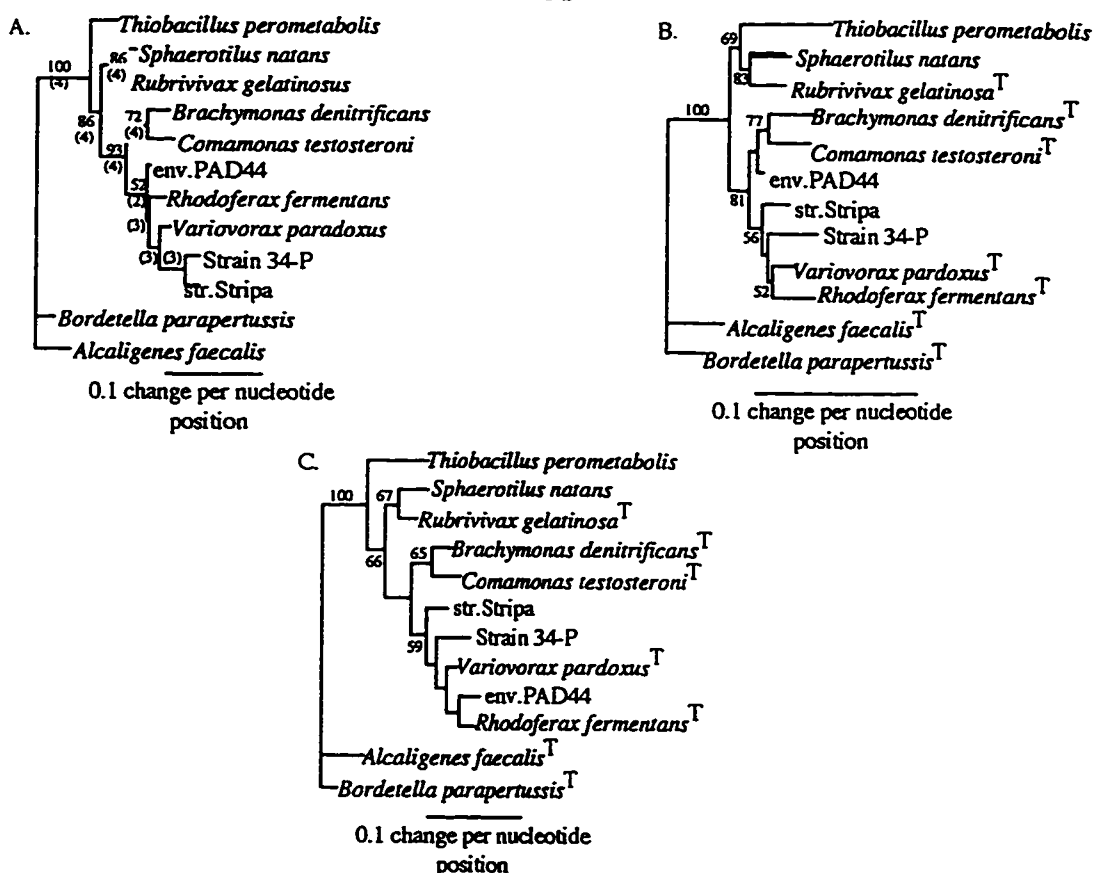


Figure 5.2. The phylogenetic relatedness of *Polaromonas vacuolata*, strain 34-P, to the most closely related species. Numbers near the branches but not in parentheses indicate percent bootstrap support for the clade. (a) Maximum parsimony tree as determined by an exact (branch and bound) search using a substitution matrix to correct for the various rates of nucleotide substitutions. This is one of four equally parsimonious trees. Numbers in parentheses near the branches indicate how many of the four equally most parsimonious trees shared that branch structure. (b) Neighbor joining tree. (c) Maximum likelihood tree for which all branches are significantly positive with $P<0.01$.

Each of these methods produced slightly different trees. For all the trees, however, it is clear that strain 34-P is most closely related to *Rhodoferax fermentans*, *Variovorax paradoxus*, "str. Stripa", and "env. PAD44". On the other hand, strain 34-P is not photosynthetic and does not grow as a non-sulfur purple bacterium under conditions used for the growth of *R. fermentans*. Also, strain 34-P differs by 5% and 7% in 16S rDNA base homology from *V. paradoxus* and *R. fermentans*, respectively. Furthermore, other genotypic and phenotypic differences indicate marked differences among *P. vacuolata*, *V. paradoxus*, and *R. fermentans* (Table 5.4). For

example, the mol% G + C values are 52-57 versus 67-69 and 60, respectively. In addition, *V. paradoxus* and *R. fermentans* are both pigmented and there are differences in cell shape and motility.

TABLE 5.4. Phenotypic comparison of *Polaromonas vacuolata* to the two phylogenetically most closely related species[1]

	Polaromonas vacuolata	*Rhodoferax fermentans*[T]	*Variovorax paradoxus*[T]
Shape of cells	rod	curved rods	straight or curved rods
Photosynthetic	-	+	-
Flagella	polar	polar	peritrichous
O_2 requirement	obligate aerobe	facultative aerobe	obligate aerobe
Temperature relations	psychrophilic	mesophilic	mesophilic
Colony pigmentation	white	peach-brown	yellow
Mol% G + C	52-57	60	67-69

[1] Data from this work and (Hiraishi et al., 1991; Willems et al., 1991)

This appears to be the first report of a gas vacuolate member of the beta Proteobacteria. This is not surprising, however, in that some members of both the alpha and gamma Proteobacteria are known to be gas vacuolate. A logical conclusion is that this feature is widespread among this phylogenetic group, many members of which are found in aquatic habitats where gas vacuolate bacteria most commonly reside.

On the basis of its phenotypic features and an analysis of its 16S rDNA base homology as discussed heretofore, 34-P is sufficiently different from other bacteria to warrant the creation of a new genus. We therefore propose that a new genus be declared and described as follows:

Polaromonas gen. nov. (Po'lar.o.mo'nas; M. L. adj. *polaris*, pertaining to the geographic poles; Gr. fem. n. *monas* unit; M. L. fem. n. *Polaromonas* polar bacterium).

Rod-shaped, gram negative rods, 0.8 x 2.0 - 3.0 μm; encapsulated. Aerobic. Chemoorganotrophic, catalase and oxidase positive. Require amino acids, but not vitamins for growth. Motile by polar flagellum. Growth temperature maximum up to 15°C. Mol % G + C is 52-57 (thermal denaturation).

The sole and type species is *Polaromonas vacuolata*, strain 34-P (= ATCC #51984).

The type species, *Polaromonas vacuolata*, is described as follows:

Polaromonas vacuolata (va.cu.o.la'ta. L. adj. *vacuus* empty; N. L. part. adj. *vacuolata* equipped with gas vacuoles)

Cells contain gas vesicles. Temperature for optimum growth is 4°C with a range of 0 to 12°C. Colonies are snowy white, circular, convex with a smooth surface and an entire edge. The more gas vesicles within the cells, the whiter the colony. There is good growth in media with the NaCl concentration ranging from 0 to 6.0% but no growth at 7.0%.

Tests for catalase, oxidase, urease, deaminase and lipase were positive. Amylase, protease (gelatin), tryptophanase (indole), nitrate reductase, cysteine desulfurase and agarase tests were negative.

The following carbon sources are utilized: acetate, lactate, malate, fumarate, pyruvate, propionate, citrate, succinate, oxaloacetate, butyrate, 2-oxoglutarate, glucose, glycerol, sorbitol, DL-alanine, DL-glutamate, DL-proline, DL-aspartate, and DL-asparagine.

The following carbon sources are not utilized: maltose, D-fructose, sucrose, lactose, D-xylose, D-ribose, formate, cellobiose, D-mannose, L-fucose, melibiose, melezitose, L-rhamnose, sorbose, trehalose, methanol, ethanol, erythritol, propanol, benzoate, malonate, DL-arginine, glycine, DL-serine, DL-isoleucine, DL-lysine, DL-histidine, DL-ornithine, DL-methionine, DL-valine, DL-threonine, and DL-tryptophan.

Sensitive to novobiocin, tetracycline, neomycin and kanamycin. Resistant to bacitracin, streptomycin and gentamicin.

The Mol% G+C ratio is 52.0%

The type strain is *Polaromonas vacuolata*, strain 34-P, ATCC number 51984.

CHAPTER 6.

Octadecobacter arcticensis gen. et. sp. nov., and *O. antarcticensis*, sp. nov., Nonpigmented Gas Vacuolate Bacteria from Polar Sea Ice and Water

Abstract. Heterotrophic, psychrophilic, gas vacuolate bacteria were recovered from arctic and antarctic sea ice and water. Cellular fatty acid analysis was used to group these isolates. One group herein described as the new genus *Octadecobacter*, had octadecenoic acid (18:1) in excess of 70% of their total fatty acid content. Phylogenetic analysis of the 16S rRNA of several strains from this group revealed that they were members of the alpha Proteobacteria and were most closely related to the genus *Roseobacter*. Further phenotypic and genotypic tests showed that these strains can be distinguished from *Roseobacter* on the basis of low levels of DNA/DNA hybridization, lack bacteriochlorophyll *a*, and because they are psychrophiles. *Octadecobacter* gen. nov., contains both a north polar species, *O. arcticensis* sp. nov. str. 238, and a south polar species, *O. antarcticensis* sp. nov. str. 307.

Introduction. Recently, a large number and variety of heterotrophic gas vacuolate (gas vesicle-producing) bacteria were found associated with arctic and antarctic sea ice and water (Irgens et al., 1989; Staley et al., 1989; Gosink et al., 1993). This finding was unexpected as gas vacuolate heterotrophs had not been previously reported from marine environments (Krieg and Holt, 1984; Walsby, 1994)

A set of over 250 heterotrophic gas vacuolate isolates have been obtained from annual sea ice and sea water from 35 separate sites over two field seasons in the Arctic and three field seasons in Antarctica. These isolates were grouped on the basis of whole cell fatty acid composition prior to further analysis. One group of 18 strains was distinguished by a very high level of octadecenoic acid (70-80%). Phylogenetic analysis of the 16S rRNA of an antarctic member of this group showed that it was a member of the α subclass of Proteobacteria and that it was most closely related to the the genus *Roseobacter* (Gosink and Staley, 1995).

The three described species of *Roseobacter* are pigmented marine heterotrophs found associated with green seaweed or dinoflagellates (Shiba, 1991; Lafay et al., 1995). Unlike most purple photosynthetic bacteria two of the three *Roseobacter* species produce bacteriochlorophyll *a* under dark aerobic conditions but not under light or anaerobic conditions (Shiba, 1991). Members of the alpha Proteobacteria are also noted for their high levels of octadecenoic acid (18:1). This phenotype is found in both pigmented and nonpigmented, bacteriochlorophyll *a*-producing and non bacteriochlorophyll *a*-producing, psychrotrophic, and mesophilic, acidophilic and non acidophilic species of α-3 and α-4 Proteobacteria (Fuerst et al., 1993; Kishimoto et al., 1995).

The fatty acid compositions of the 18 polar strains were very similar to each other and to that of *Roseobacter denitrificans*; however, unlike *Roseobacter*, these strains lack pigments, grow only at low temperature, and have a different cell shape. Also, unlike the three species of *Roseobacter*, all 18 poiar isolates produce gas vesicles. To determine the relationship of this group of polar gas vacuolate bacteria to *Roseobacter* and the other Proteobacteria, four members of the group were investigated in greater taxonomic detail. Specifically, the 16S rDNA of a second member, isolate 238 from the arctic, was sequenced, and along with isolate 307 from the antarctic (Gosink and Staley, 1995), was phylogenetically analyzed. Genomic DNA from these two strains were also hybridized with each other, and with that of *R. denitrificans*. Two other members (strains 308 and 309) from the group of 18, along with isolates 238 and 307 and *R. denitrificans* were also examined for additional phenotypic characteristics.

Results and Discussion. Gas vacuolate isolates of heterotrophic bacteria were obtained from the sea ice and sea water in the Beaufort Sea, appoximately 350 km north of Deadhorse, Alaska, and from McMurdo Sound, Antarctica. Refractile areas in cells observed under phase microscopy were revealed as gas vesicles by electron microscopy (Figure 6.1).

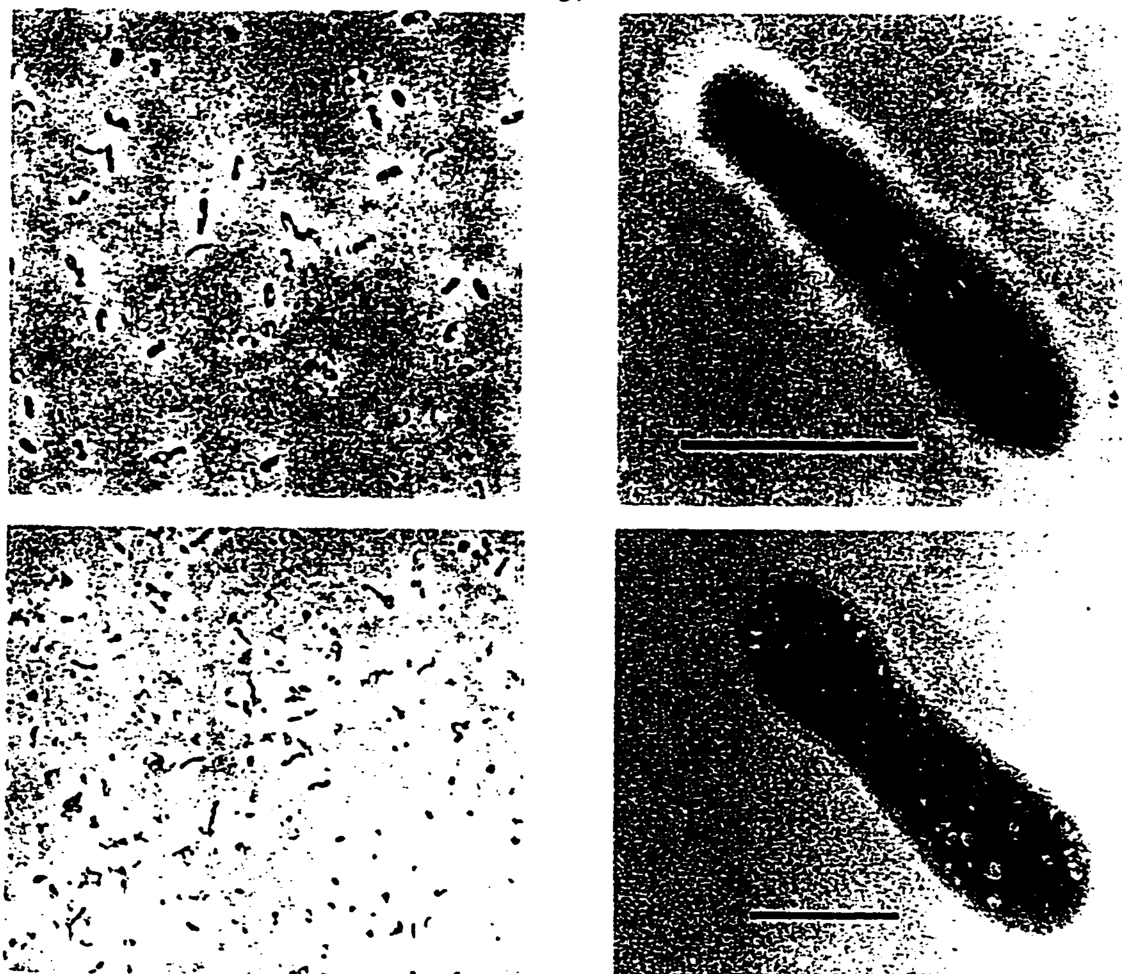


Figure 6.1. Phase micrographs (left), and electron micrographs (right) of *Octadecobacter* strains 238 (top) and 307 (bottom). Scale bars for the phase and electron micrographs equal equal 5 μm and 1 μm respectively.

Fatty acid grouping. One set of polar heterotrophic gas vacuolate isolates was distinguished by an unusually high level of octadecenoic acid (70-80%) (Table 6.1). The exact identity of this fatty acid, (either 18:1 ω7cis, ω9t, or ω12t) could not be resolved with the instrumentation used in the analysis. The remainder of the fatty acids in this group were mostly 16:1 ω7cis, 16:0, and 10:0 3OH along with several other fatty acids, each comprising 3% or less of the total. *Roseobacter denitrificans* and *R. algicola* showed similar types and levels of fatty acids. The concentration of octadecenoic acid in *R. agicola*, in fact, are the highest we are aware of for any single fatty acid in a bacterium. Similar concentrations of fatty acids were obtained when the isolates were grown on SWCm or marine media 2216 or, in the case of *R. denitrificans*, at

4°C or 10°C (data not shown). Four isolates, one Arctic (238), and three Antarctic (307, 308, 309), were investigated by further phenotypic methods. Isolates 238 and 307 were also examined by genotypic and phylogenetic methods described below.

Table 6.1. Fatty Acid Composition of *Octadecobacter* and *Roseobacter* Strains

	% of Total Fatty Acids			
Strain	18:1 ω7cis, ω9trans, ω12trans	16:1 ω7cis	16:0	10:0 3OH
238	75	8	6	4
307	77	12	6	2
308	77	11	6	2
309	77	11	6	2
Roseobacter denitrificans	93	1	1	3
Roseobacter algicola[a]	93	0	2	2

[a] *R. algicola* was grown on SWCm at room temperature

Phylogenetic analyses. Preliminary analysis of the 16S rDNA sequences of strains 238 and 307 showed that they were different in 11 nucleotide positions, and that the sequence of strain 307 possesed two more nucleotides in the V8 loop (Gutell et al., 1985) than the sequence of strain 238. Two of the 11 differences were accounted for by cannonical changes across a stem structure. The electropherograms were unambiguous about all of the differences and none of them violated the types of non-cannonical pairing seen in other rRNA sequences (Gutell, 1994). The Similarity_Rank program (Larsen et al., 1993) of the RDP and a BLAST search (Altschul et al., 1990) of GenBank, indicated that strains 238 and 307 were closely related to the *Roseobacter* genus of the alpha subdivision of Proteobacteria (Purple Bacteria). A set of 36 aligned sequences representing the entire group 2.13.1.3 of the RDP, of which *Roseobacter* is a member, along with four sequences from the adjacent groups 2.13.1.4 and 2.13.1.5 were retrieved from the RDP. The aligned sequences of strains 238 and 307 were integrated into this dataset. Alignment to closely related sequences in the dataset was checked a final time both by "eye" and by comparison to secondary structure diagrams.

This dataset was analyzed repeatedly with a neigbor-joining (Felsenstein, 1989) algorhithm under a Kimura 2 parameter model (Kimura, 1980). Different transition to transversion ratios and different sets of taxa were included in the analysis to determine the phylogenetic relationships of the sequences most similar to those of 238 and 307. These preliminary results reduced the dataset to a minimal set of 17 taxa representing the sequences most closely related to the sequences of 238 and 307, taxonomically important species of *Paracoccus, Rhodobacter, and Roseobacter*, and three outgroup species, *Hirschia baltica, Erythrobacter longus* str. OCh 101, and *Porphyrobacter neustonensis* (Table 6.2).

Table 6.2. List of Organisms and Environmental Clone 16S rDNA Sequences and their Nucleotide Differences from *Octadecobacter* 16S rDNA Sequences

Species or environmental clone name	GenBank Number	Source	16S rRNA sequence differences from strain 238 differences/ %differences	307 differences/ %differences
Roseobacter litoralis	X78312	Och 149, ATCC 49566	75/ 5.5	72/ 5.3
Roseobacter denitrificans	M59063	Och 114, ATCC 33942 (T)	85/ 6.3	82/ 6.1
Roseobacter algicola	X78315	ATCC 51440 (T)	90/ 6.7	89/ 6.7
str. 36 (Nielson)	NA	NA	79/ 5.9	77/ 5.8
str. LFR	L15345	ATCC 51258	85/ 6.8	83/ 6.6
str. NF18	M79390-M79392	NA	63/ 7.5	62/ 7.4
str. SAR83	M63810	NA	96/ 7.6	99/ 7.9
Rhodobacter veldkampii	D16421	ATCC 35703 (T)	107/ 7.8	109/ 8.0
Paracoccus alkaliphilus	D32238	JCM 7364	112/ 8.2	110/ 8.0
Paracoccus denitrificans	X69159	LMG 4218 (T)	118/ 8.6	116/ 8.5
str. AG33	M79372-M79374	NA	73/ 8.7	72/ 8.6
Rhodobacter sulfidophilus str. W12	D16430	DSM 2351	120/ 8.8	120/ 8.8
Hirschia baltica	X52909	ATCC 49814 IFAM 1418	164/ 12.2	161/ 12.0
Porphyrobacter neustonensis	M96745	ACM 2844	184/ 14.2	182/ 14.0
Erythrobacter longus str. *OCh 101*	M59062	ATCC 33941 IFO 14126 (T)	197/ 14.5	195/ 14.3

NA, Not available

Character positions near the start and end of the dataset were removed because only a few of the taxa had nucleotide data for those positions. Two regions in the middle of the alignment also were removed because they could not be unambiguously aligned. The remaining alignment retained 402 varying characters.

A series of phylogenetic analyses were then employed to resolve the relationship of strains 238 and 307 to the 15 other taxa in the dataset. First, a distance analysis was conducted by neighbor joining under a Kimura 2-parameter model using a (default) transition to transversion ratio (R) of 2. The resulting tree had an inferred R of 1.15. Another neighbor joining analysis based on this value produced an identical tree to that in the first analysis. 100 boostrap replicates were also analyzed under an R of 1.15 in a Kimura 2-parameter neighbor joining framework using jumbled sequence addition.

The neighbor joining tree was analyzed by MacClade to produce two "cost matrices" and a "weight set" for use in parsimony analyses to correct for uneven transition probabilities and varying substitution rates among the character positions. Another cost matrix was constructed which weighted each transition twice as much as each transversion (to approximate the observed R value of 1.15). A second set of phylogenetic analyses was then conducted using either weighted or unweighted Fitch (no cost matrix) or Wagner (with either cost matrix applied) parsimony in PAUP 3.0s. Both Fitch analyses (weighted and unweighted) were conducted on 100 boostrap resamplings, but computational expense prohibited a similar analysis for Wagner parsimony.

Finally, likelihood analysis was conducted on the dataset using fastDNAml. Set parameters included empirically derived nucleotide frequencies, and an R of 1.15. 100 bootstrap replicates were also searched using jumbled sequence addition. The log likelihood of the resultant tree was -6687 and it was topologically the same as both Fitch parsimony trees.

Most of the trees obtained by these various methods were different depending on which evolutionary framework (distance, parsimony, or likelihood) and what specific assumptions (transition/transversion ratio, weighting sets, step matrices etc) were applied. These methods produced a total of six distinct tree topologies. In all of these cases strains 238 and 307 rooted deeply within the RDP's group 2.13.1.3, but were not within the *Paracoccus* or *Rhodobacter* genera. These trees were evaluated under a likelihood framework by the Kishino-Hasegawa test (Kishino and Hasegawa, 1989) to see if any of them were statistically more likely than any of the others. This analysis showed that a tree produced under the unweighted Wagner

parsimony with a transition to transversion cost of 2:1 produced the best likelihood tree (Figure 6.2). Only the tree produced under an unweighted Wagner parsimony model with a cost matrix derived from the neighbor joining tree had a statistically lower likelihood than the best tree. The remaining four tree topologies, while having lower ln likelihood scores than the best tree, were not significantly worse than the best tree.

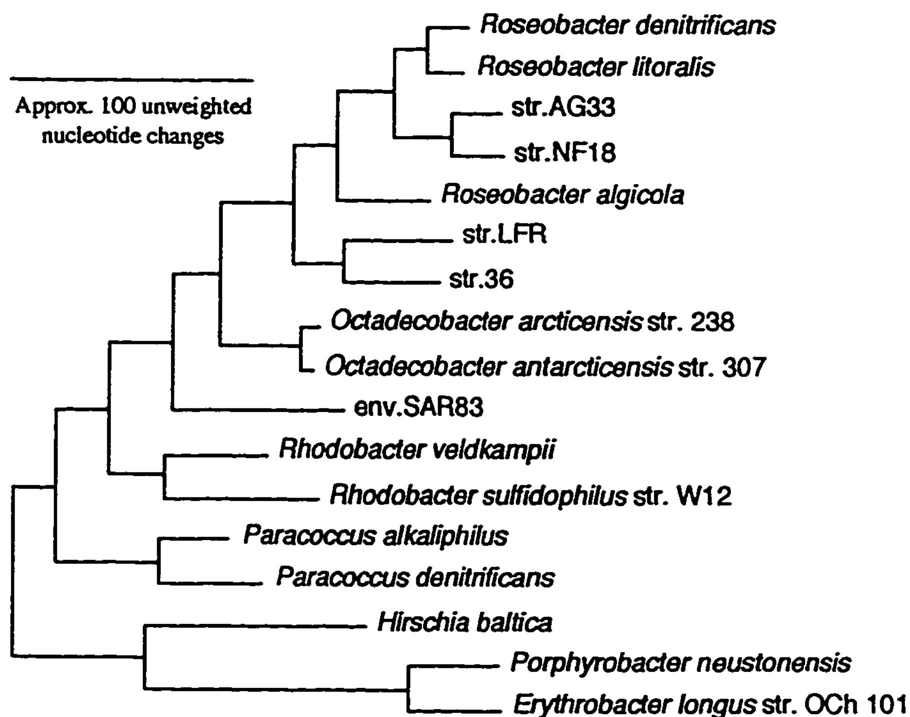


Figure 6.2. Phylogenetic Position of *Octadecobacter* in Relation to *Roseobacter* and Closely Related Species. This tree was produced by unweighted Wagner parsimony employing a cost matrix based on a transition to transversion cost of 2:1. Scale bar represents approximately 100 unweighted nucleotide changes. No bootstrap values are given as computational expense prohibited bootstrap resampling.

Phenotypic and genotypic analyses. The polar marine gas vacuolate strains were then compared by phenotypic and genotypic tests to the type species of *Roseobacter*, *R. denitrificans*. Strains 238, 307, 308, and 309 lack bacteriochlorophyll *a* and associated pigments (Table 6.3). *R. denitrificans* was brick red to brown on SWCm and 2216, whereas 238, 307, 308, and 309 were all white. *In vivo* spectroscopy did not reveal any peaks at 802-807 nm, 863-867 nm, or 868-873 nm characteristic of *Erythrobacter longus* and *R. denitrificans*

(Shiba, 1991). Spectroscopy of methanol extracts of 238, 307, 308, and 309 were likewise devoid of any peaks between 650 and 900 nm (Fuerst et al., 1993). These peaks were, however, observed in preparations of *R. denitrificans*, and in the case of the methanolic extracts, acidification caused an absorbance shift characteristic of bacteriochlorophyll *a* conversion to bacteriophaeophytin. Unlike *R. algicola* (Ledyard et al., 1993), and the DMSP-degrading isolate LFR (Lafay et al., 1995), none of the methanolic extracts of the polar isolates displayed an absorbance peak at 413 nm , thought to correspond to Mg-porphyrin compounds (Lafay et al., 1995).

Table 6.3. Physiological and Nutritional Characteristics of *Roseobacter* and *Octadecobacter* Strains

		Roseobacter denitrificans	*O. arcticensis* str. 238	*O. antarctic-ensis* str. 307	308	309
Cell morphology		rod	rod	rod	rod	rod
Cell size		0.6-1 × 1-2.4 μm	0.6-0.8 × 2.4-4 μm	0.6-0.8 × 1.6-4.8 μm	0.6-1.6 × 2.4-15 μm	0.6-0.8 × 1.6-3.2 μm
Motility		+	-	-	-	-
Gas vesicles		-	+	+	+	+
Colony color		red-brick red	white	white	white	white
Colony shape		circular, convex, entire	circular, convex, entire	circular, convex, entire	circular, convex, entire	circular, convex, entire
pH growth range						
	5.5	-	-	-	-	-
	6.5	+	+	+	+	+
	7.6	+	+	+	+	+
	8.5	+	±	+	+	+
	9.5	+	-	+	+	+
Temperature growth range						
	4°C	+	+	+	+	+
	10°C	+	+	+	+	+
	15°C	+	±	-	-	-
	19°C	+	-	-	-	-
	37°C	+	-	-	-	-
Salinity growth range[a]						
	0 ppt	-	-	-	-	-
	1 ppt	-	-	-	-	-
	3.5 ppt	-	-	-	-	-
	17 ppt	+	+	+	+	+
	35 ppt	+	+	+	+	+
	70 ppt	W	W	+	+	+
Utilization as a carbon source[b]						
	acetate	-	-	-	-	-
	pyruvate	+	±	-	±	±
	succinate	±	±	±	-	±

Table 6.3 (continued)

citrate	+	±	-	-	-
propionate	+	±	-	+	+
butyrate	-	-	-	-	-
glutamate	+	±	+	±	+
aspartate	+	±	-	-	+
leucine	±	-	-	-	-
proline	-	-	-	-	-
Cas Amino Acids	+	+	+	+	+
glycolic acid	+	±	±	-	+
glycerine	-	+	±	+	+
ribose	+	±	-	-	-
fructose	+	-	-	-	-
glucose	±	±	±	-	±
sucrose	-	-	-	-	-
N-acetyl-glucosamine	-	-	±	-	+
ethanol	-	-	-	-	-
methanol	-	-	-	-	-
Requirement for					
biotin	+	-	?[c]	+	+
thiamine	+	+	?	+	+
nicotinic acid	+	+	?	+	+
pantothenic acid	-	+	?	+	+
Hydrolysis of					
gelatin	+	-	-	-	-
starch	-	-	-	-	-
Gram stain	-	-	-	-	-
Catalase	+	+	+	+	+
Oxidase	+	-	-	-	-
NO_3^- reduction	+	-	-	-	-
Glucose	+	+	+	+	+
oxidation					
fermentation	-	W[c]	-	-	-
Mole % G+C	59.6	57	56	ND	ND
Absorbance bands *in vivo*					
802-807 nm	+	-	-	-	-
863-867 nm	-	-	-	-	-
868-873 nm	+	-	-	-	-
Absorbance band in methanolic extracts (769-773 nm)	+	-	-	-	-
Absorbance band in methanolic extracts after acidification (752-754 nm)	+	-	-	-	-

a Weak result

b + = three fold or greater increase in A_{600} over cultures without the carbon source. ± = two to three fold increase in A_{600} over cultures without the carbon source. - = less than two fold increase in A_{600} over cultures without the carbon source.

c Another cofactor or vitamin is required other than biotin, thiamine, nicotinic acid, or pantothenic acid.

Cultures of 238 and 307 grown aerobically in the light showed a slightly faster onset of turbidity than cultures grown in the dark, and although they were grown side by side in a 6° C cold room, the possibility that low level heating from the light increased the growth rate of 238 and 307 could not be ruled out.

Another major difference between the polar sea ice isolates and the genus *Roseobacter* is the presence of gas vesicles. Phase contrast microscopy of cultures of *R. denitrificans* and *R. algicola* under different growth conditions at different stages of growth never revealed the presence of gas vesicles.

Other physiological and nutritional parameters of the polar gas vacuolates are listed in Table 6.3. It was impossible to grow these strains on any of the carbon sources listed in Table 6.3 as sole carbon sources at 0.2% concentration. Even when a small amount of yeast extract (0.2 g/l) was incorporated into the basal medium, cells rarely grew to optical densities greater than 0.1 at 600 nm (see Chapter 2, "Nutritional requirements" for details).

Unlike *Roseobacter denitrificans*, the polar gas vacuolate strains lacked the ability to reduce NO_3^- to NO_2^-, the ability to grow at 15°C or higher (except in the case of strain 238 which could not grow at 19°C or higher), the ability to utilize fructose as a carbon source, the ability to hydrolyze gelatin, and they lacked oxidase activity. None of the polar gas vacuolate strains grew on either egg yolk SWCm agar or Tween 80 SWCm agar, so the presence of lipase in these strains was not determined.

The polar gas vacuolate isolates grew in anaerobic fermentation tubes under 10 mm of mineral oil, and they typically grew several millimeters below the surface in the semisolid NO_3^- reduction tubes. This suggests that they are microaerophilic. They were not capable of growth in anaerobic gas pack jars (BBL) on SWCm agar plates at 10°C.

A few differences were noted in some tests between our results and those of Shiba (Shiba, 1991). Particularly, in our study *Roseobacter denitrificans* was found to grow at pH 6.5 and was unable to utilize acetate as a carbon source.

DNA/DNA hybridization experiments showed that strains 238 and 307 are 42% ± 7% similar to one another. Strain 238 and *Roseobacter denitrificans*

showed DNA/DNA hybridization values of 35% ± 9%. Strains 307 and *R. denitrificans* are 42% ± 15% similar by DNA/DNA hybridization. All of these values are below 70%, the value defined for isolates of the same species (Wayne et al., 1987).

Taxonomy. Strains 238, 307, 308, and 309 are related to the genus *Roseobacter*. However, phylogenetic differences, the absence of bacteriochlorophyll *a* and other photopigments, the presence of gas vesicles, and low DNA/DNA hybridization values between the 238, 307 and *R. denitrificans* indicate that these strains should be considered members of a new genus descbribed below (Table 6.4).

Table 6.4 Features that Differentiate Between *Octadecobacter* strains and *Roseobacter* strains.

	Roseobacter denitrificans	O. arcticensis str. 238	O. antarcticensis str. 307
Motility	+	-	-
Gas vesicles	-	+	+
Colony color	red-brick red	white	white
Growth at pH 9.5	+	-	+
Growth at 19 °C	+	-	-
Utilization as a carbon source[a]			
pyruvate, citrate, propionate, ribose	+	W	-
leucine	W	-	-
glycerine	-	+	W
fructose	+	-	-
N-acetyl-glucosamine	-	-	W
Requirement for			
biotin	+	-	?[b]
thiamine	+	+	?
nicotinic acid	+	+	?
pantothenic acid	-	+	?
other growth factors	-	-	+
Gelatin hydrolysis	+	-	-
Oxidase	+	-	-
NO_3^- reduction	+	-	-
Glucose fermentation	-	W	-
Absorbance bands *in vivo* (802-873 nm) or in methanolic extracts (769-773 nm)	+	-	-

W Weak

? Unknown

Octadecobacter oc.ta.dec´.o.bac.ter. Gr. prefix *okto* meaning eight, Gr. prefix *deka* meaning ten, Gr. neut. n. *bakterion* a rod, M. L. masc. n. *Octadecobacter* an 18 (carbon fatty acid-containing) rod. Cells are gram negative, non motile rods or pleomorphic rods. Facultatively aerobic or microaerobic but do not reduce NO_3^- to NO_2^-. Do not produce bacteriochlorophyll *a*. Form white, circular, convex, entire colonies on SWCm agar. Grow at pH 6.5 to 8.5 at temperatures down to 4°C in media from 17 to 70‰ salinity. Catalase positive but oxidase negative. Grow on few (if any) organic compounds as sole carbon sources at 0.2% concentration. In the presence of small amounts of yeast extract they grow to low turbidities on selected carbon sources including glutamate, glycerine, and mixed amino acids. DNA base composition is 56 to 57 mol% G+C (by HPLC). Octadecenoic acid (18:1) is the predominant fatty acid. Members of the α subclass of Proteobacteria on the basis of small subunit rRNA sequences. The type species is the arctic isolate, 238, hereinafter referred to as *O. arcticensis*.

Octadecobacter arcticensis arc.tic.en´.sis. M. L. gen *arcticensis* of the arctic. The cells of the type strain, *Octadecobacter arcticensis* form long irregular or pleiomorphic rods from 0.6-0.8 x 2.4-4 μm in size. Produces gas vesicles. *O. arcticensis* requires thiamine, nicotinic acid, and pantothenic acid for growth. Ferments glucose weakly. Grows well on glycerol and mixed amino acids only in the presence of small amounts of yeast extract. Grows at temperatures from 4 to 15°C. Predominant fatty acids when grown on marine medium 2216 at 10°C are 18:1 ω7cis, ω9trans, or ω12trans (75%), 16:1 ω7cis (8%), 16:0 (6%), and 10:0 3OH (4%). G+C content is 57%. Isolated from sea ice 350 km offshore of Deadhorse, Alaska. Type strain of the species is strain 238.

Octadecobacter antarcticensis ant.arc.tic.en´.sis. M. L. gen. *antarcticensis* of Antarctica. *Octadecobacter antarcticus* forms long irregular or pleiomorphic rods from 0.6-0.8 x 1.6-4.8 μm in size. Members of the species grow well on glutamate and mixed amino acids in the presence of small amounts of yeast extract. Do not grow on vitamin-free media supplemented with only biotin, thiamine, nicotinic acid, and pantothenic acid. Growth

temperature range is from 4 to 10°C. On marine media 2216 at 10°C its predominant fatty acids are 18:1 ω7cis, ω9trans, or ω12trans (77%), 16:1 ω7cis (12%), 16:0 (6%), and 10:0 3OH (2%). G+C content is 56%. Isolated from sea ice in McMurdo Sound, Antarctica. Type strain of the species is strain 307.

Chapter 7.

Polarobacter gen. nov, with Three New Species, *P. filamentus* sp. nov., *P. irgensii* sp. nov., and *P. franzmannii* sp. nov., Gas Vacuolate Polar Marine Bacteria of the Cytophaga/Flavobacterium/Bacteroides Group and Reclassification of *Flectobacillus glomeratus* as *Polarobacter glomeratus* comb. nov.

Abstract. A group of gas vacuolate members of the CFB phylogenetic group were isolated from sea ice and water from the Arctic and Antarctica. The closest taxonomically defined species by 16S rRNA sequence analysis is *Flectobacillus glomeratus* . However, *Flc. glomeratus* is phylogenetically distant from the *Flectobacillus* type strain, *F. major*. On the basis of phenotypic, genotypic, and 16S rRNA sequence analyses we propose a new genus, *Polarobacter* with three new species, *P. irgensii* str. 23-P, *P. filamentus* str. 215, and *P. franzmannii* str. 301. This is the first taxonomic description of gas vacuolate bacteria in the CFB group. Additionally, we propose that *F. glomeratus* should be reclassified to *Polarobacter glomeratus*, comb. nov.

Introduction. Species from seven of the twelve major phylogenetic groups of Bacteria are known to produce gas vesicles. These include the alpha, beta, and gamma Proteobacteria, cyanobacteria, gram positive bacteria, green sulfur bacteria, and Planctomyces (Walsby, 1994). Archaea of the group Euryarcheota including species of extreme halophiles and methanogens also produce gas vesicles. In spite of this diversity, only about 2% of taxonomically defined species are described as producing gas vesicles (Walsby, 1974; Krieg and Holt, 1984). Recently, a large number of gas vacuolate bacteria have been isolated from arctic and antarctic sea ice and water. These isolates were initially grouped on the basis of fatty acid composition. A member from one of these groups (strain 301) was found to belong to the CFB group by 16S rRNA phylogenetic analysis (Davis, 1990; Gosink and Staley, 1995). This study examines the phenotypic, genotypic, and phylogenetic identitiy of three

members of this group, two strains from Antarctica (23-P and 301) and one strain from the Arctic (215). In the course of this investigation it was also found that *Flectobacillus glomeratus* , while closely related to the polar gas vacuolate strains on the basis of its 16S rDNA sequence, is phylogenetically separate from the type strain of the genus, *F. major*^{T}.

Specific materials and methods. Strains were collected from the sea ice and water of the Arctic and Antarctica (Figure 2.1) as previously described. Strain 23-P was isolated between 10 and 25 m depth in Penola Strait, Antarctica in November of 1986 (Irgens et al., 1989). Strain 215 was isolated from surface sea water in a small lead, 350 km offshore of Deadhorse, Alaska. Strain 301 was isolated at 25-50 cm from the bottom of an ice core at site 5 in McMurdo Sound, Antarctica in November of 1992 (Gosink and Staley, 1995). Strain 84-W(gv)1 was isolated from water 18 m beneath the ice at site 7 in Antarctica in December of 1987 (Staley et al., 1989).

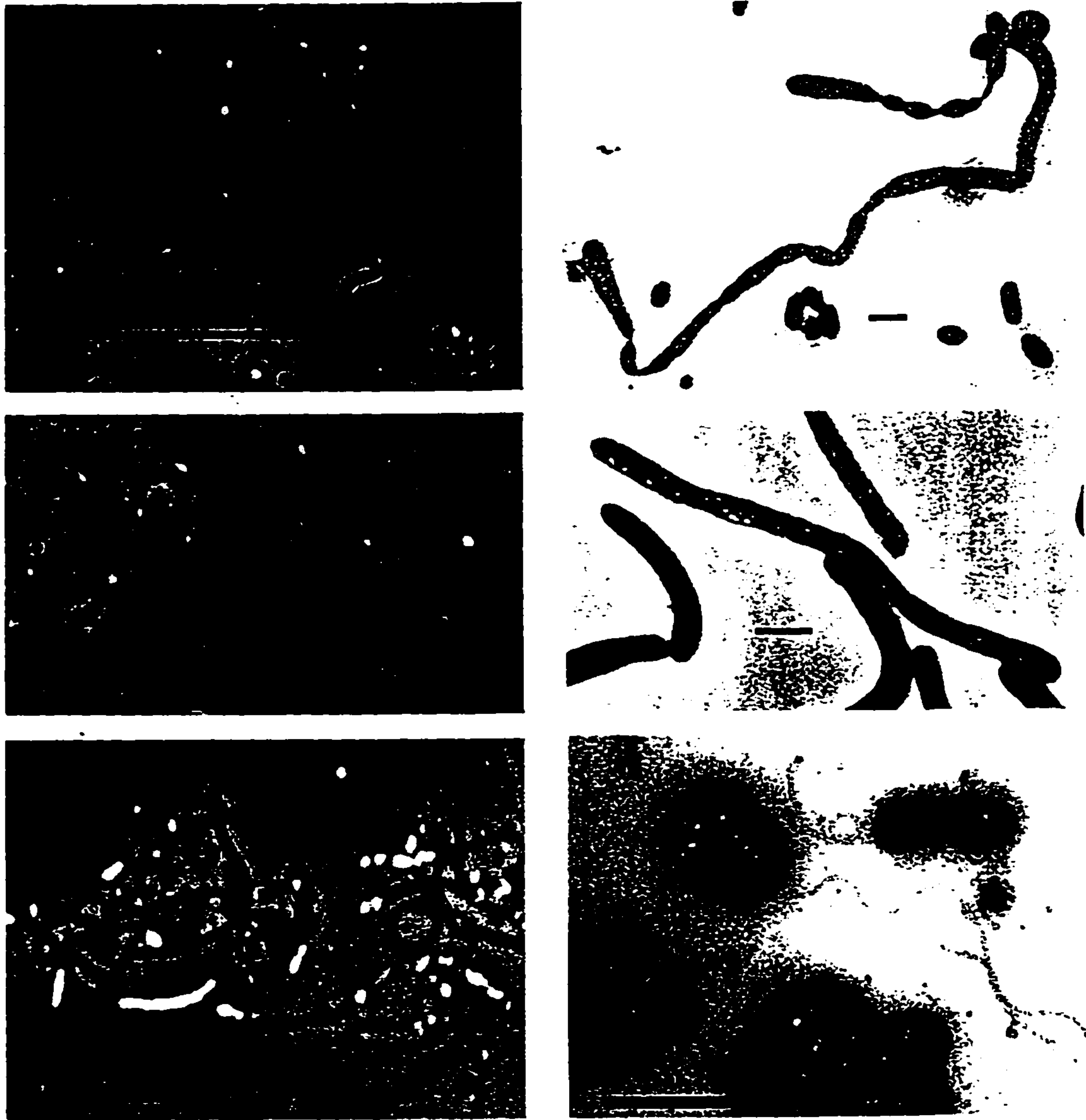


Figure 7.1 Phase Micrographs (left) and Electron Micrographs (right) of (a) strain 215, (b) strain 301, and (c) strain 23-P. Scale bars on the phase micrographs and electron micrographs are 10 μm and 1 μm respectively.

Strains were propagated on SWCm and fatty acid methyl ester analyses were conducted as described in Chapter 2.

The sequences of strains 215 and 301 were obtained as described in chapter 2. The sequences of strains 23-P and 84-W(gv)1 were determined in the lab of Carl Woese (Woese, 1991). The sequence of strain 23-P had been previously deposited in GenBank under the name 'Vesiculatum antarcticum', or 'Antarcticum vesiculatum' (GenBank accesion # M61002).

The 16S rRNA sequences listed in Table 7.1 were also included in the analysis. Phylogenetic analyses were conducted using distance, parsimony, and likelihood methods as described in chapter 2.

Table 7.1. List of Organisms and Environmental Clone 16S rDNA Sequences used in Phylogenetic Analyses.

Species or Environmental Clone Name	GenBank Number	Source
Strain 215	NA	
Strain 301	U14586	
Strain 23-P	M61002	
Strain 84-W(gv)1	NA	
Flectobacillus glomeratus	M58775	ATCC 43844; UQM 3055
Flexibacter maritimus	M64629	ATCC 43398; NCIMB 2145
marine snow associated clone agg50	L10947	NA
Cytophaga marina	D12667	JCM 8137
Cytophaga johnsonae str. MYX.1.1.1	M59051	ATCC 17061; DSM 2064; NCIMB 11054
Flavobacterium aquatile	M28236	ATCC 11947; DSM 1132
Capnocytophaga canimorsus str. 2340-2-61	L14637	ATCC 35979; CDC 7120
Flavobacterium odoratum[T]	M58777	ATCC 4651; NCTC 11036
Cytophaga lytica str. LIM-21	M28058	ATCC 23178; DSM 2039
Flavobacterium gondwanense	M92278	DSM 5423; ATCC 51278
Cytophaga marinoflava	M58770	NCIMB 397
Weeksella virosa	M93152	ATCC 43766; NCTC 11634
Weeksella zoohelcum	M93153	ATCC 43767; NCTC 1160
Microscilla aggregans subsp. *catalatica* str. HI-3	M58791	ATCC 23190
Ornithobacterium rhinotracheale	L19156	LMG 9086
Cytophaga fermentans	M58766	ATCC 19072; NCIMB 2218
Cytophaga salminocolor subsp. *agarovoran*	D12654	ATCC 19043; NCIMB 2217
Bacteroides splanchnicus	L16496	NCTC 10825
Cytophaga marinoflava	M58770	NCIMB 397
Flexibacter flexilis str. Lewin	M62794 M28056	ATCC 23079; IFO 15060
Flectobacillus major	M62787	ATCC 29496; DSM 103
Flectobacillus marinus str. WH-A	M62788, M27801	ATCC 43824

NA, Not available

Phenotypic tests were conducted at 10°C for strains 23-P, 215, 301. *Flectobacillus glomeratus* was incubated at 15°C for the carbon source, macromolecule hydrolysis, multiple media, and biochemical tests. *F. glomeratus* was incubated at 10°C for the oxidation fermentation tests. All other strains were incubated at room temperature unless otherwise noted.

The ability of these strains to grow on various carbon sources was tested as described in chapter 2 except that the yeast extract concentration was 0.05 g/l, and in the case of *Flectobacillus major*, the basal salts solution (SWCm salts) was diluted 1:10 as it would not grow in full strength SWCm.

Growth was assayed at 7, 14, 21, and 28 days post inoculation. Growth was scored as negative if the turbidity at 600 nm did not increase two fold above the intial level, it was scored plus/minus if turbidity increased to between two and four fold greater than the initial level, and it was scored as positive if the turbidity increased four fold or more over the initial level.

For the macromolecule hydrolysis tests, strains were grown on a SWCm base (*Flectobacillus marinus*, *Flc. glomeratus*, 23-P, 215 and 301), or on a 956 medium base (*Flc. major*) as described in chapter 2.

Colony color, colony shape, cell morphology, coccoid body formation, and coil formation were all examined in cells grown on SWCm or medium 956 plates (*Flectobacillus major*).

Results and discussion. Strains were initially grouped on the basis of whole cell fatty acid composition. The principal fatty acids of one group of polar gas vacuolate strains included high levels of several branched hexadecanoic (palmitic) acid derivatives. These fatty acids are often found in *Flavobacterium* and *Cytophaga* (Krieg and Holt, 1984). Two of these strains, 215 and 301, were investigated in further detail. Two other isolates, 23-P and 84-W(gv)1 had also been partially characterized previously (Irgens et al., 1989; Davis, 1990; Woese, 1991). The average fatty acid compositions of three different preparations of each of the four strains are shown in Table 7.2.

Table 7.2 Fatty acid Composition of *Polarobacter* and Related Strains

	% of Total Cellular Fatty Acids			
	23-P	301	215	84-W(gv)1
13:0 iso	2	5	5	2
15:1 iso F	ND[a]	ND	ND	22
15:1 iso G	6	11	12	ND
15:1 iso I/H /13:0 3 OH	ND	ND	6	0.4
15:0 iso	12	9	22	20
15:0 anteiso	6	4	6	16
15:1 ω6c	3	9	9	ND
16:1 ω7cis	2	9	4	1
15:0 iso 3OH	38	17	22	18
15:0 3OH	10	7	2	ND

a ND, not detected

The phylogenetic relationship of these strains to each other, and to other previously described species was determined by 16S rDNA sequence analysis. The sequences of strains 307 (Gosink and Staley, 1995), 23-P (Davis, 1990), and 84-W(gv)1 (Woese, 1991) had been determined earlier. The approximate phylogenetic identity of these strains was determined through the RDP's SIMILARITY_RANK program (Olsen, 1992). Closely related 16S rRNA sequences were retreived from the RDP and aligned with the sequences of the polar gas vacuolate strains as described in chapter 2.

An initial phylogenetic analysis was performed using DNADIST, and NEIGHBOR (Felsenstein, 1989) under a Kimura 2 parameter model (Kimura, 1980) with the transition to transversion ratio (R) set at 1.0. The R value of the resulting tree,1.17, with *Flectobacillus glomeratus*, *Flc. marinus*, and *Flexibacter flexilis* as the outgroup, did not change upon subsequent iterations of the analysis. Taxa not closely related to the *Polarobacter*/ *Flectobacillus glomeratus* group, and taxa not necessary to define the major groups of closely related CFB were removed from the aligned dataset leaving only the 25 sequences shown in Table 7.1. The V1 loop (Woese et al., 1983; Gutell et al., 1985) of the aligned dataset was also removed because these positions could not be unambiguously aligned.

"Final" phylogenetic analyses were then performed on this reduced dataset. First, another round of iterative neighbor joining analyses were performed under a Kimura 2-parameter model until a stable R value of 1.05 (using the same outgroup as above) was reached. 100 bootstrap datasets using jumbled sequence additon order and the same parameters as above were likewise analyzed. Likelihood analysis using fastDNAml (Felsenstein, 1981; Olsen et al., 1994) was also performed with the R value set to 1.05 and using the "empirical base frequencies" option (Figure 7.2). 100 bootstrap analyses were likewise performed using fastDNAml_boot (Felsenstein, 1981; Olsen et al., 1994). Generalized, unweighted (Fitch) parsimony analysis was also conducted on the reduced dataset and 100 bootstrap resampled datasets using a heuristic search method (Swofford, 1991). The reduced dataset gave rise to four equally parsimonius trees. These trees were analyzed with MacClade 3.05 to produce a "typeset" to correct for uneven rates of the 12 types of transitions and transversions. This typeset was calculated using the equation $K_{ij} = -\ln(X_{ij}/X)$ where K_{ij} was the correction for an $i \rightarrow j$ transition or

transversion, X_{ij} was the number of $i \rightarrow j$ transitions or transversions in the trees, and X was the total number of steps in the trees. Because of computational expense, however, it was only possible to analyze the topology of taxa that were closely related to the *Polarobacter* strains and 84-W(gv)1. MacClade 3.05 was used to collapse these clades to polytomies. Thus the typeset described above was then applied to the dataset and a tree with the partially collapsed clades was analyzed using PAUP 3.0s (Figure 7.2).

Each of these tree-building methods produced different topologies. The Kishino-Hasegawa test (Kishino and Hasegawa, 1989) was employed to evaluate the relative strength of these topologies under a likelihood framework with an R value of 1.05. The results show that the toplogy of the tree produced under a maximum likelihood framework with R set to 1.05, and the tree produced under a parsimony framework with the applied typeset both were the same (Figure 7.2). This tree is both the shortest tree and the most likely tree of all those produced by the above methods. The Kishino-Hasegawa test rules out the neighbor joining and the bootstrap neighbor joining trees as significantly worse than the best tree. The Fitch parsimony, bootstrap parsimony, and bootstrap likelihood trees were all longer and less likely (but not significantly so) than the best tree.

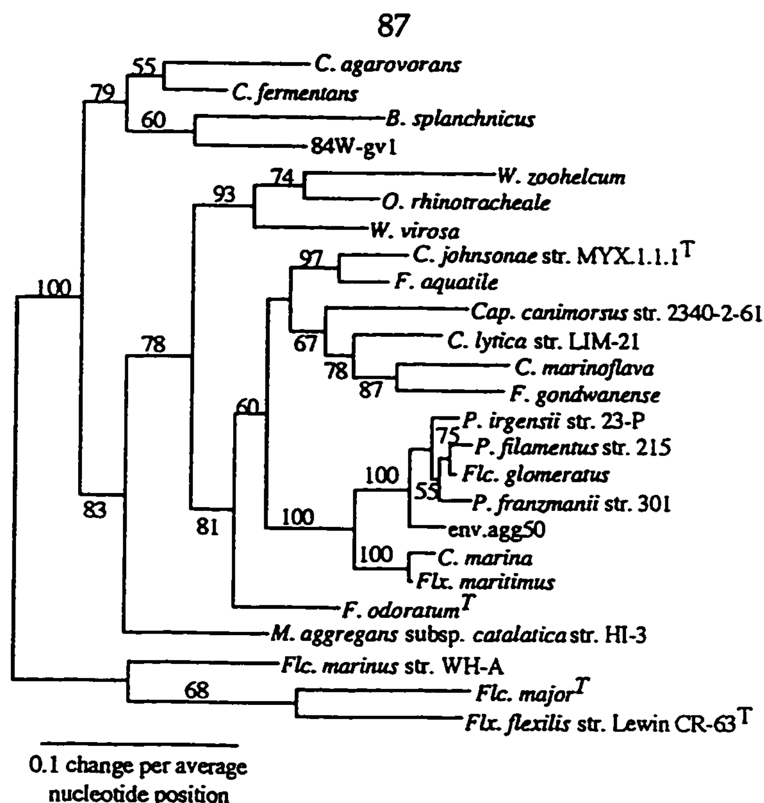


Figure 7.2 Phylogenetic Relationship of *Polarobacter* Species and Related Species. This tree was produced using fastDNAml (Felsenstein, 1981; Olsen et al., 1994) with an R value of 1.05. The scale bar represents 0.1 changes per average nucleotide position. Percent bootstrap support is indicated next to the branches. The same tree topology was produced using weighted parsimony as described in the text.

The results of these analyses clearly show that strains 23-P, 215, and 301 are most closely related to *Flectobacillus glomeratus*. These results also show that *Flc. glomeratus* is only distantly related to the type strain, *Flc. major*^{T}. *Flexibacter maritimus* and *Cytophaga marina* are similarly distant from the the type strains of their respective genera, but these relationships were not investigated in further detail in this study. The high degree of similarity between *Flc. glomeratus* and strains 23-P, 215, and 301 as evidenced in Figure 7.2 warranted further investigation by phenotypic and genotypic methods. These results are shown in Table 7.3.

Unfortunately, in many cases the test strains (23-P, 215, and 301) did not grow on the same media or at the same temperatures as the control strains (*Flectobacillus glomeratus*, *Flc. marinus*, and *Flc. major*). However most of

these tests, score only for the absence or presence of a physiological or metabolic function which occurs irrespective of temperature. Furthermore the results in Table 7.3 are robust as most tests were done in triplicate or quadruplicate.

Table 7.3 Nutritional, Physiological, and Genotypic Properties of *Polarobacter* and *Flectobacillus* strains.

	23-P	215	301	Flc. glomeratus	Flc. major	Flc. marinus
Cell morphology	filamentous	filamentous	irregular rod	curved rods	curved rods	coils
Cell size						
length (μm)	0.8-48	1.6-32	4-16	1.6-3.2	5-240	1.6-2.4
diameter (μm)	0.25-0.5	0.5-1.2	0.8-1.6	0.6-0.9	0.8-1.6	0.6-0.8
Coil formation	-	-	-	+	+	+
Coccoid cells	+	+	+	-	-	+
Motility	-	-	-	-	-	-
Gas vesicles	+	+	+	-	-	-
Gram stain	-	-	-	-	-	-
Colony color	orange	salmon	orange	orange	orange	pink/ orange
Colony morphology						
form	circular	circular	circular	circular	circular	circular
elevation	convex	flat/convx	flat/convx	convex	convex	convex
margin	entire	entire	entire	entire	entire	entire
appearance	smooth	smooth	smooth	smooth	smooth	smooth
opacity	translucent	opaque	opaque	opaque	translucent	opaque
texture	butyrous	butyrous	viscous	butyrous	butyrous	butyrous
phase variation	-	-	-	-	-	-
Utilization as a carbon source						
yeast extract	+	+	+	+	+	+
tartaric acid	-	-	-	-	-	-*
ethanol	-	-	-	-	-	-
cas amino acids	+	+	+	+	+	-
malate	+	-	-	-	+	-
sucrose	-	-	-	+	+	+
leucine	-	-	-	-	-	-
proline	-	-	-	-	-	-
glutamate	-	+	-	+	+	-
ribose	-	-	-	-	-	+
aspartate	-	-	-	-	-	-
α-ketoglutarte	-	-	-	-	-	-
fumarate	-	-	-	-	-	-
citrate	-	-	-	-	-	-*
succinate	-	-	-	-	+	-*
pyruvate	-	-	-	-	-	-*
propionate	-	-	-	-	-	-
acetate	-	-	-	-	-	-*
benzoate	-	-	-	-	-	-
glycolic acid	-	-	-	-	-	-
glycerol	-	+	+	-	W	+
methanol	-	-	-	-	-	-
N-aceytl-glucosamine	-	-	+	-	-	+
Growth on various liquid media						
MSB	-	-	-	-	++	-
MSB+1.5%NaCl	-	-	-	-	-	+
MSB+3% NaCl	-	-	-	-	-	+
2216	++	++	++	++	-	++
SWCm	+	++	++	++	-	++

Table 7.3 (continued)

Growth on various solid media						
NA	-	-	-	-	++	-
NA+2.5% NaCl	-	+	-	-	-	++
TSA	-	-	-	+	+	++
TSAS	-	-	-	+	-	+
TSAG	-	-	-	-	-	-
TSA+2.5% NaCl	-	-	-	-*	-	++
2216	+	++	++	++	-*	++
CLED	-	-	-	-	++*	-
MacConkey	-	-	-	-	-	-
EMB	-	-	-	-	+*	-
SWCm	++	++	++	++	-	++
Mueller Hinton	-	-	-	-	+	++
PMA	-	-	-	-	-*	-
MS	-	-	-	-	++	-
MS+3.0% NaCl	-	-	-	-	-	+
956	-	-	-	-	++	-
Oxidation/ Fermentation of various carbon sources						
arabinose	-	F	-	-	+	F
ribose	-	NG	NG	-	+	F
xylose	-	-	NG	-*	+	F
rhamnose	NG	F	NG	F*	+	F
fructose	NG	-	F	-	+	F
galactose	F	F	O	F*	+	F
glucose	F	F	F	F*	+	F
mannose	F	F	F	F*	+	F
cellobiose	-	-	-	-	**	F
lactose	-	-	O	-	+	F
maltose	-	-	O	F*	+	F
sucrose	-	-	F	F*	+	F
trehalose	-	-	F	-	+	F
dextrin	-	-	-	F*	+	F
inulin	NG	-	-	-	d	F
glycerol	F	F	-	-	-	-
erythritol	-	-	NG	-	-	-
dulcitol	-	-	-	-	-	-
mannitol	-	-	-	-	-	F*
sorbitol	-	-	-	-	-	-
adonitol	-	-	-	-	NA	-
salicin	-	-	NG	-	+	F
raffinose	-	-	-	-	+	F
methyl-α-D-glucopryanoside	-	-	-	-	+	F
melibiose	-	-	-	-	+	F
myoinositol	-	-	-	-	NA	-
Macromolecule hydrolysis						
esculin	-	+	+	-	**	+
casein	NG	NG	NG	NG	-	NG
starch	W	W	W	+	+	-
gelatin	-	W	+	+	+	-
chitin	-	-	-	-	-	-
cellulose	-	-	-	-	-	-
Biochemical tests						
urease	-	-	-	-	+	-
lysine decarboxylase	NG	NG	NG	-	NA	NG
ornithine decarboxylase	NG	NG	NG	-	-	W
β galactosidase	-	-	+	-	**	+

Table 7.3 (continued)

oxidase	W	-	+	+	+	+
catalase	+	+	+	W	W	+
indole formation	-	-	-	-	-	-
Voges Proskauer	-	-	-	-	-	-
nitrate reduction	-	-	-	+*	-	-
H_2S formation	NG	-	NG	-	-	-
Temperature growth range						
4°C	+	+	+	+	NA	NA
10°C	+	+	+	+	NA	NA
15°C	-	+	-	+	NA	NA
19°C	-	W	-	+	NA	NA
21°C	-	-	-	+	+	+
25°C	-	-	-	-	+	+
Absorbance wavelength of ethanolic extracts (nm)	450 475 506	451 475 506	451 506	451 476 505	455 481 505	NA
Mole % G+C	31	32±1	32±1	33.2	39.5-40.3	34-38

Data from this study and others (Larkin and Borrall, 1984; McGuire et al., 1987; Irgens et al., 1989)
F = fermentation/acid production in anaerobic tubes
O = oxidation/acid production in aerobic tubes but not anaerobic tubes
W = weak result
NG = no growth
NA = results not available
* = difference between the results of this study and previously reported results (Larkin and Borrall, 1984; McGuire et al., 1987)
** = difference between the results reported by McGuire (McGuire et al., 1987) and Larkin (Larkin and Borrall, 1984)

All polar gas vacuolate strains grew as long rods or filaments. Unlike the genus, *Flectobacillus*, however, these rods were not coiled or spiral shaped (Figure 7.1). Older cultures of the polar strains often gave rise to coccoid cells. Growth of these strains was difficult, often requiring one to three weeks' incubation on SWCm at 10°C. As with all psychrophilic strains, care had to be taken to avoid leaving the cultures at room temperature for extended periods.

Strains 23-P, 215, and 301 grew as orange or salmon colored colonies on SWCm agar plates. Pigments extracted from these cells showed absorption spectra similar to those of carotenoids (Figure 7.3). Alkalinization of these extracts did not lead to a bathychromatic shift indicating that these pigments are not flexirubins, a feature typical of *Flavobacterium* species (Weeks, 1981).

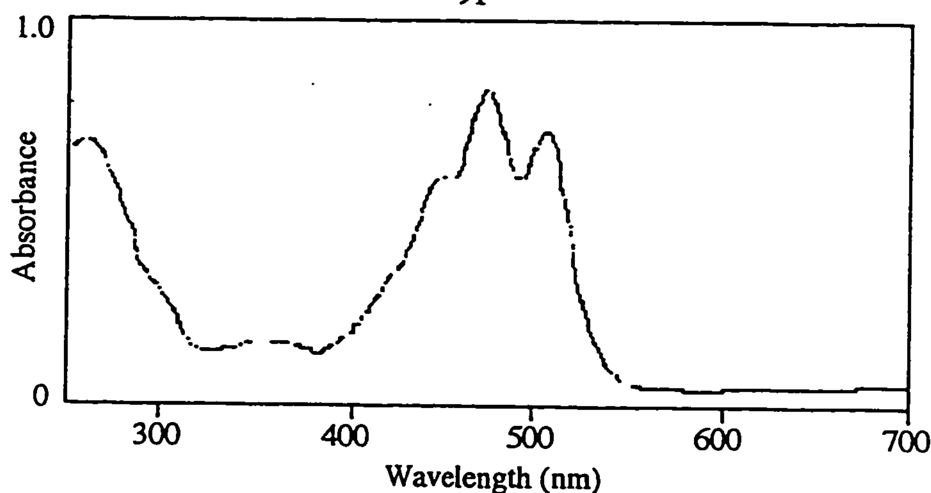


Figure 7.3 *In vitro* absorption spectrum of ethanol extract of strain 215 showing the charcteristic absorption peaks of carotenoids at approximately 450, 475, and 505 nm with small shoulder peak at approximately 425 nm. Alkalinization with 1/10 volume 0.1M NaOH did not lead to any shift in peak positions.

One of the most notable differences between strains 23-P, 215 and 301 and *Flectobacillus glomeratus* is that the former all produce gas vesicles, whereas the latter does not. This is the first taxonomic description of a gas vacuolate member of the CFB phylogenetic group of bacteria. Gas vesicles have been found in a number of evolutionarily diverse bacteria including 6 of the 12 major radiations of Bacteria and both the Crenarchaeota and Euryarchaeota (Walsby, 1994). The results in this study suggest that many species of the CFB phylogenetic group may produce gas vesicles.

The ecological role of gas vesicles in the polar strains described here is unknown. These bacteria were isolated from polar marine waters and sea ice. Gas vacuolate bacteria are usually found in calm, vertically stratified aquatic environments (Walsby, 1994). Sea ice forms a strong vertical stratification that reduces surface mixing (Maykut, 1985) of underlying marine waters. In any event it is known that the undersurface of sea ice provides a habitat for a bloom of algae, diatoms, and bacteria in the spring and fall. This region of high biological activity may be easily reached by bacteria bouyed with gas vesicles.

The ability of these strains to grow on various carbon sources was tested using a basal medium with 0.05 g/l yeast extract to stimulate growth. All strains grew well on yeast extract and Casamino Acids (Detroit, MI). The

only other carbon source that strain 23-P also grew on was malate. Strain 215 grew on glutamate and glycerol. Strain 301 grew on glycerol and N-acetyl-glucosamine. In contrast to the results shown in (Larkin and Borrall, 1984; McGuire et al., 1987), *Flectobacillus marinus* was not capable of growth on tartaric acid, malate, citrate, succinate, pyruvate, or acetate. This could be the result of differences in growth conditions between this study and previous work. It should be noted, however, that *Flc. marinus* was capable of excellent growth on other carbon sources. This lack of growth is particularly troubling in that all tests were conducted in quadruplicate and the results were scored quantitatively with a sensitive automated microtiter plate reading system.

Macromolecule hydrolysis tests show that all of the strains can weakly hydrolyze starch, and strains 215 and 301 can hydrolyze gelatin, albeit strain 215 only weakly. Strain 301 is also capable of hydrolyzing esculin. None of the strains was capable of growing on the casein medium provided. Although all of the strains were capable of growing on the chitin and cellulose overlay plates, none of them showed degradation of these macromolecules.

All of the strains demonstrated the ability to ferment a number of sugars, alcohols, and other carbon sources. Low level acid production on the basal medium (no carbon source added) was often seen under both aerobic and anaerobic conditions. However, none of the strains grew on SWCm agar plates at 10° C in an anaerboic Gas-Pak jar (BBL). All of the polar gas vacuolate strains were able to ferment or oxidize fructose, glucose, and mannose. None of the strains were able to ferment or oxidize cellobiose, lactose, dextrin, dulcitol, sorbitol, adonitol, raffinose, methyl-α-D-glucopryanoside, melibiose, or myoinositol. *Flectobacillus glomeratus* and *Flc. marinus* showed distinct acid production on a number of carbon sources which were in contrast to previous studies (Larkin and Borrall, 1984; McGuire et al., 1987). *Flc. glomeratus* was also shown to be able to grow on xylose in contrast to the results of McGuire (McGuire et al., 1987). Again, this may be the result of different growing conditions between our study and and those of previous studies.

The polar gas vacuolate strains were tested for the ability to grow on various types of media. Both marine media 2216 and SWCm as agar plates and liquid broth allowed good growth of these strains. In addition, strain 215

was also capable of limited growth on NA + 2.5% NaCl plates. Again, the results for *Flectobacillus glomeratus*, *Flc. major*, and *Flc. marinus* were slightly different from those listed in the literature (Larkin and Borrall, 1984; McGuire et al., 1987). As shown in Figure 7.3, these differences included the inability to grow on some types of media, and the presence of growth which was not previously reported on other types of media.

None of the strains showed activity for any of the biochemical tests employed, with the exception of strain 301 on β-galactosidase. This result was not unexpected as the oxidation/fermentation tests revealed oxidation of lactose by this strain. Unfortunately, neither of the decarboxylase test media allowed the growth of these polar gas vacuolate strains. Likewise, the H_2S medium did not permit the growth of strains 23-P or 301. Again, in contrast to the results of McGuire (McGuire et al., 1987), *Flectobacillus glomeratus* demonstrated a distinct, albeit low level ability to reduce nitrate to nitrite.

Table 7.4 Distinguishing Features of *Polarobacter* Strains and *Flectobacillus glomeratus*.

	23-P	215	301	Flc. glomeratus
Cell morphology	filamentous	filamentous	irregular rod	curved rods
Gas vesicles	+	+	+	-
Utilization as a carbon source				
malate	+	-	-	-
glutamate	-	+	-	+
glycerol	-	+	+	-
N-aceytl-glucosamine	-	-	+	-
Oxidation/ Fermentation of:				
arabinose	-	F	-	-
ribose	-	NG	NG	-
xylose, erythritol, and salicin	-	-	NG	-
rhamnose	NG	F	NG	F
fructose	NG	-	F	-
lactose	-	-	O	-
maltose	-	-	O	F
sucrose	-	-	F	F
trehalose	-	-	F	-
dextrin	-	-	-	F
inulin	NG	-	-	-
glycerol	F	F	-	-
Hydrolysis of:				
esculin	-	W	+	-
gelatin	-	W	+	+
β galactosidase	-	-	+	-
Growth at 21°C	-	-	-	+
Absorbance wavelength of ethanolic extracts (nm)	450 475 506	451 475 506	451 506	451 476 505

F = Fermentation, NG = No growth, O = Oxidation, and W = Weak result

In addition to the discrepancies noted above between the results of this study and previously reported work, there were also several differences between the reported results of McGuire (McGuire et al., 1987) and Larkin (Larkin and Borrall, 1984) for *Flectobacillus major* including cellobiose use, esculin hydrolysis, and β-galactosidase activity. As with the other discrepancies noted above, the test conditions employed in our experiments differed slightly from the conditions used in the previous reports.

The mole % G+C of strains 215 and 301 as determined by thermal denaturation (Smibert and Kreig, 1994) was 32±1%, very similiar to the value of 31% as reported earlier for strain 23-P (Irgens et al., 1989). These values are typical for members of the CFB phylogenetic group (Krieg and Holt, 1984;

Balows et al., 1992), and similar to the value obtained for *Flectobacillus glomeratus* (33.2%) (McGuire et al., 1987).

Finally, strains 23-P, 215, 301, and *Flectobacillus glomeratus* were tested to determine the degree to which their DNAs would hybridize with each other. As shown in Table 7.5, none of these strains shows greater that 34% DNA/DNA hybridization with any of the other strains. Pairs of bacteria that have 70% or greater DNA/DNA hybridization are defined as being members of the same species. However, members of the same genus may have much lower levels of hybridization (Wayne et al., 1987). Therefore all of these bacteria appear to be members of the same genus, but are not the same species.

Table 7.5. DNA/DNA Hybridization Between Strains 23-P, 215, 301, and *Flectobacillus glomeratus*

	23-P	215	301
23-P	-		
215	26 ±15%	-	
301	34 ±12%	13 ±5%	-
Flc. glomeratus	14 ±6%	31 ±8%	18 ±5%

Description of *Polarobacter* gen nov., and three new species *P. filamentus* str. 215 sp. nov., *P. irgensii* str. 23-P sp. nov., and *P. franzmannii* str. 301sp. nov.

Po·lar·o·bac´·ter - *Polaro*, M. L. adj. *polaris*, pertaining to the geographic poles. *bacter*, from the Greek, *baktron*, for rod or staff. *Polarobacter* - rod shaped bacterium from a polar region. Psychrophilic or psychrotrophic gram negative nonmotile rods. Filament size varies between 2.0 and 48 µm in length and 0.25 to 1.6 µm in diameter depending on growth medium, temperature, and age of the culture. Coccoid bodies are often seen in aging cultures. Cells produce orange, salmon, or pink colored pigments which are not flexirubins. Gas vesicles are produced in some species. Cells grow well in media which have NaCl. Cells produce acid from a number of carbohydrates. Starch is hydrolyzed by these bacteria. Cells can grow on yeast extract and Casamino acids. Mole% G+C ranges from 31 to 33%. All strains have been isolated from polar marine environments and grow well at low temperatures. 16S rRNA sequence analysis clearly indicates that this genus of

bacteria forms a distinct clade of the CFB phylum. *Polarobacter filamentus* str. 215 described below is the type species of the genus.

Po·lar·o·bac´·ter fil·a·men´·tus str. 215 - filamentus from *filare*, L., to spin. *Polarobacter filamentus* str. 215 a filamentous bacterium of the genus *Polarobacter*. Extremely long rods or filaments measuring 0.5 to 1.2 μm in width, and 1.6 to 32 μm in length. Cells are orange to salmon colored and produce gas vesicles. This species can use glutamate, and glycerol as carbon sources and it can hydrolyze gelatin. They also produce acid from arabinose, rhamnose, galactose, glucose, mannose, and glycerol. These cells can grow from 4 to 19°C, but not at 21°C. Mole % G+C is 32±1%. Principal fatty acids include 22% 15:0 iso 3OH, 22% 15:0 iso, and 12% 15:1 iso G when grown on SWCm agar at 10°C. Isolated from surface sea water, 350 km north of Deadhorse, Alaska. Type strain of the species is strain 215.

Po·lar·o·bac´·ter ir·gen´·sii str. 23-P - *irgensii*, in honor of Roar L. Irgens, microbiologist and polar researcher who first observed polar marine gas vacuolate bacteria. This species forms long thin rods or filaments from 0.25 to 0.5 μm in width and 0.8 to 48 μm long. Coccoid bodies are often found in most cultures, particularly aging cultures. Cells are orange colored and are gas vacuolate. Strain 23-P can grow on malate. This species produces acid from a number of carbohydrates including galactose, glucose, mannose, and glycerol. It is capable of growth from -1.5 to 12°C (Irgens et al., 1989). Mole % G+C is 31 (Irgens et al., 1989). Principal fatty acids include 38% 15:0 iso 3OH, 12% 15:0 iso, and 10% 15:0 3OH when grown on SWCm agar at 10°C. Isolated from sea water near the ice edge in Penola Strait, Antarctica. The type strain of this species is strain 23-P.

Po·lar·o·bac´·ter franz´·mann·ii str. 301 - *franzmannii*, in honor of Peter D. Franzmann, microbiologist and polar researcher. Members of this species grow as large rods from 0.8 to 1.6 μm in width, and 4 to 16 μm in length. Gas vesicles are present in these light to dark orange colored cells, but sometimes sparingly depending on the growth medium. Cells are capable of growth on glycerol, and N-acetyl-glucosamine. Acid is produced from growth on fructose, galactose, glucose, mannose, lactose, maltose, sucrose, and trehalose.

Esculin and gelatin are hydrolyzed by this species. Cells can grow from 4 to 10°C, but not at 15°C. Principal fatty acids include 17% 15:0 iso 3OH, 11% 15:1 iso G, and 9% each of 15:0 iso, 15:1 ω6cis, and 16:1 ω7cis when grown on SWCm agar at 10°C. The mole %G+C as determined by thermal denaturation is 32±1%. Isolated from sea ice in McMurdo Sound, Antarctica. The type strain of this species is strain 301.

Reclassification of *Flectobacillus glomeratus* to *Polarobacter glomeratus*, comb. nov.

Phylogenetic, phenotypic, and genotypic similarities between *Flectobacillus glomeratus* and members of the genus *Polarobacter* suggest that this species should be reclassified as *Polarobacter glomeratus*. In particular, DNA/DNA hybridization between this species and *Polarobacter* species ranges from 31 to 14, within the range of values for members of the same genus. This species can grow on sucrose and glutamate. Produces acid from rhamnose, galactose, glucose, mannose, maltose, sucrose, and dextrin. Hydrolzes gelatin. Reduces nitrate to nitrite. Oxidase and (weakly) catalase positive. Cells can grow at temperatures from 4 - 21 °C. Does not produce coccoid bodies or gas vesicles. Mole % G+C is 33 - 33.2 as determined by thermal denaturation. Additionally, phylogenetic considerations based on 16S rRNA sequence analysis shows that this species is only distantly related to the type species of *Flectobacillus*, *Flc. major*, but that it is very closely related to the genus *Polarobacter*.

Chapter 8.

Phylogenetic Analysis of *GvpA*

Abstract Partial nucleotide sequences of *gvpA* genes were determined for 12 strains of north and south polar marine bacteria of the CFB group, and the alpha, beta, and gamma Proteobacteria groups. Phylogenetic analysis of the *gvpA* sequences parallels the phylogenetic relationship of these strains as determined by 16S rRNA sequence analysis. The *gvpA* sequences of the CFB form a monophyletic group not specifically associated with any other phyletic groups. Other closely related pairs of strains from the Arctic and Antarctica had similar gas vesicle sequences, congruent with the hypothesis that each of these groups acquired the *gvpA* gene from recent common ancestors. In the case of the CFB sequences, multiple clones of *gvpA* genes from the same isolates revealed small sequence heterogeneities. This suggested that these strains harbored multiple copies of *gvpA* which differed at one or more nucleotide positions. Use of the alternate sequences in phylogenetic analysis did not lead to changes in tree topology. This obviated the concern that gene paralogy affected the phylogenetic analysis of the sequences. Finally, the mole percent G+C in the *gvpA* sequences were similar to the mole percent G+C of the strains from which they were obtained. Phylogenetic and mole percent G+C considerations support the hypothesis that *gvpA* evolved concurrently in these strains and is not the result of recent horizontal gene transfer.

Introduction. Several gas vesicle producing strains recovered from polar sea ice and water are members of the CFB phylum of Bacteria. Prior to this study no member of this well described phylum had been reported as gas vacuolate. There are two possible explanations for this observation. First, it is possible that the genes for gas vesicle formation were inherent to the common ancestor of the CFB but that these genes have been subsequently lost in all other subgroups of this phylum except the polar strains. Alternatively,

it is possible that the polar sea ice bacteria acquired these genes through a horizontal gene transfer in the recent evolutionary past. To test these two hypotheses, the gas vesicle genes were cloned and sequenced from 12 type strains representative of the major groups of polar sea ice bacteria. These sequences were then subjected to phylogenetic anaylsis to see whether the resulting tree topology was congruent with the tree topology as determined by 16S rRNA sequences (indicating vertical or parental inheritance) or whether there were significant differences in the tree topologies indicating horizontal gene transfer.

Previous investigators have reported difficulty in cloning the *gvpA* gene from various gas vesicle producing bacteria (Hayes, 1992). It has been speculated that the extreme hydrophobicity of GVPa leads it to fold and aggregate incorrectly leading to cell death when expressed in *E. coli* (Hayes, 1992). Analysis of existing GVPa and *gvpA* sequences revealed an 88 bp region within the gene that is flanked by areas that are highly conserved across all of the known bacterial *gvpA* sequences. Other investigators reported success in using oligonucleotide primers specific for this region to amplify the *gvpA* gene using PCR (Hayes, 1992). Preliminary analysis of existing *gvpA* sequences showed that this region of this gene contains enough variable nucleotide sites to be phylogenetically informative. Although only a small number of nucleotide positions could be analyzed and the encoded proteins showed strong homology to one another, the second and third site codon positions within this region were highly variable (see Table 8.1).

With regard to the CFB group, there are only six relevant outgroup rooted tree topologies that can be returned using the *gvpA* sequences. These alternatives are described below and in Figure 8.1. The hypothesis of horizontal gene transfer can be tested by producing a phylogenetic tree from *gvpA* sequences and seeing which of the six topologies below that it matches.

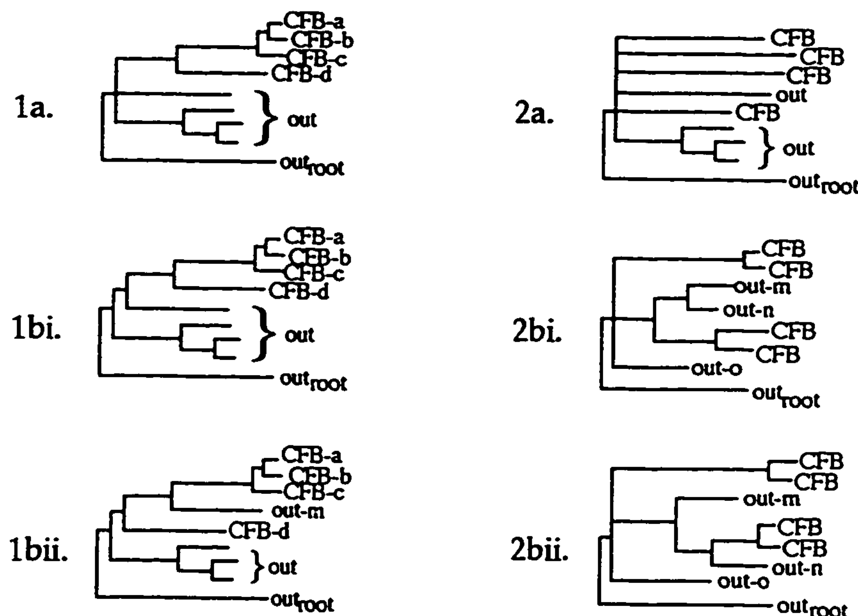


Figure 8.1 Possible alternatives to the phylogenetic tree topology returned from analysis of *gvpA* genes of CFB and non-CFB sequences. CFB-x = a Cytophaga/Flavobacterium/Bacteroides taxon, out-x = a non-CFB taxon, For clarity, some non-CFB branches have been included by left unnamed and a specific outgroup taxon (out$_{root}$) is used to root the trees. See text for discussion.

1) All the CFB *gvpA* sequences branch together:

a. If no other sequences form an ingroup to the CFB sequences then this supports the hypothesis that the CFB group possessed the *gvpA* gene before they speciated.

b. If the *gvpA* sequence of a non-CFB isolate specifically associates with the CFB sequences it can be in one of two topological forms:

i. If the non-CFB taxon forms an outgroup to the CFB group then it is evidence that both the CFB and the non-CFB *gvpA* sequences came from a recent common ancestor.

ii. If the taxon forms an ingroup to the CFB group then it is evidence for a horizontal gene transfer event wherein the non-CFB taxon had recently acquired the *gvpA* gene from a member of the CFB group.

2) Some or all of the CFB *gvpA* sequences do not specifically form a clade together:

a. If there is no specific affiliation between any of the non-CFB taxa then this is evidence that there is not enough sequence data to make any specific hypothesis regarding the evolution of *gvpA* in the multifurcating taxa. (A *multifurcation* or, *polytomy,* is a branch split in a phylogenetic tree which gives rise to more than two lineages. Multifurcations are assigned to branch splits where the relative order of the splits are uncertain)

b. If some (but not all) CFB sequences form a close affiliation with a group of non-CFB sequences it can be in one of two topological forms:

i. If one or more CFB sequence(s) form an outgroup to the non-CFB sequences then it indicates that these two groups had a recent common ancestor. The relationship between this common ancestor and the ancestor of the rest of the CFB sequences is still uncertain.

ii. If some (but not all) CFB sequence(s) form an ingroup to non-CFB sequences it is evidence supporting the hypothesis that these CFB taxa have acquired the *gvpA* gene through horizontal gene transfer from this other group of bacteria. If not all of the CFB taxa form part of this ingroup then this is evidence against the hypothesis that all of the CFB taxa possesed the *gvpA* gene before they speciated.

Results and discussion. The TYLKAE and DRILDKG primers (chapter 2) were used in a PCR to amplify an internal region of the *gvpA* genes from 12 strains of arctic and antarctic marine bacteria. These strains are listed in Table 8.1. The PCR primers were designed with a large number of degenerate sites to permit amplification of diverse *gvpA* genes. In all cases a DNA fragment of approximately 130 bp in length was amplified. A second fragment of approximately 250 bp in length was also amplified from some of the strains. DNA sequence analysis of the larger product revealed that it was unrelated to any of the known gas vesicle genes.

Table 8.1. Strains from which an internal region of the *gvpA* gene was cloned and sequenced

Arctic Strains		Antarctic Strains	
# of clones sequenced	Strain Name	# of clones sequenced	Strain Name
2	37	8	*Polarobacter franzmannii* str. 301
2	174	m,10	*Polarobacter irgensii* str. 23-P
m,3	206	2	*Octadecobacter antarcticus* str. 307
m,8	214.6	m,7	*Polaromonas vacuolata* str. 34-P
9	*Polarobacter filamentus* str. 215	2	S51-W(gv)1
		2	84-W(gv)1
		2	90-P(gv)1

m, multiple clones were pooled and sequenced

In all cases however, it was possible to clone the 120 bp fragment from each of these strains. BLAST analysis (Altschul et al., 1990) indicated that they were *gvpA* genes. In some cases pools of clones from a given strain were sequenced to average the effects of potential PCR induced errors or potential small heterogeneities between different alleles of the gene. For *Polarobacter irgensii* str. 23-P, *Polaromonas vacuolata* str. 34-P, and the unnamed strains 206 and 214.6, a pool of 3 to 10 clones was sequenced on both the 5'→3' and 3'→5' strands.

Some strains of CFB type bacteria appear to have two or more copies of the *gvpA* gene. Both the 5'→3' and the 3'→5' strands of a pool of 10 *gvpA* clones from *Polarobacter irgensii* str. 23-P were sequenced. In both cases, the electrophoregram revealed an A/G doublet at nucleotide position 53 (position 1 corresponding to the first nucleotide after the "DRILDKG" primer). An A at position 53 would give rise to a codon for threonine, a G at position 53 would give rise to a codon for alanine. For *P. filamentus* str. 215, nine separate clones were were sequenced in either the 5'→3' or the 3'→5' direction. One of the clones had an A at nucleotide position 2 thereby coding for isoleucine. The other eight clones had a G at nucleotide position 2 thereby coding for valine. The two clones of strain 84-W(gv)1 had three synonymous nucleotide differences at positions 13 (T/C), 37 (T/C), and 43 (C/T). Position 8 of strain 84-W(gv)1 had a nonsynonymous C/G difference which would produce either leucine or valine. *P. franzmannii* str. 301 produced four different sequences from eight separate clones. These differences were at the following

positions: 4 (A/G), 7 (G/A), 32 (T/C), 49 (A/G), 52 (T/C), and 70 (T/C). All of these differences were synonymous at the amino acid level. No nucleotide differences were noted in different clones from any of the strains of Proteobacteria. In total, 3 out of the 12 nucleotide differences (within individual species) led to amino acid differences. This is different from the results of Marsac et al. (Marsac et al., 1985). Their results suggested that if multiple copies of *gvpA* exist within a single strain of bacteria, then the alternate copies would code for synonymous amino acids. In contrast, others have found that different gas vesicle structural proteins can coexist in a single species (Surek et al., 1988).

For strains which had different sequences from different clones, the consensus of the different sequences were obtained for use in further analyses. These sequences along with "reference" sequences from GenBank were also analyzed for compositional biases as shown in Table 8.2. As can be seen from this table, the mole % G+C of the *gvpA* of the strains is closely matched by the mole % G+C of their 16S rRNA and their genomic DNA. Attempts to confirm or deny any relationship between codon usage patterns in the *gvpA* genes versus standard tables of codon usage patterns (Nakamura et al., 1996) for closely related bacteria were inconclusive (data not shown).

Table 8.2. Mole % G+C of *gvpA* Sequences and Corresponding Bacterial and Archaeal species

Strain	Mole % G+C[a]	
	gvpA	genomic
37	40	40
174	38	40
90-P(gv)1	40	39
S51-W(gv)1	38	NA
206	39	NA
214.6	42	36
Polaromonas vacuolata str. 34-P	53	52
Octadecobacter antarcticensis str. 307	50	56
Polarobacter filamentus str. 215	36	32
Polarobacter irgensii str. 23-P	38	31
Polarobacter franzmannii str. 301	36	32
84-W(gv)1	36	NA
Anabaena flos-aquae [a]	43	≈41[b]
Calothrix PCC 7601[a]	40	≈42[b]
Pseudanabaena sp.[a]	41	≈45[b]
Halobacterium halobium[a]	67	≈67[c]

NA, not available

a Data from GenBank and (Krieg and Holt, 1984)

b No data is available for these species so an average for the genus is given instead

c Average of the two genomic components of *H. halobium*

The consensus sequences for each strain were then manually aligned with one another (Figures 8.2 and 8.3). Several amino acid residues are noteworthy from Figure 8.3. Strains 37, 90-P(gv)1, and 174 have a phenylalanine at position 6, *Polarobacter* strains 23-P and 301 have a tyrosine, and all the others have a tryptophan. All of the beta and gamma Proteobacteria from this analysis, along with the sequence for *Amoebobacter* sp. (Griffiths et al., 1992) (not shown) have lysine at position 8 whereas the rest of the sequences have arginine. Position 18 gives a serine, threonine, or an alanine for the different strains. *Octadecobacter* strain 307 has a glycine at position 27 whereas the rest of the sequences have a serine.

```
                    10        20        30        40        50        60
                     .         .         .         .         .         .
215          ARTTGTTGTAGACGCATGGGTAAGAGTTTCATTAGTAGGTATTGAACTTCTTGCAATTGA
23P          .G.A........T..G.AT...C.T..A..T.....T.....C.....AG..R.T..C..
301          .G.R..R.....T..T.AC..TC.T..A..TY..........C...T.RG.Y..T..C..
84-Wgv1      TG....AR.T..Y..............A........Y.....Y...T.A...........
37           CA.C..AA.C.......TC..T.A...A...C..........C..GT.A..AT....C..
90-P(gv)1    CA.C..AA.C.......TC..T.A...A...C..........C..GT.A..AT....C..
174          TA.C..AA.C..T....TC..T.A.......C..........C..GT.A..AT....C..
206          TA.C.....T.....T...A.T.AG....................GT.AT.GT.T..C..
214.6        TA.C.....T.....T.....T.AG........G...........GT.AT.GT.T.....
S51-Wgv1     CA.....A.T..T..T.....T.A............C..A.....GT.A...T..G....
34-P         CA.C..CA.C.....T.....C.A...G...C.G..C...........G..GT....C..
Str.307      TG....GA.T..T.........C.C..A..GC.T..T.....C..GT.GA....CG.C..
Ana          TA.C..AA.T.....T.....TC.T.....C.....T.....C.....A..A........
Cal          TA.C........T..T......C.T..A..TC....T.........T.A.....T.....
Pse          CA.....A.T..T..............C..TC....G..A..C.....AT.GT.......
Hal          TG.C.....G....TG....CTC.T..G..GC.T..C..C..C...A.C..GA.CG.C..

                      70        80
                       .         .
215          AGCAAGAATTACAGTAGCTTCAGTTGAG
23P          .........AGTT....A..T.......
301          .........YGTT....A.........A
84-W(gv)1    .......G.AGTT..T..M..T.....A
37           ....C.TG..GTTA.C...........A
90-P(gv)1    ....C.TG..GTTA.C...........A
174          ....C.TG..GTTA.C...........A
206          ...GC.TG..GTTA.C........A..A
214.6        ...GC.CG..GTTA.C.....C..A..A
S51-Wgv1     .ATTC.CG.GGT.A.T..G..G.....A
34-P         G...C.TG.AGTG..C..A..G..C..A
307          ...GC.TG.GGTCA.....GG......T
Ana          ...TC.G..CGTTA.C.....C.....A
Cal          ...TC.G..CGTTA.C.....T.....A
Pse          ...TC.TG.CGTTA.T..A..T..G..A
Hal          G..GC.GG.CGTC.CC..C..G..G..C
```

Figure 8.2 *GvpA* nucleotide sequence alignment from polar sea ice bacteria and other gas vacuolate procaryotes. For the nucleotide alignment, R = A or G, Y = C or T, and M = A or C. Species abbreviations are as follows: 215 is *Polarobacter filamentus* str. 215, 23P is *P. irgensii* str. 23-P, 301 is *P. franzmannii* str. 301, 34-P is *Polaromonas vacuolata* str. 34-P, 307 is *Octadecobacter antarcticensis* str. 307, Ana is *Anabaena flos-aquae*, Cal is *Calothrix* PCC 7601, Pse is *Pseudanabaena* sp., and Hal is *Halobacterium halobium*.

```
215          VVDAWVRVSLVGIELLAIEARITVASVE
23P          V...Y..........V@.....V.....
301          V...Y..........V......V.....
84-W(gv)1    V*...................VV.....
37           II..F.K.........S....VVI....
90-P(gv)1    II..F.K.........S....VVI....
174          II..F.K.........S....VVI....
206          I....IK.........S....VVI....
214.6        I.....K.........S....VVI....
S51-Wgv1     II....K.........SV.I.VVI....
34-P         II....K.........S....VV.....
307          VI.............I.V...VVI.G.D
Ana          II....................VI....
Cal          I.....................VI....
Pse          II..............S....VVI....
Hal          V..V.A........I.TV...VVA...D
```

Figure 8.3 GVPa amino acid sequence alignment from polar sea ice bacteria and other gas vacuolate procaryotes. For the amino acid alignment, * = I or V, and @ = A or T. Species abbreviations are as follows are as in Figure 8.3 above.

Table 8.3 shows the number of nucleotide and amino acid differences between these strains. The nucleotide sequence of the antarctic gamma Proteobacteria strain 90-P(gv)1 was identical to that of the arctic gamma Proteobacteria strain 37 (Table 8.3). These sequences were very similar (85/88 nucleotides) to the sequence of the arctic gamma Proteobacteria strain 174 (Table 8.3). Also note in Table 8.3 the high level of identity between the sequences at the amino acid level but the low level of identity at the nucleotide level, particularly among closely related species. This observation along with the observation that different alleles within the same strain usually coded for identical or similar amino acids suggests a strong selection pressure to maintain the amino acid coding sequence.

Table 8.3 Pairwise number of nucleotide differences out of 88 positions (above diagonal) and number of amino acid differences out of 29 positions (below diagonal). Species abbreviations are as in Figure 8.2. Sequences are grouped by 16S rRNA derived phylogenetic relationships of the strains. Sequences 1-4 are from members of the CFB group, 5-12 are from the Proteobacteria, 13-15 are from the cyanobacteria, and sequence 16 is from an Archaea.

		Strains															
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1	215	-	21	2	16	27	27	27	27	27	29	28	32	25	24	28	38
2	23-P	3	-	9	17	32	32	32	33	34	34	37	30	26	19	28	34
3	301	3	0	-	18	23	23	23	27	29	32	34	27	23	18	29	34
4	84-W(gv)1	2	3	3	-	21	21	21	25	24	23	21	24	19	18	18	32
5	37	7	8	8	5	-	0	3	17	20	20	24	28	21	25	23	36
6	90-P(gv)1	7	8	8	5	0	-	3	17	20	20	24	28	21	25	23	36
7	174	7	8	8	5	0	0	-	16	19	22	23	27	20	24	23	36
8	206	6	8	8	5	3	3	3	-	5	24	23	30	22	22	25	33
9	214.6	5	7	7	4	2	2	2	1	-	24	21	32	20	21	25	33
10	34-P	5	7	7	3	2	2	2	3	2	-	26	35	27	29	24	29
11	S51-W(gv)1	8	10	10	6	3	3	3	4	3	3	-	31	25	26	21	38
12	307	8	8	8	5	8	8	8	9	8	8	7	-	29	26	29	31
13	*Anabaena*	3	5	5	3	4	4	4	5	4	4	5	6	-	13	22	30
14	*Calothrix*	2	4	4	3	5	5	5	4	3	5	6	7	1	-	21	30
15	*Pseudoanabaena*	5	7	7	3	2	2	2	3	2	2	3	6	2	3	-	34
16	*Halobacterium*	9	9	10	7	11	11	11	9	9	10	10	8	10	9	9	-

Preliminary analyses using distance, parsimony, and likelihood methods were done to confirm that the small differences in the nucleotide sequences for some of the strains did not affect the phylogenetic placement of the taxa (Figure 8.4). To reduce computational expense, the dataset using the consensus sequences and no sequence for 90-P(gv)1 (it was identical to the sequence of strain 37), was used for all subsequent phylogenetic analyses.

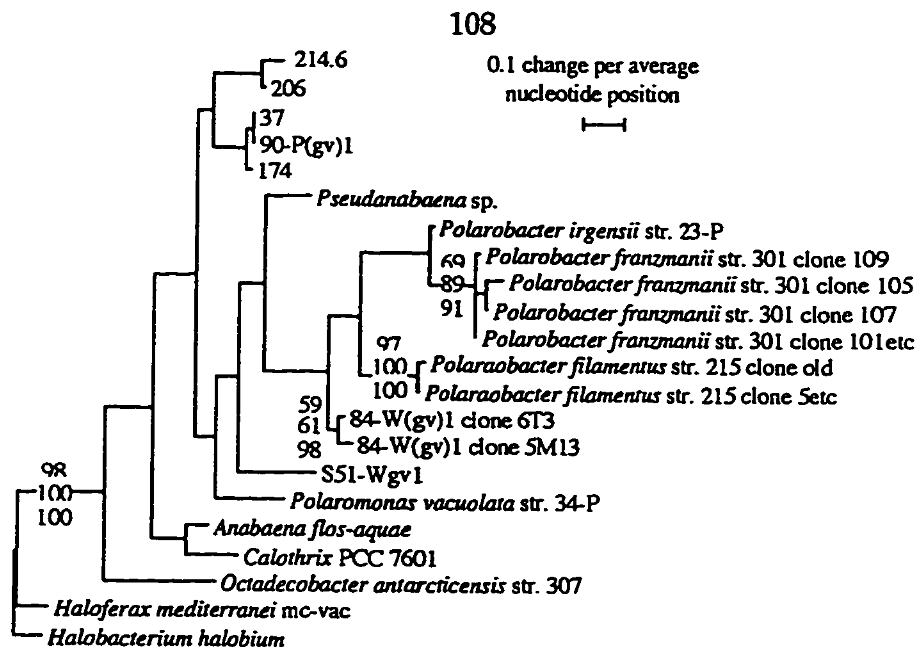


Figure 8.4. Premliminary Phylogenetic Analysis of Multiple *gvpA* Sequences from Polar Marine Bacteria and Other Gas Vesicle Sequences. This tree was produced by maximum likelihood analysis (Felsenstein, 1981; Olsen et al., 1994) with R = 0.98. Numbers by the branches indicate bootstrap support for that branch under maximum likelihood (top), parsimony (middle), and distance (bottom) frameworks. The sequence for *Haloferax mediterranei* was obtained from GenBank (X56027).

The aligned dataset (with consensus sequences) was first analyzed using a neighbor joining algorithm under a maximum likelihood model for pairwise distance calculation with the transition to transversion ratio (R) set to 2.0. Using MacClade 3.05 (Maddison and Maddison, 1992), the R value of the resulting distance tree was calculated to be 0.98 when *Halobacterium halobium* was selected as the outgroup. This value was applied to another neighbor joining analysis and the same tree topology was obtained. 100 bootstrap resampled datasets were then analyzed using the same parameters (R = 0.98, maximum likelihood distances) but with jumbled sequence addition.

An iterative process was also used in parsimony analysis. First, the most parsimonius tree was determined for the dataset using a heuristic search (Swofford, 1991). The resulting tree was then analyzed with MacClade (Maddison and Maddison, 1992) to determine the frequencies of the 12 types of transitions and transversions under the conditon of *Halobacterium*

halobium as an outgroup. A substitution matrix was then constructed to equalize the effect of each of these types of changes using the equation, $K_{ij} = -\ln(X_{ij}/X)$. Here, K_{ij} is the weight given to the $i \rightarrow j$ mutation type, X_{ij} is the number of $i \rightarrow j$ mutations in the tree, and X is the total length of the tree. The substitution matrix was then reapplied to the dataset to calculate another phylogenetic tree using a heuristic parsimony tree searching method. The resulting tree topology was again analyzed and the process repeated until the same tree topology was obtained (and hence no further changes would occur in the substitution matrix).

The dataset was also analyzed using a likelihood method (Felsenstein, 1981; Olsen et al., 1994). Like the neighbor joining analysis, the maximum likelihood analysis was conducted under an R value of 0.98 and nucleotide frequencies were determined empirically. 100 bootstrap datasets constructed with jumbled sequence addition, were likewise analyzed.

The three types of methods (distance, parsimony, and likelihood) each produced slightly different trees. A Kishino-Hasegawa test (Kishino and Hasegawa, 1989) using DNAML was employed to determine whether any of these trees were significantly more likely under a likelihood framework. Setting R to 0.98, the trees had a range of ln likelihoods from -870.7 to -879.6. Suprisingly, the most likely tree was one of the intermediate trees from the iteration process in the parsimony analysis. None of the trees (likelihood, parsimony, or distance), however, was significantly worse (in a likelihood sense) than any of the other trees. Also, the second best tree was the consensus tree produced by bootstrap maximum likelihood and bootstrap neighbor joining analysis (both trees were topologically the same).

Finally, a number of other tree topologies were manually constructed (using MacClade (Maddison and Maddison, 1992)) and examined with the Kishino-Hasegawa test. None of them were as likely as the trees produced by the standard tree building methods (likelihood, parsimony and distance) and several of them were significantly worse (Figure 8.5). In particular, a phylogenetic tree corresponding to that produced by 16S rRNA analysis of the gas vacuolate organisms, and several trees wherein *Polarobacter* species were placed with the Proteobacteria, cyanobacteria, or *Halobacterium* were all significantly worse than the best tree.

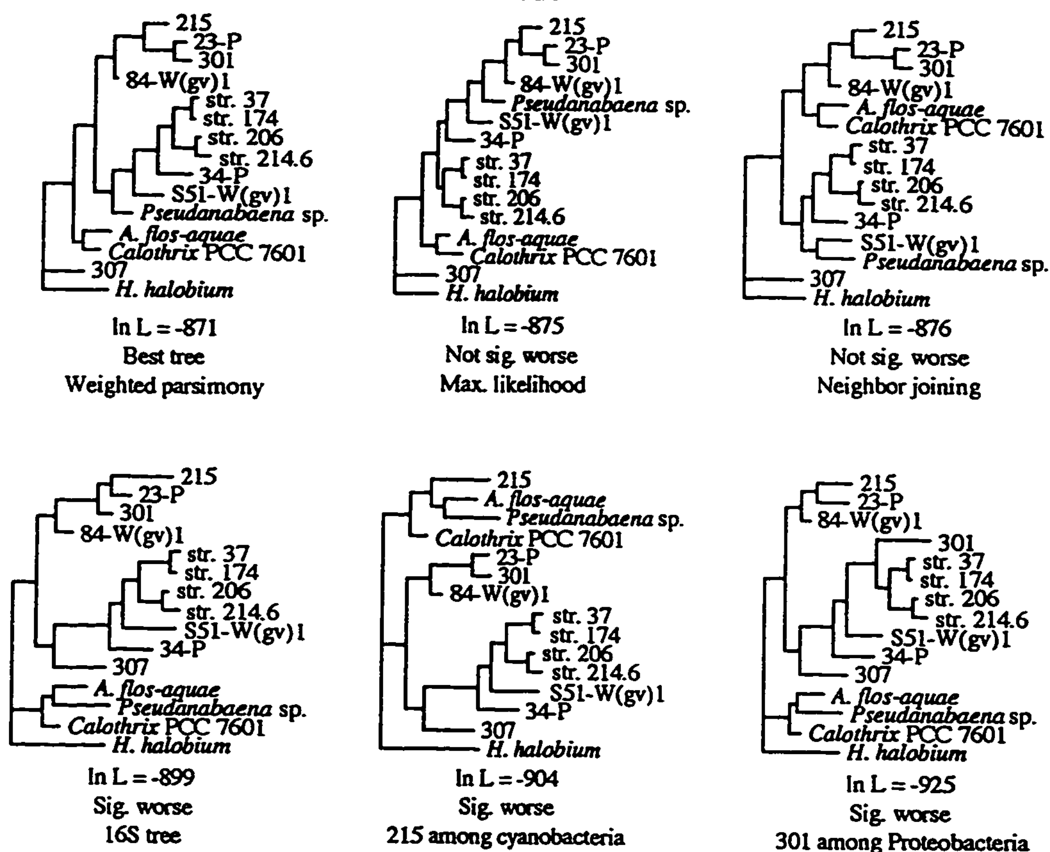


Figure 8.5. Some Alternate Topologies from the Phylogenetic Analysis of *gvpA* Sequences from Polar Gas Vacuolate Bacteria and Other *gvpA* Sequences. These trees are depicted with basal trifurcations for purposes of the Kishino-Hasegawa analysis. The ln likelihood score, the evaluation of whether each tree is significantly worse than the best tree, and the origin of each tree are given.

In order to determine if the gas vesicle genes were acquired as the result of horizontal gene transfer it was also necessary to produce a phylogenetic tree based on their 16S rRNA genes and compare it to the *gvpA* tree. The 16S rDNA sequences of strains 84-W(gv)1, *Octadecobacter arcticus* str. 238 and *O. anatarcticus* str. 307, *Polaromonas vacuolata* str. 34-P, *Polarobacter irgensii* str. 23-P, *P. filamentus* str. 215, and *P. franzmannii* str. 301 had been obtained previously. The sequences of strains 37, 174, 90-P(gv)1, 206, 214.6, S51-W(gv)1 were similarly determined. These sequences, along with the sequences of *Halobacterium halobium* (GenBank #M38280), *Calothrix "desertica"* PCC 7102 (GenBank #X99213), *Pseudanabaena* sp. PCC

6903 (Giovannoni et al., 1988), and *Anabaena "cylindrica"* PCC 7122 (Giovannoni et al., 1988), were aligned as described in chapter 2. Although extremely disparate sequences were used in the alignment, hypervarible regions were left in place so that fine scale differences in the closely related taxa could be discerned.

The aligned dataset was then analyzed using distance, parsimony, and likelihood methods. For distance analysis, an iterative neighbor joining process as described above yielded a tree with a final R value of approximately 1.0. Likelihood analysis was performed using an R value of 1.0. Bootstrap resampling and analysis were also done for the distance and likelihood analyses using the same parameters described above, plus random sequence addition. Parsimony analysis was performed without character position or transformation type weighting (Fitch parsimony) on both the original dataset and 100 bootstrap resampled datasets. The resulting three trees (distance, parsimony, and likelihood) were then evaluated under an unweighted parsimony framework to compare treelengths, and a likelihood framework using the Kishino-Hasegawa test (Kishino and Hasegawa, 1989) with an R value of 1.0 to compare ln likelihoods. The Kishino-Hasegawa test did not rule out any of the other tree topologies as being significantly worse than any of the others. The best tree was the maximum likelihood tree. The parsimony and distance trees were e^7 and e^5 less likely, respectively, than the maxiumum likelihood tree. The maximum likelihood tree when evaluated under a parsimony framework had a total length of 2057 steps as compared to 2055 steps for the parsimony tree and 2088 steps for the distance tree. The topology of the maximum likelihood tree is shown on the right of Figure 8.5 under an unweighted parsimony framework. Branches with less than 50% bootstrap support are represented as polytomies.

The left side tree in Figure 8.6 (Figure 8.6a) shows the tree resulting from the neighbor joining analysis depicted under a parsimony framework. This polytomous reconstruction was congruent with the results from bootstrap reanalysis using the likelihood and distance analyses described above. This tree was chosen to represent the phylogenetic reconstruction from *gvpA* data because it had the highest bootstrap support for several groups of sequences. Other methods (parsimony and likelihood) produced similar trees with similar bootstrap values.

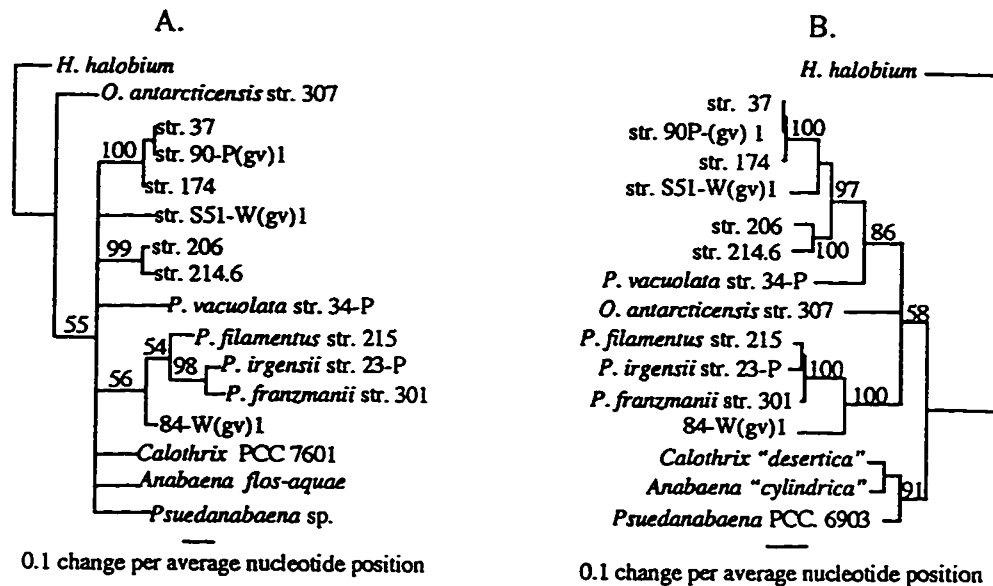


Figure 8.6 Phylogenetic relationship of *gvpA* sequences (a) and of 16S rDNA sequences from corresponding strains (b). The construction of both trees is described in the text above. Scale bars represent an average of 0.1 changes per nucleotide position. Numbers above the branches indicate bootstrap support for that branch from 100 bootstrap resamplings. Some of the bootstrap values on the 16S tree have been omitted for clarity. Branches with less than 50% bootstrap support are shown as polytomies.

Some differences were observed between the phylogenetic trees based on *gvpA* sequences and the phylogenetic trees based on 16S rRNA sequences. Most notable is the low level of bootstrap support for any most branch structures within the *gvpA* tree. This is the result of a small number of nucleotides being analyzed. It should be noted that different analysis methods applied to the *gvpA* dataset repeatedly produced tree topologies similar to the topology of the 16S tree (Figure 8.5). Another difference between the 16S and *gvpA* trees is the segregation of the *Octadecobacter antarcticensis* strain 307 sequences from the rest of the Bacterial sequences. Both the 307 gvpA sequence and the *Halobacterium halobium gvpA* sequence have a high mole %G+C. However, the *gvpA* sequence of *Polaromonas vacuolata* str. 34-P also has a high mole %G+C but it does not segregate with 307 and *H. halobium*. Given that the gas vesicle phenotype is found in a variety of diverse alpha, beta, and gamma Proteobacteria, it seems unlikely

that the phenotype was the result of recent horizontal gene transfer to this phylum. Another possibility is that the placement of the 307 *gvpA* sequence outside of the rest of the *gvpA* sequences may be the result of a statistical anomaly arising from the the small sample size (88 nucleotide positions) and the stochastic nature of evolutionary change.

Figure 8.6 shows that the *gvpA* genes in the *Polarobacter* isolates and strain 84-W(gv)1 are all closely related to each other. Furthermore, they share no specific affiliation with the sequences of any of the other strains. This is topology 1 described in Figure 8.1, above. This strongly suggests the the *gvpA* sequence found in these strains originated from a common ancestor and is probably not the result of recent horizontal gene transfer.

As shown in Table 8.2, the mole % G+C content of the *gvpA* sequences of the *Polarobacter* strains and strain 84-W(gv)1 are all closely related and very similar to the mole % G+C content of genomic DNA from the respective strains, and very similar to the value for Cytophaga, Flavobacteria, and Bacteroides in general (Krieg and Holt, 1984; Balows et al., 1992). Unfortunately there was not enough data available (88 nucleotide positions for 29 amino acid residues) to confirm or deny any particular relationship between the codon preferences of these sequences and the codon preferences of closely related bacteria (as determined by 16S rRNA phylogeny).

Finally, the observation of small nucleotide heterogeneities between different clones of *Polarobacter* strains 215, 301, and strain 84-W(gv)1 that did not affect tree topology obviates the concern that the analyses were skewed by testing paralogous gene sequences. The use of highly degenerate primers in the PCR reaction ensured that if multiple, significantly different, gene copies were in a single strain that they would be amplified and subsequently cloned.

Chapter. 9

Conclusions

In this dissertation I have examined the taxonomy, biogeography, and evolutionary history of gas vacuolate bacteria isolated from in and around the annual sea ice of the arctic and antarctic. Originally, three hypotheses were proposed to investigate the identity, distribution, and origin of these sea ice, gas vesicle producing bacteria. These hypotheses are addressed below.

The first hypothesis was that there are a several different phylogenetic groups of gas vacuolate bacteria associated with polar marine environments. We have shown that gas vacuolate bacteria are abundant in and near the polar sea ice environment. Ice cores and water columns from 16 different sites, 6 in the Arctic and 10 in Antarctica, yielded gas vacuolate bacteria when plated on SWCm agar plates. Previous work showed that these type of bacteria comprise up to 91% of the culturable bacteria in the sea ice of McMurdo Sound, Antarctica. Work reported in this dissertation shows that they comprise up to 5% of the cultured bacterial population from around Pt. Barrow and Deadhorse, Alaska.

These bacteria are evolutionarily diverse. The 16S rRNA gene nucleotide sequences of 14 isolates have been determined. Phenotypically and phylogenetically our isolates fall into four major groups of Eubacteria, the alpha, beta, and gamma Proteobacteria, and the CFB (Figure 9.1). However, none of the strains examined in detail are of the same species as any of the others. Phylogenetic considerations suggest that there are at least 14 different species of polar gas vacuolate bacteria. This study likely underestimates the true phylogenetic diversity of gas vesicle producing bacteria in the sea ice for two reasons. First, the 16S rRNA genes of only a small number of strains representing major fatty acid groups were sequenced. A number of isolates of unique fatty acid composition which did not cluster with any of the major groups were not investigated. Second, the strains characterized in this study represent only the strains which could be grown under the given culture conditions. The variety of procaryotes in an environment often greatly

exceeds the number that can be readily cultivated (for example see (Britschgi and Giovannoni, 1991; DeLong et al., 1994; Ruff-Roberts et al., 1994)). Thus the true diversity of gas vacuolate polar sea ice bacteria is likely much higher than reported here.

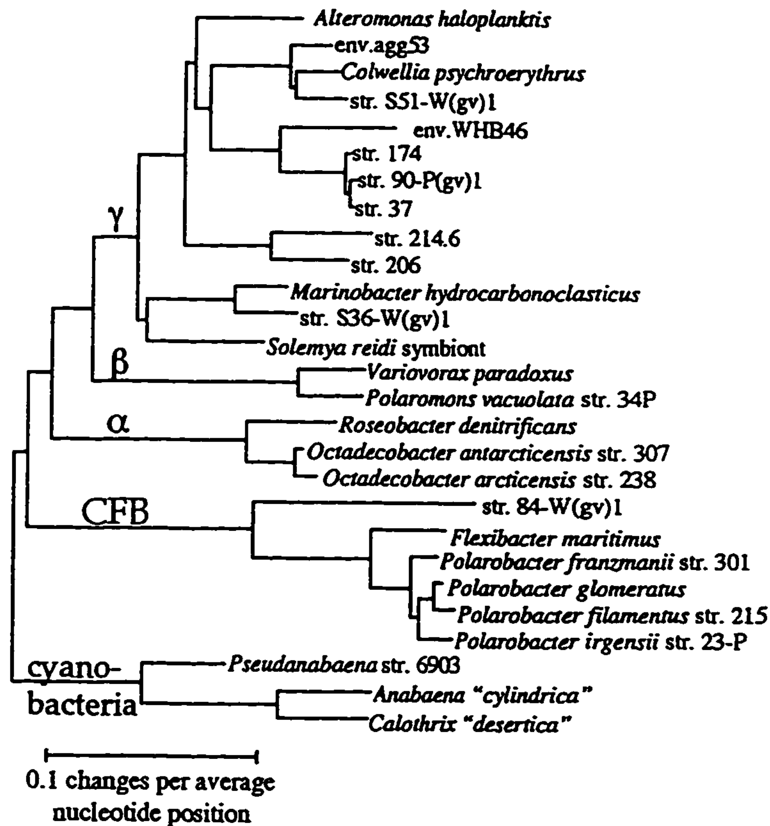


Figure 9.1. Phylogenetic Tree of Polar Gas Vacuolate Strains and Closely Related Species. This tree was produced using neighbor joining with pairwise distances determined by a maximum likelihood method with the R=2.0 (Felsenstein, 1989). This tree is for illustrative purposes only and detailed phylogenetic analyses were not persued.

The second hypothesis was that groups of these bacteria would prove to be endemic to one pole or the other. None of the 16S rRNA sequences determined in this study were identical with previously determined sequences in the sequence databases, or with each other. In particular, the closest North-South sequence pairs differed by 6-11 nucleotides. DNA/DNA hybridization experiments between closely related *Octadeocbacter* pairs (11-13 nucleotide differences) and between closely related *Polarobacter* pairs (18 - 50

nucleotide differences) indicated relationships no closer than the genus level between isolates from the Arctic and Antarctica.

None of the taxonomically described species in this work were found at both poles. Also, none of the polar marine bacteria shared closer than genus level affiliation with any other taxonomically classified species of bacteria. Thus, we have no evidence yet that any of these strains are cosmopolitan at the species level. On the other hand, the same species of polar gas vacuolate bacteria was also never obtained more than once at different locations from the same pole. Species from either pole were not repeatedly obtained since the polar gas vacuolate species diversity was higher than had been originally anticipated. Evidence for endemic strains requires that the same taxa be repeatedly obtained from a given area, but never obtained from an outside area. This suggests that we may not have a sufficient sample size of any one group from the Arctic or Antarctica to have identified an endemic or cosmopolitan species.

The third hypothesis was that horizontal transfer of gas vesicle genes into existing species of non-gas vacuolate bacteria may have given rise to some species of polar gas vacuolate bacteria. This hypothesis has been demonstrated to be false. The nucleotide sequences of the *gvpA* genes of the 14 gas vacuolate type strains were determined and subjected to phylogenetic analysis. Specific clades (as determined by 16S rRNA phylogenetics) corresponded to specific clades as determined by *gvpA* phylogenetics. In particular, the *gvpA* genes of the CFB types formed a monophyletic group. Other phylogenetically similar strains of bacteria (based on 16S rRNA sequences) also share *gvpA* genes which are monophyletic. Finally, these genes had a mole % G+C that reflected the mole % G+C of the strains from which they were isolated. The only plausible explanation for these observations is that *gvpA* existed in these groups before they diverged into separate species.

Future experiments to address the taxonomy, biogeography, and evolution of polar sea ice gas vacuolate bacteria should on the following four goals. First, a complete taxonomic description should be made of the strains which have been analyzed by 16S rDNA phylogenetics, but otherwise not described (strains 37, 174, 90-P(gv)1, 206, 214.6, S36-W(gv)1, S51-W(gv)1, and

84-W(gv)1). Particular attention should be placed on DNA/DNA hybridization studies with strains 37, 174, and 90-P(gv)1 as they are closely related to each other (by 16S rDNA) and represent north and south polar isolates. Second, the 16S rDNA sequences of a several more members from each of the fatty acid groups along with the sequences of some of the isolates which lie outside of the major fatty acid groups should be determined. This will give a greater perspective on the diversity of cultured gas vacuolate bacteria within and near the sea ice. Third, all of the members of the one of the fatty acid groups should be tested against each other in DNA/DNA hybridization experiments. Such work would more rigorously test whether some gas vacuolate species are cosmopolitan in distribution. The best candidate fatty acid group for such a study should have a large number of isolates from the Arctic and Antarctic and have a narrow distribution of fatty acid compositions (such as the "*Octadecobacter*" group). Likewise, any future sampling field trips should focus on obtaining the largest number of gas vacuolate isolates possible from just that one group. Finally, experiments on the phylogeny of *gvpA* gene sequences should expand to include all of the nucleotide positions of *gvpA*. This could be accomplished by genomic DNA cloning using the *gvpA* PCR clones from this dissertation as hybridization probes. The greater number of nucleotides available from complete gvpA sequences (≈210 nt versus 88 nt) should allow for more accurate phylogenetic trees with higher bootstrap values on the branches.

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