

# ***Octadecabacter arcticus* gen. nov., sp. nov., and *O. antarcticus*, sp. nov., Nonpigmented, Psychrophilic Gas Vacuolate Bacteria from Polar Sea Ice and Water**

J. J. GOSINK<sup>1</sup>, R. P. HERWIG<sup>2</sup>, and J. T. STALEY<sup>1</sup>

<sup>1</sup> University of Washington, Microbiology, Seattle, USA

<sup>2</sup> School of Fisheries, University of Washington, Seattle, USA

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## **Summary**

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 *Octadecabacter*, 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 of bacteriochlorophyll *a*, and because they are psychrophiles. *Octadecabacter* gen. nov., contains both a north polar species, *O. arcticus* sp. nov. str. 238, and a south polar species, *O. antarcticus* sp. nov. str. 307. This genus may be useful for examining the extent of prokaryotic biogeographic dispersal.

**Key words:** *Octadecabacter arcticus* – *Octadecabacter antarcticus* – Marine bacteria – Phylogeny – Gas vesicles – Arctic bacteria – Antarctic bacteria – Biogeography

## **Introduction**

Over 250 heterotrophic gas vacuolate (gas vesicle producing) strains were isolated from annual sea ice and sea water from 35 separate sites over two field seasons in the Arctic and three field seasons in Antarctica (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). The relationship of the arctic and antarctic strains to each other is of particular interest in addressing the question of bacterial biogeography.

These isolates were clustered on the basis of whole cell fatty acid composition prior to further analysis. One group of 18 strains was distinguished by having octadecenoic acid (18 : 1) comprise a very high proportion (70–80%) of their total cellular fatty acids. Phylogenetic analysis of the 16 S rRNA of an antarctic member of this group, strain 307, showed that it was a member of the  $\alpha$  subclass of *Proteobacteria* and that it was most closely related to 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 photosynthetic *Proteobacteria* 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. This fatty acid is found in relative abundance in both pigmented and nonpigmented, bacteriochlorophyll *a*-producing and non bacteriochlorophyll *a*-producing, psychrotrophic and mesophilic, acidophilic and non acidophilic species of  $\alpha$  *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

**Abbreviations:** SWCm – Seawater Cytophaga medium; PCR – Polymerase chain reaction; G + C – Guanine + cytosine

have a different cell shape. Also, unlike the three species of *Roseobacter*, all 18 polar strains produce gas vesicles (unpublished results). In this study the relationship of this group of polar gas vacuolate bacteria to *Roseobacter* and to other *Proteobacteria* was investigated. Specifically, the 16S rDNA nucleotide sequence of strain 238 from the arctic was determined, and along with the 16S rDNA nucleotide sequence of strain 307 from the antarctic (GOSINK and STALEY, 1995), was phylogenetically analyzed. Genomic DNA from strains 238 and 307 were also hybridized with each other, and with that of *R. denitrificans*. Strains 238 and 307, along with two other members (strains 308 and 309) of the group of 18 polar strains, were also examined for additional phenotypic characteristics.

## Materials and Methods

**Bacterial strains and cultivation:** Strains were collected from the sea ice and water of the Arctic and Antarctica as previously described (STALEY et al., 1989). Strain 238 was isolated in April of 1992 from between 38 and 48 cm from the bottom of the ice in a small, refrozen lead, 350 km offshore of Deadhorse, Alaska ( $73^{\circ} 01' N$ ,  $148^{\circ} 31' W$ ). Strains 307, 308, and 309 were obtained in November of 1992 from between 25 and 50 cm for strains 307 and 309, and between 0 and 25 cm for strain 308 from the bottom of the ice in McMurdo Sound, Antarctica ( $77^{\circ} 53' S$ ,  $166^{\circ} 35' E$ ).

**Colony and cellular morphology:** Cells were grown on Sea Water Cytophaga medium (SWCm) (IRGENS et al., 1989) or Marine Agar 2216 (Difco) plates at  $10^{\circ} C$  for two to three weeks and scored for visual appearance. Electron micrographs were obtained of unstained whole cells using a JEOL-100B transmission electron microscope at 60 kV.

**Fatty acid methyl ester analysis:** Whole cell fatty acid compositions were determined using the MIDI system (Newark, DE) as previously described (GOSINK and STALEY, 1995). Cells were grown on either SWCm or Marine Agar 2216 at 4 or  $10^{\circ} C$ .

**Physiological tests:** Strains were tested for the ability to grow at various pHs using SWCm buffered with 20 mM MES (2-[N-morpholino] ethanesulfonic acid), ACES (2[2-amino-2-oxoethyl]amino]ethanesulfonic acid), TAPSO (3-[N-tris(hydroxymethyl)methylamino]-2-hydroxypropanesulfonic acid), TAPS (N-tris[hydroxymethyl]methyl-3-aminopropanesulfonic acid), or CHES (2-[N-cyclohexylamino]ethanesulfonic acid), prepared at pH 5.5, 6.5, 7.6, 8.5, and 9.5 respectively. Growth was scored after 17 to 28 days incubation at  $10^{\circ} C$ . Salinity ranges required for growth were examined using SWCm media broth formulations which had increasing or decreasing total amounts of NaCl,  $MgCl_2 \cdot 6H_2O$ ,  $MgSO_4 \cdot 7H_2O$ , KCl,  $CaCl_2 \cdot 2H_2O$ , and ferric ammonium citrate while keeping their relative proportions constant. These components were varied from 0 times standard SWCm concentration (0 ppt salinity) to 4 times standard SWCm concentration (70 ppt). The other components of the media were kept at standard SWCm concentrations (IRGENS et al., 1989). Growth was scored after incubation at  $10^{\circ} C$  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  $37^{\circ} C$ . Oxidase, catalase, and Gram stain reactions were determined by standard methods (GERHARDT et al., 1994). 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  $10^{\circ} C$  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 oxidation/fermentation medium of Leifson (25.3.2) (GERHARDT et al., 1994). Anaerobic growth was tested by streaking cells on SWCm agar plates with or without 0.1% D-glucose added and incubating either aerobically or in anaerobic gas pack jars (BBL) at  $10^{\circ} C$ . Mobility 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. Bacteriophaeophytin spectra were determined after the addition of 1/10 volume of 1 M HCl.

**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. The following carbon sources were examined at 1 g/l: acetate, pyruvate, succinate, citrate, propionate, butyrate, L-glutamate, DL-aspartate, L-leucine, L-proline, Casamino Acids (Difco), glycolic acid, glycerine, D-ribose, D-fructose, D-glucose, sucrose, N-acetyl-glucosamine, ethanol, and methanol. Tests were conducted in Falcon Microtest III™ 96-well tissue culture plates. Plates were incubated at  $10^{\circ} C$  and growth was scored 0, 24 and 35 days after inoculation by measuring turbidity with a EL311sx microtiter plate reader (BIO-TEK, Winooski, VT) at 600 nm.

Vitamin requirements (biotin, nicotinic acid, thiamine, and pantothenic acid) were assayed in a similar manner except that the basal medium consisted of SWCm without tryptone, beef extract, yeast extract, vitamins, or trace elements solution. A carbon source was provided by Casamino Acids (Difco, Detroit, MI) at 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  $\mu$ g/ml biotin, 1  $\mu$ g/ml nicotinic acid, 1  $\mu$ g/ml thiamine HCl, or 1  $\mu$ g/ml pantothenic acid. A fifth preparation was made without any vitamins and a sixth preparation had all of the vitamins present. Washed cells were inoculated into the wells of the microtiter plates. Inocula were sequentially transferred twice more into fresh growth media after 34 and 19 days. Growth was scored 15 days after the last transfer using a microtiter plate reader as described above. Both the carbon source utilization and the vitamin source requirement tests were prepared in quadruplicate in the microtiter plates.

**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 of the DNA (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 strains 238, 307, and *Roseobacter denitrificans*. The initial denaturation temperature was set at  $97^{\circ} C$  and the reannealing temperature was set at  $76^{\circ} C$ . All pairwise reassessments were run six to ten times.

**16S rRNA gene sequence determination and phylogenetic analysis:** The 16S rDNA of strain 238 was cloned and sequenced as previously described (GOSINK and STALEY, 1995). Multiple clones were pooled and sequenced to reduce the effect of gene copy microheterogeneities or PCR artifacts (CLAYTON et al., 1995; CILIA et al., 1996). The sequence of strain 307 was obtained from GenBank under accession number U14583 (GOSINK and STALEY, 1995).

Sequences were first aligned by the ALIGEN-SEQUENCE program of the Ribosomal Database Project (RDP, version 5.0) (LARSEN et al., 1993). Small manual adjustments were made on the basis of secondary structure considerations (GUTELL, 1994). Similar, prealigned 16S rRNA sequences were also obtained from the RDP. A BLAST (ALTSCHUL et al., 1990) search of GenBank revealed no additional sequences closely related to strains 238 or 307. Phylogenetic trees were generated using PAUP 3.0s (SWOFFORD and OLSEN, 1990) 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. McClade 3.05 (MADDISON and MADDISON, 1992) was used to determine transition and transversion frequencies.

**Nucleotide sequence accession number:** The 16S rDNA sequence of strain 238 has been deposited in GenBank under accession number U73735.

## Results and Discussion

Gas vacuolate strains of heterotrophic bacteria were obtained from the sea ice and sea water in the Beaufort

Sea, approximately 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 1).

One set of polar heterotrophic gas vacuolate strains was distinguished by an unusually high level of octadecenoic acid, i.e. 70–80% of total cellular fatty acids (Table 1) (see also GOSINK and STALEY, 1995). The exact location of the double bond in this fatty acid, (either 18 : 1  $\omega$ 7cis,  $\omega$ 9t, or  $\omega$ 12t) could not be resolved with the method of analysis. The remainder of the fatty acids in this group were mostly 16 : 1  $\omega$ 7cis, 16 : 0 4OH along with several other fatty acids, each comprising 3% or less of the total. *Roseobacter denitrificans* and *R. algicola* showed similar types and proportions of fatty acids. The concentration of octadecenoic acid in *R. algicola*, in fact, is the highest we are aware of for any single fatty acid in a bacterial species. Similar concentrations of fatty acids were obtained when the strains were grown on SWCm or marine medium 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,

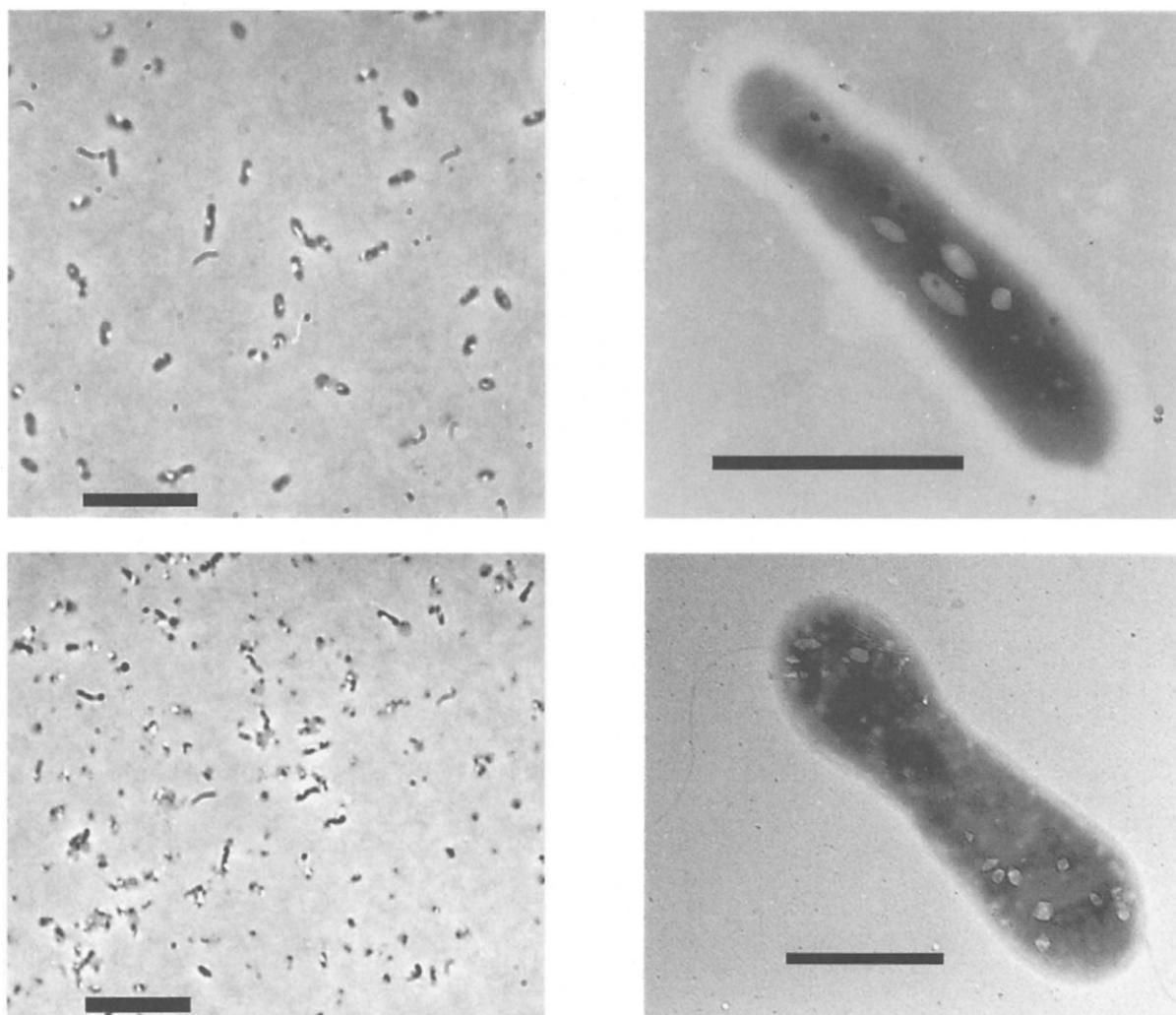


Fig. 1. Phase micrographs (left), and electron micrographs (right) of *Octadecabacter* strains 238 (top) and 307 (bottom). Scale bars for the phase and electron micrographs equal 10 µm and 1 µm, respectively.

**Table 1.** Fatty acid composition of polar gas vacuolate and *Roseobacter* strains.

Strain	% of Total Fatty Acids <sup>a</sup>			
	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
<i>Roseobacter denitrificans</i> Och 114	93	1	1	3
<i>Roseobacter algicola</i> (ATCC 51440) <sup>b</sup>	93	0	2	2

<sup>a</sup> Fatty acids each comprising less than 1% of the total were not included in the table<sup>b</sup> *R. algicola* was grown on SWCm at room temperature**Table 2.** List of organisms and environmental clone 16S rDNA sequences and their nucleotide differences from *Octadecabacter* 16S rDNA sequences.

Species or environmental clone name	16S rRNA sequence differences from strain		
	GenBank Number	238 differences/ % differences	307 differences/ % differences
<i>Roseobacter litoralis</i> Och 149	X78312	75/ 5.5	72/ 5.3
<i>Roseobacter denitrificans</i> Och 114	M59063	85/ 6.3	82/ 6.1
<i>Roseobacter algicola</i> ATCC 51440 (T)	X78315	90/ 6.7	89/ 6.7
str. 36 (Nielson)	NA	79/ 5.9	77/ 5.8
str. LFR ATCC 51258	L15345	85/ 6.8	83/ 6.6
str. NF18	M79390–M79392	63/ 7.5	62/ 7.4
str. SAR83	M63810	96/ 7.6	99/ 7.9
<i>Rhodobacter yeldkampii</i> ATCC 35703 (T)	D16421	107/ 7.8	109/ 8.0
<i>Paracoccus alkaliphilus</i> JCM 7364	D32238	112/ 8.2	110/ 8.0
<i>Paracoccus denitrificans</i> LMG 4218 (T)	X69159	118/ 8.6	116/ 8.5
str. AG33	M79372–M79374	73/ 8.7	72/ 8.6
<i>Rhodobacter sulfidophilus</i> str. W12 DSM 2351	D16430	120/ 8.8	120/ 8.8
<i>Hirschia baltica</i> IFAM 18	X52909	164/12.2	161/12.0
<i>Porphyrobacter neustonensis</i> ACM 2844	M96745	184/14.2	182/14.0
<i>Erythrobacter longus</i> str. OCh 101	M59062	197/14.5	195/14.3

NA – Not available

309), were investigated by further phenotypic methods. Isolates 238 and 307 were also examined by genotypic and phylogenetic methods.

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 possessed 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 canonical changes across a stem structure. The sequence electrophoregrams were unambiguous about all of the differences and none of the differences violated the types of

non-canonical 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*. A phylogenetic analysis of strains 238 and 307 was performed with a set of 36 aligned sequences representing the entire group 2.13.1.3 of the RDP (LARSEN et al., 1993), of which *Roseobacter* is a member, and four sequences from the adjacent groups 2.13.1.4 and 2.13.1.5.

This dataset was analyzed repeatedly with a neighbor joining algorithm (FELSENSTEIN, 1989) under a Kimura 2 parameter model (KIMURA, 1980). Different transition to transversion ratios ( $T$ ) 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, closely related species of *Paracoccus*, *Rhodobacter*, and *Roseobacter*, and three outgroup species, *Hirschia baltica*, *Erythrobacter longus* str. OCh 101, and *Porphyrobacter neustonensis* (Table 2).

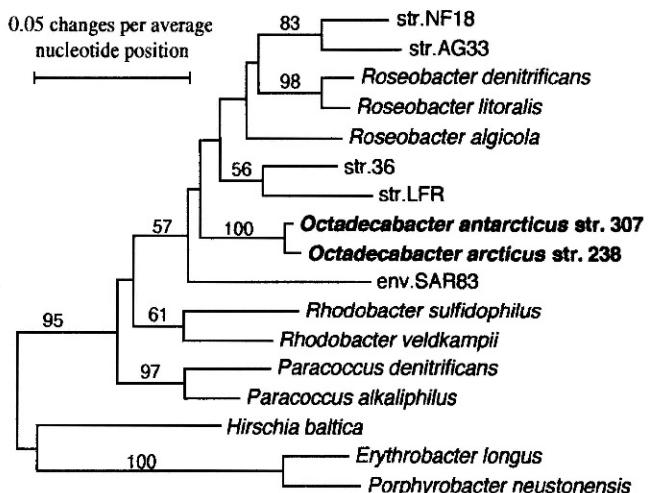
Character positions near the start and end of the dataset were removed because only a few of the taxa had nucleotide data for those positions. Two regions in the middle of the alignment also were removed because they could not be unambiguously aligned. The remaining alignment retained 402 varying characters.

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)  $T$  value of 2. The resulting tree had an inferred  $T$  of 1.15. Another neighbor joining analysis based on this value produced an identical tree to that in the first analysis. 100 bootstrap replicates were also analyzed under an  $T$  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  $T$  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 bootstrap 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 a  $T$  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



**Fig. 2.** Phylogenetic position of *Octadecabacter* in relation to *Roseobacter* and closely related species. This tree is the "best" tree from the Kishino-Hasegawa test of six alternate tree topologies and corresponds to the unweighted Wagner parsimony tree. This tree is depicted under a likelihood framework with a transition to transversion ratio of 1.15. The scale bar represents approximately 0.05 changes per average nucleotide position. Number above the branches indicate percent bootstrap support for that branch out of 100 likelihood bootstrap resamplings.

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 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 in likelihood scores than the best tree, were not significantly worse than the best tree.

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 3). *R. denitrificans* was pigmented 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 bacteriopheophytin. Unlike *R. algicola* (LEDYARD et al., 1993), and the DMSP-degrading isolate LFR (LAFAY et

**Table 3.** Physiological and nutritional characteristics of *Roseobacter* and *Octadecabacter* strains.

	<i>O. arcticus</i> str. 238	<i>O. ant- arcticensis</i> str. 307	<i>Octadecaba- cter</i> sp. str. 308	<i>Octadecaba- cter</i> sp. str. 309	<i>Roseobacter denitrificans</i> Och 114
Cell morphology	rod	rod	rod	rod	rod
Cell size	0.6–0.8× 4.4–4 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	0.6–1× 1–2.4 µm
Motility	–	–	–	–	+
Gas vesicles	+	+	+	+	–
Colony color	white	white	white	white	red-brick red
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 <sup>a</sup>					
0 ppt	–	–	–	–	–
1 ppt	–	–	–	–	–
3.5 ppt	–	–	–	–	–
18 ppt	+	+	+	+	+
35 ppt	+	+	+	+	+
70 ppt	W	+	+	+	W
Utilization as a carbon source <sup>b</sup>					
acetate	–	–	–	–	–
pyruvate	±	–	±	±	+
succinate	±	±	–	±	±
citrate	±	–	–	–	+
propionate	±	–	+	+	+
butyrate	–	–	–	–	–
L-glutamate	±	+	±	+	+
DL-aspartate	±	–	–	+	+
L-leucine	–	–	–	–	±
L-proline	–	–	–	–	–
Casamino Acids	+	+	+	+	+
glycolic acid	±	±	–	+	+
glycerine	+	±	+	+	–
D-ribose	±	–	–	–	+
D-fructose	–	–	–	–	+
D-glucose	±	±	–	±	±
sucrose	–	–	–	–	–
N-acetyl-glucosamine	–	±	–	+	–
ethanol	–	–	–	–	–
methanol	–	–	–	–	–
Requirement for					
biotin	–	?	+	+	+
thiamine	+	?	+	+	+
nicothinic acid	+	?	+	+	+
pantothenic acid	+	?	+	+	–
Hydrolysis of					
gelatin	–	–	–	–	+
starch	–	–	–	–	–

Table 3. (Continued).

	<i>O. arcticus</i> str. 238	<i>O. antarcticensis</i> str. 307	<i>Octadecabacter</i> sp. str. 308	<i>Octadecabacter</i> sp. str. 309	<i>Roseobacter</i> <i>denitrificans</i> Och 114
Gram stain	-	-	-	-	-
Catalase	+	+	+	+	+
Oxidase	-	-	-	-	+
$\text{NO}_3^-$ reduction	-	-	-	-	+
Anaerobic growth	-	-	-	-	-
Mole % G + C	57	56	ND	ND	59.6
Absorbance bands <i>in vivo</i>					
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.

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).

Another major difference between the polar sea ice strains 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 vacuolate strains are listed in Table 3. It was impossible to grow these strains on any of the carbon sources listed in Table 3 as sole carbon sources at 0.2% concentrations. 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 in microtiter plates. All of the polar gas vacuolate strains grew on L-glutamate, glycerine, and mixed amino acids in the presence of 0.2 g/l yeast extract.

Unlike *Roseobacter denitrificans*, the polar gas vacuolate strains lacked the ability to reduce  $\text{NO}_3^-$  to  $\text{NO}_2^-$ , grow at temperatures of 15 °C or higher (except in the case of strain 238 which could not grow at 19 °C or higher), utilize D-fructose as a carbon source, and hydrolyze gelatin. The polar strains were also oxidase negative. 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 several millimeters below the surface in the semisolid  $\text{NO}_3^-$  reduction tubes. This suggested that they are microaerophilic. Strain 238 showed weak acid production in a semisolid SWCM agar deep with 0.1% glucose and a mineral oil overlay. However, none of the strains were capable of growth on SWCM agar plates either with or without 0.1% D-glucose at 10 °C in anaerobic gas pack jars (BBL). It may be that the low temperatures and long incubation times allowed oxygen to diffuse into the agar deeps and permit limited growth of strain 238.

A few differences were noted in some tests between our results and those of Shiba (SHIBA, 1991). Under the conditions employed in our study, *Roseobacter denitrificans* could 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 were 42% ± 7% similar to each other. Strain 238 *Roseobacter denitrificans* showed DNA/DNA hybridization values of 35% ± 9%. Strains 307 and *R. denitrificans* have 42% ± 14% DNA/DNA hybridization. All of these values are below 70%, the value defined for isolates of the same species (WAYNE et al., 1987).

Strains 238, 307, 308, and 309 are related to the genus *Roseobacter*. However, phylogenetic differences,

**Table 4.** Features that differentiate between *Octadecabacter* strains and *Roseobacter* strains.

	<i>O. arcticus</i> str. 238	<i>O. antarcticus</i> 307	<i>Roseobacter denitrificans</i>
Motility	–	–	+
Gas vesicles	+	+	–
Colony color	white	white	red-brick red
Growth at pH 9.5	–	+	+
Growth at 19 °C	–	–	+
Utilization as a carbon source <sup>a</sup>			
pyruvate, citrate, propionate, ribose	W	–	+
leucine	–	–	W
glycerine	+	W	–
fructose	–	–	+
N-acetyl-glucosamine	–	W	–
Requirement for			
biotin	–	? <sup>a</sup>	+
thiamine	+	?	+
nicotinic acid	+	?	+
pantothenic acid	+	?	–
other growth factors	–	+	–
Gelatin hydrolysis	–	–	+
Oxidase	–	–	+
NO <sub>3</sub> <sup>–</sup> reduction	–	–	+
Absorbance bands	–	–	+
<i>in vivo</i> (802–873 nm) or in methanolic extracts (769–773 nm)			

W – Weak

? – Unknown

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

the absence of bacteriochlorophyll *a* and other photopigments, the presence of gas vesicles, and low DNA/DNA hybridization values between 238, 307 and *R. denitrificans* indicate that these strains should be considered members of a new genus described below with key properties summarized in Table 4.

*Octadecabacter oc.ta.de.ca.bac’ter.* Gr. numeral *octadeca*, eighteen, Gr. neut. n. *bacterion* a rod, M. L. masc. n. *Octadecabacter* an “eighteen rod” pointing out that it has C<sub>18</sub> fatty acids. Cells are gram negative, non motile rods or pleomorphic rods. Aerobic or microaerophilic but do not reduce NO<sub>3</sub><sup>–</sup> to NO<sub>2</sub><sup>–</sup>. 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 L-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 se-

quences. The type species is the arctic isolates, 238, hereinafter referred to as *O. arcticus*.

*Octadecabacter arcticus arc’ti.cus* M. L. adj. *arcticus*, arctic, pertaining to the arctic. The cells of the type strain, *Octadecabacter arcticus* form long irregular or pleomorphic rods from 0.6–0.8 × 2.4–4 µm in size. Known strains produce gas vesicles. *O. arcticus* requires thiamine, nicotinic acid, and pantothenic acid for growth. 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. The type strain of the species is strain 238.

*Octadecabacter antarcticus ant.arc’ti.cus* M. L. adj. *antarcticus*, pertaining to the antarctic. *Octadecabacter antarcticus* forms long irregular or pleomorphic rods from 0.6–0.8 × 1.6–4.8 µm in size. Members of the species grow well on L-glutamate and mixed amino acids in the presence of small amounts of yeast extract. Do not grow on vitamin-free media supplemented with only bi-

otin, thiamine, nicotinic acid, and pantothenic acid. Growth temperature range is from 4 to 10 °C. On Marine medium 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%. Known strains produce gas vesicles. Isolated from sea ice in McMurdo Sound, Antarctica. The type strain of the species is strain 307.

Surprisingly few systematic studies have been undertaken to address the question of prokaryotic biogeography. One study suggests that 3-chlorobenzoate degrading bacteria do not have uniform dispersal (TIEDJE et al., 1994). Another study suggests that very similar strains of denitrifying, toluene degrading *Azoarcus* could be isolated from different continents and continental regions (FRIES et al., 1994). Unfortunately, DNA/DNA hybridization studies were not conducted with these stains to determine if they were members of the same or different species. Likewise several 16S rRNA molecular probe-based studies have been conducted to determine the distribution and uniformity of bacteria [for example see (MCARTHUR et al., 1988; WOOD and TOWNSEND, 1990; BRITSCHGI and GIOVANNONI, 1991; DELONG, 1992; RUFF-ROBERTS et al., 1994)]. Again, however, no objective measure exists to gauge the level of species diversity that differences in various molecular characters reveal.

The polar sea ice associated gas vacuolate strains, particularly pairs of closely related strains from the opposite poles, may make good candidates for studies of bacterial biogeography. Several *Octadecabacter* strains were investigated in this study including strains 238 and 307 from the north and south polar regions, respectively. Although these two strains are closely related to each other by 16S rRNA based phylogeny, they were found to comprise two separate species as evidenced by their low DNA/DNA reassociation values. Additional species of this genus will need to be isolated from both poles to determine whether the species of this genus shows bi-polar endemism. However, the results of this study are consistent with recent studies of another new bi-polar genus from sea ice communities, "Polarobacter" (manuscript in preparation). In that genus, one arctic species differed from phylogenetically closely related antarctic species by DNA/DNA reassociation values which were similar to the reassociation values of the two *Octadecabacter* species to each other. Again, however, additional strains of this genus need to be examined to address the biogeography question more thoroughly.

The purpose that gas vesicles serve in these heterotrophic bacteria in the polar environment is currently unknown. Gas vesicles are produced by some marine cyanobacteria such as *Trichodesmium* (WALSBY, 1994), however marine heterotrophic gas vacuolate bacteria had not been reported prior to our studies (IRGENS et al., 1989; STALEY et al., 1989; GOSINK et al., 1993; GOSINK and STALEY, 1995; IRGENS et al., 1996). Gas vacuolate prokaryotes are commonly found in freshwater environments such as thermally stratified lakes where they use gas vesicles for buoyancy to reach a favorable depth for growth in the water column (WALSBY, 1994). Seasonal

sea ice provides a habitat for a vast microbial community that develops in the spring (HORNER, 1985). One possibility is that the polar gas vacuolate strains produce gas vesicles to rise up in the water column to the bottom of the sea ice so that they can reside close to the primary producers of the sea ice microbial community. An alternative hypothesis is that the gas vesicles allow the cells to rise to the surface of the water column during the time of year when sea ice is forming so they can be present in the ice when the sea ice microbial community develops. Further research will be needed to investigate these possibilities.

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#### Corresponding author:

J. J. GOSINK, University of Washington, Microbiology, Box 357242, Seattle, WA 98195-7242, USA.  
Tel.: (206) 543-3376; Fax: (206) 543-8297;  
email: gosink@u.washington.edu