APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Sept. 1995, p. 3486–3489 Vol. 61, No. 9
0099-2240/95/$04.00+0

Biodiversity of Gas Vacuolate Bacteria from Antarctic Sea Ice and Water

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Received 30 March 1995/Accepted 21 June 1995

Psychrophilic, gas vacuolate, heterotrophic bacteria indigenous to sea ice communities in Antarctica have been isolated. Phylogenetic analysis of representative members of these bacteria shows that they belong to the alpha, beta, and gamma *Proteobacteria* and the *Flavobacteria-Cytophaga* group. This is the first report of gas vacuolate bacteria from the beta *Proteobacteria* and the *Flavobacteria-Cytophaga* groups.

Approximately 23 million km^2 of the ocean surface freezes to produce sea ice on a seasonal basis (14). In the spring and summer months an extensive community of microorganisms develops in the sea ice at the ice-seawater interface (11). One of the principal types of bacteria associated with the sea ice community is a group of heterotrophic gas vacuolate organisms (Fig. 1) which occurs at both the North and South Poles (9, 17). Heterotrophic gas vacuolate bacteria from other marine habitats have not been reported. 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.

Gas vacuolate bacteria were recovered from antarctic sea ice and water by plating samples on seawater-*Cytophaga* medium, a dilute medium containing beef extract, yeast extract, tryptone, and succinate in half-strength artificial seawater (12). Plates were incubated at 4 to 6°C for 3 to 8 weeks and scored for the presence of gas vacuolate colonies by visual appearance. The gas vacuolate phenotype of these strains was confirmed by phase-contrast and transmission electron microscopy (Fig. 1). Strains were obtained in 1986 (17) from the U.S. Palmer Station (64°S, 64°W) (strain 34-P) and in 1987 from the U.S. McMurdo Station (78°S, 167°E) [strains S36-W(gv)1, S51-W(gv)1, and 90-P(gv)1]. Similar techniques were used to obtain additional strains from McMurdo Station in the austral summer (November and December) of 1992 (strains 301 and 307). Thus, isolates were obtained during three field seasons from multiple sites at two widely separated areas of Antarctica,

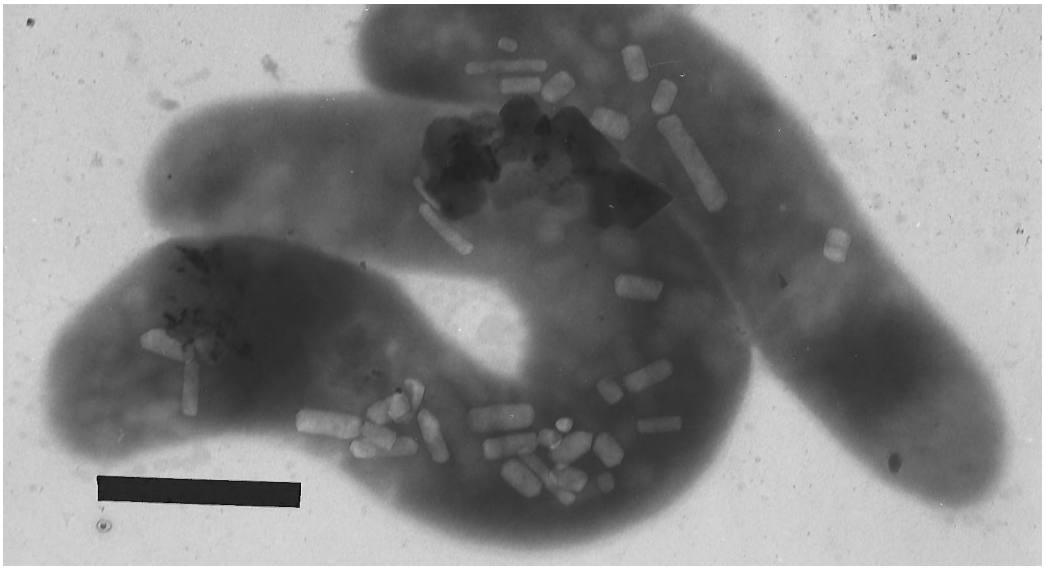

FIG. 1. Transmission electron micrograph showing an example of two unstained cells of gas vacuolate sea ice bacteria. The bright intracellular areas are gas vesicles. The bar represents 1.0 μm.

thereby indicating that gas vacuolate sea ice bacteria are members of the normal microbiota of this habitat (9, 12, 17).

Our collection of South Polar gas vacuolate bacteria contains 34 strains. In order to assess the diversity of these isolates, they were first characterized by whole-cell fatty acid composi-

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TABLE 1. Predominant fatty acids and percent compositions of type strains[a]

Strain	No. of isolates in fatty acid group	% of fatty acid in composition													
		13:0 iso	12:0 3OH	14:O	15:1 isoG	15:1 w8c	15:1 w6c	16:1 w9c	16:1 w7c	16:O	15:0 iso 3OH	15:0 3OH	17:1 w9c	18:1 w9c	18:1 w9c/w12t/w7c
301	8	5			11		9		9		17	7			
307	3							14		5					76
34-P	5								75	15					7
S51-W(gv)1	2			5					43	27					
S36-W(gv)1	1		11					13	22	12				16	7
90-P(gv)1	1					6			38	9			9		14

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

tion. Strains were grown on seawater-*Cytophaga* plates at 5°C in the dark for 3 to 4 weeks. Colonies were harvested and lysed, and the whole-cell fatty acids were saponified with methanolic base. Saponified fatty acids were converted to fatty acid methyl esters and quantitated with a Hewlett-Packard model 5890 Series II gas chromatograph according to recommended procedure (15) (Table 1). Because our isolates were incapable of growth under the standard conditions employed with the commercial database used in this study, it was necessary to use an artificial seawater medium (seawater-*Cytophaga* medium) and grow them at low temperature (17). The strains were initially grouped on the basis of whole-cell fatty acid composition by using the MIDI, Inc. (Newark, Del.), system. Despite the fact that the fatty acid compositions of these isolates could not be compared with those of known bacteria in the MIDI database, the cluster analysis program proved useful in grouping the strains (Fig. 2). The fatty acid compositions of isolates were reproducible even though many strains grew slowly (data not shown).

To determine the relationship of these groups to each other and to other known bacteria, the 16S rRNA gene of a representative from each group was subjected to sequencing and phylogenetic analysis. Genomic DNA was purified by a hexadecyltrimethylammonium bromide miniprep method described previously (1). 16S rRNA 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 the genomic DNAs by using the universal primers 8 FPL and 1492 RPL (16). The PCR product was digested with *Not*I and cloned into pBluescript II KS+ (Stratagene, La Jolla, Calif.). Multiple clones [except in the case of 90-P(gv)1 and 34-P] of the same orientation were pooled and sequenced with 16S rRNA-specific forward and reverse primers as previously described (5). Additional primers were required to sequence part of the 16S rRNA gene for some of the strains: sp3Rgamma (positions 358 to 340 [*E. coli* rRNA sequence numbering (2)]; ACTGCTGCCTCCCGTAGGA) for strain 90-P(gv)1, sp7R gamma (positions 802 to 785; TACCGGGGTATCTAATCC) for strain 90-P(gv)1, sp11rhod (positions 1096 to 1114; CCG GCAACGAGCGCAACCC) for strains 307 and S51-W(gv)1, sp11flav (positions 1096 to 1114; CCTATAACGAGCGCA ACCC) for strain 301, and sp13Rgamma (positions 1406 to 1390; ACGGGCGGTGTGTACAA) for strain S51-W(gv)1.

Sequence contigs were assembled (8) and aligned with the most similar sequences in Ribosomal Database Project (RDP) release 4.0 first by the ALIGN_SEQUENCE program (13) and second by manual comparison to secondary structures provided by the RDP (10). Additional, prealigned 16S rRNA sequences were also obtained from the RDP (GenBank nucleotide sequence 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 by using PAUP 3.0s (18). The search parameters included a simple addition sequence with one tree held at each step with the tree bisection-reconnection swapping algorithm. Similar branching order was obtained by using PAUP 3.0s (18) for transversion parsimony,

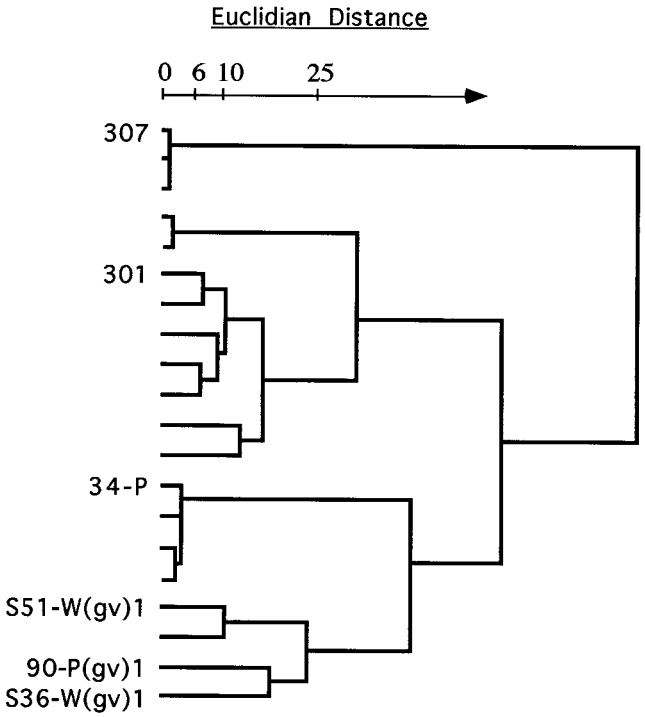

FIG. 2. Cluster analysis dendrogram of 20 antarctic gas vacuolate sea ice bacteria based on whole-cell fatty acid composition obtained by using the MIDI system. Strains that were further characterized by 16S rRNA gene sequencing and phylogenetic analysis (Fig. 3) are indicated by their designations. The designations of the other strains have been omitted for clarity.

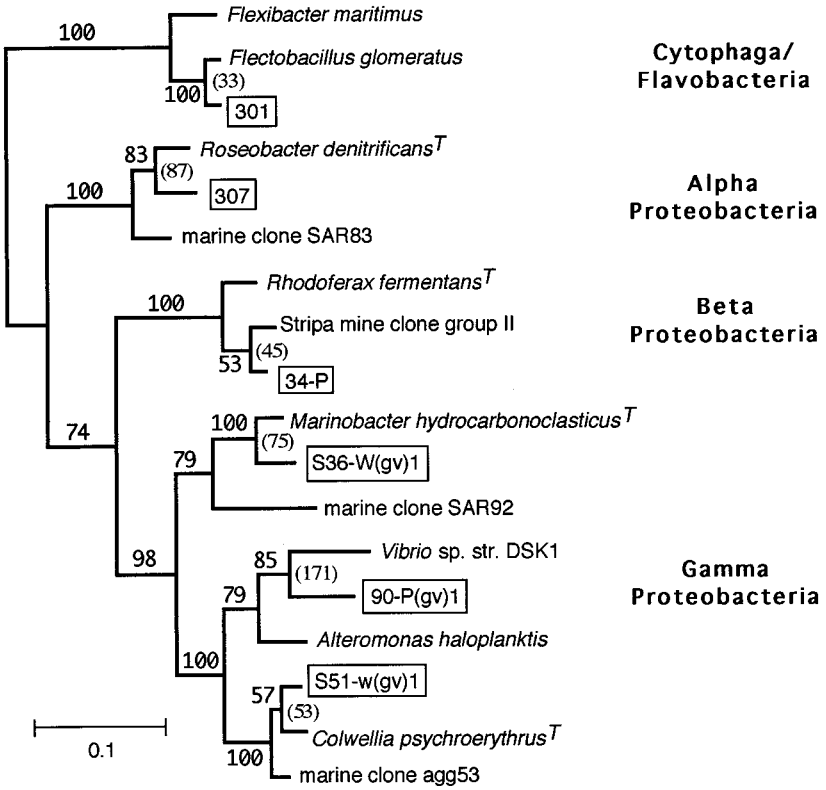

FIG. 3. Phylogenetic relationship based on 16S rRNA sequences of representative gas vacuolate sea ice bacteria (indicated by their strain numbers in boxes) and the most closely related species or environmental sequences from uncultivated bacteria listed in RDP release 4.0. This tree was the most parsimonious tree generated with PAUP 3.0s (18) in a generalized, unweighted, heuristic search. Numbers near the nodes represent the percent bootstrap values for that node (500 bootstrap resamplings). Numbers in parentheses are the numbers of nucleotide differences between the type strain and its nearest phylogenetic neighbor. The scale bar represents 0.1 nucleotide difference per sequence position.

FITCH (6) for distance, and fastDNAml (13) for maximum-likelihood analyses (data not shown).

One of the surprising results from these analyses is that the isolated polar gas vacuolate bacteria do not compose a single species or genus but are instead phylogenetically diverse (Fig. 3). They fall into four distinct bacterial groups, including the alpha, beta, and gamma *Proteobacteria* class and the *Flavobacteria-Cytophaga* group. None of these organisms is identical in 16S rRNA sequence to any known prokaryotic species, including other polar gas vacuolate strains in the same fatty acid group (unpublished data). Only one strain appears to belong to a known genus: strain S51-W(gv)1 is closely related to *Colwellia psychroerythrus*, a psychrophilic marine bacterium (3, 4). Although strain 301 is closely related to *Flectobacillus glomeratus* (7), *F. glomeratus* is phylogenetically very different from the type strain of *Flectobacillus*. This is the first report of gas vacuolate bacteria from the beta *Proteobacteria* and the *Flavobacteria-Cytophaga* groups of bacteria. Thus, now 6 of the 11 bacterial phylogenetic groups or classes, as well as some archaean methanogens and extreme halophiles, are known to contain gas vesicles (19).

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 display it. Gas vesicles are produced by many aquatic prokaryotes that use them to provide buoyancy in order to regulate their position in vertically stratified water columns (19). Clearly, this habitat has a strong vertical stratification due to the phase discontinuity between the sea ice and water. Several hypotheses for the significance of gas vesicles to sea ice bacteria could be proposed, 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.

Nucleotide sequence accession numbers. Nucleotide sequences for the antarctic gas vacuolate isolates have been deposited in the GenBank database under accession numbers U14581 to U14586.

This research was supported in part by an NSF grant (BSR 90006 788) to J.T.S. J.J.G. was supported in part by a PHS National Research Service Award from the NIGMS.

We thank R. L. Irgens and R. P. Herwig, who helped collect some of the strains studied, and appreciate the helpful comments of Peter Hirsch, John Baross, and Sarah Boomer in reviewing the manuscript. Sequencing primers were kindly provided by Romesh K. Gautom and Thomas R. Fritsche. In addition, we thank Sue Barns for advice on molecular cloning and phylogenetic analysis.

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