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

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Several strains of a novel heterotrophic gas vacuolate bacterium were isolated from antarctic marine waters. The results of phylogenetic analyses in which 16S ribosomal DNA sequencing was used, coupled with phenotypic tests, indicated that strain 34-P^T (T = type strain) belongs to a new genus and species of the beta subgroup of the *Proteobacteria*, for which the name *Polaromonas vacuolata* is proposed. Although the other four strains studied probably belong to this new species, DNA-DNA hybridization tests were not conducted. The closest phylogenetic relatives of *P. vacuolata* are the photosynthetic nonsulfur purple bacterium *Rhodospirillum rubrum* and the hydrogen autotroph *Variovorax paradoxus*.

Although gas vacuolate heterotrophic bacteria are well-known inhabitants of aquatic ecosystems, until recently none of these organisms had been observed in or isolated from marine habitats. In 1989 several types of gas vacuolate bacteria were found in Antarctica growing in association with the sea ice microbial community (9, 13). Sequencing and analyses of 16S ribosomal DNAs (rDNAs) of a variety of these Antarctic gas vacuolate bacteria revealed that they were members of the *Proteobacteria* and the *Flavobacterium-Cytophaga-Bacteroides* phylogenetic groups (6). Within the *Proteobacteria*, the alpha, beta, and gamma subgroups were represented.

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

All strains were isolated from Antarctic waters off the Palmer Peninsula near the U.S. Palmer Station, Anvers Island, Antarctica. Several strains, including 34-P^T (T = type strain), 41-P, 54-P, J-A, and J-B, were isolated from samples collected beneath sea ice (9). The presence of gas vesicles was confirmed by phase-contrast microscopy (Fig. 1) and by transmission electron microscopy (Fig. 2). The strains were grown and characterized as described previously (9), with the addition of tests for assessing generation time and urease activity (5). Resistance to various antibiotics was tested by placing a paper disk containing an antibiotic onto an SWC-m agar plate onto which the test strain had been spread. The plates were incubated at 4°C, and susceptibility was determined by the presence of a zone of clearing that was more than 40 mm wide. The fatty acid compositions of all of the strains and the 16S rDNA nucleotide sequence of strain 34-P^T were also determined as described previously (6).

Our 16S rDNA sequence was compared with the sequence determined independently in the laboratory of C. R. Woese (17). The 16S rDNA sequence of 34-P^T was aligned with the most similar sequences in Ribosomal Database Project (RDP) release 5.0 by using the ALIGN_SEQUENCE program (10) and by manually comparing the structure with secondary structures provided by the RDP (7). Preamplified 16S rRNA sequences for the following organisms were also obtained from the RDP (GenBank accession numbers are given in parentheses): *Thiobacillus perometabolis* ATCC 23370 (M79421 to M79423), *Sphaerotilus natans* (Z18534), *Rubrivivax gelatinosus*

ATCC 17011^T (D16213), *Brachymonas denitrificans* JCM 9216^T (D14320), *Comamonas testosteroni* ATCC 11996^T (M11224), Stripa-derived bacterium (L20811), *Variovorax paradoxus* IAM 12373^T (D30793), str. PAD44 (D26231), *Rhodospirillum rubrum* JCM 7819^T (D16211), *Alcaligenes faecalis* ATCC 8750^T (M22508), and *Bordetella parapertussis* ATCC 15311^T (U04949). Finally, a BLAST search was also performed with the 1 October 1995 release of GenBank to determine if there were any sequences that were closely related to the sequence of 34-P^T but were not included in RDP release 5.0. Phylogenetic trees were generated by using PAUP, version 3.0s (14), for parsimony analysis, DNADIST and NEIGHBOR (4) for distance analysis, and fastDNAm1 (3, 12) for likelihood analysis. MacClade, version 3.05 (11), was used to determine transition and transversion frequencies.

The results of phenotypic tests for growth, carbon source utilization, and various physiological features, as well as G+C contents, have been reported previously (9); in addition, the following characteristics were also determined. The bacteria which were studied were short, unicellular, gram-negative rods (0.8 by 2 to 3 µm) that typically produced gas vacuoles which appeared as bright refractile areas within the cells (Fig. 1). Although the cells were nonmotile under usual culture conditions, they produced polar flagella in addition to gas vacuoles (Fig. 2). These bacteria produced circular, convex colonies with smooth, glistening surfaces and entire edges on agar plates. The colonies were chalky white in pigmentation. No growth occurred on SWC plates containing glucose (0.1%) or L-arginine (0.1%) as a carbon source when the plates were incubated in anaerobic jars (GasPak; BBL, Baltimore, Md.). The generation time, calculated from A₆₀₀ values at 4°C, was 40 h. Strain 34-P^T grew well at 0 to 12°C. It did not grow at 15°C. This strain grew when the initial pH of the medium was 6.0 to 9.5. The pH of the spent medium was approximately 7.0 in all cases, except when sugars were present, in which case the pH was between 6.0 and 7.0. Good growth occurred in the presence of 0 to 6.0% NaCl. No growth occurred in the presence of 7.0% NaCl.

P. vacuolata 34-P^T was positive for urease and deaminase activities; susceptible to novobiocin (30 µg), tetracycline (30 µg), and neomycin (30 µg); and resistant to streptomycin (10 µg) and gentamicin (10 µg).

Strain 34-P^T grew when the tryptone, yeast extract, beef extract, and vitamins of SWC-m were replaced with vitamin-free Casamino Acids (Difco Laboratories, Detroit, Mich.), indicating that vitamins are not required for growth. Good

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FIG. 1. Phase-contrast photomicrograph showing several cells of strain 34-P^T. Note the bright, irregular gas vacuoles in the cells. Bar = 5 μ m.

growth occurred in nutrient broth containing only 4.0 g/liter, but there was no growth in nutrient broth containing 8.0 g/liter.

In addition to the carbon sources indicated previously (9), the following carbon sources were utilized by strain 34-P^T: fumarate, citrate, succinate, 2-oxoglutarate, D-glucose, oxaloacetate, butyrate, DL-alanine, pyruvate, DL-glutamate, glycerol, DL-proline, propionate, DL-aspartate, DL-asparagine, acetate, and sorbitol.

The following carbon sources were not utilized: maltose,

D-fructose, xylose, D-ribose, L-fucose, formate, glycine, DL-serine, malonate, DL-isoleucine, DL-lysine, DL-histidine, DL-methionine, DL-valine, cellobiose, mannose, melibiose, melezitose, rhamnose, sorbose, trehalose, methanol, propanol, benzoate, erythritol, DL-threonine, and DL-tryptophan.

Whole-cell fatty acid analyses were performed on all strains. All strains contained large amounts of 16:1 ω 7c (74 to 79%) and smaller amounts of 16:0 (14 to 17%). In addition, a third fatty acid was present in smaller amounts (7 to 9%). This fatty acid was identified as 18:1 ω 7c, 18:1 ω 9t, or 18:1 ω 12t or possibly a combination of more than one of these compounds; its actual identity could not be determined by the procedure and instruments used. Such predominance of a single fatty acid is unusual in bacteria, and this is the highest level of 16:1 ω 7c that we are aware of in any bacterial species.

The 16S rDNA sequence of strain 34-P^T was compared with the sequences of other bacteria included in the RDP database (Table 1). On the basis of simple sequence homology, the most closely related previously described organisms are *Rhodospirillum rubrum*, a nonsulfur purple bacterium (8), and *V. paradoxus*, a chemoorganotroph and facultative lithoautotroph (1, 16). As determined by the same method, however, strain 34-P^T is most closely related to the environmental 16S rDNA sequences str. Stripa and str. PAD44. The Stripa-derived 16S rDNA sequence was obtained from deep groundwater in the Stripa mine in Sweden (2). The str. PAD44 or env. PAD44 sequence is a sequence that was obtained from a paddy field (15). However, both of these sequences are environmental sequences without organisms available for comparison, and so it is impossible to determine how similar the actual organisms are to one another in other respects. In addition, these sequences are only partial 16S rDNA sequences, and so the actual levels of relatedness might be different if the complete sequences were available.

A phylogenetic analysis of the sequences revealed that the relationship of the organisms was uncertain. A preliminary

FIG. 2. Electron micrograph of 34-P^T showing a cell containing several gas vesicles. The flagella are not attached. Bar = 0.5 μ m.

TABLE 1. Levels of homology of the *P. vacuolata* 16S rDNA sequence and the most closely related 16S rDNA sequences obtained from the RDP

Sequence	Mean distance or no. of base differences ^a											
	34-P ^T	str. Stripa	<i>Variovorax paradoxus</i>	env. PAD44	<i>Rhodoferrax fermentans</i>	<i>Sphaerotilus natans</i>	<i>Brachymonas denitrificans</i>	<i>Rubrivivax gelatinosus</i>	<i>Comamonas testosteroni</i>	<i>Thiobacillus perometabolis</i>	<i>Bordetella parapertussis</i>	<i>Alcaligenes faecalis</i>
34-P ^T		0.05	0.052	0.059	0.065	0.074	0.083	0.084	0.085	0.113	0.121	0.124
str. Stripa	44		0.033	0.055	0.057	0.076	0.053	0.066	0.046	0.096	0.092	0.103
<i>Variovorax paradoxus</i>	75	29		0.03	0.042	0.078	0.08	0.079	0.066	0.11	0.116	0.12
env. PAD44	16	15	8		0.03	0.067	0.037	0.074	0.037	0.097	0.071	0.078
<i>Rhodoferrax fermentans</i>	94	50	61	8		0.087	0.079	0.088	0.08	0.113	0.115	0.117
<i>Sphaerotilus natans</i>	100	66	105	18	117		0.091	0.048	0.096	0.094	0.101	0.113
<i>Brachymonas denitrificans</i>	120	46	116	10	114	122		0.071	0.052	0.109	0.097	0.123
<i>Rubrivivax gelatinosus</i>	122	57	114	20	128	65	103		0.087	0.082	0.097	0.12
<i>Comamonas testosteroni</i>	123	40	96	10	116	129	76	126		0.112	0.115	0.127
<i>Thiobacillus perometabolis</i>	97	56	94	23	97	80	93	70	96		0.115	0.126
<i>Bordetella parapertussis</i>	173	80	165	19	164	135	139	139	164	98		0.058
<i>Alcaligenes faecalis</i>	176	89	170	21	166	150	174	171	181	107	82	

^a The numbers on the upper right are the mean distances between sequence pairs adjusted for missing data, and the numbers on the lower left are total numbers of base differences for the pairs of sequences.

phylogenetic set of the four most parsimonious trees obtained with PAUP, version 3.0s (14), was analyzed by using MacClade, version 3.05 (11), to produce a substitution matrix to correct for the different rates obtained for 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 determined to be 1.3. A rescaled consistency index weighting mask was also constructed from these trees by using MacClade, version 3.05 (11).

The substitution matrix was reapplied to the aligned data set in PAUP, version 3.0s (14), and the most parsimonious trees were determined by using the branch and bound option with both the original data set and 100 bootstrap-resampled data sets. The original data set (with the applied substitution matrix) yielded four equally parsimonious trees whose length was 638. These trees differed in the relationships among env. PAD44, *Rhodoferrax fermentans*, *V. paradoxus*, str. Stripa, and strain 34-P^T. The consistency index for these trees was 0.386. The bootstrap values only weakly supported any particular branching structure near 34-P^T (Fig. 3). Three similar trees were obtained when corrections for the substitution matrix and the rescaled consistency index weighting mask were used simultaneously (data not shown).

The aligned data set used in the parsimony analysis was also resampled to make 100 bootstrap replicates by using SEQBOOT (4). Distance matrix tables for both the original data set and the bootstrap resampled data sets were constructed by using DNADIST (4) with a Kimura 2 parameter correction, a jumbled input order, and a transition-to-transversion ratio of 1.3. The original and bootstrap distance matrices were analyzed by NEIGHBOR (4). Again, the exact relationship between strain 34-P^T and its nearest phylogenetic neighbors was not certain. This method identified 34-P^T as a sister taxon of a clade containing *V. paradoxus* and *Rhodoferrax fermentans* and all three of these organisms as members of the sister clade closest to *Brachymonas denitrificans* and *C. testosteroni*. While there is clear bootstrap support (bootstrap value, 81%) for placing 34-P^T in this group of beta subgroup *Proteobacteria*, with *Brachymonas denitrificans* and *C. testosteroni*^T as members

of a sister clade (bootstrap value, 77%), the level of support for an exclusive relationship to *V. paradoxus* and *Rhodoferrax fermentans* is quite low (bootstrap value, 52%).

Finally, the aligned data set and 100 bootstrap replicates were analyzed by using fastDNAm1 (3, 12), with the base frequencies determined empirically and the ratio of transition to transversion set at 1.3. Again, the more distantly related taxa showed a branching topology similar to that determined with

FIG. 3. Phylogenetic relatedness of *P. vacuolata* 34-P^T and the most closely related species. This maximum-parsimony tree was determined by an exact (branch-and-bound) search method, using a substitution matrix to correct for the various rates of nucleotide substitutions. This is one of four equally parsimonious trees. The numbers in parentheses near the branch points indicate how many of the four equally most parsimonious trees shared that branch structure. The numbers not in parentheses near the clades indicate the percentages of bootstrap support for the clades based on 100 bootstrap resamplings. Only bootstrap values of 50% or more are shown.

TABLE 2. Phenotypic comparison of *P. vacuolata* and the two phylogenetically most closely related species^a

Organism	Cell shape	Gas vesicles	Photo-synthesis	Position of flagella	O ₂ requirement	Temperature relationship	Colony pigmentation	G+C content (mol%)
<i>Polaromonas vacuolata</i>	Rods	+	—	Polar	Obligate aerobe	Psychrophilic	White	52–57
<i>Rhodoferrax fermentans</i>	Curved rods	—	+	Polar	Facultative aerobe	Mesophilic	Peach-brown	60
<i>Variovorax paradoxus</i>	Curved or straight rods	—	—	Peritrichous	Obligate aerobe	Mesophilic	Yellow	67–69

^a Data are from references 1, 8, and 9.

the parsimony and distance trees. As in the neighbor-joining analysis, strain 34-P^T was between the Stripa mine clone and a clade containing *Rhodoferrax fermentans* and *V. paradoxus*. This is in contrast to the results of the parsimony analysis, in which strain 34-P^T was identified (with weak support) as a sister taxon of the Stripa mine clone. Also, like the parsimony and distance methods, the maximum-likelihood bootstrap analysis produced no significant support for any particular branching order near strain 34-P^T and only 59% support for a clade that included only strain 34-P^T, *V. paradoxus*, *Rhodoferrax fermentans*, env. PAD44, and str. Stripa.

Each of these methods produced slightly different trees. From all of the trees, however, it is clear that strain 34-P^T is most closely related to *Rhodoferrax fermentans*, *V. paradoxus*, str. Stripa, and env. PAD44. On the other hand, strain 34-P^T is not photosynthetic and does not grow as a nonsulfur purple bacterium under conditions used for the growth of *Rhodoferrax fermentans*. Also, strain 34-P^T differs by 5 and 7% in 16S rDNA base homology from *V. paradoxus* and *Rhodoferrax fermentans*, respectively. Furthermore, other genotypic and phenotypic data indicate that *P. vacuolata*, *V. paradoxus*, and *Rhodoferrax fermentans* differ markedly (Table 2); for example, the G+C contents of these organisms are 52 to 57, 67 to 69, and 60 mol%, respectively. In addition, *V. paradoxus* and *Rhodoferrax fermentans* are pigmented, are not gas vacuolate, and differ from *P. vacuolata* in cell shape and motility (Table 2).

This appears to be the first report of a gas vacuolate member of the beta subgroup of the *Proteobacteria*. This is not surprising, however, because some members of both the alpha and gamma subgroups of the *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 the results of an analysis of its levels of 16S rDNA base homology, as discussed above, 34-P^T is sufficiently different from other bacteria to warrant creation of a new genus. We therefore propose that the new genus *Polaromonas* should be described as follows.

Description of *Polaromonas* gen. nov. *Polaromonas* (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). Cigar-shaped, gram negative rods that are 0.8 by 2.0 to 3.0 μ m. Encapsulated. Aerobic. Chemorganotrophic and catalase and oxidase positive. Requires amino acids, but not vitamins, for growth. Motile by means of a polar flagellum. Cells may contain gas vesicles. Psychrophilic. The maximum growth temperature of known strains is 15°C.

The G+C contents are 52 to 57 mol% (as determined by the thermal denaturation method) (9).

The only species is the type species, *Polaromonas vacuolata*.

Description of *Polaromonas vacuolata* sp. nov. *Polaromonas vacuolata* (va.cu.o.la'ta. L. adj. *vacuus*, empty; N. L. part. adj. *vacuolata*, equipped with gas vacuoles). Cells contain gas ves-

icles. The optimum temperature for growth is 4°C, and the growth temperature range is 0 to 12°C. Colonies are snowy white, circular, and convex with smooth surfaces and entire edges. The more gas vesicles within the cells, the whiter the colony. Good growth occurs in media containing NaCl at concentrations ranging from 0 to 6.0%, but no growth occurs in the presence of 7.0% NaCl.

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

The fatty acid composition is 75% 16:1 ω 7c, 17% 16:0, and 8% 18:1 ω 7c, 18:1 ω 9t, or 18:1 ω 12t.

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

The G+C content is 52.0 mol% (as determined by the thermal denaturation method).

The type strain is *P. vacuolata* 34-P (= ATCC 51984).

It is likely that the other strains included in this study are members of the same species on the basis of the results of the phenotypic tests that were performed, including the whole-cell fatty acid analysis. However, DNA-DNA hybridization tests were not conducted, and so this has not been verified.

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