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Albidovulum inexpectatum gen. nov., sp. nov., a Nonphotosynthetic and Slightly Thermophilic Bacterium from a Marine Hot Spring That Is Very Closely Related to Members of the Photosynthetic Genus *Rhodovulum*

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Several bacterial isolates, with an optimum growth temperature of about 50°C, were recovered from the marine hot spring at Ferraria on the island of São Miguel in the Azores. The geothermal water emerged from a porous lava flow and rapidly cooled in contact with seawater except at low tide. The bacterial species represented by strains FRR-10^T and FRR-11 was nonpigmented, strictly aerobic, and organotrophic. Several genes, *bchZ*, *pufB*, *pufA*, *pufL*, or *pufM*, encoding the photosynthetic reaction center proteins and the core light-harvesting complexes were not detected in these strains. The organism oxidized thiosulfate to sulfate with enhancement of growth. The organism did not require additional NaCl in the culture medium for growth, but NaCl at 1.0% enhanced growth. Phylogenetic analyses using the 16S rRNA gene sequence of strain FRR-10^T indicated that the new organism represented a new species of the α-3 subclass of the *Proteobacteria* and that it branches within the species of the genus *Rhodovulum*. The contradiction of classifying an organism which branches within the radiation of the genus *Rhodovulum* but does not possess the hallmark characteristics of this genus is discussed. However, the absence of several of these characteristics, namely, the lack of photosynthesis and pigmentation, which could be related to colonization of dark environments, and growth at high temperatures, leads to our proposal that strains FRR-10^T and FRR-11 should be classified as a new species of a novel genus, *Albidovulum inexpectatum*, representing, at present, the most thermophilic organism within the α-3 subclass of the *Proteobacteria*.

A large number of thermophilic organisms have been isolated from continental hydrothermal areas where the levels of sodium are very low, as well as from shallow or abyssal marine hydrothermal vents where NaCl can reach the levels of seawater. Most of the organisms isolated and described from marine hydrothermal sites are slightly halophilic and have optimum growth temperatures above 70°C and some, namely *Methanopyrus kandleri* and *Pyrolobus fumarii*, have optimum temperatures for growth above 100°C (2, 20). However, many marine hot springs have lower vent temperatures and the organisms isolated from these are slightly or moderately thermophilic. These organisms do not elicit as much interest as those that grow at temperatures around 100°C, although new, slightly or moderately thermophilic species increase our perception of microbial biodiversity and may have characteristics and phylogenetic affiliations that are sometimes unexpected.

We recently isolated several slightly thermophilic nonpigmented organisms from the marine hot spring at Ferraria on the island of São Miguel in the Azores that did not produce carotenoids, bacteriochlorophyll *a* (Bchl *a*), or *puf* genes that encode the photosynthetic reaction center proteins and the core light-harvesting complexes. These organisms were not phototrophic but they are, based on 16S rRNA gene sequence analysis, members of the genus *Rhodovulum*, which comprises the species *Rhodovulum sulfidophilum*, *Rhodovulum euryhalinum*, *Rhodovulum adriaticum*, *Rhodovulum strictum*, *Rhodovulum iodosum*, and *Rhodovulum robiginosum* (10, 12, 13, 19, 33, 45). The species of the genus *Rhodovulum*, along with the species of *Rhodobacter*, are canonical phototrophic organisms of the α-3 subclass of the *Proteobacteria* (13, 17); these organisms are similar and have optimum growth temperatures in the neighborhood of 30°C, but species of the former inhabit marine and euryhalinal environments while the latter are freshwater inhabitants. Closely related species of the α-3 subclass of the *Proteobacteria* have been isolated primarily from saline environments but, unlike the species of *Rhodovulum*, are incapable of photoautotrophic growth in artificial media. Some species may be photoorganotrophic, namely, *Rhodobaca bogoriensis* (31). Other species, such as "*Roseinatronobacter thiooxidans*" and those of the genera *Roseobacter* and *Roseovarius*, produce Bchl *a* under aerobic conditions, but photoautotrophic or photoorganotrophic growth has not been demonstrated (22, 40, 43). Others still, including the species of genera *Si-*

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licibacter, *Staleya*, *Sagittula*, and *Antarctobacter*, are obligately organotrophic (9, 21, 23, 34) or can be chemolithotrophic (44).

With the exception of *Silicibacter lacuscaerulensis* (34), which has an optimum growth temperature of about 45°C, all other organisms have optimum growth temperatures of about 30°C, and none grow at temperatures above about 40°C. We recently isolated several nonpigmented slightly thermophilic strains from the marine hot spring on the island of São Miguel in the Azores that were very closely related to the species of the genus *Rhodovulum*. These isolates were strictly organotrophic and lacked the characteristic photosynthetic reaction center genes. Since this organism lacks some of the hallmark characteristics of the species of the genus *Rhodovulum*, we conclude that this species represents a new genus for which we propose the name *Albidovulum inexpectatum*.

MATERIALS AND METHODS

Isolation and bacterial strains. Strains FRR-10^T (T = type strain) and FRR-11 were isolated from the marine hot spring at Ferraria on the island of São Miguel, the Azores. Water samples were collected in sterile screw-cap glass bottles, transported without temperature control, and filtered through membrane filters (Gelman type GN-6; pore size, 0.45 μm; diameter, 47 mm) after diluting water samples to obtain well-isolated colonies on filters. The filters were placed on the surface of agar-solidified Degryse 162 medium containing 3% NaCl (6). Medium 162 contained (per liter) the following: yeast extract, 2.5 g; tryptone, 2.5 g; nitrilotriacetic acid, 1.0 g; $CaSO_4 \cdot 2H_2O$, 0.4 g; $MgSO_4 \cdot 7H_2O$, 2.0 g; 15 ml of 0.2 M $Na_2HPO_4 \cdot 12H_2O$; 10 ml of 0.2 M KH_2PO_4; 0.5 ml of 0.01 M Fe(III) citrate·$5H_2O$; and 5.0 ml of trace element solution, pH 7.5. The trace element solution contained (per liter) the following: $MnSO_4 \cdot H_2O$, 0.22 g; $ZnSO_4 \cdot 7H_2O$, 0.05 g; H_3BO_3, 0.05 g; $CuSO_4 \cdot 5H_2O$, 0.0025 g; $Na_2MoO_4 \cdot 2H_2O$, 0.0025 g; and $CoCl_2 \cdot 6H_2O$, 0.0046 g. The organisms were grown in medium containing 10.0 g of NaCl liter^{-1} unless otherwise stated. The plates were wrapped in plastic bags and incubated at 50°C for up to 7 days in the dark. Cultures were purified by subculturing and were maintained at −80°C in Degryse 162 medium containing 1% NaCl and 15% glycerol. The type strains of *R. sulfidophilum* (DSM 1374), *Rhodovulum euryhalinum* (DSM 4868), *Rhodobacter sphaeroides* (DSM 158), *Roseococcus thiosulfatophilus* (DSM 8511), and *Paracoccus versutus* (DSM 582) were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), Braunschweig, Germany, and used to compare several characteristics of the new organisms.

Morphological, biochemical, and tolerance characteristics. Electron microscopy was performed on exponential-phase cultures in Degryse 162 liquid medium containing 1% NaCl as previously described (32). Cell morphology and motility were examined by phase-contrast microscopy during the exponential growth phase. Unless otherwise stated, all biochemical and tolerance tests were performed as described previously (26, 39) in Degryse 162 liquid medium or Degryse 162 agar containing 1% NaCl at 50°C for up to 5 days. Catalase and oxidase activities were examined as described previously (41). The salt range of the organisms was determined in liquid medium with NaCl ranging between 0.0 and 9.0%. The growth temperature range of the organisms was examined by measuring the turbidity (610 nm) of cultures incubated in 300-ml metal-capped Erlenmeyer flasks containing 100 ml of medium in a reciprocal water bath shaker. The pH range for growth was examined at 50°C in the same medium by using 20 mM morpholineethanesulfonic acid for pH values between 5.0 and 6.5, 20 mM Tris for pH values between 7.0 and 8.5, and 20 mM 3-cyclohexylamino-2-hydroxy-1-propanesulfonic acid for pH values between 9.0 and 10.5; the pH of each buffer was adjusted with HCl or NaOH. Anaerobic growth was assessed in Degryse medium 162 with 1.0-g liter^{-1} concentrations of KNO_3, dimethyl sulfoxide (DMSO), or trimethylamine-*N*-oxide (TMA) with incubation in anaerobic chambers with a CO_2 atmosphere (Biomerieux) in the dark.

Single-carbon-source assimilation tests were performed in a minimal medium composed of Degryse 162 basal salts containing 1% NaCl to which filter-sterilized ammonium sulfate (0.5 g liter^{-1}) and the carbon sources (2.0 g liter^{-1}) were added. Growth of the strains on single carbon sources was examined by measuring the turbidity of cultures incubated at 50°C in 20-ml screw-cap tubes containing 10 ml of medium for up to 5 days of growth. Positive and negative control cultures were grown in 1% NaCl Degryse 162 medium and in minimal medium without a carbon source, respectively.

Fermentation was examined using Degryse 162 basal salts medium containing 1% NaCl, yeast extract (0.5 g liter^{-1}), agar (3.0 g liter^{-1}), bromothymol blue (0.03 g liter^{-1}), and the carbon source (5.0 g liter^{-1}). The color change of the medium from blue to yellow was regarded as acidification by fermentation of the carbon source (41).

Phototrophic growth, autotrophic growth, and growth on reduced sulfur compounds. Phototrophic growth was assessed in Degryse 162 medium and medium 27 (lacking ethanol and vitamin B_{12}) containing 1% NaCl supplemented with neutralized sodium sulfide solution (0.1 g liter^{-1}) or with sodium thiosulfate (0.1 g liter^{-1}) in completely filled screw-cap bottles under incandescent illumination (1,000 lx). *Rhodovulum sulfidophilum* and *Rhodobacter sphaeroides* were used as controls.

Autotrophic growth was also assessed in medium 27 with H_2, sodium thiosulfate (1.0 g liter^{-1}), sodium tetrathionate (1.0 g liter^{-1}), sodium sulfite (1.0 g liter^{-1}), and sodium sulfide (0.5 g liter^{-1}) without any organic components (DSMZ [www.dsmz.de/media/med027.htm]) under aerobic conditions.

Chemolithoheterotrophic growth on H_2, sodium thiosulfate, sodium sulfite, and sodium sulfide was assessed under aerobic conditions at the optimum growth temperature of the FRR strains in medium 27 lacking sulfate and containing 0.3 g of yeast extract liter^{-1}, 1.0 g of succinate liter^{-1}, and 0.5 g of acetate liter^{-1}. Filter-sterilized sulfur compounds were added to the medium at concentrations that varied between 0.5 and 5.0 g liter^{-1}. The type strain of *Paracoccus versutus*, used as a control for chemolithoheterotrophic growth, was grown at 30°C. At appropriate intervals, the turbidity of the cultures was determined and the cells were harvested and filtered through Gelman type GN-6 membrane filters. The levels of thiosulfate and sulfate in the filtrates were determined using the methods described by Westley (47) and Sörbo (42), respectively.

Polar lipid, lipoquinone, and fatty acid composition. The cultures used for polar lipid analysis were grown in 1-liter Erlenmeyer flasks containing 200 ml of medium at 50°C in a reciprocal water bath shaker until the exponential phase of growth. Harvesting of the cultures, extraction of the lipids, and single-dimensional thin-layer chromatography were performed as described previously (7, 35). Lipoquinones were extracted from freeze-dried cells, purified by thin-layer chromatography, and separated by high-performance liquid chromatography as described previously (32).

Cultures for fatty acid analysis were grown on solidified medium in sealed plastic bags submerged in a water bath at 50°C for 24 h. Fatty acid methyl esters were obtained and separated as described previously (4).

Preparation of *bis*(methylthio) derivatives. Fatty acid methyl esters were dissolved in 100 μl of hexane to which was added 100 μl of dimethydisulfide and two drops of a 6% (wt/vol) solution of iodine in diethyl ether. The reaction mixture was incubated overnight, after which iodine was removed by shaking with 0.5 ml of 5% aqueous sodium thiosulfate solution, and the derivatives were recovered by extraction (twice) with an equal volume of hexane.

Preparation of DMOX derivatives. Fatty acid methyl esters were demethylated by treatment with 0.5 ml of 1 M sodium hydroxide in 50% aqueous methanol at 60°C overnight. After cooling, the pH was reduced to below 2 with 0.5 ml of 1 M HCl, and the fatty acids were recovered by extraction with hexane chloroform (4:1). The extracts were dried, redissolved in 50 μl of chloroform, mixed with 100 μl of 2-methyl-2-amino propanol, and heated for 3 h at 285°C in a sealed tube. The cooled reaction mixture was diluted with 2 volumes of chloroform and washed with 1 ml of distilled water made alkaline with a few drops of a 1 M sodium hydroxide solution. The water layer was removed and the solution of 2-alkenyl-4,4-dimethyloxazoline (DMOX) derivatives in chloroform was washed with two further portions of water. Finally, the chloroform layer was removed to a clean tube and dried on a vacuum centrifuge.

GC/MS. Electron ionization gas chromatography/mass spectrometry (GC/MS) was performed with a Kratos MS80 RFA spectrometer (Kratos Ltd., Manchester, United Kingdom) interfaced to a Carlo-Erba 5160 chromatograph. The carrier gas was helium, and samples were introduced by splitless injection into a BPX-5 fused silica column (25 m by 0.2 mm; SGE Ltd, Milton Keynes, United Kingdom). Spectra were recorded at 70 eV ionization energy.

Pigment analyses. Cultures in the exponential phase of growth were washed twice by centrifugation using a morpholinepropanesulfonic acid (MOPS) buffer (MOPS-NaOH, 0.01 M; KCl, 0.1 M; $MgCl_2$, 0.001 M; pH 7.5). Carotenoids were extracted with chloroform-methanol (1:2, vol/vol) in the dark to avoid photooxidation of the pigments (24). Single-dimensional thin-layer chromatography was performed on silica gel G plates (catalog no. 5626; Merck) using a solvent system composed of chloroform-methanol (95:12.5, vol/vol) to separate carotenoids. Bchls were extracted with acetone-methanol (7:2, vol/vol), and the absorption spectrum of the extract was examined on a Perkin-Elmer Lambda 2 UV-Vis spectrophotometer.

Analysis of *puf* genes by PCR and Southern hybridization. The isolation of DNA from strain FRR-10^T, *Rhodovulum sulfidophilum*, and *Rhodovulum eury-*

TABLE 1. Primers used in this study

Designation of primer	Sequence 5′–3′	Strand	Location	Reference
	AGAGGGAGCTCGCATGA	+	*BchZ*	29
	CCGGGTTTGTAGTGGAA	−	*pufL*	29
L810F	TGGTGGAGCYGGTGGCTCAA	+	*pufL*	11
L810R	TTGAGCCACCAGCTCACACA	−	*pufL*	11
PUBM1	TGGCASTGGCGYCCGTGG	+	*pufB*	This study
PUBM2	CCATSGTCCAGCGCCAGA	−	*pufM*	This study
PULM1	KTTCGACTTCTGGGTSGG	+	*pufL*	This study
PULM2	CCCATSGTCCAGCGCCAG	−	*pufM*	This study
FRR1	GATCAYGACMGGGCIGGSGGITAYTGG	+	*BchZ*	This study
FRR2	GCGCTTCCTISCSCGCACIATCGACG	+	*BchZ*	This study
FRR3	ACTTCCACTAYAAICC	+	*pufL*	This study
FRR4	GGITTRTAGTGGAAGT	−	*pufL*	This study
FRR5	TCGGIACGCTYGGSATICACCG	+	*pufL*	This study
FRR6	CGGTGIATSCCRAGCGTICCGA	−	*pufL*	This study
FRR7	CATCTTCICGCACCTCGACTGG	+	*pufM*	This study
FRR8	CCAGTCGAGGTGCGIGAAGATG	−	*pufM*	This study
FRRB	GGTCTIACSGACGAGCARGCGC	+	*pufB*	This study
FRRA1	ATGIIIAARTTCTACAARATYTGG	+	*pufA*	This study
FRRA2	CAGGGCGTSTTCCTGTTCC	+	*pufA*	This study
FRRM1	TTCTGGCGCTGGACSATGGG	+	*pufM*	This study

halinum was performed as described by Marmur (27). The sequences of the genes contained in *puf* operons of *Rhodovulum sulfidophilum, Rhodobacter sphaeroides, Rhodobacter capsulatus, Roseateles depolymerans, Acidiphilium rubrum*, and *Roseobacter denitrificans* were aligned, and 20 degenerate forward and reversed primers were designed based on conserved regions within these genes (Table 1). Numerous combinations of primers were used in PCR experiments at annealing temperatures ranging from 40 to 55°C and carried out in reaction mixtures (50 μl) containing 100 ng of DNA from strain FRR-10^T, *Rhodovulum sulfidophilum*, or *Rhodovulum euryhalinum*, 100 ng of each primer, 10 mM Tris-HCl (pH 9.0), 1.5 mM $MgCl_2$, 50 mM KCl, 1 U of *Taq* polymerase, and a 0.2 mM concentration of each deoxynucleoside triphosphate (Amersham Pharmacia Biotech). The mixtures were preincubated at 94°C for 3 min and then subjected to 30 cycles of denaturation at 94°C for 1 min. Annealing was performed at temperatures mentioned above for 1 min, and primer extension was at 72°C for 1 min. The extension reaction in the last cycle was prolonged for 5 min. *Roseococcus thiosulfatophilus* DNA was used as an extra positive control for the presence of *puf* genes. Fragments obtained were separated by agarose gel electrophoresis, purified from agarose with a SpinPrep Gel DNA kit (Novagen), and ligated in the pGEM T-Easy vector (Promega). Transformation of *Escherichia coli* XL1-blue was carried out with the standard procedures described by Sambrook et al. (38). After confirmation of positive clones, cells were grown overnight in ampicillin-containing Luria-Bertani medium (50 μg/ml) and plasmids were isolated with a High-Pure plasmid isolation kit (Roche). The inserts were sequenced by Agowa GmbH, Berlin, Germany.

Total DNA from strain FRR-10^T, *Rhodovulum sulfidophilum*, and *Rhodovulum euryhalinum* was digested with *Bam*HI. A 1.5-kb fragment that corresponded to a continuous nucleotide stretch between *pufL* and *pufM* was amplified from genomic DNA of *Rhodovulum sulfidophilum* with primers PULM1 and PULM2 (Table 1) and used as a probe in Southern blotting experiments. Hybridization was performed on nitrocellulose membrane with the DNA probe labeled with a Digoxigenin DNA Detection kit (Roche Molecular Biochemicals).

Determination of mean base composition of DNA, 16S rRNA gene sequence, and phylogenetic analysis. DNA was isolated as described by Cashion et al. (3). The G+C content of the overall genome was determined by high-performance liquid chromatography as described by Mesbah et al. (30). The extraction of genomic DNA for 16S rRNA gene sequence determination, PCR amplification of the 16S rRNA gene, and sequencing of the purified PCR products were carried out as described previously (37). Purified reactions were electrophoresed using a model 310 genetic analyzer (Applied Biosystems, Foster City, Calif.). The 16S rRNA gene sequences determined in this study were aligned against representative reference sequences of members of the *Proteobacteria* using the ae2 editor (25). The method of Jukes and Cantor (18) was used to calculate evolutionary distances. Phylogenetic dendrograms were generated using various treeing algorithms contained in the PHYLIP package (version 3.5.1; J. Felsenstein, Department of Genetics, University of Washington, Seattle).

Nucleotide sequence accession numbers. The 16S rRNA gene for strain FRR-10^T was deposited with EMBL under accession number AF465833. The GenBank accession number for the *pufA, pufL*, and *pufM* genes of *Rhodovulum euryhalinum* is AF486825.

Culture accession numbers. Strain FRR-10^T has been deposited in the DSMZ as strain DSM 12048 and in the ATCC as strain ATCC BAA-387. Strain FRR-11 has also been deposited in the DSMZ (DSM 12049) and the ATCC (ATCC BAA-388) as a reference strain.

RESULTS

Sample sites and isolation of thermophilic bacteria. The organisms designated FRR-10^T and FRR-11 were isolated from a marine hot spring, located in porous lava under a solid basalt lava flow, on the southern coast of the island of S. Miguel at Ferraria that could be approached at low tide. The water temperature was 53°C, the pH was 7.8, and the salinity was 2.3%. The water temperature of the spring dropped very rapidly to about 23°C in contact with seawater, so these organisms probably colonize the geothermal aquifer or the porous lava before the water is released into the seawater. Plate cultures incubated at 50°C produced nonpigmented colonies or yellow-pigmented colonies, the latter of which were very closely related to the type strain of *Thermonema rossianum*, based on 16S rRNA gene sequence analysis.

Morphological, physiological, and biochemical characteristics. Colonies of strains FRR-10^T and FRR-11 were not pigmented. The organisms formed short rods that were 1.4 to 2.2 μm in length by 0.4 to 0.6 μm in width and stained gram negative, and motility was not observed. Strain FRR-10^T possessed cells surrounded by an envelope composed of an outer membrane with a triple-layer structure and a very thin peptidoglycan layer visible between the cytoplasmic membrane and the outer membrane (Fig. 1). Intracytoplasmic membrane systems were never observed in cultures grown aerobically in the dark.

The optimum growth temperature of the FRR strains, under aerobic conditions in the dark, was about 50°C; no growth was observed at temperatures lower than 35°C, or at temperatures higher than 60°C. The optimum pH for growth was very broad and occurred between 6.5 and 8.0, but the organism did not grow at pH 5.0 or pH 9.5. These organisms grew in Degryse medium 162, containing 6 mM Na^+, without the addition of NaCl, but the growth was enhanced in medium with 1.0 to 3.0% NaCl; no growth took place in medium containing more than 6.0% NaCl.

The organisms were cytochrome oxidase and catalase positive and hydrolyzed esculin, arbutin, hippurate, Tween 20, Tween 40, and Tween 60. Gelatin, fibrin, elastin, starch, xylan, and Tween 80 were not hydrolyzed. Vitamins or yeast extract were not required for growth. A large number of organic compounds, namely D-glucose, D-fructose, D-galactose, D-mannose, L-rhamnose, D-arabinose, L-arabinose, D-ribose, D-xylose, L-fucose, sucrose, lactose, maltose, melibiose, trehalose, glycerol, D-arabitol, ribitol, D-mannitol, D-sorbitol, *myo*-inositol, acetate, pyruvate, fumarate, malate, citrate, succinate, oxaloacetate, α-ketoglutarate, L-alanine, L-asparagine, L-glutamate, L-leucine, L-methionine, L-proline, L-arginine, and L-lysine, were assimilated. On the other hand, D-raffinose, L-sorbose, D-cellobiose, xylitol, formate, glycine, L-cysteine, and L-phenylalanine were not assimilated. Fermentation of carbohydrates was not observed, nor was anaerobic growth observed with nitrate, DMSO, or TMA, but nitrate was reduced to nitrite.

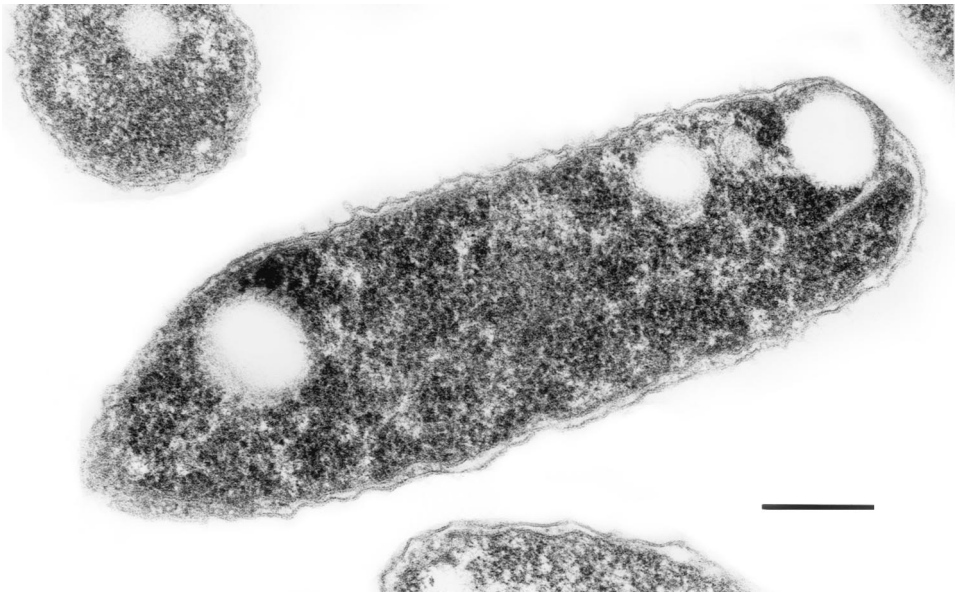

FIG. 1. Ultrastructure of strain FRR-10^T. Bar = 0.20 μm.

The addition of thiosulfate to medium 27 containing 0.3 g of yeast extract liter^{-1}, 1.0 g of succinate liter^{-1}, and 0.5 g of acetate liter^{-1} lead to an increase in the biomass of strains FRR-10^T and FRR-11, indicating that thiosulfate was used as an energy source in the presence of organic substrates (Table 2). The determination of the levels of sulfate and thiosulfate in the medium during growth of strains indicated that thiosulfate was completely oxidized to sulfate (results not shown). Sulfide, tetrathionite, and sulfite did not stimulate growth and were not oxidized to sulfate. Sulfite at concentrations above 2.0 mM inhibited growth. Photoautotrophic growth was not observed under the conditions examined in this study, and chemolithotrophic growth with H_2 was not observed either. Autotrophic growth on sodium thiosulfate, sodium tetrathionate, sodium sulfite, and sodium sulfide was not observed in a completely inorganic medium.

Pigments, Bchl *a*, and *puf* gene analysis. In contrast to the type strains of *Rhodovulum sulfidophilum*, *Rhodovulum euryhalinum*, and *Rhodobacter sphaeroides*, we did not detect the presence of Bchl *a* in any of the FRR isolates under aerobic or anaerobic growth conditions. Carotenoid pigments were not detected in extracts under any experimental conditions. The presence of *bchZ, pufB, pufA, pufL, pufM,* or *pufC* genes was not detected by PCR amplification or Southern hybridization experiments in strain FRR-10^T, although a number of PCR fragments were amplified at lower annealing temperatures; these did not correspond to *puf* gene sequences, however. On the other hand, the presence of *puf* genes was detected in *Rhodovulum euryhalinum* both by Southern analysis (data not shown) and PCR. We amplified a 1.9-kb fragment from *Rhodovulum euryhalinum* DNA (Fig. 2) that corresponded to a continuous stretch containing *pufA*, *pufL*, and *pufM*. The nu-

TABLE 2. Effect of the concentration of thiosulfate on the growth of the type strain of *P. versutus* and strains FRR-10 and FRR-11

Initial concn of thiosulfate (g liter^{-1})	Maximum turbidity (610 nm)		
	P. versutus	FRR-10	FRR-11
0.0	0.74	0.4	0.36
0.5	0.87	0.56	0.53
1.0	0.89	0.57	0.50
2.0	0.95	0.56	0.50

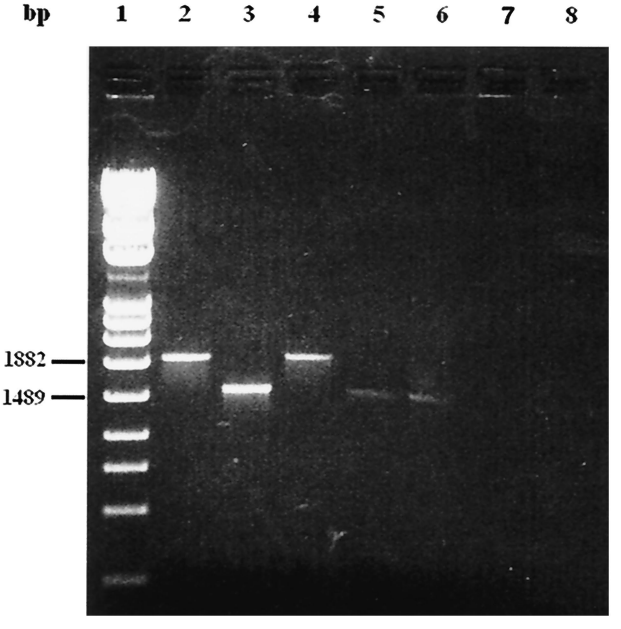

FIG. 2. Agarose gel electrophoresis of PCR-amplified partial *puf* genes with an annealing temperature of 48°C. Lane 1, marker IV (Roche Molecular Biochemicals). Amplification products were generated with primers PUBM1 and PUBM2 from *Rhodovulum sulfidophilum* DNA (lane 2), *Rhodovulum euryhalinum* DNA (lane 4), and FRR-10^T DNA (lane 7), and with PULM1 and PULM2 from *Rhodovulum sulfidophilum* DNA (lane 3), *Rhodovulum euryhalinum* DNA (lane 5), *Roseococcus thiosulfatophilus* DNA (lane 6), and FRR-10^T DNA (lane 8).

TABLE 3. Mean fatty acid composition of FRR-10^T after growth at 30 and 50°C and of *Rhodovulum sulfidophilum, Rhodovulum euryhalinum,* and *Rhodobacter sphaeroides* after growth at 30°C

Fatty acid	ECL[a]	Composition (%) in:				
		FRR-10^T		*Rhodovulum sulfidophilum,* 30°C	*Rhodovulum euryhalinum,* 30°C	*Rhodobacter sphaeroides,* 30°C
		30°C	50°C			
10:0 3OH	11.421	2.8	4.1	2.0	2.2	3.2
12:1 (5)	11.799	2.7	3.3	0	0	0
Unknown[b]	15.277	2.5	3.2	0.7	1.2	2.3
16:1 (9)	15.818	0	0.5	2.0	2.2	1.4
16:0	15.999	0.6	1.1	13.0	3.7	3.6
Unknown[b]	16.833	0	0	0	1.6	0
17:0	17.000	0.2	0.7	0.2	0.3	0.3
16:0 iso 3OH	17.142	0	0	0.7	0.5	0
18:1[11]	17.828	76.6	60.3	72.0	78.1	80.1
18:0	18.000	5.8	10.6	4.0	6.8	5.4
11-Me 18:1[11]	18.083	7.2	13.5	0.3	0.6	1.6
Unknown[b]	18.845	0	0	2.3	0.3	1.0
Unknown[b]	19.834	0	0.1	0.4	1.3	0.2

[a] Equivalent chain length.
[b] Unknown fatty acid.

cleotide sequences of *pufA pufL* and *pufM* shared about 86% sequence identity to the corresponding genes in *Rhodovulum sulfidophilum* and 58 to 76% with the corresponding genes from other α-*Proteobacteria*.

Polar lipid, lipoquinone, and fatty acid compositions. The polar lipid compositions of the FRR strains, *Rhodovulum sulfidophilum*, *Rhodovulum euryhalinum*, and *Rhodobacter sphaeroides* were dominated by phosphatidylethanolamine and phosphatidylglycerol. Phosphatidylcholine was detected in strain FRR-10^T, *Rhodovulum euryhalinum*, and *Rhodobacter sphaeroides*, while a sulfolipid, presumably sulfoquinovosyldiglyceride, was detected in all the strains except the FRR isolates (Fig. 3). Ubiquinone 10 was the major respiratory quinone of all the strains examined. 11-Octadecenoic acid (18:1[11] = 18:1ω7) was the major fatty acid in all organisms, followed by 16:0 in *Rhodovulum sulfidophilum* and 18:0 in the other organisms. The comparatively low levels of 18:1 and high levels of 18:0 in strain FRR-10^T were probably due to the high growth temperature of this organism. Strain FRR-10^T could be easily distinguished from the other organisms because this strain had a relatively high relative proportion of the very rare fatty acid, 11-methyl-11,12-octadecenoic acid (11-Me 18:1[11] = 11 met-18:1ω7), which was otherwise found in trace levels in the other organisms examined (Table 3).

The identities of all fatty acids were confirmed by GC/MS. The mass spectrum of the methyl ester of the major peak with an equivalent chain length (ECL) of 17.83 was consistent with an octadecenoic acid. Synthesis of its corresponding *bis*(methylthio) derivative confirmed this and enabled localization of the double bond to the Δ11 position from the abundant fragment ions at *m/z* 145 and *m/z* 245, originating by cleavage at the original site of unsaturation (8).

The mass spectrum of the methyl ester of the component with an ECL of 18.08 displayed a molecular ion at *m/z* 310, which would be consistent with a C_{19} acid with a single double bond or a cyclopropane ring. The spectrum was not compatible with a cyclopropane acid, but simple unsaturation was also excluded because the compound failed to form a *bis*(methylthio) derivative. To establish its structure we synthesized its DMOX derivative, since the charge stabilization conferred by the heterocyclic ring of these derivatives largely suppresses double bond migration, resulting in informative mass spectra from which the locations of features such as olefinic bonds are readily deduced (48). A molecular ion was observed at *m/z* 349, and the spectrum was characterized by a regularly spaced even mass ion series (*m/z* 126 +14n) resulting from cleavage without rearrangement at every carbon atom of the alkyl chain (Fig. 4). This regular spacing of 14 was interrupted between *m/z* 264 and 224, suggesting that the compound was 11-methyl-11,12-octadecenoic acid.

16S rRNA gene sequence comparison. Partial 16S rRNA gene sequences comprising 1,409 and 525 nucleotides were determined for strains FRR-10^T and FRR-11, respectively. The 16S rRNA gene sequences of strains FRR-10^T and FRR-11 were identical over the compared region, and so only strain FRR-10^T was included in the phylogenetic analyses. Comparing the 16S rRNA gene sequence of strain FRR-10^T with representative sequences of the main lineages within the *Proteobacteria* indicated that this strain fell within the α-3 subclass and was most closely related to species of the genus *Rhodovulum*. The highest 16S rRNA gene sequence similarity to the species of the genus *Rhodovulum* fell in the range of 92.3 to 96.0%. The phylogenetic tree, shown in Fig. 5, was reconstructed using the neighbor-joining method on the basis of a comparison of 1,023 nucleotide positions between positions 39 and 1380 (*E. coli* numbering) and indicates that strain FRR-10^T falls within the radiation of the species assigned to the genus *Rhodovulum*. The integrity of the *Rhodovulum* cluster, of which strain FRR-10^T is a member, was supported in 950 trees out of 1,000 generated in the bootstrap analysis. Strain FRR-10^T is shown in Fig. 5 to branch with *Rhodovulum euryhalinum*; however, this branching point was only recovered in 64% of the

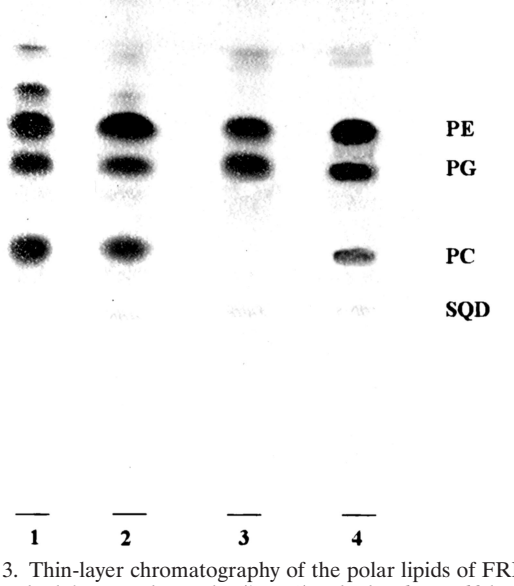

FIG. 3. Thin-layer chromatography of the polar lipids of FRR-10^T (lane 1), *Rhodobacter sphaeroides* (lane 2), *Rhodovulum sulfidophilum* (lane 3), and *Rhodovulum euryhalinum* (lane 4). PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PC, phosphatidylcholine; SQD sulfoquinovosyldiglyceride.

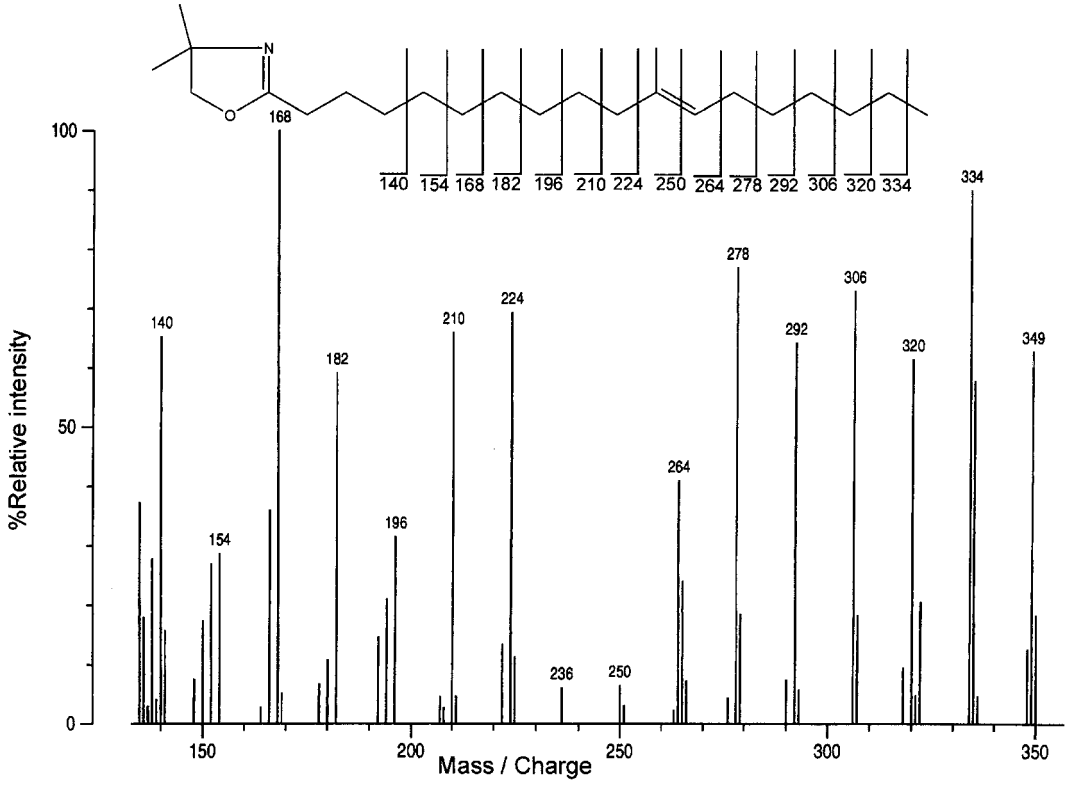

FIG. 4. Electron ionization mass spectrum of the DMOX derivative of the fatty acid with an ECL of 18.08. The interruption of the series with regularly spaced alkyl chain cleavage ions between *m/z* 264 and 224, together with the reduced abundance of the ion at *m/z* 236, identifies the compound as 11-methyl-11,12-octadecenoic acid.

trees generated, which is not statistically significant and indicates that both strain FRR-10^T and *Rhodovulum euryhalinum* can branch with other species of the genus *Rhodovulum*.

DISCUSSION

Phylogenetic analysis based on 16S rRNA gene sequence comparisons shows that strains FRR-10^T and FRR-11, isolated from a marine hot spring venting saline water, represent a new species of the α-3 subclass of the *Proteobacteria*, branching within the radiation of the species of the genus *Rhodovulum*, as currently defined. Moreover, the FRR strains are currently the most thermophilic organisms within the α-3 subclass of the *Proteobacteria*. It is noteworthy that the organism was isolated in medium containing 3% NaCl but grew well in Degryse 162 medium that contains only 6 mM Na^+, although the growth rate was enhanced with 1% (171 mM) NaCl. This species appears to be a facultative halophilic organism rather than a slightly halophilic marine organism strictly dependent on NaCl

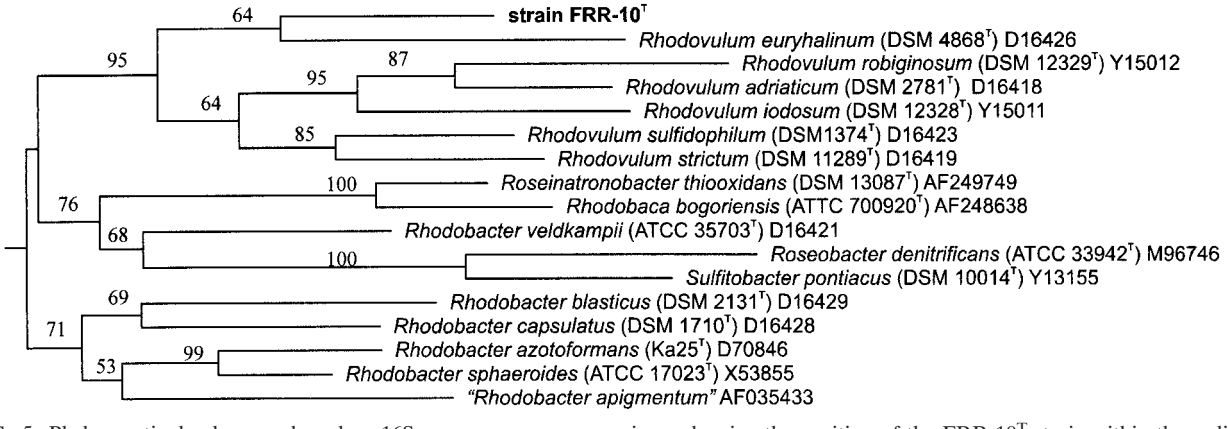

FIG. 5. Phylogenetic dendrogram based on 16S gene sequence comparisons showing the position of the FRR-10^T strain within the radiation of the species of the genus *Rhodovulum*.

for growth. Other thermophilic facultatively halophilic bacteria have been encountered before; *Thermus thermophilus* GY1211 has a slightly higher growth rate in media containing about 1% NaCl than in medium without additional salt, while all other strains of this species have higher growth rates in medium with no added NaCl (28). Many shallow marine hot springs have lower concentrations of Na^+ than seawater that sometimes approach the levels in freshwater. The ability to grow at very low levels of Na^+, as well as at levels found in seawater, may benefit colonization of many marine hot springs with very different concentrations of salt.

Basic chemical characteristics, such as the presence of phosphatidylethanolamine, phosphatidylglycerol, and phosphatidylcholine and the presence of ubiquinone 10 and straight-chain monounsaturated fatty acids, support the phylogenetic attribution of these organisms to the α-3 subclass of the *Proteobacteria*, but these characteristics are not useful in distinguishing the genera within this group (16). The rare fatty acid 11-Me 18:1[11], which is found in the FRR strains, had not been encountered before in the species of the genera *Rhodobacter* and *Rhodovulum*. This fatty acid has, however, been identified in species of *Brevundimonas*, *Caulobacter*, and *Maricaulus* (1) and may correspond to the fatty acid identified as 10-Me 18:1 in *Sulfitobacter* (36).

The Ferraria strains do not possess carotenoids or synthesize Bchl *a*. We initially thought that the FRR strains did not express Bchl *a* under the experimental conditions examined, but later concluded, on the basis of molecular data, that the FRR strains lacked *puf* genes. These genes encode the photosynthetic reaction center proteins and the core light-harvesting complexes and are highly conserved in Bchl *a*-synthesizing *Proteobacteria*, including the *Rhodovulum* and *Rhodobacter* spp. examined (29). The absence of these genes alone would preclude photosynthesis and verifies the lack of phototrophic growth of the FRR strains. The loss of photosynthesis and of photosynthetic pigments could represent an adaptation to dark geothermal environments, such as the hot spring at Ferraria. Nonpigmented strains of species of the genus *Thermus*, for example, predominate in dark man-made thermal environments, in contrast to the yellow-pigmented strains found in illuminated hot springs environments (5). Carotenoid-overproducing mutants of *T. thermophilus* are more resistant to UV irradiation than wild-type yellow-pigmented strains but grow slower at high growth temperature than the wild-type strains (14, 46). The loss of pigmentation and photosynthesis could, therefore, be a selective advantage in colonizing dark hot springs or aquifers.

The new species represented by the FRR strains does not possess hallmark characteristics of the species of the genus *Rhodovulum* to which it is phylogenetically most closely related. These isolates do not produce carotenoids, do not synthesize Bchl *a*, and are obviously not phototrophic. Like known *Rhodovulum* spp., the FRR strains were isolated from a marine environment, which may or may not be taxonomically significant, because most genera of the α-3 subclass originate from saline or hypersaline environments. The species represented by the isolates from Ferraria could, therefore, be considered to belong to a novel genus, because basic or reliable characteristics of *Rhodovulum* are not encountered in these organisms.

However, the phylogenetic analysis based on 16S rRNA gene sequence comparisons indicates that the new species falls within the radiation of the genus *Rhodovulum*. Moreover, the phylogenetic analysis shows that no explicit *Rhodovulum* species and the FRR strains form a stable branching point as indicated by the low bootstrap values (Fig. 5), although strain FRR-10^T is clearly a member of the *Rhodovulum* lineage. If we take the view that the phylogenetic relationships of the organisms should constitute a major consideration for the classification of bacteria, then we could envision that several characteristics which are present in the known species of the genus *Rhodovulum* were lost during the evolution of the FRR strains or, improbably, that these were never acquired by lateral gene transfer (15). These strains could, therefore, be considered to represent a nonphotosynthetic slightly thermophilic species of the genus *Rhodovulum*.

The difficulty of generic placement of the species represented by the FRR strains stems from the fact that these organisms do not exhibit several characteristics that define the genus *Rhodovulum*, but they are closely related to this genus from the phylogenetic analysis. Based on the higher growth temperature, growth at very low NaCl, and the absence of several hallmark characteristics of the genus *Rhodovulum*, we are of the opinion that the species represented by strains FRR-10^T and FRR-11 belongs to a novel genus and species which we propose to name *Albidovulum inexpectatum*.

Description of *Albidovulum* gen. nov. *Albidovulum* (Al.bid.o′vu.lum, L. adj. *albidus*, whitish; L neut. n. *ovulum*, small egg, N.L. neut. n. *Albidovulum*, small whitish egg). *Albidovulum* forms rod-shaped cells that stain gram negative. Endospores are not formed. Slightly thermophilic. Strictly aerobic, oxidase and catalase positive. Facultatively halophilic or slightly halophilic. Fatty acids are straight chained; major phospholipids are phosphatidylethanolamine and phosphatidylglycerol, ubiquinone 10 is the major respiratory quinone. Hydrogen and reduced sulfur compounds are not used as energy sources. Carbon dioxide is not fixed. Chemoorganotrophic; a large number of organic compounds are used for growth.

Description of *Albidovulum inexpectatum* sp. nov. *A. inexpectatum* (in. ex. pec. ta′ tum, n. L. adj. *inexpectatum*, unexpected, because the organism has characteristics that are unexpected). *A. inexpectatum* forms short rod-shaped cells 1.4 to 2.2 μm in length by 0.4 to 0.6 μm in width. The organism stains gram negative and is nonmotile. Colonies are nonpigmented. The optimum growth temperature is about 50°C, growth does not occur at 60°C; the optimum pH is between 6.5 and 8.0. Cytochrome oxidase and catalase positive. The organism is facultatively halophilic. Strictly aerobic; growth does not occur under anaerobic conditions in the dark or in the light. Bchl *a* was not detected. The predominant fatty acids are 18:1[11], 18:0; 11-Me 18:1[11] is also present. Chemoorganotrophic. Growth factors are not necessary for growth. A large number of sugars, polyols, amino acids, and organic acids serve as single carbon sources.

The mole G+C ratio of the DNA is 63.6%. This bacterium was isolated from the marine hot spring at Ferraria on the island of São Miguel. The type strain, FRR-10, has been deposited in the DSMZ as strain DSM 12048 and in the ATCC as strain ATCC BAA-387. Strain FRR-11 has also been deposited in the DSMZ (DSM 12049) and the ATCC (ATCC BAA-388) as a reference strain.

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ERRATA

Albidovulum inexpectatum gen. nov., sp. nov., a Nonphotosynthetic and Slightly Thermophilic Bacterium from a Marine Hot Spring That Is Very Closely Related to Members of the Photosynthetic Genus *Rhodovulum*

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Volume 68, no. 9, p. 4266–4273, 2002. Page 4272, column 2, lines 34 and 35: "Hydrogen and reduced sulfur compounds are not used as energy sources" should read "Hydrogen and reduced sulfur compounds are not used as energy sources under autotrophic conditions."
Line 36: "Chemoorganotrophic" should read "Facultatively chemolithoorganotrophic on reduced sulfur compounds."
Line 50: "Chemoorganotrophic" should read "Facultatively chemolithoorganotrophic."

Fumarate-Mediated Inhibition of Erythrose Reductase, a Key Enzyme for Erythritol Production by *Torula corallina*

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Volume 68, no. 9, p. 4534–4538, 2002. Page 4534, Abstract, line 9: "noncompetitively" should read "uncompetitively."
Page 4537, column 1, line 4: "noncompetitive" should read "uncompetitive."
Page 4537, column 2, line 1: "noncompetitive" should read "uncompetitive."

Isolation and Characterization of Differentially Expressed Genes in the Mycelium and Fruit Body of *Tuber borchii*

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Volume 68, no. 9, p. 4574–4582, 2002. Page 4575, column 1, line 41: "pBK-CMV" should read "P-Bluescript (SK +/−)."
Column 2, line 13: "Hybond-N" should read "Hybond-N^+."
Line 16: "dehydribized" should read "dehybridized."