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ORIGINAL PAPER

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Characterization of three spiral-shaped purple nonsulfur bacteria isolated from coastal lagoon sediments, saline sulfur springs, and microbial mats: emended description of the genus *Roseospira* and description of *Roseospira marina* sp. nov., *Roseospira navarrensis* sp. nov., and *Roseospira thiosulfatophila* sp. nov.

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Abstract Three new spirilloid phototrophic purple nonsulfur bacteria were isolated in pure culture from three different environments: strain CE2105 from a brackish lagoon in the Arcachon Bay (Atlantic coast, France), strain SE3104 from a saline sulfur spring in the Pyrenees (Navarra, Spain), and strain AT2115 a microbial mat (Tetiaroa Atoll, Society Islands). Single cells of the three strains were spiral-shaped and highly motile. Their intracellular photosynthetic membranes were of the vesicular type. Bacteriochlorophyll *a* and carotenoids of the normal spirilloxanthin series were present as photosynthetic pigments. Optimal growth occurred under photoheterotrophic conditions and in the presence of 0.5–4% w/v NaCl. These features are similar to those described for *Roseospira mediosalina*. Comparative sequence analysis of their 16S rRNA genes placed these strains within the α-subclass of Proteobacteria, in a cluster together with *Roseospira mediosalina* and *Rhodospira trueperi*. They form a closely related group of slightly to moderately halophilic spiral-shaped purple nonsulfur bacteria. However, the three new isolates exhibited some differences in their physiology and genetic characteristics. Consequently, we propose that they are members of three new species within the genus *Roseospira*, *Roseospira marina* sp. nov., *Roseospira navarrensis* sp. nov., and *Roseospira thiosulfatophila* sp. nov., with strains CE2105, SE3104, and AT2115 as the type strains, respectively. As a consequence, an emended description of the genus *Roseospira* is also given.

Keywords Phototrophic purple nonsulfur bacteria · *Roseospira* · Coastal lagoon · Saline sulfur spring · Microbial mat · Moderate halophilic bacteria

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Introduction

Purple nonsulfur bacteria are commonly encountered in anoxic waters and sediments exposed to light. Many purple nonsulfur bacterial species are found in freshwater habitats rich in organic matter such as lake and river sediments (Kaiser 1966), waste water ponds (Siefert et al. 1978), wetlands (Burke et al. 1974) or rice fields (Okuda et al. 1957). Other species of the purple nonsulfur bacteria are typical halophilic bacteria (Imhoff and Trüper 1992).

Among the spiral-shaped purple nonsulfur bacteria some are considered as halophilic bacteria (Caumette et al. 1999; Imhoff 2001). They have been isolated from hypersaline habitats such as evaporitic seawater ponds (Drews 1981), salterns (Nissen and Dundas 1984; Pfennig et al. 1997; Glaeser and Overmann 1999), the Dead Sea (Mack et al. 1993) or hot saline springs (Kompantseva and Gorlenko 1985). These bacteria were described as new species and named *Rhodothalassium salexigens*, *Rhodovibrio salinarum*, *Rhodovibrio sodomense*, *Roseospira mediosalina* (Imhoff et al. 1998), *Rhodospira trueperi* (Pfennig et al. 1997), and *Roseospirillum parvum* (Glaeser and Overmann 1999). The freshwater species are now grouped into the genera *Rhodospirillum*, *Phaeospirillum*, and *Rhodocista* (Imhoff et al. 1998).

Spiral-shaped purple nonsulfur bacteria were isolated during investigations on eutrophic coastal lagoons (Guyoneaud et al. 1996), saline springs, and microbial mats (unpublished data). Strain CE2105 was isolated from the Certes Fishponds (Arcachon Bay, France), strain SE3104 from the Salinas de Oro (Navarra Pyrénées, Spain), and strain AT2115 from the Tetiaroa Atoll (French Polynesia); these three strains have been studied in detail. The present paper describes their phenotypic properties and their genetic relationships with halophilic species of the spiral-shaped purple nonsulfur bacteria. Based on comparative analyses of their 16S rRNA gene sequences, these three strains cluster together with the species *Roseospira mediosalina* and *Rhodospira trueperi*. However, due to differences in phenotypic characteristics and genetic data, the three strains can be readily separated from the existing species and from each other. Therefore, they are described as new representatives of the genus *Roseospira*, with the names *Roseospira marina* sp. nov. for strain CE2105, *Roseospira navarrensis* sp. nov. for strain SE3104, and *Roseospira thiosulfatophila* sp. nov. for strain AT2115. As a result of their characteristics, an emended description of the genus *Roseospira* was necessary.

Material and methods

Source of strains

Strain CE2105 was isolated during ecological studies on the brackish Certes Fishponds (Arcachon Bay, French Atlantic coast). These man-made brackish shallow lagoons are periodically flooded with seawater from the Arcachon Bay (Guyoneaud et al. 1996). Liquid enrichment cultures were prepared from the upper layer of the anoxic sediments.

Strain SE3104 was isolated from the surface of a sulfide-rich sediment in a small saline pond in the Spanish Pyrenees. This pond results from the outflow of a saline spring (Salinas de Oro, Navarra, Spain) with salinity varying from 2 to 10% (total salinity). This spring water is rich in chloride (46% w/v), sodium (28% w/v), sulfate (15% w/v), calcium (5% w/v), and potassium (4% w/v).

Strain AT2115 was isolated from microbial mats in French Polynesia (Tetiaroa Atoll, Society Islands). These mats consisted of stratified structures dominated by cyanobacteria at the surface and profuse anoxygenic phototrophs in the deeper layers (Mao Che et al. 2001).

Media, isolation, and culture conditions

During enrichment and isolation procedures for strain CE2105, cultures were obtained by using a basal medium containing: filtered (0.2 µm pore size) seawater, 750 ml; distilled water, 250 ml; NH_4Cl, 0.035% (w/v); yeast extract, 0.04% (w/v); and Fe-citrate, 0.001% (w/v). The medium was autoclaved and cooled under a gas mixture of N_2/CO_2 (90/10, v/v). Vitamin V7 solution (Pfennig and Trüper 1992; 1 ml·l^{-1}), phosphate buffer (0.1 M, pH 6.8, 36 ml·l^{-1}), and Na-ascorbate/cysteine-HCl (0.25% (w/v)/0.5% (w/v) solution at pH 7.0, 0.2 ml·l^{-1}) were then aseptically added to the medium. For enrichment and isolation of strain SE3104, the culture medium was prepared according to the method of Pfennig and Trüper (1992) and supplemented with 5% (w/v) NaCl and 1% (w/v) $MgCl_2 \cdot 6H_2O$. The culture medium used for enrichment and isolation of strain AT2115 contained: filtered (0.2-µm pore size) seawater, 1,000 ml; NH_4Cl, 0.05% (w/v); KH_2PO_4, 0.02% (w/v); yeast extract 0.05% (w/v). The medium was autoclaved and cooled under N_2/CO_2 (90/10, v/v). Vitamin V7 solution (1 ml·l^{-1}), $NaHCO_3$ (0.15% w/v), and $Na_2S \cdot 9H_2O$ (0.02% w/v) were then aseptically added to the medium.

The final pH for all media was adjusted to 6.8. The media were dispensed into sterile 50-ml screw-capped bottles. Organic substrates (5 mM sodium acetate and 5 mM di-sodium succinate) were added just before utilization.

Pure cultures were obtained by repeated application of the deep-agar dilution method (Pfennig 1978). Deep-agar tubes were incubated at 25 °C under a light/dark cycle (16 h light/8 h dark) using tungsten lamps. Purity of the strains was checked by both microscopic observations and growth tests in deep-agar AC medium (Difco) supplemented with NaCl (2% or 5% w/v), sodium thiosulfate (0.05% w/v), and glucose (0.05% w/v) and incubated in the dark under air or a N_2/CO_2 (90/10, v/v) atmosphere.

Table 1 Utilization of substrates as electron donors and/or carbon sources by strains CE2105, SE3401, and AT2115. Unless otherwise indicated, the concentration was 5 mM. Growth was checked by optical density measurements at 650 nm against a control without substrate: – no utilization (similar or less than control), (+) poor utilization (1–1.5 times more than control), + good utilization (more than 1.5 times more than control), *nd* not determined

Substrates	CE2105	SE3401	AT2115
Sulfide (1 mM)	–	+	+
Sulfur (0.05%)	–	–	–
Thiosulfate	–	–	+
Sulfite	–	–	–
Formate	(+)	–	–
Acetate	+	+	+
Propionate	+	+	–
Butyrate	+	+	+
Valerate	+	+	+
Crotonate	+	+	+
Caprylate (2 mM)	–	–	–
Pelargonate (2 mM)	–	–	–
Lactate	+	+	+
Glycolate	–	–	–
Benzoate (2 mM)	–	+	–
Tartrate (2 mM)	–	–	–
Pyruvate	+	+	+
Malate	+	+	–
Fumarate	+	+	–
Succinate	+	+	–
Citrate	–	+	–
2-oxoglutarate	+	+	+
Glucose	–	–	+
Fructose	+	–	–
Gluconate	+	+	+
Glycerol	+	+	+
Mannitol	+	+	–
Cysteine (2 mM)	(+)	nd	nd
Methionine (2 mM)	–	–	nd
Aspartate	+	+	+
Glutamate	+	+	+
Peptone (0.05%)	+	+	+
Casamino acids (0.05%)	+	+	+
Yeast extract (0.05%)	+	+	+

The following substrates (concentration in mM) were tested but not utilized by strains CE2105 and SE3401: tetrathionate (2), thioglycolate (2), thioacetamide (2), glycine-betaine (2), palmitate (2), cyclohexane-carboxylate (2), nicotinate (2), ascorbate (2), gallate (2), trehalose (2), sucrose (2), methanol (5), ethanol (5), propanol (5), butanol (5), catechol (2)

The composition of final synthetic media for maintenance and characterization of the pure cultures contained (per liter of distilled water): KH_2PO_4, 0.03% (w/v); NH_4Cl, 0.05% (w/v); $CaCl_2{\cdot}2H_2O$, 0.005% (w/v); $MgCl_2{\cdot}6H_20$, 0.1% (w/v) (0.3% w/v for strain SE3104); $MgSO_4{\cdot}7H_2O$, 0.05% (w/v) (0.2% w/v for strain SE3104); NaCl, 2% (w/v) (5% w/v for strain SE3104); trace element solution SL12 (Overmann et al. 1992), 1 ml; yeast extract, 0.05% (w/v). Media were autoclaved and cooled under N_2/CO_2 (90/10, v/v). Vitamin V7 solution (1 $ml{\cdot}l^{-1}$), Na-ascorbate (0.05% w/v), and $NaHCO_3$, (0.15% w/v) were then aseptically added to the medium. The final pH was adjusted to 6.8–7.0 and the medium was dispensed into sterile 50-ml screw-capped bottles. Organic substrates (5 mM Na-acetate and/or 5 mM di-Na-succinate) were added as substrates before use. In addition, for strains SE3104 and AT2115, $Na_2S{\cdot}9H_2O$ (0.02% w/v) was also added to the medium prior to utilization. Pure cultures were grown in 50-ml screw-capped bottles and stored at +4 °C in the dark for preservation.

Microscopy

Microscopy observations and photomicrographs were made with an Olympus OM 2 photomicroscope according to the method of Pfennig and Wagener (1986). Flagella were observed by transmission electron microscopy after negative staining with 1% phosphotungstic acid neutralised to pH 7.2. The fine structure of the cells was studied by electron microscopy after fixation of a cell pellet by the method of Ryter and Kellenberger (1958) and ultrathin sectioning of the cells according to Glazer et al. (1971). The observations were made with a JEOL 1200 ES electron microscope.

Pigments

In vivo absorption spectra were analyzed with a Perkin Elmer Lambda 12 spectrophotometer after suspension of a cell pellet in a sucrose solution (Pfennig and Trüper 1992). Absorption spectra after ethanol or acetone extraction were recorded with the same spectrophotometer. The precise carotenoid composition was determined by HPLC analysis according to the method of Buffan-Dubau et al. (1996). For identification of carotenoids, strains with known carotenoid composition were used as reference.

Physiological tests

Optimal NaCl concentrations, pH, temperature, light intensity, and sulfide tolerance were determined with the final synthetic media. Utilization of carbon sources and electron donors was tested with the final synthetic media without substrates and supplemented with only 0.01% yeast extract. Substrates were added aseptically from stock solutions to the final concentrations indicated in Table 1. The experiments were carried out in triplicate in completely filled 25-ml screw-capped tubes. Growth was measured by optical density at 650 nm over a period of 10–15 days.

Capacity for aerobic or micro-aerobic growth and anaerobic growth in the dark was tested according to the method of Kämpf and Pfennig (1980). Vitamin requirements were tested after four consecutive transfers, in the final synthetic media without yeast extract, according to an experimental protocol of Goupy (1988). Assimilatory sulfate-reduction was tested through five consecutive transfers in 60-ml screw-capped bottles filled with final synthetic medium with sulfate as the only sulfur source. Hydrogen utilization was tested in aluminum-cap-sealed bottles with gas-impermeable butyl stoppers. Bottles were filled one third with liquid medium without electron donor and two-thirds with H_2/CO_2 (80/20, v/v, 100 kPa). Capacity for dinitrogen fixation was checked in rubber-stopper bottles half-filled with synthetic media lacking fixed nitrogen sources under a N_2/CO_2 (90/10, v/v) gas atmosphere; four consecutive transfers were tested. Catalase was checked by adding a few drops of 3% (v/v) H_2O_2 to a cell pellet obtained by centrifugation.

Determination of the genomic DNA G+C content, comparative 16S rDNA analysis, and DNA-DNA hybridization

The G+C content of the DNA of the three strains was determined by HPLC according to the methods of Mesbah et al. (1989) and Tamaoka and Komagata (1984).

Genomic DNA was isolated and the almost complete 16S rDNA was amplified via PCR as described by Liesack and Finster (1994), Mouné et al. (2000), and Guyoneaud et al. (1998) for strains CE2105, SE3104, and AT2115, respectively. The 16S rDNA/rRNA sequences of 13 related species, including representatives of the purple nonsulfur bacteria and the non-photosynthetic species *Aquaspirillum itersonii*, obtained from the EMBL database were used in the phylogenetic analysis (Table 2). The 16S rDNA sequences were aligned with the CLUSTAL W program (Thompson et al. 1994) from position 71 to 1,372 according to the *Escherichia coli* numbering scheme, including gaps. Distance matrices were calculated using the DNADIST program with the algorithm of Jukes and Cantor (1969) within the PHYLIP package (Felsenstein 1993). The phylogenetic tree was inferred from evolutionary distances with the FITCH program of the PHYLIP package (Fitch and Margoliash 1967). The *Allochromatium vinosum* 16S rDNA sequence was in-

Table 2 Strain designations and accession numbers of 16S rDNA sequences for all organisms used in the phylogenetic analysis

Organism	Strain designation	Accession number
Roseospira marina CE2105	ATCC BAA-447	AJ298879
Roseospira navarrensis SE3104	ATCC BAA-448	AJ298880
Roseospira thiosulphatophila AT2115	ATCC BAA-449	AJ401208
Roseospira mediosalina	BN280^T	AJ000989
Rhodospira trueperi	ATCC 700224^T	X99671
Rhodospirillum photometricum	E 11	D30777
Rhodospirillum rubrum	ATCC 11170^T	D30778
Roseospirillum parvum	DSM 12498^T	AJ011919
Phaeospirillum fulvum	DSM 113^T	D14433
Phaeospirillum molischianum	ATCC 14031^T	M59067
Rhodocista centenaria	ATCC 43720^T	D12701
Rhodothalassium salexigens	ATCC 35888^T	D14431
Rhodovibrio salinarum	ATCC 35394^T	M59069
Aquaspirillum intersonii	NCIMB 9070	Z29620
Rhodovibrio sodomensis	DS1	M59072
Allochromatium vinosum	DSM 180^T	M26629

cluded to root the tree. The confidence level of the phylogenetic tree topology was evaluated by performing 100 bootstrap replications with the programs SEQBOOK and CONSENSE of the same package. The three sequences obtained in the present work were deposited with EMBL (Table 2).

For DNA-DNA hybridization studies, DNA was isolated as described by Cashion et al. (1977). DNA-DNA hybridization was carried out as described by De Ley et al. (1970), with the modifications of Huss et al. (1983) and Escara and Hutton (1980), using a Gilford System model 2600 spectrometer equipped with a Gilford model 2527-R thermoprogrammer and plotter. Renaturation rates were computed with the TRANSFER.BAS program by Jahnke (1992).

Analysis of compatible solutes by ^{13}C-NMR spectroscopy

The strains were grown in 8-l batch cultures in basal medium supplemented with 100 mM Na-acetate and 6% (w/v) NaCl. Cultures were incubated at room temperature and continuously sparged with oxygen-free nitrogen to maintain anoxic conditions. Mid-exponential phase (OD_{650}=0.7) cultures were harvested, extracted and analyzed by ^{13}C-NMR spectroscopy according to the methods of Welsh and Herbert (1994).

Results

Natural habitat and isolation

Strain CE2105

The black silty sediments in the man-made fishponds of Certes are rich in organic matter and sulfide, mainly FeS and FeS_2. During ecological investigations in 1992, enrichment cultures obtained from the top layers of the sediments and incubated in the light gave rise to the growth of spiral-shaped purple nonsulfur bacteria (Guyoneaud et al. 1996). These enrichments served as an inoculum for agar-dilution series. Strain CE2105 was isolated in pure culture and maintained for further characterization.

Strain SE3104

In 1994, a sample of black sediment was collected on the shore of a saline pond (Salinas de Oro) and enriched in completely filled 120-ml screw-capped bottles containing synthetic medium. After 1–2 weeks, a red bloom of phototrophic bacteria appeared in the bottles. Microscopy observations revealed the presence of vibrioid and highly motile cells often forming half circles. Several strains have been isolated from these enrichments. Strain SE3104, which grew the best, was further characterized.

Strain AT2115

Samples from a Polynesian microbial mat were collected in 1997 and directly inoculated in agar-dilution series. Some of the red colonies obtained after 15 days of incubation consisted of spiral-shaped phototrophic bacteria. Several strains were isolated from different agar series

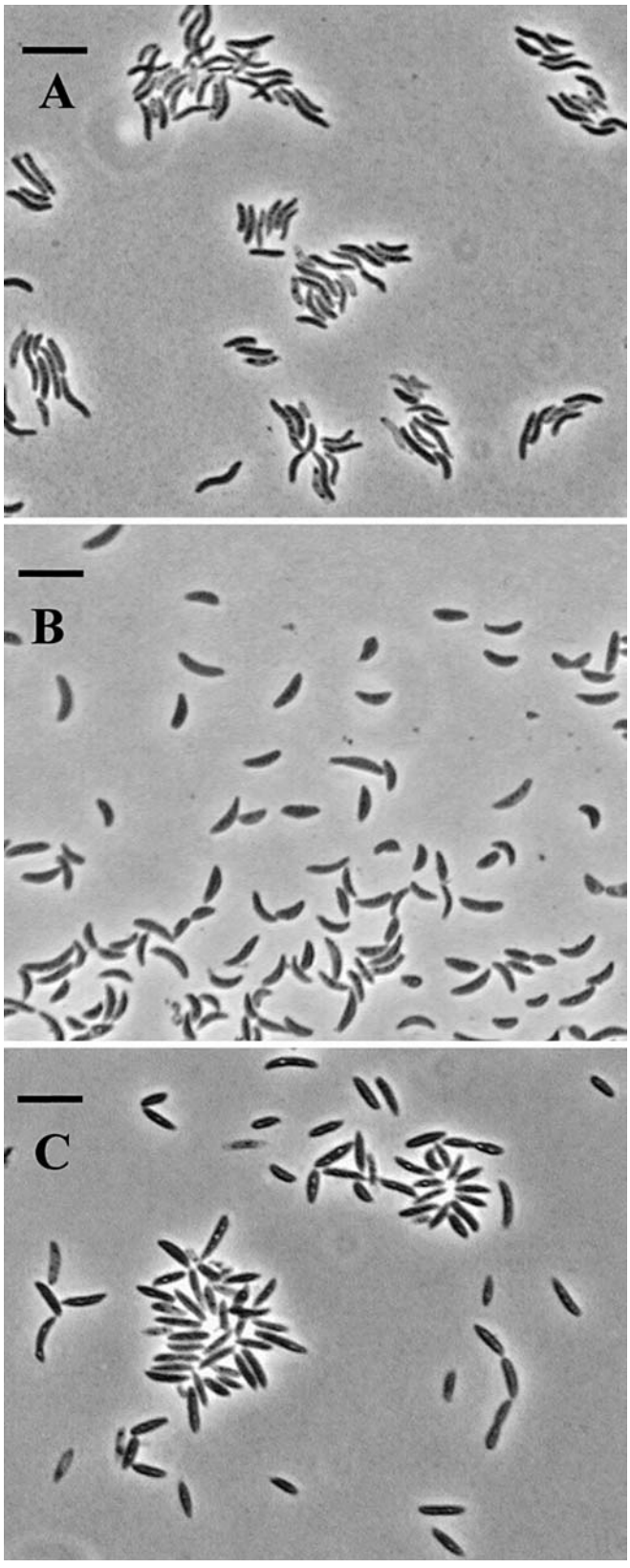

Fig. 1 Phase-contrast photomicrographs of *Roseospira marina* strain CE2105 (**A**), *Roseospira navarrensis* strain SE3104 (**B**), and *Roseospira thiosulfatophila* strain AT2115 (**C**). *Bar* 4 μm

and isolated in pure culture. Strain AT2115 was further characterized.

Morphology and fine structure

Individual cells of strain CE2105 were spiral-shaped, 0.4–0.8 µm wide (Fig. 1A). The length of the cells varied from 1.5–2.5 µm (curved cells) to 4.0–6.0 µm (one complete turn of a spiral). Longer cells (10–20 µm) rarely occurred. In liquid medium, the strain mostly developed as single cells but occasionally occurred in aggregates depending on the substrate. The cells of strain SE3104 were vibrioid rods, 0.6–0.9 µm wide and 3.5–6.5 µm long, often by pair, forming half to complete circles (Fig. 1B). Cells rarely occurred in rosettes and inclusions were often visible in the cells (Fig. 1B). Individual cells of strain AT2115 were spiral-shaped, 0.5–0.8 µm wide and 2.5–6.5 µm long (Fig. 1C).

The three strains divided by binary fission. Cells were highly motile by means of bipolar tufts of flagella in strains CE2105 and SE3104 or polar tuft of flagella in strain AT2115. In thin sections, a vesicular intracellular membrane system was visible. All three strains stained Gram-negative and a cell wall typical of Gram-negative bacteria was visible on ultrathin section.

Photosynthetic pigment composition

Cell suspensions of strains CE2105 and AT2115 were red while suspensions of strain SE3104 were brown-red. The absorption spectra of living cells (Fig. 2) showed similar characteristic absorption peaks of bacteriochlorophyll *a* (Bchl *a*) with maxima at 370/376, 589/591, 799/805 and 863/864 nm for strains CE2105 and SE3104, respectively. Strain AT2115, by contrast, exhibited a rather unusual in vivo absorption spectrum (Fig. 2C), with, in addition to the characteristic Bchl *a* peaks at 370, 590, 800, and 883 nm, a large shoulder in the infra-red at 909/910 nm. The acetone extracts spectrum revealed that normal Bchl *a* with maximum absorption at 770 nm was present (not shown).

Absorption peaks of carotenoids ranged from 470 to 540 nm with a maximum at about 500 nm for all strains, indicating the presence of carotenoids of the normal spirilloxanthin series. HPLC analyses confirmed the biosynthetic pathway with rhodovibrin or rhodopine as major carotenoids depending on the strains (Table 3).

Physiological properties

Optimal growth for all strains occurred photoheterotrophically under anoxic conditions in the light with various organic substrates (Table 1). Strain CE2105 was not capable of photolithotrophy. In contrast, sulfide could serve as electron donor for strains SE3104 and AT2115, the latter being also able to use thiosulfate. These two strains needed a reduced sulfur source whereas strain CE2105 was able

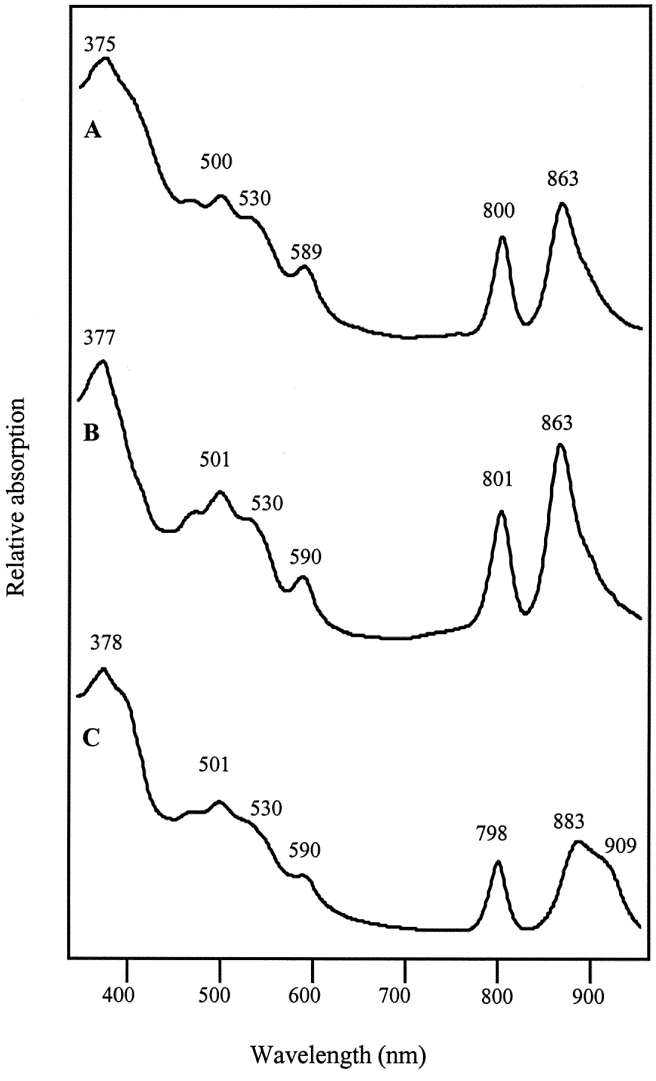

Fig. 2 In vivo absorption spectra of *Roseospira marina* strain CE2105 (**A**), *Roseospira navarrensis* strain SE3104 (**B**), and *Roseospira thiosulfatophila* strain AT2115 (**C**)

to use sulfate as sole sulfur source. The strains tolerated free sulfide concentrations up to 3 mM for strain CE2105, 2 mM for strain SE3104, and 3.5 mM for strain AT2115.

Chemoorganotrophic growth in the dark with acetate was possible under oxic conditions in strain CE2105 and micro-oxic conditions in strains SE3104 and AT2115. Catalase was present in the three strains and strongly positive in strain CE2105 when grown under oxic conditions. No respiratory or fermentative metabolism was observed under anoxic conditions in the dark.

Strain CE2105 required niacin, thiamine, and *p*-aminobenzoic acid as growth factors but addition of yeast extract (0.01% w/v) strongly increased growth. Strains SE3104 and AT2115 required yeast extract as a growth factor. Although not confirmed by acetylene reduction, the strains grew phototrophically with N_2 as sole nitrogen source after four consecutive transfers, indicating their ability to fix dinitrogen.

Table 3 Main characteristics of *Roseospira marina* strain CE2105, *Roseospira navarrensis* strain SE3104, *Roseospira thiosulfatophila* strain AT2115, and *Roseospira mediosalina*. Data for *R. mediosalina* are from Kompantseva and Gorlenko (1985). (a) μ Growth under micro-oxic conditions, + growth under oxic conditions

	Strain CE2105 *R. marina*	Strain AT2115 *R. thiosulfatophila*	Strain SE3401 *R. navarrensis*	*Roseospira mediosalina*
Cell size (μm)	0.4–0.8×1.5–6.0	0.5–0.8×2.5–6.5	0.6–0.9×3.5–6.5	0.8–1.0×2.2–6.0
Color of cell suspensions	Red	Red	Brown-red	Brown-red
Main carotenoids	Rhodovibrine, rhodopine	Rhodovibrine, spirilloxanthin	Rhodopine, lycopene	Rhodopine, lycopene
Intracytoplasmic membrane system	Vesicular	Vesicular	Vesicular	Vesicular
DNA G+C content (mol%)	68.8–69.4	71.9–72.3	66.8	66.6
Growth factors	Niacin, thiamine, p-aminobenzoate	Yeast extract	Yeast extract	Niacin, thiamine, p-aminobenzoate
Optimal NaCl concentration (range)	2–4% (0.5–10)	0.5% (0.2–5)	3–4% (1–10)	4–7% (0.5–15)
Assimilatory SO_4^{2-} reduction	+	–	–	+
Dark aerobic growth[(a)]	+	μ	μ	μ
Photolithoautotrophic growth (electron donor)	–	+ (H_2S, S_2O_3)	+ (H_2S)	+ (H_2S)
Photoorganotrophic growth on:				
Fumarate, malate, succinate	+	–	+	+
Citrate	–	–	+	–
Propionate	+	–	+	+
Benzoate	–	–	+	–
Glucose	–	+	–	–
Fructose	+	–	–	–
Mannitol	+	–	+	–

The three strains have similar ecophysiological requirements. They grow, for at least three consecutive transfers at the same salinities, at a NaCl concentration between 0.5 and 10% (optimum 2–4%) for strain CE2105, between 1 and 10% (optimum 3–4%) for strain SE3104, and between 0.2 and 5% (optimum 0.5%) for strain AT2115. The three strains exhibited the same NMR spectrum with respect to the compatible solutes accumulated, with ectoine and trehalose as the principal components. Their optimum pH was 6.7–6.8 (pH range, 5.3–8.4) for strain CE2105, 6.8–7.0 (pH range, 6.0–8.5) for strain SE3104, and 6.8–7.0 (pH range, 5.6–8.6) for strain AT2115. The strains were mesophilic with optimum temperature of about 30–35 °C. When grown under optimal conditions, the growth rates of the three strains ranged from 0.06 h^{-1} to 0.08 h^{-1}.

G+C content of genomic DNA, phylogenetic analysis and DNA-DNA hybridization

The DNA base compositions were 69.1±0.3 mol% G+C for strain CE2105, 66.8 mol% G+C for strain SE3104, and 72.1±0.2 mol% G+C for strain AT2115.

Comparative 16S rDNA sequence analysis placed strains CE2105, SE3104, and AT2115 within the α-subclass of the Proteobacteria, which comprises most of the spiral-shaped purple nonsulfur bacteria. Within this subgroup, the three strains formed a separate line of descent together with *Roseospira mediosalina* and *Rhodospira trueperi* (Fig. 3). The 16S rDNA sequences of strains CE2105, SE3104, and AT2115 differed by 4.4%, 4.6%, and 5.3%, respectively, from that of *Roseospira mediosalina*. The strains were more distantly related to *Rhodospira trueperi*, with differences in the 16S rDNA sequences of 6.0%, 6.2%, and 6.4%, respectively. Between the three strains, the percent differences in the 16S rDNA ranged from 2.4 to 3.5%, values around the limits for reliable differentiation between species (Stackebrandt and Goebel 1994).

DNA-DNA hybridization values were 28% between strains CE2105 and SE3104, 40% between strains CE2105 and AT2115, and 54% between strains AT2115 and SE3104.

Discussion

Strains CE2105, SE3104, and AT2115 are typical members of the purple nonsulfur bacteria (Imhoff and Trüper 1989). According to phylogenetic relatedness, they belong to the α-subclass of the Proteobacteria within the spirilloid purple nonsulfur bacteria. These bacteria, primarily grouped in the genus *Rhodospirillum*, were reclassified

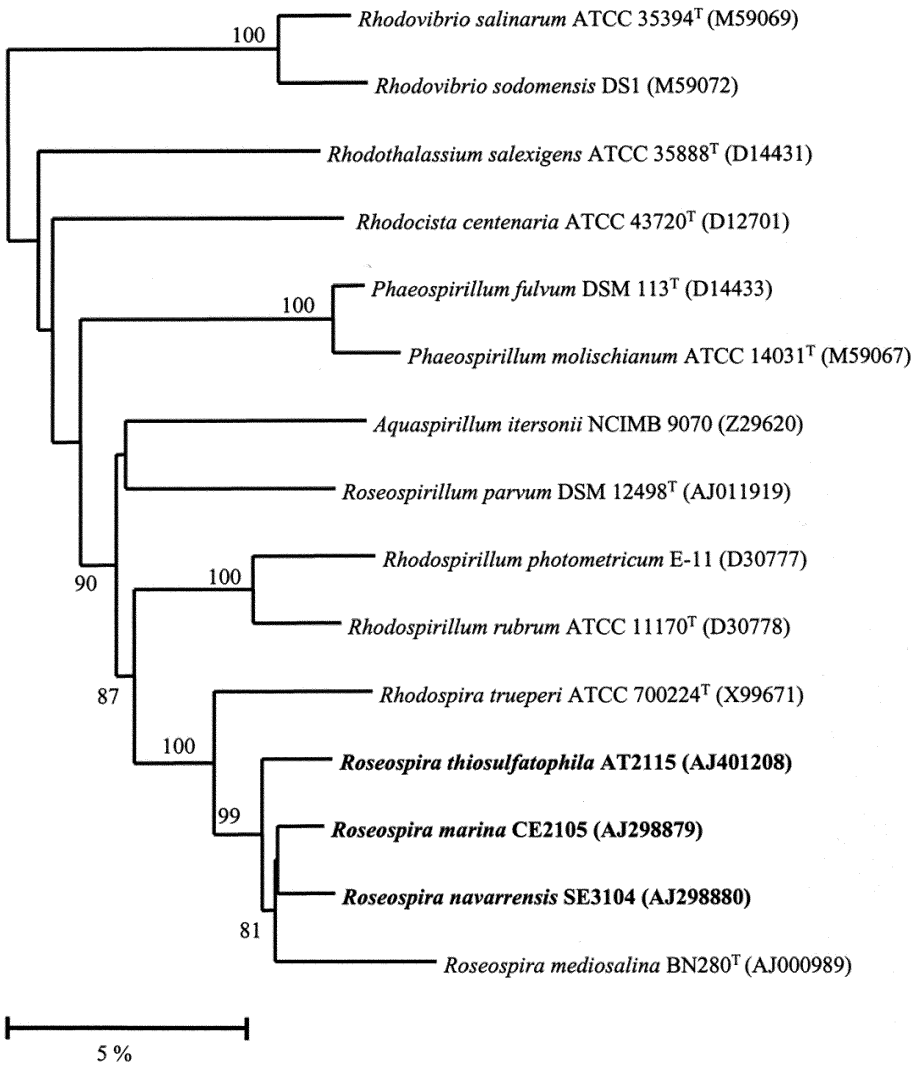

Fig. 3 Dendrogram showing the relationships between 16S rDNA sequences of *Roseospira marina* strain CE2105, *Roseospira navarrensis* strain SE3104, *Roseospira thiosulfatophila* strain AT2115, and other phototrophic purple non sulfur bacteria (strain numbers and EMBL accession numbers are indicated). The 16S rDNA sequence of *Allochromatium vinosum* (DSM 180^T) was included in the sequence analysis to root the tree (not shown). *Bar* indicates 5% difference in nucleotide sequence. The *numbers* in the dendrogram indicate the significance (percent of outcomes) of the branches (bootstrap analysis, see methods)

into several genera on the basis of comparative 16S rDNA sequence analyses (Imhoff et al. 1998). The genera can be differentiated according to their salt requirements (Imhoff 2001): *Rhodospirillum*, *Phaeospirillum*, and *Rhodocista* comprise the freshwater to halotolerant bacteria. *Roseospira*, *Rhodospira*, and *Roseospirillum* contain the slightly halophilic bacteria. *Rhodovibrio* and *Rhodothalassium* comprise moderate halophiles isolated from hypersaline environments. Strains CE2105, SE3104, and AT2115 do not exhibit high NaCl requirements and must be considered as slightly halophilic bacteria. The absence of close phylogenetic relationships between these isolates and freshwater or moderate halophilic species confirms that salt requirement is a strong taxonomic feature at the genus level for the spiral-shaped purple nonsulfur bacteria (Imhoff et al. 1998). The dendrogram constructed (Fig. 3) from the 16S rDNA comparisons revealed that the new isolates are closely related to the slightly halophilic species *Roseospira mediosalina*, and to a lesser extent *Rhodospira trueperi*. In contrast, they were more distantly related with *Roseospirillum parvum*.

Rhodospira trueperi is a spiral-shaped marine purple nonsulfur bacterium containing bacteriochlorophyll *b* and tetrahydrospirilloxanthin as main pigments (Pfennig et al. 1997). This difference in pigment composition with our three isolates, which contain Bchl *a* and carotenoids of the normal spirilloxanthin series, confirms, in addition to the phylogenetic relatedness, that the three new strains cannot be included in the genus *Rhodospira*.

Phylogenetic analysis (Fig. 3) suggested a common ancestor for strains CE2105, SE3104, and AT2115 together with *Roseospira mediosalina*. Our isolates, which have similar salt requirements (Table 3), are phylogenetically closely related, with dissimilarity values ranging from 2.4% to 3.5% (Fig. 3). In contrast, *Roseospira mediosalina* exhibits a higher optimal salinity (Table 3) and is 4.4–5.3% distant from the new isolates. Because of strong similarities between our three isolates and *Roseospira mediosalina*, we must consider them as representatives of the genus *Roseospira*. However, on the basis of phylogenetic properties, these three isolates are distant enough from the type species *Roseospira mediosalina* to be con-

sidered as different species. In addition, the results of the DNA-DNA hybridization studies between our three strains, showing very low percentages of homology, are consistent with the description of the three isolates as three new species.

The three isolates also showed differences in their morphology, pigment composition, G+C content of the DNA, and physiological capacities (Tables 1 and 3). Strain CE2105 is not capable of photolithotrophic growth but can grow chemoorganotrophically in the dark under oxic conditions. In contrast, strain SE3104 uses sulfide as electron donor for photolithotrophic growth but is unable to grow chemoorganotrophically under full oxic conditions. Strain AT2115 is able to use sulfide and thiosulfate as electron donors for photolithotrophic growth. In addition, strain AT2115 exhibits unusual Bchl *a* absorption in the infra-red with a large band from 883 nm to 909 nm. Such absorption in the infra-red was found in *Roseospirillum parvum* (Glaeser and Overmann 1999), which is distantly related with our isolate. Finally, differences in the assimilation of some organic substrates were found between the three strains and *Roseospira mediosalina* (Table 3). Therefore, according to these phenotypic and genetic differences, we propose to describe strain CE2105, strain SE3104, and strain AT2115 as members of three new species of the genus *Roseospira*, with the names *Roseospira marina* sp. nov., *Roseospira navarrensis* sp. nov., and *Roseospira thiosulfatophila* sp. nov., respectively. An emended description of the genus *Roseospira* is given below.

Roseospira gen. emend.

Full description as in Imhoff et al. (1998), with additional features

Cells are vibrioid to spiral-shaped, 0.4–1.0 µm wide, motile by means of polar or bipolar flagella. DNA G+C content between 65 and 72.3 mol%. Slightly halophilic bacteria that require NaCl or sea salt for growth. Salt concentration optima for growth between 0.5 and 7% (w/v) NaCl. Growth is possible under micro-oxic to oxic conditions in the dark.

Description of Roseospira marina *sp. nov.*

ma.ri'na L. fem. adj. *marina* marine

Cells are spiral to vibrioid-shaped, 0.4–0.8 µm wide, 1.5–6.0 µm long. Multiplication by binary fission. Gram-negative. Motile by bipolar tufts of flagella (2–5 fibrils). Intracytoplasmic membrane system is of vesicular type. Color of anaerobically grown cultures is red. Contains bacteriochlorophyll *a* and carotenoids of the normal spirilloxanthin series with rhodovibrin as major carotenoid.

Photoorganotrophic under anoxic conditions; substrates used are pyruvate, malate, succinate, fumarate, 2-oxoglutarate, formate, acetate, propionate, butyrate, valerate, crotonate, lactate, fructose, gluconate, mannitol, glycerol, aspartate, glutamate, cysteine. Peptone, yeast extract, and casamino acids also used as substrates. Not capable of photolithotrophic growth. Chemotrophic growth under oxic conditions. Not capable of anaerobic chemotrophic growth. Niacin, thiamine, and *p*-aminobenzoic acid required as growth factors. Optimal pH for growth 6.7–6.8, range 5.3–8.4. Optimal NaCl concentration for growth 2–4 %, range 0.5–10%. Optimal temperature for growth 30–35 °C.

DNA base composition of the type strain is 68.8–69.4 mol% G+C (HPLC).

Habitat: anoxic sediments and water from coastal and marine environments.

Type strain: Strain CE2105^T (ATCC number BAA-447) isolated from the anoxic sediments of the brackish lagoons of Certes, Arcachon Bay, France. 16S rDNA sequence deposited in EMBL under accession number AJ298879.

Description of Roseospira navarrensis *sp. nov.*

na.var'ren.sis M.L. fem. adj. *navarrensis* pertaining to Navarra, a Spanish region

Cells are vibrioid-rods, often in pairs, 0.6–0.9 µm wide, 3.5–6.5 µm long. Multiplication by binary fission. Gram-negative. Motile by bipolar tufts of flagella. Intracytoplasmic membrane system is of vesicular type. Color of anaerobically grown cultures is brown-red. Contains bacteriochlorophyll *a* and carotenoids of the normal spirilloxanthin series with rhodopine as major carotenoid.

Photoorganotrophic growth under anoxic conditions; substrates used are pyruvate, malate, succinate, fumarate, citrate, 2-oxoglutarate, acetate, propionate, butyrate, valerate, crotonate, benzoate, lactate, gluconate, mannitol, glycerol, aspartate, glutamate. Peptone, yeast extract, and casamino acids also used as substrates. Photoautotrophic growth with sulfide as electron donor. Chemoorganotrophic growth under micro-oxic conditions. Not capable of anaerobic chemotrophic growth. Not capable of assimilatory sulfate reduction. Yeast extract required as growth factor. Optimal pH for growth 6.8–7.0, range 6.0–8.5. Optimal NaCl concentration for growth 3–4%, range 1–10%. Optimal temperature for growth 30–35 °C.

DNA base composition of the type strain is 66.8 mol% G+C (HPLC).

Habitat: anoxic sediments exposed to light in inland saline springs.

Type strain: Strain SE3104^T (ATCC number BAA-448) isolated from the spring Salinas de oro, Navarra, Spain. 16S rDNA sequence deposited in EMBL under accession number AJ298880.

Description of Roseospira thiosulfatophila *sp. nov.*

thi.o.sul.fa.to'phi.la. M.L. n. *thiosulfatum* thiosulfate; Gr. adj. *philos* loving; M. L. fem. adj. *thiosulfatophila* thiosulfate-loving

Cells are spiral to vibrioid-shaped, 0.5–0.8 µm wide, 2.5–6.5 µm long. Multiplication by binary fission. Gram-negative. Highly motile by polar tufts of flagella. Intracy-

toplasmic membrane system is of vesicular type. Color of anaerobically grown cultures is red. Contains bacteriochlorophyll *a* with unusual absorption shoulder at 909 nm. Carotenoids are of the normal spirilloxanthin series.

Photoorganotrophic under anoxic conditions; substrates used are pyruvate, 2-oxoglutarate, acetate, butyrate, lactate, glucose, glycerol, glutamate. Peptone, yeast extract, and casamino acids also used as substrates. Photoautotrophic growth with sulfide and thiosulfate as electron donors. Chemotrophic growth under micro-oxic conditions. Not capable of anaerobic chemotrophic growth. Yeast extract required as growth factor. Optimal pH for growth 6.8–7.0, range 5.6–8.6. Optimal NaCl concentration for growth 0.5%, range 0.2–5%. Optimal temperature for growth 30–35 °C.

DNA base composition of the type strain is 71.9–72.3 mol% G+C (HPLC).

Habitat: Microbial mats in coastal and marine environments.

Type strain: Strain AT2115^T (ATCC number BAA-449) isolated from a microbial mat located in a large pond in Tetiaroa Atoll, French Polynesia. 16S rDNA sequence deposited in EMBL under accession number AJ401208.

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