***Sulfitobacter mediterraneus* sp. nov., a new sulfite-oxidizing member of the α -Proteobacteria**

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Analysis of PCR products of 16S rDNA of 680 isolates from Mediterranean Sea mesocosm experiments with taxon-specific 16S rDNA oligonucleotides revealed that 262 isolates belonged to the α subclass of the class *Proteobacteria*. Partial 16S rDNA sequence analysis of selected isolates and oligonucleotide probing with a *Sulfitobacter*-specific 16S rDNA probe affiliated 33 strains to the genus *Sulfitobacter*. Analysis of the *Hae*III digest pattern of 16S rDNA revealed the presence of two groups; while 30 strains showed a pattern identical with that obtained for *Sulfitobacter pontiacus* DSM 10014^T, a second group of three strains had a unique pattern that was different from that of the type strain. Five isolates of group 1 and one isolates of group 2, strain CH-B427^T, were selected for detailed taxonomic analysis. All six isolates closely resembled the type strain *Sulfitobacter pontiacus* DSM 10014^T in physiological reactions. However, strain CH-B427^T differed quantitatively in the composition of fatty acids from *Sulfitobacter pontiacus* DSM 10014^T and showed only 98.2% 16S rDNA sequence similarity with strain DSM 10014^T. DNA–DNA reassociation value obtained for strains DSM 10014^T and CH-B427^T revealed 46% similarity. Based on the results of DNA–DNA reassociation and discrete differences in the nucleotide composition of 16S rDNA, a new species of the genus *Sulfitobacter* is proposed, designated *Sulfitobacter mediterraneus* sp. nov., the type strain being strain CH-B427^T (= DSM 12244^T).

Keywords: *Sulfitobacter mediterraneus*, α subclass of *Proteobacteria*, chemotaxonomy, 16S rDNA analysis

INTRODUCTION

The past years have shown an increasing interest in the isolation and description of novel bacterial isolates from the marine environment. Several of these new isolates are members of group 3 of the α subclass of the class *Proteobacteria* in which they are phylogenetically moderately related to members of the genus *Roseobacter*. Besides many as yet undescribed novel isolates from the Pacific Ocean (Suzuki *et al.*, 1997; Gonzales *et al.*, 1997), Antarctic Sea (Bowman *et al.*, 1997) and the Mediterranean Sea (Benlloch *et al.*, 1995), some

have been described as members of novel genera, such as *Sulfitobacter* from the H₂S/O₂ interface of the Black Sea (Sorokin, 1995), *Octadecabacter* from Antarctic and Arctic polar sea-ice water (Gosink *et al.*, 1997) and *Sagittula* from the coast of Georgia, USA (Gonzales *et al.*, 1997).

To increase the chance of obtaining a broad range of culturable aerobic and heterotrophic marine prokaryotes, four mesocosms were established that contained natural seawater from a coastal region at Banyuls-sur-Mer, France (Mediterranean Sea), three of which were subjected to three different eutrophying conditions. A total of 680 strains was isolated from the mesocosm at different time-points and the phylogenetic position of most of them was determined by either partial 16S rDNA sequence analysis or oligo-

Abbreviation: ARDRA, amplified ribosomal DNA restriction analysis.

The EMBL accession number for the 16S rDNA sequence of CH-B427^T is Y17387.

nucleotide probing using taxon-specific 16S rDNA probes (Stackebrandt *et al.*, 1998). The PCR-amplified 16S rDNA of 33 isolates that hybridized with the α -*Proteobacteria* probe (Manz *et al.*, 1992) also hybridized with a *Sulfitobacter*-specific probe, designed in this study on the basis of the almost complete 16S rDNA sequence of *Sulfitobacter pontiacus* DSM 10014^T and partial 16S rDNA sequence information obtained from the Mediterranean isolates that shared high sequence similarity with the 16S rDNA of strain DSM 10014^T. Three of the 33 strains differed significantly from the type strain of *Sulfitobacter pontiacus* in molecular properties, which indicated that it should not be affiliated to *Sulfitobacter pontiacus*. In this paper we describe *Sulfitobacter mediterraneus* sp. nov. and designate strain CH-B427^T (DSM 12244^T) as the type strain.

METHODS

Experimental set-up. The mesocosms, originally set up for studies on the effect of eutrophication processes on the microbial diversity (MAST project CHABADA, project no. MAS3-CT96-0047) and the treatment of the mesocosms have been described previously (Stackebrandt *et al.*, 1998). Each mesocosm was filled with 300 l natural seawater collected from 1 m depth at a station (42° 31' N, 03° 11' E) located about 2400 m off Banyuls-sur-Mer, France (Mediterranean Sea) and filtered through a 200 µm nylon mesh.

Sampling. Samples were taken daily from each mesocosm. Inoculation was done by spreading 100 µl of seawater onto Marine Agar Difco 2216 (ZoBell's medium) plates. Dilutions were 10⁻¹, 10⁻² and 10⁻³. Every dilution was inoculated in triplicate. Between 35 and 50 colonies were selected from each sampling time. Sampling criteria were (i) uniqueness in morphotype and (ii) to verify their similarity, multiple samples (2–4) were selected for those colonies that appeared similar in morphology (3 or 4). Purification of each colony was done after two successive subcultures in laboratories at Banyuls and Barcelona. Isolates were preserved by (i) freezing in triplicate at –80 °C in 40% glycerol, and (ii) at 20 °C on marine agar. Strains, abbreviated to CH followed by the letter of the mesocosm and strain number (e.g. CH-B427^T), were shipped on marine agar to the DSMZ for subsequent analysis.

Molecular identification. Preparation of genomic DNA from pure cultures was done as described previously (Rainey *et al.*, 1996). Amplified ribosomal DNA restriction analysis (ARDRA) was done by PCR amplification of 16S rDNA using 50 ng genomic DNA as target and the primer pair 27f (5' GAGTTTGATCCTGGCTCAG 3') and 1385r (5' CGG-TGTGT[A/G]-CAAGGCC 3'). PCR was performed as described by Pukall *et al.* (1998). 16S rDNA amplicons were cleaved using three different restriction enzymes (*Hae*III, *Bst*UI and *Hha*I; New England Biolabs). Separation of fragments was performed by agarose gel electrophoresis (2% Metaphor agarose; FMC Bioproducts) and stained with ethidium bromide. The primer pair 27f and 1500r (5' AGAAAGGAGGTGATCCAGCC 3') was used for the amplification of the almost complete 16S rRNA gene (Lane, 1991). PCR and sequence analysis of 16S rDNA was performed as described by Rainey *et al.* (1996). Partial sequence analysis was performed using the primer 530r (5' G[T/A]ATTACCG-CGGC[T/G]GCTG 3'). Purified

sequence reactions were electrophoresed using an Applied Biosystems model 373A automated DNA sequencer.

Phylogenetic analyses. Sequences were aligned manually with sequences published previously. These were stored in the DSMZ database consisting of more than 5000 16S rDNA sequence entries, including those from the Ribosomal Database Project (Maidak *et al.*, 1997) and EMBL. Similarity values were transformed into phylogenetic distance values that compensate for multiple substitutions at any given site in the sequence (Jukes & Cantor, 1969). The algorithm of DeSoete (1983) and the neighbour-joining method contained in the PHYLIP package (Felsenstein, 1993) were used in the construction of phylogenetic dendrograms. All analyses were done on a SUN SparcII workstation.

Oligonucleotide hybridization. PCR products were purified with the Prep-A-Gene DNA Purification Kit and eluted in 50 µl water. Three microlitres of each of the 16S rDNA PCR products was denatured by heating and 1 µl of each sample was applied to a nylon membrane (Hybond-N; Amersham) and fixed by UV (260 nm) for 100 s. Hybridization and detection were performed according to the manufacturer's instructions using the DIG Luminescent Detection Kit (Boehringer Mannheim). Oligonucleotide probes were synthesized by the Eurogentec Oligo-Synthesis-Service (Eurogentec) and labelled using the DIG Oligonucleotide 3'-End Labelling Kit (Boehringer Mannheim). All membranes were processed simultaneously in the same roller tube to ensure reproducible hybridization and detection conditions. Pre-hybridization was done at 37 °C for 4 h according to the manufacturer's instructions (Boehringer Mannheim). The prehybridization solution was replaced by a solution containing the DIG-labelled probe and hybridization was continued at 37 °C for 16 h. The temperature of the stringency wash in 6 × SSC, 0.1% SDS buffer depended upon the specific *T_m* of the oligonucleotide probe used. Exposure to X-ray film (Fuji 51209) was up to for 2 h. Probes used were (i) a universal probe G[T/A]ATTACCGCGGC-[T/G]CTG (*T_m* 59 °C) (Lane, 1991) to determine the accessibility of 16S rDNA at the filter, (ii) an α -subclass-specific probe CGTTCG[C/T]TCTGAGCCAG (*T_m* 58 °C) (Manz *et al.*, 1992), and (iii) a *Sulfitobacter*-specific probe CGCCTGCCCCCGAAGG (*T_m* 62 °C) (this study). *T_m* values were determined as described previously (Lane, 1991).

Determination of base composition of DNA. Isolation of DNA (Cashion *et al.*, 1977) and determination of the DNA mol% G + C values by HPLC (Mesbah *et al.*, 1989) followed described procedures.

DNA–DNA hybridization. DNA–DNA similarity were performed by the renaturation method (Escara & Hutton, 1980; Huß *et al.*, 1983). Similarity values were calculated according to the methods of Jahnke (1992).

Extraction and analysis of fatty acids. Fatty acids were determined in cells grown in TSB medium containing 3% NaCl and on Marine agar. Fatty acid methyl esters were obtained from freeze-dried biomass (approx. 10 mg) by saponification, methylation and extraction using the modifications (Kuykendall *et al.*, 1988) of the method of Miller (1982). The fatty acid methyl ester mixtures were separated using a model 5898A microbial identification system (Microbial ID) which consisted of a model 3392 gas chromatograph fitted with a 5% phenyl-methyl silicone capillary column (0.2 mm × 25 m), a flame-ionization detector, a model 3392 integrator, model 7673A automatic sampler and a model 900/300 computer (all from Hewlett

Packard). Peaks were automatically integrated and fatty acid names and percentages were calculated by the identification system. The gas-chromatographic parameters were as follows: carrier gas, ultrahigh-purity hydrogen; column head pressure, 60 kPa; injection volume, 2 µl; column split ratio, 100:1; septum purge, 5 ml min⁻¹; column temperature, 170–270 °C at 5 °C min⁻¹; injection port temperature, 250 °C; and detector temperature, 300 °C.

Scanning electron microscopy. Bacteria grown on liquid broth were adsorbed onto poly-L-lysine-coated cover slips, fixed with 3% glutaraldehyde in PBS for 15 min at room temperature and washed three times with PBS. Samples were dehydrated with a graded series of acetone (10, 30, 50, 70, 90, 100% acetone), critical-point dried with CO₂ (Balzers CPD030), and sputter-coated with a gold film to approximately 10 nm thick (Balzers SCD040). Samples were examined in a Zeiss FESEM DSM962 Gemini microscope with 4 mm working distance and at an acceleration voltage of 5 kV using the In-lens and Everhart-Thornley SE detector in a 75:25 ratio. Pictures were stored digitally and processed using Corel PhotoPaint 7.0.

Physiological studies. Strains tested for the ability to grow at pH 6.0, 6.5, 7.5 and 8.5 and for NaCl requirement (2, 5, 20, 50 and 80 g l⁻¹) were cultivated on marine broth medium (Difco). Results were recorded after 3 d incubation. Growth temperatures were tested on marine agar plates which were incubated in the dark for 1 week at 4, 17, 28 and 37 °C. Anaerobic growth was tested on marine agar plates using Anaerocult A (Merck) incubated in an anaerobic jar. Oxidase, catalase and Gram stain reactions, and accumulation of polyhydroxybutyric acid were determined by standard methods (Gerhardt *et al.*, 1994). The existence of flagella was determined by the method of Heimbrook *et al.* (1989). Following extraction of pigments with 7:2 acetone/methanol, determination of bacteriochlorophyll *a* was done spectrophotometrically (350–950 nm) with a Perkin Elmer model Lambda2 UV/VIS spectrophotometer. Biochemical tests were done using the API 20NE identification system (API bioMérieux) with bacterial suspensions that were washed in 20 g l⁻¹ sea salt solution (Sigma).

The ability to grow on a variety of carbon sources was tested using (i) a minimal medium with or without addition of yeast extract (0.1 g l⁻¹) containing in g l⁻¹: MgCl₂·6H₂O, 0.6 g; CaCl₂·2H₂O, 0.3 g; Na₂SO₄, 0.7 g; NaCl, 20.0 g; pH 7.3, and (ii) a modified, HEPES-buffered medium consisting of (g l⁻¹): HEPES (*N*-[2-hydroxyethyl]piperazine-*N'*-[ethanesulfonic acid], 8.0 g; K₂HPO₄, 1.0 g; NH₄Cl, 0.5 g; NaCl, 15 g; MgSO₄·7 H₂O, 1.0 g; CaCl₂·2 H₂O, 0.05 g; metal salts solution '44', 1 ml and vitamin solution no. 6 (Schlesner *et al.*, 1994), 1 ml; biotin (1 mg ml⁻¹), 100 µl. The following carbon sources were examined at 1 g l⁻¹: gluconate, citrate, aspartate, acetate, aconitate, propionate, butyrate, pyruvate, lactate, glutarate, serine, proline, arginine, ornithine, alanine, asparagine, glutamate and glycerol. Ability to oxidize sulfite was tested in 50 ml HEPES medium supplemented with acetate (10 mM) under a gradual increase of concentration of 5, 10 and 20 mM, respectively. Oxidation of thiosulfate and elemental sulfur was tested as described by Sorokin (1995).

16S rDNA accession numbers of reference organisms. *Octadecabacter antarcticus*, U14583; *Octadecabacter arcticus*, U73735; *Roseobacter algicola*, X78315; *Roseobacter denitrificans*, L01784; *Roseobacter litoralis*, X78312; *Sagittula stellata*, U58356; *Sulfitobacter pontiacus*, Y13155; Sargasso Sea isolate S34, U87407; Antarctic Sea strain IC

146, AF001377; and '*Methylosulfonas methylovorum*', U62893.

RESULTS

Molecular identification of *Sulfitobacter* strains

To obtain a glimpse of the phylogenetic diversity of 680 Mediterranean Sea isolates, partial 16S rDNA sequences were analysed for 40 randomly chosen isolates followed by determination of their closest phylogenetic neighbours. The majority of these organisms belong to the α and γ subclass of *Proteobacteria* (38 and 44%, respectively), while the number of Gram-positive bacteria was smaller (15%). Many strains clustered close to described species, e.g. *Sulfitobacter pontiacus* (97.6–99.7% similarity) and *Roseobacter algicola* (97.0–100% similarity) in the α subclass of *Proteobacteria*, and members of *Pseudoalteromonas haloplanktis* (99.4–100% similarity), *Alteromonas macleodii* (95.5–97.1% similarity), *Marinobacter hydrocarbonoclasticus* (95.6–96% similarity) and *Vibrio splendidus* (95.3–97.6% similarity) in the γ subclass of *Proteobacteria* (Stackebrandt *et al.*, 1998). Oligonucleotide probing of PCR-amplified 16S rDNA with an α -subclass-specific probe (alpha-probe, Manz *et al.*, 1992) showed that 280 isolates (41%) were members of this subclass. Subsequently, the number of isolates belonging phylogenetically to the genus *Sulfitobacter* was determined by using an oligonucleotide probe generated to specifically identify members of the genus *Sulfitobacter* and isolate S34 from the Sargasso Sea (Suzuki *et al.*, 1997). Thirty-three isolates were identified as members of this species. Thus 12.5% of all members of the α subclass of *Proteobacteria*, isolated from nine sampling points from all four mesocosms could be assigned to this taxon.

When the PCR-amplified partial 16S rDNA of about 1400 nucleotides was cleaved with the enzymes *Bst*UI and *Hha*I the restriction patterns of strain DSM 10014^T, strain S34 and the 33 *Sulfitobacter*-like isolates were identical. However, the *Hae*III digest clearly indicated the presence of two strain groups. While 30 strains of group 1 showed a pattern consisting of five clearly visible fragments (571, 278, 158, 152 and 78 bp), six fragments defined the pattern of group 2 strains CH-B427^T, CH-D522 and CH-D620. The latter result can be explained by the presence of an additional *Hae*III restriction site at position 416 in the 16S rDNA of these strains (*E. coli* numbering system; Brosius *et al.*, 1978), verified in strain CH-B427^T, which results in the cleavage of the 571 bp fragment into two smaller fragments of 486 and 85 bp. It can be deduced from the presence of *Hae*III restriction sites in the 16S rDNA of *Sulfitobacter* strains that three additional fragment of size 66, 48 and 34 bp must be formed; however, these were not detectable on Metaphore agarose. Based upon the results of the ARDRA analysis, five strains of group 1 and one strain of group 3 were subjected to additional taxonomic analysis.

Partial 16S rDNA sequence analyses indicated that the

Fig. 1. Dendrogram of 16S rDNA relatedness showing the position of strain CH-B427^T next to its nearest identified neighbours within the α subclass of the class Proteobacteria. The tree was calculated by the neighbour-joining method from almost complete sequences. The percentage of 500 bootstrap resembling that support branching points above 70% confidence is indicated. The scale bar corresponds to five nucleotide substitutions per 100 sequence positions. The tree was rooted with *E. coli* as an outgroup.

five strains of group 1, i.e. strains CH-A24, CH-D261, CH-B420, CH-B423 and CH-D520, shared 100% similarity among each other and 99.7% similarity with the sequences of *Sulfitobacter pontiacus* DSM 10014^T and strain S34. With 97.6% similarity, the group 2 representative strain CH-B427^T was slightly less related to strain DSM 10014^T. For a more detailed phylogenetic analysis the almost complete sequence was analysed for isolate CH-B427^T, consisting of 1415 nucleotides (according to the *E. coli* nomenclature; Brosius *et al.*, 1978). The similarity value determined for strain CH-B427^T and strain DSM 10014^T was 98.2%. Similarity values determined for the pair *Sulfitobacter pontiacus* and strain CH-B427^T and members of *Roseobacter* and *Octadecabacter* ranged between 93 and 96%. A dendrogram of 16S rDNA relationships, displaying the position of strain CH-B427^T to its phylogenetic neighbours, is shown in Fig. 1. The branching pattern did not change with any of the algorithm applied (Maidak *et al.*, 1997; Felsenstein, 1993).

When the almost complete sequence of strain CH-B427^T was compared to those of strains DSM 10014^T and S34 several nucleotide positions could be identified which can be considered signature nucleotides (Table 1). Most of these signatures comprise base-pair exchanges and can thus be considered genetically stable. The two signatures present in the 5' 500 nucleotides of the 16S rDNA of the other five *Sulfitobacter* isolates for which partial sequences have been generated, match those of *Sulfitobacter pontiacus* and strain S34.

The G+C content of DNA was determined to be 59 mol% for strain CH-B427^T; for group 1 strains the

Table 1. 16S rDNA signature positions of *Sulfitobacter pontiacus* DSM 10014^T and strain CH-B427^T

Position	DSM 10014 ^T *	CH-B427 ^T
Helical region 80–82/87–89	3 bp	4 bp
418–425	U-A	C-G
599–639	U-A	A-U
591–648	U-A	C-G
592–647	G-C	A-U

* Same signature is present in strain S34.

values were 60 mol% (DSM 10014^T, CH-D261 and CH-D520) and 61 mol% (for strains S34, CH-B437, CH-B420 and CH-B423).

Cultural and physiological properties

All of the six isolates of groups 1 and 2, selected from the total of 33 strains that hybridized with the *Sulfitobacter*-specific probe and the Sargasso isolate S34, which in this study was identified as a phylogenetic relative of *Sulfitobacter pontiacus*, were phenotypically very similar to the type strain, DSM 10014^T, which was included in the tests for comparison.

All strains tested were Gram-negative, non-spore-forming, strictly aerobic and heterotrophic bacteria. Strains were both catalase- and oxidase-positive. Cells growing on marine agar at 25 °C were rod-shaped 1–3 µm in length and 0.5–0.8 µm in diameter (Fig. 2a). Cells were motile by means of 1–5 subpolar flagella. Bacteria grown on marine agar, supplemented with acetate, tended to form rosettes (Fig. 2b, c) and contained PHB-granula.

All strains were mesophilic (4–35 °C, optimum at 17–28 °C), neutrophilic (pH 6.5–8.5, optimum 7.0–7.5) and required NaCl for growth (2–80 g l⁻¹, optimum 15–20 g l⁻¹). Bacteriochlorophyll *a* was not detected. All strains were able to grow in minimal media with and without additions of yeast extract or vitamins incubated at 28 °C with the following compounds as sole sources of carbon and energy: glucose, mannitol, gluconate, adipate, acetate, malate, pyruvate, lactate, propionate, butyrate, serine, proline, ornithine, alanine, asparagine, glutamate, phenylacetate and glycerol. Growth of strain CH-B427^T at 25 °C on marine agar plates supplemented with acetate was slower compared to the other strains. Strains were not able to reduce nitrate; glutarate, *cis*-aconitate, tryptophan, caprate, urea, arabinose and mannose were not utilized. Glucose was not fermented. Weak growth was detected with aspartate. No oxidation of thiosulfate or elemental sulfur was observed. Utilization of citrate and maltose was stimulated in media containing yeast extract or biotin. Utilization of *N*-acetylglucosamine and activity of arginine hydrolase, β -glucosidase and gelatine hydrolase were induced after a longer incubation period (> 3 d).

Fig. 2. Electron micrographs of strain CH-B427^T showing (a) different cell morphology (bar, 2 µm) and (b, c) rosette formation (bars, 1 µm). Cells were grown overnight on marine broth supplemented with acetate.

Compared to *Sulfitobacter pontiacus* DSM 10014^T, strain CH-B427^T showed higher cell density in acetate (10 mM) – supplemented HEPES medium after short adaptation to sulfite concentrations of 5 mM and of 10 mM. None of the *Sulfitobacter* strains were able to grow in media containing 20 mM sulfite.

Fatty acid analysis

The main fatty acids determined for *Sulfitobacter pontiacus* DSM 10014^T and five related strains grown in TBS/NaCl medium were *cis*-11 octadecenoic acid (>72%), followed by hexadecanoic acid (4–7%) (Table 2). All other fatty acids occurred in less than 5%. While in strain CH-B427^T the growth medium used had no influence on the relative ratio of the fatty acid composition, a significant change was observed in the other strains of *Sulfitobacter* when grown on Marine agar. Here the percentage of *cis*11-18:1 fatty acid decreased from about 80% to 65–75%, while the percentage of 10:0-3-OH, *cis*9-16:1 and 16:0 fatty acids increased (not shown). The dendrogram of fatty acid relatedness, based upon the data obtained from cells grown in NaCl-supplemented TSB medium, clearly indicates the separate position of strain CH-B427^T (Fig. 3).

DNA–DNA hybridization

Determination of DNA relatedness by the spectrophotometric method was performed with the type strain of *Sulfitobacter pontiacus* DSM 10014^T and the six representatives of ARDRA groups 1 and 2. The

Table 2. Percentage fatty acid composition of some *Sulfitobacter* strains

Fatty acid*	DSM 10014 ^T	CH-A24	CH-D261	CH-D520	S34	CH-B427 ^T
10:0-3-OH	3.5	2.6	2.6	2.5	3.3	2.5
12:0-3-OH	0.7	0.6	0.7	0.6	0.7	0.6
ECL13-521			1.0	3.9		3.3
14:0						0.7
15:0		0.4	0.3		0.4	0.5
ECL 15-278	2.6	2.0	1.9	1.8	2.4	1.9
<i>cis</i> 7-16:1						1.9
<i>cis</i> 9-16:1	1.3	0.8	1.1	1.0	1.0	1.0
16:0	4.8	4.5	5.1	4.7	5.1	6.1
<i>cis</i> 9-17:1	1.0	1.3	1.1	1.2	1.0	1.2
17:0	1.2	1.4	1.1	1.2	1.2	0.9
<i>cis</i> 9-18:1						1.7
<i>cis</i> 11-18:1	81.5	80.4	78.6	76.7	79.1	72.4
18:0	0.7	0.7	0.7	0.6	0.6	0.7
10met-18:1	2.7	4.7	5.0	5.1	4.0	2.6
18met-18:1						1.4
ECL18-847		0.6	0.8	0.7	0.9	
10met-19:0					0.3	

* 16:0, hexanecanoic acid (palmitic acid); *cis*11-18:1, *cis*-11 octadecenoic acid (*cis*-vaccenic acid); 10met-18:1, 10-methyl octadecenoic acid; 12:0-3-OH, 3-hydroxy decanoic acid (β -hydroxy lauric acid). ECL15-278, unknown fatty acid showing an equivalent chain-length of 15:278.

Fig. 3. Cluster analysis of fatty acid profiles of *Sulfitobacter pontiacus* and related isolates. The branching pattern was constructed from the Euclidean distance coefficients by the unweighted pair group method with arithmetic averages. Analysis was done using the standard MIS software (Microbial ID).

Table 3. Percentage DNA–DNA homology between *Sulfitobacter*-like isolates and *Sulfitobacter pontiacus* DSM 10014^T as determined by spectroscopic DNA–DNA hybridization

Strain	Homolgy with DSM 10014 ^T
CH-A24	100
CH-D261	82.4
CH-B420	92.3
CH-B423	100
CH-D520	88.9
CH-B427 ^T	46.1

data revealed that those isolates that shared with DSM 10014^T identical ARDRA patterns, high fatty acid relatedness, and almost identical partial 16S rDNA similarities (group 1 strains) also shared DNA–DNA similarity values of higher than 82 %. The only lower value of 46 % similarity was obtained for *Sulfitobacter pontiacus* DSM 10014^T and strain CH-B427^T (group 2 strain).

DISCUSSION

The occurrence of a high number of isolates from mesocosm experiments with Mediterranean coastal seawater that phylogenetically resembled *Sulfitobacter pontiacus* was unexpected as this species was hitherto represented only by two strains from the Black Sea (Sorokin, 1995). The fact that this taxon has not been reported among the cultured strains from other recently studied marine environments may be due to the unavailability of the 16S rDNA sequence of *S. pontiacus* from a public database. The inclusion of published sequences from the studies of the Sargasso Sea isolate S34 (Suzuki *et al.*, 1997) indeed indicates

that bacteria related to *Sulfitobacter* also occur in other oceans.

Of the 33 strains identified to be closely related to *Sulfitobacter pontiacus* DSM 10014^T by oligonucleotide probing, 30 strains share with the type strain identical ARDRA patterns generated by three different restriction enzymes. As the five strains selected from the 30 strains share with *Sulfitobacter pontiacus* high phylogenetic (including DNA–DNA similarity values above 80 %), chemotaxonomic and phenotypic similarities, it can be deduced that all of these strains belong to the same species. The other three strains differ from the type strain and related isolates in the ARDRA pattern generated by restriction with *Hae*III. Of these three strains, CH-B427^T was investigated more thoroughly and the data reveal its taxonomic separatedness in that it differs from the type strain of *S. pontiacus* in the primary structure of 16S rDNA, quantitative composition of fatty acids and moderate DNA–DNA similarity of only 46 %. Clear-cut phenotypic differences, traditionally used to differentiate species from each other, have not been found. As we consider each nucleotide of the primary structure of a evolutionary conservative gene, such as the 16S rDNA, to be a genetically stable and reproducibly identifiable phenotypic property, strain CH-B427^T can clearly be differentiated from the type strain of *Sulfitobacter pontiacus* and related strains (Table 1). We therefore propose to classify strain CH-B427^T as the type strain of a new species of the genus *Sulfitobacter*, *Sulfitobacter mediterraneus*.

Description of *Sulfitobacter mediterraneus* sp. nov.

Sulfitobacter mediterraneus (med.i.ter.ra.ne'us. N.L. adj. *mediterraneus* of the Mediterranean Sea).

Gram-negative, non-spore-forming, strictly aerobic and heterotrophic bacteria. Catalase- and oxidase-positive. Cells grown on marine agar at 25 °C are rod-shaped, 1–3 µm in length and 0.5–0.8 µm in diameter. Colonies on Marine agar are 1.2–1.4 mm in diameter, circular, convex, with entire or undulate margin, translucent and cream-coloured. Cells are motile by means of 1–5 subpolar flagella. Bacteria grown on marine agar, supplemented with acetate, tend to form rosettes and contain PHB-granula. Mesophilic. Growth occurs between 4–35 °C, with an optimum at 17–28 °C. Neutrophilic (pH 6.5–8.5, optimum 7.0–7.5). NaCl (2–80 g l⁻¹, optimum 15–20 g l⁻¹) is required for growth. In minimal media with or without the addition of yeast extract or vitamins, growth occurs at 28 °C with glucose, mannitol, gluconate, adipate, acetate, malate, pyruvate, lactate, propionate, butyrate, serine, proline, ornithine, alanine, asparagine, glutamate and glycerol. Glutamate, *cis*-aconitate, tryptophan, caprate, urea, arabinose and mannose are not utilized. Weak growth is detected with aspartate. Nitrate is not reduced. Glucose is not fermented. No growth with thiosulfate or elemental sulfur. Utilization of citrate and maltose is stimulated in media containing yeast

extract or biotin. Utilization of *N*-acetylglucosamine and activity of arginine hydrolase, β -glucosidase and gelatine hydrolase are induced after a longer incubation period (> 3 d). Phenylacetate is utilized after 24 h incubation at 25 °C. Growth on 10 mM sulfite in acetate (10 mM)-supplemented HEPES medium. In TSB medium containing 3 % NaCl the main fatty acid is *cis*-11 octadecenoic acid. Hexadecanoic acid occurs in smaller amounts. Bacteriochlorophyll *a* is not present. The G+C content is 59 mol %. Signature nucleotides for 16S rDNA, are located at positions 418–425 (C-G), 591–648 (C-G), 592–647 (A-U) and 599–639 (A-U). Type strain CH-B427^T (= DSM 12244^T) isolated from coastal waters of the west Mediterranean Sea.

We thank Josef Vives-Rego and Theresa Guindulain for providing us with strains isolated during the CHABADA project and Marcelino T. Suzuki for providing us with strain S34. This work was supported by a grant from the European Commission, no. MAS3-CT96-0047.

REFERENCES

- Bowman, J., McCammon, S. A., Brown, M. V., Nichols, D. S. & McMeekin, T. A. (1997). Diversity and association of psychrophilic bacteria in Antarctic sea ice. *Appl Environ Microbiol* **63**, 267–280.
- Benlloch, S., Rodriguez-Valera, F. & Martinez-Murcia, A. J. (1995). Bacterial diversity in two coastal lagoons deduced from 16S rDNA PCR amplification and partial sequencing. *FEMS Microbiol Ecol* **18**, 267–280.
- Brosius, J., Palmer, M. L., Kennedy, P. J. & Noller, H. F. (1978). Complete nucleotide sequence of the 16S ribosomal RNA gene from *Escherichia coli*. *Proc Natl Acad Sci USA* **75**, 4801–4805.
- Cashion, P., Holder-Franklin, M. A., McCully, J. & Franklin, M. (1977). A rapid method for the base ratio determination of bacterial DNA. *Anal Biochem* **81**, 461–466.
- DeSoete, G. (1983). A least squares algorithm for fitting additive trees to proximity data. *Psychometrika* **48**, 621–626.
- Escara, J. F. & Hutton, J. R. (1980). Thermal stability and renaturation of DNA in dimethylsulphoxide solutions: acceleration of renaturation rate. *Biopolymers* **19**, 1315–1327.
- Felsenstein, J. (1993). PHYLIP (phylogenetic inference package) version 3.5.1. Department of Genetics, University of Washington, Seattle, USA.
- Gerhardt, P., Murray, R. G. E., Wood, W. A. & Krieg, N. R. (1994). *Methods for General Molecular Bacteriology*. Washington, DC: American Society for Microbiology.
- Gonzales, J. M., Mayer, F., Moran, M. A., Hodson, R. E. & Whitman, W. B. (1997). *Sagittula stellata* gen. nov., sp. nov., a lignin-transforming bacterium from a coastal environment. *Int J Syst Bacteriol* **47**, 773–780.
- Gosink, J. J., Herwig, R. P. & Staley, J. T. (1997). *Octadecabacter arcticus* gen. nov., spec. nov. and *O. antarcticus* sp. nov., nonpigmented, psychrophilic gas vacuolated bacteria from polar sea ice and water. *Syst Appl Microbiol* **20**, 356–365.
- Heimbrook, M. E., Wong, W. L. & Campbell, G. (1989). Staining bacterial flagella easily. *J Clin Microbiol* **27**, 2612–2615.
- HuB, V. A. R., Festl, H. & Schleifer, K. H. (1983). Studies on the spectrophotometric determination of DNA hybridization from renaturation rates. *Syst Appl Microbiol* **4**, 184–192.
- Jahnke, K.-D. (1992). BASIC computer program for evaluation of spectroscopic DNA renaturation data from GILFORD SYSTEM 2600 spectrophotometer on a PC/XT/AT type personal computer. *J Microbiol Methods* **15**, 61–73.
- Jukes, T. H. & Cantor, C. R. (1969). Evolution of protein molecules. In *Mammalian Protein Metabolism*, vol. 3, pp. 21–132. Edited by H. N. Munro. New York: Academic Press.
- Kuykendall, L. D., Roy, M. A., O'Neill, J. J. & Devine, T. E. (1988). Fatty acids, antibiotic resistance, and deoxyribonucleic acid homology groups of *Bradyrhizobium japonicum*. *Int J Syst Bacteriol* **38**, 358–361.
- Lane, D. J. (1991). 16S/23S rRNA sequencing. In *Nucleic Acid Techniques in Bacterial Systematics*, pp. 125–175. Edited by E. Stackebrandt & M. Goodfellow. Chichester: Wiley.
- Maidak, B. L., Olsen, G. J., Larsen, N., Overbeek, R., McCaughey, M. J. & Woese, C. R. (1997). The RDP (Ribosomal Database Project). *Nucleic Acids Res* **25**, 109–111.
- Manz, W., Amann, R., Ludwig, W., Wagner, M. & Schleifer, K.-H. (1992). Phylogenetic oligonucleotide probes for the major subclasses of proteobacteria: problems and solutions. *Syst Appl Microbiol* **15**, 593–600.
- Mesbah, M., Premachandran, U. & Whitman, W. B. (1989). Precise measurement of the G+C content of deoxyribonucleic acid by high-performance liquid chromatography. *Int J Syst Bacteriol* **39**, 159–167.
- Miller, L. T. (1982). A single derivatization method for bacterial fatty acid methyl esters including hydroxy acids. *J Clin Microbiol* **16**, 584–586.
- Pukall, R., Brambilla, E. & Stackebrandt, E. (1998). Automated fragment length analysis of fluorescently-labeled 16S rDNA after digestion with 4-base cutting restriction enzymes. *J Microbiol Methods* **32**, 55–64.
- Rainey, F. A., Ward-Rainey, N., Kroppenstedt, R. M. & Stackebrandt, E. (1996). The genus *Nocardiopsis* represents a phylogenetically coherent taxon and a distinct actinomycete lineage: proposal of *Nocardiopsaceae* fam. nov. *Int J Syst Bacteriol* **46**, 159–167.
- Schlesner, H. (1994). The development of media suitable for the microorganisms morphologically resembling *Planctomyces* spp., *Pirellula* spp., and other *Planctomycetales* from various aquatic habitats using dilute media. *Syst Appl Microbiol* **17**, 295–305.
- Sorokin, D. Y. (1995). *Sulfitobacter pontiacus* gen. nov., sp. nov. – a new heterotrophic bacterium from the Black Sea, specialized on sulfite oxidation. *Microbiology* (English translation of *Mikrobiologiya*) **64**, 295–305.
- Stackebrandt, E., Lebaron, P., Troussilier, M., Courties, C., Servais, P., Vives-Rego, J., Muyzer, G., Pukall, R., Lehnberg, B., BuntetuB, D., Puker, O., Ulrichs, G., Bernard, L., Guindulain, T. & Schafer, H. (1998). Assessment of changes of diversity among Mediterranean prokaryotes subjected to eutrophying conditions. In *Third European Marine Science and Technology Conference*, pp. 764–777. Edited by K.-G. Barthel, H. Barth, M. Bohle-Carbonell, C. Fragakis, E. Lipiatou, P. Martin, G. Ollier & M. Weydert. Brussels: European Commission.
- Suzuki, M. T., Rappe, M. S., Haimberger, Z. W., Winfield, H., Adair, N., Strobel, J. & Giovannoni, S. (1997). Bacterial diversity among small-subunit rRNA gene clones and cellular isolates from the same seawater sample. *Appl Environ Microbiol* **63**, 2612–2615.