

# *Rubritalea marina* gen. nov., sp. nov., a marine representative of the phylum 'Verrucomicrobia', isolated from a sponge (Porifera)

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A marine bacterium, strain Pol012<sup>T</sup>, was isolated from the Mediterranean sponge *Axinella polypoides* and subsequently characterized as belonging to subphylum 1 of the phylum 'Verrucomicrobia'. Strain Pol012<sup>T</sup> was non-motile, Gram-negative, coccoid or rod-shaped and red in colour. The menaquinones MK-8 and MK-9 were detected. The G + C content of the genomic DNA was 50.9 mol%. Growth was possible at temperatures between 8 and 30 °C and at pH values between 6.8 and 8.2. The closest cultured relative of strain Pol012<sup>T</sup> was *Akkermansia muciniphila* (83 % sequence similarity), while the closest environmental 16S rRNA gene sequence was the marine clone Arctic96BD-2 (95 % sequence similarity). Strain Pol012<sup>T</sup> is the first marine pure-culture representative of 'Verrucomicrobia' subphylum 1 and represents a novel genus and species, for which the name *Rubritalea marina* gen. nov., sp. nov. is proposed. The type strain is Pol012<sup>T</sup> (= DSM 177716<sup>T</sup> = CIP 108984<sup>T</sup>).

At present, six monophyletic subphyla are recognized (albeit not yet taxonomically validated formally) for the phylum 'Verrucomicrobia' on the basis of clone library studies (Hugenholtz *et al.*, 1998; Vandekerckhove *et al.*, 2000). While more than 500 different verrucomicrobial 16S rRNA gene sequences have been identified using 16S rRNA gene-based techniques, only a handful of them represent cultivated strains (Schlesner, 1987; Chin *et al.*, 2001; Joseph *et al.*, 2003; Sangwan *et al.*, 2004). In fact, only two marine 'Verrucomicrobia' species, *Alterococcus agarolyticus* (Shieh & Jean, 1998; Sangwan *et al.*, 2004) and '*Fucophilus fucoidanolyticus*' (Sakai *et al.*, 2003), have been cultivated so far; both belong to subphylum 4. The epixenosomes, ectosymbionts of marine ciliates of the genus *Euplotidium*, also belong to this subphylum (Petroni *et al.*, 2000). In addition, verrucomicrobial 16S rRNA gene sequences have been recovered from marine animals and plants (Weidner *et al.*, 2000; Alain *et al.*, 2002; Bowman & Nowak, 2004), from bacterioplankton (Bano & Hollibaugh, 2002; Harris *et al.*, 2004; O'Sullivan *et al.*, 2004), from coastal Antarctic sediments (Bowman *et al.*, 2000; Bowman & McCuaig, 2003; Powell *et al.*, 2003) and from seawater

(Schäfer *et al.*, 2000; Madrid *et al.*, 2001; Suzuki *et al.*, 2001; Bano & Hollibaugh, 2002; O'Sullivan *et al.*, 2004). The respective organisms are mainly affiliated with subphyla 1 and 4. In this study, we describe the cultivation and analysis of strain Pol012<sup>T</sup>, the first marine member of subphylum 1 of the phylum 'Verrucomicrobia'. In addition, 16S rRNA gene sequences of various members of the 'Verrucomicrobia' were isolated from sponges and seawater.

Strain Pol012<sup>T</sup> was isolated from the Mediterranean sponge *Axinella polypoides* on M1 agar (Mincer *et al.*, 2002). Sponges of the species *Axinella polypoides*, *Axinella damicornis* and *Aplysina aerophoba* were collected by means of scuba-diving offshore from Banyuls-sur-Mer, France (42° 29' N 03° 08' E) in May 2003. Sponges of the species *Agelas conifera* and *Aplysina fistularis* were collected in July 2000 offshore from Little San Salvador Island in the Bahamas (GPS: 24° 34.39' N 75° 58.00' W) at depths of 5–15 m. Mechanical separation of the sponge tissue and storage of the bacterial cells were performed as described by Fieseler *et al.* (2004). For further cultivation of strain Pol012<sup>T</sup>, *Verrucomicrobium spinosum* medium 607 (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany) was modified to contain the following: 1.5 g yeast extract, 1.5 g peptone, 1.5 g glucose, 20 ml Hutner's basal salts solution (Cohen-Bazire *et al.*, 1957), 5 ml Tris/HCl (1 M, pH 7.5) and 975 ml artificial seawater (ASW) (Lyman & Fleming, 1940). The new medium designed for the cultivation of marine

Abbreviation: ASW, artificial seawater.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain Pol012<sup>T</sup> is DQ302104, and those for verrucomicrobial 16S rRNA gene sequences from sponges and seawater are DQ302105–DQ302120.

members of the phylum 'Verrucomicrobia' was named 'Ver100'. Strain Pol012<sup>T</sup> was incubated at 30 °C on plates and in liquid cultures shaken at 200 r.p.m. On the newly designed marine medium (Ver100), strain Pol012<sup>T</sup> formed soft, red colonies, while the liquid cultures were pink to red in colour. Growth was observed at temperatures between 8 and 30 °C, but not at 4 or 35 °C. Cell growth was optimal at temperatures between 25 and 30 °C. Strain Pol012<sup>T</sup> was not able to grow on Ver100 agar plates incubated in an anaerobic jar. Vitamins were not required for growth.

Strain Pol012<sup>T</sup> showed a strict requirement for seawater. Growth was possible in liquid culture with media formulations containing 60–160 % (v/v) ASW, while growth was inhibited below 40 % or above 180 % ASW. Replacement of ASW with NaCl was not possible, since no growth could be observed in medium containing 0, 200, 400, 510, 610, 800, 1070 or 1600 mM NaCl. Growth was possible at pH values between 6.8 and 8.2, but not at 6.0; pH values above 8.2 could not be measured, since unknown salts precipitated in Ver100 culture medium. The cells grew poorly on ZoBell agar (Oppenheimer & ZoBell, 1952) and slightly better on M1 agar (Mincer *et al.*, 2002), on which strain Pol012<sup>T</sup> was originally isolated. In liquid culture, strain Pol012<sup>T</sup> was able to grow in minimal Ver100 medium (lacking peptone, yeast extract and glucose) with glucose, xylose, melibiose, cellobiose, lactose, pyruvate or pectin serving as the sole energy source. Growth was not possible with galactose, fructose, mannose, mannitol, arabinose, sucrose, ribose, sorbose, fucose, rhamnose, melitose, ethanol, methanol, glycerol, galacturonic acid, malate, tartrate, citrate, glycine, alanine, leucine, isoleucine, proline, lysine, glutamate, starch, xylan or chitin. The polysaccharides were tested at 0.1 % (w/v). All other substances were tested at a concentration of 10 mM.

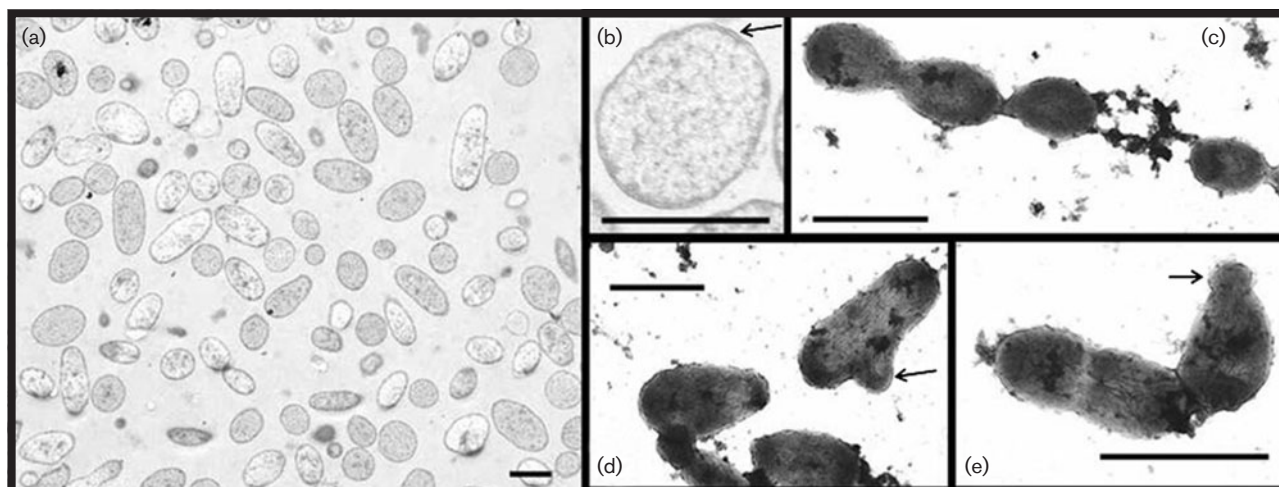
For further phenotypic testing, Pol012<sup>T</sup> was grown on Ver100 agar plates for 48 h at 30 °C and resuspended in either ASW or Ver100 minimal medium. Using the API ZYM system (bioMérieux), the enzymes alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, trypsin, acid phosphatase and naphthol-AS-BI-phosphohydrolase gave positive results. Using the API 20NE system (bioMérieux),  $\beta$ -glucosidase activity on aesculin,  $\beta$ -galactosidase activity on 4-nitrophenyl  $\beta$ -D-galactopyranoside and oxidase activity were found. Furthermore, reduction of nitrate to nitrite was measurable. Using Biolog GN2 microplates, strain Pol012<sup>T</sup> was shown to oxidize dextrin, D-cellobiose, D-fructose, D-galactose,  $\alpha$ -D-glucose, D-mannose, D-melibiose, acetic acid and formic acid. Here, cells were suspended in Ver100 minimal medium to an OD<sub>600</sub> of 0.3. All results were read after incubation at 30 °C ( $n=3$ ).

Strain Pol012<sup>T</sup> was able to grow in Ver100 culture medium supplemented with vancomycin, kanamycin, streptomycin, gentamicin, neomycin or tetracycline (each at a concentration of 100  $\mu$ g ml<sup>-1</sup>), but not with chloramphenicol, ampicillin, lincomycin, erythromycin or oxacillin (each at a

concentration of 10  $\mu$ g ml<sup>-1</sup>). Strain Pol012<sup>T</sup> was tested for antimicrobial activity against Gram-positive *Staphylococcus aureus* and Gram-negative *Escherichia coli* reference strains. Neither soft-agar overlays performed with Pol012<sup>T</sup> following growth on agar plates nor ethyl acetate extracts of liquid cultures provided evidence of antimicrobial activity.

Cells of strain Pol012<sup>T</sup> produced a red pigment that did not diffuse into the agar and which could be easily extracted with methanol or ethanol. Cell-free ethanol extracts displayed an absorption maximum at 495 nm. Cells were catalase-negative and oxidase-positive. Quinones MK-8 (23 %) and MK-9 (77 %) could be detected. This is consistent with other members of the phylum 'Verrucomicrobia', in which menaquinones have also been identified (Sittig & Schlesner, 1993; Hedlund *et al.*, 1996; Sakai *et al.*, 2003; Sangwan *et al.*, 2004). The G+C content of the genomic DNA was 50.9 mol%. The peptidoglycan structure of strain Pol012<sup>T</sup> contained direct cross-linkages of the A1 $\gamma$  meso-diaminopimelic acid type that is also present in '*Chthoniobacter flavus*' from subphylum 2 of the phylum Verrucomicrobia (Sangwan *et al.*, 2004); meso-diaminopimelic acid has also been isolated from *Prostheco bacter* strains (Hedlund *et al.*, 1996). The major cellular fatty acids were hexadecanoic acid (C<sub>16:0</sub>; 23 % of the total), 12-methyltridecanoic acid (i-C<sub>14:0</sub>; 22 % of the total), *cis*-9-hexadecenoic acid and/or 2-hydroxy-13-methyltetradecanoic acid (C<sub>16:1 $\omega$ 7c</sub> and/or 2-OH i-C<sub>15:0</sub>; 21.5 % of the total), 12-methyltetradecanoic acid (a-C<sub>15:0</sub>; 6 % of the total) and pentadecanoic acid (C<sub>15:0</sub>; 4.5 % of the total). Extraction and analysis of the cellular fatty acids and the isoprenoid quinones, analysis of the peptidoglycan structure and determination of the G+C content of the genomic DNA were performed by the Deutsche Sammlung von Mikroorganismen und Zellkulturen.

Transmission electron microscopy of ultrathin sections of strain Pol012<sup>T</sup> was performed essentially as described by Pimentel-Elardo *et al.* (2003). For transmission electron microscopy of negatively stained whole cells, bacteria were grown in liquid culture and fixed with 2.5 % glutaraldehyde. A drop of culture was incubated on a copper grid, the liquid was removed and the cells were stained with a drop of 0.5 % uranyl acetate for 5 min. The cells were examined under an EM 10 electron microscope (Zeiss). For scanning electron microscopy, colonies with surrounding material were fixed in 6.25 % glutaraldehyde, washed three times with 0.1 M cacodylate buffer and dehydrated with increasing concentrations of acetone (from 30 to 100 %). After critical-point drying and platinum coating of the dried material, colonies were examined with a DSM 962 scanning electron microscope (Zeiss). Strain Pol012<sup>T</sup> grew in various forms including almost coccoid cells ranging from 0.5 to 1.1  $\mu$ m in diameter and rod-shaped cells ranging in length from 0.6 to 1.8  $\mu$ m and in width from 0.4 to 1.0  $\mu$ m (Figs 1 and 2). Electron microscopic images of 35 negatively stained cells were used to measure cell size. Single cells and cell clusters were found. Cell division might occur by budding (see



**Fig. 1.** (a, b) Transmission electron micrographs of ultrathin sections of cells of strain Pol012<sup>T</sup>. (c, d, e) Transmission electron micrographs of negatively stained whole cells of strain Pol012<sup>T</sup>. Bars, 1 μm.

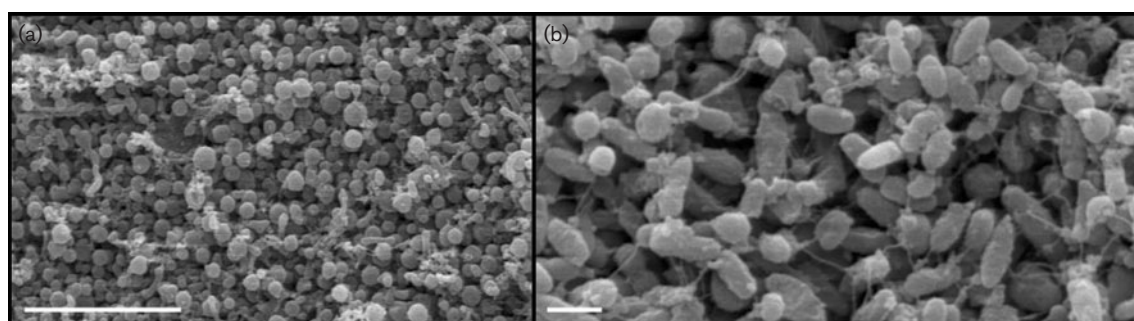
arrows in Fig. 1d, e). Motile cells, flagella and prosthecae were not observed. Strain Pol012<sup>T</sup> stained Gram-negative, and two separate membranes could be detected by electron microscopy (see arrow in Fig. 1b).

16S rRNA gene amplification, cloning and sequencing were performed according to the methods of Hentschel *et al.* (2001), using universal bacterial primers 27f and 1492r (Lane, 1991). For the amplification of verrucomicrobial 16S rRNA genes, the 'Verrucomicrobia'-specific forward primer Ver53F (Stevenson *et al.*, 2004) and the reverse primer 1492r were used. Sequences were checked for chimaeras (Huber *et al.*, 2004), and phylogenetic analyses were performed using the ARB software package (Strunk *et al.*, 2000) after alignment of the sequences by using CLUSTAL W. All sequences contained at least 1250 nt. Phylogenetic-tree construction was performed using the neighbour-joining method with bootstrap values based on 1000 replications. Phylogenetic analysis of the 16S rRNA gene sequence of strain Pol012<sup>T</sup> revealed a sequence similarity of 83 % with respect to its closest cultured relative, *Akkermansia*

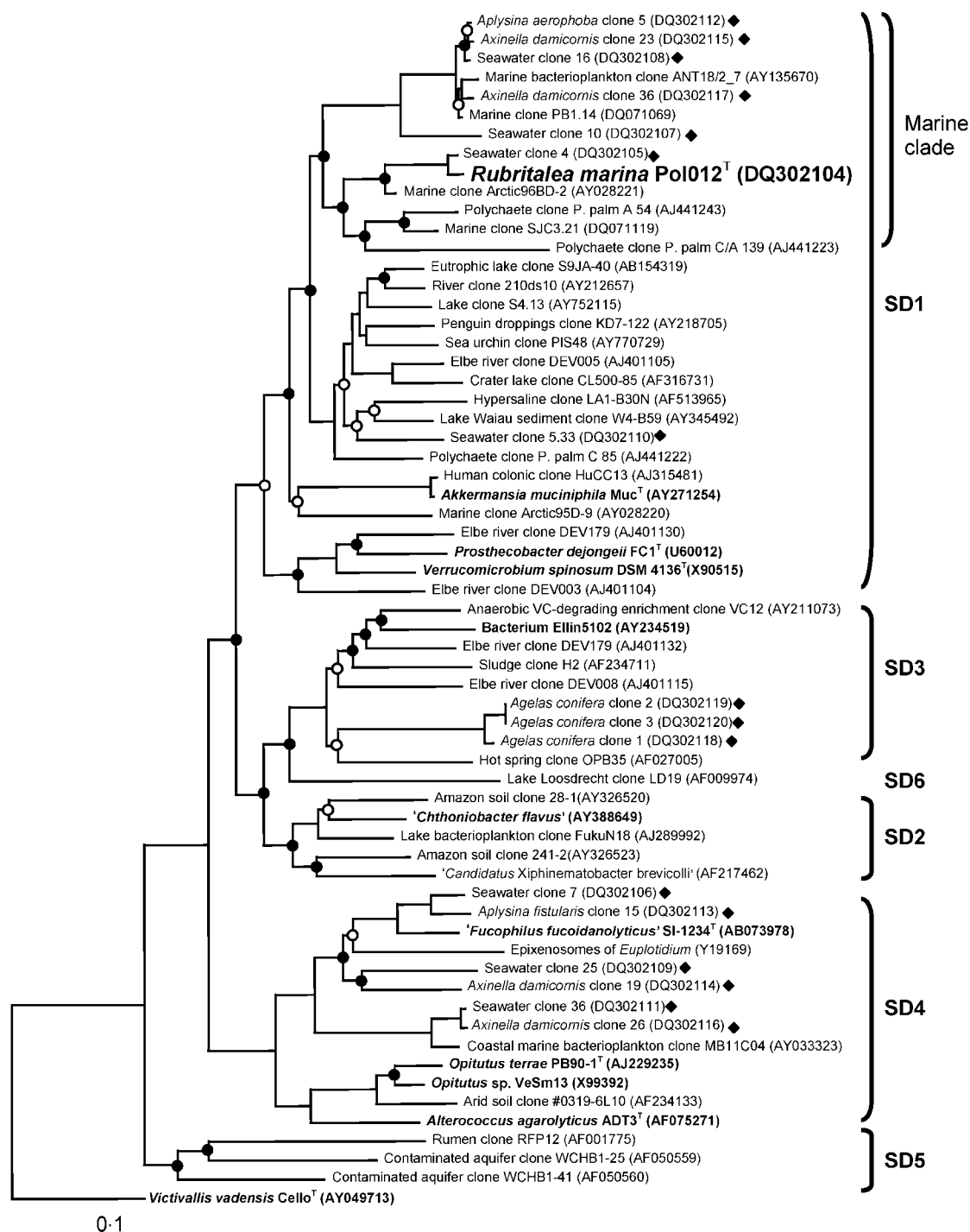
*mutiniphila* (Derrien *et al.*, 2004), and a sequence similarity of 95 % with respect to the closest environmental clone, Arctic96BD-2 (Bano & Hollibaugh, 2002). It was concluded that strain Pol012<sup>T</sup> is affiliated to verrucomicrobial subphylum 1. This clade includes the freshwater isolate *V. spinosum* (Schlesner, 1987), four freshwater *Prostheco bacter* species (Hedlund *et al.*, 1997), the human intestinal isolate *Akkermansia mutiniphila* (Derrien *et al.*, 2004) and several environmental 16S rRNA gene sequences. Strain Pol012<sup>T</sup> represents the first cultivated marine isolate of this subphylum (Fig. 3).

### Natural habitat of isolate Pol012<sup>T</sup>

Members of the phylum 'Verrucomicrobia' have been reported as symbionts of eukaryotes (Petroni *et al.*, 2000; Vandekerckhove *et al.*, 2000) and as being associated with marine animals and plants (Alain *et al.*, 2002; Bowman & Nowak, 2004; Weidner *et al.*, 2000). In this study, a novel marine isolate, strain Pol012<sup>T</sup>, was isolated from a marine sponge. In addition, several verrucomicrobial 16S rRNA



**Fig. 2.** (a, b) Scanning electron micrographs of cells of strain Pol012<sup>T</sup> growing on agar plates. Bars: (a) 10 μm; (b) 1 μm.



**Fig. 3.** Phylogenetic analysis of 16S rRNA gene sequences from cultured and uncultured representatives of the phylum '*Verrucomicrobia*'. The tree was generated by the neighbour-joining method from the ARB package and was visualized with TREEVIEW. Subphyla are labelled according to Hugenholtz *et al.* (1998) (SD1–SD5) and Vandekerckhove *et al.* (2000) (SD6). The marine clade within subphylum 1 is indicated by a bracket. Open circles indicate bootstrap values between 50 and 75% and closed circles indicate bootstrap values above 75% (1000 replicates). Diamond symbols indicate sponge- or seawater-derived environmental sequences obtained in this study. Sequences from cultured members of the phylum '*Verrucomicrobia*' are shown in bold. *Victivallis vadensis* (Zoetendal *et al.*, 2003) was used as an outgroup. Bar, 10% sequence divergence.

gene sequences were recovered from marine sponges and seawater, thus extending the range of environmental sources from which these bacteria can be isolated.

Sponges are filter-feeders that are capable of efficiently removing bacteria and small eukaryotes from seawater and translocating the food particles into their interior, where they are digested by means of phagocytosis. Sponges are also known to contain phylogenetically complex microbial communities within their tissues: these communities can amount to up to 40 % of the animal's biomass (Hill, 2004; Hentschel *et al.*, 2006; Scheuermayer *et al.*, 2006). The formation of monophyletic, sponge-specific sequence clusters in at least eight different bacterial phyla has been taken as evidence of a stable, and possibly symbiotic, association between the corresponding micro-organisms and their sponge host (e.g. Hentschel *et al.*, 2002; Fieseler *et al.*, 2004; Montalvo *et al.*, 2005). In contrast to previous studies on sponge–microbe associations, monophyletic cluster formation is not evident for the verrucomicrobial sequences recovered in this study (Fig. 3; sequences indicated by diamond symbols). Instead, the Pol012<sup>T</sup> 16S rRNA gene sequence is most closely related to a seawater 16S rRNA gene sequence from the collection site (>98 % sequence similarity). Similarly, other sponge-derived verrucomicrobial 16S rRNA gene sequences were also more closely related to seawater sequences than to those from various sponges. This indicates that marine members of the phylum 'Verrucomicrobia' can principally be isolated from sponges, but that seawater is also a likely source for this novel clade of micro-organisms. Further investigation is needed to determine whether the association of marine strain Pol012<sup>T</sup> with *Axinella polypoides* is coincidental or whether Pol012<sup>T</sup> and related strains should be considered symbionts.

### Description of *Rubritalea* gen. nov.

*Rubritalea* (Ru.bri.ta'lea. L. adj. *ruber* red; L. fem. n. *talea* a rod, staff; N.L. fem. n. *Rubritalea* a red-coloured rod).

Cells are Gram-negative, non-motile, coccoid or rod-shaped, obligately marine and red in colour. Metabolism is aerobic and chemo-organotrophic.

### Description of *Rubritalea marina* sp. nov.

*Rubritalea marina* (ma.ri'na. L. fem. adj. *marina* of or belonging to the sea, marine).

Cells are Gram-negative, non-motile, coccoid or rod-shaped, and red in colour. Able to grow with glucose, xylose, melibiose, cellobiose, lactose, pyruvate or pectin as the sole energy source under aerobic conditions. Does not grow anaerobically. The DNA G + C content is 50.9 mol%. The major cellular fatty acids are hexadecanoic acid, 12-methyltridecanoic acid, *cis*-9-hexadecenoic acid and/or 2-hydroxy-13-methyltetradecanoic acid, 12-methyltetradecanoic acid and pentadecanoic acid. Menaquinones MK-8 and MK-9 are present. The peptidoglycan of the cell wall

contains direct cross-linkages of the A1γ *meso*-diaminopimelic acid type. Strain Pol012<sup>T</sup> tests negative for catalase and positive for oxidase, alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, trypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, β-glucosidase and β-galactosidase. Strain Pol012<sup>T</sup> is capable of nitrate reduction to nitrite and oxidation of dextrin, D-cellobiose, D-fructose, D-galactose, α-D-glucose, D-mannose, D-melibiose, acetic acid and formic acid. Strain Pol012<sup>T</sup> is able to grow in medium containing 60–160 % ASW, at pH values between 6.8 and 8.2 and at temperatures between 8 and 30 °C.

The type strain, Pol012<sup>T</sup> (= DSM 177716<sup>T</sup> = CIP 108984<sup>T</sup>), was isolated from the Mediterranean sponge *Axinella polypoides*.

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