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Nautilia lithotrophica gen. nov., sp. nov., a thermophilic sulfur-reducing ε-proteobacterium isolated from a deep-sea hydrothermal vent

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A novel, strictly anaerobic, thermophilic sulfur-reducing bacterium, strain 525^T, was isolated from tubes of the deep-sea hydrothermal vent polychaete *Alvinella pompejana*, collected on the East Pacific Rise (13° N). This organism grew in the temperature range 37–68 °C, the optimum being 53 °C, and in the pH range 6·4–7·4, the optimum being 6·8–7·0. The NaCl range for growth was 0·8–5·0 %, the optimum being 3·0 %. Strain 525^T grew lithoautotrophically with H_2 as energy source, S^0 as electron acceptor and CO_2 as carbon source. Alternatively, strain 525^T was able to use formate as an energy source. The G+C content of the genomic DNA was 34·7 mol %. Phylogenetic analysis of the 16S rDNA gene sequence placed strain 525^T in the ε-subclass of the *Proteobacteria*, where it forms a deep cluster with recently isolated relatives. On the basis of phenotypic and phylogenetic differences between strain 525^T and its closest phylogenetic relatives, it is proposed that the new isolate should be described as a member of a new genus, *Nautilia*, for which the name *Nautilia lithotrophica* gen. nov., sp. nov. is proposed. The type strain is strain 525^T (= DSM 13520^T).

Keywords: deep-sea hot vents, sulfur reduction, thermophile, lithotroph, epsilon-*Proteobacteria*

INTRODUCTION

Deep-sea hydrothermal vents represent a unique microbial habitat characterized by extreme temperature gradients and high concentrations of hydrogen sulfide, gases and toxic heavy metals. The vents are colonized by highly specific invertebrate fauna, the growth of which is supported by symbiotic and non-symbiotic chemosynthetic micro-organisms (Jannasch & Mottle, 1985).

Alvinella pompejana is a tube-dwelling annelid polychaete endemic to the East Pacific Rise; it inhabits the hot areas of active deep-sea hydrothermal vent chimneys. One of the most striking features of this worm is its obligate association with a highly diverse and dense assemblage of epibiotic micro-organisms. Dorsal epidermal expansions are covered by filamentous morphotypes that dominate the worm–bacteria association. To date, these dominant morphotypes have eluded all attempts at culture. However, molecular methods have identified constant features of the associated microflora as being those of representatives of the ε-subclass of the *Proteobacteria* (Haddad *et al.*, 1995; Cary *et al.*, 1997). Very recently, four thermophilic ε-*Proteobacteria* associated with the worm's epidermis, but phylogenetically distant from its epibionts, were isolated and characterized (Campbell *et al.*, 2001). In this study, we report the isolation, from tube fragments of *A. pompejana*, of a closely related organism and propose that it represents the type species of a new genus.

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The GenBank accession number for the 16S rDNA sequence of strain 525^T is AJ404370.

METHODS

Sampling. The new strain was isolated from tube fragments of an *A. pompejana* specimen sampled at the 13° N hydrothermal vent field (12° 48′ N, 103° 56′ W), during the Amistad cruise (in 1999), on the East Pacific Rise at a depth of 2600 m.

Enrichment and isolation. For the enrichment of thermo-

philic sulfur-reducing bacteria, the following basal medium was used: NH_4Cl, 0·33 g l^{-1}; KCl, 0·33 g l^{-1}; KH_2PO_4, 0·33 g l^{-1}; $CaCl_2.2H_2O$, 0·33 g l^{-1}; $MgCl_2.6H_2O$, 0·33 g l^{-1}; NaCl, 25·0 g l^{-1}; yeast extract, 0·1; $Na_2S.9H_2O$, 0·5 g l^{-1}; $NaHCO_3$, 0·5 g l^{-1}; resazurin, 0·002 g l^{-1}; trace elements (Balch *et al*., 1979), 1 ml l^{-1}; vitamins (Wolin *et al*., 1963), 1 ml l^{-1}. The medium was prepared anaerobically (Balch *et al*., 1979) and dispensed in 15 ml Hungate tubes, the headspaces (10 ml) being filled with an H_2/CO_2 mixture (8:2, v/v; atmospheric pressure). Elemental sulfur was added as a sulfur flower aqueous suspension to a final concentration of 10 g l^{-1}. The pH of the medium was adjusted with 2·5 M H_2SO_4 or with 5 M NaOH to 6·8–7·0. When substrates other than molecular hydrogen were tested, the headspaces were filled with N_2/CO_2 (8:2, v/v; atmospheric pressure). Colonies were obtained on the same basal medium with 0·3 % formate and without yeast extract, solidified by 1·5 % agar (Difco). In this case, sulfur was substituted by polysulfides (Widdel & Pfennig, 1992). Agar shake tubes were incubated at 55 °C for 3–5 days.

Morphological and ultrastructural studies. The morphology of the new isolate was examined using a light microscope (Mikmed-1; LOMO). The ultrastructure of the whole cells and thin sections was studied as described by Bonch-Osmolovskaya *et al*. (1990).

Physiological studies. Potential growth substrates and electron acceptors were added at concentrations of 0·3 and 0·2 % (w/v), respectively. Tests for growth with ferric iron as an electron acceptor were performed in sulfide-free medium with 90 mM amorphous ferric oxide replacing the elemental sulfur. For carbon-source examination, the headspace was filled with 100 % H_2 (atmospheric pressure), and $NaHCO_3$ was omitted from the medium. The concentration of each possible carbon source was 0·05 %. When possible nitrogen sources were tested, NH_4Cl in the medium was replaced by $NaNO_3$, glutamate, yeast extract, gelatin, tryptone and urea (final concentration 0·04 %). Inoculated tubes were incubated at 55 °C. Bacterial growth was followed by examining the turbidity, and checked by visualization under a light microscope and by measurement of the hydrogen sulfide concentration in the medium.

Analytical methods. The cell density was determined by direct cell counting using a light microscope. Gaseous and liquid fermentation products were detected by means of gas–liquid chromatography (Miroshnichenko *et al*., 1994). Hydrogen sulfide was measured by a colorimetric method (Trüper & Schlegel, 1964). All experiments were done in triplicate. Determination of the G+C content of the DNA was performed using the HPLC method of Mesbah *et al*. (1989), using conditions as described by Labrenz *et al*. (2000).

Antibiotic susceptibility. The sensitivity of strain 525^T to rifampicin, chloramphenicol, vancomycin, penicillin and streptomycin (at final concentrations of 10, 25, 50 and 100 μg ml^{-1}, respectively) was tested at 55 °C.

16S rDNA-based phylogenetic analysis. Extraction of genomic DNA, PCR-mediated amplification of the 16S rDNA and direct sequencing of the purified PCR product were carried out according to Rainey *et al*. (1996). The 16S rDNA was assembled from overlapping PCR fragments obtained in the forward and reverse directions. About 70 % of DNA was double-stranded and 30 % single-stranded. The sequence reaction mixtures were electrophoresed using a model 373A automatic DNA sequencer (Applied Biosystems). The 16S rDNA sequences were aligned with published sequences obtained from the EMBL Nucleotide Sequence Database (Cambridge) and the Ribosomal Database Project (RDP) using the ae2 editor (Maidak *et al*., 1996). Evolutionary distances were calculated by the methods of De Soete (1983), Jukes & Cantor (1969) and Felsenstein (1993), and included neighbour-joining and maximum-likelihood and consensus analyses. Bootstrap analysis was used to evaluate the tree topology of the neighbour-joining data by performing 500 resamplings (Felsenstein, 1993).

RESULTS

Enrichments and isolation

Anaerobic medium with H_2 serving as an electron donor and elemental sulfur as an electron acceptor was inoculated with tube fragments of *A. pompejana* and incubated at 55 °C. After 3 days incubation, an intense growth of morphologically diverse micro-organisms

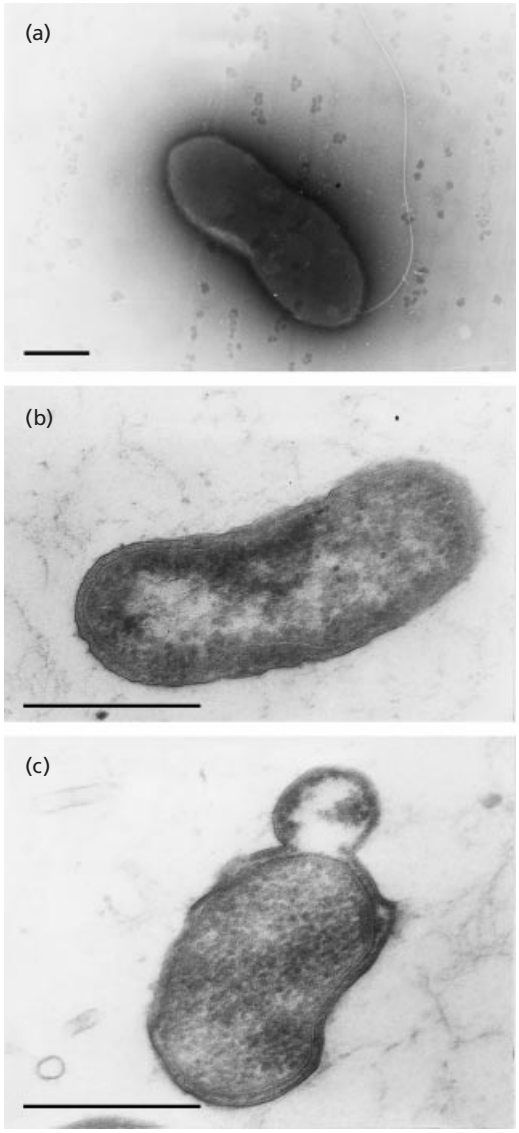

Fig. 1. Negatively stained whole cells (a) and thin sections (b, c) of strain 525^T. Bars, 0·5 μm.

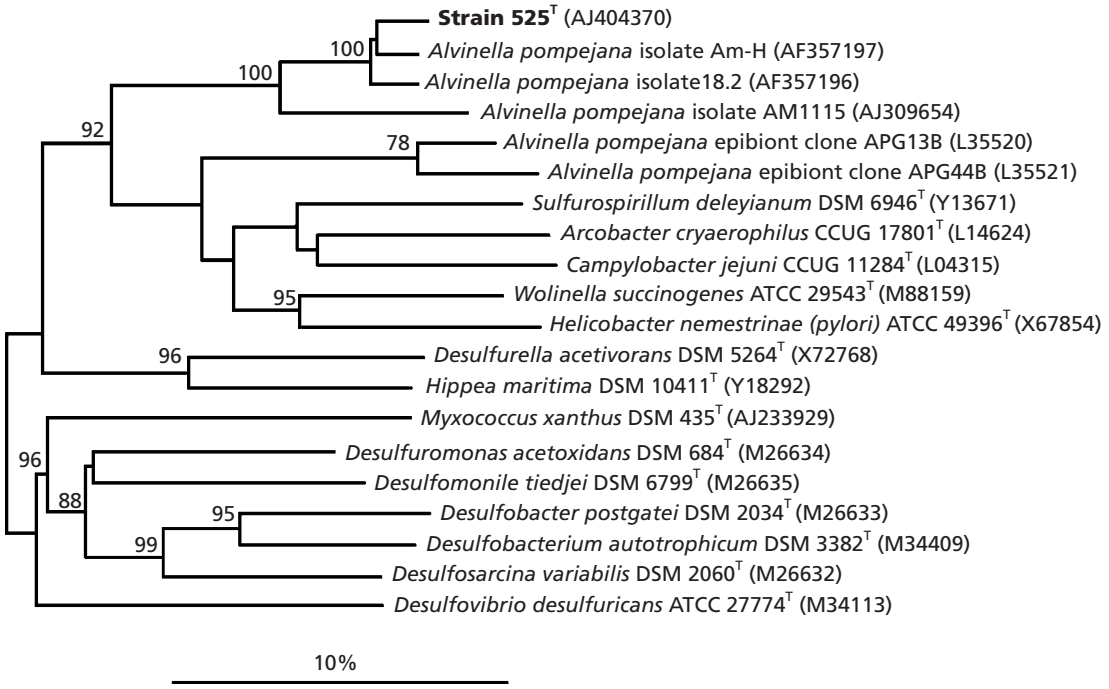

Fig. 2. Phylogenetic position of strain 525^T, as derived by comparative 16S rDNA analyses using the neighbour-joining algorithm (Felsenstein, 1993). The bar indicates 10% sequence divergence. Numbers at branching points refer to bootstrap values (only those values greater than 75% are shown).

was observed, among them cocci and long and short motile rods. Growth was accompanied by the production of large amounts of H_2S (up to 25 mM). Cultures were diluted and transferred into a formate-containing medium solidified with agar. After 5 days incubation at 55 °C, single colonies were visible in the tubes inoculated with the highest dilutions. Colonies were round, milk-white and 0·5–1 mm in diameter. When cultures were transferred into mineral medium without yeast extract, the growth of short, extremely motile rods was observed after 2–3 days cultivation. The purification procedure was repeated twice. The purity of the culture was checked microscopically and by the absence of growth in a non-selective glucose- and peptone-containing medium (each 0·3%). The pure strain designated strain 525^T was studied in detail.

Morphology and ultrastructure of the new isolate

The cells of strain 525^T were short rods (0·4–0·75 μm wide × 1·1–2·0 μm long), motile by means of single polar flagellum (Fig. 1a). The formation of spores was never observed. The shape of cells varied from almost round to rod-like, sometimes with empty spaces at the ends. At the stationary phase of growth, giant cells (three times longer than usual) were observed. Thin sectioning revealed the Gram-negative structure of the cell wall (Fig. 1b, c).

Growth characteristics

Strain 525^T is an obligately anaerobic micro-organism. No growth was observed in aerated medium and in the medium containing low levels of oxygen. Growth was observed in the temperature range 37–68 °C, the optimum being around 53 °C. The pH range for growth was 6·4–7·4, the optimum being 6·8–7·0. The optimal NaCl concentration for growth was 3·0% (w/v); no growth was observed below 0·8% NaCl or above 5·0% NaCl in the medium. Under optimal conditions (53 °C, pH 6·8, 3% NaCl), the doubling time of strain 525^T on the medium with formate and elemental sulfur was 140 min.

Strain 525^T grew on molecular hydrogen or sodium formate as energy sources with elemental sulfur as electron acceptor. The only products detected during the growth on both substrates were H_2S and CO_2. Acetate, butyrate, propionate, methanol, ethanol, pyruvate, lactate, fumarate, malate, succinate, monomethylamine, glucose, sucrose, starch, peptone and yeast extract did not support growth in the presence or the absence of elemental sulfur. Weak and slow growth was obtained when sulfite and colloidal sulfur (Sigma–Aldrich Chimie) were tested as electron acceptors. Strain 525^T did not grow when other possible electron acceptors like sulfate, thiosulfate, nitrate, malate and ferric iron were provided.

To examine possible carbon sources other than CO_2, acetate, pyruvate, formate, methylamine, methanol and malate were tested. None of them, except formate, supported growth of strain 525^T.

NH_4Cl as a nitrogen source could be substituted by $NaNO_3$, glutamate, yeast extract, gelatin, tryptone

and urea. Significant growth was observed with NH_4Cl, urea, tryptone and yeast extract. Growth was lower and slower with glutamate and nitrate.

Strain 525^T was sensitive to rifampicin (50 μg ml^{-1}), chloramphenicol (25 μg ml^{-1}), vancomycin, penicillin (10 μg ml^{-1}) and streptomycin (100 μg ml^{-1}).

DNA base composition

The G+C content of strain 525^T was 34·7 mol%, as determined by the HPLC method.

Phylogenetic analysis

Comparison of the almost complete 16S rDNA sequence of strain 525^T against the database indicated membership of the *ε-Proteobacteria*. The highest similarity values (90·4–96·7%) were found with sequences of recently isolated organisms associated with the *Alvinella* microbial community and hydrothermal chimneys (Campbell *et al*., 2001). Phylogenetic analyses showed that these sequences formed a monophyletic unit whose internal branches were supported by bootstrap analysis (Fig. 2). The lowest similarity values (83·7–85·4%) were shared with *Hippea maritima* and *Desulfurella* strains, a lineage branching intermediate to the *ε*- and *δ*-subclasses of the *Proteobacteria* but which has not yet received the status of a subclass. Similarity values among strain 525^T and other members of the *ε*-subclass, e.g. *Sulfospirillum*, *Campylobacter* and related taxa and the as yet uncultured strains from the hydrothermal vent polychaete *A. pompejana* (Haddad *et al*., 1995), ranged between 81·3 and 83·5%.

DISCUSSION

Dissimilatory sulfur reduction has been shown to be one of the major catabolic reactions in hyperthermophilic archaea and was found in representatives of numerous taxa isolated from terrestrial, shallow-water and deep-sea hydrothermal habitats (Stetter, 1996; Huber *et al*., 2000a). The metabolic capacity is also shared by some thermophilic bacteria from terrestrial and shallow-water submarine hot vents (Bonch-Osmolovskaya, 1994) that encompass several phylogenetic lineages. Among them, representatives of the genera *Desulfurella* (Bonch-Osmolovskaya *et al*., 1990; Miroshnichenko *et al*., 1994, 1998) and *Hippea* (Miroshnichenko *et al*., 1999) do not belong to any of the known subclasses of the *Proteobacteria* (Rainey *et al*., 1993) and form independent branches.

In this study, we report the isolation and characterization of a novel, chemolithotrophic sulfur-reducing organism from deep-sea hydrothermal vents. Numerous thermophilic, strictly lithoautotrophic prokaryotes have been isolated from this extreme environment (Prieur *et al*., 1995). Most of them are hyperthermophilic archaea such as methanogens of the genera *Methanopyrus* (Kurr *et al*., 1991) and *Methanococcus* (Jones *et al*., 1983, 1989; Jeanthon *et al*., 1998, 1999a, b; Zhao *et al*., 1988), hydrogen-oxidizers of the genus *Pyrolobus* (Blöchl *et al*., 1997) and sulfur-reducers of the genus *Ignicoccus* (Huber *et al*., 2000b). In the domain *Bacteria*, extremely thermophilic sulfur-reducing representatives of *Desulfurobacterium thermolithotrophum* (L'Haridon *et al*., 1998; L'Haridon & Jeanthon, 2001) and the recently described sulfate-reducer *Thermodesulfobacterium hydrogeniphilum* (Jeanthon *et al*., 2002) complete the list of strictly thermophilic lithotrophs thriving at deep-sea vents.

Isolate 525^T described here is a strictly anaerobic thermophilic bacterium capable of chemolithoautotrophic growth with molecular hydrogen and elemental sulfur. Alternatively, this organism uses formate as electron donor and carbon source. Analysis of the 16S rDNA of the new organism revealed that it belongs to the *ε*-subclass of the *Proteobacteria* and that its closest phylogenetic relatives are strains recently isolated from deep-sea hydrothermal vents but not yet formally described (Campbell *et al*., 2001). Strain 525^T shares some characteristics with strain Am-H, its closest phylogenetic relative (which was roughly characterized), but also exhibits significant physiological differences. In contrast to strain Am-H, strain 525^T is unable to use pyruvate as a carbon source and electron donor. Moreover, its maximum and optimum growth temperature are 13 and 8 °C higher than those of strain Am-H, respectively. It is evident from the degree of 16S rDNA similarity between strain 525^T and any of the reference strains represented in the 16S rDNA databases that this strain represents a novel species and a novel genus. The decision to describe a higher taxon should await complete analysis of additional members of this new lineage. On the basis of 16S rDNA analysis and phenotypic differences with respect to its closest phylogenetic relatives, we propose to classify strain 525^T as *Nautilia lithotrophica* gen. nov., sp. nov.

Assessment of the microbial diversity, using molecular methods, revealed that *ε-Proteobacteria* dominate in various deep-sea hydrothermal habitats such as the microbial mats of Loihi Seamount (Moyer *et al*., 1995), the surfaces of the invertebrates (Haddad *et al*., 1995; Polz & Cavanaugh, 1995; Cary *et al*., 1997) and sulfides from the Mid-Atlantic Ridge (Reysenbach *et al*., 2000) and the South-East Pacific Rise (Longnecker & Reysenbach, 2001). Unexpectedly, our study revealed that some of the *ε-Proteobacteria* detected by molecular methods display a thermophilic way of life, a physiological trait unknown, to date, among members of this subclass.

Description of *Nautilia* gen. nov.

Nautilia (Nau.ti'li.a. N.L. fem. n. *Nautilia* from *Nautile*, the name of the French submersible used for the exploration and investigation of deep-sea hydrothermal areas).

Cells are short, very motile rods with single polar flagella. Cell wall is of the Gram-negative type. Obligate anaerobe. Moderate thermophile. Neutrophile. Requires NaCl for growth. Grows chemolithoautotrophically on molecular hydrogen, elemental sulfur and CO_2. Does not utilize sugars, peptides, organic acids or alcohols both in the absence and presence of sulfur. Weakly uses sulfite and colloidal sulfur as electron acceptors; sulfate, thiosulfate, nitrate, fumarate and ferric iron are not used. The type species is *Nautilia lithotrophica*.

Description of *Nautilia lithotrophica* sp. nov.

Nautilia lithotrophica (li.tho.tro′phi.ca. Gr. masc. n. *lithos* stone; Gr. masc. n. *trophos* consuming; Gr. fem. adj. *lithotrophica* inorganic-substrate-consuming).

Cells are short, motile rods with single polar flagella, 0·4–0·75 × 1·1–2·0 μm in size and have the Gram-negative type of cell wall. On solid medium white colonies are formed. Obligate anaerobe. Moderate thermophile growing in the range 37–68 °C, the optimum being 53 °C. Neutrophile growing in the pH range 6·4–7·4, the optimum being pH 6·8–7·0. Grows at NaCl concentrations from 0·8 to 5·0 % (w/v), the optimum concentration being 3·0 %. Utilizes H_2 or formate as energy source, elemental sulfur as electron acceptor and CO_2 as carbon source. Sugars, peptides, organic acids and alcohols do not support growth, either in the absence or presence of elemental sulfur. Does not utilize sulfate, thiosulfate, nitrate, fumarate and ferric iron as electron acceptors, but uses sulfite and colloidal sulfur weakly. Uses only CO_2 and formate, but not acetate, pyruvate, methylamine, methanol or malate as carbon sources. Uses NH_4Cl, $NaNO_3$, glutamate, yeast extract, gelatin, tryptone and urea as nitrogen sources. Sensitive to rifampicin, chloramphenicol, vancomycin, penicillin and streptomycin. The G + C content of the DNA is 34·7 mol %. Isolated from tube fragments of an *A. pompejana* specimen from the 13° N deep-sea hydrothermal vent site on the East Pacific Rise. The type strain is *Nautilia lithotrophica* 525^T (= DSM 13520^T).

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