Nautilia abyssi sp. nov., a thermophilic, chemolithoautotrophic, sulfur-reducing bacterium isolated from an East Pacific Rise hydrothermal vent

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A novel strictly anaerobic, thermophilic, sulfur-reducing bacterium, designated PH1209T, was isolated from an East Pacific Rise hydrothermal vent (13°N) sample and studied using a polyphasic taxonomic approach. Cells were Gram-negative, motile rods (approx. 1.60×0.40 μm) with a single polar flagellum. Strain PH1209T grew at temperatures between 33 and 65 °C (optimum 60 °C), from pH 5.0 to 8.0 (optimum 6.0–6.5), and between 2 and 4 % (w/v) NaCl (optimum 3 %). Cells grew chemolithoautotrophically with H2 as an energy source, S0 as an electron acceptor and CO2 as a carbon source. Strain PH1209T was also able to use peptone and yeast extract as carbon sources. The G+C content of the genomic DNA was 35 mol%. Phylogenetic analyses based on 16S rRNA gene sequencing showed that strain PH1209T fell within the order Epsiloniiales, in the class Epsilonproteobacteria. Comparative 16S rRNA gene sequence analysis indicated that strain PH1209T belonged to the genus Nautilia and shared 97.2 and 98.7 % 16S rRNA gene sequence identity, respectively, with the type strains of Nautilia lithotrophica and Nautilia profundica. It is proposed, from the polyphasic evidence, that the strain represents a novel species, Nautilia abyssi sp. nov.; the type strain is PH1209T (=DSM 21157T = JCM 15390T).

Epsilonproteobacteria are widely distributed in marine and terrestrial ecosystems (Campbell et al., 2006). They are particularly common and abundant in 30–70 °C areas of deep-sea hydrothermal vents, as indicated by their prevalence in clone libraries (Polz & Cavanaugh, 1995; Longnecker & Reysenbach, 2001; López-Garcia et al., 2002; Alain et al., 2004), the results of fluorescence in situ hybridization (Moussard et al., 2006) and the isolation of several representatives (Alain et al., 2002; Miroshnichenko et al., 2002; Inagaki et al., 2003; Takai et al., 2003, 2005, 2006; Voordekers et al., 2005). Within this singular ecosystem, members of the class Epsilonproteobacteria are retrieved in various habitats, thriving (i) as free-living organisms on chimney structures, within vent plumes and in sediments, (ii) as epi- or endosymbionts of hydrothermal invertebrates or (iii) embedded in mats on the surfaces of chimney rocks or animals. Cultured isolates from deep-sea vents are all mesophilic to thermophilic chemolithoautotrophs coupling the oxidation of hydrogen or sulfur compounds to the reduction of nitrate, sulfur compounds or oxygen (Takai et al., 2003; Campbell et al., 2006). Because of their abundance and metabolic abilities, epsilonproteobacteria are likely to be key players in carbon, sulfur and nitrogen biogeochemical cycling at deep-sea vents.

Two orders are currently described within the class Epsilonproteobacteria Garrity et al. 2006 (Garrity et al., 2005a, 2006), namely the Nautiliales (Miroshnichenko et al., 2004) and the Campylobacterales Garrity et al. 2006 (Garrity et al., 2005b, 2006). The order Nautiliales includes the genera Nautilia (Miroshnichenko et al., 2002), Caminibacter (Alain et al., 2002) and Lebetimonas (Takai et al., 2005) which are composed exclusively of thermophilic strains isolated from deep-sea hydrothermal vents and which have been found in association with invertebrates or with chimney edifices. At present, the genus Nautilia is composed of two species, Nautilia lithotrophica (Miroshnichenko et al., 2002) and Nautilia profundica.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of Nautilia abyssi PH1209T is AM937002.

Scanning electron micrographs of cells of strain PH1209T (Fig. S1) and a graph showing the maximum growth rate of strain PH1209T at various temperatures, pH and NaCl concentrations (Fig. S2) are available with the online version of this paper.
Both strains are strictly anaerobic, sulfur-reducing mixotrophs that are able to grow on hydrogen and carbon dioxide, or, alternatively, on formate. In this study, a novel marine bacterium belonging to the genus *Nautilia* is described.

In April–May 2002, during the PHARE oceanographic cruise, fragments of active hydrothermal chimney rocks covered with colonies of the tubeworm polychaete *Alvinella* spp. were collected from a depth of 2620 m at the Elsa vent field on the East Pacific Rise 13°N (12°48′N 103°56′W). Sample collection, subsampling and storage procedures were as described elsewhere (Alain et al., 2004). One subsample collected from the Ph01 chimney was used to inoculate a series of media, including KA22 medium (Alain et al., 2002), which were incubated at 60°C under a gas phase of H₂/CO₂ (80:20; 200 kPa). After 24 h incubation, dense populations of short, rod-shaped, motile cells were observed and purified by repeated dilution-to-extinction series. One isolate, strain PH1209T, is described in this study. The purity of this isolate was confirmed routinely by microscopic examination and by repeated partial sequencing of the 16S rRNA gene using several primers. Stock cultures were stored at −80°C in KA22 medium supplemented with 5% (v/v) DMSO.

Morphological characteristics of the cells were determined by light microscopy (CX40; Olympus) and scanning electron microscopy (Quanta 200; FEI). Cells of strain PH1209T were Gram-negative, straight rods of 1.0–2.2 μm in length (mean 1.6 ± 0.3 μm; n = 11) and 0.3–0.5 μm in width (mean 0.4 ± 0.1 μm; n = 11) in the mid-exponential phase of growth (see Supplementary Fig. S1 in IJSEM Online). They generally occurred singly and were highly motile by a polar flagellum (Supplementary Fig. S1a). Division was by constriction (Supplementary Fig. S1b). Formation of spores was never observed.

Physiological characterization of the novel isolate was carried out in a basal medium, ‘NPKsalts’, which contained (per litre): 0.33 g NH₄Cl, 0.33 g KCl, 0.33 g CaCl₂, 2H₂O, 0.33 g MgCl₂·6H₂O, 25 g NaCl, 1.0 g NaNO₃, 1.95 g MES buffer (Sigma) and 1 mg resazurin (Sigma). The pH was adjusted to 6.0. Once prepared, this medium was autoclaved and then cooled to room temperature under a stream of O₂-free N₂ gas. Concentrated anaerobic filter-sterilized solutions of vitamins and trace elements were added to the medium after autoclaving. Just before inoculation, Na₂S, 9H₂O, KH₂PO₄ and elemental sulfur were provided from sterile stocks to final concentrations of 0.04% (w/v), 20 mM and 1.2% (w/v), respectively. Unless stated otherwise, experiments were carried out anaerobically under a gas phase of H₂/CO₂ (80:20; 200 kPa) and incubations were performed in the dark and under agitation. Growth was routinely monitored by direct cell counting using a modified Thoma chamber (depth 10 μm) or by counting after fixation with 1% (v/v) glutaraldehyde and storage at −20°C. Growth rates were calculated using linear regression analysis of four to nine points along the logarithmic portions of the resulting growth curves. The determination of the temperature range for growth was tested over the range 30–80°C (i.e. 30, 33, 37, 45, 50, 55, 60, 65, 70, 75 and 80°C). No growth was observed at 30°C or at 70°C and above. The novel isolate grew from 33 to 65°C, with an optimum growth rate at 60°C (see Supplementary Fig. S2a in IJSEM Online). The pH range for growth was tested at 60°C in basal medium buffered and adjusted to the required initial pH as described elsewhere (Alain et al., 2002). Growth was observed from pH 5.0 to 8.0, the optimum being around pH 6.0–6.5 (Supplementary Fig. S2b). No growth was observed at pH 4.0 or 8.5. Salt tolerance was tested at 60°C in NPKsalts medium prepared with various concentrations of NaCl (0, 0.5, 1, 2, 3, 4, 5, 6, 8 and 10%, w/v). Growth was observed at salt concentrations ranging from 2 to 4% (w/v) NaCl, with optimum salinity being around 3% (Supplementary Fig. S2c). No growth was observed in 1 or 5% (w/v) NaCl. Under optimal growth conditions, the generation time of strain PH1209T was around 120 min.

Strain PH1209T was a strictly anaerobic, chemolithoautotrophic bacterium that used sulfur, hydrogen and carbon dioxide as primary electron acceptor, electron donor and carbon source, respectively. Its ability to use alternative electron acceptors was tested by adding colloidal sulfur (5 g l⁻¹; Sigma Aldrich), l-cystine (12 g l⁻¹), sulfate (1 mM), thiosulfate (20 mM), sulfate (20 mM), nitrate (10 mM), nitrite (1 mM) and oxygen (1%, v/v) to nitrate- and sulfur-depleted media, under an atmosphere of H₂/CO₂ (80:20; 200 kPa). Quantitative determination of hydrogen sulfide was carried out as described elsewhere (Cord-Ruwisch, 1985). The novel isolate grew with elemental sulfur and colloidal sulfur, with concomitant production of H₂S, but did not grow when l-cystine, sulfate, thiosulfate, sulfate, nitrate or oxygen were used as electron acceptors. To examine possible carbon sources other than CO₂, a variety of organic carbon sources was tested in the presence of sulfur, under a 100% H₂ (200 kPa) atmosphere. Formate (10 mM), acetate (10 mM), butyrate (10 mM), propionate (10 mM), methanol (0.5%, v/v), pyruvate (10 mM), lactate (0.5%, v/v), fumarate (10 mM), glucose (10 mM), peptone (2 g l⁻¹) and yeast extract (2 g l⁻¹) were tested as potential substrates. Heterotrophic growth (with concomitant H₂S production) was observed exclusively with yeast extract and peptone and was probably the result of decarboxylation of amino acids. Growth rates with yeast extract and peptone were of the same order of magnitude as that measured with carbon dioxide as the carbon source. To test the ability of the strain to use electron donors other than molecular hydrogen, the strain was cultivated under a gas phase of N₂/CO₂ (80:20; 200 kPa) in the presence of formate (20 mM), acetate (20 mM), methanol (0.5%, v/v) and yeast extract (2 g l⁻¹), with sulfur as a terminal electron acceptor. No growth was observed with the alternative energy sources, indicating that strain PH1209T was a strict hydrogen-oxidizer. Nitrogen sources for
growth were also examined in nitrogen-depleted medium. The novel isolate was found to grow on both organic and inorganic nitrogen sources. Significant growth was observed when NH₄Cl (20 mM), glutamate (10 mM), yeast extract (0.2 g l⁻¹), tryptone (0.2 g l⁻¹), gelatin (0.05 %, v/v) and urea (0.05 %, v/v) were provided as sole nitrogen sources.

Antibiotic resistance was tested in the presence of a variety of antibiotics that differed in their chemical nature, targets and mechanisms of operation. Resistance to vancomycin, streptomycin, chloramphenicol, kanamycin, rifampicin, penicillin, ampicillin and tetracycline was investigated at concentrations of 10, 25, 50 and 100 μg ml⁻¹. When the antibiotic was diluted in ethanol (chloramphenicol) or DMSO (rifampicin), the same volume of solvent was added to control cultures. All antibiotics were added aseptically before inoculation and the cultures were incubated at 60 °C for 1 week. Strain PH1209ᵀ was sensitive to vancomycin, streptomycin, chloramphenicol, penicillin, ampicillin and tetracycline, all at 10 μg ml⁻¹. It grew in the presence of 10 μg rifampicin ml⁻¹ and 25 μg kanamycin ml⁻¹, but was sensitive to higher concentrations of these two antibiotics.

The genomic DNA G+C content was determined by the Identification Service of the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany) by HPLC analysis of deoxyribo-
nucleosides as described by Mesbah et al. (1989). The G+C content of strain PH1209ᵀ was 35 mol%.

The almost complete 16S rRNA gene (1369 bp) of the strain was double-strand sequenced, as described elsewhere (Alain et al., 2002). This sequence was compared to those in available databases by using the BLAST program (Altschul et al., 1990) and then aligned to its nearest neighbours using the CLUSTAL_X program (Thompson et al., 1997). Alignments were refined manually using the SEAVIEW program (Galtier et al., 1996). Distance matrices were calculated with LASERGENE version 6 software. Phylogenetic trees were constructed with PHYLIP version 3.63 software (http://evolution.genetics.washington.edu/phylip/getme.html) on the basis of evolutionary distance (neighbour-joining method with Jukes and Cantor corrections) (Saitou & Nei, 1987) and maximum-likelihood (Felsenstein, 1981). The robustness of the inferred topologies was assessed by bootstrap analyses based on 1000 bootstrap resamplings for the neighbour-joining and 100 replications for the maximum-likelihood method (Felsenstein, 1985). Comparison of the 16S rRNA gene sequence with sequences of 'Bacteria' indicated that the novel isolate belonged to the class Epsilonproteobacteria Garrity et al. 2006 (Garrity et al., 2005a, 2006) and, more particularly, to the order Nautiliales (Miroshnichenko et al., 2004). Within this lineage, which is composed exclusively of taxa from deep-sea hydrothermal vents, the novel isolate was most closely related to a group of

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequences of strain PH1209ᵀ and representative members of related genera within the class Epsilonproteobacteria. Sequence data of reference strains were obtained from the GenBank/EMBL and/or the Ribosomal Database Project. Two species from the class Gammaproteobacteria were chosen as outgroups. Accession numbers are indicated in parentheses. The topology shown corresponds to an unrooted tree obtained by the maximum-likelihood algorithm established using the PHYLIP package. Bootstrap values (from 100 replicates) are indicated at the branch nodes. The positioning of the novel isolate was confirmed by the neighbour-joining method. Bar, 2 nt substitutions per 100 nt.
moderately thermophilic sulfur reducers, all, like PH1209\textsuperscript{T}, isolated from the East Pacific Rise 13\textdegree N (Fig. 1). The novel isolate shared 98.7\% 16S rRNA gene sequence similarity with \textit{N. profundicola} AmHT\textsuperscript{1} (Smith et al., 2008), 97.2\% with \textit{N. lithotrophica} 525\textsuperscript{T} (Miroshnichenko et al., 2002) and 97.4\% with strain Ex-18.2, a third isolate not formerly described (Campbell et al., 2001). These three closest relatives were all isolated from tubes of the worm \textit{Alvinella pompejana} and belonged to the genus \textit{Nautilia}. The novel isolate was more distantly related to members of the genera \textit{Lebetimonas} and \textit{Caminibacter}, sharing 91.9–93.2\% 16S rRNA gene sequence similarity with strains of representative species of these genera (Table 1). Based on sequence similarity and phylogenetic analyses, the novel isolate could be assigned to the genus \textit{Nautilia}. The level of 16S rRNA gene sequence dissimilarity with \textit{N. profundicola} and \textit{N. lithotrophica} suggests that the new isolate belongs to a novel species (Stackebrandt & Ebers, 2006).

The phenotypic and genotypic properties of the novel isolate described herein generally met the minimal characteristics described for the order \textit{Nautiliales} (Miroshnichenko et al., 2004). Indeed, strain PH1209\textsuperscript{T} is a marine, thermophilic, sulfur-reducing bacterium that grows chemolithoautotrophically from \textsubscript{2}H\textsubscript{2} oxidation. It branches unambiguously with other members of the order \textit{Nautiliales}. Nevertheless, strain PH1209\textsuperscript{T} can be distinguished easily from other species of the order \textit{Nautiliales} on the basis of a number of phylogenetic, genotypic and physiological features. These distinctive criteria are detailed in Table 1. In addition to the phylogenetic distance, the novel taxon differs from its closest relatives in its temperature, \textit{NaCl} and \textit{pH} ranges for growth. The generation time under optimal growth conditions of the novel strain is also slightly different from those of its relatives. Furthermore, differences in the utilization profiles of carbon sources, electron donors and electron acceptors are also observed. The novel isolate is unable to utilize formate as energy and carbon source, in contrast to \textit{N. lithotrophica} and \textit{N. profundicola}, which are able utilize formate. Finally, another distinctive criterion is the DNA G+C content. In conclusion, in view of all the above-mentioned distinctive features, it is proposed that strain PH1209\textsuperscript{T} should be assigned as the type strain of a novel species, for which the name \textit{Nautilia abyssi} sp. nov. is proposed.

\begin{table}[h]
\centering
\caption{Differential characteristics of strain PH1209\textsuperscript{T} and related species of the order \textit{Nautiliales}}

\begin{tabular}{lccccc}
\hline
Species/strains & 1 & 2 & 3 & 4 & 5 & 6 & 7 \\
\hline
\textit{Growth temperature (\degree C):} & \\
Optimum & 60 & 40 & 53 & 60 & 55 & 55 & 50 \\
\textit{Growth pH:} & \\
Range & 5.0–8.0 & 6.0–9.0 & 6.4–7.4 & 5.5–7.5 & 6.5–7.4 & 4.5–7.5 & 4.2–7.0 \\
Optimum & 6.0–6.5 & 7.0 & 6.8–7.0 & 5.5–6.0 & 6.9–7.0 & 5.5 & 5.2 \\
\textit{NaCl concentration for growth (%):} & \\
Range & 2.0–4.0 & 2.0–5.0 & 0.8–5.0 & 1.0–4.0 & 0.5–5.0 & 1.0–4.0 & 0.6–5.0 \\
Optimum & 3.0 & 3.0 & 3.0 & 2.0–2.5 & 3.0 & 3.0 & 2.0 \\
\textit{Generation time (min)} & 120 & 360 & 140 & 90 & 40 & 50 & 120 \\
\textit{Utilization of C source other than CO\textsubscript{2}:} & \\
Complex organic substrates & + & ND & ND & + & ND & ND & – \\
\textit{Utilization of electron donor other than H\textsubscript{2}:} & \\
\textit{Utilization of electron acceptor other than S\textsubscript{0}:} & \\
Nitrate & – & – & – & + & + & + & – \\
Sulfite & – & ND & W & ND & – & – & – \\
Colloidal sulfur & + & ND & W & ND & ND & ND & ND \\
\textit{DNA G+C content (mol\%)} & 35.0 & 33.5 & 34.7 & 29.1 & 32.1 & 25.6 & 34.0 \\
\textit{16S rRNA gene sequence similarity*} & 100 & 98.7 & 97.2 & 92.8 & 92.4 & 93.2 & 91.9 \\
\hline
\end{tabular}

*The 16S rRNA gene sequence similarity (%) is calculated in reference to the 16S rRNA gene sequence of the novel isolate PH1209\textsuperscript{T}.

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Description of Nautilia abyssi sp. nov.

Nautilia abyssi (a.bys’si. L. gen. n. abyssi of an abyss, of the great deep).

Cells are Gram-negative, motile rods, approximately 1.6 μm in length and 0.4 μm in width, with a single polar flagellum. Optimal growth occurs at 60 °C, with a growth range of 33–65 °C. The pH and NaCl ranges are 5.0–8.0 (optimum 6.0–6.5) and 2–4% (w/v) (optimum, 3% w/v NaCl), respectively. Growth occurs under strictly anaerobic conditions using H₂ as electron donor, elemental sulfur (or colloidal sulfur) as a terminal electron acceptor and CO₂ as a carbon source. Yeast extract and peptone can be used as alternative carbon sources, but not formate, acetate, methanol, lactate, propionate, fumarate, malate, citrate, pyruvate, glucose or glycogen. L-Cystine, thiosulfate, sulfate, sulfite, nitrate, nitrite and oxygen are not utilized as electron acceptors. Formate, acetate, methanol and yeast extract are not used as electron donors. Sensitive to the following antibiotics at 10 μg ml⁻¹: vancomycin, streptomycin, chloramphenicol, penicillin, ampicillin and tetracycline. Sensitive to 25 μg rifampicin ml⁻¹ and 5 μg kanamycin ml⁻¹.

The type strain, PH1209T (= DSM 21157T = JCM 15390T), was isolated from the walls of an active deep-sea hydrothermal chimney colonized with alvinellid worms, on the East Pacific Rise (12° 48’ N 103° 56’ W). It is also available under request at the ‘Souchothèque de Bretagne’ (catalogue LMBE) culture collection (http://www.ifremer.fr/souchothèque/). The genomic DNA G+C content of strain PH1209T is 35 mol%.

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