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

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Footnote: The GenBank/EMBL/DDBJ accession number for the 16S rDNA sequence of *Nautilia abyssi* PH1209^T is AM937002.

Scanning electron micrographs of cells of strain PH1209^T (*Nautilia abyssi* sp. nov.) (Fig S1) and a graph showing the maximum growth rate of strain PH1209^T at varying temperatures, pH and NaCl concentrations (Fig. S2) are available in IJSEM online.

A novel, strictly anaerobic, thermophilic, sulfur-reducing bacterium designated PH1209^T, was isolated from an East Pacific Rise hydrothermal vent (13°N) sample and subjected to a polyphasic taxonomic analysis. The cells were Gram-negative motile rods (approximately 1.60 x 0.40 µm) with a single polar flagellum. Strain PH1209^T 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 H_2 as an energy source, S° as an electron acceptor and CO_2 as a carbon source. Alternatively, strain PH1209^T was 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 PH1209^T fell within the order *Nautiliales*, in the class *Epsilonproteobacteria*. Comparative 16S rRNA gene sequence analysis indicated that strain PH1209^T belonged to the genus *Nautilia*, and shared, respectively, 97.2 and 98.7% 16S rRNA gene sequence identity with *Nautilia lithotrophica* and *Nautilia profundicola*. It is proposed, from the polyphasic evidence, that the strain should be placed into a novel species, *Nautilia abyssi* sp. nov. The type strain is PH1209^T (= DSM 21157^T = JCM 15390^T).

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 the clone libraries (Polz & Cavanaugh, 1995; Longnecker & Reysenbach, 2001; Lopez-Garcia, 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; Voordeckers *et al*., 2005). Within this singular ecosystem, *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 of the carbon, sulfur and nitrogen biogeochemical cycling at deep-sea vents.

Two orders are currently described within the class *Epsilonproteobacteria* Garrity *et al.* 2006 (Validation List n°107, Garrity *et al*., 2005), namely the *Nautiliales* (Miroshnichenko *et al*., 2004) and the *Campylobacterales* Garrity *et al*. 2006 (Validation List n°107, Garrity *et al*., 2005). The order *Nautiliales* comprises the genera *Nautilia* (Miroshnichenko *et al*., 2002), *Caminibacter* (Alain *et al*., 2002) and *Lebetimonas* (Takai *et al*., 2005) which are exclusively composed 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 profundicola* (Smith *et al*., 2008). Both strains are strictly anaerobic sulfur-reducing mixotrophs 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. Based on the results of a polyphasic taxonomic analysis, the strain PH1209^T represents a novel species, *Nautilia abyssi* sp. nov.

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 2620m depth 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 on the Ph01 chimney was used to inoculate series of media, including KA22 medium (Alain *et al*., 2002), and incubated at 60°C under a gas phase of H_2/CO_2 (80/20; 200 kPa). After 24h incubation, dense populations of short, rod-shaped, motile cells were observed and purified by repeated dilution to extinction series. One isolate, referenced as strain PH1209^T, is described in this publication. 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 (Olympus CX40) and by scanning electron microscopy (FEI Quanta 200). Cells of strain PH1209^T were Gram-negative, straight rods of 1.05-2.21 μm in length (mean 1.63μm ± 0.34, n=11) and 0.30-0.51 μm in width (mean 0.39 ± 0.05, n=11) in the mid-exponential phase of growth (see Supplementary Fig. S1 in IJSEM online). They occurred mainly singly and were highly motile by a polar flagellum (Fig. S1A). Division was by constriction (Fig. S1B). Formation of spores was never observed.

The physiological characterization of the novel isolate was carried out in a basal medium referenced as "NPKsalts" and containing (per liter): 0.33 g NH_4Cl, 0.33 g KCl, 0.33 g $CaCl_2.2H_2O$, 0.33 g $MgCl_2.6H_2O$, 25 g NaCl, 1.0 g $NaNO_3$, 1.95 g MES buffer (Sigma) and 1 mg resazurin (Sigma). Its pH was adjusted to

6.0. Once prepared, this medium was autoclaved and then cooled to room temperature under a stream of O_2-free N_2 gas. Concentrated anaerobic filter-sterilized solutions of vitamins and trace elements were added to the medium after autoclaving. Then, just before inoculation, $Na_2S.9H_2O$, KH_2PO_4 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, the experiments were carried out anaerobically, under a gas phase of H_2/CO_2 (80/20; 200 kPa), and incubation were done 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, 80°C). No growth was observed at 30°C, 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 pH 8.0, the optimum being around pH 6.0-6.5 (Fig. S2B). No growth was observed at pH 4.0 and pH 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, the optimum salinity being around 3% (Fig. S2C). No growth was observed at 1 and 5% (w/v) NaCl. Under optimal growth conditions, the generation time of strain PH1209^T was around 120 minutes.

Strain PH1209^T was a strictly anaerobic, chemolithoautotrophic bacterium that used sulfur, hydrogen and carbon dioxide as respective primary electron acceptor, electron donor and carbon source. Its ability to use alternative electron acceptors was tested by adding colloidal sulfur (Sigma Aldrich) (5 g l^{-1}), L-cystine (12 g l^{-1}), sulfite (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 H_2/CO_2 (80/20; 200 kPa). Quantitative determination of hydrogen sulfide was as described elsewhere (Cord-Ruwisch, 1985). The novel isolate was found to grow with elemental sulfur and colloidal sulfur, with concomitant production of H_2S, but did not grow when L-cystine, sulfite, thiosulfate, sulfate, nitrate, nitrite and oxygen were used as electron acceptors.

To examine possible carbon sources other than CO_2, a variety of organic carbon sources were tested in the presence of sulfur, under an atmosphere of H_2 100% (200 kPa). 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 H_2S production) was observed exclusively with yeast extract and peptone and was probably the result of the decarboxylation of amino acids. The growth rates with yeast extract and peptone were in the same order of magnitude than the one measured with carbon dioxide as carbon source. To test for the capability of the strain to use electron donors other than molecular hydrogen, the strain was cultivated under a gas phase of N_2/CO_2 (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}), and with sulfur as a terminal electron acceptor. No growth was observed with the alternative energy sources, indicating that strain PH1209^T was a strict hydrogen-oxidizer. The nitrogen sources for growth were also examined in a nitrogen-depleted medium. The novel isolate was found to grow on organic and inorganic nitrogen sources. Significant growth was observed when NH_4Cl (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 source.

Antibiotic resistance was tested in the presence of a variety of antibiotics from different chemical nature and with different targets and mechanisms. The 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 one week. Strain PH1209^T was found to be sensitive to vancomycin, streptomycin, chloramphenicol, penicillin, ampicillin and tetracycline, all at 10 μg ml^{-1}. It grew in the presence of 10 μg ml^{-1} rifampicin and 25 μg ml^{-1} kanamycin, 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 deoxyribonucleosides as described by Mesbah *et al*. (1989). The G+C content of strain PH1209^T 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 use of the BLAST program (Altschul *et al*., 1990) and then aligned to its nearest neighbours using the CLUSTALX program (Thompson *et al*., 1997). Alignments were refined manually using the SEAVIEW program (Galtier *et al*., 1996). Distance matrixes were calculated with the Lasergene 6 version software. Phylogenetic trees were constructed by the PHYLIP (PHYlogeny Inference Package) 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 and 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 (Validation List n°107, Garrity *et al*., 2005) and more especially to the order *Nautiliales* (Miroshnichenko *et al*., 2004). Within this lineage that is composed exclusively of taxa from deep-sea hydrothermal vents, the novel isolate was found to be most closely related to a group of moderately thermophilic sulfur reducers, all isolated from the EPR 13°N like PH1209^T (Fig. 1). The novel isolate shared 98.7% 16S rRNA gene sequence identity with *Nautilia profundicola* strain AmH^T (Smith *et al.*, 2008), 97.2% 16S rRNA gene sequence identity with *Nautilia lithotrophica* strain 525^T (Miroshnichenko *et al*., 2002) and 97.4% 16S rRNA gene sequence identity 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 *Alvinella pompejana* and belonged to the genus *Nautilia*. Otherwise, the novel isolate was most distantly related to members of the genera *Lebetimonas* and *Caminibacter*, sharing 91.9% to 93.2% 16S rRNA gene sequence identity with representative species of these genera (Table 1). Based on the sequence similarity and phylogenetic analyses, the novel isolate could be assigned to the genus *Nautilia.* The level of 16S rDNA sequence

dissimilarity with *N. profundicola* and *N. lithotrophica* suggests that the novel isolate belongs to a novel species (Stackebrandt and Ebers, 2006).

The phenotypic and genotypic properties of the novel isolate described herein generally met the minimal characteristics described for the order *Nautiliales* (Miroshnichenko *et al*., 2004). Indeed, strain PH1209^T is a marine thermophilic sulfur-reducing bacterium growing chemolithoautrophically from H_2 oxidation. It unambiguously branches with other *Nautiliales*. Nevertheless, strain PH1209^T can be easily distinguished from other *Nautiliales* species in terms of a number of phylogenetic, genotypic and physiological features. These distinctive criteria are detailed in Table 1. In brief, in addition to the phylogenetic distance, the novel taxon differs from its closest relatives by its temperature, NaCl and pH ranges for growth. Its generation time under optimal growth condition is also slightly different from the ones of its relatives. Furthermore, differences in the utilization profiles of carbon sources, electron donors and electron acceptors are also observed. In contrast to its congeners *N. lithotrophica* and *N. profundicola* which are able to use formate as energy and carbon source, the novel isolate is unable. Finally, another distinctive criterion is the DNA G+C content. In conclusion, in view of all the above-mentioned distinctive features, we propose that the isolate PH1209^T should be assigned as the type strain of a novel species, for which the name *Nautilia abyssi* sp. nov. is proposed.

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 from 33 to 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 H_2 as an electron donor, elemental sulfur (or colloidal sulfur) as a terminal electron acceptor and CO_2 as a carbon source. Yeast extract and peptone can be used as alternative carbon sources, but formate, acetate, methanol, lactate, propionate, fumarate, malate, citrate, pyruvate, glucose and glycogen can not. The following are not utilized as electron acceptors: L-cystine, thiosulfate, sulfate, sulfite, nitrate, nitrite, oxygen. The following are not used as electron donors: formate, acetate, methanol and yeast extract. Sensitive to 10 μg ml^{-1} of the following antibiotics: vancomycin,

streptomycin, chloramphenicol, penicillin, ampicillin, tetracycline; sensitive to 25 μg ml^{-1} rifampicin and 50 μg ml^{-1} kanamycin. Genomic DNA G+C content of the type strain PH1209^T is 35 mol%.

The type strain, PH1209^T (DSM 21157^T, JCM 15390^T) was isolated from the walls of an active deep-sea hydrothermal chimney colonized with alvinellid worms, on the East Pacific Rise (103°56'W, 12°48'N). It is also available under request at the "Souchothèque de Bretagne" (catalogue LMBE) culture collection (http://www.ifremer.fr/souchotheque/).

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TABLES and FIGURES

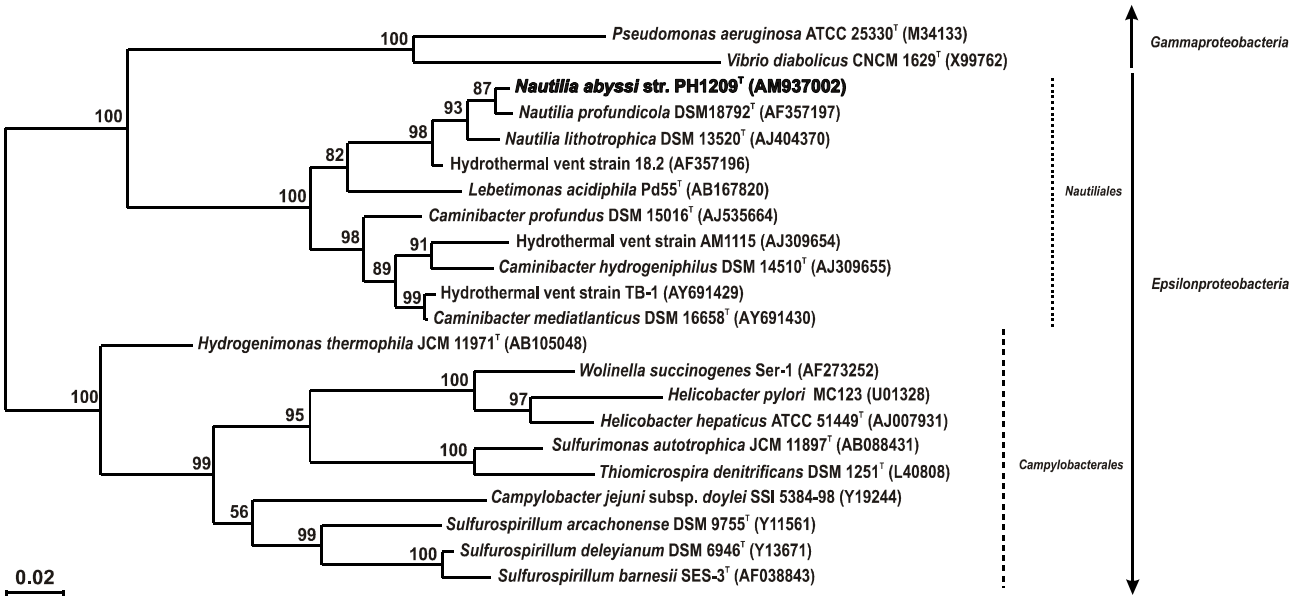

Fig. 1. **Phylogenetic tree based on 16S rRNA gene sequences of strain PH1209^T and representative members of related genera within the class *Epsilonproteobacteria***. Sequence data of reference strains were obtained from the GenBank/EMBL and/or RDP databases. Two species from the *Gammaproteobacteria* 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. The scale bar indicates 2 nt substitutions per 100 nt.

Table 1. Characteristics differentiating strain PH1209^T from related species of the order *Nautiliales*. Species: 1, *Nautilia abyssi* PH1209^T (this study); 2, *Nautilia profundicola* AmH^T (Smith *et al*., 2008); 3, *Nautilia lithotrophica* 525^T (Miroshnichenko *et al*., 2002); 4, *Caminibacter hydrogeniphilus* AM1116^T (Alain *et al*., 2002); 5, *Caminibacter profundus* CR^T (Miroshnichenko *et al*., 2004); 6, *Caminibacter mediatlanticus* TB-2^T (Voordeckers *et al*., 2005); 7, *Lebetimonas acidiphila* Pd55^T (Takai *et al*., 2005).
Legend: +, positive; ─, negative; w, weak growth; ND, not determined. The percentage of 16S rRNA gene sequence identity is calculated in reference to the 16S rRNA gene sequence of the novel isolate PH1209^T.

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Characteristic	1	2	3	4	5	6	7
Temperature range for growth (°C) [optimum]	33-65 [60]	30-55[40]	37-68 [53]	50-70 [60]	45-65 [55]	45-70 [55]	30-68 [50]
pH range for growth [optimum]	5.0-8.0 [6.0-6.5]	6.0-9.0 [7.0]	6.4-7.4 [6.8-7.0]	5.5-7.5 [5.5-6.0]	6.5-7.4 [6.9-7.0]	4.5-7.5 [5.5]	4.2-7.0 [5.2]
NaCl concentration range for growth (%) [optimum]	2.0-4.0 [3.0]	2.0-5.0 [3.0]	0.8-5.0 [3.0]	1.0-4.0 [2.0-2.5]	0.5-5.0 [3.0]	1.0-4.0 [3.0]	0.6-5.0 [2.0]
Generation time (min.)	120	360	140	90	40	50	120
Utilization of C source other than CO_2							
Formate	–	+	+	–	–	–	–
Complex organic substrates	+	ND	ND	+	ND	ND	–
Utilization of electron donor other than H_2							
Formate	–	+	+	–	–	–	–
Utilization of electron acceptor other than S°							
Oxygen	–	–	–	–	+	–	–
Nitrate	–	–	–	+	+	+	–
Sulfite	–	ND	w	ND	–	–	–
Colloidal sulfur	+	ND	w	ND	ND	ND	ND
DNA G+C content (mol%)	35.0	33.5	34.7	29±1	32.1	25.6	34.0
16S rRNA gene sequence identity (%)	100	98.7	97.2	92.8	92.4	93.2	91.9

310

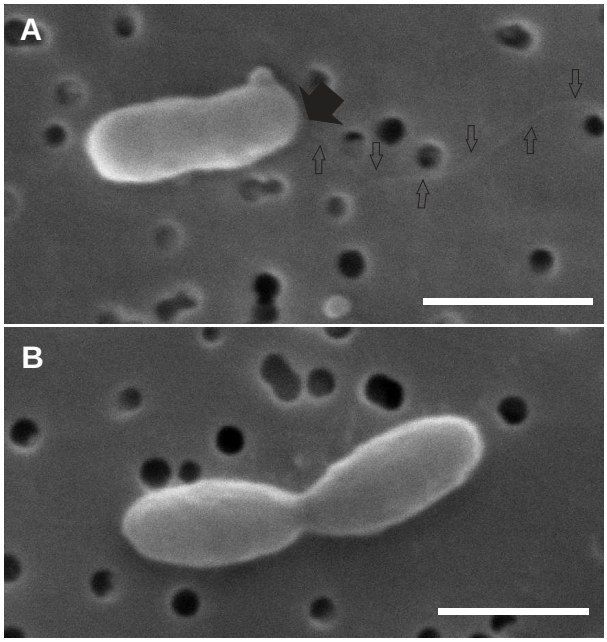
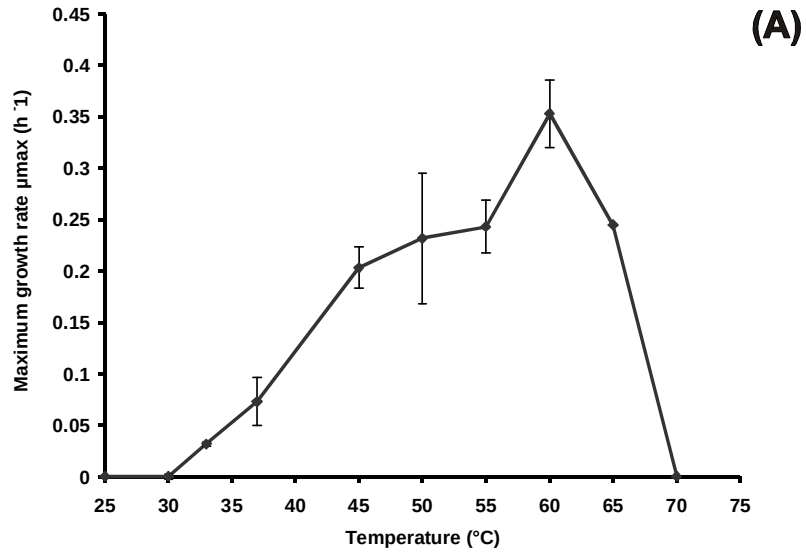
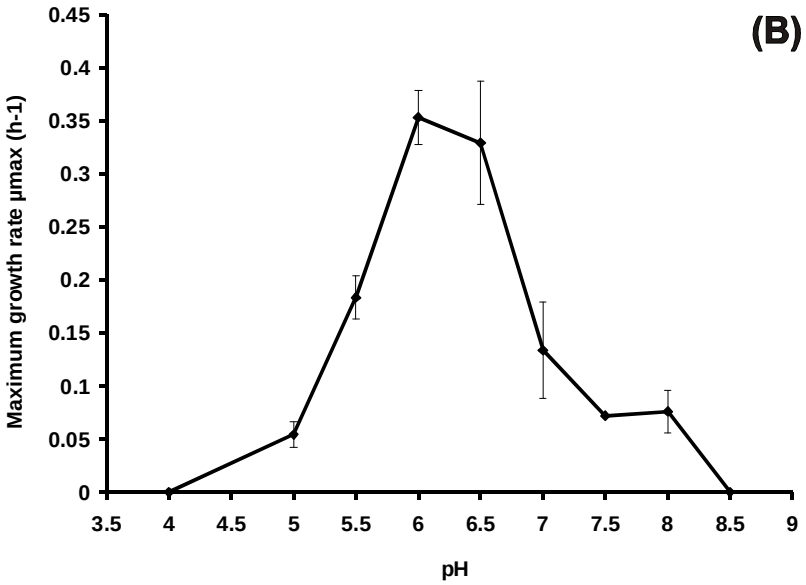
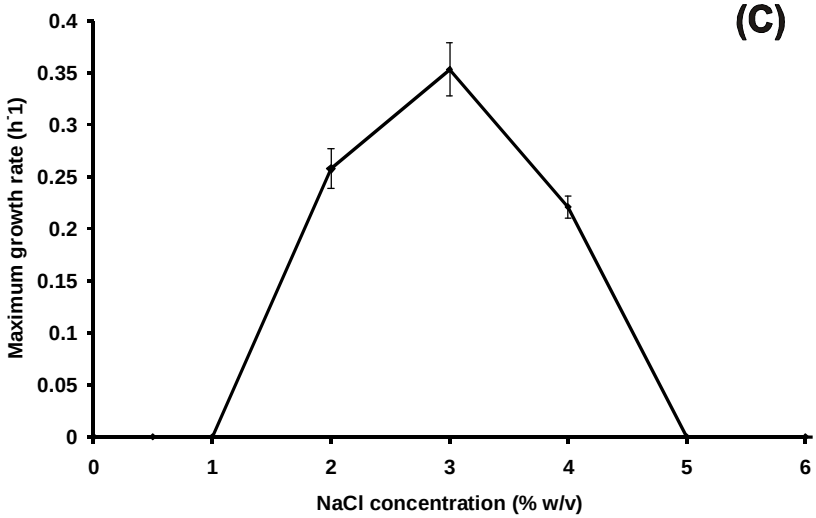


Fig. S1. Scanning electron micrographs of cells of strain PH1209^{T} in the mid-exponential phase of growth, showing the polar flagellum (A) and division by constriction (B). Bar, 1.0 μm.


(A)
Maximum growth rate µmax (h-1)
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
25 30 35 40 45 50 55 60 65 70 75
Temperature (°C)


(B)
Maximum growth rate µmax (h-1)
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
3.5 4 4.5 5 5.5 6 6.5 7 7.5 8 8.5 9
pH


(C)
Maximum growth rate (h-1)
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
0 1 2 3 4 5 6
NaCl concentration (% w/v)

315

316

317 **Fig. S2. Maximum growth rate (h^{-1}) of strain PH1209^{T} (*Nautilia abyssi* sp. nov.) at varying**
318 **temperatures (A), pH (B) and NaCl concentrations (C).** Bars indicate confidence intervals.

319

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