

***Nautilia abyssi* sp. nov., a thermophilic, chemolithoautotrophic, sulfur-reducing bacterium isolated from an East Pacific Rise hydrothermal vent**Karine Alain^{1,*}, Nolwenn Callac¹, Marianne Guégan¹, Françoise Lesongeur¹, Philippe Crassous², Marie-Anne Cambon-Bonavita¹, Joël Querellou¹ and Daniel Prieur¹¹ UMR6197, Laboratoire de Microbiologie des Environnements Extrêmes, IUEM, Technopôle Brest-Iroise, F-29280 Plouzané, France² Laboratoire Environnement Profond, Ifremer EEP/LEP, BP 70, F-29280 Plouzané, France*: Corresponding author : Karine Alain, Phone number : +33-(0)2-98-49-88-53 Fax : +33-(0)2-98-49-87-05, email address : Karine.Alain@univ-brest.fr**Abstract:**

A novel strictly anaerobic, thermophilic, sulfur-reducing bacterium, designated PH1209^T, was isolated from an East Pacific Rise hydrothermal vent (1 ° N) sample and studied using a polyphasic taxonomic approach. Cells were Gram-negative, motile rods (approx. 1.60x0.40 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 H₂ as an energy source, S⁰ as an electron acceptor and CO₂ as a carbon source. Strain PH1209^T 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 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 97.2 and 98.7 % 16S rRNA gene sequence identity, respectively, with the type strains of *Nautilia lithotrophica* and *Nautilia profundicola*. It is proposed, from the polyphasic evidence, that the strain represents 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 sulphur 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

59 deep-sea hydrothermal vents and which have been found in association with invertebrates or with chimney
60 edifices. At present, the genus *Nautilia* is composed of two species, *Nautilia lithotrophica* (Miroshnichenko
61 *et al.*, 2002) and *Nautilia profundicola* (Smith *et al.*, 2008). Both strains are strictly anaerobic sulfur-
62 reducing mixotrophs able to grow on hydrogen and carbon dioxide, or alternatively on formate.

63 In this study, a novel marine bacterium belonging to the genus *Nautilia* is described. Based on the results of
64 a polyphasic taxonomic analysis, the strain PH1209^T represents a novel species, *Nautilia abyssi* sp. nov.

65

66 In April-May 2002, during the PHARE oceanographic cruise, fragments of active hydrothermal chimney
67 rocks covered with colonies of the tubeworm polychaete *Alvinella* spp. were collected from 2620m depth at
68 the Elsa vent field, on the East Pacific Rise 13°N (12°48'N, 103°56'W). Sample collection, subsampling and
69 storage procedures were as described elsewhere (Alain *et al.*, 2004). One subsample collected on the Ph01
70 chimney was used to inoculate series of media, including KA22 medium (Alain *et al.*, 2002), and incubated
71 at 60°C under a gas phase of H₂/CO₂ (80/20; 200 kPa). After 24h incubation, dense populations of short,
72 rod-shaped, motile cells were observed and purified by repeated dilution to extinction series. One isolate,
73 referenced as strain PH1209^T, is described in this publication. Purity of this isolate was confirmed routinely
74 by microscopic examination and by repeated partial sequencing of the 16S rRNA gene using several
75 primers. Stock cultures were stored at -80°C in KA22 medium supplemented with 5% (v/v) DMSO.

76

77 Morphological characteristics of the cells were determined by light microscopy (Olympus CX40) and by
78 scanning electron microscopy (FEI Quanta 200). Cells of strain PH1209^T were Gram-negative, straight rods
79 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)
80 in the mid-exponential phase of growth (see Supplementary Fig. S1 in IJSEM online). They occurred mainly
81 singly and were highly motile by a polar flagellum (Fig. S1A). Division was by constriction (Fig. S1B).
82 Formation of spores was never observed.

83

84 The physiological characterization of the novel isolate was carried out in a basal medium referenced as
85 “NPKsalts” and containing (per liter): 0.33 g NH₄Cl, 0.33 g KCl, 0.33 g CaCl₂·2H₂O, 0.33 g MgCl₂·6H₂O,
86 25 g NaCl, 1.0 g NaNO₃, 1.95 g MES buffer (Sigma) and 1 mg resazurin (Sigma). Its pH was adjusted to

87 6.0. Once prepared, this medium was autoclaved and then cooled to room temperature under a stream of O₂-
88 free N₂ gas. Concentrated anaerobic filter-sterilized solutions of vitamins and trace elements were added to
89 the medium after autoclaving. Then, just before inoculation, Na₂S·9H₂O, KH₂PO₄ and elemental sulfur were
90 provided from sterile stocks, to final concentrations of 0.04% (w/v), 20 mM and 1.2% (w/v), respectively.
91 Unless stated otherwise, the experiments were carried out anaerobically, under a gas phase of H₂/CO₂
92 (80/20; 200 kPa), and incubation were done in the dark and under agitation. Growth was routinely
93 monitored by direct cell counting using a modified Thoma chamber (depth 10 µm), or by counting after
94 fixation with 1% (v/v) glutaraldehyde and storage at -20°C. Growth rates were calculated using linear
95 regression analysis of four to nine points along the logarithmic portions of the resulting growth curves. The
96 determination of the temperature range for growth was tested over the range 30-80°C (i.e. 30, 33, 37, 45, 50,
97 55, 60, 65, 70, 75, 80°C). No growth was observed at 30°C, 70°C and above. The novel isolate grew from 33
98 to 65°C, with an optimum growth rate at 60°C (see Supplementary Fig. S2A in IJSEM online). The pH
99 range for growth was tested at 60°C in basal medium buffered and adjusted to the required initial pH as
100 described elsewhere (Alain *et al.*, 2002). Growth was observed from pH 5.0 to pH 8.0, the optimum being
101 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
102 60°C in NPKsalts medium prepared with various concentrations of NaCl (0, 0.5, 1, 2, 3, 4, 5, 6, 8 and 10%
103 w/v). Growth was observed at salt concentrations ranging from 2 to 4% (w/v) NaCl, the optimum salinity
104 being around 3% (Fig. S2C). No growth was observed at 1 and 5% (w/v) NaCl. Under optimal growth
105 conditions, the generation time of strain PH1209^T was around 120 minutes.

106

107 Strain PH1209^T was a strictly anaerobic, chemolithoautotrophic bacterium that used sulfur, hydrogen and
108 carbon dioxide as respective primary electron acceptor, electron donor and carbon source. Its ability to use
109 alternative electron acceptors was tested by adding colloidal sulfur (Sigma Aldrich) (5 g l⁻¹), L-cystine (12 g
110 l⁻¹), sulfite (1 mM), thiosulfate (20 mM), sulfate (20 mM), nitrate (10 mM), nitrite (1 mM) and oxygen (1%
111 v/v) to nitrate and sulfur-depleted media, under an atmosphere of H₂/CO₂ (80/20; 200 kPa). Quantitative
112 determination of hydrogen sulfide was as described elsewhere (Cord-Ruwisch, 1985). The novel isolate was
113 found to grow with elemental sulfur and colloidal sulfur, with concomitant production of H₂S, but did not
114 grow when L-cystine, sulfite, thiosulfate, sulfate, nitrate, nitrite and oxygen were used as electron acceptors.

115 To examine possible carbon sources other than CO₂, a variety of organic carbon sources were tested in the
116 presence of sulfur, under an atmosphere of H₂ 100% (200 kPa). Formate (10 mM), acetate (10 mM),
117 butyrate (10 mM), propionate (10 mM), methanol (0.5% v/v), pyruvate (10 mM), lactate (0.5% v/v),
118 fumarate (10 mM), glucose (10 mM), peptone (2 g l⁻¹) and yeast extract (2 g l⁻¹) were tested as potential
119 substrates. Heterotrophic growth (with concomitant H₂S production) was observed exclusively with yeast
120 extract and peptone and was probably the result of the decarboxylation of amino acids. The growth rates
121 with yeast extract and peptone were in the same order of magnitude than the one measured with carbon
122 dioxide as carbon source. To test for the capability of the strain to use electron donors other than molecular
123 hydrogen, the strain was cultivated under a gas phase of N₂/CO₂ (80/20, 200 kPa) in the presence of formate
124 (20 mM), acetate (20 mM), methanol 0.5% (v/v) and yeast extract (2 g l⁻¹), and with sulfur as a terminal
125 electron acceptor. No growth was observed with the alternative energy sources, indicating that strain
126 PH1209^T was a strict hydrogen-oxidizer. The nitrogen sources for growth were also examined in a nitrogen-
127 depleted medium. The novel isolate was found to grow on organic and inorganic nitrogen sources.
128 Significant growth was observed when NH₄Cl (20 mM), glutamate (10 mM), yeast extract (0.2 g l⁻¹),
129 tryptone (0.2 g l⁻¹), gelatin (0.05% v/v) and urea (0.05% v/v) were provided as sole nitrogen source.

130

131 Antibiotic resistance was tested in the presence of a variety of antibiotics from different chemical nature and
132 with different targets and mechanisms. The resistance to vancomycin, streptomycin, chloramphenicol,
133 kanamycin, rifampicin, penicillin, ampicillin and tetracycline was investigated at concentrations of 10, 25,
134 50 and 100 µg ml⁻¹. When the antibiotic was diluted in ethanol (chloramphenicol) or DMSO (rifampicin),
135 the same volume of solvent was added to control cultures. All antibiotics were added aseptically before
136 inoculation and the cultures were incubated at 60°C for one week. Strain PH1209^T was found to be sensitive
137 to vancomycin, streptomycin, chloramphenicol, penicillin, ampicillin and tetracycline, all at 10 µg ml⁻¹. It
138 grew in the presence of 10 µg ml⁻¹ rifampicin and 25 µg ml⁻¹ kanamycin, but was sensitive to higher
139 concentrations of these two antibiotics.

140

141 The genomic DNA G+C content was determined, by the Identification Service of the DSMZ (Deutsche
142 Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig,

143 Germany), by HPLC analysis of deoxyribonucleosides as described by Mesbah *et al.* (1989). The G+C
144 content of strain PH1209^T was 35 mol%.

145

146 The almost complete 16S rRNA gene (1369 bp) of the strain was double-strand sequenced, as described
147 elsewhere (Alain *et al.*, 2002). This sequence was compared to those in available databases by use of the
148 BLAST program (Altschul *et al.*, 1990) and then aligned to its nearest neighbours using the CLUSTALX
149 program (Thompson *et al.*, 1997). Alignments were refined manually using the SEAVIEW program (Galtier
150 *et al.*, 1996). Distance matrixes were calculated with the Lasergene 6 version software. Phylogenetic trees
151 were constructed by the PHYLIP (PHYlogeny Inference Package) version 3.63 software
152 (<http://evolution.genetics.washington.edu/phylip/getme.html>) on the basis of evolutionary distance
153 (neighbour-joining method with Jukes and Cantor corrections) (Saitou and Nei, 1987) and maximum
154 likelihood (Felsenstein, 1981). The robustness of the inferred topologies was assessed by bootstrap analyses
155 based on 1000 bootstrap resamplings for the neighbour-joining and 100 replications for the maximum
156 likelihood method (Felsenstein, 1985). Comparison of the 16S rRNA gene sequence with sequences of
157 *Bacteria* indicated that the novel isolate belonged to the class *Epsilonproteobacteria* Garrity *et al.* 2006
158 (Validation List n°107, Garrity *et al.*, 2005) and more especially to the order *Nautiliales* (Miroshnichenko *et*
159 *al.*, 2004). Within this lineage that is composed exclusively of taxa from deep-sea hydrothermal vents, the
160 novel isolate was found to be most closely related to a group of moderately thermophilic sulfur reducers, all
161 isolated from the EPR 13°N like PH1209^T (Fig. 1). The novel isolate shared 98.7% 16S rRNA gene
162 sequence identity with *Nautilia profundicola* strain AmH^T (Smith *et al.*, 2008), 97.2% 16S rRNA gene
163 sequence identity with *Nautilia lithotrophica* strain 525^T (Miroshnichenko *et al.*, 2002) and 97.4% 16S
164 rRNA gene sequence identity with strain Ex-18.2, a third isolate not formerly described (Campbell *et al.*,
165 2001). These three closest relatives were all isolated from tubes of the worm *Alvinella pompejana* and
166 belonged to the genus *Nautilia*. Otherwise, the novel isolate was most distantly related to members of the
167 genera *Lebetimonas* and *Caminibacter*, sharing 91.9% to 93.2% 16S rRNA gene sequence identity with
168 representative species of these genera (Table 1). Based on the sequence similarity and phylogenetic
169 analyses, the novel isolate could be assigned to the genus *Nautilia*. The level of 16S rDNA sequence

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

172

173 The phenotypic and genotypic properties of the novel isolate described herein generally met the minimal
174 characteristics described for the order *Nautiliales* (Miroshnichenko *et al.*, 2004). Indeed, strain PH1209^T is a
175 marine thermophilic sulfur-reducing bacterium growing chemolithoautotrophically from H₂ oxidation. It
176 unambiguously branches with other *Nautiliales*. Nevertheless, strain PH1209^T can be easily distinguished
177 from other *Nautiliales* species in terms of a number of phylogenetic, genotypic and physiological features.
178 These distinctive criteria are detailed in Table 1. In brief, in addition to the phylogenetic distance, the novel
179 taxon differs from its closest relatives by its temperature, NaCl and pH ranges for growth. Its generation
180 time under optimal growth condition is also slightly different from the ones of its relatives. Furthermore,
181 differences in the utilization profiles of carbon sources, electron donors and electron acceptors are also
182 observed. In contrast to its congeners *N. lithotrophica* and *N. profundicola* which are able to use formate as
183 energy and carbon source, the novel isolate is unable. Finally, another distinctive criterion is the DNA G+C
184 content. In conclusion, in view of all the above-mentioned distinctive features, we propose that the isolate
185 PH1209^T should be assigned as the type strain of a novel species, for which the name *Nautilia abyssi* sp.
186 nov. is proposed.

187

188 **Description of *Nautilia abyssi* sp. nov.**

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

190 Cells are Gram-negative motile rods, approximately 1.6 µm in length and 0.4 µm in width, with a single polar flagellum.
191 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
192 6.0-6.5) and 2-4% (w/v) (optimum, 3% w/v NaCl), respectively. Growth occurs under strictly anaerobic conditions
193 using H₂ as an electron donor, elemental sulfur (or colloidal sulfur) as a terminal electron acceptor and CO₂ as a carbon
194 source. Yeast extract and peptone can be used as alternative carbon sources, but formate, acetate, methanol, lactate,
195 propionate, fumarate, malate, citrate, pyruvate, glucose and glycogen can not. The following are not utilized as electron
196 acceptors: L-cystine, thiosulfate, sulfate, sulfite, nitrate, nitrite, oxygen. The following are not used as electron donors:
197 formate, acetate, methanol and yeast extract. Sensitive to 10 µg ml⁻¹ of the following antibiotics: vancomycin,

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

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

203

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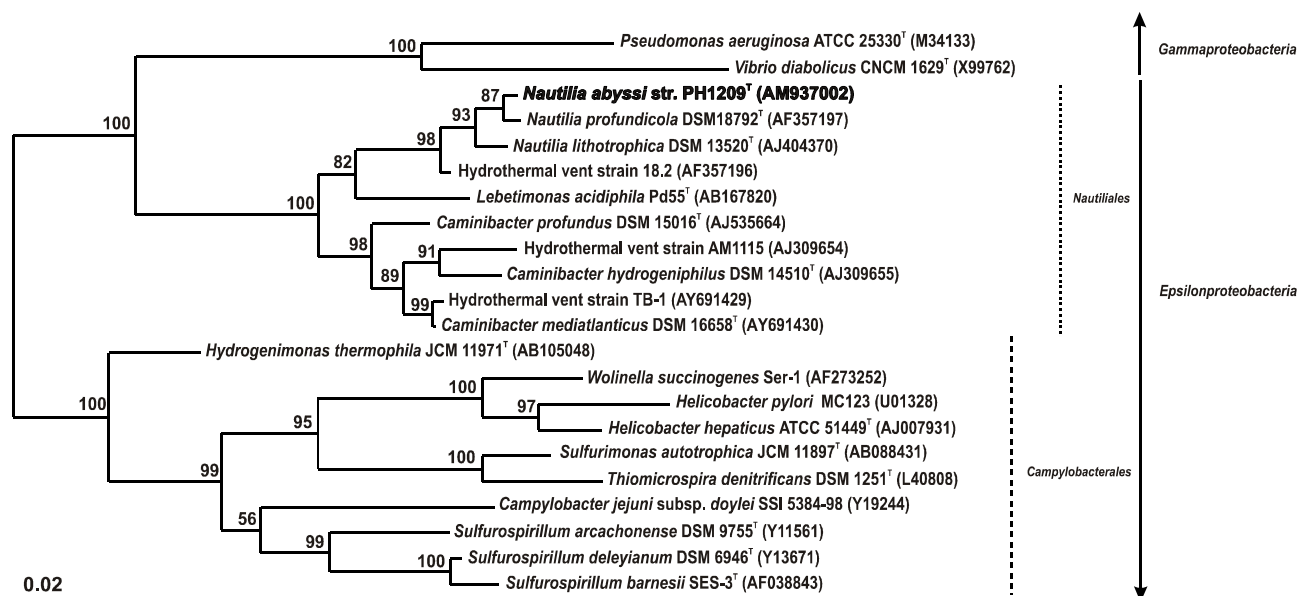
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290 **TABLES and FIGURES**

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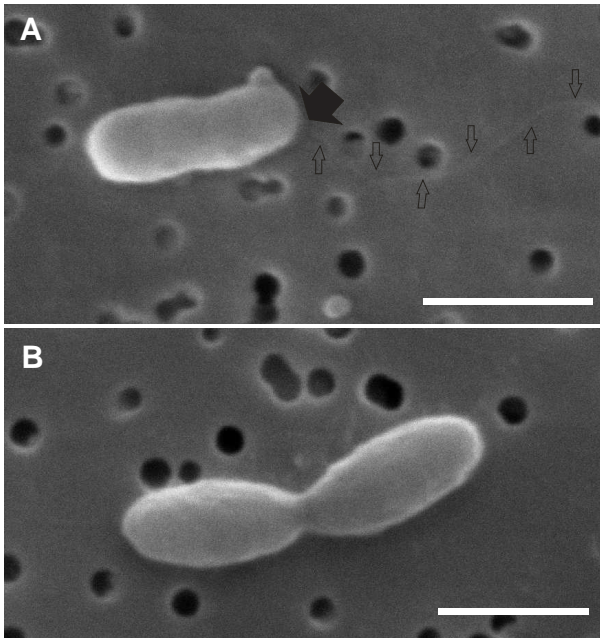
293 **Fig. 1. Phylogenetic tree based on 16S rRNA gene sequences of strain PH1209^T and representative**
 294 **members of related genera within the class Epsilonproteobacteria.** Sequence data of reference strains
 295 were obtained from the GenBank/EMBL and/or RDP databases. Two species from the
 296 *Gammaproteobacteria* were chosen as outgroups. Accession numbers are indicated in parentheses. The
 297 topology shown corresponds to an unrooted tree obtained by the maximum likelihood algorithm, established
 298 using the PHYLIP package. Bootstrap values (from 100 replicates) are indicated at the branch nodes. The
 299 positioning of the novel isolate was confirmed by the neighbour-joining method. The scale bar indicates 2 nt
 300 substitutions per 100 nt.

301

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

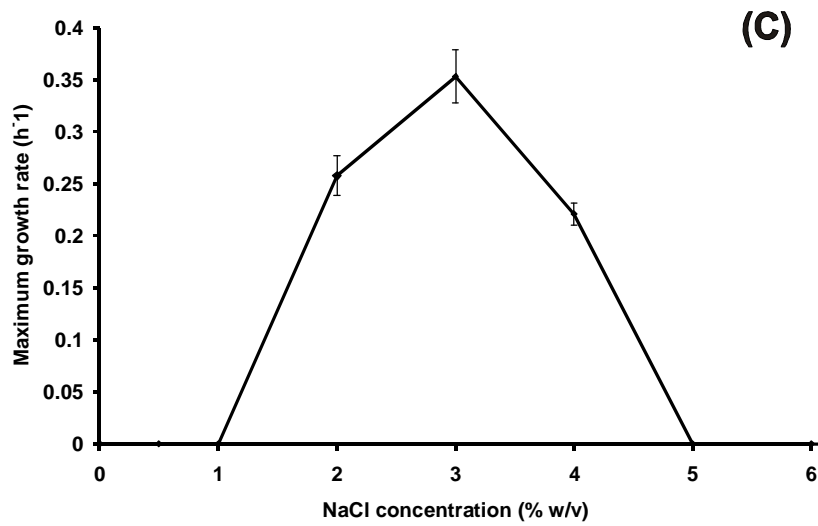
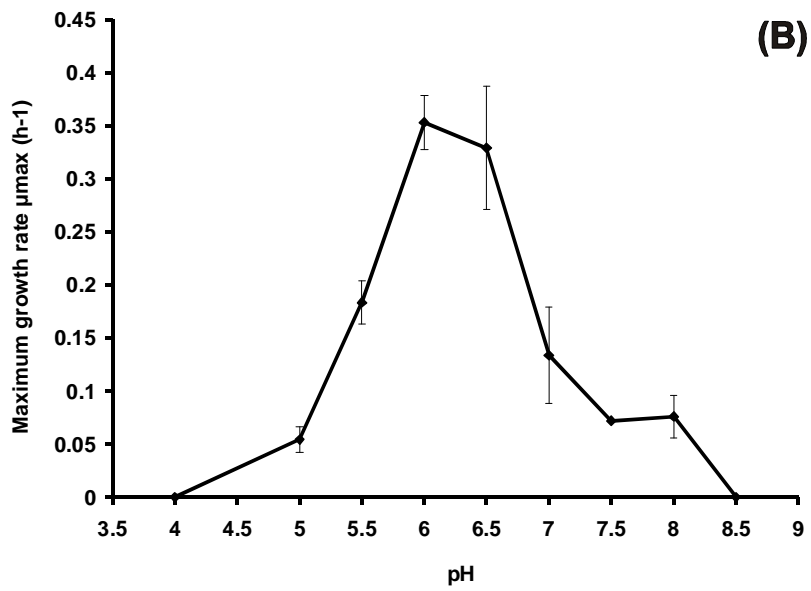
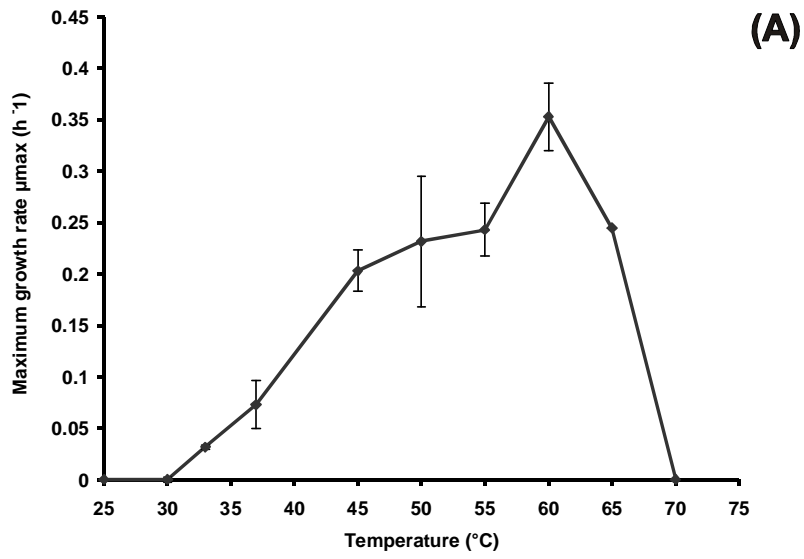
Characteristic	1	2	3	4	5	6	7
Temperature range for growth (°C) [optimum]	33-65 [60]	30-55[40]	37-68 [53]	50-70 [60]	45-65 [55]	45-70 [55]	30-68 [50]
pH range for growth [optimum]	5.0-8.0 [6.0-6.5]	6.0-9.0 [7.0]	6.4-7.4 [6.8-7.0]	5.5-7.5 [5.5-6.0]	6.5-7.4 [6.9-7.0]	4.5-7.5 [5.5]	4.2-7.0 [5.2]
NaCl concentration 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 CO₂							
Formate	—	+	+	—	—	—	—
Complex organic substrates	+	ND	ND	+	ND	ND	—
Utilization of electron donor other than H₂							
Formate	—	+	+	—	—	—	—
Utilization of electron acceptor other than S⁰							
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

311



312

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



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.**

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320