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Isolation and physiological characterization of microorganisms involved in the sulfur cycle, from under-explored deep-sea hydrothermal vents

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1 Introduction

1.1 Deep-sea hydrothermal vent systems

The deep-sea usually refers to seawaters lying below one kilometer depth, which represents 75% of the total volume of the oceans. It is a dark and cold (about 2 ℃) extreme environment with high hydrostatic pressures, which was believed to be inhabited by sparse organisms before the middle of the 20th century (Jannasch and Taylor 1984). Our views of deep-sea biology have been profoundly changed by the discovery of hydrothermal vents on the seafloor. The first deep-sea hydrothermal vent was discovered in 1977 in the Galápagos area, with the aid of the deep-sea submersible "*Alvin*" (Corliss *et al.* 1979). Since then, more than 632 vent fields have been found in numerous tectonically active zones of the sea floor (at mid-oceanic ridges or in back-arc basins, Figure 1), and about 900 vent fields remain to be explored, as suggested previously (Beaulieu 2013, Beaulieu *et al.* 2015).

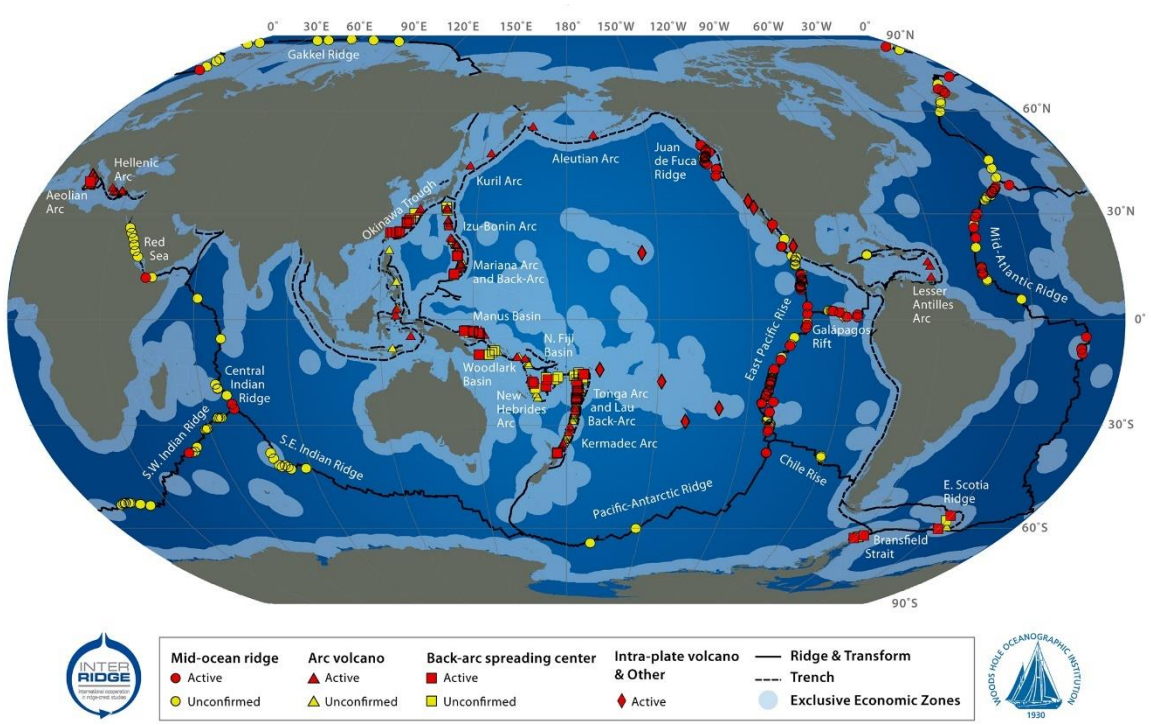

Figure 1 | Global distribution of known hydrothermal vent fields (Source: InterRidge).

The most explored and studied hydrothermal systems are located in the Pacific Ocean and in the Atlantic Ocean. Only few hydrothermal sites of the Indian Ocean (Edmond and Kairei vent

fields) have been explored so far. Indian Ocean was predicted to host most of the undiscovered vents on Earth (Beaulieu *et al.* 2015), thus it remains to be surveyed in the future.

1.2 Physical and chemical characteristics of deep-sea hydrothermal vents

Hydrothermal vents appear to be an indirect consequence of extension and accretion processes. They occur commonly at sea-floor spreading zones or at volcanic hotspots both in shallow regions close to the water surface and in deeper waters throughout the oceans. They form when seawater is overheated in oceanic crust by magma, then rises up due to the increased pressure and washes away the rocks before to be emitted explosively as hydrothermal fluid to the seafloor through rock fissures and cracks (Figure 2).

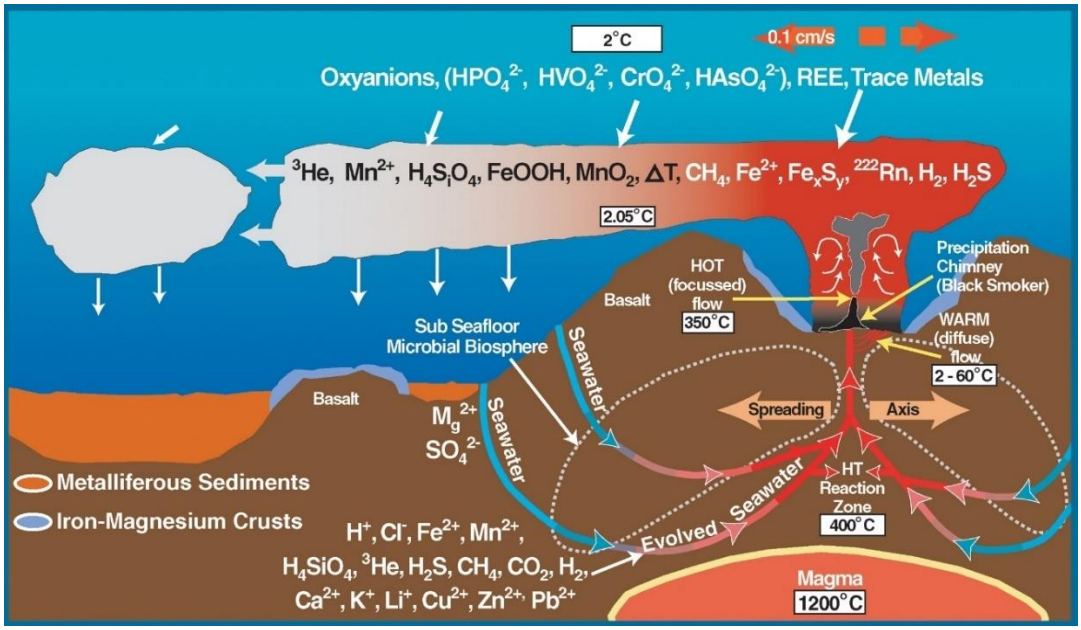

Figure 2 | Hydrothermal circulation.

Source: https://en.wikipedia.org/wiki/Hydrothermal_vent

Deep-sea hydrothermal vents are characterized by extraordinary physical and chemical gradients between the hot, anoxic, and reduced hydrothermal fluids and the cold oxic seawater (Takai and Nakamura 2011). Chemical analysis of hydrothermal vents shows that they are generally characterized by low pH, and high concentration of inorganic materials, including H_2S, Mn^{2+}, H_2, CH_4, CO_2 and CO. The composition and concentrations of dissolved gases and metal ions vary depending on the vent location and its subsurface interactions between the seawater and the host

rock composition (Prieur 2005). The distinct geological settings of different hydrothermal vents accordingly lead to highly diverse microorganisms inhibiting these environments. Deep-sea vents were also predicted to be a reservoir of novel extreme microorganisms with unique functions and bioactive natural products (Thornburg *et al.* 2010).

1.3 Diversity of prokaryotes isolated from deep-sea hydrothermal vents

Light does not reach deep-sea hydrothermal vents, so the hydrothermal ecosystem does not rely on photosynthesis. Deep-sea hydrothermal vents are among the most biologically active regions of the deep ocean, and support highly productive ecosystems fueled by chemosynthesis (Takai and Nakamura 2011, Sievert and Vetriani 2012). Chemolithoautotrophic microorganisms support primary production in the presence of reduced hydrothermal fluids, which are enriched in minerals (including sulfate, sulfite, nitrate, nitrite, *etc*.), reduced compounds (including Fe^{2+}, H_2S, H_2, CH_4), and CO_2 (Pettit 2011, Rutherford 2014, Jebbar *et al.* 2015). Since the first discovery of deep-sea hydrothermal vent, enrichment and isolation studies have been performed, and a remarkable diversity of archaea and bacteria ranging from aerobic mesophiles to anaerobic hyperthermophiles has been isolated in clonal cultures (Tables 1 and 2), exhibiting a considerable phylogenetic and physiological diversity (Takai and Nakamura 2011, Chown 2012, Jaeschke *et al.* 2012).

1.3.1 *Archaea*

Archaea represent one of the three domains (*Archaea*, *Bacteria* and *Eukarya*) of cellular organisms. This domain is currently divided into five distinct phyla, the *Euryarchaeota*, the *Crenarchaeota*, the *Thaumarchaeota*, the *Korarchaeota* and the *Nanoarchaeota* (Takai and Nakamura 2011, Parte 2015), but this subdivision might be revised in a near future as many publications suggest the existence of other phyla ("*Aigarchaeota*" (Nunoura *et al.* 2011), "*Bathyarchaeota*" (Meng *et al.* 2014), "*Diaforarchaea*" (Petitjean *et al.* 2015), "*Lokiarchaeota*" (Spang *et al.* 2015), etc.). The first archaea isolated and identified from hydrothermal vent was reported to be *Methanocaldococcus jannaschii* (Jones *et al.* 1983). Since then, a great diversity of archaea was isolated and characterized from deep-sea hydrothermal vents (Table 1).

Table 1 | Archaea isolated from deep-sea hydrothermal vents.

Modified from the PhD theses of Alain (2003), Byrne (2008), and Callac (2013).

Class	**Genera**	**Species**	**Metabolism**	**Origin**	**T (℃)**	**Reference**
-	*Nanoarchaeum*	*N. equitans*	Enriched in anaerobic, and autotrophic conditions	EPR 9 °N, Pacific Ocean	90	(Huber *et al.* 2002)
Thermoprotei	*Desulfurococcus*	*D.* sp. S and SY	Anaerobic, heterotrophic, sulfur-reducing	EPR 11 °N, Pacific Ocean	85-90	(Jannasch *et al.* 1988)
	Ignicoccus	*I. pacificus*	Anaerobic, autotrophic, sulfur-reducing	EPR 9 °N, Pacific Ocean	90	(Huber *et al.* 2000)
	Staphylothermus	*S. marinus*	Anaerobic, heterotrophic, sulfur-reducing	EPR 11 °N, Pacific Ocean	85-92	(Fiala *et al.* 1986)
	Pyrodictium	*P. abyssi*	Anaerobic, heterotrophic, fermentation, sulfur-reducing	Guaymas basin, Pacific Ocean	97	(Pley *et al.* 1991)
		P. sp 121	Oxidation of formate coupled with reduction of ferric iron (magnetite formation)	Mothra (Finn), Juan de Fuca, Pacific Ocean	85-121	(Kashefi and Lovley 2003)
	Pyrolobus	*P. fumarii*	Anaerobic, autotrophic H_2-oxidizing and reducing nitrate, thiosulfate, and low concentration oxygen	TAG, MAR, Atlantic Ocean	106	(Blochl *et al.* 1997)
	Aeropyrum	*A. camini*	Heterotrophic, aerobic	Suyio Seamount, Izu-Bonin Arc, Pacific Ocean	85	(Nakagawa *et al.* 2004)
Methanococci	*Methanocaldococcus*	*M. bathoardescens*	Obligate anaerobic, autotrophic via methanogenesis using H_2 and CO_2.	Marker 113 vent, Axial Seamount, Pacific Ocean	82	(Stewart *et al.* 2015)
		M. indicus	Anaerobic, methanogenic	Site Kairei, Central Indian Ridge	85	(L'Haridon *et al.* 2003)
		M. infernus	Anaerobic, methanogenic	Logatchev, Atlantic Ocean	85	(Jeanthon *et al.* 1998)

		M. jannaschii	Anaerobic, methanogenic	EPR 21 °N, Pacific Ocean	85	(Jones *et al.* 1983)
		M. jannaschii FS406-22	Anaerobic, methanogenic fixing of nitrogen	Axial volcano Juan de Fuca, Pacific Ocean	90	(Mehta and Baross 2006)
		M. villosus	Anaerobic, chemolithoautotrophic, reduction of CO_2 with H_2 for production of methane	Kolbeinsey Ridge, north of Iceland	80	(Bellack *et al.* 2011)
		M. vulcanius	Anaerobic, methanogenic	EPR 13 °N, Pacific Ocean	80	(Jeanthon *et al.* 1999)
	Methanothermococcus	*M. okinawensis*	Anaerobic, methanogenic	Iheya Ridge, Okinawa Trough, Pacific Ocean	60-65	(Takai *et al.* 2002)
	Methanotorris	*M. formicicus*	Anaerobic, methanogenic	Central Indian Ridge	75	(Takai *et al.* 2004)
Methanopyri	*Methanopyrus*	*M. kandleri*	Anaerobic, chemolithoautotrophic, methanogenic, sulfur-reducing	Guaymas basin, Pacific Ocean	98	(Huber *et al.* 1989, Kurr *et al.* 1991)
Thermococci	*Thermococcus*	*T. aggregans*	Anaerobic, heterotrophic, fermentation, sulfur-reducing	Guaymas basin, Pacific Ocean	88	(Canganella *et al.* 1998)
		T. barossii	Anaerobic, heterotrophic, fermentation, sulfur-reducing	Juan de Fuca, Pacific Ocean	82	(Duffaud *et al.* 1998)
		T. eurythermalis	Strictly anaerobic. Obligate chemoorganotrophic.	Guaymas Basin, Pacific Ocean (Gulf of California)	85	(Zhao *et al.* 2015)
		T. fumicolans	Anaerobic, heterotrophic, fermentation, sulfur-reducing	North Fiji Pacific Ocean basin	85	(Godfroy *et al.* 1996)
		T. guaymasensis	Anaerobic, heterotrophic, fermentation, sulfur-reducing	Guaymas basin, Pacific Ocean	88	(Canganella and Jones 1994, Canganella *et al.* 1998)
		T. hydrothermalis	Anaerobic, heterotrophic, fermentation, sulfur-reducing	EPR 21 °N, Pacific Ocean	85	(Godfroy *et al.* 1997)

		T. nautili	Anaerobic. Sulfur or cystine is necessary for growth. Chemoorganotrophic.	'La chainette' vent, East Pacific Ocean Ridge	87.5	(Gorlas *et al.* 2014)
		T. paralvinellae	Obligate anaerobic, and produce H_2S in the presence of sulfur and H_2. H_2 produced (> 2 mM) at the absence of sulfur. Heterotrophic.	Endeavour Segment, Juan de Fuca Ridge, northeastern Pacific	82	(Hensley *et al.* 2014)
		T. cleftensis	Obligate anaerobic, sulfur-reducing. Hydrogen produced (> 2 mM) at the absence of sulfur. Heterotrophic growth	Cleft Segment, Juan de Fuca Ridge Pacific (44° 30′ N, 130° 30′ W).	88	(Hensley *et al.* 2014)
		T. peptonophilus	Anaerobic, heterotrophic, fermentation, sulfur-reducing	Ditch the Marianas Pacific Ocean	85-90	(Gonzalez *et al.* 1995)
		T. siculi	Anaerobic, heterotrophic, fermentation, sulfur-reducing	Okinawa Japan, Trough, Pacific Ocean	85	(Grote *et al.* 1999)
		T. chitonophagus	Anaerobic, heterotrophic, fermentation, sulfur-reducing	Guaymas basin, Pacific Ocean	85	(Huber *et al.* 1995)
		T. barophilus	Anaerobic, heterotrophic, fermentation, sulfur-reducing	Snake Pit, MAR, Atlantic Ocean	85	(Marteinsson *et al.* 1999)
		T. atlanticus	Anaerobic, heterotrophic, fermentation, sulfur-reducing	TAG, MAR, Atlantic Ocean	85	(Cambon-Bonavita *et al.* 2003)
		T. gammatolerans	Anaerobic, heterotrophic, fermentation, sulfur-reducing	Guaymas basin, Pacific Ocean	88	(Jolivet *et al.* 2003)
		T. marinus	Anaerobic, heterotrophic, fermentation, sulfur-reducing	Snake Pit, MAR, Atlantic Ocean	88	(Jolivet *et al.* 2004)
		T. radiotolerans	Anaerobic, heterotrophic, fermentation, sulfur-reducing	Basin, Guyamas Pacific Ocean	88	(Jolivet *et al.* 2004)
		T. coalescens	Anaerobic, heterotrophic, fermentation, sulfur-reducing	Suiyo Seamount, Izu-Bonin Arc, Pacific Ocean	87	(Kuwabara *et al.* 2005)

		T. celericrescens	Anaerobic, heterotrophic, fermentation, sulfur-reducing	Suiyo Seamount, Izu-Bonin Arc, Pacific Ocean	80	(Kuwabara *et al.* 2007)
		T. thioreducens	Anaerobic, heterotrophic, fermentation, sulfur-reducing	Rainbow, MAR, Atlantic Ocean	83-85	(Pikuta *et al.* 2007)
		T. prieurii	Anaerobic, heterotrophic, fermentation, sulfur-reducing	Sarah Spring, PRT, Pacific Ocean	80	(Gorlas *et al.* 2013)
		T. sp. ES1	Anaerobic, heterotrophic, fermentation, sulfur-reducing	Juan de Fuca, Pacific Ocean	82	(Pledger and Baross 1989)
		T. sp. DT-1331	Anaerobic, heterotrophic, fermentation, sulfur-reducing	Minami-teaching Knoll, Mid-Okinawa, Pacific Ocean		(Kwak *et al.* 1995)
		T. sp.	Anaerobic, heterotrophic, fermentation, sulfur-reducing	Trough. Pacific OceanEPR 11, 13 and 21 °N, Atlantic Ocean		(Raguénes *et al.* 1995)
		T. sp.	Anaerobic, heterotrophic, fermentation, sulfur-reducing	Guyamas Pacific Ocean basin		(Antoine *et al.* 1995)
		T. sp.	Anaerobic, heterotrophic, fermentation, sulfur-reducing	Guyamas Pacific Ocean basin		(Lepage *et al.* 2004)
		T. sp.	CO oxidation with H_2	East Pacific Rise		(Sokolova *et al.* 2004)
		T. sp.	Anaerobic, heterotrophic, fermentation, sulfur-reducing	Juan de Fuca Ridge, Pacific Ocean		(Holden *et al.* 2001)
	Pyrococcus	*P. abyssi*	Anaerobic, heterotrophic, fermentation, sulfur-reducing	North Basin-Fiji, Pacific Ocean	96	(Erauso *et al.* 1993)
		P. glycovorans	Anaerobic, heterotrophic, fermentation, sulfur-reducing	Site Totem, EPR 13 °N, Pacific Ocean	95	(Barbier *et al.* 1999)
		P. horikoshii	Anaerobic, heterotrophic, fermentation, sulfur-reducing	Okinawa Japan, Trough, Pacific Ocean	98	(Gonzalez *et al.* 1998)

		P. yayanosii	Anaerobic, piezophilic, heterotrophic, fermentation, sulfur-reducing	Ashadze, MAR, Atlantic Ocean	98	(Birrien *et al.* 2011)
		P. sp. GB-D	Anaerobic, heterotrophic, sulfur-reducing	Guaymas basin, Pacific Ocean	95	(Jannasch *et al.* 1992)
		P. sp. ES4	Anaerobic, heterotrophic, fermentation, Sulfur-reducing	Juan de Fuca, Pacific Ocean	90-99	(Pledger and Baross 1991)
		P. sp.	Anaerobic, heterotrophic, fermentation, Sulfur-reducing	13 °N PRT, Pacific Ocean		(Lepage *et al.* 2004)
	Palaeococcus	*P. ferrophilus*	Anaerobic, heterotrophic. Sulfur-reducing (iron as a cofactor for growth)	Ogasawara, Pacific Ocean	83	(Takai *et al.* 2000)
		P. pacificus	Anaerobic, piezophilic, organoheterotrophic, facultatively sulfur and sulfate-reducing	Hydrothermal Sediments, PRT, Pacific Ocean	80	(Zeng *et al.* 2013)
Archaeoglobi	*Archaeoglobus*	*A. profundus*	Anaerobic, mixotrophic, Sulfate-reducing	Guaymas basin, Pacific Ocean	82	(Burggraf *et al.* 1990)
		A. veneficus	Anaerobic, grow lithoautotrophically with sulfite or thiosulfate as electron acceptor or organotrophic with sulfite.	Snake Pit, Atlantic Ocean(MAR)	75-80	(Huber *et al.* 1997)
		A. infectus	Anaerobic, mixotrophic, thiosulfate and sulfites-reducing	Suiyo Seamount, Izu-Bonin Arc, Pacific Ocean	70	(Mori *et al.* 2008)
	Geoglobus	*G. ahangari*	Anaerobic, mixotrophic, lithoautotrophic or organoheterotrophic with ferric iron as electron acceptor	Guaymas basin, Pacific Ocean	88	(Kashefi *et al.* 2002)
		G. acetivorans	Anaerobic, mixotrophic, lithoautotrophic or organoheterotrophic with ferric iron as an electron acceptor	Ashadze, MAR, Atlantic Ocean	81	(Slobodkina *et al.* 2009)
unclassified Euryarchaeota	*Aciduliprofundum*	*A. boonei*	Anaerobic, heterotrophs, thermoacidophilic, sulfur and ferric-reducing	Mariner vent field basin of Lau, Pacific Ocean	70	(Reysenbach *et al.* 2006)

The majority of deep-sea hydrothermal vent *Archaea* belong to two main phyla, the *Euryarchaeota*, and the *Crenarchaeota*. The euryarchaeal lineages most commonly isolated from deep-sea hydrothermal vent environments are methanogens (*Methanococcales*, *Methanopyrales*), *Thermococcales*, and *Archaeoglobales* (Takai and Nakamura 2011). The crenarchaeal lineages isolated from deep-sea hydrothermal vents mainly belong to the order *Desulfurococcales*.

Methanogens are a diverse group of *Archaea* producing methane by reducing carbon dioxide, acetate or methylated compounds (methanol, methylsulfides, choline, glycine-betaine, etc.) under anoxic conditions. Methanogens isolated from deep-sea hydrothermal vents mostly belong to the orders *Methanococcales* and *Methanopyrales*. *Methanococcales* methanogens use carbon dioxide and dihydrogen or formate, to produce methane (Liu and Whitman 2008, Stewart *et al.* 2015). The order *Methanopyrales* is composed of only one species, *Methanopyrus kandleri*, reducing carbon dioxide with hydrogen as energy source (Kurr *et al.* 1991).

Almost all the *Thermococcales Archaea* isolated from deep-sea hydrothermal vents are anaerobic, obligate heterotrophic, and hyperthermophilic, with an optimal temperature for growth around 80-95 ℃ (Rutherford 2014, Zhao *et al.* 2015). *Thermococcus* species are fermentative thermophilic archaea able to reduce sulfur species; Elemental sulfur stimulates significantly the growth of most of them, or is even required. Some *Thermococcus* species are also able to oxidize carbon monoxide (Table 1). They are common inhabitants of the hot areas of hydrothermal vents. They represent the most cultured representatives within the order *Thermococcales* (which encompass also the genera *Pyrococcus* and *Palaeococcus*). (Hensley *et al.* 2014, Zhao *et al.* 2015).

Archaeoglobales archaea identified from deep-sea hydrothermal vents encompass three genera, *Archaeoglobus*, *Geoglobus* and *Ferroglobus*. They represent a key group in biogeochemical cycling of sulfur or iron. *Archaeoglobus* species are able to respire sulfite, thiosulfate, or sulfate under anaerobic conditions (Burggraf *et al.* 1990, Huber *et al.* 1997, Mori *et al.* 2008). Members of the genus *Geoglobus* are mixotrophs, which were observed either lithoautotrophic or organoheterotrophic with ferric iron as electron acceptor (Kashefi *et al.* 2002, Slobodkina *et al.* 2009). Another *Archaeoglobales* archaea, *Ferroglobus placidus*, isolated from a shallow submarine hydrothermal vent can reduce ferric iron (Hafenbradl *et al.* 1996).

Desulfurococcales archaea isolated from deep-sea hydrothermal vents are hyperthermophilic, with optimal growth temperatures between 85 and 106 ℃ (Table 1). Most of them are anaerobes or facultative anaerobes, while *Aeropyrum camini* is an aerobic heterotroph, isolated from deep-sea hydrothermal vent chimney sample from the Suiyo Seamount in the Izu-Bonin Arc (Nakagawa *et al.* 2004). Under autotrophic conditions, they are able to fix carbon dioxide by reducing nitrate, nitrite, elemental sulfur, or thiosulfate with hydrogen as an energy source (Blochl *et al.* 1997, Huber *et al.* 2000, Anderson *et al.* 2009). They can also grow organotrophically by anaerobic sulfur respiration or aerobic respiration, or also by fermentation of organic substrates (Pley *et al.* 1991, Nakagawa *et al.* 2004).

Archaea inhabiting hydrothermal vents include numerous physiotypes. Most of the isolated *Archaea* from deep-sea vents are thermophilic or even hyperthermophilic, including *Archaeoglobales*, *Desulfurococcales*, *Methanococcales*, *Methanopyrales* and *Thermococcales* (Jebbar *et al.* 2015). The upper temperature limit for growth, 122 ℃, has been reported in *Methanopyrus kandleri* strain 116 (Takai *et al.* 2008). There are also numerous psychrophilic and mesophilic archaea found in deep-sea hydrothermal vents systems (Nelson *et al.* 1995, Takai and Nakamura 2011). *Archaea* from deep-sea vents display a wide range of physiologies ranging from piezophilic to piezotolerant, anaerobic to aerobic, acidophilic to alkaliphilic, and halophilic to extremely halophilic (Pettit 2011, Takai and Nakamura 2011).

1.3.2 *Bacteria*

Bacteria from deep-sea hydrothermal vents spans most of the currently defined lineages, including *Aquificae*, *Thermotogae*, *Thermodesulfatebacteria*, *Acidobacteria*, *Deinococcus-Thermus*, *Deferribacteres*, *Firmicutes*, *Actinobacteria*, *Bacteroidetes*, *Spirochaetes*, *Proteobacteria*, and unclassified *Bacteria* (Table 2) (Thornburg *et al.* 2010). *Proteobacteria* form the largest bacterial phylum isolated from the deep-sea vent environments, followed by *Firmicutes*, *Aquificae*, and *Thermotogae*.

Table 2 | Bacteria isolated from deep-sea hydrothermal vents.

Modified from the PhD theses of Alain (2003), Byrne (2008), and Callac (2013).

Class	**Genera**	**Species**	**Metabolism**	**Origin**	**T (℃)**	**Reference**
Aquificae	*Persephonella*	*P. marina*	Microaerophilic, autotrophic. Sulfur, thiosulfate and hydrogen-oxidizing, denitrifying	EPR 9 °N, Pacific Ocean	70	(Gotz *et al.* 2002)
		P. guaymasensis	Microaerophilic, autotrophic, Sulfur, thiosulfate and hydrogen-oxidizing, denitrifying	Guaymas basin, Pacific Ocean	70	(Gotz *et al.* 2002)
		P. hydrogeniphila	Microaerophilic, autotrophic, hydrogen-oxidizing, denitrifying	Suiyo Seamount, Izu-Bonin Arc, Pacific Ocean	70	(Nakagawa *et al.* 2003)
	Phorcysia	*P. thermohydrogeniphila*	Anaerobic, lithoautotrophic, denitrifying and sulfur-reducing	EPR 13 °N, Pacific Ocean	75	(Perez-Rodriguez *et al.* 2012)
	Thermosulfidibacter	*T. takaii*	Anaerobic, lithoautotrophic sulfur-reducing and hydrogen-oxidizing	Yonaguni Knoll IV, Southern Okinawa Trough, Pacific Ocean	70	(Nunoura *et al.* 2008)
	Hydrogenivirga	*H. okinawensis*	Facultative aerobic, (denitrifying) lithoautotrophic sulfur and thiosulfate-oxidizing	Yonaguni Knoll IV, Southern Okinawa Trough, Pacific Ocean	70-75	(Nunoura *et al.* 2008)
	Desulfurobacterium	*D. thermolithotrophum*	Anaerobic, autotrophic, Sulfur-reducing	Snake Pit, MAR, Atlantic Ocean	70	(L'Haridon *et al.* 1998)

		D. crinifex	Anaerobic, autotrophic, Sulfur-reducing and denitrifying	CASM, Juan de Fuca, Pacific Ocean	60-65	(Alain *et al.* 2003)
		D. atlanticum	Anaerobic, chemolithoautotrophic, Sulfur and nitrate-reducing	23 °N MAR, Atlantic Ocean	70-75	(L'Haridon *et al.* 2006)
		D. pacificum	Anaerobic, chemolithoautotrophic, Sulfur, thiosulfate and nitrate-reducing	EPR 13 °N, Pacific Ocean	75	(L'Haridon *et al.* 2006)
	Balnearium	*B. lithotrophicum*	Anaerobic, autotrophic, hydrogen-oxidizing, Sulfur-reducing	Suiyo Seamont, Izu-Bonin Arc, Pacific Ocean	70-75	(Takai *et al.* 2003)
	Thermovibrio	*T. ammonificans*	Anaerobic, autotrophic, nitrate and Sulfur-reducing	EPR 9 °N, Pacific Ocean	75	(Vetriani *et al.* 2004)
		T. guaymasensis	Anaerobic, lithoautotrophic, nitrate and sulfur-reducing	Guaymas basin, Pacific Ocean	75-80	(L'Haridon *et al.* 2006)
Thermotogae	*Marinitoga*	*M. camini*	Anaerobic, heterotrophic, fermentation, sulfur-reducing	Menez Gwen, MAR, Atlantic Ocean	55	(Wery *et al.* 2001)
		M. piezophila	Anaerobic, heterotrophic, fermentation, sulfur-reducing	Grandbonum, EPR 13 °N, Pacific Ocean	65	(Alain *et al.* 2002)
		M. okinawensis	Anaerobic, heterotrophic, sulfur reduction stimulates growth	Southern Okinawa Trough, Pacific Ocean	55-60	(Nunoura *et al.* 2007)
		M. hydrogenitolerans	Anaerobic, heterotrophic, fermentation, sulfur-reducing	Rainbow, MAR, Atlantic Ocean	60	(Postec *et al.* 2005)
	Mesoaciditoga	*M. lauensis*	Anaerobic, chemo-organotrophic. Reduce sulfur to hydrogen sulfide	Eastern Lau Spreading Center, South-Western Pacific	57-60	(Reysenbach *et al.* 2013)
	Kosmotoga	*K. pacifica*	Strictly anaerobic, obligate chemoorganoheterotrophic. Reduce sulfur and L-cystine to hydrogen sulfide.	East Pacific Ocean Rise (102°55'W, 3°58'S)	70	(L'Haridon *et al.* 2014)

	Thermotoga	*T.* sp.	heterotrophic, fermentation, Sulfur-reducing	Snake Pit, MAR, Atlantic Ocean	80	(Marteinsson *et al.* 1997)
	Thermosipho	*T. melanesiensis*	Anaerobic, heterotrophic, fermentation, Sulfur-reducing	Lau basin, Pacific Ocean	70	(Antoine *et al.* 1997)
		T. japonicus	Anaerobic, heterotrophic, fermentation, Sulfur- and thiosulfate-reducing	Iheya basin (Okinawa), Pacific Ocean	72	(Takai and Horikoshi 2000)
		T. atlanticus	Anaerobic, heterotrophic, fermentation	Menez Gwen, MAR, Atlantic Ocean	65	(Urios *et al.* 2004)
		T. affectus	Anaerobic, heterotrophic, fermentation	MAR, Atlantic Ocean	70	(Podosokorskaya *et al.* 2011)
		T. globiformans	Anaerobic, Obligate chemoorganotrophic. Sulfur-reducing. Reduction of Fe_2O_3 to Fe(II)	Suiyo Seamount, Izu-Bonin Arc, western Pacific Ocean	68	(Kuwabara *et al.* 2011)
		T. activus	Thermophilic. Obligate organotrophic. Anaerobic. Sulfur and ferric-reducing	Guaymas Basin, Gulf of California, Pacific	65	(Podosokorskaya *et al.* 2014)
Thermodesulfobacteria	*Thermodesulfobacterium*	*T. hydrogeniphilum*	Anaerobic, autotrophic, sulfate-reducing	Guaymas basin, Pacific Ocean	75	(Jeanthon *et al.* 2002)
	Thermodesulfator	*T. indicus*	Anaerobic, sulfate-reducing, autotrophic	Central Indian Ridge	70	(Moussard *et al.* 2004)
		T. atlanticus	Anaerobic, lithoautotrophic, sulfate-reducing	Rainbow, MAR, Atlantic Ocean	65-70	(Alain *et al.* 2010)
	Thermosulfurimonas	*T. dismutans*	Anaerobic, lithoautotrophic, Disproportionation of S °to thiosulfate and sulfite	Eastern Lau Spreading Center (Lau basin) Pacific	74	(Slobodkin *et al.* 2012)

unclassified Bacteria	*Caldithrix*	*C. abyssi*	Anaerobic, mixotrophic, fermentation, denitrification coupled with oxidation of hydrogen or acetate	Logatchev, MAR, Atlantic Ocean	60	(Miroshnichenko *et al.* 2003)
unclassified Acidobacteria	*Thermotomaculum*	*T. hydrothermale*	Anaerobic, heterotrophic, fermentation, Sulfur-reducing	Okinawa Trough, Pacific Ocean	55	(Izumi *et al.* 2012)
Deinococci	*Thermus*	*T. thermophiles* GY1211	Aerobic, heterotrophic	Guaymas basin, Pacific Ocean	75	(Marteinsson *et al.* 1999)
		T. sp.	Aerobic, heterotrophic	Guaymas basin, Pacific Ocean and Snake Pit, MAR, Atlantic Ocean	70-80	(Marteinsson *et al.* 1995)
	Marinithermus	*M. hydrothermalis*	Aerobic, heterotrophic	Suiyo Seamount, Pacific Ocean	67	(Sako *et al.* 2003)
	Oceanithermus	*O. profundus*	Microaerophilic, heterotrophic, facultative chemolithotrophic, hydrogen-oxidizing	EPR 13 °N, Pacific Ocean	60	(Miroshnichenko *et al.* 2003)
		O. desulfurans	Microaerophilic, chemoheterotrophic, reduced O_2, NO_2^-, NO_3^- and S^0	Suiyo Seamount, Pacific Ocean	60	(Mori *et al.* 2004)
	Vulcanithermus	*V. mediatlanticus*	Microaerophilic, lithoheterotrophic, O_2 and nitrate-reducing	Rainbow, MAR, Atlantic Ocean	70	(Miroshnichenko *et al.* 2003)
	Rhabdothermus	*R. arcticus*	Heterotrophic, aerobic	Soria Moria vent, 71 °N Arctic Mid-Ridge	65	(Steinsbu *et al.* 2011)
Deferribacteres	*Deferribacter*	*D. desulfuricans*	Anaerobic, heterotrophic, Sulfur, nitrate, and arsenate-reducing	Suiyo Seamount, Pacific Ocean	60-65	(Takai *et al.* 2003)
		D. abyssi	Anaerobic, litho or organoautotrophic, reduce S °, NO_3^-, and Fe (III)	Rainbow and Menez Gwen, MAR, Atlantic Ocean	60	(Miroshnichenko *et al.* 2003)

		D. autotrophicus	Anaerobic, autotrophic litho, Fe (III), Mn (IV), S °and nitrate-reducing	Ashadze, MAR, Atlantic Ocean	60	(Slobodkina *et al.* 2009)
Clostridia	*Abyssivirga*	*A. alkaniphila*	Strictly anaerobic, syntrophic alkane-degrading. Proteinous substrates, sugars, organic acids and hydrocarbons were utilized. Thiosulfate was used as external electron acceptor with crude oil.	a black smoker chimney at the Loki ś Castle Vent Field on the Arctic Mid-Ocean Ridge	37	
	Anoxybacter	*A. fermentans*	Piezophilic, thermophilic, anaerobic, heterotrophic, and fermentative. Sulfur and Fe(III)-reducing.	East Pacific Rise (102.66 ° W 3.16 °S)	60-62	(Zeng *et al.* 2015)
	Bacillus	*B.* sp.	Aerobic, heterotrophic	Basins of Guaymas and Lau, Pacific Ocean and Snake Pit, MAR, Atlantic Ocean	60-80	(Marteinsson *et al.* 1996)
	Caloranaerobacter	*C. azorensis*	Anaerobic, chemoorganotrophic, fermentation	Lucky Strike, MAR, Atlantic Ocean	65	(Wery *et al.* 2001)
		C. ferrireducens	Strictly anaerobic and obligate chemoorganoheterotrophic. Thermophilic, iron-reducing, fermentative hydrogen-producing.	East Pacific Ocean (W102.55 ◦S3.59 ◦)	60	(Jiang *et al.* 2015, Zeng *et al.* 2015)
		C. sp. H363	Strictly anaerobic, thermophilic, fermentative hydrogen-producing	East Pacific Ocean (W102.65 ◦S2.15 ◦)	60	(Jiang *et al.* 2015)
		C. sp. H53214	Strictly anaerobic, thermophilic, fermentative hydrogen-producing	Southwest Indian Ridge (E50.47 ◦S37.66 ◦)	60	(Jiang *et al.* 2015)

	Caminicella	*C. sporogenes*	Anaerobic, fermentation	ELSA, EPR 13 °N, Pacific Ocean	55-60	(Alain *et al.* 2002)
	Clostridium	*C. caminithermale*	Anaerobic, fermentation, Stickland fermentation	Menez Gwen, MAR, Atlantic Ocean	45	(Brisbarre *et al.* 2003)
		C. tepidiprofundi	Anaerobic, fermentation, form of spores	PRT 1 °N, Pacific Ocean	50	(Slobodkina *et al.* 2008)
	Carboxydobrachium	*C. pacificum*	Anaerobic, fermentation. lithotrophic on CO	Okinawa, Pacific Ocean	70	(Sokolova *et al.* 2001)
	Crassaminicella	*C. profunda*	Anaerobic, forming terminal endospores. Obligate heterotrophic, fermentation. Yeast extract is required for growth.	Guaymas basin (Gulf of California, Mexico)	30	(Lakhal *et al.* 2015)
	Exiguobacterium	*E. profundum*	Facultative anaerobic, moderate halophilic, lactic acid-producing, , nitrate-reducing	EPR 13 °N, Pacific Ocean	45	(Crapart *et al.* 2007)
	Tepidibacter	*T. thalassicus*	Anaerobic, fermentation, sulfur-reducing	EPR 13 °N, Pacific Ocean	50	(Slobodkin *et al.* 2003)
		T. formicigenes	Anaerobic, fermentation	Menez Gwen, MAR, Atlantic Ocean	45	(Urios *et al.* 2004)
	Vallitalea	*V. guaymasensis*	Anaerobic, fermentation, non-spore-forming	Overlying Sediments, basin of Guaymas, Pacific Ocean	30-35	(Lakhal *et al.* 2013)
	Vulcanibacillus	*V. modesticaldus*	Anaerobic, fermentation (carbohydrate, protein and organic substrates), reduced nitrate to nitrite.	Rainbow, MAR, Atlantic Ocean	55	(L'Haridon *et al.* 2006)
Actinobacteria	*Janibacter*	*J. indicus*	Aerobic, nitrate reduction	Indian Ocean	28-30	(Zhang *et al.* 2014)

	Nocardioides	*N.* sp. ERP26w, 28w	Aerobic growth on *n*-alkane (Dodecane)	Diffuse flow vent, Mk119, EPR, Pacific Ocean (9 °N, 104 °W)	28-30	(Bertrand *et al.* 2013)
	Kribbella	*K.* sp. EPR178	Aerobic, mesophilic. Growth in anaerobic ASW with NO^{3-}, Nitrate-reducing	Alvinella worm, East Pacific Ocean Rise	30	(Perez-Rodriguez *et al.* 2013)
	Rhodococcus	*R.* sp. EPR110	Aerobic, mesophilic.	Microbial biofilm, Bio9 Vent, East Pacific Ocean Rise	30	(Perez-Rodriguez *et al.* 2013)
-	*Rhodothermus*	*R. profundi*	Aerobic, heterotrophic, non-spore-forming	EPR 13 °N, Pacific Ocean	70-75	(Marteinsson *et al.* 2010)
Spirochaetia	*Exilispira*	*E. thermophila*	Anaerobic, heterotrophic (only from yeast extract)	Iheya North field, Okinawa Trough, Japan, Pacific Ocean	50	(Imachi *et al.* 2008)
Alphaproteobacteria	*Aurantimonas*	*A.* sp. AE01-7	MN (II)-oxidizing	P-vent, EPR 9 °N, Pacific Ocean	37	(Anderson *et al.* 2009)
	Brevirhabdus	*B. pacifica*	Aerobic, heterotrophic, nitrate-reducing	East Pacific Rise (102 °33'W, 3 °06'S)	30-35	(Wu *et al.* 2015)
	Citromicrobium	*C. bathyomarinum*	Aerobic, chemoorganotrophic and facultative photoheterotrophic	Juan de Fuca Ridge, Pacific Ocean	20-42	(Yurkov and Beatty 1998, Yurkov *et al.* 1999)
	Defluviimonas	*D. indica*	Aerobic, non-phototrophic, chemoheterotrophic	Southwest Indian Ridge (E 50.64 °, S 37.78 °)	25-28	(Jiang *et al.* 2014)
	Fulvimarina	*F. manganoxydans*	Aerobic, Mn(II)-oxidizing, nitrate-reducing	Southwest Indian Ocean	28	(Ren *et al.* 2014)
	Glycocaulis	*G. abyssi*	Aerobic, chemoheterotrophic, moderate halophilic	Pen hydrothermal (Pacific Ocean), Vancouver Island		(Abraham *et al.* 2013)

	Methyloceaniba cter	*M. caenitepidi*	Aerobic, methanol-oxidizing, facultative methylotrophic. Nitrogen source: nitrate and ammonia.	Kagoshima Bay, Japan. Pacific Ocean	35	(Takeuchi *et al.* 2014)
	Salipiger	*S.* sp. EPR135	Aerobic, mesophilic.	Colonization device, East Pacific Ocean Rise	30	(Perez-Rodriguez *et al.* 2013)
	Thioclava	*T.* sp. EPR65	Aerobic, mesophilic.	Tica Vent, East Pacific Ocean Rise	28	(Perez-Rodriguez *et al.* 2013)
		T. sp. EPR74	Aerobic, mesophilic.	East Wall, East Pacific Ocean Rise	28	(Perez-Rodriguez *et al.* 2013)
	Oceanibaculum	*O. pacificum*	Aerobic.	Hydrothermal Sediments of the Lau basin, Pacific Ocean	28-37	(Dong *et al.* 2010)
	Parvibaculum	*P. hydrocarboniclast icum*	Aerobic, carbon and energy source: *n*-alkanes in a medium supplemented with peptone and yeast extract	Diffuse flow vent EPR, Tica, Pacific Ocean (9 °N, 104 °W)	35	(Rosario-Passapera *et al.* 2012)
	Piezobacter	*P. thermophilus*	Facultative aerobic, mixotrophic, sulfur and hydrogen-oxidizing	TAG, MAR, Atlantic Ocean(isolated under hydrostatic pressure)	50	(Takai *et al.* 2009)
Betaproteobac teria	*Thiobacillus*	*T. hydrothermalis*	Aerobic, autotrophic. Oxidize thiosulfate, tetrathionate, sulfur and hydrogen sulfide	North Fiji, Pacific Ocean basin	35	(Durand *et al.* 1993)
Deltaproteoba cteria	*Geothermobacte r*	*G. ehrlichii*	Anaerobic, ferric iron and nitrate-reducing	"Bag city", Juan de Fuca, Pacific Ocean	55	(Kashefi *et al.* 2003)
	Desulfovibrio	*D. hydrothermalis*	Anaerobic, mixotrophic, sulfate, sulfite, and thiosulfate-reducing	Grandbonum, EPR 13 °N, Pacific Ocean	35	(Alazard *et al.* 2003)
	Desulfonauticus	*D. submarinus*	Anaerobic. Electron donor: H_2, CO_2 or formate; Electron acceptor: sulfate, sulfite,	Alvinella and Riftia, EPR 13 °N, Pacific Ocean	45	(Audiffrin *et al.* 2003)

			thiosulfate and sulfur; Carbon source: acetate.			
	Desulfothermus	*D. naphthae*	Anaerobic, sulfate reduction coupled with oxidation of hydrocarbons (*n*-alkanes)	Guaymas basin, Pacific Ocean	55-65	(Rueter *et al.* 1994)
		D. okinawensis	obligate heterotrophic and reduced sulfate	Yonaguni Knoll IV in the Southern Okinawa Trough, Pacific Ocean	50	(Nunoura *et al.* 2007)
	Dissulfuribacter	*D. thermophilus*	Anaerobic, autotrophic (sources of carbon: CO_2 and bicarbonate), disproportionation of S °, thiosulfate and sulfite to produce sulfate and H_2S	Won Fa, Eastern Lau Spreading Center (Lau basin) Pacific Ocean	61	(Slobodkin *et al.* 2013)
	Deferrisoma	*D. camini*	Anaerobic, organotrophic, ferric-reducing and sulfur-reducing	Eastern Lau Spreading Center (Lau basin), Pacific Ocean	50	(Slobodkina *et al.* 2012)
	Hippea	*H. jasoniae*	Anaerobic, thermoacidophilic, heterotrophic, sulfur-reducing	Lucky Strike, MAR, Atlantic Ocean	60-65	(Flores *et al.* 2012)
		H. alviniae	Anaerobic, thermoacidophilic, heterotrophic, sulfur-reducing	EPR 9 °N, Pacific Ocean	60	(Flores *et al.* 2012)
Gammaproteo bacteria	*Acinetobacter*	*A.* sp. EPR111, EPR17	Aerobic, mesophilic	Mk 119 Vent, East Pacific Ocean Rise	28	(Perez-Rodriguez *et al.* 2013)
	Alcanivorax	*A.* sp. EPR7	Grow aerobically on *n*-alkanes (Octane, dodecane, hexadecane). Nitrate-reducing. Ferment glucose	Mk119, East Pacific Ocean Rise (EPR), 9 °N, 104 °W	37	(Perez-Rodriguez *et al.* 2013)
		A. sp. EPR8,	Nitrate-reducing. Ferment glucose	Mk 119 Vent, East Pacific Ocean Rise	37-45	(Perez-Rodriguez *et al.* 2013)

		A. sp. EPR12	Nitrate-reducing. Ferment glucose	Mid-Atlantic Ocean Ridge	28	(Perez-Rodriguez *et al.* 2013)
		A. sp. MAR14	Grow aerobically on *n*-alkane (Dodecane)	Mid-Atlantic Ocean Ridge, 37 °N, 32 °W	28-30	(Bertrand *et al.* 2013)
	Alteromonas	*A. macleodii* subsp. *fijiensis*	Aerobic, heterotrophic	North Fiji, Pacific Ocean basin	25-35	(Raguenes *et al.* 1996)
		A. infernus	Aerobic, heterotrophic	Guaymas basin, Pacific Ocean	25-35	(Raguenes *et al.* 1997)
		A. macleodii subsp. *fijiensis*	Aerobic, heterotrophic	Snake Pit, MAR, Atlantic Ocean	35-40	(Raguenes *et al.* 2003)
	Amphritea	*A. atlantica*	Aerobic, heterotrophic	Logatchev, MAR, Atlantic Ocean	31-34	(Gartner *et al.* 2008)
	Halomonas	*H. axialensis*	Aerobic, heterotrophic	Juan de Fuca, Pacific Ocean	30	(Kaye *et al.* 2004)
		H. hydrothermalis	Aerobic, heterotrophic	South Pacific Ocean	30	(Kaye *et al.* 2004)
		H. neptunia	Aerobic, heterotrophic	Juan de Fuca, Pacific Ocean	30	(Kaye *et al.* 2004)
		H. profundus	Aerobic, heterotrophic	Rainbow, MAR, (Mid-Atlantic Ridge)	32-37	(Simo*n*-Colin *et al.* 2008)
		H. sulfidaeris	Aerobic, heterotrophic	Juan de Fuca, Pacific Ocean	20-35	(Kaye *et al.* 2004)
		H. sp. EPR84	Aerobic, mesophilic.	Mk 119 Plume, East Pacific Ocean Rise	30	(Perez-Rodriguez *et al.* 2013)
	Idiomarina	*I. loihiensis*	Aerobic, heterotrophic, and facultative psychrophilic.	Lōʻihi Seamount, Hawai'i, Pacific Ocean	30	(Donachie *et al.* 2003)

	Klebsiella	*K.* sp.	Aerobic, mesophilic. Growth in anaerobic ASW with NO^{3-}, and reduce nitrate to nitrite; ferment glucose	Crab Spa vent, East Pacific Ocean Rise	30	(Perez-Rodriguez *et al.* 2013)
	Marinobacter	*M.* sp. EPR21	Grow aerobically on *n*-alkane (Dodecane)	Diffuse flow vent, Mk119, EPR, Pacific Ocean	37	(Bertrand *et al.* 2013)
		M. sp.	Aerobic, mesophilic. Growth in anaerobic, ASW with NO^{3-}. Nitrate-reducing.	Mk 119 Plume, East Pacific Ocean Rise.	28-45	(Perez-Rodriguez *et al.* 2013)
		M. sp. EPR35	Growth in anaerobic ASW with NO^{3-}. Ferment glucose	Mk 119 Plume, East Pacific Ocean Rise	28	(Perez-Rodriguez *et al.* 2013)
	Pseudomonas	*P.* sp.	Aerobic, mesophilic. Growth in anaerobic ASW with $NO^{3-,}$ nitrate-reducing.	Mk 119 Plume, East Pacific Ocean Rise	28-30	(Perez-Rodriguez *et al.* 2013)
	Psychrobium	*P. conchae*	Strictly aerobic, chemoorganotrophic	Iheya North hydrothermal field, Okinawa Trough, Pacific Ocean	9-12	(Nogi *et al.* 2014)
	Salinisphaera	*S. hydrothermalis*	Aerobic, heterotrophic, facultative lithoautotrophic using thiosulfate, and CO_2	ERP 9 °N, Pacific Ocean	30-35	(Crespo-Medina *et al.* 2009)
	Thiogranum	*T. longum*	Aerobic, Obligate chemolithoautotrophic, sulfur, thiosulfate, sulfite, sulfide and tetrathionate-oxidizing	Suiyo Seamount, Izu-Bornin Arc, western Pacific Ocean	32	(Mori *et al.* 2015)
	Thiolapillus	*T. brandeum*	Facultative anaerobic. Sulfur-oxidizing, facultative chemolithoautotrophic. Electron donors: sulfur, thiosulfate and tetrathionate. Electron acceptors: oxygen and nitrate.	Minami-Ensei Knoll hydrothermal field, Okinawa Trough, Pacific Ocean	40	(Nunoura *et al.* 2014)
	Thiomicrospira	*T. crunogena*	Aerobic, autotrophic, oxidizing thiosulfate, sulfur and hydrogen sulfide to sulfate and elemental sulfur	EPR 21° N, Pacific Ocean	28-32	(JANNASCH *et al.* 1985)

		T. thermophila	Microaerophilic, chemolitho-mixotrophic, sulfur-oxdizing	Mariana Arc, West Pacific Ocean	35-40	(Takai *et al.* 2004)
		T. sp. MA-3	Aerobic, autotrophic, oxidize thiosulfate, sulfur and hydrogen sulfide to sulfate and elemental sulfur	TAG, MAR, Atlantic Ocean	28-32	(Wirsen *et al.* 1998)
		T. sp. L-12	Aerobic, autotrophic, oxidize thiosulfate, sulfur and hydrogen sulfide to sulfate and elemental sulfur	Galapagos Islands, Pacific Ocean	25	(Ruby and Jannasch 1982)
	Thioprofundum	*T. hispidum*	Facultative anaerobic, autotrophic. Electron donors: elemental sulfur, thiosulfate, or tetrathionate. Electron acceptors: oxygen or nitrate.	Suiyo Seamount, Izu-Bonin Arc, Pacific Ocean	39	(Mori *et al.* 2011)
		T. lithotrophica	Facultative aerobic, lithoautotrophic. Electron donors: elemental sulfur, thiosulfate, or tetrathionate. Electron acceptors: oxygen or nitrate.	TAG, MAR, Atlantic Ocean(isolated under hydrostatic pressure)	50	(Takai *et al.* 2009)
	Vibrio	*V. diabolicus*	Facultative anaerobic, heterotrophic	EPR 13 °N, Pacific Ocean	30-45	(Raguenes *et al.* 1997)
Epsilonproteo bacteria	*Caminibacter*	*C. hydrogeniphilus*	Anaerobic, autotrophic, sulfur-reducing, denitrifying	ELSA, EPR 13 °N, Pacific Ocean	60	(Alain *et al.* 2002)
		C. profundus	Microaerophilic, autotrophic. Anaerobic, sulfur-reducing, denitrifying	Rainbow, MAR, Atlantic Ocean	55	(Miroshnichenko *et al.* 2004)
		C. mediatlanticus	Anaerobic, autotrophic, sulfur-reducing, denitrifying	Rainbow, MAR, Atlantic Ocean	55	(Voordeckers *et al.* 2005)

	Cetia	*C. pacifica*	Thermophilic, anaerobic, and chemolithoautotrophic. Sulfur and nitrate-reducing. Hydrogen-oxidizing.	East Pacific Rise at Bio 9" site (9° 49' N, 104° 17' W).	55-60	(Grosche *et al.* 2015)
	Nautilia	*N. lithotrophica*	Anaerobic, autotrophic, sulfur-reducing	EPR 13 °N, Pacific Ocean	53	(Miroshnichenko *et al.* 2002)
		N. abyssi	Anaerobic, heterotrophic and lithoautotrophic, sulfur-reducing	ELSA, EPR 13 °N, Pacific Ocean	60	(Alain *et al.* 2009)
		N. profundicola	Anaerobic mixotroph, sulfur-reducing using hydrogen and formate as electron donor.	EPR 13 °N, Pacific Ocean	40	(Smith *et al.* 2008)
		N. nitratireducens	Anaerobic mixotroph using NO_3^-, $S_2O_3^-$, S ° and selenate as electron acceptor	L-vent, EPR 9 °N, Pacific Ocean	55	(Perez-Rodriguez *et al.* 2010)
	Sulfurospirillum	*S.* sp. Am-N	Anaerobic, heterotrophic	EPR 13 °N, Pacific Ocean	41	(Campbell *et al.* 2001)
	Sulfurimonas	*S. autotrophica*	Aerobic, autotrophic, sulfur and thiosulfate oxidation	Mid-Okinawa Trough Pacific Ocean	25	(Inagaki *et al.* 2003)
		S. paralvinellae	Facultative anaerobic chemolithoautotroph using H_2, S °or thiosulfate as energy source.	Mid-Okinawa Trough, Pacific Ocean	30	(Takai *et al.* 2006)
	Hydrogenimonas	*H. thermophila*	Microaerophilic, anaerobic. Autotrophic, sulfur-reducing, denitrifying.	Central Indian ridge, Indian Ocean	55	(Takai *et al.* 2004)
	Sulfurovum	*S. lithotrophicum*	Microaerophilic, anaerobic. Autotrophic, sulfur and thiosulfate-oxidizing	Okinawa Trough, Japan, Pacific Ocean	28-30	(Inagaki *et al.* 2004)
		S. aggregans	Mesophilic, chemolithoautotrophic, strictly anaerobic, hydrogen-oxidizing, sulfur, nitrate and thiosulfate-reducing.	Central Indian Ridge	33	(Mino *et al.* 2014)
	Thioreductor	*T. micantisoli*	Autotrophic, anaerobic. Sulfur-reducing, denitrifying	Okinawa Trough, Japan, Pacific Ocean	32	(Nakagawa *et al.* 2005)

	Lebetimonas	*L. acidiphila*	Autotrophic, anaerobic, sulfur-reducing	Mariana Arc, Pacific Ocean	50	(Takai *et al.* 2005)
	Thiofractor	*T. thiocaminus*	Anaerobic, lithoautotrophic using hydrogen as energy source, S °as electron acceptor.	Nikko Seamount, Mariana Arc, Pacific Ocean	37	(Makita *et al.* 2012)
Zetaproteobacteria	*Mariprofundus*	*M. ferrooxydans*	Aerobic. Obligate chemolithotrophic, requires Fe^{2+} as the energy source	Lōʻihi Seamount in the Pacific Ocean	30	(Emerson *et al.* 2007)

The cultivated *Bacteria* from deep-sea hydrothermal vent environments regroup a large fraction of *Proteobacteria* (*Alphaproteobacteria*, *Gammaproteobacteria*, *Epsilonproteobacteria* and *Zetaproteobacteria*) (Pettit 2011, Jebbar *et al.* 2015). *Proteobacteria* are Gram-negative bacteria, comprising half of all cultured bacteria, and represent a diverse range of microorganisms with numerous genome sizes and life histories (Thornburg *et al.* 2010).

Epsilonproteobacteria are widely distributed in nature, and increasingly recognized as an ecologically significant bacterial group, particularly in deep-sea hydrothermal vents (Nakagawa *et al.* 2005). The majority of the *Epsilonproteobacteria* from deep-sea hydrothermal vents are anaerobic, and only a few of them are facultative anaerobic or microaerophilic, like *Caminibacter profundus* and *Sulfurovum lithotrophicum* isolated from the Atlantic and Pacific Oceans (Inagaki *et al.* 2004, Miroshnichenko *et al.* 2004). Most of them can grow autotrophically with hydrogen, sulfur, or thiosulfate as an energy source and sulfate, sulfur, thiosulfate, selenate or nitrate as a terminal electron acceptor, while some of them are mixotrophic, like *Nautilia profundicola* using formate as a source of carbon for heterotrophic and mixotrophic growth (Smith *et al.* 2008). Several publications suggested that *Epsilonproteobacteria* play a significant role in the nitrogen, sulfur, and hydrogen fluxes at deep-sea hydrothermal vents (Nakagawa *et al.* 2005, Campbell *et al.* 2006).

The *Gammaproteobacteria* class is also ubiquitous in deep-sea vent environments. In contrast to the *Epsilon-Proteobacteria*, most of the *Gammaproteobacteria* are aerobic, and only a few are facultative anaerobic or microaerophilic like the facultative anaerobic bacterium *Thiolapillus brandeum* isolated from Minami-Ensei Knoll hydrothermal field, Okinawa Trough, Pacific Ocean (Nunoura *et al.* 2014). They can be heterotrophic, facultative chemolithoautotrophic, obligately chemolithoautotrophic or mixotrophic. For example, members of genus *Halomonas* are heterotrophic (Gaboyer *et al.* 2014). *Thiogranum longum* gps52^{T} is an obligate chemolithoautotrophic, sulfur-oxidizing bacterium isolated from a rock sample collected near the hydrothermal vents of the Suiyo Seamount in the Pacific Ocean (Mori *et al.* 2015). Finally, *Thiomicrospira thermophila* is a chemolitho-mixotroph, capable of using not only elemental sulfur, thiosulfate, or sulfide as the only energy source, and oxygen as electron acceptor, but also various organic substrates (complex proteinaceous compounds, carbohydrates, organic acids, amino acids and sugars) as the carbon source with sulfur oxidation (Takai *et al.* 2004).

Alphaproteobacteria from deep-sea hydrothermal environments are aerobic or facultative aerobic, heterotrophic or mixotrophic. *Piezobacter thermophiles* 108^T is a mixotroph, capable of both organotrophic growth with complex organics or organic acids using nitrate and O_2 as the terminal electron acceptors and able also to grow chemolithoautotrophically with H_2 or S as electron donors. The moderately thermophilic bacterium *Methyloceanibacter caenitepidi* Gela4^T is a methanol oxidizer and facultative methylotroph utilizing methylamine, methanol, trimethylamine and a variety of multi-carbon compounds (Takeuchi *et al.* 2014). Some *Alphaproteobacteria* from deep-sea hydrothermal environments are Mn(II)-oxidizing bacteria, like *Aurantimonas* sp. AE01-7 isolated from a hydrothermal vent tubeworm (Anderson *et al.* 2009) and *Fulvimarina manganoxydans* 8047^T isolated from a deep sea hydrothermal vent plume in the Southwest Indian Ocean (Ren *et al.* 2014). *Parvibaculum hydrocarboniclasticum* EPR92^T is a mesophilic, alkane-oxidizing alpha-proteobacterium able to grow on *n*-alkanes as sole carbon and energy sources (Rosario-Passapera *et al.* 2012, Bertrand *et al.* 2013).

A novel class of the *Proteobacteria*, the *Zetaproteobacteria*, was proposed by *Emerson et al* (2007). *Mariprofundus ferrooxydans* PV-1^T, isolated from Loihi Seamount in the Pacific Ocean, is an obligate chemolithotroph which grows microaerobically with Fe^{2+} as the energy source and CO_2 as the only carbon source (Emerson *et al.* 2007).

The majority of the *Firmicutes* isolated from deep-sea hydrothermal environments are anaerobic, heterotrophic and fermentative (Table 2). Several novel *Firmicutes* bacteria were described recently. *Anoxybacter fermentans* DY22613^T, isolated from a deep-sea hydrothermal sulfide deposit at the East Pacific Rise, was found to be a novel piezophilic, thermophilic, anaerobic, fermentative bacterial strain, which was able to reduce Fe(III) compounds and elemental sulfur (Zeng *et al.* 2015). A thermophilic, anaerobic, hydrogen-producing bacterium, *Caloranaerobacter ferrireducens* DY22619^T, isolated from a sulfide sample collected from an East Pacific Ocean hydrothermal field, is an obligate chemoorganoheterotroph which is facultatively dependent on various forms of Fe(III) as an electron acceptor (Jiang *et al.* 2015, Zeng *et al.* 2015). *Crassaminicella profunda* Ra1766H^T, isolated from sediments of the Guaymas basin, is a novel, anaerobic, chemo-organotrophic bacterium fermenting carbohydrates (glucose and mannose) and organic acids (pyruvate and succinate).

Aquificae bacteria are anaerobic or microaerophilic, thermophilic autotrophs using sulfate, thiosulfate or sulfur as electron acceptor, which could play an important role in deep-sea hydrothermal ecosystems as primary producers of organic matter in anaerobic zones (Miroshnichenko and Bonch-Osmolovskaya 2006). Members of genera *Persephonella*, *Phorcysia*, *Thermosulfidibacter* and *Thermovibrio* are able to reduce sulfate or thiosulfate, but not sulfur. Representatives of the *Desulfurobacterium* and *Balnearium* genera reduce sulfur or thiosulfate, but not sulfate (Table 2). *Hydrogenivirga okinawensis* LS12-2^T, isolated from a deep-sea hydrothermal field at the Southern Okinawa Trough, is an obligately chemolithoautotrophic bacterium using thiosulfate or elemental sulfur as an electron donor and oxygen or nitrate as an electron acceptor (Nunoura *et al.* 2008).

In contrast to *Aquificae*, most of *Thermotogae* are hetetrophic fermentative bacteria, using sulfate, or elemental sulfur as an electron acceptor. *Thermotogae* bacteria are anaerobic, thermophilic heterotrophs, and several novel *Thermotogae* bacteria were isolated and characterized recently. A novel moderately thermophilic, heterotrophic sulfur-reducing bacterium, *Mesoaciditoga lauensis* cd-1655R^T, was isolated from a deep-sea hydrothermal vent deposit from the Pacific Ocean (Reysenbach *et al.* 2013). *Kosmotoga pacifica* SLHLJ1^T, isolated from a Pacific hydrothermal sediment, is a novel strictly anaerobic thermophilic heterotrophic bacterium (L'Haridon *et al.* 2014). *Thermosipho globiformans* MN14^T isolated from Pacific Suiyo Seamount and *T. activus* Rift-s3^T isolated from Guaymas Basin in Gulf of California are anaerobic, thermophilic, obligately chemoorganotrophic sulfur and ferro-reducing bacteria (Kuwabara *et al.* 2011, Podosokorskaya *et al.* 2014).

Strains belonging to *Thermodesulfobacteria*, *Acidobacteria*, *Deinococcus-Thermus*, *Deferribacteres*, *Actinobacteria*, *Bacteroidetes*, *Spirochaetes*, and unclassified Bacteria were also isolated from deep-sera hydrothermal vents, all of which are thermophiles with optimal temperature ranging from 50-80 ℃. *Thermodesulfobacteria* are autotrophs, while *Deinococcus-Thermus*, *Acidobacteria*, *Deferribacteres*, *Bacteroidetes*, and *Spirochaetes* are heterotrophs (Table 2).

As shown in table 2, most of the deep-sea vent chemolithoautotrophic bacteria can get energy from diverse sources: hydrogen, reduced metals, sulfur compounds, and NH_4^+. They coupled the oxidation of these compounds to the reduction of electron acceptors like nitrate, sulfur compounds,

sulfate, Fe (III), and oxygen. The majority of the cultured Bacteria from deep-sea vents are thermophiles with optimal temperatures for growth of 50 ℃ or more (Table 2). There are also psychrophilic and mesophilic bacteria in deep-sera hydrothermal environments. For example, the bacteria *Psychrobium conchae* BJ-1^{T}, *Idiomarina loihiensis* L2-TR and *Mariprofundus ferrooxydans* PV-1 are psychrophilic isolates from hydrothermal vents (Donachie *et al.* 2003, Hou *et al.* 2004, Siddiqui *et al.* 2013, Nogi *et al.* 2014).

In summary, more than two hundred species of Bacteria and Archaea were isolated and cultured from deep-sea hydrothermal vent systems. Most of them were isolated from vents located in the Pacific (about 73 % of the isolates) and Atlantic (20 % of the isolates) Oceans, but only a few are originating from the Indian (5 % of the isolates) Ocean (Tables 1 and 2). Although our knowledge of the diversity and roles of hydrothermal vents isolates has remarkably expanded over the past decade, extensive investigations of microbiology and physiology in deep-sea hydrothermal vents remain imperative, especially in the Indian Ocean.

First and next-generation sequencing surveys based on 16S rRNA genes and functional genes have revealed an unexpected diversity of uncultured bacterial and archaeal lineages at deep-sea hydrothermal vents (Sogin *et al.* 2006, Anderson *et al.* 2015), which remain for now without cultured representatives that could serve as a reference for metabolic inferences to design cultivation strategies. The wide physiological diversity discovered so far within the microorganisms isolated from hydrothermal samples may just be the tip of the iceberg.

1.4 Sulfate and sulfur-reducing prokaryotes

Sulfur is abundant on Earth, and is present as organic sulfur (like coenzymes and amino acids), inorganic sulfur (like sulfides, elemental sulfur, sulfites, thiosulfate, and sulfates), and a series of intermediaries of minor environmental relevance (Sanz 2015). The sulfur cycle is complex and transformations occur both chemically and biologically; Microorganisms play important roles in the chemical transformations of sulfur species, through their metabolism notably (Figure 3). Sulfate is thermodynamically stable and more abundant than other form of sulfur in our oxic biosphere. Thus, sulfate reduction represents the basis of the biological sulfur cycle (Muyzer and Stams 2008). Sulfur reduction is also important to cycle the sulfur transformation, as there are intermediate

oxidation states such as elemental sulfur or thiosulfate. A great diversity of sulfur and sulfate-reducing prokaryotes, found in several phyla within the domains *Archaea* and *Bacteria*, has been isolated from aquatic habitats (Rabus *et al.* 2013). Sulfur disproportionation and sulfide oxidation are the others important reactions of the sulfur cycle but they will not be addressed in this manuscript.

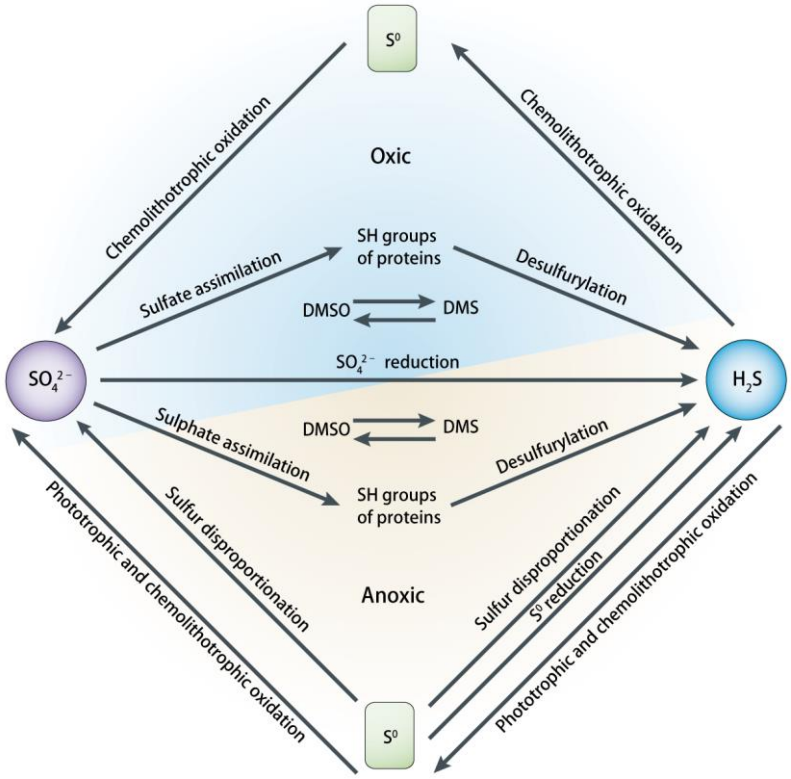

Figure 3 | The sulfur cycle.

Sulfate and sulfur-reducing prokaryotes play a key role in the sulfur transformations (Muyzer and Stams 2008).

1.4.1 Sulfate-reducing prokaryotes

Sulfate is the major bioavailable form of sulfur on Earth, and is particularly abundant in sea waters (Sanz 2015). Sulfate-reducing prokaryotes gain energy for growth by oxidation of organic compounds or hydrogen coupled with reduction of sulfate to sulfide. The detection of high concentrations of sulfide usually indicates the activity of sulfate-reducing microorganisms in natural environments (Muyzer and Stams 2008, Rabus *et al.* 2013). A great variety of sulfate-reducing microorganisms has been isolated from aquatic environments.

Sulfate-reducing bacteria fall into three major groups: (i) *Firmicutes*, Gram-positive bacteria with the genera *Desulfosporosinus* and *Desulfotomaculum*, (ii) the large majority of the genera of *Deltaproteobacteria* and (iii) deep bacterial branches formed by *Thermodesulfobacterium*, *Thermodesulfatator* and *Thermodesulfovibrio* (Rabus *et al.* 2013). Sulfate-reducing bacteria isolated from deep-sea hydrothermal vent environments belonged to several genera including *Thermodesulfobacterium*, *Thermodesulfatator*, *Desulfovibrio*, *Desulfonauticus*, and *Desulfothermus* (Table 2). Most of these sulfate-reducers are anaerobic thermophiles using sulfite or thiosulfate as alternative electron acceptors. The sulfate-reducer *Desulfonauticus submarinus* $6N^T$ was reported to be able use elemental sulfur as electron acceptor for growth (Audiffrin *et al.* 2003). However, the growth of many sulfate-reducers could be inhibited by sulfur, which is an oxidant shifting the potential of redox couples in the medium and cells to unfavorable positive values (Rabus *et al.* 2013).

In contrast to sulfate-reducing bacteria, only few sulfate-reducing archaea have been isolated from the hydrothermal ecosystem. The archaeal sulfate-reducers *Archaeoglobus profundus* $DSM5631^T$ and *Palaeococcus pacificus* $DY20341^T$ were reported to be isolated from deep-sea hydrothermal vent environments. *Archaeoglobus profundus* $DSM5631^T$ was obligate mixotrophs, using sulfate, thiosulfate and sulfite as electron acceptors and strictly requiring H_2 and an organic carbon source (like acetate) (Burggraf *et al.* 1990). *Palaeococcus pacificus* $DY20341^T$ was a hyperthermophilic, anaerobic, piezophilic chemoorganoheterotroph, facultatively using elemental sulfur or sulfate as electron acceptors (Zeng *et al.* 2013).

1.4.2 Sulfur-reducing prokaryotes

The oxidation processes of sulfide produce not only sulfate but also intermediate oxidation forms of sulfur such as thiosulfate or elemental sulfur. Elemental sulfur can serve as electron acceptor for anaerobic prokaryotes that cannot reduce sulfate. The first sulfur-reducer, *Desulfuromonas acetoxidans*, was identified as an obligately anaerobic mesophile using acetate as an electron donor (Pfennig and Biebl 1976). Since then, a great diversity of sulfur-reducers has been isolated reducing elemental sulfur but not sulfate.

Sulfur-reducing bacteria isolated from deep-sea hydrothermal vent environments mainly belong to several classes, including *Aquificae*, *Thermotogae*, *Deferribacteres*, *Epsilonproteobacteria*, and *Gammaproteobacteria* (Table 2). Most of these sulfur-reducers are thermophilic anaerobes, and a few of them are able to grow under microaerobic conditions. For example, *Hydrogenimonas thermophila* JCM 11971^{T}, isolated form a black smoker in a Central Indian Ridge hydrothermal field, is a microaerobic-to-anaerobic chemolithoautotroph using molecular oxygen, nitrate or elemental sulfur as electron acceptor (Takai *et al.* 2004). Most sulfur-reducing bacteria can alternatively use nitrate, Fe (III), or thiosulfate as electron acceptor.

Sulfur-reducing bacteria are mesophilic or thermophilic, while sulfur-reducing archaea isolated so far are all hyperthermophiles with optimal growth temperatures of 80-99 ℃. Sulfur-reducing archaea isolated from deep-sea hydrothermal vent environments mainly belong to several classes, including *Thermococci*, *Methanopyri*, *Thermoprotei*, *Aciduliprofundum*, and *unclassified Euryarchaeota* (Table 1). Members of the genera *Pyrococcus*, *Palaeococcus*, *Desulfurococcus*, *Staphylothermus*, *Pyrodictium*, *Aciduliprofundum* and *Thermococcus* are anaerobic sulfur-reducing heterotrophs, while the sulfur-reducer *Ignicoccus pacificus* is an anaerobic chemolithoautotroph using molecular hydrogen as the electron donor.

1.5 Cultivation efforts remain crucial

Up to date, there are 159 (see the tables 1 and 2) novel genera/species that have been isolated from deep-sea hydrothermal vents, representing only a minor fraction of the microbial diversity of this ecosystem, like in most natural environments. The number of prokaryotes on earth is extremely high, exceeding 10^{30} cells, and the overwhelming majority of them remain uncultured. However, even the most metabolically active and the most numerous microbes from a well-known environment are often difficult to grow in the laboratory. Estimations indicate that more than 99% of all existing prokaryotes have resisted cultivation in the laboratory (Amann *et al.* 1995), limiting the investigation of their physiology and ecological role in Nature. This is notably due to the incredible array of physiological capabilities that microorganisms possess. Indeed, we still discover novel microbial yielding energy metabolisms and novel physiological capabilities every year. The low success in cultivation efficiency is also due to the fact that cultivation is time-consuming (Alain

and Querellou 2009). Today, cultures in Petri dishes onto solidified media and liquid cultures in tubes or Erlenmeyers are still applied in most microbiology laboratories, like in the early age of microbiology. The last two decades, the advent of high-throughput cultivation techniques, the development of miniaturized cultivation methods, the used of oligotrophic media and the creativity of microbiologists to design new media improved cultivation efficiency and allowed the cultivation of previously uncultured taxa (Connon and Giovannoni 2002, Konneke *et al.* 2005, Ingham *et al.* 2007, Alain and Querellou 2009, Zengler 2009). In addition, next-generation sequencing provided unprecedented insights into the metabolisms of microorganisms by sequencing microbial metagenomes, metatranscriptomes and single cell genomes; information encoded in genomes and transcriptomes was used for directing the design of culturing conditions based on the *in situ* metabolism and allowed cultivating not-yet cultured microorganisms (Giovannoni and Stingl 2007, Bomar *et al.* 2011). In this era of omics, the cultivation of microorganisms remains crucial in several respects. It makes it possible to perform a direct and easy study of the microbial morphology, physiology, genetics and pathogenicity (Zengler 2009). From an ecological point of view, it is also very helpful to have isolates to integrate at the cell level ecological data got *via* top-down (meta-omics, rate measurements, etc.) and bottom-up (single-cell techniques, metabolomics, transcriptomics, etc.) approaches. Finally, microbial isolates allow testing hypotheses that arise from (meta-)genomic data.

First and next-generation sequencing surveys based on 16S rRNA genes and functional genes have revealed an unexpected diversity of uncultured bacterial and archaeal lineages at deep-sea hydrothermal vents (Flores and Reysenbach 2011, Takai and Nakamura 2011), which remain for now without cultured representatives that could serve as a reference for metabolic inferences to design cultivation strategies. Some of these lineages are distantly related to well-defined phyla and represent deeply branching lineages that emerge close to the first delineation between bacterial and archaeal branches. It is obvious that some of the hydrothermal prokaryotic lineages without cultured representatives (candidates divisions) encompass key ecological players of this ecosystem. The wide physiological diversity discovered so far within the microorganisms isolated from hydrothermal samples may just be the tip of the iceberg. For all these reasons, much effort should be made to grow presently uncultured microorganisms.

1.6 Genomic studies of deep-sea hydrothermal vents

Traditional cultivation approaches and molecular approaches have significantly expanded our understanding of microbial diversity and ecology (DeLong 2005). Recent advances in genomic technologies similarly had great impact on microbiology, providing further insights into microbial evolution, adaptations, physiology and ecology (Schleper *et al.* 2005). Deep-sea hydrothermal environments are the most ancient continuously inhabited ecosystems on Earth. The geochemistry of deep-sea hydrothermal vents influenced the evolution of life. In turn, biological processes in deep-sea hydrothermal vents also influenced geochemistry. Genome sequences could be useful tools for analyzing the relationships between microbial genotypes, phenotypes and environments in deep-sea hydrothermal systems, as shown in figure 4 (Reysenbach and Shock 2002).

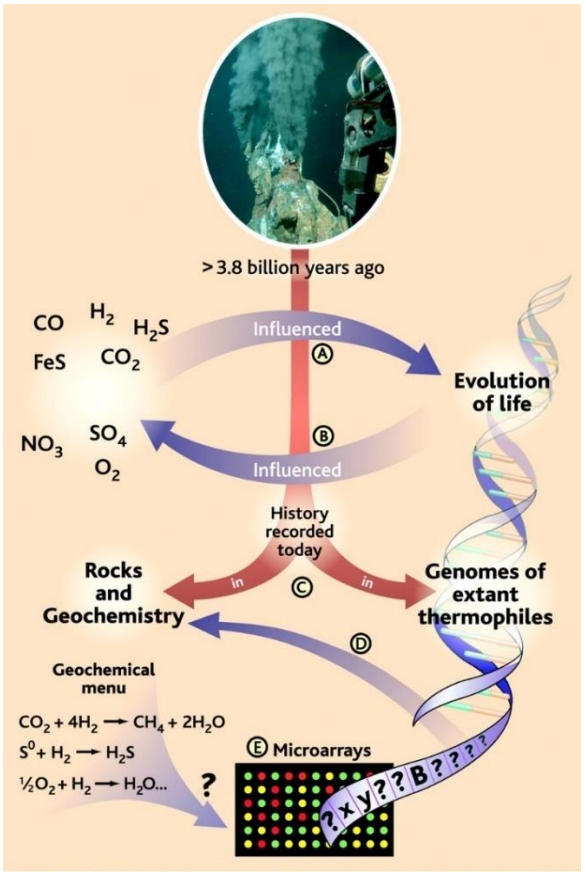

Figure 4 | Biological process and geochemistry interaction in hydrothermal ecosystems.

Genome sequences are useful tools for studying the relationship between microbial genotype, phenotype and environments (Reysenbach and Shock 2002).

The first genome sequence of a prokaryote from the deep-sea hydrothermal vent environment belonged to a methanogenic archaeon, *Methanocaldococcus jannaschii*, which was isolated from a "white smoker" chimney on the East Pacific Rise (Bult *et al.* 1996). Since then, many bacterial and archaeal genomes from deep-sea hydrothermal vents have been sequenced and annotated.

For example, the genome sequence of the deep-sea *Gamma-proteobacterium Idiomarina loihiensis* revealed an integrated mechanism of metabolic adaptation to the constantly changing deep-sea hydrothermal ecosystem (Hou *et al.* 2004). The genome of the deep-sea vent chemolithoautotroph *Thiomicrospira crunogena* XCL-2 was shown to have characteristics consistent with an obligately chemolithoautotrophic lifestyle, including few transporters predicted to have organic allocrits, and Calvin-Benson-Bassham cycle coding sequences scattered throughout the genome (Scott *et al.* 2006). The genome of the *Epsilon-proteobacterial* chemolithoautotroph *Sulfurimonas denitrificans* was analyzed to get a better understanding of the ecology and roles of sulfur-oxidizing *Epsilon-proteobacteria* in biogeochemical cycles (Sievert *et al.* 2008). Several other *Epsilon-proteobacteria* genomes (*Caminibacter mediatlanticus* TB-2^{T}, *Sulfurovum* sp. NBC37-1 and *Nitratiruptor* sp. SB155-2) were also sequenced and analyzed (Nakagawa *et al.* 2007, Giovannelli *et al.* 2011).

The genome of the hyperthermophilic archaeon *Pyrococcus abyssi* was annotated to explore its phylogeny, molecular biology and physiology, which provided an almost complete map of the key metabolic pathways (Cohen *et al.* 2003). Several other *Pyrococcus* genomes (*P.* sp. NA2, *P. yayanosii*, *P.* sp. ST04, and *P. horikoshii*) have also been completed to study their distinct characteristics, piezoadaptation strategy and evolution scenarii of metabolic pathways (Kawarabayasi *et al.* 1998, Jun *et al.* 2011, Lee *et al.* 2011, Jung *et al.* 2012). Several archaea of the genus *Thermococcus* (*T. kodakaraensis*, *T. onnurineus*, *T. gammatolerans*, *T.* sp. strain 4557, *T. sibiricus*, and *T. barophilus*) have been sequenced to investigate the metabolism, adaptation, and ecology in deep-sea hydrothermal ecosystem (Fukui *et al.* 2005, Lee *et al.* 2008, Mardanov *et al.* 2009, Zivanovic *et al.* 2009, Vannier *et al.* 2011, Wang *et al.* 2011). Genome contents and physiological studies revealed for example that some *Thermococcus* species, a taxon generally known for its capacity to grow by fermentation and to reduce sulfur species, are able to grow by carboxydotrophy (Sokolova *et al.* 2004).

Although many deep-sea vent prokaryotes genomes have been sequenced and analyzed, genomic approaches for studying natural microbiology become only now more widespread. Many more bacterial and archaeal genome sequencing projects are now underway (DeLong 2005). Furthermore, the presently available powerful “omic” (metagenomics, metatranscriptomics, etc.) and single-cell techniques are providing a new perspective on the naturally occurring microbial world (DeLong 2005, Sievert and Vetriani 2012).

2 Study scope and objectives

Contextual information about the PhD thesis. *Initially, I was supposed to perform this thesis in co-supervision and co-awarding of degree between the Laboratory of Microbiology of Extreme Environments, UMR 6197 UBO-CNRS-Ifremer, located in Brest (France), and the Harbin Institute of Technology, located in Harbin (China). For administrative reasons, the co-awarding of degree is not possible and I have to perform two PhD theses in parallel, one in two years in France, and one in four years in China. The initial objective of this thesis was to perform genetic manipulations with the strain Palaeococcus pacificus in order to design a genetic tool, and to isolate in parallel novel taxa involved in the sulfur cycles from Indian hydrothermal vents. Considering that the development of a novel genetic tool was the heart of the project and was time-consuming, we choose to use only traditional culturing strategies to isolate novel taxa. Unfortunately, we were not able to reach the initial objective and this thesis was redirected only on the cultural part. A summary of the efforts done to design the genetic tool is given at the end of the manuscript.*

Deep-sea hydrothermal vents are among the most biologically active regions, and support highly productive ecosystems fueled by chemosynthesis (Sievert and Vetriani 2012). Sulfate and sulfur-reducing prokaryotes, ubiquitous in anoxic habitats, play an important role in both the sulfur and carbon cycles (Muyzer and Stams 2008). Although our knowledge of the diversity and roles of hydrothermal vents isolates has remarkably expanded, extensive investigation of the microbiology and physiology remain imperative, especially in the Indian Ocean.

In this thesis, microorganisms involved in the sulfur cycle were enriched and isolated from deep-sea hydrothermal vent samples of the Indian Ocean. Six anaerobic prokaryotes involved in the sulfur cycle were isolated by repeated dilutions-to-extinction series from these samples. We carried out taxonomic studies of three novel strains were carried out based on 16S rRNA gene phylogenetic analysis, phenotypic and chemotaxonomic characterizations. The genomes of these three novel strains were sequenced and annotated, with focus on the genes involved in the sulfur cycle, to gain insights into the molecular basis of the sulfur metabolism.

3 Materials and Methods

3.1 Collection of bulk samples

A deep-sea serpentinized peridotite sample was collected at a depth of 3173 m in a hydrothermal area of the Indian Ocean (27° 88’ S, 63° 53’ E; site 30I-TVG05) in December 2013, during the cruise DY30/I of *Da Yang Yi Hao*.

A deep-sea sulfide sample (site JL-Dive94-S01) was collected at a depth of 2771.2 m from a hydrothermal vent (active vent, 360.9 ℃) in the Indian Ocean (37° 78’S, 49° 65’E) in January 2015, during the DY35 cruise of *Xiang Yang Hong Jiu Hao*. Another deep-sea sulfide sample (site JL-Dive90-S01) was collected at a depth of 2736.7 m from a deep-sea chimney wall (active vent, 145 ℃) in the Indian Ocean (37° 78’S, 49° 65’E) in January 2015, during the DY35 cruise of *Xiang Yang Hong Jiu Hao*.

The samples were collected using a grabber and anaerobically preserved in sterilized seawater. Once in the lab, subsamples were used to isolate anaerobic microorganisms involved sulfur cycle. Environmental physical-chemical characteristics of the sampling sites were not monitored.

3.2 Solutions and Media

Trace element solution

Add 1.5 g nitrilotriacetic acid to approximately 500 mL of water and adjust to pH 6.5 with KOH to dissolve. Add the following: 3.0 g $MgSO_4.7H_2O$, 0.5 g $MnSO_4.H_2O$, 1.0 g NaCl, 0.1 g $FeSO_4.7H_2O$, 0.1 g $CoCl_2.6H_2O$, 0.1 g $CaCl_2$, 0.1 g $ZnSO_4.7H_2O$, 0.01 g $CuSO_4.5H_2O$, 0.01 g $AlK(SO)_4.12H_2O$, 0.01 g H_3BO_3, and 0.01 g $Na_2MoO_4.2H_2O$. Bring volume to 1 L with distilled water. Sterilize by filtration (Alain *et al.* 2010).

Vitamin solution

Dissolve the following compounds in 1 L distilled water: 10 mg Pyridoxine hydrochloride, 5.0 mg Thiamine-HCl, 5.0 mg Riboflavin, 5.0 mg Nicotinic acid, 5.0 mg Calcium D-(+)-pantothenate, 5.0 mg p-Aminobenzoic acid, 5.0 mg Thioctic acid, 2.0 mg Biotin, 2.0 mg Folic Acid, and 0.1 mg Vitamin B12. Sterilize by filtration (Alain *et al.* 2010).

Selenite-tungstate solution

Dissolve the following compounds in 1 L distilled water: NaOH 500.00 mg, 3.00 mg $Na_2SeO_3.5H_2O$, and 4.00 mg $Na_2WO_4.2H_2O$. Sterilize by filtration (Alain *et al.* 2010).

TRM (*Thermococcales* Rich Medium)

Composition: NaCl 23.00 g, $MgCl_2.6H_2O$ 5,00 g, KCl 0.70 g, $(NH_4)_2SO_4$ 0.50 g, NaBr 0.05 g, $SrCl_2$ 0.01 g, PIPES 3.30 g, Yeast Extract 1.00 g, Tryptone 4.00 g, Sodium lactate 2.20 g, 4% resazurin 4 drops, Distilled water 1 L.

The medium is adjusted to pH of 6.8 with HCl or NaOH and autoclaved at 121 ℃ for 20 min. After autoclaving, these solutions are added to the mineral basis (for 1L): 5% K_2HPO_4 1 mL, 5% KH_2PO_4 1 mL, 2% $CaCl_2.2H_2O$ 1 mL, 10 mM Na_2WO_4 1 mL (Zeng *et al.* 2009).

KA22 medium

Sea salts 30 g, $Mg(NO_3)_2.6H_2O$ 2.54 g, MES buffer 1.95 g, 4% Resazurin 2 drops, Distilled water 1 L.

The medium is adjusted to pH of 6.0 with HCl or NaOH and autoclaved at 121 ℃ for 20 min. After autoclaving, these solutions are added to the mineral basis (for 1L): 1 mL of trace element solution, 1 mL of vitamin solution, 8 mL of 5% KH_2PO_4 (Alain *et al.* 2003).

SO4PNsalts medium:

NH_4Cl 0.33 g, KCl 0.5 g, $CaCl_2.2H_2O$ 0.5 g, $MgCl_2.6H_2O$ 3.0 g, NaCl 22 g, Na_2SO_4 3.0 g, PIPES buffer 5 g, 4% resazurin 2 drops, distilled water 1 L.

The medium is adjusted to pH of 6.7 with HCl or NaOH and autoclaved at 121 ℃ for 20 min. After autoclaving, these solutions are added to the mineral basis (for 1 L): 1 mL of trace element solution, 1 mL of vitamin solution, 1 mL of selenite-tungstate solution, 8 mL of 5% KH_2PO_4 (Alain *et al.* 2010).

YTG medium

Yeast extract 1 g, peptone 1 g, glucose 2.5 g, PIPES 6 g, sea salts 30g, 9% resazurin 2 drops, distilled water 1 L.

The medium is adjusted to pH of 7.0 with HCl or NaOH and autoclaved at 121 ℃ for 20 min. After sterilization, these solutions are added to the medium (for 1L): trace element solution 5 mL, vitamin solution 0.5 mL (Zeng *et al.* 2015).

The medium is degassed and aliquoted in vials (let 2/3 of gas phase), and reduced with 0.1 mL of Na_2S solution at 10%(m/v) for 10 mL of medium before inoculation. For the growth of autotroph, an atmosphere of H_2/CO_2 (80/20, v/v, 200 kPa) is applied.

3.3 Enrichment cultures and isolation of microorganisms

For the enrichment cultures of sulfate-reducing autotrophs, a subsample was used to inoculate a SO4PNsalts medium (Alain *et al.* 2010), prepared with a gas phase of H_2/CO_2 (80/20, v/v, 200 kPa) and incubate at different temperatures (30, 60, and 80 ℃).

For the enrichment of sulfur-reducing autotrophs, a subsample was used to inoculate a KA22 medium (Alain *et al.* 2003), prepared with a gas phase of H_2/CO_2 (80/20, v/v, 200 kPa) and incubate at different temperatures (30, 60, and 80 ℃).

For the enrichment cultures targeting thermophilic sulfur-reducer heterotrophs, a subsample was used to inoculate a TRM medium supplemented with elemental sulfur (Zeng *et al.* 2009), and incubate at different temperatures (55 and 80 ℃).

For all the enrichment cultures, populations of various morphotypes were generally observed after 5 days of incubation and then subcultured under exactly the same conditions. They were then purified by at least 3 repeated dilutions-to-extinction series. The purity of the isolate was confirmed routinely by microscopic examination (including observations of cultures on rich media) and by repeated partial sequencing of the 16S rRNA gene using four different primers. Stock cultures were stored at -80°C with 5% (v/v) DMSO.

From the 14 enrichment cultures that were performed, six enrichment cultures led to the isolation of six species referenced as J2 (site 30I-TVG05, 27° 88’ S, 63° 53’ E), S606 (site JL-Dive90-S01, 37° 78’ S, 49° 65’ E), J856 (site JL-Dive90-S01, 37° 78’ S, 49° 65’ E), K6013 (site JL-Dive94-S01, 37° 78’ S, 49° 65’ E), J5513 (site JL-Dive94-S01, 37° 78’ S, 49° 65’ E), and J8513 (site JL-Dive94-S01, 37° 78’ S, 49° 65’ E).

3.4 DNA extraction

Genomic DNA was extracted with the QIAGEN Genomic-tip 20/G (QIAGEN, Düsseldorf, Germany) kit following the manufacturer’s standard protocol.

3.5 Analysis of the 16S rDNA sequence

The 16S rRNA gene of bacterium was amplified from purified genomic DNA by PCR using primers B8F (5'-AGA GTT TGA TCC TGG CTC AGA-3') and U1492R (5'-GGT TAC CTT GTT ACG ACT T-3') (Alain *et al.* 2002). The 16S rRNA gene of archaeon was amplified by PCR using primers A4F (5' TCC GGT TGA TCC TGC CGG-3') and U1492R (5'-GGT TAC CTT GTT ACG ACT T-3') (Gorlas *et al.* 2013).

The initial denaturation step was 3 min at 94 ℃; this was followed by 30 cycles of denaturation at 94 ℃ for 45 s, annealing at 50 ℃ for 45 s and extension at 72 ℃ for 1.5 min. A final primer extension step was carried out at 72 ℃ for 5 min.

The 16S rRNA gene of bacterium was sequenced by Sanger method using the primers Bac8F (5'-AGA GTT TGA TCA TGG CTC AGA-3'), S8dir (5'-GTA GCG GTG AAA TGC GTA GA-3'), U1492R (5'-GGT TAC CTT GTT ACG ACT T-3') and W34 (5'-TTA CCG CGG CTG CTG GCA C-3'). The 16S rRNA gene of archaeon was sequenced by Sanger method using the primers A4F (5' TCC GGT TGA TCC TGC CGG-3') and U1492R (5'-GGT TAC CTT GTT ACG ACT T-3'). Sequencing was performed by the society Beckman Coulter Genomics (Essex). Pairwise 16S rRNA sequence similarity was calculated using global alignment algorithm implemented at the EzTaxon-e server (http://eztaxon-e.ezbiocloud.net/; (Kim *et al.* 2012). Phylogenetic analysis was performed using the software MEGA version 5.0 (Tamura *et al.* 2011). Distances were calculated using the Kimura two-parameters model and clustering was performed with the neighbor-joining algorithm (Saitou and Nei 1987). The robustness of the inferred topologies was assessed by bootstrap analyses based on 1000 bootstrap resamplings.

3.6 Phenotypic, physiological and chemotaxonomical characterization

The morphological characteristics of the cells of the novel strains (morphology, arrangement, size, etc.) were determined by using light microscopy (Olympus BX60 and CX40) and scanning electron microscopy (FEI Quanta 200).

Unless stated otherwise, physiological characterization of strain J2^T *(Desulfovibrio indicus* sp. nov.) was carried out anaerobically in TRM medium reduced with sodium sulfide (Zeng *et al.* 2009),

in duplicate, using sulfate as a terminal electron acceptor. This medium was selected because it allowed efficient growth of the strain, notably when this medium was supplemented with 20 mM lactate. Growth experiments were generally carried out as described elsewhere (Khelaifia *et al.* 2011). Growth was routinely monitored by direct cell counting by using a modified Thoma chamber (depth 10 µM) and growth rates were calculated using linear regression analysis of logarithmically transformed growth curves. Salt tolerance was tested at 35 °C in TRM medium prepared with various concentrations of NaCl (0, 0.2, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 5.0, 6.0, 7.0 and 8.0 %, w/v). The pH range for growth was tested from pH 4.0 to pH 9.0 (initial pH at 20 °C) with increments of 0.5 units. Determination of the temperature range for growth was tested at 4, 9, 16, 20, 25, 30, 35, 37, 40, 45 and 50 °C. Determination of the hydrostatic pressure range for growth was tested at 0.1, 10, 20, 30 and 40 MPa.

Unless stated otherwise, physiological characterization of strain K6013^T (*Desulfurobacterium indicum* sp. nov.) was carried out anaerobically in SO4PNsalts medium (Alain *et al.* 2010) depleted of sulfate, in duplicate, using elemental sulfur as a terminal electron acceptor, and a gas phase of H_2/CO_2 (80/20, v/v, 200 kPa) as energy and carbon sources. Growth experiments were generally carried out as described elsewhere (Alain *et al.* 2003). Growth was routinely monitored by direct cell counting by using a modified Thoma chamber (depth 10 µm). Salt tolerance was tested at 65 °C with various concentrations of NaCl (0, 0.2, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, and 6.0 %, w/v). The pH range for growth was tested from pH 4.0 to pH 9.0 (initial pH at 20 °C) with increments of 1 units. Determination of the temperature range for growth was tested at 35, 40, 45, 50, 55, 60, 65, 70, 75 and 80 °C.

For the determination of the content in fatty acids, biomass was produced from 1 L of culture stopped at the end of the exponential growth phase. Fatty acids in whole cells were saponified, methylated and extracted using the standard protocol of MIDI (Sherlock Microbial Identification System, version 6.0B). The fatty acids were analyzed by GC (Agilent Technologies 6850) and identified by using the TSBA6.0 database of the Microbial Identification System (Sasser 1990).

3.7 Determination of the pressure range for growth

Determination of the hydrostatic pressure range for growth was tested in 5 mL glass syringes containing TRM medium (Strain J2^T) or YTG medium (Strains DY22613^T, DY22619^T and DY2627C^T) inoculated with 1% late-exponential phase culture. Syringes were incubated in high-pressure, high-temperature reactors custom-built by the society Top-Industrie (Vaux-Le-Pénil, France). Hydrostatic pressure test for strain J2^T was carried out at 0.1, 10, 20, 30 and 40 MPa at 35 °C. Hydrostatic pressure test for strain DY22613^T was carried out at 0.1, 10, 20, 30, 40, 55 and 70 MPa at 60 °C. Hydrostatic pressure test for strain DY22619^T was carried out at 0.1, 10, 20, 30 and 40 MPa at 60 °C. Hydrostatic pressure test for strain DY2627C^T was carried out at 0.1, 10, 20 and 30 MPa at 37 °C. Cells were collected at regular intervals and growth was estimated by direct cell counting using a modified Thoma chamber (depth 10 µm). All samples were tested in duplicate.

3.8 Genome sequencing and analysis

Whole-genome shotgun sequencing was carried out using PacBio (Pacific Biosciences, Menlo Park, CA) single-molecule-real-time (SMRT) sequencing technology (Duke University). Genomes were assembled using the tool SPAdes (http://bioinf.spbau.ru/spades) and software suites Orione (https://orione.crs4.it) of Galaxy. Genomes were annotated using NCBI Prokaryotic Genome Annotation Pipeline (PGAP, http://www.ncbi.nlm.nih.gov/genome/annotation_prok/) and "Rapid Annotation using Subsystem Technology" (RAST, http://rast.nmpdr.org/rast.cgi).

3.9 Genetic manipulations of *Palaeococcus pacificus*

3.9.1 Construction of a suicide vector

The flanking regions of the gene targeted for deletion were amplified from genomic DNA by two successive PCR amplification steps, in a process known as splicing by overlap extension. The primers used for this first amplification were called BamHI-D6710-1F/D6710-1R and D6710-2F/NdeI-D6710-2R. BamHI-D6710-1F: 5′- AAA AAA GGA TCC ttt aga aat tcc tga agt tga tat gcc cat ca -3′; D6710-1R: 5′- cct aat aag gga aat gca tgg atg tga taa att gga tag aga tga gat g -3′; D6710-2F: 5′- cca att tat cac atc cat gca ttt ccc tta tta ggg gaa aat aaa aag ag -3′; NdeI-D6710-2R: 5′- AAA

AAA CAT ATG cga cat cca cgg cgg ag -3′. The second PCR amplification was carried out using the two flanking regions as the matrix and primers BamHI-D6710-1F and NdeI-D6710-2R, to produce a resulting DNA fragment of 2 kb composed of the two merged homologous regions. The fragment was inserted into the plasmid pUPH at the restriction sites NdeI and BamHI, producing the suicide vector pUPH-6710. The plasmid pUPH-6710 was confirmed by PCR using BamHI-D6710-1F / NdeI-D6710-2R and pUPH-F / pUPH-R as primer pairs. pUPH-F: 5′- CAG GTA GTC GCA GTA GAG TC-3′; pUPH-R: 5′-ACA ATG TTG GCA AAG TGA -3′.

3.9.2 Transformation of *Palaeococcus pacificus*

The transformation method previously described by Thiel *et al.* (2014) was used in this study.

1. Cells were cultivated in TRM liquid medium with sulfur for 16 h at 80 ℃, at atmospheric pressure.
2. An aliquot of 1 ml of this overnight culture was introduced into 50 mL of fresh TRM medium with sulfur and incubated for 6 h at 80 ℃.
3. Cells were then harvested by centrifugation (8,000 g, 6 min, anaerobiosis), concentrated in 1 ml of fresh TRM medium without sulfur, and kept on ice for 30 min under an anaerobic chamber.
4. An aliquot of 4 to 5 μg of plasmid DNA was added to 200 μL of concentrated cells, and the mixture was incubated on ice for 1 h.
5. A heat shock at 80 ℃ was carried out for 10 min, followed by incubation for 10 min on ice.
6. The transformants were then used to inoculate 20 mL of fresh TRM medium with sulfur and incubated at 80 ℃ for 18 h.
7. The cells were harvested by centrifugation (8,000 g, 6 min), resuspended in 100 μL of fresh TRM, and spread on plated TRM containing the antibiotic simvastatin (final concentration of 2.5 μg/mL) under anaerobic conditions. (Medium solidification was achieved using Phytagel (Sigma) at a concentration of 10 g/L). The plates were incubated for 5 days at 80 ℃ under anaerobic conditions.
8. The resulting Sim^r strains were analyzed by PCR using genomic DNA as a matrix and pUPH-F and pUPH-R as primers.

4 Results

4.1 Isolates

4.1.1 Isolate from the serpentinized peridotite deep-sea site 30I-TVG05

After 5 days of incubation on SO4PNsalts medium (Alain *et al.* 2010), prepared with a gas phase of H_2/CO_2 (80/20, v/v, 200 kPa) and incubate at 30 ℃, populations of highly motile vibrioid cells were observed. They were then subcultured under the same conditions, and then purified by 6 repeated dilutions-to-extinction series. One isolate, strain J2^T, was isolated. The purity of this isolate was confirmed routinely by microscopic examination (including observations of cultures on rich media) and by repeated partial sequencing of the 16S rRNA gene using 4 different primers. A nearly full-length 16S rDNA sequence (1491 bp) of strain J2^T was obtained. The 16S rRNA gene-based analysis located the novel isolate within the class *Deltaproteobacteria*, in the bacterial domain. Comparative 16S rDNA sequence analysis showed that strain J2^T formed a robust cluster with the genus *Desulfovibrio*, within the family *Desulfovibrionaceae*. Strain J2^T shared the highest sequence similarity of 98.05% to *Desulfovibrio dechloracetivorans* SF3^T, followed by *D. portus* MSL79^T (96.96%), *D. aespoeensis* Aspo-2^T (96.11 %) and *D. piezophilus* C1TLV30^T (96.04 %). This isolate was subjected to a full characterization (see §4.2).

4.1.2 Isolates from the deep-sea sulfide site JL-Dive94-S01

After 5 days of incubation on KA22 medium (Alain *et al.* 2003), prepared with a gas phase of H_2/CO_2 (80/20, v/v, 200 kPa) and incubated at 60 ℃, populations were composed of short-rod cells. They were subcultured under the same conditions, and then purified by 7 repeated dilutions-to-extinction series. One isolate, strain K6013^T, was isolated. The purity of this isolate was confirmed routinely by microscopic examination and by repeated partial sequencing of the 16S rRNA gene using 4 different primers. A nearly full-length 16S rDNA sequence (1517 bp) of strain K6013^T was obtained. The 16S rRNA gene-based analysis located the novel isolate within the class *Aquificae*, in the bacterial domain. Comparative 16S rDNA sequence analysis showed that strain K6013^T formed a robust cluster with the genus *Desulfurobacterium*, within the family

Desulfurobacteriaceae. Strain K6013^T shared the highest sequence similarity of 96.93% to *Desulfurobacterium atlanticum* SL22^T, followed by *D. pacificum* SL17^T (95.46%), *Thermovibrio guaymasensis* SL19^T (93.99 %) and *D. thermolithotrophum* DSM 11699^T (93.74 %). The phenotypic and physiological characterization of this strain was done and is described in §4.3.

The deep-sea sulfide sample (site JL-Dive94-S01) was also used to inoculate a TRM medium and incubated at 55 ℃ and 85 ℃. After 2 days of incubation, populations of rod and motile cocci cells were observed at 55 ℃ and 85 ℃, respectively. They were subcultured under the same conditions, and then purified by 5 repeated dilutions-to-extinction series. Strain J5513 was isolated at 55 ℃. The purity of this isolate was confirmed routinely by microscopic examination and by repeated partial sequencing of the 16S rRNA gene using 4 different primers. A nearly full-length 16S rDNA sequence (1425 bp) of strain J5513 was obtained. The 16S rRNA gene-based analysis located the isolate within the class *Clostridia*, in the bacterial domain. Comparative 16S rDNA sequence analysis showed that strain J5513 formed a robust cluster with the genus *Caloranaerobacter*, within the family *Clostridiaceae*. Strain J5513 shared the highest sequence similarity of 98.55% to *Caloranaerobacter azorensis* MV1087^T. Another isolate, referenced as strain J8513, was isolated at 85 ℃. The purity of this isolate was confirmed routinely by microscopic examination and by repeated partial sequencing of the 16S rRNA gene using two different primers. A nearly full-length 16S rDNA sequence (1366 bp) of strain J8513 was obtained. The 16S rRNA gene-based analysis located the isolate within the class *Thermococci*, in the archaeal domain. Comparative 16S rDNA sequence analysis showed that strain J8513 formed a robust cluster with the genus *Thermococcus*, within the family *Thermococcaceae*. Strain J8513 shared the highest sequence similarity of 99.41% to *Thermococcus nautili* 30-1^T.

4.1.3 Isolates from the deep-sea sulfide site JL-Dive90-S01

After 5 days of incubation at 60 ℃ on SO4PNsalts medium (Alain *et al.* 2010), under an atmosphere of H_2/CO_2 (80/20, v/v, 200 kPa), populations of short-rod-shaped cells were observed. They were subcultured under the same conditions, and then purified by 7 repeated dilutions-to-extinction series. One isolate, strain S606^T, was isolated. The purity of this isolate was confirmed routinely by microscopic examination and by repeated partial sequencing of the 16S rRNA gene

using 4 different primers. A nearly full-length 16S rDNA sequence (1514 bp) of strain S606^T was obtained. The 16S rRNA gene-based analysis located the novel isolate within the class *Thermodesulfobacteria*, in the bacterial domain. Comparative 16S rDNA sequence analysis showed that strain S606^T formed a robust cluster with the genus *Thermodesulfatator*, within the family *Thermodesulfobacteriaceae*. Strain S606^T shared the highest sequence similarity of 98.15% to *Thermodesulfatator indicus* DSM 15286^T, followed by *Thermodesulfatator atlanticus* AT1325^T (97.38%) and *Thermosulfurimonas dismutans* S95^T (91.26 %). This strain was fully characterized (§4.4).

The deep-sea sulfide sample (site JL-Dive90-S01) was also used to inoculate a TRM medium and incubated at 85 ℃. After 2 days of incubation, populations of motile cocci cells were observed, subcultured under the same conditions, and then purified by repeated dilutions-to-extinction series. Strain J856 was isolated. The purity of this isolate was confirmed routinely by microscopic examination and by repeated partial sequencing of the 16S rRNA gene using two different primers. A nearly full-length 16S rDNA sequence (1363 bp) of strain J856 was obtained. The 16S rRNA gene-based analysis located the isolate within the class *Thermococci*, in the archaeal domain. Comparative 16S rDNA sequence analysis showed that strain J856 formed a robust cluster with the genus *Thermococcus*, within the family *Thermococcaceae*. Strain J856 shared the highest sequence similarity of 99.71% to *Thermococcus hydrothermalis* AL662^T.

4.2 *Desulfovibrio indicus* sp. nov.

The characterization of this strain was submitted to the Journal *International Journal of Systematics and Evolutionary Microbiology*.

An article of description of its genome is in preparation and will be submitted to the journal *Genome Announcements*.

***Desulfovibrio indicus* sp. nov., a piezophilic sulfate-reducing bacterium from the Indian Ocean**

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Subject: NEW TAXA *Proteobacteria*

Running title: *Desulfovibrio indicus* sp. nov.

Abbreviations: MCCC, Marine Culture Collection of China; SRP, sulfate-reducing prokaryotes; DMSO, Dimethylsulfoxide

The GenBank[/EMBL/DDBJ] accession number for the 16S rRNA sequence of *Desulfovibrio indicus* J2^T sp. nov. is KT750867.

A novel sulfate-reducing bacterium, strain J2^T, was isolated from a serpentinized peridotite sample collected at a depth of 3173 m in the Indian Ocean. Phylogenetic analysis based on 16S rRNA gene sequences showed that strain J2^T falls into the genus *Desulfovibrio* within the class *Deltaproteobacteria*, with highest sequence similarity of 98.05% to *Desulfovibrio dechloracetivorans* SF3^T. Cells were Gram-negative, anaerobic, motile vibrios (2-6×0.4-0.6 µm). Growth was observed at salinities ranging from 0.2 to 6% (optimum 2.5%), from pH 5 to 8 (optimum 6.5-7), and at temperatures between 9 and 40 ℃ (optimum 30-35 ℃). Strain J2^T was piezophilic, growing optimally at 10 MPa (range 0–30 MPa). Strain J2^T used lactate, malate, pyruvate, formate and hydrogen as energy sources. Sulfate, thiosulfate, sulfite, fumarate, and nitrate, were used as terminal electron acceptors, but not elemental sulfur nor nitrite. Lactate and pyruvate were fermented. The main fatty acids were iso-$C_{15:0}$ (25.57%), anteiso-$C_{15:0}$ (8.28%), iso-$C_{17:1}$ (Summed feature 9, 15.57%) and iso-$C_{17:0}$ (14.85%). Strain J2^T was piezophilic, growing optimally at 10 MPa (range 0–30 MPa). The DNA G+C content of the chromosomal DNA of strain J2^T was 63.5 mol%, determined using the whole genome sequence (1 contig of 3,966,573 bp, coverage 299X, sequenced using the PacBio technology). The combined genotypic and phenotypic data show that strain J2^T represents a novel species of the genus *Desulfovibrio*, for which the name *Desulfovibrio indicus* sp. nov. is proposed, with the type strain J2^T (=MCCC 1A01867^T = DSM 101483^T).

Sulfate-reducing prokaryotes (SRP) are anaerobic prokaryotes, using sulfate as a terminal electron acceptor for respiration and hydrogen or various organic acids as energy sources (Heidelberg *et al.* 2004). Members of the bacterial genus *Desulfovibrio* are SRP of geomicrobiological significance (Heidelberg *et al.* 2004, Khelaifia *et al.* 2011). Most representatives of this genus oxidize simple organic compounds incompletely, leading to acetate as an end product. At the time of writing, the genus *Desulfovibrio*, within the family *Desulfovibrionaceae*, comprises 66 species, which have been isolated ubiquitously in nature,

mainly from freshwater and marine habitats (Sun *et al.* 2000, Khelaifia *et al.* 2011, Thabet *et al.* 2011). Among *Desulfovibrio* isolates, only few strains were reported to be piezophilic; they include *Desulfovibrio profundus* (Bale *et al.* 1997), *Desulfovibrio hydrothermalis* (Alazard *et al.* 2003) and *Desulfovibrio piezophilus* (Khelaifia *et al.* 2011).

In this paper, a novel mesophilic, piezophilic SRP belonging to the genus *Desulfovibrio*, strain $J2^T$ is described. Phenotypic and 16S ribosomal DNA (rDNA) phylogenetic studies indicate that it represents a novel species.

Strain $J2^T$ was isolated from a deep-sea serpentinized peridotite sample collected at a depth of 3173 m in a hydrothermal area of the Indian Ocean (27° 88' S, 63° 53' E; site 30I-TVG05) in December 2013, during the cruise DY30/I of *Da Yang Yi Hao*. The sample was collected using a grabber and anaerobically preserved in sterilized seawater onboard. Once in the lab, a subsample was used to inoculate a SO4PNsalts medium (Alain *et al.* 2010), prepared with a gas phase of H_2/CO_2 (80/20, v/v, 200 kPa) and incubate at 30 ℃. After 5 days of incubation, populations of highly motile vibrioid cells were observed, subcultured under the same conditions, and then purified by repeated dilutions-to-extinction series. One isolate, strain $J2^T$, is described in this study. The purity of this isolate was confirmed routinely by microscopic examination (including observations of cultures on rich media) and by repeated partial sequencing of the 16S rRNA gene using 4 different primers. Stock cultures were stored at -80°C with 5% (v/v) DMSO.

Genomic DNA was extracted with the QIAGEN Genomic-tip 20/G (QIAGEN, Düsseldorf, Germany) kit following the manufacturer's standard protocol. The 16S rRNA gene was sequenced by Sanger method using the primers Bac8F (5'-AGA GTT TGA TCA TGG CTC AG-3'), S8dir (5'-GTA GCG GTG AAA TGC GTA GA-3'), U1492R (5'-GGT TAC CTT GTT ACG ACT T-3') and W34 (5'-TTA CCG CGG CTG CTG GCA C-3') (Alain *et al.* 2002). Pairwise 16S rRNA sequence similarity was calculated using global alignment algorithm implemented at the EzTaxon-e server (http://eztaxon-e.ezbiocloud.net/; (Kim *et al.* 2012). Phylogenetic analysis was performed using the software MEGA version 5.0 (Tamura *et al.* 2011). Distances were calculated using the Kimura two-parameters model and clustering was performed with the neighbor-joining algorithm (Saitou and Nei 1987). The robustness of the inferred topology was assessed by bootstrap analyses based on 1000 bootstrap resamplings.

A nearly full-length 16S rDNA sequence (1491/1504 bp) of strain J2^T was obtained. The 16S rRNA gene-based analysis located the novel isolate within the class *Deltaproteobacteria*, in the bacterial domain. Comparative 16S rDNA sequence analysis showed that strain J2^T formed a robust cluster with the genus *Desulfovibrio*, within the family *Desulfovibrionaceae* (Figure 5).

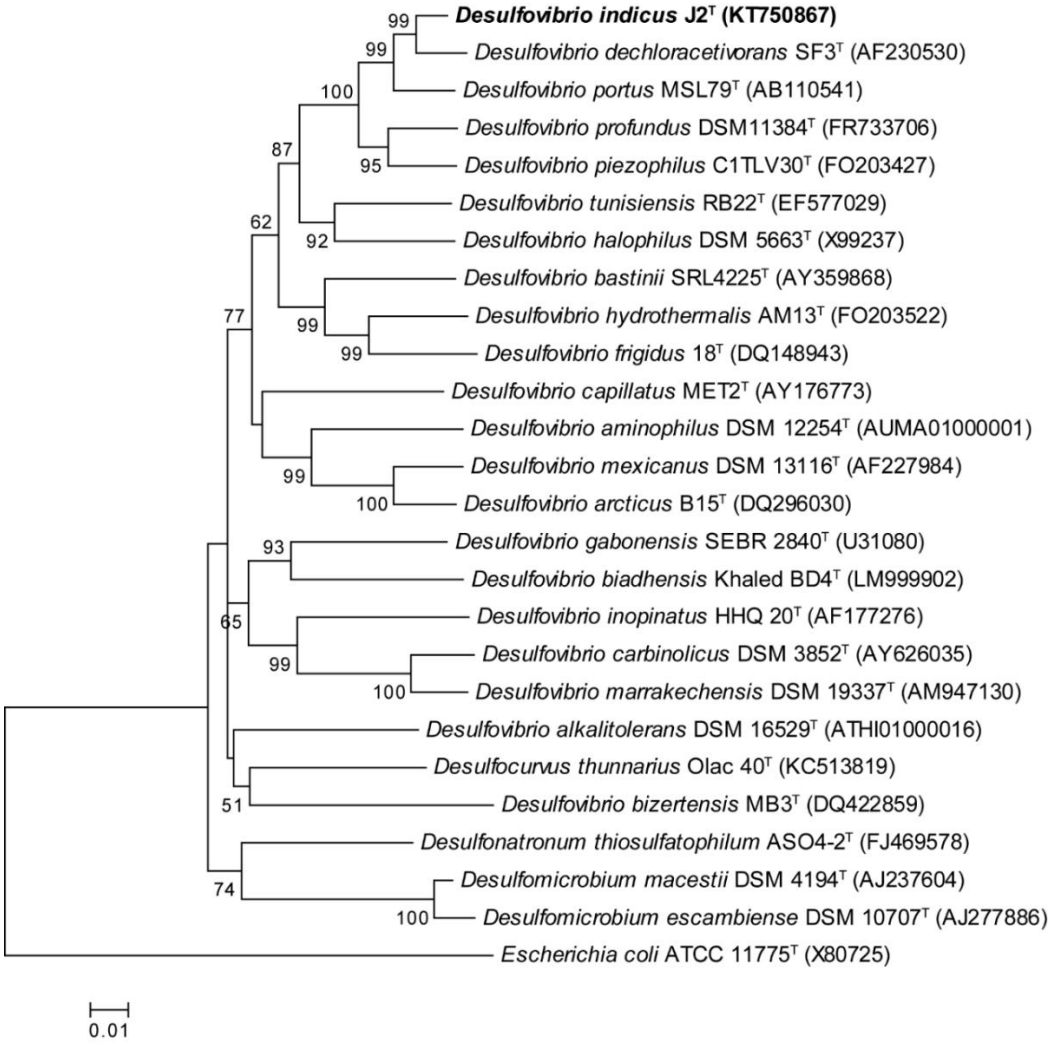

Figure 5 | Neighbor-joining tree showing the phylogenetic positions of strain J2^T and representatives of some other related taxa, based on 16S rRNA gene sequences.

Bootstrap values (expressed as percentages of 1000 replications) are shown at branch nodes. Bar, 0.01 nucleotide substitution rate (K_{nuc}) units.

Strain J2^T shared the highest sequence similarity of 98.05% with *Desulfovibrio dechloracetivorans* SF3^T, followed by *D. portus* MSL79^T (96.96%), *D. aespoeensis* Aspo-2^T (96.11 %) and *D. piezophilus* C1TLV30^T (96.04 %). Other available sequences shared less than

95.17% 16S rRNA gene sequence similarity. The level of 16S rRNA gene sequence similarity with *D. dechloracetivorans* showed that strain J2^T displayed sufficient molecular differences for delineation at the species level, because it falls well below the threshold value (98.65-98.7%) currently recommended for two species demarcation (Stackebrandt and Ebers 2006, Kim *et al.* 2014). It was not possible to confirm this result by DNA-DNA hybridization, because public culture collections and the persons who have isolated the closest relative *Desulfovibrio dechloracetivorans* could not provide it upon request.

The whole genome of the novel isolate was recently sequenced by the PacBio technology, and will be soon available. The DNA G + C content of strain J2^T was 63.5 mol%, as determined using the whole genome sequence.

Morphological characteristics of cells of strain J2^T were determined by using light microscopy (Olympus BX60 and CX40) and scanning electron microscopy (FEI Quanta 200). Cells were motile, vibrios (2-6 µm in length and 0.4-0.6 µm in width, *n*=10, Figure 6) that appeared singly. They stained Gram-negative.

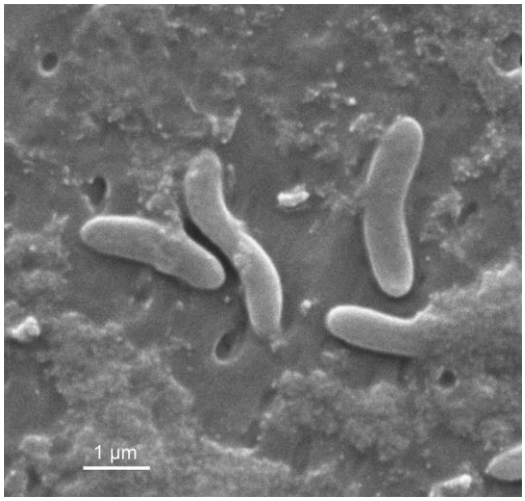

Figure 6 | Scanning electron micrographs of cells of strain J2^T.

Unless stated otherwise, physiological characterization was carried out anaerobically in TRM medium reduced with sodium sulfide (Zeng *et al.* 2009), in duplicate, using sulfate as a terminal electron acceptor. This medium was selected because it allowed efficient growth of the strain, notably when this medium was supplemented with 20 mM lactate. Growth experiments were generally carried out as described elsewhere (Khelaifia *et al.* 2011). Growth was routinely

monitored by direct cell counting by using a modified Thoma chamber (depth 10 µM) and growth rates were calculated using linear regression analysis of logarithmically transformed growth curves. Salt tolerance was tested at 35 ℃ in TRM medium prepared with various concentrations of NaCl (0, 0.2, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 5.0, 6.0, 7.0 and 8.0 %, w/v). Strain J2^T required NaCl for growth and growth was observed at 0.2–6% NaCl (optimum: 2.5%). The pH range for growth was tested from pH 4.0 to pH 9.0 (initial pH at 20 ℃) with increments of 0.5 units. Growth was observed at pH 5.0–8.0, the optimum being around 6.5-7.0. Determination of the temperature range for growth was tested at 4, 9, 16, 20, 25, 30, 35, 37, 40, 45 and 50 ℃. The isolate was mesophilic and grew at 9–40 ℃ (optimum 30-35 ℃). Determination of the hydrostatic pressure range for growth was tested at 0.1, 10, 20, 30 and 40 MPa, using a procedure and an equipment described previously (Alain *et al.* 2002). The novel isolate J2^T was piezophilic and grew within a pressure range of 0.1 to 30 MPa, with an optimum at 10 MPa (Figure 7).

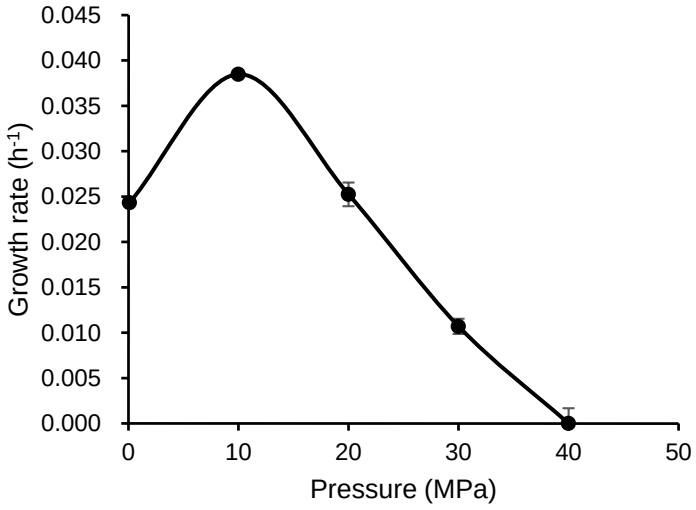

Figure 7 | Effect of hydrostatic pressure on growth of strain J2^T.

Strain J2^T was a strictly anaerobic bacterium that used lactate and sulfate as primary electron donor and acceptor, respectively. Its ability to use alternative electron acceptors was tested on the mineral base of the SO4PNsalts medium depleted of sulfate, but supplemented with a gas phase of H_2/CO_2 (80/20, v/v, 200 kPa). In order to ensure an adequate electron donor and carbon source, elemental sulfur (12 g L^{-1}), fumarate (20 mM) sulfite (2 mM), thiosulfate (20 mM), nitrate (10 mM), nitrite (2 mM) or oxygen (1% v/v) were added. Hydrogen sulfide production was determined as

described elsewhere (Cord-Ruwisch 1985). Sulfate, thiosulfate, sulfite, fumarate, and nitrate, were used as terminal electron acceptors, but not elemental sulfur nor nitrite. When using sulfate as terminal electron acceptor, strain $J2^T$ grew on hydrogen, malate, formate, pyruvate and lactate. The ability of the strain to grow by fermentation was tested on sulfate-depleted mineral base of the SO4PNsalts medium, after addition of lactate (20 mM), yeast extrac (1 g L^{-1}), tryptone (4 g L^{-1}), fumarate (20 mM), formate (20 mM), malate (20 mM), and pyruvate (20 mM). Lactate and pyruvate were fermented.

Fatty acids in whole cells were saponified, methylated and extracted using the standard protocol of MIDI (Sherlock Microbial Identification System, version 6.0B). The fatty acids were analyzed by GC (Agilent Technologies 6850) and identified by using the TSBA6.0 database of the Microbial Identification System (Sasser 1990). The predominant fatty acids of strain $J2^T$ were iso-$C_{15:0}$ (25.57%), anteiso-$C_{15:0}$ (8.28%), iso-$C_{17:1}$ (Summed feature 9, 15.57%) and iso-$C_{17:0}$ (14.85%), which were in the same range than the values got for *D. piezophilus* and *D. portus* (Table 3). A distinguishing feature was that *D. piezophilus* contained higher amounts of $C_{16:0}$ (17%) than strain $J2^T$ and *D. portus* (4.8%, 7.3%, respectively).

Table 3 | Whole-cell fatty acid profiles of strain $J2^T$ and related species of the genus *Desulfovibrio*.

Strains: 1, $J2^T$; 2, *D. piezophilus*; 3, *D. portus*. Values are percentages of total fatty acids. Data for strain $J2^T$ were obtained in this study. Data for strains 2 and 3 were obtained from Khelaifia *et al.* (2011) and Suzuki *et al.* (2009).

Fatty acid	1	2	3
iso-$C_{15:0}$	25.57	31	12
anteiso-$C_{15:0}$	8.28		12.4
iso-$C_{16:0}$	3.17	3	2.1
$C_{16:0}$	4.79	17	7.3
iso-$C_{17:1}$ ω9*c* (Summed feature 9†)	15.57	16	15.8
anteiso-$C_{17:1}$ B (Summed feature 4†)	4.52		4.5
iso-$C_{17:0}$	14.85	9	11.8
$C_{18:1}$ ω7*c* (Summed feature 8†)	3.59	6	4.5
$C_{18:0}$	3.28	8	4.2

†Summed feature 4 contains anteiso-$C_{17:1}$ B, summed feature 8 contains $C_{18:1}$ ω7*c* and summed feature 9 contains iso-$C_{17:1}$ ω9*c*.

The results of the phylogenetic, phenotypic and chemotaxonomic analyses described in this article support the view that strain $J2^T$ should be assigned to the genus *Desulfovibrio* in the family *Desulfovibrionaceae*. However, the strain could be distinguished from the closely related species by some phenotypic characteristics given in Tables 3 and 4. *D. piezophilus* and *D. portus* are capable of using fumarate and ethanol as electron donors, while strain $J2^T$ and *D. profundus* are not. Strain $J2^T$, *D. piezophilus* and *D. portus* are capable of using formate and malate as electron donors, while *D. profundus* is not. The genomic DNA G+C content of strain $J2^T$ is much higher than the ones of its closest relatives. Therefore, from the polyphasic evidence, strain $J2^T$ represents a novel species for which the name *Desulfovibrio indicus* sp. nov. is proposed.

Table 4 | Physiological characteristics of strain $J2^T$ and related species of genus *Desulfovibrio*.

Strains: 1, $J2^T$; 2, *D. piezophilus*; 3, *D. profundus*., 4, *D. portus.* Characteristics are scored as: +, positive; -, negative; w, weak positive; ND, not determined. Data for strains 1 were obtained during this study; data for strains 2, 3 and 4 were obtained from Khelaifia *et al.* (2011), Bale *et al.* (1997) and Suzuki *et al.* (2009).

Characteristics	1	2	3	4
T (℃) (optimal)	9-40 (30-35)	15-45(30)	15-65(25)	10-40 (35)
pH (optimal)	5-8 (6.5-7)	5.4-8.6(7.3)	4.5-9(7)	5.7-8.5 (6.5)
NaCl (%) (optimal)	0.2-6 (2.5)	0.1-8 (2.5)	0.2-10	0.15-6.5 (2.0)
Pressure (MPa)	0-30 (10)	0-30 (10)	0-40 (15)	ND
Length (μm)	2-6	2-4	1-2	1.8-2.3
Width (μm)	0.4-0.6	0.5	0.5-1	0.7-1
G+C (%)	63.5	49.6	53	62.1
Electron donor				
Fumarate	-	+	-	+
Ethanol	-	+	-	+
Formate	+	+	-	+
Malate	+	+	-	+
Electron acceptor				
NO_3^-	w	-	+	ND
Fumarate	w	-	+	-
Fermentation				
Malate	-	-	-	+
Fumarate	-	+	-	+
Lactate	+	-	+	-

Description of *Desulfovibrio indicus* sp. nov.

Desulfovibrio indicus (in'.di.cus. L. masc. adj. indicus. Indian, referring to the Indian Ocean, from where the type strain was isolated).

Cells were Gram-negative, anaerobic, motile vibrios (2-6×0.5 μm). Growth was observed at salinities from 0.2 to 6% (optimum 2.5%), from pH 5 to 8 (optimum 6.5-7), and at temperatures between 9 and 40 ℃ (optimum 30-35 ℃). Strain J2^T used lactate, malate, pyruvate, formate and hydrogen as energy sources. Sulfate, thiosulfate, sulfite, fumarate, and nitrate, were used as terminal electron acceptors, but not elemental sulfur nor nitrite. Lactate and pyruvate were fermented. The principal fatty acids were iso-$C_{15:0}$ (25.57%), anteiso-$C_{15:0}$ (8.28%), iso-$C_{17:1}$ (Summed feature 9, 15.57%) and iso-$C_{17:0}$ (14.85%). Strain J2^T was piezophilic, growing optimally at 10 MPa (range 0–30 MPa). The G+C content of the chromosomal DNA was 63.5 mol%.

The type strain J2^T (=MCCC 1A01867^T = DSM 101483^T) was isolated from a deep-sea serpentinized peridotite sample collected at a depth of 3173 m in a hydrothermal area of the Indian Ocean (27° 88’ S, 63° 53’ E; site 30I-TVG05).

Genome of *Desulfovibrio indicus*, a piezophilic sulfate-reducing bacterium from the Indian Ocean

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Here, we report the complete genome sequence of *Desulfovibrio indicus* J2^T of the family *Desulfovibrionaceae*.

Desulfovibrio indicus J2^T is a type strain isolated from a deep-sea serpentinized peridotite sample collected at a depth of 3173 m in a hydrothermal area of the Indian Ocean (27° 88' S, 63 ° 53' E; site 30I-TVG05) (this study). It is a piezophilic sulfate-reducing bacterium, growing optimally at 10 MPa (range 0–30 MPa). This anaerobic, motile vibrios can use lactate, malate, pyruvate, formate and hydrogen as energy sources when using sulfate, thiosulfate, sulfite, fumarate, and nitrate as terminal electron acceptors.

Genomic DNA was extracted with the QIAGEN Genomic-tip 20/G (QIAGEN, Düsseldorf, Germany) kit following the manufacturer's standard protocol. Whole-genome shotgun sequencing was carried out using PacBio (Pacific Biosciences, Menlo Park, CA) single-molecule-real-time (SMRT) sequencing technology (Duke University) (Eid *et al.* 2009). Genomes were assembled using in-house scripts, the tool SPAdes (http://bioinf.spbau.ru/spades) (Bankevich *et al.* 2012) and

the software suites Orione (https://orione.crs4.it) of Galaxy (Cuccuru *et al.* 2014). The final assembly had ~299 coverage for the 1 contig of 3,966,573-bp genome with a G+C content of 63.5%.

Genomes were annotated using the NCBI Prokaryotic Genome Annotation Pipeline (http://www.ncbi.nlm.nih.gov/genome/annotation_prok/). 3611 genes were identified, of which 3461 were protein-coding sequences (CDS), 90 were pseudogenes, and 60 were RNA genes. The 60 RNA genes comprise 5 noncoding RNA (ncRNA), 3 rRNAs (5S, 16S, and 23S), and 52 tRNAs. Additionally, 1 clustered regularly interspaced short palindromic repeat (CRISPR) arrays was identified in the genome.

Genes involved in sulfate reduction (Meyer and Kuever 2007) were identified in the genome, for example, sulfate adenylyltransferase gene (s*at*, AWY79_04190 and AWY79_13965); adenosine phosphosulfate reductase genes (*aprBA*, AWY79_04195 and AWY79_04200); dissimilatory sulfite reductase genes (*dsrAB*, AWY79_17895 and AWY79_17900; *dsrC*, AWY79_11020); sulfate transporter gene (AWY79_06665, AWY79_10840 and AWY79_14480); Genes that mediate the electron transport between the cytoplasmic AprBA and DsrAB and the membrane-integral quinol/quinone pool (Meyer and Kuever 2007, Pereira *et al.* 2011) were also found, for instance, quinone-interacting membrane-bound oxidoreductase genes (*qmoABC*, AWY79_04205 AWY79_04210 and AWY79_04215); sulfite reduction-associated complex protein genes (*dsrMKJOP*, AWY79_04535, AWY79_04540, AWY79_04545, AWY79_04550, and AWY79_04555). The genome also contains a large number of genes encoding hydrogenases, cytochromes c and cytochrome c-associated membrane redox complexes, which may be possibly involved in electron-transfer and energy conserving pathways (Pereira *et al.* 2011).

Previous study indicated that energy metabolism of SRB is far more versatile than we considered, so that SRB can use different alternative strategies for energy conservation (Pereira *et al.* 2011, Morais-Silva *et al.* 2014). The genome sequence will allow comprehensive comparisons with other SRB and pave the way for further understanding of SRB in anaerobic marine environments.

Nucleotide sequence accession number. The genome sequence has been deposited in GenBank under the accession no. CP014206.

4.3 *Desulfurobacterium indicum* sp. nov.

The article of characterization of this strain is in preparation for the Journal *International Journal of Systematics and Evolutionary Microbiology*.

Its genome was sequenced using the PacBio technology but we did not receive the data so far.

***Desulfurobacterium indicum* sp. nov., a thermophilic sulfur-reducing bacterium from the Indian Ocean**

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Subject: NEW TAXA Other bacteria

Running title: *Desulfurobacterium indicum* sp. nov.

Abbreviations: MCCC, Marine Culture Collection of China; DMSO, Dimethylsulfoxide

The GenBank[/EMBL/DDBJ] accession number for the 16S rRNA sequence of *Desulfurobacterium indicum* K6013^{T} sp. nov. is KT750866.

A novel sulfur-reducing bacterium, strain K6013^{T}, was isolated from a sulfide sample collected at a depth of 2771 m from a high-temperature hydrothermal vent in the Indian Ocean. Cells were Gram-negative, anaerobic, motile rods (0.9-2.2 × 0.4-0.6 µm). The strain grew at NaCl concentrations ranging from 1 to 4.5% (w/v) (optimum 2.5%), from pH 5 to 8 (optimum 6), and at temperatures between 40 and 75 ℃ (optimum 65 ℃). Strain K6013^{T} was an obligate chemolithoautotroph using thiosulfate, sulfur, and nitrate as terminal electron acceptors in the presence of H_2, but not sulfate, sulfite nor nitrite. The major cellular fatty acids were $C_{16:0}$ (17.4%), $C_{18:1}$ *ω7c* (Summed feature 8, 37.91%), $C_{18:0}$ (18.29%) and $C_{14:0}$ 3-OH (Summed feature 2, 8.56%). The DNA G+C content was XX mol%. Phylogenetic 16S rRNA gene sequence analyses showed that strain K6013^{T} falls into the genus *Desulfurobacterium* within the class *Aquificae*, with highest sequence similarity of 96.93% to *Desulfurobacterium atlanticum* SL22^{T}. On the basis of genotypic and phenotypic data, strain K6013^{T} is considered to represent a novel species of the genus *Desulfurobacterium*, for which the name *Desulfurobacterium indicum* sp. nov. is proposed, with the type strain K6013^{T} (= DSM 101677^{T}= MCCC 1A01868^{T}).

Sulfur-reducing prokaryotes are anaerobic or facultative anaerobic prokaryotes, using sulfur (or other lower oxidation states of this element, S^0, S_8) as a terminal electron acceptor but not sulfate (Rabus *et al.* 2013). Members of the genus *Desulfurobacterium* are sulfur-reducing chemolithoautotrophs using hydrogen as sole electron donor (L'Haridon *et al.* 1998, Alain *et al.* 2003, L'Haridon *et al.* 2006). They represent a deep-branching lineage of the bacterial phylogenetic tree which play an important role in deep-sea hydrothermal ecosystems as primary producers of organic matter in anaerobic zones (Miroshnichenko and Bonch-Osmolovskaya 2006). At the time of writing, the genus *Desulfurobacterium*, within the family *Desulfurobacteriaceae*, comprises 4 species, *D. pacificum*, *D. atlanticum*, *D. thermolithotrophum* and '*D. crinifex*', which have been isolated exclusively from deep-sea hydrothermal systems (L'Haridon *et al.* 1998, Alain *et al.* 2003, L'Haridon *et al.* 2006).

In this study, we describe a novel thermophilic sulfur-reducer, strain K6013^T, isolated from a hydrothermal sulfide sample in the Indian Ocean. Phenotypic and 16S ribosomal DNA (rDNA) phylogenetic studies indicate that it represents a novel species of the genus *Desulfurobacterium*.

A deep-sea sulfide sample was collected at a depth of 2771 m from a hydrothermal vent in the Indian Ocean (37° 78'S, 49° 65'E; site JL-Dive94-S01) in January 2015, during the DY35 cruise of *Xiang Yang Hong Jiu Hao*. The sample was collected using a benthic seabed grab and anaerobically preserved in sealed sterile vials at 4 ℃ onboard. Once in the lab, a subsample was used to inoculate a KA22 medium (Alain *et al.* 2003), prepared with a gas phase of H_2/CO_2 (80/20, v/v, 200 kPa) and incubated at 60 ℃. After 5 days of incubation, populations were composed of short rod-shaped cells. They were subcultured under the same conditions, and purified by 7 repeated dilutions-to-extinction series. One isolate, strain K6013^T, was isolated. The purity of this isolate was confirmed routinely by microscopic examination, by repeated partial sequencing of the 16S rRNA gene using 4 different primers and by sequencing of its genome. Stock cultures were stored at -80°C with 5% (v/v) DMSO.

Genomic DNA was extracted with the QIAGEN Genomic-tip 20/G (QIAGEN, Düsseldorf, Germany) kit following the manufacturer's standard protocol. The 16S rRNA gene was sequenced by Sanger method using the primers Bac8F (5'-AGA GTT TGA TCA TGG CTC AG-3'), S8dir (5'-GTA GCG GTG AAA TGC GTA GA-3'), U1492R (5'-GGT TAC CTT GTT ACG ACT T-3') and W34 (5'-TTA CCG CGG CTG CTG GCA C-3') (Alain *et al.* 2002). Pairwise 16S rDNA sequence similarity was determined using the EzTaxon-e server (http://eztaxon-e.ezbiocloud.net/; (Kim *et al.* 2012). Phylogenetic analysis was performed using the software MEGA version 5.0 (Tamura *et al.* 2011). Distances were calculated using the Kimura two-parameters model and clustering was performed with the neighbor-joining algorithm (Saitou and Nei 1987). The robustness of the inferred topology was assessed by bootstrap analyses based on 1000 replications.

Almost a full-length 16S rRNA gene sequence (1517 bp) of strain K6013^T was determined. Based on the 16S rRNA gene phylogenetic analysis, the novel isolate was affiliated with the class *Aquificae*, in the bacterial domain. Comparative 16S rDNA sequence analysis showed that strain K6013^T formed a robust cluster with the genus *Desulfurobacterium*, within the family *Desulfurobacteriaceae* (Figure 8). Strain K6013^T shared the highest sequence similarity of 96.93%

with *Desulfurobacterium atlanticum* SL22^T, followed by *D. pacificum* SL17^T (95.46%), *Thermovibrio guaymasensis* SL19^T (93.99 %) and *D. thermolithotrophum* DSM 11699^T (93.74 %). The level of 16S rRNA gene sequence similarity with *D. atlanticum* showed that strain K6013^T displayed sufficient molecular differences for delineation at the species level, because it falls well below the threshold value (98.65-98.7%) currently recommended for two species demarcation (Stackebrandt and Ebers 2006, Kim *et al.* 2014).

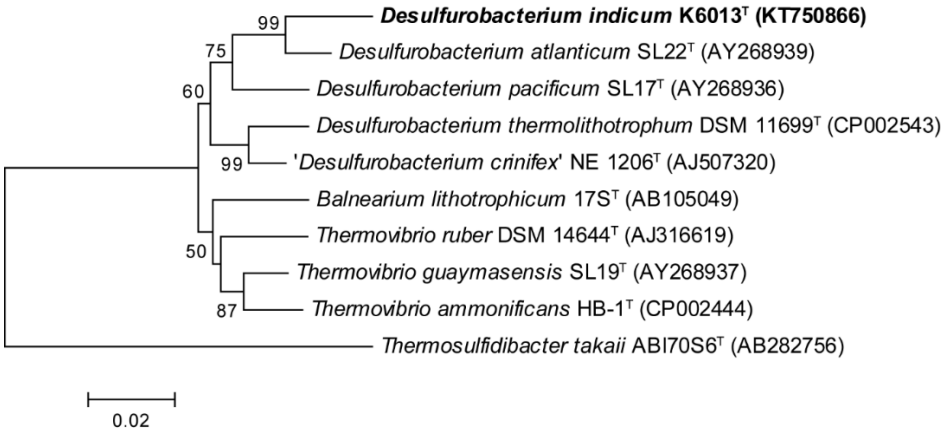

Figure 8 | Neighbor-joining tree showing the phylogenetic positions of strain K6013^T and representatives of some other related taxa, based on 16S rRNA gene sequences.

Bootstrap values (expressed as percentages of 1000 replications) are shown at branch nodes. Bar, 0.01 nucleotide substitution rate (K_{nuc}) units.

Morphological characteristics of strain K6013^T were observed by using light microscopy (Olympus BX60 and CX40) and scanning electron microscopy (FEI Quanta 200). Cells were Gram-negative, motile rods (0.9-2.2 μm in length and 0.4-0.6 μm in diameter, *n*=10, Figure 9) that occurred generally singly. Some cells became spherical in the late stationary growth phase.

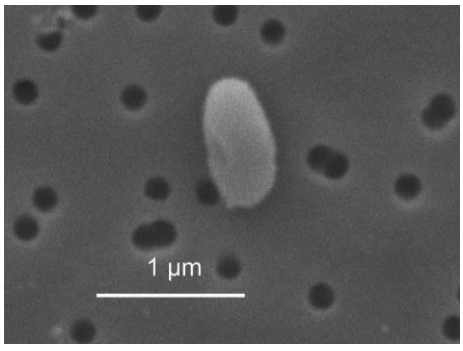

Figure 9 |Scanning electron micrographs of cells of strain K6013^T.

The whole genome of the novel isolate was recently sequenced by the PacBio technology, and will be soon available. The DNA G + C content of strain K6013^T was XX mol%, as determined using the whole genome sequence.

Unless noted otherwise, physiological tests were carried out anaerobically in SO4PNsalts medium (Alain *et al.* 2010) depleted of sulfate, in duplicate, using elemental sulfur as a terminal electron acceptor, and a gas phase of H_2/CO_2 (80/20, v/v, 200 kPa) as energy and carbon sources. Growth tests were generally carried out as described previously (Alain *et al.* 2003). Cells were routinely counted by direct cell counting by using a modified Thoma chamber (depth 10 µm). Determination of the temperature range for growth was carried out at 35, 40, 45, 50, 55, 60, 65, 70, 75 and 80 °C. The isolate was thermophilic and grew between 40 and 75 °C with an optimum around 65 °C. Salt tolerance was tested at 65 °C with various concentrations of NaCl (0, 0.2, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, and 6.0 %, w/v). Strain K6013^T required NaCl for growth and grew at NaCl concentrations between 1.0 and 4.5% (optimum: 2.5%). The pH range for growth was tested from pH 4.0 to pH 9.0 (initial pH at 20 °C) with increments of 1 units. Growth of the isolate was observed between pH 5.0 and 8.0 with an optimum around 6.0.

Strain K6013^T was a strictly anaerobic bacterium using hydrogen and sulfur as primary electron donor and acceptor, respectively. The ability of the isolate to use various electron acceptors was tested with sulfite (2 mM), thiosulfate (20 mM), elemental sulfur (12 g L^{-1}), nitrate (10 mM), nitrite (2 mM) or oxygen (1% v/v). Hydrogen sulfide production was tested as described previously (Cord-Ruwisch 1985). Sulfur, thiosulfate and nitrate were used as terminal electron acceptors, but not sulfate, sulfite nor nitrite. Strain K6013^T grew exclusively on hydrogen and carbon dioxide, when using elemental sulfur as a terminal electron acceptor.

Chemotaxonomic analyses were performed on mid- to late-exponential-phase of growth cultures grown for 90 hours on KA22 medium prepared with S °as a terminal electron acceptor, and incubated under an atmosphere of H_2/CO_2 (80/20, 200 kPa). The cellular fatty acids in whole cells were saponified, methylated and extracted using the standard protocol of MIDI (Sherlock Microbial Identification System, version 6.0B). The cellular fatty acids were analyzed by GC (Agilent Technologies 6850) and identified by using the TSBA6.0 database of the Microbial Identification System (Sasser 1990). The major fatty acids were $C_{16:0}$ (17.4%), $C_{18:1}$ $\omega 7c$ (Summed feature 8,

37.91%), $C_{18:0}$ (18.29%) and $C_{14:0}$ 3-OH (Summed feature 2, 8.56%), which were in the same range than the values got from *D. pacificum*, *D. atlanticum* and *D. thermolithotrophum* (Table 5). Under our growth conditions, the novel isolate K6013^T did not contain $C_{18:1}$, but this fatty acid was detected in low amounts in *D. pacificum*, *D. atlanticum* and *D. thermolithotrophum*.

Table 5 | Whole-cell fatty acid profiles of strain K6013^T and related species of the genus *Desulfurobacterium*.

Strains: 1, K6013^T; 2, *D. pacificum* SL17^T (L'Haridon *et al.* 2006); 3, *D. atlanticum* SL22^T (L'Haridon *et al.* 2006); 4, *D. thermolithotrophum* BSA^T (L'Haridon *et al.* 1998). Values are percentages of total fatty acids. Data for strain K6013^T were obtained in this study. Data for strains 2, 3 and 4 were obtained from L'Haridon *et al.* (2006).

Fatty acid	1	2	3	4
$C_{9:0}$	1.37			
Summed feature 2 ($C_{14:0}$ 3-OH/iso-$C_{16:1}$ I)	8.56	6.87	10.98	6.82
Summed feature 3 ($C_{16:1}$ ω7*c*/$C_{16:1}$ ω6*c*)	2.13		1.42	1.29
$C_{16:0}$	17.4	14.38	16.16	5.7
Summed feature 5 ($C_{18:2}$ ω6,9*c*/ante-$C_{18:0}$)	4.14			
$C_{18:1}$	ND	2.68	2.16	3.28
$C_{18:1}$ ω9*c*	4.84	3.37	1.95	
Summed feature 8 ($C_{18:1}$ ω7*c*/$C_{18:1}$ ω6*c*)	37.91	31.99	42.08	44.98
$C_{18:0}$	18.29	31.01	26.85	35.31

The phylogenetic, phenotypic, physiological and chemotaxonomic data shown in this article support the view that strain K6013^T should be assigned to the genus *Desulfurobacterium* in the family *Desulfurobacteriaceae*. However, there are several important phenotypic differences, given in Tables 5 and 6, between the novel isolate and its closely related species. Strain K6013^T and *D. pacificum* are both able to reduce sulfur, nitrate and thiosulfate, but *D. atlanticum* is unable to reduce sulfur, *D. thermolithotrophum* is unable to reduce nitrate and '*D. crinifex*' is unable to reduce thiosulfate. Strain K6013^T and *D. thermolithotrophum* can grow at 40 ℃, while *D. pacificum*, *D. atlanticum* and '*D. crinifex*' cannot grow at temperature lower than 50 ℃. Therefore, from the phylogenetic, phenotypic, physiological and chemotaxonomic evidence, we proposed to assign

strain K6013^T to a novel species of the genus *Desulfurobacterium*, for which the name *Desulfurobacterium indicum* sp. nov. is proposed.

Table 6 | Differential characteristics of strain K6013^T and related species of genus *Desulfurobacterium.*

Strains: 1, K6013^T; 2, *D. pacificum*; 3, *D. atlanticum*; 4, *D. thermolithotrophum*; 5, '*D. crinifex*'. Characteristics are scored as: +, positive; -, negative. Data for strain 1 were obtained in this study; data for strains 2 or 3, 4 and 5 were obtained from L'Haridon *et al.* (2006), L'Haridon *et al.* (1998) and Alain *et al.* (2003) and differences were confirmed in this study.

Characteristics	1	2	3	4	5
Cell shape	Straight to curved rods	Straight to curved rods	Straight rods	Straight rods	Straight to curved rods
Length (um)	0.9-2.2	1-2	2.5-3.5c	1-2	0.9-3.5
Wide (um)	0.4-0.6	0.4-0.5	0.4-0.5	0.4-0.5	0.4-0.7
T (℃) (optimal)	40-75 (65)	55-85 (75)	50-80 (70-75)	40-75 (70)	50-70 (60-65)
pH (optimal)	5-8 (6)	5.5-7.5 (6-6.2)	5.5-7 (5.8-6)	4.4-8 (6)	5-7.5 (6-6.2)
NaCl (%) (optimal)	1-4.5 (2.5)	1.5-5 (3)	1.5-5 (3)	1-4.6 (2.3)	2-4 (3)
Flagellation	Undetermined	Monopolar	Monopolar	Monopolar	Bipolar
G+C (%)	XX	42	41	36	37
Electron acceptor					
S^0	+	+	-	+	+
NO^{3-}	+	+	+	-	+
$S_2O_3^{2-}$	+	+	+	+	-
SO_3^{2-}	-	-	-	+	-

Description of *Desulfurobacterium indicum* sp. nov.

Desulfurobacterium indicum (in'.di.cum. L. masc. adj. indicum. Indian, referring to the Indian Ocean, from where the type strain was isolated).

Cells were Gram-negative, anaerobic, motile rods (0.9-2.2 ×0.4-0.6 μm). Growth was observed at temperatures between 40 and 75 ℃ (optimum 65 ℃), at NaCl concentration from 1.0 to 4.5% (optimum 2.5%), and at pH from 5 to 8 (optimum 6.0). Strain K6013^T used sulfur, thiosulfate and nitrate as terminal electron acceptors, but not sulfate, sulfite nor nitrite. The predominant fatty acids

were $C_{16:0}$ (17.4%), $C_{18:1}$ $\omega 7c$ (Summed feature 8, 37.91%), $C_{18:0}$ (18.29%) and $C_{14:0}$ 3-OH (Summed feature 2, 8.56%). The genomic DNA G+C content was XX mol%.

The type strain K6013^T (= DSM 101677^T= MCCC 1A01868^T) was isolated from a deep-sea sulfide sample collected at a depth of 2771 m from a hydrothermal area in the Indian Ocean (37 ° 78’S, 49° 65’E; site JL-Dive94-S01).

4.4 *Thermodesulfatator lithotrophica* sp. nov.

The article of characterization of this strain is in preparation for the Journal *International Journal of Systematics and Evolutionary Microbiology*

Its genome was sequenced using the Illumina MiSeq 2x300 bp technology, but we did not receive the data so far.

***Thermodesulfatator autotrophica* sp. nov., a thermophilic sulfate-reducing bacterium from the Indian Ocean**

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[†]Qiliang Lai and Junwei Cao contributed equally to this work.

Subject: NEW TAXA - Thermodesulfobacteria

Running title: *Thermodesulfatator autotrophica* sp. nov.

Abbreviations: MCCC, Marine Culture Collection of China

The GenBank[/EMBL/DDBJ] accession number for the 16S rRNA sequence of *Thermodesulfatator autotrophica* S606^T sp. nov. is XXXX.

A novel sulfate-reducing bacterium, strain S606^T, was isolated from a sulfide sample collected at a depth of 2764 m from a deep-sea chimney wall in the Indian Ocean. Phylogenetic 16S rRNA gene sequence analyses located strain S606^T within the genus *Thermodesulfatator*, with highest sequence similarity of 98.15% to *Thermodesulfatator indicus* DSM 15286^T, followed by *Thermodesulfatator atlanticus* AT1325^T (97.4%), others were all below 91.3%. The ANI values between strain S606^T and two type strains (*T. indicus* DSM 15286^T and *T. atlanticus* AT1325^T) are XX% and XX%, respectively. The digital DNA-DNA hybridization estimate values between strain S606^T and two type strains are XX±2.4% and XX±2.3%, respectively. Cells were Gram-negative, anaerobic, motile rods (1-1.8 × 0.5-0.7 µm). It grew at NaCl concentrations ranging from 1.5 to 4.5% (optimum 2.5-3%), from pH 5.5 to 8 (optimum 6.5-7), and at temperatures between 50 and 80 ℃ (optimum 65-70 ℃). Strain S606^T grew chemolithoautotrophically in an H_2/CO_2 atmosphere (80/20, v/v; 200 kPa), using sulfate as an electron acceptor. The predominant fatty acids were $C_{16:0}$ (24.17%), Summed feature 8 ($C_{18:1}$ *ω6c* and/or $C_{18:1}$ *ω7c*, 26.33%), $C_{18:0}$ (22%) and $C_{18:1}$ *ω9c* (9.17%). The DNA G+C content was XX mol%, determined using the whole genome sequence. The combined genotypic and phenotypic traits show that strain S606^T should be described as a novel species of the genus *Thermodesulfatator*, for which the name *Thermodesulfatator autotrophica* sp. nov. is proposed, with the type strain S606^T (= DSM 101864^T = MCCC 1A01871^T).

Sulfate-reducing prokaryotes (SRP) are anaerobic microorganisms, both bacteria and archaea, using sulfate as a terminal electron acceptor in their energy metabolism and hydrogen or various organic acids as energy sources (Heidelberg *et al.* 2004). At the time of writing, the genus *Thermodesulfatator*, within the family *Thermodesulfobacteriaceae*, comprises 2 species, *T. indicus* and *T. atlanticus*, both of which have been isolated from deep-sea hydrothermal systems (Moussard *et al.* 2004, Alain *et al.* 2010). Members of the genus *Thermodesulfatator* are sulfate-reducing chemolithoautotrophs using hydrogen as sole electron donor. In addition, *T. atlanticus* is able to use several organic compounds (methylamine, peptone and yeast extract) (Alain *et al.* 2010).

In this study, we report on the isolation of a novel thermophilic sulfate-reducer, strain S606^T, belonging to the genus *Thermodesulfatator*. The characteristics of this strain indicate that it represents a novel species of the genus *Thermodesulfatator*.

In January 2015, a deep-sea sulfide sample was collected at a depth of 2771 m from a chimney wall in the Indian Ocean (37° 78' S, 49° 65' E; site JL-Dive90-S01), during the DY35 cruise of *Xiang Yang Hong Jiu Hao*. The sample was collected using a benthic seabed grab and anaerobically preserved in sterilized seawater at 4 ℃ onboard. Once in the lab, a subsample was used to inoculate a SO4PNsalts medium (Alain *et al.* 2010), prepared with a gas phase of H_2/CO_2 (80/20, v/v, 200 kPa) and incubated at 60 ℃. After 5 days of incubation, cultures of short-rod cells were observed. The enrichments were subcultured under the same conditions, and purified by 6 repeated dilutions-to-extinction series. One pure culture, strain S606^T, is described in this study. The purity of this isolate was checked routinely by microscopic examination and by repeated partial sequencing of the 16S rRNA gene using 4 different primers. Stock cultures were stored at -80°C with 5% (v/v) dimethylsulfoxide (DMSO).

Genomic DNA was extracted with the QIAGEN Genomic-tip 20/G (QIAGEN, Düsseldorf, Germany) kit following the manufacturer's standard protocol. The 16S rDNA sequence was determined by Sanger method using the primers Bac8F (5'-AGA GTT TGA TCA TGG CTC AG-3'), S8dir (5'-GTA GCG GTG AAA TGC GTA GA-3'), U1492R (5'-GGT TAC CTT GTT ACG ACT T-3') and W34 (5'-TTA CCG CGG CTG CTG GCA C-3'). Pairwise 16S rDNA sequence similarity was calculated using global alignment algorithm implemented at the EzTaxon-e server (http://eztaxon-e.ezbiocloud.net/; (Kim *et al.* 2012). Phylogenetic 16S rDNA sequence analysis was performed using the software MEGA version 5.0 (Tamura *et al.* 2011). Distances were calculated using the Kimura two-parameters model and clustering was performed with the neighbor-joining algorithm (Saitou and Nei 1987). The robustness of the inferred topology was assessed by bootstrap analyses based on 1000 replications.

A nearly full-length 16S rDNA sequence (1514 bp) of strain S606^T was obtained. The 16S rRNA gene-based analysis located the novel isolate within the class *Thermodesulfobacteria*, in the bacterial domain. Comparative 16S rDNA sequence analysis showed that strain S606^T formed a robust cluster with the genus *Thermodesulfatator,* within the family *Thermodesulfobacteriaceae* (Figure 10). Strain S606^T shared the highest sequence similarity of 98.2% with *Thermodesulfatator indicus* DSM 15286^T, followed by *Thermodesulfatator atlanticus* AT1325^T (97.4%) and *Thermosulfurimonas dismutans* S95^T (91.3 %).

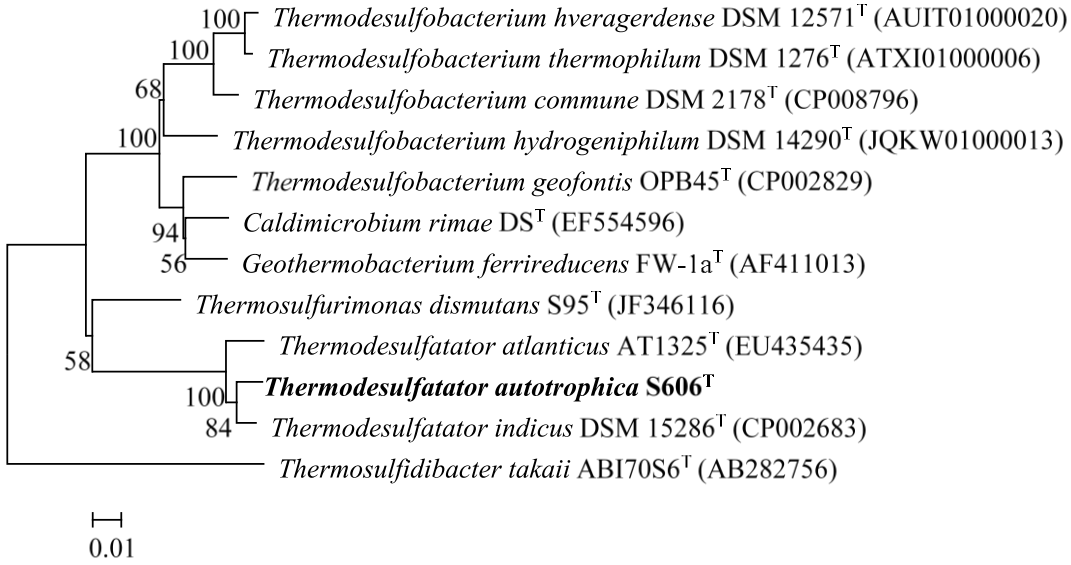

Figure 10 | Neighbor-joining tree showing the phylogenetic positions of strain S606^T and all species of the family *Thermodesulfobacteriaceae*, based on 16S rRNA gene sequences.

Bootstrap values (expressed as percentages of 1000 replications) are shown at branch points. Bar, 0.01 nucleotide substitution rate (K_{nuc}) units. *Thermosulfidibacter takaii* ABI70S6^T was used as outgroup.

The genome sequence of strain S606^T was determined by Shanghai Majorbio Bio-pharm Technology Co., Ltd. (Shanghai, China), using Solexa paired-end (500 bp library) sequencing technology. The genome sequence of *T. indicus* DSM 15286^T (NC_015681.1, Anderson *et al.* (2012)) and *T. atlanticus* AT1325^T (NZ_ATXH01000001, unpublished) were downloaded from NCBI. The G+C content of the chromosomal DNA was determined from the draft genome sequence. The average nucleotide identity (ANI) was calculated using the algorithm of Goris *et al.* (2007) using the EZGenome web service. DNA-DNA hybridization (DDH) estimate values were analyzed using the genome-to-genome distance calculator (GGDC2.0) (Auch *et al.* 2010, Auch *et al.* 2010, Meier-Kolthoff *et al.* 2013). The DNA G+C content of strain S606^T was XX mol%, which was close to two type strains (42.4%-45.0%). The ANI values between strain S606^T and two type strains (*T. indicus* DSM 15286^T and *T. atlanticus* AT1325^T) are XX% and XX%, respectively, which are below standard ANI criteria for species identity (95–96%) (Richter and Rossello-Mora 2009). The digital DNA-DNA hybridization estimate values between strain S606^T and two type strains are XX±2.4% and XX±2.3%, respectively, which are far below the standard criteria (70%) for

delineation of prokaryotic species (Wayne *et al.* 1987). These results confirmed that strain S606^T represents a novel species of the genus *Thermodesulfatator*.

Morphological characteristics of cells of strain S606^T were examined by using light microscopy (Olympus BX60 and CX40) and transmission electron microscopy (100 CXII, JEOL). Cells were motile, rods (1-1.8 µm in length and 0.5-0.7 µm in width, *n*=10, Figure 11) that occurred singly. Some cells became spherical in the late stationary growth phase. They stained Gram-negative.

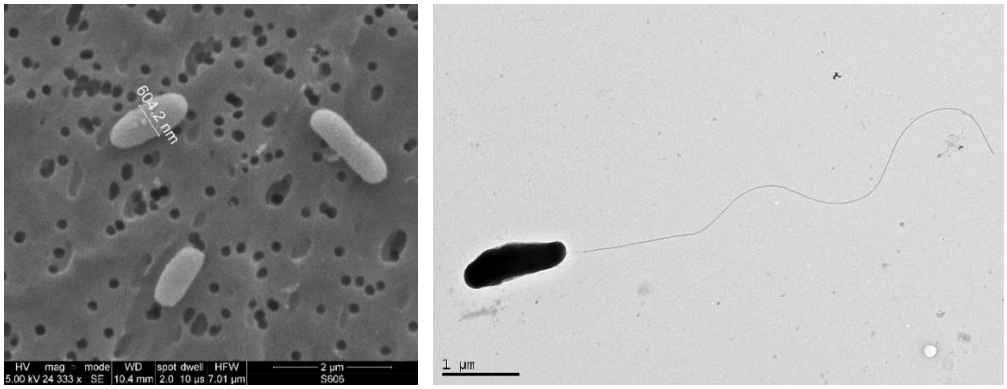

Figure 11 | Scanning electron and transmission electron micrograph of cells of strain S606^T.

Unless otherwise stated, physiological characterization was performed anaerobically in SO4PNsalts medium (Alain *et al.* 2010), in duplicate, using sulfate as a terminal electron acceptor, and a gas phase of H_2/CO_2 (80/20, v/v, 200 kPa) as energy and carbon sources. Growth experiments were generally performed as described previously (Alain *et al.* 2003). Growth of the isolate was routinely monitored by direct cell counting by using a modified Thoma chamber (depth 10 µm). Determination of the temperature range for growth was tested at 45, 50, 55, 60, 65, 70, 75, 80 and 85 ℃. The isolate grew at 50–80 ℃ (optimum 65-70 ℃). Salt tolerance was tested at 65 ℃ with various concentrations of NaCl (0, 0.2, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5 and 5.0 %, w/v). Growth of strain S606^T occurred at NaCl concentration between 1.5 and 4.5% (optimum: 2.5-3%). The pH range for growth was tested from pH 5 to pH 9.0 (initial pH at 20 ℃) with increments of 0.5 units. Growth occurred between pH 5.5 and 8.0, the optimum being around 6.6-7.

The new isolate was a strictly anaerobic bacterium using hydrogen and sulfate as primary electron donor and acceptor, respectively. The ability to use alternative electron acceptors was tested in SO4PNsalts medium depleted of sulfate, but supplemented with elemental sulfur (12 g/L),

sulfite (2 mM), thiosulfate (20 mM), nitrate (10 mM), nitrite (2 mM) or oxygen (1% v/v). Hydrogen sulfide production was carried out as described previously (Cord-Ruwisch 1985). Sulfate were used as terminal electron acceptors, but not sulfur, sulfite, nitrate nor thiosulfate. When using sulfate as a terminal electron acceptor, strain S606^T grew exclusively on hydrogen.

The cellular fatty acids were saponified, methylated and extracted using the standard protocol of MIDI (Sherlock Microbial Identification System, version 6.0B). The fatty acids were analyzed by GC (Agilent Technologies 6850) and identified by using the TSBA6.0 database of the Microbial Identification System (Sasser 1990). The principal fatty acids were $C_{16:0}$ (24.17%), Summed feature 8 ($C_{18:1}$ ω6*c*/$C_{18:1}$ ω7*c*, 26.33%), $C_{18:0}$ (22.22%) and $C_{18:1}$ ω9*c* (9.17%), and the minor fatty acids were shown in Table 7. All strains contain $C_{16:0}$, Summed feature 8 and $C_{18:0}$ as the major fatty acid, but strain S606^T can differentiate from two type strains by the major fatty acid of $C_{18:1}$ ω9*c*.

Table 7 | Cellular fatty acid profiles of strain S606^T and two species of the genus *Thermodesulfatator*.

Strains: 1, S606^T; 2, *T. atlanticus* AT1325^T (Alain *et al.* 2010); 3, *T. indicus* CIR29812^T (Moussard *et al.* 2004). Values are percentages of total fatty acids. ND, not detected; tr, trace amount (<1.0%).

Fatty acid	1	2	3
$C_{14:0}$	1.04	ND	
$C_{16:0}$	24.17	6.37	8.7-10.2
$C_{17:0}$	1.7	1.12	2.2-2.8
$C_{18:0}$	22.22	16.14	42.7-50.9
iso-$C_{10:0}$	2.11	ND	
iso-$C_{11:0}$	1.50	ND	
$C_{16:0}$ 3OH	1.21	1.9	0-3.2
$C_{16:1}\omega$5*c*	1.36	0.82	
$C_{17:1}\omega$6*c*	ND	2.23	
$C_{18:1}\omega$5*c*	1.12	2.07	
$C_{18:1}\omega$9*c*	9.17	1.16	
$C_{20:1}\omega$7*c*	ND	1.23	
$C_{19:0}$ cyclo ω8*c*	2.75	6.4	14.5-20.5
Sum In Feature 3 ($C_{16:1}$ ω6*c* and/or $C_{16:1}$ ω7*c*)	1.84	1.13	
Sum In Feature 5 ($C_{18:2}\omega$6,9*c* and/or ante-$C_{18:0}$)	3.23	ND	
Sum In Feature 8 ($C_{18:1}$ ω6*c* and/or $C_{18:1}$ ω7*c*)	26.33	59.43	19.2-23.6

Based on a combination the results of the phylogenetic, phenotypic and chemotaxonomic analyses (Tables 7 and 8) described in this article, strain S606^T should be assigned to the genus

Thermodesulfatator in the family *Thermodesulfobacteriaceae*. Nevertheless, the novel isolate can be distinguished from the closely related species based on some phenotypic characteristics and their low ANI and DDH values. Therefore, based on the polyphasic data, we propose that strain S606^T represents a novel species, for which the name *Thermodesulfatator autotrophica* sp. nov. is proposed.

Table 8 | Differential characteristics of strain S606^T and related species of genus *Thermodesulfatator*.

Strains: 1, S606^T; 2, *T. atlanticus* AT1325^T; 3, *T. indicus* CIR29812^T. Characteristics are scored as: +, positive; -, negative. Data for strains 1 were obtained during this study; data for strains 2 and 3 were obtained from Alain *et al.* (2010) and Moussard *et al.* (2004), and differences were confirmed in this study.

Characteristics	1	2	3
Cell shape	Rods	Rods	Rods
Length (um)	1-1.8	1.04-6.08	0.8-1
Wide (um)	0.5-0.7	0.3-0.75	0.4-0.5
T (℃) (optimal)	50-80(65-70)	55–75(65–70)	55–80(70)
pH (optimal)	5.5-8.0 (6.5-7.0)	5.5–8.0(6.5–7.5)	6.0–6.7(6.25)
NaCl (%) (optimal)	1.5-4.5(2.5-3)	1.5–4.5(2.5)	1.0–3.5(2.5)
Flagellation	Monopolar	Monopolar	Monopolar
NO^{3-}	-	-(+**)	-
SO_4^{2-}	+	+	+
Organic compounds	-	+	-
Genome size (Mb)		2.30	2.32
Gene number		2270	2282
G+C (mol %)	XX	45.6 (45.0*)	46 (42.4*)

*Data were obtained according to its genome sequence.

**Data was obtained in this study.

Description of *Thermodesulfatator autotrophica* sp. nov.

Thermodesulfatator autotrophica (au.to.tro'phi.ca. Gr. n. *autos* self; Gr. adj. *trophikos* nursing, tending or feeding; N.L. fem. adj. *autotrophica* autotroph).

Strictly anaerobic. Cells were Gram-negative., motile rods (1-1.8 ×0.5-0.7 μm). Growth occurs at salinities from 1.5 to 4.5% (optimum 2.5-3%), from pH 5.5 to 8 (optimum 6.5-7), and at temperatures between 50 and 80 ℃ (optimum 65-70 ℃). Strictly chemolithoautotrophic using sulfate as terminal electron acceptor and hydrogen as electron donor. The predominant fatty acids

were $C_{16:0}$, Summed feature 8 ($C_{18:1}$ $\omega 6c$ and/or $C_{18:1}$ $\omega 7c$), $C_{18:0}$ and $C_{18:1}$ $\omega 9c$. The DNA G+C content was XX mol%.

The type strain S606^T (= DSM 101864^T = MCCC 1A01871^T) was isolated from a deep-sea sulfide sample collected at a depth of 2764 m from a chimney wall in the Indian Ocean (37° 78’ S, 49° 65’ E; site JL-Dive90-S01).

4.5 Determination of the pressure range for growth of isolates from the deep-sea

4.5.1 Piezophilic bacterium *Anoxybacter fermentans* DY22613^{T}

Anoxybacter fermentans DY22613^{T} was originally isolated from a deep-sea hydrothermal sulfide deposit at a depth of 2891 m at the East Pacific Rise (GPS position: 102.6 °W, 3.1 °S), and was identified to be a novel thermophilic, anaerobic, fermentative bacterial strain (Zeng *et al.* 2015) (article is given in annex 1). Sampling and storage of the sample was not done under isobaric conditions. The determination of the hydrostatic pressure range for growth was tested in 5 mL glass syringes containing YTG medium inoculated with 1% late-exponential phase culture. Hydrostatic pressure test for strain DY22613^{T} was carried out at 0.1, 10, 20, 30, 40, 55 and 70 MPa at 60 ℃. Growth was also observed under high hydrostatic pressure, from 0.1 to 55 MPa, with optimum growth pressure of 20 MPa (Figure 12). *Anoxybacter fermentans* DY22613^{T} grows faster at 20 MPa than at atmospheric pressure (0.1 MPa), thus demonstrating it is a piezophilic bacterium.

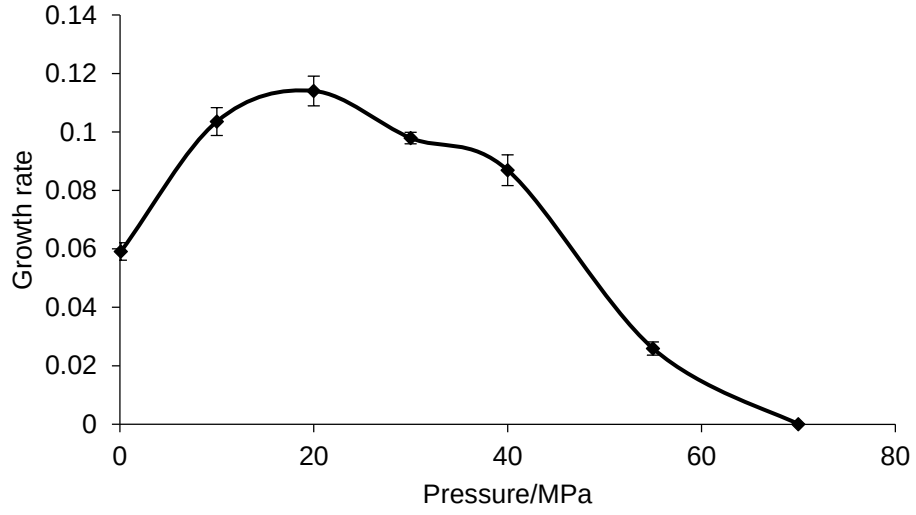

Figure 12 | Pressure curve of *Anoxybacter fermentans* DY22613^{T}.

4.5.2 Piezosensitive bacterium *Caloranaerobacter ferrireducens* DY22619^{T}

Caloranaerobacter ferrireducens DY22619^{T} is a novel thermophilic, anaerobic, iron-reducing bacterium, isolated from a sulfide sample collected from an East Pacific Ocean hydrothermal field

at a depth of 2901 m (Zeng *et al.* 2015) (see annex 2). Sampling and storage of the sample was not done under isobaric conditions. The hydrostatic pressure range for growth was determined in 5 mL glass syringes containing YTG medium inoculated with 1% late-exponential phase culture. Hydrostatic pressure test for strain DY22619^T was carried out at 0.1, 10, 20, 30 and 40 MPa at 60 ℃. The strain is able to grow from 0.1 to 40 MPa, with optimum growth pressure of 0.1 MPa (Figure 13). *Anoxybacter fermentans* DY22619^T is a novel piezosensitive bacterium, which tolerates hydrostatic pressure up to 40 MPa.

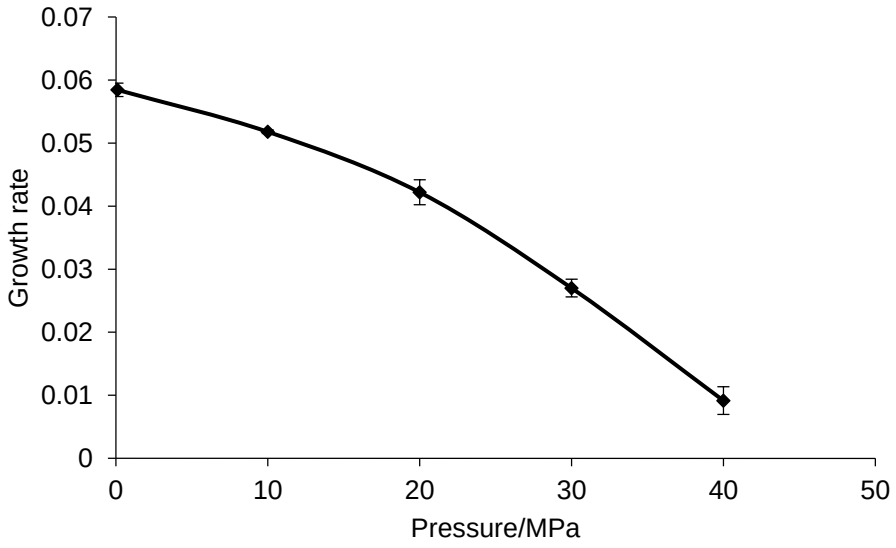

Figure 13 | Pressure curve of *Caloranaerobacter ferrireducens* DY22619^T.

4.6 Genetic manipulations of *Palaeococcus pacificus* DY20341^T

Palaeococcus pacificus DY20341^T, isolated from a sediment sample collected from an East Pacific Ocean hydrothermal field (1°38 S, 102°46 W) at a depth of 2737 m, is a hyperthermophilic, anaerobic, piezophilic archaeon (Zeng *et al.* 2013). It is one of the few thermopiezophilic organisms isolated so far, which can grow at temperatures ranging from 50 ℃ to 90 ℃ (optimally at 80 ℃) and optimally under 30 MPa. The genome of *Palaeococcus pacificus* is now available (GenBank: CP006019) (Zeng *et al.* 2015), and the development of genetic tools for the analysis of its adaptation to pressure would be very useful.

Some archaeal genetic systems have been described in members of the *Thermococcaceae*, such as *Thermococcus barophilus* (Thiel *et al.* 2014), *Thermococcus kodakaraensis* (Santangelo *et al.* 2010) and *Pyrococcus furiosus* (Kreuzer *et al.* 2013), which are phylogenetically related to *Palaeococcus pacificus*. Simvastatin was used to screen for transformed cells as *Palaeococcus pacificu* was sensitive to simvastatin (final concentration of 2.5 μg/mL), and 6-methyl purine (6-MP) was used for counterselection. The suicide vector pUPH (Figure 14), with 6-MP sensitive gene (*TK0664* gene in *Thermococcus kodakarensis*) inserted, was used as a tool for gene disruption by homologous recombination in this study. Gene *PAP_06710* in *Palaeococcus pacificus* was annotated as hypoxanthine/guanine phosphoribosyltransferase, a homolog of the *TK0664* gene (77% of identity). In order to use the plasmid pUPH as a suicide vector for counterselection, gene *PAP_06710* was firstly subjected to gene deletion, as loss of *TK0664* resulted in resistance to 6-methyl purine (6MP^r) (Santangelo *et al.* 2010).

4.6.1 Construction of the suicide vector

The suicide vector pUPH was used to clone the flanking regions of the targeted gene *PAP_06710* and the resulting plasmid was named pUPH-6710. The flanking regions of the gene targeted for deletion were amplified from genomic DNA by two successive PCR amplification steps, in a process known as splicing by overlap extension. The primers used for this first amplification were named BamHI-D6710-1F/D6710-1R and D6710-2F/NdeI-D6710-2R (see § 3.9 in the Materials and Methods). The second PCR amplification was carried out using the two flanking

regions as the matrix and primers BamHI-D6710-1F and NdeI-D6710-2R, to produce a resulting DNA fragment of 2 kb composed of the two merged homologous regions. The fragment was inserted into the plasmid pUPH at the restriction sites NdeI and BamHI, producing the suicide vector pUPH-6710. The plasmid pUPH-6710 was confirmed by PCR using BamHI-D6710-1F / NdeI-D6710-2R and pUPH-F / pUPH-R as primer pairs.

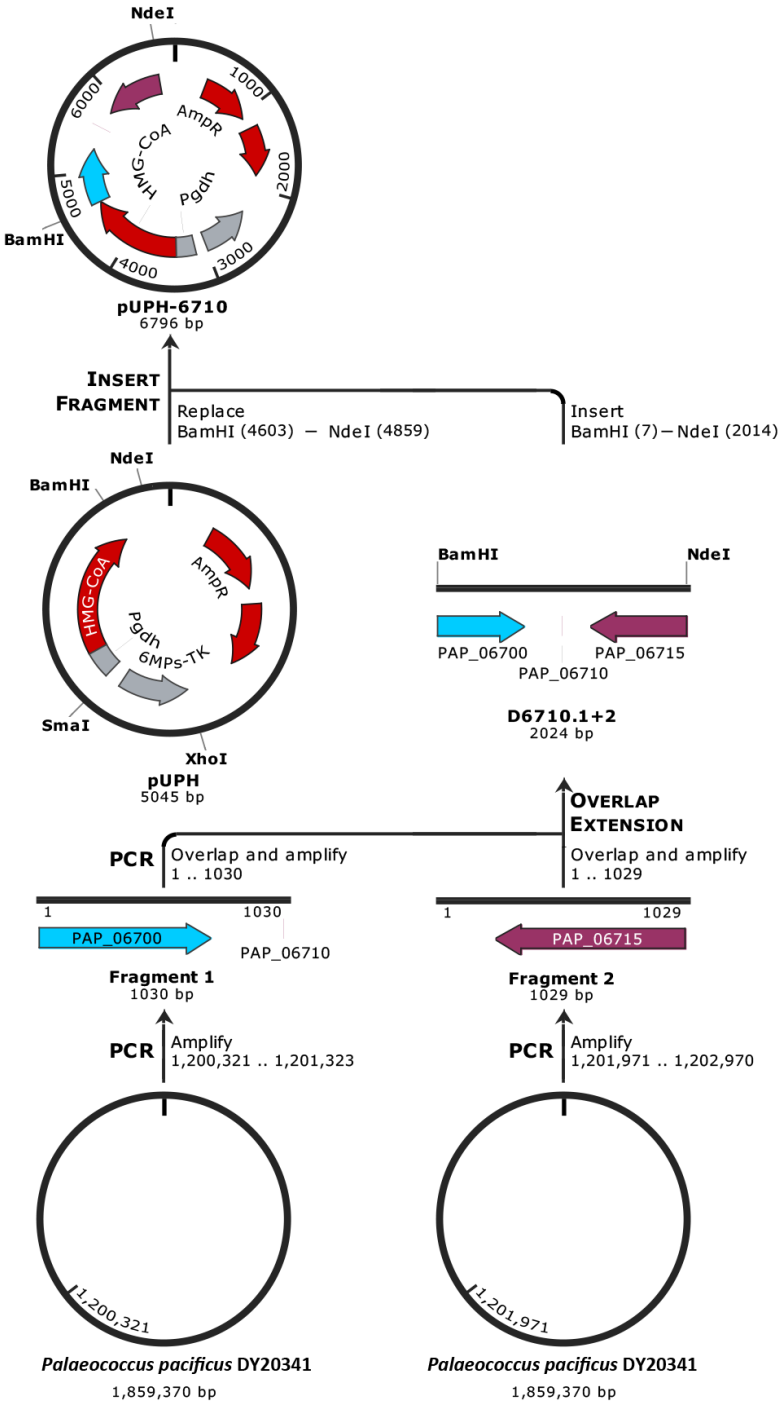

Figure 14 | Construction of pUPH-6710 plasmid.

4.6.2 Transformation of *Palaeococcus pacificus*

The plasmid pUPH-6710 was used to transform *Palaeococcus pacificus* DY20341 using simvastatin as the resistant marker. The resulting Sim^r strains were analyzed by PCR using genomic DNA as a matrix and pUPH-F and pUPH-R as primers. However, the plasmid pUPH-6710 was not found in the resulting Sim^r strains. It seems like that the plasmid cannot be transformed into *Palaeococcus pacificus* DY20341.

5 Discussion and Conclusion

Deep-sea hydrothermal vents are extreme habitats, characterized by extraordinary physical and chemical gradients. They are among the most biologically active regions, and support highly productive ecosystems fueled by chemosynthesis (Takai and Nakamura 2011, Sievert and Vetriani 2012). The most explored and studied hydrothermal systems are located in the Pacific Ocean and in the Atlantic Ocean, while only few hydrothermal sites of the Indian Ocean have been explored so far. This ocean may host most of the undiscovered vents on Earth (Beaulieu *et al.* 2015). Although our knowledge of the diversity and functions of hydrothermal vents isolates has remarkably expanded, extensive investigation of the microbiology and physiology remain imperative, especially in the poorly-documented hydrothermal vents of the Indian Ocean. Indeed, before this study, only 8 prokaryotic strains were isolated from deep-sea vents of the Indian Ocean: two methanogens, one sulfate-reducing autotroph, one fermentative, one nitrate-reducing strain, one heterotrophic aerobe and autotrophs respiring sulfur or nitrate.

Three deep-sea samples were collected from hydrothermal vents in the Indian Ocean, and investigated in this study. Once in the lab, subsamples were used to isolate anaerobic microorganisms involved the sulfur cycle. In total six anaerobic sulfur- or sulfate-reducers prokaryotes have been isolated from deep-sea hydrothermal vents from the Middle Indian Ocean or from the Southwest Indian Ridge. This study increased by nearly two-folds the number of species known from the Indian Ocean.

5.1 Isolates from the deep-sea hydrothermal samples

A novel species, *Desulfovibrio indicus* J2^T, was isolated from the serpentinized peridotite deep-sea site 30I-TVG05. Strain J2^T was a piezophilic sulfate-reducer, growing optimally at 10 MPa (range 0–30 MPa). As it comes from a sample collected at a depth of 3173 m in the Indian Ocean, it was probably not active in its natural environment of sampling. Members of the bacterial genus *Desulfovibrio* are SRP of geomicrobiological significance (Heidelberg *et al.* 2004, Khelaifia *et al.* 2011). Strain J2^T was able to reduce sulfate, thiosulfate and sulfite in the presence of H_2/CO_2 (80/20, v/v, 200 kPa), and was likely to play a role in the sulfur cycle at deep-sea hydrothermal vents.

The deep-sea sulfide sites JL-Dive94-S01 and JL-Dive90-S01 are characterized by high temperature, with fluids emitted at 361 and 145 ℃, respectively. As a result, a hyperthermophilic archaeon, *Thermococcus nautili* J8513, and a thermophilic bacterium, *Caloranaerobacter azorensis* J5513, were isolated from the deep-sea sulfide sites JL-Dive94-S01, and another hyperthermophilic archaeon, *Thermococcus hydrothermalis* J856, was isolated from the deep-sea sulfide site JL-Dive90-S01.

A novel thermophilic sulfur-reducing bacterium, *Desulfurobacterium indicum* K6013^T was isolated from the high-temperature sulfide sites JL-Dive94-S01 in the Indian Ocean. It grew chemolithoautotrophically in the presence of thiosulfate or sulfur as terminal electron acceptor and hydrogen as the electron donor, with optimal temperature of 65 ℃. Members of the genus *Desulfurobacterium* were supposed to play an important role in deep-sea hydrothermal ecosystems as primary producers of organic matter in anaerobic zones (Miroshnichenko and Bonch-Osmolovskaya 2006).

The novel sulfate-reducing bacterium, *Thermodesulfatator autotrophica* S606^T, grew chemolithoautotrophically with an atmosphere of H_2/CO_2 (80/20, v/v; 200 kPa), using sulfate as a terminal electron acceptor. It grew optimally at temperatures of 65-70 ℃, in agreement with the high temperature of the original sample (sites JL-Dive94-S01).

In summary, only a few isolates were obtained from these deep-sea samples. This is notably due to the fact that it was difficult to design culture conditions because the chemical parameters of the hydrothermal sites were not measured during the cruise. This is also due to the fact that the "conventional" culture approaches that we used are time-consuming. It might have been interesting to use high-throughput cultivation approaches. Enrichment cultures into microplates might have increase the number and diversity of isolated strains. We did not decide to perform molecular inventories of the diversity with our hydrothermal samples, because the main aim of this work was initially the design of a genetic tool and because the time was short (2 years). Nevertheless, such molecular approaches would have given clues on the chemical parameters of the different sites and be very helpful to direct culture conditions.

5.2 The cultivation of novel microorganisms

First and next-generation sequencing surveys based on 16S rRNA genes and functional genes have revealed an unexpected diversity of uncultured bacterial and archaeal lineages at deep-sea hydrothermal vents (Sogin *et al.* 2006, Anderson *et al.* 2015). Recent advances in genomic technologies similarly had great impact on microbiology, providing further insights into microbial evolution, adaptations, physiology and ecology (Schleper *et al.* 2005). Up to date, there are 159 (see the tables 1 and 2) novel genera/species that have been isolated from deep-sea hydrothermal vents, representing only a minor fraction of the microbial diversity of this ecosystem. The uncultured bacterial and archaeal lineages remain for now without cultured representatives that could serve as a reference for metabolic inferences to design cultivation strategies. The cultivation of microorganisms from deep-sea hydrothermal vents will undoubtedly be an important technology providing further understanding of the microbial physiology and biochemistry.

In order to optimize prokaryote cultivation efficiency microbiologists developed different novel cultivation strategies in recent years, which can be classified in to four categories: Refinement of standard cultivation strategies, cultures *in situ* or cultures in simulated natural conditions, cultures of microbial communities and high-throughput automatable microbial culture formats (Alain and Querellou 2009). The repeated dilution-to-extinction culturing has been demonstrated to be a successful strategy to isolate novel species (Alain and Querellou 2009). Other isolation methods were also often used, such as filtration methods, flow cytometry and cell sorting (FACS), and density-gradient centrifugation or elutriation (Vartoukian *et al.* 2010).

However, it is still difficult to identify required nutrients for microbes and to design growth media and conditions. And microbial cultivation is also a low-throughput process and time-consuming. So, one of the main tasks in microbiology is to develop culture techniques and strategies to cultivate the uncultured majority (Vartoukian *et al.* 2010). The key points for microbial cultivation do not depend only of a single technical advance but depend on our understanding of the natural microbial systems. And the application of combinatorial approaches and the development of novel approaches will be required in the future to come close to natural conditions and to further improve cultivation efficiency.

5.3 Novel microorganisms from underexplored deep-sea vents and their potential

Microorganisms from deep-sea hydrothermal vents represent an untapped reservoir of biomolecules with tremendous biotechnological potential (Pettit 2011). Numerous studies reported microbial natural products or new functions from microorganisms inhabiting extremely acidic, high temperature or high pressure environments (Thornburg *et al.* 2010, Pettit 2011). Microorganisms produce a variety of secondary metabolites, which could have bioactive properties, particularly in the anticancer and anti-infective areas. Nearly 10% of microbial genomes are devoted to secondary metabolism and the pathways responsible for the biosynthesis of these metabolites have been identified and characterized.

In this study, three novel species (*Desulfovibrio indicus*, *Desulfurobacterium indicum* and *Thermodesulfatator autotrophica*) from deep-sea hydrothermal vents were isolated and characterized. All of the three novel species are supposed to play an important role in deep-sea hydrothermal ecosystems as primary producers of organic matter in anaerobic zones. The genomes of these three novel species have been sequenced and will provide further insights into its adaptations, physiology and ecology. In the future, to determine if these strains present a biotechnological potential (and even if that was not the aim of our study), that would be interesting to perform genome mining and search for key enzymes (such as PolyKetide Synthases (PKS)), Non-Ribosomal Peptide Synthetases (NRPS) or TerPene Synthases (TPS)) involved in the production of secondary metabolites, some of which possess bioactive properties. Enzymes involved in recalcitrant substrates degradation (keratin, chitin, cellulose and /or hydrocarbons) could also be targeted, in addition to polymers like exopolysaccharides, and metabolites (lipids, compatible solutes, etc.), as all these compounds might find biotechnological applications.

5.4 Perspectives

In order to understand the microbial adaptations, physiology and ecology, more studies should be carried out. Physiological and metabolic experiments, molecular studies, and genomic and transcriptomic analyses could be combined to investigate the pathways of sulfur and sulfate

reduction. Another question is how the isolates adapt to the deep-sea hydrothermal environments and in which way they are involved in the biogeochemical cycling. The mechanism of interaction between microbes and the abiotic environments is another interesting topic needed to be studied in the future.

6 Annexes

6.1 Annex 1: Article *Anoxybacter fermentans* gen. nov., sp. nov., a piezophilic, thermophilic, anaerobic, fermentative bacterium isolated from a deep-sea hydrothermal vent.

6.2 Annex 2: Article Physiological features of *Halomonas lionensis* sp. nov., a novel bacterium isolated from a Mediterranean Sea sediment.

6.1 Annex 1: Article *Anoxybacter fermentans* gen. nov., sp. nov., a piezophilic, thermophilic, anaerobic, fermentative bacterium isolated from a deep-sea hydrothermal vent.

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Anoxybacter fermentans gen. nov., sp. nov., a piezophilic, thermophilic, anaerobic, fermentative bacterium isolated from a deep-sea hydrothermal vent

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A novel piezophilic, thermophilic, anaerobic, fermentative bacterial strain, designated strain DY22613^T, was isolated from a deep-sea hydrothermal sulfide deposit at the East Pacific Rise (GPS position: 102.6° W 3.1° S). Cells of strain DY22613^T were long, motile rods (10 to 20 μm in length and 0.5 μm in width) with peritrichous flagella and were Gram-stain-negative. Growth was recorded at 44–72 °C (optimum 60–62 °C) and at hydrostatic pressures of 0.1–55 MPa (optimum 20 MPa). The pH range for growth was from pH 5.0 to 9.0 with an optimum at pH 7.0. Growth was observed in the presence of 1 to 8 % (w/v) sea salts and 0.65 to 5.2 % (w/v) NaCl, with optimum salt concentrations at 3.5 % for sea salts and at 2.3 % for NaCl. Under optimal growth conditions, the shortest generation time observed was 27 min (60 °C, 20 MPa). Strain DY22613^T was heterotrophic, able to utilize complex organic compounds, amino acids, sugars and organic acids including peptone, tryptone, beef extract, yeast extract, alanine, glutamine, methionine, phenylalanine, serine, threonine, fructose, fucose, galactose, gentiobiose, glucose, mannose, melibiose, palatinose, rhamnose, turanose, pyruvate, lactic acid, methyl ester, erythritol, galacturonic acid and glucosaminic acid. Strain DY22613^T was able to reduce Fe(III) compounds, including Fe(III) oxyhydroxide (pH 7.0), amorphous iron(III) oxide (pH 9.0), goethite (α-FeOOH, pH 12.0), Fe(III) citrate and elementary sulfur. Products of fermentation were butyrate, acetate and hydrogen. Main cellular fatty acids were iso-$C_{15:0}$, iso-$C_{14:0}$ 3-OH and $C_{14:0}$. The genomic DNA G+C content of strain DY22613^T was 36.7 mol%. Based on 16S rRNA gene sequence analysis, the strain forms a novel lineage within the class *Clostridia* and clusters with the order *Haloanaerobiales* (86.92 % 16S rRNA gene sequence similarity). The phylogenetic data suggest that the lineage represents at least a novel genus and species, for which the name *Anoxybacter fermentans* gen. nov., sp. nov. is proposed. The type strain is DY22613^T (=JCM 19466^T=DSM 28033^T=MCCC 1A06456^T).

Abbreviations: AQDS, 9,10-anthraquinone-2,6-disulfonate; S°, elemental sulphur.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA sequence of strain DY22613T is KC794015.

Two supplementary figures and one supplementary table are available with the online Supplementary Material.

Molecular inventories revealed a wide diversity of thermophilic prokaryotes at deep-sea hydrothermal vents, only some of which have been cultivated (Miroshnichenko & Bonch-Osmolovsaya, 2006). Some thermophiles of the class *Clostridia* have been isolated from deep-sea vents, including representatives of the genera *Caloranaerobacter* (Wery *et al.*, 2001), *Caminicella* (Alain *et al.*, 2002), *Tepidibacter* (Slobodkin *et al.*, 2003; Urios *et al.*, 2004), *Caldanaerobacter* (Fardeau *et al.*, 2004) and *Clostridium*. Two species of the genus *Clostridium* have also been isolated from deep-sea vents, namely, *Clostridium caminithermale* (Brisbarre *et al.*, 2003) and *Clostridium tepidiprofundi* (Slobodkina *et al.*, 2008). Recently, one novel species, *Vallitalea pronyensis*, has been isolated from a shallow hydrothermal vent chimney (Ben Aissa *et al.*, 2014). These six genera fall into the class *Clostridia*, a highly polyphyletic class of obligate anaerobes. Most of them ferment carbohydrates to acetate, ethanol, H_2 and CO_2. At the time of writing, the class *Clostridia* encompasses four orders including *Clostridiales*, *Halanaerobiales*, *Natranaerobiales* and *Thermoanaerobacterales* and the suborder *Eubacteriineae* (Rainey, 2009). The orders *Haloanaerobiales* and *Natranaerobiales* were created to accommodate halophilic anaerobes (Rainey *et al.*, 1995; Mesbah *et al.*, 2007). The order *Thermoanaerobacterales* is polyphyletic, encompassing species able to survive in environments of extreme elevated temperature (Hogan, 2010). The order *Clostridiales* is highly polyphyletic, not a natural group, with diverse clades. We describe in this report the characterization of a novel piezophilic, anaerobic, thermophilic, fermentative bacterium (designated strain DY22613^T) isolated from a deep-sea hydrothermal vent environment. On the basis of the physiological and phylogenetic evidence presented, we propose a novel genus, *Anoxybacter* gen. nov., to accommodate this micro-organism.

Strain DY22613^T was isolated from hydrothermal sulfides collected in July 2011 at a depth of 2891 m at the East Pacific Rise (GPS position: 102.6° W 3.1° S), during the DY125-22 cruise of R/V *Da Yang Yi Hao*. Sulfide samples were collected using a benthic seabed grab and stored hermetically in sealed sterile vials. Samples were transported at 4 °C to the laboratory. A sample composed of hydrothermal chimney fragments bearing polychaete tubes and tube worms was chosen to perform enrichment cultures of thermophilic heterotrophic anaerobes. X-ray diffraction analysis indicated that this sample was mainly composed of pyrite (FeS_2) and sphalerite (ZnS).

One subsample was used to inoculate (1/10, w/v) a sterile liquid medium called FRPFO, which was prepared anaerobically and kept under an atmosphere of highly purified 100 % nitrogen. FRPFO medium contained (g l^{-1}, unless stated otherwise): peptone (10), sea salts (30; Sigma), PIPES (6.05), cysteine hydrochloride (0.5), resazurin (1 mg) and amorphous Fe(III) oxyhydroxide (50 mM, pH 7.0) as an electron acceptor. Enrichment cultures were incubated at 60 °C. Between 3 to 5 days of incubation, the colour of the precipitates changed from brown to black, indicating Fe(III) reduction. The enriched microbial community was composed of motile long and small rods. One strain, designated DY22613^T, was unable to form colonies in solidified medium containing 1.5 % (w/v) agar or 0.2 % (w/v) Gelrite, therefore, strain DY22613^T was purified by three repeated dilution-to-extinction series. The purity of this isolate was confirmed routinely by microscopic examination and by repeated partial sequencing of the 16S rRNA gene using several PCR primers (Lane, 1991) (Bac8F, Bac27F, 1100R, U1492R). Stock cultures were stored at −80 °C in FRPFO medium supplemented with 5 % (v/v) DMSO.

The morphological characteristics of cells of the novel isolate were determined by using light microscopy (CX21; Olympus) and transmission electron microscopy (JEM-1230; JEOL). For ultrathin section examination of the cell wall, bacterial cells were fixed with osmic acid and embedded in araldite; the samples were then sliced and stained with lead citrate (Reynolds, 1963). Cells of strain DY22613^T were regular to long rods (10 to 20 μm in length and 0.5 μm in width), motile, bearing flagella (Fig. S1a, available in the online Supplementary Material). Cells occurred mainly singly or formed short chains. The cells stained Gram-negative (Hangzhou Tianhe Micro-organism Reagent), and electron microscopy of ultrathin sections of cells revealed the presence of two layers characteristic of Gram-stain-negative bacteria (Fig. S1b). Moreover, the KOH reaction was positive, confirming the Gram-stain-negative type of the cells. Spores were not observed.

Physiological characterization of the novel isolate was carried out in FRPFO medium dispensed anaerobically in 50 ml vials sealed with butyl-rubber stoppers, reduced with 0.05 % (w/v) cysteine hydrochloride sterile solution, just before inoculation. Unless stated otherwise, experiments were carried out anaerobically under an atmosphere of N_2 (100 %, 1 bar) and incubations were performed in the dark at 60 °C and pH 7.0. Growth was routinely monitored by direct cell counting using a modified Thomas chamber (depth 10 μm). Growth rates were calculated using linear regression analysis of eight points along the linear portions of the growth curves that were exponentially transformed. The determination of the temperature range for growth was tested over the range 40–74 °C at 2 °C intervals. Growth was observed from 44 to 72 °C, with an optimum growth rate at 60–62 °C. Growth was also observed under high hydrostatic pressure, from 0.1 to 55 MPa (optimum 20 MPa; Fig. S2). The pH range for growth was tested from initial pH 4.0 to initial pH 10.0 at 60 °C in basal medium buffered and adjusted to the required pH (initial pH at 20 °C) with MES buffer (pH 4.0–6.0), PIPES buffer (pH 7.0–8.0), HEPES buffer (pH 8.0–9.0), AMPSO buffer (pH 9.0–10.0). Growth was observed from pH 5.0 to 9.0 and the optimum pH for growth was pH 7.0. Salt tolerance was tested at 60 °C in FRPFO medium prepared with various concentrations of NaCl (0–10 %, w/v, at 0.5 % intervals) or various concentrations of sea salts (0–100 g l^{-1} at 5 g l^{-1} intervals). Strain DY22613^T required salt and

grew at concentrations ranging from 0.65–5.20 % (w/v) NaCl (optimum 2.3 %, w/v, NaCl). Growth of strain DY22613^T was observed at sea salt concentration of 10–80 g l^{-1}, with an optimum sea salt concentration of 35 g l^{-1}. Under optimal growth conditions, the generation time was around 54 min at atmospheric pressure and around 27 min under 20 MPa.

Strain DY22613^T was an obligate chemoorganoheterotroph, utilizing complex organic compounds including peptone, tryptone, beef extract and yeast extract. The ability of the isolate to use single carbon sources for growth was tested in triplicates at the optimal growth temperature using Biolog AN microplates in anaerobic jars as per the manufacturer's instructions. The Biolog AN plate results showed that strain DY22613^T was able to utilize amino acids (including L-alanine, L-alanyl-L-glutamine, L-glutamic acid, L-glutamine, L-methionine, L-phenylalanine, L-serine and L-threonine), sugars (including D-fructose, L-fucose, D-galactose, gentiobiose, D-glucose, D-glucose 6-phosphate, D-mannose, melibiose, 3-methyl D-glucose, palatinose, L-rhamnose and turanose) and organic acids (including pyruvate, D-lactic acid, methyl ester, erythritol, D-galacturonic acid and D-glucosaminic acid). Strain DY22613^T was not able to utilize D-gluconic acid, lactose, maltose, D-mannitol, melezitose, raffinose, salicin, D-sorbitol, stachyose, sucrose, trehalose, acetic acid, formic acid, fumaric acid, glyoxylic acid, malic acid, succinic acid, alaninamide, L-asparagine, L-valine, inosine, thymidine or uridine. The major fermentation products of glucose, determined by gas chromatography (QP2010; Shimadzu) were butyrate, acetate and hydrogen.

The ability of the novel isolate to use electron acceptors was tested by adding elemental sulfur (12 g l^{-1}), sulfite (1 mM), thiosulfate (20 mM), nitrate (10 mM), 9,10-anthraquinone-2,6-disulfonate (AQDS; 5 mM), Fe(III) oxyhydroxide (pH 7.0, 50 mM), amorphous iron(III) oxide (pH 9.0, 50 mM), goethite (α-FeOOH; pH 12.0, 50 mM); Fe(III) citrate (20 mM), Fe(III) chlorite (20 mM), Fe(III) EDTA (20 mM) or oxygen (0.05–0.5 %, v/v) to the medium. Various forms of Fe(III) were synthesized by using modifications of previously described methods (Lovley & Phillips, 1986a). Strain DY22613^T was found to be strictly anaerobic. The strain grew only by fermentation with complex organic compounds and glucose, and also with Fe(III) as an electron acceptor. Strain DY22613^T was facultatively dependent on different forms of Fe(III) including Fe(III) oxyhydroxide (pH 7.0), amorphous iron(III) oxide (pH 9.0), goethite (α-FeOOH; pH 12.0), Fe(III) citrate or AQDS as an electron shuttle; but was unable to reduce Fe(III) chloride or Fe(III) EDTA. The cell yields of strain DY22613^T with peptone as an electron donor reached 10^8 cells ml^{-1}, both in the presence and in the absence of Fe(III). Reduced Fe(II) was measured by the accumulation of HCl-soluble Fe(II) over time with ferrozine (Lovley & Phillips, 1986b). The maximum concentration of reduced Fe(II) reached 16.82, 14.40, 14.29 and 4.51 mM (in the late stationary growth phase) when grown on amorphous iron(III) oxide (pH 9.0), Fe(III) citrate, Fe(III) oxyhydroxide (pH 7.0) and goethite (pH 12.0), respectively (experiments were performed in triplicate). The novel isolate reduced elemental sulfur (S°) to hydrogen sulfide (checked by colorimetry in the presence of $CuSO_4$/HCl), but did not reduce sulfite, sulfate, thiosulfate or nitrate.

The determination of the whole-cell fatty acid composition was performed on cultures grown on YTG medium at 60 °C. Cells were harvested at the end of the exponential growth phase (36 h of incubation). Fatty acids were extracted and analysed following the instructions of the Sherlock Microbial Identification System (MIDI). The fatty acids in strain DY22613^T comprised iso-$C_{15:0}$ (36.26 %), iso-$C_{14:0}$ 3-OH (20.61 %) and $C_{14:0}$ (7.36 %) and differed from the type strain *Halothermothrix orenii* H168^T in the proportion of several fatty acids including iso-$C_{15:0}$ (54.3 % in H168^T), $C_{16:0}$ (9.94 %), anteiso-$C_{15:0}$ (9.79 %) and $C_{14:0}$ (7.96 %) (Cayol *et al.*, 1994). The fatty acid profiles of both species are given in Table S1.

The genomic DNA G+C content of strain DY22613^T was 36.7 mol% as determined by genome sequencing using Illumina GAIIX (Meiji Company, Shanghai). An almost complete 16S rRNA gene sequence (1471 nt) was determined by double strand DNA sequencing. The identification of phylogenetic neighbours was initially carried out using BLAST (Altschul *et al.*, 1997) and MEGABLAST (Zhang *et al.*, 2000) against the database of type strains with validly published prokaryotic names (Chun *et al.*, 2007). A search of the most similar 16S rRNA gene sequences was also done against the web-based EzTaxon-e Server (Kim *et al.*, 2012). The 16S rRNA gene sequence of strain DY22613^T was found to be very distantly related to species in the orders *Halanaerobiales*, *Natranaerobiales*, *Thermoanaerobacterales* and *Clostridiales* in the phylum Firmicutes, with similarity below 87.0 %. The closest relative was *H. orenii* H168^T (Cayol *et al.*, 1994), with 86.92 % 16S rRNA gene sequence similarity, followed by *Natranaerobius trueperi* JW/NM-WN-LH1^T (85.73 %) (Mesbah & Wiegel, 2009) and *Moorella humiferrea* 64-FGQ^T (85.63 %) (Nepomnyashchaya *et al.*, 2012).

A phylogenetic tree of representative members in the class *Clostridia* was reconstructed from 16S rRNA gene sequences using 1239 homologous gene sequence positions (Fig. 1). Alignment of all sequences was performed using the software CLUSTAL_X (version 2.3) and the phylogenetic tree was reconstructed using the neighbour-joining method with the software MEGA (version 5.1). Bootstrap analysis was performed with 1000 replications to provide confidence estimates for the tree topology. Based on this analysis, strain DY22613^T clearly belongs to the class *Clostridia*, but is not affiliated closely with any of the described lineages. Its closest neighbours belong to the order *Halanaerobiales* but are distantly related to the novel isolate (Fig. 1). Furthermore, strain DY22613^T could be differentiated from its closest relatives *H. orenii*, *N. trueperi* and *M. humiferrea*

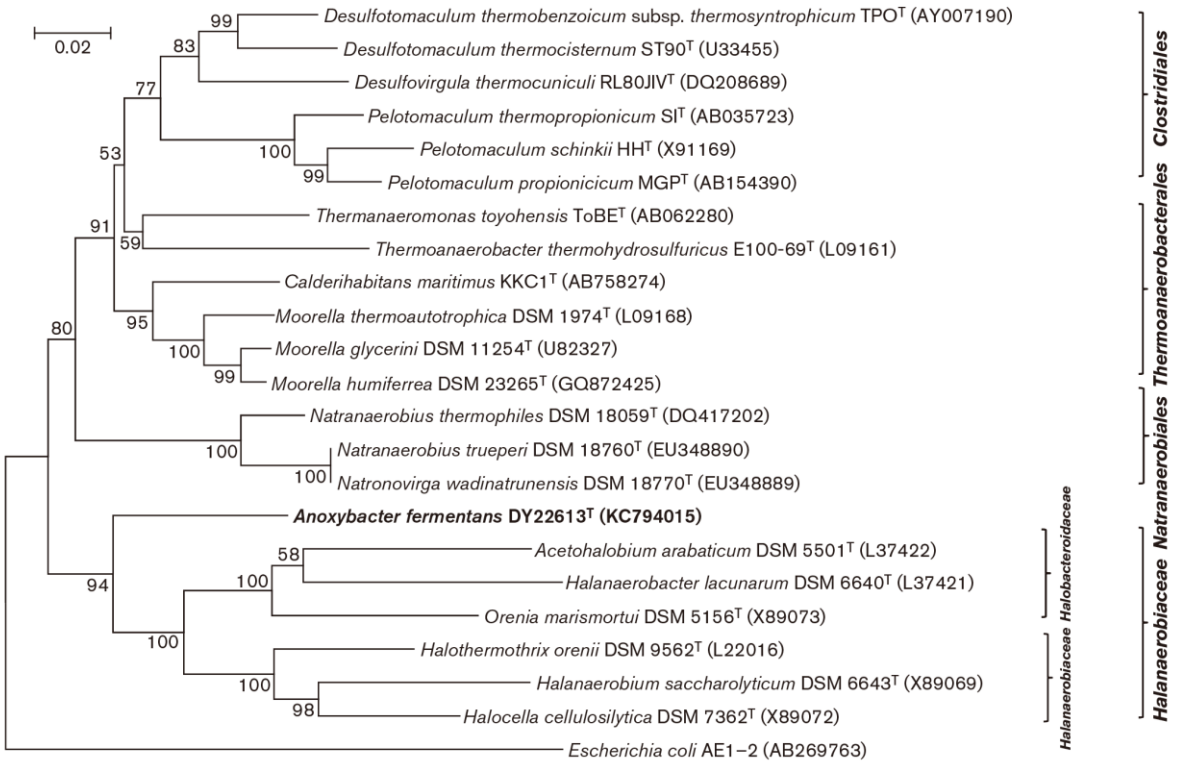

Fig. 1. Phylogenetic dendrogram obtained by neighbour-joining analysis based on 16S rRNA gene sequences (1239 bp, omitting unaligned regions), showing the position of strain DY22613^T within the class *Clostridia*. Bar, expected number of changes per sequence position.

Table 1. Differential characteristics between strain DY22613^T and its phylogenetically closest relatives

Strains: 1, DY22613^T; 2. *H. orenii* H168^T; 3. *N. trueperi* JW/NM-WN-LH1^T; 4. *M. humiferrea* 64-FGQ^T. ND, No data.

Characteristic	1	2	3	4
Geographical origin	Sulfide of deep-sea hydrothermal vent, East Pacific rise	Sediment of salted lake, Tunisia	Sediment of alkaline, hypersaline lake, Egypt	Sediment of terrestrial hydrothermal spring, Russia
Cell size (μm)	10.0–20.0 × 0.5	10.0–20.0 × 0.4–0.6	4.0–5.0 × 0.3–0.4	2.0–5.0 × 0.3–0.5
NaCl range, %, w/v (optimum)	0.65–5.20 (2.3)	4.0–20.0 (10)	9.9–21.6 (13.4)	0–15.0 (0)
Temp. range, °C (optimum)	45.0–70.0 (60.0–62.0)	45.0–68.0 (60.0)	26.0–56.0 (51.0)	46.0–70.0 (65.0)
pH range (optimum)	5.0–9.0 (7.0)	5.5–8.2 (6.5–7.0)	8.5–11.5 (9.9)	5.5–8.5 (7.0)
DNA G+C content (mol%)	36.7	39.6	41.7	51.0
Major fatty acids (>7 %)	iso-$C_{15:0}$ (36.26 %); iso-$C_{14:0}$ 3-OH (20.61 %); $C_{14:0}$ (7.36 %)	iso-$C_{15:0}$ (54.30 %); $C_{16:0}$ (9.94 %); anteiso-$C_{15:0}$ (9.79 %); $C_{14:0}$ (7.96 %)	iso-$C_{15:0}$ (80.40 %); anteiso-$C_{15:0}$ (9.40 %)	ND
16S rRNA gene sequence similarity (%)*	100.00	86.92	85.73	85.63

*Calculated in reference to the 16S rRNA gene sequence of strain DY22613^T.

based on a number of physiological characteristics such as NaCl range for growth and optimal salinity for growth, genomic DNA G+C content (mol%) and fatty acid profile (Table 1).

In conclusion, on the basis of the wide phylogenetic distance from its closest relatives (far below the threshold level of 94.5 % identity for the delineation of a new genus and close to the threshold level of 86.5 % for a new family delineation) (Yarza *et al.*, 2014), and with phenotypic differences with the closest neighbours, we propose to place strain DY22613^T as the type strain of a novel species within a new genus, for which the name *Anoxybacter fermentans* gen. nov., sp. nov., is proposed. A novel family will have to be established in the future to encompass this genus and related genera yet to be described, when more isolates are available.

Description of *Anoxybacter* gen. nov.

Anoxybacter (An.o.xy.bac′ter. Gr. pref. *an* without; M.L. *oxy* shortened from *oxygenium* oxygen; N.L. *bacter* masc. equivalent of Gr. neut. n. *bakterion* rod or sta; N.L. masc. n. *Anoxybacter* rod growing without oxygen).

Cells are Gram-stain-negative. Endospores are not observed. Thermophilic. Strictly anaerobic. Chemoorganoheterotrophic. The genomic DNA G+C content is approximately 37 mol%.

Description of *Anoxybacter fermentans* sp. nov.

Anoxybacter fermentans (fer.men′tans. L. part. adj. *fermentans* fermenting).

Cells are motile, round-ended rods with flagella. Cells grow in the temperature range of 44 to 72 °C (optimum 60–62 °C), in the hydrostatic pressure ranging from 0.1 to 55 MPa (optimum 20 MPa), pH range of 5.0 to 9.0 (optimum pH 7.0) and with 10 to 85 g l^{-1} sea salts (optimum 35 g l^{-1}). The shortest doubling time is 27 min at 60 °C under 20 MPa. It can utilize complex organic compounds, amino acids, sugars and organic acids including peptone, tryptone, beef extract, yeast extract, alanine, glutamine, methionine, phenylalanine, serine, threonine, fructose, fucose, galactose, gentiobiose, mannose, melibiose, glucose, palatinose, rhamose, rhamnose, turanose, pyruvate, lactic acid, galacturonic acid, glucosaminic acid, methyl ester and erythritol. Insoluble Fe(III) compounds, including amorphous Fe(III) oxyhydroxide (pH 7.0), amorphous iron (III) oxide (pH 9.0), goethite (α-FeOOH; pH 12.0) and Fe(III) citrate can be reduced to Fe(II). Reduces S° but does not reduce sulfite, sulfate, thiosulfate or nitrate.

The type strain, DY22613^T (=JCM 19466^T=DSM 28033^T=MCCC 1A06456^T), was isolated from a hydrothermal sulfide sample collected from an East Pacific Ocean hydrothermal field (GPS position: 102.6° W 3.1° S) at a depth of 2891 m. The genomic DNA G+C content of the type strain is 36.70 mol%.

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6.2 Annex 2: Article Physiological features of *Halomonas lionensis* sp. nov., a novel bacterium isolated from a Mediterranean Sea sediment.

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Original article

Physiological features of *Halomonas lionensis* sp. nov., a novel bacterium isolated from a Mediterranean Sea sediment

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Abstract

A novel halophilic bacterium, strain RHS90^T, was isolated from marine sediments from the Gulf of Lions, in the Mediterranean Sea. Its metabolic and physiological characteristics were examined under various cultural conditions, including exposure to stressful ones (oligotrophy, high pressure and high concentrations of metals). Based on phylogenetic analysis of the 16S rRNA gene, the strain was found to belong to the genus *Halomonas* in the class *Gammaproteobacteria*. Its closest relatives are *Halomonas axialensis* and *Halomonas meridiana* (98% similarity). DNA–DNA hybridizations indicated that the novel isolate is genotypically distinct from these species. The DNA G + C content of the strain is 54.4 mol%. The main fatty acids ($C_{18:1}$ $\omega 7c$, 2-OH iso-$C_{15:0}$, $C_{16:0}$ and/or $C_{19:0}$ cyclo $\omega 8c$), main polar lipids (diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, phosphatidylcholine and an unidentified phosphoglycolipid) and major respiratory quinone (ubiquinone Q9) were determined. The novel isolate is heterotrophic, mesophilic, euryhaline (growth optimum ranging from 2 to 8% w/v NaCl) and is able to grow under stressful conditions. The strain accumulates poly-β-hydroxyalkanoates granules and compatible solutes. Based on genotypic, chemotaxonomic and phenotypic distinctiveness, this isolate is likely to represent a novel species, for which the name *Halomonas lionensis* is proposed. The type strain of *H. lionensis* is RHS90^T (DSM 25632^T = CIP 110370^T = UBOCC 3186^T).

Keywords: Halomonas; Taxonomy; Environmental adaptation; Metal tolerance; Poly-β-hydroxyalkanoate; Compatible

1. Introduction

At the time of writing, the genus *Halomonas*, within the class *Gammaproteobacteria*, encompasses more than 76 recognized species [31]. It comprises mostly marine halophilic aerobic heterotrophs well known for their metabolic versatility [1,6]. Microorganisms belonging to the genus *Halomonas* were initially found in hypersaline environments such as the Dead Sea, hypersaline lakes, hypersaline soils and solar salterns [10,26,32,43]. Later, culture-based and molecular-based studies revealed that *Halomonas* microorganisms are also present in numerous non-hypersaline environments such as animal tissues [35], factories [8], non-marine biofilms [13], human blood [17] and in environments considered as stressful from an anthropocentric point of view, such as highly polluted/alkaline waters [3,45] and non-hypersaline ices from Antarctica [34]. The use of molecular techniques in microbial ecology has also enlarged the list of environments associated with *Halomonas* species, as they have been found in deep oceans [40], hydrothermal vents [15,16,38], subsurface environments [9] and crustal fluids and rocks [37]. Thus, members of the genus *Halomonas* are widespread in the biosphere and colonize common to extreme environments. This distribution

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suggests that these bacteria display broad physiological plasticity and metabolic versatility and have developed specific adaptations that allow them to maintain or grow under extreme physical (pressure), chemical (pollutants, high concentrations of metals) and energetic (starvation) conditions, thus allowing them to colonize a variety of habitats.

For instance, different halophilic archaea and bacteria, including several *Halomonas* species, accumulate poly-β-hydroxyalkanoates (PHA) (carbon and energy storage materials) to cope with nutrient-depleted conditions [20,38]. Also, some halophilic strains develop specific osmoadaptation mechanisms to prevent molecular damage from cellular freezing and dehydration. These mechanisms include (i) transmembrane exchange of salts to balance osmotic pressure through specific membrane transport proteins and (ii) accumulation of protective compatible solutes such as betaine or ectoine. *Halomonas* species are known to accumulate compatible solutes by uptake and/or by synthesis [46]. Comparative genomic analyses have shown that gene clusters *pha* (responsible for PHA synthesis) and *ect* (responsible for ectoine synthesis) are subject to horizontal gene transfer (HGT) events within halophilic species and that the genomic organization of *phaC* (coding for PHA synthase) and *phaP* (coding for phasin) is conserved in *Halomonas elongata* and *Halomonas* sp. TD01 [4]. This conservation suggests that selective pressure is exerted on these genes, which may be partly responsible for the adaptive success and colonization capabilities of *Halomonas* species.

Even though the metabolic diversity of several *Halomonas* species has been described, very few studies have focused on the capacity of these microorganisms to confront various physical, chemical and nutritional conditions. In this study, we report the isolation and physiological characterization of a novel *Halomonas* species, strain RHS90^T, isolated from Mediterranean Sea sediments, which exhibits wide physiological flexibility.

2. Materials and methods

2.1. Bacterial isolation

In October 2008, a sediment core was recovered in the Gulf of Lions (42°41′.596 N, 03°50′.493 E; water depth: 291 m), in the western Mediterranean Sea and subsampled for microbiological analyses, as described elsewhere [5]. A sediment sample from 84 cm below the seafloor was spread on an agar plate composed of modified R2A medium [5] and then incubated at 25 °C. After 10 days of incubation, a beige colony was picked, purified by repeated streaking on marine agar 2216 (MA; Difco) plates and referenced as strain RHS90^T. Stock cultures were stored at −80 °C, in marine broth 2216 (MB, Difco) supplemented with 5% (v/v) DMSO, until characterization.

2.2. Culture conditions

Unless stated otherwise, cultures were carried out aerobically in sterile MB 2216 medium (Difco) aliquoted into 50 mL vials or 10 mL aerobic tubes. Fifty or 25 µL of an overnight preculture were inoculated in 10 mL of MB 2216 medium and then incubated at 30 °C in the dark with shaking at 90 or 100 rpm. All solutions and media used for microbiological experiments were sterile and all reagents used for molecular biology experiments were of molecular biology grade.

2.3. Growth monitoring

Growth of strain RHS90^T was routinely monitored by optical density measurement and ATP assay. The correlation ($n = 81$, $r^2 = 0.92$) between cell counting and optical density was determined by measuring the optical density at 600 nm of cultures diluted at different dilution factors (1/10th, 1/100th, 1/1,000th) with a spectrophotometer (Genesys 20, Thermo Scientific). The same diluted cultures were counted in parallel in a modified Thoma chamber (depth 10 µm, Preciss Europe). The ATP content of cultures was determined with a Kikkoman Lumitester C-110 (Isogen Life Science) using the BacTiter-Glo Microbial Cell Viability assay (Promega) according to the manufacturer's instructions with a few modifications: 75 µL of culture and 75 µL BacTiter-Glo buffer were used; internal calibration was performed with 10 µL of a 100 nM ATP solution and maximal fluorescence emissions values were considered.

2.4. Microscopic observations of PHA inclusions and viability assay

Cells were observed with a phase-contrast light microscope (Olympus BX60) at 40× and 100× magnifications. PHA cytoplasmic inclusions were stained with oxazine dye Nile Blue A following a modified procedure of the Gram-negative viable-colony staining technique of Spiekermann [39]: 0.5 µg Nile Blue A (Sigma) were added per mL of liquid culture medium. After one day of cultivation, cells were observed under ultraviolet light with an epifluorescence microscope (Olympus BX60). *Escherichia coli* CM237T, which does not produce PHA, was used as a negative control. Cell viability and structural integrity of cultures grown under high hydrostatic pressure were determined using the LIVE/DEAD® BacLight Bacterial Viability kit (Invitrogen). A volume of 200 µL culture exposed to 60 MPa hydrostatic pressure for 9 h was stained in the dark for 15 min with 3 µL propidium iodide/SYTO®9 (Invitrogen) and then observed under UV. Scanning electron microscopy (FEI Quanta 200) observations of cultures were done with standard HMDS-based (HexaMethylDiSilasane) preparation. Transmission electron microscopy (Jeol JEM 100 CX II) observations were made after negative staining with uranyl acetate (2% v/v).

2.5. Determination of optimal growth parameters

Determinations of temperature, pH and NaCl ranges for growth were performed in triplicate in 10 mL aerobic tubes incubated with shaking (90 or 100 rpm) in the dark. Growth rates were calculated using linear regression analysis of 5–9

points along the linear portions of the logarithmically transformed growth curves. Determinations of the temperature, NaCl concentration and pH ranges for growth were tested over the range 4–45 °C (4 °C, 10 °C, 16 °C, 22 °C, 30 °C, 37 °C, 40 °C, 43 °C and 45 °C) at pH 7 and with 2% (w/v) NaCl for temperature determination; over the range 0–30% (w/v) NaCl (0%, 0.5%, 2%, 4%, 6%, 8%, 15%, 20% and 30%) at 20 °C and pH 7 for NaCl concentration analysis; and over the range pH 3–11 (3, 3.5, 4, 5, 6, 7, 8, 9, 10 and 11) at 20 °C and with 2% NaCl for pH determination. Exposure to hydrostatic pressure (0.1, 20, 40, 50 and 60 MPa) was done in 0.6 L autoclaves (TopIndustrie, Vaux le Penil, France), in triplicate, at room temperature, with 5 mL syringes containing 3 mL MB medium and 1 mL tetradecafluorohexane (Sigma Aldrich) to facilitate oxygen diffusion.

2.6. Substrate utilization

To investigate the capacity of the strain to catabolize different substrates as sole carbon and energy sources with oxygen as a terminal electron acceptor, the strain was grown in the dark on the mineral basis of MB medium (depleted of all carbon and energy sources) supplemented with one substrate for each test. Carbon utilization tests were performed at concentrations of 1 mM for amino acids, 1 mM for organic acids, 1% (w/v) for alcohols and 10 mM for sugars except for cellulose, D(+)cellobiose, dextrin, D(+)galactose, poly-D(+)galacturonic acid, D(−)fructose, D(+)lactose, pectin and xylan, which were all tested at 1 g L^{-1}. Tween 80 degradation was investigated on Noble agar (Sigma–Aldrich) plates prepared with the mineral basis of MB medium and covered with the substrate (0.75 mM). The ability of the strain to grow anaerobically and to ferment complex organic matter or carbohydrates (yeast-extract 1 g L^{-1}, peptone 5 g L^{-1} and glucose 10 mM) was investigated under an N_2 atmosphere (100% w/v) on an MB mineral basis degassed and reduced with 0.05% (w/v) $Na_2S{\cdot}9H_2O$. The ability of the strain to reduce nitrate, nitrite, sulfate or DMSO was investigated on an MB mineral basis prepared with 10 mM nitrate, 10 mM nitrite, 10 mM sulfate or 10 mM DMSO, respectively, and reduced with 10 µL of $Na_2S{\cdot}9H_2O$ 5% (v/v). *Aminomonas paucivorans* (DSM 12260^T) and *Shewanella profunda* (DSM 15900^T), which are respectively fermentative and nitrate-reducing microorganisms, were used as positive controls for fermentation and nitrate reduction tests. The utilization of amino acids as sole nitrogen sources was tested in artificial sea water with fumarate and D(−)fructose (2 mM each) as carbon sources.

2.7. Growth under oligotrophic conditions

The capacity of strain RHS90^T to grow under oligotrophic conditions was investigated in duplicate with 20 mL of late-exponential phase cultures centrifuged at 6000× *g* for 15 min at 4 °C. Cell pellets were then washed and suspended in 200 mL artificial sea water (pH = 6.8) and stored at 4 °C for 30 days. Cellular density and cellular activity were measured every 3 days by cell counting and by ATP content measurements as described above. To discriminate between hypothetical ATP released after cellular lysis and intracellular ATP representative of cellular activity, the extracellular ATP content was also measured: 1 mL of cells suspended in artificial sea water and stored at 4 °C was filtered onto 0.2 µm syringe filters (Millipore) to retain cells and the total ATP content of the filtrate was measured as described above. The viability of stored cells was further evaluated by inoculation of 50 mL vials containing 10 mL MB 2216 medium with 1 mL of the stored suspension diluted at different factors (1/100th, 1/1,000th, 1/10,000th, 1/100,000th, 1/1,000,000th) and then incubated as described above.

2.8. Metal exposure

Tolerance to metal exposure of the novel isolate was investigated in triplicate in MB medium supplemented with different metals [$AgSO_4$, $CdCl_2$, $CrK(SO_4)_2$, $CuSO_4$, $CoSO_4$, $ZnSO_4$, $MnSO_4$, CsCl] at several concentrations (0.0005, 0.001, 0.005, 0.01 and 0.05 mM for $AgSO_4$; 0.05, 0.2, 0.4, 0.6 and 0.8 mM for $CdCl_2$; 0.5, 0.75, 1, 1.5 and 2 mM for $CrK(SO_4)_2$; 0.5, 1, 1.5, 2 and 2.5 mM for $CuSO_4$; 1, 1.5, 2 and 2.5 mM for $CoSO_4$; 0.5, 1, 1.5, 2, 3 and 4 mM for $ZnSO_4$; 10, 20, 30, 40, 50 and 60 mM for $MnSO_4$; 80, 100, 125, 150 and 200 mM for CsCl). Growth was monitored by ATPmetry after 12–15 h incubation at 30 °C with shaking (100 rpm). Minimal inhibitory concentrations (MICs) of metals were defined by the concentration of metals leading to the same ATP content as the inoculum after 12 h of incubation.

The multiresistant strain *Cupriavidus metallidurans* CH34^T, used as a control, was grown in DSMZ medium *n*°1 (http://www.dsmz.de/microorganisms/medium/pdf/DSMZ_Medium1.pdf) supplemented with different concentrations of metals. Its growth and MIC values were determined as described above.

2.9. Chemotaxonomic analyses

Chemotaxonomic analyses were performed on mid- to late-exponential phases of growth cultures grown for 1 day in MB medium at 30 °C with shaking (100 rpm). The determination of whole-cell fatty acid composition was made by the standard protocol of the Sherlock Microbial Identification System (MIDI Inc., Newark, NJ, USA) and separation of polar lipids was performed by two-dimensional silica gel thin layer chromatography followed by development of total lipids and specific functional groups, as described previously. The analysis of respiratory quinones was carried out by thin-layer chromatography and then HPLC, as described previously [42].

2.10. Susceptibility to antibiotics

Susceptibility to ampicillin, vancomycin, streptomycin, chloramphenicol nitrofurantoin, nalidixic acid, erythromycin, ampicillin (diluted in ethanol), kanamycin, rifampicin (diluted in DMSO), penicillin G and tetracycline was investigated at 10, 30 and 100 ng at 30 °C on MA plates, using the diffusion disc method.

2.11. RMN spectroscopy

Intracellular accumulation of organic compatible solutes was analyzed on cells grown on a rich medium containing 1 g L^{-1} yeast extract and 5 g L^{-1} peptone on a mineral basis of MB medium prepared with or without NaCl. It was studied by ^{13}C NMR spectroscopy on 4 L of culture either with and without NaCl 12.5% (w/v), incubated at 30 °C with shaking. Cells were harvested by centrifugation (6000× *g*, 15 min at 4 °C) in late-exponential growth phase. Cell pellets were suspended in 20 mL RNase-free water mixed with 80 mL absolute ethanol, and then shaken for 2 h at room temperature. These suspensions were then pelleted (15,000× *g*, 20 min at 4 °C) and supernatants were transferred into 50 mL tubes before being dried in a rotary evaporator. One-dimensional ^{13}C NMR spectra were recorded at 25 °C on a BRUKER DRX 300 spectrometer equipped with a 5 mm QNP probehead 1H/13C/31P/19F. NMR analyses were performed on samples dissolved in 700 µL D_2O at 99.96%. The spectra were obtained with BRUKER pulse programs, using standard pulse sequences of 2 s delay, a 30° pulse and 5000 scans. Chemical shifts were expressed in ppm relative to TMS (tetramethylsilane) as an external reference.

2.12. DNA extraction and amplification

Briefly, DNA was extracted after centrifugation (20 min, 10,000× *g* at 4 °C) of 10 and 20 mL of mid-log phase culture. The pellet was suspended in 1 mL buffer (Tris 100 mM-pH8, EDTA 50 mM-pH8, NaCl 100 mM) and cellular lysis was achieved with 50 µL sarkosyl 20%, 100 µL SDS 10% and 20 µL proteinase K at 20 mg/mL (1 h, 55 °C). One mL phenol/chloroform/isomaylic acid (25/24/1; Sigma) was added and gently mixed with the lysis buffer. After centrifugation (10,000× *g*, 15 min at 4 °C), the aqueous phase was gently mixed with 1 mL chloroform (Carlo Erba) and centrifuged (10,000× *g*, 15 min at 4 °C). The aqueous phase was then transferred, mixed with 400 µL of sodium acetate (3 M, pH = 5.2) and a 0.8 volume of isopropanol. DNA pellet was precipitated 30 min at −20 °C, centrifuged (15,000× *g*, 10 min at 4 °C), dried and finally resuspended in 50 µL DEPC water. Amplification by polymerase chain reaction (PCR) was performed with Go*Taq*® Flexi DNA polymerase (Promega), following the manufacturer's instructions. The 16S rRNA gene was amplified with the Bac8F and Bac1492R primers [7] using the following protocol: 3 min at 95 °C; 30 cycles of 1 min at 95 °C, 1 min 30 s at 52 °C and 2 min at 72 °C; 6 min at 72 °C. The amplification of genes encoding ectoine synthase (*ectC*), PHA synthase (*phaC*) and phasin (*phaP*) was performed using degenerated oligonucleotide primers (Eurogentec) designed with *H. elongata* sequences as references: ectc_R_141 (TAC−CGA−GAC−SCA−YAT−CCA−YT), ectc_F_7 (GTT−CGC−AAB−MTB−GAA−GAA−GC), phaC_F_767 (CGC−CCT−GGA−TCA−ACA−AGT−AT), phaC_R_998 (CCG−ACA−CAG−TAG−CTC−AGC−AG), phaC_F_727 (AGC−ACC−GAG−AAG−GTC−TTC−AA), phaC_R_1037 (CTG−GTC−AGG−TAG−GCC−ACT−GT), phaP_F_69 (CAA−TGC−CTT−GAT−GCT−GGA−C), phaP_R_251 (AGC−ATR−TGS−TTG−GAC−AGC−TC). The program used for PCR amplification was the same as that described above except that the hybridization temperatures were 60 °C, 64 °C and 62 °C for gene *ectC*, *phaP* and *phaC*, respectively.

2.13. Genotypic and phylogenetic analyses

DNA−DNA hybridization experiments were performed by the Identification Service of the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany), with *Halomonas axialensis* (DSM-15723) and *Halomonas meridiana* (DSM-5425), using a Cary 100 Bio UV/VIS-spectrophotometer.

Blast-based research of most similar 16S rRNA sequences was done against the GenBank database and against the web-based EzTaxon-e server [19]. Phylogenetic analyses were done with SeaView4 [12] using the Muscle Multiple Alignment option to align sequences. Sequences of the nearest neighbors used to perform the alignment were imported from the Ribosomal Database Project (RDP) website (http://rdp.cme.msu.edu/). Phylogenetic trees were constructed using SeaView4 software, on the basis of Neighbor Joining and PhyML (GTR model) algorithms. The robustness of the inferred topologies was assessed by bootstrap analyses based on 1000 replications. The 19 nucleotidic signatures of the family *Halomonadaceae* [8] were manually investigated with SeaView4 using the *E. coli* 16S rRNA gene as reference numbering (Accession number NR_102804). The 16S rRNA gene sequence of *Halomonas lionensis* RHS90^T was deposited in the GenBank/EMBL/DDBJ databases under the accession number HE661586.

The genomic DNA G + C content of the isolate was determined by the Identification Service of the DSMZ, by HPLC analysis.

3. Results and discussion

3.1. Genotypic and phylogenetic analyses

Based on a BLASTN search against GenBank and the EzTaxon-e Server, the 16S rRNA gene of strain RHS90^T shared highest sequence similarity with *H. axialensis* (97.96%), *H. meridiana* (98.03%) and *Halomonas aquamarina* (97.89%). The 19 nucleotidic signatures of the family *Halomonadaceae* defined by [8] were also all found in RHS90^T 16S rRNA gene. Phylogenetic analyses performed with this gene confirmed these results, positioning the novel isolate RHS90^T close to *H. axialensis* and *H. meridiana*, within the genus *Halomonas*, in the family *Halomonadaceae*, class *Gammaproteobacteria* (Fig. 1).

To further determine whether or not strain RHS90^T represents a novel species, DNA−DNA hybridizations were performed with the two closest relatives. Levels of DNA−DNA relatedness with *H. axialensis* and *H. meridiana* were 57.1% and 62.4%, respectively, and were therefore

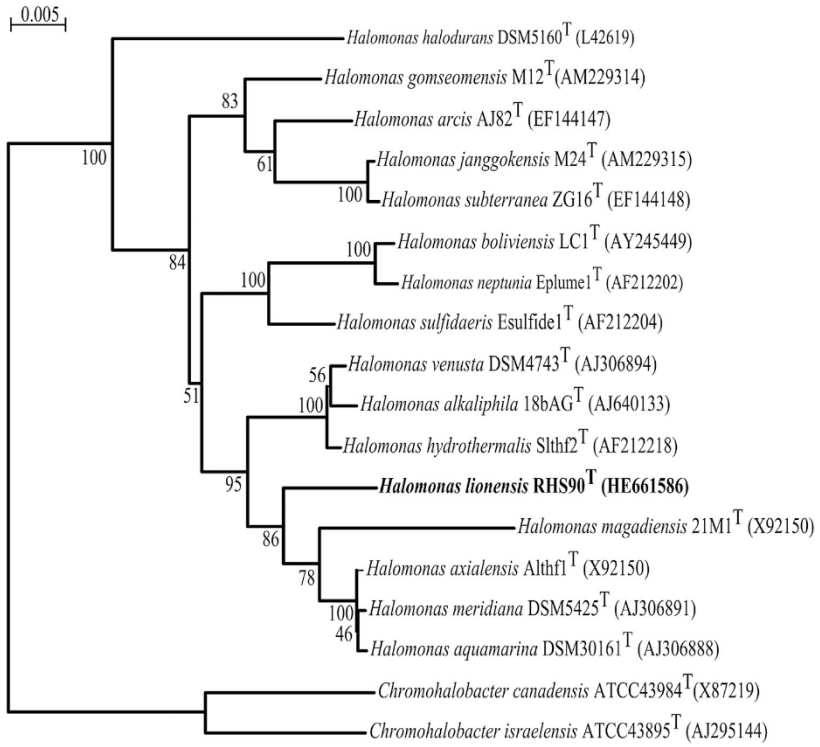

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequences showing the relationships between *Halomonas lionensis* RHS90^T and its related phylogenetic neighbors. The topology shown was calculated with the neighbor-joining algorithm. Accession numbers are indicated in brackets. Bootstrap values (%) are indicated at the branch nodes and were calculated from 1000 resampled datasets. *Chromohalobacter canadensis* and *Chromohalobacter israelensis* were used as outgroups.

below the threshold value of 70% for species delineation [44], indicating that the novel isolate was likely a novel *Halomonas* species.

3.2. Morphology

Cells were rod-shaped, with a size of 4.4–2.2 × 0.8–0.6 μm (n = 30). They were motile. This motility feature is characteristic of the genus *Halomonas* as the vast majority of *Halomonas* species are flagellated.

3.3. Physiological characteristics

Strain RHS90^T is mesophilic and moderately alkaliphilic, since its optimal temperature is 30 °C (upper limit 45 °C and positive growth at 4 °C, the minimal tested temperature) and has a pH range from 6 to 10 (optimum 7–9). It has a euryhaline phenotype, growing at NaCl concentrations from 0 to 20% NaCl (w/v) with a wide optimum of 2–8%. The strain was shown to be a heterotrophic and obligate aerobic bacterium. It was able to use the following substrates as sole energy and carbon sources, with O_2 as a terminal acceptor: the carbohydrates D(−)fructose, D(−)ribose, sucrose, D(+) galacturonate, pectin, D(−)trehalose, N-acetylglucosamine, and xylan; the alcohols glycerol and mannitol; the organic acids propionate, fumarate and succinate; the amino acids L-alanine, L-arginine, L-asparagine, L-glutamine, L-glutamate, L-methionine, L-proline, L-serine, L-valine, L-cysteine, L-glycine, L-leucine and L-aspartate; and creatine. The strain did not use nitrate and nitrite as terminal electron acceptors with lactate or acetate as the carbon source, which is in agreement with the fact that no amplification of the *nirK* and the *nirS* genes could be obtained. It respired neither sulfate nor DMSO. Growth was not observed under fermentative conditions. No amino acid could be used as a sole nitrogen source. Its metabolic versatility regarding carbon and energy sources may allow the strain to use refractory organic matter and detrital macromolecules such as proteins, polypeptides and polysaccharides from dead marine organisms, that may become available to them. Strain RHS90^T presents a distinctive carbon source utilization profile compared with its closest relatives (Table 1): it cannot, for example, use glucose or ethanol as its sole carbon source, whereas *H. axialensis*, *H. meridiana* and *H. aquamarina* are able to use these compounds. On the contrary, strain RHS90^T is able to grow on a minimal medium with D(−) ribose, while its closest relatives cannot.

Table 1
Phenotypic characteristics that differentiate strain RHS90^T from related species of the genus *Halomonas*.

Characteristic	1	2	3	4
Isolation source	Mediterranean Sea sediments	Temperate ocean	Cold hypersaline lake	Low temperature hydrothermal fluid
Motility	Y	Y	Y	Y
Size (μm)	4.4–2.2 × 0.8–0.6	4–6 × 0.4–0.6	1.9–4.5 × 0.6–1.0	ND
Temperature range (opt)	≤4–45 (30)	5–40 (20–25)	−5 – 45 (28–40)	−1 – 35 (30)
pH range (opt)	6–10 (7–9)	5–10	5–10	5–12
NaCl range %w:v (opt)	0–20 (2–8)	0–20 (7.5–10)	0.01–2 5 (1–3)	0.5–24 (4)
Hydrolysis of:				
Tween 80	−	+	+	−
Growth with:				
L(+)Arabinose	−	+	−	+
D(−)Fructose	+	−	−	+
D(+)Galactose	−	−	+	−
D(+)Glucose	−	+	+	+
D(+)Lactose	−	+	−	−
D(+)Maltose	−	−	+	+
D(−)Ribose	+	−	−	−
Citrate	−	+	−	−
Lactate	−	+	+	−
Malonate	−	+	−	−
Propionate	+	+	−	−
Succinate	+	+	+	−
Ethanol	−	+	+	+
Glycerol	+	+	+	−
Mannitol	+	+	−	−
L-Alanine	+	+	+	−
L-Arginine	+	−	ND	ND
L-Asparagine	+	−	ND	−
L-Glutamine	+	−	ND	ND
L-Glutamate	+	−	−	+
Lysine	−	+	+	−
Proline	+	−	+	−
Serine	+	−	+	−
Valine	+	−	−	−
DNA G + C content (mol %)	54.4	57–58	58.2–59.9	54.4

Taxa: 1, strain RHS90^T (this study); 2, *H. aquamarina* [1,14,15]; 3, *H. meridiana* [1,14,15]; 4, *H. axialensis* [14,15]; +, Positive; −, Negative; ND, no data available; Y, Yes.

Similarly to numerous other *Halomonas* species, this euryhaline strain, isolated from a marine sediment with an interstitial water salinity of 4% (w/v) [5], was able to grow under strict halophilic conditions. Indeed, it was shown to be able to grow at concentrations from 0 to 20% NaCl and its upper and optimal salinities (2–8%) for growth were higher than the values generally accepted to discriminate halophilic from halotolerant microorganisms (optimum NaCl concentration ≥5%; upper NaCl concentration ≥10%) [30]. Strain RHS90^T was also able to grow under high hydrostatic pressure. Its growth rate was optimal at atmospheric pressure, but was slightly affected by an increase in hydrostatic pressure up to 40 MPa (Fig. 2). Above 40 MPa, its growth rate decreased sharply. When grown under 50 or 60 MPa, the growth rate of the novel isolate was about one-fifth of its growth rate under atmospheric pressure, but microscopic observations confirmed that cells were still dividing. However, these cells were non-motile and exhibited atypical elongated cellular shapes. LIVE/DEAD® staining of cells exposed to high pressure demonstrated that cells remained intact and that membranes

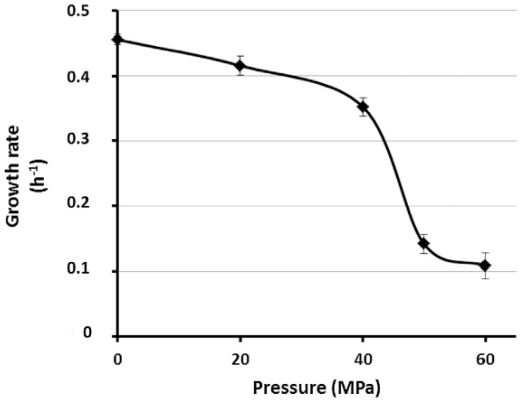

Fig. 2. Effects of hydrostatic pressure on the growth rate of strain RHS90^T. Bars indicate standard deviation (n = 3).

were not permeabilized (Fig. S1). Since its growth rate is higher under atmospheric pressure, this strain can be considered as piezotolerant. Even though *H. meridiana* has already been reported to be capable of growing under 55 MPa [14], this is the first time that growth of a *Halomonas* species has been described under 60 MPa. Piezotolerant strains have already been described among *Gammaproteobacteria* and *Halomonas* species. For example, enrichment cultures under high pressure have already been performed and efficient growth of *Halomonas*-related organisms has been described under 30 MPa [40]. As the pressure of 60 MPa is much higher than the pressure measured in situ, it can be hypothesized that strain RHS90^T would be capable of growing in deeper environments, at 6000 m depth where hydrostatic pressure reaches this level of high pressure. The effects of hydrostatic pressure have already been studied in *H. axialensis*, *H. meridiana* and *Halomonas hydrothermalis*, showing a change in membrane lipid composition and in the protein expression level [14]. These properties may explain the fact that several *Halomonas* species have also been isolated from deep marine environments [15,38].

Antibiotics have many roles in natural environments, shaping microbial physiology such as motility or biofilm formation at low concentrations [33]. Considering these multiple effects, we considered it would be interesting to find out whether the novel isolate was resistant to antibiotics. The strain presented variable sensitivities towards different antibiotics. On solid medium, it was sensitive to nalidixic acid, chloramphenicol, ampicillin, rifampicin and penicillin G at 10 ng, to streptomycin, kanamycin and tetracycline at 30 ng and to vancomycin at 100 ng. The strain was resistant to nitrofurantoin, erythromycin and ampicillin at 100 ng. This variability in antibiotic sensitivities of strain RHS90^T may reflect complex cellular communication mediated by diffusive secondary metabolites within natural communities.

3.4. Fatty acids, polar lipids and quinone composition

The main fatty acid component of strain RHS90^T was $C_{18:1}$ $\omega7c$(48.6%). The fatty acids $C_{16:1}$ $\omega7c$/$C_{15:0}$ iso-2-OH (13%), $C_{16:00}$ (11.9%),$C_{19:0}$ cyclo $\omega8c$(9.3%), $C_{12:0}$ 3-OH (6.3%) and $C_{17:0}$ cyclo (4.3%) were also present in significant proportions (Table S1). The polar lipid pattern indicated the presence of phosphatidylglycerol, diphosphatidylglycerol, phosphatidylethanolamine, one phosphoglycolipid, two glycolipids and two phospholipids (Fig. S2). The major respiratory quinone was ubiquinone 9 (90%), which is the typical dominant quinone in *Halomonas* species. In a previous study [11], showed that there was no clear distinction between the genera *Halomonas* and *Deleya*, 2 genera of the family *Halomonadaceae*, on the sole basis of respiratory quinones, polar lipids and fatty acid composition. All species of these genera were described as containing $C_{16:1}$ cis 9, $C_{16:0}$, $C_{17:0}$ cyclo, $C_{18:1}$ and $C_{19:0}$ $cyclo_{11-12}$ as major fatty acid components. Interestingly, $C_{16:1}$ cis 9 and $C_{19:0}$ $cyclo_{11-12}$ were not detected in strain RHS90^T, although a $C_{19:0}$ cyclo $\omega8c$ fatty acid represented a significant proportion.

3.5. Tolerance to metals

Metals can have beneficial or deleterious effects on cells, mainly depending on which metal is considered and at what concentration. Metals become toxic for a cell when they disturb molecular and cellular functions and structures. In environments such as polluted sites or hydrothermal vents, metals can be present at high concentrations and can diffuse more rapidly into cells. Over their evolution, cells have developed strategies to overcome these problems [28] and the tree of life contains microorganisms with a range of metal sensitivities that are more or less adapted to metal-rich environments. To investigate the capacity of strain RHS90^T to grow in the presence of metals, the MICs of strain RHS90^T were determined for 9 metals and compared with the MICs of *C. metallidurans* strain CH34^T (determined in this study), a highly metal-resistant bacterium [23], and to MICs of the model bacterium *E. coli* strain CM237^T determined by [24] (Table 2). The novel isolate was highly sensitive to Ag (MIC: 0.01 mM) and Cd (MIC: 0.75 mM), which inhibited its growth at very low concentrations, but grew very well at high concentrations of Cs (MIC: 200 mM). Strain RHS90^T was also particularly resistant to Mn (MIC: 60 mM). Metal MIC values of strain RHS90^T differed substantially from those of *C. metallidurans* CH34^T and from those of *E. coli* CM237^T. They were higher overall than those of *E. coli* CM237^T, but lower than those of *C. metallidurans*. This trend was observed after exposure to Cd, Cu, Co, Ni and Cs. However, strain RHS90^T had higher MIC for Mn than *C. metallidurans* CH34^T and *E. coli* CM237^T, higher and lower MIC values for Ag than *C. metallidurans* and *E. coli* CM237^T respectively, and was as resistant to Cr as *C. metallidurans*.

Strain RHS90^T might possess specific mechanisms to detoxify cells of an excess of metals. In *C. metallidurans* str. CH34^T, it has been shown that metal tolerance is conferred by different plasmid encoded-systems such as the czc

Table 2
Comparison of minimal inhibitory concentrations (MIC) of different metals for *H. lionensis* strain RHS90^T, *C. metallidurans* CH34^T and *E. coli* CM237^T.

	$AgSO_4$	$CdCl_2$	$CrK(SO_4)_2$	$CuSO_4$	$CoSO_4$	$NiCl_2$	$ZnSO_4$	$MnSO_4$	CsCl
Halomonas lionensis RHS90^T	0.01	0.75	1.75	2	3	8	3	60	200
Cupriavidus metallidurans CH34^T	0.0005	8	1.75	3	35	13	12	30	250
E. coli strain CM237^T[a]	0.02	0.5	0.2	1	1	1	1	20	50

Values are expressed in mM.
[a] The MIC values of *E. coli* strain CM237^T correspond to those previously determined by Ref. [25].

(cobalt–zinc–cadmium) or cnr (cobalt–nickel) tolerance systems [25,27]. These highly regulated systems involve the sensing of metals and gene expression activation in order to release metals into the extracellular medium through efflux pumps. Many *Halomonas* species have been reported to harbor plasmids of ~600 Mbp and ~70 Mbp, as well as other extrachromosomal elements [2]. These plasmids could be responsible for some of the adaptive advantages in the genus *Halomonas*, including tolerance to metals. Interestingly, plasmid extraction could be performed on cells of strain RHS90^T, revealing one or several plasmids >10 kbp (data not shown) that might possibly be involved in metal tolerance. CMI values of strain RHS90^T were higher (Ag, Cu, Cd) or comparable (Co and Cr) to those previously determined for *H. elongata* and *Halomonas subglaciescola* [29], two organisms harboring ~600 kbp and ~70 kbp plasmids [2].

3.6. Growth under oligotrophic conditions

The isolation of *Halomonas* species and detection of *Halomonas*-related sequences from oligotrophic environments have been extensively described. For example, sediments from the Arctic and Antarctic seas, Mediterranean sea, deep-sea waters or deep-sea bed [9,16] have been shown to harbor representatives of the genus *Halomonas*. This widespread representation of *Halomonas* species in nutrient-depleted habitats raises questions about their adaptation to oligotrophic conditions and may reflect a strong capability to thrive in such conditions. In order to ascertain whether strain RHS90^T can survive in extremely nutrient-depleted environments, the strain was stored for 4 weeks in artificial sea water without any carbon source at 4 °C (Fig. 3). During this storage period, cellular density remained constant (~8.10^6 cells mL^{-1}). The viability of counted cells was demonstrated by the positive growth of starved cultures when these were transferred to nutrient-rich media (MB2216) inoculated with the stored cell suspension diluted from the 1/100th to the 1/100,000th. Total ATP content (with 85–97% representing intracellular ATP) determination showed that cellular activity remained relatively constant after 15 days storage and dropped off sharply after 25 day storage. This can be explained by (i) a decrease in metabolic activity and/or (ii) a decrease in cell size, as was microscopically observed (data not shown). These results show that strain RHS90^T remained viable and maintained its population size under extremely oligotrophic conditions and at low temperatures over a period of one month.

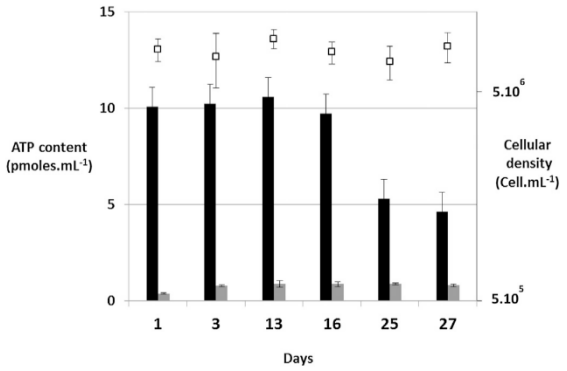

Fig. 3. Cellular activity, as determined by total ATP content and cellular density of cells of strain RHS90^T stored in carbon source-depleted artificial sea water. The total and extracellular ATP contents of artificial sea water are represented by black and gray bars, respectively. Cellular densities determined by cell counts are shown by white squares.

3.7. Amplification of PHA synthesis genes

Many prokaryotes respond to starvation or to imbalanced ratios between carbon and nitrogen through the accumulation of carbon substrates in the form of polyhydroxyalkanoate (PHA) granules. PHA metabolism relies mostly on PHA synthase (*phaC*), PHA depolymerase (*phaZ*) and phasin, a protein associated with PHA granule inclusions [22]. PHA granules are synthesized by *phaC* when carbon sources are abundant and used under starvation. The phasin gene is generally located upstream of *phaC* and this genomic organization is conserved in many *Proteobacteria* [4]. It is likely that PHA accumulation confers a strong adaptive advantage in natural environments where carbon source concentrations fluctuate. To determine whether strain RHS90^T had the genetic potential to synthesize PHA granules, PCR amplifications of the *phaC* gene were performed on DNA extracts. A single stretch of 234 nucleotides was obtained with the primer pairs phaCF998–phaCR767. Sequence comparison showed that this sequence was highly similar to poly(R)-hydroxyalkanoic acid synthase of some other *Halomonas* species. The highest similarity (96% identity) was shared with *Halomonas* sp. HAL1, *Halomonas* sp. GFAJ and *Halomonas* sp. TD01, isolated from a gold mine and from two salt lakes (California, USA and Xinjiang, China), respectively [18,21,41]. This suggests that the genome of strain RHS90^T encodes a PHA synthase gene. Four classes of PHA synthases have been described, differing in subunit numbers and product chain-lengths [4]. Phylogenetic reconstruction demonstrated that this sequence belongs to class I of *phaC* genes (Fig. S3). Class I *phaC* comprises enzymes with one subunit that synthesizes short chains (3–5 carbon atoms) and medium chains (6–14 carbon atoms). Interestingly, other *phaC* genes belonging to class I have been sequenced in *Halomonas* sp. TD01 and *H. elongata* [4].

Intracytoplasmic granules of PHA were observed by microscopy after Nile Blue A staining, suggesting that the amplified *phaC* gene is functional and allows the synthesis of PHA.

3.8. Production of ectoine

Compatible solutes such as ectoine, hydroxyectoine, betaine or glutamate are commonly produced by halophilic microorganisms to adapt to osmotic pressure caused by high extracellular salt concentrations. These compatible solutes prevent molecular and cellular structures from dehydration or

freezing [46]. In order to discover whether such compatible solutes are produced by strain RHS90^T, the metabolites of cells grown in MB without or with 12.5% NaCl were analyzed with nuclear magnetic resonance (NMR) spectroscopy (Fig. S4).

When grown in rich medium without NaCl, cells did not accumulate ectoine (Fig. S4A). On the contrary, when cells were grown in rich medium supplied with 12.5% NaCl, peaks attributed to ectoine, glycine betaine and glutamate were detected and represented the vast majority of metabolites accumulated (Fig. S4B). These results demonstrate that ectoine is accumulated by biosynthesis under our hypersaline growth conditions and suggest that the genome of strain RHS90^T carries the ectoine biosynthetic pathway genes. Similar results were previously obtained with *Halomonas pantelleriense*. Ectoine was indeed the most abundant compatible solute detected in *H. pantelleriense* when grown in rich medium, and hydroxyectoine, betaine, glycine and glutamate were also detected [36]. The proportion of ectoine increased with increasing NaCl concentration. This phenomenon was observed in rich (yeast-extract) medium but appeared less pronounced in minimal (glucose) medium [36]. In another study [46], showed that the presence of ectoine or hydroxyectoine increased the cellular growth of the halophile *Halomonas ventosae* DL7^T after both thermal and osmotic stresses.

In order to confirm that an ectoine synthase (*ectC*) encoding gene is indeed borne by the genome of strain RHS90^T, PCR amplifications were performed. Unfortunately, no positive amplification could be obtained. This lack of amplification may be attributed to the use of a non-specific primer, since primer sequences were determined on the basis of the *H. elongata ectC* gene sequence (NCBI Accession number: YP_003897659). The corresponding regions might not be conserved in strain RHS90^T, thus leading to mismatches.

In conclusion, this study demonstrates the physiological plasticity of strain RHS90^T. From the results of polyphasic taxonomic analysis and based on genetic, physiological and chemotaxonomic distinctness, it is proposed that strain RHS90^T be considered a novel species within the genus *Halomonas*, for which the name *H. lionensis* is proposed. This novel species presents interesting growth features, especially in terms of salinity, metal concentration and hydrostatic pressure tolerance. It has developed adaptive mechanisms based notably on PHA and ectoine accumulation, to overcome extreme environmental conditions. This flexibility might allow strain RHS90^T to colonize environments associated with a variety of environmental conditions and may be related to the ecological success and the ubiquitous presence of *Halomonas* species in natural settings. More studies focusing on the adaptive mechanisms are needed to fully understand the interaction of *Halomonas* species with their natural biogeochemical environments. It would be, for instance, relevant to undertake comparative genomics studies within the genus *Halomonas*, notably to investigate the role of plasmids in the ecological success of this genus.

3.8.1. Description of H. lionensis *sp. nov*

(li.on.en'sis. N.L. fem. adj. lionensis, of or belonging to *Golfe du Lion* [Gulf of Lions], in reference to the origin of the type strain).

Cells are Gram-negative, rod-shaped, motile, 0.7–2.5 μm in length × 0.4–1 μm in width. Colonies on MA are white, regularly circular, convex, translucent, smooth with an entire edge, creamy and do not produce exopolysaccharides. Grows aerobically at ≤4–45 °C with an optimum at 30 °C, pH 6–10 with an optimum at 7–9 and with NaCl concentrations ranging from 0 to 20% (w/v) with an optimum at 2–8%. Negative for nitrate and nitrite reduction, fermentation of peptone or yeast extract, Voges–Proskauer test and methyl red test, indole formation, β-galactosidase (ONPG), arginine dihydrolase, gelatinase, β-glucosidase, lysine decarboxylase, ornithine decarboxylase, tryptophane deaminase, potassium gluconate assimilation, capric acid assimilation, adipic acid assimilation. Positive for urease, oxidase and catalase. The following substrates can be used as a sole carbon source: citrate, fumarate, propionate, succinate, glycerol, D-mannitol, pectin, xylan, D(−)fructose, poly-D-(+)galacturonic acid, N-acetylglucosamine, D(+)mannose, D(+)rhamnose, D(−)ribose, sucrose, D(−)trehalose, L-alanine L-arginine, L-asparagine, L-glutamate L-glutamine, L-glycine, L-leucine L-proline, L-serine, L-valine, creatine. The following substrates cannot be used as sole carbon source: collagen, elastine, keratine, tween 80, acetate, ascorbate, benzoate, betain, caprylate, citrate, formate, gluconate, hippurate, lactate, malate, malonate, tartrate, *myo*-inositol, ethanol, isopropanol, sorbitol, D-melezitose, threalose, L(+)arabinose, cellulose, dextrine, D(+)cellobiose, D(+)glucose, D(+)galactose, D(+)lactose, D(+)maltose, D(+)xylose, L-aspartate, L-cysteine, L-glycine, L-histidine, L-isoleucine, L-lysine, L-methionine, L-ornithine, L-phenylalanine, L-threonine, L-tryptophane, L-tyrosine and L-valine. None of the 20 proteic amino acids can be used as a sole nitrogen source.

The main fatty acids are C_{16} (11.85%), $C_{17:00}$ cyclo (4.32%), $C_{19:0}$ Cyclo $\omega 8c$ (9.32%), $C_{18:1}$ $\omega 7c$ (48.6%), 3-OH $C_{12:0}$ (6.25%) and $C_{16:1}$ $\omega 7c$ and/or 2-OH iso-$C_{15:0}$ (13%). The main polar lipids are diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, phosphatidylcholine and an unidentified phosphoglycolipid. Ubiquinone 9 (Q-9) is the major quinone (90%). The DNA G + C content is 54.4 mol %.

The type strain RHS90^T (DSM 25632^T, CIP 110370^T, UBOCC3186) was isolated from surficial sediments (84 cm below the seafloor) of the Gulf of Lions, in the western Mediterranean Sea.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.resmic.2014.07.009.

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Abstrcact

Deep-sea hydrothermal vents are among the most biologically active regions, and support highly productive ecosystems fueled by chemosynthesis. Although our knowledge of the diversity and roles of hydrothermal vents isolates has remarkably expanded, extensive investigation of the microbiology and physiology remain imperative for several reasons, especially in the poorly-documented hydrothermal vents of the Indian Ocean. In this era of omics, the cultivation of microorganisms remains crucial in several respects. Estimations indicate that more than 99% of all existing prokaryotes have resisted cultivation in the laboratory, limiting the study of their physiology and ecological role. The cultivation makes it possible to perform a direct and easy study of the microbial morphology, physiology, genetics and pathogenicity. From an ecological point of view, it is very helpful to have isolates to integrate at the cell level ecological data got via top-down (meta-omics, rate measurements, etc.) and bottom-up (single-cell techniques, metabolomics, transcriptomics, etc.) approaches. Finally, microbial isolates allow testing hypotheses that arise from (meta-)genomic data. Sulfate/sulfur-reducing prokaryotes, ubiquitous in anoxic habitats, play an important role in both the sulfur and carbon cycles. These cycles are particularly important at deep-sea hydrothermal vents and our study focused aimed at cultivating novel taxa involved in these cycles from the poorly documented Indian Ocean.

Six anaerobic prokaryotes were isolated from deep-sea hydrothermal samples from the Indian Ocean. For taxonomic study of 3 novel strains, 16S rRNA gene phylogenetic analysis, phenotypic and chemotaxonomic characterizations were carried out. The genomes of these three isolates were sequenced and annotated.

The first characterized isolate was strain J2^T, a novel sulfate-reducing bacterium. It was isolated from a serpentinized peridotite sample collected at a depth of 3173 m. Phylogenetic analysis based on 16S rRNA gene sequences showed that strain J2^T falls into the genus *Desulfovibrio* within the class *Deltaproteobacteria*, with highest sequence similarity of 98.05% to *Desulfovibrio dechloracetivorans* SF3^T. The combined genotypic and phenotypic data showed that strain J2^T represents a novel species of the genus *Desulfovibrio*, for which the name *Desulfovibrio indicus* sp. nov. was proposed.

The second characterized isolate was a novel sulfur-reducing bacterium, strain K6013^T. It was isolated from a sulfide sample collected at a depth of 2771 m from a high-temperature hydrothermal vent. Phylogenetic 16S rRNA gene sequence analyses showed that strain K6013^T falls into the genus *Desulfurobacterium* within the class *Aquificae*, with highest sequence similarity of 96.93% to *Desulfurobacterium atlanticum* SL22^T. On the basis of genotypic and phenotypic data, strain K6013^T is considered to represent a novel species of the genus *Desulfurobacterium*, for which the name *Desulfurobacterium indicum* sp. nov. is proposed.

Another characterized isolate was a novel sulfate-reducing bacterium, strain S606^T. It was isolated from a sulfide sample collected at a depth of 2764 m from a deep-sea chimney wall in the Indian Ocean. Phylogenetic analysis based on 16S rRNA gene sequences showed that strain S606^T falls into the genus *Thermodesulfatator* within the class *Thermodesulfobacteria*, with highest sequence similarity of 98.15% to *Thermodesulfatator indicus* DSM 15286^T. The combined genotypic and phenotypic data show that strain S606^T represents a novel species of the genus *Thermodesulfatator*, for which the name *Thermodesulfatator autotrophica* sp. nov. was proposed.

Finally, experiments aimed at trying to design a genetic tool with the archaea *Paleococcus pacificus* and another part aimed at testing the response of a deep-sea microbial isolates to hydrostatic pressure.