Lentibacter algarum gen. nov., sp. nov., isolated from coastal water during a massive green algae bloom

Zhao Li, Zhe Qu, Xiuming Zhang and Xiao-Hua Zhang

College of Marine Life Sciences, Ocean University of China, Qingdao, PR China

Correspondence

Xiao-Hua Zhang

xhzhang@ouc.edu.cn

Two novel Gram-stain-negative, aerobic strains (ZXM100^T and ZXM098) were isolated from seawater in the coastal region of Qingdao (36.027° N 120.184° E), China, during a massive green algae bloom. Cells were ovoid to irregular short rods and lacked flagella. Poly- β -hydroxybutyrate was accumulated. Cells did not contain bacteriochlorophyll *a*. The isolates grew at NaCl concentrations of 3–9‰ and 22–28 °C. C_{18:1} ω 7c, C_{18:0} and C_{16:0} were the major fatty acid components. The polar lipids of ZXM100^T were phosphatidylglycerol, phosphatidylethanolamine, phosphatidylcholine, a single lipid and an unidentified aminolipid. The predominant isoprenoid quinone of ZXM100^T was Q-10. Phylogenetic analysis based on 16S rRNA gene sequences showed that strains belonged to the family *Rhodobacteraceae*. Based on the results obtained in this study, strain ZXM100^T was considered to represent a novel species in a new genus, for which the name *Lentibacter algarum* gen. nov., sp. nov. is proposed. The type strain is ZXM100^T (=LMG 24861^T=CGMCC 1.10234^T); the DNA G+C content of the type strain is 54.6 mol%.

Members of the family *Rhodobacteraceae*, classified within the class *Alphaproteobacteria*, are exclusively marine or hypersaline, with characterized isolates demonstrating salt tolerance (Buchan *et al.*, 2005); at the time of writing, the family contained 84 phenotypically, metabolically and ecologically diverse genera. Their global distribution seems to be related to oceanic water masses and their environmental and biogeochemical properties (Gao *et al.*, 2006). Two novel bacterial strains, designated ZXM100^T and ZXM098, were isolated from seawater samples in the coastal region of Qingdao, China, during a massive green algae bloom. The aim of the present study was to determine the exact taxonomic position of strains ZXM100^T and ZXM098 by a polyphasic taxonomic approach.

A seawater sample was collected from the coastal region of Qingdao (36.027° N 120.184° E), China in June 2008, during a massive green algae (*Enteromorpha prolifera*) bloom. The sample was diluted with sterile saline and spread onto marine agar 2216 (MA; Difco) plates incubated at 28 °C for 7 days. Strains ZXM100^T and ZXM098 were purified by streaking three times on MA. Cultures were maintained on MA plates and stocks were kept in sterile

0.9% (w/v) saline supplemented with 15% (v/v) glycerol at –80 °C. *Sulfitobacter pontiacus* DSM 10014^T obtained from the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany) was used as the reference strain and was cultured under the same conditions as ZXM100^T and ZXM098 [MA or marine broth 2216 (MB) at 28 °C], unless otherwise specified.

Cell morphology was determined by transmission electron microscopy (JEOL; JEM-1200EX) after cells had been negatively stained with 1% (w/v) phosphotungstic acid. Gram and endospore staining were carried out using the standard method (Beveridge *et al.*, 2007). The presence of poly- β -hydroxybutyrate (PHB) was assessed using Nile Blue A stain under a fluorescence microscope (BH2; Olympus) (Tindall *et al.*, 2007).

Growth on nutrient agar (NA) and tryptone soy agar (TSA; Difco) supplemented with 3% (w/v) NaCl was tested for 7 days at 28 °C. Growth under anaerobic conditions was determined after incubation in a dry-seal glass vacuum desiccator on MA for 3 days at 28 °C, with 10 g gallic acid and 100 ml 10% NaOH to consume oxygen. Halotolerance was investigated using synthetic marine ZoBell broth [5 g Bacto peptone, 1 g yeast extract and 0.1 g FePO₄ in 1 l modified artificial seawater (Lyman & Fleming, 1940)] supplied with various concentrations of NaCl (0–15%, w/v, in increments of 1%); all Na⁺ was replaced by the appropriate K⁺ in the modified artificial seawater. The temperature range for growth was determined on MA plates incubated at 0–37 °C (0, 4, 16, 22, 28 and 37 °C) for 20 days. The pH range for growth was

Abbreviations: Bchl, bacteriochlorophyll; PHB, poly- β -hydroxybutyrate.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of *Lentibacter algarum* ZXM100^T and ZXM098 are FJ436732 and FJ436731, respectively.

Three supplementary figures and a supplementary table are available with the online version of this paper.

tested at pH 2–10 (in steps of 1 pH unit) in MB with citrate/phosphate buffer (pH 2.0–7.0), Tris/HCl (pH 8.0–9.0) (Breznak & Costilow, 1994) or sodium carbonate/sodium bicarbonate (pH 10.0). Susceptibility to antibiotics was investigated on MA plates by using discs (Hangzhou Microbiology Reagent) containing different antibiotics.

Standard protocols (Tindall *et al.*, 2007) were used to assess catalase (method 2) and oxidase activities, degradation of casein, starch, gelatin (method 2) and Tween 80 (method 2), nitrate reduction (method 1) and H₂S production from thiosulfate (method 1). The ability to hydrolyse alginate was tested as described by Smibert & Krieg (1994). The ability to oxidize sulfite was tested as described by Pukall *et al.* (1999). Bacteriochlorophyll (Bchl) *a* of cells was determined in acetone/methanol (7:2, v/v) extracts using a TU-1810 spectrophotometer (Pgeneral) as described previously (Martens *et al.*, 2006). Activities of constitutive enzymes, substrate oxidation, carbon source utilization and other physiological properties were determined by using the API 20E, API 20NE, API 50CH, API ZYM strips (bioMérieux) and Gram-negative MicroPlates (Biolog), according to the manufacturer's instructions, except that sterile 3 % (w/v) NaCl was used to prepare the inocula. For Biolog MicroPlates, seawater was used instead of 3 % (w/v) NaCl.

The detailed morphological, physiological and biochemical characteristics of strain ZXM100^T and ZXM098 are given in the species description (see below) or are shown in Table 1; transmission electron micrographs of strain ZXM100^T are shown in Fig. 1.

Cell mass of ZXM100^T and ZXM098 was obtained from MB after cultivation at 28 °C for 72 h and from *S. pontiacus* DSM 10014^T after cultivation for 20 h, when all of the communities reached the exponential stage. Cellular fatty acids were prepared and analysed according to the standard protocol described in the Sherlock System (MIDI) (Sasser, 1990) with the Sherlock version 6.0 and the included database. C_{18:1}ω7c, C_{18:0} and C_{16:0} were the major fatty acid components in both ZXM100^T and ZXM098. Detailed fatty acid compositions are listed in Table S1 (available in IJSEM Online).

Analyses of polar lipids and respiratory quinones of ZXM100^T were carried out by the Identification Service of the DSMZ and Dr B. J. Tindall, DSMZ, Braunschweig, Germany, using the two stage method described by Tindall (1990a, b). The predominant polar lipids of ZXM100^T were phosphatidylglycerol, phosphatidylethanolamine, phosphatidylcholine, a single lipid and an unidentified aminolipid (Figs S2 and S3, available in IJSEM Online). The predominant isoprenoid quinone of ZXM100^T was Q-10. The polar lipid and respiratory quinone compositions are basically consistent with those of members of related genera (Table 1; Martens *et al.*, 2006; Choi & Cho, 2006; Wagner-Döbler *et al.*, 2004; Kurahashi & Yokota, 2007).

The G+C content of the chromosomal DNA was determined as described by Mesbah *et al.* (1989) using

reverse-phase HPLC. The DNA G+C contents of strain ZXM100^T and ZXM098 were 54.6 and 56.7 mol%, respectively.

DNA was extracted from strain ZXM100^T and ZXM098 and purified using a kit (BioDev). 16S rRNA genes were amplified by PCR using universal primers 8f (5'-AGAGTTTGATC-CTGGCTCAG-3') and 1492r (5'-GGTTACCTTGTTAC-GACTT-3') (Bosshard *et al.*, 2000). Purified PCR products were ligated to the pMD 18-T (TaKaRa) and cloned according to the manufacturer's instructions. Sequencing reactions were carried out using ABI BigDye 3.1 Sequencing kits (Applied BioSystems) and an automated DNA sequencer (model ABI3730; Applied BioSystems). Pairwise comparison showed that strains ZXM100^T and ZXM098 shared 100 % 16S rRNA gene sequence similarity. The near-complete 16S rRNA gene sequence (1429 nt) of strain ZXM100^T was submitted to GenBank/EMBL to search for similar sequences using the BLAST algorithm. The identification of phylogenetic neighbours and the calculation of pairwise 16S rRNA gene sequence similarities were achieved using the EzTaxon server (<http://www.eztaxon.org/>; Chun *et al.*, 2007). Sequences were aligned using CLUSTAL X1.8 (Thompson *et al.*, 1997; the sequence alignment is available as Fig. S3). Phylogenetic trees were constructed using the neighbour-joining (Fig. 2) and maximum-likelihood (Fig. S1) methods with Kimura 2-state parameter model analyses implemented in the program MEGA version 5 (Tamura *et al.*, 2007). In each case, bootstrap values were calculated based on 1000 replicates. ZXM100^T was circumscribed to the family *Rhodobacteraceae* on the basis of phylogenetic analysis of 16S rRNA gene sequences and showed 95.6 % sequence similarity to *Sulfitobacter delicatus* LMG 20554^T, 95.5 % to *Nautella italica* LMG 24365^T and 95.3 % to *Sulfitobacter dubius* LMG 20555^T (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>). Members of 10 genera (*Sulfitobacter*, *Nautella*, *Thalassobacter*, *Leisingera*, *Roseobacter*, *Shimia*, *Marinovum*, *Phaeobacter*, *Oceanibulbus* and *Tateyamaria*) of the family *Rhodobacteraceae* that showed the highest 16S rRNA gene sequence similarities (92.0–95.6 %) were chosen to construct the phylogenetic trees. However, strain ZXM100^T did not form a robust lineage in the phylogenetic trees with any species in the family *Rhodobacteraceae*, but formed a phylogenetically distinct lineage alongside related strains.

In conclusion, the proposal that strain ZXM100^T represents a novel species in a new genus based on phylogenetic analysis was supported by its differential morphological and physiological characteristics, i.e. (1) colourless colonies, (2) lack of flagella, (3) narrow growth temperature range, (4) better adaptation to acidic environments, (5) inability to oxidize sulfite to sulfate (sulfite oxidation is a very important property enabling members of the genus *Sulfitobacter* to be distinguished from other genera), (6) ability to reduce nitrate to nitrite, (7) a lower level of C_{18:1}ω7c and the presence of the unique component C_{17:0} and (8) a lower DNA G+C content. Other characteristics that differentiate strain ZXM100^T from members of related genera are shown in Table 1.

Table 1. Characteristics that differentiate ZXM100^T and ZXM098 from members of related genera

Taxa: 1, ZXM100^T (data from this study); 2, ZXM098 (data from this study); 3, *Sulfitobacter* [*S. pontiacus* DSM 10014^T (data from this study; Sorokin, 1995; Ivanova *et al.*, 2004), *S. mediterraneus* DSM 12244^T (Pukall *et al.*, 1999; Labrenz *et al.*, 2000; Yoon *et al.*, 2007a), *S. guttiformis* DSM 11458^T (Labrenz *et al.*, 2000), *S. brevis* DSM 11443^T (Labrenz *et al.*, 2000; Ivanova *et al.*, 2004), *S. delicatus* LMG 20554^T (Ivanova *et al.*, 2004), *S. dubius* LMG 20555^T (Ivanova *et al.*, 2004), *S. litoralis* DSM 17584^T (Park *et al.*, 2007), *S. donghicola* KCTC 12864^T (Yoon *et al.*, 2007a), *S. marinus* KCTC 12738^T (Yoon *et al.*, 2007b)]; 4, *Nautella* [*N. italica* LMG 24365^T (Vandecastelaere *et al.*, 2009)]; 5, *Thalassobacter* [*T. stenotrophicus* DSM 16310^T (Macián *et al.*, 2005; Pujalte *et al.*, 2005); *T. arenae* DSM 19593^T (Kim *et al.*, 2009)]; 6, *Leisingera* [*L. methylohalidivorans* DSM 14336^T (Schaefer *et al.*, 2002; Martens *et al.*, 2006)]; 7, *Roseobacter* [*R. litoralis* ATCC 49566^T (Shiba, 1991; Ruiz-Ponte *et al.*, 1998; Park *et al.*, 2007; Sass *et al.*, 2010), *R. denitrificans* ATCC 33942^T (Shiba, 1991; Ruiz-Ponte *et al.*, 1998; Park *et al.*, 2007; Sass *et al.*, 2010)]; 8, *Shimia* [*S. marina* KCCM 42117^T (Choi & Cho, 2006)]; 9, *Marinovum* [*M. algicola* ATCC 51440^T (Lafay *et al.*, 1995; Martens *et al.*, 2006)]; 10, *Phaeobacter* [*P. gallaeciensis* DSM 17395^T (Ruiz-Ponte *et al.*, 1998; Martens *et al.*, 2006), *P. inhibens* DSM 16374^T (Martens *et al.*, 2006)]; 11, *Oceanibulbus* [*Oceanibulbus indolifex* DSM 14862^T (Wagner-Döbler *et al.*, 2004)]; 12, *Tateyamaria* [*T. omphalii* KCTC 12333^T (Kurahashi & Yokota, 2007; Sass *et al.*, 2010); *T. pelophila* DSM 17270^T (Sass *et al.*, 2010)]. +, Positive; −, negative; w, weak reaction; v, varies between species and/or strains; ND, not described or data are not available for the type strain.

Characteristics	1	2	3	4	5	6	7	8	9	10	11	12
Colony colour*	C	C	Y/Wh/Br/B/P	B	Br/P	C	P	O/C/B	B/P	Br	Wh	B/P
Cell shape	Ovoid to irregular short rods	Ovoid to irregular short rods	Rods	Rods	Ovoid to irregular rods	Rods or ovoid rods	Rods or ovoid rods	Rods	Ovoid rods	Ovoid rods	Irregular rods	Coccus to short rod
Flagella	−	−	+	+	+	+	+	+	+	+	−	v
PHB	+	+	+	+	v	−	ND	ND	−	ND	+	ND
Bchl <i>a</i>	−	−	v	ND	+	−	+	−	−	−	−	v
Ranges for growth												
Temperature (°C)	22–28	22–28	4–37	4–45	5–37	ND	2–30	15–35	10–37	4–37	ND	4–37
NaCl (% w/v)	3–9	3–9	0.2–15.0	1–5	0.85–8.0	1.5–6.5	ND	3–7	0.1–2.0	0.059–11.7	1–7	0.2–10.2
pH	2.0–9.0	2.0–9.0	5.0–11.0	5.5–9.0	ND	ND	ND	6–10	6–9	6.0–10.0	ND	5.4–9.0
Oxidase	+	w	+	+	+	+	+	+	+	+	w	+
Catalase	+	+	+	+	+	+	+	+	+	+	+	+
Sulfite oxidation	−	−	v	ND	ND	ND	ND	ND	ND	ND	ND	v
Nitrate reduction	+	+	v	−	−	−	v	+	−	−	−	v
Hydrolysis of:												
Tween 80	+	−	v	+	v	−	+	+	−	−	−	−
Gelatin	−	−	v	−	−	ND	+	+	ND	−	−	−
Oxidation of:												
Citrate	+	+	v	−	v	−	+	+	+	v	+	+
D-Mannitol	+	w	v	−	−	ND	+	−	−	+	+	+
Maltose	w	w	v	−	−	ND	−	ND	+	+	−	+
D-Glucose	+	+	v	−	v	−	+	−	+	+	+	v
Major polar lipids												
Phosphatidylglycerol	+	ND	v	ND	+	+	+	ND	+	+	+	ND
Phosphatidylethanolamine	+	ND	+	ND	v	+	−	ND	+	+	+	ND
Phosphatidylcholine	+	ND	+	ND	+	−	v	ND	+	+	+	ND
Diphosphatidylglycerol	−	ND	v	ND	+	−	+	ND	−	−	+	ND
DNA G + C content (mol%)	54.6	56.7	55.5–63.5†	61.0 ± 0.8	56.0–59.1	60.5	56–60	57.2	60	55.7–58.0	60.0	56.4–61.6

*Abbreviations: B, beige; Br, brown; C, colourless; P, pink; Y, yellow; Wh, white.

†Data for *S. pontiacus* DSM 10014^T from Ivanova *et al.* (2004).

Fig. 1. Transmission electron micrographs of negatively stained cells of *Lentibacter algarum* ZXM100^T. Bars: a, 200 nm; b, 1 μ m.

Therefore, on the basis of the phenotypic and phylogenetic evidence, the two strains ZXM100^T and ZXM098 should be classified as representatives of a novel species in a new genus in the family *Rhodobacteraceae*, for which the name *Lentibacter algarum* gen. nov., sp. nov. is proposed.

Description of *Lentibacter* gen. nov.

Lentibacter (Len.ti.bac'ter. L. adj. *lentus* slow; N.L. masc. n. *bacter* a rod; N.L. masc. n. *Lentibacter* a slow-growing rod bacterium).

Cells are Gram-negative and aerobic with no flagella. Colonies are colourless. Growth temperature range is narrow and growth occurs very slowly. Growth occurs in media containing 3–9 % NaCl. Cells do not contain Bchl *a*. PHB is accumulated. Oxidase and catalase are positive. Cannot oxidize sulfite to sulfate. Nitrate is reduced to nitrite. C_{18:1} ω 7c, C_{18:0} and C_{16:0} are the major fatty acid components. The polar lipids are phosphatidylglycerol, phosphatidylethanolamine, phosphatidylcholine, a single lipid and an unidentified aminolipid. The predominant respiratory quinone is Q-10. The type species is *Lentibacter algarum*.

Description of *Lentibacter algarum* sp. nov.

Lentibacter algarum (al'ga.rum. L. gen. pl. n. *algarum* of from sea-weeds, isolated from Qingdao, China).

In addition to the characteristics that define the genus, the following characteristics are observed. Cells are ovoid to

Fig. 2. Phylogenetic dendrogram of *Lentibacter algarum* ZXM100^T, ZXM098 and strains of related species based on 16S rRNA gene sequence similarities. The tree was constructed using the neighbour-joining method with Kimura 2-state parameter model analyses implemented in the program MEGA version 5. Bar, 0.01 nt substitutions per site.

irregular short rods, approximately 1.0–3.0 µm long by 0.8–1.0 µm wide. Does not form endospores. Colonies are uniformly round, 0.2–1.0 mm in diameter, regular, convex and smooth. Grows on NA and forms colourless colonies; cannot grow on TSA. No diffusible pigment is produced in the medium.

The temperature range for growth is 22–28 °C. The pH range for growth is 2.0–9.0. Hydrolysis of Tween 80 is strain-dependent (positive for type strain). Gelatin, agar, starch, casein and alginate are not hydrolysed. Antibiotic susceptibilities (amount of antibiotic/diameter of inhibition zone) are as follows: susceptible to vancomycin (30 µg/28 mm), erythromycin (15 µg/54 mm), ampicillin (10 µg/19 mm) and carbenicillin (100 µg/29 mm); slightly susceptible to benzylpenicillin (10 IU/24 mm); resistant to polymyxin B (100 IU/0 mm), gentamicin (10 µg/10 mm), kanamycin (30 µg/0 mm), tetracycline (30 µg/0 mm), neomycin (30 µg/0 mm) and oxacillin (1 µg/0 mm).

According to API 20E: β -galactosidase-positive; negative for arginine dihydrogenase, lysine decarboxylase, ornithine decarboxylase and tryptophan deaminase; citrate is utilized; H₂S and indole are not produced; urease and Voges–Proskauer reaction are strain-dependent (positive for type strain); produces acid from D-glucose, D-mannitol and sucrose; does not produce acid from inositol, D-sorbitol, L-rhamnose, melibiose, amygdalin or L-arabinose. According to API 20NE: aesculin ferric citrate is degraded; D-glucose is oxidized; N-acetylglucosamine, potassium gluconate, capric acid, adipic acid, malic acid, trisodium citrate and phenylacetic acid are not utilized; and maltose is weakly utilized; D-mannitol, L-arabinose and D-mannose oxidation are strain-dependent (positive for the type strain). According to API ZYM, alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, acid phosphatase and naphthol-AS-BI-phosphohydrolase are positive; lipase (C14), valine arylamidase, cystine arylamidase, trypsin, α -chymotrypsin, α -galactosidase, β -glucosidase, β -glucuronidase, α -glucosidase, β -N-acetylglucosaminidase, α -mannosidase and α -fucosidase are negative. According to API 50CH, glycerol, D-xylose, arbutin, aesculin ferric citrate, cellobiose, sucrose, melezitose, turanose, gluconate, 2-ketogluconate and 5-ketogluconate are oxidized; D-galactose, D-fructose, maltose, amygdalin, glycogen, gentiobiose and D-lyxose are only oxidized weakly. According to Biolog, glycogen, Tween 40, Tween 80, L-arabinose, cellobiose, α -D-glucose, D-mannose, sucrose, turanose, citric acid, formic acid, α -hydroxybutyric acid, β -hydroxybutyric acid, α -ketovaleric acid, DL-lactic acid, L-phenylalanine and glycerol are oxidized; D-fructose, D-galactose, gentiobiose, maltose, succinic acid monomethyl ester and L-alaninamide are only weakly oxidized.

Of the 11 identified fatty acids in the type strain, C_{18:1}ω7c is the major component, with smaller amounts of C_{18:0} and C_{16:0}. C_{17:0}, C_{16:1}ω7c and/or C_{16:1}ω6c, C_{10:0} 3-OH and C_{18:1}ω7c 11-methyl are present as minor components. Other cellular fatty acids present at levels greater than 1 %

include C_{14:0}, C_{17:1}ω8c, C_{18:1}ω9c, unknown C_{18.846} and/or C_{19:1}ω6c.

The type strain, strain ZXM100^T (=LMG 24861^T=CGMCC 1.10234^T), and one additional strain [ZXM098 (=DSM 24677=CGMCC 1.8996)] were isolated from seawater collected from a coastal region of Qingdao, PR China, during a massive green algae (*Enteromorpha prolifera*) bloom.

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