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Kiloniella laminariae gen. nov., sp. nov., an alphaproteobacterium from the marine macroalga *Laminaria saccharina*

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A novel alphaproteobacterium, strain LD81^T, was isolated from the marine macroalga *Laminaria saccharina*. The bacterium is mesophilic and shows a typical marine growth response. It is a chemoheterotrophic aerobe with the potential for denitrification. Growth optima are 25 °C, pH 5.5 and 3 % NaCl. Strain LD81^T has a unique phylogenetic position, not fitting any of the known families of the *Alphaproteobacteria*. The 16S rRNA gene sequence revealed a distant relationship to species of several orders of the *Alphaproteobacteria*, with less than 90 % sequence similarity. Phylogenetically, strain LD81^T is related to the type strains of *Terasakiella pusilla* (88.4 % 16S rRNA gene sequence similarity) and the three *Thalassospira* species (88.9–89.2 %). It forms a cluster with these bacteria and a novel as-yet undescribed isolate (KOPRI 13522; 96.6 % sequence similarity). Strain LD81^T has a relatively low DNA G+C content (51.1 mol%) and, due to its distant phylogenetic position from all other alphaproteobacteria, strain LD81^T (=NCIMB 14374^T =JCM 14845^T) is considered as the type strain of a novel species within a new genus, for which the name *Kiloniella laminariae* gen. nov., sp. nov. is proposed. The genus *Kiloniella* represents the type of the new family *Kiloniellaceae* fam. nov. and order *Kiloniellales* ord. nov.

The *Alphaproteobacteria* is one of the most well-represented bacterial groups observed in marine habitats (Giovannoni & Rappé, 2000), with members of the orders *Caulobacterales*, *Sphingomonadales*, *Rhizobiales*, *Rickettsiales*, *Rhodobacterales*, *Rhodospirillales*, *Kordiimonadales* and '*Parvularculales*' being reported (Garrity *et al.*, 2005; Kwon *et al.*, 2005). In a study concerning the phylogenetic analysis of bacteria that are associated with the marine brown alga *Laminaria saccharina* from the Baltic Sea, strain LD81^T was isolated.

Pieces of *Laminaria saccharina* tissue were suspended in sterile seawater and homogenized using an Ultraturrax T25 (IKA Werke). The suspension was diluted in sterile seawater and plated on TSB medium (l^{-1}: 3 g Difco tryptic soy broth, 7 g NaCl, 15 g Bacto agar; pH 7.2). The plates were incubated at 22 °C in the dark for 20 days. After good growth was obtained, an overlay containing TSB medium (with 8 g l^{-1} Bacto agar) and 10 % (v/v) overnight culture of *Candida glabrata* DSM 6425 was poured onto the plates and incubated for 24 h at 22 °C in order to detect inhibition zones against *C. glabrata* by individual colonies. Antibiotically active colonies were repeatedly streaked on agar plates with TSB medium to obtain pure cultures. One of the pure cultures obtained was strain LD81^T, which was stored at −80 °C using the Cryobank System (Mast Diagnostica GmbH) for maintenance.

Cell morphology was examined by scanning electron microscopy. Strain LD81^T was cultivated for 24 h in marine broth (MB; Difco 2216) at 28 °C on a rotary shaker with shaking at 95 r.p.m., followed by fixation with a final concentration of 1 % formol and filtration through 0.2 μm polycarbonate filters (Sarstedt). The filters were applied in a subsequent ethanol series for dehydration (50, 70 and 90 % and three times in 100 % for 10 min each) (Boyde & Wood, 1969), critical-point dried with CO_2 and sputter-coated with Au/Pb and examined with a Zeiss DSM 940 scanning electron microscope. Light microscopy was used for determination of the cell size and to study motility.

The temperature (4–50 °C) and pH (pH 3.5–10) ranges as well as the optima for growth of strain LD81^T were examined by cultivation in MB. The temperature and pH

Abbreviations: ME, minimum evolution; ML, maximum-likelihood; NJ, neighbour-joining; PHB, poly-β-hydroxybutyrate.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain LD81^T is AM749667.

16S rRNA gene sequence similiarities between strain LD81^T and related type strains are available as supplementary material in IJSEM Online.

optima were assessed after incubation for 3 days. Ranges were ascertained after prolonged incubation for 3 weeks. Growth was measured photometrically at OD_{600}. Salt relations (0–10 % NaCl, w/v) were determined after incubation at 25 °C for 10 days on a basal medium (l^{-1}: 1 g Bacto peptone, 5 g yeast extract, 15 g Bacto agar, pH 7.0) supplemented with NaCl.

Well-grown fresh colonies of overnight cultures [grown on half-strength MB agar (l^{-1}: 17 g Difco 2216, 15 g Bacto agar) at 28 °C] were used for the Gram reaction using KOH according to Gregersen (1978), for poly-β-hydroxybutyrate (PHB) staining with Sudan black following Smibert & Krieg (1994) and for the catalase reaction (detected with 5 % H_2O_2). The presence of PHB was confirmed by phase-contrast microscopy (Axiophot; Zeiss). Luminescence was tested in liquid and on solid half-strength MB supplemented with 3 % glycerol. The adsorption spectrum of disrupted cells was measured using a UV–Vis spectrophotometer Lambda 2 (Perkin Elmer) to determine the presence of photosynthetic pigments.

The aerobic oxidation of organic carbon compounds was tested using the Biolog GN2 system. Strain LD81^T was inoculated in half-strength MB (17 g Difco 2216 l^{-1}) and incubated overnight. Cells were centrifuged at 8000 ***g*** for 10 min, resuspended in 1 % NaCl solution and adjusted to an OD_{600} of 0.8–1.3. Three microplates were inoculated with this suspension and incubated at 22 °C for 48 h. Utilization of compounds was scored as positive when three positive reactions were observed. In addition, further physiological characteristics including enzyme activities were tested using API 20E strips for Gram-negative bacteria (bioMérieux) and API ZYM strips (bioMérieux) according to the manufacturer's instructions. The inoculum was prepared as described above and the test systems were incubated at 32 °C for 3 days. Both tests were run in triplicate.

The DNA base composition (G+C content) of strain LD81^T was determined by the HPLC method of Mesbah *et al.* (1989). The profile of cellular fatty acids was studied using GC analysis according to the Microbial Identification System (MIDI Inc.) (Sasser, 1990). Both determinations were carried out by the German Collection of Microorganisms and Cell Cultures (DSMZ GmbH, Braunschweig, Germany). Extraction of genomic DNA and amplification and sequencing of the 16S rRNA gene were performed according to Gärtner *et al.* (2008).

Phylogenetic classification was performed with the Naive Bayesian rRNA Classifier (Wang *et al.*, 2007) version 2.0 of the Ribosomal Database Project (RDP) release 9.56 (http://rdp.cme.msu.edu/index.jsp).

For phylogenetic study, the nearest bacterial relatives of strain LD81^T were determined by comparison to 16S rRNA gene sequences in the NCBI GenBank and EMBL databases using BLAST (Altschul *et al.*, 1997) and the Seqmatch program of the RDP II (http://rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp), restricted to type strains. Sequences were aligned using the FASTALIGN function of the alignment editor implemented in the ARB software package (http://www.arb-home.de) (Ludwig *et al.*, 2004) and refined manually employing secondary structure information. For phylogenetic calculations, PhyML Online (Guindon *et al.*, 2005) and MEGA version 3.1 (Kumar *et al.*, 2004) were used. Trees were calculated by the maximum-likelihood (ML) (Felsenstein, 1981), neighbour-joining (NJ) (Saitou & Nei, 1987) and minimum-evolution (ME) (Rzhetsky & Nei, 1993) methods. The ML tree was calculated using the GTR model and estimated proportion of invariable sites as well as the gamma distribution parameter. The NJ and ME trees were calculated based on distances corrected by Kimura's two-parameter nucleotide substitution model, using sites corresponding to the 'pairwise deletion' option, respectively including transition and transversion substitutions and uniform substitution rates. Sequence similarity values were determined using the BLAST 2 SEQUENCES tool of the NCBI database (http://www.ncbi.nlm.nih.gov/BLAST/bl2seq/wblast2.cgi; Tatusova & Madden, 1999).

Colonies grown on MB agar for 7 days at 22 °C are cream-coloured, smooth and soft, 1–2 mm in diameter. Cells grown in MB for 24 h at 22 °C are motile with one polar flagellum. The cells are slender, slightly curved spirilla, and their size measured in the light microscope is 0.5–0.6 × 2.5–5.0 μm. Short rod-like cells and also longer filamentous cells were occasionally observed (Fig. 1).

Strain LD81^T grows as a chemoheterotrophic, aerobic bacterium in complex media. It can use nitrate as an alternative electron acceptor, which is reduced to gaseous products (N_2O is the major product; the gene for N_2O reductase, *nosZ*, is lacking). The temperature for growth is 4–40 °C with an optimum at 25 °C. The pH for growth of

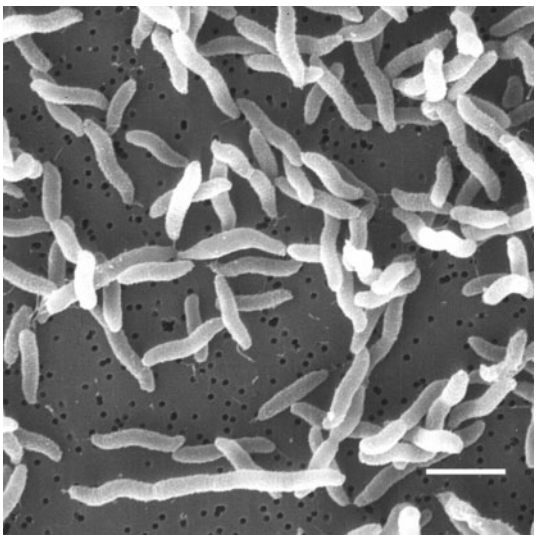

Fig. 1. Scanning electron photomicrograph of cells of strain LD81^T after cultivation in MB for 48 h at 28 °C. Bar, 2 μm.

the isolate is pH 3.5–9.5, with an optimum at pH 5.5. Growth is observed in media containing 0.3–8.0 % NaCl (optimum 3.0 %) or 0.3–10 % artificial sea salts (optimum 4.0 %), indicating a typical marine growth response. Catalase and oxidase reactions are positive and PHB is accumulated. Luminescence is negative.

Details concerning the physiological characteristics of strain LD81^T including substrate utilization (according to Biolog GN2) and enzyme activities are given in the species description. Pigments are not produced under any growth conditions applied in this study.

The components of the fatty acid profile are listed in Table 1; the major cellular fatty acids are $C_{18:1}\omega7c$ (49 %), $C_{16:1}\omega7c$ (31 %), $C_{16:0}$ (9 %), $C_{18:0}$ (3 %) and $C_{19:0}$ cyclo $\omega8c$ (1 %). The DNA G+C content of strain LD81^T was 51.1 mol%.

Phylogenetic classification using the Naive Bayesian rRNA Classifier led to the assignment of strain LD81^T to the class *Alphaproteobacteria* (100 % confidence). Sequence similarity values are below 91 % to any of the 20 closest sequences of type strains of species with validly published names (Supplementary Table S1, available in IJSEM Online). BLAST searches revealed strain KOPRI 13522 as the closest non-type strain relative, sharing 96.6 % sequence similarity. Sequences of the 16S rRNA genes of the five closest type strains, of strain KOPRI 13522 as well as of representatives of all orders of the *Alphaproteobacteria* were used for phylogenetic analysis. All resulting trees confirm the close phylogenetic relationship of strain LD81^T to strain KOPRI 13522. The two sequences form a distinct group not included in any of the known alphaproteobacterial orders with 100 % bootstrap values. They are related distantly to the group consisting of *Terasakiella pusilla* IFO 13613^T and the type strains of the three known *Thalassospira* species (*Thalassospira xiamensis* M-5^T, *Thalassospira lucentensis* DSM 14000^T and *Thalassospira profundimaris* WP0211^T). Though members of the genus *Thalassospira* were provisionally assigned to the family *Rhodospirillaceae* (López-López *et al.*, 2002), the phylogenetic analysis of our study does not confirm this affiliation (Fig. 2). *Thalassospira* species together with *Terasakiella pusilla*, strain KOPRI 13522 and strain LD81^T form a strongly supported cluster (>90 % bootstrap values) clearly separated from the *Rhodospirillaceae* and *Acetobacteraceae* (<90 % sequence similarity). However, isolate LD81^T and species of the genera *Thalassospira* and *Terasakiella* share 16S rRNA gene sequence similarities below 90 % (Supplementary Table S1). Therefore, strain LD81^T is supposed to represent the type of a novel species within a new genus, which is the type of a new family and order.

Table 1. Fatty acid profile of strain LD81^T

Values are percentages of total fatty acids. ECL, Equivalent chain-length.

Fatty acid	Proportion (%)
$C_{12:0}$ ALDE	1.7
$C_{13:1}$ AT 12–13	0.1
Unknown (ECL 14.502)	0.7
$C_{15:1}\omega8c$	0.3
Unknown (ECL 14.959)	1.2
$C_{15:0}$	0.1
$C_{14:0}$ 3-OH/iso-$C_{16:1}$ I	1.2
$C_{16:1}\omega7c$	30.7
$C_{16:0}$	8.5
$C_{17:1}\omega8c$	0.3
$C_{17:1}\omega6c$	0.1
$C_{17:0}$	0.9
$C_{18:1}\omega7c$	48.6
$C_{18:0}$	3.0
$C_{17:0}$ 3-OH	0.2
Unknown (ECL 18.814)	0.4
$C_{19:0}$ cyclo $\omega8c$	1.4
$C_{18:0}$ 3-OH	0.5
$C_{20:1}\omega9c$	0.3

Strain LD81^T was also different morphologically, chemotaxonomically and physiologically from other members of the class *Alphaproteobacteria*. Strains belonging to the family *Rhodospirillaceae* and *Acetobacteraceae* exhibit significantly higher DNA G+C contents, generally well above 60 mol%, mostly between 62 and 67 mol% and, in some clusters of the *Acetobacteraceae* related to *Craurococcus*, above 70 and even up to 75 mol% (Shi *et al.*, 2002). Representatives of the family *Acetobacteraceae* show ellipsoid, rod or coccoid cell morphology and usually do not require salt for growth. Many members of the *Rhodospirillaceae* produce photosynthetic pigments.

The nearest relatives of strain LD81^T within the order *Rhodobacterales* are *Pseudovibrio denitrificans* DN34^T and *Pseudovibrio ascidiaceicola* NBRC 100514^T (approx. 91 % 16S rRNA gene sequence similarity), which are rod-shaped and produce gelatinase (Shieh *et al.*, 2004; Fukunaga *et al.*, 2006). The nearest relatives within the order *Rhizobiales*, *Mesorhizobium chacoense* PR5^T (90 % similarity), *Ensifer terangae* LMG 7834^T (89.7 % similarity) and *Pseudaminobacter salicylatoxidans* BN12^T (89.5 % similarity), exhibit DNA G+C contents of 62, 61.6 and 63.9 mol%, respectively (Velázquez *et al.*, 2001; Young, 2003; de Lajudie *et al.*, 1994; Kämpfer *et al.*, 1999). To date, only one representative of the '*Parvularculales*' has been described (Cho & Giovannoni, 2003). The production of pigments and the DNA G+C content of 60.8 mol% clearly distinguish *Parvularcula bermudensis* HTCC2503^T from isolate LD81^T. *Kordiimonas gwangyangensis* GW14-5^T, the sole member of the order *Kordiimonadales*, is not able to reduce nitrate and, quite unusually for the *Alphaproteobacteria*, has a DNA G+C content of only 39.3 mol% and produces iso-$C_{17:1}$ as the predominant fatty acid (Kwon *et al.*, 2005).

Common properties of strain LD81^T and its closest neighbours in the phylogenetic tree, *Terasakiella pusilla* and the three *Thalassospira* species, are the salt requirement

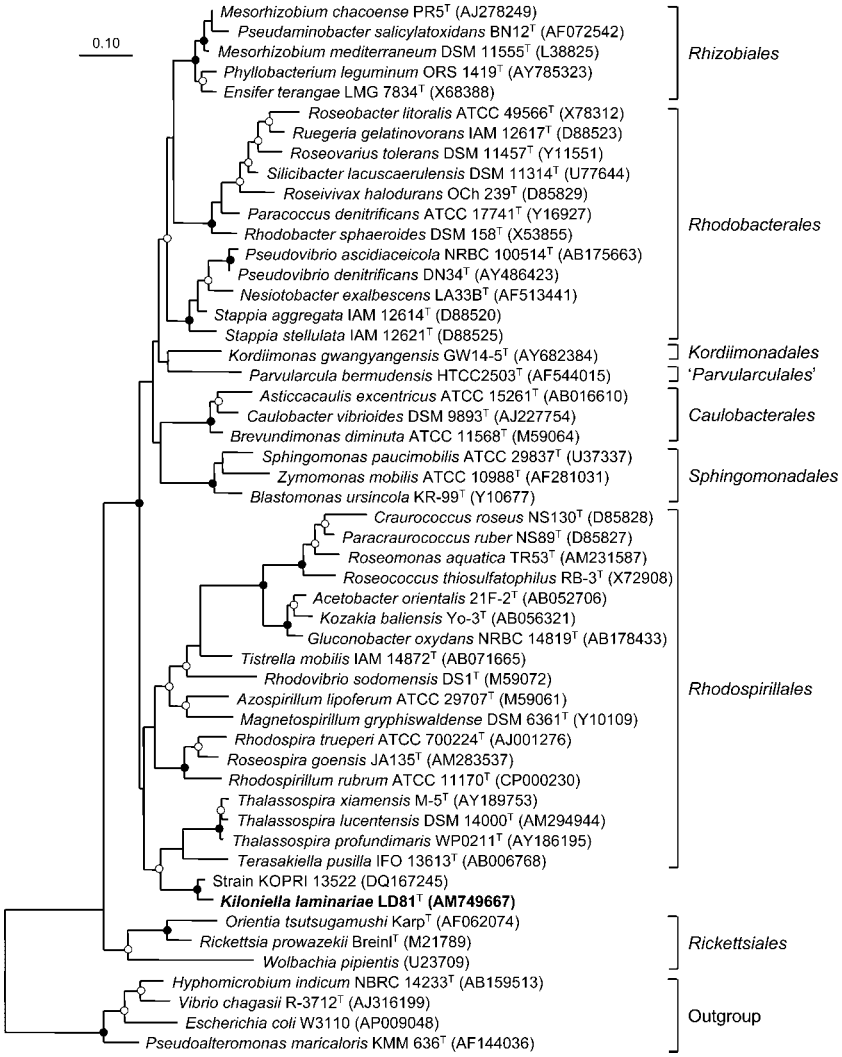

Fig. 2. Phylogenetic tree showing the relationships of strain LD81^T to representative members of the *Alphaproteobacteria*. The calculation was based on the ML, NJ and ME method with 1000 bootstraps. Filled circles indicate bootstrap values >95 %; open circles indicate bootstrap values >50 %.

and tolerance of up to approx. 8–10 % NaCl, the ability to reduce nitrate, the G+C content of the DNA (48–55 mol%) and the spiral to vibrioid cell shape (Table 2). Differences from these bacteria, in addition to clear differences in 16S rRNA gene sequences, are the proportions of fatty acids, the production of 3-hydroxyheptadecanoic acid by isolate LD81^T and the reduction of nitrate to N_2O by strain LD81^T rather than nitrite, as produced by the other bacteria (Table 2). Also, *Terasakiella pusilla* possesses bipolar single flagella, in contrast to the single monopolar flagellum of LD81^T.

Because of its isolated phylogenetic position, its low G+C content of 51 mol%, the absence of pigments, the salt requirement and other distinguishing properties as outline above and in Table 2, strain LD81^T is considered as the representative of a novel species and genus within a new family and order of the *Alphaproteobacteria*. The name *Kiloniella laminariae* gen. nov., sp. nov. is proposed, and *Kiloniella* is defined as the type genus of the new family *Kiloniellaceae* fam. nov. and new order *Kiloniellales* ord. nov. Due to their distant relationship to *Kiloniella*, the species of *Terasakiella* and *Thalassospira* are not considered members of the family *Kiloniellaceae*. They may be included in the order *Kiloniellales* as members of a separate family or families. However, determination of their exact taxonomic standing requires further studies with a larger number of representatives, and their taxonomic position should be defined when more data are available.

Description of *Kiloniella* gen. nov.

Kiloniella [Ki.lo′ni.el′la. L. n. *Kilonium* Latin name of the northern German city of Kiel; N.L. fem. dim. n. *Kiloniella* arbitrary name for a bacterium found in marine waters close to Kiel, the place of an important institution of marine research (the IFM-GEOMAR), in which the first strain of the genus was discovered].

Mesophilic, chemoheterotrophic bacteria with typical marine and moderately halotolerant growth response. Metabolism is aerobic and facultatively anaerobic with nitrate as electron acceptor. Major fatty acids are mono-

Table 2. Differential characteristics of strain LD81^T and phylogenetically related species of the genera *Terasakiella* and *Thalassospira*

Data for reference taxa are derived from Sakane & Yokata (1994), Terasaki (1979), Satomi *et al.* (2002), López-López *et al.* (2002) and Liu *et al.* (2007). +, Positive; −, negative; W, weak; ND, no data available; BChl, bacteriochlorophyll. All taxa require salt for growth and are positive for oxidase and growth on carbohydrates.

Characteristic	Strain LD81^T	*Terasakiella pusilla*	*Thalassospira*
Cell morphology	Spiral (occasionally rod or filamentous)	Spiral	Vibrioid to spiral
Flagella	+ (Single polar)	+ (Bipolar single)	+ (Single polar)/−
Pigment	−	−	+/−
BChl *a*	−	ND	−/ND*
Salt tolerance (%)	Up to 8	Up to 8	Up to 10
Catalase	+	W/−	+
Reduction of nitrate	+ (to N_2O)	+ (to nitrite)	+ (to nitrite)/–
Quinone type	Not tested	Q10	ND
DNA G+C content (mol%)	51.1	48/51†	47–54.7
Non-polar fatty acids (%)‡			
$C_{18:1}$	49	58	43–45
$C_{16:1}$	31	18	3–16
$C_{16:0}$	8	15	15–18
$C_{18:0}$	3	1	3–9
3-Hydroxy fatty acids (%)§			
$C_{14:0}$ 3-OH	64‖	87	25–41
$C_{16:0}$ 3-OH	0	2	51–61
$C_{17:0}$ 3-OH	11	0	0
$C_{18:0}$ 3-OH	25	10	8–15
Oxygen requirement	Aerobe/anaerobe	Aerobe	Aerobe/anaerobe
Anaerobic phototrophic growth	−	ND	−/ND*

*Result for *Thalassospira lucentensis*. No data available for *Thalassospira xiamenensis* or *Thalassospira profundimaris*.
†Sakane & Yokota (1994) reported 48 mol%; Terasaki (1979) reported 51 mol%.
‡Percentages of total fatty acids.
§Percentages of total 3-hydroxy fatty acids. Percentages shown for *Thalassospira* species are calculated from the data given by Liu *et al.* (2007).
‖$C_{14:0}$ 3-OH and/or iso-$C_{16:1}$ I.

unsaturated, even-numbered, straight-chain C_{18} and C_{16} fatty acids, with $C_{18:1}\omega7c$ as the dominant component. Cells have spiral to vibrioid cell shape, occasionally rod-like or filamentous, and are motile by means of flagella. Gram-negative, oxidase- and catalase-positive. PHB is accumulated. The G+C content of the DNA of the type strain of the type species is 51.1 mol%. The type species is *Kiloniella laminariae*.

Description of *Kiloniella laminariae* sp. nov.

Kiloniella laminariae (la.mi.na′ri.ae. N.L. fem. n. *Laminaria* botanical name of a genus of macroalgae; N.L. gen. fem. n. *laminariae* pertaining to the alga *Laminaria*, from which the type strain was isolated).

Displays the following properties in addition to those described above for the genus. Cells are slender, slightly curved spirilla, 0.5–0.6 μm wide and 2.5–5.0 μm long. Cells carry monopolar flagella. Pigments are not produced. Colonies are cream in colour and grow up to 1–2 mm in diameter on MB agar. Grows at 4–40 °C, pH 3.5–9.5 and from 0.3–10 % artificial sea salts. Salt is required for growth. Optimal growth at 25 °C, pH 5.5 and 3 % NaCl. Growth occurs chemoheterotrophically under oxic conditions. Nitrate is used as an alternative electron acceptor under anoxic conditions. Nitrate is reduced to N_2O. Carbon sources (Biolog GN2) used are glycogen, α-D-glucose, monomethyl succinate, acetic acid, β-hydroxybutyrate, 2-oxoglutarate, DL-lactate, succinamate, alaninamide, D-alanine, L-alanine, L-alanyl glycine, L-asparagine, L-aspartate, L-glutamate, glycyl L-aspartate, glycyl L-glutamate, L-histidine, hydroxy-L-proline, L-leucine, L-proline, L-pyroglutamate, L-serine, L-threonine, urocanate, inosine, uridine and glycerol. Enzyme activities are observed for alkaline phosphatase, leucine arylamidase, valine arylamidase, trypsin, acid phosphatase and naphthol-AS-BI-phosphohydrolase. Negative reactions are obtained in tests for esterase, esterase lipase, lipase, cystine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase. Furthermore, the API 20NE test system shows strong activities of arginine dihydrolase, citrate utilization and

tryptophan deaminase. Negative reactions occur in tests for indole production, β-galactosidase, lysine decarboxylase, ornithine decarboxylase, urease, H_2S production and gelatinase. Major cellular fatty acids are $C_{18:1}\omega7c$, $C_{16:1}\omega7c$, $C_{16:0}$, $C_{18:0}$ and $C_{19:0}$ cyclo $\omega8c$.

The type strain, LD81^T (=NCIMB 14374^T =JCM 14845^T), was isolated from a specimen of *Laminaria saccharina* collected from the Baltic Sea in the Kiel Bight (Germany).

Description of *Kiloniellaceae* fam. nov.

Kiloniellaceae (Ki.lo′ni.el.la′ce.ae. N.L. fem. n. *Kiloniella* name of a bacterial genus; *-aceae* ending to denote the name of a family; N.L. fem. pl. n. *Kiloniellaceae* the *Kiloniella* family).

Bacteria of this family are Gram-negative and cells have spiral to vibrioid cell shape. Mesophilic, chemoheterotrophic bacteria with typical marine and moderately halotolerant growth response. Major fatty acids are monounsaturated, even-numbered, straight-chain C_{18} and C_{16} fatty acids. The G+C content of the DNA is approximately 50 mol%. The type genus is *Kiloniella*.

Description of *Kiloniellales* ord. nov.

Kiloniellales (Ki.lo′ni.el.la′les. N.L. fem. n. *Kiloniella* name of a bacterial genus; *-ales* ending to denote an order; N.L. fem. n. *Kiloniellales* the order of *Kiloniella*).

The description is the same as for the family *Kiloniellaceae*. The type genus is *Kiloniella*.

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