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TAXONOMIC DESCRIPTION
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Litoribacterium kuwaitense gen. nov., sp. nov., isolated from a Kuwait tidal flat

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Abstract

A Gram-stain-positive, strictly aerobic, spore-forming, rod-shaped and non-motile bacterium designated strain SIJ1^T was obtained from tidal flat sediment collected from the northern shore of Kuwait Bay, northwest of the Arabian Gulf. Strain SIJ1^T grew optimally at 30 °C and pH 7–8 in the presence of 6% (w/v) NaCl. The cell-wall peptidoglycan was based on *meso*-diaminopimelic acid and an unsaturated menaquinone with seven isoprene units (MK-7) was the predominant respiratory quinone. It contained anteiso-$C_{15:0}$, iso-$C_{16:0}$ and iso-$C_{15:0}$ as the major fatty acids and ribose as the major whole-cell sugar. The polar lipids were diphosphatidylglycerol, phosphatidylglycerol, unidentified phospholipid, an unidentified glycolipid, phosphoglycolipid and an unidentified lipid. Phylogenetic analysis based on 16S rRNA genes revealed that SIJ1^T showed a distinct evolutionary lineage within the Firmicutes. The DNA G+C content was 43.1 mol% and the full genome analysis for strain SIJ1^T showed that it had a genome size of 3989945 bp and contained 4085 predicted protein-encoding genes. The SIJ1^T annotated genome showed more stress resistance encoding genes in comparison to its closely related strains. The amino acid identity and average nucleotide identity data for the whole genome proved that strain SIJ1^T does indeed represent a novel genus. The strain was distinguishable from the phylogenetically related genera through differences in several phenotypic properties. On the basis of the phenotypic, phylogenetic and genetic data, strain SIJ1^T represents a novel genus and species in the family Bacillaceae, for which the name *Litoribacterium kuwaitense* gen. nov., sp. nov. is proposed. The type strain is SIJ1^T (=DSM 28862^T=LMG 28316^T).

INTRODUCTION

Marine tidal flats shores are interface regions between marine and terrestrial habitats [1]. They are representatives of thalassohaline environments and are highly dynamic systems due to the rapid change in water during high tides [2] and periodic fluctuations in environmental parameters, such as temperature, ion concentration, desiccation, UV-irradiation and wave action [1]. Nevertheless, tidal flat sediments are characterized by high primary production, high microbial activities [3], high sedimentation rate, vertical chemical gradients and gradual decrease in oxygen concentration within a few millimetres below the sediment surface [4]. All these factors participate in the presence of a diverse bacterial community in tidal flat sediment. Various members of the phylum Firmicutes, family Bacillaceae were reported among the tidal flat bacteria [5]. The family Bacillaceae is an ecophysiologically diverse group with members able to survive at different temperature and salinity levels [6]. There are various mechanisms by which the family members can survive such conditions, one of them is the ability to produce compatible solutes such as glycine betaine that functions as a heat, cold and osmosis stress protectant simultaneously [7]. Other compatible solutes have a specific function, such as ectoine, which can act as an osmoprotectant [8] and has many applications in industry and medicine [9]. Here we report *Litoribacterium kuwaitense* gen. nov., sp. nov., typified by strain SIJ1^T, as a new genus of the family Bacillaceae that is rich with stress resistance encoding genes and isolated from a tidal flat of the Arabian Gulf, a water body known for its extreme environmental conditions [10].

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Keywords: *Litoribacterium kuwaitense*; Kuwait; tidal flat; Arabian Gulf; Firmicutes.
Abbreviations: AAI, amino acid identity; ANI, average nucleotide identity; BSA, basal marine agar; MA, marine agar; ML, maximum-likelihood; MP, maximum-parsimony; NJ, neighbour-joining; TYGS, Type (Strain) Genome Server.
The GenBank accession number for the 16SrRNA gene sequence of strain SIJ1^T is MT003183. The GenBank/DDBJ/ENA accession number of the whole genome sequence of strain SIJ1^T is JAALFC000000000.
Ten supplementary figures are available with the online version of this article.

ISOLATION AND ECOLOGY

In the current study, the taxonomic position of strain SIJ1^T, isolated from tidal flat sediment collected from the Sabiya coast (north of Kuwait Bay, Kuwait; northwest Arabian Gulf; 29°34'28.7"N 48°02'09.9"E) was determined. Culturable bacteria were originally isolated from homogenized sediment treated with lysozyme as part of a study targeting isolating Archaea from mudflats shores [11]. One gram of the homogenized sediment samples was serially diluted with 6% sterile saline (NaCl; equivalent to the salinity level measured at the sampling sites) and diluents of 10^{-1}, 10^{-3} and 10^{-5} were used in plating. One hundred microlitres of each diluent was inoculated into modified marine agar (MA) supplemented with vitamins (g l^{-1}: 0.7 KCl, 0.1 KBr, 0.04 $SrCl_2{\cdot}6H_2O$, 0.03 H_3BO_3, 0.005 $NaSiO_3{\cdot}9H_2O$, 0.003 NaF, 0.002 NH_4NO_3, 0.001 $Fe_3PO_4{\cdot}4H_2O$, 1.013 $CaCl_2{\cdot}6H_2O$, 4.0 Na_2SO_4, 11.0 $MgCl_2{\cdot}6H_2O$, 60.0 NaCl, 1.0 yeast extract, 5.0 peptone, 10.0 tryptone and 15.0 agar). The pH was adjusted to pH 7.6 and two vitamins were added [0.8 ml of 1 mg ml^{-1} thiamine (Sigma) and 0.1 ml of 1 mg ml^{-1} biotin (Sigma)] [12]. The media pH and salinity as well as the incubation temperature were equivalent to that of the natural environment from which the samples were collected. All plates were incubated at 37 °C for 48 h. The novel strain SIJ1^T was purified and maintained at 37 °C on 6% (w/v) NaCl MA. Stocks of the purified culture were preserved as suspensions at Microbank (Pro-Lab diagnostics, Wirral, UK) and stored at −80 °C.

Several morphologically distinct single colonies were individually recultured into new media and purified. Strain SIJ1^T developed small colonies that were 1–2 mm in diameter, entire and convex in shape, and opaque and cream in colour (Fig. S1, available in the online version of this article).

PHENOTYPIC AND CHEMOTAXONOMIC CHARACTERIZATION

Cell morphology was determined by phase contrast microscopy (Zeiss Axio), variable-pressure field-emission scanning electron microscopy (Leo supra 50V, Zeiss) and transmission electron microscopy (JEM-1200EX II, JEOL) (Fig. S2). Cells of strain SIJ1^T were non-flagellated, sporulating rod shape. The cell length (μm; average (minimum–maximum) SD] was 3.6 (2–6) 1.3 and the cell width was 1.2 (0.9–2) 0.3 (Figs. S2a, b). The spores were ellipsoidal in parasporal position within a non-swollen sporangium (Fig. S2c). For comparison, some of the investigations were performed with *Aureibacillus halotolerans* S1203^T and *Bacillus acidicola* 105-2^T (from the Deutsche Sammlung von Mikroorganismen und Zelkulturen) as reference strains (Tables 1 and 2). The motility test was executed using the hanging-drop technique. Strain SIJ1^T and *B. acidicola* 105-2^T were non-motile and had no flagella when cells were stained using the flagella staining method [13, 14], while *A. halotolerans* S1203^T was motile with a peritrichous flagella type.

Growth of strain SIJ1^T was tested under different NaCl concentrations in the range of 0–15% (w/v), in increments of 1%. Growth of the strain was also tested at temperatures ranging from 5–55°C and pH values ranging from pH 4 to 11. The isolate grew successfully with NaCl concentrations from 0 to 15%, pH between pH 6 and 10 and temperature between 25 and 42 °C (failed to grow above 42 °C), with optimal growth with 6% (w/v) NaCl, at 30 °C and at pH 7–8. In comparison to SIJ1^T, the reference strain *A. halotolearns* S1203^T was unable to grow at pH 6 and the minimum pH it grew at was pH 7. *A. halotolearns* S1203^T was able to grow at 4 °C. On the other hand, the reference strain *B. acidicola* 105-2^T was able to grow at temperatures from 15 to 45 °C, pH from pH 4 to 7 and NaCl concentration from 0 to 3% only.

The detailed physiological and biochemical characteristics of strain SIJ1^T are given in Tables 1 and 2, as well as the genus and species descriptions. A series of physiological and biochemical characteristics could distinguish strain SIJ1^T from the reference strains *A. halotolerans* S1203^T and *B. acidicola* 105-2^T as well as other related genera. The phenotypic characterization was carried out using GEN III MicroPlates (Biolog) and API 20NE, API ZYM and API 50CH strips (bioMérieux) following the manufacturers' methods. The ability of strain SIJ1^T to grow on crude oil in addition to various carbon sources equivalent to those tested by the Biolog system was confirmed by growing the strain on basal marine agar (BMA) prepared as described in González *et al.* [15] and supplemented with 0.1 % w/v or v/v of the tested carbon source and NH_4Cl as a nitrogen source. The inoculated plates were incubated at 30 °C for 1 week. Furthermore, strain SIJ1^T was inoculated on MA and incubated at 30 °C inside a Mitsubishi Gas Chemical anaerobic system for 2 weeks to determine the strain anaerobic growth ability. In addition, catalase and oxidase activities were determined according to Smibert and Krieg [16]. In the API 20NE strip, there were positive results for aesculin hydrolysis, maltose assimilation and β-galactosidase. The API 50 CHE strip showed fermentation and production of acid from the following carbohydrates ribose, D-xylose, methyl α-d-glucoside, amygdalin, arbutin, aesculin, salicin, cellobiose, maltose, xylitol and gentiobiose in addition to weak positive reactions for glucose, methyl α-d-mannoside, *N*-acetyl-glucose, lactose and trehalose. The API ZYM result showed a weak positive result for β-galactosidase. In the Biolog GEN III MicroPlate system, there were positive results for maltose, trehalose, cellobiose, gentiobiose, melibiose, D-salicin, inosine, 1% sodium lactate, glycerol, nalidixic acid, lithium chloride, sodium butyrate, *myo*-inositol and D-glucuronic acid; and weak positive results for glucose, raffinose, turanose, D-serine, stachyose, *N*-acetyl-D-galactosamine, dextrin, acetic acid, acetoacetic acid, troleandomycin, aztreonam, guanidine HCl, tetrazolium violet, tetrazolium blue and potassium tellurite. The BMA results confirmed the Biolog GEN III results and showed the inability of strain SIJ1^T to grow on BMA supplied with crude oil as a sole carbon source.

The ability of strain SIJ1^T to precipitate calcium carbonate was tested by growing it on B4 at pH 7.6 [17]. The plates were incubated for 1 month at 30 °C and were examined for calcium carbonate precipitation under stereomicroscope

Table 1. Key characteristics of strain SIJ1^T in comparison with the reference strains

Strains: 1, SIJ1^T; 2, *Aureibacillus halotolerans* S1203^T; 3, *Bacillus acidicola* 105-2^T; 4, *Bhargavaea cecembensis* DSE10^T; 5, *Bacillus marinisedimentorum* KCTC 33721^T; 6, *Virgibacillus sediminis* YIM kkny3^T. +, Positive; w, weak positive; –, negative; ND, not determined.

Characteristic	1	2	3	4	5	6
Source	Current study	Current study and [38]	Current study and [24]	[65]	[55]	[66]
Colony colour	Cream	Creamy white and shiny	White and little bit creamy	Brown	ND	Creamy white to pale yellow
Sporulation	+	+	+	–	–	+
Cell shape	Rods	Rods	Rods	Rods	Rods	Rods
Gram stain	+	+	+	+	+	+
Motility	–	+	–	–	+	+
Flagella	No	Peritrichous	No	No	Peritrichous	Peritrichous
Anaerobic	–	–	–	–	+	–
Growth in/at:						
4 °C	–	+	–	–	–	–
42 °C	+	+	+	+	+	+
0% NaCl	+	+	+	+	–	–
15% NaCl	+	+	–	–	–	+
pH 6	+	–	+	–	–	+
pH 10	+	+	–	–	–	+
Nitrate reduction	–	–	–	+	+	+
Oxidase	+	+	–	+	–	+
Catalase	+	+	+	+	–	+
Urease	–	–	–	+	–	–
Gelatinase	–	+	–	–	–	+
Amylase	–	+	+	–	ND	–
Caseinase	–	–	–	ND	ND	–
Hydrolysis of:						
Aesculin	+	+	–	–	+	+
Tween 80	–	w	–	–	–	–
Indole production	–	–	–	–	–	–
Citrate utilization	–	–	+	+	–	–
Acid production from:						
D-Glucose	w	–	+	–	+	+
D-Mannose	–	–	+	–	+	+
D-Fructose	–	–	+	–	+	+
Maltose	+	–	+	–	+	NR
Major isoprenoid quinones	MK-7	MK-7	MK-7, MK-6, MK-5, MK-4, MK-3	MK-8, MK-6	MK-7	MK-7

Continued

Table 1. Continued

Characteristic	1	2	3	4	5	6
Major lipids‡	PG, DPG, GL, PL, PGL,L	PG, DPG, GL	PG, DPG, GL, PE	PG, DPG	DPG, PG, PE	DPG, PG, PE, PL
DNA G+C content (mol%)	43.1*	47.7†	43.2†	59.5†	46.3*	40.9†

*G+C content determined from whole genome sequence.
†G+C content determined by reverse-phase HPLC [67].
‡DPG, diphosphatidylglycerol; PG, phosphatidylglycerol; GL, glycolipid; PL, phospholipid; PGL, phosphoglycolipid; PE, phosphatidylethanolamine; L, lipid.

supplied with camera. The calcium carbonate crystals were harvested following the method described by Mahmoud [18] and examined directly under the Leo Supra 50V variable-pressure field-emission scanning microscope (Zeiss). The isolate was able to precipitate $CaCO_3$ crystals of various shapes and sizes (Fig. S3) and could be exploited in various biotechnological applications including the removal of heavy metals, biodegradation of pollutants, sequestering atmospheric CO_2 and remediation of buildings [19].

The resistance of strain SIJ1^T to antibiotics was tested by the Identification Service of the Deutsche Sammlung von Mikroorganismen und Zelkulturen (DSMZ) in Germany and following their protocol [20]. SIJ1^T cells were found to be resistant to the following antibiotics (μg disk^{-1}): erythromycin (15), oflaxin (5), norfloxacin (10), colistin (10), pipemidic acid (20), fosfomycin (50), moxifloxacin (5), polymyxin B (300 IU disk^{-1}) and nystatin (100 IU disk^{-1}).

The cellular fatty acid composition was determined using Sherlock Microbial Identification System (MIDI) as described by Sasser [21]. The cellular fatty acids were analysed in culture at the late exponential stage of growth after growing the isolate on MA incubated at 30 °C for 48 h. The fatty acid profiles of strain SIJ1^T and two reference strains *A. halotolerans* S1203^T and *B. acidicola* 105-2^T are presented in Table 2. The major

Table 2. Comparison of the fatty acids of strain SIJ1^T and the reference strains

Strains: 1, SIJ1^T; 2, *Aureibacillus halotolerans* S1203^T; 3, *Bacillus acidicola* 105-2^T. Only fatty acids with results higher than 0.5% in at least one of the tested strains are listed

Cellular fatty acids	1	2	3
Straight-chain:			
$C_{14:0}$	0.7	2.3	1.3
$C_{16:0}$	2.8	7.5	4.4
$C_{17:0}$	–	1.4	–
Branched-chain:			
iso-$C_{14:0}$	7.1	4.7	3.3
iso-$C_{15:0}$	11.0	13.3	43.1
anteiso-$C_{15:0}$	47.4	66.3	18.7
iso-$C_{16:0}$	19.4	1.9	8.5
anteiso-$C_{17:1}$ A	0.7	–	–
iso-$C_{17:0}$	0.7	0.6	7.9
anteiso-$C_{17:0}$	3.2	1.0	12.4
Unsaturated:			
$C_{16:1}$ ω11*c*	–	0.6	–
Summed feature 2*	2.0	–	–
Summed feature 3*	4.5	–	–

*Summed feature 2: iso-$C_{16:0}$ I, $C_{14:0}$-3OH; summed feature 3: $C_{16:1}\omega 6c$, $C_{16:1}\omega 7c$.

fatty acids (>10%) of strain SIJ1^T were anteiso-$C_{15:0}$ (47.4%), iso-$C_{16:0}$ (19.4%) and iso-$C_{15:0}$ (11.0%). The major fatty acids of *A. halotolerans* S1203^T were anteiso-$C_{15:0}$ (66.3%) and iso-$C_{15:0}$ (13.3%), while *B. acidicola* 105-2^T was dominated by iso-$C_{15:0}$ (43.1%), anteiso-$C_{15:0}$ (18.7%) and anteiso-$C_{17:0}$ (12.4%).

The extraction and the analysis of polar lipids and respiratory quinones of strain SIJ1^T were carried out by the Identification Service of the DSMZ in Germany by Dr. Susanne Verbarg following Tindall's [22, 23] methods. Strain SIJ1^T possessed diphosphatidylglycerol, phosphatidylglycerol, an unidentified glycolipid, an unidentified phospholipid, phosphoglycolipid and an unidentified lipid as polar lipids (Fig. S4). The last three types of lipid were only detected in SIJ1^T and not in the reference strains (Table 1).

Strain SIJ1^T contained an unsaturated menaquinone with seven isoprene units (MK-7) as the predominant respiratory quinone. Comparing the data obtained for SIJ1^T with *B. acidicola* 105-2^T published elsewhere [24] showed differences in their major isoprenoid quinones where the reference strain contains MK-3, MK-4, MK-5, Mk-6 and MK-7.

The peptidoglycan of the SIJ1^T cell wall was analysed according to Schumann [25] and it showed that the cell-wall peptidoglycan was based on *meso*-diaminopimelic acid. The strain SIJ1^T whole-cell sugars were also analysed using Schumann's protocol [25]. The results showed that strain SIJ1^T contains ribose and, in smaller amounts, xylose, mannose and glucose.

16S RNA GENE PHYLOGENY

The complete 16S rRNA gene sequence of SIJ1^T was determined by direct sequencing of its PCR-amplified 16S rRNA gene. Genomic DNA extraction was carried out using the MasterPure Gram Positive DNA Purification Kit (Epicenter Biotechnologies) according to the manufacturer's instructions. PCR-mediated amplification of the 16S rRNA gene and purification of the PCR product was carried out as describe in Rainey *et al.* [26]. Purified PCR products were sequenced using the CEQ DTCS-Quick Start Kit (Beckman Coulter) as directed in the manufacturer's protocol. Sequence reactions were electrophoresed using the CEQ 8000 Genetic Analysis System (Beckman Coulter.

The length of the 16S rRNA gene sequence of strain SIJ1^T was a continuous stretch of 1540 bp. Comparing the 16S rRNA gene of the SIJ1^T with corresponding sequences in the GenBank, EMBL and RDP II databases indicated that strain SIJ1^T is a member of the phylum Firmicutes, class Bacilli, order Bacillales, family Bacillaceae. A comparative sequence analysis of 16S rRNA genes demonstrated 95.8% similarity between strain SIJ1^T and *A. halotolerans* S1203^T and 94.3% similarity between SIJ1^T and *B. acidicola* 105-2^T. A phylogenetic tree was reconstructed by multiple sequence alignment of the strain SIJ1^T sequence with its closest relatives in the GenBank. Pairwise sequence similarities were calculated using the method recommended by Meier-Kolthoff *et al.* [27] for the 16S rRNA gene available via the Genome-to-Genome Distance Calculator (GGDC) web server [28] available at http://ggdc.dsmz.de/. Phylogenies were inferred by the GGDC web server available at http://ggdc.dsmz.de/ using the DSMZ phylogenomics pipeline [29] adapted to single genes. A multiple sequence alignment was created with MUSCLE [30]. Maximum-likelihood (ML) and maximum-parsimony (MP) trees were inferred from the alignment with RAxML [31] and TNT [32], respectively. For ML analysis, rapid bootstrapping in conjunction with the autoMRE bootstopping criterion [33] and subsequent search for the best tree was used; for MP, 1000 bootstrapping replicates were used in conjunction with tree-bisection-and-reconnection branch swapping and ten random sequence addition replicates. The sequences were checked for a compositional bias using the X^2 test as implemented in PAUP* [34]. The input nucleotide matrix comprised 24 operational taxonomic units and 1579 characters, 315 of which were variable and 239 of which were parsimony-informative. The base-frequency check indicated no compositional bias (P=1.00, α=0.05). ML analysis under the GTR+GAMMA model yielded a highest log likelihood of −6372.3, whereas the estimated alpha parameter was 0.1. The ML bootstrapping converged after 1000 replicates; the average support was 74.1%. MP analysis yielded a best score of 869 (consistency index 0.5, retention index 0.6) and 16 best trees. The MP bootstrapping average support was 78.8% (Fig. 1). Furthermore, all sequences were edited manually using BioEdit [35] then aligned with their nearest neighbour from the GenBank using ClustalX [36]. Neighbour-joining (NJ) method including bootstrap (2000×) analysis [37] was used via PAUP version 4 software [34] to construct the phylogenetic tree (Fig. S5).

The phylogeny analysis showed that strain SIJ1^T forms a distinct phylogenetic lineage with respect to the closely related genera *Bacillus*, *Bhargavaea*, *Planocomicrobium* and *Virgibacillus* (Figs 1 and S5). Strain SIJ1^T shared common ancestor with *A. halotolerans* S1203^T.

GENOME FEATURES AND COMPARATIVE GENOMICS

The whole genome of SIJ1^T was determined. The genome was extracted using the PrepMan Ultra Sample Preparation Reagents (ThermoFisher) according to the manufacturer's instructions and the sample quality check was performed using Qubit DNA BR assay kit. The NEBNEXT Fast DNA Library Prep Set for Ion Torrent (Biolabs Inc.) and the KAPA Library Quantification Kit were used. The whole-genome was sequenced with Ion Torrent using Ion PI Hi-Q sequencing 200 kit and Ion PI chip kit version 3 (ThermoFisher Scientific) at SEQme (Dobříš, Czech Republic). The G+C content of the strain SIJ1^T DNA based on the whole genome sequencing was 43.1 mol%, close to *A. acidicola* 105-2^T (42 mol%) [24] but lower than that of *A. halotolerans* S1203^T (47.7%) [38].

In total, 4.3 million single reads were produced with an average length of 177 bases. The SPAdes genome assembler version 3.1.0 was used to perform a *de novo* genome assembly,

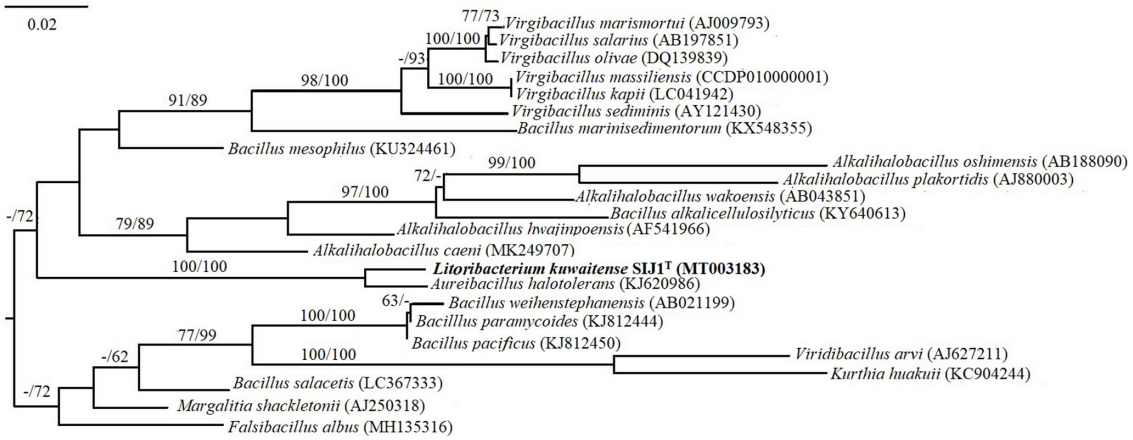

Fig. 1. Maximum-likelihood (ML) and maximum-parsimony (MP) phylogenetic analysis based on 16S rRNA gene sequences for strain *Litoribacterium kuwaitense* SIJ1^T (MT003183) and related genera. ML/MP tree inferred under the GTR+GAMMA model and rooted by midpoint-rooting. The branches are scaled in terms of the expected number of substitutions per site. The numbers above the branches are support values when larger than 60% from ML (left) and MP (right) bootstrapping.

which yielded 949 contigs where 108 contigs had length larger than 500 bp, N50 values equal to 78202 bp and the longest contig measured 348688 bp. The whole genome analysis of strain SIJ1^T showed that the bacterium has a genome size of 3989945 bp and contains 4085 predicted protein-encoding genes and 149 predicted RNAs. The authenticity and contamination of the whole genome sequence of strain SIJ1^T were checked as indicated by Chun *et al.* [39] and executed using EzBioCloud Contamination Estimator by 16S (ContEst16S) [40], where the SIJ1^T genome was confirmed to be free of contamination. The quality of the strain SIJ1^T genome was analysed using CheckM version 1.1.18 [41], which showed 97.3% completeness and only 0.2% contamination in the genome (Fig. S6). Genome features were visualized using the CGView server [42]

The amino acid identity (AAI) of strain SIJ1^T and its relation to various Uniport species was investigated using an AAI profiler [43]. The results showed that the closest match to SIJ1^T is *A. halotolerans* S1203^T (Fig. S7a), with a 69.8 % AAI value. However, when the comparison was made between SIJ1^T and *A. halotolerans* S1203^T on a one-to0one basis using the Kostas lab AAI calculator [44], the results showed that the two genomes share 63.7% AAI only (Fig. S7b). The Kostas lab averagenucleotide identity (ANI) calculator made it easy to compare the genome of SIJ1^T with that of *A. halotolerans* S1203^T, and the similarity between them was 75.3%. Both AAI and ANI readings are below the threshold of 70 and 80%, respectively, for genus delineation described by Rodriguez-R and Konstantinids [44].

The relatedness of strain SIJ1^T on the whole genome level to its nearest match from the GenBank was investigated. The genome sequence data were uploaded to the Type (Strain) Genome Server (TYGS), a free bioinformatics platform available under https://tygs.dsmz.de, for a whole genome-based taxonomic analysis [45]. Determination of closest type strain genomes was done in two complementary ways. First, all user genomes were compared against all type strain genomes available in the TYGS database via the MASH algorithm, a fast approximation of intergenomic relatedness [46], and the ten type strains with the smallest MASH distances chosen per user genome. Second, an additional set of ten closely related type strains was determined via 16S rRNA gene sequences. These were extracted from the user genomes using RNAmmer [47], and each sequence was subsequently BLASTed [48] against the 16S rRNA gene sequence of each of the currently 13787 type strains available in the TYGS database. This was used as a proxy to find the 50 best-matching type strains (according to the bitscore) for each user genome and to subsequently calculate precise distances using the genome BLAST distance phylogeny (GBDP) approach under the algorithm 'coverage' and distance formula *d5* [28]. These distances were used to determine the 10 closest type strain genomes for each user genomes. For phylogenomic inference, all pairwise comparisons among the set of genomes were conducted using GBDP and accurate intergenomic distances inferred under the algorithm 'trimming' and distance formula *d5* [28]. 100 distance replicates were calculated each. Digital DNA–DNA hybridization values and confidence intervals were calculated using the recommended settings of the GGDC 2.1 [28]. The resulting intergenomic distances were used to infer a balanced minimum-evolution tree with branch support via FASTME 2.1.4, including SPR postprocessing [49]. Branch support was inferred from 100 pseudobootstrap replicates each. The trees were rooted at the midpoint [50] and visualized with PhyD3 [51]. The result of the phylogenomic tree clustered strain SIJ1^T with the *Bacillus marinisedimentorum* KCTC 33721^T and far away from *A. halotolerans* S1203^T (Fig. 2) despite the 16S rRNA tree produced by the TYGS database clustering strain SIJ1^T with *A. halotolerans* S1203^T (Fig. 1). It

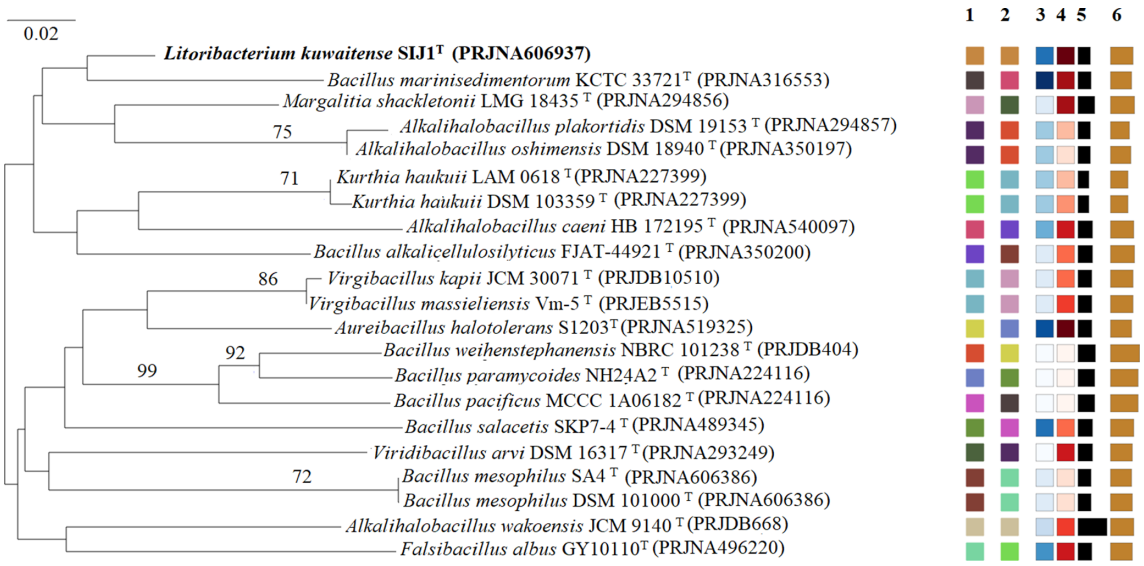

Fig. 2. Tree inferred with FastME 2.1.6.1 from GBDP distances calculated from whole genome sequences of strain *Litoribacterium kuwaitense* SIJ1^T (PRJNA606937) and 20 related genomes. Sequence length was 1540 bp. The branch lengths are scaled in terms of GBDP distance formula *d5*. The numbers above branches are GBDP pseudo-bootstrap support values >60% from 100 replications, with an average branch support of 35.2%. The tree was rooted at the midpoint. The bar is equal to 0.02 of the phylogenetic distance. The corresponding genome project numbers are included between brackets. 1, Species cluster; 2, subspecies cluster; 3, G+C content; 4, delta statistics; 5, genome size; 6, protein count.

is worth mentioning that the AAI value between strain SIJ1^T and *B. marinisedimentorum* KCTC 33721^T is only 54.5%. The AAI and ANI results and the phylogenomic and phylogenetic analysis for the genome all proved that strain SIJ1^T does indeed a novel genus.

The functional annotation for the SIJ1^T genome was achieved by the Rapid Annotation using Subsystem Technology (RAST) server [52–54]. Among the coding sequences, the dominant sequences belong to conserved processes such as those of metabolic, cellular and regulatory processes (Fig. S8). The majority of the annotated genes belong to carbohydrates>amino acids and derivatives>proteins metabolism subsystems. The majority of the carbohydrate sequences belong to the central carbohydrate metabolism and was dominated by the glycolysis and the gluconeogenesis coding sequences. The second major group of sequences in the subsystems, i.e. amino acids and derivatives, was dominated by lysine, threonine, methionine, cysteine biosynthesis and degradation genes. Protein biosynthesis genes dominated the third major group, i.e. proteins metabolism subsystem.

The strain SIJ1^T genome contained stress responses coding genes within six subcategories, i.e. heat shock, cold shock, detoxification, osmotic stress, oxidative stress and unknown subcategories. Upon comparison to *A. halotolerans* S1203^T and *B. marinisedimentorum* KCTC 33721^T genomes using RAST annotation, the SIJ1^T genome was the only one that contained cold shock and heat shock coding genes, and in total, it included 114 coding genes within the six subcategories of the stress-resistant subsystem while *A. halotolerans* S1203^T contained 49 and *B. marinisedimentorum* KCTC 33721^T contained 57 genes. *A. halotolerans* S1203^T was isolated from marine sediment at a water depth of almost 865 m in the Okinawa Trough [38] while *B. marinisedimentorum* was isolated from a coastal sediment sample from Weihai, PR China [55]. On the other hand, strain SIJ1^T was isolated from an Arabian Gulf's tidal flat, an environment known for its extreme high fluctuations in temperature and high salinity level due to a low precipitation (low rainwater fall) and high evaporation rates [10]. Harbouring higher diversity of stress-encoding genes may present a potential survival mechanism for microflora in such harsh conditions.

Although both SIJ1^T and *A. halotolerans* S1203^T can grow with NaCl ranging from 0 to 15%, the SIJ1^T genome includes more genes related to osmotic stress, especially choline and betaine uptake and betaine biosynthesis as well as ectoine biosynthesis and regulation genes (Fig. S9). Both betaine and ectoine are organic solutes that can be accumulated intracellularly when osmolarity rises and is released extracellularly when it decreases helping the bacteria survive [56]. SIJ1^T genome analysis revealed the presence of genes encoding enzymes of the ectoine biosynthesis pathway, i.e. l-2,4-diaminobutyric acid acetyltransferase (EC 2.3.1.-), diaminobutyratepyruvate aminotransferase (EC 2.6.1.46) and L-ectoine synthase (EC 4.2.1.-) [57]. Ectoine is of special importance in industry where it is used as a protecting agent for macromolecules, cells and tissues, and as a therapeutic agent for specific diseases [58]. Furthermore, strain SIJ1^T showed a higher number of choline sulfatase coding genes, i.e. 18, compared to *A. halotolerans* S1203^T and *B. marinisedimentorum* KCTC 33721^T that included only one coding gene each. Choline sulfatase

encoded by the betC gene is the key enzyme for degrading choline-*O*-sulphate to choline, converted to glycine betaine, and catabolized further into ammonia and pyruvate [59]. Glycine betaine is an osmoprotectant found in many bacteria [60] and used as carbon, nitrogen, and energy sources like in the Gram-negative bacterium *Synrhizobium meliloti* [59]. Research done on *Bacillus subtilis* proved that glycine betaine can protect against cold and heat stress [7].

The annotated genome of strain SIJ1^T also suggested that it can uptake the environment's glycine betaine by specific transporter systems. The genome includes the operon that encodes a high-affinity ATP-binding cassette (ABC) transport system consisting of three proteins: (1) ProV, peripheral membrane protein found on the cytoplasmic side; (2) ProW, the integral membrane component of the transport system; and (3) the ProX periplasmic glycine betaine-binding protein (GBBP) [61–63]. Also, the genome includes OpuA, and OpuD ABC uptake systems. The OpuA system is the predominant transporter for glycine betaine. It consists of three components: an ATPase (OpuAA), an integral membrane protein (OpuAB) and a hydrophilic polypeptide (OpuAC), which functions as the GBBP, and all are found in SIJ1^T annotated genome. OpuD is essential for glycine betaine uptake and osmoprotection [61–63].

One interesting finding in the annotated SIJ1^T genome using RAST is the presence of motility and chemotaxis genes. The majority are related to flagellar motility, but morphological and microscopic examinations showed with with no doubt the absence of flagella in the examined strain. The functional annotations of the genome conducted using the KEGG Automatic Annotation Server [64], where GHOSTX homology software and KEGG orthology bi-directional best hit assignment method were used, showed that the flagellar assembly and bacterial chemotaxis gene clusters within the SIJ1^T genome are almost complete (Fig. S10).

Based on the comprehensive analysis of the phenotypic, chemotaxonomic and phylogenetic characteristics and the whole genome sequencing, strain SIJ1^T should be considered as representing new genus and new species for which we propose the name *Litoribacterium kuwaitense* gen. nov., sp. nov.

DESCRIPTION OF *LITORIBACTERIUM* GEN. NOV.

Litoribacterium (Li.to.ri.bac.te'ri.um. L. neut. n. *litus* shore; N.L. neut. n. *bacterium* a small rod; N.L. neut. n. *Litoribacterium* small rod isolated from shore).

Cells are Gram-stain-positive, non-motile, spore-forming and strictly aerobic rods. Oxidase- and catalase-positive. Unable to hydrolyse starch and Tweens 20, 40, 60 and 80. Unable to hydrolyse casein and gelatin. The predominant ubiquinone is MK-7 and the cell-wall peptidoglycan is based on *meso*-diaminopimelic acid. The major fatty acids are anteiso-$C_{15:}$, iso-$C_{16:0}$ and iso-$C_{15:0}$ and the major whole-cell sugar is ribose. The major polar lipids are diphosphatidylglycerol, phosphatidylglycerol, unidentified phospholipid, unidentified glycolipid, phosphoglycolipid and unidentified lipid. Phylogenetically, the genus *Litoribacterium* belongs to the family Bacillaceae in the class Bacilli. The type species is *Litoribacterium kuwaitense*.

DESCRIPTION OF *LITORIBACTERIUM KUWAITENSE* SP. NOV.

Litoribacterium kuwaitense (ku.wait.en'se, N.L. neut. adj. *kuwaitense* pertaining to the State of Kuwait, from where the type strain was isolated).

Litoribacterium kuwaitense exhibits the following properties to those given in the genus description. Cells are 2–6 µm long and 0.9–2 µm wide. Colonies on modified MA are small colonies that are 1–2 mm in diameter and are entire and convex in shape as well as being opaque and cream in colour. Growth occurs at 25–42 °C with optimum growth at 30 °C. Growth is observed at pH 6–10 with optimal growth at pH 7–8. Furthermore, the NaCl range for growth is 0–15% (optimum 6%).

Strain SIJ1^T is able to ferment and produce acid from the following carbohydrates ribose, D-xylose, methyl α-d-glucoside, amygdalin, arbutin, aesculin, salicin, cellobiose, maltose, xylitol and gentiobiose in addition to weak positive reactions for glucose, α-MeDmannoside, *N*-acetyl-glucose, lactose and trehalose. The strain showed the ability to assimilate maltose, trehalose, cellobiose, gentiobiose, melibiose, D-salicin, inosine, 1% sodium lactate, glycerol, nalidixic acid, lithium chloride, sodium butyrate, myo-inositol and D-glucuronic acid; and to weakly assimilate glucose, raffinose, turanose, D-serine, stachyose, *N*-acetyl-D-glactoseamine, dextrin, acetic acid, acetoacetic acid, troleandomycin, aztreonam, guanidine HCl, tetrazolium violet, tetrazolium blue and potassium tellurite.

The type strain, SIJ1^T (=DSM 28862^T=LMG 28316^T), was isolated from Sabiya mud flat sediment collected north of Kuwait Bay. The DNA G+C content of the strain is 43.1 mol% and the draft genome showed that the bacterium has a genome size of 3989945 bp and contains 4085 predicted protein-encoding genes.

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Author contributions
Writing: original draft, review and editing: H. M. Investigation: H. M., L. J., S. E. and F. A. Strain isolation: A. A. Formal Analysis and resources: H. M.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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