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Lelliottia Rebaudianalis Sp. Nov. Isolated From Wild *Stevia Rebaudiana Bertoni*

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Abstract

A Gram-stain-negative, aerobic, chemoheterotrophic bacterium, characterized with rod shape and mobility, designated as LST-1^{T}, was isolated from wild *Stevia rebaudiana Bertoni* and subjected to polyphasic taxonomic analysis. The LST-1^{T} strain grew optimally at 37 °C and pH 6.0–7.0 in the presence of 0.5% (w/v) NaCl. Phylogenetic sequence analysis based on 16S rDNA from LST-1 indicated that it is close to *Lelliottia jeotgali* (99.85%), *Lelliottia nimipressuralis* (98.82%), and *Lelliottia amnigena* (98.54%). Multi-locus sequence typing analysis of concatenated partial *recA*, *atpD*, and *infB* was performed to improve resolution, and clear distinctions between the closest related type strains were exhibited. Meanwhile, the results from average nucleotide identify analyses and DNA–DNA hybridization with four species (16S rDNA similarity > 98.65%) were less than 90% and 40% respectively, verifying the distinct characteristics from other species of *Lelliottia*, The cellular fatty acid profile of the strain consisted of C16:0, Summed Feature3, and Summed Feature8 (may be 16:1 w6c/16:1 w7c and 18:1 w6c) as major components. The major polar lipids included phosphatidylethanolamine, phosphatidylglycerol, aminophospholipid, three non-characteristic phospholipids, and a non-characteristic lipid. The genome of LST-1^{T} is 4,611,055 bp, with a DNA G + C content of 55.02%. Combination of several phenotypic, chemotaxonomic, and genomic characteristics proved that the LST-1^{T} strain does represent a novel genus, for which the name *Lelliottia sp.* LST-1 was proposed. The type strain is LST-1^{T} (= CGMCC 1.19175^{T} = JCM 34938^{T}).

Introduction

Lelliottia cells are straight rods, motile by peritrichous flagella, facultative anaerobic, and Gram-stain-negative. They have been identified in natural environments and foods. The taxonomy of *Enterobacter* has a complicated history, with several species transferred to and from this genus over the past 20 years. Based on the results of multi-locus sequence typing (MLST) analysis, DNA–DNA hybridization (DDH), phenotypic carbohydrate fermentation characteristics, and fatty acid profiles in cell-wall membranes, the species *E. amnigena* and *E. nimipressuralis* were assigned to a novel genus, for which the name *Lelliottia* was proposed in 2013 (Brady, Cleenwerck, Venter, Coutinho, & De Vos, 2013). Although they are in the same genus, their habitats are quite different. *L. nimipressuralis* is often found in trees or plants, and *L. amnigena* has been isolated from onion, raw milk, cream, cheese, onion, and Spanish pork sausage. Both are notably suspected to have pathogenic possibilities (Chow et al., 1991; Kämpfer et al., 2018; Mezzatesta, Gona, & Stefani, 2012; Morand et al., 2009). *L. amnigena* is a food contamination marker (García Fontán, Lorenzo, Parada, Franco, & Carballo, 2007; Liu & Tang, 2016), while *L. nimipressuralis* has clinical relevance, suggesting that it may be associated with pseudobacteremia (Kim et al., 2010). In addition, *L. amnigena* was isolated from a heart transplant recipient and a patient with endophthalmitis (Bollet, Elkouby, Pietri, & de Micco, 1991; Westerfeld, Papaliodis, Behlau, Durand, & Sobrin, 2009).

This study focused on the description of strain LST-1^{T}, isolated from wild *Stevia rebaudiana Bertoni* obtained from the stevia planting and production base in Dongtai City, Yancheng City, Jiangsu Province. *Lelliottia jeotgali* JCM31901 and *Lelliottia nimipressuralis* DSM 18955 were used as reference strain. The

new isolate represented a novel species of the genus *Lelliottia* on the basis of the taxonomical analysis and microbial/genomic characterizations presented in this study.

Materials And Methods

Isolation and cultivation

For isolation of strain LST-1^T, inorganic salt medium (KH_2PO_4 3 g/L, $MgSO_4$ 0.1 g/L, K_2HPO_4 1.5 g/L, $CaCl_2$ 0.01 g/L, EDTA disodium 0.01 g/L, pH 7.5) containing 10 g/L stevioside as the only carbon source was adopted as the selection medium to screen a mass of endophytic bacterium from *S. rebaudiana Bertoni* that could use stevioside (Xu, Feng, Wang, & Lin, 2013). Then, esculin-MS medium was used for a second screening campaign to isolate β-glucosidase-producing endophytic bacteria in accordance with the method described by Xuan et al., with some modifications (Xuan, 2012). MS medium with double agar was supplemented with 0.2% esculin (6,7-2-hydroxy-coumarin–β-D-glucoside) and 0.6% $FeCl_3$ of the same volume. Small wells (0.3–0.6 cm) were punched out in plates, loaded with 100 μL of bacterial broths, and incubated at 37°C until the wells were surrounded by black or dark brown (Pérez et al., 2011), and then a strain named LST-1^T was obtained. The purified strain culture was added with glycerol to the concentration of 25% (v/v) and frozen at −80°C for long-term storage.

Morphological, physiological, and biochemical characteristics

Cell morphology was determined using scanning electron microscopy (SEM) (Jung et al., 2013). Individual bacterial colonies were activated to the logarithmic stage, and 1 mL broths were taken out and centrifuged at 8000 rpm for 1 min. Then, 850 μL of the medium was discarded, and 150 μL of polypeptide was added. The mixtures were incubated at 37°C for 30 min. After centrifugation at 8000 rpm for 1 min, the supernatant was discarded, and 2.5% glutaraldehyde was added. The solutions were mixed, fixed overnight at 4°C, and then centrifuged at 8000 rpm for 1 min to remove the supernatants. The precipitates were washed three times with PBS and then dehydrated by 30%, 50%, 70%, 80%, and 90% gradient ethanol. Finally, the precipitates were washed twice and suspended with 100% ethanol. After CO_2 critical point drying and ion sputtering were conducted, the bacteriological samples were observed by SEM.

The growth at different temperatures was measured at 4°C, 10°C, 15°C, 20°C, 25°C, 28°C, 30°C, 35°C, 37°C, 40°C, 42°C, 45°C, and 48°C in LB medium, with 2 weeks of incubation. The tolerance with different NaCl concentrations (0%, 0.5%, 1.0%, 1.5%, 2.0%, 2.5%, 3.0%, 3.5%, 4.0%, 5.0%, 6.0%, 7.0%, 8.0%, 9.0%, 10.0%, 11.0%, and 12.0%, w/v) on LB medium, which was prepared in accordance with LB formula but without NaCl. The pH range for growth was tested at pH 4.0–10.0 with intervals of 0.5 pH units by using different buffers (40 mM, acetate buffer solution for pH 4.0–4.5, MES for pH 5.0–5.5, MOPS for pH 6.0–7.5, tricine buffer for pH 8.0–8.5, and CAPSO for pH 9.0–10.0) to maintain the pH (Pu et al., 2021). All experiments were carried out in triplicate, the absorbance at OD 600nm was measured in a UV/visible

spectrophotometer (UV756CRT, Shanghai Youke Instrument Co., Ltd.), and the condition or range of the highest absorbance value was set as the optimum growth condition for the strain LST-1^{T}.

Catalase activity was evaluated by assessing bubble formation in 3 % (v/v) hydrogen peroxide, and oxidase activity was tested by 1% N,N-dimethyl-p-phenylenediamine dihydrochloride, with observations on changes in color. Other physiological and biochemical tests were performed using API 20NE.

Chemotaxonomic analyses

Strain LST-1^{T}, *L. jeotgali* JCM31901, and *L. nimipressuralis* DSM 18955, which were grown on LB medium for 24 h at 37°C, were used for polar lipid and isoprenoid quinone analysis. Polar lipids were extracted using a chloroform/methanol system and analyzed using one- and two-dimensional TLC in accordance with Cui et al. (Cui, Gao, Yang, & Xu, 2011) and Han et al. (Han et al., 2016). The first dimension of the solvent system was chloroform:methanol:water (13:5:0.8, v/v), and the second dimension was chloroform:methanol:acetic acid:water (16:2.4:3:0.8, v/v). In accordance with Tindall (Tindall, 1990), α-naphthol, ninhydrin, molybdenum blue, and 5% phosphomolybdic acid were used to detect glycolipids, aminolipids, phospholipids, and total lipids, respectively. Isoprenoid quinones were extracted from freeze-dried cells with chloroform:methanol (2:1, v/v) and analyzed by reversed-phase HPLC. Cellular fatty acid methyl esters (FAMEs) were prepared in accordance with Kuykendall et al. (KUYKENDALL, ROY, apos, NEILL, & DEVINE, 1988), and their identification and quantification were performed using the Sherlock Microbial Identification System (MIDI) with standard MIS Library Generation software (Microbial ID).

Genome features

Whole genome (estimated size of 4 M) was sequenced on the basis of the Nanopore sequencing technology platform (Beijing Biomarker Biotechnology Co., Ltd.) (Deamer, Akeson, & Branton, 2016). The sequencing depth was ≥ 100×, with 0 gap. The test was performed in accordance with the standard provided by Oxford Nanopore Technologies (ONT). Complete genome sequence analysis was performed by Biomarker Technologies.

Canu v1.5 software was used to assemble the filtered subreads, and Pilon software was utilized to use the second-generation data to further correct the assembled genome to obtain the final more accurate genome.

In accordance with the minimum standard proposed by Chun et al. (Chun et al., 2018), the average nucleotide identity (ANI) and DDH values were calculated using the ANI calculator (http://enve-omics.ce.gatech.edu/ani/) and DDH calculator (http://ggdc.dsmz.de/ggdc.php), respectively. The G + C content of strain LST-1^{T} was analyzed with the RAST server using the complete genome sequence (Aziz et al., 2008).

16S rRNA phylogeny

The genomic DNA was extracted in accordance with the method described in the quick bacterial genomic DNA extraction kit. The 16S rRNA gene was amplified using the universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') (Weisburg, Barns, Pelletier, & Lane, 1991), and sequencing analysis was conducted by Sangon Biotech. On the basis of 16S rRNA gene sequence analysis using NCBI and EzBioCloud (Yoon et al., 2017), closely related organisms were determined. Comparative phylogenetic tree analysis of 16S rRNA gene sequences was performed with mega6 program (Tamura et al., 2011; Tamura, Stecher, Peterson, Filipski, & Kumar, 2013) using the maximum-likelihood method, neighbor-joining method, and the maximum-parsimony method (Bryant, Fischer, Linz, & Semple, 2017; Hall, 2013). The bootstrap value was calculated on the basis of 1000 replicates to obtain the confidence levels for the branches.

MLST analysis

MLST analysis with three housekeeping genes, namely, recombinase (*recA*), ATP synthase β subunit (*atpD*), and initiation translation factor 2 (*infB*), was performed to distinguish and further understand the taxonomic relationship between *Lelliottia* and *Enterobacter* and support the 16S rDNA phylogenetic trees of strain LST-1^T. The DNA sequences of the three genes were assembled in the order of *recA-atpD-infB*.

Results And Discussion

Phylogenetic analysis

Phylogenetic analysis based on 16S rRNA gene indicated that the strain LST-1^T shared the highest similarity with *L. jeotgali* (99.85%), followed by *L. nimipressuralis* (98.82%) and *Lelliottia amnigena* (98.54%). *Lelliottia* and *Enterobacter* belong to the same family. However, the phylogenetic tree using 16S rRNA gene sequences (Fig. 1a) did not differentiate *Lelliottia* strains from *Enterobacter* strains, probably due to low resolution.

The MLST-based comparative phylogenetic tree analysis method based on *recA-atpD-infB* sequence was conducted using the same methods as the 16S rRNA gene sequence analysis. The strain LST-1^T was closest to *L. jeotgali* in a separate small group of the 16S rRNA gene and MLST-based phylogenetic trees (Figs. 1a and 1b). In accordance with the phylogenetic tree, two type strains were obtained from culture collections, used for comparison, and characterized in parallel with the novel isolates *L. jeotgali* JCM31901 and *L. nimipressuralis* DSM 18955.

Genomic features

The genome of *Lelliottia sp.* LST-1^T is composed of a chromosome with 4,611,055 base pairs and a plasmid with 39,319 base pairs. The total length of the predicted coding gene sequence is 4,140,156. Gene function annotation analysis was conducted after BLAST with functional databases, such as COG, Kyoto Encyclopedia of Genes and Genomes, Swiss-Prot, and Non-Redundant Protein Database. The

whole genome sequence was uploaded to NCBI (https://www.ncbi.nlm.nih.gov/), and the accession number is CP063663.

ANI analysis, DDH analysis, and G + C content calculation (Yuk, Kim, Huh, & Lee, 2018) were conducted for strains with similarities > 98.5%. ANI values < 95% correspond to DDH values < 70% for identification of a novel bacterial species (Lawrence, G., & Wayne %J Zentralblatt für Bakteriologie, 1988; Wayne, 1988). The results showed that the ANI values between strain LST-1^T and *L. jeotgali* and *L. nimipressuralis* were 89.21% and 83.11%, respectively. The DDH values between strain LST-1^T and *L. jeotgali* and *L. nimipressuralis* were 38.80% and 25.80%, respectively (Table 1).

In addition, the G + C content of the genomic DNA from LST-1^T was 55.02%, which was different from that of *L. jeotgali* (54.2%) but close to that of *L. nimipressuralis* (55.2%). These differences indicated that the taxonomic status of LST-1^T lies between *L. nimipressuralis* and *L. jeotgali* (Table 1).

Phenotypic characteristics

The cells of strain LST-1^T were Gram-negative, aerobic, catalase-positive, rod-shaped, and without flagella, and the surface of the bacteria was rough. The colony of strain LST-1^T was milky white, round, and smooth. Compared with two reference strains, LST-1^T could grow in a wide temperature range from 4 °C to 45 °C. The salt tolerance and pH growth range of strain LST-1^T was similar to those of other strains. It could grow with the presence of 0.5% NaCl but not with 9% NaCl. It could also grow at pH 5.0–9.0 but not at pH 4.5 or 9.5.

API 20NE strips (bioMérieux) were used in conjunction with AUX medium (bioMérieux) for carbohydrate fermentation to further distinguish strain LST-1^T from other *Lelliottia* species strains. In accordance with the analysis of the MIDI Microbial Identification System, the phenotypic differences between the strain LST-1^T and other *Lelliottia* species are shown in Table 3. Only LST-1^T could ferment with capric acid, and adipic acid could only be used for LST-1^T and *L. nimipressuralis* DSM 18955.

Chemotaxonomic characterization

LST-1^T respiratory quinones mainly include menaquinone (MK) 8 and coenzyme Q8, while polar lipids mainly include phosphatidylethanolamine (PE), phosphatidylglycerol (PG), aminophospholipid (APL), three non-characteristic phospholipids (PL1–3), and a non-characteristic lipid (PL), which is quite different from the other two strains (Table 2 and Fig. 2). It is especially different from *L. nimipressuralis* DSM 18955, which has four types of unidentified aminolipids, whereas LST-1^T does not have one. In addition, DPG and GL were detected from *L. nimipressuralis* DSM 18955, but it was absent in LST-1^T.

The overall fatty acids profile of LST-1^T was similar to those of the reference taxa of the genus *Lelliottia*, but the respective proportions of some fatty acid components had some differences (Table 4). The data showed that LST-1^T has three main fatty acids, C16:0, Summed Feature3, and Summed Feature8 (may be

16:1 w6c, 16:1 w7c, and 18:1 w6c). In addition, many differences were observed in the fatty acid distribution, especially on $C_{13:0}$, $C_{11:0\ 2\text{-}OH}$, $C_{14:1\ w5c}$, and $C_{19:0\ cyclo\ w8c}$. The three strains have their own unique fatty acid composition.

Taxonomic conclusion

Combination of the analysis of physiological and biochemical characteristics provided evidence that LST-1^T represents a novel species independent of genus *L. jeotgali*, for which the name *Lelliottia rebaudianalis sp. nov.* was proposed.

Description of *L. rebaudianalis sp. nov.*

L. rebaudianalis sp. nov. (re'bau.dia.na.lis N.L.; L. fem. suff. -alis, suffix denoting pertaining to, from which the type strain was isolated from *S. rebaudiana Bertoni*).

The cells of *Lelliottia sp.* LST-1^T are Gram-stain-negative, aerobic, and chemoheterotrophic. SEM results (Fig. 3) showed that it was rod-shaped and without flagella, and the surface of the bacteria was rough. After incubation for 24 h on LB solid medium, the colonies were circular and flat, the surface was smooth and moist, and the edges of the colonies were neat, milky white-pigmented, and opaque. Growth occurred at pH 5.0–9.0, with optimum growth at 6.0–7.0, but not at pH 4.5 or 9.5. Growth also occurred at 4 °C–45 °C, with optimum growth at 37 °C, but no growth was detected at 48 °C. The NaCl range for growth was 0%–8% (w/v, optimum of 0.5%) but not at 9% NaCl (w/v) in LB medium. The cells were positive for catalase activity and weakly positive for oxidase activity. It assimilated with D-glucose, L-arabinose, D-mannose, D-mannitol, N-acetyl-glucosamine, D-maltose, potassium gluconate, malic acid, trisodium citrate, and phenylacetic acid. However, the assimilation of capric acid and adipic acid were weak and not clear. The major components of cellular fatty acids included C16:0, Summed Feature3, and Summed Feature8 (may be 16:1 w6c/16:1 w7c and 18:1 w6c). The polar lipids found in *Lelliottia sp.* LST-1 were PE, PG, APL, three non-characteristic phospholipids, and a non-characteristic lipid. MK8 and coenzyme Q8 were the predominant isoprenoid quinones detected in LST-1.

The type strain LST-1^T (= CGMCC 1.19175^T = JCM 34938^T) was isolated from the stevia planting and production base in Dongtai City, Yancheng City, Jiangsu Province (32°84' N, 120°31' E).

The GenBank accession numbers for the 16S rRNA gene and genome sequences of strain LST-1^T are MZ497264 and CP063663, respectively.

Abbreviations

LB, Luria Broth; MLST, multi-locus sequence typing; ANI, average nucleotide identity; DDH, DNA–DNA hybridization; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PL, unidentified phospholipid; GL, glycolipid; APL, unidentified aminophospholipid; DPG, diphosphatidylglycerol.

Statements & Declarations

Repositories:

The GenBank accession numbers for the 16S rRNA gene and genome sequences of strain LST-1^T are MZ497264 and CP063663, respectively.

Conflicts of interest

The authors declare that they have no conflict of interest.

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Tables

Table 1 ANI, DDH, and G + C content analysis for *Lelliottia* and *Enterobacter* genus

Strain	*Lelliottia jeotgali*	*Lelliottia nimipressuralis*	*Lelliottia amnigena*	*Enterobacter soli*
ANI	89.21%	83.11%	85.05%	83.64%
DDH	38.80%	25.80%	28.90%	26.60%
DNA G + C content (mol%)	54.2%	55.2%	52.9%	53.9%
Similarity based on 16S rDNA	99.85%	98.82%	98.54%	97.78%

Table 2 Phenotypic characteristics of LST-1^T and two known species of genus

Polar lipids	LST-1^T	*Lelliottia jeotgali* JCM31901	*Lelliottia nimipressuralis* DSM 18955
AL1	-	-	+
AL3	-	-	+
AL4	-	-	+
AL5	-	-	+
APL1	+	+	-
APL2	+	+	+
APL4	-	+	-
DPG	-	-	+
GL	-	-	+
L	+	+	-
PE	+	+	+
PG	+	+	+
PL1	+	+	+
PL2	+	+	-

+: Positive; –: negative.

Table 3 Phenotypic characteristics of LST-1^T and two known species of genus *Lelliottia*

Characteristic	LST-1	*Lelliottia jeotgali* JCM31901	*Lelliottia nimipressuralis* DSM 18955
Growth at			
4 °C	+	-	-
45 °C	+	+	-
pH 10	-	-	-
Motility	-	+	+
Enzyme activities:			
Reduction of nitrate to nitrite	+	+	+
Reduction of nitrate to nitrogen	+	+	+
Indole production	-	-	-
Fermentation of glucose	+	+	+
Arginine dihydrolase	+	+	+
Hydrolysis of urea	-	-	-
Hydrolysis of esculin	+	+	+
Hydrolysis of gelatin	-	-	-
β-galactosidase	+	+	+
Carbon utilization:			
D-glucose	+	+	+
L-arabinose	+	+	+
D-mannose	+	+	+
D-mannitol	+	+	+
N-acetyl-glucosamine	+	+	+
D-maltose	+	+	+
Potassium gluconate	+	+	+
Capric acid	W	-	-
Adipic acid	W	-	W
Malic acid	+	+	+
Trisodium citrate	+	+	+

Phenylacetic acid	+	+	+
DNA G+C content (mol%)	55.02	54.2%	55.2%

+: Positive; W: weakly positive; –: negative.

Table 4 Composition of cellular fatty acids of LST-1^{T} and two species of genus *Lelliottia*

Fatty acid	LST-1[T]	*Lelliottia jeotgali* JCM31901	*Lelliottia nimipressuralis* DSM 18955
Straight-chain saturated			
$C_{10:0}$	0.18	0.40	0.14
$C_{12:0}$	3.65	3.78	3.47
$C_{13:0}$	/	/	0.14
$C_{14:0}$	6.65	5.80	8.48
$C_{16:0}$	**27.88**	**28.69**	**33.65**
$C_{17:0}$	0.20	0.48	0.52
$C_{18:0}$	0.50	0.62	0.26
Hydroxy			
$C_{8:0}$ 3-OH	0.37	0.33	/
$C_{11:0}$ 2-OH	/	0.09	/
Unsaturated			
$C_{14:1}$ w5c	/	0.34	/
$C_{16:1}$ w5c	0.23		0.18
$C_{17:0}$ cyclo	6.40	8.71	6.61
$C_{19:0}$ cyclo w8c	0.18	/	/
Summed Feature			
1	/	0.20	/
2	6.48	6.21	6.52
3	**25.92**	**22.30**	**30.50**
8	**21.38**	**22.07**	**10.04**

Figures

Figure 1

Phylogenetic tree analysis of LST-1T. (a) Phylogenetic tree constructed based on 16S rRNA gene sequence using maximum likelihood method. (b) MLST analysis based on partial recA, atpD, and infB.

Figure 2

TLC analysis of polar lipids in LST-1^T and two strains of *Lelliottia.* a–c represent LST-1^T, *Lelliottia jeotgali* JCM31901, and *Lelliottia nimipressuralis* DSM 18955, respectively.

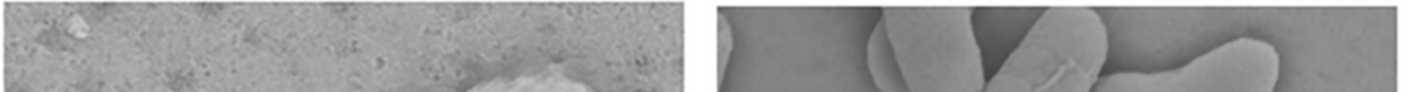

Figure 3

Scanning electron microscopic results of LST-1^T