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Rhizobium daejeonense sp. nov. isolated from a cyanide treatment bioreactor

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A polyphasic study was carried out to determine the taxonomic position of two aerobic, cyanide-degrading bacterial strains, designated L61^T and L22, which had been isolated from a bioreactor for the treatment of nickel-complexed cyanide. The two isolates exhibited almost identical taxonomic characteristics. Phylogenetic analysis inferred from comparative 16S rRNA gene sequences indicated that the isolates fall in a sublineage of the genus *Rhizobium* comprising the type strains of *Rhizobium giardinii*, *Rhizobium radiobacter*, *Rhizobium rubi*, *Rhizobium larrymoorei*, *Rhizobium vitis*, *Rhizobium undicola*, *Rhizobium loessense*, *Rhizobium galegae* and *Rhizobium huautlense.* Cells of the two isolates are Gram-negative, aerobic, motile and non-spore-forming rods (0·6–0·7 × 1·1–1·3 μm), with peritrichous flagella. The DNA G + C content is 60·1–60·9 mol%. Cellular fatty acids are $C_{16:0}$ (2·2–3·3 %), $C_{18:0}$ (2·1–3·2 %), $C_{19:0}$ cyclo $\omega 8c$ (9·9–16·8 %), $C_{20:3}\omega 6,9,12c$ (2·7–3·3 %), summed feature 3 (7·2–7·7 %) and summed feature 7 (67·8–73·7 %). The strains formed nodules on a legume plant, *Medicago sativa*. A *nifH* gene encoding denitrogenase reductase, the key component of the nitrogenase enzyme complex, was detected in L61^T by PCR amplification by using a *nifH*-specific primer system. Strains L61^T and L22 were distinguished from the type strains of recognized *Rhizobium* species in the same sublineage based on low DNA–DNA hybridization values (2–4 %) and/or a 16S rRNA gene sequence similarity value of less than 96 %. Moreover, some phenotypic properties with respect to substrate utilization as a carbon or nitrogen source, antibiotic resistance and growth conditions could be used to discriminate L61^T and L22 from *Rhizobium* species in the same sublineage. Based on the results obtained in this study, L61^T and L22 are considered to be representatives of a novel species of *Rhizobium*, for which the name *Rhizobium daejeonense* sp. nov. is proposed. The type strain is L61^T (= KCTC 12121^T = IAM 15042^T = CCBAU 10050^T).

Since the first description of the genus *Rhizobium* for root- and/or stem-nodule bacteria by Frank (1889), a number of revisions and additions of novel species into this genus have been made. Currently, the genus *Rhizobium* includes 20 recognized species: *Rhizobium galegae*, *R. huautlense*, *R. vitis*, *R. undicola*, *R. rubi*, *R. radiobacter*, *R. loessense*, *R. larrymoorei*, *R. mongolense*, *R. sullae*, *R. hainanense*, *R. leguminosarum*, *R. etli*, *R. tropici*, *R. rhizogenes*, *R. giardinii*, *R. gallicum*, *R. indigoferae*, *R. lupini* and *R. phaseoli*.

Cyanides have been regarded as toxic pollutants because of their inhibitory effect on cytochrome oxidase in respiratory electron transport chains; therefore, most countries request the complete removal of cyanide from wastewater effluent before discharge. Toxic cyanides have been known to be oxidized by various species of bacteria, fungi, yeast and plants. Isolations were made here of the cyanide-degrading bacterial population found in a bioreactor. Comparative

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The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains L61^T and L22 are AY341343 and DQ089696, respectively. The GenBank/EMBL/DDBJ accession number for the partial *nifH* gene sequence of strain L61^T is AY428644.

A transmission electron micrograph of cells of strain L61^T, a minimum-evolution phylogenetic tree and a table detailing the fatty acid compositions of L61^T, L22 and related *Rhizobium* species are available as supplementary material in IJSEM Online.

16S rRNA gene sequence analysis indicated that the two strains isolated, L61^T and L22, were members of the clade representing the genus *Rhizobium*. The strains were the subject of further study. Many aerobic bacteria and fungi capable of degrading cyanides have been isolated, including *Pseudomonas* sp. NCIB 11764 (Harris & Knowles, 1983), *Burkholderia cepacia* C-3 (Adjei & Ohta, 1999) and *Alcaligenes xylosoxidans* DF-3 (Ingvorsen *et al.*, 1991). However, to our knowledge, no *Rhizobium* species has been reported in this population. It is also unusual for rhizobia to inhabit an activated sludge. In order to determine the precise taxonomic position of strains L61^T and L22, a polyphasic study was carried out. Based on the results obtained, we suggest that L61^T is the type strain of a novel species of *Rhizobium*.

Strains L61^T and L22 were isolated from a nickel-complexed cyanide treatment bioreactor that had been inoculated with an activated sludge from a municipal sewage treatment plant (Daejeon, Korea). The bioreactor had been run by supplying a basal medium (BM) comprising 1·25 g K_2HPO_4, 0·38 g KH_2PO_4, 0·2 g $NaSO_4$, 0·06 g $CaCl_2.2H_2O$, 0·06 g $MgCl_2$, 0·72 g glucose and 82·5 mg $K_2[Ni(CN)_4]$ in 1 l distilled water. For the isolation of cyanide-degrading bacteria, a sample of the consortium (0·1 g) taken from the reactor on day 30 of the operation was suspended in 1 ml BM. After serially diluting the suspension with BM, 0·1 ml diluted suspension was spread on BM agar plates [BM components plus 1·5 % (w/v) agar] and was incubated at 28 °C in the dark. After 2 weeks of incubation, single colonies that appeared on the plates were transferred on to newly prepared plates, and were incubated again under the same conditions for the purification of colonies. The cyanide degradation activity of the purified isolates was tested in liquid BM containing cyanides. Reference organisms used in this study, *R. giardinii* H152^T (=KACC 10720^T), *R. huautlense* S02^T (=KACC 10738^T), *R. galegae* ATCC 43677^T (=KACC 10639^T), *R. radiobacter* DSM 30148^T (=KACC 10736^T), *R. rubi* IFO 13261^T (=KACC 10739^T) and *R. vitis* NCPPB 3554^T (=KACC 10777^T), were obtained from Korean Agricultural Culture Collection (KACC).

Morphological features of the cells grown on YMA for 3 days were determined with a phase-contrast microscope and a transmission electron microscope (EM912Ω; Leo Zeiss Inc.) after negative staining with 1 % (w/v) phosphotungstic acid. Gram staining and catalase and oxidase tests were performed following the protocols outlined by Smibert & Krieg (1981). Some physiological properties and substrate utilizations were determined by means of the API 20 NE and API 32 GN systems, in accordance with the manufacturer's instructions (bioMérieux). An antibiotic resistance test and determination of the NaCl concentration and pH range for growth were performed in YMA, as described by Gao *et al.* (1994). Utilization of nitrogen sources, including cyanides KCN and $K_2[Ni(CN)_4]$ was tested in modified White's medium (White, 1972). Cellular fatty acids were extracted from cells grown on TY medium (Beringer, 1974) for 3 days, as described by Jarvis *et al.* (1996). The fatty acids were analysed by means of a gas chromatograph (Hewlett Packard 6890) equipped with the Microbial Identification software package (Sasser, 1990).

Genomic DNA was extracted by using a Qiagen DNeasy tissue kit in accordance with the manufacturer's protocols. RNA was removed from the DNA solution by treating with a mixture of RNases A and T1 (each at 20 U ml^{-1}) at 30 °C for 1 h. PCR amplification of the 16S rRNA gene was performed by using a bacterial universal primer set, 9F (5′-GAGTTTGATCCTGGCTCAG-3′) and 1512R [5′-ACGG(A/T/C)TACCTTGTTACGACTT-3′]. The thermal profile used was an initial denaturation step at 94 °C for 5 min, 30 cycles consisting of 1 min denaturation at 94 °C, 30 s of primer annealing at 55 °C and 2 min of extension at 72 °C, plus a 7 min final extension at 72 °C. The PCR product was purified with a QIAquick PCR purification kit and was then sequenced by using an ABI Prism BigDye Terminator cycle sequencing ready reaction kit and a 3700 DNA Analyser (both Applied Biosystems). For full sequencing, primers 519F (5′-CAGCAGCCGCGGTAATAC-3′), 907F [5′-AAACTCAAA(G/T)GAATTGACGG-3′], 536R (5′-GTATTACCGCGGCTGCTG -3′) and 1100R (5′-GGGTTGCGCTCGTTG-3′) were used. The partial sequences were aligned and combined by using the BIOEDIT program (Hall, 1999). Sequences of related *Rhizobium* species were obtained from GenBank. These collected sequences were aligned with the CLUSTAL X program (Thompson *et al.*, 1997). Evolutionary distances were calculated using the Kimura two-parameter model (Kimura, 1983) and the phylogenetic tree was constructed by using three tree-building methods, neighbour-joining (Saitou & Nei, 1987), minimum-evolution (Rzhetsky & Nei, 1992) and maximum-parsimony (Swofford, 1993), in the MEGA 2 program (Kumar *et al.*, 2001). A bootstrap method was used to obtain confidence levels with 1000 replications (Felsenstein, 1985). A *nifH* gene was amplified by means of PCR using a PolF–PolR primer system designed by Poly *et al.* (2001).

DNA–DNA hybridization values were estimated fluorometrically using photobiotin-labelled DNA probes and microdilution wells according to the method of Ezaki *et al.* (1989). From five repetitions, three values, excluding the highest and the lowest, were used for the estimation. The hybridization temperature was 55 °C. The DNA G+C content was determined by using the procedure described by Mesbah *et al.* (1989).

A nodulation test was carried out in a test tube (50 × 200 mm) containing a quarter-strength nitrogen-free plant nutrient solution (Vincent, 1970), which had been autoclaved for 3 h. Surface-sterilized germinated seedlings of *Glycine max*, *Phaseolus vulgaris*, *Pisum sativum* and *Medicago sativa* were planted aseptically in each tube. The tubes were inoculated with the culture broth (YM broth, 1·5 ml) of strain L61^T with approximately 100 cells ml^{-1}. The plants were grown at 25 °C for 6 weeks in a greenhouse under natural radiation that was supplemented with fluorescent lamps to lengthen the photoperiod to 12 h.

Table 1. Characteristics of strains L61^T and L22 and related *Rhizobium* species

Strains: 1, L61^T; 2, L22; 3, *R. giardinii* H152^T; 4, *R. huautlense* S02^T; 5, *R. galegae* ATCC 43677^T; 6, *R. radiobacter* DSM 30148^T; 7, *R. rubi* IFO 13261^T; 8, *R. vitis* NCPPB 3554^T (unless indicated, data in columns 1–8 were obtained in this study); 9, *R. undicola* LMG11875^T (data from de Lajudie *et al.*, 1998); 10, *R. loessense* CCBAU 7190B^T (Wei *et al.*, 2003; and personal communication from Dr G. H. Wei); 11, *R. larrymoorei* AF3-10^T (Bouzar & Jones, 2001). +, Positive; ±, weakly positive; −, negative; ND, no data available.

Characteristic	1	2	3	4	5	6	7	8	9	10	11
Origin	Bioreactor treating cyanide (Korea)	Bioreactor treating cyanide (Korea)	*Phaseolus vulgaris* (France)[a]*	*Sesbania herbacea* (Mexico)[b]	*Galega orientalis* (Finland)[c]	ND	ND	ND	*Neptunia natans* (Senegal)	*Astragalus campanatus* (China)	*Ficus benjamina* (Florida)
Cell morphology	Rods (0·7 × 1·3 μm)	Rods	Rods (0·5–0·7 × 1·6–2·2 μm)[a]	ND	Rods (0·9–1·0 × 1·5-1·8)[c]	ND	ND	ND	Rods (0·5–0·7 × 2–4 μm)	Rods (0·5–0·7 × 1·8–2·1 μm)	Rods
Flagella	Several, peritrichous	Several, peritrichous	ND	ND	1–2, Polar or subpolar[c]	Several, peritrichous[d]	Several, peritrichous[d]	Several, peritrichous[d]	+	ND	Several peritrichous
DNA G+C content (mol%)	60·1	60·9	60·2	57·0[b]	63·0[c]	57·1	58·1	56·9	60·1	59·5	ND
Nodulation	+	+	+[a]	+[b]	+[c]	−[d]	−[d]	−[d]	+	+	−
pH range for growth	5·0–10·0	5·0–10·0	4·0–8·5[a]	5·0–9·0[b]	5·0–9·5[c]	ND	ND	ND	ND	7·0–10·0	ND
Growth at/in:											
40 °C	+	+	−[a]	+[b]	−[c]	ND	−[d]	ND	ND	−	−
1 % NaCl	+	+	d[a]†	+[b]	ND‡	+[d]	ND	−[d]	ND	+	+
2 % NaCl	+	+	−[a]	−[b]	−[c]	+[d]	−[d]	+[d]	ND	+	+
Utilization as carbon source:											
D-Melibiose	−	−	+	+	+	+	+	+	−	+	+
Propionate	+	±	±	−	−	+	−	−	−	−	ND
Caprate	+	+	−	−	−	−	−	−	−	ND	ND
Valerate	+	+	−	−	−	−	−	−	−	−	ND
Histidine	+	+	+	+	−	+	+	+	+	ND	+
2-Ketogluconate	−	−	+	+	+	+	−	−	−	ND	ND
N-Acetylglucosamine	−	−	+	+	±	+	+	+	−	ND	+
Malonate	−	−	−	+	−	−	+	−	−	−	−
Acetate	+	+	±	+	+	+	+	±	+	−	−
L-Alanine	+	+	+	+	+	+	+	+	+	+	ND
5-Ketogluconate	−	−	±	−	+	+	+	−	−	ND	ND
3-Hydroxybenzoate	+	+	−	+	+	−	−	−	−	ND	ND
Maltose	+	+	+	±	±	+	+	+	+	−	+
Gluconate	−	−	+	±	+	+	+	±	−	−	ND
Citrate	−	−	−	−	−	−	−	+	−	−	−

Table 1. cont.

Characteristic	1	2	3	4	5	6	7	8	9	10	11
Utilization as nitrogen source:											
KCN	+	+	−	+	±	±	−	±	ND	ND	ND
$K_2[Ni(CN)_4]$	+	+	−	±	−	−	−	−	ND	ND	ND
L-Phenylalanine	+	+	+	+	+	+	−	+	+	−	−
L-Cysteine	+	+	+	±	−	+	+	+	−	+	ND
Glycine	−	−	+	−	−	+	−	−	−	+	ND
Antibiotic resistance ($\mu g\ ml^{-1}$):											
Ampicillin (100)	+	+	−	+	+	−	+	+	ND	− (100)	ND
Kanamycin (20)	−	−	−	+	+	+	−	−	ND	− (50)	ND
Erythromycin (20)	+	+	+	+	−	+	−	−	ND	+ (5)	ND
Erythromycin (100)	−	−	−	+	−	±	−	−	ND	− (100)	ND

*Data taken from: *a*, Amarger *et al.* (1997); *b*, Wang *et al.* (1998); *c*, Lindström (1989); *d*, Young *et al.* (2001) (data taken from original descriptions).

†Amarger *et al.* (1997) reported that 76 % of 29 strains of *R. giardinii* tested grew in 1 % NaCl, but the specific result for the type strain is not given.

‡Strains grow in 0·5 % NaCl; growth in 1 % NaCl was not reported by Lindström (1989).

Cells of strains L61 and L22 were aerobic, Gram-negative, motile and non-spore-forming rods. The isolates utilized 2 mM KCN or $K_2[Ni(CN)_4]$ as a nitrogen source. Peritrichous flagella were observed under transmission electron microscopy (Supplementary Fig. S1 in IJSEM Online). Available carbon and nitrogen source utilizations and antibiotic resistance are summarized in Table 1. Fatty acid analysis showed that L61^T and L22 contained low levels of $C_{16:0}$ (2·2–3·3 %) compared with related *Rhizobium* species (6·6–13·4 %) (Supplementary Table S1 in IJSEM Online). Other morphological, phenotypic and chemotaxonomic characteristics of strains L61^T and L22 obtained in this study are given in the species description below.

The nearly complete 16S rRNA genes of strains L61^T (1421 nt) and L22 (1396 nt) were sequenced for phylogenetic analysis. The two strains showed identical 16S rRNA gene sequences. Fig. 1 shows the phylogenetic tree constructed by the neighbour-joining method using these sequences and those of closely related organisms obtained from GenBank. Strains L61^T and L22 fall in a sub-branch of the genus *Rhizobium* that comprises the type strains of *R. giardinii* (16S rRNA gene sequence similarity of 96·9 %), *R. radiobacter* (96·1 %), *R. rubi* (96·0 %), *R. larrymoorei* (95·7 %), *R. vitis* (95·4 %), *R. undicola* (94·4 %), *R. loessense* (94·4 %), *R. galegae* (94·1 %) and *R. huautlense* (94·9 %). Members of this group also clustered on the same phylogenetic branch when the minimum-evolution (Supplementary Fig. S2 in IJSEM Online) and maximum-parsimony (not shown) tree-building methods were used, indicating that the phylogenetic positions of the two isolates were not affected by the choice of algorithm.

A sequence (360 nt) from strain L61^T was obtained by amplification with *nifH* primers. This gene sequence showed similarities of 87·6, 87·1 and 85·7 %, respectively, when it was aligned with *nifH* gene sequences from *Azospirillum lipoferum* 4VI (GenBank accession no. AY786992), *Rhizobium* sp. TJ167 (AJ505316) and *Sinorhizobium americanum* CFNEI 54 (AF506515), indicating the presence of *nifH* in strain L61^T.

DNA–DNA reassociation is considered to be a standard method for determining species differences (Wayne *et al.*, 1987; Stackebrandt *et al.*, 2002). A species is considered to be represented by a population whose strains share levels of DNA–DNA hybridization of more than 70 %. DNA–DNA hybridization values between strains L61^T and L22 were 86 %, suggesting that they represent the same species. Reference species (*R. giardinii*, *R. radiobacter*, *R. rubi*, *R. vitis*, *R. galegae* and *R. huautlense*) all had DNA–DNA reassociation values of 2–4 % with strains L61^T and L22, indicating a genetic distance that supports these strains as representing a novel species. *R. undicola*, *R. loessense* and *R. larrymoorei*, which clustered with two strains L61^T and L22 on one branch of the phylogenetic tree, and other *Rhizobium* species exhibited 16S rRNA gene sequence similarity values of <96 %; therefore, they could be clearly discriminated from the two new isolates as different species.

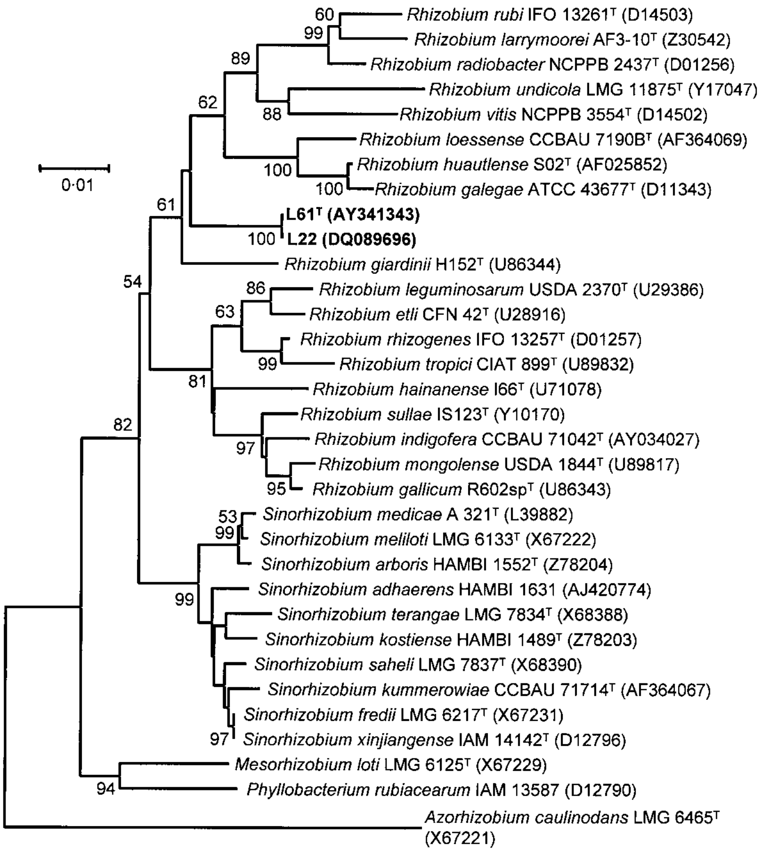

Fig. 1. Neighbour-joining tree showing the phylogenetic relationship of strains L61^T and L22, *Rhizobium* species and other related species of the '*Alphaproteobacteria*'. Bootstrap values (expressed as percentages of 1000 replications) above 50 % are shown at branch points. Bar, 0·01 substitutions per nucleotide position.

The DNA G+C content of strains L61^T and L22 was 60·1 and 60·9 mol%, respectively, values that match closely those of related *Rhizobium* species (56·9–60·2 mol%) (Table 1).

Nodulation and nitrogen-fixing ability are typical characteristics of the genus *Rhizobium*, and may be essential for their symbiotic relationship with legumes. To determine whether strains L61^T and L22 have these functional characteristics, a nodulation test was carried out using four different legumes: *G. max*, *Phaseolus vulgaris*, *Pisum sativum* and *M. sativa*. The two strains successfully formed nodules on *M. sativa*; *Medicago* includes many species known to be host plants of rhizobia, such as *Sinorhizobium meliloti* (de Lajudie *et al.*, 1994), *Sinorhizobium medicae* (Rome *et al.*, 1996) and *Rhizobium mongolense* (van Berkum *et al.*, 1998).

The phylogenetic data, chemotaxonomic features (DNA G+C content, fatty acid composition) and the functional traits of rhizobia (nodulation and *nifH*) strongly support the classification of strains L61^T and L22 within the genus *Rhizobium*. The DNA–DNA hybridization values and/or the low 16S rRNA gene sequence similarity values suggest that the two strains represent a novel species in this genus. The two strains could also be distinguished from recognized *Rhizobium* species based on several important phenotypic characteristics: substrate (carbon or nitrogen sources) utilization, antibiotic resistance, growth conditions (pH, temperature, NaCl concentrations), as summarized in Table 1, and fatty acid profile (Supplementary Table S1). Based on the results obtained, strains L61^T and L22 can be assigned to be same species, while they are sufficiently distinct from strains of recognized *Rhizobium* species as to be recognized as representing a novel species. The name *Rhizobium daejeonense* sp. nov. is proposed.

Description of *Rhizobium daejeonense* sp. nov.

Rhizobium daejeonense (dae.jeon.en′se. N.L. neut. adj. *daejeonense* pertaining to Daejeon, a city in Korea, where the type strain was isolated).

Cells are Gram-negative, aerobic, motile, non-spore-forming rods (0·6–0·7 × 1·1–1·3 μm) with peritrichous flagella. Colonies appearing on YMA within 3 days of incubation at 28 °C are circular, cream coloured, semi-translucent and 1·5–3·0 mm in diameter. Catalase, oxidase, urease, *β*-galactosidase and *β*-glucosidase are positive. Gelatin liquefaction, indole production and arginine dihydrolase are negative. No nitrate reduction to nitrite. Cells grow at 41 °C, in 0–2 % NaCl and over a pH range of 5–10; however, no growth is observed at 4 or 45 °C, in 2·5 % NaCl or at a pH >10 or <4·5. Nodules are formed in *M. sativa* by the type strain. A *nifH* gene encoding a component of the nitrogenase complex is detected. DNA G+C content is 60·1–60·9 mol%, as determined by

HPLC. Cellular fatty acids are $C_{16:0}$ (2·2–3·3 %), $C_{18:0}$ (2·1–3·2 %), $C_{19:0}$ cyclo $\omega 8c$ (9·9–16·8 %), $C_{20:3}\omega 6,9,12c$ (2·7–3·3 %), summed feature 3 (7·2–7·7 %) and summed feature 7 (67·8–73·7 %). Carbon sources used include propionate, caprate, valerate, histidine, acetate, L-alanine, 3-hydroxybenzoate, maltose, D-glucose, fucose, D-sorbitol, L-proline, rhamnose, D-ribose, inositol, DL-lactate, arabinose, mannose, mannitol, maltose and malate, but not citrate, itaconate, suberate, glycogen, phenylacetate, adipate, D-melibiose, 2-ketogluconate, *N*-acetylglucosamine, malonate, 5-ketogluconate or gluconate. L-Glutamic acid, L-methionine, L-phenylalanine, L-cysteine and free and nickel-complexed cyanides are used as nitrogen sources, but DL-tryptophan and glycine are not. Cells are resistant to ampicillin (20 and 100 μg ml^{-1}) and erythromycin (20 μg ml^{-1}), but susceptible to tetracycline (50 μg ml^{-1}), erythromycin (100 μg ml^{-1}), chloramphenicol (100 μg ml^{-1}) and kanamycin (100 μg ml^{-1}).

The type strain is $L61^T$ (=KCTC 12121^T=IAM 15042^T=CCBAU 10050^T) and a reference strain is L22 (=KCTC 12120=IAM 15041). The strains were isolated from a cyanide-degrading bioreactor originally inoculated by an activated sludge from a municipal sewage treatment plant (Daejeon, Korea).

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