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Leucobacter luti sp. nov., and *Leucobacter alluvii* sp. nov., two new species of the genus *Leucobacter* isolated under chromium stress

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Abstract

Two strains designated RF6^T and RB10^T were isolated, from activated sludge and from river sediments, respectively, both systems receiving chromium contaminated water. Phylogenetic analysis showed that strain RF6^{T}and strain RB10^T represented two new species of the genus *Leucobacter*. Strain RB10^T can be distinguished from RF6^T by its ability to grow at 37 °C, by showing a different optimum pH, by cell wall amino acids different relative amount and by having the fatty acid strait C16:0 as the third most abundant fatty acid. On the basis of the distinct peptidoglycan composition, 16S ribosomal DNA sequence analysis, DNA–DNA reassociation values, and phenotypic characteristics we are of the opinion that strain RF6^T represents a new species of the genus *Leucobacter* for which we propose the name *Leucobacter luti* (CIP 108818^T = LMG 23118) and that strain RB10^T represents an additional new species of the same genus for which we propose the name *Leucobacter alluvii* (CIP 108819^T = LMG 23117).

Keywords: *Leucobacter luti*; *Leucobacter alluvii*; Chromium contaminated environment

Introduction

The bacteria of genus *Leucobater* represents one of 12 genera of irregular rod-shaped aerobic bacteria of the family *Microbacteriaceae* which possess 2,4-diaminobutyric acid (DAB) in the peptidoglycan [6,11,21]. The species of these genera are distinguished from each other by their menaquinone types, the composition of their cell wall sugars, the mole % G+C of the DNA and key physiological features.

Despite the toxic effects of hexavalent chromium (Cr(VI)) several microorganisms have evolved resistance/detoxification mechanisms [2,16]. To increase the success of microbially based metal remediation strategies a better knowledge of the organisms belonging to microbial communities under metal stress is necessary [16]. Species from the genus *Leucobacter* turn out to be part of metal stressed communities and have been isolated from activated sludge subjected to chromium contamination [14].

In this paper, we describe two new species based on strains also isolated from chromium-contaminated

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environments. One was isolated from activated sludge and the other was isolated from sediments of a river receiving chromium-contaminated water. On the basis of phylogenetic analysis, phenotypic, and chemical properties we are of the opinion that strain RF6^T represents a new species of the genus *Leucobacter* for which we propose the name *Leucobacter luti* (CIP 108818^T = LMG 23118) and strain RB10^T represents an additional new species of the same genus for which we propose the name *Leucobacter alluvii* (CIP 108819^T = LMG 23117).

Material and Methods

Bacterial strains and culture conditions

Strains RF6^T was isolated from chromium contaminated activated sludge [7] and RB10^T was isolated from river sediments with 0.3% Cr(VI) [3]. All strains were preserved at −80 °C in Nutrient Broth (NB; Difco) containing 15% glycerol. The type strains used were obtained at the local Culture Collection and the type strain of *Leucobacter komagatae* (DSM 8803^T) was obtained from Deutsche Sammlung Von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany (DSMZ).

Morphological, physiological and biochemical tests

Cell morphology and motility were examined by phase-contrast microscopy after growth on PY-BHI medium (peptone 10.0 g, brain heart infusion 2.0 g, yeast extract, 2.0 g, D-glucose 2.0 g, NaCl 2.0 g per liter) at 28 °C. Morphology, Gram reaction, the presence of cytochrome oxidase and catalase were determined after 24 h of incubation as described by Smibert and Krieg [20]. Anaerobic growth was assessed in cultures grown in PY-BHI medium incubated in anaerobic chambers with H_2/CO_2 atmosphere (BioMerieux, Marcy LÉtoile, France) at 25 °C during 5 days. Acid production was examined in Hugh and Leifson medium (Difco) with 1% glucose. Single carbon source assimilation was determined using API 50 CH test strips (Analytab Products Inc., Biomerieux, France), with 0.2 M phosphate buffer pH 7.2 supplemented with 0.3% (w/v) agar (Difco), 0.05% Yeast Nitrogen Base (Difco) and 0.7% Yeast Extract (Difco) as described by Morais et al. [14]. Results were recorded after 24 h, 48 h and 5 days incubation at 30 °C. Nitrate reduction, hydrolysis of gelatin and urea and, the presence of arginine dihydrolase were determined using the API 20NE system according to the manufacturer instructions. The growth temperature range of the organisms was examined by measuring the turbidity (610 nm) of cultures incubated in 100 ml PY-BHI medium adjusted to pH 7.0. The pH range for growth was examined at 30 °C in PY-BHI medium with pH values adjusted by the addition of MES for pH values of 5, 6 and 7, or TAPS for pH values of 8 and 9, or CAPSO for pH value of 10. The ability to grow in the presence of NaCl was examined in PY-BHI supplemented with NaCl at final concentrations of 2.0%, 5.0%, 6.0%, 7.0%, 8.0%, 9.0% and 10%.

Lipoquinones, fatty acid composition and cell wall peptidoglycan type

Lipoquinones were extracted from freeze-dried cells, purified by TLC and identified by HPLC [23]. Cultures for fatty acids analysis were grown on PY-BHI medium plates in sealed plastic bags at 28 °C for 24 h. Fatty acid methyl esters were identified using the standard MIS Library Generation Software (Microbial ID). The purification of the cell walls and the acquisition of the amino acids and peptides in cell wall hydrolysates were performed by the method of Schleifer and Kandler [19]. The terminal amino acid of the interpeptide bridge was determined by dinitrophenylation as described by Schleifer [18].

Tolerance to chromium

Tolerance to chromium was examined under aerobic conditions in 100 ml PY-BHI medium, pH 7.0, supplemented with K_2CrO_4, in order to obtain Cr(VI) at final concentrations of 1.0, 2.0, 3.0, and 4.0 mM. The ability to reduce Cr(VI) was also determined under aerobic conditions, was followed by quantifying Cr(VI) concentration in medium by the diphenylcarbazide method [1].

Base composition of the DNA, DNA:DNA reassociation studies and 16S rRNA gene sequence analysis

The G+C content of the overall genome was determined by high-performance liquid chromatography as described by Mesbah et al. [13]. DNA for DNA–DNA reassociation studies was extracted and purified by the procedure of Marmur [12]. The degree of DNA reassociation (%D) was determined spectrophotometrically in 1 × SSC (0.15 M NaCl and 0.015 M trisodium citrate at pH 7.0) from the initial renaturation rates, according to De Ley et al. [5]. The optimal renaturation temperature used in each case was calculated from the mol% G+C [5].

The extraction of genomic DNA, PCR amplification of the 16S rRNA gene and sequencing of the purified PCR products were carried out as described by Rainey et al. [15]. Purified reactions mixtures were

electrophoresed using a model 310 Genetic Analyzer (Applied Biosystems, Foster City, Calif.).

The quality of 16S rRNA gene sequences was checked manually using Bioedit editor [8] and aligned against representative reference sequences of the most closely related members, obtained from the Ribossomal Database Project [4] and EMBL, using the multiple-alignment CLUSTAL X software package [22]. The evolutionary distances were calculated [9], phylogenetic dendrograms were constructed using the neighbor-joining method [17], and trees topologies were evaluated by performing bootstrap analysis [6] of 1000 data sets by using the MEGA2 package [10].

Results

Morphological, biochemical and physiological characteristics

Both organisms formed Gram-positive, non-motile rod-shape cells. The colonies on PY-BHI solid medium were rough, round convex and pale cream-colored. Under anaerobic conditions no growth was observed in the presence or absence of Cr(VI). Strain RF6^T was able to grow at temperatures between 4 and 30 °C with optimum temperature at 25 °C and did not grow at 37 °C but RB10^T was able to grow at temperatures between 4 and 37 °C with optimum temperature at 30 °C but did not grow at 40 °C (Table 1). The optimum pH for growth of strain RF6^T was between 7.0 and 9.0, and between 7.0 and 8.0 for strain RB10^T. The growth rates of both strains were higher in PY-BHI without NaCl, but the organisms were halotolerant and grew in medium containing 8% NaCl. These strains were resistant up to 4 mM Cr(VI) and both were able to reduce Cr(VI), although at different rates. After 15 h growth in the presence of 2 mM Cr(VI) only strain RF6^T showed Cr(VI) reducing ability (0.06 mmol of Cr(VI) reduced). Only strain RB10^T was able to hydrolyze gelatin and Tween 20, 40, 60 and 80. Cytochrome oxidase was not detected in any of the organisms, but catalase was positive in all strains. None of the strains were able to ferment glucose. The organisms could be distinguished from each other based on the carbohydrate assimilation patterns on the API50 CH (Table 2).

Chemotaxonomic parameters

The molar ratio of alanine to glycine to threonine to DAB to glutamic acid in the cell wall peptidoglican of strain RF6 was 2.4: 1.2: 0.7: 0.5: 1.0 and of strain RB10^T, was 1.0: 0.6: 0.6: 1.0: 2.0. (Table 1). Both structures are characterized by the B-type of cross-linkage with the alanine at the *N*-terminus of the interpeptide bridge. This peptidoglycan is consistent with the rare type B2δ of Schleifer and Kandler [20]. The isoprenoid quinones were MK11 (80%) and MK10 (20%) in both strains (Table 1). The major fatty acids of the organisms were iso- and anteiso-branched C15:0, C16:0 and C17:0; straight-chain C16:0 was the third most abundant fatty acid in strain RB10^T. Differences in the relative proportions of the fatty acids were useful in distinguishing both species from each other and from the type strains of the other species of the genus *Leucobacter*.

Base composition of the DNA, DNA:DNA reassociation studies and 16S rRNA gene sequence analysis

The G + C base composition of the DNA of strains RF6^T and RB10^T were 68.8 and 68.9 mol %, respectively (Table 1). The distinct species status of strains RF6^T and RB10^T were demonstrated by DNA:DNA reassociation values of 51% between them and less then 45% with the other type strains of the genus *Leucobacter* (Table 3).

The 16S rRNA gene sequences were aligned with those of representative *Actinobacteria*. The phylogenetic analysis showed that strains RF6^T and RB10^T belong within the radiation of phylum *Actinobacteria* that contain B-type peptidoglycan. Strain RF6^T cluster with *L. komagatae*, *L. albus*, *L. chromiireducens* and *L. aridicollis* at levels of 98.6%, 98.0%, 98.5% and 98.0%, respectively (Fig. 1). Strain RB10^T cluster with *L. komagatae*, *L. albus*, *L. chromiireducens* and *L. aridicollis* at levels of 97.4%, 97.9%, 97.5% and 97.9%, respectively. The phylogenetic analysis indicated a 16S rDNA sequence similarity of 97.5% between strains RF6^T and RB10^T.

Discussion

The organisms characterized in this study were isolated in environments contaminated with chromium in Central Portugal [3,7]. These strains have DAB in their peptidoglycan and MK11 as the major respiratory quinone corroborating the phylogenetic analysis, which places strains RF6^T and RB10^T within the genus *Leucobacter*. Their Cr(VI) resistance or reducing ability was similar to the other strains of the genus *Leucobacter* and was not related with the Cr(VI) concentration of the environment they came from [3,14,21]. This probably shows that in metal stress environments bacteria subsist in microenvironments with different concentrations of the metal according to their metal resistance ability. Characteristics that differentiate strain RF6^T and strain RB10^T from each other and from the type strains of the

Table 1. Differential characteristics between RF6^T, RB10^T, *L. chromiireducens* LMG 22506^T *L. aridicollis* LMG 22507^T, *L. komagatae* strain DSM 8803^T, *L. albus* strain IAM 14851^T

Characteristic	*L. komagatae* DSM 8803^T	*L. albus*[a] IAM 14851^T	*L. aridicollis* LMG22507^T	*L. chromiireducens* LMG22506^T	RF6^T	RB10^T
Urease	+	–	+	+	+	+
Gelatin	+	N.D.	–	–	–	+
Tween 20	–	N.D.	–	–	–	+
Tween 40	–	N.D.	–	–	–	+
Tween 60	–	N.D.	–	–	–	+
Tween 80	+	N.D.	–	+	–	+
Growth in/at:						
5.0% NaCl	+	N.D.	+	+	+	+
8.0% NaCl	–	N.D:	+	+	+	+
10.0% NaCl	–	N.D.	+	–	–	–
4 °C	+	N.D.	+	+	+	+
37 °C	–	N.D.	+	+	–	+
Optimum pH	7–9	N.D.	7–8	7	7–9	7–8
Chemotaxonomic characteristics						
Cell wall diamino acid	L-DAB	L-DAB	L-DAB	L-DAB	L-DAB	L-DAB
Amino acid in cell wall (%)						
DAB	0.8	0.8	1.0	1.0	1.0	0.5
Ala	1.9	1.8	4.0	2.1	1.0	2.4
Gly	0.9	1.1	2.3	1.4	0.6	1.2
Glu	1.0	1.0	2.0	1.1	2.0	1.0
GABA	0.7	0.7	–	–	–	–
Thr	–	–	–	0.7	0.6	0.7
Peptidoglycan cross-linkage	B-type	B-type	B-type variant[b]	B2δ variant	B2δ variant	B2δ variant
Major menaquinone	MK-11 (81%)	MK-11 (77%)	MK-11 (80%)	MK-11 (80%)	MK-11 (80%)	MK-11 (80%)
Cellular fatty acids:						
anteiso-C15:0	65%[c]	50%	49%	54%	61%	51%
anteiso-C17:0	22%	16%	24%	16%	14%	25%
iso-C16:0	13%	24%	12%	14%	12%	9%
C16:0	–	5%	11%	12%	7%	12%
G+C content (%mol)	66.3	66.0	67.3	66.7	68.8	68.9

All strains had catalase activity. None of the strains had cytochrome oxidase or arginine dihydrolase activity; degraded gelatin or esculin; produced indol or reduced nitrate.

Results are scored as: +, positive; –, negative.

Abreviations: N.D.-not determined; DAB-2,4-diaminobutyric acid; Ala- alanine; Gly glycine; GABA-γ-aminobutyric acid; Glu- glutamic acid; Thr- threonine

[a]Results from Lin et al. [11].

[b]Different from the B-type peptidoglican of the type strains of *L. komagatae* and *L. albus.*

[c]Fatty acids profile are from Takeuchi et al. [21].

genus *Leucobacter* are primarily related to their maximum growth temperature, distinctive carbohydrate assimilation pattern, the fatty acid composition, the different halotolerance and the peptidoglycan type. RB10^T was the only strain able to grow at 37 °C, assimilate D-glucose and both strains were less halotolerant than *L. aridicollis*. The fatty acid composition of RB10^T was different from all other species of the genus *Leucobacter*, and from strain RF6^T since the third most abundant fatty acid was C16:0 instead of iso-C16:0. The murein of strains RF6^T and RB10^T and *L. chromiireducens* is slightly different from each other, but belong to type B2δ. These peptidoglycans differ from both *L. komagatae* and *L. albus*, which belong to B-type, and from *L. aridicollis*, which belongs to undescribed B-type variant [11,14]. Furthermore, the peptidoglycan of strains RF6^T and RB10^T contained alanine as an *N*-terminal of the interpeptide bridge that makes their peptidoglycan different from all others previously described structures containing DAB. The different peptydoglycan types of these organisms coupled to the low DNA:DNA reassociation values between each other

Table 2. Differential carbohydrate assimilation patterns, on the API 50 CH, of the type strains of RF6^T, RB10^T, *L. komagatae* DSM 8803^T, *L. albus* IAM 14851^T, *L. chromiireducens* LMG 22506^T, and *L. aridicollis* LMG 22507^T

Carbon source	*L. komagatae* DSM 8803^T	*L. albus*[a] IAM 14851^T	*L. aridicollis* LMG 22507^T	*L.chromiireducens* LMG 22506^T	RF6^T	RB10^T
D-Arabinose	−	−	+^{w}	−	−	+^{w}
L-Arabinose	+^{w}	−	+^{w}	−	−	+^{w}
Glycerol	+	+	+^{w}	+^{w}	+^{w}	+
Erythritol	−	−	+^{w}	+^{w}	−	−
D-Ribose	+	+	−	−	+	+^{w}
D-Adonitol	+^{w}	−	−	−	−	−
D-Galactose	−	−	−	+^{w}	−	−
D-Glucose	−	−	−	−	−	+
D-Fructose	−	−	+^{w}	−	−	+
D-Mannose	−	−	+^{w}	−	−	+
L-Sorbose	−	−	+^{w}	−	−	−
L-Rhamnose	−	−	−	+	+	+
Inositol	−	−	−	−	+	+
D-Sorbitol	−	−	−	+^{w}	−	−
D-Cellobiose	−	−	−	+^{w}	−	−
D-Maltose	−	−	+^{w}	−	−	+
Sucrose	−	−	+^{w}	+^{w}	−	−
D-Trehalose	−	−	+^{w}	+^{w}	−	+
Inulin	−	−	+^{w}	+^{w}	−	−
D-Melezitose	−	−	−	+^{w}	−	−
D-Raffinose	−	−	−	+^{w}	−	+^{w}
Starch	−	−	−	+^{w}	−	+^{w}
Xylitol	−	−	−		−	+
D-Lyxose	−	−	−	+^{w}	−	−
D-Tagatose	−	−	−	+^{w}	−	−
D-Fucose	−	−	+^{w}	−	−	−
L-Fucose	−	+	+^{w}	−	−	−
D-Arabitol	−	−	−	+^{w}	−	−
2-Keto-gluconate	−	−	−		−	+
5-Keto-gluconate	−	−	−	+^{w}	−	+

All strains were negative for D- and L-xylose, β-methyl-xyloside, galactitol, mannitol, α-methyl-D-mannoside, α-methyl-D-glucoside, *N*-acetyl-glucosamine, amygdalin, arbutin, esculin, salicin, lactose, melibiose, glycogen, gentiobiose, D-turanose, D-arabitol.
Results are scored as: +, positive; −, negative; +^{w}, weak positive.
Results according Lin et al. [11].

Table 3. DNA:DNA reassociation values within the type strains of the genus *Leucobacter* with phylogenetic distances lower then 0.02 and strains RF6^T and RB10^T

Strain	*L. komagatae* DSM 8803^T	*L. chromiireducens* LMG 22506^T	*L. aridicollis* LMG 22507^T	RF6^T	RB10^T
L. komagatae	100				
L. chromiireducens	30	100	36		
L. aridicollis	38	21	100		
RF6^T	10	31	15	100	
RB10^T	38	41	43	51	100

and the type strains of the genus *Leucobacter* strongly support the opinion that strains RF6^T and RB10^T represent two new species of this genus.

On the basis of the distinctive peptidoglycan composition, 16S ribosomal DNA sequence analysis, DNA: DNA reassociation values, fatty acid composition, and physiological and biochemical characteristics we propose that strains RF6^T represents a new species of the genus *Leucobacter* for which we offer the name *L. luti* sp. nov., and strain RB10^T represents a new species of

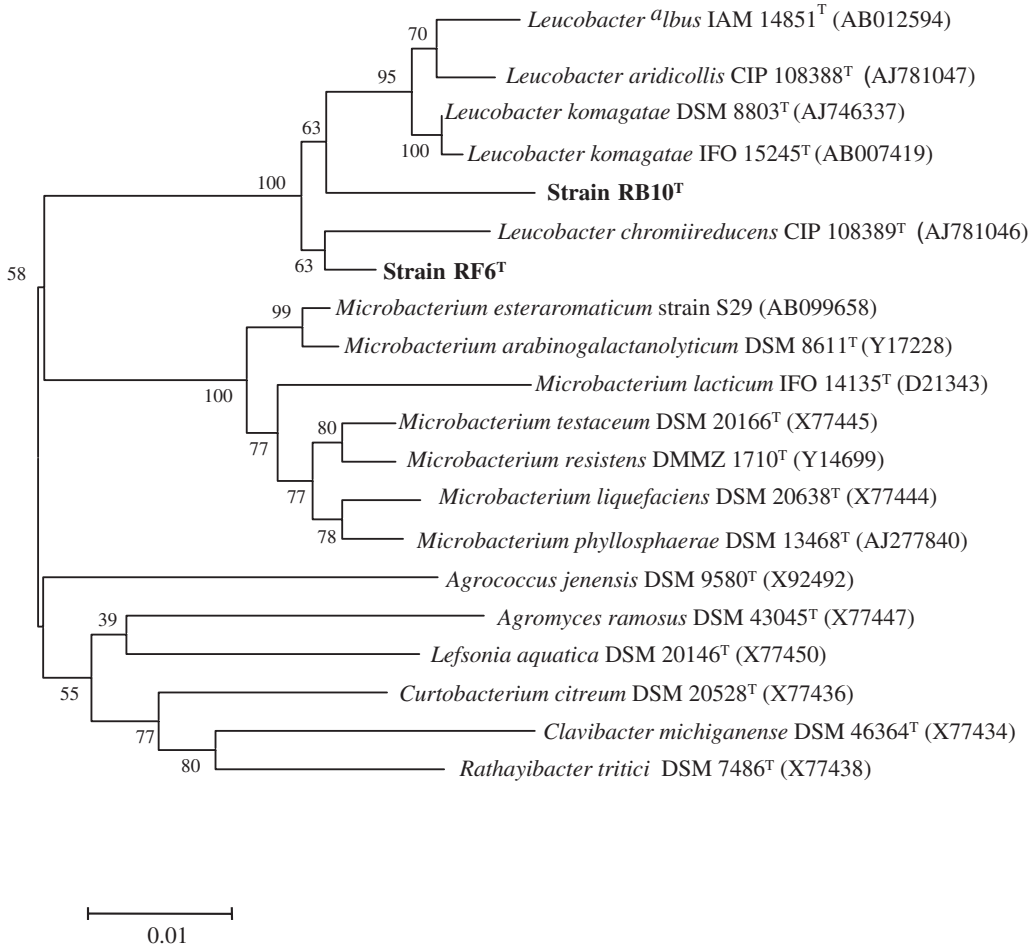

Fig. 1. Phylogenetic dendrogram based on 16S rRNA gene sequence comparisons. The dendrogram was reconstructed from evolutionary distances by using the neighbor-joining method and the topologies were evaluated by performing bootstrap analysis of 1000 data sets. The scale bar represents one inferred nucleotide substitutions per 100 nucleotides.

the same genus for which we offer the name *L. alluvii*, sp. nov.

Description of *Leucobacter luti* sp. nov.

Leucobacter luti (lu.te′um, L. neut. n. *lutum*, mud; L. gen. n. *luti*, of mud, from mud. *Leucobacter luti* forms Gram-positive, irregular rod-shaped cells. Spores are not observed; non-motile on PY-BHI. Colonies are circular, entire, low convex, smooth, opaque, and cream-colored. Optimum growth temperature is about 25 °C; does not grow at 37 °C. Optimum pH is between 7.0 and 9.0. Oxidase negative and catalase positive; urease is produced, arginine dihydrolase is not produced. Starch is hydrolized, but esculine, gelatin and tween 20, 40, 60, and 80 are not. Nitrate is not reduced. Growth occurs in medium with 8% NaCl; growth occurs in medium with 3 mM Cr(VI). The cell wall peptidoglycan contains alanine, glycine, threonine DAB, and glutamic acid. The major isoprenoid quinone is menaquinone MK-11. The major cellular fatty acids are anteiso-$C_{15:0}$, anteiso-$C_{17:0}$, iso-$C_{16:0}$ and $C_{16:0}$. Chemoorganotrophic, strictly aerobic and non-fermentative. D-ribose, L-rhamnose and inositol are assimilated; glycerol is weakly assimilated. Other carbohydrates are not utilized.

G+C content of the DNA is 68.8%. Isolated from activated sludge from a chromium polluted wastewater treatment plant in Alcanena, Portugal. The type strain, $RF6^T$, has been deposited in the Collection of the Institute Pasteur, Paris, France, as strain CIP 108818^T and in the BCCM/LMG Bacteria Collection, Ghent, Belgium as strain LMG 23118.

Description of *L. alluvii* sp. nov.

Leucobacter alluvii (al.lu'vi.i. L. neut. n. *alluvium*, alluvial deposit; L. neut. gen. n. *alluvii*, of an alluvial

deposit). Cells are gram-positive, irregular-shaped rods. Spores are not produced; non-mobile in PY-BHI. Colonies are circular, entire, low raised, smooth, opaque, and cream-colored. Optimum growth temperature is 30 °C; does not grow at 40 °C. Optimum pH is between 7.0 and 8.0. Catalase and urease are produced, but oxidase and arginine dihydrolase are negative. Esculine are not hydrolysed. Tween 20, 40, 60, 80 and gelatin are hydrolysed. Nitrate is not reduced. Growth occurs in the presence of 8% NaCl; growth occurs in the presence of 3 mM Cr(VI). The cell wall peptidoglycan contains alanine, glycine, threonine DAB, and glutamic acid. The major isoprenoid quinone is menaquinone MK-11. The major cellular fatty acids are anteiso-$C_{15:0}$, anteiso-$C_{17:0}$, linear-$C_{16:0}$ and iso-$C_{16:0}$. Chemoorganotrophic, strictly aerobic and non-fermentative. Positive growth was obtained with D-glucose, D-fructose, D-mannose L-rhamnose, inositol, D-maltose, D-trealose and xylitol.

G + C content of the DNA is 68.9%. Isolated from River Alviela sediments, Portugal. The type strain, RB10^T, has been deposited in the Collection of the Institute Pasteur, Paris, France, as strain CIP 108819^T and in the BCCM/LMG Bacteria Collection, Ghent, Belgium as strain LMG 23117.

Nucleotide sequence accession numbers: The EMBL accession numbers for the 16S rRNA gene sequences are: strain RF6^T LMG 23118^T (AM072819) and strain RB10^T LMG 23117^T (AM072820), strain *Leucobacter aridicollis* CIP 108389^T = LMG 22507^T (AJ781047), strain *Leucobacter chromiireducens* CIP 108388^T = LMG 22506^T (AJ781046), *Leucobacter komagatae* DSM 8803^T (AJ746337).

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References

[1] Anonymous, Metals, In: A.E. Greenberg, R.R. Trussell, L.S. Clesceri (Eds.), Standard Methods for the Examination of Water and Wastewater, American Public Health Association, Washington, D.C., USA, 1998, pp. 65–68.

[2] R. Branco, M.C. Alpoim, P.V. Morais, *Ochrobactrum tritici* strain 5bvl1—characterization of a Cr(VI)-resistant and Cr(VI)-reducing strain, Can. J. Microbiol. 50 (2004) 697–703.

[3] R. Branco, A.-P. Chung, A. Veríssimo, P.V. Morais, Impact of chromium contaminated wastewaters in the microbial community of a river, FEMS Microbial. Ecol (2005) online 30 March 2005.

[4] J.R. Cole, B. Chai, T.L. Marsh, R.J. Farris, Q. Wang, S.A. Kulam, S. Chandra, D.M. McGarrell, T.M. Schmidt, G.M. Garrity, J.M. Tiedje, The Ribosomal Database Project (RDP-II): previewing a new autoaligner that allows regular updates and the new prokaryotic taxonomy, Nucleic Acids Res. 31 (2003) 442–443.

[5] J. De Ley, H. Cattoir, A. Reynaerts, The quantitative measurement of DNA hybridization from renaturation rates, Eur. J. Biochem. 12 (1970) 133–142.

[6] J. Felsenstein, Confidence limits on phylogenies: an approach using the bootstrap, Evolution 39 (1985) 783–791.

[7] R. Francisco, M.C. Alpoim, P.V. Morais, Diversity of chromium-resistant and –reducing bacteria in a chromium-contaminated activated sludge, J. Appl. Microbiol. 92 (2002) 837–843.

[8] T.A. Hall (Ed.), BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT, Nucl. Acids. Symp. Ser. 41 (1999) 95–98.

[9] T.H. Jukes, C.R. Cantor, Evolution of protein molecules, In: H.N. Munro (Ed.), Mammalian Protein Metabolism, Academic Press, New York, 1969, pp. 21–132.

[10] S. Kumar, K. Tamura, I.B. Jakobsen, M. Nei, MEGA2: Molecular evolutionary genetics analysis software, Bioinformatics 17 (12) (2001) 1244–1245.

[11] Y.-C. Lin, K. Uemori, D.A. Briel, V. Arunpairojana, A. Yokota, *Zimmermannella helvola* gen. nov., sp. nov., *Zimmermannella alba* sp. nov., *Zimmermannella bifida* sp. nov., *Zimmermannella faecalis* sp. nov., and *Leucobacter albus* sp. nov., novel members of the family *Microbacteriaceae*, Int. J. Syst. Evol. Microbiol. 54 (2004) 1669–1676.

[12] J. Marmur, A procedure for the isolation of deoxyribonucleic acid from microorganisms, J. Mol. Biol. 3 (1961) 208–218.

[13] M. Mesbah, U. Premachandran, W.B. Whitman, Precise measurement of the G + C content of deoxyribonucleic acid by high-performance liquid chromatography, Int. J. Syst. Bacteriol. 39 (1989) 159–167.

[14] P.V. Morais, R. Francisco, R. Branco, A.-P. Chung, M.S. da Costa, *Leucobacter chromiireducens* sp. nov, and *Leucobacter aridicollis* sp. nov., two new species isolated from a chromium contaminated environment, Syst. Appl. Microbiol. 27 (2004) 646–652.

[15] F.A. Rainey, N. Ward-Rainey, R.M. Kroppenstedt, E. Stackebrandt, The genus *Nocardiopsis* represents a phylogenetically coherent taxon and a distinct actinomycete lineage: proposal of *Nocardiopsaceae* fam. nov., Int J. Syst. Bacteriol. 446 (1996) 1088–1092.

[16] T.M. Roane, L.L. Pepper, Microbial responses to environmentally toxic cadmium, Microb. Ecol. 28 (2000) 358–364.

[17] N. Saitou, M. Nei, The neighbor-joining method: a new method for reconstructing phylogenetic trees, Mol. Biol. Evol. 4 (1987) 406–425.

[18] K.H. Schleifer, Analysis of the chemical composition and primary structure of murein, Methods Microbiol. 18 (1985) 123–156.
[19] K.H. Schleifer, O. Kandler, Peptidoglycan types of bacterial cell walls and their taxonomic implications, Bacteriol. Rev. 36 (1972) 407–477.
[20] R.M. Smibert, N.R. Krieg, General characterization, In: P. Gerhardt, R.G.E. Murray, R.N. Costilow, E.W. Nester, W.A. Wood, N.R. Krieg, G.B. Phillips (Eds.), Manual of Methods for General Bacteriology, American Society for Microbiology, Washington, DC, USA, 1981, pp. 411–442.
[21] M. Takeuchi, N. Weiss, P. Schumann, A. Yokota, *Leucobacter komagatae* gen. nov., sp. nov., a new aerobic gram-positive, non-sporulating rod with 2,4-diaminobutyric acid in the cell wall,, Int. J. Syst. Bacteriol. 46 (1996) 967–971.
[22] J.D. Thompson, T.J. Gibson, F. Plewniak, F. Jeanmougin, D.G. Higgins, The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools, Nucleic Acids Res. 24 (1997) 4876–4882.
[23] B.J. Tindall, Fully saturated menaquinones in the archaebacterium *Pyrobaculum islandicum*, FEMS Microbiol. Lett. 60 (1989) 251–254.