Leucobacter chromiiresistens sp. nov., a chromate-resistant strain

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A Gram-positive, irregular rod-shaped, non-motile, yellow-pigmented bacterium, strain JG 31^T, was isolated in the course of identifying chromium-resistant soil bacteria. 16S rRNA gene sequence analysis of the isolated bacterium indicated its phylogenetic position within the genus *Leucobacter*. Binary 16S rRNA gene sequence alignments of the isolated bacterium with the 11 species of the genus recognized at the time of writing revealed sequence similarities of more than 97 % with *Leucobacter alluvii* (GenBank accession no: AM072820; 99.4 %), *Leucobacter iarius* (AM040493; 98.2 %), *Leucobacter aridicollis* (AJ781047; 97.8 %), *Leucobacter komagatae* (AB007419; 97.4 %), *Leucobacter chironomi* (EU346911; 97.1 %) and *Leucobacter luti* (AM072819; 97.1 %). In contrast, DNA–DNA hybridization experiments showed similarity values below 28 % for DNA samples from the most closely related type strains of *L. alluvii*, *L. aridicollis* and *L. iarius*. Protein analysis by matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) and automated RiboPrinting using the restriction enzyme *PvuII* differentiated strain JG 31^T from all type strains of the genus *Leucobacter*. The dominant fatty acids of the novel isolate were anteiso-C_{15:0}, anteiso-C_{17:0} and iso-C_{16:0}, while the quinone system consisted of menaquinones MK-11, MK-10, MK-9 and MK-8. In a B-type cross-linked peptidoglycan, the cell-wall amino acids were alanine, glycine, threonine, glutamic acid and 2,4-diaminobutyric acid. Strain JG 31^T was able to grow in a medium containing up to 300 mM K₂CrO₄ and showed cellular aggregation in response to chromate stress. From biochemical and genomic analyses, the new strain is considered to represent a novel species of the genus *Leucobacter*, for which the name *Leucobacter chromiiresistens* sp. nov. is proposed. The type strain is strain JG 31^T (=DSM 22788^T =CCOS 200^T).

The genus *Leucobacter* was described by Takeuchi *et al.* (1996). At the time of writing, the genus *Leucobacter* includes 11 species, namely *Leucobacter komagatae* (Takeuchi *et al.*, 1996), *Leucobacter chromiireducens*, *Leucobacter aridicollis* (Morais *et al.*, 2004), *Leucobacter albus* (Lin *et al.*, 2004), *Leucobacter alluvii*, *Leucobacter luti* (Morais *et al.*, 2006), *Leucobacter iarius* (Somvanshi *et al.*, 2007), *Leucobacter tardus* (Behrendt *et al.*, 2008), *Leucobacter chironomi* (Halpern *et al.*, 2009), *Leucobacter aerolatus* (Martin *et al.*, 2010) and *Leucobacter salsicium* (Yun *et al.*, 2011). All species of the genus are characterized by 2,4-diaminobutyric acid (DAB) as the diagnostic diamino acid in the B-type cross-linked peptidoglycan.

Abbreviation: DAB, 2,4-diaminobutyric acid.

The GenBank/EMBL/DBJ accession number for the 16S rRNA gene sequence of strain JG 31^T is GU390657.

Three supplementary figures and a supplementary table are available with the online version of this paper.

However, differences can be observed within the general amino acid composition of the peptidoglycan and the occurrence of γ -aminobutyric acid (GABA), which has only been found in *L. albus*, *L. komagatae* and *L. chironomi*. Species of the genus *Leucobacter* have been isolated from diverse environmental habitats, such as soil (Lin *et al.*, 2004), air (Martin *et al.*, 2010), activated sludge from chromium-contaminated wastewater (Morais *et al.*, 2006), chironomid egg masses (Halpern *et al.*, 2009) and nematodes (Somvanshi *et al.*, 2007; Muir & Tan, 2007).

Strain JG 31^T was isolated during a series of experiments aimed at identifying chromium-resistant bacteria from uncontaminated soil in July 2007. It was the only bacterium that grew with more than 5 mM chromate (K₂CrO₄) in the medium. Further investigation demonstrated a very high chromate tolerance of strain JG 31^T, which is, to the authors' knowledge, the only known bacterium that is capable of growing in medium containing up to 300 mM chromate.

Taxonomic classification was performed via 16S rRNA gene amplification using universal bacterial primers (27f and 1525r; Rainey *et al.*, 1992). Purified PCR products were sequenced using additional strain-specific primers (490f: GCGTTGTCCGGAATTATTG; 1041f: CGTGTCGTGAG-ATGTTTCG; 1049r: TTTACGAGTGTCCAAAGAG). The assembled sequence was used to determine the phylogenetic position of strain JG 31^T by means of binary sequence alignments using the AE2 alignment editor and 16S rRNA gene sequences of all recognized species of the genus *Leucobacter*. Geneious software (Biomatters) was used to calculate a phylogenetic tree using the neighbour-joining algorithm. Bootstrap values were determined after the analysis of 1000 resamplings (Fig. 1).

The sequence obtained (1460 bp) for strain JG 31^T showed more than 97 % similarity to the sequences of *L. alluvii* (99.4 %), *L. iarius* (98.2 %), *L. aridicollis* (97.8 %), *L. komagatae* (97.4 %), *L. chironomi* (97.1 %) and *L. luti* (97.1 %), while similarities to the type strains of other recognized species of the genus *Leucobacter* were between 95.9 and 97 % (Fig. 1). Although the threshold value for 16S rRNA gene sequence similarity of two separate species is proposed to be <98.7 % (Stackebrandt & Ebers, 2006), DNA–DNA hybridizations were performed using *L. iarius* (DSM 17402^T), *L. aridicollis* (DSM 17380^T) and *L. alluvii* (CIP 108819^T) as the three closest relatives to strain JG 31^T. DNA was isolated from cells disrupted by using a French pressure cell (Thermo Spectronic) and was purified by hydroxyapatite chromatography (Cashion *et al.*, 1977). DNA–DNA hybridization was carried out as described by De Ley *et al.* (1970) under consideration of the modifications described by Huß *et al.* (1983) using a Cary 100 Bio UV/VIS-spectrophotometer (Varian) equipped with a Peltier-thermostatted 6 × 6 multicell changer and a temperature controller with an *in-situ* temperature probe. The binary hybridization values and their standard deviations against strain JG 31^T were 2.1 ± 1.48 % for *L. iarius*, 8.4 ± 2.97 % for *L. aridicollis* and 28.0 ± 5.16 % for *L.*

alluvii. These similarity values were below 70 %, which differentiated strain JG 31^T from its closest phylogenetic neighbours *L. alluvii*, *L. iarius* and *L. aridicollis* at the species level (Wayne, 1988).

Sample preparation for matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) protein analysis and recording of mass spectra by using a Microflex L20 instrument (Bruker Daltonics) were carried out as described by Tóth *et al.* (2008). The dendrogram (see Supplementary Fig. S1 in IJSEM Online) was calculated from mass spectra of strain JG 31^T and type strains of the genus *Leucobacter* by using the BioTyper 2.0 software (Bruker Daltonics). The clusters that appeared in the dendrogram, *L. luti*/*L. chromiireducens* subsp. *chromiireducens*/*L. chromiireducens* subsp. *solipictus* and *L. komagatae*/*L. aridicollis*/*L. albus* agreed with the intrageneric phylogenetic structure of the genus *Leucobacter* as revealed by sequence analysis of the 16S rRNA gene (Fig. 1). The results confirmed the close relationship of strain JG 31^T to the type strain of *L. alluvii* and differentiated strain JG 31^T from the type strains of all species of the genus *Leucobacter*.

Fully automated ribotyping of *Pvu*II-digested samples was performed with a RiboPrinter system (DuPont Qualicon) as described by Bruce (1996). The dendrogram (Supplementary Fig. S2) calculated by using the BioNumerics software (Applied Maths) demonstrated that strain JG 31^T differed in its band pattern from all type strains of the genus *Leucobacter*.

General strain characteristics such as colony colour, size and shape were determined after 24 h and 48 h of growth on LB (lysogeny broth) agar (Bertani, 1951). Strain JG 31^T formed pale yellow colonies after 48 h. Microscopic examination showed that strain JG 31^T formed irregular-shaped rods. The cell size was between 0.33 µm and 0.4 µm in width and 0.74 µm and 1.4 µm in length.

In general, phenotypic characterization was conducted using LB medium containing 1 % (w/v) NaCl. Cells were

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequences. Bootstrap values >30 % are shown (1000 resamplings). GenBank accession numbers are given in parentheses. The numbers on the right show binary 16S rRNA gene sequence similarity values from alignments with strain JG 31^T. Bar, 0.8 % sequence divergence.

grown at 25 °C, 30 °C, 37 °C and 42 °C to determine the optimal growth temperature. The pH range for growth was determined using LB media with pH values ranging from pH 2 to 11 at 0.5 intervals. pH values were adjusted prior to sterilization using HCl and NaOH. No deviation from the initially adjusted pH values was detectable during the growth experiments. Determination of substrate utilization was performed in M9 medium (Ausubel *et al.*, 1987) supplemented with 2 mM MgSO₄ and 100 µM CaCl₂. Salt tolerance was tested in LB medium containing 0–11 % (w/v) NaCl.

Acid production was analysed using the API 50CH test panel (bioMérieux) according to the manufacturer's instructions using medium E (bioMérieux). The incubation temperature was set to 30 °C. Results were scored for up to 5 days according to Morais *et al.* (2004). Of all the supplied carbohydrates, only aesculin was metabolized by strain JG 31^T with a detectable concomitant acid production. Substrate utilization was determined using GP2 Micro Plates (AES Chemunex) according to the manufacturer's instructions except that incubation was carried out at 30 °C for 24 h. Strain JG 31^T showed a unique pattern of substrate utilization when compared with the type strains of *L. iarius* and *L. alluvii* grown under identical conditions (Table 1). Growth under anaerobic conditions was determined using minimal media (Schuetz *et al.*, 2009) containing 0.5 % glycerol as the carbon and electron source and 10 mM potassium nitrate or 20 mM disodium fumarate as the electron acceptor. Anaerobic cultures were grown in Hungate tubes sealed with butyl rubber stoppers. Oxygen was removed from the medium by repeatedly flushing the headspace of each tube for 1.5 min with nitrogen followed by a 1.5 min application of vacuum. Nitrogen gas and vacuum cycles were repeated 15 times before the tubes were inoculated. Citrate utilization, anaerobic growth with glucose as the sole carbon and electron source and urease activity were tested using an Oxi/Ferm Tube II (BD).

Oxidase activity was examined by using 1 % *N,N,N',N'*-tetramethyl-*p*-phenylenediamine dihydrochloride. Catalase activity was determined via bubble production after addition of 3 % (v/v) hydrogen peroxide solution.

Peptidoglycan preparations purified according to the method of Schleifer (1985) were obtained after disruption of cells by shaking with glass beads and subsequent trypsin digestion. The amino acids and peptides in cell-wall hydrolysates were analysed by two-dimensional ascending TLC on cellulose plates by using previously described solvent systems (Schleifer, 1985). The amino-terminal amino acid of the interpeptide bridge was detected by dinitrophenylation as described by Schleifer (1985). The molar ratios of amino acids were determined by GC and GC-MS of *N*-heptafluorobutyl amino acid isobutyl esters (MacKenzie, 1987; Groth *et al.*, 1996). The hydrolysate (4 M HCl, 100 °C, 16 h) of the preparation of strain JG 31^T contained DAB, alanine, glycine, threonine and

glutamic acid in a molar ratio of 0.4:1.8:1.0:0.6:1.0. The appearance of the peptide Gly-D-Glu in the partial hydrolysate (4 M HCl, 100 °C, 0.75 h) indicated a B-type cross-linked peptidoglycan. γ -Aminobutyric acid, present in some other members of the genus *Leucobacter* (*L. albus*, *L. komagatae* and *L. chironomi*), was not detected. Alanine was found to be the *N*-terminal amino acid. Polar lipids were extracted (Minnikin *et al.*, 1979) and analysed by two-dimensional TLC (Collins & Jones, 1980). Phosphatidylglycerol, diphosphatidylglycerol, an unidentified phospholipid and an unidentified glycolipid were detected (Supplementary Fig. S3).

Analysis of menaquinones was performed by HPLC as described previously (Groth *et al.*, 1996). MK-11 was found to be the main menaquinone (59 %). MK-10, MK-9 and MK-8 were present in lower amounts of 22 %, 11 % and 5 %, respectively.

Cellular fatty acids were extracted, methylated and analysed by GC using the Sherlock Microbial Identification system (MIDI) as described previously (Kämpfer & Kroppenstedt, 1996). Anteiso-C_{15:0}, anteiso-C_{17:0} and iso-C_{16:0} were found to be the major fatty acids of strain JG 31^T, which was in line with the general fatty acid pattern of the genus (see Supplementary Table S1).

Several species of the genus *Leucobacter* are known to be resistant to chromium (*L. chromiireducens*, *L. aridicollis*, *L. komagatae*, *L. luti*, *L. alluvii* and *L. chironomi*) and antibiotics (*L. chromiireducens*). Therefore, chromate tolerance of strain JG 31^T was determined by growth in LB medium supplemented with K₂CrO₄ at 30 °C. Surprisingly, strain JG 31^T showed growth at concentrations of up to 300 mM Cr(VI). Antibiotic resistance was observed in LB media containing (µg ml⁻¹): streptomycin (30), gentamicin (15), tetracycline (15), kanamycin (30) and spectinomycin (100). In contrast, addition of 100 µg ampicillin ml⁻¹, 20 µg chloramphenicol ml⁻¹ and 150 µg rifampicin ml⁻¹ resulted in growth inhibition.

In conclusion, based upon the phylogenetic and chemotaxonomic evidence, strain JG 31^T represents a novel member of the genus *Leucobacter*, family *Microbacteriaceae*, for which the name *Leucobacter chromiiresistens* sp. nov. is proposed.

Description of *Leucobacter chromiiresistens* sp. nov.

Leucobacter chromiiresistens (chro.mi.i.re.sis'tens. N.L. neut. n. *chromium* chromium; L. part. adj. *resistens* resisting; N.L. part. adj. *chromiiresistens* chromium resisting).

Cells are Gram-positive-staining, aerobic, non-sporulating, non-motile rods (0.33–0.4 × 0.74–1.4 µm). After 48 h of growth they form yellow colonies on LB agar. Optimal growth occurs at 30 °C and at pH 7–8. Cells do not grow at 4 °C or 42 °C or below pH 5 or above pH 10. Growth is observed at NaCl concentrations from 0 to 8 % (w/v).

Table 1. Phenotypic characteristics of strain JG 31^T and closely related species of the genus *Leucobacter*

Strains: 1, JG 31^T; 2, *L. alluvii* CIP 108819^T; 3, *L. iarius* DSM 17402^T. All other carbohydrates provided by the test panels were scored as negative and are not listed below. All strains were pre-grown in LB medium at 30 °C. +, Growth or positive test result; –, no growth or negative test result; w, weak growth on Biolog GP2 Micro Plates; NA, no data available.

Characteristic	1	2	3
Colony pigmentation	Yellow	Cream	White
Urease	–	+	–
Gelatinase	–	+	–
pH optimum	7–8	7–8	NA
Optimal temperature (°C)	30	30	30
Growth at/in:			
4 °C	–	+	–
37 °C	+	+	+
7 % NaCl	+	+	+
10 % NaCl	–	–	–
Amino acids in cell wall			
DAB	0.4	0.5	0.5
Alanine	1.8	2.4	1.5
Glycine	1.0	1.2	0.9
Glutamic acid	1.0	1.0	1.0
γ-Aminobutyric acid	–	–	–
Threonine	0.6	0.7	0.7
Menaquinones			
Major amounts	MK-11	MK-11	MK-11
Minor amounts	MK-10, MK-9, MK-8	MK-10	MK-10, MK-9, MK-12
Acid production from (API 50CH):			
N-Acetylglucosamine	–	–	+
Aesculin	+	–	+
D-Fructose	–	–	+
Glycerol	–	–	+
D-Ribose	–	–	+
Turanose	–	+	–
Utilization of (Biolog GP2 Micro Plates):			
Adenosine	+	+	w
L-Alaninamide	+	+	+
L-Alanine	+	–	+
L-Alanyl glycine	–	+	–
L-Asparagine	+	–	–
2'-Deoxyadenosine	+	+	–
D-Fructose	–	–	+
L-Glutamic acid	–	+	+
Glycerol	+	+	+
Glycyl L-glutamic acid	–	–	+
p-Hydroxyphenylacetic acid	+	–	+
α-Ketoglutaric acid	–	+	–
Pyruvic acid methyl ester	+	+	+
Putrescine	+	+	+
Pyruvic acid	+	+	–
D-Ribose	–	–	+

Table 1. cont.

Characteristic	1	2	3
Succinic acid	–	+	–
Succinic acid monomethyl ester	–	+	–
Thymidine	+	+	–
Xylitol	+	+	–
Uridine	–	–	w
Tween 40	+	+	+
Tween 80	+	+	w

Growth rates decrease when the NaCl concentration exceeds 2 % (w/v). Catalase activity is detected, whereas oxidase, gelatinase and urease activities are absent. Oxidative acid production is observed for aesculin but not for any other of the API 50CH carbohydrates after 5 days at 30 °C. Neither nitrate nor fumarate serves as electron acceptor under anaerobic growth conditions. Glucose is not fermented. On Biolog GP2 MicroPlates, utilization of Tween 40, Tween 80, xylitol, *p*-hydroxyphenylacetic acid, pyruvic acid methyl ester, pyruvic acid, L-alaninamide, L-alanine, L-asparagine, glycerol, adenosine, 2'-deoxyadenosine, thymidine and putrescine is detected. Polar lipids are phosphatidylglycerol and diphosphatidylglycerol. Two unidentified components, one glycolipid and one phospholipid, are also present. The B-type cross-linked peptidoglycan contains alanine, glycine, threonine, glutamic acid and DAB. Major menaquinones are MK-11 and MK-10. The dominant cellular fatty acids are anteiso-C_{15:0}, anteiso-C_{17:0} and iso-C_{16:0}. The cells are highly chromate resistant and able to tolerate up 300 mM K₂CrO₄ in the medium, showing aggregation as a phenotypic stress response. The type strain is resistant to (μg ml^{–1}): streptomycin (30), gentamicin (15), tetracycline (15), kanamycin (30) and spectinomycin (100), but sensitive to ampicillin (100), chloramphenicol (20) and rifampicin (150).

The type strain, JG 31^T (=DSM 22788^T =CCOS 200^T), was isolated from an uncontaminated soil sample at the University of Freiburg, Germany.

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