Leucobacter chironomi sp. nov., a chromate-resistant bacterium isolated from a chironomid egg mass

Malka Halpern,¹ Tamar Shakéd,¹ Rüdiger Pukall² and Peter Schumann²

Correspondence

Malka Halpern

mhalpern@research.haifa.ac.il

¹Department of Biology Education, Faculty of Science and Science Education, University of Haifa, Oranim, Tivon 36006, Israel

²DSMZ – Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Inhoffenstr. 7B, D-38124 Braunschweig, Germany

A Gram-positive, rod-shaped, aerobic, non-motile, yellow-pigmented bacterial strain, designated MM2LB^T, was isolated from a chironomid egg mass that was sampled in a waste stabilization pond in northern Israel. 16S rRNA gene sequence analysis indicated that the isolate should be placed in the genus *Leucobacter* (family *Microbacteriaceae*). Levels of 16S rRNA gene sequence similarity between strain MM2LB^T and the type strains of recognized *Leucobacter* species were 97.1 % or lower. The dominant cellular fatty acids of strain MM2LB^T were anteiso-C_{15:0}, iso-C_{16:0} and anteiso-C_{17:0}, which are typical of members of the genus *Leucobacter*. Strain MM2LB^T grew at 17–37 °C, in the presence of 0–7.0 % NaCl and at pH 4.0–9.5. Cell-wall amino acids were alanine, glycine, threonine, 2,4-diaminobutyric acid, glutamic acid and γ -aminobutyric acid in a B-type cross-linked peptidoglycan. The quinone system consisted of menaquinones MK-11, MK-10 and MK-12. The novel isolate was able to grow in medium containing 18 mM Cr(VI). On the basis of phenotypic properties and phylogenetic distinctiveness, strain MM2LB^T is considered to represent a novel species of the genus *Leucobacter*, for which the name *Leucobacter chironomi* sp. nov. is proposed. The type strain is MM2LB^T (=LMG 24399^T=DSM 19883^T).

The genus *Leucobacter* (family *Microbacteriaceae*) was erected by Takeuchi *et al.* (1996). All species of the genus are characterized by 2,4-diaminobutyric acid (DAB) as the diagnostic diamino acid in the peptidoglycan. However, there are differences in the amounts of other amino acids in the peptidoglycan. Among members of the genus, γ -aminobutyric acid has been found only in *Leucobacter albus* and *Leucobacter komagatae*. At the time of writing, the genus comprises eight recognized species: *L. komagatae* (Takeuchi *et al.*, 1996), *L. chromiireducens* (Morais *et al.*, 2004; Muir & Tan, 2007), *L. aridicollis* (Morais *et al.*, 2004), *L. albus* (Lin *et al.*, 2004), *L. alluvii* and *L. luti* (Morais *et al.*, 2006), *L. iarius* (Somvanshi *et al.*, 2007) and *L. tardus* (Behrendt *et al.*, 2008).

Strains belonging to the genus *Leucobacter* occur in a variety of ecological niches such as activated sludge (from a

treatment plant containing chromium-contaminated wastewater; Morais *et al.*, 2004, 2006), soil (Lin *et al.*, 2004), river sediment (Morais *et al.*, 2006), nematodes (Somvanshi *et al.*, 2007; Muir & Tan, 2007) and the phyllosphere of potato plants (Behrendt *et al.*, 2008). In the present study, an obligately aerobic, non-motile, Gram-positive, rod-shaped bacterium (strain MM2LB^T), isolated from a freshwater insect egg mass, is proposed as representing a novel species of the genus *Leucobacter*.

Strain MM2LB^T was isolated during the course of studies aimed at identifying culturable bacteria from chironomid (non-biting midge, Chironomidae; Diptera) egg masses. Chironomid egg masses were sampled from Tivon waste stabilization pond in northern Israel, as previously described (Halpern *et al.*, 2007a). To determine the diversity of culturable bacteria in chironomid egg masses, the masses were washed thoroughly with sterile saline (vortexing for 1 min with five changes of saline) and then their homogenates were diluted and cultured directly on Luria–Bertani (LB) agar (Himedia). More than 10⁴ faecal coliforms ml⁻¹ were counted in Tivon waste stabilization pond water samples (Halpern *et al.*, 2004). However, no faecal coliforms were isolated from the egg mass when MacConkey agar, a selective medium for *Escherichia coli*,

Abbreviation: DAB, 2,4-diaminobutyric acid.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain MM2LB^T is EU346911.

An electron micrograph of a negatively stained cell of strain MM2LB^T and a table detailing the major fatty acid contents of strain MM2LB^T and recognized species of the genus *Leucobacter* are available with the online version of this paper.

was used. This indicated that the egg masses had been rinsed thoroughly and that the novel bacterial isolate was tightly bound to the egg mass.

Strain MM2LB^T was isolated in July 2006 from a chironomid egg mass, cultured on LB agar and incubated at 30 °C for 48 h in the dark. Two other novel species that were isolated from chironomid egg masses in a previous study were recently characterized as *Oceanobacillus chironomi* (Raats & Halpern, 2007) and *Rheinheimera chironomi* (Halpern *et al.*, 2007b). The exact taxonomic position of strain MM2LB^T was determined by means of a polyphasic approach that included phenotypic characterization and phylogenetic analysis based on 16S rRNA gene sequences.

Universal bacterial primers 8f and 1512r (Felske *et al.*, 1997) were used to amplify internal fragments of the 16S rRNA gene of strain MM2LB^T. Purified PCR products were sequenced with primers 8f, 534r, 968f and 1512r as described in detail by Raats & Halpern (2007). This resulted in a sequence of approximately 1500 bp. Identification of phylogenetic neighbours and calculation of pairwise 16S rRNA gene sequence similarity were performed by using the EzTaxon server (<http://www.eztaxon.org/>; Chun *et al.*, 2007). Phylogenetic and molecular evolutionary analyses were conducted by using MEGA version 4 (Tamura *et al.*, 2007). Strain MM2LB^T showed highest levels of 16S rRNA gene sequence similarity with *L. aridicollis* CIP 108388^T (97.1%), *L. komagatae* IFO 15245^T (97.0%) and the type strains of other recognized *Leucobacter* species (96.8–96.4%). In the neighbour-joining tree based on 16S rRNA gene sequences, strain MM2LB^T formed a distinct branch within the radiation encompassing the genus *Leucobacter* (Fig. 1). Levels of 16S rRNA gene sequence similarity between strain MM2LB^T and the type strains of representative members of other genera were below 94.9%. The data from 16S rRNA gene sequence analysis thus suggest that strain MM2LB^T represents a novel species within the genus *Leucobacter* (Stackebrandt & Goebel, 1994; Stackebrandt & Ebers, 2006).

For electron microscopy, bacteria on LB agar medium were suspended in saline. The samples were adhered to a carbon-coated grid, stained with 2% uranyl acetate and photographed under a JEM-1200EX electron microscope (JEOL). Electron microscopy showed that the cells were rods measuring 0.5–0.75 µm in width and 0.95–1.4 µm in length (see Supplementary Fig. S1 in IJSEM Online).

For phenotypic characterization, LB agar containing 0.5% NaCl was used as the basal growth medium (except for determination of salt tolerance where LB agar lacking NaCl was used). Salt tolerance was determined at 30 °C on LB agar containing 0–10% (w/v) NaCl at 1.0% intervals. Growth at 5, 10, 14, 17, 25, 30, 33, 37, 40 and 45 °C was measured on LB agar. The pH range for growth was determined by using LB broth adjusted to pH 4.0–8.0 at 2.0 pH unit intervals. The pH was adjusted prior to sterilization by the addition of HCl or NaOH and was measured again following sterilization. For determination of growth on alkaline medium, LB broth was adjusted to pH 9.0, 9.5 and 10.0 with 0.1 M Na₂CO₃ or 0.1 M NaHCO₃ and then filtered through a 0.2 µm pore size filter (Corning) to obtain a sterilized medium. Growth under anaerobic conditions was determined after incubation in an anaerobic chamber on LB agar supplemented with 0.5% (w/v) glucose or 0.1% (w/v) potassium nitrate. Growth on marine, MacConkey and cetrimide agars (Himedia) was determined after 48 h incubation at 30 °C. Biochemical tests were performed by using API 20E, API 20NE, API 50CH and API ZYM identification systems (bioMérieux), according to the manufacturer's instructions, except that the incubation temperature was 30 °C. Substrate utilization was analysed by using Biolog GP2 microplates according to the manufacturer's instructions (release 3.50, version DE). The plates were incubated for 24 h at 30 °C before being read with the Microlog plate reader. Wells that changed colour to purple were marked as positive for metabolic activity. Catalase activity was determined based on bubble production in a 3% (v/v) hydrogen peroxide solution. Oxidase activity was deter-

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences obtained from strain MM2LB^T and the type strains of related species. Bootstrap values (>50%) based on 1000 replicates are indicated at branch points. *Arthrobacter globiformis* DSM 20124^T was used as an outgroup. Asterisks indicate branches of the tree that were also formed by using the maximum-parsimony method. Bar, 0.01 substitutions per nucleotide position.

mined by using 1% *N,N,N',N'*-tetramethyl-*p*-phenylenediamine dihydrochloride (T3134; Sigma-Aldrich). The phenotypic traits of the new isolate are detailed in the species description below and in Table 1.

For cellular fatty acid analysis, strain MM2LB^T was cultured on trypticase soy agar (Difco) for 24 h at 28 °C and the fatty acids were then extracted and methylated (Ben-Ze'ev *et al.*, 2005). Fatty acid methyl esters were analysed by GC by using the MIDI/Hewlett Packard microbial identification system (Analytical Services Inc.). The major fatty acids (>10% of the total) of strain MM2LB^T were anteiso-C_{15:0} (47.0%), anteiso-C_{17:0} (23.9%) and iso-C_{16:0} (22.9%) (see Supplementary Table S1 in IJSEM Online).

The composition of the cell-wall peptidoglycan is a useful diagnostic tool for characterizing novel species belonging to the family *Microbacteriaceae*. For analysis, purified peptidoglycan preparations were obtained after disruption of cells by shaking with glass beads and subsequent trypsin digestion, according to the method of Schleifer (1985). The amino acids and peptides in the cell-wall hydrolysates were analysed by two-dimensional ascending TLC on cellulose plates by using the solvent systems described by Schleifer (1985). The molar ratios of the amino acids were determined by GC (GC 14A; Shimadzu) and GC-MS (320-MS Quadrupole GC/MS; Varian) of *N*-heptafluorobutyl amino acid isobutyl esters (MacKenzie, 1987; Groth *et al.*, 1996). The hydrolysate (4 M HCl, 100 °C, 16 h) of the purified peptidoglycan of strain MM2LB^T contained the amino acids alanine, glycine, threonine, DAB and glutamic acid in a molar ratio of approximately 2.5:1.2:0.9:0.5:1.0, respectively. γ -Aminobutyric acid was also present but could not be quantified (Table 1). Strain MM2LB^T possessed a B-type cross-linked peptidoglycan (Schleifer & Kandler, 1972).

Isoprenoid quinone analysis of strain MM2LB^T was performed by HPLC as described by Groth *et al.* (1996). Strain MM2LB^T contained menaquinones MK-11, MK-10 and MK-12 in a molar ratio of approximately 85:9:6, respectively (Table 1).

As several species of the genus *Leucobacter* (*L. chromii-reducens*, *L. aridicollis*, *L. komagatae*, *L. luti* and *L. alluvii*) showed resistance to chromate, tolerance to chromium was examined by culturing strain MM2LB^T in LB broth supplemented with K₂CrO₄ at final concentrations of 1, 3, 4, 5, 6, 8, 10, 12, 16, 18 and 20 mM Cr(VI). Cells were incubated at 30 °C for 48 h. Strain MM2LB^T grew in the presence of up to 18 mM Cr(VI).

The new isolate shared the main characteristics of strains of species of the genus *Leucobacter*, namely non-motile, rod-shaped, catalase-positive cells, anteiso-C_{15:0}, iso-C_{16:0} and anteiso-C_{17:0} as the dominant fatty acids, MK-11 as the major menaquinone, and DAB, alanine, glycine and glutamic acid in a B-type cross-linked peptidoglycan. However, strain MM2LB^T differed from

recognized *Leucobacter* species based on several phenotypic traits (see Table 1). Strain MM2LB^T contained γ -aminobutyric acid, which has been described only in *L. albus* and *L. komagatae* among members of the genus *Leucobacter*. On the basis of phenotypic characterization and 16S rRNA gene sequence phylogenetic analysis, we suggest that strain MM2LB^T represents a novel species of the genus *Leucobacter*, for which the name *Leucobacter chironomi* sp. nov. is proposed.

Description of *Leucobacter chironomi* sp. nov.

Leucobacter chironomi [chi.ro'no.mi. N.L. gen. n. *chironomi* of *Chironomus*, named after the non-biting midge insect of the genus *Chironomus* (Chironomidae: Diptera) from which the type strain was isolated].

Cells are aerobic, Gram-positive, non-motile rods measuring 0.5–0.75 μ m in width and 0.95–1.4 μ m in length. Cells occur singly. After 48 h incubation on LB agar at 30 °C, colonies are opaque, circular, with entire margins and yellow-coloured. Growth is not observed under anaerobic conditions even after 8 days incubation. Growth is observed after 48 h incubation at 17–37 °C (optimum 30 °C), in the presence of 0–7.0% NaCl (optimum 0–1.0% NaCl) and at pH 4.0–9.5 (optimum pH 6.0–8.0). Good growth occurs on marine agar after 48 h incubation. Growth is not observed on MacConkey or cetrimide agars. Oxidase reaction is negative; catalase reaction is weakly positive. Although the type strain was not isolated from a chromium-enriched environment, it is able to grow in the presence of up to 18.0 mM Cr(VI). On API 50CH strips incubated for 48 h at 30 °C, acid is not produced from any of the substrates tested. On API 20NE strips incubated for 48 h at 30 °C, all the results are negative. The following results are obtained from API 20E strips after 48 h incubation at 30 °C: acetoin is produced; nitrate is reduced to nitrogen; H₂S and indole are not produced; urea and gelatin are not hydrolysed; citrate is not utilized; and β -galactosidase, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase and tryptophan deaminase activities are absent. Putrescine and glycerol are utilized in the Biolog test plates. Cell-wall amino acids are alanine, glycine, threonine, DAB, γ -aminobutyric acid and glutamic acid. Has a B-type cross-linked peptidoglycan. The major menaquinone is MK-11; MK-10 and MK-12 occur in minor amounts. The dominant cellular fatty acids are anteiso-C_{15:0}, anteiso-C_{17:0} and iso-C_{16:0}. The following fatty acids are present as minor components: C_{16:0}, C_{14:0} and iso-C_{15:0}.

The type strain, MM2LB^T (=LMG 24399^T=DSM 19883^T), was isolated from a chironomid egg mass in Israel.

Acknowledgements

Strain MM2LB^T was isolated by Maya Ben-Zion and Michal Ben-Harush during the course of a microbial ecology workshop.

Table 1. Differential phenotypic characteristics between strain MM2LB^T and recognized species of the genus *Leucobacter*

Strains: 1, MM2LB^T; 2, *L. albus* DSM 17379^T; 3, *L. chromiireducens* subsp. *chromiireducens* DSM 17381^T; 4, *L. aridicollis* DSM 17380^T; 5, *L. komagatae* DSM 8803^T; 6, *L. luti* CIP 108818^T; 7, *L. alluvii* CIP 108819^T; 8, *L. iarius* DSM 17402^T; 9, *L. tardus* DSM 19811^T. Results of acid production from various substrates were determined by using an API 50CH test panel with medium E and were scored after 2 days. All strains were negative for all other substrates provided on the API 50CH test panel. Data for reference taxa were obtained from Takeuchi *et al.* (1996), Lin *et al.* (2004), Morais *et al.* (2004), Muir & Tan (2007), Somvanshi *et al.* (2007) and Behrendt *et al.* (2008). +, Positive; –, negative; w, weakly positive; NA, data not available; +/–, different reactions reported.

Characteristic	1	2	3	4	5	6	7	8	9
Colony pigmentation	Yellow	White	Cream	Cream	Whitish brown	Cream	Cream	White	Yellow
Urease	–	–	+	+	+	+	+	–	–
Gelatinase	–	NA	–	–	+	–	+	–	–
pH optimum	6–8	NA	7	7–8	7–9	7–9	7–8	NA	NA
Growth at:									
4 °C	–	NA	–	–	+	+	+	–	–
37 °C	+	NA	+	+	–	–	+	+	–
Optimal temperature (°C)	30	30	28	28	28	25	30	30	21–30
Growth in the presence of:									
7 % NaCl	+	NA	+	+	–	+	+	+	NA
10 % NaCl	–	NA	–	+	–	–	–	–	NA
Amino acids in the cell wall (molar ratios)									
DAB*	0.5	0.8	0.9	0.5	0.8	0.5	0.5	0.5	0.5
Alanine	2.5	1.8	1.9	2.0	1.9	0.5	2.4	1.5	3.2
Glycine	1.2	1.1	1.3	1.1	0.9	0.3	1.2	0.9	2.1
Glutamic acid	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
γ-Aminobutyric acid	+†	0.7	–	–	0.7	–	–	–	–
Threonine	0.9	–	0.7	–	–	0.6	0.7	0.7	–
Menaquinone(s) (MK-)									
Major amounts	11	11	11	11	11	11	11	11	10;11
Minor amounts	10;12	12;9	10	10	10;12	10	10	10;9;12	9
Acid production from (API 50CH):									
N-Acetylglucosamine	–	–	–	–	–	–	–	+	–
D-Adonitol	–	–	–	–	w	–	–	–	–
D-Arabinose	–	w	–	–	–	–	–	–	–
L-Arabinose	–	–	–	–	w	–	–	–	–
L-Fucose	–	+	–	–	–	–	–	–	–
Fructose	–	–	–	–	–	–	–	+	+
Glycerol	–	w	–	–	+	–	–	+	–
Inositol	–	–	–	–	–	w	–	w	–
5-Ketogluconate	–	–	–	–	–	–	–	w	–
Melezitose	–	–	–	–	–	–	–	–	+
Salicin	–	–	–	–	–	–	–	w	–
Trehalose	–	+	–	–	–	–	–	–	–
Raffinose	–	–	–	–	–	–	–	–	+
L-Rhamnose	–	–	–	–	–	w	–	–	+
Ribose	–	+	–	–	+	+	–	+	–
Utilization of (Biolog GP2 micro plates):‡									
N-Acetyl-D-glucosamine	–	–	–	–	–	–	–	+	–
N-Acetyl L-glutamic acid	–	+	–	–	–	w	–	–	+
Adenosine	–	w	+	+	–	–	+	+	+
Adenosine 5'-monophosphate	–	–	–	+	–	–	–	w	–
L-Alaninamide	–	+	+	+	+	–	+	+	+
L-Alanine	–	w	+	+	–	+	+	w	–
L-Alanyl glycine	–	+	+ / –	+	–	–	+	–	–
L-Arabinose	–	–	–	–	–	–	–	–	+
L-Asparagine	–	+	–	+	–	–	–	–	–
2,3-Butanediol	–	–	–	–	–	–	–	–	+
2'-Deoxyadenosine	–	+	+	+	+	w	–	–	+
Dextrin	–	–	–	–	–	–	+	–	–

Table 1. cont.

Characteristic	1	2	3	4	5	6	7	8	9
D-Fructose	—	—	—	—	—	—	+	+	w
D-Fructose 6-phosphate	—	+	+/-	+	+	+	+	+	—
D-Gluconic acid	—	—	—	—	—	—	—	—	+
α -D-Glucose	—	—	—	—	—	—	+	—	+
D-Glucose 6-phosphate	—	—	—	—	—	—	—	—	+
L-Glutamic acid	—	+	+	+	w	w	—	w	—
Glycerol	+	+	—	+	+	—	+	+	+
DL- α -Glycerol phosphate	—	—	—	—	—	—	—	—	+
Glycyl L-glutamic acid	—	+	+	+	w	w	—	+	—
α -Hydroxybutyric acid	—	—	—	—	—	—	—	—	+
β -Hydroxybutyric acid	—	—	—	+	—	—	—	—	—
p-Hydroxyphenylacetic acid	—	—	+	+	w	+	+	+	+
α -Ketovaleric acid	—	+	—	w	—	—	—	—	w
L-Lactic acid	—	—	—	—	—	—	+	—	+
Methyl α -D-galactoside	—	—	—	—	—	—	—	—	+
Methyl β -D-galactoside	—	—	—	—	—	—	—	—	+
3-Methyl glucose	—	—	—	—	—	—	—	—	+
Methyl pyruvate	—	+	+	—	—	+	+	—	+
Putrescine	+	—	+/-	—	—	—	—	—	+
Pyruvic acid	—	—	—	—	—	—	—	—	+
D-Ribose	—	+	—	+	+	—	—	+	+
L-Serine	—	+	+/-	+	—	—	—	w	+
D-Sorbitol	—	—	—	—	—	—	—	—	+
Succinamic acid	—	—	—	—	—	—	—	—	+
Succinic acid	—	—	—	—	—	—	—	—	+
Thymidine	—	+	+	+	—	—	+	+	+
Thymidine 5'-monophosphate	—	+	—	+	—	—	—	+	—
Trehalose	—	+	—	—	—	—	—	—	—
Xylitol	—	—	—	—	—	—	+	—	—
D-Xylose	—	—	—	—	—	—	—	—	+
Uridine	—	+	+/-	+	—	—	—	+	+
Uridine 5'-monophosphate	—	w	—	+	—	—	—	+	—

*DAB, 2,4-diaminobutyric acid.

† γ -Aminobutyric acid is present but could not be quantified.

‡Data for reference species from Muir & Tan (2007), Somvanshi *et al.* (2007) and Behrendt *et al.* (2008).

References

- Behrendt, U., Ulrich, A. & Schumann, P. (2008). Description of *Leucobacter tardus* sp. nov., isolated from the phyllosphere of *Solanum tuberosum* L. *Int J Syst Evol Microbiol* **58**, 2574–2578.
- Ben-Ze'ev, I. S., Levy, E., Eilam, T. & Anikster, Y. (2005). Whole-cell fatty acid profiles – a tool for species and subspecies classification in the *Puccinia recondita* complex. *J Plant Pathol* **87**, 187–197.
- Chun, J., Lee, J.-H., Jung, Y., Kim, M., Kim, S., Kim, B. K. & Lim, Y. W. (2007). EzTaxon: a web-based tool for the identification of prokaryotes based on 16S ribosomal RNA gene sequences. *Int J Syst Evol Microbiol* **57**, 2259–2261.
- Felske, A., Rheims, H., Wolterink, A., Stackebrandt, E. & Akkermans, A. D. (1997). Ribosome analysis reveals prominent activity of an uncultured member of the class *Actinobacteria* in grassland soils. *Microbiology* **143**, 2983–2989.
- Groth, I., Schumann, P., Weiss, N., Martin, K. & Rainey, F. A. (1996). *Agrococcus jenensis* gen. nov., sp. nov., a new genus of actinomycetes with diaminobutyric acid in the cell wall. *Int J Syst Bacteriol* **46**, 234–239.
- Halpern, M., Broza, Y. B., Mittler, S., Arakawa, E. & Broza, M. (2004). Chironomid egg masses as a natural reservoir of *Vibrio cholerae* non-O1 and non-O139 in freshwater habitats. *Microb Ecol* **47**, 341–349.
- Halpern, M., Landsberg, O., Raats, D. & Rosenberg, E. (2007a). Culturable and VBNC *Vibrio cholerae*; interactions with chironomid egg masses and their bacterial population. *Microb Ecol* **53**, 285–293.
- Halpern, M., Senderovich, Y. & Snir, S. (2007b). *Rheinheimera chironomi* sp. nov., isolated from a chironomid (Diptera; Chironomidae) egg mass. *Int J Syst Evol Microbiol* **57**, 1872–1875.
- Lin, Y.-C., Uemori, K., de Briel, D. A., Arunpairojana, V. & Yokota, A. (2004). *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**, 1669–1676.

- Mackenzie, S. L. (1987).** Gas chromatographic analysis of amino acids as the *N*-heptafluorobutyl isobutyl esters. *J Assoc Off Anal Chem* **70**, 151–160.
- Morais, P. V., Francisco, R., Branco, R., Chung, A. P. & da Costa, M. S. (2004).** *Leucobacter chromiireducens* sp. nov., and *Leucobacter aridicollis* sp. nov., two new species isolated from a chromium contaminated environment. *Syst Appl Microbiol* **27**, 646–652.
- Morais, P. V., Paulo, C., Francisco, R., Branco, R., Chung, A. P. & da Costa, M. S. (2006).** *Leucobacter luti* sp. nov., and *Leucobacter alluvii* sp. nov., two new species of the genus *Leucobacter* isolated under chromium stress. *Syst Appl Microbiol* **29**, 414–421.
- Muir, R. E. & Tan, M. V. (2007).** *Leucobacter chromiireducens* subsp. *solipictus* subsp. nov., a pigmented bacterium isolated from the nematode *Caenorhabditis elegans*, and emended description of *L. chromiireducens*. *Int J Syst Evol Microbiol* **57**, 2770–2776.
- Raats, D. & Halpern, M. (2007).** *Oceanobacillus chironomi* sp. nov., a halotolerant and facultative alkaliphilic species isolated from a chironomid egg mass. *Int J Syst Evol Microbiol* **57**, 255–259.
- Schleifer, K. H. (1985).** Analysis of the chemical composition and primary structure of murein. *Methods Microbiol* **18**, 123–156.
- Schleifer, K. H. & Kandler, O. (1972).** Peptidoglycan types of bacterial cell walls and their taxonomic implications. *Bacteriol Rev* **36**, 407–477.
- Somvanshi, V. S., Lang, E., Schumann, P., Pukall, R., Kroppenstedt, R. M., Ganguly, S. & Stackebrandt, E. (2007).** *Leucobacter iarius* sp. nov., in the family *Microbacteriaceae*. *Int J Syst Evol Microbiol* **57**, 682–686.
- Stackebrandt, E. & Ebers, J. (2006).** Taxonomic parameters revisited: tarnished gold standards. *Microbiol Today* **33**, 152–155.
- Stackebrandt, E. & Goebel, B. M. (1994).** Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *Int J Syst Bacteriol* **44**, 846–849.
- Takeuchi, M., Weiss, N., Schumann, P. & Yokota, A. (1996).** *Leucobacter komagatae* gen. nov., sp. nov., a new aerobic gram-positive, nonsporulating rod with 2,4-diaminobutyric acid in the cell wall. *Int J Syst Bacteriol* **46**, 967–971.
- Tamura, K., Dudley, J., Nei, M. & Kumar, S. (2007).** MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. *Mol Biol Evol* **24**, 1596–1599.