Enterobacter turicensis sp. nov. and *Enterobacter helveticus* sp. nov., isolated from fruit powder

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Four Gram-negative, facultatively anaerobic, non-spore-forming isolates of coccoid rods were obtained from fruit powder and investigated in a polyphasic taxonomic study. Comparative 16S rRNA gene sequence analysis allocated the isolates to the family *Enterobacteriaceae*. Their phylogenetic position within the family *Enterobacteriaceae* was confirmed by *rpoB* sequence analysis and as the highest *rpoB* sequence similarities were obtained with *Enterobacter radicincitans*, *Enterobacter cowanii* and *Enterobacter sakazakii*, the isolates clearly belong to the genus *Enterobacter*. Biochemical data revealed that the isolates can be separated into two distinct groups that represent two novel species, as confirmed by DNA–DNA hybridizations. The two novel species can be differentiated from their nearest neighbours by the following characteristics: the utilization of sucrose, D-sorbitol, putrescine and mucate, the hydrolysis of aesculin and a negative result in the Voges–Proskauer reaction. It is therefore proposed that these novel isolates are classified as *Enterobacter turicensis* sp. nov. (type strain 508/05^T=LMG 23730^T=DSM 18397^T) and *Enterobacter helveticus* sp. nov. (type strain 513/05^T=LMG 23732^T=DSM 18396^T).

The current US Food and Drug Administration method for the detection of *Enterobacter sakazakii* includes a pre-enrichment procedure in buffered peptone water (BPW), enrichment in *Enterobacteriaceae* enrichment (EE) broth, plating on violet red bile glucose agar (VRBG) and the picking of five grown colonies onto tryptone soy agar (TSA) plates, which are incubated at 25 °C for 48–72 h. Yellow-pigmented presumptive colonies have to be further identified. Based on α -glucosidase activity, another biochemical property of *E. sakazakii*, several differential media have been developed recently (Iversen *et al.*, 2004; Oh & Kang, 2004). Two of these media, Oxoid chromogenic *Enterobacter sakazakii* agar (Oxoid CM1055; Oxoid), also known as the Druggan–Forsythe–Iversen (DFI) formulation (Iversen *et al.*, 2004), and *Enterobacter sakazakii* isolation agar (ESIA; AES), are commercially available.

Abbreviations: BPW, buffered peptone water; DFI, Druggan–Forsythe–Iversen formulation; EE, *Enterobacteriaceae* enrichment; VRBG, violet red bile glucose.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA and *rpoB* gene sequences of strains 508/05^T, 610/05, 513/05^T and 1159/04 are DQ273681, DQ273680, DQ273688 and DQ273683 and DQ779998, DQ780000, DQ779997 and DQ779999, respectively.

In a recent study, a set of 12 strains isolated from fruit powder showed yellow-pigmented presumptive *E. sakazakii* colonies on TSA plates as well as typical turquoise colonies on DFI medium when incubated at the recommended temperature (Lehner *et al.*, 2006). API 32E analysis of these strains revealed ambiguous results, but none of the strains were clearly identified as *E. sakazakii*. The API 32E tests suggested identifications as *Escherichia vulneris*, *Pantoea* spp. and *Buttiauxiella agrestis* (previously known as *Citrobacter* group F) for several of the strains, but with low levels of confidence. In order to obtain more information on the exact taxonomic position of these fruit powder isolates, 16S rRNA gene sequencing was performed (Lehner *et al.*, 2006). Comparative analysis revealed that all of the isolates were clearly distinct from *E. sakazakii* with gene sequence similarities <97% (Lehner *et al.*, 2004).

Four of these strains were further characterized and, based on the results of a polyphasic taxonomic study, it can be concluded that these isolates represent two novel species of the genus *Enterobacter*.

Fruit powder (150 g) samples (spray-dried powder of 100% fruit pulp or vacuum-dried granules of fruit juice) were enriched in a first step for 24 h at 37 °C in 1.5 l BPW. In a second step, 0.1 ml of BPW was subcultured in 9 ml EE

broth and incubated for a further 24 h at 37 °C. This second enrichment was then plated on VRBG agar and on Oxoid chromogenic *E. sakazakii* agar. After incubation for 24 h at 37 °C, isolates 508/05^T (=LMG 23730^T=DSM 18397^T), 610/05 (=LMG 23731), 513/05^T (=LMG 23732^T=DSM 18396^T) and 1159/04 (=LMG 23733) were recovered as typical turquoise colonies from *E. sakazakii* agar as well as yellow-pigmented colonies on TSA plates.

For morphological and physiological studies, strains were grown on BHI medium. Physiological studies were performed by using API 32E and Biotype 100 tests (bioMérieux) with Biotype Medium 1, according to the manufacturer's instructions. Tests for motility and indole and H₂S production were performed in SIM agar (BD Diagnostics) at 37 °C.

The four isolates from fruit powder consisted of facultatively anaerobic, motile, Gram-negative coccoid rods (1.0 × 1.5–2.5 µm). After 24 h aerobic incubation at 37 °C on sheep blood agar, colonies were non-haemolytic and yellow-pigmented. Yellow pigmentation increased when colonies were exposed to light. All strains were catalase-positive and weakly oxidase-positive. The strains could grow at temperatures of 10 to 44 °C. The minimal pH for growth at 37 °C was pH 5.0 for all strains.

The four strains could be separated into two groups on the basis of biochemical patterns and could be differentiated by the following characteristics: strains 508/05^T and 610/05 were positive for L-ornithine decarboxylase and for the fermentation of L-rhamnose, but were negative in tests for 5-bromo-3-indoxyl-nonanoate utilization. In contrast, strains 513/05^T and 1159/04 were positive for 5-bromo-3-indoxyl-nonanoate utilization, but negative in tests for L-ornithine decarboxylase and for the fermentation of L-rhamnose. Moreover, in contrast to strains 508/05^T and 610/05, strains 513/05^T and 1159/04 could utilize the following substrates: *trans*-aconitate, 5-keto-D-gluconate, protocatechuate, *p*-hydroxybenzoate, quinate, putrescine and DL- α -amino-n-butyrate.

Sequencing of the 16S rRNA genes was performed according to Lehner *et al.* (2006). The almost complete 16S rRNA gene sequences comprising 1266 (508/05^T), 1319 (610/05), 1245 (513/05^T) and 1267 (1159/04) nucleotides were determined and aligned to 28 000 almost full-length 16S rRNA gene sequences by using the alignment tool of the ARB program package (Ludwig *et al.*, 2004). Alignments were refined by visual inspection. Analysis of the 16S rRNA gene sequences was performed using the distance-matrix tool and a phylogenetic tree was estimated using the neighbour-joining method combined with a Felsenstein correction, all of which are included in the ARB package. The significance of branchings was evaluated by a bootstrap analysis of 1000 replicates. Phylogenetic analyses were performed using the distance matrix and the neighbour-joining dendrogram tool included in the ARB software package employing special data

structures (PT-servers) derived from the ssu-rRNA database, 'ssu_jan04.arb'.

The 16S rRNA gene sequences of strains 508/05^T and 610/05 showed 98.7 % sequence similarity to each other and formed a separate branch in the phylogenetic tree (Fig. 1), although weakly supported by bootstrap analysis. They were grouped most closely with a cluster containing *Erwinia mallotivora* LMG 2708^T with 95.7 and 94.9 % similarity, *Erwinia amylovora* LMG 2024^T with 96.6 and 95.8 % similarity and *Erwinia rhapontici* DSM 4484^T with 96.5 and 96.0 % sequence similarity, respectively. High sequence similarities were also found to *Enterobacter radicincitans* CIP 108468^T with 95.3 and 94.9 % similarity and *Enterobacter cowanii* CIP 107300^T with 96.7 and 96.4 % gene sequence similarity, respectively.

The 16S rRNA gene sequences of strains 513/05^T and 1159/04 showed 99.4 % sequence similarity to each other and formed a separate branch in the phylogenetic tree (Fig. 1), although weakly supported by bootstrap analysis. They were grouped most closely to a cluster containing *Enterobacter sakazakii* DSM 4485^T (AB004746) with 96.5 and 96.4 % gene sequence similarity and *Enterobacter sakazakii* ATCC 51329 (AY752937) with 96.9 and 96.8 % similarity, respectively. High gene sequence similarities were also found to *Escherichia coli* ATCC 11775^T (X80725) and *Shigella flexneri* ATCC 29903^T (X96963) with 96.1 % and 96.3 % similarity, respectively. The 16S rRNA gene sequence similarities between strains 508/05^T and 610/05 and 513/05^T and 1159/04 varied between 95.7 and 96.2 %. Overall, no 16S rRNA gene sequence similarities above 97 % were found with any recognized species, suggesting that the strains represent novel species (Stackebrandt & Goebel, 1994).

Since the branches representing the different genera of the family *Enterobacteriaceae* are not monophyletic and not supported by high bootstrap values and since the highest similarity values with the fruit powder strains are found with reference strains of different genera, it can be concluded that for the family *Enterobacteriaceae*, the similarity levels obtained reflect a high level of homoplasy in the 16S rRNA gene sequences.

16S rRNA gene sequence analysis indicates that the strains belong to the family *Enterobacteriaceae* but based on these results alone, the novel strains cannot be allocated unequivocally to a narrower taxonomic level. As the usefulness of *rpoB* sequence analysis for species discrimination within the family *Enterobacteriaceae* has been reported previously by several authors (Mollet *et al.*, 1997; Drancourt *et al.*, 2001; Li *et al.*, 2004; Kämpfer *et al.*, 2005), *rpoB* sequence analysis was performed with the four novel strains. Total DNA was prepared according to the protocol of Niemann *et al.* (1997). The *rpoB* gene was amplified and sequenced following the protocol of Mollet *et al.* (1997). Sequence assembly was performed by using the AutoAssembler program (Applied Biosystems). Phylogenetic analysis was performed using TREECON software (Van de Peer & De

Fig. 1. Neighbour-joining dendrogram of 16S rRNA gene sequences showing the estimated phylogenetic relationships between *Enterobacter turicensis* sp. nov., *Enterobacter helveticus* sp. nov. and related genera within the family Enterobacteriaceae. Bootstrap values (percentages of 1000 replicates) are shown. Bar, 0.01% nucleotide substitutions.

Wachter 1994) after including the consensus sequence in an alignment (CLUSTAL W, Thompson *et al.*, 1994) of *rpoB* sequences collected from EMBL. Evolutionary distances were calculated using the Jukes & Cantor evolutionary model and the resulting tree was constructed using the neighbour-joining method. Bootstrap values (500 replicates) were also calculated.

The *rpoB* sequences of strains 508/05^T and 610/05 were identical. The highest *rpoB* sequence similarities were found with *Enterobacter radicincitans* CIP 108468^T (91.9% similarity), *Enterobacter cowanii* CIP 107300^T (90.2%) and *Enterobacter sakazakii* LMG 5740^T (89.6%). The *rpoB* sequences of strains 513/05^T and 1159/04 were also identical and the highest sequence similarities to these strains were with those of *Enterobacter sakazakii* LMG 5740^T (91.0%), *Enterobacter radicincitans* CIP 108468^T (90.8%), *Enterobacter gergoviae* ATCC 33028^T (90.0%) and *Enterobacter cowanii* CIP 107300^T (89.8%). The *rpoB* sequence similarity between strains 508/05^T and 610/05 and strains 513/05^T and 1159/04 was 91.6%. The phylogenetic branch formed by *E. sakazakii*, *E. radicincitans*, *E. cowanii* and the novel strains is

supported by a high bootstrap value (86%) (Fig. 2), demonstrating that they clearly belong to the genus *Enterobacter*, taking into account that when using only one protein-coding gene, a possible genetic transfer cannot be excluded. Moreover, the similarity values found with their nearest neighbours are rather low (89.6–91.0%) compared with the intraspecies similarity range of 98–100% found in the family Enterobacteriaceae (Mollet *et al.*, 1997), confirming that the fruit powder strains represent novel species within this family.

Finally, to confirm whether these novel strains represent two independent genospecies within the genus *Enterobacter*, DNA–DNA hybridizations were performed. DNA was prepared according to a modification of the method of Wilson (1987) and hybridizations were carried out at 46 °C according to a modification of the method described by Ezaki *et al.* (1989). The DNA–DNA relatedness percentages obtained were the means of a minimum of four hybridizations. Reciprocal reactions (i.e. A × B and B × A) were performed and the variation between them was within the accepted limits of this method (Goris *et al.*, 1998).

Fig. 2. Neighbour-joining dendrogram based on *rpoB* gene sequences of *Enterobacter turicensis* sp. nov., *Enterobacter helveticus* sp. nov. and related members of the family Enterobacteriaceae. Bootstrap values (percentages of 500 replicates) of > 70 % are shown. Bar, 0.1 % nucleotide substitutions.

The DNA–DNA hybridization results revealed that strains 508/05^T and 610/05 show a DNA–DNA relatedness of 95 % while strains 513/05^T and 1159/04 show 100 % DNA–DNA relatedness. The DNA–DNA relatedness between the strains of the different genospecies falls within a range of 23–27 %, which is clearly below 70 %, the generally accepted limit for species delineation (Wayne *et al.*, 1987). On the basis of these genotypic results, it is clear that the four fruit powder strains represent two novel genomic species.

The overall DNA G + C content was determined from DNA prepared for the DNA–DNA hybridizations, according to

the HPLC method (Mesbah *et al.*, 1989). The values (means of three independent analyses of the same DNA sample) for strains 508/05^T, 610/05, 513/05^T and 1159/04 are 57.8, 57.7, 55.2 and 55.8 mol%, respectively. These values are consistent with the DNA G + C contents of other members of the genus *Enterobacter* (Richard, 1984; Inoue *et al.*, 2000).

The results of this polyphasic analysis support the recognition of two novel *Enterobacter* species, for which the names *Enterobacter turicensis* sp. nov. and *Enterobacter helveticus* sp. nov. are proposed. Details of the physiological and biochemical characteristics of the two novel species are given

Table 1. Phenotypic characteristics that differentiate *Enterobacter turicensis* sp. nov. and *Enterobacter helveticus* sp. nov. from related species of the genus *Enterobacter*

Strains: 1, *Enterobacter turicensis* sp. nov. (n=2); 2, *Enterobacter helveticus* sp. nov. (n=2); 3, *Enterobacter sakazakii*; 4, *Enterobacter cowanii*; 5, *Enterobacter radicincitans*; 6, *Enterobacter gergoviae*; 7, *Enterobacter asburiae*; 8, *Enterobacter kobei*; 9, *Enterobacter cloacae* subsp. *cloacae*; 10, *Enterobacter cloacae* subsp. *dissolvens*; 11, *Enterobacter hormaechei*; 12, *Enterobacter cancerogenus*; 13, *Enterobacter nimipressuralis*; 14, *Enterobacter amnigenus* biovar 1; 15, *Enterobacter amnigenus* biovar 2; 16, *Enterobacter intermedius*; 17, *Enterobacter ludwigii*; 18, *Enterobacter pyrinus*. –, 0–10% positive; +, 10–20% positive; v, 20–80% positive; +, 80–90% positive; +, 90–100% positive; ND, no data available. Data for other *Enterobacter* species are from Hoffmann *et al.* (2005) and Kämpfer *et al.* (2005).

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Voges–Proskauer test	–	–	+	+	+	+	+	+	+	+	+	+	+	+	ND	+	+	+
Motility	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	+	+	+
Aesculin hydrolysis	+	+	+	+	+	+	+	–	–	+	–	–	+	+	+	+	–	+
Carbon source utilization:																		
α -D-Melibiose	+	+	+	+	–	+	+	+	+	+	–	–	+	+	+	+	+	+
Sucrose	–	–	+	+	+	+	+	+	+	+	+	–	–	+	–	v	+	+
Dulcitol	+	+	–	+	ND	–	–	v	–	–	+	–	–	–	–	+	–	–
D-Sorbitol	–	–	–	+	+	–	+	+	+	+	–	–	+	–	ND	+	+	–
Putrescine	–	+	+	–	–	v	–	v	+	+	–	+	–	–	ND	–	–	+
L-Fucose	–	–	–	–	ND	v	–	–	–	v	+	+	–	–	ND	v	v	–
D-Arabitol	–	–	–	–	ND	+	–	–	–	–	–	–	–	–	ND	–	–	+
α -L-Rhamnose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	ND	+	+	+
Mucate	+	+	–	+	ND	–	v	v	v	+	v	+	+	+	ND	+	+	–
1-0-Methyl α -galactopyranoside	+	+	+	+	ND	+	+	+	+	+	–	–	–	+	ND	+	+	–
3-0-Methyl D-glucopyranose	–	–	–	–	ND	–	–	–	–	–	v	+	–	–	ND	–	+	–

below. They can be clearly differentiated from their nearest neighbours by several properties including the utilization of sucrose, D-sorbitol, putrescine and mucate, the hydrolysis of aesculin and a negative result in the Voges–Proskauer reaction (see Table 1).

Description of *Enterobacter turicensis* sp. nov.

Enterobacter turicensis (tu.ri.cens'is. L. masc. adj. *turicensis* from Turicum/Zurich, from where the species was first isolated).

Cells are Gram-negative, coccoid rods that are facultatively anaerobic and motile. Cells are 1.0 μ m wide by 1.5–2.5 μ m long and occur singly or in pairs. After 24 h aerobic incubation at 37 °C on TSA medium, colonies are yellow-pigmented and convex. Catalase-positive and weakly oxidase-positive. Colonies grow well at 10 °C (within 3 days) but poorly at 44 °C. Positive for ornithine- and malonate decarboxylase reactions and negative for urease, arginine dihydrolase and lysine decarboxylase. Tests for indole and H₂S production and the Voges–Proskauer test are negative. Acid is produced from the following compounds: galacturonate, D-mannitol, D-maltose, D-glucose, L-arabinose, D-trehalose and L-rhamnose. No acid production is observed from L-arabitol, D-arabitol, 5-ketoglucuronate, phenol red, adonitol, palatinose, sucrose, inositol, D-cellobiose or D-sorbitol. The chromogenic substrates ONPG, 4-nitrophenyl β -D-glucopyranoside, 4-nitrophenyl β -D-galactopyranoside, 4-nitrophenyl α -D-glucopyranoside, 4-nitrophenyl α -D-galactopyranoside and 4-nitrophenyl

α -D-maltopyranoside are hydrolysed. The following compounds are not hydrolysed: 5-bromo-3-indoxyl-nonanoate, 4-nitrophenyl β -D-glucuronide or L-aspartic acid 4-nitro-anilide. Positive reaction in tests for the utilization of α -D-glucose, β -D-fructose, D-galactose, D-trehalose, D-mannose, α -D-melibiose, maltotriose, maltose, α -lactose, 1-0-methyl β -galactopyranoside, 1-0-methyl α -galactopyranoside, D-cellobiose, β -gentiobiose, 1-0-methyl β -D-glucopyranoside, aesculin, D-ribose, L-arabinose, D-xylose, α -L-rhamnose, dulcitol, glycerol, D-mannitol, D-turanose, D-saccharate, mucate, L-malate, *cis*-aconitate, D-glucuronate, D-galacturonate, 2-keto-D-gluconate, *N*-acetyl-D-glucosamine, D-gluconate, DL-lactate, D-glucosamine, L-aspartate, L-glutamate, L-proline, L-alanine and L-serine. The following compounds are not utilized as sole sources of carbon: L-sorbose, sucrose, D-raffinose, lactulose, α -L-fucose, D-arabitol, L-arabitol, xylitol, D-tagatose, *myo*-inositol, maltitol, D-sorbitol, adonitol, hydroxyquinoline- β -glucuronide, i-erythritol, 1-0-methyl α -D-glucopyranoside, 3-0-methyl D-glucopyranose, L-tartrate, D-tartrate, *myo*-tartrate, *trans*-aconitate, tricarballoylate, 5-keto-D-gluconate, L-tryptophan, phenylacetate, protocatechuate, *p*-hydroxybenzoate, quinate, gentisate, *m*-hydroxybenzoate, benzoate, 3-phenylpropionate, trigonelline, betain, putrescine, DL- α -amino-n-butyrate, histamine, caprate, caprylate, L-histidine, fumarate, glutarate, DL-glycerate, DL- α -amino-n-valerate, ethanolamine, tryptamine, itaconate, DL- β -hydroxybutyrate, malonate, propionate, L-tyrosine or 2-oxoglutarate. The DNA G + C contents of strains 508/05^T and 610/05 are 57.8 and 57.7 mol%, respectively.

The type strain, strain 508/05^T (=LMG 23730^T=DSM 18397^T), was isolated from fruit powder.

Description of *Enterobacter helveticus* sp. nov.

Enterobacter helveticus (hel.ve.ti'cus. L. masc. adj. *helveticus* from Helveticum/Switzerland, from where the species was first isolated).

Cells are Gram-negative, coccoid rods that are facultatively anaerobic and motile. Cells are 1.0 µm wide by 1.5–2.5 µm long and occur singly or in pairs. After 24 h aerobic incubation at 37 °C on TSA medium, colonies are yellow-pigmented and convex. Catalase-positive and weakly oxidase-positive. Colonies grow well at 44 °C, but poorly at 10 °C. Positive in tests for the malonate decarboxylase reaction and negative in tests for urease, ornithine decarboxylase, arginine dihydrolase and lysine decarboxylase activities. Tests for indole and H₂S production and the Voges–Proskauer test are also negative. Acid is produced from the following compounds: galacturonate, D-mannitol, D-maltose, D-glucose, L-arabinose and D-trehalose. No acid production is observed for L-arabitol, L-rhamnose, 5-ketogluconate, phenol red, adonitol, palatinose, sucrose, D-arabitol, inositol, D-cellobiose or D-sorbitol. The chromogenic substrates ONPG, 5-bromo-3-indoxyl-nonanoate, 4-nitrophenyl β-D-glucopyranoside, 4-nitrophenyl β-D-galactopyranoside, 4-nitrophenyl α-D-glucopyranoside, 4-nitrophenyl α-D-galactopyranoside and 4-nitrophenyl α-D-maltopyranoside are hydrolysed. The following compounds are not hydrolysed: 4-nitrophenyl β-D-glucuronide and L-aspartic acid 4-nitroanilide. Positive reaction in tests for the utilization of α-D-glucose, β-D-fructose, D-galactose, D-trehalose, D-mannose, α-D-melibiose, maltotriose, maltose, α-lactose, 1-0-methyl β-galactopyranoside, 1-0-methyl α-galactopyranoside, D-cellobiose, β-gentiobiose, 1-0-methyl β-D-glucopyranoside, aesculin, D-ribose, L-arabinose, D-xylose, α-L-rhamnose, dulcitol, glycerol, D-mannitol, D-turanose, D-saccharate, mucate, L-malate, *cis*-aconitate, *trans*-aconitate, D-glucuronate, D-galacturonate, 2-keto-D-gluconate, 5-keto-D-gluconate, *N*-acetyl-D-glucosamine, D-gluconate, protocatechuate, *p*-hydroxybenzoate, quinate, putrescine, DL-α-amino-n-butyrate, DL-lactate, D-glucosamine, L-aspartate, L-glutamate, L-proline, L-alanine and L-serine. The following compounds are not utilized as sole sources of carbon: L-sorbose, sucrose, D-raffinose, lactulose, α-L-fucose, D-arabitol, L-arabitol, xylitol, D-tagatose, *myo*-inositol, maltitol, D-sorbitol, adonitol, hydroxyquinoline-β-glucuronide, i-erythritol, 1-0-methyl α-D-glucopyranoside, 3-0-methyl D-glucopyranose, L-tartrate, D-tartrate, *myo*-tartrate, tricarballoylate, L-tryptophan, phenylacetate, gentisate, *m*-hydroxybenzoate, benzoate, 3-phenylpropionate, trigonelline, betain, histamine, caprate, caprylate, L-histidine, fumarate, glutarate, DL-glycerate, DL-α-amino-n-valerate, ethanolamine, tryptamine, itaconate, DL-β-hydroxybutyrate, malonate, propionate, L-tyrosine or 2-oxoglutarate. The DNA G+C contents of strains 513/05^T and 1159/04 are 55.2 and 55.8 mol%, respectively.

The type strain, strain 513/05^T (=LMG 23732^T=DSM 18396^T), was isolated from fruit powder.

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