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Abstract: Six Gram-negative, facultative anaerobic, non-spore-forming isolates of coccoid rods were obtained from fruit powder (n=3), infant formula (n=2) and infant formula production environment (n=1) and investigated in a polyphasic taxonomic study. Comparative 16S rRNA gene sequence analysis combined with rpoB sequence analysis, allocated the isolates to the Enterobacteriaceae. The highest rpoB sequence similarities (91.2-95.8 %) were obtained with Enterobacter helveticus, Enterobacter radicincitans, Enterobacter turicensis and Enterobacter sakazakii and the phylogenetic branch formed by these species is supported by a high bootstrap value. Biochemical data revealed that the isolates could be differentiated from their nearest neighbours by the positive utilization of -D-melibiose, sucrose, D-arabitol, mucate and 1-0-methyl- -galacto-pyranoside as well as negative tests for D-sorbitol, and the Voges-Proskauer reaction. On the basis of the phylogenetic analyses, DNA-DNA hybridizations and the unique physiological and biochemical characteristics, it is proposed that the isolates represent a novel Enterobacter species, Enterobacter pulveris sp. nov. The type strain is 601/05T (= LMG 24057T = DSM 19144T).

Enterobacter pulveris sp. nov., isolated from fruit powder, infant formula and an infant formula production environment

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Six Gram-negative, facultatively anaerobic, non-spore-forming, coccoid rod-shaped isolates were obtained from fruit powder ($n=3$), infant formula ($n=2$) and an infant formula production environment ($n=1$) and investigated in a polyphasic taxonomic study. Comparative 16S rRNA gene sequence analysis combined with *rpoB* gene sequence analysis allocated the isolates to the family *Enterobacteriaceae*. The highest *rpoB* gene sequence similarities (91.2–95.8 %) were obtained with *Enterobacter helveticus*, *Enterobacter radicincitans*, *Enterobacter turicensis* and *Enterobacter sakazakii* and the phylogenetic branch formed by these species was supported by a high bootstrap value. Biochemical data revealed that the isolates could be differentiated from their nearest neighbours by their ability to utilize melibiose, sucrose, D-arabitol, mucate and 1-*O*-methyl-α-galactopyranoside and their negative reactions for D-sorbitol utilization and the Voges–Proskauer test. On the basis of the phylogenetic analyses, DNA–DNA hybridization data, and unique physiological and biochemical characteristics, it is proposed that the isolates represent a novel species of the genus *Enterobacter*, *Enterobacter pulveris* sp. nov. The type strain is 601/05[T] (=LMG 24057[T]=DSM 19144[T]).

Recent studies on the isolation and identification of *Enterobacter sakazakii* have revealed the existence of novel *Enterobacter* species that persist in the same ecological niches. Iversen and others described unclassified members of the family *Enterobacteriaceae* isolated from dried food ingredients that formed divergent clusters using 16S rRNA and *hsp60* gene sequence analysis (Iversen *et al.*, 2004a), but which could be mistaken phenotypically for *E. sakazakii* (Iversen *et al.*, 2004b). Lehner and others also described isolates from fruit powder that gave colony morphologies on *E. sakazakii* isolation media that were typical for the target species, but the API 32E confirmation of these strains gave ambiguous results (Lehner *et al.*, 2004). Subsequently, comparative analysis of 16S rRNA gene sequences revealed that these isolates were clearly distinct from *E. sakazakii* with sequence similarities of <97 % (Lehner *et al.*, 2006).

There is no evidence that the presence of these novel species in food products represents a health concern, but they do pose a challenge for the design of isolation methods for *E. sakazakii*, a micro-organism that is occasionally reported as an opportunistic pathogen and has been associated with infant formula (Lehner & Stephan, 2004). These novel species share several typical characteristics with *E. sakazakii*, such as resistance to desiccation, production of a yellow pigment and constitutive metabolism of 5-bromo-4-chloro-3-indolyl-α-D-glu-copyranoside, which is a significant trait used in the differentiation of presumptive *E. sakazakii* colonies (Iversen *et al.*, 2004b).

Recently, two groups from the fruit powder isolates have been described as novel species, *Enterobacter helveticus* and

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Enterobacter turicensis (Stephan *et al.*, 2007). The characterization of a third group of strains, with representative isolates from fruit powder, infant formula and an infant formula production environment, is described in this study and it is concluded that these isolates represent another novel *Enterobacter* species.

Apple fruit powder (150 g) samples (spray-dried powder of 100 % fruit pulp or vacuum-dried granules of fruit juice) were suspended in 1.35 l buffered peptone water (BPW) and incubated for 24 h at 37 °C. Samples (0.1 ml) of the BPW enrichment were subcultured in 9 ml *Enterobacteriaceae* enrichment broth (BBL EE broth; Becton Dickinson) for 24 h at 37 °C and subsequently plated on violet red bile glucose agar (Difco VRBG; Becton Dickinson) and chromogenic *E. sakazakii* agar (Oxoid CM1055). After incubation for 24 h at 37 °C, isolates 1160/04 (=LMG 24058=DSM 19146), 516/05 and 601/05[T] (=LMG 24057[T]=DSM 19144[T]) were recovered as typical turquoise colonies from *E. sakazakii* agar. All strains produced yellow-pigmented colonies on tryptone soy agar (TSA) plates.

In addition to the isolates obtained from fruit powder, three further isolates have been obtained from infant formula [E441 (=LMG 24059=DSM 19145) and E443] and an infant formula production environment (E444) using the ISO/TS for *E. sakazakii* (Anonymous, 2006).

For morphological and physiological studies, strains were grown on BHI medium (Oxoid CM0225). Physiological studies were performed using API 32E and Biotype 100 tests with Biotype Medium 1, according to the instructions of the manufacturer (bioMérieux). Motility testing and indole and H_2S production were performed in SIM agar (BD) at 37 °C.

The six isolates consisted of facultatively anaerobic, motile, Gram-negative coccoid rods ($0.9–1.0 \times 1.5–3.0$ 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 negative or weakly positive for the oxidase reaction and were catalase-positive. The strains could grow at 10–44 °C. The minimal pH for growth at 37 °C was pH 5.0 for all strains. Generally, the six strains had the same biochemical profile, but they gave variable results for the utilization of dulcitol, aesculin, putrescine, turanose, DL-α-amino-n-butyrate, DL-glycerate, lactulose, maltitol, 1-*O*-methyl-α-D-glucopyranoside and L-tartrate as sole sources of carbon (see Table 1 and species description).

Sequencing of the 16S rRNA genes was performed according to the protocol described by Lehner *et al.* (2006). The almost complete 16S rRNA gene sequences comprising 1220 (1160/04), 1317 (516/05), 1325 (601/05[T]), 1420 (E441), 1412 (E443) and 1479 (E444) nucleotides were determined and aligned to 28 000 almost full-length 16S rRNA gene sequences using the alignment tool of the ARB 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

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

Species: 1, *Enterobacter pulveris* sp. nov. (*n*=6); 2, *E. turicensis*; 3, *E. helveticus*; 4, *E. sakazakii*; 5, *E. cowanii*; 6, *E. aerogenes*; 7, *E. radicincitans*; 8, *E. gergoviae*; 9, *E. asburiae*; 10, *E. kobei*; 11, *E. cloacae* subsp. *cloacae*; 12, *E. cloacae* subsp. *dissolvens*; 13, *E. hormaechei*; 14, *E. cancerogenus*; 15, *E. nimipressuralis*; 16, *E. amnigenus* biovar 1; 17, *E. amnigenus* biovar 2; 18, *E. intermedius*; 19, *E. ludwigii*; 20, *E. pyrinus*. −, 0–10 % Positive; (−), 10–20 % positive; v, 20–80 % positive; (+), 80–90 % positive; +, 90–100 % positive; ND, no data available. Data from this study and Hoffmann *et al.* (2005), Kämpfer *et al.* (2005), Farmer *et al.* (2007) and Stephan *et al.* (2007).

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Voges–Proskauer test	−	−	−	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+	+	(+)
Methyl red test	+	+	+	−	−	−	−	−	+	−	−	−	−	−	−	−	v	−	−	−
Ornithine decarboxylation	−	−	−	+	ND	+	ND	+	+	ND	+	+	+	+	v	+	+	+	ND	+
Arginine dihydrolase	−	−	−	+	ND	−	ND	−	v	ND	+	+	v	+	−	−	v	−	ND	−
Motility	+	+	+	+	+	+	+	+	(−)	(+)	+	−	v	+	−	+	+	+	+	v
Aesculin hydrolysis	+	+	+	+	+	+	+	+	+	−	v	+	−	+	+	+	+	+	(−)	+
Carbon source utilization:																				
Melibiose	+	+	+	+	+	+	−	+	−	+	+	+	(−)	−	+	+	+	+	+	−
Sucrose	+	−	−	+	+	+	+	+	+	+	+	+	+	−	−	+	−	v	+	+
D-Arabitol	+	−	−	−	−	+	ND	+	−	−	(−)	−	(−)	−	−	−	−	−	−	−
Mucate	+	+	−	+	+	+	ND	−	v	v	+	−	−	+	v	+	+	+	+	−
D-Sorbitol	−	−	−	+	+	+	+	−	(+)	+	+	(−)	−	+	−	+	+	+	+	−
L-Fucose	−	−	−	−	−	ND	ND	v	−	−	−	v	+	+	−	−	ND	ND	v	v
1-*O*-Methyl-α-galactopyranoside	+	+	+	+	+	ND	ND	+	(+)	+	+	+	−	−	−	+	ND	+	+	−

combined with a Felsenstein correction, all included in the ARB package. The significance of branchings was evaluated by bootstrap analysis of 1000 replicates. Phylogenetic analyses were performed using 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 isolates 1160/04, 516/05, 601/05^T, E441, E443 and E444 showed 99.3–99.5 % similarity to each other and formed a separate branch in the phylogenetic tree (see Supplementary Fig. S1 available in IJSEM Online). Based on the data of Stackebrandt & Ebers (2006), this suggests that these strains may belong to the same species. The strains grouped most closely with a cluster containing *E. helveticus* LMG 23732^T (GenBank accession no. DQ273688; 98.6–99.2 % sequence similarity), *E. sakazakii* DSM 4485^T (AB004746; 96.8–97.3 % sequence similarity) and *E. sakazakii* ATCC 51329 (AY752937; 96.7–97.3 % sequence similarity).

16S rRNA gene sequence analysis indicates that the strains belong to the family *Enterobacteriaceae* but, based on these results alone, they cannot unequivocally be allocated to a finer taxonomic level. Since *rpoB* sequence analysis has been used successfully for species discrimination within the family *Enterobacteriaceae* (Mollet *et al.*, 1997; Drancourt *et al.*, 2001; Li *et al.*, 2004; Kämpfer *et al.*, 2005; Stephan *et al.*, 2007), this approach was also used to determine the taxonomic position of the six 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 program AUTOASSEMBLER (Applied Biosystems). Phylogenetic analysis was performed using TREECON software (Van de Peer & De Wachter, 1994) after including the consensus sequence in a CLUSTAL W alignment (Thompson *et al.*, 1994) of *rpoB* sequences collected from the EMBL nucleotide sequence library. Evolutionary distances were calculated using the Jukes–Cantor evolutionary model and a tree was constructed using the neighbour-joining method. Bootstrap values (500 replicates) were also calculated.

The *rpoB* sequences of the six isolates showed 99.6–100.0 % sequence similarity to each other, indicating that they probably belong to the same species. The highest *rpoB* sequence similarities were obtained with *E. helveticus* LMG 23732^T (95.6–95.8 %), *Enterobacter radicincitans* CIP 108468^T (91.5–91.7 %), *E. turicensis* LMG 23730^T (91.3–91.6 %) and *E. sakazakii* LMG 5740^T (91.2–91.5 %). The phylogenetic branch formed by *E. helveticus*, *E. turicensis*, *E. sakazakii*, *E. radicincitans*, *Enterobacter cowanii* and the six novel strains was supported by a high bootstrap value (95 %) (Fig. 1), demonstrating that they belong to the genus *Enterobacter*, taking into account that when using only one protein coding gene, a possible genetic transfer cannot be excluded. The similarity values found with their nearest neighbours were rather low (89.4–95.8 %)

compared with the intraspecies similarity range of 98–100 % found in the family *Enterobacteriaceae* (Mollet *et al.*, 1997), thus confirming that these strains represent a novel species within this family.

To confirm finally that these strains represent an independent genomospecies within the genus *Enterobacter*, DNA–DNA hybridizations with three representative strains were performed. DNA was prepared according to the method of Wilson (1987) and hybridizations were carried out at 45 °C according to the microtitre plate technique described by Ezaki *et al.* (1989). The DNA relatedness percentages are 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 acceptable limits of this method (Goris *et al.*, 1998).

The DNA–DNA hybridization results revealed that the strains 1160/04, 516/05 and 601/05^T show a very high DNA–DNA relatedness to each other (99–100 %), demonstrating that they belong to the same genomospecies. The DNA–DNA relatedness between strain 601/05^T and *E. helveticus* LMG 23732^T was 54 %, 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 concluded that the six strains represent a novel genomic species.

The overall G + C content was also determined for three strains according to the HPLC method (Mesbah *et al.*, 1989) using the DNA prepared for DNA–DNA hybridizations. The values (means of three independent analyses of the same DNA sample) for 1160/04, 516/05 and 601/05^T were 57.0, 56.4 and 57.0 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 a novel *Enterobacter* species, for which the name *Enterobacter pulveris* sp. nov. is proposed. Details on the physiological and biochemical characteristics of the novel species are given below. It can be clearly differentiated from its nearest neighbours by several properties, including the utilization of melibiose, sucrose, D-sorbitol, D-arabitol, mucate and 1-*O*-methyl-α-galactopyranoside and the Voges–Proskauer reaction (see Table 1).

Description of *Enterobacter pulveris* sp. nov.

Enterobacter pulveris (pul′ve.ris. L. n. *pulvis* -*eris* dust, powder; L. gen. n. *pulveris* of powder).

Cells are Gram-negative coccoid rods that are facultatively anaerobic and motile. Cells are 0.9–1.0 μm wide by 1.5–3.0 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 negative or weakly positive for oxidase. Colonies grow poorly at 10 °C (within 3 days), but well at 44 °C. Negative for ornithine decarboxylase, malonate decarboxylase, urease, arginine
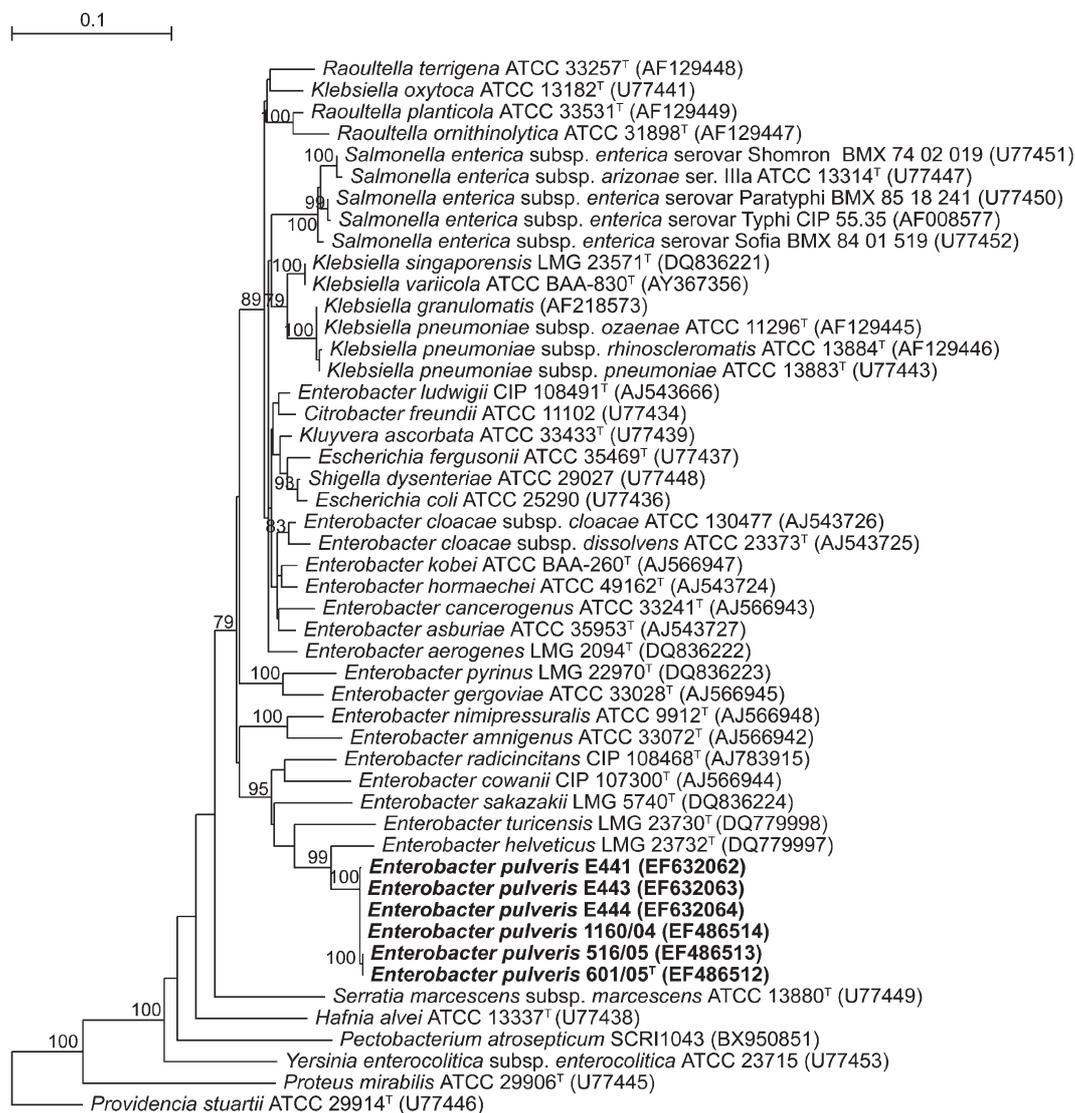
Fig. 1. Neighbour-joining tree comprising *rpoB* gene sequences of *Enterobacter pulveris* sp. nov. and related members of the *Enterobacteriaceae*. Bootstrap values (percentages of 500 replicates) of >70 % are shown. Bar, 0.1 % nucleotide substitutions.

dihydrolase and lysine decarboxylase. Voges–Proskauer test, indole production and H₂S production are also negative. Acid is produced from galacturonate, D-mannitol, maltose, D-glucose, sucrose, D-arabitol, L-arabinose, trehalose, L-rhamnose and D-cellobiose. Acid is not produced from L-arabitol, 5-ketogluconate, adonitol, palatinose, inositol or D-sorbitol. The chromogenic substrates ONPG, 4-nitrophenyl-β-D-glucopyranoside, 4-nitrophenyl-β-D-galactopyranoside, 4-nitrophenyl-α-D-glucopyranoside, 5-bromo-3-indoxyl-nonanoate (weak reaction), 4-nitrophenyl-α-D-galactopyranoside and 4-nitrophenyl-α-D-maltopyranoside are hydrolysed. 4-Nitrophenyl-β-D-glucuronide and L-aspartic acid 4-nitroanilide are not hydrolysed. Positive for utilization of α-D-glucose, β-D-fructose, D-galactose, trehalose, D-mannose, melibiose, sucrose, raffinose, maltotriose, maltose, α-lactose, 1-*O*-methyl-β-galactopyranoside, 1-*O*-methyl-α-galacto-pyranoside, D-cellobiose, β-gentiobiose, 1-*O*-methyl-β-D-glucopyranoside, D-ribose, L-arabinose, D-xylose, α-L-rhamnose, D-arabitol, glycerol, D-mannitol, 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, DL-lactate, fumarate, 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, α-L-fucose, L-arabitol, xylitol, D-tagatose, *myo*-inositol, D-sorbitol, adonitol, hydroxyquinoline-β-glucuronide, i-erythritol, 3-*O*-methyl-D-glucopyranose, D-tartrate, *meso*-tartrate,

tricarballylate, L-tryptophan, phenylacetate, gentisate, *m*-hydroxybenzoate, benzoate, 3-phenylpropionate, trigonelline, betaine, histamine, caprate, caprylate, L-histidine, glutarate, DL-α-amino-n-valerate, ethanolamine, tryptamine, itaconate, DL-β-hydroxybutyrate, malonate, propionate, L-tyrosine and α-ketoglutarate.

Variable for utilization of dulcitol, aesculin, putrescine, turanose, DL-α-amino-n-butyrate, DL-glycerate, lactulose, maltitol, 1-*O*-methyl-α-D-glucopyranoside and L-tartrate as sole sources of carbon. The DNA G + C contents of strain 601/05[T] and representative strains 1160/04 and 516/05 are 57.0, 56.4 and 57.0 mol%, respectively.

The type strain, 601/05[T] (=LMG 24057[T]=DSM 19144[T]), was isolated from fruit powder.

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