Basfia succiniciproducens gen. nov., sp. nov., a new member of the family *Pasteurellaceae* isolated from bovine rumen

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Gram-negative, coccoid, non-motile bacteria that are catalase-, urease- and indole-negative, facultatively anaerobic and oxidase-positive were isolated from the bovine rumen using an improved selective medium for members of the *Pasteurellaceae*. All strains produced significant amounts of succinic acid under anaerobic conditions with glucose as substrate. Phenotypic characterization and multilocus sequence analysis (MLSA) using 16S rRNA, *rpoB*, *infB* and *recN* genes were performed on seven independent isolates. All four genes showed high sequence similarity to their counterparts in the genome sequence of the patent strain MBEL55E, but less than 95% 16S rRNA gene sequence similarity to any other species of the *Pasteurellaceae*. Genetically these strains form a very homogeneous group in individual as well as combined phylogenetic trees, clearly separated from other genera of the family from which they can also be separated based on phenotypic markers. Genome relatedness as deduced from the *recN* gene showed high interspecies similarities, but again low similarity to any of the established genera of the family. No toxicity towards bovine, human or fish cells was observed and no RTX toxin genes were detected in members of the new taxon. Based on phylogenetic clustering in the MLSA analysis, the low genetic similarity to other genera and the phenotypic distinction, we suggest to classify these bovine rumen isolates as *Basfia succiniciproducens* gen. nov., sp. nov. The type strain is JF4016^T (=DSM 22022^T =CCUG 57335^T).

The family *Pasteurellaceae* currently contains 14 recognized genera and over 60 species with validly published names (Christensen & Bisgaard, 2008; Gregersen *et al.*, 2009). These bacteria are commonly isolated from a broad range of vertebrate species, where they belong to the normal flora of mucosal membranes, some also acting as opportunistic pathogens (Kuhnert & Christensen, 2008). Species of the *Pasteurellaceae* found in the bovine rumen include *Mannheimia ruminalis*, *Actinobacillus lignieresii*, [*Actinobacillus*] *succinogenes* and '*Mannheimia succiniciproducens*'. The latter two species were found to be efficient producers of succinic acid, as reflected by their names. Based on phylogenetic analyses using several genes, both species are suggested to be excluded from their respective genera (Korczak & Kuhnert, 2008). Moreover, whereas the name

[*Actinobacillus*] *succinogenes* was validly published by Guettler *et al.* (1999), '*Mannheimia succiniciproducens*' has no standing in nomenclature (<http://www.bacterio.cict.fr>) and the only representative strain of this species, strain MBEL55E, is a patent strain and not publicly available (Lee *et al.*, 2002). This species therefore still awaits proper description and taxonomic classification.

To investigate the presence of members of the *Pasteurellaceae* in the bovine rumen we used a selective medium and repeatedly isolated strains genetically resembling '*M. succiniciproducens*'. For further clarification of the taxonomy of these organisms we applied a polyphasic approach. Strains were phenotypically characterized by standard tests and extended conventional tube assays (Christensen *et al.*, 2007; Dousse *et al.*, 2008). Furthermore, strains were tested on the Vitek2 (bioMérieux) to check for phenotypic variation using the broad panel of reactions available on that system. The genetic characterization included 16S rRNA gene sequencing of all isolates (Kuhnert *et al.*, 2002). In addition, partial sequences of the two housekeeping genes *rpoB* (encoding the β -subunit of RNA polymerase) and *infB*

Abbreviation: MLSA, multilocus sequence analysis.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain JF4016^T is FJ463881.

Phylogenetic trees based on 16S rRNA, *rpoB*, *infB* and *recN* gene sequences, along with details of strains and the GenBank/EMBL/DDBJ accession numbers for the gene sequences determined in this study are available with the online version of this paper.

(coding for translation initiation factor 2), which previously proved useful for characterization of members of the *Pasteurellaceae*, were included in the phylogenetic analysis (Korczak *et al.*, 2004; Mayor *et al.*, 2006). The *rpoB* gene sequence has also proved generally useful for bacterial species and genus delineation (Adekambi *et al.*, 2008). Finally, the *recN* sequence (encoding a DNA repair protein) of the novel strains was determined for calculation of genome similarities to other representative species of the family, as well as for phylogenetic analysis (Kuhnert & Korczak, 2006). Screening for RTX toxin genes and cytotoxicity assays were performed for risk assessment of the species. The newly isolated strains were proposed to represent a new genus and species, *Basfia succiniciproducens* gen. nov., sp. nov.

Isolation of strains

A modified selective medium based on that described by Jacobsen & Nielsen (1995) proved highly efficient for the isolation of species of the *Pasteurellaceae*. Whereas on standard sheep blood-agar plates no species of the *Pasteurellaceae* could be isolated from rumen juice, 50 % of colonies were species of *Pasteurellaceae* on the selective medium. Besides *Basfia succiniciproducens* gen. nov., sp. nov., several previously unknown representatives of the family as well as *Bibersteinia trehalosi* were isolated. The modified *Pasteurellaceae* selective medium (PSM) contained per litre 25 g BHI (Difco) and 18 g Bacto agar (Difco), and was adjusted to pH 7.5–7.6. After autoclaving, the medium was cooled to 45 °C, 50 ml sheep blood was added and the medium was further supplemented with 3.5 ml β -NAD (200 mg ml⁻¹; Sigma), 2 ml bacitracin (50 mg ml⁻¹; Sigma), 1 ml lincomycin (1 mg ml⁻¹; Sigma), 1 ml nystatin (50 mg ml⁻¹; Sigma) and 1 ml crystal violet (1 mg ml⁻¹; Merck).

Bovine rumen juice was collected from freshly slaughtered animals or from live nursing cows providing rumen juice for therapy. The animals were of different breeds and originated from Germany or from Switzerland. A total of seven individual animals from different farms were probed and strains isolated. Rumen juice was diluted 1 : 10 with LB medium and 50 μ l was plated on PSM plates and incubated at 37 °C overnight under 5 % CO₂ atmosphere. Single colonies were picked and subcultured on trypticase soy blood agar plates. Genetic identification of each single colony was performed using the 16S rRNA gene sequence (Kuhnert *et al.*, 2002). Strains showing highest similarity to the genomic sequence of patent strain MBEL55E were repeatedly isolated (Hong *et al.*, 2004). This strain is not publicly available and could not therefore be included in our analyses, except for phylogenetic analyses based on its published genome sequence (Hong *et al.*, 2004). Single strains were kept from six animals and eight strains were isolated from one animal, resulting in a total of 14 strains included in the study (Supplementary Table S1, available in IJSEM Online).

Multilocus sequence analysis (MLSA)

Phylogenetic analysis based on 16S rRNA gene sequence clustered strains proposed as *Basfia succiniciproducens* gen. nov., sp. nov. in the proximity of the genus *Nicoletella* (Supplementary Fig. S1). However, they formed a clearly separated cluster and showed only 95 % similarity to the *Nicoletella* 16S rRNA gene sequence. Heterogeneity for the 16S rRNA genes within the species *Basfia succiniciproducens* is low, with 99 % or higher sequence similarity. Phylogeny based on the housekeeping genes showed closest relation of *Basfia succiniciproducens* to the genus *Aggregatibacter* in all three trees (Supplementary Figs S2–S4), with sequence similarities of 84.2, 82.4 and 70.2 % for *rpoB*, *infB* and *recN*, respectively. Again, the species was shown to be genetically very homogeneous, with sequence similarity between strains of >99.3 (*rpoB*), >99.0 (*infB*) and >98.4 % (*recN*). The eight strains isolated from the same individual (JF4213–JF4220) had identical 16S rRNA and *rpoB* gene sequences, and the *infB* gene sequences from the three strains analysed were identical (Supplementary Table S1).

A combined tree of all four genes resulted in the same clear monophyletic branch of *Basfia succiniciproducens* strains, nearest to *Aggregatibacter actinomycetemcomitans* (Fig. 1). On the other hand, the proposed genus *Basfia* was very distant from the genus *Mannheimia*, which was located on the opposite of the two observed main clusters (Fig. 1).

The use of *recN* gene sequence similarities proved very useful for prediction of genome similarities as an alternative to DNA–DNA hybridization within the family *Pasteurellaceae* (Kuhnert & Korczak, 2006; Christensen *et al.*, 2007; Kuhnert *et al.*, 2007; Bisgaard *et al.*, 2007). Based on these publications it can be deduced that species within genera have similarity values of 0.4 or higher, whereas similarity values between species of different genera are less than 0.4. Table 1 shows the similarity values calculated on the basis of *recN* gene sequence similarities between the type strain of *Basfia succiniciproducens* gen. nov., sp. nov. and the type species of the currently known genera of the *Pasteurellaceae* (Kuhnert & Korczak, 2006). All values were clearly below 0.4, with the highest observed value with *Aggregatibacter actinomycetemcomitans* (0.27). This was highly correlated with the phylogenetic analysis using four genes (Fig. 1), where *Basfia succiniciproducens* clustered in the vicinity of *Aggregatibacter actinomycetemcomitans*. Also, from this analysis it was obvious that *Basfia succiniciproducens* is not related to the genus *Mannheimia* since *Mannheimia haemolytica* showed one of the lowest similarity values (0.05) with *Basfia succiniciproducens*. The genetic similarity values calculated based on *recN* (Table 1) correlated well with the clustering observed in the combined tree based on four genes (Fig. 1). Genera of the cluster that includes *Basfia succiniciproducens* showed similarity values that were clearly above 0.1, whereas genera of the other cluster had values below 0.1. The correlation of the MLSA with the data derived from the *recN* gene confirmed this gene as a

Comparison of genome sequences of strains DD1 and MBEL55E

The genome sequence of strain DD1, as a representative of the novel species, was determined by high-throughput sequencing (454; Life Science, Roche) with a 20-fold coverage, and compared by BLAST to the sequence of strain MBEL55E (Altschul *et al.*, 1997; Hong *et al.*, 2004). Initial annotation was performed with Pedant-Pro 2.3–3.0

Table 1. Genetic similarity of *Basfia succiniciproducens* gen. nov. sp. nov. to strains of type species of the *Pasteurellaceae* calculated based on *recN* sequences

Species	Similarity to <i>Basfia succiniciproducens</i>
<i>Actinobacillus lignieresii</i> NCTC 4189 ^T	0.04
<i>Pasteurella multocida</i> CCUG 17976 ^T	0.18
<i>Haemophilus influenzae</i> Rd KW20	0.25
<i>Mannheimia haemolytica</i> NCTC 9380 ^T	0.05
<i>Lonepinella koalarum</i> ATCC 700131 ^T	0.21
<i>Phocoenobacter uteri</i> NCTC 12872 ^T	0.05
<i>Gallibacterium anatis</i> CCUG 15563 ^T	0.02
<i>Volucrobacter psittacida</i> JEO101	0.16
<i>Histophilus somni</i> 8025 ^T	0.18
<i>Avibacterium gallinarum</i> NCTC 11188 ^T	0.19
<i>Nicoletella semolina</i> CCUG 43639 ^T	0.06
<i>Bibersteinia trehalosi</i> NCTC 10370 ^T	0.06
<i>Aggregatibacter actinomycetemcomitans</i> HK1651	0.27

(Biomax Informatics). This software uses the Glimmer gene prediction algorithm for ORF finding (settings: allow ORFs extending off ends of sequence to be scored: true; minimum overlap length: 12; length of the smallest fragment considered to be a gene, in base pairs: 90). For tRNA annotation from within Pedant, tRNAscan-SE with default parameters was used. Both genomes were found to have a DNA G + C content of 42.5 mol%. The two strains had similar genome sizes of 2 340 000 bp for DD1 and

2 314 078 bp for MBEL55E, with 2363 and 2380 ORFs, respectively. Of these, 2006 ORFs were homologous between the two strains (core genome), whereas 145 putative genes present in strain DD1 showed no similarity to MBEL55E ORFs. The average similarity between the homologous proteins of both strains was 95 % at the DNA as well as the amino acid sequence level.

Phenotypic characterization

Phenotypic and biochemical analysis was done by conventional tube tests, as recommended by the minimal standards for the description of genera, species and subspecies of the *Pasteurellaceae* (Christensen *et al.*, 2007). Between two and eight phenotypic characters allowed separation of the genus *Basfia* gen. nov. from other genera within the *Pasteurellaceae* as listed in Table 2.

Further biochemical characterization was done on the Vitek2 system using GN and NH cards. Differences in acid production from some sugars were observed between the commercial Vitek2 system and the conventional tube assay. This has previously been seen with other commercial assays and may be due to the fact that they, as well as the Vitek2 test, use large inocula and short incubation times, thereby not necessarily measuring fermentation but rather acidification of sugars (Kuhnert *et al.*, 2004).

Strains were tested for production of succinic acid using a UV-method-based kit according to the manufacturer (R-Biopharm). All strains analysed produced similar levels of succinic acid under anaerobic conditions in liquid BHI

Table 2. Key characteristics for differentiation of *Basfia succiniciproducens* gen. nov. sp. nov. from other genera of the *Pasteurellaceae*

Genera: 1, *Actinobacillus sensu stricto* (Christensen & Bisgaard, 2004); 2, *Pasteurella sensu stricto* (Mutters *et al.*, 1985); 3, *Haemophilus sensu stricto* (includes *H. influenzae*, *H. haemolyticus* and *H. aegyptius* – results for *H. parainfluenzae* and *H. pittmaniae* are given in brackets; Nørskov-Lauritsen *et al.*, 2005); 4, *Mannheimia* (Angen *et al.*, 1999); 5, *Lonepinella* (Osawa *et al.*, 1995); 6, *Phocoenobacter* (Foster *et al.*, 2000); 7, *Gallibacterium* (Christensen *et al.*, 2003); 8, *Volucrobacter* (Christensen *et al.*, 2004); 9, *Histophilus* (Angen *et al.*, 2003); 10, *Avibacterium* (Blackall *et al.*, 2005); 11, *Nicoletella* (Kuhnert *et al.*, 2004); 12, *Bibersteinia* (Blackall *et al.*, 2007); 13, *Aggregatibacter* (Patel *et al.*, 2004; Nørskov-Lauritsen & Kilian, 2006); 14, *Basfia* gen. nov. (this study). +, Positive; –, negative; v, variable; w, weakly positive; ND, no data available.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Catalase	v	v	v	v	–	–	+	v	–	v	+	v	v	–
Urease	+	– ^{a*}	+	[v]	–	–	–	–	–	–	+	–	–	–
Oxidase	+	+	+	+	–	+	+	v	+	+	+	v	–	+
Indole	–	+	v	–	–	–	–	–	+	–	–	–	–	–
Methyl red	–	–	ND	–	–	ND	w	+	ND	–	ND	–	–	+
D-Galactose	v	+	+	+	ND	ND	+	+	ND	v	–	–	v	+
D-Mannitol	+	^b	v	–	+	–	+	–	ND	v	–	+	v	+
D-Mannose	v	+	–	[+]	–	+	+	+	ND	+	–	+	v	+
Sucrose	+	+	–	[v]	+	v	–	+	+	–	+	–	v	+
Trehalose	v	v	–	–	–	–	v	–	–	v	–	+	v	+
D-Xylose	+	^c	– ^d	v	+	ND	–	+	–	–	–	–	v	+
α-Glucosidase	v	+	–	–	–	ND	+	–	ND	+	ND	v	v	–

medium in a substrate dose-dependent manner. BHI alone without any additives resulted in about 0.5 g succinic acid l^{-1} after 19 h growth. If the standard concentration of 2 g glucose l^{-1} in BHI was increased to 20 g l^{-1} , strains produced >5 g succinic acid l^{-1} . This was double the amount of succinic acid produced by *Mannheimia glucosida* or *Escherichia coli* strains included as controls. A more detailed biochemical study has previously been carried out with strain DD1 (Scholten & Dagele, 2008).

Toxicity assays

Strain DD1, as a representative of the new species, was investigated for the presence of RTX toxin genes often found in the *Pasteurellaceae* (Frey & Kuhnert, 2002) and for its toxicity towards various host cells in comparison to *Mannheimia haemolytica*, a bovine pathogen of the family *Pasteurellaceae*. A Southern-blot screening using broad-range gene probes for RTX toxins did not indicate the presence of known or related toxin genes (Kuhnert *et al.*, 1997). This is in agreement with an *in silico* screening of the genome sequence of DD1, where no RTX toxins could be found using BLAST-searches at the DNA or amino acid levels. The same holds true for the genome sequence of strain MBEL55E (GenBank accession no. AE016827; Hong *et al.*, 2004).

Toxicity was assayed with the bovine lymphoblastoid cell line BL20, the two human Burkitt lymphoma cell lines BJAP and Ramos, as well as the fish cell line EPC using the CytoTox 96 non-radioactive cytotoxicity assay (Promega). Cell lines were incubated for 4 h with either sterile bacterial culture supernatant or bacteria themselves. *Mannheimia haemolytica* strain SH1217 known to secrete the bovine host-specific leukotoxin Lkt (Fedorova & Highlander, 1997), as well as its culture supernatant, showed 20–30% cytotoxic activity towards bovine lymphoid cells but not towards human or fish cell lines. In contrast, *Basfia succiniciproducens* gen. nov., sp. nov. strain DD1 did not show any cytotoxicity towards bovine, human or fish cell lines.

Description of *Basfia* gen. nov.

Basfia (Bas.fi'a. N.L. fem. n. *Basfia* derived from the chemical company BASF SE in Ludwigshafen, Germany, in reference to the origin of the first strain characterized).

A member of the family *Pasteurellaceae*. Gram-stain-negative, non-motile cells. Do not require β -NAD or haemin for growth. Oxidase-positive. Negative reactions for catalase, urease, indole and α -glucosidase. Positive for methyl red and negative for Voges–Proskauer reactions. In conventional tube assays, acid is produced from D-galactose, D-mannitol, D-mannose, sucrose, trehalose and D-xylose. The type species is *Basfia succiniciproducens*.

Description of *Basfia succiniciproducens* sp. nov.

Basfia succiniciproducens (suc.ci.ni.ci.pro.du'cens. N.L. n. *acidum succinicum* succinic acid; L. part. adj. *producens*

producing; N.L. part. adj. *succiniciproducens* succinic acid producing)

Grows on blood agar under facultatively anaerobic conditions at 37 °C. Capnophilic. Colonies are 0.1–0.5 mm in diameter, shiny and greyish after 24 h. Cells are coccoid to rod-shaped. Non-haemolytic. Produces succinic acid under anaerobic conditions in the presence of a substrate such as glucose. Glucose is fermented without production of gas. In conventional test tube assays, acid is produced from dextrin, maltose and D-fructose, whereas no acid is produced from adonitol, D-arabinose, cellobiose, dulcitol, *myo*-inositol, melezitose, D-sorbitol and D-salicin. Acid production from melibiose and D-rhamnose is variable. On the Vitek2 GN and NH cards the following results are obtained. Positive reactions for α -arabinosidase, β -galactosidase, leucine arylamidase, phenylalanine arylamidase, *N*-acetyl-D-glucosamine, β -galactopyranosidase, phosphorylcholine, D-mannitol, maltotriose, D-galactose, D-glucose, D-mannose, D-malate, D-ribose, sucrose, trehalose, phosphatase and phenylphosphonate. Negative reactions for adonitol, L-arabitol, Ala-Phe-Pro arylamidase, L-pyrrolidonyl arylamidase, tyrosine arylamidase, *N*-acetyl- β -glucosaminidase, γ -glutamyl transferase, glutamyl arylamidase, β -glucosidase, β -xylosidase, alanine arylamidase, L-proline arylamidase, lipase, palatinose, citrate, L-malate, urease, maltose, H₂S, D-sorbitol, D-tagatose, D-xylose, malonate, L-lactate alkalization, α -glucosidase, starch (glycogen), succinate alkalization, *N*-acetyl- β -galactosaminidase, glycine arylamidase, arginine arylamidase, ornithine decarboxylase, lysine decarboxylase, L-histidine, β -glucuronidase, Glu-Gly-Arg arylamidase, L-lysine arylamidase, pyruvate and L-glutamine. Variable reactions were observed with cellobiose, 5-keto-D-gluconate, α -galactosidase, coumarate and Ellman's reagent. No toxicity towards lymphoid bovine and human cells or fish cells, and no RTX toxin genes observed. The DNA G + C content of the type strain is 42.5 mol%. Strains have been isolated from bovine rumen as part of the normal flora.

The type strain is JF4016^T (=DSM 22022^T =CCUG 57335^T) isolated from the rumen of a Simmental cow in Switzerland.

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