***Actinobacillus succinogenes* sp. nov., a novel succinic-acid-producing strain from the bovine rumen**

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Strain 130Z^T was isolated from the bovine rumen. It is a facultatively anaerobic, pleomorphic, Gram-negative rod. It exhibits a 'Morse code' form of morphology, which is characteristic of the genus *Actinobacillus*. Strain 130Z^T is a capnophilic, osmotolerant succinogen that utilizes a broad range of sugars. It accumulates high concentrations of succinic acid (> 70 g l⁻¹). Strain 130Z^T is positive for catalase, oxidase, alkaline phosphatase and β -galactosidase, but does not produce indole or urease. Acid but no gas is produced from D-glucose and D-fructose. 16S rRNA sequence analysis places strain 130Z^T within the family *Pasteurellaceae*; the most closely related members of the family *Pasteurellaceae* have 16S rRNA similarities of 95.5% or less with strain 130Z^T. Strain 130Z^T was compared with *Actinobacillus lignieresii* and the related Bisgaard Taxa 6 and 10. Based upon morphological and biochemical properties, strain 130Z^T is most similar to members of the genus *Actinobacillus* within the family *Pasteurellaceae*. It is proposed that strain 130Z^T be classified as a new species, *Actinobacillus succinogenes*. The type strain of *Actinobacillus succinogenes* sp. nov. is ATCC 55618^T.

Keywords: *Actinobacillus succinogenes* sp. nov., succinic acid, rumen, *Pasteurellaceae*

INTRODUCTION

The family *Pasteurellaceae* is formally composed of three genera, *Pasteurella*, *Haemophilus* and *Actinobacillus*. There are more than 80 described species and taxa that are either members or potential members of this family (Dewhirst *et al.*, 1993). There are also many strains with morphological, cultural and biochemical characteristics consistent with this family that have not been fully described (Mannheim, 1984). None of these bacteria are known to be free living but have been obtained from mammalian, avian and reptilian sources. Some species cause disease but most species are recognized as commensals in the respiratory, alimentary and reproductive tracts of animals. Very little has been published regarding possible symbiotic activities

performed by these many species that live in close association with animals.

Bacteria that degrade the tannin/protein complex were recently described; they may be key to the digestion of tannin-bound protein in the alimentary tract of the koala (Osawa *et al.*, 1995). *Lonepinella koalarum* was placed within the family *Pasteurellaceae* based on analysis of its 16S rRNA sequence. This species colonized the caecum of koalas which have a diet with a high tannin content and it made up 60% of the faecal flora.

Thirty-eight years ago, Phillips (1961) used an antibiotic-containing medium to isolate actinobacillus-like micro-organisms from ruminal contents and proposed that there were 'rumen actinobacilli' with a fermentative role in microbial digestion. Phillips (1961) characterized *Actinobacillus lignieresii* and attempted to identify a symbiotic form of this species with an enhanced fermentative activity. In the rumen, bacteria convert plant carbohydrates to fatty acids and, to a very large extent, succinic acid (Stewart & Bryant,

Abbreviations: ALA, δ -aminolaevulinic acid; CSL, corn steep liquor; FAMES, fatty acid methyl esters; TMP, thiamin monophosphate.

The GenBank accession number for the 16S rRNA sequence of strain 130Z^T is AF024525.

1988). The succinic acid is decarboxylated to propionic acid, which is utilized in the gluconeogenic pathway of the ruminant host, and the gas mixture in the rumen contains 65% (v/v) CO₂ (Yokoyama & Johnson, 1988).

Twenty-nine bovine rumen strains obtained by Phillips (1961) were re-examined and only three could be identified as *A. lignieresii* (Bisgaard *et al.*, 1986). While most of the rumen strains had phenotypic characters compatible with *Pasteurellaceae*, they could not be identified as accepted species within the family. Since the 1960s, it has become increasingly apparent that classification of new strains as members of the *Pasteurellaceae* is difficult even with extended phenotypic characterization. Increased knowledge regarding the family *Pasteurellaceae* has revealed a phylogenetic complexity that may require more than seven genera to accommodate the major phenotypic divisions. Differentiating new species within the family *Pasteurellaceae* has become dependent on sequencing the genes encoding 16S rRNA (Dewhirst *et al.*, 1993; Foster *et al.*, 1996; Osawa *et al.*, 1995).

Recent physiological and biochemical studies have indicated that strain 130Z^T produces succinate-pathway enzymes in much higher amounts than other succinic-acid-producing bacteria (Van der Werf *et al.*, 1997). In this paper, a description of this novel succinic-acid-producing strain isolated from the bovine rumen is presented, including its cultural and biochemical characteristics and its phylogenetic position within the family *Pasteurellaceae* based upon complete 16S rRNA sequence information.

METHODS

Source of strains. Strain 130Z^T (ATCC 55618^T) was isolated at MBI International from bovine ruminal contents. *A. lignieresii* (ATCC 49236^T) and *Staphylococcus aureus* (ATCC 6538P) were obtained from the American Type Culture Collection (ATCC). Reference strains of Bisgaard Taxon 6 (CCUG 15568) and Bisgaard Taxon 10 (CCUG 15572) were obtained from the Culture Collection at the University of Göteborg (CCUG). The Bisgaard Taxa 6 and 10 are related to strain 130Z^T and *A. lignieresii* is a non-fastidious organism that is outwardly similar to strain 130Z^T and a normal inhabitant of the bovine rumen. All three strains were tested simultaneously with strain 130Z^T for cultural and biochemical properties. *S. aureus* was used to test for growth stimulation from cross feeding.

Enrichment and isolation. Ruminal contents were collected from surgically cannulated cattle and used as an inoculum for an enrichment medium containing the ionophoric antibiotic lasalocid (Sigma). Two subcultures were made after 18 h incubation at 37 °C; 10% inocula were used. The final enrichment culture was diluted (10⁻⁶) in PBS (0.145 M NaCl, 0.01 M sodium phosphate) and 0.1 ml was spread onto agar plates. Plates were incubated in an Oxoid anaerobic jar with a CO₂ atmosphere. After 24–48 h, single-well-isolated colonies were picked with a 22 gauge needle and washed into vials of enrichment medium without lasalocid or fumarate (Macy *et al.*, 1972; Miller & Wolin, 1974). Primary isolates

were grown for 24–36 h and samples were analysed by HPLC and screened for succinic acid production. The enrichment medium contained (l⁻¹): 20 g corn steep liquor (CSL) containing approximately 50% dry matter (Corn Products), 15.0 g glucose, 15.0 g sodium fumarate, 15.0 g MgCO₃, 3.0 g K₂HPO₄, 1.0 g NaCl, 1.0 g (NH₄)₂SO₄, 0.2 g CaCl₂·2H₂O, 0.2 g MgCl₂·6H₂O and 16 mg lasalocid (Sigma). Plates used for isolation were made similarly but contained (l⁻¹): 10.0 g Bacto peptone and 5.0 g Bacto yeast extract substituted for the CSL, 15.0 g MgCO₃ and 1.8 g Bacto agar (Difco). The plates used for isolation also contained the following additions and vitamins (l⁻¹): 1.4 g sodium acetate, 1.0 g xylose, 0.2 ml methyl butyric acid, 0.2 ml valeric acid, 0.2 ml isobutyric acid, 0.2 ml isovaleric acid, 500 µg haemin, 100 µg B₆, 50 µg lipoic acid, 20 µg thiamin, 20 µg riboflavin, 20 µg niacin, 20 µg pantothenate, 20 µg *p*-aminobenzoate, 10 µg 1,4-naphthoquinone, 1 µg B₁₂, 1 µg biotin and 1 µg folic acid. The enrichment and isolation media were prepared and sterilized anaerobically (Holdeman *et al.*, 1977; Zeikus *et al.*, 1980).

Analysis. The organic acid fermentation products were determined by HPLC (Guerrant *et al.*, 1982). Components were analysed chromatographically by elution with 0.006 M H₂SO₄ from a cation exchange resin in the hydrogen form. A Waters model 600 HPLC system with a Bio-Rad HPX-87H column and a Waters model 410 Refractive Index detector were used in this analysis. Residual glucose was determined by the same method.

Morphological and physiological characterization. To test the effects of CO₂ and N₂ atmospheres on growth, Oxoid jars were flushed and evacuated three times before filling with CO₂ or N₂. A mixture of approximately 40% (v/v) CO₂ was obtained by adding CO₂ to 69 kPa into an air- or N₂-containing jar; an Oxoid jar filled with CO₂ and topped up with N₂ to 69 kPa contained approximately 60% (v/v) CO₂ by calculation. The 5% (v/v) CO₂ and N₂ mixture was obtained commercially.

The test for fumarate reduction was done as previously described (Hollander *et al.*, 1981). A sterile sodium fumarate stock solution was added to give a 50 mM fumarate concentration in pressure tubes containing 10 ml yeast extract medium and an N₂/CO₂ atmosphere. The medium contained (l⁻¹): 5.0 g yeast extract, 0.19 g NaH₂PO₄·H₂O and 0.5 g Na₂HPO₄. A 0.1% inoculum was used and incubation was stationary at 37 °C. Turbidity was read with a Spectronic 20 spectrophotometer (Milton Roy) at 600 nm.

Biochemical characterization. For the biochemical testing, all strains were grown for 20–24 h at 37 °C on TSB agar plates made with Trypticase Soy Broth (BBL) containing 1.8% (w/v) Bacto agar (Difco). For biochemical testing, *A. lignieresii* was grown aerobically, and Bisgaard Taxon 6 and Bisgaard Taxon 10 strains were grown with 40% (v/v) CO₂. Cells of strain 130Z^T grown aerobically and with 40 and 100% (v/v) CO₂ were tested separately for comparison. The isolates were biochemically characterized using the API 50CH system with CHB medium, the API 20E system, the API OF medium (bioMérieux), and the RapID ANA II system (Innovative Diagnostic Systems) according to the manufacturers' instructions. The API 50CH, API 20E and the RapID ANA II systems were inoculated with cell suspensions with an OD_{550 nm} of approximately 0.75, and the API ZYM system was inoculated with cell suspensions of approximately 1.5 OD_{550 nm}. Each set of tests was repeated at least twice. The oxidase test was performed using a drop of a fresh preparation of 0.5% *N,N,N',N'*-tetramethyl-*p*-

Table 1. Oligonucleotide primers used for PCR amplification and sequencing of 16S rDNA

Primer	Identification	Type	Sequence (5'–3')*	Position†	Orientation
1	C70	PCR	AGAGTTTGATYCTGGCT	8–24	Forward
2	B37	PCR	TACGGYTACCTTGTTACGACT	1493–1513	Reverse
3	B34	Sequencing	ACTGCTGCCTCCCGT	344–358	Reverse
4	B35	Sequencing	GTRTTACCGCGGCTGCTG	519–536	Reverse
5	B36	Sequencing	CTACCAGGGTATCTAATC	786–804	Reverse
6	C01	Sequencing	GGTTGCGCTCGTTGCGGG	1096–1113	Reverse
7	C31	Sequencing	GGAATCGCTAGTAATCG	1337–1353	Forward
8	X91	Sequencing	CCCGGGAACGTATTCACCG	1369–1387	Reverse

* Standard International Union of Biochemistry codes are used for bases.

† Based upon *E. coli* numbering.

Table 2. Accession numbers of strains included in the similarity matrix and levels of 16S rRNA similarity between *Actinobacillus succinogenes* sp. nov. and other representatives of the family *Pasteurellaceae*

Strain	Culture collection no.	GenBank no.	Sequence similarity to <i>Actinobacillus succinogenes</i> (%)
<i>Actinobacillus succinogenes</i>	ATCC 55618 ^T	AF024525	100
<i>Actinobacillus actinomycetemcomitans</i>	ATCC 33384 ^T	M75039	92.1
<i>Actinobacillus equuli</i>	NCTC 8529 ^T	M75072	92.8
<i>Actinobacillus hominis</i>	CCUG 19800 ^T	L06076	92.7
<i>Actinobacillus lignieresii</i>	ATCC 49236 ^T	M75068	93.8
<i>Actinobacillus muris</i>	CCUG 16938	AF024526	92.9
<i>Actinobacillus seminis</i>	ATCC 15768 ^T	M75047	93.6
<i>Actinobacillus suis</i>	ATCC 15557	M75071	92.6
<i>Actinobacillus ureae</i>	Henriksen 3520/59 ^T	M75075	92.8
<i>Actinobacillus</i> / <i>Pasteurella</i> -like	CCUG 28030	AF024527	93.6
Bisgaard Taxon 10	CCUG 15572	AF024528	95.4
Bisgaard Taxon 17	CCUG 17206	AF024529	92.8
Bisgaard Taxon 6	CCUG 15568	L06081	95.5
Bisgaard Taxon 9	CCUG 24862	L06084	92.7
<i>Haemophilus aegyptius</i>	NCTC 8502 ^T	M75044	93.2
<i>Haemophilus aphrophilus</i>	ATCC 33389 ^T	M75041	93.4
<i>Haemophilus aphrophilus</i>	ATCC 7901	M75040	93.3
<i>Haemophilus haemolyticus</i>	NCTC 10659 ^T	M75045	94.1
<i>Haemophilus influenzae</i>	ATCC 33391	M35019	93.2
<i>Haemophilus influenzae</i> murium	NCTC 11146	AF024530	91.7
<i>Haemophilus paraphrophilus</i>	ATCC 29241 ^T	M75042	93.4
<i>Haemophilus segnis</i>	ATCC 33393 ^T	M75043	94.1
' <i>Haemophilus somnus</i> '	OVC 43826	M75046	93.6
<i>Pasteurella aerogenes</i>	ATCC 27883 ^T	M75048	93.9
<i>Pasteurella haemolytica</i> -like	CCUG 28028	AF024531	92.1
<i>Pasteurella mairii</i>	CCUG 27189	L06089	92.2
<i>Pasteurella pneumotropica</i>	NCTC 8141 ^T	M75083	93.2

phenylenediamine dihydrochloride (Sigma) on filter paper. The slide catalase test was performed with 1.5 % (w/v) H₂O₂ solution to confirm the API 20E catalase determination. The cells were tested for oxidase and catalase as described previously (MacFaddin, 1980). The δ -aminolaevulinic acid

(ALA; Sigma) test was done as described previously to test for porphyrin synthesis (Kilian & Biberstein, 1984). Growth stimulation by thiamin monophosphate (TMP) and by cross feeding with *S. aureus* was tested on TSB agar plates as previously described (Stephens *et al.*, 1983).

Cellular fatty acid analysis. The cellular fatty acid compositions of strain 130Z, *A. lignieresii* and the Bisgaard Taxa were determined with cells grown aerobically for 48 h on TSB agar at 28 °C. Fatty acid methyl esters (FAMES) were synthesized and analysed with the MIDI Microbial Identification System as described previously (Sasser, 1990). Briefly, whole cells were treated with NaOH in aqueous methanol at 100 °C. The whole-cell methanolysis and saponification were followed with methylation using HCl in aqueous methanol; FAMES were extracted with hexane/methyl-*tert*-butyl-ether (1:1, v/v) and washed with dilute NaOH. Chromatographic analysis was made with a Hewlett-Packard gas model 5890 Series II, with an HP 7673 automatic sampler and flame-ionization detector. Separation was done with an HP-1 capillary column (25 m × 0.2 mm × 0.33 µm) with H₂ as the carrier gas. FAME identification and analysis was done using the Microbial Identification System software for anaerobic and aerobic bacteria.

16S rRNA sequence analysis. The 16S rRNA sequence determination was done on a 'fee for service' basis by Floyd E. Dewhirst (Forsyth Dental Center, Boston, MA, USA) using previously described methods (Mendes *et al.*, 1996). Briefly, DNA was isolated from cells of strain 130Z^T by a proteinase K method. The 16S rRNA genes were amplified using primers 1 and 2 (see Table 1). The amplicon was purified by the PEG 800 purification method of Kuskawa *et al.* (1990). The amplicon was cycle-sequenced as previously described using the primers in Table 1 (Mendes *et al.*, 1996). The 16S rRNA sequence was analysed using programs written in Microsoft QuickBasic (Paster & Dewhirst, 1988). The sequence database contains over 1000 sequences determined at the Forsyth Dental Center, including over 150 sequences for strains in the family *Pasteurellaceae* and 500 sequences obtained from GenBank and the Ribosomal Database Project (Maidak *et al.*, 1997). Similarity matrices were constructed from the aligned sequences using only those sequence positions for which 90 % of the strains had data. The similarity matrices were corrected for multiple base changes at single positions by the method of Jukes & Cantor (1969). A phylogenetic tree was constructed using the neighbour-joining method of Saitou & Nei (1987) and Studier & Keppler (1988).

GenBank accession numbers. The GenBank and culture collection accession numbers for the strains examined in this report are given in Table 2.

Determination of DNA G+C content. The DNA G+C determination was performed by William B. Whitman (University of Georgia, Athens, GA, USA). The G+C content of DNA obtained from strain 130Z^T was determined by HPLC (Mesbah *et al.*, 1989).

RESULTS AND DISCUSSION

Enrichment, isolation and succinic acid production

The medium used for enrichment and isolation was an organic acid production medium that contained glucose and CSL as its primary constituents. Many of the isolated strains were obligate anaerobes that produced primarily succinic acid and shared other similarities with well-known rumen species.

Strain 130Z^T was part of a second group of isolates that were facultatively anaerobic. Strain 130Z^T pro-

duced more than 30 g succinic acid l⁻¹ in the enrichment vials containing CSL and MgCO₃. The succinic acid was produced along with acetic acid in high ratios (e.g. 5:1–8:1). Formic acid was also produced along with small amounts of pyruvic acid, oxaloacetic acid and ethanol. Growth and acid production were stimulated by additions of yeast extract to the CSL enrichment medium.

For the purpose of comparison, strain 130Z^T and *A. lignieresii* were grown in TSB medium in a 5% (v/v) CO₂ atmosphere. The two species produced similar acid profiles in this poorly buffered medium containing less than 2 g glucose l⁻¹. However, strain 130Z^T produced approximately three times as much succinic acid (data not shown). An increased CO₂ concentration stimulated growth and succinic acid production by strain 130Z. *A. lignieresii* did not grow in any media that contained insoluble MgCO₃, or with high concentrations of CO₂ in the gas phase.

General and growth characteristics

Strain 130Z^T is a non-motile, non-spore-forming, fermentative, facultatively anaerobic, pleomorphic Gram-negative rod or coccobacillus. It is a non-fastidious organism that grows rapidly on blood-free media. It grew on simple chemo-organotrophic media such as 0.4 % yeast extract with glucose and phosphate. It was positive for the ALA test and did not require NAD or other special growth factors. Growth of strain 130Z^T was not stimulated by TMP or cross-fed by *S. aureus*. Strain 130Z^T was mesophilic and grew well at 37–39 °C, whereas no growth was observed on TSB agar plates at 20 or 45 °C. The strain did not grow in TSB containing 4.5 % (w/v) NaCl.

Strain 130Z^T grew aerobically and was capnophilic since although it grew satisfactorily in air, its growth was enhanced in an atmosphere that contained an increased CO₂ concentration. After 24 h incubation at 37 °C in CO₂, colonies of strain 130Z^T on TSB agar were circular, entire, grey, translucent and 1–1.5 mm in diameter. There was slightly more growth at 5–40 % (v/v) CO₂ and isolated colonies were slightly larger than those produced at 60–100 % (v/v) CO₂. Growth produced at 5–100 % (v/v) CO₂ was smooth and did not adhere to the plate. Growth produced in the air adhered to the agar surface, while growth produced without CO₂ (100 % N₂) was sticky but non-adherent. Growth without CO₂ was poor even with several days incubation.

Cells grown with 100 % CO₂ occurred singly (0.8 × 1 µm), in pairs and in short chains. Occasional filamentous cells (0.8–1.2 × 10–85 µm) were seen in most cultures. Cells produced in an atmosphere of CO₂ (≥ 40 %, v/v) were the most uniform and the least pleomorphic. Cells produced at 5 % (v/v) CO₂ were swollen and pleomorphic. Cells produced without CO₂ were more swollen, pleomorphic and appeared to be in a poor condition.

Fig. 1. Phase-contrast micrographs of strain 130Z^T. (a) Cells grown aerobically on TSB agar. (b) Uniform and moniliform chains of cells grown in yeast extract/CSL broth with MgCO₃ and 100% CO₂. Bar, 10 µm.

Aerobically grown cultures produced large sheets of cells apparently cemented together by a matrix of extracellular material (Fig. 1a). The presence of large sheets of cells in air-grown cultures corresponded with the adherence of colonies to the agar surface. Small aggregates of cells occurred in all TSB cultures. Strain 130Z^T produced chains of coccoidal cells in rapidly growing broth cultures. 'Morse code' forms that have bacillary and coccoidal elements interspersed with each other were also easily found in broth cultures (Fig. 1b).

Strain 130Z^T grew rapidly and extensively, utilizing L-arabinose, cellobiose, fructose, galactose, glucose, lactose, maltose, mannitol, mannose, sucrose, D-xylose or salicin in media made with yeast extract and CSL.

Biochemical characterization

Strain 130Z^T was positive for catalase, oxidase, β-galactosidase (ONPG) and nitrate reduction. It was negative for arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, citrate utilization, H₂S, urease, tryptophan deaminase, tryptophanase (indole),

Table 3. Comparison of phenotypic characteristics of the bovine rumen isolate, strain 130Z^T, with the type strains of the genus *Actinobacillus* and the related Bisgaard Taxa 6 and 10

All strains produced a negative reaction for ornithine decarboxylase, lysine decarboxylase, citrate utilization, gelatin hydrolysis, H₂S, indole, tryptophan deaminase, acid from adonitol, amidon, L-arabitol dulcitol, erythritol, D-fructose, glycogen, inositol, inulin, 2-ketogluconate, D-lyxose melezitose, methyl α-D-mannoside, methyl α-D-glucoside, methyl β-xyloside, D-tagatose, D-turanose, L-sorbose, xylitol and L-xylose. All strains produced a positive test with β-galactosidase (ONPG), nitrate reduction, acid from D-ribose, galactose, D-glucose, D-fructose, D-mannose, maltose, raffinose and sucrose. 1, *A. succinogenes* sp. nov. ATCC 55618^T; 2, *A. lignieresii* ATCC 49236; 3, Bisgaard Taxon 6 CCUG 15568; and 4, Bisgaard Taxon 10 CCUG 15572.

Test	1	2	3	4
Catalase	+	—	—	—
Oxidase (TMPD)†	+	+	—	—
Urease	—	+	+	+
Acetoin (Voges-Proskauer)	—	+	+	+
D-Glucose (gas)	—	—	+	+
O/F*	F	F	F	F
Acid from:				
Glycerol	—	+	+	+
D-Arabinose	—	—	+	+
L-Arabinose	+	—	+	+
D-Xylose	+	+	+	—
L-Rhamnose	—	—	—	+
D-Mannitol	+	+	—	+
D-Sorbitol	+	—	—	—
N-Acetylglucosamine	—	+	—	—
Amygdalin	+	—	—	—
Arbutin	+	—	—	—
Aesculin	+	—	—	—
Salicin	+	—	—	—
Cellobiose	+	—	—	—
Lactose	+	—	+	+
D-Melibiose	—	—	+	+
Trehalose	—	—	+	+
β-Gentiobiose	+	—	—	—
L-Fucose	—	+	+	—
D-Arabitol	+	—	—	—
Gluconate	+	—	+	+
5-Ketogluconate	+	—	+	+

* F, Fermentative; O, oxidative.

† TMPD, Tetramethyl-*p*-phenylenediamine.

the Voges-Proskauer test, gelatin hydrolysis and gas production from glucose (Table 3).

Strain 130Z^T produced acid from L-arabinose, D-ribose, D-xylose, galactose, D-glucose, D-fructose, D-mannose, D-mannitol, D-sorbitol, amygdalin, arbutin, aesculin, salicin, cellobiose, maltose, lactose, sucrose, raffinose, β-gentiobiose, D-arabitol, gluconate and 5-

Table 4. Enzyme profiles produced by strain 130Z^T, *A. lignieresii*, Bisgaard Taxon 6 and Bisgaard Taxon 10 as determined by the API ZYM system

1, *A. succinogenes* sp. nov. ATCC 55618^T; 2, *A. lignieresii* ATCC 49236; 3, Bisgaard Taxon 6 CCUG 15568; and 4, Bisgaard Taxon 10 CCUG 15572. All strains were positive for esterase (C4), esterase-lipase (C8), leucine arylamidase and β -galactosidase, but negative for lipase (C14), valine arylamidase, cystine arylamidase, trypsin, chymotrypsin, β -glucuronidase, *N*-acetyl- β -glucosaminidase, α -mannosidase and α -fucosidase.

Enzyme activity	1	2	3	4
Phosphohydrolase	+	—	+	+
α -Galactosidase*	—	—	+	+
α -Glucosidase	—	—	+	—
β -Glucosidase	+	—	—	—
Acid phosphatase, alkaline phosphatase	+	++†	+	+

* α -Galactosidase activity was determined by the RapID ANA II System.

† Strong positive reaction.

ketogluconate (Table 3). It produced no change with glycerol, erythritol, D-arabinose, L-xylose, adonitol, methyl β -xyloside, L-sorbose, L-rhamnose, dulcitol, inositol, methyl α -D-mannoside, methyl α -D-glucoside, *N*-acetylglucosamine, D-melibiose, trehalose, inulin, melezitose, amidon, glycogen, xylitol, D-turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, L-arabitol or 2-ketogluconate (Table 3). Tests were not examined beyond 48 h so some additional late reactions could be possible.

In the API ZYM analysis, strain 130Z^T produced positive reactions for alkaline phosphatase, esterase (C4), esterase-lipase (C8), leucine arylamidase, phosphohydrolase, β -galactosidase and β -glucosidase, and it gave a weak reaction for acid phosphatase. There was no reaction for lipase (C14), valine arylamidase, cystine arylamidase, trypsin, chymotrypsin, α -galactosidase, β -glucuronidase, α -glucosidase, *N*-acetyl- β -glucosaminidase, α -mannosidase and α -fucosidase (Table 4).

The RapID ANA II system confirmed the presence of β -glucosidase and β -galactosidase activities, and the lack of tryptophanase (indole), urease, *N*-acetyl- β -glucosaminidase, α -fucosidase and α -glucosidase activities. The RapID ANA II system detected four additional activities: arginine arylamidase (strong positive), leucyl-glycine arylamidase, phenylalanine arylamidase and serine arylamidase. A positive β -glucosidase activity was consistent with acid from cellobiose, salicin and the other β -glucosides (Table 3). β -Disaccharidase, arabinosidase, α -galactosidase, leucine, glycine, proline, phenylalanine, serine and pyroglutamic acid arylamidases were absent, as determined by the RapID ANA II system.

Table 5. Methylated cellular components of cells grown aerobically for 48 h on TSB agar plates

1, *A. succinogenes* sp. nov. ATCC 55618^T; 2, *A. lignieresii* ATCC 49236; 3, Bisgaard Taxon 6 CCUG 15568; and 4, Bisgaard Taxon 10 CCUG 15572.

Component FAME	Total chromatographic area (%)			
	1	2	3	4
C _{12:0}	0.0	0.0	1.7	2.4
C _{16:1 cis9}	34.1	32.7	36.8	36.1
C _{14:0}	26.0	30.8	18.7	20.4
C _{16:0}	20.1	16.0	24.4	23.8
C _{14:0} 3-OH or C _{16:1} iso	16.4	17.5	13.3	13.2
C _{18:1 cis11/t9/t6 or}	1.3	0.0	1.7	2.0
C _{18:1 trans9/t6/c11 or}				
C _{18:1 trans6/t9/c11}				

Fumarate respiration

Strain 130Z^T had increased turbidity as a result of fumarate respiration. The turbidity increased by approximately 1 OD unit in a medium containing sodium fumarate but lacking carbohydrate. Tubes lacking sodium fumarate did not show any increase in turbidity. Fumarate is used as a terminal electron acceptor in dimethylmenaquinone-mediated anaerobic respiration which is a characteristic of bacteria within the family *Pasteurellaceae* (Hollander *et al.*, 1981). The response to fumarate was determined simultaneously for strain 130Z^T and the three reference strains. Strain 130Z^T and Bisgaard Taxon 10 produced approximately 1.0 OD units from fumarate respiration in 15 h, while Bisgaard Taxon 6 produced approximately 0.75 OD units from fumarate. *A. lignieresii* turbidity increased 0.45 OD units with added fumarate.

Cellular fatty acids

The major fatty acid derivatives of strain 130Z^T and *A. lignieresii* were C_{14:0}, C_{16:0} and C_{16:1 cis9} FAME. A fourth major component was either C_{14:0} 3-OH FAME, C_{16:1} iso FAME, or a combination of the two esters. These esters were not separated sufficiently to be reported as individual components, but are reported as a combined feature by the MIDI Microbial Identification System. Strain 130Z^T and the Bisgaard Taxa 6 and 10 produced a minor amount of either C_{18:1 cis} or C_{18:1 trans} FAMES. The Bisgaard Taxa also produced a minor amount of C_{12:0}. Cellular fatty acid data for both species are shown in Table 5. The results were consistent with reports that members of the family *Pasteurellaceae* produce a restricted profile of fatty acids with major amounts of hexadecanoate (C_{16:1}), n-tetradecanoate (C_{14:0}), 3-hydroxy-tetradecanoate (C_{14:0} 3-OH) and n-hexadecanoate (C_{16:0}), and minor amounts of octadecanoate (C_{18:1}) (Jantzen *et al.*, 1981).

Fig. 2. Dendrogram of 16S rRNA relationship of strain 130Z^T to members of the family Pasteurellaceae. It was generated from the similarity data using the neighbour-joining method of Saitou & Nei (1987).

DNA G + C base composition

The DNA obtained from strain 130Z^T had a G + C base content of 45.1 mol%, as determined by HPLC (Mesbah *et al.*, 1989). The DNA from Bisgaard Taxon 6 was reported to have a G + C content of 51.2 mol% (Bisgaard *et al.*, 1983). Although these were substantially different, the DNA G + C base compositions of actinobacilli are quite variable and are reported not to be useful for differentiating species of actinobacilli (Phillips, 1992).

Phylogenetic affiliation and 16S rRNA sequence analysis

The essentially complete 16S rRNA sequence (1441 bases) was determined for strain 130Z^T. Sequence comparison with organisms in the database indicated that strain 130Z^T was clearly a member of the family Pasteurellaceae. The sequence for strain 130Z^T was compared to 26 previously sequenced members of the Pasteurellaceae (Table 2) and a similarity matrix based upon comparisons at 1423 base positions was produced. Table 2 shows levels of 16S rRNA similarity between strain 130Z^T (*Actinobacillus succinogenes* sp. nov.) and other representatives of the family Pasteurellaceae. A phylogenetic tree was generated from the similarity data by the neighbour-joining method (Fig. 2). Strain 130Z^T is most closely related to strains

representing Bisgaard Taxon 6 (4.7% difference), Bisgaard Taxon 10 (4.8% difference) and '*Haemophilus somnus*' (6.7% difference). These organisms correspond to cluster 2B in the previously published phylogenetic description of the Pasteurellaceae (Dewhirst *et al.*, 1993).

Of the organisms within cluster 2B, '*H. somnus*' had the lowest level of sequence similarity with strain 130Z^T. '*H. somnus*' and strain 130Z^T were also phenotypically dissimilar. '*H. somnus*' is a fastidious Gram-negative rod that occurs commensally in normal cattle but also causes disease; it has been studied rather extensively (Kirkham *et al.*, 1989; Stephens *et al.*, 1983; Ward *et al.*, 1995). All '*H. somnus*' strains were fastidious in their growth requirements and grew well only in media containing blood or tissue extracts. Growth was stimulated by TMP or by cross feeding with *S. aureus*. It did not grow in air but required 10% (v/v) CO₂ for growth. Stephens *et al.* (1983) found that most strains did not grow on sugars other than glucose.

'*H. somnus*' differed from strain 130Z^T by its fastidious growth requirements, inability to grow in air, and by the production of β -glucuronidase and tryptophanase (indole). Unlike strain 130Z^T, '*H. somnus*' lacked catalase, β -galactosidase and β -glucosidase activities, and it did not hydrolyse aesculin or produce acid from L-arabinose, cellobiose, lactose, maltose, raffinose, salicin or sucrose. Unlike '*H. somnus*', strain 130Z^T was not stimulated by TMP or cross-fed by *S. aureus*.

The other taxa within cluster 2B were the Bisgaard Taxa 6 and 10 which had a low level of 16S rRNA sequence similarity ($\approx$ 95.5%) with strain 130Z^T and numerous phenotypic distinctions. Strain 130Z^T was catalase-, oxidase- and β -glucosidase-positive, urease-negative and non-aerogenic; strain 130Z^T could be distinguished from either Bisgaard Taxon 6 or Bisgaard Taxon 10 by any of these characteristics.

The Bisgaard Taxon 6 strain was originally isolated from two different colonies of healthy guinea pigs and it made up to 50% of the guinea pig pharyngeal flora (Bisgaard *et al.*, 1983). Lower portions of the alimentary tract were not investigated. It was isolated on blood-containing agar but it grew on TSB agar plates in a similar fashion to strain 130Z^T. Notably, seven strains isolated from guinea pigs and wild muskrat have been classified as *Actinobacillus* species (Boot & Bisgaard, 1994). Bisgaard Taxon 10 is also non-fastidious and can be cultured on TSB agar. It was isolated from the equine oral cavity but no publications were found regarding the circumstances surrounding its isolation. The horse's mouth is replete with indigenous members of the family Pasteurellaceae (Samitz & Biberstein, 1991). Both of the Bisgaard taxa could be grown aerobically or in high concentrations of CO₂.

Bisgaard Taxon 6 differed from strain 130Z^T by the presence of urease, α -galactosidase and α -glucosidase activities, the production of gas from glucose, and the

lack of β -glucosidase activity. Major differences regarding the substrates fermented by Bisgaard Taxon 6 and strain 130Z^T were observed (Table 3). Bisgaard Taxon 6 failed to ferment amygdalin, gentiobiose, arbutin, aesculin, salicin, cellobiose, mannitol, sorbitol or D-arabitol. Bisgaard Taxon 6 type strain produced acid from D-arabinose, and produced acid and gas from L-fucose. Trehalose and melibiose were fermented, which is consistent with the presence of α -galactosidase (Tables 3 and 4). Bisgaard Taxon 6 produced gas from a number of substrates other than glucose and L-fucose, including fructose, mannose, maltose, lactose, sucrose, trehalose, gluconate and 5-ketogluconate. Bisgaard Taxon 6 and strain 130Z^T were biochemically distinct species.

Bisgaard Taxon 10 differed from strain 130Z^T by the presence of urease and α -galactosidase activities, the production of gas from glucose, and the lack of β -glucosidase activity. There were major differences regarding the substrates fermented by Bisgaard Taxon 10 and strain 130Z^T (Table 3). Bisgaard Taxon 10 failed to ferment amygdalin, gentiobiose, arbutin, aesculin, salicin, cellobiose, sorbitol, D-arabitol and D-xylose. Bisgaard Taxon 10 fermented D-arabinose and rhamnose. Fermentation of trehalose and melibiose were consistent with the presence of α -galactosidase. Bisgaard Taxon 10 produced gas from a number of substrates other than glucose including fructose, mannose, mannitol, maltose, gluconate and 5-ketogluconate. Although both Bisgaard Taxa 6 and 10 shared some general growth characteristics with strain 130Z^T, they were phenotypically distinct from strain 130Z^T based on numerous biochemical differences (Table 3). Strain 130Z^T was also phylogenetically distinct from both Taxon 6 and Taxon 10 based on the differences in their 16S rRNA sequences (4.7 and 4.8%, respectively). Strain 130Z^T and Bisgaard Taxa DNA-DNA hybridizations were not done because 16S rRNA similarities were less than 96% and DNA-DNA reassociation values were expected to be low. Strains of the same species have at least 60–70% DNA similarity. Therefore, the DNA-DNA reassociation studies become particularly useful when 16S rRNA similarities are 97% or higher (Stackebrandt, 1994).

Of the accepted species of *Actinobacillus*, *Actinobacillus capsulatus* shared many biochemical properties of strain 130Z^T. Samitz & Biberstein (1991) used the same API batteries of reactions to biotype wild isolates and reference strains including *A. capsulatus*. The reactions of the *A. capsulatus* reference strain were similar to those of strain 130Z^T, except that 130Z^T did not produce acid from melibiose, trehalose or N-acetylglucosamine. Unlike *A. capsulatus*, strain 130Z^T did not produce α -galactosidase or α -glucosidase but did produce β -glucosidase. Strain 130Z^T lacked the capsule present in *A. capsulatus*. According to Samitz & Biberstein (1991), the capsule is so pronounced that *A. capsulatus* cells are difficult to sediment by centrifugation. Strain 130Z^T produces an extracellular slime

under certain conditions, which is typical of members of the genus *Actinobacillus* (Phillips, 1984), but strain 130Z^T is not known to form a compact capsule under any experimental conditions. Based upon 16S sequence information, strain 130Z^T lies within cluster 2B, while *A. capsulatus* lies within cluster 3C at a considerable phylogenetic distance from strain 130Z^T (Dewhirst *et al.*, 1993).

Based upon phylogenetic placement, phenotypic properties and the ability to produce succinic acid, it is proposed that strain 130Z^T be included in the genus *Actinobacillus* as the type strain of a new species, *Actinobacillus succinogenes*. Strain 130Z^T is phenotypically most similar to the genus *Actinobacillus*. Placement within the genus *Actinobacillus* will also serve to call attention to its source in the bovine rumen. Phillips (1961) proposed that there were 'rumen actinobacilli' that had an enhanced fermentative activity and that were typically catalase-positive and urease-negative. It is recognized in this study that there is a new body of knowledge concerning the family *Pasteurellaceae* that is being built from 16S rRNA sequence determinations and that the structure of the family *Pasteurellaceae* will be changed in the future based upon 16S rRNA information and other molecular approaches. It is also recognized that strains within the 2B subcluster of *Pasteurellaceae* may not fit with any *Pasteurella*, *Haemophilus*, or *Actinobacillus sensu stricto* genus definitions (Dewhirst *et al.*, 1993), and that the genus designation for *Actinobacillus succinogenes* may be changed in the future.

Description of *Actinobacillus succinogenes* sp. nov.

Actinobacillus succinogenes (suc.ci.no'ge.nes. M.L. n. *acidum succinicum* succinic acid; Gr. v. *gemmaio* to produce; M.L. adj. *succinogenes* succinic-acid-producing).

Cells are non-motile, pleomorphic, Gram-negative rods (0.8 × 1 µm). Occasional filamentous cells are seen in most cultures. 'Morse code' forms and chains are common in actively growing broth cultures. Cells within an extracellular matrix are common in aerobically grown cultures. Chemo-organotrophic. Yeast extract and CO₂ stimulate growth. Colonies produced on TSB agar are circular, entire, grey, translucent and 1–1.5 mm in diameter after 24 h incubation at 37 °C with CO₂. Growth occurs at 37–39 °C, but not at 20 or 45 °C. Catalase- and oxidase-positive. Alkaline phosphatase-positive. Acid but no gas is produced within 24 h from D-glucose and D-fructose. Acid is also produced with L-arabinose, D-ribose, D-xylose, galactose, D-mannose, D-mannitol, D-sorbitol, amygdalin, arbutin, aesculin, salicin, cellobiose, maltose, lactose, sucrose, raffinose, β -gentiobiose, D-arabitol, gluconate or 5-ketogluconate. Acid is not produced from dulcitol, inositol, inulin, glycerol, erythritol, D-arabinose, L-xylose, adonitol, methyl β -xyloside, L-sorbose, L-rhamnose, methyl α -D-mannoside, methyl

α -D-glucoside, *N*-acetylglucosamine, D-melibiose, trehalose, melezitose, amidon, glycogen, xylitol, D-turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, L-arabitol and 2-ketogluconate. β -Galactosidase, arginine- β -arylamidase and leucine arylamidase were produced in large amounts. Nitrates are reduced to nitrites. Indole and urease are not produced. Rapid and extensive growth observed utilizing arabinose, cellobiose, fructose, galactose, glucose, lactose, maltose, mannitol, mannose, sucrose, xylose or salicin in media made with yeast extract and CSL. Marked production of succinic acid in the presence of CO₂ and high substrate concentration. DNA G+C content is approximately 45 mol%. The type strain, strain 130Z^T, was isolated from the bovine rumen, preserved by freezing in glycerol vials and by lyophilization, and has been deposited in the American Type Culture Collection as ATCC 55618^T.

Occurrence and role of *Actinobacillus succinogenes* sp. nov. in the rumen

Actinobacillus succinogenes was isolated from ruminal contents. It is a capnophilic, osmotolerant and highly productive saccharolytic succinogen utilizing a broad range of sugars. These general properties are suggestive of a micro-organism that is adapted to a symbiotic role in the rumen. The present description of *A. succinogenes* may lead to additional isolations of this organism and information regarding its frequency of occurrence in the bovine rumen or possibly other ruminants.

A. succinogenes is an acidogenic strain that has a conspicuous ability to accumulate very high concentrations of succinic acid. Strain 130Z^T initiated growth in the presence of high glucose concentrations, and produced 74 g succinic acid l⁻¹ in vials that contained increased amounts of glucose and MgCO₃. Some derivatives of strain 130Z^T accumulated more than 100 g succinic acid l⁻¹ in vials containing yeast extract/CSL medium (Guettler *et al.*, 1996a, b).

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