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ORIGINAL PAPER

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Isolation and characterization of a new succinic acid-producing bacterium, *Mannheimia succiniciproducens* MBEL55E, from bovine rumen

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Abstract A novel succinic acid-producing bacterium was isolated from bovine rumen. The bacterium is a non-motile, non-spore-forming, mesophilic and capnophilic gram-negative rod or coccobacillus. Phylogenetic analysis based on the 16S rRNA sequence and physiological analysis indicated that the strain belongs to the recently reclassified genus *Mannheimia* as a novel species, and has been named *Mannheimia succiniciproducens* MBEL55E. Under 100% CO_2 conditions, it grows well in the pH range of 6.0–7.5 and produces succinic acid, acetic acid and formic acid at a constant ratio of 2:1:1. When *M. succiniciproducens* MBEL55E was cultured anaerobically in medium containing 20 g l^{-1} glucose as carbon source, 13.5 g l^{-1} of succinic acid was produced.

Introduction

Succinic acid is a dicarboxylic acid produced as an intermediate of the tricarboxylic acid (TCA) cycle and also as one of the fermentation products of anaerobic metabolism (Gottschalk 1986; Zeikus 1980). Fermentative production of succinic acid from renewable biomass has recently drawn much attention because it can be used for the manufacture of synthetic resins and biodegradable polymers and as an intermediate in chemical synthesis (Landucci et al. 1994; Zeikus et al. 1999).

The rumen is the first division of the stomach of a ruminant animal. More than 200 kinds of bacteria inhabit the bovine rumen. A number of functionally important rumen bacteria produce succinic acid during fermentation of carbohydrates (Hungate 1969), although succinic acid is seldom detected in measurable amounts in rumina because it is rapidly converted to propionic acid (Blackburn and Hungate 1963). Several anaerobic and facultative anaerobic bacteria that produce succinic acid from carbohydrates have been isolated from rumen and other sources: *Ruminococcus albus* (Leatherwood 1965), cellulolytic *Prevotella ruminicola* (Howlett et al. 1976), *Bacteroides amylophilus* (Caldwell et al. 1973), and *Bacteroides fragilis* (Caspari and Macy 1983). Recently, *Actinobacillus succinogenes*, which produces a large amount of succinic acid, was isolated from bovine rumen (Guettler et al. 1999).

In this article, we report isolation of a novel succinic acid-producing bacterium, *Mannheimia succiniciproducens* MBEL55E, from bovine rumen. We also report its cultural, physiological and fermentation characteristics, and its phylogenetic position within the family *Pasteurellaceae* based on the complete 16S rRNA sequence.

Materials and methods

Culture conditions

General anaerobic cultivation techniques were used for the growth of organisms and preparation of media (Bryant 1972; Hungate 1966) with slight modification. The enrichment medium contained, per liter: 20 g glucose, 5 g polypeptone, 5 g yeast extract, 3 g K_2HPO_4, 2 g NaCl, 2 g $(NH_4)_2SO_4$, 0.2 g $CaCl_2 \cdot 2H_2O$, 0.4 g $MgCl_2 \cdot 6H_2O$, and 15 g $MgCO_3$. Screening agar plates used for the isolation of bacteria contained, per liter: 5 g glucose, 23 g peptone, 5 g yeast extract, 12 g Bacto agar, 2 g NaCl, 0.4 g sodium bicarbonate, 0.5 g cysteine-HCl, 0.25 g soluble pyrophosphate, 10 mg Hemin and 1 mg vitamin K. Culture media were gassed with oxygen-free CO_2 and autoclaved for 15 min at 121°C (Lee et al. 1999b). Yeast extract and peptones were purchased from Difco (Detroit, Mich.), and other chemicals were from either Sigma (St. Louis, Mo.) or Junsei (Tokyo, Japan).

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Enrichment and isolation

Ruminal contents were taken from a Korean cow and were inoculated into an enrichment medium containing the ionoporic antibiotics lasalocid (16 mg l^{-1}, Sigma) and monensin (10 mg l^{-1}, Sigma), which inhibit rumen microorganisms that produce acetic acid and hydrogen, thereby favoring enrichment of those bacteria producing propionic acid (Singleton and Sainsbury 1987). Subcultures were carried out after 18 h of incubation at 37°C. The culture was diluted to 10^{-6} in PBS buffer (0.145 M NaCl, 0.01 M sodium phosphate). The diluted cultures (0.1 ml) were spread onto screening agar plates and were incubated in an anaerobic jar (Oxoid, Basingstoke, UK) under CO_2 atmosphere at 37°C. After 24–48 h, isolated colonies were picked with a sterilized 22-gauge needle and injected into vials containing enrichment medium plus lasalocid and monensin. Primary isolates were cultured for 24 h and their fermentation products were analyzed by HPLC (see below). Isolates producing succinic acid were saved for further analyses.

Growth conditions and phenotypic analysis

Cells were grown anaerobically in sealed anaerobic bottles containing 100 ml of MH medium plus 10 g l^{-1} of glucose under CO_2 atmosphere. MH medium contained, per liter: 10 g polypeptone, 5 g yeast extract, 3 g K_2HPO_4, 2 g NaCl, 2 g $(NH_4)_2SO_4$, 0.2 g $CaCl_2 \cdot 2H_2O$, 0.2 g $MgCl_2 \cdot 6H_2O$, and 10 g $MgCO_3$. $Na_2S \cdot 9H_2O$ was added to a final concentration of 1 mg l^{-1} to ensure anaerobic conditions. For flask cultures, exponentially growing cells, washed anaerobically with MH medium, were used to inoculate sealed anaerobic medium bottles containing 100 ml of MH medium plus 10 g l^{-1} of glucose or other carbon sources. MH medium with lower amounts of polypeptone (0, 2, and 5 g l^{-1}) and yeast extract (0, 1, and 2 g l^{-1}) was also examined to see the effect of complex nitrogen sources on cell growth. Utilization of various carbon sources (see Results) was examined by monitoring their concentrations during cultivation. Batch cultures were carried out at 39°C in a jar fermentor (2.5 l, Korea Fermentor Company, Incheon, Korea) containing 1 l MH medium plus 20 g l^{-1} glucose. $Na_2S \cdot 9H_2O$ was again added to a final concentration of 1 mg l^{-1}. The pH was controlled at 6.5 using 5 N NaOH. Foaming was controlled by the addition of Antifoam 289 (Sigma). During aerobic cultivation, air was sparged and dissolved oxygen concentration was maintained at over 40% of air saturation by increase of agitation speed up to 1,000 rpm. CO_2 and N_2 gases were sparged during anaerobic cultivation and gas sparging rates and agitation speed were controlled at 0.25 vvm and 200 rpm, respectively. CO_2 and N_2 gases were scrubbed free of oxygen by passing them through a gas purifier (Cobert, St. Louis, Mo.). The sensitivity of *M. succiniciproducens* MBEL55E to various antibiotics (ampicillin, kanamycin, tetracycline and chloramphenicol; Sigma) was examined by counting colony-forming units (cfu) on agar plates containing various concentrations of these antibiotics.

Analytical procedures

The concentrations of fermentation products and carbon compounds were determined by HPLC (Hitachi L-3300 RI detector, D2500 chromato-integrator, Tokyo, Japan) equipped with an ion exchange column (Aminex HPX-87H, 300 mm ×7.8 mm, Hercules, Calif.) using 0.012 N H_2SO_4 as a mobile phase (Lee et al. 1999a). Cell growth was monitored by measuring the absorbance at 660 nm (OD_{660}) using a spectrophotometer (Ultrospec3000, Pharmacia, Sweden). Dry cell weight (DCW) was calculated from a curve relating the OD_{660} to DCW. An OD_{660} of 1.0 was equivalent to 400±20 mg DCW l^{-1}. The yields of fermentation products were defined as grams of product formed from 1 g of glucose, and were expressed as a percentage. Carbon balance was calculated as previously described (Lynd et al. 1982).

Table 1 16S Ribosomal RNA similarity between strain *Mannheimia succiniciproducens* MBEL55E and bacteria belonging to related taxa

Species	Accession number	Similarity (%)
M. succiniciproducens MBEL55E (KCTC 0769BP)	AY029193	100
M. varigena CCUG 38462^T	AF053893	95.1
M. granulomatis ATCC 49244^T	AF053902	94.9
M. ruminalis CCUG 38470^T	AF053900	94.7
M. glucosida CCUG 38457^T	AF053889	94.7
Pasteurella trehalosi NCTC 11550	U57073	94.7
P. dagmatis ATCC 43325^T	M75051	94.3
M. haemolytica NCTC 9380 ^{T}	AF060699	94.1
P. avium NCTC 11297 ^{T}	M75058	94.0
P. aerogenes ATCC 27883^T	M75048	94.0
P. bettyae CCUG 2042^T	L06088	94.0
P. volantium NCTC 3438 ^{T}	M75070	93.8
P. mairii CCUG 27189 ^{T}	AF024532	93.8
P. multocida NCTC 10322 ^{T}	M35018	93.8
P. langaaensis ATCC 43328 ^{T}	M75053	93.7
P. gallinarum NCTC 11188 ^{T}	M75059	93.5
P. stomatis ATCC 43327^T	M75050	93.2
P. canis ATCC 43326^T	M75049	93.1
Haemophilus influenzae ^{T}	M35019	92.5
P. pneumotropica NCTC 8141 ^{T}	M75083	92.3
P. anatis ATCC 43329 ^{T}	M75054	92.0
P. testudinis CCUG 19802 ^{T}	L06090	91.3
Vibrio cholerae ATCC 140357	Z21856	87.8
Escherichia coli	J01695	86.6

Scanning electron microscopy

Samples were fixed for 2 h in a 2.5% paraformaldehyde-glutaraldehyde mixture buffered with 0.1 M phosphate (pH 7.2), postfixed for 1 h in 1% osmium tetroxide in the same buffer, dehydrated in graded ethanol, and finally substituted by isoamyl acetate. They were then dried under CO_2 using a critical point dryer (E3000, BioRad, Calif.). Finally, the samples were sputtered with gold using a coater (SC502, Polaron, West Sussex, UK), and were observed under a scanning electron microscope (SEM 515, Philips, The Netherlands).

16S rRNA sequence and phylogenetic analysis

The 16S rRNA gene was amplified by PCR (GeneAmp PCR system 9700, Roche, Mannheim, Germany). The sequences of the primers used for amplification were 5′-AGAGTTTGATCMTGGCTCAG-3′ and 5′-AAGGAGGTGWTCCARCC-3′, corresponding to positions 8–27 and 1,545–1,525, respectively, of the *Escherichia coli* 16S rRNA sequence (Stackebrandt and Goodfellow 1991). The PCR product was purified using a Wizard PCR DNA purification system (Promega, Madison, Wis.) and sequenced using an ABI PRISM 310 automated sequencer (Perkin-Elmer, Conn.), as described in the manual for the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit.

The accession numbers for the sequences used as references are shown in Table 1. The phylogenetic analysis was carried out by the neighbor-joining method (Saitou and Nei 1987) using the PHYLIP program package, version 3.5c (Department of Genetics, University of Washington, Seattle, Wash.).

GenBank accession number

The complete sequence of the 16S rRNA gene of *M. succiniciproducens* MBEL55E has been deposited with GenBank under the accession no. AY029193.

Results

Morphological characteristics

Colonies of strain MBEL55E appearing on the screening agar plate after 24 h of incubation were smooth and grayish, and 1–2 mm in diameter. The bacterium was non-spore-forming and gram-negative. Light microscopy and scanning electron microscopy revealed that the morphology of the cells could best be described as that of a typical *Mannheimia* cell cluster (Angen et al. 1999). Cells occurred singly (0.5×0.8 μm), and in pairs or short chains. Some filamentous cells and small cell aggregates were seen in most of the cultures. Motility was not observed.

Phylogenetic analysis

The 16S rRNA sequence (1,392 bases) was determined for strain MBEL55E and compared with that of *E. coli* and 22 previously sequenced members of *Pasteurellaceae*. The 16S rRNA similarity between strain MBEL55E and other representatives of the family *Pasteurellaceae* was determined (Table 1), followed by construction of a phylogenetic tree (Fig. 1). Strain MBEL55E is most closely related to *Mannheimia* spp. It was concluded that strain MBEL55E belongs to the new genus *Mannheimia*, which was recently reclassified based on the 16S rRNA sequence and DNA-DNA hybridization (Angen et al. 1999). Since this new bacterium produces significant amounts of succinic acid, we named it *Mannheimia succiniciproducens* MBEL55E. The strain has been deposited at the Korean Collection for Type Cultures (Taejon, Korea) as KCTC 0769BP.

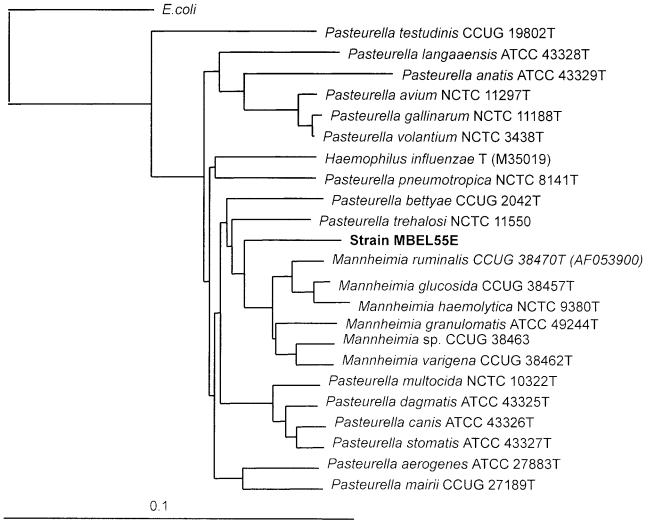

Fig. 1 Phylogenetic tree of *Mannheimia succiniciproducens* MBEL55E and related organisms based on their 16S rRNA sequences. *Scale bar* represents 10 base substitutions per 100 bases

Growth characteristics of *M. succiniciproducens* MBEL55E

M. succiniciproducens MBEL55E was found to be a non-fastidious organism that grows rapidly on blood-free medium. It could grow on semi-defined media containing glucose, yeast extract (and/or polypeptone) and mineral salts. It is mesophilic and grows well at 37–39°C, whereas no growth was observed on agar plates incubated at 20 or 45°C. It was sensitive to ampicillin, kanamycin, and chloramphenicol, but not to tetracycline. Changing the pH of the medium under CO_2-rich conditions had significant effects on cell growth, glucose consumption and end-product formation (succinic, acetic, formic and lactic acids). When the pH was maintained at 5.5, cell growth was poor, and succinic acid was produced as a major product. As the pH was increased from 6.0 to 7.5, cells grew better and the glucose consumption rate increased. When the pH was maintained at 8.0, cell growth was severely impaired (to the level observed at pH 5.5). Within the range pH 6.0–7.5, the ratio of end-products formed (succinic, acetic, formic and lactic acids) was not much affected by changing the culture pH; growth rate, on the other hand, was significantly affected. The optimum pH for cell growth was 6.5. Cell growth was also examined in MH medium with varying concentrations of polypeptone and yeast extract. Cells could grow in all media except that without yeast extract. However, cell growth was best in a medium containing 10 g l^{-1} polypeptone and 5 g l^{-1} yeast extract.

The effect of CO_2 level on cell growth and end-product formation during batch culture of *M. succiniciproducens* MBEL55E

M. succiniciproducens MBEL55E is a facultative bacterium that can grow under both anaerobic and aerobic conditions. When *M. succiniciproducens* MBEL55E was cultured under aerobic conditions, lactic acid (9.6 g l^{-1}) was produced as a major product and a small amount of acetic acid (3.4 g l^{-1}) was also produced (Fig. 2A). Interestingly, CO_2 gas was not generated during cultivation. This finding suggests that some CO_2-generating pathways, including the TCA cycle, are not active under aerobic conditions.

M. succiniciproducens MBEL55E could also grow under anaerobic conditions. Since it is well known that the level of CO_2 in the medium exerts a significant effect on the growth of the succinic acid-producing bacteria *Anaerobiospirillum succiniciproducens* (Lee et al. 2000; Samuelov et al. 1991) and *Actinobacillus* sp. 130Z (Van der Werf et al. 1997), it was expected that growth of *M. succiniciproducens* MBEL55E would be enhanced under elevated CO_2 levels. Therefore, the effect of CO_2 levels on cell growth and product formation was investigated in batch fermentations of *M. succiniciproducens* MBEL55E under CO_2 and N_2 atmospheres. Glucose (20 g l^{-1}) was used as a carbon substrate. Under CO_2 at-

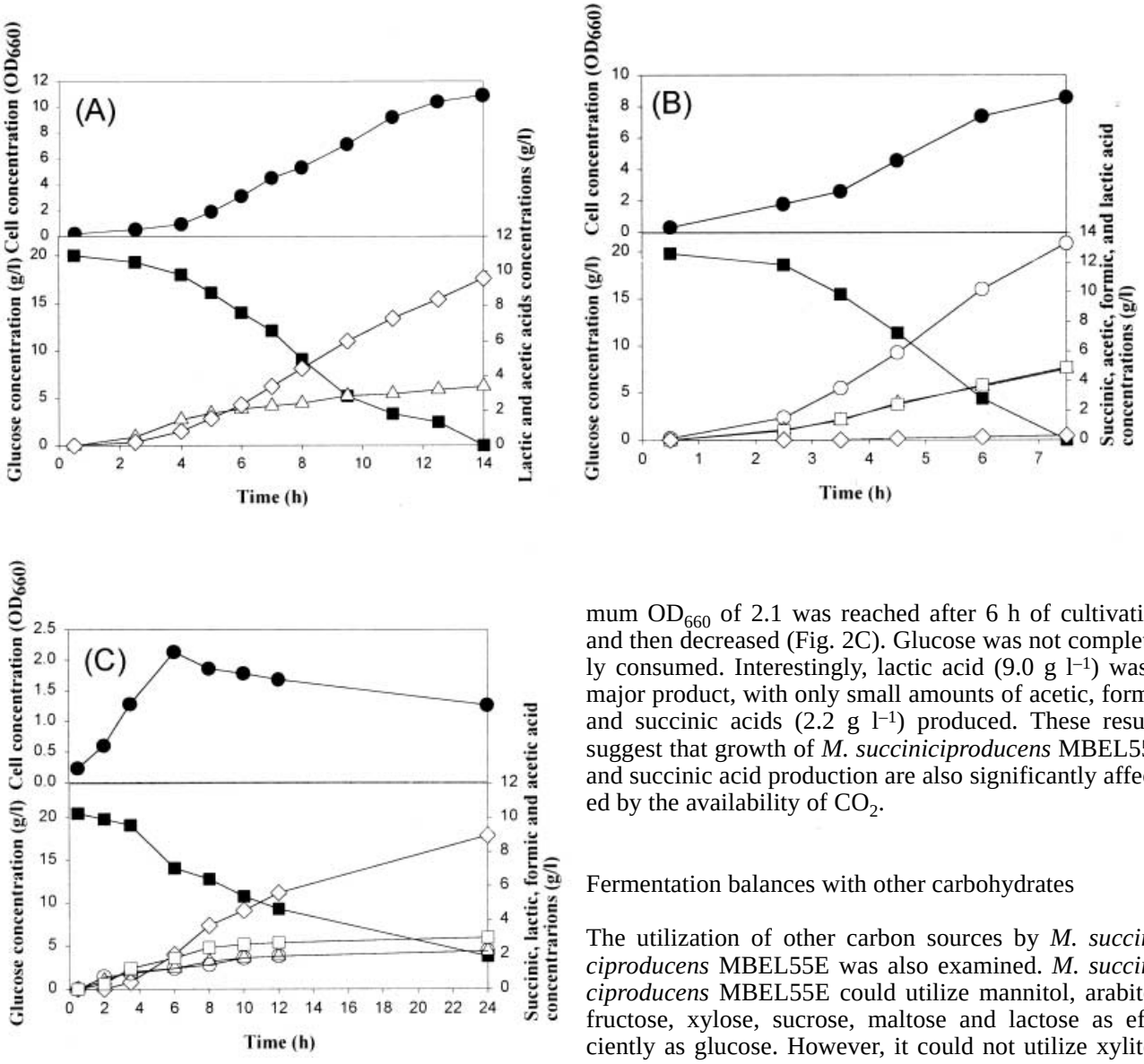

Fig. 2A–C Time profiles of the culture OD_{660} (*black circles*) and concentrations (g l^{-1}) of glucose (*black squares*), succinic acid (*open circles*), acetic acid (*open triangles*), formic acid (*open squares*) and lactic acid (*open diamonds*) during **A** aerobic batch cultures of *M. succiniciproducens* MBEL55E and during anaerobic cultures on glucose under **B** CO_2 and **C** N_2 atmospheres

mosphere, glucose was completely consumed in 7.5 h and a final culture OD_{660} of 8.6 was obtained (Fig. 2B). At the end of cultivation, succinic acid was produced as a major product (14 g l^{-1}), aceticy and formic acids as the second major products, and lactic acid as a minor product. The succinic acid yield was 0.7 g succinic acid/g glucose, and the productivity was 1.87 g succinic acid l^{-1} h^{-1}. Other acids and alcohols were not detected during fermentation on glucose. Changing from CO_2 to N_2 atmosphere had a significant effect on cell growth and end-product formation. Under N_2 atmosphere, cell growth and glucose consumption were poor. A maximum OD_{660} of 2.1 was reached after 6 h of cultivation and then decreased (Fig. 2C). Glucose was not completely consumed. Interestingly, lactic acid (9.0 g l^{-1}) was a major product, with only small amounts of acetic, formic and succinic acids (2.2 g l^{-1}) produced. These results suggest that growth of *M. succiniciproducens* MBEL55E and succinic acid production are also significantly affected by the availability of CO_2.

Fermentation balances with other carbohydrates

The utilization of other carbon sources by *M. succiniciproducens* MBEL55E was also examined. *M. succiniciproducens* MBEL55E could utilize mannitol, arabitol, fructose, xylose, sucrose, maltose and lactose as efficiently as glucose. However, it could not utilize xylitol, inositol, sorbitol, glycerol, xylan or cellulose. The fermentation balances for hexoses and pentoses of different oxidation states were analyzed (Table 2). Since the culture was CO_2 limited when external CO_2 was not provided, we calculated the carbon recovery by assuming that all CO_2 incorporated into succinic acid was provided from outside. Thus, the carbon recovery data presented in Table 2 can be considered as minimum estimates. The end-products formed during fermentation on these carbon sources, except for mannitol, were succinic, formic, acetic and lactic acids, showing the same profiles obtained from fermentation on glucose. When mannitol was used, ethanol was produced. Because mannitol is more reduced than glucose, a more reduced end-product ethanol seems to be produced to adjust the cellular redox balance (Alam and Clark 1989; Van der Werf et al. 1997). However, the exact reason why ethanol was not produced from other reduced carbohydrates, such as arabitol, cannot be explained at this time. Besides the specific end-product profiles, the mannitol-positive and

Table 2 Carbon balances during anaerobic cultivation of *M. succiniciproducens* MBEL55E on various carbohydrates

End-products	Mol product/100 mol carbohydrate				
	Glucose	Mannitol	Arabitol	Fructose	Xylose
Molecular formula	$C_6H_{12}O_6$	$C_6H_{14}O_6$	$C_5H_{12}O_5$	$C_6H_{12}O_6$	$C_5H_{10}O_5$
Cells[a]	71	61	63	56	51
Succinic acid[b]	100	99	106	85	83
Formic acid	91	101	76	97	76
Acetic acid	70	35	51	75	61
Lactic acid	4	7	10	16	28
Ethanol	0	31	0	0	0
C-recovery	1.02	1.02	1.01	1.01	0.97

[a] Carbon compound incorporated into the cells was calculated using the cell composition formula $CH_2O_{0.5}N_{0.21}$ (Samuelov et al. 1991)
[b] Carbon compound in succinic acid was calculated as 3 mol carbon/mol product because of CO_2 fixation during succinic acid formation [succinic acid – (CO_2)] (see assumption in the text; Van der Werf et al. 1997)

trehalose-negative phenotypic characteristics of *M. succiniciproducens* MBEL55E additionally support its classification as a *Mannheimia* species because mannitol and trehalose are key carbon sources used to distinguish the genus *Mannheimia* from other genera within the family *Pasteurellaceae*: *Acinobacillus*, *Pasteurella*, *Haemophilus* and *Lonepinella* (Angen et al. 1999; Osawa et al. 1995).

Discussion

A new succinic acid-producing bacterium, *M. succiniciproducens* MBEL55E, was isolated from bovine rumen, and its growth characteristics and product formation were examined in detail. As expected, the optimum growth conditions of *M. succiniciproducens* MBEL55E are similar to those of bovine rumen: 37–39°C and pH 6–7. This is the first report on the isolation of *Mannheimia* from bovine rumen. Other sources from which *Mannheimia* spp. have been isolated include sheep [*M. haemolytica* NCTC 9380^T (Bisgaard and Mutters 1986)], cattle [*M. granulomatis* ATCC 49244^T (Ribeiro et al. 1989)], ovine lung [*M. glucosida* CCUG 38457^T (Angen et al. 1997)], sheep rumen [*M. ruminalis* CCUG 38470^T (Bisgaard et al. 1986)] and bovine pneumonia [*M. varigena* CCUG 38462^T (Bisgaard and Mutters 1986)]. Based upon phylogenetic placement, it is proposed that strain MBEL55E can be included in the genus *Mannheimia* as a new species.

M. succiniciproducens MBEL55E produced various fermentation products from a wide range of carbon substrates under aerobic and anaerobic conditions. In particular, a large amount of succinic acid was produced under anaerobic conditions in the presence of CO2. Under anaerobic conditions, phosphoenolpyruvate (PEP) can be directed to C4 branch (succinic acid) and/or C2 , C3 branch (ethanol, lactic and acetic acids) pathway by PEP carboxykinase and pyruvate kinase, respectively. PEP carboxykinase is a CO2-fixing enzyme that converts PEP to oxaloacetate, which is further converted to succinic acid as a final compound via several reactions. Pyruvate kinase converts PEP to pyruvate, which is subsequently converted to several end-products including acetic, lactic, and formic acids. Therefore, PEP carboxylation catalyzed by PEP carboxykinase is a key reaction for succinic acid production (Alam and Clark 1989), and the availability of CO2 controls the partition of PEP to various metabolites such as succinic, lactic and acetic acids. This seems to be why overall cellular activities and succinic acid production were significantly repressed under N2.

In conclusion, like other succinic acid-producing bacteria (Caspari and Macy 1983; Samuelov et al. 1991; Van der Werf et al. 1997), *M. succiniciproducens* MBEL55E, isolated from bovine rumen, fermented various carbon sources mainly to succinic acid under CO_2 atmosphere. *M. succiniciproducens* MBEL55E may be suggested as a candidate strain for the production of succinic acid because of its high succinic acid productivity.

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