Appl Microbiol Biotechnol (2012) 94:461–466
DOI 10.1007/s00253-011-3751-z

Isolation, characterization, and quantification of *Clostridium kluyveri* from the bovine rumen

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Received: 15 September 2011 / Revised: 4 November 2011 / Accepted: 21 November 2011 / Published online: 9 December 2011

Abstract A strain of *Clostridium kluyveri* was isolated from the bovine rumen in a medium containing ethanol as an electron donor and acetate and succinate (common products of rumen fermentation) as electron acceptors. The isolate displayed a narrow substrate range but wide temperature and pH ranges atypical of ruminal bacteria and a maximum specific growth rate near the typical liquid dilution rate of the rumen. Quantitative real-time PCR revealed that *C. kluyveri* was widespread among bovine ruminal samples but was present at only very low levels (0.00002% to 0.0002% of bacterial 16S rRNA gene copy number). However, the species was present in much higher levels (0.26% of bacterial 16S rRNA gene copy number) in lucerne silage (but not maize silage) that comprised much of the cows' diet. While *C. kluyveri* may account for several observations regarding ethanol utilization and volatile fatty acid production in the rumen, its population size and growth characteristics suggest that it is not a significant contributor to ruminal metabolism in typical dairy cattle, although it may be a significant contributor to silage fermentation. The ability of unadapted cultures to produce substantial levels (12.8 gL^{-1}) of caproic (hexanoic) acid in vitro suggests that this strain may have potential for industrial production of caproic acid.

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Keywords Butyric acid · Caproic acid · Ethanol · *Clostridium* · Rumen · Silage

Introduction

The microbial fermentation of feedstuffs in the rumen provides volatile fatty acids (VFA) used by the host animal for both energy and biosynthetic reactions. Approximately 90–95% (molar basis) of the ~0.1 to 0.2 M total VFA in rumen contents are the straight chain C_2–C_4 acids (acetic, propionic, and butyric), which are derived primarily from carbohydrate fermentation. Smaller amounts of the C_4–C_5 branched chain VFA (isobutyric, isovaleric, and 2-methylbutyric) are also produced, primarily from amino acid fermentations (Russell 2002). Two other straight chain VFA, valeric (C_5) and caproic (C_6), are also produced in small amounts, but their origins have been less clear. The complex origins of ruminal VFA have been revealed by radiotracer studies that have shown substantial interconversions among the different VFA species (reviewed in Ungerfeld and Kohn 2006). For example, Esdale et al. (1968) reported that 62% or 72% of ruminal butyrate was derived from acetate in a cow fed lucerne hay or maize silage, respectively. The specific taxa responsible for these interconversions have remained obscure.

We hypothesized that valeric and caproic acids may be produced from shorter chain (C_2 to C_4) VFA via microbes using a pathway characteristic of *Clostridium kluyveri* (Bornstein and Barker 1948). This species oxidizes ethanol and reduces condensation products of acetyl-CoA to produce butyric and caproic acids. While *C. kluyveri* is a common inhabitant of freshwater sediments and while it is not known to inhabit the rumen, it was previously suggested by Ørskov et al. (1967) as a possible agent of ruminal

caproate production. We report here the isolation and characterization of a strain of *C. kluyveri* from the bovine rumen and the quantification of this species across individual cows by quantitative PCR that suggests a minimal role in ruminal metabolism. We also describe the effects of incubation conditions on growth and end product formation, which would impact the use of this strain as an agent of industrial caproic acid production.

Materials and methods

Culture methods Enrichments and pure cultures were routinely grown in ethanol/acetate/succinate/yeast extract (EASY) medium. This medium consisted of a basal medium prepared under CO_2 prior to autoclaving, followed by addition of N_2-gassed, sterile stock solutions of miscellaneous medium components. The basal medium consisted of the following (in 875 mL of deionized water): 0.90 g NaCl, 0.90 g $(NH_4)_2SO_4$, 0.085 g $MgCl_2{\cdot}6H_2O$, 0.066 g $CaCl_2{\cdot}2H_2O$, 0.028 g $MnCl_2{\cdot}4H_2O$, 0.020 g $FeSO_4{\cdot}7H_2O$, 0.009 g $ZnCl_2$, 0.002 g $CoCl_2{\cdot}6H_2O$, and 0.002 g resazurin. This medium (8.0 mL) was dispensed under CO_2 gassing into Balch tubes (Balch and Wolfe 1976), and the tubes were sealed with flanged butyl stoppers and aluminum crimp seals and then were autoclaved. The following components then were added using sterile hypodermic syringes: KH_2PO_4 (0.4 mL of 22.5 g L^{-1}), Na_2CO_3 (0.4 mL of 80 gL^{-1}), cysteine HCl (0.5 mL of 25 gL^{-1}), ethanol (0.15 mL of 500 mL L^{-1}), Na acetate (0.12 mL of 2.5 M), Na succinate (0.4 mL of 0.5 M), and yeast extract (0.10 mL of 100 gL^{-1}). The complete medium had an initial pH of 6.8.

Ruminal samples were collected from lactating and nonlactating Holstein cows, each surgically fitted with a permanent ruminal fistula. The cows were fed a total mixed ration that contained primarily maize (*Zea mays*) and lucerne (*Medicago sativa*) silages (29.5% and 27.7% of dietary dry matter, respectively), along with ground dry corn, soybean meal, and supplemental vitamins and minerals. Enrichment cultures were prepared by inoculating 0.3 mL of ruminal digesta (filtered through four layers of cheesecloth into a CO_2-gassed flask) into tubes of EASY medium supplemented with 0.025 mM 2-bromoethanesulfonic acid and chloroform (to 0.01 mL L^{-1}) to inhibit methanogens; incubations were conducted at 39°C. Successful enrichment cultures were obtained from five cows. After ten successive transfers (0.3 mL/tube, made at 3- to 5-day intervals), the most active enrichment culture was selected for pure culture isolation, which was accomplished by dilution into roll tubes of EASY medium supplemented with Bacto-agar (25 gL^{-1}) and picking of single colonies after 7-day incubation into liquid EASY medium. The culture has been deposited for distribution in the NRRL culture collection at the National Center for Agricultural Utilization, Agricultural Research Service, US Department of Agriculture, as strain NRRL B-59667.

16S rRNA gene sequencing DNA was isolated from cell pellets as described previously (Stevenson and Weimer 2007), and the 16S rRNA genes were amplified using bacterial primers B8F (5′-AGAGTTTGATCMTGGCTCAG-3′) and U1406R (5′-TTCCGGTTGATCCYGCCGAA-3′) (Heijs et al. 2007). The amplicons were ligated into a pGEMT-EZ vector (Promega) and transformed into *Escherichia coli* strain JM109. Recombinants were identified by blue/white screening, and the 16S rRNA gene was sequenced using M13 universal primers located on the vector. Sequencing was performed by the University of Wisconsin Biotechnology Center.

Analyses Cell growth in liquid culture was determined turbidimetrically at 525 nm in a Spectronic 21 (Bausch and Lomb) colorimeter. Culture supernatants were analyzed for alcohols and organic acids by the HPLC method of Kenealy et al. (1995). H_2 was determined by gas chromatography after collection of culture headspace samples (0.40 cm^3) into 1 mL glass syringes fitted with Pressure-Lok fittings (Supelco). Samples were injected into a Shimadzu 8A gas chromatograph fitted with a 1.9-m×3.2-mm (6-ft×1/8-in) stainless steel column containing Carbosieve S-II (Supelco) and with a thermal conductivity detector operated at a current of 120 mA. Helium (100 cm^3/min) was used as carrier gas, and injector, oven, and detector temperatures were 120°C, 70°C, and 120°C, respectively.

Quantitative (real-time) PCR (qPCR), including relative quantification against total bacterial DNA, was conducted as described previously (Stevenson and Weimer 2007), with two modifications: The primer set used was CloKly1F (5′-GAGGAGCAAATCTCAAAAACTGC-3′) and CloKly1R (5′-CCTCCTTGGTTAGACTACGGACTT-3′), and the combined annealing/extension stage within each cycle was carried out at 59°C for 90 s.

Results

Cell and colony morphology Well-isolated colonies in agar roll tubes were off-white, circular, smooth, soft, and up to 1 mm in diameter after 4-day incubation at 39°C. Four separate colonies were picked, and all showed similar cell morphologies and growth behavior in EASY medium. A single isolate, strain 3231B, was selected for further examination. In liquid culture, cells of this strain were slightly curved rods, 10–15 μm in length and ~1.5 μm in width. In older cultures, cells had a hook-like appearance at one or both ends, sometimes with the appearance of phase-bright endospores. Cultures typically reached maximum optical

density in batch culture within ~48 h and showed a gradual loss of turbidity thereafter. Under the growth conditions used here, only a small fraction of cells underwent sporulation.

16S rRNA gene sequence The nearly complete (1,370 nt) 16S rRNA gene for strain 3231B had a sequence identical to that of the type strain of *C. kluyveri* (ATCC 8527/DSM555/NCIMB10680). The sequence for strain 3231B has been deposited in GenBank under accession number JN592512.

Growth characteristics In EASY medium, strain 3231B grew over a fairly broad temperature range, with an optimum near 39°C (Fig. 1a). At 39°C, growth was observed the entire range of initial pH values tested (pH 4.88 to 9.20), with maximum growth rate observed at pH 7.6 (Fig. 1b). A defined vitamin mixture (Schaefer et al. 1980) was unable to support growth on ethanol/acetate/succinate in the absence of added yeast extract. In media adequately prepared by sparging with CO_2 and maintained under a CO_2 gas phase, a chemical reducing agent was not required to permit growth but substantially shortened the culture lag phase; thus, for the experiments described below, cysteine HCl (final concentration 1.25 gL^{-1}) was used as reducing agent, and incubations were conducted at 39°C. No growth was observed in media containing yeast extract and the cysteine reducing agent as sole organic additions.

Substrate range As indicated in Table 1, among electron donors tested with acetate as electron acceptor, only ethanol and *n*-propanol supported growth. Electron acceptors that supported growth (with ethanol as electron donor) included acetate, propionate, butyrate, succinate, and malonate. For most electron donor/acceptor combinations, caproate was the most abundant fermentation product. Substantial amounts of valerate were formed during growth on ethanol plus propionate and on *n*-propanol plus acetate. Under the latter substrate combination, significant amounts of propionate were also detected, suggesting oxidation of *n*-propanol to propionate. In addition to these products, all cultures that displayed growth also produced H_2.

Effect of substrate concentration on growth and product formation Figure 2 summarizes the conversion of ethanol and acetate to butyrate and caproate by batch cultures after 72-h incubation. At a fixed initial acetate concentration of 120 mM, strain 3231B grew most rapidly (μ=0.11 h^{-1}) within the range 200–300 mM ethanol, although good growth was observed even at 700 mM ethanol. The maximum amount of ethanol consumed was 500 mM, resulting in the accumulation of 110 mM caproate. By contrast, butyrate reached ~30 mM regardless of initial ethanol concentration. At low initial ethanol concentrations (≤200 mM), caproate molar yields averaged 0.17 mol/mol ethanol consumed, while at higher initial ethanol concentration, this value increased to an average of 0.22.

At a fixed initial ethanol concentration of 350 mM, strain 3231B grew most rapidly (μ=0.08 h^{-1}) at an acetate concentration of only 25 mM, with a nearly linearly decreasing growth rate up to the highest acetate level tested (240 mM). Under these conditions, the maximum amount of acetate converted was 110 mM, and the yield of caproate declined from 0.29 to 0.16 mol caproate per mole acetate consumed as initial acetate concentration increased to 240 mM.

Figure 3a summarizes the conversion of ethanol and succinate by batch cultures after 72 h incubation. Under the conditions tested (ethanol as electron donor at an initial concentration of 350 mM), growth rate was independent of succinate concentration within the range tested (25 to 240 mM). Fermentation end products included acetate, butyrate, and caproate. When acetate and succinate were available simultaneously with a high concentration (650 mM) of ethanol as electron donor (Fig. 3b), succinate was consumed while acetate concentration remained relatively constant,

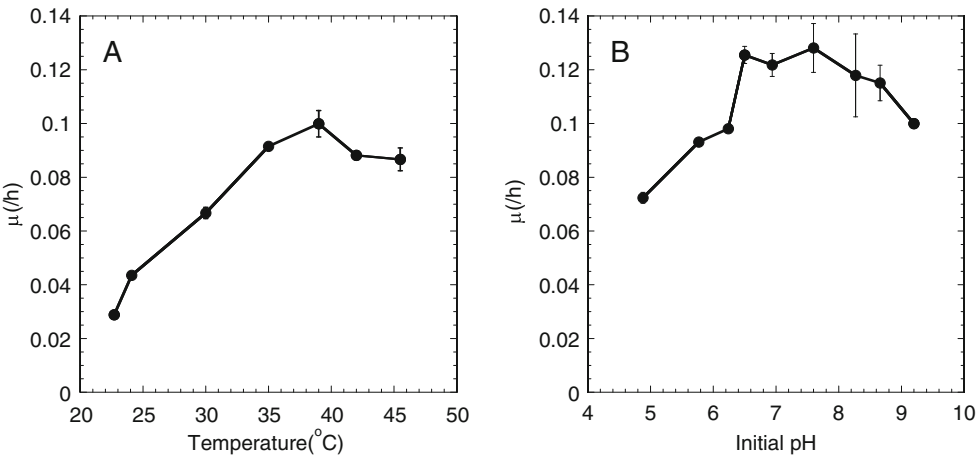

Fig. 1 Effect of growth temperature and culture pH on growth of *C. kluyveri* 3231B. **a** Effect of temperature at an initial culture pH of 6.8. **b** Effect of pH at an incubation temperature of 39°C

Table 1 Substrate utilization range of *C. kluyveri* 3231B

	μ_{max} (h^{-1})	OD_{525} increase	Fermentation products (mmol L^{-1})				
			Propionate	Butyrate	Valerate	Caproate	H_2
Electron donors[a]							
Ethanol	0.079	0.360	–	2.2	–	42.1	21.5
n-Propanol	0.069	0.269	42.4	11.6	18.2	10.1	14.6
Electron acceptors[b]							
Acetate	0.092	0.293	–	1.6	–	36.6	39.7
Propionate	0.037	0.107	–	–	5.9	6.1	18.3
Butyrate	0.029	0.119	–	–	–	36.2	26.5
Malonate	0.064	0.115	–	–	–	13.5	20.2
Succinate	0.056	0.154	–	8.4	–	30.4	23.2

[a] Tested at 130 mM concentration with acetate (30 mM) as electron acceptor. Potential electron donors not utilized included methanol, 2-propanol, *n*-butanol, and DL-lactate

[b] Tested at 25 mM concentration with excess ethanol (350 mM) as electron donor. Potential electron acceptors not utilized included crotonate, isobutyrate, *n*-valerate, isovalerate, 2-methylbutyrate, oxalate, fumarate, maleate, malate, glutarate, and pyruvate

likely due to production of acetate from succinate cleavage and ethanol oxidation (Kenealy and Wasalefsky 1985) with a simultaneous reduction of acetate to butyrate and caproate.

Abundance in ruminal and silage samples qPCR was used to assess the relative abundance of *C. kluyveri* in the rumina of 11 cows, including cow 3231 from which the strain was isolated. The species was detected in all 11 cows tested (including cow 3231). However, the populations were very low (contributing 0.00002% to 0.00020% of the total bacterial 16S rRNA gene copy number). Parallel assays indicated that *C. kluyveri* was present in similarly low quantities in maize silage (the major feed ingredient of the cows' diet) but was much more abundant in lucerne silage (0.26% of bacterial 16S rRNA gene copy number) which was also a major component of the ration.

Discussion

The present study provides the first report of an isolation of *C. kluyveri* from the rumen and its quantification in ruminal contents. The presence of *C. kluyveri* in the rumen, originally proposed by Ørskov et al. (1967), could account for several disparate observations regarding ruminal fermentation end products. Ethanol has been identified as a transient component of rumen fluid both in vitro and in vivo, particularly when diets rich in grains are fed. Allison et al. (1964) reported that 12 of 12 sheep fed cracked wheat had detectable levels of ethanol in the rumen, and in one sheep, ethanol concentrations reached 33 mM (although this sheep died a few hours later). Dietary induction of ethanol accumulation has been reported to increase butyrate and caproate concentrations in the rumen, with the latter reaching up to

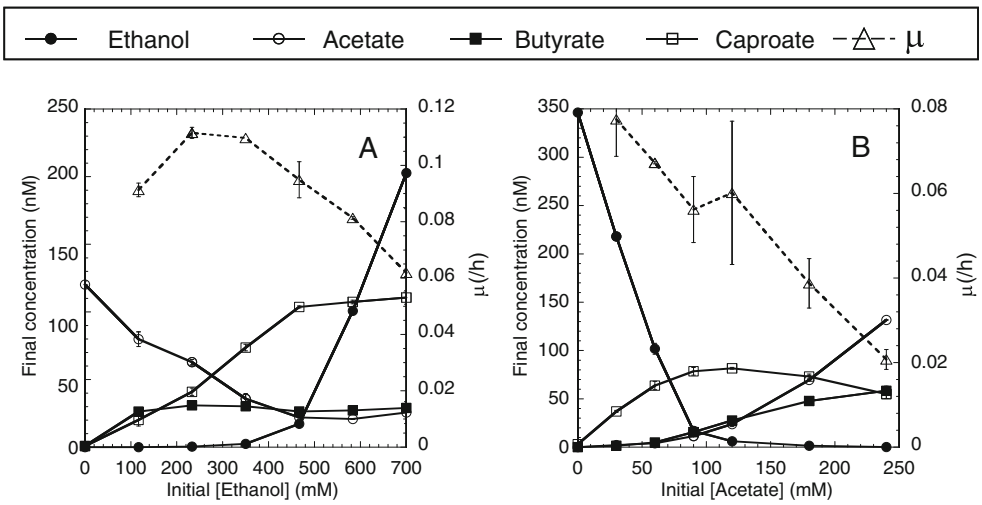

Fig. 2 Effect of ethanol and acetate concentrations on substrate consumption, product formation, and growth rate by pure cultures of ruminal *C. kluyveri* strain 3231B after 72-h incubation. **a** Effect of varying initial ethanol concentration at a fixed initial acetate concentration of 120 mM. **b** Effect of varying initial acetate concentration at a fixed initial ethanol concentration of 350 mM

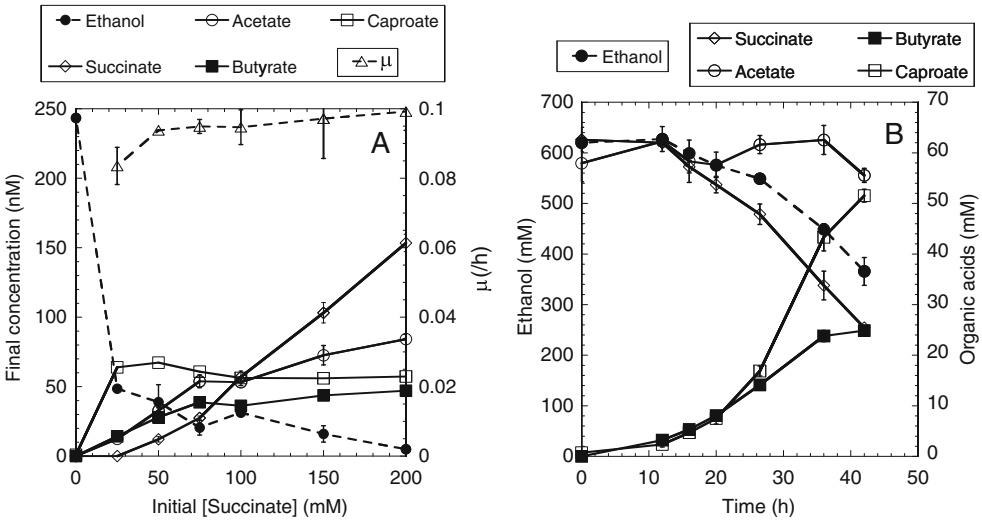

Fig. 3 Metabolism of succinate by *C. kluyveri* strain 3231B. **a** Effect of succinate concentration on substrate consumption, product formation, and growth rate after 72-h incubation in media containing 350 mM ethanol as electron donor. **b** Utilization of succinate in the presence of acetate. Succinate consumption preceded net consumption of acetate, probably due to simultaneous conversion of succinate to acetate while acetate was being consumed

20 mM (Ørskov et al. 1967). In addition, direct infusion of ethanol into the rumen has been shown to increase the ruminal proportions of valerate and caproate (Pradhan and Hemken 1970). Moomaw and Hungate (1963) reported that radiolabeled ethanol was converted to several different VFA (primarily acetate, but also butyrate and caproate) by mixed ruminal microbes in vitro. Taken together, these data suggested that ruminal ethanol accumulation precedes formation of longer chain (C_4 to C_6) VFA, as would be predicted from the pathway of butyrate and caproate synthesis from ethanol and acetate (or ethanol and succinate) known to operate in *C. kluyveri* (Kenealy et al. 1995).

Despite its ruminal origin and the ease with which its presence in ruminal metabolism may be rationalized, several observations suggest that *C. kluyveri* is not a significant component of the ruminal microbial community under normal feeding conditions. Quantitative PCR revealed that *C. kluyveri* represented only 0.00002% to 0.0002% of the 16S rRNA gene copy number in the rumens of all 11 cows tested (including the source cow from which the strain was isolated). These values, while very low, are similar to those reported for several "classical" ruminal bacteria such as *Ruminococcus albus*, *Streptococcus bovis*, and *Megasphaera elsdenii* (Stevenson and Weimer 2007). However, the ruminal *C. kluyveri* isolate displayed a variety of physiological characteristics atypical of predominant ruminal bacteria. While most ruminal bacteria are stenothermal, the isolate displayed an unexpectedly broad growth temperature range (22°C to 45°C), well outside the narrow operating temperature range of the rumen. The strain also grew over the entire pH range tested (4.8 to 9.2), well beyond the typical ruminal pH range of 5 to 7 and beyond the pH range for growth of most ruminal bacteria in pure culture. The maximum growth rate of the isolate (~0.13 h^{-1}) is similar to the fluid dilution rate of the rumen, but at the low concentrations of ethanol and high concentrations of acetate typical of the rumen, growth rate of the strain is substantially lower (Fig. 2), suggesting that the strain may have difficulty in avoiding washout from the rumen. A minimal role of *C. kluyveri* in the rumen is further suggested by the data of Raun and Kristensen (2011). These workers have reported that dairy cows fed either ethanol or *n*-propanol at 14 g/kg diet dry matter displayed differences in the ruminal proportions of expected oxidation products (higher acetate in ethanol-fed cows and higher propionate in propanol-fed cows), but there were no differences in molar proportions of butyrate, valerate, or caproate (the expected products of *C. kluyveri* metabolism). These observations are consistent with those of Connor et al. (2010) who reported that feeding corn grain to weaned calves (which promotes subacute ruminal acidosis and ruminal ethanol production) elevates expression of alcohol dehydrogenase by ruminal epithelial cells, suggesting a direct host contribution to alcohol metabolism.

We suspected that one potential source of *C. kluyveri* in the rumen could be silage, which was a major component of the total mixed ration fed cow 3231, from which the strain was isolated. Most silages contain significant quantities of the *C. kluyveri* substrates ethanol, acetate, and succinate, sometimes at several per cent each on a dry matter basis (Makoni et al. 1997). Silages also exhibit broader temperature and pH ranges than the rumen, along with prolonged static incubations that would not penalize species whose μ_{max} approximates the fluid dilution rate of the rumen. qPCR assays indicated that *C. kluyveri* was present at moderate levels in lucerne silage, but not in maize silage. The data suggest that *C. kluyveri* may be present and active in silage and thus may contribute to the undesirable formation of butyric acid observed in some silages. Moreover, *C. kluyveri* is a likely agent for production of caproic acid that has been occasionally detected in silages (Jones and Kay 1976; Ohyama et al. 1977).

The ruminal strain 3231B displayed some similarities in substrate range to described *C. kluyveri* strains. The isolate had a narrow range of electron donors (ethanol or *n*-propanol, with the latter supporting growth nearly as well as the former) and an only slightly wider range of electron acceptors (with acetate providing the best growth). This strain was unable to use crotonate (2-butenoate), which is used by most other *C. kluyveri* strains (Bader et al. 1980; Kenealy and Wasalefsky 1985).

The literature contains relatively little information on the effect of substrate concentration on growth of *C. kluyveri*. The results reported here suggest that strain 3231B grew fastest at ethanol concentrations of ~200 mM, well in excess of the concentrations found in most anaerobic habitats. By contrast, the strain exhibited a decreased growth rate when acetate was >50 mM, a concentration well below that in the rumen (where it often exceeds 100 mM in the liquid phase). This sensitivity to acetate may reflect VFA anion toxicity, which has been commonly observed in many acidogenic strict anaerobes (Russell 1992).

Regardless of its ultimate source (rumen or silage), *C. kluyveri* 3231B may have utility as an agent of industrial caproic acid production. The strain produced up to 110 mM (12.8 gL^{-1}) of caproate without adaptation, due in part to an ability to grow at the low pH values resulting from extensive caproic acid production. By comparison, an unclassified saccharolytic isolates from sludge, *Clostridium* sp. strain BS-1 (most closely related phylogenetically to *Clostridium sporosphaeroides*), has been reported to ferment a mixture of D-galactitol and Na acetate to a maximum concentration of 3 g caproic acid L^{-1}; further, caproic acid production was limited by its toxicity to this strain (Jeon et al. 2010). Currently, industrial use of caproic acid is modest, primarily as a precursor for synthesis of flavorants. Interestingly, caproic acid also has potential utility in promoting aerobic stability in silages when added at 0.12 g(kg silage DM)$^{-1}$ prior to ensiling (Ohyama et al. 1977). Perhaps more importantly in terms of commercial potential, caproic acid may also be useful as a feedstock for production of liquid alkane fuels using Kolbe electrochemistry (Levy et al. 1983).

Acknowledgments We thank Christine Odt for technical assistance, Nancy Betzold and the USDFRC barn crew for animal handling, and Richard Muck for helpful discussions. This research was supported by the USDA Agricultural Research Service thorough CRIS project 3655-31000-06-00D.

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