The enrichment of a ruminal bacterium (*Megasphaera elsdenii* YJ-4) that produces the trans-10, cis-12 isomer of conjugated linoleic acid

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Y.J. KIM, R.H. LIU, J.L. RYCHLIK AND J.B. RUSSELL. 2002. Aims: To isolate predominant ruminal bacteria that produce trans-10, cis-12 conjugated linoleic acid (CLA) from linoleic acid (LA).

Methods and Results: Mixed bacteria from ruminal contents of a cow fed grain were enriched with DL-lactate and trypticase. They produced more trans-10, cis-12 CLA than those that were not enriched (7 vs 2 μg mg protein⁻¹, P < 0.05). Enrichments had an abundance of large cocci that produced trans-10, cis-12 CLA from LA. Strain YJ-4 produced the most trans-10, cis-12 CLA (approx. 7 μg mg protein⁻¹) and 16S rDNA sequencing indicated that YJ-4 was a strain of *Megasphaera elsdenii*. *Megasphaera elsdenii* T81 produced approx. 4 μg trans-10, cis-12 CLA mg protein⁻¹ while strains B159, AW106 and JL1 produced < 0·5 μg mg protein⁻¹. The trans-10, cis-12 CLA production of YJ-4 was first order with respect to cell concentration (0–800 μg protein ml⁻¹), but kinetics were not first order with respect to substrate concentration.

Conclusions: Some *M. elsdenii* strains produce significant amounts of trans-10, cis-12 CLA.

Significance and Impact of the Study: Trans-10, cis-12 CLA appears to cause milk fat depression in cattle fed diets supplemented with grain and polyunsaturated fatty acids, but predominant ruminal bacteria that produced trans-10, cis-12 CLA from LA had not previously been isolated.

INTRODUCTION

Ruminant animals usually consume a low fat diet that is rich in polyunsaturated fatty acids and most of these fatty acids are saturated via a process known as biohydrogenation (Dawson and Kemp 1970). Polyunsaturated fatty acids are more toxic than saturated fatty acids and biohydrogenation appears to be a detoxification mechanism that protects ruminal bacteria from unsaturated fatty acids (Henderson 1973). Gram-positive ruminal bacteria are more sensitive to polyunsaturated fatty acids than Gram-negative species, but even Gram-negative bacteria can be inhibited if the concentration is high enough (Henderson 1973).

Early work indicated that small amounts of conjugated linoleic acid (CLA) could accumulate in ruminant milk and meat (Riel 1963) and later work showed that CLA was an intermediate in biohydrogenation (Kepler *et al.* 1966). Conjugated linoleic acid prevents carcinogenesis, cardiovascular diseases and obesity (Ha *et al.* 1987; Lee *et al.* 1994; Belury *et al.* 1996). There are 15 isomers of CLA, but ruminal bacteria only produce significant amounts of the cis-9, trans-11 and trans-10, cis-12 isomers (Griinari and Bauman 1999). The ruminal bacterium *Butyrivibrio fibrisolvens* A38 has been used as a model for cis-9, trans-11 CLA production, but this bacterium does not produce the trans-10, cis-12 isomer (Kepler and Tove 1967; Kim *et al.* 2000).

In lactating dairy cattle, the trans-10, cis-12 isomer of CLA appears to cause milk fat depression and this condition...
is aggravated by polyunsaturated fatty acids and grain-based diets that cause a decrease in ruminal pH (Griiniari and Bauman 1999). Propionibacterium acnes and P. freudereichii produce \textit{trans}-10, \textit{cis}-12 CLA from linoleic acid (LA), but these species are not predominant ruminal bacteria (Verhulst \textit{et al.} 1987; Jiang \textit{et al.} 1998). The following experiments examined the CLA production of mixed ruminal bacteria. It was hoped that, by adding enrichment substrates and measuring CLA production, a predominant ruminal bacterium that could produce the \textit{trans}-10, \textit{cis}-12 CLA isomer might be identified.

**MATERIALS AND METHODS**

**Cattle**

Non-lactating ruminally fistulated dairy cows were fed diets (approx. 9 kg d\(^{-1}\)) consisting of timothy hay for a period of at least 3 weeks prior to rumen sampling. Surgical procedures were approved by the Cornell Institutional Animal Care and Use Committee (Protocol 95-1-00). The timothy hay had 14% crude protein and 40% neutral detergent fibre. The cattle were then switched to a 90% grain (cracked corn)/10% timothy hay ration and acclimated to this diet for 3 weeks.

**Mixed ruminal bacteria**

Ruminal fluid was removed with a suction device via a plastic pipe (70 cm long, 25 cm diameter) with holes (7 mm). The holes in the pipe filtered the ruminal fluid so that it would not contain large feed particles. The pH of the ruminal fluid was measured and the fluid transported to the laboratory. The ruminal fluid was centrifuged at low speed to remove large feed particles (150 g, 2 min, 22°C). The mixed bacterial supernatant fluid was centrifuged again at higher speed (1000 g, 5 min, 22°C) to separate particle-attached ruminal bacteria from free-floating bacteria. The particle-attached ruminal bacteria were resuspended in a similar volume of clarified, autoclaved (121°C, 20 min) ruminal fluid. Both bacterial preparations (particle-attached and free-floating ruminal bacteria) were incubated at 39°C in 150 × 18 mm tubes (capped with butyl rubber stoppers and aluminium seals) that had been flushed with O\(_2\)-free CO\(_2\).

**Enrichment, isolation and growth of pure cultures**

Mixed ruminal bacteria (free-floating from a cow fed the 90% grain ration) were enriched in a basal medium that was prepared anaerobically under O\(_2\)-free CO\(_2\) (mg l\(^{-1}\)): K\(_2\)HPO\(_4\), 292; KH\(_2\)PO\(_4\), 292; (NH\(_4\))\(_2\)SO\(_4\), 480; NaCl, 480; MgSO\(_4\),7H\(_2\)O, 100; CaCl\(_2\),2H\(_2\)O, 64; Na\(_2\)CO\(_3\), 4000; cysteine hydrochloride, 600; vitamins and micromineral mixture (Cotta and Russell 1982). Glucose, lactic acid or trypticase (BBL Microbiology Systems, Cockeysville, MD, USA) were prepared as separate solutions and added after autoclaving. Growth was estimated from the increase in optical density (600 nm, 1 cm cuvettes). Bacterial colonies were isolated from basal medium agar plates (2 mg lactate and 15 mg trypticase ml\(^{-1}\)) that had been streaked with the enrichment and incubated anaerobically for 2 d (39°C). \textit{Megasphaera elseni} T81, B159 and JL1 were from the authors’ own culture collection. \textit{Megasphaera elseni} AW106 was obtained from Dr Forster (Lethbridge Research Centre, Lethbridge, Alberta, Canada).

**Fatty acid preparation**

The LA stock solution (100 mg LA and 200 mg bovine serum albumin ml\(^{-1}\); Sigma Chemical, St. Louis, MO, USA) was filter sterilized (pore size 0.22 μm) and added to mixed ruminal bacteria or stationary phase pure cultures to achieve a final concentration of 20 μg LA ml\(^{-1}\). The bovine serum albumin ensured that the LA remained in solution.

**Fatty acid analyses**

Bacterial cell suspensions (typically 10 ml) were extracted with a mixture of organic solvents (2 ml; one part hexane, three parts isopropanol and one part acetone; 1 min using a vortex mixer). The suspensions were centrifuged (1000 g, 3 min, 20°C) and the solvent layer (top) flushed with nitrogen until dry. The fatty acids were dissolved in toluene (1 ml) and methylated as previously described by Kim and Liu (1999). Fatty acid methyl esters were separated by a Supelcowax-10 fused silica capillary column (60 m × 0.53 mm, 0.5 μm film thickness; Supelco, Bellefonte, PA, USA) using a gas chromatograph (HP5890; Hewlett Packard, Palo Alto, CA, USA) equipped with a flame ionization detector and integrator (HP 3392; Hewlett Packard). The conditions were: helium flow, 24 ml min\(^{-1}\); injector, 200°C; detector, 250°C; column chamber temperature, initially 40°C (5 min); column temperature, increased to 220°C at 20°C min\(^{-1}\) and held for 30 min. The sample (1 μl), containing 0.5–5 μg LA or CLA, was injected into the column in a splitless mode. Heptadecanoic acid (C\(_{17:0}\)) was used as an internal standard. \textit{cis}-9,\textit{trans}-11 octadecadienoic acid and \textit{trans}-10, \textit{cis}-12 octadecadienoic acid were used as a CLA standard (>98% purity). The recovery of CLA and C\(_{17:0}\) was 83% and 80%, respectively. A known standard mixture of fatty acids (Kim \textit{et al.} 2000; Sigma) was used to identify other fatty acids. This protocol was able to separate eight isomers of CLA, but it could not differentiate \textit{cis}, \textit{trans vs trans}, \textit{cis} configurations in the same position.
**Phylogenetic analysis**

Chromosomal DNA from strain YJ-4 was purified using a Fast DNA Spin Kit (Bio101; Carlsbad, CA, USA). The 16S rDNA region was amplified using the 27F and 1492R primers (Lane 1991). The polymerase chain reaction products were cloned using the pGEM-T Vector System (Promega, Madison, WI, USA) and clones sequenced at the Cornell University Biotechnology Center (Ithaca, NY, USA) using two primers based on the plasmid cloning site and three primers based on internal conserved regions (Lane 1991). Sequences were assembled and edited using the SeqMan component of the Lasergene package (DNASTAR, Madison, WI, USA) and aligned to known *Megasphaera* 16S rRNA sequences using ClustalX (Thompson et al. 1997). Similarity to other *Megasphaera* sequences was calculated using the Similarity Matrix (version 1.1) service of the Ribosomal Database Project (Maidak et al. 2000). Sequences were checked for chimeras using the Chimera Check service (version 2.7) of the Ribosomal Database Project (Maidak et al. 2000). Phylogenetic trees were constructed using the neighbour-joining method included in the PHYLIP software (Felsenstein 1993) and a radial tree was constructed using TreeView PPC (Page 1996). The 16S rDNA sequences of *M. elsdenii* JL1, AW106, T81 and YJ-4 have been deposited in GenBank under accession nos AY038993, AY038994, AY038995 and AY038996, respectively.

**Other analyses**

Protein from NaOH-hydrolysed cells (0–2 N NaOH, 100 °C, 15 min) was assayed by the method of Lowry et al. (1951).

**Statistical analyses and design**

All incubations were performed in triplicate. Mean values and s.e. of the mean are shown. Student’s *t* test was used to determine statistical significance.

**RESULTS**

**Mixed ruminal bacteria**

When cattle were fed either hay- or grain-based diets, the ruminal pH values were 6.8 ± 0.2 and 6.0 ± 0.3, respectively. Preliminary experiments indicated that free-floating bacteria produced as much or more trans-10, cis-12 CLA as particle-attached ruminal bacteria and the free-floating bacteria from cattle fed the grain-based diet appeared to produce more trans-10, cis-12 CLA than mixed ruminal bacteria from cattle fed only hay (data not shown). However, these estimates of trans-10, cis-12 CLA production were confounded by differences in cell density and the impact of cell density vs pH could not be resolved.

When free-floating ruminal bacteria were harvested by centrifugation, resuspended in basal medium (pH 6.8), adjusted to a cell concentration of 200 µg protein ml⁻¹ and incubated with 20 µg ml⁻¹ LA, the mixed ruminal bacteria from cattle fed grain produced more trans-10, cis-12 CLA than bacteria from cattle fed hay (20 vs 0.8 µg mg protein⁻¹; *P* < 0.05). Free-floating ruminal bacteria, from cattle fed grain, that were given glucose (2 mg ml⁻¹) or trypticase (15 mg ml⁻¹) grew, but neither of these substrates caused an increase in specific trans-10, cis-12 CLA production (*P* > 0.05) (Fig. 1). However, lactate or lactate and trypticase additions caused an increase in specific trans-10, cis-12 CLA production (*P* < 0.05).

![Fig. 1 The trans-10, cis-12 conjugated linoleic acid (CLA) production of free-floating bacteria from cattle fed a grain-based diet. Mixed cultures were incubated for 24 h with substrates (glucose or lactate and trypticase at 2 and 15 mg ml⁻¹, respectively) to enrich different types of bacteria. The stationary phase bacteria were then incubated with linoleic acid (20 µg ml⁻¹, 39 °C, 30 min) and the trans-10, cis-12 CLA production determined. A control having no addition is also shown. In each case, the ruminal fluid was obtained from three cows and the error bars show the S.E. of the mean.](image)
Lactate enrichments and isolation

When the lactate and trypticase enrichment was transferred three times (10% inoculum, pH 6.8), the cells retained their ability to produce trans-10, cis-12 CLA and large cocci predominated. When agar plates containing lactate and trypticase were streaked and incubated for 2 d (39 °C), large colonies were observed. Several colonies were picked, transferred to basal broth containing lactate and trypticase and assayed for their ability to produce trans-10, cis-12 CLA. The isolate that produced the most trans-10, cis-12 CLA was designated as YJ-4.

Characterization of *Megasphaera elsdenii* YJ-4

Based on its morphology, monensin resistance (it grew with 10 μmol L⁻¹) and ability to use glucose, lactate or trypticase as an energy source for growth, YJ-4 was presumptively identified as *M. elsdenii*. 16S rDNA sequencing indicated that YJ-4 was 99.6% and 99.3% similar to *M. elsdenii* La03 and B159, respectively (Fig. 2). *Megasphaera elsdenii* S2 and S3 were less closely related, but the similarity coefficients were 98.6%.

Kinetics of trans-10, cis-12 conjugated linoleic acid production

*Megasphaera elsdenii* B159, AW106 and JL1 cells that had been grown on lactate produced < 0.5 μg trans-10, cis-12 CLA mg protein⁻¹, but *M. elsdenii* T81 produced nearly as much trans-10, cis-12 CLA from LA as YJ-4 (Fig. 3). *Megasphaera elsdenii* YJ-4 produced trans-10, cis-12 CLA very rapidly and it was maximal after only 2 min (Fig. 4). Trans-10, cis-12 CLA production was first order with respect to cell concentration (0–800 μg protein ml⁻¹), but kinetics were not first order with respect to substrate concentration. If the LA concentration was increased from 20 to 200 μg ml⁻¹, there was only a 30% increase in trans-10, cis-12 CLA (data not shown).

DISCUSSION

High producing dairy cattle are frequently fed large amounts of grain to increase ruminal fermentation rate and energy availability, but it has long been recognized that grain feeding can have a negative impact on milk fat percentage and yield (Davis and Brown 1970). Milk fat depression was, in many cases, correlated with an increased ratio of ruminal propionate to acetate and this observation led ruminant nutritionists to formulate a variety of hypotheses and questions. Was starch bypassing the rumen, being absorbed from the intestines as glucose, causing an increase in insulin and thus diverting fatty acids from the mammary gland to adipose tissue (Sutton 1989)? Was propionate itself causing an increase in glucose and insulin (Sutton 1989)? Were microbially derived vitamin B₁₂ analogues preventing normal propionate metabolism by the animal (Sutton and Elliot 1972)? Despite more than two decades of work, definitive answers were not forthcoming and the condition known as milk fat depression was still a problem.

Early work indicated that dietary oil supplements could accentuate the milk fat depression of cattle fed low fibre rations but abomasal infusions of a similar amount of oil did not affect milk fat yield (Davis and Brown 1970). These results suggested that milk fat depression was associated with changes in ruminal metabolism rather than a direct effect of the oils on the mammary gland per se. Davis and
Brown (1970) noted that oil supplements caused an increase in trans-C18 : 1 and other geometric and positional isomers of unsaturated fatty acids leaving the rumen and concluded that these ‘unacceptable substrates’ might be rejected by the mammary gland. Gaynor et al. (1994) examined the effect of trans vs cis-C18 : 1 on the milk fat yield of dairy cattle and noted that neither fatty acid inhibited stearoyl-CoA desaturase or reduced milk fat yield. However, recent work by Baumgard et al. (1999) showed that abomasal infusion of trans-10, cis-12 CLA caused a decrease in milk fat yield. Mammalian tissues can produce cis-9, trans-11 CLA via the Δ9 desaturase, but trans-10, cis-12 CLA is thought to arise only from ruminal fermentation (Griinari et al. 1997).

When Harfoot et al. (1973a, 1973b) incubated mixed ruminal bacteria from cattle fed 50% grain and 50% hay with LA, the free-floating bacteria produced more total CLA and less biohydrogenated product (C18 : 0 and C18 : 1) than particle-associated bacteria. As sucrose additions increased biohydrogenation and decreased CLA (Harfoot et al. 1973a, 1973b), it appeared that reducing equivalent availability was regulating the flux of LA through CLA to biohydrogenated products. However, the trans-10, cis-12 and cis-9, trans-11 isomers were not resolved and CLA-producing bacteria were not isolated.

It has long been recognized that grain can increase lactate turnover in the rumen, but only a few ruminal bacteria can utilize lactate (e.g. M. elsdenii and Selenomonas ruminantium; Hungate 1966). Counotte et al. (1981) demonstrated that M. elsdenii was the most important lactate-utilizing bacteria in cattle fed grain and our experiments indicated that lactate and trypticase enriched large cocci that resembled M. elsdenii. All of these large cocci utilized lactate as an energy source and were highly monensin resistant (Callaway et al. 1999). 16S-rDNA sequencing indicated that YJ-4 was closely related to other strains of M. elsdenii. Butyryrivibrio fibrisolvens has often been used as a model for ruminal CLA production, but this bacterium produces only cis-9, trans-11 CLA (Kepler et al. 1966; Kepler and Tove 1967; Kim et al. 2000). Studies with B. fibrisolvens A38 indicated that cis-9, trans-11 CLA was not produced until the LA concentration (100 µg ml⁻¹) was sufficient to inhibit biohydrogenation and the cells started to lyse (Kim et al. 2000). Megaphaera elsdenii is more resistant to LA than B. fibrisolvens (Henderson 1973). However, even relatively low concentrations of LA (20 µg ml⁻¹) triggered trans-10,

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cis-12 CLA production of *M. elsdenii* YJ-4 and higher concentrations (200 µg m\(^{-1}\)) only caused a 30% increase. *Butyrivibrio fibrisolvens* produced 18 µg cis-9, trans-11 CLA mg protein\(^{-1}\) (Kim et al. 2000) and *M. elsdenii* YJ-4 cultures that had been grown on lactate produced approx. 7 µg trans-10, cis-12 CLA mg protein\(^{-1}\).

The LA isomerase of *B. fibrisolvens* produces cis-9, trans-11 CLA very rapidly, but it cannot recycle like a normal enzyme to catalyse additional substrate (Kim et al. 2000). The *M. elsdenii* YJ-4 isomerase had similar kinetic properties. Trans-10, cis-12 CLA was produced rapidly, but only a small fraction of the LA was converted to trans-10, cis-12 CLA. The trans-10, cis-12 CLA production could be increased by adding more cells, but additional LA only caused a small increase in trans-10, cis-12 CLA. These results are consistent with the idea that CLA is a cell-bound intermediate in biohydrogenation rather than a terminal end-product per se (Kim et al. 2000).

Milk fat depression is a phenomenon that is highly animal dependent and some cows do not produce low fat milk even if grain is abundant and ruminal pH is low (Storry and Sutton 1969). The present work indicates that *M. elsdenii* strains can differ greatly in their ability to produce the trans-10, cis-12 CLA and that CLA production is not a phylogenetically conserved trait. Given these observations, it is conceivable that animals may harbour different *M. elsdenii* strains with different capacities to produce trans-10, cis-12 CLA.

Further work will be needed to define more clearly the contribution of *M. elsdenii* in the production of trans-10, cis-12 CLA, but the observation that grain feeding promotes the growth of *M. elsdenii* (Counotte et al. 1981) is consistent with the idea that this species could play a significant role in milk fat depression.

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