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Partial Inhibition of Biohydrogenation of Linoleic Acid Can Increase the Conjugated Linoleic Acid Production of *Butyrivibrio fibrisolvens* A38

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Butyrivibrio fibrisolvens A38, one of the most active rumen bacteria in conjugated linoleic acid (CLA) production, was characterized in vitro. Previous findings that some inhibitory levels of substrate for biohydrogenation (BH) by *B. fibrisolvens* A38 resulted in more CLA accumulation led to a prediction that partial inhibition of BH could increase ruminal CLA production. The inhibitory conditions for bacterial growth were less effective on the isomerization step than on the following reduction step. Linoleic acid (LA) was inhibitory not only to cell growth but also to LA hydrogenation, and this effect was greater at high concentrations. The reduction step, converting CLA to hydrogenated products (*trans*-C18:1 and C18:0), was significantly inhibited, and more CLA accumulated during aerobic incubation when LA was added along with a glycolytic inhibitor, iodoacetate (IAA), to cells that were pre-adapted to LA (1 g/OD at 600 nm/L, $P < 0.05$). Monensin was more inhibitory than IAA to cell growth but less effective for CLA accumulation. Rumen fluid in the culture medium appeared to activate BH even in an aerobic condition, resulting in a lower CLA level than the control group ($P < 0.05$). Because the isomerization and reduction steps are coupled reactions in BH of most hydrogenating bacteria including *B. fibrisolvens* A38 cells, both positive and negative modulations of the reduction steps could be key determinants for CLA accumulation in the rumen.

KEYWORDS: Conjugated linoleic acids; *Butyrivibrio fibrisolvens*; linoleic acid; biohydrogenation; antibiotic

INTRODUCTION

Conjugated linoleic acid (CLA) refers to a series of positional and geometric isomers of linoleic acid (LA) with double bonds at positions 7−9, 8−10, 9−11, 10−12, and so forth. CLA is a naturally occurring compound mainly in foods of ruminant origin and has shown a variety of biological activities in animal studies including anti-tumorigenesis and anti-atherosclerosis (*1*−*3*). Among the isomers, the *cis*-9,*trans*-11 isomer is the major one present in nature mainly produced by rumen bacteria such as *Butyrivibrio fibrisolvens* A38 as an intermediate byproduct in the biohydrogenation (BH) process. Kepler and Tove (*4*) first showed that CLA is the first intermediate in the process, and its biological effects were recognized a few decades later (*1*).

Ruminants rely on a unique host−microbial symbiosis in the rumen in which microorganisms metabolize feed material into utilizable energy sources. Feed materials ingested by ruminants are subjected to microbial conversion, and long-chain unsaturated fatty acids are hydrogenated by some rumen bacteria (*5*). Thus, a variety of fatty acid metabolites become available to ruminants for incorporation into tissue and milk (*6*). BH by *B. fibrisolvens* A38 is a two-step process: isomerization to *cis*−*trans*(*trans*−*cis*)-octadecadienoic acids (C18:2) and reduction of the isomers to *trans*(*cis*)-octadecenoic acids (C18:1) (*4*, *7*). Although bacterial growth and the complete BH of unsaturated fatty acids require anaerobic conditions, isomerization, the first step of BH, is not inhibited by an aerobiosis whereby CLA concentration can be transiently accumulated (*7*).

Supplementing some dietary source of LA for ruminants has been shown to elevate the CLA content of milk (*8*, *9*). Considering that BH is an energy-requiring process and metabolically inactive or even dead cells could produce CLA in high-LA conditions (*7*), a question arose: Will inhibitors of energy metabolism have an effect similar to that of high LA supplementation? Iodoacetate (IAA) is a specific inhibitor of the enzymatic EMP pathway and thus inhibits ATP and NADPH production. It was also speculated that inhibitors of membrane-associated energy metabolism could inhibit BH without interfering with the isomerization step. Dietary ionophores could disrupt the permeability of ions across bacterial membranes and change the ruminal fermentation pattern by selecting resistant bacteria (*10*). Monensin is a monovalent antiporter with selective binding affinity for Na^+ and has been used to enhance animal performance in feeding trials (*10*, *11*).

BH depends on a series of enzymatic reactions by certain rumen bacteria, but little attempt has been made to maximize

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CLA accumulation in the rumen by manipulating the factors affecting the enzymatic processes. To obtain high-CLA accumulation during the BH process, multiple factors should be considered simultaneously because two metabolically distinct enzymatic steps are coupled. So far, knowledge of the factors that affect the transformation of unsaturated fatty acids by rumen bacteria has not been sufficient to optimize ruminal CLA production. We previously studied some extrinsic factors such as LA concentration, pH, and cell age that affect CLA production by *B. fibrisolvens* A38. In the present study, both inhibitory and stimulating factors for BH were further characterized in vitro with the purpose of enhancing CLA accumulation by *B. fibrisolvens* A38 by partial inhibition of BH.

MATERIALS AND METHODS

Reagents and Materials. Chemicals and organic solvents used in the experiments were all of analytical grade and were purchased from Fisher Co. (Fair Lawn, NJ). A CLA standard containing >95% of *cis*-9,*trans*-11-octadecadienoic acid was supplied by Lipozen Inc. (Pyungtaek, Korea). Other fatty acid standards and antibiotics were obtained from Sigma Chemical Co. (St. Louis, MO).

Cell Growth and Harvest. *B. fibrisolvens* A38 cells were grown anaerobically in a basal medium at 39 °C with 4% glucose and 10% Trypticase. Cells were grown up to 2 OD anaerobically in 15 mL rubber-stoppered glass tubes (*12*). Typically, the approximate colony forming unit per OD was 10^8-10^9. Cultures were harvested by centrifugation (10000*g*, 5 °C, 5 min) after three washings with 0.9% NaCl and resuspended anaerobically in a N-free basal medium to inhibit additional growth. LA emulsified with twice the weight of bovine serum albumin was incubated with 10 mL of a suspension of *B. fibrisolvens* A38 in 0.1 M sterile potassium phosphate (pH 6.5). Stocks (×100) of monensin and IAA were dissolved in ethanol and sterilely filtered.

GC Analysis. Methylation of fatty acids was performed as described previously (*13*). Briefly, samples (15−30 mg fatty acids) were methylated with 1 mL of 2% sulfuric acid in methanol. The mixture was heated for 1 h in a 20 mL glass tube at 70 °C, and then 5 mL of 5% NaCl was added. The esters were extracted with 2 mL of hexane twice. The hexane layer was washed with 4 mL of 4% of potassium bicarbonate solution and dried over Na_2SO_4. The solution was filtered and the solvent removed by nitrogen flushing. The samples were analyzed for CLA isomers and total fatty acid profile by gas chromatography (GC) equipped with a flame ionization detector (FID) (Hewlett-Packard, HP 5890, Avondale, PA). Fatty acid methyl esters were separated on a Supelcowax-10 fused silica capillary column (60 m × 0.53 mm i.d., 0.5 μm film thickness, Supelco Inc., Bellefonte, PA) with a 2.4 mL/min helium flow. GC conditions were as follows: injector temperature, 200 °C; oven temperature program, hold at 40 °C for 5 min, then increase to 220 °C at 20 °C/min, hold for 40 min; detector temperature, 250 °C. A 1 μL sample containing 0.5−5 μg of fatty acids was injected into the column in the splitless mode.

Quantification of Fatty Acids. The peaks of CLA and hydrogenated products were identified by comparison with the retention time of each fatty acid standard, and the peak area was analyzed for the quantification. Hydrogenated products were mostly *trans*-11-C18:1, and C18:0 was <5% of total hydrogenated products. The CLA content was expressed as milligrams per liter of reaction mixture. Heptadecanoic acid (C17:0) as an internal standard was added before the extraction to determine the recovery of the fatty acids in the samples. The recovery of methylated fatty acids that was calculated in a comparison to the internal standard (C17:0) was >80% after analysis.

Statistical Analyses and Design. All experiments were replicated at least three times, and statistical analysis was conducted using the SigmaStat program (version 1.0; Jandel Corp., San Rafael, CA). Treatment means were compared using the Student *t* test, and the significance of difference was defined at $P < 0.05$.

RESULTS AND DISCUSSION

The bacterial cell density reached up to 2 OD at 600 nm when *B. fibrisolvens* A38 was incubated in a basal medium with 4% glucose. Stationary phase cells lysed steadily, and the OD after 48 h was significantly reduced. If LA was added to the cultures just before the cells reached stationary phase, the decline in OD was even more pronounced. The lysis rate of untreated stationary phase cells was ∼2%/h, but the rate of cultures with 100 mg of LA/L was ∼5 times higher (**Figure 1**). It was previously observed that the growth of *B. fibrisolvens* A38 was inhibited in an anaerobic condition by the initial addition of >25 mg of LA/L, and the growth rate was inversely correlated with LA concentration (*7*). However, cells that were in logarithmic phase were more resistant to LA, and bacterial growth was observed at LA concentrations as high as 50 mg/L (data not shown). Long-chain unsaturated fatty acids are toxic to some rumen bacteria. Growth of Gram-positive bacteria was particularly inhibited by long-chain fatty acids, and BH was thought to be a detoxification mechanism (*14*, *15*). The uptake of fatty acids onto the bacterial cell surface, and cell lysis by unsaturated long-chain fatty acids is known to dramatically increase with chain length (*15*).

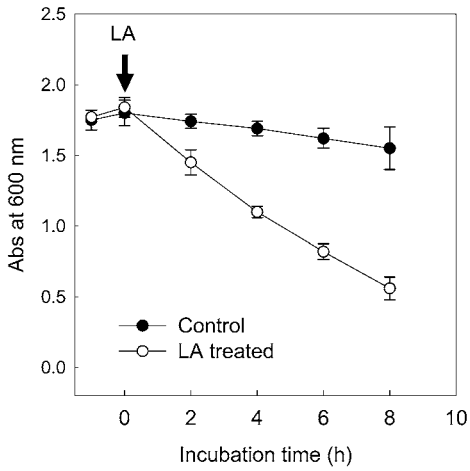

Figure 1. Lysis of *B. fibrisolvens* A38 cells by the addition of linoleic acid (LA) at stationary phase cells. Cells were incubated with 4% glucose at 39 °C for 48 h, and LA (100 mg/L) was added to the culture when the cells reached a stationary phase. The arrow indicates LA addition to the culture. Values are means of triplicate trials, and standard deviations are shown as error bars.

When cells of 2 OD were incubated with various concentrations of LA for 6 h aerobically, the time-dependent decline of CLA was more evident at lower concentrations. Approximately 95% of the CLA was the *cis*-9,*trans*-11 isomer, and most of the decrease in CLA was due to reduction to *trans*-11-C18:1 (data not shown). When the initial LA concentration was 10 mg/L, the maximal level of CLA was ∼1.0 mg/L and only 10% was left after 6 h of incubation. At 100 mg of LA/L, 40% was converted to CLA in 10 min of incubation and 50% of the CLA was left after 6 h of incubation. At 1000 mg of LA/L, however, only 22% was converted to CLA and ∼70% of the CLA was left after 1 h of incubation; no further decrease was detected during prolonged incubation (**Figure 2**). Considering the conversion rate and the further decline during a prolonged incubation, approximately 100 mg of LA/L may be the optimal concentration for CLA production with ∼2 OD of *B. fibrisolvens* A38 cells. The conversion of LA to CLA by *B. fibrisolvens* A38 was higher in an aerobic condition than in an anaerobic condition in which most of the LA was depleted at its low concentrations, and up to 80% of LA was converted to hydrogenated products. During typical rumen bacterial BH, the level of C18:0 increased slowly after the *trans*-C18:1 level peaked (*5*). However, the conversion of *trans*-C18:1 to C18:0

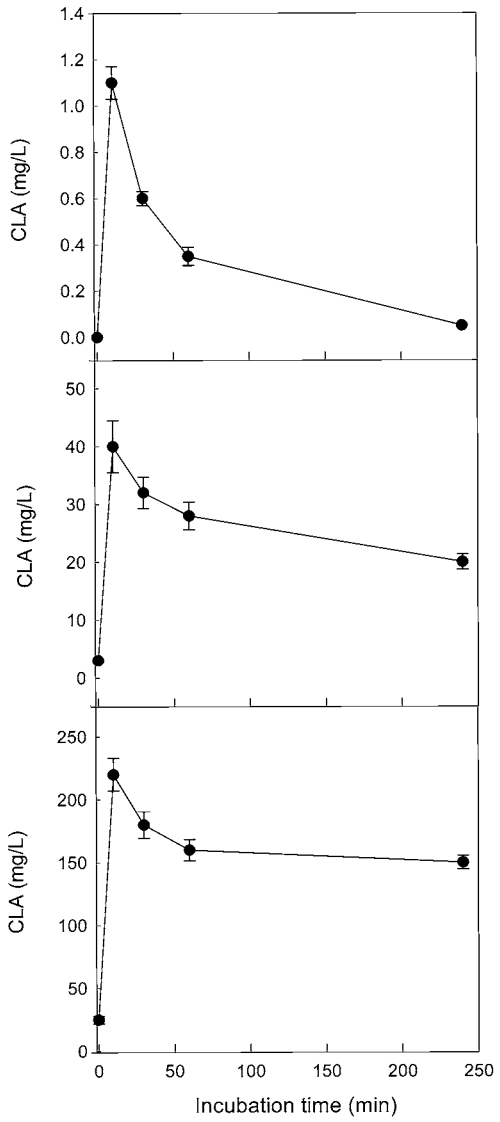

Figure 2. Production of conjugated linoleic acid by *B. fibrisolvens* A38 cells at various LA concentrations in an aerobic condition. Cells of 2 OD were incubated at 39 °C with LA at different levels (10, 100, and 1000 mg/L) for 6 h. Values are means of triplicate trials, and standard deviations are shown as error bars.

was not observed with *B. fibrisolvens* A38 (*4*). The coupled enzymatic reactions in BH may be a metabolically independent process despite the fact that the enzymes are all membrane-associated (*4*, *7*).

Growing rumen cultures were active in BH, and CLA persisted in high-LA conditions (>100 mg). This effect was more pronounced when *B. fibrisolvens* A38 cells were metabolically inactive (stationary phase cells and/or cells under aerobic incubation). In our previous study, CLA accumulation was substantially enhanced when cell growth was inhibited by high LA (*7*). In the long-term incubation, the decrease in CLA was less evident at higher LA concentrations. Thus, it is feasible that a high LA content in the rumen could interrupt the energy metabolism that is required for reduction of rumen bacteria, resulting in accumulation of CLA. At lower substrate concentration, however, all BH steps may be active, and the rate of reduction could surpass the rate of isomerization, leaving little CLA. Indeed, the time-dependent decline of CLA was negatively correlated with the amount of LA added.

When stationary phase cells (1 OD) were incubated with 100 mg of LA/L and 100 μM iodoacetate, a glycolytic inhibitor,

Table 1. Effect of IAA on Biohydrogenation of LA by *B. fibrisolvens* A38 in Aerobic or Anaerobic Condition (Mean ± SD; $n = 3$)[a]

condition	antibiotic	CLA (mg/L)	hydrogenated products (mg/L)
aerobic	untreated	22.0 ± 2.52	10.0 ± 1.57
	IAA	30.4 ± 1.84	2.8 ± 0.41
anaerobic	untreated	10.5 ± 1.30	8.5 ± 0.86
	IAA	7.0 ± 0.75	2.1 ± 0.11

[a] Iodoacetate (100 μM) was added to 1 OD cells and incubated aerobically or anaerobically at 39 °C with 100 mg of LA/L for 10 min.

Table 2. Effect of Pre-adaptation to LA and IAA on Biohydrogenation of LA by *B. fibrisolvens* A38 in an Aerobic Condition (Mean ± SD; $n = 3$)[a]

condition	antibiotic	CLA (mg/L)	hydrogenated products (mg/L)
control	untreated	22.0 ± 2.52	10.0 ± 1.57
	IAA	30.4 ± 1.84	2.8 ± 0.41
pre-adapted	untreated	17.0 ± 2.33	15.0 ± 1.32
	IAA	39.0 ± 4.51	2.5 ± 0.35

[a] Cells were cultured with or without pre-adaptation to LA in a stepwise fashion (10–100 mg), and 1 OD cells treated with iodoacetate (100 μM) were incubated aerobically with 100 mg of LA/L at 39 °C for 10 min.

CLA production was increased by 50% compared to the incubation without IAA, but only if the cells were incubated aerobically. When IAA was added either aerobically or anaerobically, fewer hydrogenated products were produced in comparison to the control. The difference in the CLA content was almost compensated by the amount of hydrogenated products in aerobic conditions. IAA efficiently inhibited BH aerobically but failed to increase the CLA level anaerobically, indicating that CLA increase by IAA treatment was due to the inhibition of reduction rather than to the isomerization of *B. fibrisolvens* A38.

To investigate the effects of pre-adaptation to a fat substrate, LA was used as a potential inducer. Cells were grown anaerobically with increasing amounts of LA (10−100 mg) for 48 h, and cells of 1 OD were harvested and incubated with 100 mg of LA/L for 10 min. Aerobic suspensions of pre-adapted cells produced less CLA and more hydrogenated products than normal cells. However, CLA concentration was increased when pre-adapted cells were treated with IAA, and fewer hydrogenated products were generated in comparison to normal cells ($P < 0.05$) (**Table 2**). Characteristically, when CLA increased, hydrogenated products declined. Our previous studies indicated that BH could be activated by the adaptation of *B. fibrisolvens* A38 cells to 10 mg of LA/L (*7*). The CLA level could be further increased by IAA treatment of pre-adapted cells with LA. Only the reduction step may be inhibited by IAA treatment in an aerobic condition when total BH is triggered by pretreatment of LA. This could maintain CLA at a higher level in the reaction mixture during the incubation, and this effect may be pronounced when the cells are in the stationary phase, because glycogen accumulation is active in stationary phase cells. Inhibition of reduction to CLA by IAA could be increased when cells are stressed by aerobic incubation.

Addition of monensin always decreased the hydrogenated products compared to the untreated control, but the CLA level was not significantly increased ($P > 0.05$) (**Table 3**). Aerobic cells without monensin treatment converted 27% of the LA to CLA in 10 min of incubation, and ~75% remained after 30 min of incubation. Monensin inhibited cell growth to the same

Table 3. Effect of Monensin on Biohydrogenation of LA by *B. fibrisolvens* A38 in Aerobic or Anaerobic Condition (Mean ± SD; $n = 3$)[a]

condition	antibiotic	CLA (mg/L)	hydrogenated products (mg/L)
aerobic	untreated	22.0 ± 2.52	4.0 ± 1.57
	monensin	25.6 ± 3.40	2.0 ± 0.12
anaerobic	untreated	11.0 ± 1.21	8.5 ± 0.85
	monensin	8.5 ± 0.75	1.7 ± 0.15

[a] Monensin (100 μM) was added to 1 OD cells incubated in aerobic or anaerobic conditions with 100 mg of LA/L at 39 °C for 10 min.

Table 4. Effects of Antibiotics on CLA Production of *B. fibrisolvens* A38 Cells in Aerobic Condition (Mean ± SD; $n = 3$)[a]

condition	time (min)	LA (mg/L)	CLA (mg/L)	CLA/LA ratio
untreated	0	103 ± 10.3	0	
	10	71.4 ± 6.9	22.5 ± 5.5	0.31
	30	59.6 ± 3.9	16.8 ± 4.0	0.28
iodoacetate	0	98.5 ± 7.6	0	
	10	61.0 ± 3.9	33.0 ± 2.0	0.54
	30	57.8 ± 4.5	35.6 ± 5.4	0.62
monensin	0	93.0 ± 6.0	0	
	10	64.6 ± 3.1	25.6 ± 3.4	0.40
	30	64.9 ± 3.8	24.4 ± 1.7	0.38

[a] Iodoacetate was treated at 100 μM, and monensin was treated at 1 μM.

extent at about $^1/_{100}$ the concentration of IAA (data not shown). Compared to untreated cells, both monensin- and IAA-treated cells had more CLA and the degree of CLA decline in further incubation was lower (**Table 4**). The increase in CLA concentration was not observed when monensin was added at a level that gave the same growth inhibition as IAA. This indicated that disrupting membrane-associated energy metabolism may inhibit not only reductase but also isomerase activity to some degree. Our previous studies showed that membrane fractions of *B. fibrisolvens* cells produced greater amounts of CLA than cell-free supernatant (*7*). Hunter et al. (*16*) also noted that the enzymes involved in BH were membrane-associated, but direct synergism between the two enzymatic activities was not demonstrated. Taken together, this coupled reaction is metabolically independent in terms of the requirement for reducing equivalent, which is generated through membrane-associated energy metabolism.

An effect of clarified rumen fluid was also observed during incubation with LA (**Figure 3**). When washed stationary phase cells (1 OD) were incubated in 30% clarified sterile rumen fluid, the initial increase in CLA was very rapid and 15% more CLA was accumulated within 10 min compared to control cells that were incubated without rumen fluid; however, the decrease in CLA concentration was also greater with rumen fluid, and the CLA concentration after 2 h of incubation was only 50% of the control cells. Rumen fluid enhanced the overall BH rate but failed to increase CLA production by *B. fibrisolvens* A38. The presence of rumen fluid appeared to activate reduction steps even aerobically. In fact, complete ruminal BH was shown to be possible when ruminal fluid was included in the culture (*17*). This effect is possibly due to the unknown factors in the rumen fluid, and it was assumed to be an iron-containing complex in our previous study (*18*). This indicated that some components in rumen fluid may be able to overcome, at least in part, the inhibition caused by aerobic incubation.

In conclusion, *B. fibrisolvens* A38 cells accumulate more CLA when reduction is partially inhibited by oxygen or the

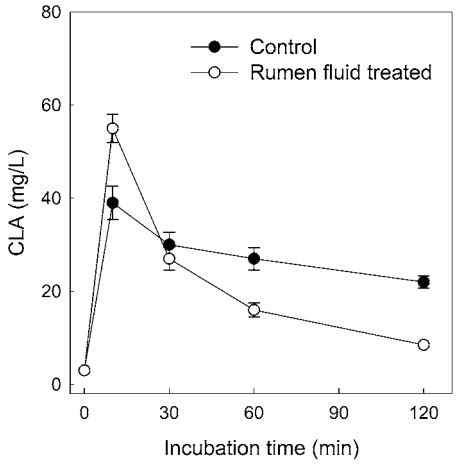

Figure 3. Effect of rumen fluid on biohydrogenation by *B. fibrisolvens* A38. Cells of 1 OD were incubated aerobically in 30% clarified rumen fluid medium with 100 mg of LA/L at 39 °C for 10 min. Values are means of triplicate trials, and standard deviations are shown as error bars.

additions of antibiotics. These factors could be coupled with positive factors such as pre-adaptation, rumen fluid, and optimum LA concentration to maximize CLA accumulation. However, the metabolically related enzymes catalyzing the BH process obscured the role of environmental factors. Efficient ruminal CLA production requires multidisciplinary investigation considering a variety of extrinsic factors affecting BH. Further metabolic studies in various conditions are clearly needed to maximize CLA production by *B. fibrisolvens* A38 in vivo.

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