Metabolism of conjugated linoleic acids and 18:1 fatty acids by ruminal bacteria: products and mechanisms

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Cultures of ruminal bacteria known to metabolize unsaturated fatty acids were grown in medium containing 50 µg ml⁻¹ of geometric and positional isomers of conjugated linoleic acid (CLA) or 18:1 fatty acids and 37.4% deuterium oxide to investigate the mechanisms responsible for fatty acid metabolism. *Butyrivibrio fibrisolvens* JW11 converted *cis*-9,*trans*-11-18:2 and *trans*-9,*trans*-11-18:2 to *trans*-11-18:1 as the main product, labelled at C-9, and metabolized *trans*-10,*cis*-12-18:2 to *trans*-10-18:1, labelled at C-13, and smaller amounts of *trans*-12-18:1 and *cis*-12-18:1. *Butyrivibrio proteoclasticus* P-18 did not grow in the presence of *cis*-9,*trans*-11-18:2 or *trans*-10,*cis*-12-18:2, but grew in medium containing *trans*-9,*trans*-11-18:2, forming 18:0. *Propionibacterium acnes*, a ruminal species that isomerizes linoleic acid to *trans*-10,*cis*-12-18:2, did not metabolize CLA isomers further. *B. fibrisolvens* metabolized small amounts of *trans*-10-18:1, *trans*-11-18:1 and *cis*-9-18:1, but the products formed were not detected. *B. proteoclasticus*, on the other hand, carried out substantial conversion of 18:1 substrates to 18:0. *P. acnes* hydrated *cis*-9-18:1 and *trans*-11-18:1 to 10-OH-18:0, which was further oxidized to yield 10-O-18:0. The deuterium enrichment in the intermediates formed during incubations with 9,11 geometric isomers of CLA was about half that of the products from *trans*-10,*cis*-12 CLA and 18:1 isomers, suggesting that the reduction of 9,11 geometric isomers CLA by ruminal bacteria occurs via different mechanisms compared with the metabolism of other unsaturated fatty acids.

Received 16 November 2009

Accepted 18 November 2009

INTRODUCTION

There is increasing evidence that nutrition plays an important role in the development of chronic diseases in the human population, including cancer, cardiovascular disease, insulin resistance and obesity. Clinical and biomedical studies have provided a large body of data to indicate that excessive intakes of saturated fatty acids increase cardiovascular disease risk (World Health Organization, 2003; Mensink *et al.*, 2003) and may lower insulin sensitivity, which is a key factor in the development of the metabolic syndrome (Nugent, 2004). Most of the 12:0 and 14:0 and a significant amount of 16:0 in the human diet comes from ruminant-derived foods, and therefore there is interest in altering the composition of milk fat, in particular, as part of nutritional approaches to preventing and reducing the economic and social impact of chronic disease. Targets for altering milk fatty acid

composition with the potential to improve long-term human health include decreasing the content of medium-chain saturated fatty acids and enhancing the concentrations of several bioactive lipids, including *cis*-9-18:1, *trans*-11-18:1 and *cis*-9,*trans*-11-18:2 (Pariza, 2004; Bauman *et al.*, 2005; Shingfield *et al.*, 2008).

Following ingestion, lipids in the ruminant diet are released from structural components, hydrolysed, and the non-esterified fatty acids are released into the rumen, adsorbed onto feed particles and hydrogenated or incorporated directly into bacterial lipids (Harfoot & Hazlewood, 1997; Jenkins *et al.*, 2008). Characterizing the formation of intermediates and production of end products arising from the biohydrogenation of lipids in the rumen is central to attempts to enhance the nutritional attributes of ruminant milk fat. Biohydrogenation has been studied for many years, most recently in attempts to identify species that form isomers of conjugated linoleic acid (CLA) as transient intermediates during the metabolism of linoleic acid (*cis*-9,*cis*-12-18:2) to 18:0 (Kim *et al.*, 2002; Paillard *et al.*, 2007; Wallace *et al.*, 2006; Nam & Garnsworthy, 2007) and the mechanisms responsible for CLA synthesis (Wallace

Abbreviations: CLA, conjugated linoleic acid; DMOX, 4,4-dimethyloxazoline; FAME, fatty acid methyl ester; MPE, moles % excess; PUFA, polyunsaturated fatty acid.

Two supplementary figures are available with the online version of this paper.

et al., 2007). Isomers of CLA also serve as a substrate for reduction to 18:1 intermediates and 18:0 (Kepler *et al.*, 1966; Harfoot & Hazlewood, 1997).

The main microbes known to influence fatty acid metabolism in the rumen are the ciliate protozoa, which effectively accumulate unsaturated fatty acids (Devillard *et al.*, 2006), at least partly by ingesting chloroplasts (Huws *et al.*, 2009), and a small number of bacterial species with a high biohydrogenating activity. *Butyrivibrio fibrisolvens* is a rather diverse group of Gram-positive bacteria, all of which metabolize linoleic acid at many times the rate of other ruminal species (Polan *et al.*, 1964; Maia *et al.*, 2007). Only a small subgroup within the *B. fibrisolvens* grouping carries out the reduction of 18:1 fatty acids, particularly *trans*-11-18:1 to 18:0 (Wallace *et al.*, 2006). This subgroup is now known as *Butyrivibrio proteoclasticus* (Moon *et al.*, 2008), formerly *Clostridium proteoclasticum* (Wallace *et al.*, 2006), which in turn is believed to be the same species as that isolated many years ago, named *Fusocillus* sp. (Kemp *et al.*, 1975). Metabolism of linoleic acid by *Butyrivibrio* results in the formation of *trans*-11-18:1 and *cis*-9,*trans*-11-18:2 as major intermediates. The same is usually true in the mixed ruminal ecosystem, but under certain conditions *trans*-10-18:1 is formed via the reduction of *trans*-10,*cis*-12-18:2 or from the isomerization of *cis*-9-18:1 and represents the major biohydrogenation intermediate of the mixed ruminal ecosystem *in vivo* (Shingfield & Griinari, 2007). Post-ruminal infusion experiments have established that *trans*-10,*cis*-12-18:2 exerts anti-lipogenic effects in the lactating cow (Baumgard *et al.*, 2000; Sæbø *et al.*, 2005; Lock *et al.*, 2006), with recent studies suggesting that *trans*-10-18:1 may also decrease mammary lipogenesis (Shingfield *et al.*, 2009). Our studies indicate that *Propionibacterium acnes* may be responsible for the formation of *trans*-10,*cis*-12-18:2 (Wallace *et al.*, 2006). The aim of the present study was to extend previous investigations on CLA formation (Wallace *et al.*, 2007) using similar techniques to examine and elucidate the mechanisms responsible for the reduction of CLA isomers and 18:1, and to characterize the production of metabolic intermediates by pure strains of ruminal bacteria.

METHODS

Bacteria and growth conditions. *Butyrivibrio fibrisolvens* JW11 was originally isolated from sheep as a proteolytic species (Wallace & Brammall, 1985). *Butyrivibrio proteoclasticus* P-18 is a recently identified 18:0-producing bacterium isolated from grazing sheep (Wallace *et al.*, 2006); its previous name was *Clostridium proteoclasticum*, but it was renamed recently (Moon *et al.*, 2008) in view of its clear genetic distance from *Clostridium* and proximity to *Butyrivibrio*. *Propionibacterium acnes* G449 was isolated from sheep in the same study (Wallace *et al.*, 2006) and found to produce *trans*-10,*cis*-12-18:2 from linoleic acid. These ruminal bacteria are held in the culture collection maintained at the Rowett Institute. A type strain of *P. acnes* (DSM 1897) was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany. All transfers and incubations were carried out under O₂-free CO₂ at 39 °C in Hungate-type tubes (Hungate, 1969). Inoculum volumes were 5% (v/v) of a fresh culture into 5 ml of

medium. The media used in these experiments were based on the liquid form of M2 medium (Hobson, 1969), and deuterium oxide and fatty acids (50 mg l⁻¹) were added as described previously (Wallace *et al.*, 2007). All bacteria were incubated at 39 °C until stationary phase was reached. With *B. fibrisolvens* and *B. proteoclasticus*, this occurred at times up to 96 h, depending on the fatty acid substrate, while *P. acnes* was grown for 96 h. Growth of bacteria was measured in triplicate from the increase in optical density at 650 nm of the control tubes using a Novaspec II spectrophotometer (Amersham Biosciences). A 1 ml sample was removed for protein determinations and analysis of the enrichment in water. Thereafter, 100 µl of 19:0 (200 mg l⁻¹ in methanol) was added and tubes were stored at -70 °C and subsequently freeze-dried. Fatty acid metabolism was assessed based on comparisons of fatty acid determinations in the inoculated medium containing fatty acids compared with uninoculated controls.

Fatty acids. *trans*-10,*cis*-12-18:2, *cis*-9,*trans*-11-18:2 and *trans*-9,*trans*-11-18:2 were obtained from Universal Biologicals, Cambridge, UK, sourced from Matreya, Pleasant Gap, PA, USA. *trans*-11-18:1 and *cis*-9-18:1 were purchased from Sigma-Aldrich, while *trans*-10-18:1 was a gift from Frédéric Destailats, Nestlé, Lausanne.

Fatty acid extraction and analysis. Extraction of total fatty acids was based on the method of Folch *et al.* (1957), with modifications (Waśowska *et al.*, 2006). Fatty acid methyl esters (FAMES) and 4,4-dimethyloxazoline (DMOX) derivatives were prepared and analysed by gas chromatography-mass spectrometry (GC-MS) according to standard procedures as described previously (Waśowska *et al.*, 2006). Enrichment in the *m/z* (M+1) and (M+2) isotopomers (molecular ion+1 and +2, respectively) was determined by GC-MS (Wallace *et al.*, 2007). Enrichment in water was determined by gas isotope ratio MS (Wallace *et al.*, 2007). Oxygen-containing FAMES were analysed by GC-MS, as follows. A gas chromatograph (model 6890, Agilent Technologies) equipped with a flame-ionization detector and linked to a quadrupole mass-selective detector (model 5973N) was fitted with a 30 m fused silica capillary column (i.d., 0.25 mm) coated with a 0.25 µm film of 100% polyethylene glycol (Zebtron ZB-Wax Plus, Phenomenex). The total FAME profile in a 1 µl sample at a split ratio of 20:1 was determined using a temperature gradient programme (initial temperature 80 °C for 1 min; increased at a rate of 25 °C min⁻¹ to 160 °C, which was held for 3 min, then increased at a rate of 1 °C min⁻¹ to 190 °C, then further increased at a rate of 10 °C min⁻¹ to 230 °C, held for 40 min). Helium was the carrier gas, operated at constant pressure (14 p.s.i., 97 kPa) and flow rate of 1.4 ml min⁻¹. Injector and MS detector temperatures were maintained at 250 and 230 °C, respectively. Peaks were routinely identified by comparison of retention times with authentic FAME standards obtained from Sigma and Matreya. Identification was validated based on electron impact ionization spectra of FAMES obtained under an ionization voltage of 2247 eV.

Data analysis. Results from replicate cultures (*n*=3) were averaged and analysed by ANOVA. Least-square means ± SE are reported and effects were considered significant at *P*<0.05. Enrichment of M+1 and M+2 isotopomers was calculated from the *m/z* ratios at M, M+1, M+2 and M+3 by deconvolution according to Campbell (1974). Natural abundance was calculated from the isotopomer distribution of linoleic acid.

RESULTS

Metabolism of CLA

Three commercial samples of positional and geometric isomers of CLA in free fatty acid form were added to the growth medium of the four bacteria. None of these

materials was of high purity as indicated from the analyses carried out on uninoculated medium (Tables 1 and 2). *cis*-9,*trans*-11-18:2 was heavily contaminated with *trans*-9,*trans*-11-18:2 (19.3 g per 100 g fatty acids) and lower amounts of *cis*-9,*cis*-11-18:2 (2.80) and *trans*-9,*cis*-11-18:2 (2.39). Similarly, the *trans*-9,*trans*-11-18:2-containing medium also contained *trans*-9,*cis*-11-18:2 (4.68 g per 100 g fatty acids) and *cis*-9,*trans*-11-18:2 (4.11). The *trans*-10,*cis*-12-18:2 material was the least pure and also contained *trans*-10,*trans*-12-18:2 (28.7 g per 100 g fatty acids), *cis*-10,*cis*-12-18:2 (3.70) and *cis*-10,*trans*-12-18:2 (3.26). Although the fatty acids were added to a nominal concentration of 50 mg l⁻¹, CLA concentrations detected were lower, the reasons for which were not established.

B. fibrisolvens converted *cis*-9,*trans*-11-18:2 and the contaminating *trans*-9,*trans*-11-18:2 almost completely to *trans*-11-18:1 (Table 1). The concentration of *trans*-18:1 formed corresponded to 92 % of the *cis*-9,*trans*-11-18:2 and *trans*-9,*trans*-11-18:2 that were removed from the medium. In isolation, *trans*-9,*trans*-11-18:2 was metabolized less extensively, mainly to *trans*-11-18:1, while the mixture of *trans*-10,*cis*-12-18:2/*trans*-10,*trans*-12-18:2 resulted in the production of *trans*-10-18:1 and to a lesser extent *trans*-12-18:1 and *cis*-12-18:1. *B. fibrisolvens* did not reduce isomers of CLA to 18:0. *B.*

proteoclasticus, on the other hand, was unable to grow in the presence of *cis*-9,*trans*-11-18:2 or *trans*-10,*cis*-12-18:2. Metabolism of *trans*-9,*trans*-11-18:2 resulted in the formation of 18:0, with only trace amounts of 18:1 intermediates (Table 2). No CLA was metabolized ($P>0.05$) by either strain of *P. acnes* (results not shown).

Metabolism of 18:1 fatty acids

The 18:1 fatty acids used as substrates, *trans*-10-18:1, *trans*-11-18:1 and *cis*-9-18:1, were much purer than the CLA, with no evidence of major contaminants (Table 3). Also unlike the CLA, concentrations detected in the medium corresponded well to the concentration added. *B. fibrisolvens* removed 25–33 % of these fatty acids from the medium (Table 3), but it was not possible to determine the products formed by GC-MS analysis. Although several small, generally broad peaks were visible, they were not suitable for integration, nor were they sufficient for MS analysis. In contrast, *B. proteoclasticus* converted substantial amounts of 18:1 substrates, *cis*-9-18:1 in particular, to 18:0. *P. acnes* DSM 1897 removed 29 % of the *trans*-10-18:1, almost all the *cis*-9-18:1, but none of the *trans*-11-18:1, incubated. No 18:0 was formed. The main product was a fatty acid that eluted at 44.35 min on the Wax column, eluting before 10-OH-18:0 (47.83 min). The

Table 1. Metabolism of conjugated linoleic acids by *B. fibrisolvens* JW11

Fatty acid	Fatty acid concentration (mg l ⁻¹)												
	Substrate ...	<i>cis</i> -9, <i>trans</i> -11-18:2				<i>trans</i> -9, <i>trans</i> -11-18:2				<i>trans</i> -10, <i>cis</i> -12-18:2			
		Initial*		Final†		Initial*		Final†		Initial*		Final†	
		Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
18:0	3.39	0.09	2.76	0.09	3.05	0.05	2.43	0.03	3.41	0.04	2.88	0.07	
18:1													
<i>trans</i> -9	0	0	0	0	0	0	0.38	0.05	0	0	0	0	
<i>trans</i> -10	0	0	0	0	0	0	0	0	0.21	0	11.52	0.22	
<i>trans</i> -11	0.22	0.01	30.64	1.15	0	0	3.22	1.14	0	0	0	0	
<i>trans</i> -12	0.04	0.02	0	0	0	0	0	0	0.06	0.03	3.93	0.24	
<i>cis</i> -9	1.53	0.01	1.55	0.05	1.18	0.01	1.21	0.01	1.29	0.04	1.49	0.02	
<i>cis</i> -10	0	0	0	0	0	0	0	0	0	0	0	0	
<i>cis</i> -11	0.09	0.01	0.48	0.04	0	0	0.45	0.13	0	0	0	0	
<i>cis</i> -12	0	0	0	0	0	0	0	0	0	0	3.57	0.95	
18:2													
<i>cis</i> -9, <i>trans</i> -11	26.60	0.16	0.39	0.04	1.70	0.09	2.26	0.37	0.21	0.10	0.18	0.18	
<i>trans</i> -9, <i>cis</i> -11	1.01	0.03	0.24	0.06	1.94	0.13	2.35	0.43	0	0	0	0	
<i>cis</i> -10, <i>trans</i> -12	0	0	0	0	0	0	0	0	1.18	0.02	0.31	0.03	
<i>trans</i> -10, <i>cis</i> -12	0	0	0	0	0	0	0	0	18.09	0.14	1.39	0.31	
<i>cis</i> -9, <i>cis</i> -11	1.18	0.05	0	0	0.32	0.05	0.27	0.04	0	0	0	0	
<i>cis</i> -10, <i>cis</i> -12	0	0	0	0	0	0	0	0	1.34	0.03	0.18	0.01	
<i>trans</i> -9, <i>trans</i> -11	8.13	0.20	1.52	0.10	33.22	1.63	26.33	7.15	0	0	0	0	
<i>trans</i> -10, <i>trans</i> -12	0	0	0	0	0	0	0	0	10.38	0.23	2.73	0.04	

*Uninoculated medium, $n=3$.

†Determined in 24 h cultures, $n=3$.

Table 2. Metabolism of conjugated linoleic acids by *B. proteoclasticus* P-18

No growth occurred with *cis*-9,*trans*-11-18:2 or *trans*-10,*cis*-12-18:2 in the medium.

Fatty acid Substrate ...	Fatty acid concentration (mg l ⁻¹) <i>trans</i> -9, <i>trans</i> -11-18:2			
	Initial*		Final†	
	Mean	SE	Mean	SE
18:0	3.05	0.05	10.22	0.27
18:1				
<i>trans</i> -9	0	0	0.23	0.04
<i>trans</i> -10	0	0	0	0
<i>trans</i> -11	0	0	0.39	0.03
<i>trans</i> -12	0	0	0	0
<i>cis</i> -9	1.18	0.01	1.22	0.03
<i>cis</i> -10	0	0	0	0
<i>cis</i> -11	0	0	0.24	0.02
<i>cis</i> -12	0	0	0	0
18:2				
<i>cis</i> -9, <i>trans</i> -11	1.70	0.09	0.84	0.11
<i>trans</i> -9, <i>cis</i> -11	1.94	0.13	0.99	0.16
<i>cis</i> -10, <i>trans</i> -12	0	0	0	0
<i>trans</i> -10, <i>cis</i> -12	0	0	0	0
<i>cis</i> -9, <i>cis</i> -11	0.32	0.05	0.14	0.07
<i>cis</i> -10, <i>cis</i> -12	0	0	0	0
<i>trans</i> -9, <i>trans</i> -11	33.22	1.63	26.95	2.93
<i>trans</i> -10, <i>trans</i> -12	0	0	0	0

*Uninoculated medium, *n*=3.

†Determined in 30 h cultures, *n*=3.

electron impact spectrum (Supplementary Fig. S1) of the FAME indicated a molecular ion at *m/z* 312 and an M-31 fragment at 281 consistent with an oxo-18:0 structure. Abundant ions at *m/z* 141, 171 and 199 corresponding to cleavage alpha to the oxo group and the occurrence of fragments at *m/z* 156 and 214 formed by beta cleavage provided clear evidence of the location of the oxo group, allowing the product formed to be identified as 10-O-18:0. Interpretation was verified by cross-referencing with earlier reports that elucidated the structure of 10-O-18:0 based on GC-MS, FTIR spectroscopy and NMR analysis (Hou, 1994). Smaller amounts of 10-OH-18:0 were also produced from *trans*-10-18:1 and from *cis*-9-18:1 (Table 3). The rumen *P. acnes* isolate, G449, similarly metabolized no *trans*-11-18:1, but was less active than the type strain with *cis*-9-18:1, metabolizing about one-tenth of the initial concentration (results not shown). Product concentrations were too low for the structures to be identified.

Labelling of products

Mass spectra of methyl esters of the products formed during the metabolism of CLA and 18:1 substrates by *B.*

fibrisolvens and *B. proteoclasticus* revealed three different patterns of incorporation of D from D₂O (Table 4). *trans*-11-18:1 produced from 9,11 geometric isomers of CLA was enriched in the M+1 isotopomer to about 25 % moles % excess (MPE). In contrast, the M+1 isotopomers formed during metabolism of 10,12 CLA isomers were labelled to 39.9–42.8 % MPE. None of the incubations with CLA yielded a labelled M+2 isotopomer in the 18:1 products. Labelling of 18:0 resulting from the reduction of 18:1 substrates was 39.9–43.1 % MPE in M+1 and 19.5–23.8 % MPE in M+2. No labelling was evident in the molecular ion from 10-O-18:0 derived from *P. acnes* incubations (Table 4). 10-OH-18:0 was found to be labelled in M+1 and M+2, as adjudged from ion fragments at *m/z* 169 and 201 due to the absence of a molecular ion.

The position of D atoms was investigated by examining mass spectra of DMOX esters. Enrichment of fragments of the DMOX derivative of *trans*-11-18:1 produced from 9,11 geometric isomers of CLA did not provide evidence for the location of the D label due to the low abundance of ion fragments in the C-10 (*m/z*=224) region (Supplementary Fig. S2a). The mass spectrum of the DMOX derivative for *trans*-11-18:1 that was produced in even smaller amounts during incubations with *trans*-9,*trans*-11-18:2 was even less clear (not shown). In contrast, the mass spectrum from *trans*-10-18:1 formed by the reduction of *trans*-10,*cis*-12-18:2 yielded more information, and revealed that the enrichment in M+1 declined markedly between C-13 (*m/z*=264) and C-12 (*m/z*=250) (Supplementary Fig. S2b), indicating that the label was located, in the main, on C-13. Mass spectra of 18:0 produced as an end product were not informative about the location of D atoms (not shown).

DISCUSSION

Previous studies on the mechanisms responsible for the formation of CLA isomers in the rumen (Wallace *et al.*, 2007) complemented earlier research carried out by ourselves and others to unravel the microbiology and biochemistry of the biohydrogenation process. Clear differences were identified between the mechanisms underlying the synthesis of *cis*-9,*trans*-11-18:2 and *trans*-10,*cis*-12-18:2 from linoleic acid (Wallace *et al.*, 2007), and also in the bacterial species that carry out the reactions. Here we provide analogous information on the subsequent metabolism of CLA isomers, revealing that there are also differences in the mechanism of reduction to 18:1 intermediates. The experiments were carried out with growing cultures of bacteria rather than cell suspensions, because active growth is required for biohydrogenation to occur (Wallace *et al.*, 2006). Fatty acid metabolism during the lag phase is essential for *Butyrivibrio* spp. to escape the toxicity of unsaturated fatty acids (Maia *et al.* 2007). Further metabolism occurs in the exponential phase, but no further transformations occur during the stationary

Table 3. Metabolism of 18:1 fatty acids by ruminal bacteria and *P. acnes* DSM 1897

Fatty acid	Substrate ...	Fatty acid concentration (mg l ⁻¹)											
		<i>trans</i> -10-18:1				<i>trans</i> -11-18:1				<i>cis</i> -9-18:1			
		Initial*		Final		Initial		Final		Initial		Final	
		Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
<i>B. fibrisolvens</i> JW11†													
18:0		3.32	0.02	2.91	0.03	3.46	0.07	2.58	0.08	3.33	0.05	3.05	0.01
<i>trans</i> -9		0	0	0	0	0	0	0	0	0.78	0.04	0.65	0.03
<i>trans</i> -10		40.54	3.15	32.25	2.39	0	0	0	0	0.27	0.07	0	0
<i>trans</i> -11		0	0	0	0	48.05	2.04	36.13	1.15	0	0	0.37	0
<i>trans</i> -12		0	0	0	0	0	0	0	0	0	0	0	0
<i>cis</i> -9		1.39	0.02	1.39	0.02	1.80	0.27	1.42	0.01	47.80	0.60	31.83	0.46
<i>cis</i> -10		1.21	0.03	0.64	0.07	0	0	0	0	0	0	0	0
<i>cis</i> -11		0	0	0	0	0.86	0.01	0.41	0.01	0	0	0	0
<i>cis</i> -12		0	0	0.20	0.01	0	0	0.21	0.02	0	0	0	0
<i>B. proteoclasticus</i> P-18‡													
18:0		3.32	0.02	19.06	0.80	3.46	0.07	13.70	0.19	3.33	0.05	34.47	1.02
<i>trans</i> -9		0	0	0.66	0.02	0	0	0.28	0.01	0.78	0.04	0.91	0.05
<i>trans</i> -10		40.54	3.15	13.89	1.51	0	0	0	0	0.38	0.07	0.71	0.07
<i>trans</i> -11		0	0	0	0	48.05	2.04	29.05	1.21	0	0	0.58	0.01
<i>trans</i> -12		0	0	0	0	0	0	0	0	0	0	0	0
<i>cis</i> -9		1.39	0.02	1.42	0.09	1.80	0.27	1.46	0.02	47.80	0.60	3.13	0.26
<i>cis</i> -10		1.21	0.03	0.34	0.02	0	0	0	0	0	0	0	0
<i>cis</i> -11		0	0	0.25	0.04	0.86	0.01	0.61	0.02	0	0	0.27	0.02
<i>cis</i> -12		0	0	0	0.80	0	0	0.10	0.02	0	0	0	0
<i>P. acnes</i> DSM 1897§													
18:0		3.32	0.02	3.41	0.04	0.32	0.04	0.46	0.02	0.30	0.01	0.41	0.03
<i>trans</i> -9		0	0	0	0	0	0	0	0	0.84	0.03	1.60	0.04
<i>trans</i> -10		40.54	3.15	28.88	1.36	0	0	0	0	0	0	0	0
<i>trans</i> -11		0	0	0	0	43.76	0.51	44.41	0.57	0	0	0	0
<i>trans</i> -12		0	0	0	0	0	0	0	0	0	0	0	0
<i>cis</i> -9		1.39	0.02	1.14	0.03	0.86	0.11	1.02	0.05	41.45	0.16	4.67	0.07
<i>cis</i> -10		1.21	0.03	1.16	0.03	0	0	0	0	0	0	0	0
<i>cis</i> -11		0	0	0	0	0.82	0.04	0.94	0.03	0	0	0	0
<i>cis</i> -12		0	0	0	0	0	0	0	0	0	0	0	0
10-O-18:0		0	0	6.07	0.89	0	0	0	0	0	0	29.16	3.40
10-OH-18:0		0	0	0.65	0.10	0	0	0	0	0	0	13.41	1.47

*Uninoculated medium.

†Final values determined in 24 h cultures, *n*=3.‡Final values determined in 30 h cultures, except *cis*-9-18:1, which was determined at 72 h, *n*=3.§Final values determined in 96 h cultures, *n*=3.

phase (Wallace *et al.*, 2006). Even though the CLA substrates were found to be impure, the occurrence of other fatty acids in these materials was not considered to influence the main conclusions of the study.

The different positional and geometric isomers of CLA are similar chemically and typically difficult to separate from each other (Christie *et al.*, 2001). They are also subject to racemization under relatively mild conditions (Christie *et al.*, 2001). Thus, although impurities were present in the substrates used here, it would in practice be difficult to improve upon their quality. Note that the impurities were

invariably geometric rather than positional isomers, and it is known that, for example, *B. fibrisolvens* produces several 9,11 geometric isomers, most likely by the same isomerization mechanism that converts linoleic acid to CLA (Kepler *et al.*, 1966; Wallace *et al.*, 2007). Thus, the reductases of these bacteria would typically be presented with several geometric isomers. Here, the *trans*-10,*cis*-12-18:2 preparation was heavily contaminated with *trans*-10,*trans*-12-18:2. It is possible that the contaminant inhibited the reduction of *trans*-10,*cis*-12-18:2 by *P. acnes*, but in view of the fact that no other CLA were reduced either, it seems more likely that *P. acnes* does not metabolize *trans*-10,*cis*-

Table 4. Enrichment in products of metabolism of CLA isomers and 18:1 fatty acids by ruminal *Butyrivibrio* spp. and *P. acnes* DSM 1897 grown in deuterium oxide-enriched medium

Data were obtained from 24 h cultures for *B. fibrisolvens*, 30 h cultures for *B. proteoclasticus* with *trans*-10-18:1 and *trans*-11-18:1 and 72 h cultures with *cis*-9-18:1, and 96 h cultures for *P. acnes*; the enrichment of D in water was 0.374.

Bacterium	Substrate	Product	Enrichment in M+1 (MPE)		Enrichment in M+2 (MPE)	
			Mean	SE	Mean	SE
<i>B. fibrisolvens</i>	<i>cis</i> -9, <i>trans</i> -11-18:2	<i>trans</i> -11-18:1	25.9	0.25	2.37	0.38
	<i>trans</i> -10, <i>cis</i> -12-18:2	<i>trans</i> -10-18:1	42.8	0.13	3.14	0.09
	<i>trans</i> -10, <i>cis</i> -12-18:2	<i>trans</i> -12-18:1	39.9	0.30	1.06	1.57
	<i>trans</i> -10, <i>cis</i> -12-18:2	<i>cis</i> -12-18:1	40.6	1.53	-11.0	4.49
	<i>trans</i> -9, <i>trans</i> -11-18:2	<i>trans</i> -11-18:1	25.4	0.60	-6.0	1.31
<i>B. proteoclasticus</i>	<i>trans</i> -10-18:1	18:0	42.0	0.19	22.6	0.08
	<i>trans</i> -11-18:1	18:0	39.9	0.18	23.8	0.50
	<i>cis</i> -9-18:1	18:0	43.1	0.16	19.5	0.25
<i>P. acnes</i>	<i>trans</i> -10-18:1	10-O-18:0	0	0	0	0
	<i>trans</i> -10-18:1	10-OH-18:0*	51.2	1.34	27.1	2.28
	<i>cis</i> -9-18:1	10-O-18:0	-4.6	4.84	-1.5	1.01
	<i>cis</i> -9-18:1	10-OH-18:0*	35.1	0.28	-1.7	0.44

*Estimate based on enrichment calculated for the abundant ion fragment at *m/z* 201 owing to the lack of molecular ion.

12-18:2 further. The main uncertainty is with *B. fibrisolvens*, where *trans*-10-18:1 was the main product, but *trans*-12-18:1 and *cis*-12-18:1 were also formed. It is impossible to say which 18:1 was derived from which CLA precursor. Otherwise, the results are not ambiguous.

The choice of initial fatty acid concentration is another important practical issue. If the concentration is too low, trace activities cannot be distinguished from significant ones and the results may be misleading. Many of the bacteria identified by Harfoot & Hazlewood (1997), on the basis of the metabolism of low-concentration, radiolabelled substrates, to play a role in biohydrogenation actually have very little activity (Maia *et al.*, 2007). On the other hand, high concentrations of polyunsaturated fatty acids (PUFAs) lead to inhibition of biohydrogenation, particularly the final step (Beam *et al.*, 2000; Troegeler-Meynadier *et al.*, 2003; Fievez *et al.*, 2007). In our experience, concentrations of 200 mg l⁻¹ and above of 18:2 and 18:3 fatty acids inhibit biohydrogenation. Such concentrations would be common in grazing animals (Dawson & Hemington, 1974; Fievez *et al.*, 2007). The concentration used here, 50 mg l⁻¹, enables activity to be determined and most products to be identified, while not presenting most of the inhibitory problems associated with higher concentrations.

The first ruminal bacterium found to carry out biohydrogenation of linoleic acid was *B. fibrisolvens* (Polan *et al.*, 1964). Further studies (Paillard *et al.*, 2007; Maia *et al.*, 2007) indicated that the high capacity to metabolize linoleic acid is associated entirely with the *Butyrivibrio* group, which includes the 18:0-producing *B. proteoclasti-*

cus (Moon *et al.*, 2008), previously named *C. proteoclasticum* (Wallace *et al.*, 2006), or *Fusocillus* sp. (Kemp *et al.*, 1975). These species produce mainly *cis*-9,*trans*-11-18:2 from linoleic acid, but also smaller concentrations of *trans*-9,*trans*-11-18:2 (Kepler *et al.*, 1966; Wallace *et al.*, 2007). Here it is shown that both 9,11 geometric isomers of CLA are metabolized to *trans*-11-18:1 by *B. fibrisolvens*. Although the concentrations of *cis*-9,*trans*-11-18:2 and *trans*-10,*cis*-12-18:2 prevented growth of *B. proteoclasticus*, previous reports (Wallace *et al.*, 2006; Maia *et al.*, 2007) using lower fatty acid concentrations tend to suggest that *B. proteoclasticus* is capable of converting CLA to 18:1 fatty acids in the same manner as *B. fibrisolvens*. Furthermore, *trans*-10,*cis*-12-18:2, which is known to exert anti-lipogenic effects in lactating ruminants (Lock *et al.*, 2006; Shingfield & Griinari, 2007; Shingfield *et al.*, 2009), is not metabolized further by the producing species, *P. acnes*, but is converted by *Butyrivibrio* spp. to a mixture of *trans*-10-18:1, *trans*-12-18:1 and *cis*-12-18:1. Early studies also demonstrated that *trans*-10,*cis*-12-18:2 is converted to *trans*-10-18:1 during incubation with pure cultures of *B. fibrisolvens* (Kepler *et al.*, 1966).

The microbial ecology of biohydrogenation seems likely not to be as simple as was once thought. Although much effort has been expended in studying biohydrogenating bacteria, their biochemistry and microbial ecology, the correlation between biohydrogenation activity in the rumen, fatty acid composition of ruminal or duodenal digesta and microbial ecology has been disappointingly weak (Boeckert *et al.*, 2008; Kim *et al.*, 2008), leading to some speculation that key species may not yet have been isolated, or indeed may be uncultivable (Boeckert *et al.*,

2008; Kim *et al.*, 2008). In particular, the species responsible for the changes in ruminal lipid metabolism resulting in *trans*-10-18:1 replacing *trans*-11-18:1 as the major intermediate remains unclear. *Megasphaera elsdenii* was identified as a possible candidate (Kim *et al.*, 2002), but no *M. elsdenii* has been found to metabolize linoleic acid in our laboratory, and only isolates of *P. acnes* have been demonstrated to exhibit this activity. *P. acnes* is one of the less numerous ruminal species, and it is questionable whether alterations in the growth of this species alone can account for the changes in ruminal fatty acid outflow known to occur in lactating cows fed diets containing low proportions of forage, high levels of starch or lipid supplements rich in PUFAs (Shingfield & Griinari, 2007). The present results do not resolve this issue, but they do illustrate the uniqueness and importance of bacteria such as *B. proteoclasticus* that are capable of metabolizing *trans*-10,*cis*-12-18:2 to 18:0 via *trans*-10-18:1. It had been shown previously (Hazlewood *et al.*, 1976) that *B. proteoclasticus* converted *trans*-11- and *cis*-9-18:1 to 18:0. Here we demonstrate that *trans*-10-18:1 is also hydrogenated. Thus, *B. proteoclasticus* and possibly other bacteria that catalyse the conversion of 18:1 intermediates to 18:0 play a vital role in determining the supply of fatty acids available for absorption and subsequent incorporation into ruminant meat and milk.

The mass spectra of deuterated DMOX esters provide some clues about the mechanisms responsible for the reduction of *cis* and *trans* double bonds. For example, although the mass spectra were not definitive, the results are consistent with the conclusion of Rosenfeld & Tove (1971) that the biohydrogenation of the *cis*-9 bond of *cis*-9,*trans*-11-18:2 occurs by a stereospecific *cis* addition to the D side of carbons 9 and 10. Metabolism of other CLA isomers has not been investigated previously. The novel information generated from this study indicates that the reduction of *trans*-9,*trans*-11-18:2 appears similar to that of *cis*-9,*trans*-11-18:2, while the reduction of *trans*-10,*cis*-12-18:2 follows a different pattern, as highlighted by the higher incorporation of deuterium from D₂O. Rosenfeld & Tove (1971) discovered that, when the *cis*-9 bond of *cis*-9,*trans*-11-18:2 was biohydrogenated, more deuterium was found on the C atom distal to the *trans* bond than on the adjacent C. Incorporation of label indicated that the same is also true for the reduction of the *cis*-12 bond of *trans*-10,*cis*-12-18:2. However, enrichment from D₂O was almost twice as high for the reduction of the *cis*-12 compared to the *cis*-9 double bond.

The formation of 10-O-18:0 from *trans*-10-18:1 and *cis*-9-18:1 by *P. acnes* may provide an insight into the synthesis of saturated oxo-fatty acids that are known to be incorporated into bovine milk fat (Brechany & Christie, 1992). Hudson *et al.* (1995) had already shown that hydration of oleic acid led to the formation of 10-OH-18:0 in *Selenomonas ruminantium* and *Enterococcus faecium*. Further studies revealed that ruminal digesta of sheep and

cows contained several strains capable of hydrating *cis*-9-18:1, the most numerous being isolates of *Streptococcus bovis* (Hudson *et al.*, 2000). Incubations of *cis*-9-18:1 with mixed rumen microbes resulted in the formation of 10-OH-18:0, with evidence that the hydroxyl group was further oxidized to yield 10-O-18:0 as a major end product (Jenkins *et al.*, 2006). Several bacteria have been identified as capable of converting *cis*-9-18:1 to 10-O-18:0, including *Staphylococcus* sp. (Lanser, 1993) and *Flavobacterium* sp. strain DS5 (Hou, 1994). The present data indicate that *P. acnes* is at least one bacterium in the rumen responsible for the hydration of *cis*-9-18:1 and further oxidation of 10-OH-18:0. *P. acnes* also metabolized *trans*-10-18:1 to 10-O-18:0 via 10-OH-18:0. Early studies have also demonstrated that an enzyme preparation from a pseudomonad converted *trans*-10-18:1 to 10-OH-18:0 (Mortimer & Niehaus, 1972). However, labelling of 10-OH-18:0 formed from both 18:1 substrates during incubations with *P. acnes* suggests that the mechanism of hydration of the *cis*-9 bond involves the incorporation of one deuterium atom from water, while the hydration of *trans*-10-18:1 results in two deuterium atoms being incorporated.

It is also notable that 18:0 formed during metabolism of 18:1 fatty acids was labelled in M+2, whereas 18:1 intermediates produced during the reduction of CLA isomers were not, which also suggests that the enzymic mechanisms are different. The latter mechanism may account for the ability of *B. proteoclasticus*, but no other known species, to reduce 18:1 fatty acids to 18:0 in the rumen. Morris (1970) concluded that the reduction of the *cis* bond of *cis*-9-18:1 was, like that of *cis*-9,*trans*-11-18:2, a stereospecific *cis* addition. Our data do not provide definitive information one way or the other on the mechanism responsible for the metabolism of 18:1 fatty acids, but the fact that all three gave the same pattern of M+1/M+2 enrichments suggests that the same enzymic mechanism may be involved.

In conclusion, there are likely to be three systems for the metabolism of unsaturated fatty acids in ruminal biohydrogenating bacteria (Fig. 1) – one that reduces geometric isomers of 9,11 CLA to *trans*-11-18:1, another that reduces 10,12 geometric isomers of CLA to a mixture of 10- and 12-18:1 fatty acids, and a third, only found in *B. proteoclasticus*, that reduces a range of monoenoic fatty acids, including *trans*-10-18:1, to 18:0. Our data also indicate that *P. acnes* hydrates 18:1 substrates to yield 10-O-18:0 as an end product. To our knowledge, this is the first time that the mechanisms of reduction of PUFAs and 18:1 fatty acids in ruminal bacteria have been analysed in this way. The results inform us about a biochemical process that has a major influence on human health, via the fatty acid composition of ruminant-derived products (Scollan *et al.*, 2001; Bauman *et al.*, 2005) and possibly, by extrapolation, to the similar reactions that occur in the human colon (Devillard *et al.*, 2007).

Fig. 1. Summary of our present understanding of the roles of *Butyrivibrio* spp. in general, *B. proteoclasticus* and *P. acnes* in the metabolism of dietary unsaturated fatty acids (linoleic and oleic acids) and their products. A dashed arrow denotes a minor route. The following observations should be considered alongside the flow chart. The fate of *cis*-12-18:1 is unknown. *Butyrivibrio* spp. other than *B. proteoclasticus* metabolize 18:1 fatty acids to products that have not been determined; the transformation occurs much less extensively than the conversion of 18:1 fatty acids to stearate by *B. proteoclasticus*. *Butyrivibrio* spp. form traces of other 9,11-18:2 isomers not shown here from linoleic acid.

ACKNOWLEDGEMENTS

The Rowett Institute of Nutrition and Health receives funding from the Rural and Environment Research and Analysis Directorate of the Scottish Government. We thank Frédéric Destailats, Nestlé, Lausanne, for the gift of *trans*-10-18:1. We also thank David Brown, Eric Milne and Donna Wallace for technical assistance and expertise. Grietje Holtrop, Gerald Lobley, Wendy Russell and Vesa Toivonen offered invaluable advice on mass spectral and isotopic analysis, for which the authors are extremely grateful.

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Edited by: H. L. Drake