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SHORT COMMUNICATION

Relation between phylogenetic position, lipid metabolism and butyrate production by different *Butyrivibrio*-like bacteria from the rumen

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Abstract The *Butyrivibrio* group comprises *Butyrivibrio fibrisolvens* and related Gram-positive bacteria isolated mainly from the rumen of cattle and sheep. The aim of this study was to investigate phenotypic characteristics that discriminate between different phylotypes. The phylogenetic position, derived from 16S rDNA sequence data, of 45 isolates from different species and different countries was compared with their fermentation products, mechanism of butyrate formation, lipid metabolism and sensitivity to growth inhibition by linoleic acid (LA). Three clear sub-groups were evident, both phylogenetically and metabolically. Group VA1 typified most *Butyrivibrio* and *Pseudobutyrivibrio* isolates, while Groups VA2 and SA comprised *Butyrivibrio hungatei* and *Clostridium proteoclasticum*, respectively. All produced butyrate but strains of group VA1 had a butyrate kinase activity <40 U (mg protein)$^{-1}$, while strains in groups VA2 and SA all exhibited activities >600 U (mg protein)$^{-1}$. The butyrate kinase gene was present in all VA2 and SA bacteria tested but not in strains of group VA1, all of which were positive for the butyryl-CoA CoA-transferase gene. None of the bacteria tested possessed both genes. Lipase activity, measured by tributyrin hydrolysis, was high in group VA2 and SA strains and low in Group VA1 strains. Only the SA group formed stearic acid from LA. Linoleate isomerase activity, on the other hand, did not correspond with phylogenetic position. Group VA1 bacteria all grew in the presence of

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200 μg LA ml^{-1}, while members of Groups VA2 and SA were inhibited by lower concentrations, some as low as 5 μg ml^{-1}. This information provides strong links between phenotypic and phylogenetic properties of this group of clostridial cluster XIVa Gram-positive bacteria.

Keywords Biohydrogenation · Linoleic acid · Rumen

Abbreviations

CLA conjugated linoleic acid
LA linoleic acid

Butyrivibrio fibrisolvens is a name that has been used to identify a genetically and functionally diverse group of motile, Gram-positive (though staining Gram-negative; Cheng and Costerton 1977) bacteria, isolated mainly from the rumen of cattle and sheep (Stewart et al. 1997). *B. fibrisolvens* is also present in the faecal flora of man, rabbits and horses (Wedekind et al. 1988; Hespell 1992; Rumney et al. 1995; Eckburg et al. 2005). All *B. fibrisolvens* strains are members of cluster XIVa of the *Clostridium* subphylum (Willems et al. 1996). The type strain, ATCC 19171, is actually atypical of the great majority of *B. fibrisolvens* isolates (Forster et al. 1996), which include species names *Butyrivibrio hungatei*, *Butyrivibrio crossotus*, *Clostridium proteoclasticum*, *Pseudobutyrivibrio ruminis* and *Pseudobutyrivibrio xylanivorans* (Moore et al. 1976; van Gylswyk et al. 1996; Kopečný et al. 2003; van de Vossenberg and Joblin 2003). The bacteria participate in several important functions in the rumen, including fibre breakdown (Hespell et al. 1987), fatty acid biohydrogenation (Polan et al. 1964; Wallace et al. 2006) and proteolysis (Hazlewood et al. 1983; Wallace and Brammall 1985; Strydom et al. 1986). To some extent, the fibrolytic activity of different strains/species is related to phylogenetic position, as are acetate production and lactate production (Kopečný et al. 2003). The aim of the present study was to understand how the metabolism of fatty acids by different strains/species is related to their phylogenetic position.

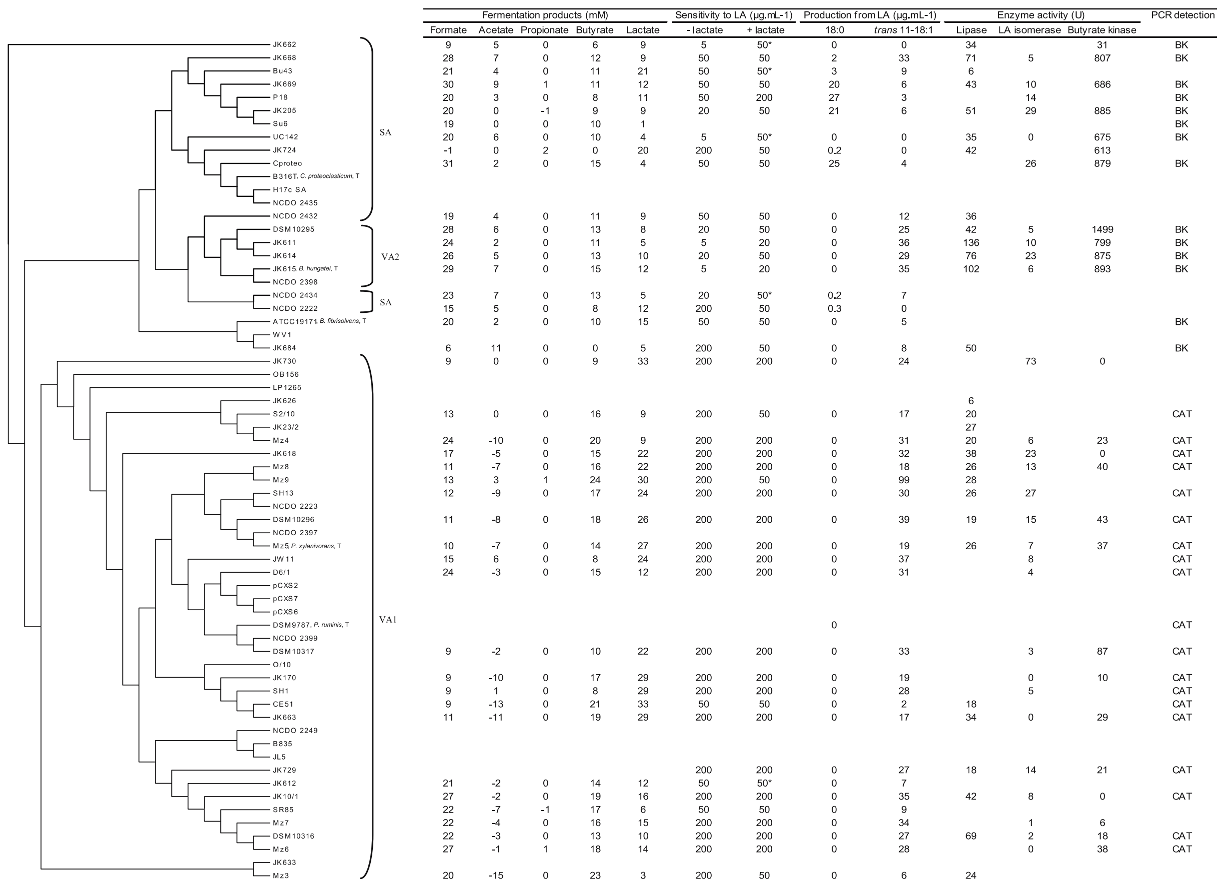
Bacterial strains were obtained from the Rowett collection (P-18, SH1, SH13 and JW11), purchased from culture collections or gifted by international colleagues. Most cultures were obtained from J. Kopečný and they are described in Kopečný et al. (2003). *C. proteoclasticum* 'C-proteo' was a gift from G.T. Attwood and is among the strains described by Attwood et al.(1996). Strain Su6 was a gift from K.N. Joblin and is described in van de Vossenberg and Joblin (2003). The bacteria were isolated from cattle and sheep in the United States, United Kingdom, Czech Republic, Slovenia, New Zealand and Australia.

Bacteria were grown on the liquid form of Hobson's medium M2 (Hobson 1969), sometimes modified by omitting sodium lactate. Sensitivity to linoleic acid (LA) was determined by inoculating 5% into both media containing different concentrations of LA and incubating for 96 h. A 10% inoculum was used in 50 μg ml^{-1} sodium lactate-free LA-containing medium to assess the products of biohydrogenation. Fermentation products were determined by capillary GC of esterified acids (Richardson et al. 1989) and the products of fatty acid metabolism by extraction of free fatty acids, methylation and separation by GC (Wąsowska et al. 2006). Butyrate kinase was measured by the acyl hydroxamate assay (Rose 1955) and LA isomerase by the release of conjugated LA, which absorbs at 233 nm (Kepler and Tove 1967). Lipase was measured by the hydrolysis of tributyrin, the rate of hydrolysis being calculated from the development of zones of clearance (Smeltzer et al. 1992). The assay was calibrated using lipase from *Candida rugosa*.

The phylogenetic relation between the strains and other *Butyrivibrio* sequences, a total of 64 strains, was based on full-length sequence analysis of 16S rDNA, as described by Wallace et al. (2006). Degenerate PCR was used to detect genes involved in butyrate production. For the preparation of PCR templates, a cell pellet from 1 ml of culture was resuspended in 50 μl sterile water. PCR reactions were performed with JumpStart Taq DNA Polymerase (Sigma) and dNTPs from Bioline according to the manufacturers' instructions, with 0.5 μl template in a total volume of 50 μl. Primers BUKfor1, BUKrev1 and PTBfor2 (Louis et al. 2004) were used for the detection of

the butyrate kinase and phosphotransbutyrylase genes, and primers CoATDF1 and CoATDR2 (Charrier et al. 2006) were used for the detection of the butyryl-CoA transferase gene. Amplification of the CoA-transferase fragment was performed with a ramped annealing approach as described previously (Charrier et al. 2006), whereas an extra annealing step at 35°C for 5 s was included for amplification of the butyrate kinase operon. Ten microliters of PCR reaction were run on a 1% agarose gel in TBE buffer and product sizes estimated from comparison with molecular weight marker hyperladder I (Bioline). Expected product sizes were approximately 771 bp for PTBfor2 and BUKrev1, 408 bp for BUKfor1 and BUKrev1 (based on the butyrate kinase operon from strain L2-50, Louis et al. 2004), and 582 bp for CoATDF1 and CoATDR2 (based on the butyryl-CoA CoA-transferase from *Roseburia* sp. A2-183, Charrier et al. 2006). Statistical analyses were performed by unpaired t-test using the Genstat 8th edition, Release 8.1 software (VSN International Ltd., Hemel Hempstead, Herts, UK). When data were not normally distributed and/or variances were unequal, the Mann-Whitney test was performed. The P-value was considered significant when ≤0.05.

Several phenotypic characteristics corresponded to the position of the different bacteria in the phylogenetic tree (Fig. 1). The clearest of



Strain	Fermentation products (mM)					Sensitivity to LA (µg.mL-1)		Production from LA (µg.mL-1)		Enzyme activity (U)			PCR detection
	Formate	Acetate	Propionate	Butyrate	Lactate	- lactate	+ lactate	18:0	*trans* 11-18:1	Lipase	LA isomerase	Butyrate kinase	
JK662	9	5	0	6	9	5	50*	0	0	34		31	BK
JK668	28	7	0	12	9	50	50	2	33	71	5	807	BK
Bu43	21	4	0	11	21	50	50*	3	9	6			
JK669	30	9	1	11	12	50	50	20	6	43	10	686	BK
P18	20	3	0	8	11	50	200	27	3		14		BK
JK205	20	0	-1	9	9	20	50	21	6	51	29	885	BK
Su6	19	0	0	10	1								BK
UC142	20	6	0	10	4	5	50*	0	0	35	0	675	BK
JK724	-1	0	2	0	20	200	50	0.2	0	42		613	
Cproteo	31	2	0	15	4	50	50	25	4		26	879	BK
B316T, *C. proteoclasticum*, T													
H17c SA													
NCDO 2435													
NCDO 2432	19	4	0	11	9	50	50	0	12	36			
DSM10295	28	6	0	13	8	20	50	0	25	42	5	1499	BK
JK611	24	2	0	11	5	5	20	0	36	136	10	799	BK
JK614	26	5	0	13	10	20	50	0	29	76	23	875	BK
JK615, *B. hungatei*, T	29	7	0	15	12	5	20	0	35	102	6	893	BK
NCDO 2398													
NCDO 2434	23	7	0	13	5	20	50*	0.2	7				
NCDO 2222	15	5	0	8	12	200	50	0.3	0				
ATCC19171, *B. fibrisolvens*, T	20	2	0	10	15	50	50	0	5				BK
WV1													
JK684	6	11	0	0	5	200	50	0	8	50			BK
JK730	9	0	0	9	33	200	200	0	24		73	0	
OB156													
LP1265													
JK626										6			
S2/10	13	0	0	16	9	200	50	0	17	20			CAT
JK23/2										27			
Mz4	24	-10	0	20	9	200	200	0	31	20	6	23	CAT
JK618	17	-5	0	15	22	200	200	0	32	38	23	0	CAT
Mz8	11	-7	0	16	22	200	200	0	18	26	13	40	CAT
Mz9	13	3	1	24	30	200	50	0	99	28			
SH13	12	-9	0	17	24	200	200	0	30	26	27		CAT
NCDO 2223													
DSM10296	11	-8	0	18	26	200	200	0	39	19	15	43	CAT
NCDO 2397													
Mz5, *P. xylanivorans*, T	10	-7	0	14	27	200	200	0	19	26	7	37	CAT
JW11	15	6	0	8	24	200	200	0	37		8		CAT
D6/1	24	-3	0	15	12	200	200	0	31		4		CAT
pCXS2													
pCXS7													
pCXS6													
DSM9787, *P. ruminis*, T								0					CAT
NCDO 2399													
DSM10317	9	-2	0	10	22	200	200	0	33		3	87	CAT
O/10													
JK170	9	-10	0	17	29	200	200	0	19		0	10	CAT
SH1	9	1	0	8	29	200	200	0	28		5		CAT
CE51	9	-13	0	21	33	50	50	0	2	18			CAT
JK663	11	-11	0	19	29	200	200	0	17	34	0	29	CAT
NCDO 2249													
B835													
JL5													
JK729						200	200	0	27	18	14	21	CAT
JK612	21	-2	0	14	12	50	50*	0	7				
JK10/1	27	-2	0	19	16	200	200	0	35	42	8	0	CAT
SR85	22	-7	-1	17	6	50	50	0	9				
Mz7	22	-4	0	16	15	200	200	0	34		1	6	
DSM10316	22	-3	0	13	10	200	200	0	27	69	2	18	CAT
Mz6	27	-1	1	18	14	200	200	0	28		0	38	CAT
JK633													
Mz3	20	-15	0	23	3	200	50	0	6	24			

Fig. 1 Phylogenetic position and fatty acid metabolism by different bacteria related to *Butyrivibrio fibrisolvens*. The tree is indicative only; the precise genetic distances can be found in Wallace et al. (2006). U—units of enzyme activity, which were nkat ml^{-1} for lipase activity, nmol min^{-1} mg protein^{-1} for linoleate isomerase activity and µmol min^{-1} mg protein^{-1} for butyrate kinase activity. BK—positive for butyrate kinase in degenerate PCR. CAT—positive for butyryl acyl-CoA CoA-transferase in degenerate PCR. *Lowest concentration tested. Where no entry is made, the strain was not tested for that property. Negative values for acetate concentration mean net uptake of acetate. Results are means obtained with three different cultures

all was the mechanism of butyrate formation, based on both the measurement of butyrate kinase activity and on butyrate kinase and butyryl-CoA CoA-transferase gene detection. The phylogenetic tree (Fig. 1) formed three main groups and a number of less closely related bacteria. The type strain *B. fibrisolvens* D1^T (ATCC 19171) was among the outliers. One group, containing *C. proteoclasticum* B316^T, produced stearic acid from LA (see below). This group (SA; stearic acid-producing) had high butyrate kinase activity and was without exception positive for the butyrate kinase gene and negative for butyryl-CoA CoA-transferase. A related group, which included the type strain of *B. hungatei* JK615^T, had similar characteristics except that they did not produce stearate (group VA2, vaccenic acid-producing). The most numerous group was named VA1 as its members also produced vaccenic acid (*trans*-11–18:1) from LA but they were genetically different from VA2. All VA1 strains had significantly lower butyrate kinase activity compared to the others ($P < 0.001$) and were positive for butyryl-CoA CoA-transferase in degenerate PCR. The type strain *B. fibrisolvens* D1^T was positive for the butyrate kinase gene and negative for butyryl-CoA CoA-transferase. The last observation corresponds with that made by Diez-Gonzalez et al. (1999). However the relationship between lactate production and the enzymic mechanism of butyrate formation made by Diez-Gonzalez et al. (1999) was not sustained here. As found with human faecal butyrate producers (Louis et al. 2004), butyrate kinase had a limited distribution among the bacteria tested. The ruminal butyrate producers had butyrate kinase or butyryl-CoA CoA-transferase, but not both, similar to the findings of Diez-Gonzalez et al. (1999). The present data indicate strongly that butyrate kinase is a strong taxonomic criterion for distinguishing the *B. hungatei* and *C. proteoclasticum* groups from related bacteria.

The concentration of fermentation products produced also corresponded well with phylogenetic position (Fig. 1). Acetate tended to be used during growth of VA1 strains, while small amounts were produced during growth of VA2 and SA bacteria (VA1 versus VA2 and SA: $P < 0.001$). Lactate production was generally greater with the VA1 group ($P < 0.001$), but growth was generally more abundant with these strains. Formate production was generally less with the VA1 strains ($P = 0.012$). Van der Toorn and Van Gylswyck (1985) found three biotypes of *Butyrivibrio*, one which grew with net production of acetate, another which showed net uptake of acetate, and a third which produced 1 mM propionate. The first may therefore correspond to SA and VA2 and the second to VA1. Although it was not demonstrated, we suspect that the propionate formed by strain JK724 may have resulted from contamination.

Lipid metabolism corresponded well with phylogenetic position in terms of lipase activity, biohydrogenation products and sensitivity to growth inhibition by fatty acids, but not linoleate isomerase activity. With only a couple of exceptions, lipase activity was greater in VA2 and SA than VA1 strains ($P = 0.002$). Virtually all strains across the entire tree formed vaccenic acid (*trans*-11–18:1) from LA (SA versus others: $P < 0.001$). Those that did not either grew very weakly or formed *cis*-9,*trans*-11–18:2. Only SA bacteria produced stearate (SA versus others: $P < 0.001$). Strain Su6 was not tested here but was shown by van de Vossenberg and Joblin (2003) to produce stearate. VA1 strains grew after 96 h in 200 $\mu g\ ml^{-1}$ LA, but VA2 and SA strains were much more sensitive ($P < 0.001$). If sodium lactate was added to the medium, the growth of some SA strains became sensitive to LA concentrations as low as 5 $\mu g\ ml^{-1}$. Fukuda et al. (2005) found that the linoleate isomerase activity of 5 strains of *B. fibrisolvens* varied and that the tolerance of the strains to LA in the medium corresponded to linoleate isomerase activity. The same relationship was not found here in a larger number of strains.

It can be concluded from these results that groups SA and VA2 are genetically and phenotypically distinct from VA1 and other related butyrate-producing bacteria. The only phenotypic characteristic separating SA and VA2 is stearate production from LA. It is tempting to speculate that the possession of butyrate kinase rather than butyryl-CoA CoA-transferase makes the VA2 and SA group strains more vulnerable to the toxic effects of unsaturated fatty acids than the VA1 strains.

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