Analysis of bacterial phospholipid markers and plant monosaccharides during forage degradation by *Ruminococcus flavefaciens* and *Fibrobacter succinogenes* in co-culture

LILIANA SALUZZI,¹ ALISTAIR SMITH² and COLIN S. STEWART^{1*}

¹Rowett Research Institute, Bucksburn, Aberdeen AB2 9SB, UK

²The Macaulay Land Use Research Institute, Craigiebuckler, Aberdeen AB9 2QJ, UK

(Received 25 March 1993; revised 25 May 1993; accepted 7 June 1993)

Marker components of the phospholipids of *Ruminococcus flavefaciens* and *Fibrobacter succinogenes* were identified for studies on the degradation of forage by these bacteria growing in mixed culture. The principal fatty acid methyl esters and dimethyl acetals detected varied between strains and were influenced by the addition of a mixture of higher volatile fatty acids and vitamins to the medium, but these effects were small compared to the differences between the species. When two strains of *R. flavefaciens* were grown on a mixture of clover and ryegrass, and on barley straw in the presence or absence of two strains of *F. succinogenes*, the solubilization of plant material tended to be lowered by the presence of *F. succinogenes*. *R. flavefaciens* was the predominant bacterium among colonies recovered from roll tubes, and the phospholipids were primarily those of *R. flavefaciens*. Analysis of the culture supernatant liquids showed that *F. succinogenes* produced greater amounts of free and bound xylose from both clover and straw than did *R. flavefaciens*. With both forages, cultures containing the two species produced more soluble free arabinose, and less soluble-bound arabinose, than either species grown alone.

Introduction

Interactions between cellulolytic micro-organisms from the rumen and from other habitats have mainly been monitored by comparing the cellulolytic or plant cell-wall-degrading activity of pure and mixed cultures in which the species are represented by single selected strains (Dehority & Scott, 1967; Stewart & Walsh, 1972; Miron, 1991; Irvine & Stewart, 1991; Bernalier *et al.*, 1992). Assessing the contribution of different bacteria to the activity of mixed cultures would be helped if the relative contributions of the component species to the microbial biomass were known.

The analysis of microbial lipids has been used for the estimation of the relative proportions of microbial species and groups in natural mixed microbial communities (Bobbie & White, 1980; McKinley & Vestal, 1984; Vestal & White, 1989). Although the composition of the lipids, including phospholipids and plasmalogens, of many rumen bacteria has been determined (Verkely *et al.*, 1975; Miyagawa, 1982; Watanabe *et al.*, 1982), little

attempt has so far been made to use lipid markers for the analysis of mixed cultures of rumen organisms.

The main aim of this study was to identify potential markers among the alkyl and alk-1-enyl components of the phospholipids of strains of *F. succinogenes* and *R. flavefaciens*, and to study the effect of the presence of *F. succinogenes* on the fermentation of fibrous substrates by *R. flavefaciens*. To reduce possible bias resulting from strain selection, and for a closer approximation to natural conditions, two strains of the relevant species (the type strain and one other) were used simultaneously in the present study. The growth substrates, a mixture of white clover and ryegrass harvested from a mixed sward, and a pure sample of barley straw, were selected as representative of mixed legume/grass pasture forage and cereal straw respectively, as such plants are known to differ in their chemical composition and susceptibility to attack by rumen bacteria (Varel *et al.*, 1989).

Methods

Bacteria. The type strains, FD1 of *Ruminococcus flavefaciens* and S85 of *Fibrobacter succinogenes* were obtained from M. P. Bryant, University of Illinois, Urbana, IL, USA. The isolation and characteristics of *F. succinogenes* strain BL2 was as described by Stewart *et al.* (1981) and *R. flavefaciens* strain 17 was isolated and characterized by Flint *et al.* (1989).

* Author for correspondence. Tel. +44 224 716 654; fax +44 224 715 349.

Abbreviations: DMA, dimethyl acetal; FAME, fatty acid methyl ester; i, iso; ai, anteiso.

Anaerobic methods and viable counts. Media were prepared and maintained under anaerobic conditions using O_2 -free CO_2 as described by Bryant (1972). The bacteria were maintained using medium M3, a modification of medium M2 of Hobson (1969) from which lactate was omitted, contained in 16×125 mm glass Hungate tubes sealed with butyl rubber septum stoppers (Bellco Glass). For some tests referred to in the text, the medium was supplemented with vitamins and volatile fatty acids. The vitamin solution contained (per 100 ml distilled H_2O): 15 mg pyridoxamine, 5 mg (each) folic acid, riboflavin and thiamine, 3 mg *p*-aminobenzoic acid (PABA), and 1 mg (each) biotin and cobalamin. The VFA solution contained (per 100 ml distilled H_2O) 4 ml *n*-butyric acid and 1 ml (each) *n*-valeric, iso-valeric, iso-butyric and DL-2-methylbutyric acids. The pH of this solution was adjusted to 7.5 with NaOH. Viable counts were performed (triplicate) in roll tubes of medium M3, by the method of Hungate (1969). Counts of bacteria detached from clover or straw were carried out following detachment with methylcellulose at a final concentration of 0.15% (w/v) (Product 29779, high degree of substitution, Merck) and chilling, as described by Minato & Suto (1981). The proportions of *R. flavefaciens* and *F. succinogenes* present in the mixed cultures was determined by the examination of Gram-stained smears of (on average) 25 colonies picked from roll tubes which received the highest countable dilutions of samples from each experimental flask. Strains 17 and FD1 of *R. flavefaciens* were distinguished on the basis of the difference in the average number of cells in the chains, in the intensity of their Gram-staining reaction, differences in their ability to solubilize cotton fibres (Stewart *et al.*, 1981) and differences in the composition of their phospholipids, as described in the text. All culture incubations were at $38 \pm 1^\circ C$.

Growth of bacteria on clover and barley straw. Winter barley straw (*Hordeum vulgare*) was the untreated straw used by Kolankaya *et al.* (1985). The mixture of white clover (*Trifolium repens*) var. Kersey (62% of dry matter) and ryegrass (38% of dry matter) was from a mixed sward harvested at the Scottish Agricultural College, Aberdeen. The forages were hammer-milled and fractionated by dry-sieving (Endecotts Filters) to a particle size of 212 to 425 μm . To reduce potential interference from plant lipids, lipids were extracted from the forages before incubation with bacteria. Extraction was performed (twice) by boiling the forages under reflux with chloroform:methanol [2:1, v/v; 30 ml (g sample) $^{-1}$] for 1 h as recommended by Christie (1982). After extraction, residual solvent was removed by air drying. Plant material (3.0 g) was added to 1000 ml capacity Duran Bottles (Gallenkamp) which received 600 ml medium M3 from which sugars were omitted. The bottles were fitted with ports for inoculation constructed from Hungate tubes (above) from which the sealed end had been cut. The inocula were pre-grown for 18–24 h on medium M3 modified to contain cellobiose (0.25% w/v) as sole energy source. The inoculum volume was 5% of the culture volume. The results reported here are the pooled observations from three separate experiments. To standardize the inoculum between experiments as far as possible, the relationship between OD_{650} and culture counts was determined for all four strains growing on medium M3, and the OD_{650} of the inoculum was adjusted to 0.35 for *R. flavefaciens* and 0.9 for *F. succinogenes*.

Isolation and analysis of bacterial phospholipids. Bacteria from liquid supernatants or detached from plant material with methylcellulose were washed twice in distilled H_2O , recovered by centrifugation (20000 g, 20 min) and resuspended in a known volume of distilled H_2O . Lipids were extracted by the method of Folch *et al.* (1957) with some modifications. Methanol was added to a final ratio methanol: H_2O of 1:33:1 (v/v). The pellets were homogenized for 1 min using an IKA Ultra-Turrax T25 homogenizer (Janke & Kunkel). A volume of chloroform, double that of the methanol, was added and the homogenization repeated for 2 min. The phases were separated by centrifugation. The interfacial material and the glass sample container

were washed twice with chloroform and, together with the lower phase containing the extracted lipids, the washings were filtered through a bed of anhydrous Na_2SO_4 on Whatman IPS filter paper which had been soaked previously with chloroform. The solvent was removed in a rotary film evaporator at $40^\circ C$ and the total lipid fraction was dried azeotropically three times under nitrogen after adding methanol to remove traces of H_2O . Lipids from bacteria attached to plant material were extracted by the same method except that after the first extraction with solvent, the extract was filtered through Whatman no. 1 filter paper and the solid residue resuspended in chloroform:methanol (2:1, v/v). The extraction was repeated. After filtering and washing the residues, the extracts were pooled, H_2O added and the procedure continued as described above.

Lipid classes were separated by adsorption column chromatography using Si Silica Bond Elut columns (Analytichem International). After conditioning the columns with methanol and chloroform, neutral lipids were removed under vacuum with three column volumes of chloroform, glycolipids with chloroform:acetone (1:1, v/v), and phospholipids and plasmalogens with methanol. Thin layer chromatography (Whatman Silica Gel KS 150 A plates, 250 μm) was used to verify the purity of the lipid fractions obtained (Christie, 1982). The plates (normally 20×20 cm) were developed with a mixture of chloroform:methanol: H_2O (65:25:4, by vol.). After drying the plates in air, further separation of free fatty acids and neutral lipids from phospholipids was obtained whilst developing the plates with a solution of 10% (v/v) diethyl ether in petroleum ether plus formic acid [10 drops (100 ml) $^{-1}$]. Glycolipids were detected by developing the plates using acetone:acetic acid: H_2O (100:2:1, by vol.). Fatty acid methyl esters (FAMES) and dimethyl acetals (DMAs) were prepared by acid-catalysed transesterification of phospholipids. The pure phospholipid fraction, dissolved in tetrahydrofuran, was heated at $100^\circ C$ in sealed tubes under N_2 with 5% methanolic HCl [10 ml (mg lipid) $^{-1}$] for 1.5 h (Christie, 1982). Water was added to the cooled samples and the derivatives were extracted with hexane, followed by diethyl ether. The extracts were pooled, washed twice with distilled H_2O and dried with anhydrous Na_2SO_4 . FAMES and DMAs were analysed by capillary GC, using a WCOT fused silica column (50 m length, 0.25 mm i.d.) coated with a polar liquid phase CP-sil-88 (film thickness 0.21 μm ; Chrompack). Injector and flame ionization detectors were at $280^\circ C$. Helium (0.5 ml min^{-1}) was the carrier gas. The column was operated at $150^\circ C$ for 4 min followed by a $4^\circ C min^{-1}$ rise to $220^\circ C$. Injection was performed in split mode. The relative retention time (RRT) of the peaks was compared with that of 2-methylhexadecanoate, synthesized at the Rowett Institute. Peaks were identified by comparison of the RRT with those of standards (Sigma; Supelchem). Peak areas of the chromatographic components determined by disk integration were calculated using an HP 3390A integrator (Hewlett Packard). Further identification of components for which standards were not commercially available was by GC-MS.

Numerical analysis of phospholipid profiles. Similarities were calculated with the Bravais–Pearson coefficient of linear correlation (Drucker, 1976). The square roots of the data were used to reduce the influence of the larger peaks on the analysis.

Chemical analysis of plant residues. The residues remaining after fermentation were recovered by filtration (Whatman no. 1 filter paper), washed twice with distilled H_2O , and lyophilized before weighing. Samples were hydrolysed in 12 M- H_2SO_4 for 30 min at $25^\circ C$ with regular vortex mixing, then at $100^\circ C$ for 2 h after dilution to 1 M- H_2SO_4 (Hoebler *et al.*, 1989). Aliquots of the hydrolysates were neutralized, reduced with sodium borohydride in dilute ammonia solution, extracted and analysed by GC using β -D-allose as internal standard (Englyst & Cummings, 1984).

Sugars in culture supernatants were analysed in the same way following clarification with zinc acetate in acetic acid (Carrez I) and

Table 1. *Phospholipid DMA and FAME profiles of strains S85 and BL2 of F. succinogenes and strains 17 and FD1 of R. flavefaciens grown in medium M3 with and without the addition of volatile fatty acids and vitamins (M3+VV)*

Results expressed as the percentage of the area of each peak in relation to the total area of peaks detected in duplicate samples. The average coefficient of variation was 0.19. —, component not detected or present at less than 0.1%.

Species:	<i>F. succinogenes</i>				<i>R. flavefaciens</i>			
Strain:	S85		BL2		17		FD1	
Medium:	M3	M3+VV	M3	M3+VV	M3	M3+VV	M3	M3+VV
DMA								
11:0	0.3	0.2	—	—	—	—	—	—
12:0	5.7	2.8	1.6	0.9	—	—	—	—
13:0	3.9	3.3	6.4	5.6	—	—	—	—
14:0	6.9	3.9	3.9	1.8	0.4	0.4	0.5	0.4
15:0	0.7	0.9	0.3	0.2	0.8	0.7	1.3	1.0
16:0	0.5	0.5	—	—	11.1	9.3	12.2	9.8
17:0	—	—	—	—	0.6	0.3	0.3	1.0
18:0	—	—	—	—	5.1	4.4	0.6	0.9
ai13:0	11.2	9.1	7.3	10.1	0.3	0.3	0.4	0.2
ai15:0	1.7	1.6	1.4	1.3	0.4	0.3	0.7	1.0
ai17:0	—	—	—	—	1.6	1.3	2.5	3.3
i12:0	1.0	0.7	0.6	0.4	—	—	—	—
i13:0	0.1	0.2	—	—	—	—	—	—
i14:0	3.5	4.5	5.1	3.3	0.1	0.1	0.3	—
i15:0	—	—	—	—	2.5	5.1	8.7	15.8
i17:0	0.2	0.1	—	—	10.9	14.1	6.1	8.4
FAME								
13:0	1.4	1.0	2.1	3.6	0.1	—	0.1	—
14:0	13.2	9.9	16.8	9.3	1.0	0.7	1.2	0.9
15:0	11.1	12.1	22.0	29.8	1.3	0.7	1.6	0.9
16:0	20.0	23.6	12.8	9.1	9.8	6.1	12.3	8.3
17:0	3.9	4.8	1.4	1.9	0.3	0.1	0.3	—
18:0	2.8	2.4	0.7	0.5	1.8	1.2	1.2	1.0
ai15:0	5.4	8.1	6.9	13.6	3.0	1.9	4.7	4.3
ai17:0	2.0	2.9	0.8	1.4	4.5	2.2	3.0	2.3
i14:0	2.1	2.1	5.9	4.5	0.1	0.2	0.3	0.2
i15:0	—	0.7	—	—	29.3	38.9	37.0	36.4
i16:0	1.3	3.2	3.9	2.5	4.2	1.8	1.5	1.4
i17:0	0.3	0.7	—	0.2	10.5	8.9	3.0	2.6
i18:0	0.4	0.3	—	—	0.3	0.3	—	—

potassium ferricyanide (Carrez II) solutions (Feeding Stuffs Regulations, 1982) to remove protein and other sources of interference.

Results

Phospholipid content and identification of marker components

Analysis of freeze-dried cells of strains of *R. flavefaciens* (Gram-positive) and *F. succinogenes* (Gram-negative) revealed that the total phospholipid content (percentage of dry matter) of *F. succinogenes* (5.4 ± 0.4 , $n = 8$) was more than twice that of *R. flavefaciens* (2.1 ± 0.3 , $n = 8$). To determine the possible effect of either cross-feeding of or competition for trace nutrients in the mixed cultures, the phospholipid content of the strains was determined in the presence and absence of vitamin and VFA supplements in the medium. Neither the strain identity

nor these additions to the medium markedly affected the phospholipid content of the cells (data not shown).

When grown on medium M3 with or without the addition of vitamins and VFA, there were a number of marked differences between the species in the relative proportions of the individual FAMES and DMAs detected, and in both species, some variation between strains (Table 1). In general, the two strains of *R. flavefaciens* contained a higher proportion of DMAs 16:0, i15:0 and i17:0, and a lower proportion of DMAs ai13:0, 13:0, i14:0 and 14:0 than *F. succinogenes*. The *R. flavefaciens* strains contained more FAMES i15:0 and i17:0, and a lower proportion of FAMES 14:0 and 15:0 than *F. succinogenes*. The addition of vitamins and VFA to the culture medium produced changes in the relative proportions of some of the DMA and FAME components of the two species, but these changes were

Table 2. Correlation between the phospholipid profiles of *R. flavefaciens* (strains 17 and FD1) and *F. succinogenes* (strains S85 and BL2) cells in the inoculum (grown on cellobiose) or recovered following growth on clover or straw for different incubation times

Results are expressed as the coefficient of linear correlation between the average profile of the same species grown on the different substrates.

Incubation time (d)	<i>R. flavefaciens</i>			<i>F. succinogenes</i>		
	Clover vs straw	Inoculum vs clover	Inoculum vs straw	Clover vs straw	Inoculum vs clover	Inoculum vs straw
2	0.99	0.92	0.95	0.99	0.95	0.93
3	0.99	0.92	0.95	1.00	0.92	0.91
7	0.99	0.96	0.98	0.98	0.94	0.91

generally small in relation to the differences between the species. Thus, the addition of vitamins and VFA to the medium led to a decrease in the proportion of DMAs 12:0, 14:0 and i12:0, and FAME 14:0 and an increase in the proportion of FAME ai15:0 in the phospholipids of both strains of *F. succinogenes*. The proportion of FAMES 15:0 and 16:0 in strain BL2, and of FAMES 16:0 and 17:0 in strain S85 were also modified by the addition of vitamins and VFA to the medium. The addition of vitamins and VFA increased the proportions of DMAs i15:0 and i17:0 in both strains of *R. flavefaciens*, and influenced the proportion of FAMES i15:0 and ai17:0 in the phospholipids of strain 17. The data in Table 1 also suggested that the two strains of *R. flavefaciens* could be distinguished on the basis of the differences in the relative proportions of DMAs i15:0 and i17:0, and FAME i17:0 present.

Following analysis of the phospholipid FAMES and DMAs in the biomass detached from the plant material with methylcellulose, coefficients of correlation were calculated to establish whether the composition of the phospholipids of either *R. flavefaciens* or *F. succinogenes* was markedly affected by either the growth substrate or the duration of incubation. This showed that the composition of the *R. flavefaciens* and *F. succinogenes* populations was relatively little affected by these variables, despite the fact that for each species, two strains were present (Table 2).

The phospholipid markers for calculation of the relative biomass of the two species in mixed cultures were chosen on the basis that the marker occurred in concentrations of less than 1% in the second species and none of the markers were present at concentrations of more than 1% of the residual lipids in the plant material and nutrient medium. The DMAs ai13:0, 13:0, i14:0 and 14:0 were selected as markers for *F. succinogenes*, and DMAs i15:0, 16:0, i17:0 and 18:0, and FAME i15:0 were selected for *R. flavefaciens*. Although 18:0

Fig. 1. Total viable cell counts recovered from cultures inoculated with *R. flavefaciens* and *F. succinogenes* and grown on clover/ryegrass (□) or barley straw (●). Bars indicate SD.

DMA was detected in only small amounts in *R. flavefaciens* strain FD1 in the experiment shown in Table 1, this component was routinely detected in this strain subsequently, constituting around 3.4% of the DMA.

Mixed cultures – biomass analysis and viable counts

When *R. flavefaciens* strains 17 and FD1 were grown together in the presence of *F. succinogenes* strains S85 and BL2, the viable counts and examination of colonies picked from the roll tubes suggested that *R. flavefaciens* quickly became predominant. Thus, *F. succinogenes* contributed 59% of the colonies recovered after 1 d incubation on clover, and none of the colonies recovered after 2, 3 or 7 d incubation on clover. Of the colonies recovered from cultures grown on straw, *F. succinogenes* contributed 13% after 2 d incubation, none after 3 d incubation, and 20% after 7 d incubation. The viable counts from the cultures inoculated with both species, corrected for the number of cells in the chains of the ruminococci, are shown in Fig. 1. The average ($n = 50$) chain length was 16.8 ± 10.6 cells for strain FD1, and

Fig. 2. Relative proportions of *R. flavefaciens* (○) and *F. succinogenes* (■) in mixed culture at different stages of incubation on clover/ryegrass (a) or barley straw (b). Results are presented as the percentage of the total biomass from phospholipid analysis (—), and as the percentage of total viable cells recovered (···). Bars indicate SD.

4.8 ± 2.4 for strain 17. Particularly when clover was the substrate, cultures inoculated with ruminococci alone and incubated for 2 or 3 d tended to contain more recoverable viable cells than those shown in Fig. 1 (between 3.6×10^9 and 1.5×10^{10} cells ml^{-1}), though this tendency was not always statistically significant. No significant increase was found in the viable numbers of *F. succinogenes* recovered from either clover or straw during the incubations, the viable counts ranging from 5×10^7 to 5×10^8 ml^{-1} during the first 3 d incubation with either substrate, then falling to between 2×10^7 and 4×10^7 ml^{-1} at 7 d. However, this species degraded both clover and straw (Fig. 3 below). When Gram-stained smears of the ruminococci picked from the highest countable dilutions were examined, the majority of the colonies (75% for clover and 56% for straw) were found to belong to strain 17. No attempt was made to distinguish between the two strains of *F. succinogenes*: neither strain formed chains

Fig. 3. Solubilization of dry matter (%) of clover/ryegrass (a) and barley straw (b) by *R. flavefaciens* (●), *F. succinogenes* (■) and the co-culture containing both species (○) at different stages of incubation. Bars indicate SD.

or clumps and they could not readily be differentiated by microscopic examination.

The analysis of the phospholipids in the bacterial population detached from the plant material using methylcellulose suggested that with both clover and straw, *R. flavefaciens* became predominant in the early stages of the incubations despite forming a smaller proportion of the biomass in the inoculum than *F. succinogenes*, the attempts to standardize the inoculum having failed to ensure precisely matching numbers of the two species (Fig. 2a, b). The relative proportions of each species in the cultures containing both *R. flavefaciens* and *F. succinogenes* shown in Fig. 2 were calculated by comparing the fatty acid profiles with those of the cultures containing single species incubated with the same forage for the same time. The estimates of the relative viable numbers of the two species in mixed culture (dotted lines in Fig. 2a, b) followed a similar pattern to the relative proportions of the bacteria in the biomass calculated from analysis of phospholipids.

Table 3. Fate of monosaccharides of clover/ryegrass and barley straw following incubations with *R. flavefaciens* strains 17 and FD1, *Fibrobacter succinogenes* strains S85 and BL2, and cultures containing all four strains

Results are the means of two replicates. The average coefficient of variation was 0.16.

Fraction	Starting amount (mg)	Amount remaining after incubation for 7 d (mg)		
		<i>R.</i>	<i>F.</i>	Co-culture
		<i>flavefaciens</i>	<i>succinogenes</i>	
Clover				
Arabinose				
insoluble	86.9	14.4	21.1	14.8
soluble monosacc.	8.6	33.9	25.6	57.6
soluble-bound	30.6	79.2	91.5	54.6
Total	126.1	127.5	138.2	127.0
Xylose				
insoluble	233.7	43.4	96.7	60.6
soluble monosacc.	19.3	1.1	9.4	0.7
soluble-bound	0.0	50.4	109.1	63.8
Total	253.0	94.8	215.2	125.1
Glucose				
insoluble	740.0	100.5	199.3	141.4
soluble monosacc.	37.7	0.4	1.1	1.4
soluble-bound	382.2	234.6	264.6	152.7
Total	1159.9	335.5	465.0	295.5
Straw				
Arabinose				
insoluble	132.2	47.8	87.5	88.8
soluble monosacc.	5.9	34.9	22.7	52.9
soluble-bound	44.9	36.0	32.8	18.7
Total	183.0	118.7	143.0	160.4
Xylose				
insoluble	929.5	417.1	638.8	583.9
soluble monosacc.	16.1	1.8	23.4	1.8
soluble-bound	0.0	76.3	203.8	70.2
Total	942.6	495.2	866.0	655.9
Glucose				
insoluble	1505.0	899.5	1224.2	1096.4
soluble monosacc.	40.9	1.1	0.7	1.5
soluble-bound	403.3	143.7	94.7	84.4
Total	1949.2	1044.3	1319.6	1182.3

To check whether the process of detaching cells with methylcellulose favoured the detection of one or other of the species, a few analyses were performed of bacterial phospholipids associated with the residual plant material after 3 d incubation following its treatment with methylcellulose. Analysis of these samples (data not shown) suggested that the population which remained adherent during this treatment also consisted predominantly of *R. flavefaciens*, in that for both clover and straw, the phospholipids extracted from the plant residues were very largely (97–98%) those of *R. flavefaciens*.

Analysis of plant material

After incubation with plant material for up to 7 d, it was clear that the *R. flavefaciens* strains were more active than the strains of *F. succinogenes* in solubilizing clover

(Fig. 3*a, b*). When straw was the substrate, the effect of the presence of *F. succinogenes* varied according to the incubation time, but from 3 d onwards tended to depress solubilization (Fig. 3*b*). The negative effect of the presence of *F. succinogenes* was also evident from the analysis of the insoluble plant residues. *R. flavefaciens* removed more of the major monosaccharides, glucose, arabinose and xylose from both forages than did *F. succinogenes* or the cultures containing both species (Table 3). However, the fate of soluble monosaccharides, and of soluble compounds from which monosaccharides could be released by acid hydrolysis (di- or oligosaccharides, or glycosides, shown as 'soluble-bound' in Table 3) showed some contrasting trends. Glucose was largely removed from the monosaccharide pool in all of the cultures. When grown on clover, the mixed culture containing both bacterial species tended to be more

effective in removing glucose from the soluble-bound pool than either of the pure cultures. Arabinose accumulated in the monosaccharide pool of all cultures, but for both forages this accumulation was greatest with the cultures inoculated with the two bacterial species. Although *F. succinogenes* removed rather less xylose from clover and straw than did *R. flavefaciens*, a high proportion of the xylose solubilized by *F. succinogenes* appeared in the soluble-bound pool (Table 3).

To check whether the negative effect of the presence of *F. succinogenes* on the solubilization of forage by *R. flavefaciens* could be due to competition for growth factors, lipid-extracted clover/ryegrass and barley straw were incubated for 7 d with the two strains of *R. flavefaciens* in the presence and absence of the two strains of *F. succinogenes*, with and without supplementation of the medium with vitamins and VFA as described in Methods. These tests confirmed that the addition of vitamins and VFA did not influence the degradation of either forage, or reduce the negative effect of the presence of *F. succinogenes* (data not shown).

Discussion

The composition of the phospholipids of *F. succinogenes* and *R. flavefaciens* was consistent with the data from the more limited chemical analysis of these species reported by Miyagawa *et al.* (1979) and Minato *et al.* (1990). Although the phospholipid composition of the two species studied here varied between strains and was affected by the composition of the nutrient medium and by growth on different substrates, these effects were small compared with the differences between the species. The selected FAME and DMA marker components allowed the detection of the two species in mixed culture, and showed the same general trends as the viable counts (Fig. 2), despite the fact that phospholipid analysis detects both viable and non-viable cells. Analysis of phospholipids confirmed the continued presence of *F. succinogenes* during the later stages of incubations on clover when this bacterium was not recovered from the roll tubes.

In the cultures containing both species, the analysis of phospholipids showed that *R. flavefaciens* rapidly outgrew *F. succinogenes*. Most earlier studies with mixed cultures of rumen cellulolytic micro-organisms have involved observations after 4 or (more commonly) 7 d incubation (Dehority & Scott, 1967; Morris & Van Gylswyk, 1980; Miron, 1991), by which time many of the faster growing species would have completed their degradation. The barley straw used here was the same as that used by Kolankaya *et al.* (1985), who reported that *R. flavefaciens* strain 007 degraded untreated barley straw more rapidly than did *F. succinogenes* strain S85;

the maximum rate of straw solubilization during the first 48 h incubation with *R. flavefaciens* was approximately double that for *F. succinogenes*. Cell protein also accumulated more rapidly in supernatant liquids of the cultures containing *R. flavefaciens*, suggesting that this bacterium grew faster than *F. succinogenes* (Kolankaya *et al.*, 1985), consistent with the results of the present study. After incubation for 8 d, Kolankaya *et al.* (1985) found that the strains of *R. flavefaciens* and *F. succinogenes* showed the same extent of straw solubilization (around 32%). The present study, which was carried out with much greater quantities of plant material per incubation (3.5 g versus 50 mg) could reasonably be expected to be more accurate, but the two strains of *F. succinogenes* used solubilized straw and clover both less rapidly and less extensively than the two strains of *R. flavefaciens*. The effects of the presence of two strains of the species were not investigated and possible interstrain interactions are an obvious topic for further investigation (Stewart *et al.*, 1991), but it is considered that the use of multiple strains should more nearly approach the microbiological conditions *in vivo*.

Gram-positive cellulolytic cocci are normally recovered from the rumen in greater numbers than Gram-negative rods (Bryant & Burkey, 1953; Kistner *et al.*, 1962; Van Gylswyk, 1970; Mackie *et al.*, 1978), in line with the predominance of *R. flavefaciens* in this study. The low numbers of *F. succinogenes* recovered from incubations performed with this species alone are puzzling. Analysis of the lipids associated with the residues did not suggest that *F. succinogenes* was resistant to detachment. Dehority (1968) reported that isolated hemicellulose can be degraded by cell-free extracts of rumen bacteria, but intact plant material would be expected to be more resistant to attack. Earlier observations by electron microscopy show that *F. succinogenes* proliferates to cover exposed plant cell wall surfaces during incubation with plant material (Cheng *et al.*, 1983/84): it may be that these cells cannot be detached without losing viability.

In the rumen, the soluble products from the degradation of plant material that are not utilized by the major species of cellulolytic bacteria are presumably utilized by non-cellulolytic micro-organisms. Although *R. flavefaciens* was more active in solubilizing plant material than *F. succinogenes*, the latter, with its limited ability to utilize the products of hemicellulose hydrolysis (Morris & Van Gylswyk, 1980) would be expected on the basis of this data to provide more arabinose and xylose-containing substrates for interspecies cross-feeding. Xylose and glucose remained in the cultures after incubation mainly in the insoluble residues and as components of soluble degradation products, presumably di- or oligosaccharides, or glycosides. In contrast,

significant amounts of arabinose tended to accumulate as the free sugar in incubations with either clover/ryegrass or barley straw, and the accumulation of this free arabinose in the cultures containing the two bacterial species approximated to the sum of the amounts that accumulated in incubations that contained only one species. Significantly less soluble-bound arabinose accumulated in the cultures inoculated with both bacterial species than in cultures that received only one species. The characterization of the soluble components to which these monosaccharides were bound is an obvious topic of further interest. It should be remembered that the present study was carried out with plant material from which lipids had been extracted. In the rumen, the plant acyl lipids would be hydrolysed by lipolytic micro-organisms (Harfoot & Hazlewood, 1988). In the present study the removal of the lipid component that cannot be hydrolysed by either of the bacterial species used would be expected to increase the apparent degradability of the substrates, but there is no evidence that the removal of lipids enhances plant cell wall degradation (Van Soest, 1981).

Several studies, mainly with *Alfalfa* (lucerne) have now demonstrated that the degradative activity of mixed cultures of primary rumen cellulolytic organisms including the ruminococci and *F. succinogenes* is usually less than that of the more active species growing in pure culture (Dehority & Scott, 1967; Miron, 1991). A combined inoculum of *R. albus* and *Clostridium longisporum* was no more effective in the degradation of barley straw than *R. albus* alone (Varel *et al.*, 1989). The present study specifically shows that the presence of 2 strains of *F. succinogenes* reduced the solubilization of clover/ryegrass by *R. flavefaciens*.

It is not clear why cellulolytic bacteria do not normally show complementary or synergistic activity in the solubilization of plant material. *R. flavefaciens* and *F. succinogenes* are thought to adhere to different plant tissue types (Latham *et al.*, 1978), and these species do not show antagonistic or competitive interactions during their initial attachment to barley straw (Bhat *et al.*, 1990). Both species require certain B-vitamins and branched-chain fatty acids (Hungate, 1966), however, in the present study there was no evidence of competition between the species for these nutrients. The bacteria may compete for colonization sites when they spread beyond the areas of initial attachment during the degradation. The bacteria with lower degradative activity (in this case, *F. succinogenes*) may thus reduce the access of the ruminococci to their substrate. This might explain why the numbers of viable bacteria recovered from the incubations tended to be greater in the absence of *F. succinogenes*, especially when clover/ryegrass served as substrate. Alternatively, the reduction by *R. flavefaciens*

and *R. albus* of cellulolysis by some rumen fungi (Irvine & Stewart, 1991; Bernalier *et al.*, 1992) has been shown to involve the production by ruminococci of inhibitory compounds (Bernalier, 1991; Stewart *et al.*, 1992) and it may be that similar interactions also occur between some species of rumen bacteria.

We thank Moira Thomas, Sylvia Duncan, A. J. Richardson and P. J. S. Dewey for their advice and help. We are also grateful to The Rotary International Freedom from Hunger Campaign, for their financial support for L.A.S., and the Scottish Office Agriculture and Fisheries Department who support A.S. and C.S.S.

References

- BERNALIER, A. (1991). *Les champignons anaerobies du rumen: caracterisation et interactions avec les bacteries du rumen dans la cellulolyse, in vitro*. PhD thesis, Universite Blaise Pascal, Clermont Ferrand, France. No. d'ordre DU 281.
- BERNALIER, A., FONTY, G., BONNEMOY, F. & GOUET, F. (1992). Degradation and fermentation of cellulose by the rumen anaerobic fungi in axenic cultures or in association with cellulolytic bacteria. *Current Microbiology* **25**, 143–148.
- BHAT, S., WALLACE, R. J. & ORSKOV, E. R. (1990). Adhesion of cellulolytic ruminal bacteria to barley straw. *Applied and Environmental Microbiology* **56**, 2698–2703.
- BOBBIE, R. J. & WHITE, D. C. (1980). Characterization of benthic microbial community structure by high resolution gas chromatography of fatty acid methyl esters. *Applied and Environmental Microbiology* **39**, 1212–1222.
- BRYANT, M. P. (1972). Commentary on the Hungate technique for the cultivation of strict anaerobes. *American Journal of Clinical Nutrition* **25**, 1324–1328.
- BRYANT, M. P. & BURKEY, L. A. (1953). Numbers and some predominant groups of bacteria in the rumen of cows fed different rations. *Journal of Dairy Science* **36**, 218–224.
- CHENG, K.-J., COSTERTON, J. W., DINSDALE, D. & STEWART, C. S. (1983/84). Electron microscopy of bacteria involved in the digestion of plant cell walls. *Animal Feed Science and Technology* **10**, 93–120.
- CHRISTIE, W. W. (1982). *Lipid Analysis: Isolation, Separation, Identification and Structural Analysis of Lipids*. Oxford: Pergamon Press.
- DEHORITY, B. A. (1968). Mechanisms of isolated hemicellulose and xylan degradation by cellulolytic rumen bacteria. *Applied Microbiology* **16**, 781–786.
- DEHORITY, B. A. & SCOTT, H. W. (1967). Extent of cellulose and hemicellulose digestion in various forages by pure cultures of rumen bacteria. *Journal of Dairy Science* **50**, 1136–1141.
- DRUCKER, D. B. (1976). Gas-liquid chromatographic chemotaxonomy. *Methods in Microbiology* **9**, 51–125.
- ENGLYST, H. N. & CUMMINGS, J. H. (1984). Simplified method for the measurement of total non-starch polysaccharides by gas-liquid chromatography of constituent sugars as alditol acetates. *Analyst* **109**, 937–942.
- FEEDING STUFFS (SAMPLING AND ANALYSIS) REGULATIONS (1982). Luff-Schoorl method. 10a, p. 29. London: HMSO.
- FLINT, H. J., MCPHERSON, C. A. & BISSETT, J. (1989). Molecular cloning of genes from *Ruminococcus flavefaciens* encoding xylanase and $\beta(1,3,4)$ glucanase activities. *Applied and Environmental Microbiology* **55**, 1230–1233.
- FOLCH, J., LEES, M. & STANLEY, G. H. S. (1957). A simple method for the isolation and purification of total lipids from animal tissues. *Journal of Biological Chemistry* **226**, 497–509.
- HARFOOT, C. G. & HAZLEWOOD, G. P. (1988). Lipid metabolism in the rumen. In *The Rumen Microbial Ecosystem*, pp. 285–322. Edited by P. N. Hobson, London and New York: Elsevier Applied Science.
- HOBSON, P. N. (1969). Rumen bacteria. *Methods in Microbiology* **3B**, 133–149.
- HOEBLER, C., BARRY, J. L., DAVID, A. & DELORT-LAVAL, Y. (1989). Rapid hydrolysis of plant cell wall polysaccharides and simplified

- quantitative determination of the neutral monosaccharides by gas-liquid chromatography. *Journal of Agricultural and Food Chemistry* **37**, 360-367.
- HUNGATE, R. E. (1966). *The Rumen and its Microbes*. New York: Academic Press.
- HUNGATE, R. E. (1969). A roll tube method for cultivation of strict anaerobes. *Methods in Microbiology* **3B**, 117-132.
- IRVINE, H. L. & STEWART, C. S. (1991). Interactions between anaerobic cellulolytic bacteria and fungi in the presence of *Methanobrevibacter smithii*. *Letters in Applied Microbiology* **12**, 62-64.
- KISTNER, A., GOUWS, L. & GILCHRIST, F. M. C. (1962). Bacteria of the ovine rumen. II. The functional groups fermenting carbohydrates and lactate on a diet of lucerne (*Medicago sativa*) hay. *Journal of Agricultural Science* **59**, 85-91.
- KOLANKAYA, N., STEWART, C. S., DUNCAN, S. H., CHENG, K.-J. & COSTERTON, J. W. (1985). The effect of ammonia treatment on the solubilization of straw and the growth of cellulolytic rumen bacteria. *Journal of Applied Bacteriology* **58**, 371-379.
- LATHAM, M. J., BROOKER, B. E., PETTIPHER, G. L. & HARRIS, P. J. (1978). Adhesion of *Bacteroides succinogenes* in pure culture and in the presence of *Ruminococcus flavefaciens* to cell walls in leaves of perennial ryegrass (*L. perenne*). *Applied and Environmental Microbiology* **35**, 1166-1173.
- MACKIE, R. I., GILCHRIST, F. M. C., ROBERTS, A. M., HANNAH, P. E. & SCHWARTZ, H. M. (1978). Microbiological and chemical changes in the rumen during the stepwise adaptation of sheep to high concentrate diets. *Journal of Agricultural Science* **90**, 241-254.
- McKINLEY, V. L. & VESTAL, J. R. (1984). Biokinetic analysis of adaptation and succession: microbial activity in composting sewage sludge. *Applied and Environmental Microbiology* **47**, 933-941.
- MINATO, H. & SUTO, T. (1981). Technique for fractionation of bacteria in rumen microbial ecosystem. IV. Attachment of rumen bacteria to cellulose powder and elution of bacteria attached to it. *Journal of General and Applied Microbiology* **27**, 21-31.
- MINATO, H., MIYAGAWA, E. & SUTO, T. (1990). Techniques for analysis of rumen microbial ecosystems. In *The Rumen Ecosystem*, pp. 3-12. Edited by S. Hoshino, R. Onodera, H. Minato & H. Itabashi. Tokyo: Japan Scientific Societies Press.
- MIRON, J. (1991). The hydrolysis of lucerne cell-wall monosaccharide components by monocultures or pair combinations of defined ruminal bacteria. *Journal of Applied Bacteriology* **70**, 245-252.
- MIYAGAWA, E. (1982). Cellular fatty acid and fatty aldehyde composition of rumen bacteria. *Journal of General and Applied Microbiology* **28**, 389-408.
- MIYAGAWA, E., AZUMA, R. & SUTO, T. (1979). Cellular fatty acid composition in Gram-negative obligately anaerobic rods. *Journal of General and Applied Microbiology* **25**, 41-51.
- MORRIS, E. J. & VAN GYLSEWYK, N. O. (1980). Comparison of the action of rumen bacteria on cell walls of *Eragrostis tef*. *Journal of Agricultural Science* **95**, 313-323.
- STEWART, C. S. & WALSH, J. H. (1972). Cellulolytic activity of pure and mixed cultures of fungi. *Transactions of the British Mycological Society* **58**, 527-531.
- STEWART, C. S., FLINT, H. J. & NEKREP, F. V. (1991). Degradation of plant cell wall polymers by the anaerobic rumen bacterium *Ruminococcus flavefaciens*. In *Production and Utilization of Lignocellulosics*, pp. 387-399. Edited by G. C. Galletti. London and New York: Elsevier Applied Science.
- STEWART, C. S., PANIAGUA, C., DINSDALE, D., CHENG, K.-J. & GARROW, S. H. (1981). Selective isolation and characteristics of *Bacteroides succinogenes* from the rumen of a cow. *Applied and Environmental Microbiology* **41**, 504-510.
- STEWART, C. S., DUNCAN, S. H., RICHARDSON, A. J., BACKWELL, C. & BEGBIE, R. (1992). The inhibition of fungal cellulolysis by cell-free preparations from ruminococci. *FEMS Microbiology Letters* **97**, 83-88.
- VAN GYLSEWYK, N. O. (1970). The effect of supplementing a low protein hay on the cellulolytic bacteria in the rumen of sheep and on the digestibility of cellulose and hemicellulose. *Journal of Agricultural Science* **74**, 169-180.
- VAN SOEST, P. J. (1981). Limiting factors in plant residues of low biodegradability. *Agriculture and Environment* **6**, 135-143.
- VAREL, V. H., RICHARDSON, A. J. & STEWART, C. S. (1989). Degradation of barley straw, ryegrass, and alfalfa cell walls by *Clostridium longisporum* and *Ruminococcus albus*. *Applied and Environmental Microbiology* **55**, 3080-3084.
- VERKLEY, A. J., VERVERGAERT, P. H. J. T., PRINS, R. A. & VAN GOLDE, L. M. G. (1975). Lipid-phase transitions of the strictly anaerobic bacteria *Veillonella parvula* and *Anaerovibrio lipolytica*. *Journal of Bacteriology* **124**, 1522-1528.
- VESTAL, J. R. & WHITE, D. C. (1989). Lipid analysis in microbial ecology. *Bioscience* **39**, 535-541.
- WATANABE, T., OKUDA, S.-I. & TAKAHASHI, H. (1982). Physiological importance of even numbered fatty acids and aldehydes in plasmalogen phospholipids of *Selenomonas ruminantium*. *Journal of General and Applied Microbiology* **28**, 23-33.