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Co-Utilization of Polymerized Carbon Sources by *Bacteroides ovatus* Grown in a Two-Stage Continuous Culture System

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Bacteroides ovatus **NCTC 11153 was grown in a two-stage continuous culture system at various growth rates (vessel 1, D = 0.06 to 0.19 h^{-1}; vessel 2, D = 0.03 to 0.09 h^{-1}) on media containing mixtures of starch and arabinogalactan as carbon sources. The cell-associated enzyme activities needed to hydrolyze both substrates (amylase, arabinogalactanase, α-glucosidase, β-galactosidase, and α-arabinofuranosidase) were variously influenced by growth rate and polysaccharide availability but were detected under all growth conditions tested. Measurements of residual carbohydrate in spent culture media showed that both polysaccharides were co-utilized during growth under putative C-limited conditions. The arabinogalactan was partly depolymerized in N-limited chemostats, and significant amounts of arabinose- and galactose-containing oligosaccharides accumulated in the cultures, indicating that starch was being preferentially utilized. Acetate, propionate, and succinate were the major fermentation products formed by C-limited bacteria, but under N limitation, lactate was also produced. Molar ratios of succinate increased concomitantly with the dilution rate in C-limited chemostats, whereas molar ratios of propionate decreased. During N-limited growth, however, decarboxylation of succinate to propionate was relatively independent of growth rate. Cell viability was higher in C-limited cultures compared with those grown under N limitation and was greatest at high dilution rates, irrespective of nutrient limitation.**

Bacteroides fragilis-type bacteria are the single most numerous group of microorganisms that inhabit the human large intestine. They constitute between 20 and 30% of the gut microflora (13, 24), with cell population densities typically being in excess of 10^{11} per gram (dry weight) of contents (10). These gram-negative anaerobes are thought to be of special significance in the colonic ecosystem through their ability to initiate the breakdown of the polysaccharides and proteins that are the principal sources of carbon and nitrogen entering the large gut (4). A wide variety of polysaccharides of plant and human origin are degraded by human colonic bacteria. The principal end products of these processes are short-chain fatty acids, which play an important role in the large intestine physiology of the host (3).

Although the ability to ferment polysaccharides appears to be variable within the *B. fragilis* group (25, 26), *B. ovatus* is exceptionally versatile with respect to the range of polymerized carbon sources on which it is able to grow (17). Previous studies have demonstrated that starch and hemicelluloses such as arabinogalactan and xylan are co-utilized by mixed populations of fecal bacteria (9); however, it is not known whether *B. ovatus* or other colonic bacteroides are able to simultaneously utilize more than one type of polysaccharide substrate or whether selective uptake of carbohydrate occurs. In this study, we investigated the ability of continuous cultures of *B. ovatus* NCTC 11153 to grow on mixtures of two polysaccharides that are present in dietary residues that enter the large gut, namely starch and arabinogalactan (7, 23). The experiments were carried out by using a two-stage continuous culture system to enable physiological measurements to be made under the different conditions of nutrient availability that characterize the right and left colons.

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MATERIALS AND METHODS

Bacterium. *B. ovatus* NCTC 11153 (ATCC 8483) was obtained from the National Collection of Type Cultures, Public Health Laboratory Service, Colindale Avenue, London, England.

Continuous culture system. *B. ovatus* was grown over a range of dilution rates in a two-stage continuous culture system. The glass chemostats (vessel 1, 0.31 liters [working volume] and vessel 2, 0.67 liters [working volume]) were arranged in series. Sterile media were fed to vessel 1, which sequentially fed vessel 2 via a weir. The growth temperature was 37°C, and the pH (6.5) was automatically controlled as described by Macfarlane and Englyst (16). Anaerobic conditions were maintained by sparging the vessels with a gas mixture (0.6 liters h^{-1}) containing H_2, CO_2, and N_2 (10:10:80). The bacteria were equilibrated for at least seven turnovers in vessel 2 at each dilution rate before sampling. The growth medium consisted of the following (grams per liter): KH_2PO_4, 4.0; $Na_2H_2PO_4$, 2.0; NH_4Cl, 0.75; NaCl, 9.0; $MgCl_2 \cdot 6H_2O$, 0.15; $CaCl_2 \cdot 2H_2O$, 0.1; $MnCl_2 \cdot 4H_2O$, 0.1; $CoCl_2 \cdot 6H_2O$, 0.1; cysteine, 0.8; $NaHCO_3$, 1.5; hemin, 0.01; vitamin B12, 0.005; $FeSO_4 \cdot 7H_2O$, 0.001; and resazurin, 0.001. Starch and arabinogalactan were used as carbon sources (each 2 g $liter^{-1}$ for C-limited growth and 5 g $liter^{-1}$ for N-limited growth).

Polysaccharides. Lintner starch, an α(1,4)- and α(1,6)-linked glucan, was obtained from BDH Ltd. Larchwood arabinogalactan (Sigma grade II) consists of a β(1,4)- or β(1,3)-linked galactose backbone with α-linked arabinose side chains. Chemical measurements (by gas chromatogra-

TABLE 1. Effect of dilution rate on cell-associated polysaccharidase and glycosidase activities[a]

D (h^{-1})	Vessel	Polysaccharidase activity				Glycosidase activity					
		Amylase		Arabinogalactanase		α-Glucosidase		β-Galactosidase		α-Arabinofuranosidase	
		C-LIM	N-LIM	C-LIM	N-LIM	C-LIM	N-LIM	C-LIM	N-LIM	C-LIM	N-LIM
0.06	1	8.1	5.9	2.4	0.07	2.5	2.1	2.1	1.2	1.9	0.7
0.03	2	6.0	5.8	1.7	0.14	2.0	2.2	5.4	1.6	0.5	0.8
0.13	1	6.1	4.4	1.5	1.6	1.9	1.4	4.6	1.9	1.8	0.4
0.06	2	5.4	4.0	1.2	1.4	1.7	1.3	4.5	1.7	1.3	0.4
0.19	1	2.4	2.0	0.3	1.9	1.1	0.9	8.0	1.7	1.8	0.5
0.09	2	2.1	2.3	1.0	1.3	1.1	1.0	10.6	2.3	1.6	0.9

[a] *B. ovatus* was grown under anaerobic conditions in a two-stage continuous culture system. Starch and arabinogalactan concentrations were each 2 g $liter^{-1}$ in C-limited medium (C-LIM) and 5 g $liter^{-1}$ each in N-limited medium (N-LIM). NH_4Cl (14.2 mM) was the nitrogen source. Values are means of two measurements from separate chemostat runs. Polysaccharidase activity was measured as micromoles of reducing sugar released per hour per milligram of protein; glycosidase activity was measured as micromoles of *p*-nitrophenol released per hour per milligram of protein.

phy) showed that galactose and arabinose constituted approximately 85 and 15% of the preparation, respectively.

Enzyme activities. Bacteria were harvested from the chemostats by centrifugation (20,000 × *g*, 30 min). Supernatants were retained for measurements of extracellular enzyme activities. Cell pellets were washed once and resuspended in 0.1 M sodium phosphate buffer, pH 6.5, containing 0.1 M NaCl. The bacterial suspensions were disrupted by two passages through a French pressure cell (1.1×10^5 kPa). The crude cell extracts were centrifuged at 23,000 × *g* for 30 min to remove cell debris. Amylase, arabinogalactanase, α-glucosidase, α-arabinofuranosidase, and β-galactosidase activities were determined in culture supernatants and cell extracts as described by Englyst et al. (9). For estimations of enzyme specific activity, proteins were measured by the method of Lowry et al. (15).

Carbohydrate measurements. The carbohydrate composition of the feed media and residual carbohydrates in spent media from the chemostats were determined as alditol acetates by gas chromatography, by using procedures developed by Englyst and Cummings (6, 8).

Measurement of fermentation products. Short-chain fatty acids, succinate, and lactate were detected by gas chromatography as described by Holdeman et al. (12).

Enumeration of bacteria. Cell population densities of *B. ovatus* were determined by standard plating procedures and by direct microscope counting. The N-limited growth medium described earlier, solidified with 20 g of purified agar (Oxoid Ltd.) per liter, was used as the plating medium. All manipulations and incubations (2 to 5 days) were carried out in an anaerobic cabinet containing an atmosphere of H_2, CO_2, and N_2 (10:10:80). Total microscope counts were done in a Neubauer counting chamber, as described by Cruickshank (2). The difference in plate and microscope counts was used to estimate the viability of *B. ovatus* populations in the chemostats.

Chemicals. Unless otherwise stated, all chemicals were obtained from Sigma Chemical Co.

RESULTS

Polysaccharide-degrading enzymes. The hydrolytic enzyme activities involved in depolymerization of starch (amylase and α-glucosidase) and arabinogalactan (arabinogalactanase, β-galactosidase, and α-arabinofuranosidase) were principally cell bound in *B. ovatus*. Trace levels of α-glucosidase and β-galactosidase activity were detected in culture supernatants, but only at the lowest dilution rate in vessel 2. During C- and N-limited growth, amylase and α-glucosidase activities were inversely related to dilution rate, with no significant difference in their activities being observed between vessels 1 and 2 (Table 1). At low dilution rates, amylase and α-glucosidase activities were consistently higher in vessel 1 of the C-limited chemostat than in the corresponding N-limited vessel. Arabinogalactanase activities showed a similar response to amylase during C-limited growth but increased with the dilution rate in N-limited chemostats (Table 1). Activities of β-galactosidase were maximal during growth under C limitation and were strongly growth rate related under these culture conditions. β-Galactosidase activities were between two- and fourfold lower in the same N-limited cultures. α-Arabinofuranosidase synthesis was only influenced by the dilution rate in bacteria growing in vessel 2 of the C-limited culture. Activities of this enzyme were generally lower in N-limited bacteria and were unaffected by growth rate.

Polysaccharide utilization. Analysis of residual carbohydrates in culture effluents showed that starch was almost completely utilized by *B. ovatus* in vessel 1 of the C-limited cultures (Table 2; Fig. 1a, d, and g). The arabinose side chains of the arabinogalactan were depolymerized (Fig. 1b, e, and h), and approximately 37 to 57% of this carbohydrate was assimilated by the bacteria (Table 2). The galactose backbone was also extensively depolymerized, and large amounts of galactose-containing oligosaccharides accumulated in the cultures (Fig. 1c, f, and i). Between 36 and 51% of the galactose was utilized (Table 2). These results show that most polysaccharide degradation occurred in vessel 1 and that the amounts of arabinose and galactose assimilated by *B. ovatus* were not significantly influenced by the growth rate. Starch was never completely utilized in vessel 1 of the N-limited chemostats, and free sugars and oligosaccharides accumulated in the culture effluents (Fig. 2a, d, and g). They were subsequently fermented by bacteria in the second vessel. Starch utilization was maximal at low dilution rates, and between 67 and 94% of the polysaccharide was assimilated (Table 2). The arabinogalactan was less extensively depolymerized in N-limited chemostats. Nevertheless, significant amounts of arabinose (Fig. 2b, e, and h)-

TABLE 2. Utilization of starch and arabinogalactan by *B. ovatus*[a]

D (h^{-1})	Vessel	Percent total carbohydrate utilized (mean ± SD)					
		C limited			N limited		
		Glucose	Arabinose	Galactose	Glucose	Arabinose	Galactose
0.06	1	91.4 ± 5.1	36.7 ± 6.3	36.2 ± 0.9	77.2 ± 5.0	12.5 ± 7.1	2.2 ± 0.3
0.03	2	90.4 ± 2.6	40.0 ± 7.8	39.1 ± 1.3	93.7 ± 2.4	20.8 ± 6.6	7.9 ± 4.5
0.13	1	93.9 ± 6.7	43.3 ± 3.4	40.8 ± 2.8	66.9 ± 8.3	1.4 ± 0.2	0
0.06	2	96.0 ± 1.0	56.7 ± 10.2	51.1 ± 6.1	86.7 ± 3.6	11.1 ± 1.4	2.5 ± 2.4
0.19	1	94.9 ± 5.2	43.3 ± 6.6	41.4 ± 7.0	67.9 ± 6.5	15.3 ± 8.8	3.4 ± 3.4
0.09	2	100.0 ± 3.9	43.3 ± 9.3	47.7 ± 3.3	87.9 ± 6.2	26.4 ± 9.0	16.0 ± 2.1

[a] Bacteria were grown as described in the legend in Table 1. Values are mean (± SD) of two measurements from separate chemostat runs.

and galactose (Fig. 2c, f, and i)- containing oligosaccharides were found in the cultures. At all dilution rates, the assimilation of both arabinose and galactose by N-limited bacteria was lower compared with their C-limited counterparts (Table 2).

Fermentation products. Acetate, propionate, and succinate were the major fermentation products in C-limited chemostats, but in the N-limited cultures, lactate was also produced (Table 3). The relative proportion of acetate was higher in C-limited than in N-limited cultures, but molar ratios of this short-chain fatty acid were comparatively unaffected by the dilution rate. Propionate formation decreased markedly with the growth rate in both vessels of the C-limited system, but this effect was considerably less

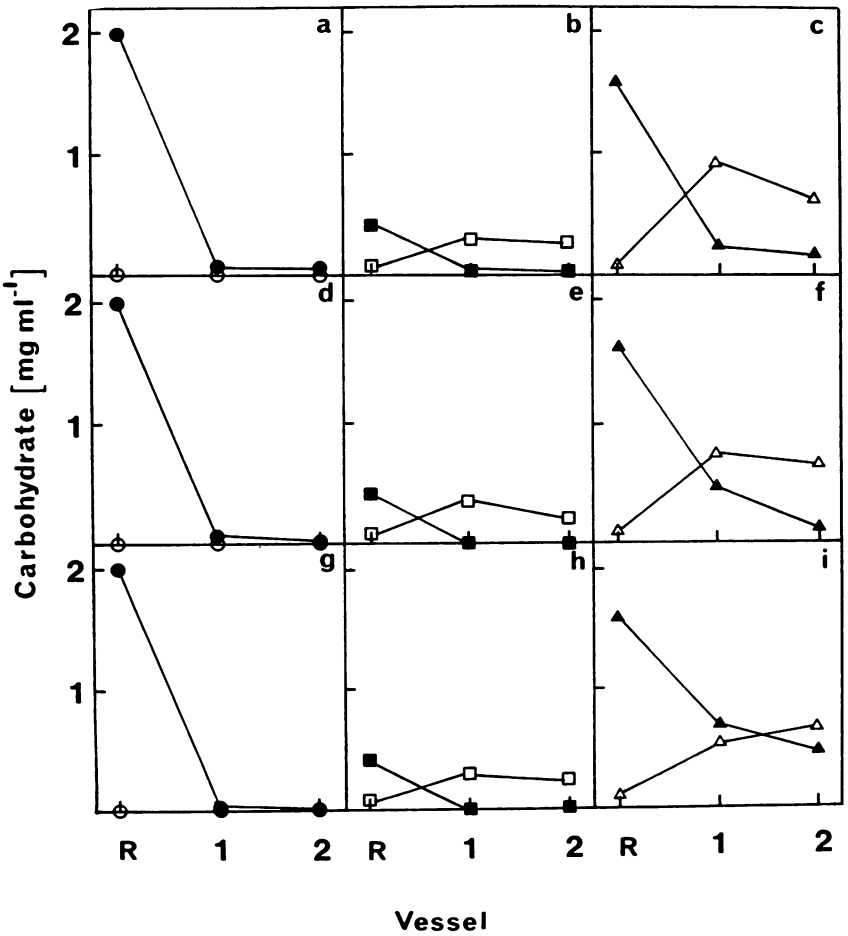

FIG. 1. Carbohydrate utilization by *B. ovatus* in C-limited chemostats. (a to c) V1, $D = 0.06\ h^{-1}$; V2, $D = 0.03\ h^{-1}$. (d to f) V1, $D = 0.13\ h^{-1}$; V2, $D = 0.06\ h^{-1}$. (g to i) V1, $D = 0.19\ h^{-1}$; V2, $D = 0.09\ h^{-1}$. R, Medium reservoir. Symbols: ●, starch polymer; ○, glucose-containing oligosaccharides and glucose monomers; ■, arabinose polymer; □, arabinose-containing oligosaccharides and arabinose monomers; ▲, galactose polymer; △, galactose-containing oligosaccharides and galactose monomers. Results are means from two separate experiments.

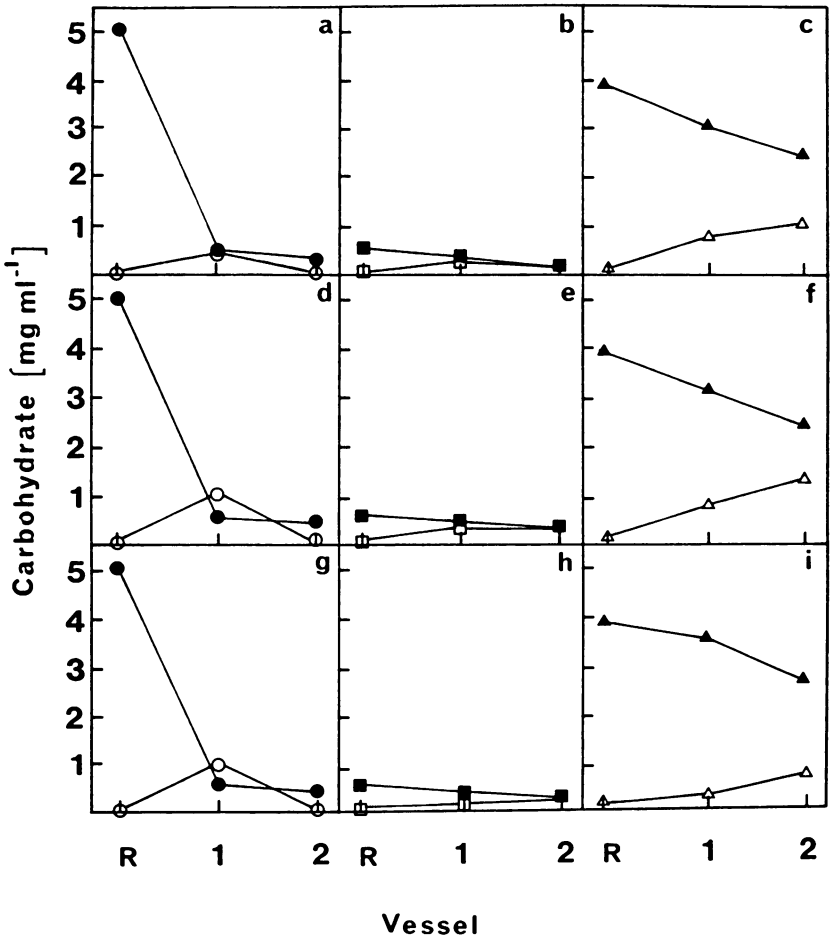

FIG. 2. Carbohydrate utilization by *B. ovatus* in N-limited chemostats. (a to c) V1, $D = 0.06\ h^{-1}$; V2, $D = 0.03\ h^{-1}$. (d to f) V1, $D = 0.13\ h^{-1}$; V2, $D = 0.06\ h^{-1}$. (g to i) V1, $D = 0.19\ h^{-1}$; V2, $D = 0.09\ h^{-1}$. R, Medium reservoir. Symbols: ●, starch polymer; ○, glucose-containing oligosaccharides and glucose monomers; ■, arabinose polymer; □, arabinose-containing oligosaccharides and arabinose monomers; ▲, galactose polymer; △, galactose-containing oligosaccharides and galactose monomers. Results are means from two separate experiments.

pronounced under N limitation. In contrast, succinate production increased with the dilution rate in C-limited chemostats, but only in vessel 2 of the N-limited system was succinate formation significantly influenced by the growth rate.

Effect of nutrient availability and dilution rate on cell counts. In both C- and N-limited chemostats, cell viability increased with the growth rate (Table 4). Except in vessel 1 at $D = 0.06\ h^{-1}$, viable counts did not vary significantly with the type of nutrient limitation. The percentage of cell viability was slightly higher in N-limited cultures at the lowest dilution rate but was lower than the C-limited cultures at

TABLE 3. Influence of substrate availability and growth rate on fermentation products[a]

D (h^{-1})	Vessel	mM fermentation product (molar ratio)							
		Acetate		Propionate		Succinate		Lactate	
		C-LIM	N-LIM	C-LIM	N-LIM	C-LIM	N-LIM	C-LIM	N-LIM
0.06	1	6.3 (58)	15.0 (49)	3.6 (33)	4.6 (15)	1.0 (9)	9.5 (31)	ND	1.6 (5)
0.03	2	8.9 (56)	18.5 (51)	5.2 (33)	5.4 (15)	1.8 (11)	8.9 (24)	ND	3.7 (10)
0.13	1	7.7 (65)	14.5 (49)	1.8 (15)	3.8 (13)	2.4 (20)	9.7 (33)	ND	1.5 (5)
0.06	2	10.2 (64)	19.8 (52)	2.4 (15)	6.1 (16)	3.3 (21)	10.3 (27)	ND	1.9 (5)
0.19	1	6.8 (53)	14.0 (49)	1.0 (8)	3.0 (11)	5.0 (39)	10.1 (36)	ND	1.2 (4)
0.09	2	10.6 (53)	17.5 (45)	2.2 (11)	5.7 (15)	7.1 (36)	14.0 (36)	ND	2.0 (5)

[a] Bacteria were grown as described in legend to Table 1. Values are the means of results from two separate experiments. Molar ratios are given in parentheses. C-LIM, C limiting; N-LIM, N limiting; ND, not detected (<0.1 mM).

TABLE 4. Viability of *B. ovatus* populations at different dilution rates[a]

D (h^{-1})	Vessel	Mean (± SEM) viable count (cells/ml, 10^9)[b]		Mean (± SEM) microscope count (cells/ml, 10^9)[b]		Mean percentage of viable cells	
		C-LIM	N-LIM	C-LIM	N-LIM	C-LIM	N-LIM
0.06	1	1.9 ± 0.2	2.8 ± 0.5	4.0 ± 0.7	4.6 ± 0.9	47.5	60.1
0.03	2	2.6 ± 0.7	2.6 ± 0.4	5.2 ± 1.4	4.8 ± 0.7	50.0	54.2
0.13	1	6.7 ± 0.3	5.9 ± 0.1	7.3 ± 1.2	6.9 ± 1.5	91.8	85.5
0.06	2	6.8 ± 0.5	6.1 ± 0.4	7.6 ± 1.2	7.4 ± 1.0	89.5	82.4
0.19	1	5.4 ± 1.6	6.7 ± 0.5	5.8 ± 1.4	8.0 ± 1.7	93.1	83.8
0.09	2	6.9 ± 0.7	6.8 ± 0.9	7.2 ± 0.7	8.2 ± 1.3	95.8	82.9

[a] Bacteria were grown as described in the legend in Table 1. C-LIM, C limiting; N-LIM, N limiting.
[b] Values are means from five measurements ± the standard errors of the means.

higher growth rates. The viability of the cells was not appreciably different in the two vessels under either C- or N-limited growth.

DISCUSSION

Largely because of the anatomy of the organ itself, marked regional differences occur in the activities of bacteria that inhabit the human large intestine. Those growing in the right colon receive substrates directly from the small bowel, and as a consequence, bacterial growth rates and activities are greatest in this part of the gut. However, colonic contents become increasingly nutrient depleted as they traverse the large bowel, and bacterial activities decrease markedly in the left colon (5). This provided the rationale for investigating polysaccharide utilization by *B. ovatus* in a two-stage continuous culture system, where studies could be made at different growth rates under carbon excess and exceptionally carbon-limited conditions.

Total residence times of bacteria in the continuous culture system ranged from 50 h at the lowest dilution rates to 16.4 h at the fastest dilution rates, but large populations of bacteria were maintained in the chemostats under all growth conditions tested. Although viabilities of *B. ovatus* were significantly influenced by growth rate, surprisingly little difference in viability was found between populations in vessels 1 and 2 (Table 4). These results show that *B. ovatus* can remain viable for long periods in nutrient-poor environments and suggest that the survival characteristics of *B. ovatus* at low growth rates are probably important determinants of the success of these bacteria in the gut.

Bacteria regulate carbohydrate metabolism in a variety of ways, such as catabolite repression, in which the synthesis of enzymes not needed for growth on a particular substrate is repressed in the presence of a preferred nutrient (21). Alternatively, they can control the uptake of carbohydrate into the cells by catabolite inhibition (20) or inducer exclusion (22) mechanisms. Many polysaccharide-degrading enzymes produced by *B. ovatus*, including those involved in starch and arabinogalactan hydrolysis, are repressed during growth on glucose and induced in the presence of polymerized carbohydrates or certain other monosaccharides (17).

In this study, amylase and α-glucosidase activities were generally highest in C-limited cultures at low dilution rates, indicating that partial repression occurred as more carbohydrate entered the chemostats. Although synthesis of the enzymes involved in arabinogalactan degradation (arabinogalactanase, β-galactosidase, and α-arabinofuranosidase) was variously influenced by growth rate and carbohydrate availability (Table 1), carbohydrate analysis showed that sufficient amounts of these enzymes were always produced to hydrolyze at least part of the polysaccharide (Fig. 1 and 2). However, synthesis of β-galactosidase and α-arabinofuranosidase was partly repressed in N-limited chemostats, and this correlated with a decrease in the relative amount of depolymerization of the arabinogalactan in these cultures (Fig. 1 and 2).

Extensive depolymerization of arabinogalactan occurred in the C-limited chemostats, and arabinose- and galactose-containing oligosaccharides accumulated. However, starch, the arabinose side chains, and the galactose backbone sugars of the arabinogalactan were co-utilized by *B. ovatus*. In contrast, neither starch nor arabinogalactan was completely utilized in the N-limited chemostats, and although the arabinogalactan was partly hydrolyzed, little of the arabinose and even less galactose were assimilated by the bacteria (Table 2). These data indicate that in the presence of high concentrations of carbohydrate, starch and its hydrolysis products were used in preference to the arabinogalactan. *B. ovatus* may therefore be able to control polysaccharide utilization at the level of uptake of carbohydrate into the cells, as well as by regulating synthesis of the enzymes involved in the initial hydrolysis of the polymer. However, monosaccharide uptake experiments have shown that batch cultures of these bacteria can simultaneously assimilate glucose, galactose, xylose, mannose, and arabinose (unpublished results).

The partial breakdown of polymerized carbohydrates and the production of oligosaccharides by polysaccharide-degrading species such as *B. ovatus* are likely to be of great ecological significance in the gut. By making carbohydrate available to saccharolytic bacteria that are unable to break down polymers by themselves, this process undoubtedly plays an important role in generating species diversity in the colonic ecosystem.

Active fermentation occurred in both vessels of the continuous culture system, albeit at a much reduced rate in vessel 2. Molar ratios of individual fermentation products were strongly influenced by growth rate and the form of nutrient limitation. This was particularly evident with propionate and succinate, where propionate formation decreased with increasing dilution rate (Table 3). A number of human colonic bacteroides produce propionate and CO_2, which is needed in small amounts for growth, via the succinate pathway (19), and a similar relationship between propionate and succinate formation and growth rate has previously been reported in C-limited continuous cultures of *B. thetaiotaomicron* (14) and *B. fragilis* (1).

Interestingly, however, succinate production was relatively unaffected by the dilution rate in N-limited *B. ovatus* chemostats. The high levels of this fermentation product found at all growth rates in these cultures demonstrate that in the presence of excess carbohydrate, sufficient CO_2 was generated from other fermentation reactions (18) to obviate the need to generate CO_2 through extensive decarboxylation of succinate. Conversely, the small quantities of succinate that accumulated at low dilution rates in the C-limited cultures indicate that CO_2 was limiting under these growth conditions.

D-Lactate was detected only in N-limited cultures of *B. ovatus* and was probably produced through the reduction of pyruvate, as occurs with *B. fragilis* (18). This reaction results in oxidation of NADH to NAD^+, indicating that lactate formation could be used to dispose of excess reducing power when the flow of carbon through the cell is high.

Lactate and succinate production by carbohydrate-fermenting species in the human colon is significant from an ecological viewpoint, since these fermentation intermediates are growth substrates for other members of the gut community (11, 27). It is evident, therefore, from results obtained in this study that environmental and physiological factors which regulate the amounts of lactate and succinate produced from bacteria such as *B. ovatus* can subsequently have important consequences for the growth and activities of other cross-feeding species in the gut.

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