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Xylose, Arabinose, and Rhamnose Fermentation by *Bacteroides ruminicola*

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Metabolism and growth yields of *Bacteroides ruminicola* grown on D-xylose, L-arabinose, and L-rhamnose were studied. Growth yields were 62, 68, and 35.5 g (dry weight) per mol of carbohydrate fermented after correction for storage polysaccharide. Experiments with [1-^{14}C]arabinose indicated that pentose was fermented by a pentose phosphate cycle plus glycolysis, with some indication of a minor phosphoketolase-type pathway. The product ratios from pentose were similar to those previously described for hexose. Rhamnose was fermented mainly to 1,2-propanediol, succinate, and acetate, although the latter was quantitatively less than expected. Estimates of adenosine 5'-triphosphate (ATP) molar growth yields could not be calculated with any certainty, as ATP generation by electron transport-linked phosphorylation cannot yet be assessed. If ATP were generated by substrate-level phosphorylation reactions alone, ATP molar growth yields for xylose, arabinose, and rhamnose would be 30, 28, and 35 g/mol. If calculations are based on an assumption that two ATP are generated by electron transport-linked phosphorylation per succinate, ATP molar growth yields become 15, 14, and 22 g/mol; if the assumption is also made that the pathway of lactaldehyde reduction is coupled to production of one ATP per 1,2-propanediol by electron transport-linked phosphorylation, the ATP molar growth yield for rhamnose fermentation becomes 14 g/mol. No preference can be expressed between these alternatives at present.

Bacteroides ruminicola produces high growth yields during hexose fermentation (14). This phenomenon has been described in several anaerobic organisms which produce succinate, propionate, or both (3, 5, 9, 13, 16, 17). It has been suggested that synthesis of extra adenosine 5'-triphosphate (ATP) by electron transport-linked phosphorylation (ETLP), in addition to the ATP formed by substrate-level phosphorylation (SLP), can explain these high yields. These studies used hexose as the energy source.

Comparisons of media used to enumerate anaerobic bacteria from sheep rumens (8) have shown that xylose is almost as effective an energy source as glucose, cellobiose, or starch and that counts are highest when a source of pentose (polymer) is added to nonselective medium (12). The energetics of pentose metabolism in such bacteria are not well understood. Here we present data showing that pentose and 6-deoxyhexose fermentation by *B. ruminicola* also give high growth yields. We have investigated the pathways used and the product stoichiometries to determine whether the quantity of a particular product such as succinate can be correlated with extra ATP and high growth yields.

MATERIALS AND METHODS

Bacterium and growth medium. *B. ruminicola* subsp. *brevis* strain B_14 was maintained as previously described (14). For growth yield and product studies, glucose in the liquid medium (14) was replaced by pentose or 6-deoxyhexose. Cells for the inoculum were subcultured until minimum doubling times (t_d) were obtained. A series of batch cultures was grown in which final growth was limited by the concentration of the carbohydrate energy source (0 to 13 mM) added after autoclaving. The t_d values given are minimum values obtained during logarithmic growth phase. As soon as growth ceased, cultures were cooled and centrifuged; cell dry weights and polysaccharide levels were measured in washed pellets, and the supernatant was frozen for later analysis of products (14).

Analysis of carbohydrates. D-Xylose was measured with *o*-toluidine reagent (10), L-arabinose was measured with 0.01% (wt/vol) anthrone reagent, and L-rhamnose was measured with 0.05% (wt/vol) anthrone reagent (1, 14). Cellular polysaccharide was measured as described previously (14).

Analysis of fermentation products. Analyses for formate, acetate, and succinate were carried out by methods previously outlined, and results were confirmed by celite column chromatography (14, 20). 1,2-

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Propanediol was measured on a Varian Aerograph series 1700 gas-liquid chromatograph with a column (2 m by 4.5-mm ID) packed with Chromosorb 101 (100–120 mesh). The column temperature was 160°C, the N_2 carrier gas flow rate was 50 ml/min, and samples were measured by flame ionization detection. The culture supernatant (0.9 ml) was mixed with 0.1 ml of 0.01% (vol/vol) 1,3-propanediol, and 10 μl was injected into the column. 1,2-Propanediol was estimated from its peak area relative to the 1,3-propanediol standard. These compounds were well separated from commonly occurring bacterial fermentation products. The ratios of the retention times of standards relative to 1,2-propanediol were as follows: acetaldehyde, 0.05; ethanol, 0.08; acetone, propan-2-ol, 0.14; acetic acid, *n*-propanol, 0.21; butan-2-ol, 0.34; propionic acid, butan-1-ol, 0.52; 1,2-propanediol, 1.0; 1,3-propanediol, 1.9; and 1,3-butanediol, 2.6. The retention time of 1,2-propanediol was approximately 24 min. In later work an 87-cm column was found to give an effective and shorter assay for the two propanediols.

Radiochemical experiments and measurements. Radiochemical experiments and measurements were carried out as previously described (20). [1-^{14}C]arabinose was bought from Radiochemical Centre, Amersham, United Kingdom.

RESULTS

D-Xylose fermentation. The t_d of cells grown at 37°C was 1.7 h, although initial subcultures from glucose-containing stabs had t_d values four times larger. The molar growth yield uncorrected for polysaccharide accumulation by cells was 61 g (dry weight) of cells per mol of xylose disappearing from the medium (Fig. 1). The cell polysaccharide was analyzed (data not shown) and consisted of 4.2 g of ribose and 5.3 g of glucose per mol of xylose disappearing; 55% of the glucose disappeared when bacteria were incubated for 5 h beyond full growth (data not shown). To calculate the molar growth yield (14), the weight of metabolizable polysaccharide (2.9 g) was subtracted from the cell dry weight (61 g), and the total weight of polysaccharide (9.5 g) was subtracted from 1 mol of xylose (150 g), giving a corrected molar growth yield of 62 g (dry weight) of cells per mol of xylose fermented. The fermentation products per mole of xylose disappearing (Fig. 1) were 0.96 mol of succinate and 0.53 mol of volatile fatty acids, i.e. 1.06 mol of succinate and 0.58 mol of volatile fatty acids per mol of xylose fermented. Celite column chromatographic analysis of the products was in good agreement, with 0.12 mol of formic acid and 0.49 mol of acetic acid found per mol of xylose fermented.

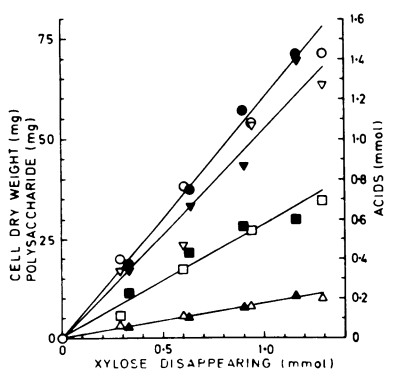

FIG. 1. *Growth of B. ruminicola on limiting amounts of* D-*xylose in batch culture. Symbols;* ○ *and* ●, *dry weight of cells uncorrected for cellular polysaccharide;* ▽ *and* ▼, *succinate;* □ *and* ■, *volatile fatty acids (acetate plus formate);* △ *and* ▲, *cellular polysaccharide measured by using 0.05% anthrone reagent. Open and closed symbols represent two different experiments.*

L-Arabinose fermentation. The cells grew in the first subculture with a t_d of 1.05 h on arabinose, and this did not decrease subsequently. The molar growth yield uncorrected for polysaccharide accumulation by cells was 66 g (dry weight) per mol of arabinose disappearing from the medium (Fig. 2). When the arabinose utilization was corrected for the total polysaccharide content of the cells (6.4 g of ribose and 8.0 g of glucose per mol of arabinose disappearing) and cell yields were corrected for storage polysaccharide (assumed to be about 33% of the total measured polysaccharide [see above]), the corrected molar growth yield became 68 g (dry weight) per mol of arabinose fermented. Product analysis showed that 1.06 mol of succinate and 0.69 mol of volatile fatty acids were produced per mol of arabinose disappearing (1.17 mol of succinate and 0.76 mol of volatile fatty acids per mol of arabinose fermented). Celite column chromatographic analysis showed the volatile fatty acids to be 0.13 mol of formate and 0.62 mol of acetate per mol of arabinose fermented.

Fermentation of [1-^{14}C]arabinose. The fermentation was stopped just before maximum growth of the culture to minimize exchange reactions. The specific radioactivities of acidic fermentation products were calculated after separation by celite column chromatography (Table 1). The acetate, formate, and succinate were 47, 18, and 54% of the specific radioactivity of the arabinose on a radioactivity per mole basis, and some 16% of the initial counts were incorporated into cells.

L-Rhamnose fermentation. We examined the ability of *B. ruminicola* to utilize carbohydrates with oxidation states different from those of hexoses and pentoses. The carbohydrates were autoclaved separately, and 5 mM (final concentration) amounts were added. D-Gluconate, D-glucuronate, sorbitol, ribitol, dulcitol, and L-fucose were not fermented, but L-rham-

nose was used. The first subculture grew slowly (t_d, 4.1 h), but fully induced cells grew with a t_d of 1.73 h. Figure 3 shows that 36 g (dry weight) of cells, including 5 g of cellular polysaccharide, 0.28 mol of succinate, and 0.31 mol of volatile fatty acids, was formed per mol of rhamnose disappearing. Celite column chromatography showed that 0.35 mol of acetate and 0.02 mol of formate were formed per mol of rhamnose disappearing from the medium.

These data indicate that only 1.84 g-atoms of carbon was recovered in acidic fermentation products from 6 g-atoms of rhamnose carbon. Since rhamnulose-1-P is cleaved in *Escherichia coli* to dihydroxyacetone phosphate and L-lactaldehyde, we tested for the latter product in the fermentation supernatant with *o*-aminobenzaldehyde reagent; the results were negative. The supernatant also did not react with Fehling or Benedict solution. Gas-liquid chromatography of the supernatant on Chromosorb 101 gave a major peak which cochromatographed with 1,2-propanediol. 1,3-Propanediol was used as an internal standard to measure 1,2-propanediol production as a function of rhamnose disappearance (Fig. 4), and 0.89 mol of 1,2-propanediol was detected per mol of rhamnose disappearing. The identification of 1,2-propanediol was confirmed by gas-liquid chromatography on Porapak Q, on which the product cochromatographed with the standard. In addition, the product was extracted (15) and then converted to acetaldehyde and assayed (2). The 560-nm-absorbing material indicated that either lactaldehyde (see above) or 1,2-propanediol was present.

The corrected molar growth yield (assuming by analogy with xylose that one-third of total polysaccharide is metabolizable) was 35.5 g (dry weight) per mol of rhamnose fermented. After rhamnose disappearance was corrected for production of cellular polysaccharide, the products recovered per mole of rhamnose fermented were 0.36 mol of acetate, 0.02 mol of formate, 0.29 mol of succinate, and 0.92 mol of 1,2-propanediol.

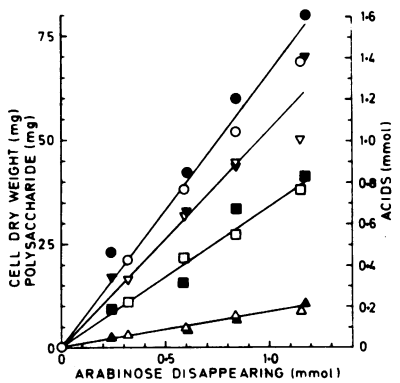

FIG. 2. *Growth of B. ruminicola on limiting amounts of L-arabinose in batch culture. For symbols, see legend to Fig. 1.*

DISCUSSION

The distribution of isotope in products from [1-^{14}C]arabinose fermentation (Table 1) was almost that expected if the fermentation pathway is composed of the pentose phosphate cycle plus glycolysis. This sequence should give phosphoenolpyruvate with 60% of the specific radioactivity per mole of the pentose, one third in C1 and two-thirds in C3. From this phosphoenolpyruvate succinate, acetate, and formate should be formed with 40 to 60, 40, and 0 to 20%, respectively, of the specific radioactivity per mole of the pentose, depending on the extent of CO_2 exchange into the carboxyl of phosphoenolpyruvate and pyruvate (18, 20). Peptide catabolism may also dilute the radioactivity in succinate, as occurs during glucose metabolism (20). The results shown in Table 1 lie within the ranges given above, except for acetate, which is slightly

TABLE 1. *Specific radioactivities of products formed during fermentation of [1-^{14}C]arabinose*

Parameter	Substrate (S) or product (P)	Amt in: Expt 1 μmol	Expt 1 Radioactivity (×10^{-4} dpm)	Expt 1 Specific radioactivity (×10^{-4} dpm/μmol)	Expt 2 μmol	Expt 2 Radioactivity (×10^{-4} dpm)	Expt 2 Specific radioactivity (×10^{-4} dpm/μmol)
Arabinose	S	112.9[a]	905	8.02	112.9[a]	905	8.02
Acetate	P	51.6	213	4.12	62.0	210	3.38
Formate	P	10.6	15	1.36	11.0	17	1.53
Succinate	P	75.7	339	4.48	80.9	332	4.11
CO_2	P		62			62	
Bacteria	P		141			147	

[a] The reaction was stopped before maximum growth. See text.

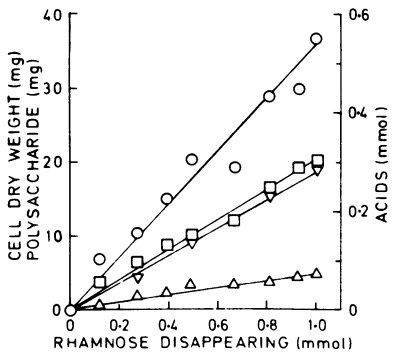

FIG. 3. *Growth of B. ruminicola on limiting amounts of L-rhamnose in batch culture. For symbols see legend to Fig. 1.*

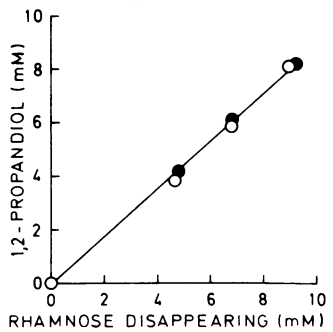

FIG. 4. *Production of 1,2-propanediol during L-rhamnose fermentation in batch culture. Open and closed symbols represent two different experiments.*

higher. This observation would be consistent with the possibility of a minor phosphoketolase-type fermentation pathway. However, this cannot be the major pathway, as CO_2, formate, and succinate would not become labeled. Wallnöfer et al. (25), working with rumen fluid from three cows, concluded that, on average, 75% of xylose was metabolized via the pentose phosphate cycle plus glycolysis and 25% was metabolized via a phosphoketolase-type pathway. However, little can be deduced about pathways in individual species from their data.

Fermentation pathway partial stoichiometries (CO_2 fixation and evolution were not measured) calculated from Fig. 1 and 2 can be used to obtain estimates of ATP yields by SLP. One xylose molecule was fermented to 1.06 succinate, 0.49 acetate, and 0.12 formate, and one arabinose molecule gave rise to 1.17 succinate, 0.62 acetate, and 0.13 formate. Theoretically, the stoichiometry of pentose fermentation by the pentose phosphate cycle plus glycolysis, SLP, and metabolism to CO_2, formate, acetate, and succinate should lie between the following limits: (i) 3 pentose + 2.5 CO_2 → 2.5 succinate + 2.5 acetate + 2.5 formate + 7.5 ATP; and (ii) 3 pentose + 1.67 CO_2 → 3.33 succinate + 1.67 acetate + 6.67 ATP, where pyruvate is metabolized to (i) acetate and formate and (ii) acetate and CO_2. The data suggest about 15% of pathway (i) and 85% of pathway (ii) for both pentoses. ATP yields calculated on the basis of 1 ATP per succinate and 2 ATP per acetate suggest that xylose fermentation makes 2.04 ATP per mol of pentose and that arabinose makes 2.41 ATP per mol of pentose by SLP. The corrected molar growth yields found were 62 and 68 g (dry weight) for xylose and arabinose respectively. It is not possible to determine accurate ATP molar growth yields (Y_{ATP}) from these data, since the contribution of ETLP coupled to fumarate reduction is uncertain. The composition of the electron transfer chain in *B. ruminicola* has been previously described (19). If SLP alone occurs Y_{ATP} would be 30 g/mol for xylose and 28 g/mol for arabinose (cf. the glucose fermentation value of 23 [14]), whereas if two ATP are formed by ETLP per succinate, Y_{ATP} would become 15 and 14 g/mol, respectively. The possible contribution of a minor phosphoketolase pathway has been ignored in these calculations since the ATP yield per succinate and acetate in products is the same.

The pentose fermentation partial stoichiometry in this work differs from that published by Dehority (7), whose strains of *B. ruminicola* made propionate in slightly larger quantities than succinate. The difference may be due to the 40% rumen fluid in the medium of Dehority, although the *B. ruminicola* subsp. *brevis* type strain, GA33, made little propionate in the experiments of Dehority.

The fermentation of rhamnose by *B. ruminicola* subsp. *brevis* has been noted previously (unpublished results of S. R. Elsden, quoted by Bryant et al. [4]). This fermentation was of interest because rhamnose (6-deoxy-L-mannose) is at a different oxidation state than are hexoses and pentoses and might be expected to give different product ratios; 1 mol of rhamnose gave rise to 0.29 mol of succinate, 0.36 mol of acetate, 0.02 mol of formate, and 0.92 mol of 1,2-propanediol. The probable pathway, by analogy to metabolism in other bacteria (6, 11, 22, 23), should involve rhamnose isomerization, phosphorylation at C1, and cleavage by an aldolase to dihydroxyacetone phosphate and L-lactaldehyde. The former should be metabolized by glycolysis, and the latter should be reduced to 1,2-propanediol. One would expect a theoretical stoichiometry as follows: 1 rhamnose → 1 propanediol + 0.33 succinate + 0.66 acetate + 0.33 CO_2.

The low acetate level (0.36 mol) was the main

difference in the measured stoichiometry. Some or all of the discrepancy can be explained if the 0.34 mol of missing 3-carbon intermediate were used for biosynthesis of cell material. This missing intermediate would weigh 30 g, slightly less than the observed mass of cells.

ATP yields, based on measured succinate and acetate production, can be calculated to give widely different values, depending on the assumptions made. SLP would give 1.01 ATP per mol of rhamnose, and in theory this could be increased up to 1.59 ATP per mol if two ATP were formed by ETLP per succinate. Values for Y_{ATP} would become 35 and 22, respectively. The high latter value leaves open an additional possibility, namely that lactaldehyde reduction could theoretically be linked to ATP synthesis by ETLP. The pathway of reducing equivalents to lactaldehyde is presently unknown. Y_{ATP} would become 14 if one ATP were formed per propanediol.

Our present results demonstrate that *B. ruminicola* gives high growth yields not only during hexose fermentation (14) but also with pentoses and rhamnose. The finding that pentoses were fermented mainly by a glycolytic terminal pathway, thus forming product ratios roughly similar to those of glucose, has meant that little extra information was gained on the relative importance of acetate and succinate to ATP production. Rhamnose fermentation proved more informative in that a reduction-oxidation pathway not involving fumarate was the major route for disposal of reducing equivalents; yet the growth yields were still very high. The roles of pyruvate oxidation to CO_2 and acetate, the reduction of L-lactaldehyde, and the reduction of fumarate are poorly understood aspects of the energetics of this problem. It is premature to decide with any certainty whether such reactions, although probably linked to or in membrane-bound electron transfer chains, are coupled to formation of stoichiometric amounts of ATP. A possible way to answer this question would be to measure the change in the observed molar growth yield (Y_s) with respect to growth rate (21) in succinate-producing anaerobes giving high growth yields. From such data the "true" or maximum molar growth yield (Y_s^{max}) could be calculated. The latter value could then be compared with values calculated by assuming that Y_{ATP}^{max} equals 28.6 g (dry weight) of bacteria per mol of ATP (24) and assuming an ATP yield consisting of SLP plus either zero, one, or two ATPs per succinate (or propanediol or both) by ETLP. Only one of the latter values should give an estimate for Y_s^{max} near that found empirically. The importance of ATP formation by ETLP might then be evaluated.

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