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Some Growth and Metabolic Characteristics of Monensin-Sensitive and Monensin-Resistant Strains of *Prevotella* (*Bacteroides*) *ruminicola*†

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New strains with enhanced resistance to monensin were developed from *Prevotella* (*Bacteroides*) *ruminicola* subsp. *ruminicola* 23 and *P. ruminicola* subsp. *brevis* GA33 by stepwise exposure to increasing concentrations of monensin. The resulting resistant strains (23MR2 and GA33MR) could initiate growth in concentrations of monensin which were 4 to 40 times greater than those which inhibited the parental strains. Resistant strains also showed enhanced resistance to nigericin and combinations of monensin and nigericin but retained sensitivity to lasalocid. Glucose utilization in cultures of the monensin-sensitive strains (23 and GA33) and one monensin-resistant strain (23MR2) was retarded but not completely inhibited when logarithmic cultures were challenged with monensin (10 mg/liter). Monensin challenge of cultures of the two monensin-sensitive strains (23 and GA33) was characterized by 78 and 51% decreases in protein yield (milligrams of protein per mole of glucose utilized), respectively. Protein yields in cultures of resistant strain 23MR2 were decreased by only 21% following monensin challenge. Cell yields and rates of glucose utilization by resistant strains GA33MR were not decreased by challenge with 10 mg of monensin per liter. Resistant strains produced greater relative proportions of propionate and less acetate than the corresponding sensitive strains. The relative amounts of succinate produced were greater in cultures of strains 23, GA33, and 23MR2 following monensin challenge. However, only minor changes in end product formation were associate with monensin challenge of resistant strain GA33MR. These results suggest that monensin has significant effects on both the growth characteristics and metabolic activities of these predominant, gram-negative ruminal bacteria.

It is generally believed that the beneficial effects associated with the use of ionophores in ruminant diets can be explained by their effects on the activities of the microbial population in the rumen (1, 13, 19, 22, 25). While many effects of ionophores on ruminal fermentations have been described, few studies have examined the effects of these agents on the growth and activities of specific organisms from this environment. For the most part, these studies have focussed on the selective antimicrobial activities of these agents against representative strains of ruminal bacteria (6, 10, 14, 17) and have not examined the specific physiological effects of ionophores. Recent studies have described the effects of monensin on the growth, metabolism, and membrane function of *Streptococcus bovis* (7, 20, 21, 23), but effects of these antimicrobial compounds on the predominant, gram-negative bacteria from the rumen have been largely ignored.

Several investigators have demonstrated enhanced ionophore resistance in gram-negative bacteria after exposure or adaptation of sensitive strains to increasing concentrations of monensin (6, 9, 14). The ability of these organisms to grow in the presence of the ionophores could enhance their overall contributions to fermentations in the rumens of animals receiving monensin-supplemented diets. This study examined the effects of monensin on the growth characteristics and metabolic activities of monensin-sensitive and -resistant strains of *Prevotella* (*Bacteroides*) *ruminicola*.

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MATERIALS AND METHODS

Growth conditions and organisms. All of the media used in these experiments were prepared and inoculated under a CO_2 gas phase by using modifications of the anaerobic culture techniques described by Bryant (3). Stock cultures of all organisms were maintained in the rumen fluid-containing medium described by Bryant and Robinson (4). Cultures were incubated at 37°C in an incubator or a circulating water bath. *Prevotella* (*Bacteroides*) *ruminicola* subsp. *ruminicola* 23 and subsp. *brevis* GA33 were originally obtained from M. J. Allison, National Animal Disease Center, Ames, Iowa, and are maintained in the culture collection at the Department of Animal Sciences, University of Kentucky, Lexington.

Sensitivity of organisms to ionophores. The abilities of organisms to initiate growth in the presence of individual ionophores (monensin, lasalocid, valinomycin, and nigericin) and combinations of ionophores (nigericin-monensin, nigericin-lasalocid, nigericin-valinomycin, and monensin-lasalocid) were measured in a basal medium (medium A) containing (in grams per liter) glucose (1.0), Trypticase peptones (BBL Microbiology Systems, Cockeysville, Md.) (5.0), hemin (0.001), resazurin (0.001), KH_2PO_4 (0.9), NaCl (0.9), $CaCl_2$ (0.02), $MgCl_2 \cdot 6H_2O$ (0.02), $MnCl_2 \cdot 4H_2O$ (0.01), $CoCl_2 \cdot 6H_2O$ (0.001), $FeSO_4$ (0.018), $(NH_4)_2SO_4$ (0.9), Na_2CO_3 (4.0), and cysteine hydrochloride (0.5). The medium was also supplemented with vitamins, methionine (0.05 g/liter), and volatile fatty acids containing (in milliliters per liter) acetate (1.6), propionate (0.67), *n*-butyrate (0.41), isobutyrate (0.08), *n*-valerate (0.098), isovalerate (0.098), and 2-methylbutyrate (0.098) (5). The medium pH was adjusted to 7.0 prior to sterilization. Ionophores were prepared to give final concentrations in the medium of 0, 0.625,

1.25, 2.5, 5.0, 10.0, 20.0, and 40.0 mg/liter in 95% ethanol under a CO_2 gas phase. Sensitivity of the organisms to the combination of two ionophores was determined with similar concentrations of each ionophore in an ethanol solution containing both ionophores. Culture tubes containing the medium (5 ml) and the appropriate ionophore solution (0.01 ml) were inoculated with 0.1 ml of a 24-h culture of the organisms grown in the ionophore-free basal medium. The MIC was determined in duplicate tubes and was defined as the lowest concentration of an ionophore which completely inhibited growth during a 24-h period. Growth of all cultures was monitored for 72 h to evaluate the possibility of an adaptive growth response. Growth was evaluated by monitoring optical density by using a Bausch & Lomb Spectronic 70 spectrophotometer at 600 nm in culture tubes measuring 13 by 100 mm. MIC values were confirmed several times during these studies to ensure consistency of this response.

Development of resistant strains. *P. ruminicola* 23MR2 and GA33MR were adapted to high concentrations of monensin from cultures of *P. ruminicola* 23 and GA33, respectively, by using the procedure described by Dawson and Boling (9). After 24 h, the MIC for strain 23 was less than 0.625 mg of monensin per liter. However, after extended incubation this strain could initiate growth in the presence of 0.625 mg of monensin per liter. Successive transfers of the organism into medium containing increasing concentrations of monensin resulted in a strain of *B. ruminicola* (23MR2) that could initiate growth in 40.0 mg of monensin per liter. The MIC for strain GA33 (10 mg of monensin per liter) was initially greater than that for strain 23. However, adaptation also occurred in this strain, resulting in a strain (GA33MR) which could initiate growth in 40 mg of monensin per liter. Adapted strains were streaked on roll tubes containing the basal medium modified to contain 2% agar and 10 mg of monensin per liter. Isolates from these roll tubes were maintained as monensin-resistant stock cultures in medium which did not contain ionophores.

Challenge of actively growing cultures with monensin. Erlenmeyer flasks containing 600 ml of basal medium A, modified to contain 0.5 g of glucose per 100 ml, were used for growth of batch cultures in ionophore challenge experiments. These cultures were inoculated with 5.0 ml of a 24-h culture of the organism grown in the basal medium and were constantly bubbled with oxygen-free CO_2 to maintain anaerobic conditions. After the cultures reached an A_{600} (light path, 1 cm) of approximately 0.4, the cultures were divided into separate flasks. Cultures were challenged with ethanol solutions (0.5 ml/100 ml) prepared to provide final monensin concentrations of 0, 10, and 40 mg/liter. Samples were taken from these cultures prior to challenge and at 0.25, 0.5, 1, 3, 6, 18, 24, and 36 h after challenge to evaluate optical density, protein concentration, and glucose concentration. End product concentrations were determined at the end of the 36-h incubation period.

Analysis. Concentrations of fermentation end products were determined by using gas chromatography. Volatile fatty acids, prepared as described by Erwin et al. (11), using cyclohexanone as an internal standard, were quantitatively separated on a 5880A gas chromatograph with an integrator (Hewlett Packard Co., Avondale, Pa.) using GP 10% SP-1000–1% H_3PO_4 on 100/200 Chromosorb WAW (Supelco, Inc., Bellefonte, Pa.) in a column measuring 1.83 m by 2 mm. The temperatures used for separation were as follows: injector, 185°C; detector, 190°C; oven, 140°C. Nonvolatile fatty acids were assayed as methyl esters by using oxalic acid as the internal standard (15). An Aerograph 2100 chromatograph (Varian Instrument Division, Palo Alto, Calif.) using the same packing material as described above and a column measuring 4 mm by 1.82 m was used to measure nonvolatile acid concentrations. The temperatures required for separation were as follows: injector, 200°C; detector, 200°C; oven, 155°C. Glucose concentrations were determined enzymatically by using a glucose hexokinase single-reagent system (16-50; Sigma Chemical Co., St. Louis, Mo.). Samples for glucose determination were centrifuged at 10,000 × *g* for 5 min and filtered by using a 0.2-μm-pore-size membrane filter to remove cells and cell debris. Protein concentrations were determined by using the Coomassie blue dye-binding assay system (2).

TABLE 1. MICs of representative ionophores for monensin-resistant and monensin-sensitive strains of *P. ruminicola*

Compound[a]	MIC[b] (mg/liter) for:			
	Sensitive strains		Resistant strains	
	23	GA33	23MR2	GA33MR
Monensin	<0.625	10.0	40.0	40.0
Lasalocid	<0.625	10.0	<0.625	10.0
Nigericin	10.0	20.0	40.0	40.0
Valinomycin	40.0	40.0	40.0	40.0
Nigericin-monensin	<0.625	<0.625	10.0	10.0
Nigericin-lasalocid	<0.625	<0.625	<0.625	<0.625
Nigericin-valinomycin	<0.625	<0.625	<0.625	<0.625
Monensin-lasalocid	<0.625	<0.625	<0.625	<0.625

[a] Concentrations of ionophores were equal in mixtures.
[b] MICs were tested in duplicate tubes. The average A_{600} was used to evaluate the growth of each organism.

Statistics. All treatments, except for the MICs, were examined in duplicate cultures. Means were determined from four replicate samples. Differences in treatment means were evaluated by using Student's *t* test (24).

RESULTS

Ionophore susceptibility of sensitive and resistant strains. The ability to initiate growth in the presence of high concentrations of monensin (>20 mg/liter) distinguished the monensin-resistant strains (23MR2 and GA33MR) from the monensin-sensitive strains (23 and GA33). Monensin-resistant strain 23MR2 initiated growth in a monensin concentration which was at least 40-fold greater than that which inhibited the growth of the parental strain (Table 1). Strain GA33 was initially resistant to greater concentrations of monensin than was strain 23. The concentration of monensin required to inhibit resistant strain GA33MR was four times greater than that required to inhibit sensitive strain GA33. Selection for monensin resistance did not alter sensitivity to lasalocid or valinomycin. However, both resistant strains were able to initiate growth in concentrations of nigericin (40 mg/liter) or a combination of nigericin with monensin which inhibited the growth of the sensitive parental strains. In general, exposure to combinations of two ionophores tended to be more effective at inhibiting growth than was exposure to a single agent. As a result, strains that were resistant to high concentrations (>5 mg/liter) of monensin or lasalocid were sensitive to lower concentrations (0.625 mg/liter) when both agents were added to the medium.

Resistance to monensin in the resistant strains was a stable characteristic which was maintained when the organisms were grown in cultures without monensin. The resistant strains were maintained for more than 10 successive trans-

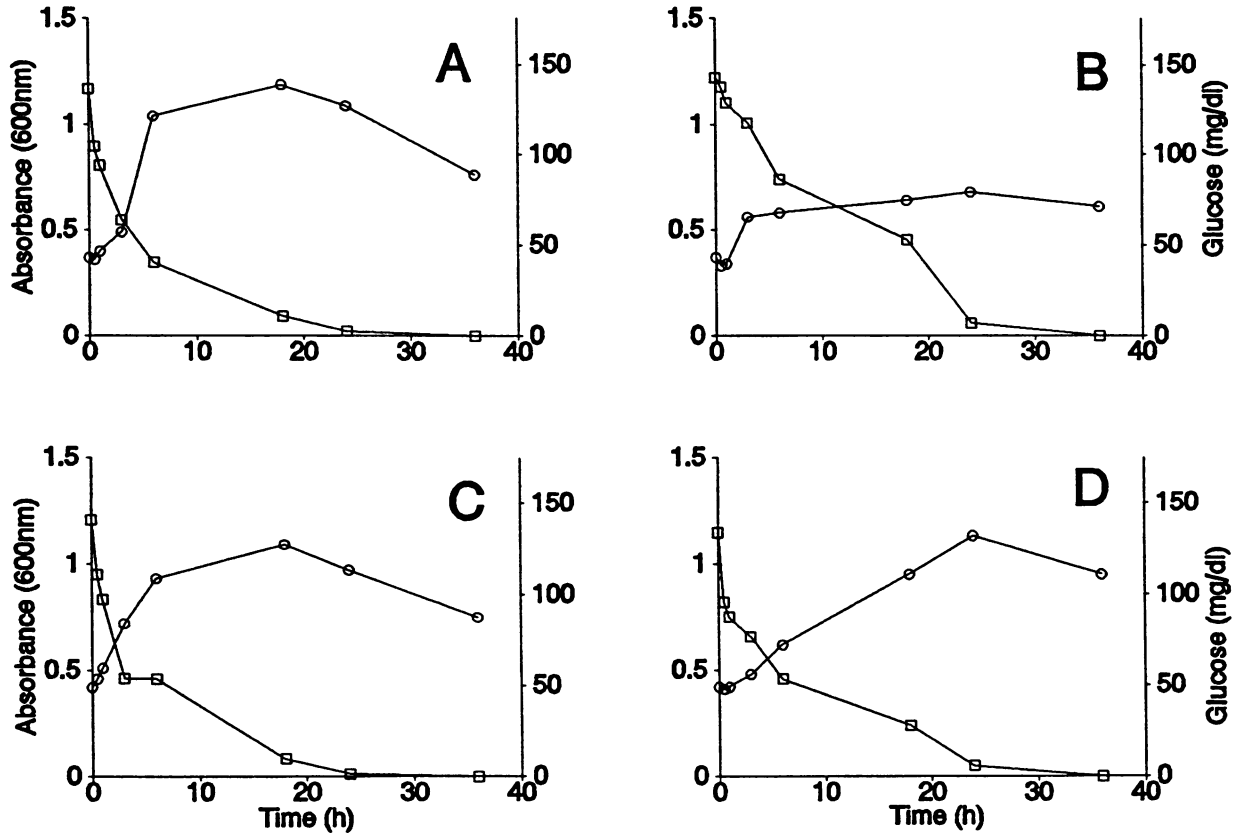

FIG. 1. Effects of monensin challenge (10 mg/liter) on glucose utilization (□) and growth (○) of monensin-sensitive (A and B) and monensin-resistant (C and D) strains originating from *P. ruminicola* subsp. *ruminicola* 23. Control cultures (A and C) were challenged with an ethanol solution containing no monensin. The ionophore was added to monensin-challenged cultures (B and D) in an ethanol solution.

fers in monensin-free medium without appreciable changes in their sensitivity to monensin.

Glucose utilization and growth of strains 23 and 23MR2. Similar growth and glucose utilization patterns were observed when sensitive strain 23 and resistant strain 23MR2 were grown in the defined glucose medium and challenged with ethanol in control batch cultures (Fig. 1A and C). There were no differences ($P > 0.05$) in the rates of growth and glucose utilization after ethanol challenge. Maximum cell yields were observed in these cultures 18 h after challenge, at which time over 95% of the glucose had been degraded in the control cultures. Glucose utilization and growth in cultures of both the sensitive and resistant strains were retarded after challenge with 10 mg of monensin per liter (Fig. 1B and C). Similar responses were seen in cultures challenged with 40 mg of monensin per liter (data not shown). Growth in cultures of sensitive strain 23 had stopped within 3 h of challenge (Fig. 1B). Glucose utilization continued in these cultures but at a rate that was lower ($P < 0.05$) than that observed in monensin-free controls (Fig. 1A). Growth rates of resistant strain 23MR2 also declined after challenge with monensin (10 mg/liter), but growth continued for up to 24 h (Fig. 1D). Maximum cell yields in monensin-challenged cultures of the resistant strain were observed 24 h after challenge. At this point, more than 90% of the glucose in the cultures had been depleted. Rates of glucose utilization in cultures of the resistant strain after challenge were slower ($P < 0.05$) than those observed in the monensin-free controls but faster ($P < 0.05$) than those observed in cultures of the sensitive strain after monensin challenge.

Protein yields and end product formation by strains 23 and 23MR2. Glucose utilization by sensitive strain 23 was decreased by more than 50% during the 18 h following challenge with 10 or 40 mg of monensin per liter (Table 2). Even though glucose utilization continued, protein concentration only increased very slowly, resulting in at least a 78% decrease in the protein yield in cultures of the sensitive strain following monensin challenge. Glucose utilization in cultures of the resistant strain was not influenced by monensin challenge. However, protein yields decreased ($P <$

TABLE 2. Effects of monensin challenge on glucose utilization and protein yields of monensin-sensitive (strain 23) and monensin-resistant (strain 23MR2) strains of *P. ruminicola* subsp. *ruminicola*[a]

Monensin concn (mg/liter)	Mean glucose utilization (mg/dl/18 h) ± SD		Mean protein yield (g/mol of glucose) ± SD	
	23	23MR2	23	23MR2
0	121.2 (0.2)	141.6 (8.4)	12.9 (1.2)	16.6 (3.0)
10	59.9 (1.7)	151.1 (3.1)	2.9 (0.3)	13.1 (0.2)
40	40.7 (1.6)	143.3 (0.8)	2.2 (0.1)	8.7 (1.4)

[a] Glucose utilization and yields represent net changes during the 18 h following challenge. The values shown are means from duplicate cultures.

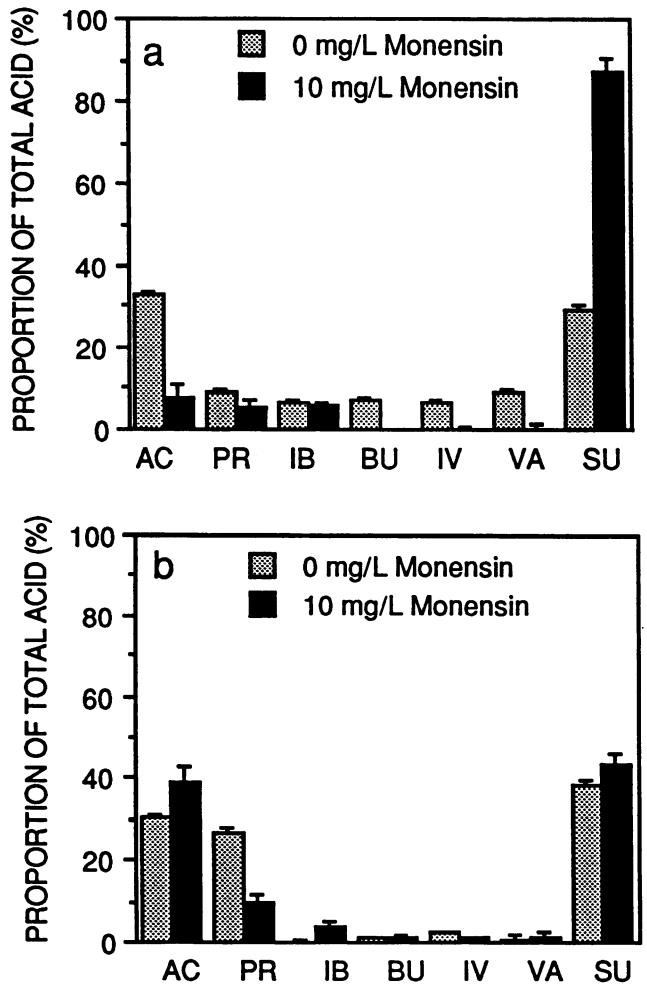

FIG. 2. Relative end product production by monensin-sensitive strain 23 (a) and monensin-resistant strain 23MR2 (b) 36 h after monensin challenge. The end products measured were acetic acid (AC), propionic acid (PR), isobutyric acid (IB), butyric acid (BU), isovaleric acid (IV), valeric acid (VA), and succinic acid (SU).

0.05) as monensin concentrations increased from 10 to 40 mg/liter.

The total amount of acidic end products produced 36 h after challenge averaged 26.2 mmol/liter and was lower ($P < 0.05$) in monensin-challenged (10 mg/liter) cultures of strain 23 than in the ethanol-challenged control (28.2 mmol/liter). However, acid production was greater ($P < 0.05$) in cultures of the resistant strain after challenge (23.4 versus 28.8 mmol/liter). Total acidic end product production was greater ($P < 0.05$) in cultures of the sensitive strain than in cultures of the resistant strain. In the absence of monensin challenge, the resistant strain produced considerably more propionate and succinate than did the sensitive strain (Fig. 2). Challenge with monensin altered patterns of end product formation by both the sensitive and resistant strains. The relative amounts of acetate, propionate, isobutyrate, butyrate, isovalerate, and valerate produced were smaller ($P < 0.05$) and the relative amounts of succinate produced were greater ($P < 0.05$) in the cultures containing the sensitive strain after challenge than in the monensin-free control. In comparison, more acetate and succinate and less propionate were associated with monensin-challenged cultures containing strain 23MR2, but these changes were not as great as those associated with the challenge of the sensitive strain.

Glucose utilization and growth of strains GA33 and GA33MR. Even though parental strain GA33 could initiate growth in a monensin concentration of 10 mg/liter (Table 1), both the rate of glucose utilization and the rate of growth were decreased by challenge with monensin during the logarithmic growth phase (Fig. 3A and B). Resistant strain GA33MR grew more rapidly than the sensitive strain in the defined glucose medium in the absence of monensin (Fig. 3C). Growth rates and glucose utilization were greatest in the resistant cultures (strain GA33MR) immediately following challenge with ethanol and were greatest in the monensin-challenged cultures (Fig. 3C and D).

Protein yields and end product formation from strains GA33 and GA33MR. Glucose utilization by the sensitive strain during the 18 h following challenge with 10 or 40 mg of monensin per liter was decreased by 81 and 60%, respectively (Table 3). This was accompanied by decreases in protein yields of 50 to 80%, respectively. Glucose utilization by resistant strain GA33MR was not inhibited by challenge with a monensin concentration of 10 mg/liter but was decreased by challenge with 40 mg/liter. Decreased glucose utilization was not accompanied by a decrease in the production of bacterial protein in these culture and resulted in greater protein yields when the resistant strain was exposed to 40 mg of monensin per liter.

The average total amount of acidic end products produced in cultures of sensitive strain GA33 36 h after monensin challenge (10 mg/liter) was 23.6 mmol/liter and was greater ($P < 0.05$) than that observed in the ethanol-challenged control cultures (19.3 mmol/liter). In contrast, resistant strain GA33MR tended to produce more acidic end products in the ethanol-challenged control culture (23.9 mmol/liter) than in the monensin-challenged culture (22.9 mmol/liter) after 36 h. Propionate made up a greater proportion of the end products produced in cultures of strains GA33 and GA33MR than in cultures of strains 23 and 23MR2. Acetate production was decreased and succinate production was greater in cultures of sensitive strain GA33 after monensin challenge (Fig. 4). However, such shifts in metabolic activities were not associated with the growth of resistant strain GA33MR when it was challenged with monensin (10 mg/liter). Unlike strains 23, 23MR, and GA33, the relative amounts of succinate produced were not influenced when cultures of resistant strain GA33MR were challenged with monensin.

DISCUSSION

A number of investigators have suggested that gram-negative bacteria are resistant to the antimicrobial effects of ionophores (6, 10, 14, 17). This resistance has been related to inability of ionophores to permeate the outer cell membrane in gram-negative bacteria (18, 21). However, our data suggest that there is considerable variation in the ionophore susceptibility of various strains of *P. ruminicola*. This is consistent with other studies which have shown that the monensin sensitivity of some groups of gram-negative bacteria can be altered by exposure to increasing concentrations of ionophores in a stepwise process (6, 9, 14). The present study confirms the existence of mechanisms which resulted in the development of increased monensin resistance in at least two physiologically distinct strains of *P. ruminicola*.

Resistant strains had growth patterns and ionophore sus-

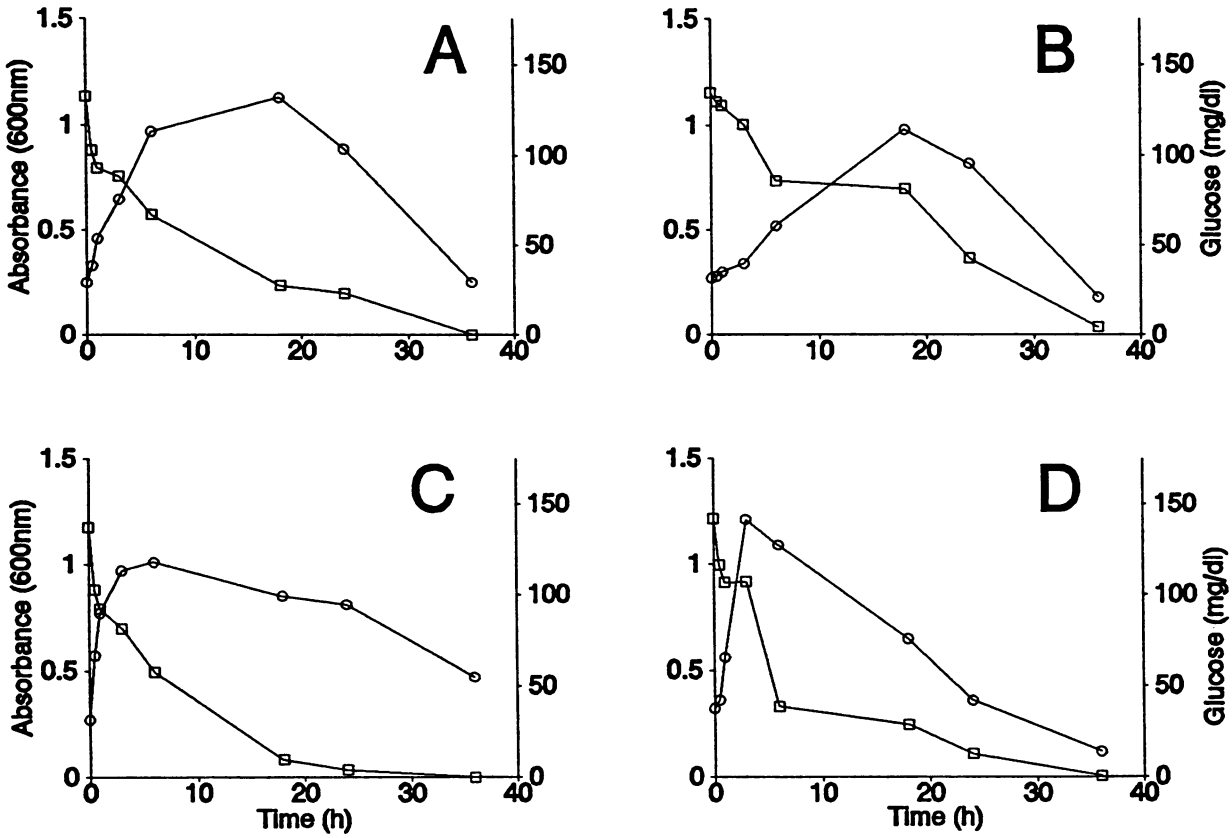

FIG. 3. Effects of monensin challenge (10 mg/liter) on glucose utilization (□) and growth (○) of monensin-sensitive (A and B) and monensin-resistant (C and D) strains originating from *P. ruminicola* subsp. *brevis* GA33. Control cultures (A and C) were challenged with an ethanol solution containing no monensin. The ionophore was added to monensin-challenged cultures (B and D) in an ethanol solution.

ceptibility patterns which were distinctly different from those of the sensitive parental strains. The resistant strains were able to initiate growth in monensin concentrations which consistently inhibited the initiation of growth in cultures of the parental strain. In addition, the growth of these resistant strains was not as readily influenced when actively growing cultures were challenged with monensin. However, selection for resistance to monensin did not result in strains that were universally resistant to other ionophores or combinations of ionophores. This suggests that resistance mechanisms are very specific for certain ionophores or classes of ionophores.

The monensin susceptibility pattern of at least one strain of *P. ruminicola* was altered by exposure to as little as 0.625 mg of monensin per liter. This concentration was much lower than the typical monensin concentration (5.5 to 33 mg/kg) used in ruminant feeds. Even when considering dilution of feed materials with water and saliva, it is easy to predict absolute monensin concentrations which could significantly alter the susceptibility patterns of these gram-negative bacteria in the rumen.

TABLE 3. Effects of monensin challenge on glucose utilization and protein yields of monensin-sensitive (GA33) and monensin-resistant (GA33MR) strains of *P. ruminicola* subsp. *brevis*[a]

Monensin concn (mg/liter)	Mean glucose utilization (mg/dl/18 h) ± SD		Mean protein yield (g/mol of glucose) ± SD	
	GA33	GA33MR	GA33	GA33MR
0	112.0 (0.5)	114.5 (0.4)	23.0 (1.6)	24.0 (0.2)
10	21.2 (1.8)	108.5 (2.0)	11.4 (1.7)	25.7 (0.8)
40	44.1 (0.3)	40.6 (0.5)	4.7 (1.2)	41.4 (1.7)

[a] Glucose utilization and yields represent net changes during the 18 h following challenge. The values shown are means from duplicate cultures.

The addition of monensin to logarithmic cultures of the sensitive strains did not completely inhibit growth when used at concentrations which prevented initiation of growth in freshly inoculated cultures. Such differences in sensitivity patterns may be related to the relative amounts of ionophore required to react with and influence the membrane material present at different stages of growth. This observation is in agreement with previous work which suggested that the ratio of ionophore to cell material may be more important than the absolute ionophore concentration in determining the physiological effects of monensin on ruminal bacteria (7).

Monensin challenge of both resistant and sensitive strains of *P. ruminicola* resulted in distinct changes in growth and metabolism, even though many of these strains were not considered to be sensitive to the antimicrobial activities of monensin in the initial MIC studies. In all of the organisms examined in this study, the rate of glucose utilization was decreased when actively growing cells were challenged with monensin at concentrations of 10 and 40 mg/liter. This was particularly apparent in monensin-sensitive strains. However, there was no instance in which glucose utilization was

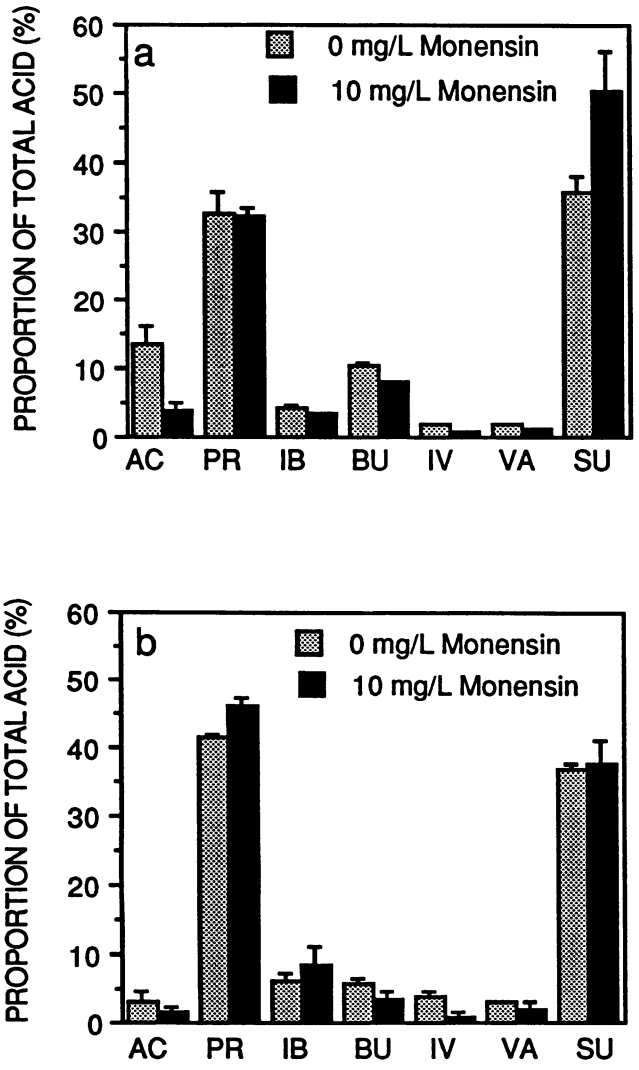

FIG. 4. Relative end product production by monensin-sensitive strain GA33 (a) and monensin-resistant strain GA33MR (b) 36 h after monensin challenge. The end products measured were acetic acid (AC), propionic acid (PR), isobutyric acid (IB), butyric acid (BU), isovaleric acid (IV), valeric acid (VA), and succinic acid (SU).

completely inhibited by monensin challenge. The mechanism by which monensin influences glucose utilization is not clear. However, studies with another gram-negative ruminal bacterium, *Fibrobacter succinogenes*, have demonstrated that glucose uptake by bacterial cells can be inhibited by monensin and lasalocid (12, 16). These effects appear to be directly related to the ability of the ionophore to alter membrane function and the energetics of glucose transport mechanisms.

Monensin challenge also resulted in decreased protein yields in both of the sensitive strains and in one of the resistant strains. However, unlike other studies with gram-positive organisms (7, 20), growth of strains of *P. ruminicola* did not appear to be completely inhibited when actively growing cultures were challenged with monensin. Measurable but impaired protein production was associated with all of the cultures for up to 18 h after monensin challenge. Observations in this and similar studies of *Streptococcus bovis* support the hypothesis that ionophores act to uncouple bacterial growth from fermentation processes (7, 20, 21). This may be related to energy expenditure to maintain membrane functions rather than support normal growth and protein synthesis.

The patterns of end product formation were also altered during selection for resistance and, in many cases, by exposure to monensin. In all but one of the strains examined in this study, challenge with monensin enhanced succinate formation at the expense of acetate and propionate production. Few studies have provided evidence that exposure to ionophores can alter the metabolic activities. However, Henderson et al. reported that the proportion of succinate relative to acetate was increased in one strain of *P. ruminicola* when it was grown in a monensin concentration of 10 mg/liter (14). Such changes in end product formation may not in themselves completely account for the enhanced propionate formation associated with ionophore supplementation in the rumen but suggest that ionophores have an impact on the metabolic activities in some succinate-producing ruminal bacteria.

Ionophores are generally believed to influence ruminal microbial activities by selectively inhibiting the growth of specific groups of hydrogen-, butyrate-, and lactate-producing bacteria while allowing the growth of resistant propionate-producing bacteria (6, 10, 14, 17). Such a model is consistent with the observed ionophore susceptibility patterns of ruminal bacteria. However, this study suggests that ionophores also alter the metabolic activities of some of the predominant, gram-negative anaerobic bacteria in the rumen. Such metabolic changes could provide alternate mechanisms for modifying rumen function based on altered pathways for end product formation. This mechanism would provide an explanation of earlier studies which failed to correlate changes in ruminal volatile acid production with increased proportions of monensin-resistant bacteria in the rumens of steers receiving a monensin supplement (8).

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