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Influence of Hydrogen-Consuming Bacteria on Cellulose Degradation by Anaerobic Fungi

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The presence of methanogens *Methanobacterium arboriphilus*, *Methanobacterium bryantii*, or *Methanobrevibacter smithii* increased the level of cellulose fermentation by 5 to 10% in cultures of several genera of anaerobic fungi. When *Neocallimastix* sp. strain L2 was grown in coculture with methanogens the rate of cellulose fermentation also increased relative to that for pure cultures of the fungus. Methanogens caused a shift in the fermentation products to more acetate and less lactate, succinate, and ethanol. Formate transfer in cocultures of anaerobic fungi and *M. smithii* did not result in further stimulation of cellulolysis above the level caused by H_2 transfer. When *Selenomonas ruminantium* was used as a H_2-consuming organism in coculture with *Neocallimastix* sp. strain L2, both the rate and level of cellulolysis increased. The observed influence of the presence of methanogens is interpreted to indicate a shift of electrons from the formation of electron sink carbon products to H_2 via reduced pyridine nucleotides, favoring the production of additional acetate and probably ATP. It is not known how *S. ruminantium* exerts its influence. It might result from a lowered production of electron sink products by the fungus, from consumption of electron sink products or H_2 by *S. ruminantium*, or from competition for free sugars which in pure culture could exert an inhibiting effect on cellulolysis.

The microbial population of the rumen consists of many species of strictly anaerobic bacteria, protozoa, and fungi (3, 8). When grown in pure culture, many of these organisms, including all known species of anaerobic fungi, produce, in addition to acetate, one or more of the fermentation products H_2, ethanol, lactate, and succinate, which are formed as electron sink products during the reoxidation of reduced pyridine nucleotides (NADH and NADPH) formed during glycolysis. The fermentation products of the mixed ruminal population are usually CO_2, CH_4, acetate, propionate, and butyrate, while other products do not normally accumulate (27). Hungate (8) has suggested that the disposal of electrons via CH_4 might be energetically more favorable than the production of other electron sink products in the mixed ruminal population.

It has been shown that interspecies H_2 transfer between the cellulolytic H_2-producing anaerobic fungus *Neocallimastix frontalis* and methanogens (among these a *Methanobrevibacter* species) resulted in increased CO_2 and acetate formation and decreased ethanol and lactate production (1, 16). This phenomenon is comparable with the effect of methanogens on H_2-producing bacteria (26). The shift in fermentation is made possible by the presence of a hydrogenase in the H_2-producing organism, which catalyzes the production of H_2 from reduced pyridine nucleotides at low partial pressures of H_2 ($P_{H_2} < 10^{-3}$ atm [0.1 kPa]) (25). At a higher P_{H_2}, this reaction is thermodynamically unfavorable (1, 25). As methanogens maintain low H_2 levels in these cocultures, the production of H_2 is facilitated, resulting in a shift away from products like lactate, succinate, and ethanol. As ATP synthesis is coupled to the formation of acetate in many anaerobes (6), higher acetate production in the presence of methanogens could have a profound effect on energy metabolism and growth of the fungus. In cocultures of *N. frontalis* and methanogens, a higher rate and degree of cellulose fermentation were indeed observed with a higher cellulolytic activity (1, 16, 28). The role of formate transfer in this type of coculture has not been studied yet. It was suggested (2) that despite the higher diffusion coefficient of H_2 in microbial flocs (4), formate transfer could also be of considerable importance because of the fact that formate might reach fairly high concentrations relative to H_2 within such close microbial associations.

In addition to methanogens, some sugar-fermenting ruminal bacteria, such as *Selenomonas ruminantium*, are capable of consuming extracellular H_2 (7). Hydrogen can be used by *S. ruminantium* in the formation of propionate, which is produced via the succinate pathway (21). In addition, this bacterium can utilize succinate produced by other microorganisms, and several strains of *S. ruminantium* are capable of fermenting lactate (20, 24). Although these bacteria form an important group of bacteria in the rumen, interactions between these bacteria and anaerobic fungi have not been studied.

In this paper, we describe the effect of methanogens *Methanobacterium bryantii*, *Methanobacterium arboriphilus*, and *Methanobrevibacter smithii* on cellulose fermentation by several anaerobic fungi. We also describe the influence of *S. ruminantium* on cellulose fermentation by a *Neocallimastix* strain isolated from llama feces.

MATERIALS AND METHODS

Organisms. *M. smithii* PS was provided by M. P. Bryant (University of Illinois, Urbana), *M. arboriphilus* AZ was provided by J. Macy (University of California, Davis), and *M. bryantii* OGC 110 was provided by D. R. Boone (Oregon Graduate Center, Beaverton). *S. ruminantium* subsp. *ruminantium* (strain JW2) and *S. ruminantium* subsp. *lactilytica*

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(strain JW13) were isolated from the rumen of a sheep as described previously (24). *Piromonas communis* P and *Neocallimastix patriciarum* CX were provided by C. G. Orpin (Commonwealth Scientific and Industrial Organisation, Queensland, Australia). *Sphaeromonas communis* FG10 was kindly donated by G. Fonty (Institut National de la Recherche Agronomique, Theix, France). *N. frontalis* RE1 was isolated from the rumen of a sheep as described previously (22). Strain L2 was isolated by F. D. Marvin-Sikkema from llama (*Lama guanicoë glama*) feces by the method of Joblin (9) with 10 mM cellobiose as a carbon and energy source and was characterized by phase-contrast microscopy, using a Carl Zeiss G42-110-e Axioscope.

Media and growth conditions. Three different media were used in the experiments. Basal medium contained 150 ml of solution I, 150 ml of solution II, 10 ml of a fatty acid solution, 10 ml of a trace elements solution, 10 ml of a vitamin solution, 10 ml of a hemin solution, 6 g of $NaHCO_3$, 0.1 mg of resazurine, and distilled water to a final volume of 1 liter. Cysteine hydrochloride was added as a reducing agent (1.0 g $liter^{-1}$) to all culture media prior to heat sterilization. Solution I contained 3.0 g of K_2HPO_4 per liter. Solution II contained (per liter) 3.0 g of KH_2PO_4, 3.0 g of $(NH_4)_2SO_4$, 6.0 g of NaCl, 0.6 g of $MgSO_4 \cdot 7H_2O$, and 0.6 g of $CaCl_2 \cdot 2H_2O$. The fatty acid solution was prepared by the method of Lowe et al. (13). The trace elements solution was prepared in 0.2 M HCl and contained (per liter) the following: 0.25 g of $MnCl_2 \cdot 4H_2O$, 0.25 g of $NiCl_2 \cdot 6H_2O$, 0.25 g of $NaMoO_4 \cdot 2H_2O$, 0.25 g of H_3BO_3, 0.20 g of $FeSO_4 \cdot 7H_2O$, 0.05 g of $CoCl_2 \cdot 6H_2O$, 0.05 g of $Na_2SeO_3 \cdot 5H_2O$, 0.05 g of $NaVO_3 \cdot 4H_2O$, 0.025 g of $ZnSO_4$, and 0.025 g of $CuSO_4 \cdot 2H_2O$. The vitamin solution was prepared in distilled water and contained (per liter) the following: 0.01 g of thiamin, 0.2 g of riboflavin, 0.6 g of calcium pantothenate, 0.6 g of nicotinic acid, 1.0 g of nicotinamide, 0.05 g of folic acid, 0.02 g of cyanocobalamin, 0.2 g of biotin, 0.1 g of pyridoxamine, and 0.05 g of *p*-aminobenzoic acid. Hemin solution was prepared by dissolving 0.10 g of hemin in 1 liter of 0.05 N NaOH.

A peptone yeast extract (PY) medium was the same as basal medium except it was supplemented with yeast extract (2.4 g $liter^{-1}$) (BBL, Cockeysville, Md.) and gelysate peptone (1.0 g $liter^{-1}$) (BBL).

A ruminal fluid (RF) medium was the same as basal medium to which centrifuged (twice for 30 min at 30,000 × *g*) ruminal fluid (20% [vol/vol]) and trypticase peptone (0.5 g $liter^{-1}$; BBL) were added. Vitamin and hemin solutions were omitted from the RF medium.

Growth experiments were performed in small Hungate tubes (16 by 125 mm) with 10-ml aliquots of medium, containing 2 mg of ball-milled filter paper (Schleicher and Schuell) per ml, fitted with butyl septum stoppers (Bellco Glass Inc., Vineland, N.J.), flushed with O_2-free CO_2, and subsequently autoclaved (121°C, 20 min). The vitamin and hemin solutions were sterilized by membrane filtration (0.15-μm pore diameter) and added to the medium after autoclaving.

The fungi were cultured in basal medium with filter paper as the carbon and energy source (pH = 6.8) and subcultured every 3 to 4 days. *M. smithii* and *M. bryantii* were grown in basal medium under an O_2-free 80% H_2–20% CO_2 gas phase (pH = 7.0). *M. arboriphilus* was grown in the PY medium under the same gas phase (pH = 7.0). *S. ruminantium* JW2 and JW13 were grown in the RF medium with 15 mM glucose (filter sterilized; 0.15-μm pore diameter) (pH = 6.8). Coculture experiments with anaerobic fungi and *M. smithii* or *M. bryantii* were carried out in basal medium with 2 mg of ball-milled filter paper per ml as a source of cellulose (pH = 6.8). Experiments with anaerobic fungi and *M. arboriphilus* were carried out in the PY medium (pH = 6.8). Coculture experiments with anaerobic fungi and *S. ruminantium* were carried out in 10 ml of RF medium with 3 mg of filter paper (Whatman no. 1) per ml inoculated with 3×10^7 bacteria and fungal cultures with 10^2 to 10^3 viable zoospores (final titers) (pH = 6.8). All incubations were carried out at 39°C. All transfers were made by using plastic syringes with hypodermic needles to pierce the butyl rubber septa.

Analytical methods. Alcohols (11) and short-chain fatty acids, both volatile and nonvolatile (18), were analyzed by gas chromatography. Formate was determined by the method of Lang and Lang (12). H_2 and CH_4 in the gas phase of the cultures were analyzed as described before (5). CO_2 formation could not be analyzed, because the gas phase in the growth experiments consisted of 100% CO_2. Therefore, it was approximated on the basis of the fermentation products formed, assuming the following relationship (quantities in moles): formation of CO_2 = acetate + ethanol − formate − succinate − CH_4 formation. Cellulose was determined by the method of Updegraff (23) and glucose by the glucose oxidase method (15). Protein was measured by the method of Lowry et al. (14) in cocultures with methanogens and with the Bio-Rad assay system (Bio-Rad, Watford, United Kingdom) for the experiments with *S. ruminantium* after the samples were boiled in 0.5 N NaOH to release intracellular proteins.

RESULTS

Isolation of the anaerobic fungus strain L2. An anaerobic fungus was isolated from llama feces, according to the methods described by Joblin (9) with 10 mM cellobiose as a carbon and energy source. After 3 days, visible growth was observed in roll tubes. One colony was picked and cultured in liquid medium. This isolate, L2, proved to be a pure culture and was assigned to the genus *Neocallimastix* (3) on the basis of its structural characteristics: the thallus of strain L2 consisted of a sporangium with a highly branched rhizoid, its zoospores were polyflagellated, and the organism formed monocentric colonies in roll tubes.

Fermentation of cellulose in pure and mixed cultures. The major characteristics of the cellulose fermentation by some anaerobic fungi grown in pure culture or in coculture with *M. arboriphilus*, *M. bryantii*, or *M. smithii* are shown in Table 1. In all cases, the fermentation of cellulose by the fungi alone resulted in the formation of H_2, CO_2, formate, acetate, lactate, succinate, and ethanol. In cocultures with *M. arboriphilus* or *M. bryantii*, CH_4, CO_2, formate, and increased amounts of acetate were practically the only products formed. Only traces of succinate and ethanol could be detected, while lactate was not detected. When *M. smithii* was used in the cocultures, a largely similar shift in fermentation products was observed, together with a rise in the production of CO_2 and complete consumption of formate by the methanogen. In none of the cocultures could H_2 be detected during or after growth. The amounts of cellulose fermented and protein formed after 14 days of incubation were higher in all cocultures than in pure cultures of the fungi (Table 1). In similar experiments in which glucose was used instead of cellulose (data not shown), no such differences in the extent of substrate consumption were detected, although very similar shifts in the fermentation product pattern were found.

TABLE 1. Fermentation of filter paper cellulose by anaerobic fungi in the absence or presence of methanogenic bacteria[a]

Organisms	Fermentation products (mol/10 mol of h.u. fermented)								Cellulose fermented (%)	Cell protein (g/10 mol of h.u. fermented)	Carbon recovery[b] (%)
	H_2	CO_2[c]	CH_4	Formate	Acetate	Lactate	Succinate	Ethanol			
N. frontalis RE1	1.80	3.75		6.30	6.48	6.73	0.59	4.16	89.5	74	100.1
N. frontalis RE1 + *M. arboriphilus* AZ		3.72	5.79	5.83	15.25		0.03	0.09	93.4	109	92.0
N. frontalis RE1 + *M. bryantii* OCG 110		3.63	6.01	6.01	15.55		0.01	0.10	93.4	121	95.1
N. frontalis RE1 + *M. smithii* PS		8.02	6.96		15.00		0.02		95.6	119	91.6
N. patriciarum CX	3.67	3.27		6.52	7.06	5.61	0.39	3.85	85.1	79	94.3
N. patriciarum CX + *M. arboriphilus* AZ		3.47	5.83	6.09	15.30		0.02	0.11	94.1	118	93.5
N. patriciarum CX + *M. bryantii* OCG 110		3.51	5.81	5.88	15.15		0.04	0.09	93.2	112	92.0
N. patriciarum CX + *M. smithii* PS		8.00	7.05		15.01		0.01	0.05	95.0	120	92.0
Neocallimastix sp. strain L2	1.68	3.22		6.04	5.50	7.21	0.48	4.24	88.3	81	98.4
Neocallimastix sp. strain L2 + *M. arboriphilus* AZ		3.63	5.95	6.01	15.50			0.09	94.2	124	95.2
Neocallimastix sp. strain L2 + *M. bryantii* OCG 110		3.35	5.80	5.99	15.10		0.04	0.08	93.8	123	93.2
Neocallimastix sp. strain L2 + *M. smithii* PS		7.66	7.11		14.76		0.01	0.02	96.5	120	90.6
P. communis P	3.87	3.46		7.32	7.17	4.27	0.81	4.41	80.2	83	94.8
P. communis P + *M. arboriphilus* AZ		3.06	5.67	6.38	15.37		0.01	0.07	95.1	119	93.2
P. communis P + *M. bryantii* OCG 110		3.10	5.99	6.20	15.17			0.12	94.3	125	93.8
P. communis P + *M. smithii* PS		7.74	7.58		15.23		0.01	0.10	93.1	121	93.5
S. communis FG10	4.67	6.78		8.07	9.56	1.51	0.26	5.55	79.1	80	95.5
S. communis FG10 + *M. arboriphilus* AZ		2.80	5.81	7.11	15.60			0.12	94.1	121	95.4
S. communis FG10 + *M. bryantii* OCG 110		2.68	5.93	6.95	15.43		0.01	0.14	93.5	115	93.9
S. communis FG10 + *M. smithii* PS		7.02	7.69		14.60	0.01	0.01	0.12	95.6	120	90.4

[a] Fermentation products, protein and cellulose were analyzed after 14 days of growth. h.u., Hexose units from cellulose.
[b] Carbon recoveries were estimated assuming that the protein content of the cell approximately equaled its carbon content on the basis of gram per gram (dry weight). This approximation was necessary, since cell carbon could not be measured directly because of attachment of the cells to cellulose particles.
[c] CO_2 was calculated on the basis of the other fermentation products formed (see Materials and Methods).

The time courses of cellulose degradation and formation of fermentation products in a pure culture of *Neocallimastix* sp. strain L2 and in cocultures of *Neocallimastix* sp. strain L2 and *M. bryantii* or *M. smithii* are shown in Fig. 1. The observed pattern illustrates the close coupling between cellulose hydrolysis and fermentation product formation. With the anaerobic fungus alone, the rate of cellulose degradation was lower (81 μM hexose units fermented per h) than in the cocultures. In the presence of *M. bryantii* or *M. smithii*, a 15 to 25% increase in the rate of cellulose degradation was observed: 101 and 95 μM hexose units fermented per h, respectively. A similar pattern was observed for the rates of fermentation product formation, which were expressed as micromolar carbon in fermentation products produced per hour, 520, 615, and 620 μM carbon h^{-1}, respectively.

In mixed cultures containing *Neocallimastix* sp. strain L2 and the lactate-utilizing *S. ruminantium* JW13, cellulose was mainly fermented to CO_2, formate, acetate, and propionate (Table 2). In comparable mixed cultures of *Neocallimastix* sp. strain L2 with *S. ruminantium* JW2, which is unable to ferment lactate, the fermentation pattern was very much the same. So, despite its inability to use lactate, the presence of *S. ruminantium* JW2 prevented accumulation of lactate in the mixed culture with *Neocallimastix* sp. strain L2. In both mixed cultures, concentrations of ethanol and succinate were lower and the amounts of cellulose fermented and protein produced were somewhat higher than in the pure culture of *Neocallimastix* sp. strain L2. When the results of experiments with pure cultures of *Neocallimastix* sp. strain L2 in basal or PY medium (Table 1) and in RF medium (Table 2) were compared, differences could be observed in the stoichiometry of the fermentation products, while cell protein yields were lower in the RF medium compared with the other two media.

The rates of cellulose fermentation were also monitored, and it was found that the rate of cellulolysis was significantly higher in the mixed cultures of *S. ruminantium* JW13 or JW2 with *Neocallimastix* sp. strain L2 than in the pure culture of the fungus (approximately 500, 560, and 350 μM hexose units fermented per h, respectively). The higher growth rate of *Neocallimastix* sp. strain L2 in RF medium compared with growth in basal medium (Fig. 1) is apparent.

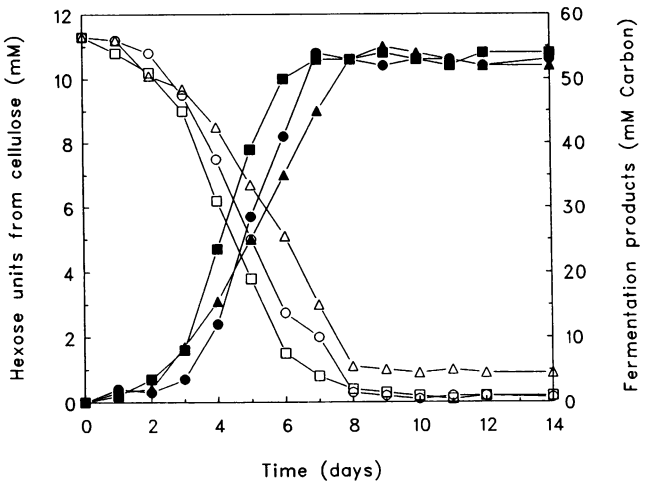

FIG. 1. Cellulose degradation (open symbols) and fermentation product formation (solid symbols) by an axenic culture of *Neocallimastix* sp. strain L2 (△ and ▲) and cocultures of this organism with *M. bryantii* (□ and ■) or *M. smithii* (○ and ●). Organisms were cultured in basal medium as described in Materials and Methods. Cellulose was expressed as millimolar hexose units obtained after hydrolysis of residual cellulose. Fermentation products were expressed as millimolar carbon. This excludes cell carbon which accounts for approximately 10 mM carbon (see Table 1).

DISCUSSION

This study describes the influence of H_2-consuming bacteria on cellulose fermentation by anaerobic fungi.

When methanogens were used as the H_2-consuming organisms, a shift in fermentation pattern to more acetate and less electron sink products was found. This shift appeared to be a general phenomenon in cocultures of H_2-CO_2 or H_2-CO_2 plus formate-consuming methanogens and representatives of several known genera of anaerobic fungi isolated from various ruminants. The rate of cellulose fermentation was higher in cocultures of *Neocallimastix* sp. strain L2 and methanogens than in pure cultures of the anaerobic fungus, confirming earlier observations (1, 16). The results shown in Fig. 1 also indicated that the level of cellulose hydrolysis was higher in the cocultures. However, this remains to be proven more firmly as the possibility that unknown products that accumulated only in the pure culture interfered with the method used to quantify residual cellulose cannot be excluded.

When cocultures of anaerobic fungi and *M. arboriphilus* or *M. bryantii* are compared with cocultures in which *M. smithii* was used, it is evident that interspecies formate transfer in the latter case did not result in additional stimulation of cellulose fermentation by the fungi. Although Orpin et al. (19) reported that *N. patriciarum* did not produce formate, in the present study strain CX did produce formate in quantities similar to those of the other fungal species tested. Whether this is the result of differences in growth conditions or the development of mutant strains cannot be decided.

The stimulatory effect of methanogens on cellulose fermentation by all anaerobic fungi tested suggests that these fungi contain hydrogenases which catalyze the production of H_2 from reduced pyridine nucleotides. The presence of this reaction sequence allows a relative increase in acetate production and concomitant ATP formation. The presence of such a hydrogenase was demonstrated in the anaerobic fungus *N. patriciarum* and was localized in microbodies designated as hydrogenosomes (29). The presence of hydrogenosomes in all other genera of anaerobic fungi (17) suggests that a similar system is operative and explains the comparable results found with all anaerobic fungi used in our experiments.

Compartmentalization of H_2 formation in the cell could facilitate the production of H_2 from reduced pyridine nucleotides by creating distinct pools of enzymes, substrates, or both between the compartment and the cytosol (10). Positioning of both this organelle and methanogens in close contact with the cell surface of the fungus, one on the inside and the other on the outside of the cell, may effectively reduce the distance for diffusion of H_2 from the hydrogenosome to the hydrogenotrophic bacteria. This may explain why no additional stimulatory effect by formate transfer was observed on cellulose fermentation in cocultures of the fungi and *M. smithii*, assuming that formate is not produced in the hydrogenosome. Close association of methanogens and the anaerobic fungus *N. frontalis* was observed by Bauchop et al. (1).

When *S. ruminantium* was used as a H_2-consuming organism in coculture with *Neocallimastix* sp. strain L2, the rate of cellulose degradation was enhanced. As *S. ruminantium* is capable of consuming free sugars, succinate and (strain JW13) lactate, it is not clear whether cellulolysis was enhanced by lowered concentrations of H_2 or other electron sink products or by competition for free sugars which in pure culture could exert an inhibiting effect on cellulolysis.

TABLE 2. Fermentation of filter paper cellulose and glucose[a]

Organism(s)	Fermentation products (mol/10 mol of h.u. fermented)								Cellulose or glucose fermented (%)	Cell protein (g/10 mol of h.u. fermented)	Carbon recovery[b] (%)
	H_2	CO_2[b]	Formate	Acetate	Propionate	Lactate	Succinate	Ethanol			
Neocallimastix sp. strain L2	3.19	2.10	7.32	6.39		5.55	0.94	3.97	82.5	35.8	91.2
Neocallimastix sp. strain L2 + *S. ruminantium* JW2	0.01	1.06	6.84	6.92	5.97	0.89	0.07	1.05	84.5	40.1	79.8
Neocallimastix sp. strain L2 + *S. ruminantium* JW13	0.02	1.84	7.01	7.66	7.26	0.08		1.18	86.6	36.5	85.9
S. ruminantium JW2					1.12	15.32	0.04		97.2	31.2	86.8
S. ruminantium JW13		9.94		9.94	12.96				97.8	37.5	119.0

[a] Fermentation products, protein, and cellulose were determined after 14 days of growth. Analyses of the fermentation pattern of pure cultures of *S. ruminantium* JW2 and JW13 were done after growth for 2 days on glucose. h.u., Hexose units from cellulose or glucose (in the case of *S. ruminantium*).

[b] Carbon recoveries were estimated assuming that the protein content of the cell approximately equaled its carbon content on the basis of gram per gram (dry weight). This approximation was necessary, since cell carbon could not be measured directly because of attachment of the cells to cellulose particles.

[c] CO_2 was calculated on the basis of the other fermentation products formed (see Materials and Methods).

It was shown that with all anaerobic fungi tested, interspecies H_2 transfer resulted in shifts in the fermentation, as has been amply demonstrated for anaerobic bacterial mixed cultures (25, 26). The possibility of manipulating H_2 flow by coculturing the fungi with methanogens, which would probably result in differences in numbers or enzymatic activities in the hydrogenosomes, could enlarge our knowledge of the function of compartmentalization of H_2 production and other parts of the fermentation pathway in hydrogenosomes of anaerobic fungi.

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