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Characterization of a Naturally Occurring Diamine Auxotroph of *Veillonella alcalescens*

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Veillonella alcalescens strain ATCC 17745 was shown to require putrescine or cadaverine for growth. None of the other compounds tried, including magnesium and spermidine, were able to substitute for the diamines. Studies with labeled diamines showed that spermidine was made from putrescine in this organism. A polyamine analogous to spermidine, but made from cadaverine, was not found. A combination of growth experiments and chemical assays suggested that protein synthesis was limited in diamine-starved cells. Protein synthesis occurred prior to nucleic acid synthesis when putrescine was added to starved cells.

The diamine putrescine, and the polyamines spermidine and spermine, have been found in every form of biological material. Many functional roles have been assigned to them (2, 17). These include the regulation of transcription (2, 16), regulation of translation (5, 10, 18, 19), and control of cellular osmotic pressure (13). The isolation of *Escherichia coli* mutants deficient in the synthesis of putrescine, and thereby spermidine (4, 9, 11), has been valuable in the investigation of polyamine function.

The genus *Veillonella* includes gram-negative, anaerobic cocci which do not utilize carbohydrates as an energy source. Some of the members of this genus, particularly the strains of *Veillonella alcalescens*, have been reported to have an absolute requirement for putrescine or cadaverine (15; D. Kafkewitz, M. S. thesis, Cornell Univ., Ithaca, N.Y., 1967). In view of the current interest in polyamines, it seemed useful to examine a naturally occurring diamine auxotroph and compare some of its properties with those of the isolated *E. coli* mutants.

MATERIALS AND METHODS

Organism and cultivation. *V. alcalescens* strain 17745 was obtained from the American Type Culture Collection. It was cultivated and maintained on a complex medium of the following composition: yeast extract (1%), tryptone (1%), sodium lactate (1%, vol/vol, using 60% syrup), potassium carbonate (0.01%), and sodium thioglycolate (0.075%). Putrescine was present at a final concentration of 5 μg/ml when added. A semidefined medium containing inorganic salts, casein hydrolysate, and a number of vitamins (15) was also used. Incubation was at 37 C. Anaerobiosis was maintained by using freshly autoclaved or steamed and rapidly cooled media, tightly capped growth vessels that were nearly full, or an anaerobic incubator. When it was necessary to take several samples from one culture over a period of time, the culture was continuously sparged with "prepurified" nitrogen. Cells were washed with a solution of 0.01% sodium sulfide to aid in maintaining reduced conditions. Absorbance was measured with a Fisher electrophotometer II or a Beckman DU spectrophotometer fitted with a Gilford absorbance indicator.

Polyamine analysis. For polyamine analysis, cells were harvested at the appropriate time at 4 C by centrifugation and washed three times in a small volume of 0.1 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer, pH 7.2. The cell pellet was weighed and extracted overnight at 4 C with a solution of 5% trichloroacetic acid containing hydrochloric acid to 0.05 N (10 ml/g [wet weight]). The pellet was washed with the same solution, and the two supernatant fluids were combined. Trichloroacetic acid was removed from the supernatant fluid by several ether extractions, and the ether was removed by bubbling with nitrogen. Polyamines were then extracted by the butanol method of Raina (14), dried with nitrogen, and resuspended in 0.1 N HCl. Polyamines were identified by the methods of Hammond and Hebst (3) and Karrer et al. (6). Amounts were estimated by the method of Hammond and Hebst (3).

Protein and RNA analyses. Protein was estimated by the method Lowry et al. (7). Ribonucleic acid (RNA) was estimated with the orcinol procedure (1). Samples for protein and RNA analyses were taken in the following manner. An appropriate volume of cell culture was killed by adding 10 N perchloric acid to a final concentration of 0.5 N and incubated in an ice bath for at least 30 min. The cells were collected by centrifugation and washed once with 0.5 N perchloric acid. The pellet was resuspended in the washing

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solution, and hydrolysis was carried out for 20 min at 70 C. After chilling and centrifuging, the supernatant fluid was used for RNA analysis, and the pellet was used for protein analysis. Duplicate determinations were made for each sample.

Polyamine uptake and synthesis. Cells were grown on the semidefined medium with putrescine and cadaverine at a concentration of 5 μg/ml and spermidine, when added, at 7.8 μg/ml. [1,4-^{14}C]putrescine-dihydrochloride (New England Nuclear Corp., 9 mCi/mmol), [1,5-^{14}C]cadaverine-dihydrochloride (Calbiochem, 4.05 mCi/mmol), and [1,4-^{14}C]spermidine-trihydrochloride (New England Nuclear Corp., 8.3 mCi/mmol) were added at 1 to 2 μCi/100 ml of medium. Inocula were taken from cells previously transferred once or twice in the same medium, but without radioactive components. Cells were harvested when the increase in mass had terminated or was nearly complete, unless otherwise indicated. They were washed, extracted, and analyzed as previously described. Radioactive spots were located by placing the thin-layer plates on X-ray film. Radioactive material was scraped from the plates and counted in a Packard Tri-Carb liquid scintillation spectrometer using a solution of 0.4% 2,5-bis[2-(5-*t*-butylbenzoxazolyl)]-thiopene.

RESULTS

Growth of V. alcalescens on putrescine and substitutes. Figure 1 shows the growth of *V. alcalescens* strain 17745 on complex medium with putrescine and various substitutes. Both putrescine and cadaverine supported a high level of growth. Without any supplements or with spermidine, some increase in mass was observed, but most of it was lost by the end of the growth period. Magnesium at a high level supported a certain amount of growth, but the rate was slower than with the diamines, and the cells appeared abnormal. Under the phase-contrast microscope they were seen to be swollen and lysing. The concentration of magnesium was almost 100 times higher than that present normally in the semidefined medium. Table 1 shows the final absorbance reached when the organism was grown on complex medium with varying levels of magnesium. Magnesium in any concentration does not serve to support growth as well as the diamines. It does appear, however, that higher densities are reached with higher levels of magnesium. It may be that magnesium is retarding the lysis of cells to some extent. Lysine and ornithine, the diamine precursors, were also unable to support growth above the level of the unsupplemented medium. When the same experiments were repeated using the semidefined medium rather than the complex medium, only putrescine and cadaverine were able to support growth. No increase in mass was observed with other substitutes during the normal growth period.

By using a large inoculum and transferring each day, it is possible to maintain these cells in complex medium without a diamine for at least

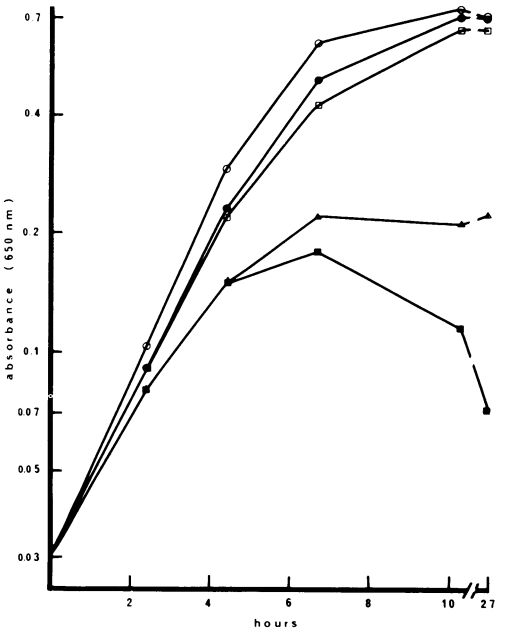

FIG. 1. *Growth with putrescine and substitutes. Cells were grown on complex medium with putrescine·HCl (5 μg/ml) and inoculated into complex medium with the following additives:* (○) *putrescine·HCl (5 μg/ml);* (●) *$MgSO_4 \cdot 7H_2O$ (40 mM) plus putrescine·HCl (5 μg/ml);* (□) *cadaverine·HCl (5 μg/ml);* (△) *$MgSO_4 \cdot 7H_2O$ (40 mM);* (■) *no additives or spermidine·HCl (7.5 μg/ml).*

TABLE 1. *Growth of V. alcalescens ATCC 17745 on complex medium plus varying levels of magnesium*

Additive	Final concn (mM)	OD_{650}[a]
$MgSO_4 \cdot 7H_2O$	0.4	0.08
$MgSO_4 \cdot 7H_2O$	1.0	0.07
$MgSO_4 \cdot 7H_2O$	2.0	0.10
$MgSO_4 \cdot 7H_2O$	4.0	0.10
$MgSO_4 \cdot 7H_2O$	20.0	0.16
$MgSO_4 \cdot 7H_2O$	40.0	0.21
$MgCl_2 \cdot 6H_2O$	0.5	0.06
$MgCl_2 \cdot 6H_2O$	1.2	0.08
$MgCl_2 \cdot 6H_2O$	2.5	0.09
$MgCl_2 \cdot 6H_2O$	5.0	0.10
$MgCl_2 \cdot 6H_2O$	25.0	0.15
$MgCl_2 \cdot 6H_2O$	50.0	0.19

[a] Growth was monitored every 2 h for 10 h and then again at 24 h. The optical density (OD) represents the maximum possible density reached by each culture. This was reached by 10 h.

17 days. The maximum density is 0.12. The small amount of growth is most likely due to the presence of small amounts of diamines in the complex medium. On defined medium with vitamin-free casein hydrolysate, it is not possible to transfer these cells at all without putrescine or cadaverine.

Figure 2 shows the maximum density possible for *V. alcalescens* strain 17745 in the presence of various levels of diamines. The response is fairly linear, with maximum growth supported by 3 to 4 μg/ml of either diamine per ml.

Uptake and synthesis of polyamines. Table 2 shows that this organism was able to take up the two diamines, putrescine and cadaverine. The polyamine spermidine was incorporated to a degree. It appears that spermidine is biosynthesized from putrescine. Little, if any, spermidine was converted to putrescine, but this may be a result of the fact that putrescine had to be present initially in the medium in order to obtain growth. In addition, it appears that very little, if any, cadaverine is converted to a polyamine. Extracts of cells grown on a medium with cadaverine have not been found to contain either spermidine or spermine. The polyamine content of different strains of *Veillonella* has been described (M. Ritchey and E. A. Delwiche, manuscript in preparation).

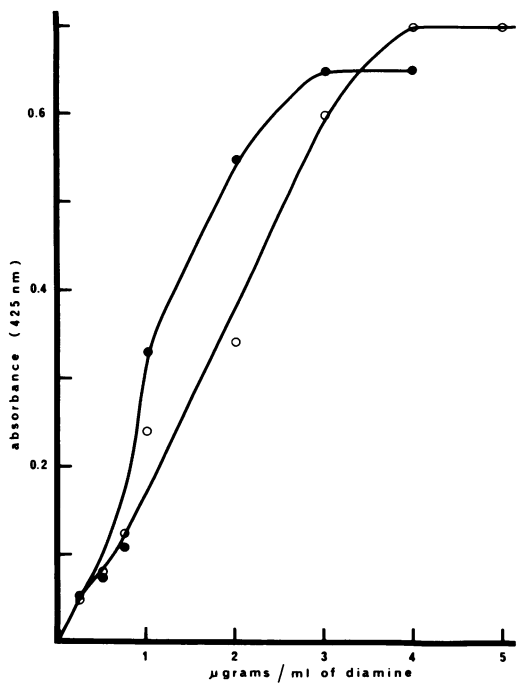

FIG. 2. *Growth versus concentration of diamines. Cells were grown in semidefined medium with the appropriate diamine (5 μg/ml) and then inoculated into semidefined medium with various amounts of* (○) *putrescine·HCl and* (●) *cadaverine·HCl.*

TABLE 2. *Synthesis and uptake of polyamines*

Culture conditions	Labeled compound	μmol/g (wet wt)	Uptake (%)[a]
[1,4-^{14}C]putrescine	Putrescine	1.8	5.9
	Spermidine	0.4	1.5
[1,5-^{14}C]cadaverine (early log)	Cadaverine	1.0	0.8
	Polyamine	0.0	0.04
[1,5-^{14}C]cadaverine (midlog)	Cadaverine	0.7	1.5
	Polyamine	0.0	0.02
[1,5-^{14}C]cadaverine (late log)	Cadaverine	2.7	6.1
	Polyamine	0.0	0.03
[1,4-^{14}C]spermidine[b]	Putrescine	0.0	0.05
	Spermidine	0.3	1.5

[a] Percentage of uptake was calculated as the amount of isotope taken up by all the cells in the culture at the time of harvest over the original amount of isotope added.

[b] The medium here also contained unlabeled putrescine.

Putrescine starvation. Figure 3 describes the growth of cells that had been cultured overnight in a complex medium containing putrescine, washed, and then resuspended in a complex medium with and without putrescine. Both cultures began to grow without an apparent lag and at the same rate for an hour. Then the cells without putrescine displayed an increasingly diminished growth rate. The generation time for the putrescine-supplemented cells was about 75 min, whereas that of the unsupplemented cells diminished to a generation time of approximately 150 min. They never displayed the optical density obtained by the normal culture, and eventually they lost about half of the density they did reach. Lysis was observed by phase-contrast microscopy at the end of the growth period, when these cells were losing their optical density. Figure 4 shows the analyses of RNA and protein for these cultures. The putrescine-free culture demonstrated a small net increase in RNA which eventually was lost. The protein was increased threefold. The culture with putrescine increased its net RNA content more than twofold and its net protein content sevenfold. The RNA-to-protein ratios were somewhat higher for the putrescine-starved culture during the course of the growth period.

Figure 5 shows the growth of cells that were collected after the maximum absorbance had been reached in the putrescine-free medium (grown as in Fig. 3), washed, and then resuspended in media with or without putrescine.

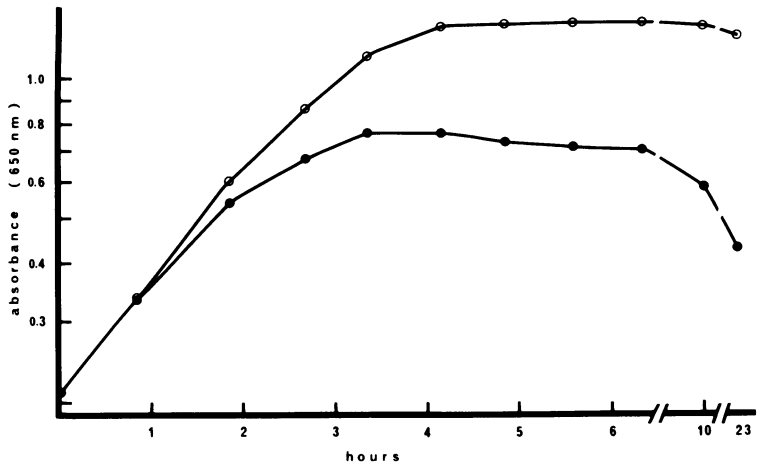

FIG. 3. *Growth on complex medium. Cells were grown overnight in complex medium with putrescine, washed as described, and then resuspended in complex medium* (○) *with and* (●) *without putrescine·HCl (5 μg/ml).*

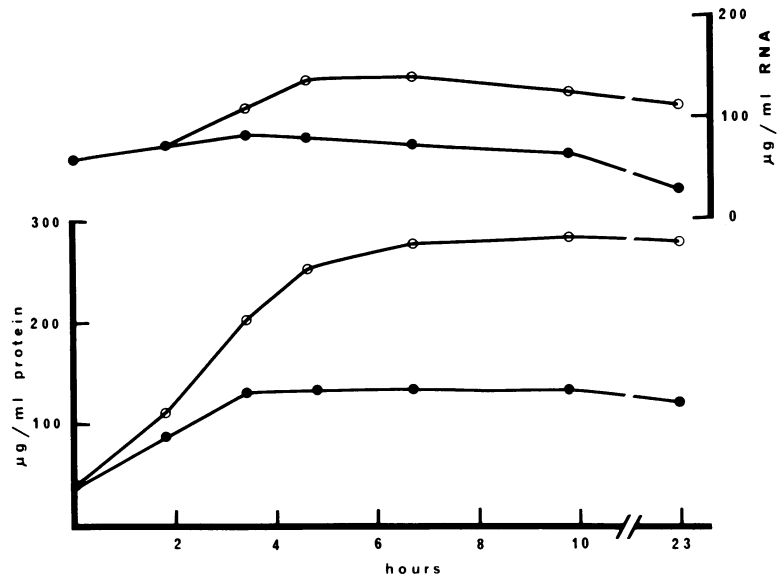

FIG. 4. *RNA and protein on complex medium. Cultures sampled for Fig. 3 were sampled for RNA and protein as described in Materials and Methods;* (○) *with putrescine,* (●) *without putrescine.*

These cells were now starved for polyamines and presumably had depleted internal pools of polyamines. Both cultures had a lag of about 1 h before growth began. The pattern of growth was similar to that described above, except that the putrescine-deficient cells had a slower growth rate than the supplemented cells over the whole growth period, rather than during the middle and latter part of the period. The putrescine-supplemented cells had a generation time of around 75 min, whereas the unsupplemented cells had a maximum generation time of 165 min. Also, the difference in mass between the two cultures was more pronounced here than in the set described above. Figure 6 shows the RNA and protein content of these cells. The unsupplemented culture had little net increase in RNA or protein. The supplemented culture, however, more than doubled its net RNA content and increased its net protein content 13-fold. Again the RNA-to-protein ratios were higher in the putrescine-free culture. These two sets of experiments indicate that protein synthesis is limited in putrescine-starved cells.

Figure 7 illustrates the effect of adding putrescine to a putrescine-free culture grown as

described in Fig. 5. At the time of addition of putrescine, the starved cells were growing at a slow rate, which represented the maximum rate that they would be able to attain. With the

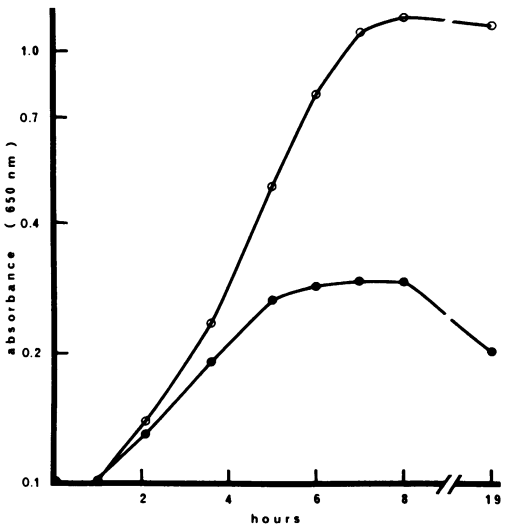

FIG. 5. *Growth on complex medium with starved cells. Cells were grown overnight in complex medium with putrescine, washed as described, and resuspended in complex medium without putrescine. After maximum absorbance was reached, the cells were washed and resuspended in complex medium* (○) *with and* (●) *without putrescine·HCl (5 μg/ml).*

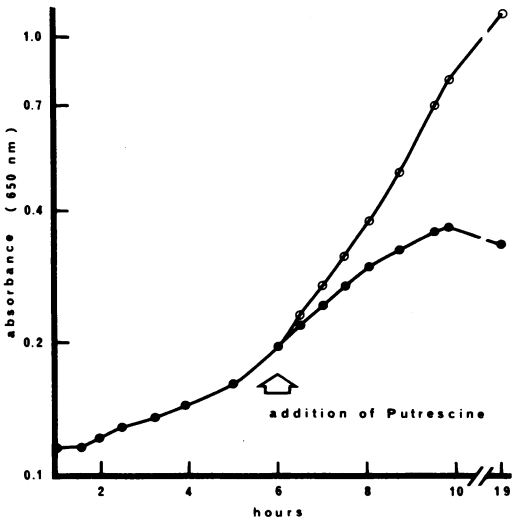

FIG. 7. *Addition of putrescine to starved cells. Cells were grown overnight in complex medium plus putrescine, washed as described, and resuspended in complex medium without putrescine. After maximum absorbance was reached, cells were washed, resuspended in complex medium without putrescine, and allowed to grow for a few hours. Then the culture was divided in two, and one part received putrescine·HCl (5 μg/ml). Symbols:* (○) *with putrescine,* (●) *without putrescine,* (△) *addition of putrescine.*

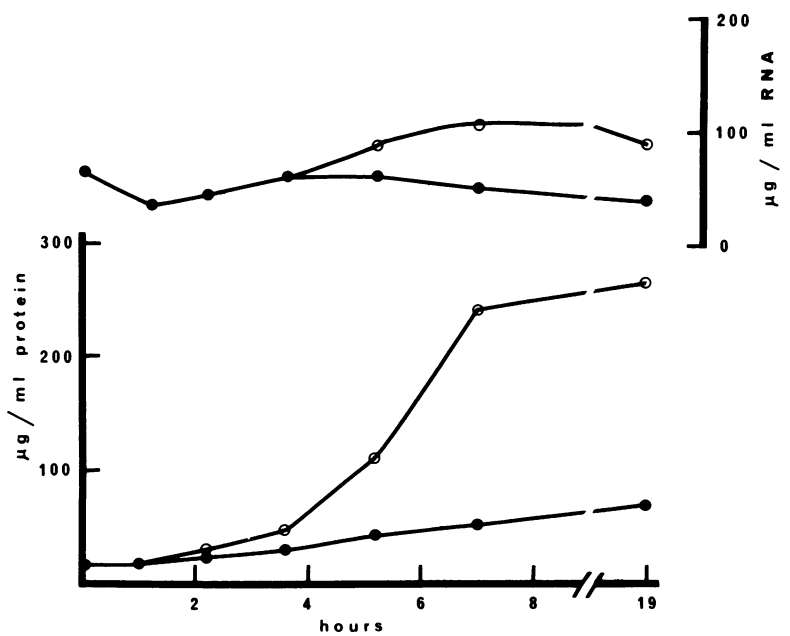

FIG. 6. *RNA and protein on complex medium with starved cells. Cultures sampled for Fig. 5 were also sampled for RNA and protein as described in Materials and Methods. Symbols:* (○) *with putrescine,* (●) *without putrescine.*

addition of putrescine, the cells began to grow at a new, more rapid rate. The maximum doubling time for the supplemented cells was 90 min, and for the unsupplemented cells it was 120 min. Figure 8 illustrates the RNA and protein analyses for these cultures. It appears as though an increase in protein content occurred before an increase in RNA was observed. (It should be noted, however, that a more sensitive technique, such as analysis with radioactive precursors, could reveal that a small amount of RNA synthesis occurs before protein synthesis.) This is in contrast to a normal shift-up experiment in which RNA increases before protein when nutrients are added to the medium (9). The rise in protein seen here paralleled the rise in optical density. The unsupplemented culture had a very small net increase in RNA and protein contents. The RNA-to-protein ratios were higher in the putrescine-free culture.

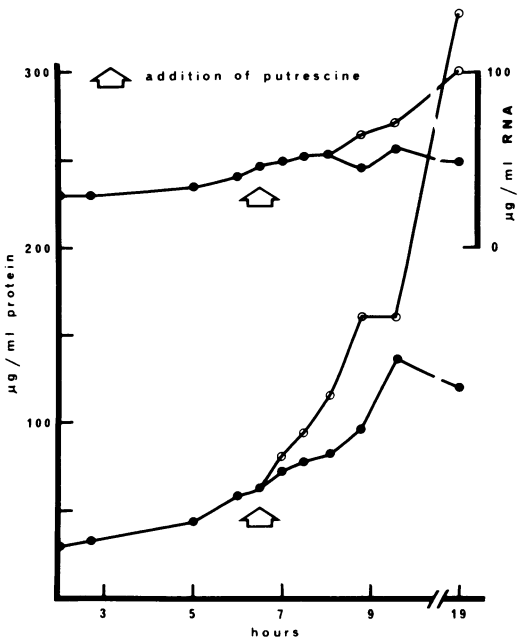

FIG. 8. *Addition of putrescine to starved cells: RNA and protein. Cultures sampled for Fig. 7 were also sampled for RNA and protein. Analysis was done as described in Materials and Methods. Symbols:* (○) *with putrescine,* (●) *without putrescine,* (△) *addition of putrescine.*

The polyamine content of starved and normal cells is shown in Table 3. Also included are the corresponding optical densities and RNA-to-protein ratios. These cells were grown as described for Fig. 3. The putrescine-supplemented cells were sampled once while the mass was still increasing and again at the end of the increase in mass. The younger cells had a higher polyamine content and a higher RNA-to-protein ratio than the older cells. The unsupplemented cells were harvested just after maximum absorbance was reached. In these cells the RNA-to-protein ratio was characteristic of normal cells that were still increasing in mass, although their maximum mass had already been reached. These results suggest that protein synthesis is limited in polyamine-starved cells, and that starvation is somehow unbalancing the normal growth pattern. The total polyamine content in the starved cells is only 50% of that observed in the normal cells that have reached their maximum mass and only 20% of that found in the normal, growing cells. In addition, there was only a trace of putrescine and a detectable amount of spermine, which is normally found only in trace quantities in this medium (M. Ritchey and E. A. Delwiche, manuscript in preparation). The pattern of putrescine and spermidine depletion observed here is similar to that found in *E. coli*, in that spermidine is retained more rigorously than putrescine (12). Although the cells here are not completely deprived of polyamines, the amount of starvation is sufficient to produce the abnormal growth described.

DISCUSSION

The growth experiments indicate that *V. alcalescens* 17745 has a stringent diamine requirement. The levels of diamines supporting growth and the inability to substitute other compounds confirms and extends earlier reports by Rogosa and Bishop on *V. alcalescens* (15). The diamine deficiency is different than that in *E. coli* in that a higher level is needed to support growth (0.1 μg/ml is found to be sufficient for

TABLE 3. *RNA, protein, and polyamines of cells grown on complex medium*

Culture	OD[a]	RNA/protein	Polyamines (μmol/g [wet wt])			
			Putrescine	Spermidine	Spermine	Total
+Putrescine	0.743	0.47	2.5	2.0	Trace	4.5
+Putrescine	1.212	0.35	0.5	1.4	Trace	1.9
−Putrescine	0.561	0.50	Trace	0.5	0.4–0.5	0.9

[a] OD, Optical density.

some *E. coli* mutants [9]), and spermidine will substitute for putrescine in the *E. coli* studies (9, 12). The fact that the lysine and ornithine do not meet the diamine requirement may indicate that the organism does not possess the appropriate decarboxylases, or that it is deficient in the transport mechanisms for these amino acids. At present, little is known about the amino acid requirements or metabolism of *Veillonella*. It has been shown that *E. coli* is able to decarboxylate both lysine and ornithine to make the corresponding diamine.

The fact that *V. alcalescens* will not utilize spermidine as a substitute may be a result of inefficient uptake compared to the diamines (Table 2), or to the fact that the diamine requirement is truly a diamine requirement, and not merely a requirement for a precursor for a polyamine. The latter possibility is supported by the fact that the organism is able to grow in the presence of cadaverine apparently without synthesizing a polyamine from it or any other polyamine as far as can be determined. This is in contrast to *E. coli* in which mutants exist that can lose 99% of their putrescine and still behave normally (11). These mutants have higher levels of spermidine than normal.

The starvation experiments suggest that protein synthesis is limited by putrescine starvation. This is based on the fact that the RNA-to-protein ratios were higher in the starved cells than in the normal cells, and that protein synthesis occurred before nucleic acid snythesis after relief of starvation. At what level protein synthesis is being harmed has yet to be determined for *Veillonella*. In *E. coli* it has been shown that protein synthesis is limited by putrescine starvation (5, 10, 18, 19). In particular, the evidence indicates that the block is at the level of elongation of proteins. The importance of the *Veillonella* study is that it provides another vehicle for the examination of polyamine function, especially with regard to protein synthesis. This organism is sufficiently different from *E. coli* so that similarities between the two in regard to polyamine function may indicate more general phenomena.

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