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Putrescine and Cadaverine Are Constituents of Peptidoglycan in *Veillonella alcalescens* and *Veillonella parvula*

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Veillonella alcalescens **ATCC 17745, a strictly anaerobic, gram-negative small coccus, requires putrescine or cadaverine for growth (M. B. Ritchey, and E. A. Delwiche, J. Bacteriol. 124:1213–1219, 1975). Both putrescine and cadaverine were demonstrated to be incorporated exclusively into the peptidoglycan layer of *V. alcalescens* ATCC 17745. *V. parvula* GAI 0574 also proved to contain putrescine as a component of peptidoglycan. The primary chemical structure of the peptidoglycan common to the two *Veillonella* species is *N*-acetylglucosamine–*N*-acetylmuramic acid–L-alanine–D-glutamic acid–*meso*-diaminopimelic acid–D-alanine. Putrescine or cadaverine links covalently to the α-carboxyl group of the D-glutamic acid residue of the peptidoglycan is necessary for normal cell growth. In *V. alcalescens* ATCC 17745, above 40% saturation at cadaverin linked to the α-carboxyl group of the D-glutamic acid residue of the peptidoglycan is necessary for normal growth.**

Diamines and polyamines are implicated in a wide variety of biological reactions, including synthesis of DNA, RNA, and protein, and also involved in maintaining cell envelope integrity of bacteria (7). It is known that *Veillonella alcalescens* ATCC 17745, a gram-negative, anaerobic coccus (6), has a stringent growth requirement for putrescine or cadaverine (5). In *V. alcalescens* ATCC 17745 grown with either putrescine or cadaverine, no significant cell lysis was observed even after addition of a large amount of EDTA; however, diamine starvation in *V. alcalescens* caused considerable retardation of cell growth and resulted in cell lysis (unpublished data). These observations closely resemble our previous findings in *Selenomonas ruminantium*, which contains neither the free nor the bound form of murein lipoprotein in its envelope but does contain cadaverine as a component of its peptidoglycan (2–4). These observations led us to assume two possibilities. (i) *V. alcalescens* ATCC 17745 requires putrescine or cadaverine to form the diamine-containing peptidoglycan, or (ii) *V. alcalescens* does not contain the murein lipoprotein. Therefore, we analyzed the peptidoglycan fraction, which was obtained by the methods described previously (2), and examined the presence of diamines covalently linked to the peptidoglycan in *V. alcalescens* ATCC 17745 and *V. parvula* GAI 0574. The former contained neither lysine decarboxylase nor ornithine decarboxylase, and the latter possessed only ornithine decarboxylase and grew in a medium without diamine (unpublished data).

TABLE 1. Distribution of radioactivity in *V. alcalescens* ATCC 17745[a]

Fraction	[1,5-^{14}C]cadaverine-grown cells		[1,4-^{14}C]putrescine-grown cells	
	Total radioactivity (10^6 cpm)	% Radioactivity (% of total)	Total radioactivity (10^6 cpm)	% Radioactivity (% of total)
Culture (original amt of isotope added)	9.0	100	9.0	100
Whole cells	5.1	56.7 (100)	4.8	53.3 (100)
5% trichloroacetic acid-soluble fraction	0.2	2.2 (3.7)	1.0	11.1 (20.8)
Peptidoglycan	4.8	53.3 (94.1)	3.5	38.9 (72.9)

[a] *V. alcalescens* cells were grown at 37°C in medium A supplemented with [1,5-^{14}C]cadaverine (5 μg/ml) or [1,4-^{14}C]putrescine (5 μg/ml). The yeast extract and tryptone in medium A were pretreated with purified putrescine oxidase. Polyamines, including putrescine, cadaverine, spermidine, and spermine, were not detected in the medium A. At mid-exponential phase, the cells were harvested and washed twice with 50 mM Tris hydrochloride (pH 7.4). The cells were treated with trichloroacetic acid at 0°C for 30 min. After centrifugation, the supernatants were removed, and the insoluble fractions were treated twice with 4% sodium dodecyl sulfate at 100°C for 30 min. The insoluble fractions obtained were designated as the peptidoglycan fractions.

V. alcalescens cells were grown in a yeast extract (1%) (Difco Laboratories, Detroit, Mich.)-tryptone (1%) (Difco)-sodium lactate (1%) medium (medium A) supplemented with either [1,4-^{14}C]putrescine (CEA, Gif-Sur-Yvette Cedex, France) or [1,5-^{14}C]cadaverine (CEA), and the location of diamine which was taken up by the cells was determined. In [^{14}C]cadaverine-grown cells, about 95% of the total radioactivity incorporated into the cells was recovered in the peptidoglycan preparation. In contrast to the fate of cadaverine, the percent distribution of [^{14}C]putrescine in the peptidoglycan preparation was 73% of the total incorporated activity (Table 1). The reason why the percentage of putrescine incorporated into the peptidoglycan was lower than that of cadaverine might be due to a limited supply of putrescine in the cells because putrescine incorporated into

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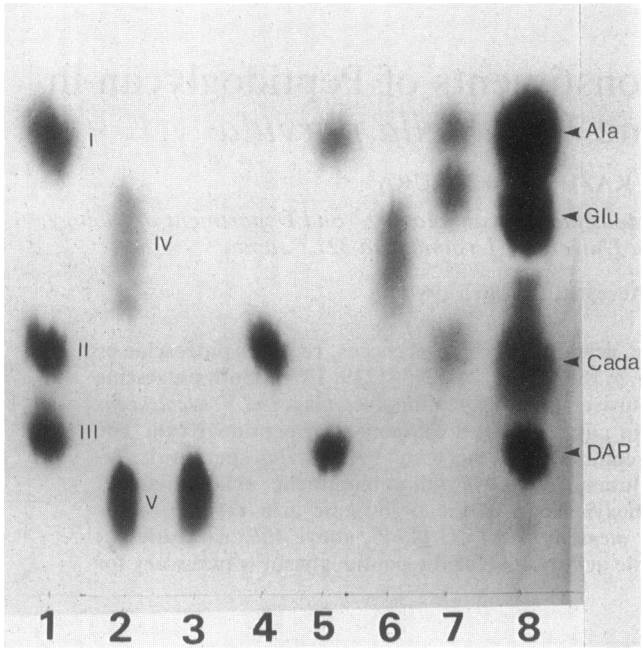

FIG. 1. Paper chromatograms of the peptide fragments from peptide A after partial acid hydrolysis and of the components of spots II and IV. The hydrolysate was applied to a Dowex 50 (H^+ type) column. The amino acids and peptides containing cadaverine were eluted with 2 N NH_4OH. Free cadaverine was not eluted with 2 N NH_4OH. The eluate was evaporated to dryness, and the residue was dissolved in water and applied to a Dowex I (CH_3COO^- type) column. The neutral amino acids and peptides and basic peptides were eluted with water. Finally, the eluate was applied to the Dowex 50 (NH_4^+ type) column. The neutral amino acids and basic peptides were eluted with water and 2 N NH_4OH, respectively. All fractions were examined by paper chromatography. The upper phase of *n*-butyl alcohol–acetic acid–water (65:25:4 [vol/vol]) was used as a solvent system. Spots were detected by spraying with ninhydrin reagent. Samples 1 and 2 are products eluted into the neutral and basic fractions, respectively, from the Dowex 50 (NH_4^+ type) column. Samples 3, 4, and 6 are the purified spots V, II, and IV, respectively. Samples 5 and 7 are the acid hydrolysates of spots II and IV, respectively. Sample 8 is a mixture of authentic alanine (Ala), glutamic acid (Glu), cadaverine (Cada), and DAP.

the cells is known to be converted to spermidine immediately (5).

The [^{14}C]putrescine- or [^{14}C]cadaverine-labeled peptidoglycan preparation was hydrolyzed with 6 N HCl and subjected to chemical analysis with an IRIKA (Kyoto, Japan) automatic amino acid analyzer and a high-performance liquid chromatograph (HLC-830 system; Toyo Soda, Tokyo, Japan) equipped with an RS-8000 detector. Glutamic acid, alanine, diaminopimelic acid (DAP), muramic acid, glucosamine, and cadaverine were found. However, no other amino acid was found. The chemical composition of the peptidoglycan preparation of *V. alcalescens* grown with cadaverine was determined to be as follows: alanine-glutamic acid-cadaverine-DAP-muramic acid-glucosamine (2.0:1.0:1.0:1.0:1.0:1.0). On the other hand, from the elution profile of the acid hydrolysate on the high-performance liquid chromatograph all of the ^{14}C counts were found intact as putrescine and cadaverine in the acid hydrolysates (data not shown). These results clearly indicate that *V. alcalescens* does not contain the murein lipoprotein and that the ^{14}C-labeled diamines which were added to the medium were incorporated into the peptidoglycan and kept chemically intact as a constituent of the peptidoglycan of *V. alcalescens*. These findings led us to study which component of the peptidoglycan putrescine or cadaverine links covalently. We determined the peptide sequence of the peptidoglycan of *V. alcalescens*. The peptidoglycan preparation from cadaverine-grown cells was digested with *N*-acetylmuramyl-L-alanine amidase and endopeptidase from *Streptomyces albus* G, and a peptide preparation (designated peptide A) was obtained by the methods described previously (1). The peptide A preparation was partially hydrolyzed with 2 N HCl for 3 h at 100°C, and the degradation products were separated by ion-exchange and paper chromatography. Five ninhydrin-positive spots were obtained (Fig. 1, lanes 1 and 2). Spots I, III, and V, respectively, were identified as alanine, DAP, and intact peptide A which was not degraded. The components corresponding to spots II and IV were obtained and hydrolyzed with 6 N HCl at 110°C for 12 h, and the amino acid and cadaverine in the hydrolysate were examined by paper chromatography. Spot II was composed of alanine and DAP. Spot IV consisted of alanine, glutamic acid, and cadaverine (Fig. 1, lanes 5 and 7). Alanine was determined to be of D configuration in spot II and L

TABLE 2. Amount of cadaverine in peptidoglycan of *V. alcalescens* ATCC 17745 cells grown with various amount of cadaverine added to the medium[a]

Amt of cadaverine added to medium		Maximum growth (OD_{550})	Cell no. (10^{10})/ml of culture at maximum growth point	Amt of cadaverine incorporated into peptidoglycan		% Saturation
μg/ml	Molecules/ml (10^{16})			No. (10^{15}) of molecules/ml of culture	No. (10^5) of molecules/cell	
10	3.42	0.55	1.43	10.3	7.2	100
3	1.03	0.52	1.35	7.2	5.3	74
1.5	0.52	0.51	1.34	4.2	3.1	43
0.9	0.31	0.45	1.14	2.5	2.2	30
0.6	0.21	0.40	1.01	1.7	1.7	24
0.4	0.14	0.25	0.64	0.9	1.4	19
0.2	0.070	0.17	0.44	0.28	0.63	8.8
0.1	0.035	0.15	0.35	0.22	0.63	8.8
0.05	0.018	0.15	0.32	0.09	0.28	3.9
0	0	<0.01	ND[b]	ND	ND	ND

[a] The organism was preincubated overnight in medium A supplemented with cadaverine (5 μg/ml). The cells were harvested and washed twice with medium A. The optical density at 550 nm (OD_{550}) of the resulting suspension was adjusted to 0.1, and 0.1 ml of this suspension was used as an inoculum for 10-ml cultures containing various amounts of [1,5-^{14}C]cadaverine hydrochloride.

[b] ND, Not determined.

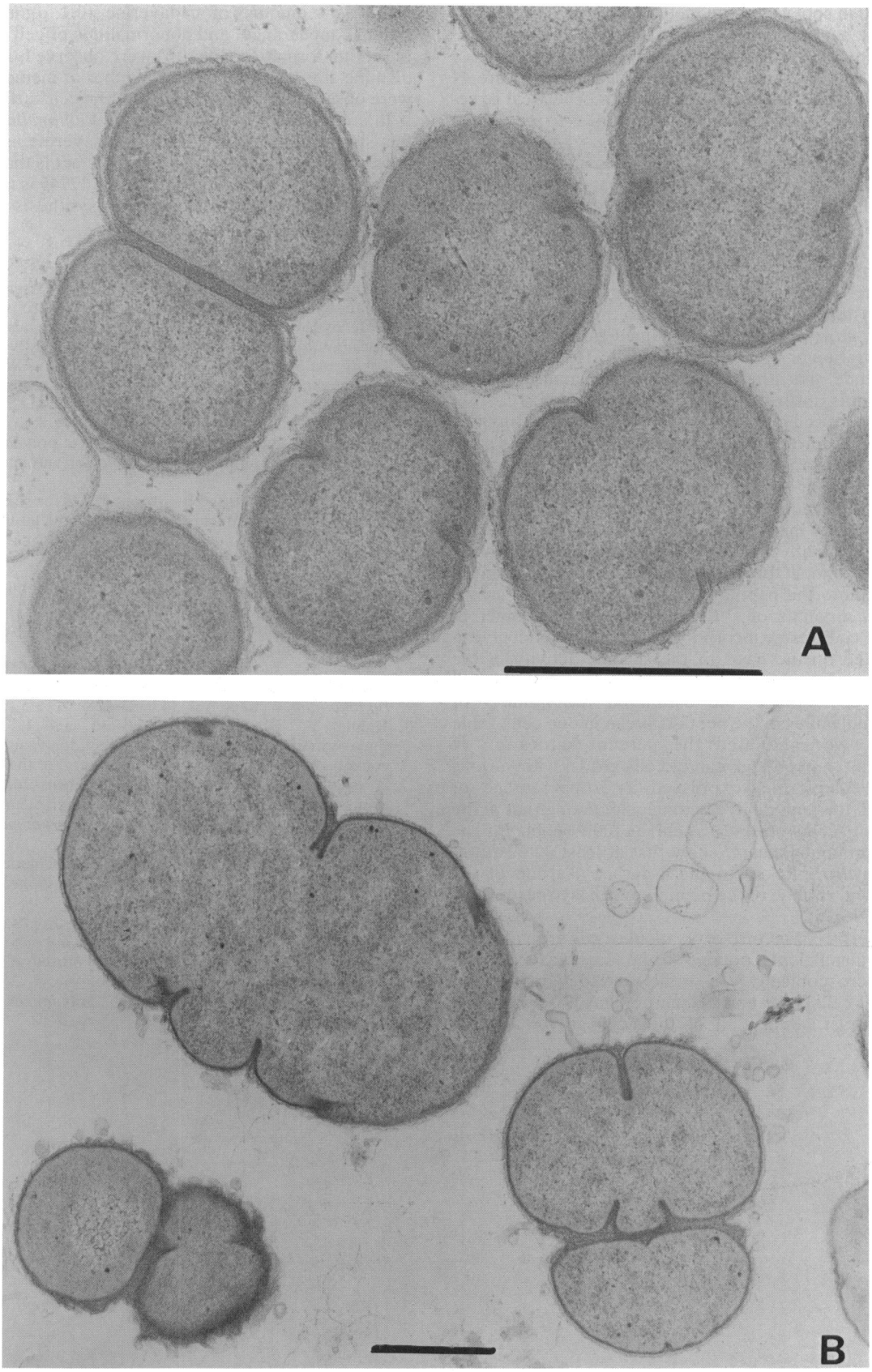

FIG. 2. Electron micrographs of ultrathin sections of *V. alcalescens*. For sectioning, samples were fixed and embedded by the methods described previously (3). Examination was carried out on a Hitachi HU-11A electron microscope at an acceleration of 75 kV. Panels: A, control cells (grown with cadaverine [10 μg/ml]); B, cadaverine-starved cells. Bars, 0.5 μm.

configuration in spot IV by the method described previously (1). Glutamic acid in spot IV was of D configuration. DAP was determined to be *meso* type. In addition, DAP was N terminal in spot II, and alanine and cadaverine were N terminal in spot IV. From these results, the chemical structures of spots II and IV were determined to be *meso*-diaminopimelyl-D-alanine, and L-alanyl-D-glutamyl cadaverine, respectively. It is well known that the γ-glutamyl linkage is more labile to acid treatment than is the α linkage. From these results, we propose the primary structure of the peptidoglycan of *V. alcalescens* ATCC 17745 to be *N*-acetylglucosamine–*N*-acetylmuramic acid–L-alanine–D-glutamic acid–($^{\alpha}$cadaverine)$^{\gamma}$meso-DAP–D-alanine. The same result, except for the finding that putrescine instead of cadaverine links to the D-glutamic acid residue with an α linkage, was obtained from the peptidoglycan preparation of *V. parvula* GAI 0574. These results led us to conclude that the occurrence of diamines in the peptidoglycan is not restricted to *S. ruminantium*.

The maximum cell growth response of *V. alcalescens* was almost linear according to the concentration of cadaverine over a range of 0.2 to 0.5 μg/ml. No significant differences in maximum growth or doubling speed were observed when the concentration of cadaverine exceeded 1.5 μg/ml. This suggests that cell growth should be dependent on the amount of cadaverine covalently linked to the peptidoglycan. Therefore, we determined the number of cadaverine molecules incorporated into the peptidoglycan per cell in cells grown with various amounts of [^{14}C]cadaverine. The number of molecules of cadaverine incorporated into the peptidoglycan was calculated on the basis of the specific activity of the labeled cadaverine added to the medium. By using the cell number in the culture, we estimated the number of cadaverine molecules in the peptidoglycan in one cell (Table 2). Finally, we calculated the percent saturation of cadaverine in the peptidoglycan of cells grown with various amount of cadaverine, expressed as a percentage of cadaverine in the peptidoglycan molecule, taking that in the cell grown with cadaverine (10 μg/ml) as 100% (Table 2). The results thus obtained clearly show that at least 40% saturation of cadaverine linked to the α-carboxyl group of the D-glutamic acid residue of the peptidoglycan is necessary for normal cell growth.

The cell surface structure of *V. alcalescens* grown with a sufficient or insufficient amount of cadaverine was examined electron microscopically. In sufficient-cadaverine-grown cells (control cells), the normal septum was observed (Fig. 2A). In contrast, almost all of the cells grown with an insufficient amount of cadaverine (0.1 μg/ml) showed a swollen appearance, and abnormalities of cell structure and of septum formation (Fig. 2B) were observed. In addition, in diamine-starved cells, a large number of membrane vesicles were observed to be released from the cell surface (Fig. 2B).

In conclusion, the two strains of *Veillonella* examined in this study contain putrescine or cadaverine as an essential constituent of the peptidoglycan. The fact is that the diamine requirement in *V. alcalescens* ATCC 17745 is a true requirement for diamine itself for the synthesis of diamine-containing peptidoglycan.

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