JOURNAL OF BACTERIOLOGY, Apr. 1982, p. 332–347
0021-9193/82/040332-16$02.00/0

Vol. 150, No. 1

Novel Peptidoglycans in *Caulobacter* and *Asticcacaulis* spp.

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Received 7 August 1981/Accepted 16 November 1981

Peptidoglycan sacculi free of poly-β-hydroxybutyric acid were prepared from whole cells of four species of *Caulobacter* and two species of *Asticcacaulis* and from morphological mutants of *Caulobacter crescentus* and *Caulobacter leidyi*. Acid hydrolysates of the sacculi were analyzed quantitatively, and each of the hydrolysates was found to contain significant amounts of only five ninhydrin-reactive compounds: alanine, glutamic acid, α, ε-diaminopimelic acid, muramic acid, and glucosamine. Four types of peptidoglycans were distinguishable on the basis of the molar ratios among these five compounds. The respective ratios were as follows: in *C. leidyi*, 2:1:1:1:0.8; in *Asticcacaulis biprosthecum*, 1.7:1.6:1:1.1:0.7; in the cells of the remaining species, 2:1:1:1.2:0.8; and in stalks shed by the abscission mutant 2NY66, 2:1:1:1:1.67. Thus, in addition to some species differences among these caulobacters, it was found that the peptidoglycan sacculus of the stalked *C. crescentus* cell is chemically differentiated; the cellular peptidoglycan is richer in muramic acid than is the peptidoglycan of typical gram-negative bacteria, and the peptidoglycan of the stalk is correspondingly rich in glucosamine. Empirical formulas for the repeating units of the peptidoglycans have been inferred on the basis of the molar ratios of their amino components.

The peptidoglycan sacculus of unicellular bacteria has long been known to retain the shape of the cell even after all other components of the cell have been removed (15, 38, 57) and so can be recognized as the structural skeleton of the bacterial cell. Although it seems unlikely that a structure such as the sacculus could determine its own shape, it is equally clear that whatever determines the shape of the sacculus determines the shape of the cell.

This recognition has derived from a variety of experimental approaches, including studies of (i) synchronously dividing populations and autoradiograms of labeled sacculi (12, 17, 40, 47, 55), (ii) enzymatic and morphogenetic roles of penicillin-binding and penicillin-sensitive proteins (2, 3, 10, 46, 49, 51, 52), and (iii) peptidoglycan composition and murein hydrolase activities in naturally dimorphic bacteria and in mutants in which cell shape is conditioned by environmental factors (8, 14, 18, 19, 24–26, 39, 42). In several instances, it has been possible to correlate changes in cell (or sacculus) shape with changes in the structure of the peptidoglycan or in the activity or localization of murein hydrolases (or in both). Shape change can be associated with cell membrane modification without a dramatic change in the primary structure of the peptidoglycan (14), but sacculus morphology is altered as a consequence of the change in membrane composition. Accordingly, the generalization stated above still applies; i.e., a change in cell shape is not achieved without a change in sacculus shape.

The information available at present implies that although a change in the primary structure of the peptidoglycan may not be necessary to achieve a change in the shape of the cell, structural change in the peptidoglycan may nevertheless serve as a mechanism for reorienting the direction of peptidoglycan growth and thereby of altering the shape of the sacculus and of the cell. It seems clear, further, that effectors of sacculus shape change would need to act enzymatically on the peptidoglycan, whether or not their action resulted in a permanent alteration of the primary structure.

Accordingly, it is reasonable to ask whether cell shape change in a particular morphogenetic system involves a (transient or permanent) change in the structure of the peptidoglycan as well as a change in the shape of the sacculus. In the case of natural dimorphisms, sporulating systems such as in bacilli and myxobacteria represent a shape transition that is complicated by modifications required not just for shape change, but also for establishment of or release from dormancy. In two types of bacteria, shape transition occurs in the growing, vegetative cells. In one of these (*Arthrobacter* spp.), shape change is dependent on environmental conditions (30). In the other (prosthecate bacteria),

the basic shape alteration, prostheca initiation, is an endogenous event in most isolates, although the extent of prosthecal development is influenced by environmental factors (6, 13, 16, 27, 34, 45, 58).

The caulobacters (genera *Caulobacter* and *Asticcacaulis*) are prosthecate bacteria in which initiation of prosthecal (here, stalk) development is a discrete morphogenetic event in the life of the cell (32, 34, 48, 54). Since the prostheca includes the murein layer (53), its shape must be reflected in its peptidoglycan sacculus; this has been illustrated (11, 34, 36). Accordingly, determination of the primary structure of the peptidoglycan of the cell proper and of the prostheca is important to elucidation of the mechanism by which the growth of the peptidoglycan is reoriented during localized, outward extension of the sacculus. Further, identification of the effectors of sacculus shape change during prostheca initiation should assist in developing an understanding of the regulation of stalk morphogenesis by identifying one or more specific biosynthetic processes that must be subject to such regulation.

The composition of peptidoglycan is highly conserved, particularly in gram-negative bacteria (43), and previous studies (1, 9, 11) of peptidoglycan composition in *Caulobacter crescentus* led to the conclusions that (i) its peptidoglycan was a typical gram-negative A1γ type that did not significantly differ among populations grown in media in which morphology was markedly different, and (ii) the peptidoglycan of the stalk sacculus was not different from that of the cell body sacculus, since peptidoglycan composition was similar among peptidoglycans extracted from populations enriched in swarmers, dividing cells, or stalked cells. However, isolated stalk sacculi were not analyzed. (The observation that swarmer cell peptidoglycan lacks diaminopimelic acid [Dpm] [11] has not been confirmed by other laboratories.)

In the most detailed report (1), the amino acid ratios were found to vary among peptidoglycan preparations from cells cultivated in different media, especially with respect to the proportion of glutamic acid (Glu). Agabian and Unger (1) expressed doubt that the excess Glu was present within the peptidoglycan molecule and suggested that it could reflect the presence of polyglutamic acid that copurified with the peptidoglycan. Of particular relevance to the present studies, the authors interpreted their amino sugar ratios as indicating equimolar amounts of muramic acid (Mur) and glucosamine (Glc). However, in peptidoglycan from populations grown in three of the four media tested (the media in which the glutamic acid was equimolar with Dpm), the molar ratios reported implied that Mur accounted for 58 to 63% of the amino sugar content, a proportion that is closer to a Mur/Glc ratio of 3:2 than 1:1.

In the present studies, the peptidoglycan composition of 12 wild-type and mutant strains of *Caulobacter* spp. and of two wild-type strains of *Asticcacaulis* spp. has been analyzed. Our findings imply that the peptidoglycan of five of the six species studied differs from the A1γ type of peptidoglycan by containing an excess of Mur relative to Glc in the glycan chain. We have also taken advantage of the stalk abscission phenotype (33) to prepare sufficient amounts of stalk sacculus material uncontaminated with cell debris to allow its analysis in the absence of cellular peptidoglycan; in such preparations, we have detected that the relative proportions of the two amino sugars are reversed in the stalk peptidoglycan. Accordingly, stalk development appears to be an instance of cell shape change that involves alteration of the primary structure of the peptidoglycan. Primary structures of the novel peptidoglycans are proposed.

MATERIALS AND METHODS

Bacterial strains. *C. crescentus* CB2 (ATCC 15252), CB13, and CB15 (ATCC 19089); *Caulobacter bacteroides* CB11a (ATCC 19090); *Caulobacter fusiformis* CB27 (ATCC 15257); *Caulobacter leidyi* CB37 (ATCC 15260); and *Asticcacaulis excentricus* AC48 (ATCC 15261) were from our collection (32). *Asticcacaulis biprosthecum* AC-2 (a copy of C-19 [34]) was kindly provided by J. L. Pate. The mutant strains 15NY87 (Cds) and 2NY66 (Abs) were described previously (33); strain 2NY66R is a spontaneous revertant of 2NY66 that does not shed its stalk. *C. leidyi* 37NY2 is a spontaneous non-rosette-forming derivative of CB37.

Media and cultivation. Peptone (0.2%)–yeast extract (0.1%) medium was prepared in distilled water and supplemented with vitamin-free Hutner base (5; PYH medium [36]) or in tap water supplemented with 0.02% $MgSO_4 \cdot 7H_2O$ (PYE medium [32]).

Hutner base-imidazole buffered basal solution (Hi) containing 0.05% NH_4Cl (45) and 0.2 mM each Na_2HPO_4 and KH_2PO_4 phosphate at pH 7.0 was supplemented with glucose and monosodium glutamate·H_2O at 0.05 and 0.05% (wt/vol), 0.1 and 0.1%, or 0.3 and 0.3%; these defined media were designated Higg 0.1, 0.2, or 0.6%, respectively.

All cultures were incubated at 30°C in a model G25 New Brunswick Gyrotory shaker at 200 rpm. Before harvesting, cultures were chilled to 0°C in an ice-water slush. Centrifugation was performed at 4°C in a Sorvall RC2-B refrigerated centrifuge.

Harvesting of stalks and formerly stalked cells. Five 400-ml cultures of 2NY66 in Higg 0.6% medium were incubated until 3.5 h after the population had reached maximum turbidity. At the onset of the stationary phase of turbidity, more than 90% of the population consisted of stalked cells; between 2 and 4 h after this point, 75 to 80% of the cells assembled flagella and shed their stalks (J. S. Poindexter, Abstr. Annu. Meet.

Am. Soc. Microbiol. 1981, I51, p. 95). The stalks and the cells that had shed their stalks were harvested separately by a procedure based on that devised for segregation of stalked and nonstalked populations (54), as follows. The cultures were chilled, and the formerly stalked cells were sedimented as a dense pellet by centrifugation at 17,000 × *g* for 15 min; the supernatant and opalescent surface layer of the sediment were aspirated for harvesting of stalks (below), and the loosely packed layer consisting of cells that had retained their stalks, some nonstalked cells, and some shed stalks was decanted and discarded. The surface of the dense pellet was rinsed vigorously with several changes of Hi basal solution; the rinses were discarded. The dense pellet was suspended in basal solution and centrifuged at 12,000 × *g* for 15 min, the surface of the pellet was rinsed again to eliminate any remaining stalked cells, and the cells were finally suspended in basal solution and frozen at −70°C for extraction of the cell sacculi.

Stalks were sedimented from the culture supernatant and the aspirated opalescent layer by centrifugation at 39,000 × *g* for 30 min; the supernatant was discarded. The top, opalescent layer was gently suspended in basal solution and centrifuged at 12,000 × *g* for 15 min to sediment any remaining cells. The supernatant and opalescent layer were aspirated, and the purified stalks were sedimented at 39,000 × *g* for 60 min. The final opalescent pellet was suspended in basal solution and stored at −70°C for extraction of the stalk sacculi.

To prepare stalks artificially detached from their cells, five parallel 400-ml cultures of 2NY66 in Higg 0.6% were chilled at the onset of maximum turbidity, before stalk abscission. Ten 200-ml samples were treated in an ice-wrapped Waring blender at high speed for three 30-s intervals in a cold (4°C) room. The stalks (and other cell fragments; see Fig. 4B and C.) were harvested by differential centrifugation as in the harvesting of shed stalks and stored at −70°C for extraction of sacculi.

Preparation of murein sacculi. Murein sacculi were prepared from whole cells and stalks by a procedure based on that of Schwarz et al. (46) as described previously (36). The principal steps in the procedure were as follows. (i) Cells were chilled when the growing population reached half-maximum turbidity and harvested from 400 ml of culture by centrifugation at 17,000 × *g* for 15 min, washed once with distilled water, and finally suspended in distilled water and stored at −70°C. (ii) The frozen suspension was thawed once at 0°C and refrozen. (iii) The twice-frozen suspension was thawed, extracted with two volumes of 4% sodium dodecyl sulfate (SDS; Fisher Scientific Co.) at 80°C, washed with distilled water, and then treated overnight with trypsin and DNase. (iv) The enzyme-treated sacculi were washed with distilled water and then subjected to a second SDS extraction, washed, and suspended in distilled water. At this stage, the cell sacculi typically contained granules of poly-β-hydroxybutyrate (PHB), a problem also encountered in preparations of sacculi from *Hyphomicrobium* spp. (21) and *Spirillum* spp. (4). (v) The granules were removed by extraction into 2.5 N NaOH at 70°C, and the PHB-free sacculi were washed with distilled water and finally suspended in distilled water. (vi) Sacculus suspensions were mounted for electron microscopy (below) and then stored at −70°C until hydrolyzed.

Hydrolysis of sacculi and analysis of amino acids and amino sugars. Purified sacculi were hydrolyzed in vacuo in 4 N HCl and 0.2% phenol for 2 h at 110°C. Under these conditions, hydrolysis was complete. Longer heating times or higher HCl concentrations did not alter the amino acid content, but did result in a slight loss of the two amino sugars (see Table 2). Since there was no progressive change in ratios among the amino acids or between the two amino sugars, the lower amino sugar/amino acid ratios observed on prolonged hydrolysis do not appear to have resulted from cochromatography of peptides with either of the sugars in hydrolysates prepared by the milder conditions (7). Hydrolysates were evaporated to dryness (60 to 75 min) in a Savant Speed Vac concentrator in vacuo at 40 to 45°C. The residue was suspended in 0.5 to 1 ml of sodium citrate buffer (7) and stored at −70°C when not immediately applied to the chromatography column of the analyzer (below) and between iterated analyses. Just before application to the chromatography column, each hydrolysate was centrifuged in a Beckman Microfuge B at 9,000 × *g* for 5 min; 100 μl of the supernatant (or appropriate dilution in sodium citrate buffer) containing 50 to 150 nmol of ninhydrin-reactive compounds was used for the analysis.

Quantitative analysis of ninhydrin-reactive substances was performed by the method of Fauconnet and Rochemont (7) modified by chromatography on a 6- by 370-mm column and by employing a Beckman model 119CL automatic amino acid analyzer. Concentrations were calculated by using both Beckman-prepared amino acid standards and standard solutions of L-alanine (Ala), L-Glu, and glycine (each from Fluka AG) and (LL, DD, *meso*-)α,ε-Dpm, Mur, D-(+)-galactosamine, and D-(+)-Glc (each from Sigma Chemical Co.) prepared at 0.25 mM in 10 mM HCl. Molar concentrations were calculated on the basis of peak area.

Preparation of envelopes and envelope membranes for thin sectioning. Cells of *C. crescentus* CB15 or 15NY87 growing in PYH were harvested at half-maximum turbidity; the pellet was suspended in Hi basal solution to 10 mg (dry weight) per ml. The same suspension was used to prepare membranes and complete envelopes.

To prepare murein-free envelope membranes, the cell suspension was diluted 10-fold into Tris buffer (0.05 M, pH 7.8) containing polyethylene glycol (10%, wt/vol) and EDTA (0.01 M, pH 7.8); lysozyme (egg white; Sigma) was added to a final concentration of 50 μg/ml, and the suspension was incubated at 30°C. After 7.5 min, when 100% of the cells had been converted to spheroplasts, the mixture was diluted into an equal volume of Hi basal solution at 0°C and allowed to stand at 0°C overnight for lysis to occur. The ghosts were then collected by centrifugation at 39,000 × *g* for 60 min. The pellet was suspended in Hi basal solution containing 5 mM $MgSO_4$ and DNase I (Sigma) at 50 μg/ml and incubated at 30°C for 10 min. The mixture was then added to 10 volumes of tryptone-salt broth (41) and thereafter processed as described previously for whole cells (35) and finally embedded in Epon 812 (Fisher). A sample of the harvested cells was fixed and embedded by the same procedure immediately after harvest.

Complete envelopes were prepared from CB15 by mixing the cell suspension with an equal volume of glass beads and vibrating in a Braun disintegrator for three 45-s intervals with intermediate periods of rechilling to 0°C; one 30-s treatment was sufficient to rupture 100% of the cells of 15NY87. The beads were removed by centrifugation at 3,000 × *g* for 10 min. $MgSO_4$ (5 mM) and DNase I (50 μg/ml) were added to the opalescent supernatant, and the mixture was incubated at 30°C for 12 min. This mixture was added to five volumes of tryptone-salt broth, as above, and fixed and embedded for sectioning.

Electron microscopy. To prepare sacculi for electron microscopy, the sacculus suspension was mixed with cytochrome *c* (to 0.7 mg/ml), picked up on nitrocellulose-coated copper grids, positively stained with uranyl acetate, air dried, and rotary shadowed with Pt:Pd as described previously (36). Before freezing, washed whole cells were mounted on nitrocellulose-coated copper grids, air dried, and shadowed at an angle of 27°. Stalk suspensions were mounted similarly and shadowed at 10°. Shadowed specimens were examined in a Philips EM300 microscope operated at 80 kV with a 50-μm objective aperture in place.

Thin sections were cut on a Porter-Blum MT-2 microtome fitted with a diamond knife, mounted without support on 400-mesh copper grids, poststained with lead citrate (37), and examined in the EM300 microscope operated at 60 kV with a 30-μm objective aperture in place.

Assay of PHB. To identify and quantitate the PHB content of sacculi, 200 μl of sacculus suspension was centrifuged at 39,000 × *g* for 30 min, the supernatant was carefully aspirated, and the liquid was drained from the wall of the tube with a filter paper strip. The sediment (sacculi) was suspended in 0.5 to 1 ml of $CHCl_3$ and heated at 60°C for a few minutes until optically clear. Measured samples were removed to clean tubes, and the $CHCl_3$ was evaporated at 60°C. Sulfuric acid (96%) was added to dissolve the residue, the solution was heated at 100°C for 10 min and then cooled to room temperature, and the optical density was read at 5- to 10-nm intervals from 200 to 350 nm. Crotonic acid, β-hydroxybutyric acid, and purified *C. crescentus* PHB samples treated in the same manner exhibited the crotonic acid spectrum and the extinction coefficient (E_{235} = 0.175 optical density unit per μg per ml) previously reported (28), allowing calculation of the PHB content of the sacculus suspension.

Assay of protein. A sample of sacculus suspension was heated in 0.2 N NaOH at 100°C for 30 min and then assayed by the method of Lowry et al. (29). Bovine plasma albumin served as the reference protein.

RESULTS

Purity of sacculi. In all sacculus hydrolysates, there were five principal ninhydrin-reactive substances: Ala, Glu, Dpm, Mur, and Glc. Quantitatively minor reactive substances were identifiable as known protein amino acids. The source of the minor amino acids appeared to be the membrane of the PHB granules (see Fig. 8 and reference 35). Alkali extraction of SDS-prepared sacculi removed the granules (Fig. 1), most of the minor amino acids (Fig. 2), and some of the Ala and Glu (Fig. 2). However, the quantities of the three compounds that would not be expected in proteins (namely, Dpm, Mur, and Glc) were not affected by the alkali treatment (Fig. 2). The effectiveness of the NaOH extraction in removing PHB and protein from sacculi is illustrated in Table 1 for *C. leidyi* CB37. This strain proved the most refractory; in other suspensions, no more than 1 μg of Lowry protein per μg of Dpm remained after the NaOH extraction. Further, sacculi prepared from purified shed stalks lacked PHB granules and minor amino acids (see Fig. 5B, C, and D and Fig. 6), even without the NaOH extraction step. Accordingly, we infer that the five principal substances comprised the units of the peptidoglycan of these caulobacters.

Composition of sacculi. The molar ratios among the five peptidoglycan components are listed in Tables 2, 3, and 4. The molar ratios of total cellular peptidoglycan did not change with growth medium (Fig. 3 and Table 3), even though cell morphology varied considerably among populations grown in these several media. For example, the cells of *C. leidyi* grown in PYH or Higg 0.1% were short (1 to 1.5 μm) and fusiform with very short (maximum 0.7-μm) stalks or none at all, whereas in Higg 0.6%, the cells were long (3-μm) rods with stalks at least equal to cell length. In *C. crescentus*, the short (1.3- to 1.6-μm) vibrioid cells with short stalks (0.8 to 1.5 μm) in PYH or Higg 0.1% medium become long (2.5- to 2.8-μm) vibrioid cells with stalks of 4.2 to 4.9 μm in length when grown in Higg 0.6% medium. Similarly, the peptidoglycan composition did not change upon entry into the stationary phase (Table 4). The ratios were also not affected by either of the stalk abnormality mutations (structurally irregular stalks of the Cds mutant, or stalk abscission of the Abs mutant). However, alterations in peptidoglycan composition of the stalk alone are probably not detectable in sacculus preparations from stalked cells, since the relatively slender stalk sacculus must be at least as long as the cell sacculus to contribute at least 10% of the peptidoglycan. Stalk peptidoglycan is further diluted relative to cellular peptidoglycan in preparations from heterogeneous populations that include swarmers as well as stalked cells.

The data listed in Tables 2, 3, and 4 reveal four different quantitative sets. In *C. bacteroides*, *C. crescentus*, *C. fusiformis*, and *A. excentricus* cell sacculi, the ratio was generally 2:1:1:1.2:0.8 for Ala/Glu/Dpm/Mur/Glc, respectively. This ratio implies that, as is usual in gram-negative peptidoglycans, the amino sugars occurred as one pair per Dpm-containing peptide chain; however, Mur regularly accounted for 60% of the amino sugar, so that the Mur/Glc ratio was

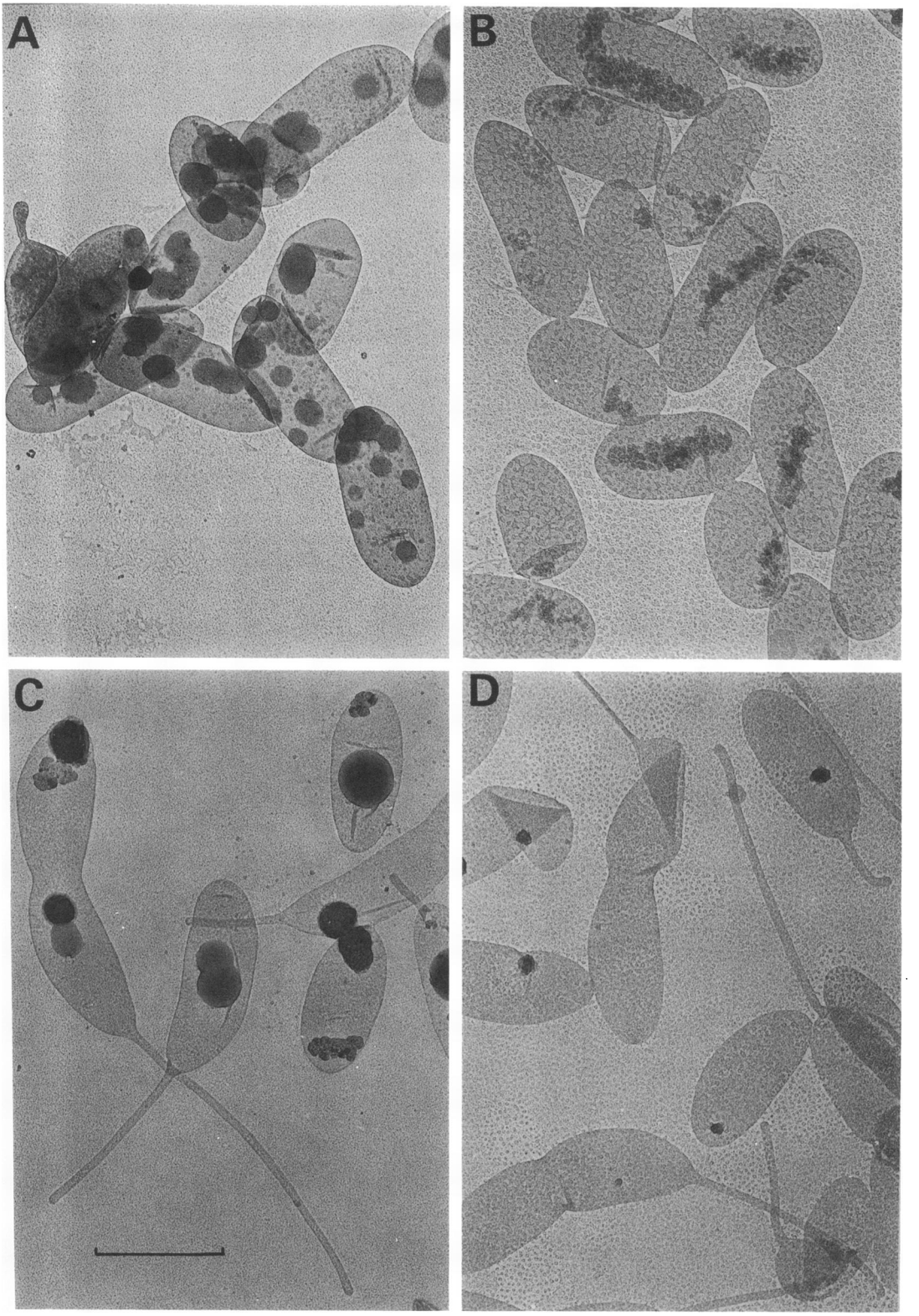

FIG. 1. Sacculi before (A and C) and after (B and D) NaOH extraction of PHB granules in *C. leidyi* CB37 (A and B) and *C. crescentus* 2NY66R (C and D). Bar, 1 μm; here, as in Fig. 4, 5, and 8, the bar indicates the magnification for all panels.

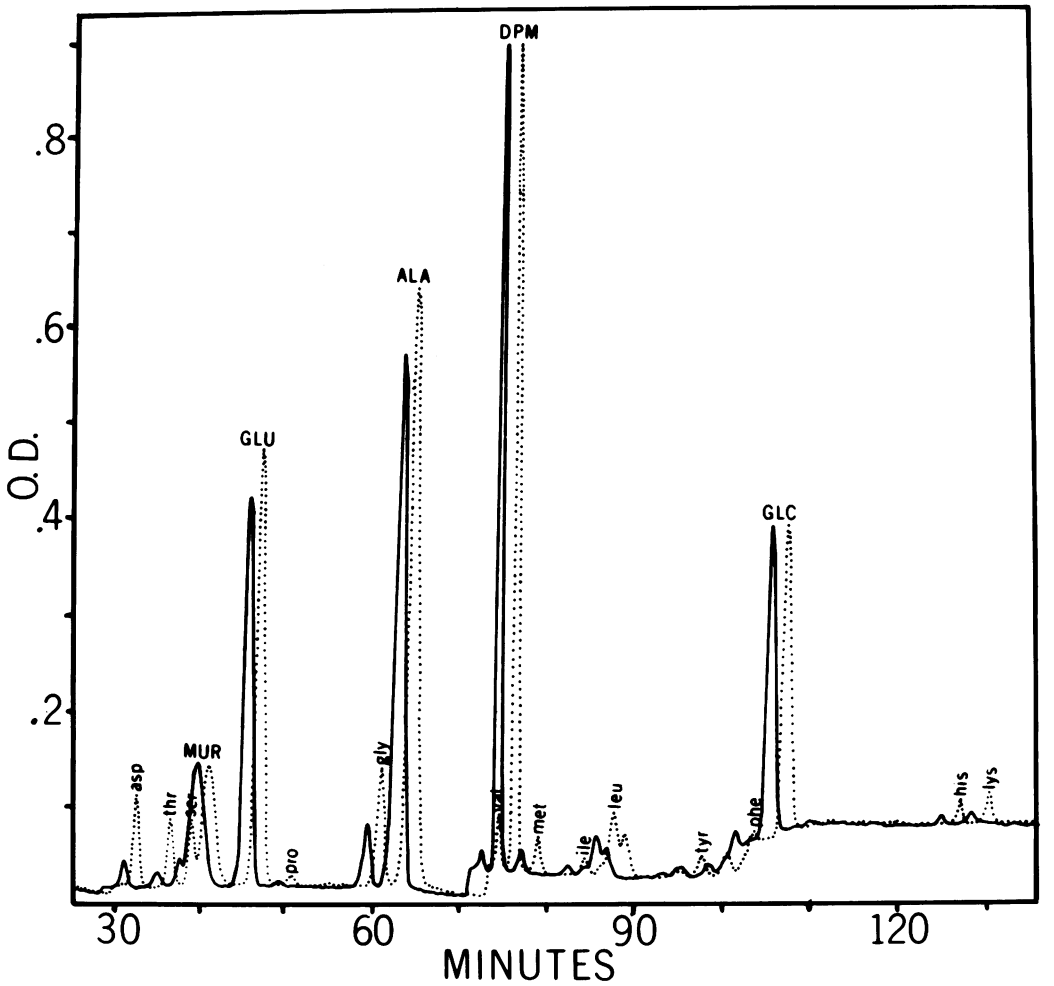

FIG. 2. Chromatographic elution profiles of ninhydrin-reactive components (see text) in hydrolysates of *C. leidyi* CB37 sacculi prepared with (—) and without (·····) NaOH extraction. The dotted line profile has been offset approximately 2 min so that both profiles are fully visible. The cells were grown in Higg 0.2%. The molar ratios (not listed in Table 3) were 1.9:1.0:1.0:1.1:0.9 with NaOH extraction and 2.1:1.1:1.0:1.0:0.9 without NaOH extraction. The proportion of amino sugar accounted for by Mur was 54.6% with NaOH extraction and 53.4% without NaOH extraction. Ammonia and arginine (not shown) eluted at 150 and 182 min, respectively.

3:2. Such a ratio implies that one of every five pairs of amino sugars consisted of two Mur moieties, only one of which was linked to a peptide chain.

In contrast, the sacculi extracted from stalks shed by *C. crescentus* 2NY66 (Fig. 4A and Fig. 5B, C, and D) exhibited a molar ratio of 2:1:1:1:1.67 (Table 4). This ratio would be observed if one of approximately every five pairs of amino sugars consisted of two Glc units, so that neither member of the pair was linked to a peptide chain. Sacculi prepared from the cells that had shed these stalks (Fig. 5E) exhibited the ratio 2:1:1:1:0.9, a ratio intermediate between those of shed stalks and heterogeneous populations. The elution profile of the peptidoglycan of

TABLE 1. Protein and PHB content of *C. leidyi* CB37 sacculus suspensions

Extraction	μg of protein/μg of Dpm	μg of PHB/μg of Dpm	μg of Dpm/ml of suspension	% Recovery of Dpm
SDS	2.46	4.84	235.6	
SDS plus NaOH	1.29	0	232.6	98.7

TABLE 2. Molar ratios of peptidoglycan components under different conditions of hydrolysis[a]

Strain	Medium	HCl (N)	Hydrolysis time (h)	Dpm (nmol/sample)	Molar ratio to Dpm of:				% of amino sugar accounted for by Mur
					Ala	Glu	Mur	Glc	
C. crescentus									
CB13	PYH	4	2	19.6	1.85	0.92	1.28	0.84	60.3
		5	5	8.7	1.80	0.90	0.97	0.69	58.4
CB15	PYH	4	2	8.7	1.91	1.08	1.20	0.80	58.7
			3	16.8	1.86	1.00	1.13	0.76	59.7
			4.5	16.0	1.84	0.99	1.19	0.79	59.9
			6	24.5	1.89	1.02	1.29	0.88	59.5
15NY87	PYE	4	2	26.3	1.86	0.91	1.14	0.84	57.5
		5	5	27.6	1.84	0.89	0.85	0.60	58.6
A. biprosthecum									
AC-2	PYE	4	2	43.7	1.72	1.62	1.10	0.74	59.6
		5	5	45.9	1.76	1.65	0.83	0.59	58.4

[a] All sacculi were prepared from exponential phase cultures. The Dpm content is given to allow calculation of quantities of the other compounds.

these cells is compared in Fig. 6 with the profile of the peptidoglycan of the stalks they had shed. Although not matched in total amount of peptidoglycan per sample, it is clear from the relative respective peak heights (in stalks to cells: Mur, 0.41; Glu, 0.45; Ala, 0.44; Dpm, 0.43, and Glc, 0.76) that the stalk hydrolysate contained a considerably higher proportion of Glc than did

TABLE 3. Molar ratios of peptidoglycan components[a]

Strain	Medium	Dpm[b] (nmol/sample)	Molar ratio[b] to Dpm of:				% of amino sugar accounted for by Mur[c]
			Ala	Glu	Mur	Glc	
C. bacteroides							
CB11a	PYH	66.8	1.9	1.0	1.3	0.9	58
C. crescentus							
CB2	PYH	23.7	1.9	0.9	1.3	0.8	61
	Higg 0.1%	25.0	1.9	1.0	1.3	0.8	61
	Higg 0.6%	9.1	1.9	0.9	1.2	0.8	60
2NY66	PYH	28.8	1.8	0.9	1.3	0.8	60
	Higg 0.1%	17.6	1.9	0.9	1.3	0.9	59
	Higg 0.6%[d]	24.3	1.9	0.9	1.2	0.8	61
2NY66R	PYH	12.8	1.9	0.9	1.3	0.9	59
	Higg 0.1%	46.2	1.9	0.9	1.2	0.8	61
	Higg 0.6%	13.8	1.9	0.9	1.2	0.8	60
CB15	PYH[e]	8.7	1.9	1.0	1.1	0.8	59
	Higg 0.6%	16.7	1.9	1.0	1.3	0.8	61
15NY87	PYH[e]	26.3	1.9	0.9	1.1	0.8	58
	Higg 0.6%	42.4	1.8	0.9	1.1	0.8	58
C. fusiformis							
CB27	PYH	33.6	2.0	0.9	1.1	0.8	58
A. excentricus							
AC48	PYH	32.7	1.9	0.9	1.3	0.8	61
C. leidyi							
CB37	PYH	18.7	1.7	0.9	0.8	0.7	54
37NY2	Higg 0.1%	9.3	1.9	1.0	1.0	0.8	54
	Higg 0.6%	20.4	1.9	1.0	1.0	0.9	55

[a] All sacculi were prepared from exponential phase cultures and hydrolyzed in 4 N HCl for 2 h. See Table 2 for *C. crescentus* CB13 and *A. biprosthecum* AC-2.

[b] Ratios are rounded off to one decimal place, which suitably represents averages of iterated assays. The Dpm content of a representative assay is given to allow calculation of representative quantities of the other compounds.

[c] Calculated directly from molarities, not from rounded-off ratios, hence some apparent inconsistencies between ratios and percentages given for different hydrolysates.

[d] Also in Table 4.

[e] Also in Table 2.

TABLE 4. Molar ratios of peptidoglycan components of *C. crescentus* 2NY66 cells and stalks[a]

Source of sacculi	Growth phase at harvest	Dpm (nmol/sample)	Molar ratio to Dpm of:				% of amino sugar accounted for by Mur
			Ala	Glu	Mur	Glc	
Total population[b]	Exponential	24.3	1.89	0.91	1.20	0.77	61.0
Total population	Stationary	9.9	1.84	0.94	1.17	0.83	58.6
Shed stalks[c]	Stationary	5.5	1.98	1.07	1.05	1.67	38.7
Formerly stalked cells[c]	Stationary	13.6	1.93	0.96	0.99	0.88	53.1
Sheared stalks[d]	Late exponential	1.7	2.00	1.35	(0.65)	1.53	(29.7)

[a] Cells were grown in Higg 0.6% medium. Hydrolysis was in 4 N HCl for 2 h.

[b] Also in Table 3.

[c] Shed stalks and formerly stalked cells were harvested from the same culture. The sacculi of sheared stalks were subjected to NaOH extraction, resulting in a considerable loss of material. Since PHB was not present in suspension of shed stalk sacculi, this step was omitted in their purification.

[d] The small amount of material available for this assay contained Mur just at the level of detectability, and the calculated quantity of this compound may not be meaningful. The quantity of Glc was, however, sufficient to provide a significant calculation, since it reacts more intensely with ninhydrin than does muramic acid (Fig. 2, 3, and 6).

the cell hydrolysate. Stalks sheared from late exponential phase cells of 2NY66 also exhibited a high Glc/Dpm ratio (1.5), even though there was a low level of contamination of this preparation with sacculi of other cell fragments (Fig. 4C). The peptidoglycan composition of the sacculi extracted from unfractionated populations of 2NY66 was the same as that seen in the parental strain, CB2, and in the nonshedding revertant, 2NY66R (compare Tables 3 and 4).

Two species exhibited singular ratios among the caulobacter strains examined. In *C. leidyi* cell sacculi prepared from cells grown in any of four media, the ratio was 2:1:1:1:0.8 (Fig. 2; Table 3), a ratio closer to that considered typical of A1γ gram-negative bacterial peptidoglycan

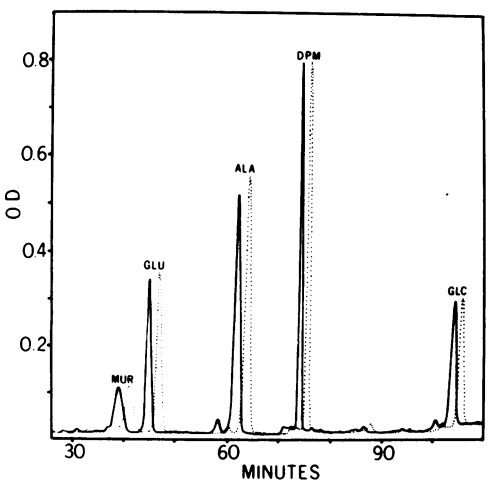

FIG. 3. Elution profiles of hydrolysates of *C. crescentus* 2NY66R sacculi prepared from cells grown in PYH (—) and Higg 0.6% (·····) media. The dotted line has been offset as in Fig. 2.

(43). Mur nevertheless accounted for significantly more than 50% of the amino sugar. In *A. biprosthecum*, the lateral-stalked caulobacter, as in the first four species (above), Mur accounted for 60% of the amino sugar units. However, this peptidoglycan differed from the others by appearing to contain a slightly lower proportion of Ala and a distinctly higher proportion of Glu (1.7:1.6:1:1.1:0.8; Table 2). This amino acid ratio, unusual for gram-negative bacteria, suggests that some cross-linking may involve a Glu unit as an interpeptide bridge, or that some of the peptide chains include more than one Glu unit.

Morphology of sacculi. Previous illustrations of sacculi prepared by the SDS-alkali extraction procedure demonstrated that general cell and stalk shape (34) and the septa in *A. excentricus* (36) are preserved and readily visible in preparations containing only peptidoglycan. Stalk bands, known to contain lysozyme-soluble material (44), did not copurify with the sacculi, implying that they are not continuous with the stalk sacculus and do not constitute septa within the stalk, as once suggested (20). However, in both *Asticcacaulis* species, indentations appeared at intervals along each stalk sacculus that might mark the former sites of bands (Fig. 7). Such indentations were not discernible in *Caulobacter* spp. stalk sacculi.

Stalks are shed by the Abs mutants by subpolar constriction so that the cell-proximal end of a shed stalk is typically larger in diameter than the rest of the stalk (Fig. 4A; see also reference 33). This difference in diameter, like every other feature of cell and appendage profile, was also observable in stalk sacculi (Fig. 5A through D).

Morphology of the peptidoglycan within the cell envelope. As seen in thin sections, the lysozyme-soluble layer (Fig. 8E) of *Caulobacter* cells appeared unaltered in thickness or in staining

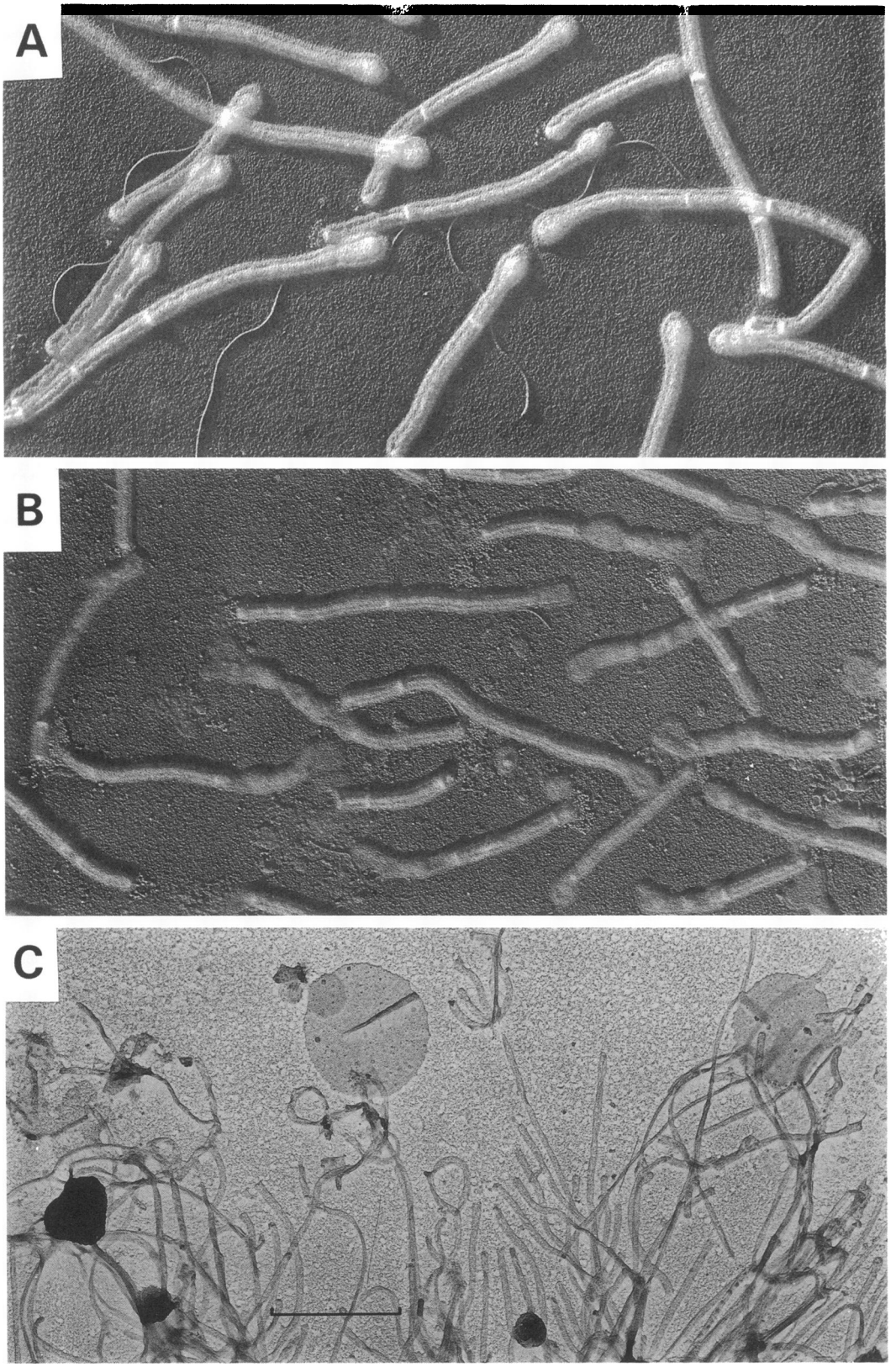

FIG. 4. Stalks shed (A) or sheared (B) from *C. crescentus* 2NY66. The granular material in (A) is holdfast material; when present, it is invariably at the once-distal end of the shed stalk. Some flagella are present. The granular material in B includes both holdfast material and cell debris. Flagellar fragments are also present. C, Sacculi prepared from the sheared stalks of B. The stalk sacculi typically adhered to each other in clumps; the round flat pieces are sacculi of fragments of cells disrupted by the shearing process. Bar, 1 μm.

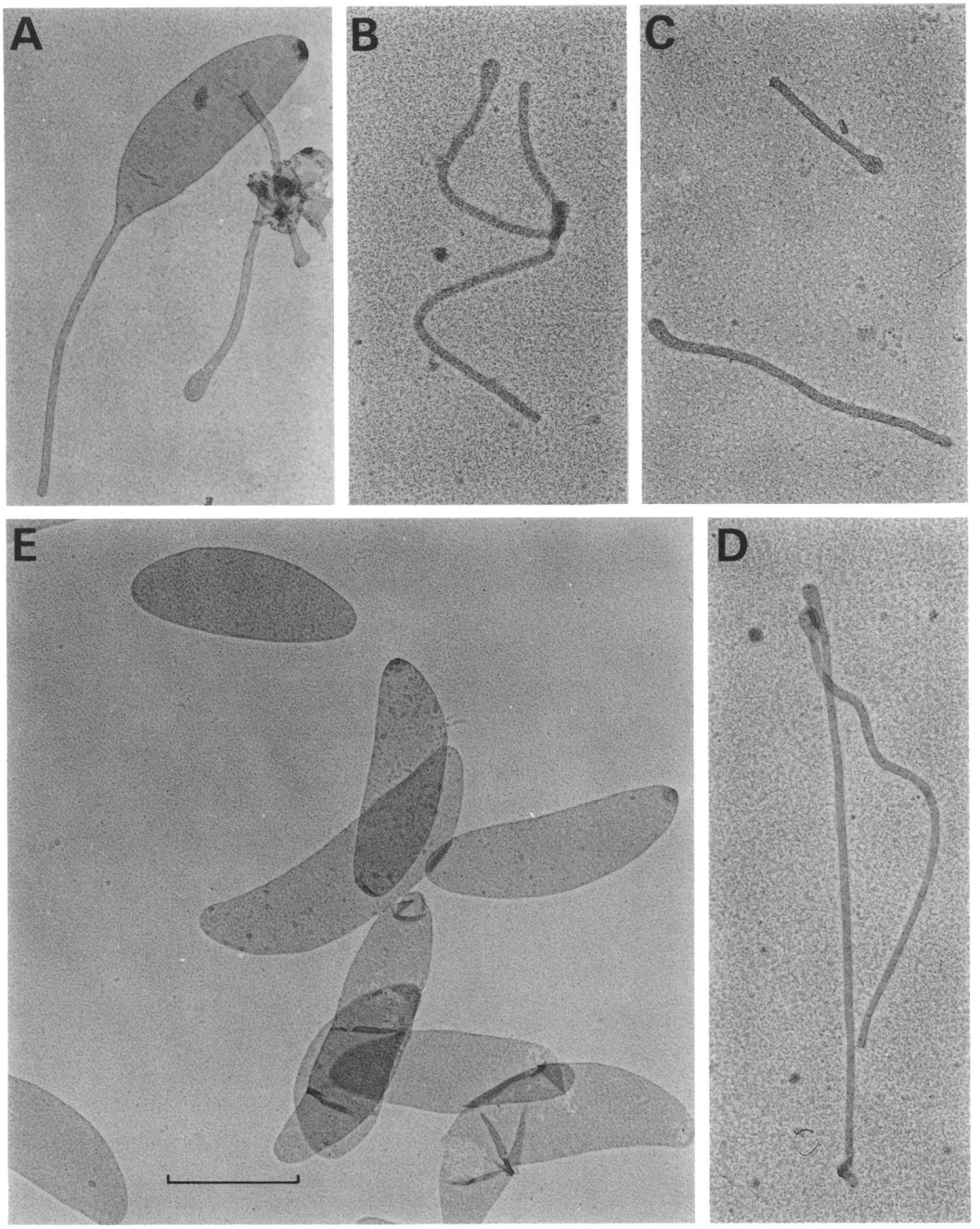

FIG. 5. Sacculi preparations from stationary phase populations of *C. crescentus* 2NY66. A, Total population; like sacculi of sheared stalks (Fig. 4C), sacculi of shed stalks appeared predominantly in adherent clumps. B, C, and D, Purified shed stalks (Fig. 4A). The once-proximal end is wider than the remainder of the stalk sacculus. The outline of the stalk sacculi appears smooth (compare with Fig. 7). The diameter of the stalk sacculus (50 to 75 nm) is equal to the diameter of the stalk core as seen in shadowed specimens (Fig. 4A). E, Cells that had recently shed their stalks. The dense polar straining visible in two of the sacculi is not peculiar to the Abs mutant and was seen as frequently at nonstalked poles of sacculi of the parent (CB2) and revertant (2NY66R) strains. Bar, 1 μm.

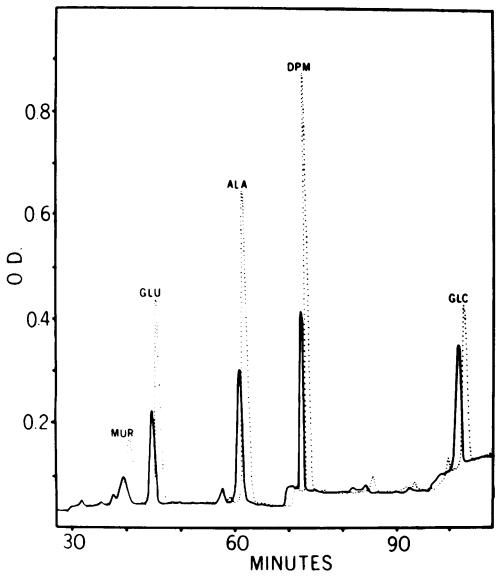

FIG. 6. Elution profiles of hydrolysates of shed stalks (—) and of the cells from which they were shed (·····). The dotted line has been offset as in Fig. 2.

intensity as it passed through the cell-stalk juncture (Fig. 8A). Thus, the change in peptidoglycan composition in this region was not discernible in thin sections. Further, there was not a detectable site of structural weakness or discontinuity in the sacculus, since the peptidoglycan of the juncture region was not especially susceptible to disruption by mechanical rupture of the cell, even though peptidoglycan was sometimes lost from other regions of the broken cell (Fig. 8B). The absence of the peptidoglycan layer from extensive regions of broken cells was typical of the Cds mutant 15NY87 (Fig. 8D), whose cells were susceptible to breakage in less than one-fourth the time required to disrupt cells of its parent strain, CB15. The mechanical fragility of the cells of this mutant was not reflected in any peculiarity of its peptidoglycan composition (Tables 2 and 3).

DISCUSSION

Gram-negative bacteria typically possess peptidoglycan in which the glycan chain consists of alternating units of Glc and Mur and the peptide linked to the Mur is a four-unit chain of Ala-Glu-Dpm-Ala. Hydrolysates of such peptidoglycans contain Ala, Glu, Dpm, Mur and Glc in molar ratios of 2:1:1:1:1 (43). Exceptions have been reported in which the Mur and Glc are not present in equimolar amounts, but neither amino sugar was found to exceed the proportion of Dpm (22, 56).

In the present studies, only one type of caulobacter, *C. leidyi*, appeared to have a peptidoglycan composition that approximated this type; its peptidoglycan contained only a moderate excess of Mur (54 to 55% of the amino sugar). In the other five species studied, the molar ratios of the sacculus hydrolysates revealed an unequal amount of the two amino sugars corresponding to a 50% excess of Mur over Glc. This ratio could be accounted for by the primary structure illustrated in Fig. 9A. Alternatively, the sacculus could be chemically heterogeneous, comprising some regions of typical glycan chains and other regions in which Mur occurred in Glc-free tracts.

A third type of glycan structure was implied by the molar ratios detected in hydrolysates of stalk peptidoglycans. These hydrolysates contained an approximately 50% excess of Glc over Mur, suggesting the primary structure illustrated in Fig 9B. Glc-rich peptidoglycan was detected in two types of stalk preparations—stalks shed by a stalk abscission mutant and stalks artificially sheared from cells of the same strain. The peptidoglycan of shed stalks was somewhat richer in Glc than that of the sheared stalks (Glu/Dpm, 1.67 and 1.53, respectively). The major structural difference between stalks prepared by these two methods is the presence on shed stalks of a swollen end derived from the cell-stalk juncture. It consequently appears significant that the peptidoglycan extracted from cells immediately after they had shed their stalks was higher in Mur than in Glc but nevertheless contained 10% more Glc than it did before abscission. These observations imply that the peptidoglycan synthesized within the subpolar constriction that culminates in stalk abscission is, at least initially, relatively high in Glc content and raise the possibility that a similar, possibly transient, change in peptidoglycan composition occurs during the development of the equatorial constriction that culminates in cell division. This possibility was not explored in the present studies.

A Glc/Mur/Dpm ratio of 3:2:2 has not been reported in the peptidoglycan of vegetative cells of wild-type gram-negative bacteria, but it has been found in the spheroid form of the pH-conditional cell shape mutant of *Klebsiella pneumoniae*, MirM7 (see Table 3 of reference 8). This mutant is interpreted as conditionally defective in lateral wall elongation; at the restrictive pH (pH 7), the wall consists entirely of cell poles (42). In *C. crescentus*, if stalk peptidoglycan of wild-type cells is similarly Glc-rich, the stalk could be interpreted chemically, as well as structurally, as an extension of the cell pole. Determi-

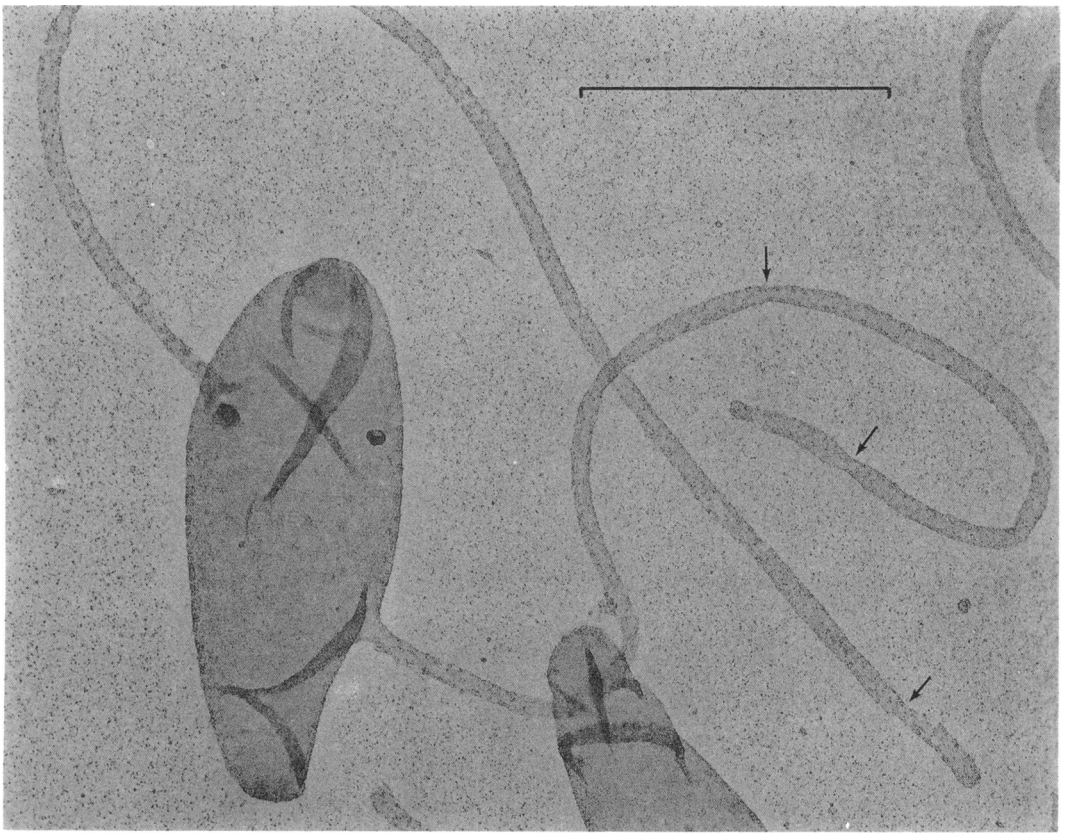

FIG. 7. Biprosthecate sacculus prepared from *A. biprosthecum*. Arrowheads indicate three indentations, possibly previous sites of stalk bands. Bar, 1 μm.

nation of stalk peptidoglycan composition needs yet to be extended to *Asticcacaulis*, in which stalks arise subpolarly (in *A. excentricus*) or laterally (in *A. biprosthecum*).

Among peptidoglycans, it is especially uncommon to find a peculiar glycan composition, since the majority of variations in composition comprise differences in peptide components (43). Only one species (*A. biprosthecum*) among the six studied was found to exhibit a unique amino acid composition. Assuming that not every cross-linkage involved Glu as an interpeptide bridge, the molar ratios of the *A. biprosthecum* peptidoglycan components would be consistent with the primary structure proposed in Fig. 9C. Alternatively, the excess Glu units could occur within some of the peptide chains, rather than as interpeptide bridges, and the occasional truncated peptide chain implied by the relatively low Ala content need not occur only on the Mur-Mur pairs.

Changes in cell shape such as sphere-rod and filament-rod conversions are known that involve alteration of glycan chain length (14), frequency and composition of interpeptide bridges (8, 19), or both (25, 26), alteration of proportions of penicillin-binding proteins (18), or alteration of autolysins (24). In each such case, changes in the amount or structure (or both) of the peptidoglycan appear to provide mechanisms for change in the shape of the cell. It seems plausible that modification of the primary structure of the glycan chain could be a means for reorienting the growing peptidoglycan.

We infer from the results of the present studies that modification of the primary structure of the peptidoglycan is involved in stalk morphogenesis in *Caulobacter* spp. (and possibly also in pole formation during cell division). It is tempting, upon examination of the proposed primary structures, to regard the Mur-Mur pairs of the cell sacculus as corresponding (biosynthetically) to the Glc-Glc pairs of the stalk sacculus. The homogeneous amino sugar pairs would thus constitute sites that could be altered during stalk morphogenesis; the reduced possibilities for

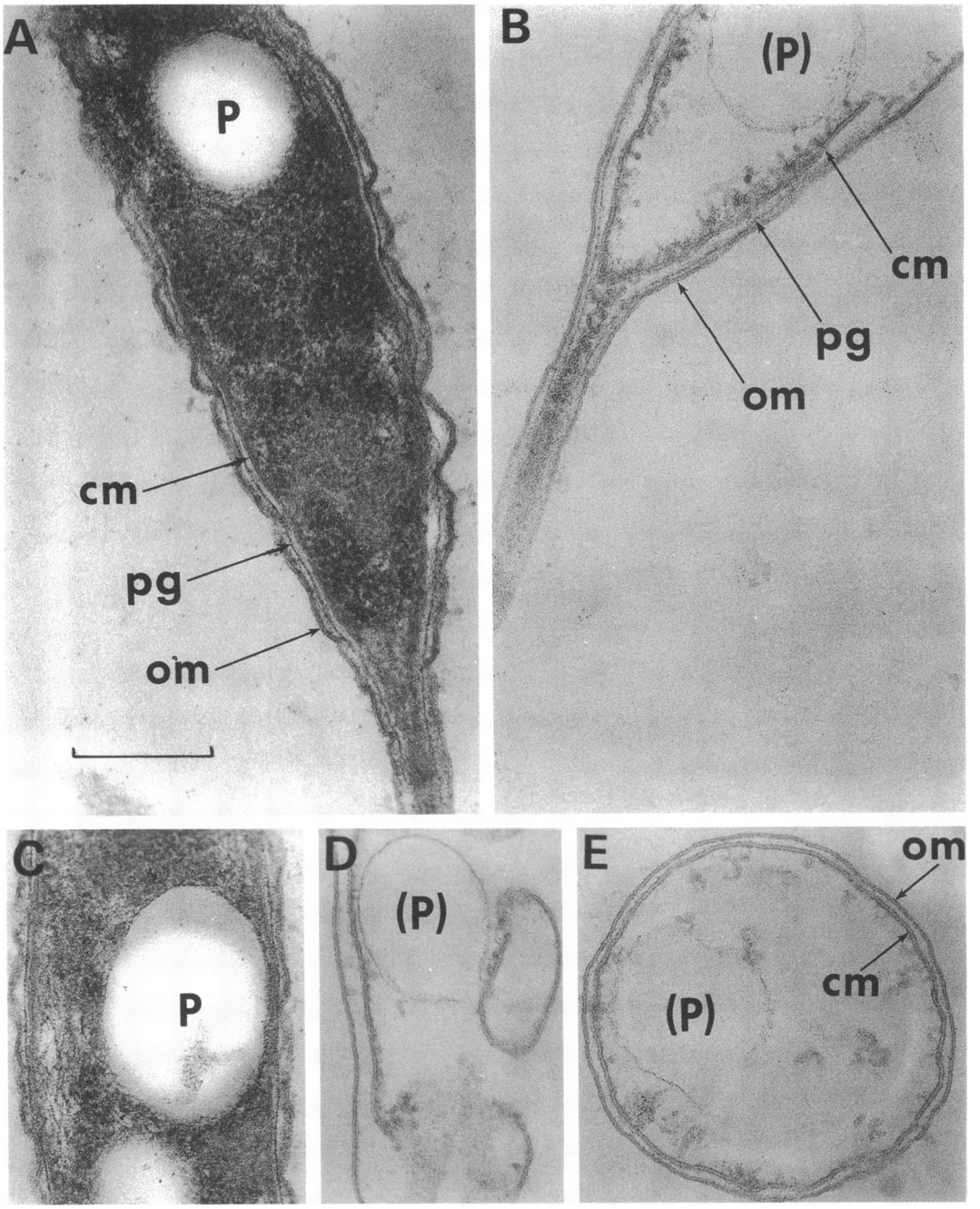

FIG. 8. Sections prepared from *C. crescentus* CB15 (A, B, C, and E) and 15NY87 (D). Longitudinal sections through the cell-stalk juncture of intact (A) and mechanically disrupted (B) cells. C, Longitudinal section through an intact cell and normal to the single-layered membrane of the PHB granule. D, Longitudinal section through a disrupted cell from which practically all of the peptidoglycan layer has been lost. E, Section through a lysed lysozyme-treated cell. Abbreviations: cm, cell membrane; pg, peptidoglycan layer; om, outer membrane; P, intact PHB granule; (P), previous site of PHB, surrounded by the single-layered membrane. Bar, 0.25 μm.

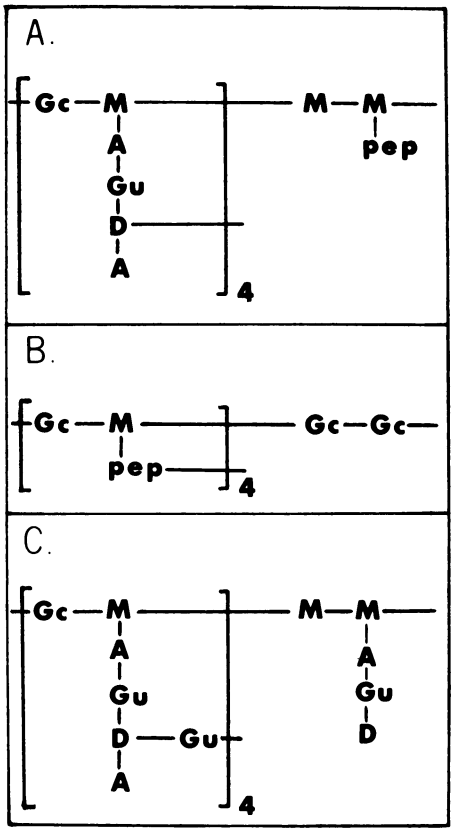

FIG. 9. Proposed primary structures of repeating units of caulobacter peptidoglycans. A, Predominant type of cellular peptidoglycan. B, Stalk peptidoglycan. C, *A. biprosthecum* cellular peptidoglycan. Abbreviations: M, Mur; Gu, Glu; A, Ala; D, Dpm; Gc, Glc; pep, peptide as shown in brackets in A.

cross-linking within a Glc-rich peptidoglycan would confer greater flexibility on the peptidoglycan and allow its arrangement as a narrower cylinder, the stalk sacculus. In this way, most of the peptidoglycan structure could remain essentially the same as in the majority of gram-negative bacteria, and only relatively small genetic changes would have allowed the derivation of these prosthecate bacteria from nonprosthecate ancestors. According to this view, those changes would have involved the synthesis of two (sets of) murein synthetases or hydrolases, whose regulation and localization should prove of central importance in the initiation of stalk development.

The recent reports of studies on the identity and localization of penicillin-binding proteins of *C. crescentus* (23, 23a) encourage the anticipation that one or more of the proteins will prove, as in *Escherichia coli* (49–52), to be enzymes involved in murein synthesis. Two of the cellular penicillin-binding proteins cannot be detected in stalks shed by Abs mutant 15NY106, whereas two others are practically undetectable in cells, but prominent in stalks (S. Koyasu, personal communication). In addition, the availability of *C. crescentus* mutants in which cell division is temperature-conditional (31) may provide an independent approach to the identification of the effectors of constriction in this species.

These related studies, as well as the present findings regarding peptidoglycan composition, justify further characterization of caulobacter peptidoglycans and initiation of investigations of the enzymes that effect their synthesis and possible differentiation. The elucidation of the determinants of stalk development—both at the genetic and at the murein level—may provide considerable insight into the more general phenomena of shape determination in bacteria and of cell morphogenesis during cell division.

ACKNOWLEDGMENTS

We are indebted to Carol Lusty for her instructions and assistance in the analysis of the amino compounds.

This work was supported in part by Public Health Service grant AI-15467 from the National Institutes of Health and by grant PCM-7903659 from the National Science Foundation.

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