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Alterations in Peptidoglycan Chemical Composition Associated with Rod-to-Sphere Transition in a Conditional Mutant of *Klebsiella pneumoniae*

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Klebsiella pneumoniae Mir M7 is a spontaneous parentless morphology mutant which grows as cocci at pH 7 and as rods at pH 5.8. This strain has been characterized as defective in lateral wall formation (at pH 7). Data suggest that the cell wall is mainly made up of poles of the rods (G. Satta, R. Fontana, P. Canepari, and G. Botta, J. Bacteriol. **137**:727-734, 1979). In this work the isolation and the biochemical properties of the peptidoglycan of both Mir M7 rods and cocci and a nonconditional rod-shaped Mir M7 revertant (strain Mir A12) are described. The peptidoglycan of Mir M7 (both rods and cocci) and Mir A12 strains carried covalently bound proteins which could be easily removed by pronase treatment in Mir M7 rods and Mir A12 cells, but not in Mir M7 round cells. However, when the sodium dodecyl sulfate-insoluble residues of Mir M7 cocci were pretreated with ethylenediaminetetraacetic acid (EDTA), pronase digestion removed the covalently bound proteins, and pure peptidoglycan was obtained. EDTA treatment of the rigid layer of Mir M7 cocci removed amounts of Mg^{2+} and Ca^{2+}, which were 10- and 50-fold higher, respectively, than the amount liberated from the rigid layer of Mir M7 rods and Mir A12 cells. Amino acid composition was qualitatively similar in both strains, but Mir M7 cocci contained a higher amount of alanine and glucosamine. Mir M7 cocci contained approximately 50% less peptidoglycan than rods. Under electron microscopy, the rigid layer of the Mir M7 rods and Mir A12 cells appeared to be rod-shaped and their shape remained unchanged after EDTA and pronase treatment. On the contrary, the Mir M7 cocci rigid layer appeared to be round, and after EDTA treatment it collapsed and lost any definite morphology. In spite of these alterations, the peptidoglycan of Mir M7 cocci still appeared able to determine the shape of the cell and protect it from osmotic shock and mechanical damages. The accumulation of divalent cations appeared necessary for the peptidoglycan to acquire sufficient rigidity for shape determination and cell protection. We concluded that the coccal shape in Mir M7 cells is not due to loss of cell wall rigidity but is a consequence of the formation of a round peptidoglycan molecule. The possibility that the alterations found in the Mir M7 cocci rigid layer may reflect natural differences in the biochemical composition of the septa and lateral wall of normally shaped bacteria is discussed.

In bacteria, the events that lead to determination and maintenance of cell shape during the division cycle are complex and poorly understood. Conditional and nonconditional mutants, which undergo rod-to-sphere conversion, offer the opportunity of comparing cells of the same strain with differing morphologies and have been used to confront this problem (1, 2, 5, 11, 16, 21, 24, 29, 40, 41).

It has been proposed recently that rod-shaped bacteria bear two sites for peptidoglycan assembly, one responsible for lateral wall elongation and the other responsible for septum formation. In this view strains defective in the site for septum formation would form filaments, whereas those defective in the site for lateral wall elongation would grow as cocci (35; G. Satta, P. Canepari, G. Botta, and R. Fontana, submitted for publication).

Klebsiella pneumoniae Mir M7 is a wild-type strain with a round shape at pH 7 and a rod shape at pH 5.8 (22, 33-37). Recent studies on this strain have led to the conclusion that its site for lateral wall formation is altered in such a

way that, although it works normally in media at pH 5.8, its activity is not expressed at pH 7. This would suggest that the cell wall of strain Mir M7 grown at pH 7 is made up mainly of the two polar portions of the rod cell wall (35).

Other morphological mutants with a coccal shape have already been analyzed for the peptidoglycan chemical composition to determine whether the altered shape was associated with alterations in the chemical composition of the peptidoglycan (3, 16, 18, 19, 20, 25, 30, 32). No damage to this polymer, which could clearly be considered responsible for shape alteration, was found. However, none of these mutants was conditional for pH, nor were they characterized as defective in the site for lateral wall extension. For these reasons we felt that it was important to study the peptidoglycan composition of cocci and rods of the morphology mutant Mir M7, which shows several peculiarities compared with previously described mutants (33–37). In particular, given that the cell walls of the cocci in Mir M7 are most likely made up of the poles of the rods, this analysis could show possible differences between the peptidoglycan of the lateral wall and that of the septa.

We show in this work that strain Mir M7 cocci contain approximately 50% less peptidoglycan than rods. Moreover, amounts of alanine, Mg^{2+}, and Ca^{2+} are significantly higher in the peptidoglycan of cocci than that in rods. Finally, for the peptidoglycan of cocci to have a rigid structure with a defined shape, divalent cations are necessary.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The strains of *K. pneumoniae* Mir M7 and Mir A12 used in this work have been described elsewhere in detail (33–37). Briefly, *K. pneumoniae* Mir M7 is a parentless spontaneous, morphologically conditional mutant of *K. pneumoniae* which grows as round cells at pH 7 and as rods at pH 5.8. *K. pneumoniae* Mir A12 is a revertant of strain Mir M7 with the physiological and morphological characteristics of wild-type *K. pneumoniae* (8, 34).

Bacteria were grown with shaking at 37°C in peptone-lactose (PL) medium either at pH 7 or at pH 5.8. This medium contains per liter: peptone (Difco), 20 g and lactose, 20 g. To these base components, we added either $Na_2HPO_4 \cdot 2H_2O$, 6.3 g–NaH_2PO_4, 1.8 g, to obtain a medium of pH 7, or $Na_2HPO_4 \cdot 2H_2O$, 0.7 g and NaH_2PO_4, 6.9 g, to obtain a medium of pH 5.8. When necessary, peptidoglycan was specifically labeled with [^{3}H]diaminopimelic acid (DAP) (specific activity 300 mCi/mmol; Radiochemical Centre, Amersham, England) or with [^{14}C]DAP (44 mCi/mmol; Radiochemical Centre), as described previously (35).

Isolation of peptidoglycan. Peptidoglycan purification was performed essentially as described by Braun and Rehn (4). Cells growing exponentially (10 g, dry weight) were suspended in 1 liter of distilled water. A 20% stock solution of sodium dodecyl sulfate (SDS) was added to the suspension, drop by drop, to a final concentration of 4%. Then the suspension was heated for 45 min at 100°C and incubated overnight with stirring at 37°C. The suspension was centrifuged at 8,000 × *g* for 15 min to remove unbroken cells, and the resulting supernatant was centrifuged at 78,000 × *g* for 30 min at 20°C. The sediment (SDS-insoluble residue) was then washed three to five times with water. After the last centrifugation, the pellet was resuspended in 100 ml of 10 mM Tris-hydrochloride buffer (pH 7.4) containing 50 mg of pronase and incubated at 60°C for 150 min. This treatment is more effective in removing the proteins covalently bound to peptidoglycan of *K. pneumoniae* than the more frequently used trypsin treatment (8). The suspension was then heated at 100°C, and SDS was added to a final concentration of 1% to stop the reaction and solubilize proteins. After washing it three times with water, the peptidoglycan was resuspended in distilled water and stored at −25°C or lyophilized.

Preparation of radioactive cell envelopes. Cells were grown in minimal medium (35) supplemented with 2 μCi of [^{3}H]DAP per ml and harvested in mid- to late-exponential-growth phase. After they were cooled on ice the cells were centrifuged at 8,000 × *g* for 5 min, resuspended in ice-cold 1 mM sodium phosphate buffer (pH 7), and broken by three 30-s pulses of sonication at maximal amplitude (Vibrason KG 150, Kerry Vitrasonics Ltd., Hitchin, England) with intervening 30-s periods of cooling. Unbroken cells were removed by centrifugation at 8,000 × *g* for 15 min at 4°C, and the cell envelopes were pelleted out of the supernatant by centrifugation at 78,000 × *g* for 30 min at 4°C. The cell envelopes were resuspended in ice-cold phosphate buffer (as above) and washed twice by centrifugation at 78,000 × *g*. Peptidoglycan hydrolase activity in these cell envelopes was then evaluated as described by Hartmann et al. (15).

Analytical procedures. Amino acid analysis was performed with a Unichrom (Beckman) amino acid analyzer as described by Braun and Rehn (4).

Estimations of Mg^{2+} and Ca^{2+} concentrations in ethylenediaminetetraacetic acid (EDTA) extracts were evaluated with the help of a Jarrel ASH-82-271 atomic adsorption spectrophotometer.

Peptidoglycan cross-linkages were determined both by fluorodinitrobenzene technique, as described by Ghuysen et al. (12), and by deamination of the intact peptidoglycan by $NaNO_2$, as described by Fordham and Gilvarg (9). In the first technique, 2 mg of purified peptidoglycan was treated with FDNB and then hydrolyzed in 6 N HCl and analyzed for amino acid composition as described above. DAP was the only amino acid reacting with FDNB in all samples. The amount of DAP engaged in peptide cross-linking was calculated from the molar ratios of non-dinitrophenylated DAP found in treated samples. In the second technique [^{14}C]DAP-labeled cells were treated with $NaNO_2$ and then hydrolyzed in 6 N HCl as described (9). Radioactive DAP and radioactive α-hydroxy-ε-aminopimelic acid formed by the nitrous acid reaction

from non-cross-linked DAP were separated by descending paper chromatography and quantitatively evaluated as described (9).

Other procedures. Extraction of whole cells and SDS-insoluble residues with 34 mM EDTA were made as described by Gray and Wilkinson (14).

Radioactive aqueous samples were measured in Triton-toluene scintillator (0.5-ml sample in 5-ml of scintillator containing 10 g of 2,5-diphenyloxazole [PPO] in a mixture of 1 liter of toluene and 0.5 liter of Triton X-100). Radioactive samples on paper were counted in 5 ml of toluene scintillator (5 g of PPO per liter of toluene). A Beckman LS-133 liquid scintillation counter was used.

Electron micrographs were made with a Siemens electron microscope. All samples were suspended in 1% SDS and incubated at 100°C for 1 min and at 37°C for 2 h with stirring. These suspensions were then applied to a Formvar-coated grid and removed after 1 min. A small drop of 2% aqueous phosphotungstic acid (pH 7.4) was applied and then removed after 0.5 min.

RESULTS

Peptidoglycan content of Mir M7 cocci and rods. Loss of peptidoglycan rigidity appears to be the first possible candidate causing shape alteration in morphology mutants. In previous studies we found that, at pH 7, Mir M7 cocci incorporate approximately 50% less radioactive DAP than rods, and we have obtained preliminary results showing that cocci contain 50% less peptidoglycan than Mir M7 rods at pH 5.8 (35).

To confirm this last finding, we calculated the peptidoglycan content in the cells by two different methods—one based on the amount of DAP present in the whole cells and the other consisting of the direct isolation of the polymer from a known weight of cells. As shown in Table 1, Mir M7 cocci contained approximately half the peptidoglycan contained in both Mir M7 rods and Mir A12 cells. However, this finding did not necessarily mean that cocci had a thinner peptidoglycan layer than rods since the coccal shape requires the minimal surface to cover a fixed volume. By using microphotographs we therefore calculated the surface area of cocci and rods, assuming cocci to be spheres and rods to be cylinders with hemispherical ends (Fig. 1). As shown in Fig. 1, cocci appeared to have an external surface area approximately 20% smaller than that of the rods, thus indicating that their peptidoglycan layer might be no more than 30% thinner than that of the rods.

Extent of peptide side-chain cross-linking in the peptidoglycan layer. Another cause for possible loss of rigidity in the peptidoglycan could be reduction in the peptidoglycan cross-linkages. Therefore, the cross-linkages in rods and cocci were calculated both by reacting isolated peptidoglycan with fluorodinitrobenzene and by assaying the dinitrophenylated product for the amount of residual DAP not converted into mono-dinitrophenylated-DAP (see Materials and Methods) and by the method described by Fordham and Gilvarg (9) which is based on the reaction of nitrous acid with the unprotected amino groups of non-cross-linked DAP. Cocci were found to have at least the same amount of cross-linkages as rods (Table 2). The percent of cross-linkages in strain Mir M7 was virtually the same as that in strain Mir A12. Moreover, in Mir strains, the amount of cross-linkages was higher during the stationary phase of growth.

TABLE 1. *Peptidoglycan content, expressed as percent of the whole cells, of K. pneumoniae Mir M7 and Mir A12 grown at pH 7 and 5.8*

Strain	pH	Cell shape	Peptidoglycan in whole cells (%, dry wt) determined by:	
			Direct isolation[a]	Amt of DAP in whole cell[b]
Mir M7	7	Cocci	0.8	1.1
	5.8	Rods	1.9	2.1
Mir A12	7	Rods	1.8	2.0
	5.8	Rods	1.8	2.0

[a] Three samples of exponential cells (10 g, salt-free dry weight) from every strain were suspended separately in 1 liter of boiling 4% SDS solution. Peptidoglycan was then purified as described in Materials and Methods. The sediments containing pure peptidoglycan were lyophilized and carefully weighed.

[b] Three samples of exponential cells (2 mg, salt-free dry weight) from every strain were analyzed for amino acid content as described in Materials and Methods, and the amount of DAP per milligram of whole cells was carefully evaluated. The percent of polymer in the salt-free dry weight of the cell then was calculated with the help of the data obtained in the present study on the ratios of the peptidoglycan amino acids of the two strains.

Amino acid and amino sugar analysis of the peptidoglycan layer. The amino acid and amino sugar compositions of the peptidoglycan of strains Mir M7 and Mir A12, both grown at pH 7 and 5.8, are shown in Table 3. All the pure peptidoglycan preparations contained only those amino acids characteristic of the peptidoglycan of gram-negative bacteria. Quantitative analysis also showed that the molar ratios of these amino acids were similar for the peptidoglycan isolated from Mir M7 rods and Mir A12 cells. On the contrary, Mir M7 cocci contained a higher level of alanine and glucosamine.

Mg^{2+} and Ca^{2+} content of rigid layers isolated by SDS treatment of whole cells. While trying to obtain pure peptidoglycan we

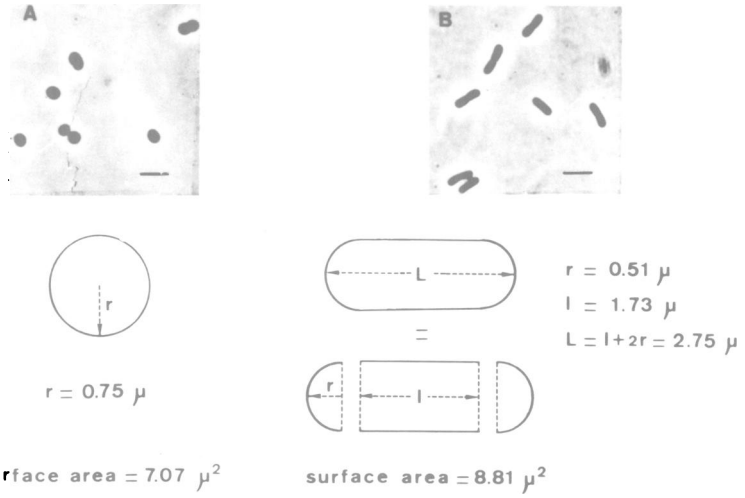

FIG. 1. *Phase-contrast micrographs of K. pneumoniae Mir M7 cells grown at pH 7 (A) and pH 5.8 (B) and the calculation of the surface area of both cocci and rods. The dimensions of 100 septated cocci and 100 septated rods were accurately measured, and the average values are reported in the diagrams. To calculate the surface area, round cells were considered as spheres and rods as cylinders with round ends. The surface of the round ends together was calculated to be equal to that of a sphere having a radius of one-half the transverse diameter of the cells. The length of the cylinder was considered to be equal to the length of the rods minus their transverse diameter (equivalent to the cell poles). Bar = 5 μm.*

TABLE 2. *Peptide side-chain cross-linkages in peptidoglycan of K. pneumoniae Mir M7 and Mir A12 grown at pH 7 and 5.8*

Strain	pH	Cell shape	DAP engaged in cross-linkages (%) as determined by:		
			FDNB technique[a] (exponential cells)	Nitrous acid technique[b]	
				Exponential cells	Stationary cells
Mir M7	7	Cocci	36	33	47
	5.8	Rods	30	28	36
Mir A12	7	Rods	32	30	35
	5.8	Rods	31	30	34

[a] A 2-mg amount of pure peptidoglycan was treated with fluorodinitrobenzene as described in Materials and Methods. Amino acid analysis of dinitrophenyl-peptidoglycan and untreated samples were then carried out. The amount of non-dinitrophenylated-DAP found in treated samples compared with the amount of DAP found in untreated sample indicate the amount of DAP engaged in cross-linkages.

[b] Bacteria were grown in 25 ml of minimal salt medium (35) containing 1 μCi of [^{14}C]DAP per ml at 37°C. Exponential-phase cells were obtained at a turbidity of 0.8 optical units (OU)/ml and solubilized by adding 15 ml of the culture to 15 ml of 8% SDS; stationary-phase cells were collected at a turbidity of 1.6 OU/ml 2 h after the end of exponential growth by diluting the remaining 10 ml with 10 ml of 8% SDS. Cross-linkages were determined by using one-half of each sample as described in Materials and Methods.

TABLE 3. *Amino acid and amino sugar composition of the peptidoglycan of K. pneumoniae Mir M7 and Mir A12*

Strain	pH	Cell shape	Amino acid and amino sugar (molar ratio)[a]				
			Muramic acid	Glutamic acid	Alanine	DAP	Glucosamine
Mir M7	7	Cocci	1.05	1.32	2.72	1.00	1.59
	5.8	Rods	0.86	1.15	2.05	1.00	1.03
Mir A12	7	Rods	0.97	1.12	1.86	1.00	0.82
	5.8	Rods	0.92	1.09	1.92	1.00	0.85

[a] The amino acid residues are referred to as 1.00 residues of DAP.

found that this polymer could be purified from cocci only if the SDS-insoluble residues were treated with EDTA before pronase digestion. Pure peptidoglycan could, on the contrary, be obtained without such treatment from both Mir M7 rods and Mir A12 cells (both grown at pH 5.8 and 7).

To understand the mechanism by which EDTA made possible purification of peptidoglycan, we analyzed the compounds removed by EDTA from SDS-insoluble residues and tested their sensitivity to pronase before and after EDTA treatment. As Table 4 shows, EDTA removed amounts of Mg^{2+} and Ca^{2+} from the SDS-insoluble residues of cocci that were 10- and 50-fold higher, respectively, than the

TABLE 4. *Divalent cations removed by EDTA from SDS-insoluble residues and whole cells of K. pneumoniae Mir M7 and Mir A12 grown at pH 7 and pH 5.8*

Strain	pH	Cell shape	Divalent cations (mmol/g, dry wt) removed from:			
			Whole cells[a]		SDS-insoluble residues[a]	
			Mg^{2+}	Ca^{2+}	Mg^{2+}	Ca^{2+}
Mir M7	7	Cocci	0.08	0.40	0.47	2.82
Mir A12	5.8	Rods	0.07	0.25	0.05	0.06
	7	Rods	0.08	0.31	0.03	0.13
	5.8	Rods	0.07	0.20	0.02	0.12

[a] Lyophilized cells and SDS-insoluble residues were resuspended at a concentration of 1 mg/ml in 34 mM EDTA in 0.5 M borate buffer (pH 9.2). The mixture was kept at room temperature for 60 min and then centrifuged at 78,000 × *g* for 45 min. The supernatant was analyzed to evaluate Mg^{2+} and Ca^{2+} content as described in Materials and Methods.

amount removed from the SDS-insoluble residues of Mir M7 rods and Mir A12 cells. The higher amount of divalent cations removed from the rigid layer of cocci did not reflect a characteristic of the whole cells, but appeared to be specific of the SDS-insoluble residues. In fact, EDTA removed approximately the same amount of divalent cations from whole cells of Mir M7 cocci as from whole cells of Mir M7 rods and Mir A12 cells (Table 4).

SDS-insoluble residues isolated from Mir M7 cocci became fully sensitive to pronase and trypsin digestion after treatment with EDTA. On the contrary, the sensitivity of the SDS-insoluble residues isolated from Mir M7 rods and Mir A12 cells was not influenced by such treatment (Fig. 2). These findings gave a possible explanation for the specific need for EDTA treatment during purification of peptidoglycan and raised the possibility that Mir M7 cocci could require a relatively high concentration of divalent cations in the growth medium.

Growth in PL medium added with EDTA. To confirm the above possibility, both Mir M7 cocci and rods and Mir A12 cells were grown in the presence of 0.1 mM EDTA and the effect of this substance on cell growth, autolysis, and properties of the SDS-insoluble residues was studied. The growth rate of Mir M7 cocci was significantly inhibited by an EDTA concentration which did not influence Mir M7 rods (Fig. 3) or Mir A12 cells (not shown). Furthermore, cocci grown in the presence of 0.1 mM EDTA were much more prone to autolysis (Fig. 4) and peptidoglycan digestion by cell wall hydrolases than both rods and Mir A12 cells grown in the same medium (Fig. 5).

The SDS-insoluble residues of both Mir M7 cocci and rods and Mir A12 cells grown in the presence of 0.1 mM EDTA were tested for sensitivity to proteolytic enzymes and susceptibility to purification without EDTA treatment. The

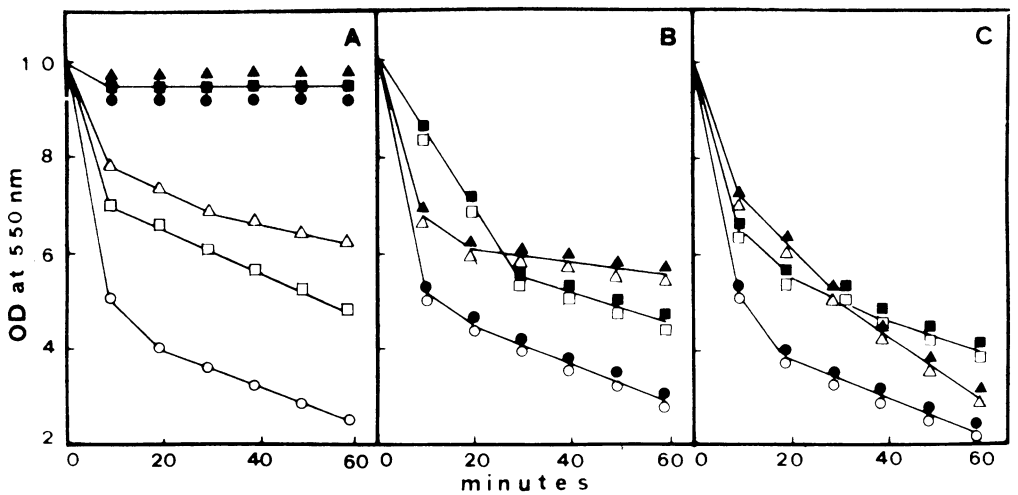

FIG. 2. *Effect of the EDTA on sensitivity to digestion by proteolytic enzymes of SDS-insoluble residues from K. pneumoniae Mir M7 cocci (A), Mir M7 rods (B), and Mir A12 cells grown at pH 7 (C). Exponentially growing cells were treated with 4% SDS as described in Materials and Methods. SDS-insoluble residues were then subdivided into two parts, one of which (open symbols) was treated with 34 mM EDTA in borate buffer (pH 9) at room temperature for 60 min and then washed twice in distilled water and the other of which (closed symbols) was not treated. Each portion of the SDS-insoluble residues was resuspended in 10 mM Tris-hydrochloride buffer at pH values of 7.4 for pronase treatment (●, ○) or 8.2 for trypsin (■, □) and chymotrypsin (▲, △) treatment. The turbidity of the rigid-layer suspensions was measured at 550 nm with a Beckman DB spectrophotometer.*

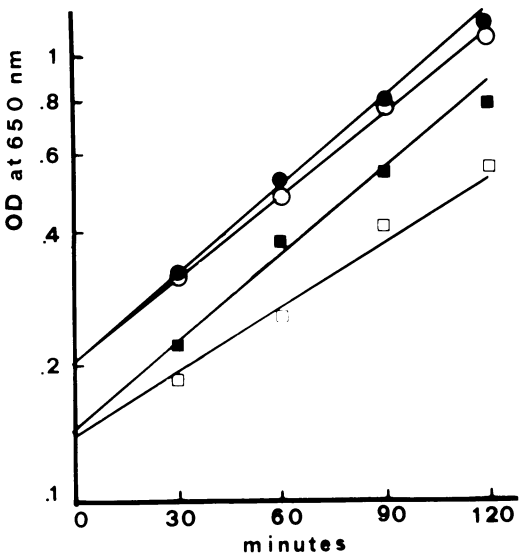

FIG. 3. *Effect of EDTA on the growth rate of K. pneumoniae strain Mir M7. Cells exponentially growing in PL medium at pH 7 and pH 5.8 were collected by centrifugation and transferred in PL medium (closed symbols) and in PL medium containing 0.1 mM EDTA (open symbols). The optical density (OD) was monitored at 650 nm with a Beckman DB spectophotometer. Mir M7 pH 7* (■, □); *Mir M7 pH 5.8* (●, ○).

substances released from these SDS-insoluble residues by EDTA treatment and the effect of EDTA on their shapes were also studied. As shown in Table 5, the SDS-insoluble residues isolated from cocci were sensitive to pronase and trypsin digestion, and EDTA treatment was not necessary to obtain pure peptidoglycan. Finally, the amount of divalent cations removed by EDTA from both the SDS-insoluble residues and the pure peptidoglycan was much lower than the amount removed from cocci grown in media without EDTA and was not greater than the quantity removed from SDS-insoluble residues and peptidoglycan of rods.

Role of divalent cation accumulation in rigidity of the Mir M7 cocci peptidoglycan. We then studied, under the electron microscope, the shape of the SDS-insoluble residues from rods and cocci grown in both PL medium and PL medium containing 0.1 mM EDTA. The SDS-insoluble residues from Mir M7 rods appeared always as rod-shaped structures, regardless of the absence or presence of EDTA in the growth media (Fig. 6A and B). On the contrary, the SDS-insoluble residues from cocci appeared as regular coccoid structures when isolated from cells grown in PL medium (Fig. 6C), but appeared as clumps of amorphous material when grown in PL medium containing EDTA (Fig. 6D).

To study the possible role of divalent cations in the rigidity and shape maintenance of the peptidoglycan of Mir M7 cocci, the effect of EDTA on the shape of SDS-insoluble residues from cells grown in media not containing EDTA was analyzed. The SDS-insoluble residues from Mir M7 cocci lost their spheroidal shape immediately after EDTA treatment and appeared as bulks of amorphous material resembling the SDS residues from cocci grown in the presence of EDTA (not shown). On the contrary, EDTA had no effect on the shape of the SDS-insoluble residues isolated from Mir M7 rods and Mir A12 cells (not shown).

The EDTA treatment invariably caused complete loss of shape in the peptidoglycan of Mir M7 cocci was also demonstrated by the finding that pure peptidoglycan prepared both from cocci grown in normal media and in media containing EDTA was always amorphous (Fig. 6E), whereas the one isolated from both Mir M7 rods and Mir A12 cells was rod shaped (Fig. 6F).

Evaluation of the mechanical strength of Mir M7 cocci. The last findings clearly indicated that, to acquire rigidity, the peptidoglycan

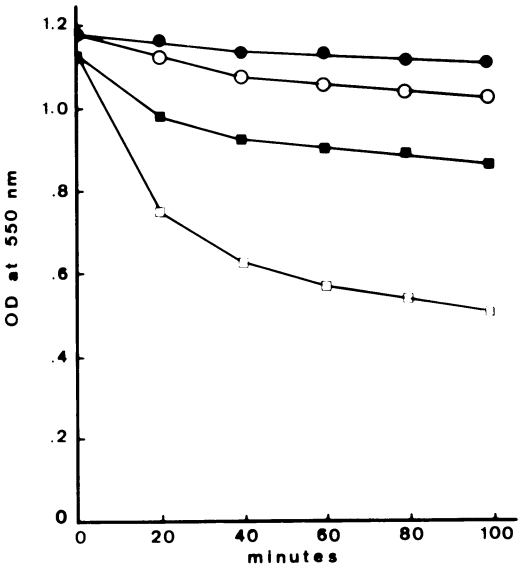

FIG. 4. *Effect of EDTA on autolysis of K. pneumoniae Mir M7 cocci and rods. Cells exponentially growing in PL medium (closed symbols) and in PL medium containing 0.1 mM EDTA (open symbols) at both pH 7 and pH 5.8 were collected by centrifugation and resuspended in 10 mM Tris-hydrochloride buffer (pH 7). The cell suspensions were incubated at 37°C, and at intervals samples were taken to monitor the optical density (OD) as described (see Fig. 2). Mir M7 pH 7* (■, □); *Mir M7 pH 5.8* (●, ○).

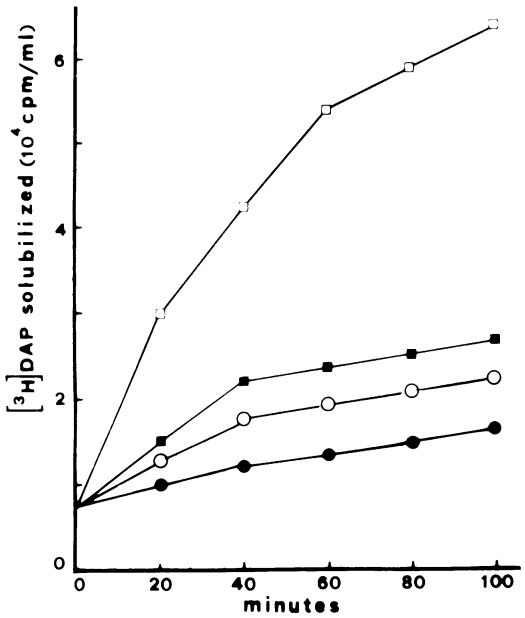

FIG. 5. *Peptidoglycan digestion by the cell wall hydrolases of K. pneumoniae strains Mir M7 grown in PL medium and in PL medium containing 0.1 mM EDTA at both pH 5.8 and 7. Radioactive cell walls were prepared from cells grown in the presence of [^{3}H]DAP as described in Material and Methods. A sample of wall from cells grown in PL medium (closed symbols) and a sample from cells grown in PL medium containing EDTA (open symbols) were resuspended in 10 mM Tris-hydrochloride buffer (pH 7) at a concentration of 0.5 mg/ml (2.5 × 10^6 cpm/ml) and incubated at 37°C. At intervals, 200-μl portions were placed in ice; sequentially 20 μl of an aqueous 5% solution of bovine serum albumin and 20 μl of a 50% trichloroacetic acid solution were added. After sedimentation of precipitates (2 min at 12,000 × g), radioactivity in 100 μl of supernatant was counted as described in Materials and Methods. Mir M7 pH 7* (■, □); *Mir M7 pH 5.8* (●, ○).

of Mir M7 cocci had a specific requirement for incorporation of divalent cations. We therefore questioned whether this peptidoglycan was still able to confer a certain degree of mechanical strength to the cell wall. We studied whether conditions of growth allowing the accumulation of divalent cations were necessary for the cocci to become as resistant to mechanical stress as the Mir M7 rods and Mir A12 cells. As shown in Table 6, Mir M7 cocci grown in PL medium neither lysed nor died when resuspended in distilled water and submitted to shearing blender treatment for 10 min. Furthermore, Mir M7 cocci appeared no more sensitive to freezing and thawing than rods and Mir A12 cells. On the contrary, cocci grown in the presence of EDTA were highly fragile, dying and lysing rapidly in distilled water and being rapidly killed by Waring blender treatment, freezing and thawing.

Finally, envelopes of Mir M7 cocci isolated by breaking the cells with glass beads were found to maintain cell shape (data not shown).

DISCUSSION

Round morphology can be assumed by rod-shaped bacteria either as a consequence of the loss of rigidity of the cell wall or of alteration(s) in the shape-determining mechanism.

The data presented in this paper clearly indicate that the envelope of Mir M7 cocci is rigid and has a defined shape. The fact that EDTA causes loss of shape and transformation into the amorphous material of the SDS-insoluble residues does not mean that the peptidoglycan of Mir M7 cocci is not rigid, but only that rigidity and mechanical strength, in this polymer, are due specifically to the presence of divalent cations. When Mir M7 cocci are grown in a medium containing EDTA to prevent accumulation of divalent cations in the peptidoglycan, this poly-

TABLE 5. *Characteristics of SDS-insoluble residues from K. pneumoniae Mir M7 cells grown in PL and in PL containing 0.1 mM EDTA (PL + EDTA) at pH 7 and 5.8*

pH	Medium	Cell shape	Divalent cations removed by EDTA (mmol/g, dry wt)[a]		Sensitivity to proteolytic enzymes[b]	Peptidoglycan purification
			Mg^{++}	Ca^{++}		
7	PL	Cocci	0.47	2.85	Resistant	Not possible
	PL + EDTA	Cocci	0.11	0.32	Sensitive	Possible
5.8	PL	Rods	0.06	0.06	Sensitive	Possible
	PL + EDTA	Rods	0.04	0.04	Sensitive	Possible

[a] SDS-insoluble residues were prepared as described in Materials and Methods. EDTA treatment was performed as described in Table 4.

[b] The sensitivity to proteolytic enzymes (trypsin and pronase) was tested as described in the legend to Fig. 2.

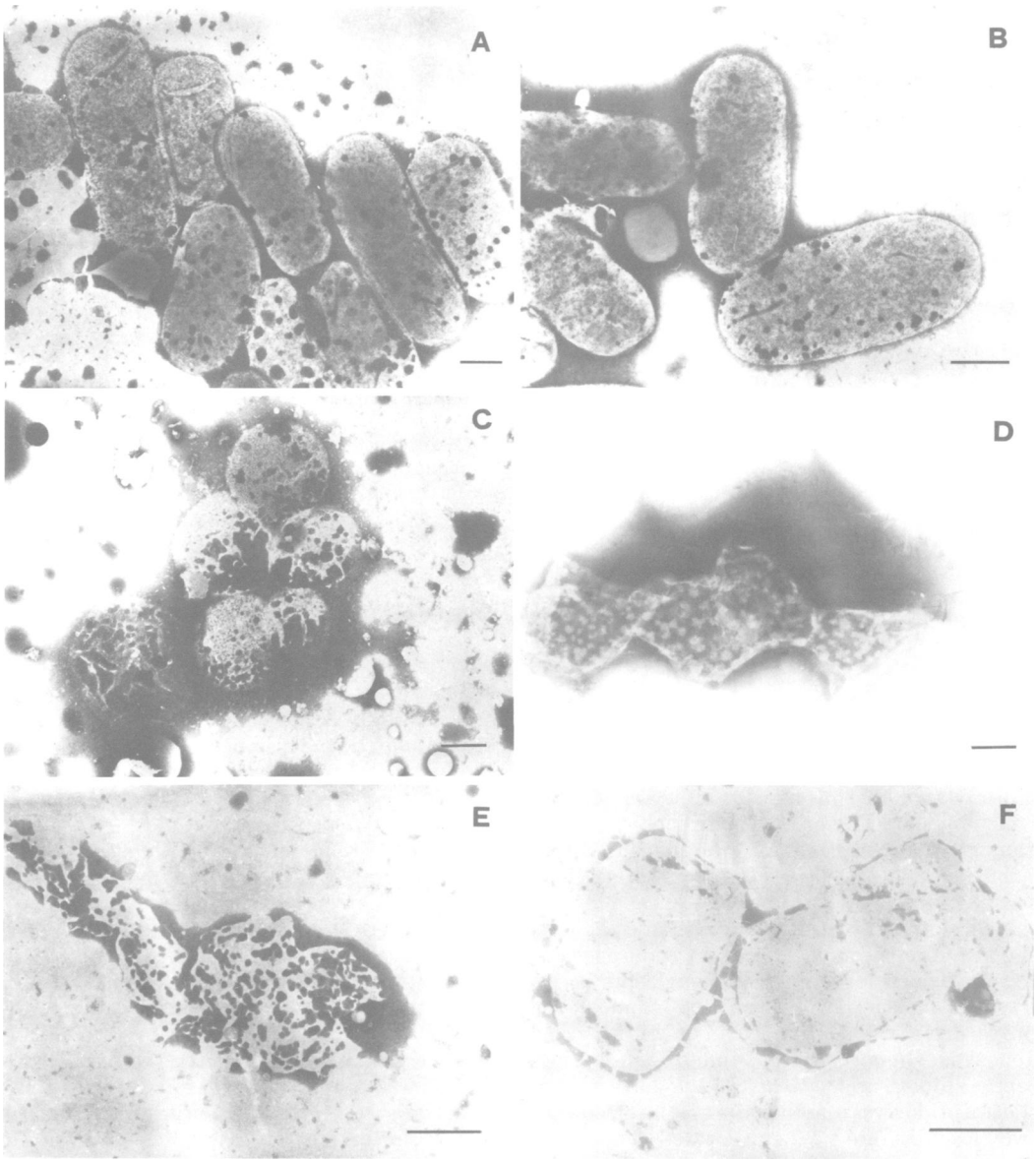

FIG. 6. *Electron micrographs of SDS-insoluble residues and purified peptidoglycan layers from K. pneumoniae strain Mir M7. SDS-insoluble residues and purified peptidoglycan were prepared as described in Materials and Methods and negatively stained as described. SDS-insoluble residues of Mir M7 rods grown in PL not containing EDTA (A) and containing EDTA (B). SDS-insoluble residues of Mir M7 cocci grown in PL not containing EDTA (C) and containing EDTA (D). Pure peptidoglycan of Mir M7 cocci (E) and rods (F). Bar = 0.5 μm.*

mer does not show defined shape or rigidity. Under such conditions cells grow significantly more slowly and are fragile and prone to autolysis.

It is interesting to note that in another gram-negative microorganism, a marine pseudomonad which contains an amount of peptidoglycan similar to that in Mir M7 cocci, divalent cations have been shown to be necessary in conferring upon the peptidoglycan the mechanical strength necessary for making the cell wall sufficiently rigid (10, 28). A model also has been presented to show the possible mechanism of interaction of Mg^{2+} with free amino and carboxyl groups of

TABLE 6. *Effect of osmotic and mechanical shocks on K. pneumoniae Mir M7 and Mir A12 grown in PL and in PL containing 0.1 mM EDTA (PL + EDTA) at pH 7 and 5.8*

Strain	pH	Medium	Cell shape	Treatment					
				Resuspension in distilled water[a]		Shearing in a Waring blender[b]		Freezing and thawing[c]	
				OD[d]	CFU[e]	OD[d]	CFU[d]	OD[d]	CFU[e]
Mir M7	7	PL	Cocci	16	87	42	45	45	39
		PL + EDTA	Cocci	40	22	95	0.07	92	0.5
	5.8	PL	Rods	5	95	25	57	30	51
		PL + EDTA	Rods	10	90	32	48	37	43
Mir A12	7	PL	Rods	5	100	20	61	18	58
		PL + EDTA	Rods	5	94	27	49	15	49
	5.8	PL	Rods	2	98	18	63	12	61
		PL + EDTA	Rods	2	94	20	58	21	48

[a] Exponentially growing cells were harvested by centrifugation, washed twice in distilled water, and resuspended in distilled water at a concentration of 1×10^8 cells/ml. After incubation at 0°C for 60 min, turbidity was measured at 550 nm with a Beckman DB spectrophotometer, and colony-forming units (CFU) were determined.

[b] Cells prepared as in *a* were sheared in a Waring blender for 10 min.

[c] Cells prepared as in *a* were frozen and thawed three times.

[d] Values indicate the percentage of reduction of initial turbidity.

[e] Values indicate the percentage of surviving cells calculated as CFU.

two adjacent peptide side chains (28). The same model may apply to the peptidoglycan of Mir M7 cocci.

The change in shape of Mir M7 cells grown at pH 7 appears much more likely to depend on an alteration in the shape-determining mechanism. This conclusion agrees with other data which indicate that the mechanism in strain Mir M7 which regulates the balance between lateral wall elongation and septation is altered; thus, at pH 7, lateral wall extension is prevented and only septa are made (35). These differences between the peptidoglycan of Mir M7 cocci and the rods should therefore indicate that, in gram-negative rods, the peptidoglycan of growing septa is different from that of the lateral wall. Some of the properties of the peptidoglycan of Mir M7 cocci appear to support this possibility. Autoradiography studies of *Escherichia coli* have shown that peptidoglycan precursors are inserted into two close discrete sites and located at the base of nascent septa (38). Therefore the septa and the zones around it are the portions of the peptidoglycan which grow at a faster rate and must contain a particularly high number of acceptor sites, which implies a reduced number of covalent bonds. It is possible that divalent cations are accumulated specifically in this portion to form linkages, thus increasing the rigidity of the polymer. It is also plausible that the polymer just inserted into the expanding part of the peptidoglycan is subjected to further handling and rearrangement before becoming the mature peptidoglycan of the cell. The possible handling and rearrangements could be facilitated in a polymer where some linkages are mediated by divalent cations instead of by covalent bonds. Another property of the Mir M7 cocci peptidoglycan which suggests that it is made up of septa only is its high alanine content. This defect could be due to the persistence of the terminal D-alanine in the peptides not involved in cross-linkages and could depend on damage to D-alanine-carboxypeptidase activity. It has been suggested by Mirelman et al. (23) that this enzyme plays an important role in the regulation of lateral wall extension and septum formation of bacterial rods.

Minicells are thought to consist only of the pole material of mother cells (13). It was recently found that their peptidoglycan does not differ from that of normal rods (13). This finding does not necessarily contradict the suggestion that the cell wall of Mir M7 cocci can be made of two bacterial poles. Minicells neither increase their surface nor divide, whereas the cell wall of Mir M7 cocci grows rapidly and is more likely to be made of newly formed septa ("new poles"). The properties of the peptidoglycan of Mir M7 could be typical of the still-expanding or just-completed septa. The finding that, in *Bacillus subtilis* the peptidoglycan of the new septa has properties different from those of old septa (7) supports this suggestion. The fact that the cell wall of Mir M7 cocci is made of two growing poles also explains the finding that the diameter of cocci was larger than that of the poles of the rods (Fig. 1). Based on studies on *Streptococcus faecalis*, Higgins and Shockman (17) and Daneo-Moore and Shockman (5) have suggested

that the increase in surface area of cocci is achieved through the extension of septa.

Interesting analogies exist between strain Mir M7 and another microorganism, *Arthrobacter crystallopoietes*, which is widely used for morphogenesis studies (6). During the transition from the rod to the coccal shape the *A. crystallopoietes* cells divide and increase in number without elongating, exactly as shown for strain Mir M7 (6). As for peptidoglycan composition, the transition to coccal shape in this species is accompanied by an increase of both D-alanine and *N*-acetylglucosamine and by substitution of diaminopimelic acid by lysine. It has been proposed that the substitution of a tetrafunctional amino acid like DAP with a trifunctional one (lysine) could be responsible for the change of shape of the peptidoglycan (26, 27). The first two alterations also have been found in Mir M7 cocci. Furthermore, it is possible that divalent cations in these cells mask one of the functional groups of DAP, making it act like a trifunctional amino acid. The masking of one of the DAP functional groups could then be responsible for the prevalence of septation over lateral wall elongation and could cause a transition to the coccal shape (35). In this view divalent cations, aside from conferring rigidity to the polymer, could also be important in regulating septal peptidoglycan formation.

The peptidoglycan composition of other coccal-shaped mutants of gram-negative and positive rods has already been described (3, 16, 18, 19, 20, 25, 30, 32). Among the mutants analyzed, most showed a normal peptidoglycan. Only some were found to involve changes in the peptidoglycan consisting of alterations in the percent of cross-linkages (18, 20). Coccal-shaped mutants requiring high Mg^{2+} levels for growth have been described recently in both *E. coli* (39) and *B. subtilis* (31). Therefore strain Mir M7, which already has peculiar genetic and physiological properties (33–37) also appears to have unique biochemical properties in its rigid layer of cocci.

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LITERATURE CITED

1. **Anton, D. N., and L. Orce.** 1976. Envelope mutation-promoting autolysis in *Salmonella typhimurium*. Mol. Gen. Genet. **144**:97–105.
2. **Boylan, R. J., and N. H. Mendelson.** 1969. Initial characterization of a temperature-sensitive rod⁻ mutant of *Bacillus subtilis*. J. Bacteriol. **100**:1316–1321.
3. **Boylan, R. J., N. H. Mendelson. D. Brooks, and F. E. Young.** 1972. Regulation of the bacterial cell wall: analysis of a mutant of *Bacillus subtilis* defective in biosynthesis of teichoic acid. J. Bacteriol. **110**:281–290.
4. **Braun, V., and K. Rehn.** 1969. Chemical characterization, spatial distribution and function of a lipoprotein (murein-lipoprotein) of *Escherichia coli* cell wall. The specific effect of trypsin on the membrane structure. Eur. J. Biochem. **10**:426–438.
5. **Daneo-Moore, L., and G. D. Shockman.** 1977. The bacterial cell surface in growth and division, p. 597–715. *In* G. D. Poste and G. Nicholson (ed.), Cell surface reviews, vol. 4. Elsevier-North Holland Publishing Co., Amsterdam.
6. **Ensign, J. C., and R. S. Wolfe.** 1964. Nutritional control of morphogenesis in *Arthrobacter crystallopoietes*. J. Bacteriol. **87**:925–932.
7. **Fan, D. P., M. C. Pelvit, and W. P. Cunningham.** 1972. Structural difference between walls from ends and sides of the rod-shaped bacterium *Bacillus subtilis*. J. Bacteriol. **109**:1266–1272.
8. **Fontana, R.** 1978. The chemical composition of the peptidoglycan of *Klebsiella pneumoniae* strain Mir A12 and ATCC 13883. FEMS Microbiol. Lett. **3**:77–80.
9. **Fordham, W. D., and C. Gilvarg.** 1974. Kinetics of cross-linking of peptidoglycan in *Bacillus megaterium*. J. Biol. Chem. **249**:2478–2482.
10. **Forsberg, C. W., M. K. Rayman, J. W. Costerton, and R. A. MacLeod.** 1972. Isolation, characterization, and ultrastructure of the peptidoglycan layer of a marine pseudomonad. J. Bacteriol. **109**:895–905.
11. **Fujiawara, T., and S. Fukui.** 1972. Isolation of morphological mutants of *Agrobacterium tumefaciens*. J. Bacteriol. **110**:743–746.
12. **Ghuysen, J. M., D. J. Tipper, and J. L. Strominger.** 1966. Enzymes that degrade bacterial cell walls. Methods Enzymol. **8**:685–699.
13. **Goodell, E. W., U. Schwarz, and R. M. Teather.** 1974. Cell envelope composition of *Escherichia coli* K12: a comparison of the cell poles and the lateral wall. Eur. J. Biochem. **47**:567–572.
14. **Gray, G. W., and S. G. Wilkinson.** 1965. The effect of ethylenediaminotetra-acetic acid on the cell walls of some gram-negative bacteria. J. Gen. Microbiol. **39**: 385–399.
15. **Hartmann, R., S. B. Bock-Hennig, and U. Schwarz.** 1974. Murein hydrolases in the envelope of *Escherichia coli*. Eur. J. Biochem. **41**:203–208.
16. **Henning, U., K. Rehn, V. Braun, B. Hohn, and U. Schwarz.** 1972. Cell envelope and shape of *Escherichia coli* K12. Properties of a temperature-sensitive *rod* mutant. Eur. J. Biochem. **26**:570–586.
17. **Higgins, M. L., and G. D. Shockman.** 1971. Procaryotic cell division with respect to wall and membranes. Crit. Rev. Microbiol. **1**:29–72.
18. **Kamiryo, T., and J. K. Strominger.** 1974. Penicillin-resistant temperature-sensitive mutants of *Escherichia coli* which synthesize hypo- or hyper-cross-linked peptidoglycan. J. Bacteriol. **117**:568–577.
19. **Lazdunski, C., and B. M. Shapiro.** 1972. Relationship between permeability, cell division, and murein metabolism in a mutant of *Escherichia coli*. J. Bacteriol. **111**: 499–509.
20. **Matsuhashi, S., T. Kamiryo, P. M. Blumberg, P. Linett, E. Willoughby, and J. L. Strominger.** 1974. Mechanism of action and development of resistance to a new amidino penicillin. J. Bacteriol. **117**:578–587.
21. **Matsuzawa, H., K. Hayakawa, T. Sato, and K. Imahori.** 1973. Characterization and genetic analysis of a mutant of *Escherichia coli* K-12 with rounded morphology. J. Bacteriol. **115**:436–442.
22. **Meloni, G. A., and C. Monti-Bragadin.** 1962. Osservazioni sull'isolamento dell'uomo di sferoplasti "spontanei" derivati da un ceppo di *Klebsiella*. Ann. Sclavo **4**: 143–152.

23. **Mirelman, D., Y. Yashouv-Gan, and U. Schwarz.** 1977. Regulation of murein biosynthesis and septum formation in filamentous cells of *Escherichia coli* PAT 84. J. Bacteriol. **129:**1593–1600.
24. **Normark, S.** 1969. Mutation in *Escherichia coli* K-12 mediating sphere-like envelopes and changed tolerance to ultraviolet irradiation and some antibiotics. J. Bacteriol. **98:**1274–1277.
25. **Olden, K., S. Ito, and T. H. Wilson.** 1975. D-alanine-requiring cell wall mutant of *Escherichia coli*. J. Bacteriol. **122:**1310–1321.
26. **Previc, E. P.** 1970. Biochemical determination of bacterial morphology and the geometry of cell division. J. Theor. Biol. **27:**471–497.
27. **Previc, E. P., and N. Lowell.** 1975. Peptidoglycan composition of a new strain of *Arthrobacter crystallopoietes* during sphere-rod morphogenesis. Biochim. Biophys. Acta **411:**377–385.
28. **Rayman, M. K., and R. A. MacLeod.** 1975. Interaction of Mg^{2+} with peptidoglycan and its relation to the prevention of lysis of a marine pseudomonad. J. Bacteriol. **122:**650–659.
29. **Rogers, H. J., M. McConnel, and I. D. J. Burdett.** 1970. The isolation and characterization of mutants of *Bacillus subtilis* and *Bacillus licheniformis* with disturbed morphology and cell division. J. Gen. Microbiol. **61:**155–171.
30. **Rogers, H. J., M. McConnel, and R. C. Hughes.** 1971. The chemistry of the cell walls of *rod* mutants of *Bacillus subtilis*. J. Gen. Microbiol. **66:**297–308.
31. **Rogers, H. J., and P. F. Thurman.** 1978. Temperature-sensitive nature of the *rodB* mutation in *Bacillus subtilis*. J. Bacteriol. **133:**298–305.
32. **Rogers, H. J., P. F. Thurman, C. Taylor, and J. N. Reeve.** 1974. Mucopeptide synthesis by *rod* mutants of *Bacillus subtilis*. J. Gen. Microbiol. **85:**335–350.
33. **Satta, G., and R. Fontana.** 1974. Characterization of a conditional mutant with altered envelope showing pH-dependent morphology and temperature-dependent division. J. Gen. Microbiol. **80:**51–63.
34. **Satta, G., and R. Fontana.** 1974. Cell division, macromolecular synthesis and morphology dependent on the state of the envelope in a mutant of *Klebsiella pneumoniae*. J. Gen. Microbiol. **80:**65–75.
35. **Satta, G., R. Fontana, P. Canepari, and G. Botta.** 1979. Peptidoglycan synthesis in cocci and rods of a pH dependent morphologically conditional mutant of *Klebsiella pneumoniae*. J. Bacteriol. **137:**727–734.
36. **Satta, G., C. Pruzzo, E. Debbia, and R. Fontana.** 1978. Close association between shape alteration and loss of immunity to superinfection in a wild-type *Klebsiella pneumoniae* stable lysogen which can be both immune and nonimmune to superinfection. J. Virol. **28:**772–785.
37. **Satta, G., G. C. Schito, and G. A. Meloni.** 1969. Transizione bastoncinosfera in un ceppo di *Klebsiella pneumoniae*. Ultrastruttura delle forme coccoidi tipiche ed abnormi, p. 247–253. XV Congresso Nazionale Microbiologia. Torino- Saint Vincent.
38. **Schwarz, U., A. Ryter, A. Rambach, R. Hellio, and Y. Hirota.** 1975. Process of cellular division in *Escherichia coli*: differentiation of growth zones in the sacculus. J. Mol. Biol. **98:**749–759.
39. **Sonntag, I., H. Schwarz, Y. Hirota, and U. Henning.** 1978. Cell envelope and shape of *Escherichia coli*: multiple mutants missing the outer membrane lipoprotein and other major outer membrane proteins. J. Bacteriol. **136:**280–285.
40. **Spratt, B. G.** 1975. Distinct penicillin-binding proteins involved in the division, elongation, and shape of *Escherichia coli*. Proc. Natl. Acad. Sci. U.S.A. **74:**2999–3003.
41. **Spratt, B. G., and A. B. Pardee.** 1975. Penicillin binding proteins and cell shape in *E. coli*. Nature (London) **254:**516–517.