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Biochemical and Cytochemical Evidence for the Polar Concentration of Periplasmic Enzymes in a "Minicell" Strain of *Escherichia coli*

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A number of "surface" enzymes of *Escherichia coli* (i.e., among those selectively released by osmotic shock) all displayed higher specific activities in extracts of minicells than in extracts of typical rod forms; these enzymes included alkaline phosphatase, cyclic phosphodiesterase, acid hexose monophosphatase, 5'-nucleotidase, and ribonuclease I. In addition, alkaline phosphatase, cyclic phosphodiesterase, and acid hexose monophosphatase were cytochemically localized to regions of minicell periplasm that resembled reactive polar enlargements of the periplasm in rod forms. In contrast, a number of "internal" cytoplasmic enzymes (inorganic pyrophosphatase, β-galactosidase, glutamine synthetase, polynucleotide phosphorylase, and ribonuclease II) showed elevated or similar specific activities in extracts of rod forms versus extracts of minicells. A specific heat-labile inhibitor for 5'-nucleotidase, known to occur in the cytoplasm, also showed no enrichment in minicells. These findings indicate that the "surface" enzymes are segregated in vivo into the terminal minicell buds, possibly because these enzymes are concentrated in the polar enlargements of the periplasm in typical rod forms.

Evidence has been reviewed that *Escherichia coli* contains a group of enzymes which occupy a peripheral location in the cell and yet do not seem firmly bound to the cell envelope (10, 11, 30). Our previous cytochemical studies (30) affirmed the localization of several phosphatases in this group external to the cytoplasmic membrane (5, 15, 21, 27, 28); we have also demonstrated an unusual concentration of these enzymes in polar caplike enlargements of the periplasmic space. An opportunity to confirm this polar localization by an independent method arose with the discovery of a "minicell" mutant of *E. coli* (1) which buds small spherical bodies, termed minicells, from the poles of the cell throughout the growth cycle.

This study shows the enrichment of five of these "surface" enzymes in minicells, in comparison with five "internal" or cytoplasmic, enzymes which show similar specific activities in extracts of minicells and of rod forms. In addition, cytochemical methods applied to minicells demonstrate three of these surface phosphatases in enlarged regions of periplasmic space that resemble reactive polar caps of rod forms.

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MATERIALS AND METHODS

Bacteria and growth media. The minicell-producing strain P 678-54 was kindly supplied by H. I. Adler and was maintained on nutrient agar slants. Cells were grown to mid-exponential or stationary phase in the medium of Fraser and Jerrell (7) which contains (per liter): 10.5 g of Na_2HPO_4, 4.5 g of KH_2PO_4, 1 g of NH_4Cl, 0.3 g of $MgSO_4$, 15 g of Casamino Acids (Difco), 30 g of glycerol, 1 ml of 1% gelatin, and 0.3 ml of 1 M $CaCl_2$. Cells grown in this medium could be tested for 5'-nucleotidase and acid hexose phosphatase without interference by alkaline phosphatase, the formation of which was repressed by the high concentration of inorganic phosphate. The synthesis of alkaline phosphatase could be induced by maintaining the cells on the following medium (8), low in inorganic phosphate: 0.12 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride, 0.08 M NaCl, 0.02 M KCl, 0.02 M NH_4Cl, 0.003 M Na_2SO_4, 0.001 M $MgCl_2$, 2×10^{-4} M $CaCl_2$, 2×10^{-6} M $ZnCl_2$, and 0.5% bactopeptone (Difco), adjusted to *p*H 7.5. Induction of β-galactosidase was carried out

by addition of isopropyl thio-β-galactoside to the growth medium at a final concentration of 1 mM.

Separation of minicells from rod forms. Cultures of the minicell strain P-678-54 were centrifuged, washed with 0.15 M NaCl and weighed. The pellet was resuspended in 0.15 M NaCl, and a sample was removed for study. The remainder (1.5 g, wet weight) was diluted to 200 ml in 0.15 M NaCl and centrifuged for 10 min at 1,935 × *g* in the SS34 head of the RC-2 Sorvall centrifuge. Further treatment of supernatant fluid and pellet was as follows. The supernatant fraction, which contained most of the minicells was divided into 40-ml portions and centrifuged twice more at 1,935 × *g* for 10 min. Each time the sedimented material was rejected, thereby removing the contaminating rod-forms. Finally, the minicells were collected by centrifuging for 15 min at 14,500 × *g*. The original pellet was resuspended in 0.15 M NaCl and centrifuged for 3 min at 1,935 × *g*. This procedure was repeated, and the pellet of rod-shaped cells was finally suspended in a small volume of 0.15 M NaCl.

Enzyme assays. Sonically treated extracts were made by treatment of bacterial suspensions for three 30-sec intervals with a Branson Sonifier, model LS75. The few remaining unbroken cells and cell fragments were removed from the sonically treated extracts by centrifugation for 10 min at 9,000 × *g*. With the surface enzymes, values for specific activity were the same whether sonically treated extracts were centrifuged or not. Assays for cyclic phosphodiesterase (20, 22), acid hexose phosphatase (20), β-galactosidase (20), inorganic pyrophosphatase (2), glutamine synthetase (14), 5′-nucleotidase inhibitor (6), ribonuclease (19), and alkaline phosphatase (20) have been published. Protein was determined by the method of Lowry et al. (16).

Electron microscopy. For electron microscopy, whole cultures and minicell concentrates were washed finally with 0.15 M NaCl containing 5% bovine fibrinogen (Armour Pharmaceutical Co., Chicago, Ill.) and were centrifuged once more. After removing the supernatant fluid, one drop of 1% thrombin (Mann Research Laboratories, New York, N.Y.) was layered on each pellet for 10 min at room temperature. Clotted pellets for cytochemical studies were fixed for 90 min in 6% glutaraldehyde (24) and 40-μm cryostat sections were prepared and incubated for 90 min at 37 C in various cytochemical media as described elsewhere (30). The reaction mixtures were as follows: cyclic phosphodiesterase—2 ml of 0.1 M uridine 2′,3′-cyclic phosphate, 8 ml of 0.2 M Tris-maleate buffer (*p*H 6), 1.2 ml of 2% Pb $(NO_3)_2$, 0.2 ml of 0.1 M $CoCl_2$, 0.5 ml of 0.05 M $MgCl_2$, and 7.8 ml of distilled water; alkaline phosphatase—8 ml of 0.2 M Tris-hydrochloride (*p*H 9), 8 ml of 0.05 M ethanolamine phosphate, 1.2 ml of 2% $Pb(NO_3)_2$, 0.5 ml of 0.05 M $MgSO_4$, and 2.8 ml of distilled water; acid hexose phosphatase—8 ml of 0.005 M glucose 6-phosphate, 8 ml of 0.2 M Tris-maleate (*p*H 6), 1.2 ml of 2% $Pb(NO_3)_2$, and 2.8 ml of distilled water. Cytochemical specimens were then postfixed in osmium tetroxide (18).

Clotted pellets destined for morphological study were fixed overnight in sodium Veronal-buffered osmium tetroxide at room temperature (13) or for 90 min in 6.25% glutaraldehyde (24), followed by 45 min in osmium tetroxide (18), both at 4 C. They were stained en bloc overnight in 0.5% uranyl acetate (13).

All tissue samples were dehydrated in ethyl alcohol and embedded in Maraglas (25). Some ultrathin sections were stained with a sequence of uranyl acetate (29) and lead citrate (Venable, 26). The tissue was examined with an RCA-EMU3G electron microscope.

RESULTS

Specific activities of various enzymes in minicells and rod forms. In Table 1 we have recorded the specific activities of a number of enzymes in extracts of whole cultures and of separated minicells and rod forms. The rod forms presumably included cells before and after minicells had budded from their poles. Preparations of separated minicells and rod forms were routinely stained with Safranine O and examined under oil immersion to assure freedom from cross-contamination (1).

On the basis of previous evidence, the enzymes assayed in this study were categorized either as "internal" cytoplasmic enzymes or as "surface" enzymes that seem to occur outside the plasma membrane. For example, inorganic pyrophosphatase and β-galactosidase were previously identified among the internal enzymes which remain within the cell after osmotic shock or spheroplast formation with lysozyme and ethylenediaminetetraacetate (EDTA) (12, 17, 20); moreover, β-galactosidase hydrolysis of *o*-nitrophenyl-*p*-galactoside proceeds at a negligible rate in intact cells unless a permease is present to enable the substrate to penetrate the plasma membrane (23). The localization of glutamine synthetase has been less thoroughly studied, but presumably it accompanies other synthetic enzymes which, so far, have all been localized within the cytoplasm (10). These three internal enzymes each showed similar or higher specific activities in extracts of rod forms, as compared with extracts of minicells (Table 1). Preliminary studies on two other internal enzymes, polynucleotide phosphorylase and ribonuclease II (10), also demonstrated equivalent specific activities in rod forms and minicells. Thus, all five presumably cytoplasmic enzymes examined in this study showed similar or reduced activity in extracts of minicells versus rod forms.

In contrast, extracts of minicells displayed higher specific activities than extracts of rod forms when assayed for surface or periplasmic enzymes such as alkaline phosphatase, 2′,3′-cyclic phosphodiesterase, acid hexose monophosphatase, and 5′-nucleotidase (Table 1). These

TABLE 1. *Specific activities of various enzymes in minicells and rod-shaped cells of Escherichia coli P-678-54*[a]

Type cell	Cell fraction	5'Nucleotidase[b]	Inhibitor[c]	Acid hexose phosphatase	Alkaline phosphatase	Cyclic phosphodiesterase	β-Galactosidase	Glutamine synthetase	Inorganic pyrophosphatase	Ribonuclease I
Stationary	Whole culture				0.088	0.105	3.15	0.025	7.00	71
	Separated rods				0.081	0.087	3.5	0.028	7.00	72
	Minicells				0.132	0.202	2.5	0.019	6.83	52
	Ratio of minicells to, rod forms				1.6	2.3	0.7	0.7	1.0	0.7
Exponential	Whole culture	0.32		0.0145		0.127	1.95	0.060	7.17	44
	Separated rods	0.19	2.67	0.0062		0.095	2.0	0.061	7.17	24
	Minicells	0.44	0.025	0.0217		0.140	1.4	0.043	7.33	37
	Ratio of minicells to rod forms	2.3	0.009	3.5		1.5	0.7	0.7	1.0	1.5

[a] Values are expressed as units per milligram. The minicell-producing strain of *E. coli*, P-678-54, was grown to stationary phase in the low-phosphate medium of Garen and Levinthal (8) and was harvested and washed with 0.15 M NaCl. After separation of minicells and rod forms by differential centrifugation, suspensions were made in 0.15 M NaCl and sonically treated extracts were prepared. Enzyme assays are described in Materials and Methods. Cells grown in the high-phosphate medium of Fraser and Jerrel (7) were harvested in mid-exponential phase and were similarly treated. Data are presented for extracts of the whole culture before fractionation, for extracts of the rod forms separated from minicells, and for extracts of the minicell fraction. The ratios of specific activities of minicells divided by those of separated rod forms are also given, Enzyme activity is reported in international units (1 IU = 1 μmole/min at 37 C), except in the cases of β-galactosidase, glutamine synthetase, and ribonuclease I for which the units are defined as in previous studies (*see* references 20, 14, and 19, respectively).

[b] With whole cultures and separated rods, the extracts were first activated by heating; this destroys the 5'-nucleotidase inhibitor.

[c] Heat-labile protein that specifically inhibits 5'-nucleotidase.

enzymes have all been identified among those that are selectively released from *E. coli* by osmotic shock or by spheroplast formation with lysozyme and EDTA (10). In other experiments, cyclic phosphodiesterase and 5'-nucleotidase were studied in cells harvested during early exponential growth, and the minicell extracts showed even greater (10-fold) specific activity than extracts of rod forms ribonuclease I was another surface hydrolase (20) which showed enrichment in minicells harvested during exponential growth (Table 1). Thus five presumed periplasmic enzymes displayed higher specific activities in minicell extracts than in extracts of rod forms.

The distribution of the specific heat-labile 5'-nucleotidase inhibitor (6, 9) between minicells and rod forms was especially interesting. This inhibitor has been identified as an internal protein that is not released by osmotic shock (6), whereas 5'-nucleotidase has appeared as a surface enzyme (22); the specific inhibitor was confined to the rod forms, whereas the enzyme was enriched in the minicells.

Release of enzymes by osmotic shock. When subjected to osmotic shock (22), the minicells behaved much like the rod forms. Release of 5'-nucleotidase, a surface enzyme, was quantitatively complete from both rods and minicells; similarly, osmotic shock released 95% of the measurable cyclic phosphodiesterase activity from rod forms and 86% of this activity from minicells. On the other hand, release of an internal enzyme, inorganic pyrophosphatase, was below the limits of detection (under 0.5%) for both rod forms and minicells. With uridine 2',3'-cyclic phosphate as substrate, the ratio of activity expressed by intact cells over that of an equivalent sonically treated extract was 70% for both minicells and rod forms. This indicates that a similar "wall barrier" to the penetration of substrate exists in both forms.

Electron microscopy and cytochemistry. As previously described by Adler and co-workers (1), cultures of this strain comprised typical rod-shaped cells (Fig. 1) and numerous small, round profiles which generally lacked nuclear material and mostly represented minicell buds (Fig. 2, 3); in addition, a small number (<1%) of extremely long (more than 15 μm) filaments were evident. In our preparations, the rods all showed polar enlargements of the periplasmic space between the cell wall and the plasma membrane (Fig. 1, 4).

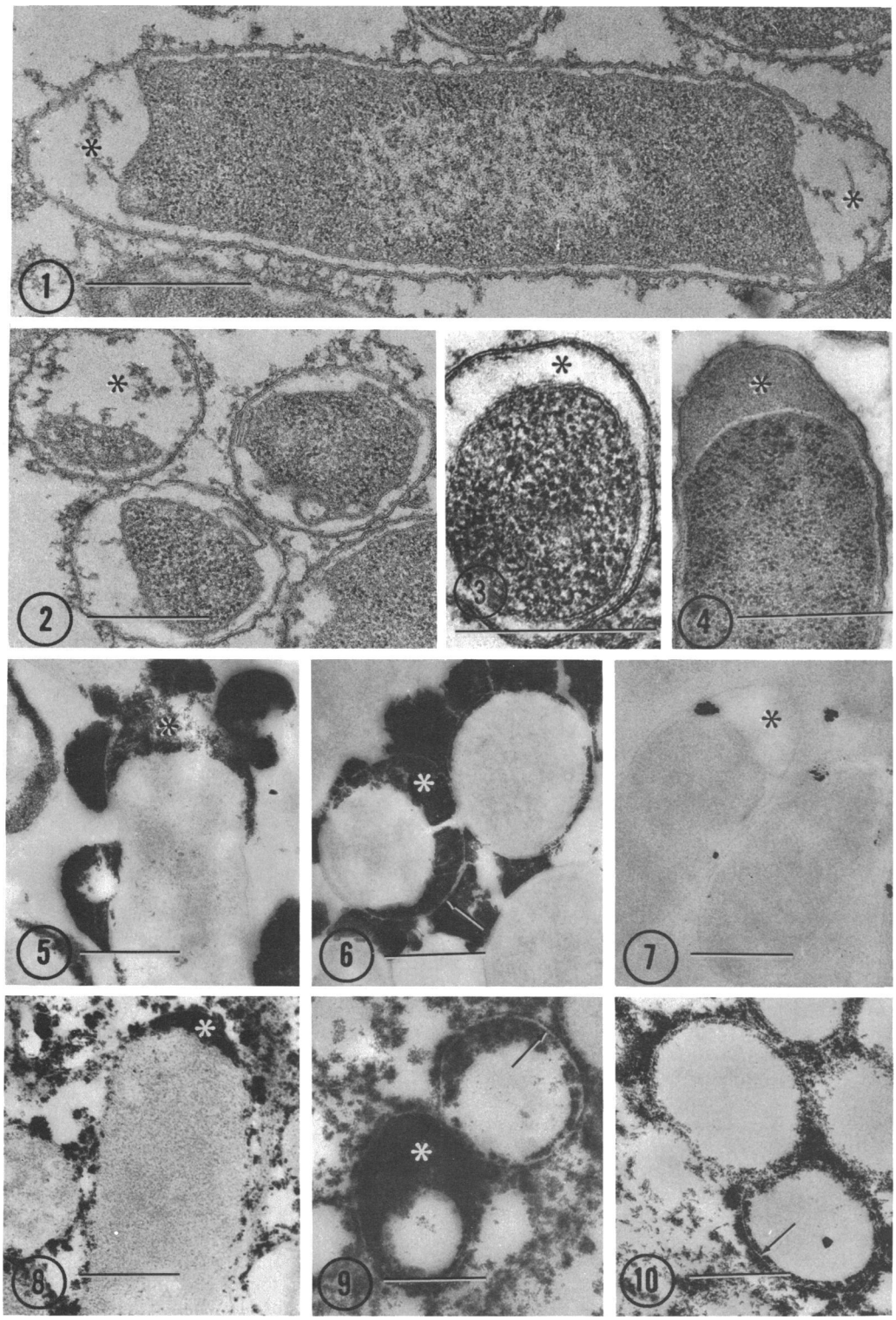

FIG. 1–10

Such polar caps have been found in all other strains of *E. coli* examined in this laboratory (C-90, U-7, and K-37; *see* reference 30); this configuration has often been ascribed to plasmolytic shrinkage of the protoplast in hypertonic media (e.g., reference 4). Small indentations of the lateral plasma membrane were also seen occasionally. In some samples, a homogeneous or flocculent substance of moderate density filled the periplasmic space of most cells (Fig. 4), but this compartment often appeared structureless and electron lucent. The purified minicell fractions were virtually free of rod forms, and minicells could thus be identified with certainty. Most minicells in purified fractions and the corresponding population of presumed minicell profiles in whole cultures displayed an enlarged periplasmic space which resembled the polar caps of the rod forms.

Reaction product indicative of alkaline phosphatase was evident in the periplasmic space and along the surfaces of cells induced for this enzyme (on low-phosphate media). The staining appeared to be concentrated in the prominent polar caps of the rod forms (Fig. 5) and in the similarly enlarged periplasmic space of the minicells (Fig. 6). The protoplasts were generally free of precipitate. Cells whose alkaline phosphatase had been repressed by growth on high-phosphate medium appeared unreactive with this procedure (Fig. 7). Cells induced for alkaline phosphatase and incubated at *p*H 6.7 with uridine 2′,3′-cyclic phosphate as substrate again showed reaction product concentrated in enlargements of the periplasmic space and scattered on the cell surface (Fig. 8, 9); much weaker, less-consistent staining was observed in repressed cells incubated in this manner for cyclic phosphodiesterase, suggesting that alkaline phosphatase is responsible for much of the reaction product (by hydrolysis of the phosphomonoester intermediate). Repressed cells incubated for acid hexose monophosphatase activity also showed periplasmic and surface staining in both minicells (Fig. 10) and rod forms. In all cytochemical preparations, a very sharp staining gradient was observed and only the bacteria along the surfaces of the cryostat sections were consistently reactive.

DISCUSSION

The examination of purified minicells now enables one to study the properties of the polar regions of *E. coli* in comparison with the entire cell. Cohen et al. (3) summarized results from their laboratory (1) and from Hurwitz and Gold (*unpublished data*) which show that many cytoplasmic constituents occur in similar concentrations in rod forms and minicells (ribonucleic acid methylase and deoxyribonucleic acid polymerase) or are absent from the minicells (ribonucleic acid

FIG. 1–10. *Electron micrographs of thin sections of fibrin-enmeshed pellets of Escherichia coli. Scale markers represent approximately 0.5 μm. (Fig. 1) Typical rod form from an unfractionated minicell culture. The protoplast is indented at each pole, producing terminal enlargements (*) of the periplasmic space (between the cell wall and the plasma membrane). These polar caps are continuous with the narrower lateral portions of this compartment. Kellenberger-Ryter fixation; thin section stained. × 49,000. (Fig. 2) Three probable minicells from an unfractionated culture. Such profiles are extremely numerous in sections of unfractionated cultures and are representative of minicells seen in purified fractions. Note the large volume of periplasmic space, comparable to the polar regions of rod forms. The outer contour of the minicell remains circular and the protoplast appears flattened or indented. The contents of the periplasmic space in minicells resemble the contents of polar caps in rod forms of the same specimen (compare Fig. 1). Kellenberger-Ryter fixation; thin section stained. × 36,000. (Fig. 3) As in Fig. 2. The membrane surrounding the protoplast and constituting the inner boundary of the periplasmic space can be discerned (*). × 60,000. (Fig. 4) Portion of a rod form from a minicell culture. In some specimens, a homogeneous substance of moderate density is evident in the periplasmic space of the cells and is especially prominent in the polar caps and in the enlarged periplasmic space of minicells. In this case, the plasma membrane displays a relatively low density and appears as a negative image. Glutaraldehyde-osmium tetroxide fixation; thin section stained. × 46,000. (Fig. 5) Rod form from minicell culture. Alkaline phosphatase cytochemistry. A granular precipitate occupies most of the polar cap (*) and large, discrete crystalline deposits are seen at several points along the periphery of the bacterium. No precipitate is seen in the protoplast. Thin section unstained. × 30,000. (Fig. 6) Minicells from a purified fraction. Alkaline phosphatase cytochemistry. Precipitate is seen within the periplasmic space and along the outer surfaces of two minicells. The cell wall often appears in negative image (e.g., arrow). Thin section unstained. × 20,000. (Fig. 7) Portion of a rod form from a whole minicell culture grown on high-phosphate medium. Alkaline phosphatase cytochemistry. Precipitate is very sparse in these repressed cells and the polar caps (*) are unreactive. Thin section unstained. × 30,000. (Fig. 8) Rod form cell from an unfractionated minicell culture. Cyclic phosphodiesterase cytochemistry. Precipitate is found at the cell surfaces and in the periplasmic space. Note the reaction product in the polar cap (*). Thin section unstained. × 30,000. (Fig. 9) Two minicells from a purified fraction. Cyclic phosphodiesterase cytochemistry. Reaction product appears in the enlarged periplasmic space of the minicells and on the surfaces of the cells. The cell walls of these cells are often seen in negative image (arrow). Thin section unstained. × 30,000. (Fig. 10) Minicells from a purified fraction. Hexose-6-monophosphatase cytochemistry. The appearance of the cell wall in negative image (arrow) delineates reaction product in the periplasmic space of these cells; considerable surface staining is also present. Thin section unstained. × 20,000.*

polymerase and deoxyribonucleic acid methylase). Our investigation establishes that five more cytoplasmic enzymes occur in both rod forms and minicells and that three of these are concentrated in the rod forms. In contrast, the so-called surface enzymes, alkaline phosphatase, cyclic phosphodiesterase, 5'-nucleotidase, and acid hexose monophosphatase, all show higher specific activities in the minicells than in the rod forms from which they budded. An even distribution of these enzymes along the bacterial envelope, either as a constituent of the cell wall or in a uniform periplasmic layer, would not account for their enrichment in the minicells. Instead, these results indicate the segregation of these enzymes in vivo into the terminal minicell buds, as though these enzymes had been concentrated in the polar regions of the rod forms.

The three of these surface enzymes which were examined cytochemically indeed appeared concentrated both in the polar caps of rod-forms [*see* Wetzel et al. (30)] and in the similarly enlarged periplasmic space of the minicells. Comparable cytoplasmic shrinkage (plasmolysis) of rods and minicells during tissue preparation would not account for the disproportionately large periplasmic space seen in minicells. The direct incorporation of preexisting polar caps into minicells as they bud would simply and plausibly explain the commensurate distribution of enzymes and periplasmic space observed in these sites.

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