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Distribution of Alkaline Phosphatase Within the Periplasmic Space of Gram-Negative Bacteria

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Both reaction-product localization and ferritin-coupled antibody studies have shown that alkaline phosphatase is evenly distributed throughout the periplasmic space of *Escherichia coli* and a marine pseudomonad. This space is not locally enlarged except in cases where plasmolysis has occurred.

Biochemical studies have established that a number of degradative enzymes are located in the area outside the cytoplasmic membrane in the gram-negative bacterial cell (1, 7, 8). Cytochemical techniques have been used to show that these enzymes are found either at the cell surface (4, 12) or in the space between the cytoplasmic membrane and the double-track layer of the cell wall (2, 10). The recently established equivalence of the double-track layer of the cell wall with the "molecular sieve" (5; and Forge, Costerton, and Kerr, *unpublished observations*), which was originally proposed by Mitchell (11), confirms the latter's suggestion that the gram-negative cell is surrounded by a discrete periplasmic space which contains many of the degradative enzymes of the cell. More recently, several workers have turned their attention to the distribution of these enzymes within this space (6, 15) and to the association of the enzymes with structural components of the cell wall (4).

Cheng et al. (4) have established that alkaline phosphatase is associated with a structural cell wall component which is present both inside and outside the double-track layer of the cell wall, and further studies have indicated that this component is lipopolysaccharide. Cheng et al. (2) have concluded that this enzyme is evenly distributed in the periplasmic space or on the cell surface of *Pseudomonas aeruginosa*, and an even distribution of alkaline phosphatase and of 5'-nucleotidase has also been noted in *Escherichia coli* (10, 12).

On the other hand, Heppel has concluded that periplasmic enzymes are not tightly bound to structural components of the cell wall (8), and Wetzel et al. (15) have reported that these enzymes are concentrated in "polar caps" where the periplasmic space is radically enlarged by a separation of the cytoplasmic membrane from the inner aspect of the cell wall. These authors have proposed that polar caps containing concentrations of periplasmic enzymes are present in live cells, and Dvorak et al. (6) have suggested that "minicells" are especially well endowed with these enzymes because they are formed preferentially from the polar regions of the cell.

It is clear that the pattern of cytochemical localization of enzymes by reaction-product deposition is heavily dependent on the technique used. Cheng et al. (2) have shown that glutaraldehyde fixation causes an apparent shift of alkaline phosphatase from the periplasmic space of *P. aeruginosa* to the cell surface, whereas Wetzel et al. (15) noted a similar shift after Formalin fixation or the use of Ca^{2+} as a "capture reagent." Wetzel et al. (15) also found that glutaraldehyde fixation "drastically impaired" the activity of alkaline phosphatase, but they found that this fixative retained the perplasmic location of the enzyme when Pb^{2+} was used as a capture reagent.

Because of this variability and because exposure of cells to such stresses as suspension in $MgSO_4$ may cause a shift (L. Thompson, M.Sc. thesis, McGill University, 1972) or a release (3) of the enzyme, we have correlated cytochemical localization with enzyme release data. We have found excellent correlation with this data when we have used unfixed cells with Ca^{2+} as a capture reagent, in both *P. aeruginosa* (2, 4) and the marine pseudomonad (L. Thompson, M.Sc. thesis, McGill University, 1972). In 298 preparations of cells of the above species, of *E. coli*, and of various strains of *Salmonella* in which alkaline phosphatase-3'-

nucleotidase and 5'-nucleotidase have been localized by reaction-product precipitation, we have seen an even distribution of the enzyme activity (Fig. 1), and we have never observed polar caps containing concentrations of the reaction product. Even in rare instances where random plasmolysis has caused a separation of the cytoplasmic membrane from the cell wall (Fig. 2), we have never seen a concentration of enzyme activity in the distended periplasmic space.

We have used the ferritin-coupled antibody technique to localize the alkaline phosphatase of *E. coli* because the basis of this method is very different from reaction-product localization. In this method, the enzyme is localized by its antigenic reaction with specific ferritin-coupled antibody after the cells had been induced to produce alkaline phosphatase, fixed in glutaraldehyde, embedded in glycol methacrylate, and sectioned for electron microscopy. The fixative is the same as that used by Wetzel et al. (15), and localization does not depend on the precipitation of a diffusable reaction product but on the antigenic reaction of the actual enzyme molecule in the site where it has been immobilized by fixation. These preparations show an even distribution of the enzyme at the periphery of the cells (Fig. 3) and no sign of polar concentrations. Control preparations, in which uninduced cells were exposed to the ferritin-coupled antibody to alkaline phosphatase, were negative (Fig. 4). The activity in the cytoplasm of the reactive cells (Fig. 3) is attributed to the presence of enzyme subunits (13).

Thus both the ferritin-coupled antibody technique and reaction-product localization, in our hands, indicate that the alkaline phosphatase of *E. coli* and of several other gram-negative organisms, is evenly distributed within the periplasmic space or over the surface of the cell (Fig. 5). But this fails to explain why polar caps with concentrated enzyme activity were seen in *E. coli* in the very careful studies of Wetzel et al. (15). Thompson et al. (14) have shown that cells of the marine pseudomonad (B16) become plasmolyzed when suspended in a distinctly hypotonic (0.05 M) solution of $MgSO_4$ because of membrane collapse due to a lack of other cations, and Knowles (9) has reported that cells of *E. coli* are plasmolyzed in 0.15 M NaCl. Since Wetzel et al. (15) washed their cells three times in a buffer containing 0.15 M NaCl, before fixation and incubation with the reaction mixture, it is likely that the cells were plasmolyzed. It is also possible that the periplasmic alkaline phosphatase had

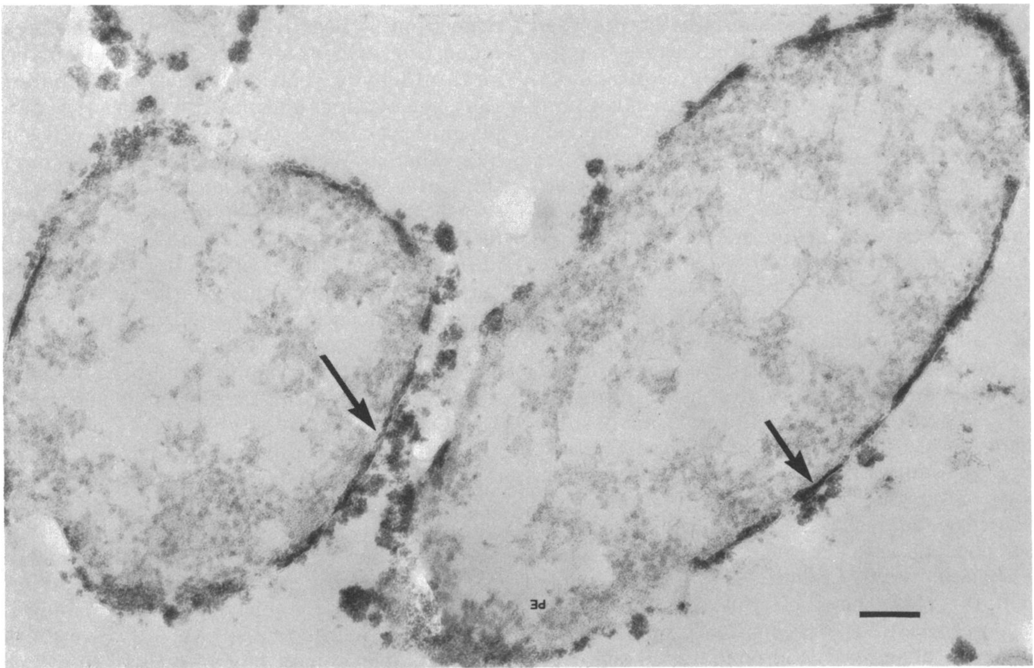

FIG. 1. *Reaction-product localization of alkaline phosphatase in cells of the marine pseudomonad (B16) showing the even distribution of the enzyme which is largely localized in the periplasmic space (arrows). The bar indicates 0.1 μm.*

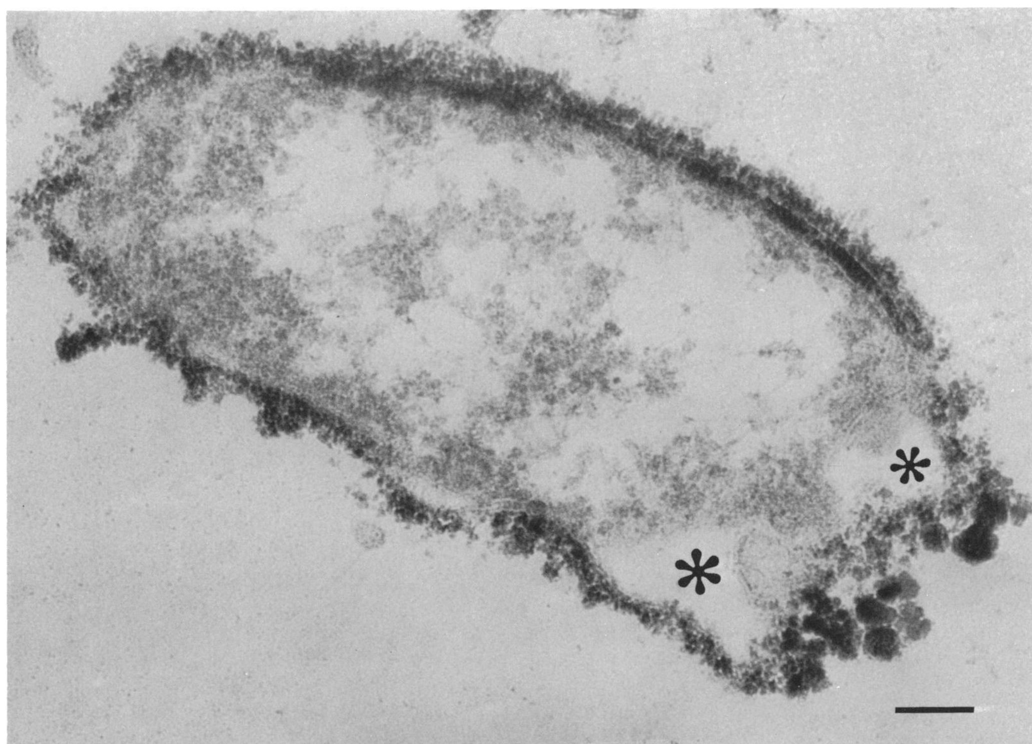

FIG. 2. *One of the very rare accidentally plasmolyzed cells from the same preparation showing the lack of reaction product in the areas (*) where the periplasmic space has been fortuitously enlarged. Bar indicates 0.1 μm.*

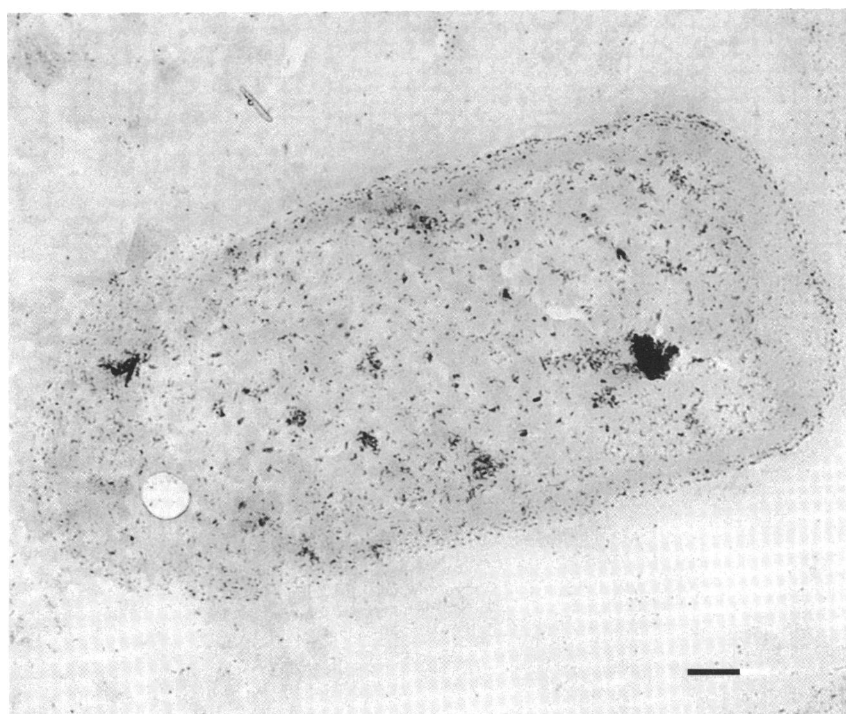

FIG. 3. *Localization of the alkaline phosphatase of Escherichia coli by the use of specific ferritin-coupled antibody. Note the even distribution of the enzyme within the cell envelope. The positive reaction in the cytoplasm is attributed to the presence of enzyme subunits. Bar indicates 0.1 μm.*

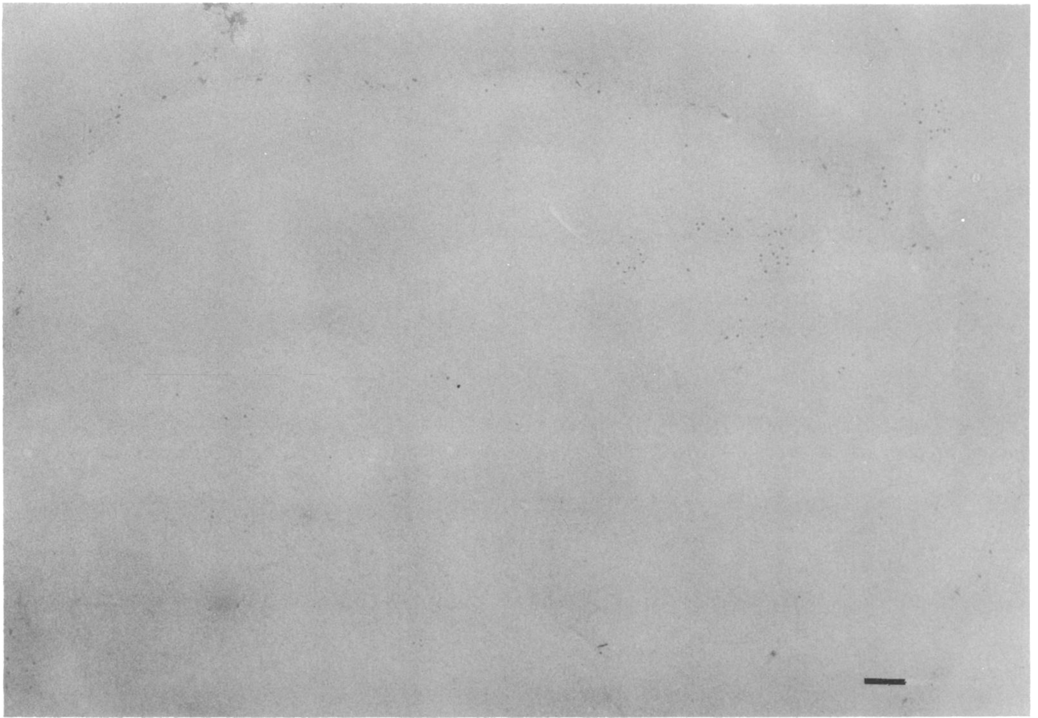

FIG. 4. *A control preparation showing a section of an uninduced cell which had been exposed to the same ferritin-coupled antibody to alkaline phosphatase. Bar indicates 0.1 μm.*

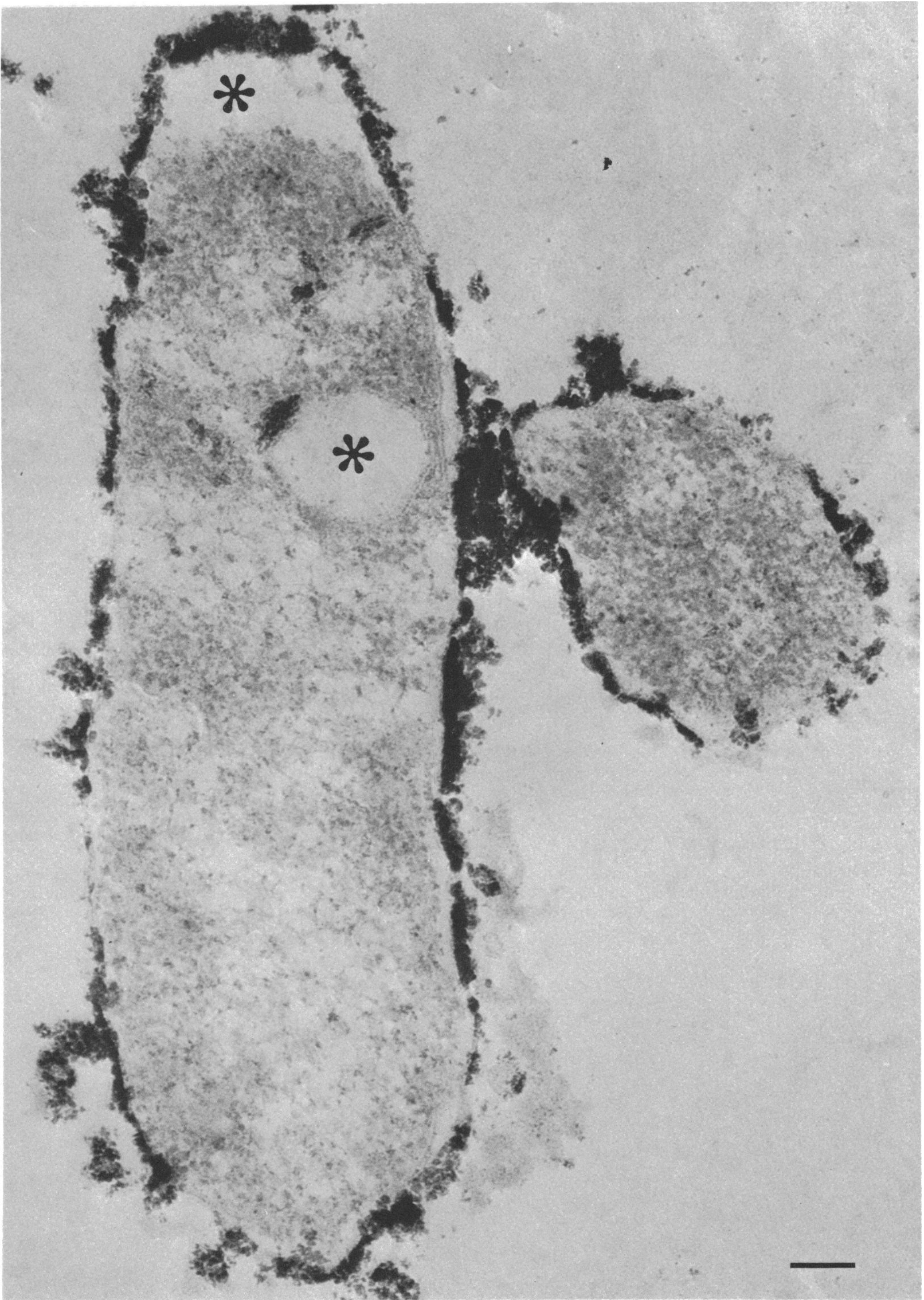

FIG. 5. *Reaction-product localization of alkaline phosphatase in cells of the marine pseudomonad (B16) which have been plasmolyzed by the method of Thompson et al. (14). Note that the enzyme is now associated with the outer aspect of the double-track layer of the cell wall, and that there is no significant concentration of the enzyme in the vesicles (*) which are derived from the "bays" produced by plasmolysis. Bar indicates 0.1 μm.*

migrated, with associated structural components of the cell wall, to the characteristic polar plasmolysis "bays," since Thompson et al. (14) noted that electron-dense aggregates of cell wall material collected in the bays of plasmolyzed cells of the marine pseudomonad (B16), and Wetzel et al. (15) have noted similar material in their polar caps. When we used the plasmolysis system of Thompson et al. in the marine pseudomonad (B16), we found no concentration of alkaline phosphatase in the plasmolysis bays (Fig. 5), some of which were polar, but the enzyme had shifted to the outer cell surface because $MgSO_4$ was present (0.05 M) in the plasmolyzing solution (14) and was therefore no longer present in the periplasmic space. Because of the presence of Na^+ and K^+ in the incubation mixture, these cells have been partially deplasmolyzed.

Thus we conclude that alkaline phosphatase is distributed evenly throughout the periplasmic space of the gram-negative bacteria studied herein. In the living cell, the periplasmic space is not distended by the departure of the cytoplasmic membrane from the general contour of the cell envelope, except when plasmolysis is induced, but it is maintained in close juxtaposition to the cell wall by normal turgor pressure.

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