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Relationship of a Wall-Associated Enzyme with Specific Layers of the Cell Wall of a Gram-Negative Bacterium

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In untreated cells of the marine pseudomonad studied here, alkaline phosphatase was found to be located in the periplasmic space, at the cell surface, and in the medium into which it had been shed during growth. Washing in 0.5 M NaCl, which removed the loosely bound outer layer, caused a shift of periplasmic enzyme to the outer aspect of the double-track layer and released some of the cell surface-associated enzyme. When the double-track layer of the cell wall was partially deranged, large amounts of this cell wall-associated enzyme were released, and, when the double-track was removed from the cells to produce mureinoplasts, alkaline phosphatase was released into the menstruum. There was no significant association of the enzyme with the peptidoglycan layer of the cell wall, which is the outermost structure of the mureinoplast, and no association of the enzyme with the cytoplasmic membrane of these modified cells. This study has shown that alkaline phosphatase is specifically associated with the outer layers of the cell walls of cells of this organism and is retained within the cell wall by virtue of this association.

Cell wall-associated enzymes have been localized at the cell surface (5, 19) and in the periplasmic space (3, 16) of a large number of gram-negative bacteria (6). This localization was effected by biochemical means (1), by reaction product deposition (3, 26), and by the use of ferritin-coupled antibodies (18). Dvorak et al. (11) suggested that periplasmic enzymes are concentrated in polar caps, but MacAlister et al. (18) challenged these data and concluded that these enzymes are evenly distributed throughout the periplasmic space.

It is important that we understand the nature of the association of these enzymes with the structural framework of the gram-negative cell wall. Cheng et al. (5) suggested that cell wall-associated enzymes are bound to lipopolysaccharide on both the inner and outer aspects of the double-track layer of the cell wall, and this group also showed that this association protects alkaline phosphatase from low pH denaturation (2). This conclusion is based on the retention of the enzyme by sucrose spheroplasts and on the demonstration of a molecular association between lipopolysaccharide and alkaline phosphatase, when both are removed from the cell (J. M. Ingram, K.-J. Cheng, and J. W. Costerton, submitted for publication). Similarly we have shown that an alkaline phosphatase-lipopolysaccharide complex is shed during the growth of rough strains of *Salmonella typhimurium* (S. S. Lindsay, B. Wheeler, K. E. Sanderson, and J. W. Costerton, Can. J. Microbiol., in press), and that washing with Tris buffer removes a similar complex from these cells (S. Lindsay, unpublished results). The double track has been identified as the barrier layer of the cell wall of the marine pseudomonad used in this study, and it has been concluded that this layer is instrumental in retaining the enzyme-lipopolysaccharide complex in the periplasmic area (12).

Because of these suggestions that enzymes are associated with structural components, we undertook a "chemical dissection" (7, 13) of the cell wall of a marine pseudomonad, and we used reaction product localization to determine the extent to which alkaline phosphatase is bound to, or contained by, each cell wall layer.

MATERIALS AND METHODS

The marine pseduomonad (B-16) used in this study has been classified as a *Pseudomonas* species type IV. It is deposited in the American Type Culture Collec-

tion (ATCC 19855) and in the National Collection of Marine Bacteria, Aberdeen, Scotland (NCMB19). Studies on the chemistry (14, 20), ultrastructure (9), and molecular architecture (12) of the cell walls of this organism were reported in previous communications.

The media used for the maintenance of stock cultures and for growth are described by Costerton et al. (7), and "5 h" cells (13) were produced by the following sequence of cultures. A 10-ml volume of the liquid medium in a 50-ml Erlenmeyer flask was inoculated from a slant and incubated at 25 C for 8 h on a rotary shaker. The entire contents of this flask was used as inoculum for a 250-ml volume of liquid medium in a 2-liter Erlenmeyer flask, and this was incubated for 5 h. Forty-milliliter volumes of this culture served as inocula for 250-ml volumes of media in 2-liter flasks, and these cultures were incubated for 5 h at 25 C on a rotary shaker. This procedure yielded large quantities of cells in early logarithmic phase.

Cells were harvested from the growth medium by centrifugation at 16,300 × *g* for 20 min at 4 C and were washed four times in "complete salts" (0.3 M NaCl, 0.05 M $MgSO_4$, and 0.01 M KCl) by suspension in, and centrifugation from, volumes of washing solution equal to that of the growth medium.

Cells were harvested from the growth medium by centrifugation at 16,300 × *g* for 20 min at 4 C and were washed four times in volumes of 0.5 M NaCl equal to that of the growth medium. This treatment removes the loosely bound outer layer of the cell wall (13).

The NaCl-washed cells were suspended in sufficient 0.5 M sucrose to provide a concentration of approximately 10 mg (dry wt) of cells per ml and were incubated for 30 min on a rotary shaker at 25 C. The mureinoplasts which result from this treatment were harvested by centrifugation at 35,000 × *g* for 20 min.

The "complete salts"-washed cells were suspended in 0.5 M sucrose and incubated and harvested under the same conditions as those described for mureinoplast production.

The cells to be tested were carefully suspended in the appropriate incubation solution for 20 min at 22 C with occasional mixing. The basic incubation solution contained the following: sodium β-glycerophosphate, 0.017 M; sodium barbital, 0.024 M; $Ca(NO_3)_2 \cdot 4H_2O$, 0.02 M; $MgCl_2 \cdot 6H_2O$, 0.01 M; and tris(hydroxymethyl)aminomethane, 0.1 M at pH 8.4. These solutions were made up in distilled water or the menstruum in which the cells had previously been suspended (complete salts, 0.5 M NaCl, or 0.5 M sucrose), and the pH was checked. Controls in which only the substrate had been omitted from the incubation solution were included for each preparation. The cells were recovered by centrifugation at 16,300 × *g* for 10 min, suspended in a 2% aqueous solution of $Pb(NO_3)_2$, and allowed to stand for 10 min so that calcium phosphate was converted to lead phosphate. The cells were then washed twice by suspension for 10 min in Veronal acetate buffer (22) (Na^+, 0.17 M; Mg^{2+}, 0.01 M; K^+, 0), fixed for 2 h at 22 C in 5% glutaraldehyde (70% under Argon, Ladd Industries, Burlington, Vt.) in Veronal acetate buffer, and enrobed in 4% agar. After five 10-min washes in Veronal acetate buffer, the agar "cores" were fixed for 90 min at 22 C in 2% OsO_4 in Veronal acetate buffer with the addition of tryptone broth (0.2%) and $MgCl_2$ (0.002 M). Five 10-min washes in Veronal acetate buffer were used to remove residual osmium, and the specimens were dehydrated in a graded series of acetone solutions and in propylene oxide before being embedded in Vestopal W (Polysciences, Rydal, Pa.). Thin sections were cut, stained with uranyl acetate and lead citrate (24), and examined using an A.E.I.-EM (801) electron microscope.

RESULTS

When cells were harvested from the medium and suspended directly in the incubation solution, alkaline phosphatase was found to be located both at the cell surface and in the periplasmic space (Fig. 1). The reaction product at the cell surface is both in the form of an even crust and in the form of discrete crystals, which suggests that the enzyme which caused its deposition is both bound to the cell surface and free in the menstruum (5). Control preparations, in which only the substrate had been omitted, showed a very small amount of reaction product (Fig. 2). Thus it is established that the reaction product seen in association with the cell wall of the cells in Fig. 1 is due to enzyme activity and not to the presence of inorganic phosphate in the menstruum. Similar controls were carried out for all preparations described below, and all showed an absence of reaction product.

Forsberg et al. (13) established that washing in complete salts does not affect the cell walls of cells of this organism, and we see no morphological changes in complete salts-washed cells (Fig. 3). The cell wall-associated alkaline phosphatase is seen to be localized in the periplasmic space and at the cell surface where it forms a very even "crust" of reaction product. The absence of individual and discrete crystals of lead phosphate in these preparations indicates an absence of cell-free enzyme (5).

When the loosely bound outer layer of the cell wall was removed by washing with 0.5 M NaCl, almost all of the alkaline phosphatase activity of the cell envelope was seen to be associated with the outer aspect of the double-track layer (Fig. 4), and very little enzyme activity was seen in the periplasmic space. The reaction product formed an even crust over the cell surface, and the presence of a few discrete crystals at the cell surface indicated that small amounts of cell-free enzyme are present in the menstruum. When the incubation solution was made up in distilled water rather than in 0.5 M NaCl, the enzyme was seen to be associated with the outer surface of the double-track layer in the same pattern.

When the double-track layer of the cell wall was removed to produce mureinoplasts, alkaline

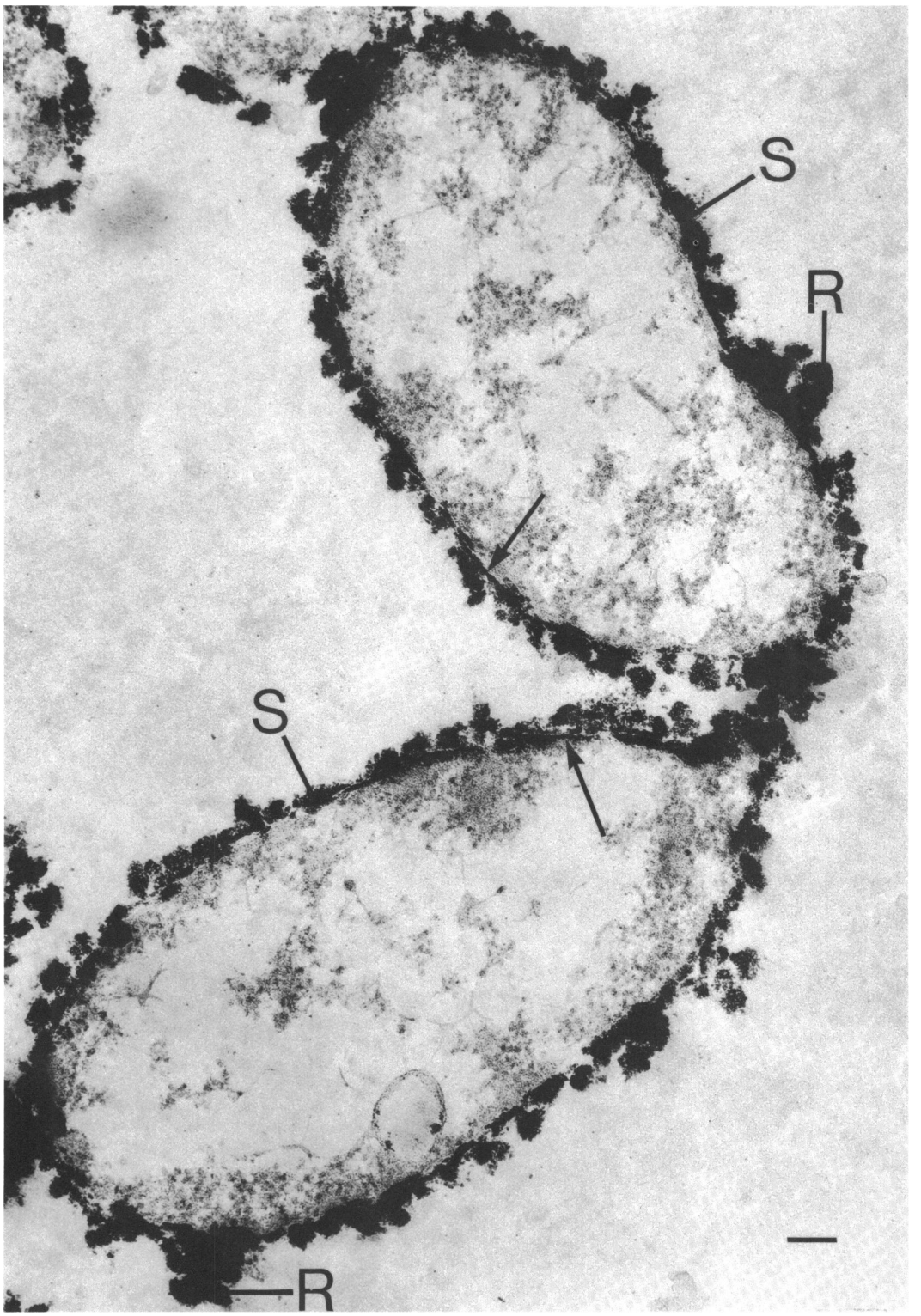

FIG. 1. *Reaction product localization of alkaline phosphatase in cells of the marine pseudomonad harvested from the growth medium. The reaction product is located in the periplasmic space (arrows), in a surface "crust" (S), and in individual and discrete crystals at the cell surface (R). The bar in this and subsequent micrographs indicates 0.1 μm.*

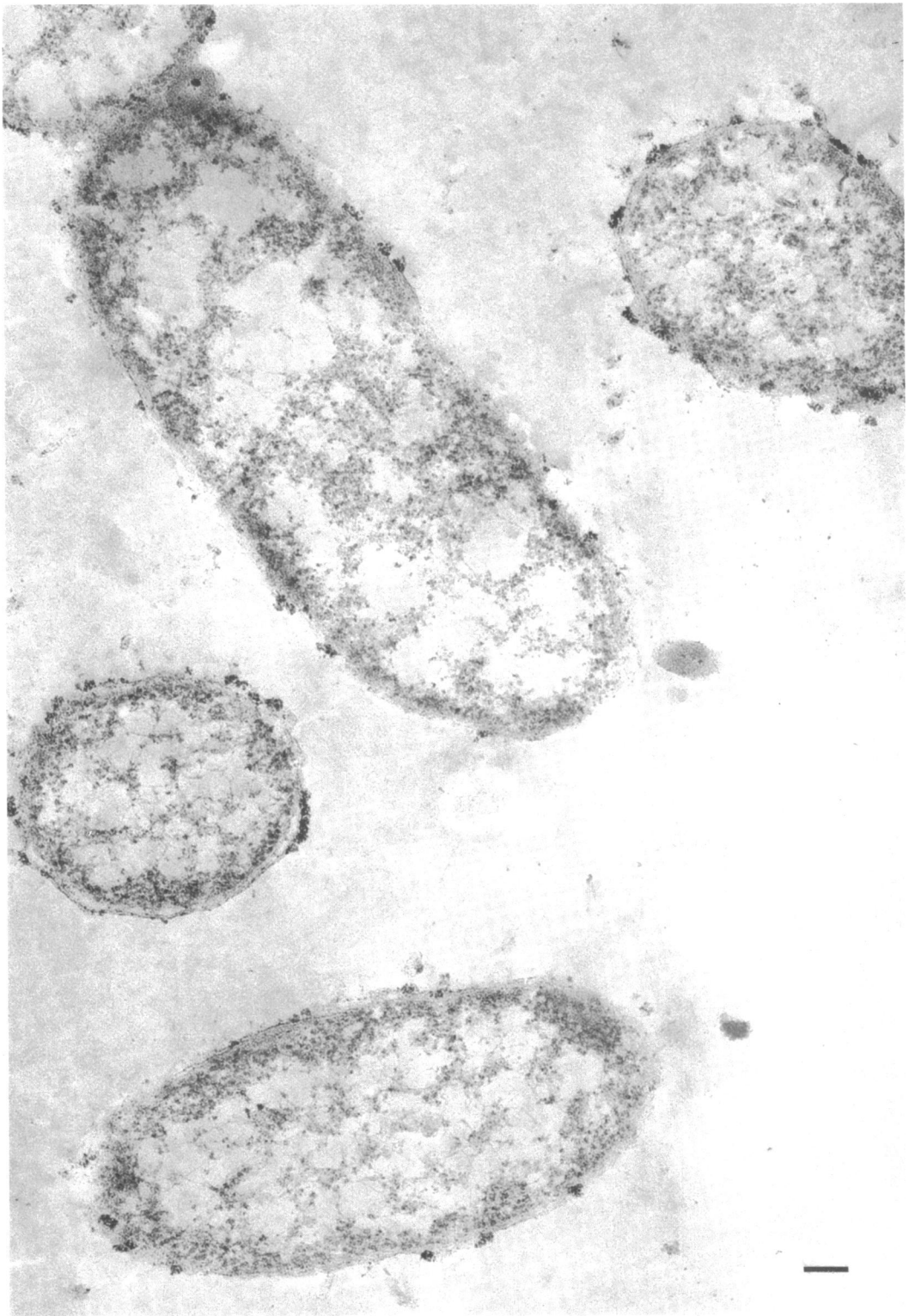

FIG. 2. *Control preparation of cells harvested from the growth medium in which the substrate was omitted from the incubation solution. Note the absence of significant amounts of reaction product.*

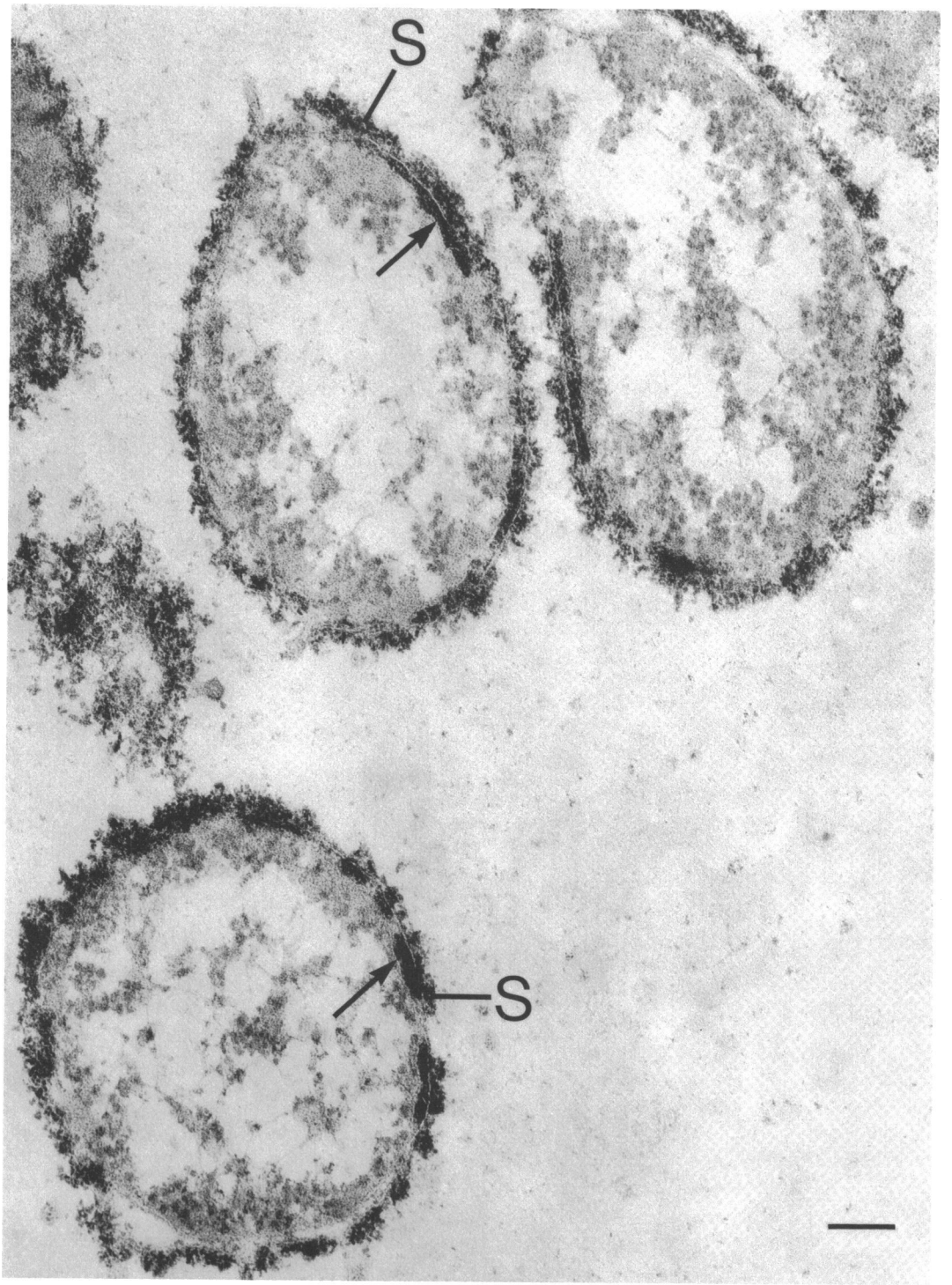

FIG. 3. *Reaction product localization of alkaline phosphatase in "complete salts"-washed cells. Note that the reaction product is located in the periplasmic space (arrows) and in a surface crust (S), and that individual and discrete crystals are not formed in significant numbers.*

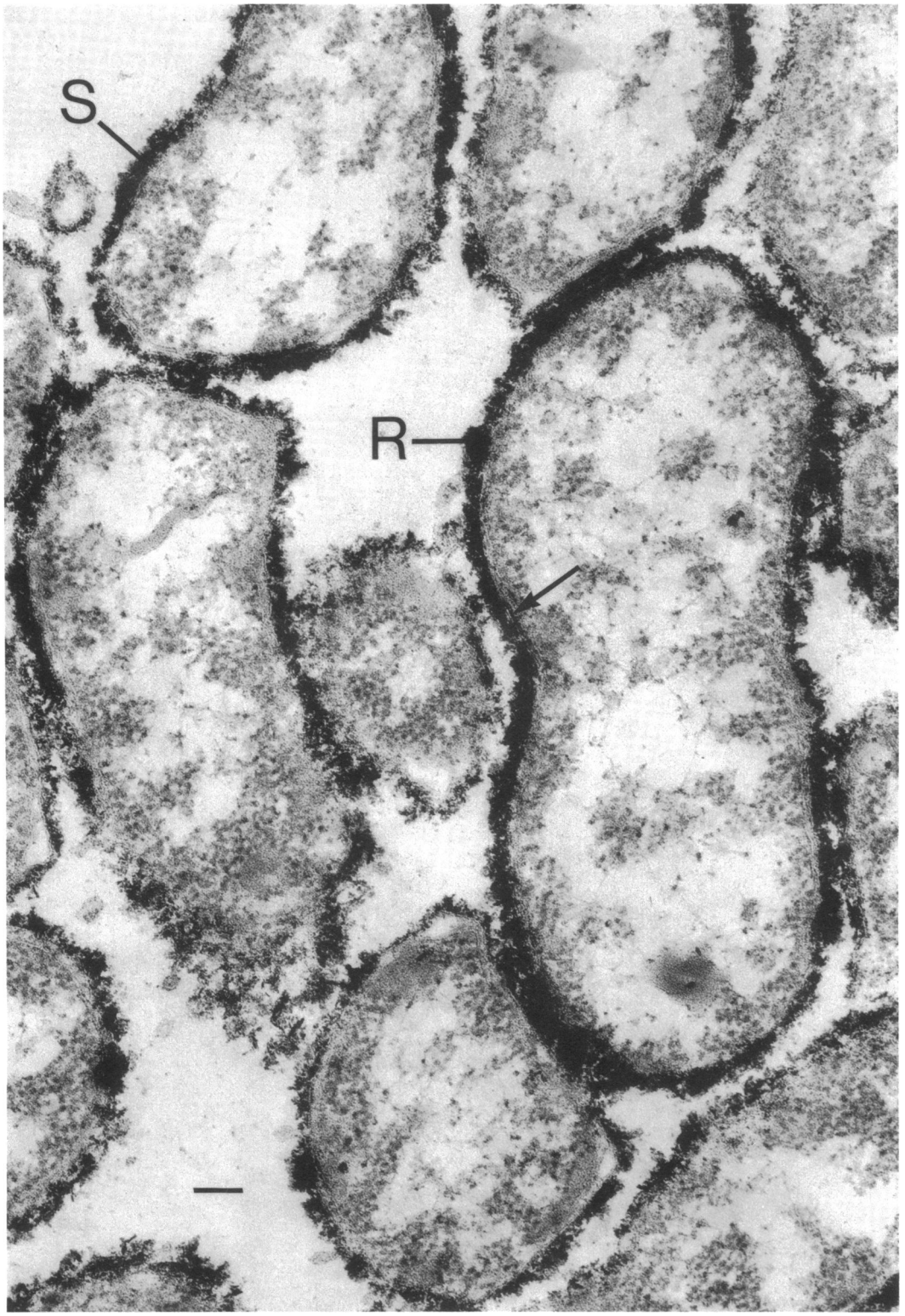

FIG. 4. *Reaction product localization of alkaline phosphatase in cells which were washed in complete salts and then in 0.5 M NaCl so that the loosely bound outer layer of the cell wall was removed. Note that the reaction product is predominantly in an even surface crust (S) on these cells and that there is very little activity in the periplasm (arrows). Individual and discrete crystals are present in only very small numbers (R).*

phosphatase was no longer associated with the cells, but was present in the menstruum as indicated by the large numbers of individual and discrete crystals seen surrounding clumps of mureinoplasts (Fig. 5). In this case the bulk of the "recrystallization" (5) has taken place on detached double-track fragments, which have served as foci for crystal formation (Fig. 6, arrows), and not on the cell surface. In the few cells from which the double-track layer has not been removed, alkaline phosphatase is still seen to be associated with the outer aspect of this layer (Fig. 5, I, and Fig. 6, I). Careful examination of these preparations at higher magnification (Fig. 6) shows that the removal of the double-track layer has been accomplished in most cells, and that the chemical indignities involved in reaction product deposition have not altered the cells in any discernible morphological respect (compare with Forsberg et al. [13], Fig. 6 and 7). When the incubation solution was made up using distilled water rather than 0.5 M sucrose, the cells were less well preserved but the enzyme distribution was very similar.

Washing of the cells in complete salts, followed by exposure to 0.5 M sucrose, leads to the release of the loosely bound outer layer of the cell wall and to a derangement of the double-track layer (13). The enzyme distribution after four washes in complete salts is seen in Fig. 3, and the enzyme distribution after incubation in sucrose is seen in Fig. 7. In these latter cells, protrusions of the double-track layer are clearly seen (Fig. 7, D), as are areas in which the double-track layer is separated from the cell to enlarge the periplasmic space (Fig. 7, P). A small amount of enzyme activity is retained in the periplasmic space (Fig. 7, arrows), but most of the reaction product is associated with the outer surface of the cell where it is in the form of individual and discrete crystals. Reaction product crystals are also seen in the menstruum where they may have formed on projections from the cells (Fig. 7, C), and the preponderance of discrete crystals indicates that most of the alkaline phosphatase in this preparation was free in the menstruum.

DISCUSSION

Reaction product localization is heavily dependent on the technique used, and Cheng et al. (3) noted that fixation in glutaraldehyde causes a shift of alkaline phosphatase from the periplasmic space to the cell surface, whereas Wetzel et al. (26) noted a similar shift due to Formalin fixation and to the use of Ca^{2+} as a capture reagent. In previous studies we found excellent correlation between direct biochemical assays of enzyme concentration and the localization of reaction product, using unfixed cells with calcium nitrate as the capture reagent (3, 4, 5). Reaction product localization does, however, depend on sites of crystal formation by a diffusible salt, and the results must be interpreted with this fact in mind if the conclusions are to be meaningful. If the diffusible salt is formed by cell-free enzyme, the reaction product will form individual and discrete crystals on the closest available surface. The surface may be that of the cell, or of cell debris, or of the reaction vessel, but individual and discrete crystals are formed because the first aggregates act as "seed crystals" for subseqeunt deposition. On the other hand, if the enzyme is present at the cell surface, the diffusible salt will form a continuous crystalline crust because it is being produced at many loci right at the surface. This distinction has been borne out in systems where reaction product localization has been conducted in parallel with assays of cell-free enzymes (5).

In this study the salt requirements for the structural integrity of the modified cells sometimes differed from the chemical requirements for reaction product deposition. Thus it was necessary, as an example, to take cells from a sucrose solution into an incubation mixture containing 0.058 M Na^+, 0.01 M Mg^{2+} and 0.02 M Ca^{2+}, and the effects of these ions on the localization of the enzyme must be borne in mind. After the original deposition of calcium phosphate, which serves to localize the alkaline phosphatase, the cells were suspended in a 2% aqueous solution of $Pb(NO_3)_2$ so that the calcium phosphate would be converted to lead phosphate, and the cells were washed twice in Veronal acetate buffer (0.17 M Na^+, 0.01 M Mg^{2+}) before fixation. The effects of these treatments on the morphology of the cells were assessed by comparison of reaction product deposition preparations and controls from which substrate was omitted with untreated controls and with previously published reports on these cells (7, 10, 13, 14, 25). Only very minor morphological differences resulted from the treatments described above.

Although reaction product deposition shows the location of enzymes very accurately, the technique is only roughly quantitative, and comparison with enzyme assay data shows that the amount of reaction product at the cell surface does not decrease significantly when more than 50% of the enzyme is removed from the cells (S. Lindsay, unpublished results).

When whole cells of the marine pseudomonad

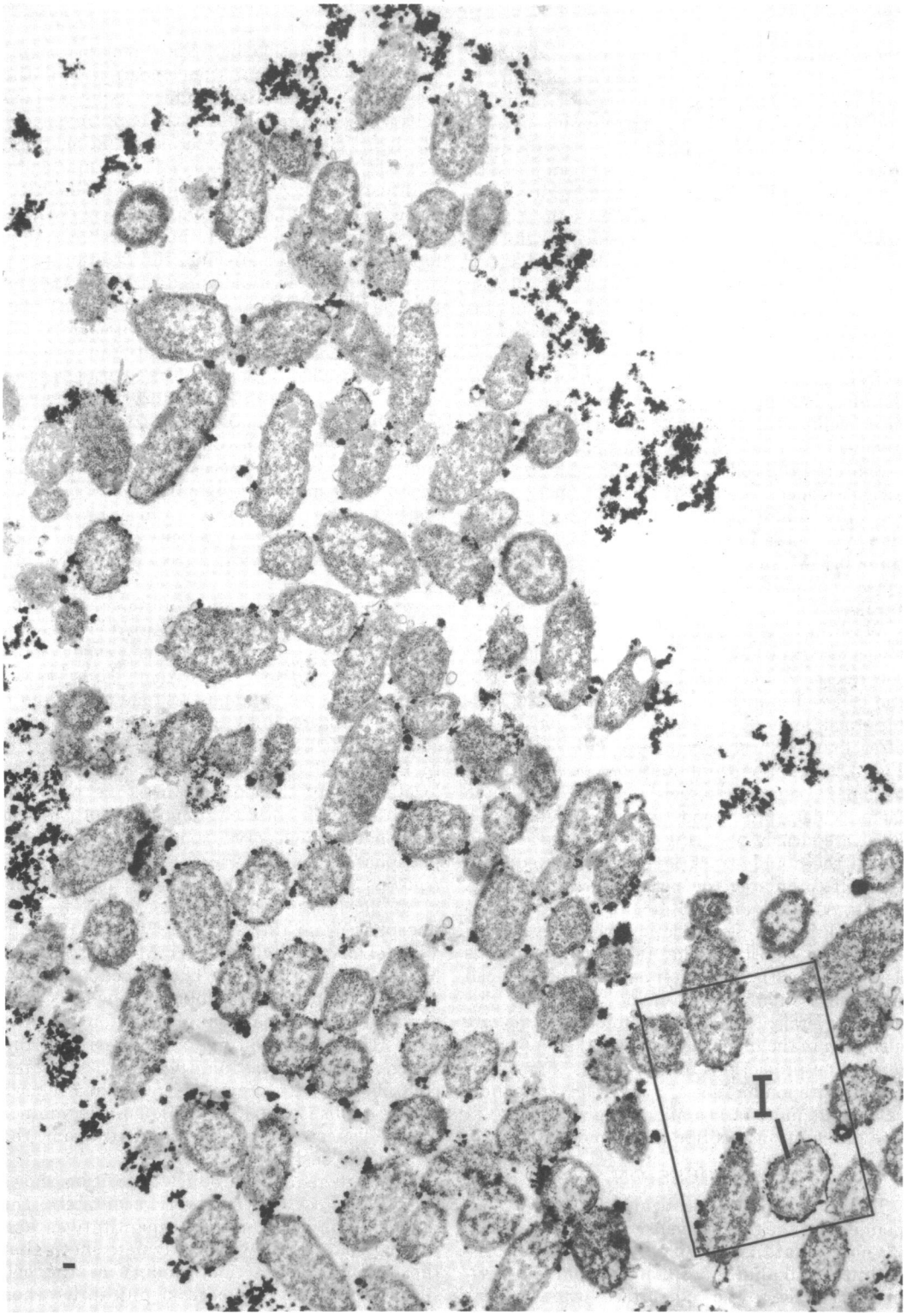

FIG. 5. *Reaction product localization of alkaline phosphatase in mureinoplasts. Note that the reaction product produced by cell-free enzyme has formed discrete crystals at the periphery of the clump of cells, and that there is little enzyme activity associated with the cells except for the one cell (I) whose double-track layer has not been removed (see Fig. 6).*

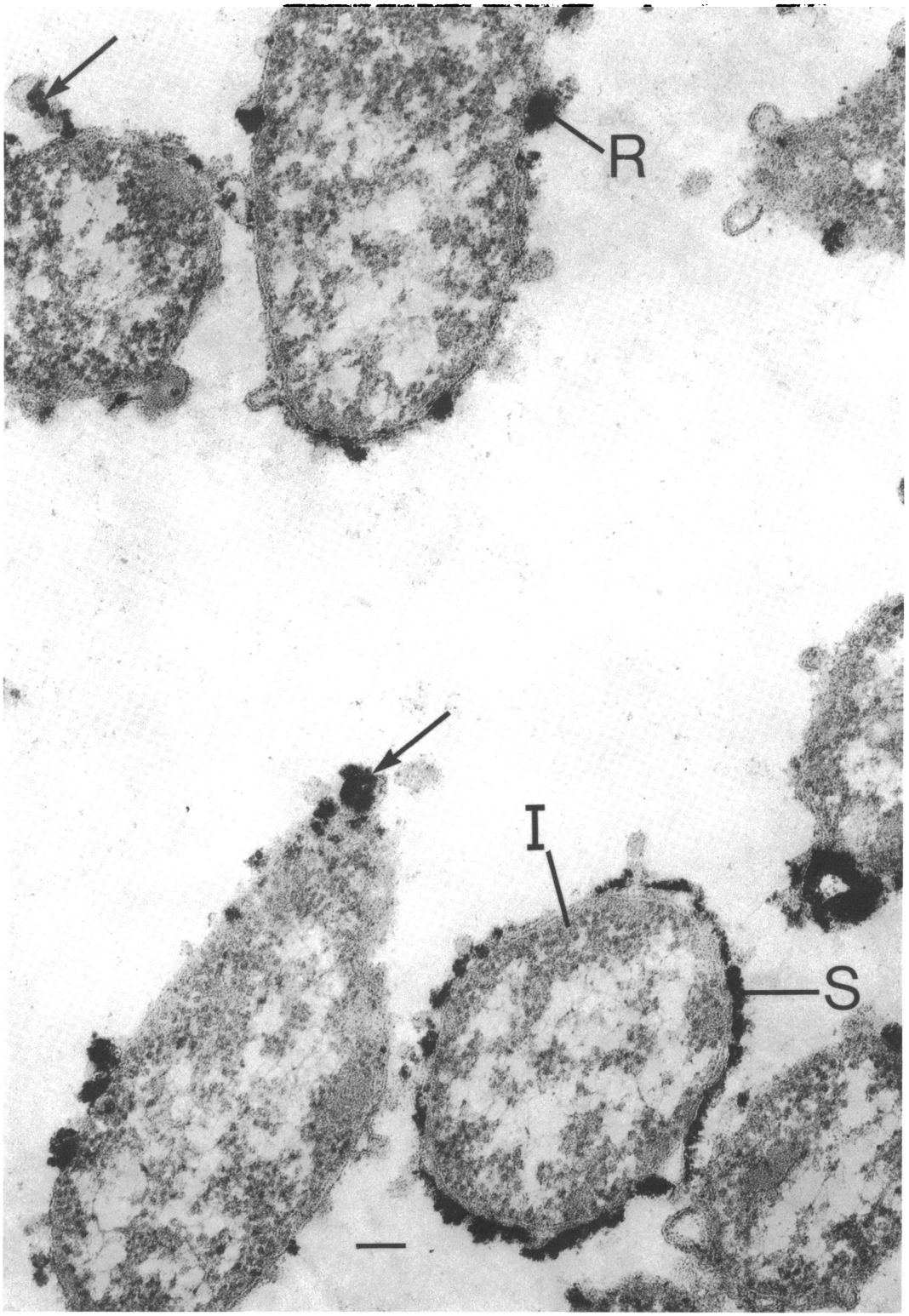

FIG. 6. *Higher magnification of the cell (I) in Fig. 5 which retained its double-track layer. Note that the double-track layer of the cell wall is fully retained by this cell, whereas the other cells have only fragments of this layer at their surfaces. The reaction product on the intact cell has formed a continuous crust (S), whereas that on the mureinoplasts is clearly in the form of discrete crystals (R) which are often associated with detached fragments of the double-track layer (arrows).*

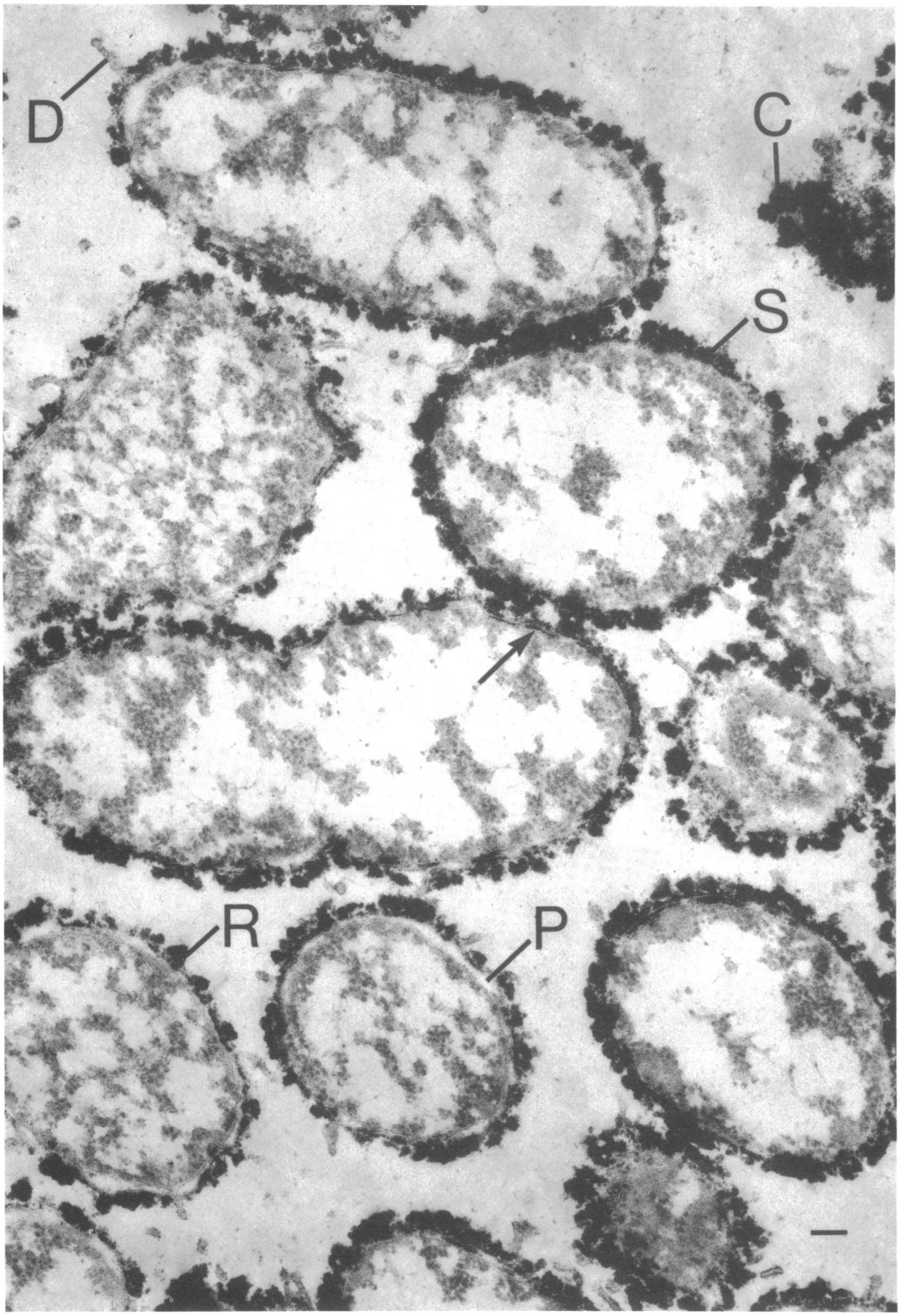

FIG. 7. *Reaction product localization of alkaline phosphatase in cells which were washed in complete salts and suspended in sucrose. Note that the double-track layer is deranged to produce protrusions from the cells (D) and areas where the double track is separated from the cytoplasmic membrane (P). Cell-free enzyme has produced large numbers of discrete reaction product crystals on the surfaces of the cells (R). Small amounts of reaction product are seen in the periplasmic space (arrows) and in small areas of "crust" at the cell surface (S). Reaction product crystals (C) are also seen in the menstruum where they may have formed on projections from the cells.*

were treated for reaction product deposition directly from the medium, their alkaline phosphatase was seen to be localized in the periplasmic space and at the cell surface. Discrete crystals were seen at the cell surface, indicating that the enzyme had been released into the medium during growth. The absence of reaction product in controls from which the substrate had been omitted established that this reaction was not due to a carry-over of inorganic phosphate from the medium. It is unlikely that the ion balance in the incubation solution influenced the enzyme distribution or the cell envelope structure of these whole cells since Thompson et al. (25) showed that Na^+ is essential to membrane stability in these cells and Na^+ is present (0.58 M) in this solution. The cell-free alkaline phosphatase was removed by four washes in complete salts, and the cell wall-associated enzyme was seen both in the periplasmic area and at the cell surface in washed cells.

This distribution of cell wall-associated enzyme is similar to that found in a great variety of gram-negative bacteria (3, 16, 18; Lindsay et al., in press), and the release of cell wall-associated enzymes during growth is also reported in many of these organisms (4, 17, 24; Lindsay et al., in press).

Enzyme assays indicate that the removal of the loosely bound layer of the cell wall leads to the release of large amounts of alkaline phosphate from these cells (S. Lindsay, unpublished results). Because reaction product deposition is not quantitative at high concentrations, the decrease in cell wall-associated enzyme is not obvious in the illustrations presented here (compare Fig. 3 and 4), but it is clear that the exposure of these cells to four washes in 0.5 M NaCl caused a shift of periplasmic enzyme to the outer aspect of the double-track layer where it formed an even crust of reaction product. This enzyme shift is very similar to that caused by washing cells of this marine pseudomonad in 0.05 M $MgSO_4$ (18), and by glutaraldehyde fixation (before reaction product deposition) (3). It is clearly not the result of the ionic content of the incubation solution, since the same results were obtained when it was made up with distilled water (0.058 M Na^+) or with the 0.5 M NaCl solution (0.558 M Na^+). If the alkaline phosphatase is associated directly or indirectly with the double track, this shift entails a simple movement of the enzyme from the inner to the outer aspect of this layer.

The double-track layer is fragmented and released when these NaCl-washed cells are incubated in 0.5 M sucrose, and the structural dissociation of this layer is paralleled by the release of cell wall-associated alkaline phosphatase. The fact that enzyme release is a corollary of the structural dissociation of the double-track layer is indicated by the observation that the small number of cells whose double-track layer is intact have retained the associated enzyme, even though the cells have been exposed to the same sucrose washing and to the same procedures for reaction product deposition. DeVoe et al. (10) have shown that Na^+ and Mg^{2+} have a stabilizing influence on mureinoplasts, and this accounts for the favorable preservation of these cells after treatment with the incubation solution (0.058 M Na^+, 0.01 M Mg^{2+}).

The double-track layer is also partially deranged by incubation in sucrose after four washes in complete salts (7, 13), and this treatment avoids exposure of the cells to the 0.5 M NaCl solution which causes the enzyme shift noted above. When complete salts-sucrose-washed cells were examined by the reaction product deposition technique, their alkaline phosphatase had been largely shed into the menstruum and its reaction product had formed very discrete and individual crystals on available surfaces. Only very small amounts of periplasmic and of cell surface-associated enzyme were seen in the cells in these preparations. The morphological consequence of the derangement of the double-track layer in these cells was the production of protrusions similar to those noted by Costerton and Thompson (8) in cells plasmolyzed in 0.05 M $MgSO_4$, and the broader protrusions noted by Forsberg et al. (13) were not seen in these preparations.

This system has allowed us to understand the association between a specific cell wall-associated enzyme and the specific layers of the cell wall. Alkaline phosphatase is localized both in the periplasmic space and at the cell surface in whole cells, and the removal of the loosely bound outer layer causes the release of a part of this cell wall-associated enzyme. When the double-track layer is disturbed, a large part of the cell's alkaline phosphatase is released into the menstruum, and the enzyme is totally liberated by the structural dissociation of this cell wall layer. Mureinoplasts retain the peptidoglycan component of their cell wall as well as their cytoplasmic membranes (13, 15), but these cells do not retain significant amounts of alkaline phosphatase.

These findings suggest that alkaline phosphatase, which has been found to be an exceptionally hydrophobic protein molecule (D. F. Day and J. M. Ingram, submitted for publication), is

associated with the outer layers of the cell wall in such a way that it is retained within the cell envelope by this association.

The double-track layer of the marine pseudomonad studied here has been found to be largely composed of protein and phospholipid (14), as has that of *Escherichia coli* (23), and biophysical studies (12) have shown that the phospholipid forms a membrane-like, hexagonally close packed bilayer which functions as a barrier layer within the cell wall. Schnaitman's suggestion (23) that lipopolysaccharide is an integral part of the double-track layer is supported by Lindsay et al. in press), who reported that genetic defects in the lipopolysaccharide influence both the penetrability of the cell walls of *S. typhimurium* and the shedding of an alkaline phosphatase-lipopolysaccharide complex during growth. Alkaline phosphatase has been shown to exist as an enzyme-lipopolysaccharide complex in the cell walls of *Pseudomonas aeruginosa* (Ingram et al., submitted for publication) and of *S. typhimurium* (S. Lindsay, personal communication), and Cheng et al. (5) have suggested that the enzyme is bound to lipopolysaccharide both in the periplasmic space of *P. aeruginosa* and at the cell surface. This suggests that the association of alkaline phosphatase with the double-track layer of the cell wall of gram-negative bacteria may be either direct or mediated by lipopolysaccharide. This association may influence the activity of the enzyme significantly, as indicated by the observations by Cheng et al. (2) that cell wall-associated alkaline phosphatase is protected from low pH denaturation which affects cell-free enzyme in the same preparations.

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