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Expression and Localization of *Escherichia coli* Alkaline Phosphatase Synthesized in *Salmonella typhimurium* Cytoplasm

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The *Escherichia coli* structural gene for alkaline phosphatase was inserted into *Salmonella typhimurium* by episomal transfer in order to determine whether this enzyme would continue to be localized to the periplasmic space of the bacterium even though it was formed in a cell that does not synthesize alkaline phosphatase. The *S. typhimurium* heterogenote synthesized alkaline phosphatase under conditions identical to that observed with *E. coli*. This enzyme appeared to be identical to that synthesized by *E. coli*, and was quantitatively released from the bacterial cell by spheroplast formation with lysozyme. These results showed that localization is not a property unique to the *E. coli* cell and suggested that, in *E. coli*, enzyme location is related to the structure of the protein. Formation of alkaline phosphatase in the *S. typhimurium* heterogenote was repressed in cells growing in a medium with excess inorganic phosphate, even though only one of the three regulatory genes for this enzyme is on the episome. Thus, *S. typhimurium* can supply the products of the other two regulatory genes essential for repression even though this bacterium seems to lack the structural gene for alkaline phosphatase.

Alkaline phosphatase from *Escherichia coli* is a stable, dimeric zinc-metalloprotein localized to the periplasmic space of the bacterial cell (6, 15, 17, 19, 22). Recent experimental evidence suggests that assembly of the subunits to form the active dimer takes place in the periplasmic space after the subunits have been released from polyribosomes in the cell cytoplasm and moved across the cell membrane (27; A. Torriani, *in preparation*).

We wanted to know whether there is a specific transport process in *E. coli* that facilitates the movement of the large polypeptide subunit (molecular weight, 43,000). One means of testing this possibility is to establish a situation in which the *E. coli* gene for alkaline phosphatase can be expressed in a cell that itself does not have an alkaline phosphatase and which, presumably, would not contain a specific transport system for this enzyme. If the *E. coli* enzyme is found exterior to the cell cytoplasm in this heterogenote, then the case for a specific carrier is considerably weakened.

A heterogenote suitable for the proposed test was prepared with *E. coli* K-12 and *Salmonella typhimurium* LT·2. These two enteric organisms show extensive genetic homology (23, 31), and genetic hybrids of them can be produced (1, 11, 16, 33); furthermore, *S. typhimurium* does not make an alkaline phosphatase. For the experiments described, the episomal element F'13 of *E. coli* K-12 which carries the structural gene for alkaline phosphatase was transferred to *S. typhimurium* LT·2. The results reported here show that the *S. typhimurium* heterogenote could form an alkaline phosphatase that was indistinguishable from the *E. coli* enzyme and that this enzyme was quantitatively released from the bacterium when spheroplasts were prepared. Thus, the *E. coli* enzyme was localized in the periplasmic space despite its formation in a foreign cytoplasm.

MATERIALS AND METHODS

Bacterial strains and preparation of the heterogenote. *E. coli* CW 3747 was obtained from the laboratory of C. Levinthal, Massachusetts Institute of Technology. It requires methionine for growth and carries the episomal element F'13 which contains the *lac* operon as well as the structural gene for alkaline phosphatase (5, 30). *S. typhimurium* LT·2 was obtained from B. Ames. Heterogenotes were selected on plates that contained M-63 salts medium (18) and 0.2% lactose as the carbon source. Neither the strain of *E. coli* nor the strain of *S. typhimurium* can grow on this medium because the former requires methionine and the latter cannot utilize lactose as a carbon source.

About 2×10^7 cells of each strain were taken from cultures growing exponentially on Penassay medium and were mixed in 2 ml of the same medium. After 3 hr at 37 C with occasional shaking, 0.1 ml of a 10-fold dilution was plated and colonies were permitted to develop for 48 hr. Of the *S. typhimurium* cells in the cross, approximately 10^{-4} developed into colonies (heterogenotes), as indicated by growth on the M-63 lactose agar. Several of these colonies were streaked onto M-63 lactose agar plates and were stored on this medium.

Tests for S. typhimurium. Cultures of the heterogenotes gave a positive agglutination test with *Salmonella* polyvalent diagnostic serum (antigens 1 to 10, 15, 19, and vi; Lederle Laboratories, Pearl River, N.Y.) and were unable to form indole. These cultures were compared with *S. typhimurium* LT·2 in a large variety of routine diagnostic and nutritional tests (carried out by A. Sonnenwirth, The Jewish Hospital, St. Louis, Mo.), and, except for the ability of the heterogenote to ferment lactose and form β-galactosidase, the heterogenote was indistinguishable from *S. typhimurium*.

The heterogenote was treated with acridine orange under conditions that led to curing of episomes (10). Survivors of this treatment could no longer form colonies on lactose M·63 medium and did not produce alkaline phosphatase. Ten colonies were tested.

Growth and preparation of cell extracts. Overnight cultures were diluted 20-fold into 100 ml of fresh tris(hydroxymethyl)aminomethane (Tris)-salts medium (26) with lactose as carbon source, and were incubated with shaking at 37 C. Samples (1.0 ml) were removed periodically and the optical density (OD) at 540 nm was measured. A portion of this sample was subjected to brief sonic treatment, and the clear extract was assayed for alkaline phosphatase activity. When the culture density reached about 4×10^8 cells/ml (OD at 540 nm = 0.5), the entire culture was centrifuged at room temperature and the pelleted cells were resuspended in 100 ml of fresh medium that lacked inorganic phosphate. The culture was returned to the 37 C bath, and 1-ml samples were removed at intervals over a 2-hr period. Then the entire culture was harvested at 0 C, washed with 25 ml of 0.05 M Tris-chloride (*p*H 8.0), and suspended in 2 ml of buffer containing 15% sucrose and 0.05 M Tris-chloride, *p*H 8.0. Lysozyme (final concentration, 200 μg/ml) and 4 μmoles of ethylenediaminetetraacetic acid were added and the cells were incubated for 30 min at 23 C. The spheroplasts were removed by high-speed centrifugation and the supernatant solution was saved. The spheroplasts were suspended in 2 ml of 0.05 M Tris-chloride (*p*H 8.0), and were disrupted by sonic treatment. Unbroken cells and debris were removed by high-speed centrifugation and the clear extract was used for further tests.

Assays. Alkaline phosphatase activity was measured with *p*-nitrophenyl phosphate as substrate (26); one unit equals 1 μmole of *p*-nitrophenol formed per min at 37 C measured at λ = 410 nm. Glucose 6-phosphate dehydrogenase was measured according to Kornberg's procedure (13); one unit equals 1 μmole of nicotinamide adenine dinucleotide phosphate reduced per min at 37 C. β-Galactosidase was assayed with *o*-nitrophenylgalactopyranoside in 0.1 M sodium phosphate (*p*H 7.0), 0.001 M $MgSO_4$, 0.001 M $MnSO_4$, and 0.1 M mercaptoethanol; one unit equals 1 nmole of *o*-nitrophenol formed per min at 28 C, measured at λ = 420 nm. Protein was determined by micromodification of the Folin-Ciocalteu procedure (12).

Chemicals. *p*-Nitrophenylphosphate, *o*-nitrophenyl β-galactopyranoside, nicotinamide adenine dinucleotide phosphate, and glucose-6-phosphate were purchased from Sigma Chemical Co., St. Louis, Mo.

RESULTS AND DISCUSSION

In *E. coli* K-12, the formation of alkaline phosphatase begins after the culture medium is depleted of inorganic phosphate (P_i). In contrast, no alkaline phosphatase activity is detected in *S. typhimurium* when the culture fluid is free from P_i (Fig. 1A). Furthermore, no protein is produced by *S. typhimurium* that can cross-react with antibodies directed against active *E. coli* alkaline phosphatase or with those directed against inactive subunits (M. Schlesinger, *unpublished data*). Apparently, the structural gene for this enzyme does not exist or is incapable of functioning in *S. typhimurium*. In the heterogenote, however, the *E. coli* gene carried on the episome functions normally and activity appeared as soon as the cells were starved on P_i (Fig. 1B), as in the *E. coli* donor strain (Fig. 1C).

When examined by starch gel electrophoresis (Fig. 2), the enzyme formed by the heterogenote was indistinguishable from that synthesized by *E. coli*. Both the electrophoretic mobility at *p*H 8.0 and the isozyme patterns of the two prepara-

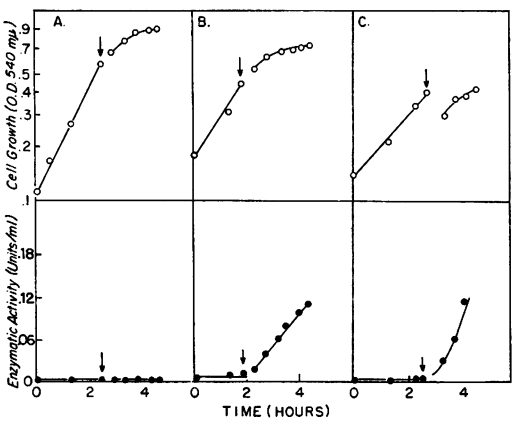

FIG. 1. *Alkaline phosphatase formation in (A) S. typhimurium LT·2, (B) the heterogenote LT·2/F'13, and (C) E. coli W-3747. The arrows indicate times when cultures were harvested and resuspended in the medium lacking inorganic phosphate (see Materials and Methods); there was some loss of cells in this step in culture (C), leading to the decreased OD before starvation.*

tions were identical. In addition, Ouchterlony double diffusion in agar showed complete antigenic identity of the two enzymes when tested with an antiserum prepared against the *E. coli* alkaline phosphatase.

To test for localization of this enzymatic activity in the heterogenote, spheroplasts were prepared with lysozyme. Under conditions in which 90 to 95% of the cytoplasmic enzymes (β-galactosidase and glucose-6-phosphate dehydrogenase) remained within the spheroplasts, 98% of the alkaline phosphatase was released into the medium (Table 1); this result is identical to that obtained with spheroplasts of *E. coli*. By this criterion, then, the enzyme made in the *S. typhimurium* heterogenote is localized to the periplasmic space of the bacterial cell.

Localization of *E. coli* alkaline phosphatase is not unique to the *E. coli* cell. To account for the presence of this enzyme in the periplasma of *S. typhimurium*, three possibilities can be considered. (i) Some type of carrier transfers this protein from the cytoplasm to the cell wall space, but the carrier is relatively nonspecific in that the transport system of *S. typhimurium* can recognize *E. coli* proteins. (ii) A protein is present in *E. coli* that specifically transports the alkaline phosphatase polypeptide chains across the cell membrane, and the gene that codes for its structure is linked to the structural gene of alkaline phosphatase on the F'13 episome. (iii) The transfer of newly formed protein from the cytoplasm to outside the cell membrane is a direct result of the unique chemical structure of the polypeptide chain (i.e., the amino acid sequence).

TABLE 1. *Release of alkaline phosphatase from spheroplasts*[a]

Strain	Alkaline phosphatase	β-Galactosidase	Glucose-6-phosphate dehydrogenase
S. typhimurium LT·2/F'13.....	98	4	10
E. coli W-3747.....	92	1	<1

[a] Values are expressed as per cent of total enzymatic activity (measured in extracts of spheroplast plus the supernatant solution) in the supernatant solution after removal of the spheroplasts. For strain LT·2/F'13, the total alkaline phosphatase activity was 4.4 units, the total β-galactosidase activity was 2,530 units, and the total glucose-6-phosphate dehydrogenase activity was 0.22 unit. The comparable values for strain W-3747 were 16.3 units, 3,800 units, and 0.42 unit, respectively. The total protein in the extracts of the spheroplasts of strain LT·2/F'13 was 5.4 mg and that in the supernatant solution was 0.62 mg. The extracts and supernatant solutions of strain W-3747 had comparable amounts of protein.

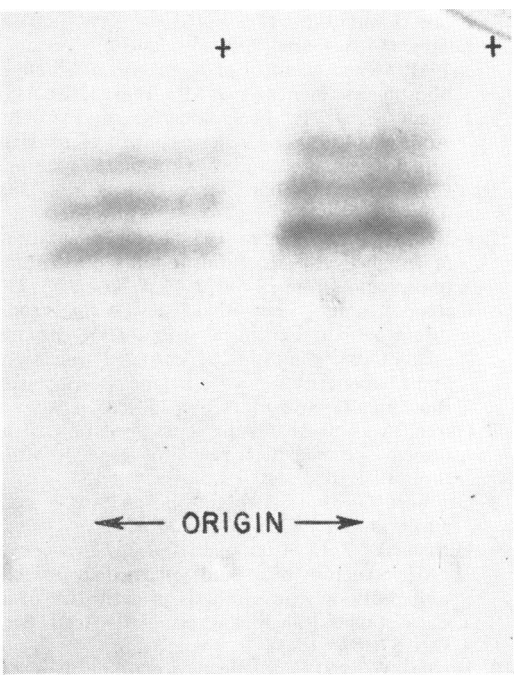

FIG. 2. *Starch gel electrophoresis of alkaline phosphatase in extracts from E. coli W-3747 (right) and LT·2/F'13 (left). The presence of enzyme is revealed by a histochemical stain (8). Electrophoresis was at pH 8.0 for 1 hr, according to published procedures (28).*

There is evidence that similar, if not identical, lipid carriers are involved in the transport of small molecular weight precursors of bacterial cell walls in *S. anatium* (32) and in *Micrococcus lysodeikticus* (9), two organisms that are more distantly related than the two organisms studied here. This type of carrier, however, may not be adequate for the transport of a large polypeptide chain. Thus far, no data have appeared which suggest that there are proteins that act as highly specific carriers of large polypeptide chains. However, if there were such a protein for alkaline phosphatase, then there would be a reasonable possibility that its gene would be linked to the phosphatase structural gene. Genetic analyses of mutants with altered enzymes that are closely related metabolically have shown that, in about half the cases, the genes for these proteins are closely linked on the bacterial chromosome (23, 31).

The hypothesis that localization of alkaline phosphatase is a consequence of its structure could be considered as an extension of the theory that the quarternary structure of a protein is

determined by its amino acid sequence (2, 4, 24). Recent experiments showed that phosphatase subunits were secreted into the culture medium by spheroplasts that continually made phosphatase-like protein (27). However, the information obtained thus far from studies on the properties and conformation of the alkaline phosphatase subunit and dimer offers only indirect evidence for a possible role of the subunit in transport. The subunit is more stable in the presence of anionic detergents (25) and, unlike the dimer, adsorbs strongly to surfaces (J. A. Reynolds and M. J. Schlesinger, *unpublished data*). Titrimetric studies indicate that almost all of the polar groups in the subunit are exposed to solvent (21), although there are significant differences in protein conformation between the alkaline phosphatase subunit and dimer (20). It may be possible to determine whether there is a correlation between protein structure and enzyme location by utilizing mutationally altered forms of the enzyme and by inserting amino acid analogues into the protein. Analyses of one mutant (26) and one analogue (29) have shown that changes in structure that prevented the subunits from dimerizing did not affect localization of the protein to the periplasmic space, and these altered subunits were released from cells by spheroplast formation. Experiments of this type are being continued in order to relate protein conformation to the transport process.

On the basis of electron micrographs of cells making phosphatase, an extrusion process has been proposed to account for the location of *E. coli* alkaline phosphatase (14). Enzymatic activity was also observed in nodules on the bacterial cell surface. Although in the cell this enzyme is accessible to small substrate molecules, recent attempts to bind fluorescent-labeled antibodies to bacteria containing alkaline phosphatase have failed (D. Attardi and M. J. Schlesinger, *unpublished data*). Apparently, this protein is not immediately at the surface of the bacterium.

Our results also show that the isozymes of alkaline phosphatase are not caused strictly by formation of the protein in the *E. coli* cytoplasm. Arguments similar to those cited above could be made for the appearance of isozymes, since they are found in the *S. typhimurium* heterogenotes. However, other data strongly suggest that the isozyme pattern is more closely related to the growth conditions of the culture, and that isozymes result from chemical modifications that occur after active enzyme has been formed (28).

Finally, it is noteworthy that phosphatase formation is repressed in the *S. typhimurium* heterogenote during the exponential phase of growth. In *E. coli*, repression requires (3, 7) the product of tree genes (phospho R_1, R_{2A}, R_{2B}) but only the R_1 gene is present on the episome introduced into *S. typhimurium*. If the other two genes are also required for repression in the heterogenote, then *S. typhimurium* must supply the products of the other regulatory genes. It is unlikely that a cell would make "regulatory" molecules for a protein it does not synthesize; it seems more probable that the R_2 regulator genes are not specific to the control of alkaline phosphatase formation in *E. coli*.

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