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# Conditions Leading to Secretion of a Normally Periplasmic Protein in *Escherichia coli*

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**The phosphate-binding protein (PhoS) is a periplasmic protein which is part of the high-affinity phosphate transport system of *Escherichia coli*. Hyperproduction of PhoS in strains carrying a multicopy plasmid containing *phoS* led to partial secretion of the protein. By 6 h after transfer to phosphate-limiting medium, about 13% of the total newly synthesized PhoS was secreted to the medium. Kinetic studies demonstrated that this secretion consists of newly synthesized PhoS. This secretion occurs in PhoS-hyperproducer strains but not in a PhoS-overproducer strain. Another type of secretion concerning periplasmic PhoS was observed in both PhoS-hyperproducer and PhoS-overproducer strains. This mode of secretion depended upon the addition of phosphate to cells previously grown in phosphate-limiting medium.**

Periplasmic and outer membrane proteins of *Escherichia coli* are synthesized as precursors that contain an amino-terminal signal sequence of 15 to 30 residues which is removed during export from the cell (22). These proteins are synthesized on polysomes that are associated with the inner membrane (26, 28, 32). Periplasmic proteins do not diffuse across the outer membrane, and only recently have a number of gram-negative bacteria, including *E. coli* K-12, been recognized for their ability to secrete proteins (18, 24). To date, the secretion mechanisms have not been determined.

In this study, we determined conditions which promote the release of a normally periplasmic protein into the medium. The phosphate-binding protein PhoS is a periplasmic protein which belongs to a high-affinity phosphate transport system, which also comprises several other proteins (1, 30, 31). The synthesis of these proteins is induced under conditions of phosphate starvation, and the entire sequence of the *phoS* gene has been reported (19, 29). In strains carrying a multicopy plasmid containing the *phoS* gene (20), hyperproduction of the phosphate-binding protein results in saturation of export sites (consistent with the idea of a limited number of these sites [12]). Overproduction of PhoS reduces its own rate of maturation as well as that of other periplasmic proteins (4). Under these conditions, pre-PhoS is accumulated in the inner membrane and in the cytoplasm. This was demonstrated both by electron microscopy and by cell fractionation (6, 23). We now demonstrate that beyond a threshold overproduction of periplasmic proteins, newly synthesized PhoS is released into the cell medium. This type of secretion has been observed both in the presence and in the absence of $P_i$ in the medium. Another type of secretion, which does not specifically concern newly synthesized PhoS, has been obtained in bacteria that produce large amounts of PhoS when phosphate is added to previously phosphate-starved cells.

In this paper, to avoid confusion, the extracellular protein is called secreted PhoS, in contrast to that located in the periplasmic space, which is exported PhoS.

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## MATERIALS AND METHODS

[$^{14}$C]serine (150 mCi/mmol) and $^{14}$C-labeled (1.75 Ci/g) amino acids were purchased from the Commissariat à l'Energie Atomique. All reagents were of the best grade available.

**Bacterial strains and media.** *E. coli* ANCC75 (*leu purE trp his argG rpsL met thi phoS*) and C600 (F$^-$ *leu thr thi lacY*) and the plasmids pSN5182, pSN507, pTD101, and pAJ202 have been previously described (2, 4, 10, 20). High-phosphate medium (0.64 mM) and low-phosphate medium (0.064 mM) containing 10 μg of tetracycline per ml, as well as the conditions for induction of PhoS synthesis, were previously described (20, 23).

**Biosynthesis and secretion of PhoS to the medium.** Cells grown overnight in Tris-glucose high-phosphate medium were harvested by centrifugation and washed. They were then suspended in low-phosphate medium at an optical density at 600 nm of 0.5 and incubated at 37°C in a gyratory water bath. Samples ($10^8$ cells) were collected by centrifugation. Proteins in the supernatant fluid (extracellular fluid) were immediately treated with 10% cold (4°C) trichloroacetic acid. The trichloroacetic acid precipitates were washed twice with 90% acetone and dried. Proteins were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and stained with Coomassie brilliant blue. The protein patterns were analyzed by densitometer scanning.

**Pulse-labeling and chase.** At various times after transfer to low-phosphate medium, samples ($10^9$ cells) were removed and $^{14}$C-labeled amino acids (2 μCi/ml) or [$^{14}$C]serine (2.5 μCi/ml) was added. Cells pulse-labeled for 2 min were removed by centrifugation, and the supernatant fluids were precipitated with trichloroacetic acid. Chases were done by adding a nonradioactive amino acid mixture (final concentration, 10 mg/ml) or serine (25 mM) in the absence or presence of 5 mM phosphate. Samples were removed at intervals and treated as described above. Cell pellets and trichloroacetic acid precipitates were treated by the method of Anba et al. (4).

**SDS-polyacrylamide gel electrophoresis, staining, fluorography, and immunoblot techniques.** SDS-polyacrylamide gel electrophoresis, staining, fluorography, and densitometer scanning were carried out as previously described (3, 4, 23).

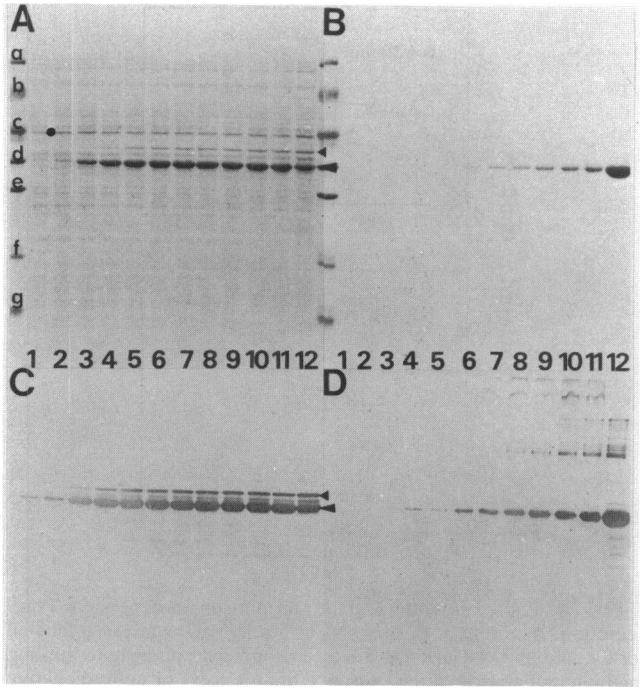


FIG. 1. Secretion of PhoS into the medium by hyperproducing cells. Cell samples of *E. coli* ANCC75(pSN5182) at intervals after transfer to low-phosphate medium were removed by centrifugation. Proteins from the cell pellet (A and C) and from the supernatant medium (B and D) were analyzed by SDS-polyacrylamide gel electrophoresis with Coomassie brilliant blue staining (panels A and B). The immunoblots obtained with a serum directed against PhoS are shown in panels C and D. The times of sampling were 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 h (lanes 1 to 11, respectively. Lane 12: 24-h sampling in low-phosphate medium. The small and large arrowheads indicate the migrations of pre-PhoS and PhoS, respectively. The dot indicates the position of EF-Tu. Molecular size standards (in kilodaltons): a, 94; b, 67; c, 46; d, 36; e, 30; f, 20; g, 14.

Western blotting was carried out as described by Burnette (8), and immunoprinting was performed as described by Coudrier et al. (9).

## RESULTS

Growth of *E. coli* strains carrying a multicopy plasmid encoding the phosphate-binding protein in phosphate-limiting medium results in accumulation of the precursor form of this protein in addition to the mature form (20). Evidence for saturation of export sites and accumulation of two precursor pools, cytoplasmic and membrane associated, was obtained (6, 23). At late times after transfer to phosphate-limiting medium, PhoS began to be secreted into the medium, and this secretion increased with time (Fig. 1B and D). The onset of secretion correlated with the appearance of pre-PhoS accumulation within the cells (Fig. 1A and C). Cell lysis was not responsible for the release of PhoS, as clearly demonstrated by the absence of the precursor form and cytoplasmic proteins in the medium (Fig. 1B). Traces of protein of molecular weight higher than that of mature PhoS in the cell supernatant turned out to be oligomers of PhoS, as shown by immunoblotting (Fig. 1D). These forms may result from trichloroacetic acid precipitation of PhoS.

The elongation factor EF-Tu is one of the major cytoplasmic proteins in *E. coli* (13). Its level was stable in phosphate-limiting medium (Fig. 1A). This protein was suitable as an internal standard, as previously reported (21), to monitor the extent of PhoS production (Fig. 2).

The PhoS/EF-Tu ratio, deduced from densitometer scanning of stained gels, reached a value of about 13 by 10 h after induction. This suggests that individual cells might contain as many as $10^6$ molecules of PhoS.

The production of PhoS reached a certain level before release started (Fig. 2). Both plasmids pSN5182 (4.95 kilobases) and pSN507 (12.45 kilobases) carry the *phoS* gene, but PhoS is less efficiently expressed in strain ANCC75(pSN507) (referred to as an overproducer strain) than in the isogenic strain carrying pSN5182 (referred to as a hyperproducer strain) (Fig. 2). The hyperproducer strain produced over three times more PhoS than the overproducer strain did. In Coomassie blue-stained gels, there was no detectable accumulation of pre-PhoS in ANCC75(pSN507) and no secretion of PhoS (Fig. 2). The lack of PhoS secretion by ANCC75(pSN507) was demonstrated by overloading the gel [fivefold excess over the ANCC75(pSN5182) samples].

There was a delay in PhoS secretion by the hyperproducing strain after transfer to phosphate-limiting medium, and secretion only occurred after a marked accumulation of pre-PhoS was detected (Fig. 1). PhoS synthesis in ANCC75 carrying either pSN5182 or pSN507 was compared by using $^{14}$C-amino-acid pulse-labeling and chase. The extents of labeling of PhoS forms (mature and precursor cellular forms; extracellular form) and cellular EF-Tu during the pulse-chase experiment were evaluated by densitometer analyses. The various PhoS/EF-Tu ratios are presented in Fig. 3. The total PhoS/EF-Tu ratio reflecting the instantaneous rates of synthesis (5) reached a maximum of 28 for strain ANCC75(pSN5182) and 7 for ANCC75(pSN507) by 3 h after transfer, the time required to attain full induction (Fig. 3A). The lower ratios of 13 and 4, respectively, observed with Coomassie blue staining are explained by the fact that EF-Tu is produced in the cells before transfer to low-phosphate

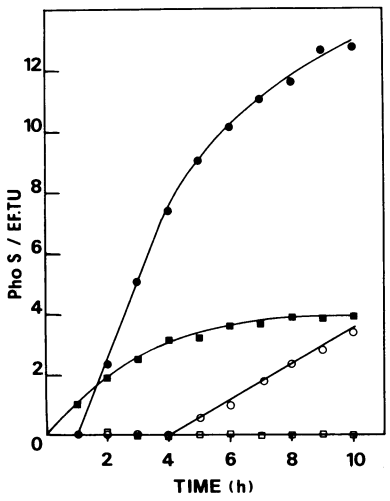


FIG. 2. Triggering of PhoS secretion. Cell samples of *E. coli* ANCC75(pSN507) and ANCC75(pSN5182) were removed at intervals after transfer to low-phosphate medium and centrifuged. Proteins from the cell pellet and from the supernatant medium were analyzed by SDS-polyacrylamide gel electrophoresis. The PhoS/EF-Tu ratios were determined by densitometer scanning analyses of Coomassie blue-stained gels from ANCC75(pSN507) (■, □) and ANCC75(pSN5182) (●, ○). Ratios measured were total PhoS/EF-Tu (■, ●) and extracellular PhoS/EF-Tu (□, ○).

medium. With increasing time of induction, the genes belonging to the *pho* regulon (31) become more highly expressed, while the expression of other genes decreases owing to the slow growth of the cells. Thus the ratio of 4 between the level of PhoS synthesis by the two plasmids, obtained from pulse-labeling, is in good agreement with the results of Coomassie blue staining.

At early times of induction, the processing of pre-PhoS in ANCC75(pSN5182) was very slow, leading to its highest accumulation at 2 h (Fig. 3B). At this time, about 20% of total PhoS was detected as precursor form (pre-PhoS/EF-Tu = 6 [Fig. 3B]; total PhoS/EF-Tu = 28 [Fig. 3A]), the rest (80%) corresponding to the mature form exported to periplasmic space. The pre-PhoS was cleaved during the chase, as previously observed (23). The overshoot in newly synthesized pre-Phos was not detected in Coomassie blue-stained gels (Fig. 1). In ANCC75(pSN507), the precursor form was detected only at 2 h after transfer and the pre-PhoS/EF Tu ratio reached a steady-state level at 4 h (Fig. 3B).

By 3 h after induction, some PhoS was secreted in the medium (Fig. 3C), and the amount secreted increased with time. This secretion was observed only with hyperproducer cells and not with overproducer cells, even when the gels were overloaded or the film was overexposed in the latter case. The amount of secreted PhoS was similar after 10 and 20 min of chase (Fig. 3C).

It is important that along with nonradioactive amino acids, 3 mM $P_i$ was added during the chase. This addition caused an arrest in de novo PhoS synthesis. Thus, the results strongly suggest that the secreted PhoS is newly synthesized protein. From the densitometer scannings of the fluorogram areas corresponding to cellular (mature and residual precursor forms) and extracellular PhoS, we calculated that about 13% of the pulse-labeled protein was secreted to the medium during the chase by 6 h after transfer in low-phosphate medium. We have checked that the $NH_2$-terminal sequence of secreted PhoS is identical to that of authentic mature PhoS (data not shown).

We also observed that not only newly synthesized PhoS but also mature PhoS, which is accumulated in large amounts in the periplasmic space under these growth conditions (3, 23), could be secreted. Cell proteins in the hyperproducer strain were pulse-labeled at 5 h after transfer to low-phosphate medium and chased in the presence or absence of $P_i$ (Fig. 4). Irrespective of the $P_i$ addition, a small percentage of labeled PhoS was secreted rapidly during the first 30 min of chase. There was almost no further release of PhoS (Fig. 4) between 30 and 180 min of chase, the amount detected in the extracellular fluid reaching 15% of the total labeled PhoS after 3 h. In contrast, in the presence of $P_i$ a slow release of PhoS was observed during this time (30 to 180 min). A similar secretion was also obtained when $P_i$ was added after only a 30-min chase (Fig. 4). This release thus corresponds to mature PhoS previously accumulated in the periplasmic space. Such a phosphate-dependent release was also observed in the overproducer strain (Fig. 5), which did not secrete PhoS in absence of phosphate.

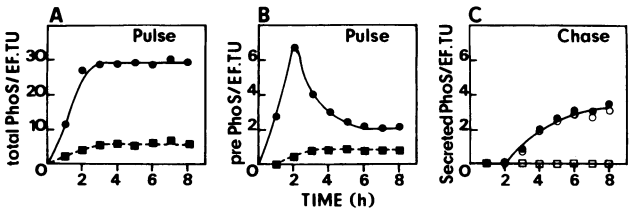


FIG. 3. Secretion of newly synthesized PhoS. Cell samples of *E. coli* ANCC75(pSN507) and ANCC75(pSN5182) were removed at hourly intervals after transfer to phosphate-limiting medium, pulse-labeled for 2 min with $^{14}C$-amino acids, and chased for 10 min (○, □) or 20 min (●, ■) in the presence of an excess of nonradioactive amino acids (10 mg/ml) containing 3 mM phosphate. The samples were centrifuged, and proteins in the supernatant and in the cell pellet were analyzed by SDS-polyacrylamide gel electrophoresis. (A) Total PhoS/EF-Tu, (B) pre-PhoS/EF-Tu, (C) secreted PhoS/EF-Tu for ANCC75(pSN507) (■, □) and ANCC75(pSN5182) (●, ○) were determined by densitometer scanning of fluorograms. Only one symbol (□) is indicated for secreted PhoS/EF-Tu (panel C) in the case of ANCC75(pSN507); no material was detected in the supernatant fluids for the 10- or 20-min chase throughout the experiment.

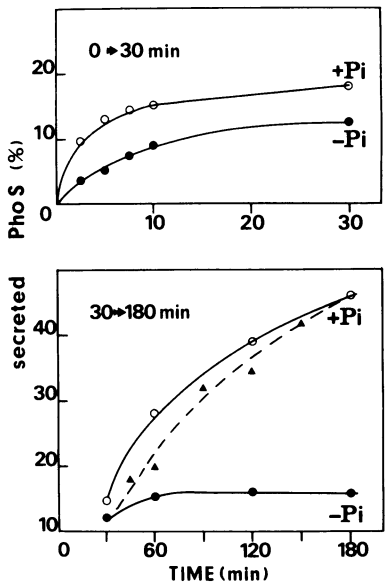


FIG. 4. Phosphate-independent and phosphate-dependent PhoS secretion. A culture of *E. coli* ANCC75(pSN5182) was pulse-labeled for 2 min with [$^{14}C$]serine at 5 h after transfer to phosphate-limiting medium and chased in the presence of an excess of nonradioactive serine (25 mM) and in the absence or presence of 5 mM phosphate. Samples were removed at intervals during the chase; proteins from the cell pellet and from the supernatant medium were analyzed by SDS-polyacrylamide gel electrophoresis and fluorography. The percentage of secreted PhoS was calculated by densitometer scanning analysis. The kinetics of release during the first 30 min and beyond this time are shown separately. Symbols: ●, chase performed in the absence of phosphate; ○, chase performed in the presence of phosphate; ▲, phosphate added after 30 min of chase.

Periplasmic-leaking mutants, altered in their cell permeability and displaying a pleiotropic pattern of protein excretion, have been previously isolated (15). One of them was transformed with pSN5182 and tested for PhoS secretion. An efficient secretion was observed. However, in contrast to strain ANCC75, the leaky mutant showed as much as 14% cellular lysis at 5 h after induction of PhoS synthesis, as monitored by assays of β-galactosidase activity in the cells and supernatant medium (not shown).

It has recently been reported that mutants carrying a *pst* or *phoS* mutation excrete periplasmic proteins into the growth medium (14). We therefore tested the secretion of PhoS in strain C600, which, in contrast to ANCC75, is altered in neither *phoS* nor *pst*. We also tested the possibility of secretion of β-lactamase, another periplasmic protein. Strain

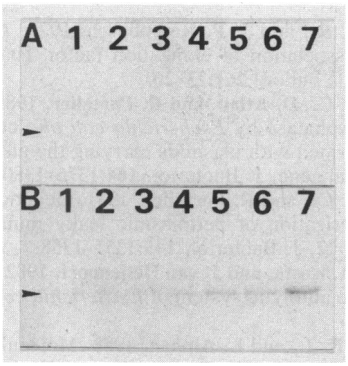


FIG. 5. Secretion of periplasmic PhoS into the medium by PhoS-overproducer cells in the presence of phosphate. A culture of *E. coli* ANCC75(pSN507) was pulse-labeled for 2 min with [$^{14}$C]serine at 6 h after transfer to phosphate-limiting medium and chased in the presence of an excess of nonradioactive serine (25 mM) and in the absence (A) or presence (B) of 5 mM phosphate. Samples were removed at intervals (5, 10, 15, 30, 60, 90, and 120 min; lanes 1 to 7, respectively) during the chase and centrifuged. Proteins from the supernatant medium were analyzed by SDS-polyacrylamide gel electrophoresis and fluorography. The arrowheads indicate the migration of PhoS.

C600 was cotransformed with two compatible high-copy-number plasmids bearing *phoS* and *bla* (plasmids pAJ202 and pTD101, respectively) (4, 10). Secretion of both PhoS and β-lactamase was observed when $P_i$ was added to phosphate-starved cells containing these plasmids (Fig. 6). This suggests that secretion is a consequence of hyperproduction of periplasmic proteins in cells grown under phosphate limitation but does not depend upon the presence of *phoS* or *pst* mutation in the host strain. The amount of secreted material in both cases appeared to be roughly proportional to the total amount of the particular protein synthesized.

## DISCUSSION

Early steps in the export process in *E. coli* are shared by outer membrane proteins and periplasmic proteins. Neither the targeting signals for outer membrane proteins nor the localization mechanisms have been defined, despite much effort (for a review, see reference 7). However, several lines of evidence indicate that the number of export sites is limited in *E. coli*. Ito et al. (12) demonstrated this feature by using hybrid maltose-binding protein β-galactosidase proteins that cannot be exported and cause jamming of the export machinery. A similar effect can be obtained by overwhelming this machinery. The production of very large amounts of PhoS leads to the accumulation of pre-PhoS (20). This accumulation occurs both in the inner membrane and in the cytoplasm (6, 23) and causes a marked reduction in the rate of maturation of other periplasmic proteins (β-lactamase and alkaline phosphatase) in addition to that of PhoS (4, 23). Under these conditions, leader peptidase is not rate limiting (4, 6). Thus we were led to conclude that the extremely high PhoS production leads to saturation of export sites, presumably by competing for the rate-limiting component of export apparatus (5).

In this paper, we demonstrate that newly synthesized PhoS is partly secreted to the medium in ANCC75(pSN5182) but not in ANNC75(pSN507). The difference in the amount of PhoS produced by these strains was first evidenced by Morita et al. (20). We now show that there is about a four-fold difference in levels of PhoS synthesis between these two strains. This must not be taken to mean that ANCC75(pSN507) produces small amounts of PhoS, since the PhoS/EF-Tu ratios indicate there are about 250,000 copies per cell. However, this is not enough to cause the accumulation of pre-PhoS detectable by staining after ordinary SDS-polyacrylamide gel electrophoresis, and pulse-labeling must be used for this purpose (Fig. 3). In contrast, so much pre-PhoS is accumulated in ANCC75(pSN5182) that it can be easily detected after Coomassie blue staining of gels (Fig. 1). We therefore believe that the secretion of newly synthesized PhoS is related to an extremely high production of PhoS in ANCC75(pSN5182). This would also explain why this secretion is only detected about 3 h after induction, since this is the time required to reach full induction.

Several hypotheses might explain the secretion of newly synthesized pre-PhoS. Since after a pulse-labeling, only about 15% of the PhoS reaches the medium, even at long chase times, it is clear that the mechanism of secretion is not a nonspecific leakage from the periplasm, because otherwise the labeled protein would continue to be released at a constant rate. Thus it seems that the protein is competent for secretion for only a limited time after its synthesis. The precursor that is slowly processed and seems to give rise to the extracellular PhoS might associate with export sites that are defective (6, 23). This defect could be the result of damage to the cell envelope by phosphate starvation (11) or overproduction of the periplasmic protein; PhoS might appear in the medium by an abortive pathway.

Alternatively, the localization processes of cell most probably being based upon kinetics of production and affinity of targeting signals for cell components (7, 25, 27), the high level of PhoS production could conceivably result in an overwhelming of these localization mechanisms, leading to an aberrent interaction with the cell envelope and release to the medium.

With regard to the secretion of periplasmic PhoS upon $P_i$ addition to the cells, the mechanism is as yet unknown. It is likely that this type of secretion is due to some osmotic effect, i.e., many molecules of water penetrate with phosphate ions to the periplasmic space and lead to cell swelling and membrane leakiness. It has been previously reported that the growth of cells under phosphate starvation causes the synthesis of defective lipopolysaccharide (17). It is possible that after $P_i$ addition, the modification of the outer membrane structure could also be involved in the release of the periplasmic proteins. The addition of $P_i$ to growing cells

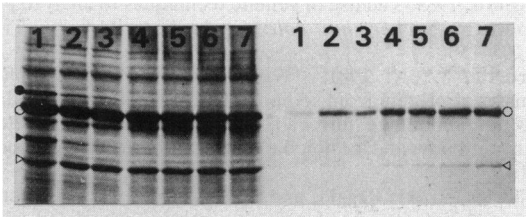


FIG. 6. Secretion of β-lactamase in addition to PhoS by phosphate-starved cells when phosphate is added to the medium. A culture of *E. coli* C600(pAJ202,pTD101) was pulse-labeled for 2 min with $^{14}$C-amino acids (lane 1) at 5 h after transfer to low-phosphate medium and chased in the presence of an excess of unlabeled amino acids (1%) and 3 mM phosphate. Samples were removed at intervals (10, 30, 45, 60, 90, and 180 min; lanes 2 to 7, respectively), and proteins from the cell pellet (left) and from the supernatant medium (right) were analyzed by SDS-polyacrylamide gel electrophoresis. Solid and open circles indicate, respectively, the migration of pre-PhoS and PhoS; solid and open triangles indicate, respectively, the migration of pre-β-lactamase and β-lactamase.

has been shown to trigger the autolytic activities (16); such a gentle autolytic phenomenon could explain the release of periplasmic proteins to the medium.

In the experiments that are described here, we demonstrated that massive overproduction of a periplasmic protein leads to a substantial extracellular release of the newly synthesized protein. It is hoped that further analysis of this aberrant localization will shed light on the nature of the sites at which proteins are normally translocated across the cytoplasmic membrane. Furthermore, the addition of $P_i$ to phosphate-starved cells that have accumulated large amounts of PhoS appears to result in a general release of periplasmic proteins. It would be interesting to know whether this release depends specifically on the overproduced protein (PhoS) or the phosphate, and experiments are in progress to answer these questions.

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