APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Nov. 1986, p. 1157–1161
0099-2240/86/111157-05$02.00/0

Vol. 52, No. 5

Localization of Inclusion Bodies in *Escherichia coli* Overproducing β-Lactamase or Alkaline Phosphatase

GEORGE GEORGIOU,[1]† JOHN N. TELFORD,[2] MICHAEL L. SHULER,[1] AND DAVID B. WILSON[2]*

School of Chemical Engineering[1] and Department of Molecular and Cell Biology,[2] Cornell University, Ithaca, New York 14853

Received 21 April 1986/Accepted 13 August 1986

High-level synthesis of the periplasmic protein β-lactamase in *Escherichia coli* caused the formation of insoluble protein precipitates called inclusion bodies. β-Lactamase inclusion bodies differed from those reported previously in that they appeared to be localized in the periplasmic space, not in the cytoplasm. The inclusion bodies contained mature β-lactamase and were solubilized more easily than has been reported for cytoplasmic inclusion bodies. In contrast, overproduction of the periplasmic protein alkaline phosphatase caused the formation of cytoplasmic inclusion bodies containing alkaline phosphatase precursor.

Proteins can be overproduced by introducing their structural genes downstream from a strong promoter in a high-copy-number plasmid. A number of plasmids containing strong promoters have been constructed and used to obtain high-level expression of the protein product of genes cloned into them by using *Escherichia coli* as a host strain. Levels of synthesis as high as 50% of the total cellular protein have been reported. Frequently, the protein product aggregates inside the cell, forming cytoplasmic inclusion bodies (17). These are formed by either covalent (15) or hydrophobic interactions between protein molecules. The proteins present in inclusion bodies are usually not biologically active. Regeneration of their activity is sometimes possible by solubilization of the protein aggregate under denaturing conditions, followed by the slow removal of the denaturing agent, allowing the protein to refold into its active conformation.

Inclusion body formation is known to result from the accumulation of cytoplasmic proteins. It has been generally assumed that intracellular protein precipitation can be avoided if the protein is secreted through the inner membrane. In gram-negative cells, secreted proteins are translocated into the periplasmic space, i.e., the aqueous compartment which is formed between the inner and the outer membranes (6, 18). Its composition, pH, and redox potential are different from those of the cytoplasm, and these factors all affect protein solubility. We show here that inclusion bodies can also result from the overproduction of secreted proteins. The inclusion bodies which are formed by the periplasmic enzyme β-lactamase are distinctly different from other inclusion bodies previously reported in *E. coli* in that they are localized predominantly in the periplasmic space. In contrast, overproduction of another periplasmic enzyme, alkaline phosphatase, results in the accumulation of the precursor (7) of this protein in cytoplasmic inclusion bodies.

MATERIALS AND METHODS

E. coli RB791 (*lacI*$^{q}_{L8}$) (1) was used as a host. The plasmid pTac11 is a 4.6-kilobase derivative of pBR322 that carries the *tac* promoter between the *Eco*RI and the *Hin*dIII restriction sites (1). Plasmid pHI-7 (8), which carries the *phoA* gene coding for the periplasmic enzyme alkaline phosphatase, was a gift from Jon Beckwith.

Growth conditions. Cells were grown in M9 medium supplemented with 0.2% fructose, 0.2% casein amino acids, and 100 μg of ampicillin per ml. A low-phosphate medium (11) supplemented as described above but containing 0.1% casein amino acids was used to induce alkaline phosphatase. β-Lactamase was induced by growing cultures at 37°C to an optical density at 600 nm of approximately 0.500, at which point isopropyl-β-D-thiogalactopyranoside (IPTG) was added to give a final concentration of 10^{-4} M.

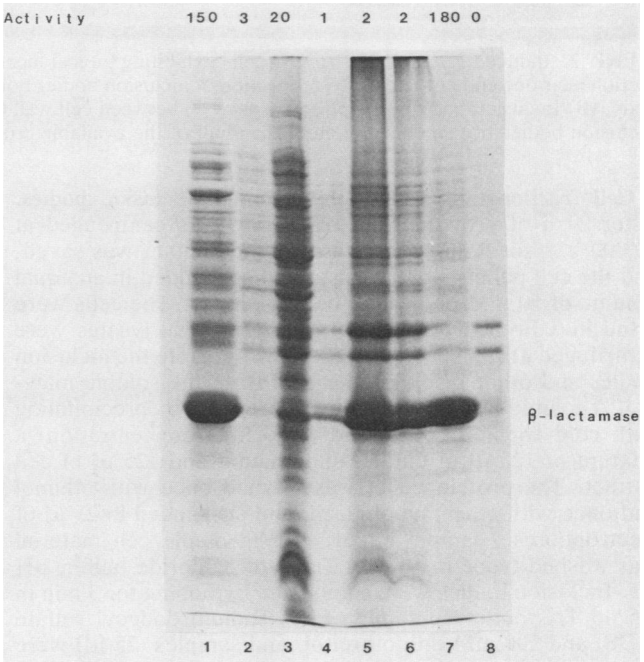

FIG. 1. Analysis of β-lactamase distribution by SDS-polyacrylamide gel electrophoresis. Each fraction corresponds to 250 μl of culture. Lanes: 1, extracellular fluid; 2, wash; 3, soluble lysate; 4 to 8, insoluble pellet treated with the following: 4, 3% chloroform; 5, 0.1% SDS; 6, 0.25% deoxycholate; 7, 6 N guanidine hydrochloride; 8, pellet from lane 7. Activities of β-lactamase in each fraction are given in units per milliliter of culture.

* Corresponding author.
† Present address: Department of Chemical Engineering, The University of Texas at Austin, Austin, TX 78712.

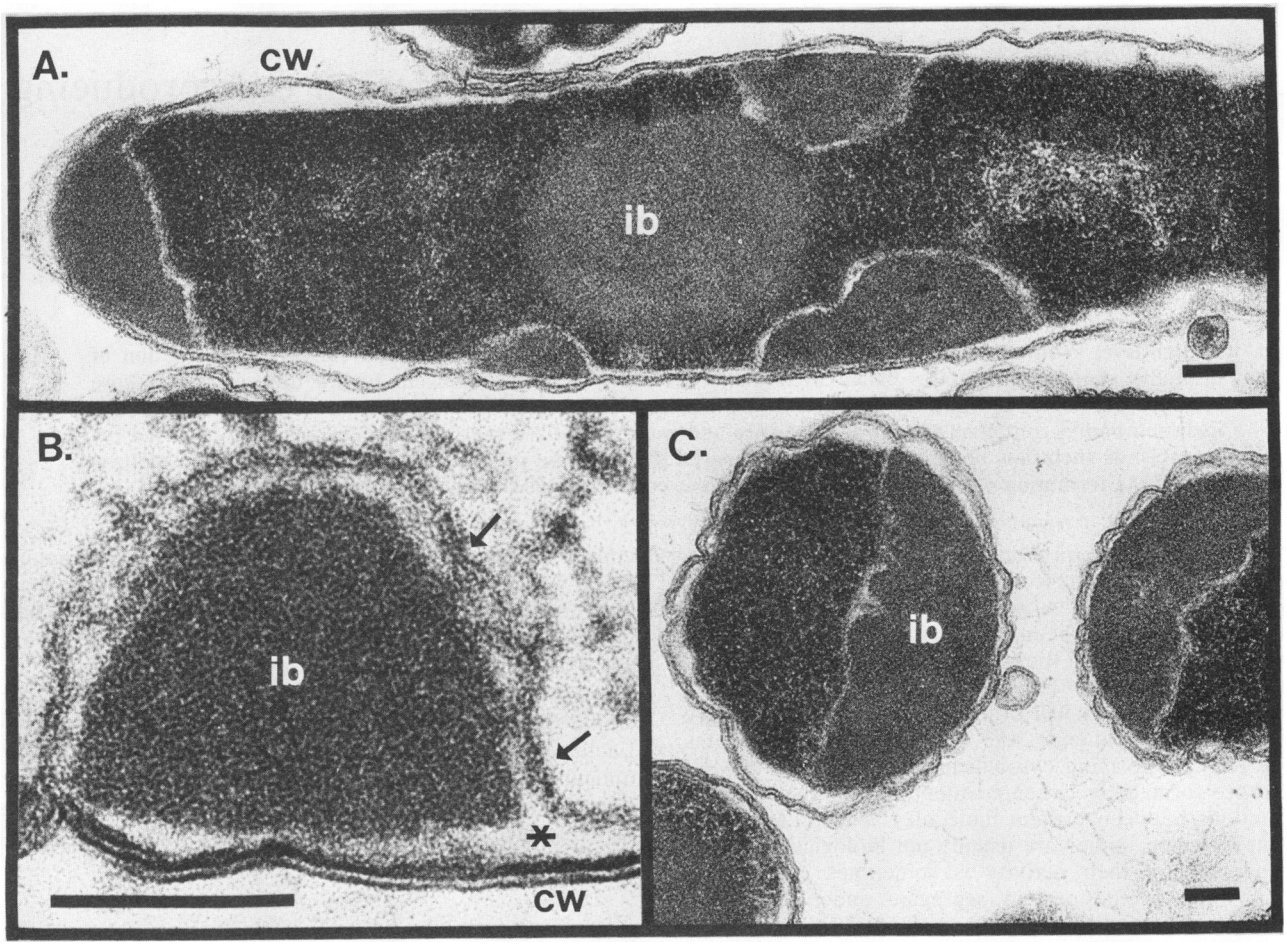

FIG. 2. Induced *E. coli* RB791(pKK) cells exhibiting typical locations of β-lactamase inclusion bodies. Bars = 100 nm. (A) Longitudinal section near one end of the cell. Note location of inclusion bodies both laterally and apically in periplasmic space. cw, Cell wall; ib, inclusion body. (B) Inclusion body in periplasmic space (*) between cell wall (cw) and plasma membrane (arrows). (C) Cross section of several cells. Inclusion bodies (ib) can occupy more than half of the available cross-sectional area of the cell.

Cell fractionation and solubilization of inclusion bodies. After 24 h of growth, 5 ml of culture was centrifuged at 10,000 × *g* for 8 min. The clarified supernatant was saved, and the cell pellets were washed and suspended in an equal volume of 50 mM phosphate buffer, pH 6.5. The cells were lysed in a French press at 20,000 lb/in^2. The lysates were centrifuged at 13,300 × *g* for 10 min to separate the inclusion bodies and other insoluble material from the soluble material. A soluble-protein fraction was prepared by precipitating with cold trichloroacetic acid (10% final concentration) a mixture of 125 μl of culture supernatant and 125 μl of cell extract. The protein pellet was washed once with ethanol and once with ether, lyophilized, and suspended in 25 μl of electrophoresis sample buffer. The insoluble cell material was washed twice in 50 mM Tris hydrochloride buffer, pH 7.5. Inclusion bodies were solubilized by boiling for 3 min in 0.5 ml Tris buffer containing 1.0% sodium dodecyl sulfate (SDS) and 100 mM dithiothreitol, and samples (25 μl) were mixed with 5× concentrated electrophoresis sample buffer. Solubilization of the inclusion bodies was also attempted by the following procedures. (i) Samples of resuspended insoluble material in Tris buffer were mixed with the following solubilizing agents: 0.1% SDS, 0.25% deoxycholate, 3.0% chloroform, or 2.0% Triton X-100. After 30 min of incubation at 37°C, the samples were centrifuged at 13,300 × *g* for 15 min. The pellets were solubilized by boiling for 5 min in electrophoresis sample buffer. Proteins from the supernatant fractions were precipitated with 10% trichloroacetic acid as described above. (ii) The insoluble cell material was resuspended in 1.0 ml of buffer I (50 mM Tris hydrochloride [pH 7.9], 250 mM KCl, 1 mM dithiothreitol, and 0.1 mM EDTA) containing 6 N guanidine hydrochloride. After 45 min of incubation at 0°C, the mixture was diluted 10-fold by dropwise addition of cold buffer I. The final solution was dialyzed against 1 liter of the same buffer. The dialysate was centrifuged at 13,300 × *g* for 25 min to separate solubilized β-lactamase from cell debris.

Other methods. The activity of β-lactamase was measured as described previously (4). Total soluble protein was determined by the method of Bradford (3).

SDS-polyacrylamide gel electrophoresis was performed in a 12% gel (10). The composition of the sample buffer was as follows: 2.0% SDS, 100 mM dithiothreitol, 50% glycerol, 0.4% Tris hydrochloride (pH 6.8). Before electrophoresis, samples were heated for 5 min at 100°C.

Electron microscopy. Late-exponential-phase cells were harvested by centrifugation and suspended in 0.1 M sodium cacodylate, pH 6.9, containing 4% glutaraldehyde and 10 mM CaCl. After centrifugation, the pellet was washed with buffer alone, postfixed in 1% OsO_4 in sodium cacodylate

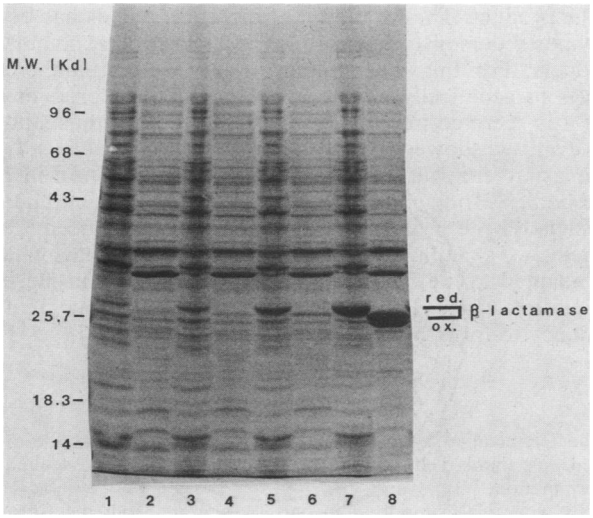

FIG. 3. Insoluble and soluble proteins in cultures induced with different concentrations of IPTG. The total activity of β-lactamase in units per milliliter of culture is given below in parentheses. Lanes: 1, 3, 5, and 7, insoluble fractions; 2, 4, 6, and 8, soluble fractions; 1 and 2, uninduced (9.6 U/ml); 3 and 4, 1×10^{-5} M IPTG (27.0 U/ml); 5 and 6, 2×10^{-5} M IPTG (68.0 U/ml); 7 and 8, 5×10^{-5} M IPTG (137.0 U/ml). Numbers at left indicate molecular weight markers.

buffer (pH 6.9), rinsed in buffer and in distilled water, dehydrated through an ascending series of ethanol and two changes of propylene oxide, and embedded in Epon 812 (5). The embedded cells were sectioned with a diamond knife. The sections, supported on bare, 300-mesh Athene-type copper grids, were poststained with uranyl acetate and lead citrate.

RESULTS

We reported previously (4) that RB791(pTac11) cells induced with 10^{-4} M IPTG excrete up to 95% of the soluble β-lactamase into the culture supernatant. The active β-lactamase constitutes approximately 15% of the total soluble protein of overnight cultures. However, we did not look for insoluble β-lactamase. The pellet from centrifugation of extracts of induced cells gave an intense protein band migrating in the position of β-lactamase after SDS-polyacrylamide gel electrophoresis (Fig. 1). This band reacts with anti-β-lactamase serum when tested by an immunoblot procedure (16). When the pellet was extracted with 6 N guanidine hydrochloride, the band was solubilized (Fig. 1, lanes 7 and 8). Finally, when the guanidine hydrochloride was removed slowly, β-lactamase activity was restored, giving a preparation of β-lactamase that was 90% pure as determined by SDS-polyacrylamide gel electrophoresis and with a specific activity about 90% of that of homogeneous β-lactamase. The presence of inclusion bodies was confirmed by electron micrographs of β-lactamase-overproducing cells. Irregular, densely staining areas were readily discerned in induced RB791(pTac11) cells. These areas were predominantly localized adjacent to the surface, although some spanned the cross section of the cell (Fig. 2B and C). There were a few β-lactamase inclusion bodies that appeared to be surrounded by the cytoplasm (Fig. 2A) and may be artifacts that arose during the fixation and sectioning steps, or it is possible that some cytoplasmic inclusion bodies were present.

Once the presence of inclusion bodies was confirmed, the culture conditions which result in protein precipitation were characterized further. Neither growth in LB media, nor the presence of divalent ions, nor the optical density at which the cultures were induced affected the formation of inclusion bodies. The effect of varying the level of β-lactamase synthesis was studied by inducing the *tac* promoter of the pTac11 plasmid with different concentrations of IPTG. The results from these experiments are shown in Fig. 3. Induction of the *tac* promoter with a 2×10^{-5} M final concentration of IPTG gave a sevenfold increase in the total (i.e., intracellular plus extracellular) β-lactamase activity over the basal level but did not cause the formation of inclusion bodies (Fig. 3, lanes 5 and 6), even though β-lactamase was estimated to be about 8% of the total soluble protein. Induction with 5×10^{-5} M IPTG resulted in the formation of inclusion bodies, and under these conditions the precipitated β-lactamase typically represents about 35% of the insoluble cellular protein. This percentage can be even higher in fully induced cultures (IPTG, $>10^{-4}$ M) because the synthesis of a number of insoluble membrane proteins is repressed (Georgiou et al., manuscript in preparation).

The β-lactamase in the insoluble fraction had a higher electrophoretic mobility than the soluble enzyme (Fig. 3) when our standard electrophoresis sample buffer was used, presumably because the β-lactamase is present in an oxidized form (12). Consistent with this conclusion, when a higher final concentration of dithiothreitol (0.25 M) was used in the electrophoresis sample buffer, the correct mobility was restored.

The ability of different compounds to solubilize the precipitated β-lactamase was examined. The ionic detergent SDS at a concentration of 0.1% gave complete solubiliza-

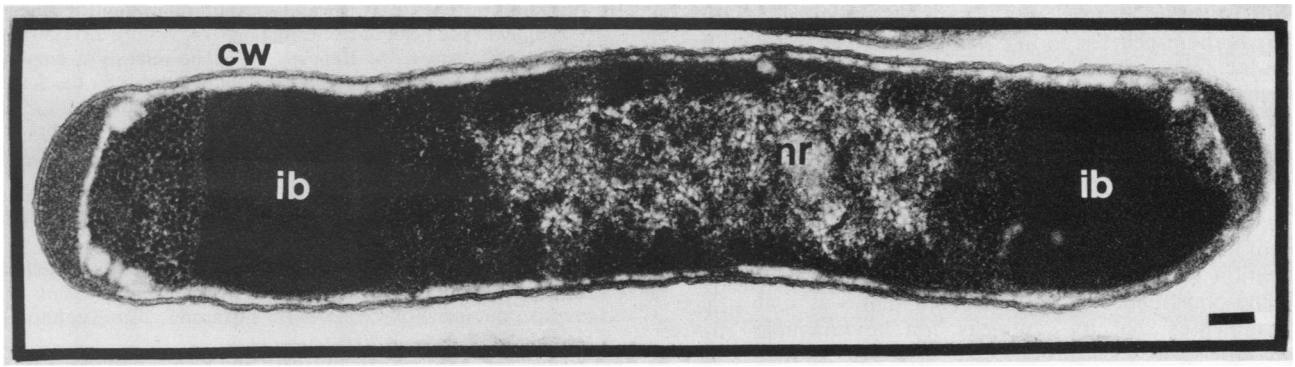

FIG. 4. Induced *E. coli* RB791(pHI-7) exhibiting typical alkaline phosphatase inclusion bodies (ib). Bar = 100 nm. nr, Nuclear region; cw, cell wall.

tion. Deoxycholate (0.25%) and Triton X-100 (2.0%) gave partial solubilization (approximately 65% as estimated by scanning the gels with a densitometer). Chloroform, a membrane-permeating agent, had no effect on the inclusion bodies (Fig. 1). In general, solubilization of the β-lactamase inclusion bodies was achieved under relatively mild conditions, compared with those used by Kawaguchi et al. (9) for the dissolution of cytoplasmic inclusion bodies.

Overproduction of the other secreted protein tested was also found to cause the formation of inclusion bodies. In RB791(pHI-7) cells grown in a low-phosphate medium, more than 85% of the alkaline phosphatase was present in insoluble form, as estimated by densitometry scanning of SDS-polyacrylamide gels stained with Coomassie blue. Attempts to obtain alkaline phosphatase activity by solubilizing the inclusion bodies in 6 N guanidine hydrochloride in the presence of 1 mM $ZnCl_2$ followed by dilution and dialysis were unsuccessful. Furthermore, electron micrographs of alkaline phosphatase-overproducing cells (Fig. 4) show clear evidence of cytoplasmic inclusion bodies. To determine whether the inclusion bodies result from the accumulation of mature alkaline phosphatase or of its precursor form, the inclusion body protein was purified and the amino acid sequence of the amino terminus was analyzed. The sequence of the first seven amino acids of the precipitated protein was determined and found to be identical to that reported for alkaline phosphatase precursor (7).

DISCUSSION

The cells which contained inclusion bodies appeared elongated compared with normal *E. coli* cells. This phenomenon has been frequently observed in protein-overproducing cultures (14, 15). Inclusions resulting from the overproduction of cytoplasmic proteins tend to have discrete boundaries with the rest of the cytoplasm but are not surrounded by membranes, and this was seen with the alkaline phosphatase inclusion bodies (Fig. 4). In contrast, the β-lactamase inclusion bodies were usually distinctly separated from the cytoplasm by a lightly staining boundary. The membranous nature of the light staining material was evident in partially lysed cells where the inclusion bodies were retained between the cell wall and what appears to be the inner membrane (Fig. 2B).

Secreted proteins are synthesized in the cytoplasm in a precursor form which consists of a signal sequence, an extension of about 15 to 20 amino acids, attached to the structural protein (2). The signal sequence contains the information necessary for secretion. This amino acid sequence is cleaved from the precursor once the protein is secreted into the periplasmic space. In the case of β-lactamase, the mature product is enzymatically active, whereas the precursor form is not (13). We have found that the β-lactamase which is present in the insoluble fraction has enzymatic activity after it is renatured. This indicates that the inclusion bodies resulted from the precipitation of secreted β-lactamase. Furthermore, the electron micrographs clearly demonstrate that most β-lactamase inclusion bodies are located in the periplasmic space. In contrast, the alkaline phosphatase inclusion bodies resulted from the accumulation of the precursor form. This result is consistent with the formation of intracellular inclusion bodies. The fact that alkaline phosphatase precursor accumulates at such a high rate indicates that its synthesis can be uncoupled from secretion.

The yield of enzymatically active recombinant proteins can be dramatically affected by the intracellular precipitation of the product. The formation of inclusion bodies has been previously demonstrated only for cytoplasmic recombinant products. For this reason, there have been a number of efforts to genetically engineer the recombinant product so that it is secreted into the periplasmic space. In this study, however, we showed that the overproduction of at least two periplasmic enzymes also results in the formation of inclusion bodies. The cellular location of these inclusion bodies depends on the protein which is being overproduced. The proteins we examined are both native *E. coli* proteins which are adapted to the conditions that typically exist inside the periplasmic space. Foreign proteins may well have a greater tendency to form inclusion bodies.

ACKNOWLEDGMENTS

The technical assistance of Diana Irwin is gratefully acknowledged. We thank Ted Tenhauser for his help in protein sequence determination.

This work was supported in part by a grant from the Cornell Biotechnology Program.

LITERATURE CITED

1. **Amann, E., J. Brosius, and M. Ptashne.** 1983. Vectors bearing a hybrid *trp-lac* promoter for regulated gene expression of cloned genes in *Escherichia coli*. Gene **25**:167–178.
2. **Benson, S. A., M. N. Hall, and T. J. Silhavy.** 1985. Genetic analysis of protein export in *Escherichia coli* K12. Annu. Rev. Biochem. **54**:101–134.
3. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248–252.
4. **Georgiou, G., J. J. Chalmers, M. L. Shuler, and D. B. Wilson.** 1985. Continuous protein production from *E. coli* capable of selective protein excretion: a feasibility study. Biotechnol. Prog. **1**:75–79.
5. **Hayat, M. A.** 1970. Principles and techniques of electron microscopy, vol. 1. Biological applications. Van Norstrand Reinhold Co., New York.
6. **Heppel, L. A.** 1971. The concept of periplasmic enzymes, p. 223–247. *In* L. I. Rothfield (ed.), Structure and function of biological membranes. Academic Press, Inc., New York.
7. **Inouye, H., W. Barnes, and J. Beckwith.** 1982. Signal sequence of alkaline phosphatase of *Escherichia coli*. J. Bacteriol. **149**:434–439.
8. **Inouye, H., S. Michaelis, A. Wright, and J. Beckwith.** 1981. Cloning and restriction mapping of the alkaline phosphatase structural gene (*phoA*) of *Escherichia coli* and generation of deletion mutants in vivo. J. Bacteriol. **146**:668–675.
9. **Kawagushi, Y., N. Shimizu, K. Nishimori, T. Uozumi, and T. Beppu.** 1984. Renaturation and activation of calf prochymosin produced in an insoluble form in *Escherichia coli*. J. Biotechnol. **1**:307–315.
10. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227**:680–685.
11. **Neu, H. C., and L. A. Heppel.** 1965. The release of enzymes from *Escherichia coli* by osmotic shock and during the formation of spheroplasts. J. Biol. Chem. **240**:3685–3691.
12. **Politt, S., and H. Zalkin.** 1983. Role of primary structure and disulfide bond formation in β-lactamase secretion. J. Bacteriol. **153**:27–32.
13. **Roggenkamp, R., H. Dargatz, and C. P. Hollenberg.** 1985. Precursor of β-lactamase is enzymatically inactive. J. Biol. Chem. **260**:1508–1512.
14. **Schoner, R. G., L. F. Ellis, and B. E. Schoner.** 1985. Isolation and purification of protein granules from *Escherichia coli* cells overproducing bovine growth hormone. Bio/Technology **4**:151–154.
15. **Shoemaker, J. M., A. H. Brasnett, and F. A. O. Marston.** 1985. Examination of calf prochymosin accumulation in *Escherichia coli*: disulphide linkages are a structural component of

prochymosin-containing inclusion bodies. EMBO J. **4**:775–780.
16. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedures and some applications. Proc. Natl. Acad. Sci. USA **76**:4350–4354.
17. **Williams, D. C., R. M. Van Frank, W. L. Muth, and J. P. Burnett.** 1982. Cytoplasmic inclusion bodies in *Escherichia coli* producing biosynthetic human insulin proteins. Science **215**:687–689.
18. **Wilson, D. B.** 1984. Osmotic shock, p. 218. *In* A. Hurst and A. Nasim (ed.), Repairable lesions in microorganisms. Academic Press, Inc., New York.