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INCREASED CELL BUOYANT DENSITIES OF PROTEIN OVERPRODUCING ESCHERICHIA COLI CELLS

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SUMMARY: Escherichia coli K12 cells carrying recombinant plasmids directing a high level accumulation of either β-galactosidase or its nonsense protomer X90 have high stationary cell buoyant densities ranging from 1.11 to 1.13 g/ml. In contrast, the buoyant densities of control cells are 1.08-1.09 g/ml. The increased cell buoyant densities are found when the cytoplasmic inclusion bodies are formed.

INTRODUCTION: Using recombinant genes to synthesize functional proteins in bacteria has been successful in many cases. Upon proper arrangement of the bacterial transcriptional and translational control elements and a structural gene, it is possible to synthesize a protein to a very high level in Escherichia coli (1,2,3,6,7,8). For example, E. coli cells carrying a recombined plasmid containing part of the insulin gene fused to the E. coli lac Z gene synthesize chimeric proteins in up to 20% of the total cellular protein (2). Similarly, a nonsense lac ZX90 allele has been cloned in a plasmid, and E. coli transformants produce X90 protomers to an extremely high level (1). These overproduced bacterial proteins appear to have unusual physical properties. For example, both the β-galactosidase-insulin A chain, β-galactosidase-insulin B chain hybrid proteins, and the X90 protomers are insoluble (1,2). Furthermore, the overproduced, insoluble X90 protomer is resistant to cellular proteolytic systems; whereas,

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the low-level soluble X90 protomer is rapidly degraded (1). The insolubility of these proteins is probably due to the formation of intracellular protein aggregates, and this structure has recently been observed as cytoplasmic inclusion bodies in E. coli cells producing excessive amounts of insulin chimeric proteins (9). This report extends these findings with the study of the physiological properties of E. coli cells producing high level β-galactosidase protomers. These cells have greater stationary cell buoyant densities than control cells.

MATERIALS AND METHODS: An E. coli K12 strain 3341: F^-, thi, CysC43, lys A22, met 2, mal A1, rps L104, lac ZX90, carrying a previously described plasmid (1), p41, was used. It was designated as 3341 (p41). The other E. coli strain used was CSH 50 : F^-, ara, Δ(lac pro), rpsL, thi. The centrifugation medium, Percoll, was used for density separation of bacterial cells; it was purchased from Pharmacia Fine Chemicals, Piscataway, New Jersey. The procedure used to separate proteins using SDS (sodium dodecylsulfate) polyacrylamide gels was described by Laemmli (5).

RESULTS AND DISCUSSION: E. coli transformants carrying the previously described plasmid, p41 (1), were unstable during storage. After a two-year storage in agar stabs, most of the recovered clones from 3341 (p41) no longer produced an excessive amount of X90 protomer. A few clones that stably produced high levels of X90 protomer were isolated and their plasmid DNA purified. One of the plasmids was called p41-14D13. Like the chromosomal lac ZX90 allele, the lac ZX90 allele in p41-14D13 could revert to a wild type allele. About 10^8 cells of 3341 (p41-14D13) were plated on McDonkey lactose plates, and Lac^+ cells were isolated. These cells were purified on tetracycline plates, and single colonies were reisolated three times. Plasmid DNA preparations were made from these Lac^+ isolates and later used to transform CSH 50 cells. The resulting transformants

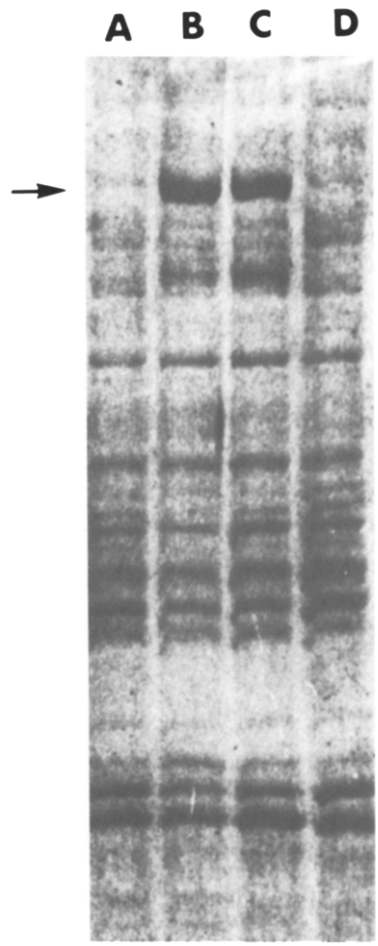

Figure 1 - Protein Profiles of 3341 Transformants

E. coli cells were cultured in LB supplemented with 10 µg/ml tetracycline and harvested at 40 hr after the end of cell growth. The bacterial cells were collected by centrifugation and lysed. Aliquots of cell lysate were run in a 10% SDS polyacrylamide gel, and proteins were detected by Coomassie Brilliant Blue staining. The position of the β-galactosidase and the X90 protomer is marked with an arrow. Cell lysates were made from 3341 (A), 3341 (p41-14D13) (B), 3341 (p41-14D2) (C), and 3341 (pOP 203 (UV-5)-3) (D).

were plated on LB plates supplemented with 10 µg/ml Xgal (5-bromo-4-chloro-3-indoly-β-D-galactoside), and 0.1 mM isopropyl β-D-thiogalacto-pyranoside, and dark blue Lac^+ colonies were isolated. Plasmid DNA was prepared from one of the blue clones, and the plasmid carrying wild type lacZ gene was called p41-14D2.

Plasmid DNA preparations of p41-14D2 and p41-14D13 were used to transform 3341. A transformant, 3341 (p41-14D13), accumulated X90 protomers to a level greater than 15% of the total cell proteins (Fig. 1B). A similar high-level protein accumulation was found in the β-galactosidase overproducer, 3341 (p41-14D2) (Fig. 1C). These cells accumulated high-level protein products in exponentially growing cells as well as in stationary cells. The high-level accumulation of the β-galactosidase protomers required the presence of the lacZ genes in a multiple copy plasmid, since neither 3341 nor 3341 (pOP 203 (UV-5)-3) (4) had a high-level accumulation of any protein (Fig. 1 A&D).

High-level accumulation of proteins was perhaps detrimental to E. coli cells because a fraction of the cellular resources was used in an apparently non-productive way. This could be particularly serious to cells if these "useless" proteins were not recycled in stationary cells when the nutrients in the media were depleted. To understand the physiological consequence of the protein overproduction in E. coli, unusual changes of stationary cells overproducing β-galactosidase protomers were studied. The most prominent difference observed between the protein overproducing bacteria and the normal bacteria was the greater cell buoyant densities of the former.

This density difference could be easily resolved using Percoll as a centrifugation medium. Different Percoll concentrations were tried under various centrifugation conditions for the separation of E. coli cells, and a 60% Percoll solution in 0.25M sucrose was found to give the best separation of the protein overproducing and the normal bacteria. The centrifugation was done by using a fixed angle

rotor, such as a Ti50 rotor, under a centrifugal force of 100,000 xg for 45 min at 10°C. To facilitate the estimation of the bacterial buoyant densities, about 10^7-10^8 cells were mixed with a small amount of density marker beads (Pharmacia) in 60% Percoll, and cell buoyant densities could be estimated from the relative positions of the bacterial bands and density markers.

E. coli cells were grown in Luria's Broth supplemented with 10 μg/ml tetracycline and were harvested at 40 hr after the end of the cell growth. They were resuspended and centrifuged in 60% Percoll. At the end of the centrifugation, cells of 3341 (pOP 203 (UV-5)-3) were concentrated in the Percoll Density gradient with a mean buoyant density of 1.085 g/ml. Stationary cells of 3341 (p41-14D2) that accumulate a high level β-galactosidase were also centrifuged in 60% Percoll and 0.25 M sucrose. These cells had higher and more heterogeneous buoyant densities than those of the normal cells. A majority of the 3341 (p41-14D2) cells had a buoyant density greater than 1.11 g/ml, and the mean buoyant density of the stationary 3341 (p41-14D2) cells was 1.12 g/ml. This density difference between the cells of 3341 (pOP 203 (UV-5)-3) and 3341 (p41-14D2) allowed a clear resolution of these two types of cells in a Percoll density gradient (Fig. 2). Similarly, higher stationary cell buoyant densities ranging 1.11-1.12 g/ml were found in 3341 (p41-14D13) cells. However, the mean buoyant densities of the stationary cells of 3341 derivatives that were transformed by either pMB9 or pBR322 were between 1.08-1.09 g/ml. Furthermore, a difference in buoyant densities between cells that accumulated a high level of β-galactosidase and those that did not was

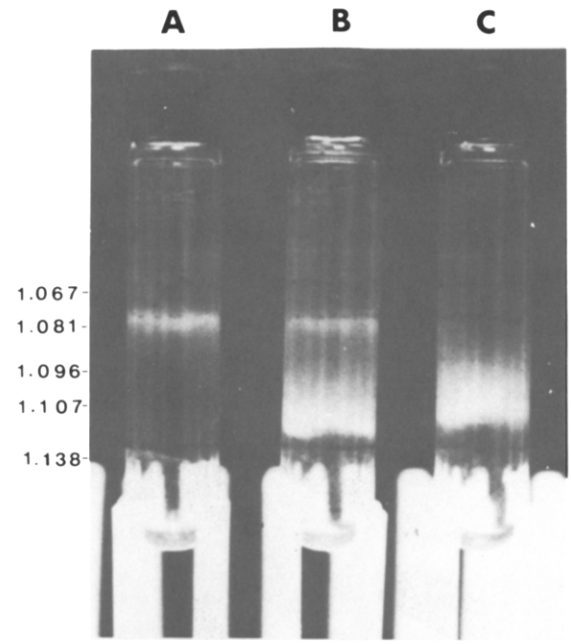

Figure 2 - Density Gradient Separation of E. coli Cells

E. coli cells were cultured in LB with 10 μg/ml tetracycline and collected at 40 hr after the end of cell growth. The collected cells were washed with phosphate buffered saline and were mixed with a small amount of density markers in 60% Percoll, 0.25 M sucrose. The centrifugation conditions used were described in the text. The numbers at the left side are densities of the density markers. Some of the density markers cannot be seen here because of their lighter colors. (A) 10^7 cells of 3341 (pOP 203 (UV-5)-3); (B) a mixture of 10^7 cells of 3341 (pOP 203 (UV-5)-3) and 10^8 cells of 3341 (p41-14D2); (C) 10^8 cells of 3341 (p41-14D2).

found in other E. coli strains. For example, CHS 50 cells were transformed by p41-14D2, and the transformants that accumulated a high level β-galactosidase always had a higher stationary cell buoyant density than that of the CSH 50 cells transformed by (pOP 203 (UV-5)-3). This difference in buoyant densities, however, was found only in stationary cells. Even though the exponentially growing 3341 (p41-41D2) cells had a high level β-galactosidase accumulation, their buoyant densities were identical to those of the exponentially growing 3341 (pOP 203 (UV-5)-3) cells.

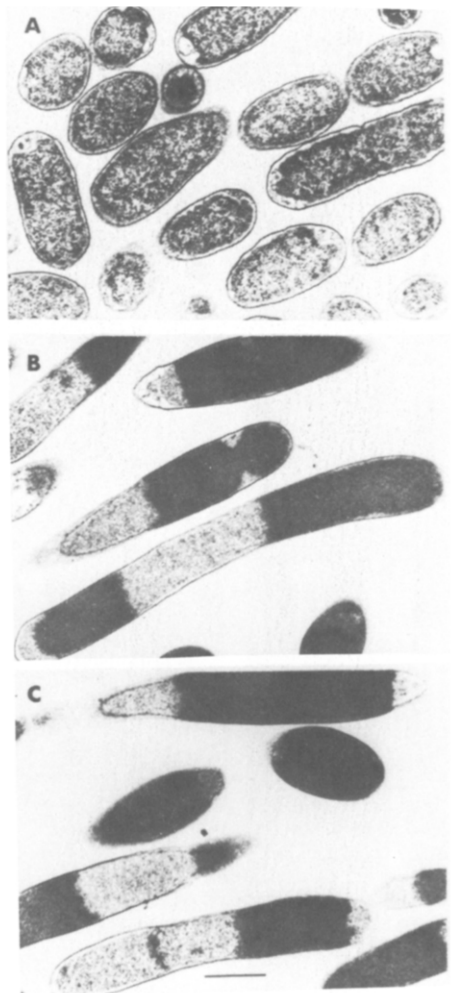

Figure 3 - Transmission Electronmicrographs of E. coli Cells

E. coli cells were collected by centrifugation from LB-tetracycline (10 µg/ml) cultures at 40 hr after the end of cell growth. The cell pellets were doubly fixed with 2% osmic acid in phosphate buffer, pH 6.8. The fixed cells were later dehydrated through an alcohol series and infiltrated with fresh Spurr® (Polyscience, Inc. Warrington, PA). Sixty nm sections were cut, stained with lead citrate and uranyl acetate and examined in a Zeiss EM10 electron microscope. A. 3341 (pOP 230 (UV-5)-3), B. 3341 (p41-14D2), C. 3341 (p41-14D13). Bar, 0.5 µm.

The increased cell buoyant densities of the stationary cells that accumulated high level β-galactosidase protomers suggested that these bacteria might have unusual structural features. E. coli cells were fixed and the sectioned cells examined. Extensive inclusion bodies were

found in the stationary cells that accumulated either β-galactosidase or X90 protomer; however, control cells that did not accumulate a high-level protein product did not have these structures (Fig. 3). The formation of the cytoplasmic inclusion bodies only in the stationary cells that had a high-level protein accumulation was consistent with their greater buoyant densities. In addition, cytoplasmic inclusion bodies were not found in any exponential cells observed.

These results suggested that the increased cell buoyant densities and the formation of the cytoplasmic inclusion bodies were closely related bacterial properties resulting from a high-level protein accumulation; however, a high-level protein accumulation alone seemed to be insufficient to produce these cellular changes. Perhaps other factors associated with a reduced cell metabolic state were also needed for these cellular changes.

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