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Identification and Characterization of Inclusion Bodies Found in pUC19 Transformed *Escherichia coli* DH5α

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Inclusion bodies are protein aggregates found in various types of bacterial cells as a result of overexpression, mutations that affect the folding pathway, or exposure of cells to certain environmental stresses. Previous studies have shown that inclusion bodies are formed when *Escherichia coli* DH5α were transformed with plasmid pUC19. We hypothesized that the inclusion bodies observed were due to overexpression of plasmid genes, specifically *lacZ'* which encodes the amino-terminal fragment of β-galactosidase, and amp^R which encodes β-lactamase. To test the hypothesis, we attempted to purify these inclusion bodies and determine their composite proteins via western blotting. Based upon the results obtained, we were unable to show that any inclusion bodies were formed due to aggregation of β-galactosidase and β-lactamase. Furthermore, it is highly possible that no inclusion bodies were present at all due to the lack of distinctive protein band patterns in the inclusion body-containing samples. However, the purification procedures used were crude and were not optimized; hence inclusion bodies, if present, may not have been separated from other components in the cells. Thus the possibility of inclusion bodies being present still exists.

Inclusion bodies, a common phenomenon in protein expression, represent insoluble protein aggregates formed within the cell. They are commonly encountered when producing recombinant proteins that are not native to the host cell, such as eukaryotic protein in bacteria, and result primarily from the fact that the host lacks the necessary post-translational mechanisms to properly process the protein (5). In addition, it has also been observed that inclusion bodies can result from the overexpression of native bacterial protein, such as β-lactamase, alkaline phosphatase and β-galactosidase (4, 9). In this case, the aggregation is the result of an overload of the host protein processing pathways, such as folding, secretion and degradation (5, 9).

This study began as a follow up to an earlier observation that *Escherichia coli* DH5α cells harbouring pUC19 produce crystal violet inclusions, as visualized by microscopic observations of gram stains (K. Lin, unpublished data). In comparison, *E. coli* DH5α cells carrying no plasmid were reported to stain in a normal fashion and did not exhibit inclusions. If these observations are correct they could bear important implications on studies using *E. coli* DH5α with pUC19. For example, it is quite possible that culture growth would be slowed due to excessive synthesis of a particular protein. Even more importantly, this process could inhibit the production of other cellular components by detracting cellular machinery toward an unnecessary production process. Thus, we set out to investigate the nature of these reported aggregates with the intention of finding the source gene that may be responsible for inclusion body formation. Identifying the source of the inclusion bodies could lead to a solution that would eliminate or reduce this unnecessary process and any negative consequences that could be associated with it.

There are various possible factors that could account for inclusion body formation, such as overexpression due to a strong promoter or a high plasmid copy number (4), or the lack of a critical factor, such as a chaperone that is needed for correct post-translational processing (5, 6, 9). Since the reported observations suggested that the inclusions were only present in pUC19 harbouring *E. coli* DH5α, but not in plasmid-free cells, we hypothesized that, in this case, the aggregation was attributable to a pUC19 encoded gene. Thus, since the plasmid only encodes two different protein moieties, we narrowed down our search to β-lactamase, encoded by amp^R and the N-terminal fragment of β-galactosidase, encoded by *lacZ'* (7). Furthermore, we reasoned that the inclusions were due overexpression since both β-lactamase and β-galactosidase have been previously reported to aggregate when overexpressed (1, 4, 9).

Our rationale for overexpression was also founded on the nature of pUC19 (7). pUC series plasmids contain a ColE1-based replicon. In this system, plasmid copy number is controlled by three components: RNA I, RNA II, and Rop protein. RNA II is a pre-primer that is cleaved by RNase H to generate the primer used in DNA plasmid replication. RNA I is a counter-transcript (anti-sense) to RNA II. The binding of RNA I to RNA II blocks the necessary cleavage by RNase H, thus preventing replication. The interaction between the two RNAs is not

particularly strong, but it is facilitated by the Rop protein. Thus, in a plasmid with a fully functional ColE1 replicon, copy number will be kept at about 15-20 per cell. However, the replicon of all pUC series plasmids contains a point mutation upstream of the transcription initiation site for RNA I that results in a truncated transcript. This truncated RNA I has a reduced binding affinity for RNA II, which allows for enhanced DNA replication and a higher plasmid copy number. Furthermore, pUC19 does not posses the *rop* gene. The absence of Rop diminishes the interaction between RNA I and RNA II even further. The combined result of these two mutations is that pUC19 replicates to about 500-700 plasmid copies per cell (7). With such a high plasmid copy number there is a strong possibility of overexpression of a plasmid-encoded gene. Indeed, pUC19 has been used previously to produce β-lactamase inclusion bodies (9). However, in that case, the host bacterium used was of a different strain and possessed different characteristics.

To test our hypothesis, we attempted to confirm the previously reported microscopic observations and then purify the presumed inclusions and determine their identity. Identification was performed by western blotting using antibodies specific for β-lactamase and β-galactosidase. We were not able to identify inclusion bodies resulting from either protein. Therefore, we were not able to confirm that the aggregates observed by K. Lin (unpublished data) were due to the presence of pUC19. Thus, a possibility still exists for an alternate source of the inclusions, such as growth conditions or the cells themselves.

MATERIALS AND METHODS

Strains and chemicals. *Escherichia coli* DH5α, with or without plasmid pUC19, were kindly provided by Dr. William Ramey. All chemicals, except when stated, were obtained from Sigma (St. Louis, MO). Rabbit anti-β-lactamase antibody and goat anti-rabbit antibody conjugated with horseradish peroxidase were both purchased from Chemicon (Temecula, CA). Mouse anti-β-galactosidase antibody was provided by Eileen Hinze, and anti-mouse antibody was obtained from Sigma (St. Louis, MO).

Growth condition. Bacteria were grown in modified Luria-Bertani (LB) broth supplemented with 0.2% glycerol at 37°C with aeration overnight. Cultures containing plasmid were supplemented with ampicillin to a final concentration of 50 μg/ml. Overnight cultures were diluted to an OD_{600} of 0.2 and grown at 37°C with aeration under specified condition (Table 1) until mid-log phase. Isopropylthiogalactoside (IPTG) was then added to selected cultures to a final concentration of 280 μg/ml and the cultures were allowed to grown to saturation (OD_{600} = 1.4) at 37°C with aeration.

TABLE 1. Growth conditions for *Escherichia coli* strains used in this experiment.

Strain	Plasmid	Growth Condition
DH5α	pUC19	LB, 0.2% glycerol
DH5α	pUC19	LB, 0.2% glycerol, IPTG
DH5α	pU19	LB, 0.2% glycerol, ampicillin
DH5α	pUC19	LB, 0.2% glycerol, ampicillin, IPTG
DH5α	pUC 19	LB, 0.2% glycerol
DH5α	pUC 19	LB, 0.2% glycerol, IPTG

Cell lysis. The cell lysis protocol is adapted from Sambrook *et al* (7). Cultures grown to OD_{600} of 1.4 were centrifuged at 500 x g for 15 minutes at 4°C and then resuspended in lysis buffer (50 mM Tris-HCl, 1 mM EDTA, 100 mM NaCl, pH 8.0) at a volume of 3 ml g • $bacteria^{-1}$. Phenylmethylsulfonyl fluoride (PMSF) at 50 mM and lysozyme (10 mg/ml) were added to the suspension to a final concentration of 8 and 80 μl g • $bacteria^{-1}$, respectively. The suspension was then stirred for 20 minutes and 5 mg g • $bacteria^{-1}$ of deoxycholic acid per gram of bacteria. The suspension was stirred at 37°C for 30 minutes or until viscous, then 20 μl of DNase I (1 mg/ml) was added to the suspension. The lysate was incubated at room temperature until it was no longer viscous.

Inclusion body purification. Cell lysate was centrifuged at 6000 x g for 15 minutes at 4°C. The supernatant was saved and pellet resuspended in 9 volumes of lysis buffer 2 (50 mM Tris-HCl, 10 mM EDTA, 100 mM NaCl, 0.5% Triton X-100, 1). The mixture was incubated at room temperature for 5 minutes then centrifuged at 14,000 x g for 15 minutes at 4°C. The pellet was resuspended in 100 μl of distilled water. The supernatant from the first centrifugation was subjected to another centrifugation at 25 000 x g at 4°C for 15 minutes and the pellet was resuspended in 100 μl of distilled water. All supernatant and pellets were stored at –20°C until ready for SDS-PAGE analysis and western blots.

Protein analysis. SDS-polyacrylamide gel electrophoresis was performed in a 12% gel. The samples were heated for 3 minutes at 95°C before electrophoresis. Coomassie Blue staining was used to visualize total protein bands. Gels were vacuum-dried and laminated.

Western blotting. Proteins in unstained gels were electroblotted to Millipore Immobilon-P (polyvinylidene difluoride) membranes and the membranes were blocked overnight at 4°C in TBST (0.2 M Tris base, 1.5 M NaCl, 0.1% (w/v) Tween-20) containing 5% (w/v) skim milk. Proteins were reacted with either rabbit anti-β-lactamase (1:2500) or mouse anti-β-galactosidase (1:2500) antibodies for 1.5 hours, followed by treatment with horseradish peroxidase-conjugated anti-rabbit or anti-mouse immunoglobulin G (1:7500) for 1 hour. The blots were blocked with 5% (w/v) skim milk. Membranes were washed using TBST, and the secondary antibody was detected with the enhanced chemiluminescence western blotting analysis system (Amersham Pharmacia Biotech) and Kodak X-ray film.

RESULTS

Observation of bacterial inclusion bodies by Gram staining. No definite identification of inclusion bodies were made using Gram staining and light microscope observations. The appearance of inclusion bodies, if any, was found to be independent of the presence of pUC19. This result was inconsistent with the initial observation made by K. Lin (unpublished data). Therefore, no concrete conclusion regarding presence of inclusion bodies could be made.

As a result, we looked towards an alternate approach, differential centrifugation, as a way of identifying inclusion bodies

Estimation of protein concentration and total protein staining of samples from inclusion body purification fractions. Estimated protein concentrations for the various culture conditions were determined by the Bradford protein assay (2). Fractions differed quite significantly in their protein content (Table 2). Most samples had a protein concentration between 1-5 mg/ml. However, some samples (1, 20 and 24) had protein concentrations as high as 25-60 mg/ml.

In order to observe the total protein content obtained from each step of the inclusion body purification procedure, SDS-PAGE gels were run and stained with Coomassie Blue (Fig. 1 and 2). Due to errors initially made in the calculation of protein concentrations, varying amounts of protein were run in each lane (Table 1). Therefore, some of the variation between lane intensity observed in the gels can be attributed to this fact. However, much of the variation between lanes seems to show no correlation with the amount of protein loaded. The supernatant of the initial supernatant lanes were all of approximately similar intensities, while the pUC19 containing strains treated with ampicillin and IPTG tended to show darker lanes in the pellet of the initial supernatant. In general, the lightest lanes tended to be from the pellet of the initial pellet, while in the supernatant of pellet, those strains grown in only LB were significantly darker than the rest.

TABLE 2. Estimated protein concentration and amount of protein loaded onto SDS-PAGE gels for samples from various stages of inclusion body purification under different conditions.

Sample Number	Growth Conditions	pUC19	Protein Concentration (mg/ml)	Protein Loaded (µg)
Supernatant of Initial Supernatant				
1	LB	+	8.40	3.0
2	LB + IPTG	+	0.29	1.1
3	LB + Ampicillin	+	2.77	9.9
4	LB + IPTG + Ampicillin	+	5.08	18.0
5	LB	-	25.87	8.9
6	LB + IPTG	-	2.40	0.9
Pellet of Initial Supernatant				
7	LB	+	2.81	10.0
8	LB + IPTG	+	1.53	5.5
9	LB + Ampicillin	+	1.89	6.8
10	LB + IPTG + Ampicillin	+	2.05	7.4
11	LB	-	4.36	15.4
12	LB + IPTG	-	3.27	11.6
Supernatant of Initial Pellet				
13	LB	+	4.31	15.2
14	LB + IPTG	+	1.15	4.1
15	LB + Ampicillin	+	1.49	5.4
16	LB + IPTG + Ampicillin	+	1.28	4.6
17	LB	-	1.72	6.2
18	LB + IPTG	-	2.36	8.4
Pellet of Initial Pellet				
19	LB	+	3.85	13.7
20	LB + IPTG	+	37.60	13.3
21	LB + Ampicillin	+	3.76	13.3
22	LB + IPTG + Ampicillin	+	4.27	1.5
23	LB	-	4.29	15.2
24	LB + IPTG	-	59.73	20.9

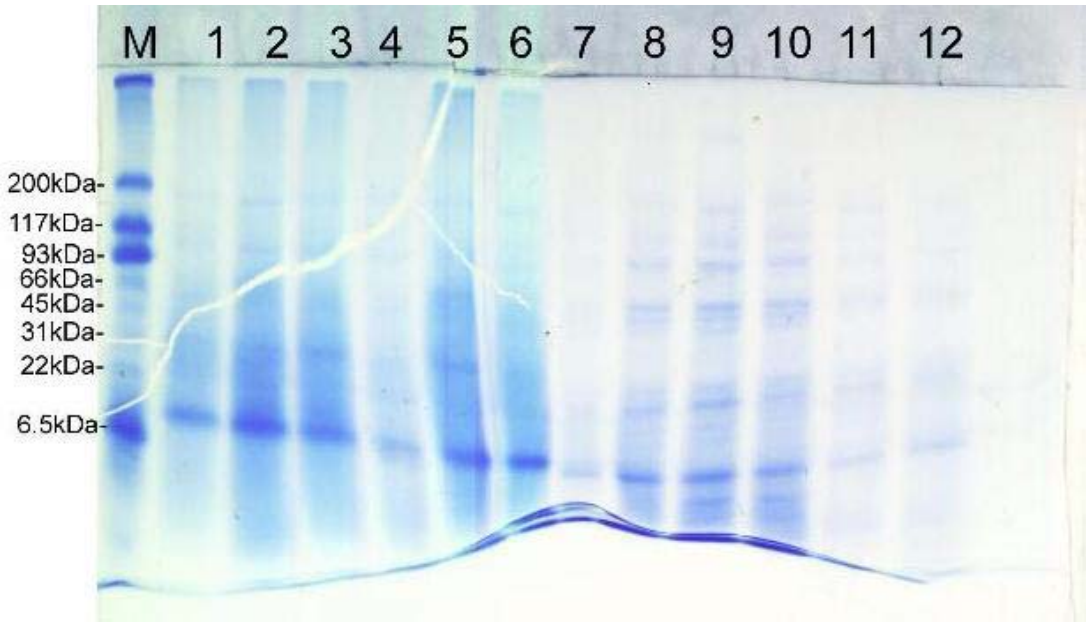

Fig. 1. Coomassie Blue total protein staining of supernatant of initial supernatant and pellet of initial supernatant fractions from inclusion body purification. Lanes are loaded according to Table 2. 'M' represents molecular weight marker.

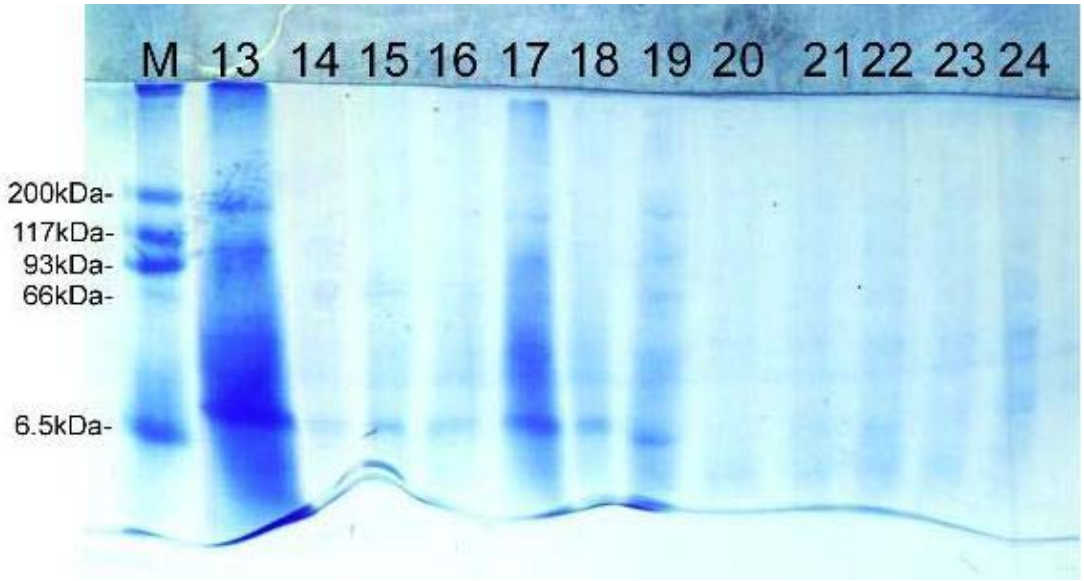

Fig. 2. Coomassie Blue total protein staining of supernatant of initial pellet and pellet of initial pellet fractions from inclusion body purification. Lanes are loaded according to Table 2. 'M' represents molecular weight marker.

Detection of β-galactosidase protein in inclusion body purification fractions. Western blots were performed on all of the samples from the inclusion body purification protocol using antibody specific for β-galactosidase (Fig. 3 and 4). The anti-β-galactosidase antibodies used are known to detect both the plasmid encoded N-terminal fragment and the larger C-terminal fragment gene product encoded by the chromosome of *E. coli* DH5α. Bands representing either the complete β-galactosidase or the truncated *lacZ'* encoded fragment, were prominent in the supernatant of initial supernatant and the supernatant of initial pellet fractions (Fig. 3), with the exception of lanes 5, 6 and 11.

Note that the full protein and the truncated protein are very close in size and thus cannot be distinguished on these membranes. Lanes 5 and 11 are both from uninduced cultures lacking pUC19, thus β-galactosidase expression is not expected. On the other hand, lane 6 is from an induced culture without pUC19 and thus β-galactosidase expression would be expected. Its absence can likely be attributed to the low protein added to this lane (0.9 μg). In general, the response is lowest in the samples lacking pUC19 or IPTG.

Only weak bands were observed in lanes representing the pellet of the initial supernatant and the pellet of pellet fractions (Fig. 4). Since even the molecular weight marker lane gives a weaker signal on this membrane, the weaker bands can be partly attributed to poor detection sensitivity obtained using this membrane. Bands corresponding to β-galactosidase are seen in lanes 14, 15, 16, 18 and 19, although it is probable that improved sensitivity in detection would reveal bands in other lanes. It may also be possible that these bands may represent non-specific binding by the detection system.

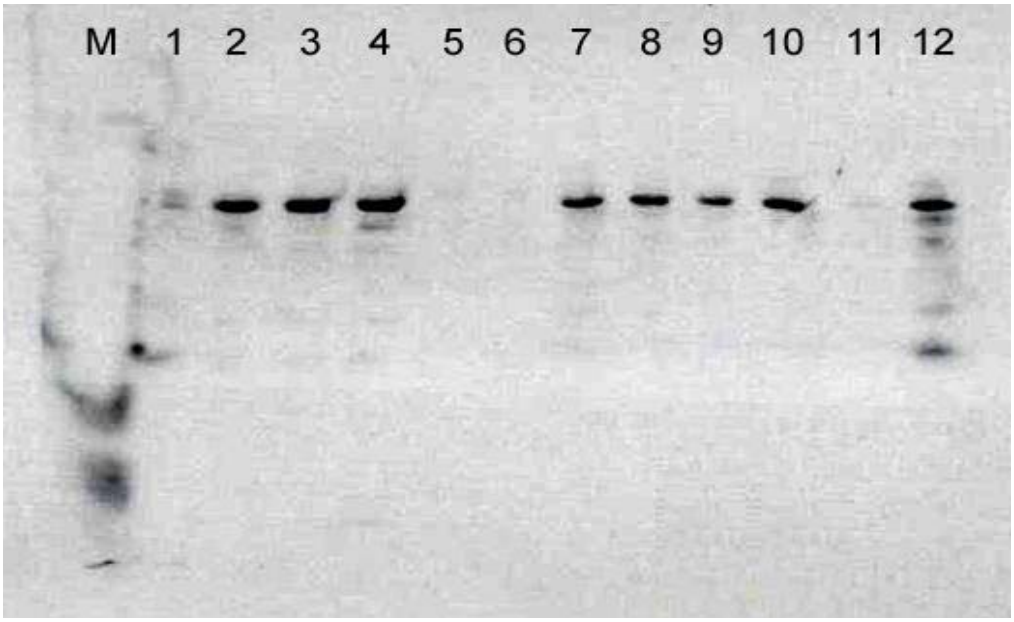

Fig 3. Western blot using anti-β-galactosidase antibody on supernatant of initial supernatant and pellet of initial supernatant fractions from inclusion body purification. Lanes are labeled according to Table 2.

Detection of β-lactamase protein in inclusion body purification fractions. Anti-β-lactamase antibody was used to detect the presence of the protein in the various fractions obtained from the inclusion body purification procedure (Fig. 5 and 6). A conserved band was seen in most of the lanes for all four fractions, except for the pellet of initial pellet samples. These fractions also contained a second higher molecular weight band. Finally, smeared lower molecular weight bands are present in most lanes of all of the fractions. All of these bands are too large or too small to represent β-lactamase, which has an expected molecular weight is 32.5 kDa, and thus likely arise from non-specific binding of the antibody to other proteins. In addition, the bands are present in lanes from cultures which lacked pUC19 and thus do not possess the amp^R gene which encodes for β-lactamase.

DISCUSSION

The presence of inclusion bodies within DH5α was originally observed as purple artifacts found within the pink cytoplasm of a cell after Gram staining by K. Lin (unpublished data). However, we did not observe any consistent results when the same protocol was repeated. Initially, only cells containing pUC19 were hypothesized to contain inclusion bodies. Nevertheless, the presence of pUC19 was not the determining factor of whether or not inclusion bodies were observed. Our microscopic observation showed that Gram-stained cells containing pUC19 had similar physical properties to cells without plasmid. Inconsistent purple artifacts were found in all cells, which suggest the

purple artifacts were a result of other sources of contamination, perhaps unrelated to protein aggregate formation. Therefore, we suggest that Gram staining may not be an effective way of determining the presence of inclusion bodies. In addition, other studies have found that cells with inclusion bodies appeared to have an elongated structure as compared to normal *E. coli* cells (3). These observations did not match with what we saw in our cells, further supporting the possibility that they did not form inclusion bodies in the first place.

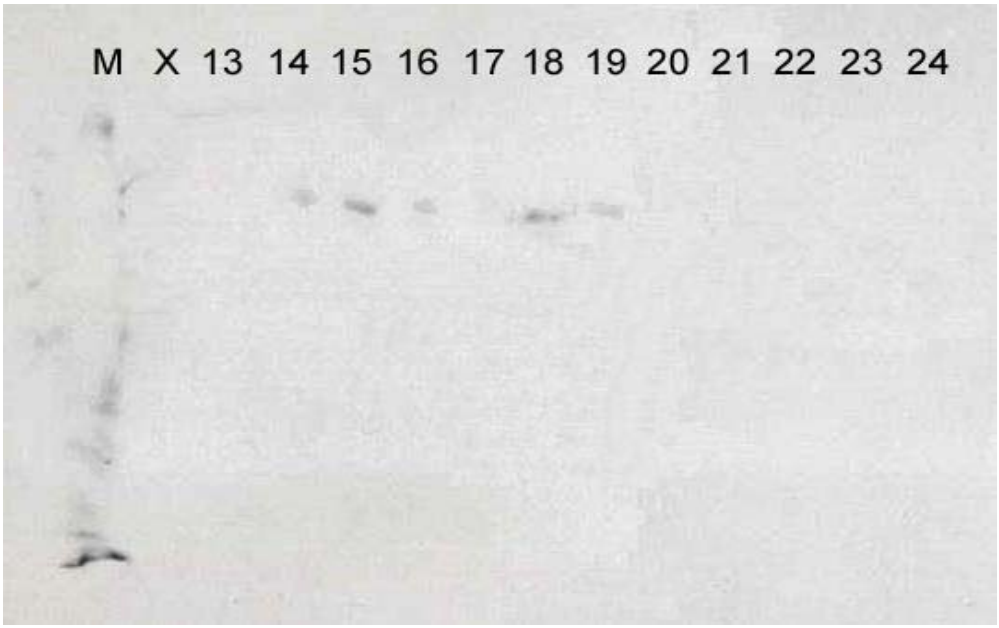

Fig 4. Western blot using anti-β-galactosidase antibody on supernatant of initial pellet and pellet of initial pellet fractions from inclusion body purification. Lanes are labeled according to Table 2. 'M' represents molecular weight marker. Lane 'X' is blank.

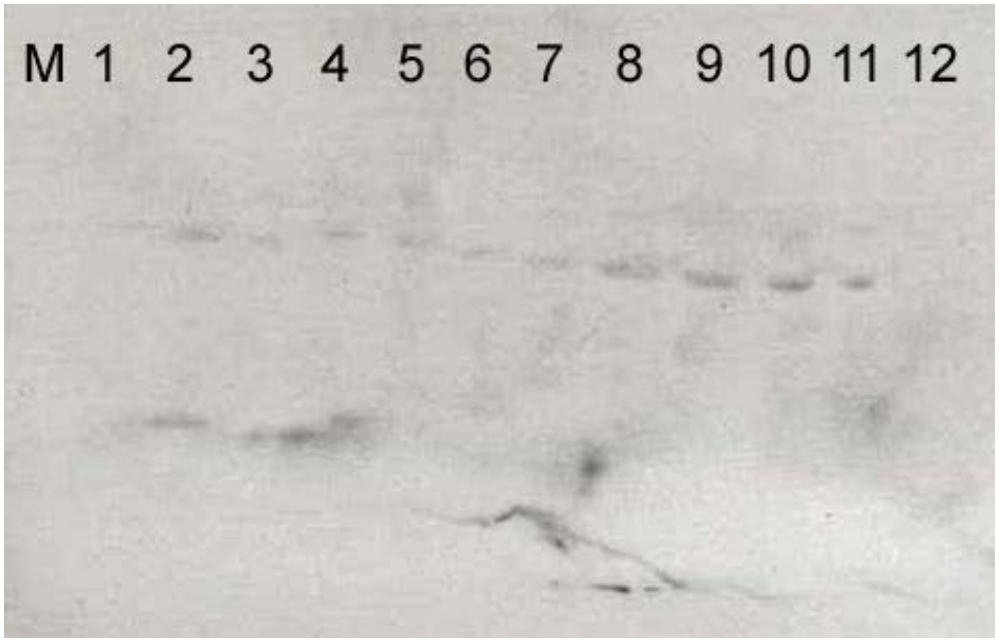

Fig. 5. Western blot using anti-β-lactamase antibody on supernatant of initial supernatant and pellet of initial supernatant fractions from inclusion body purification. Lanes are labeled according to Table 2. 'M' represents molecular weight marker.

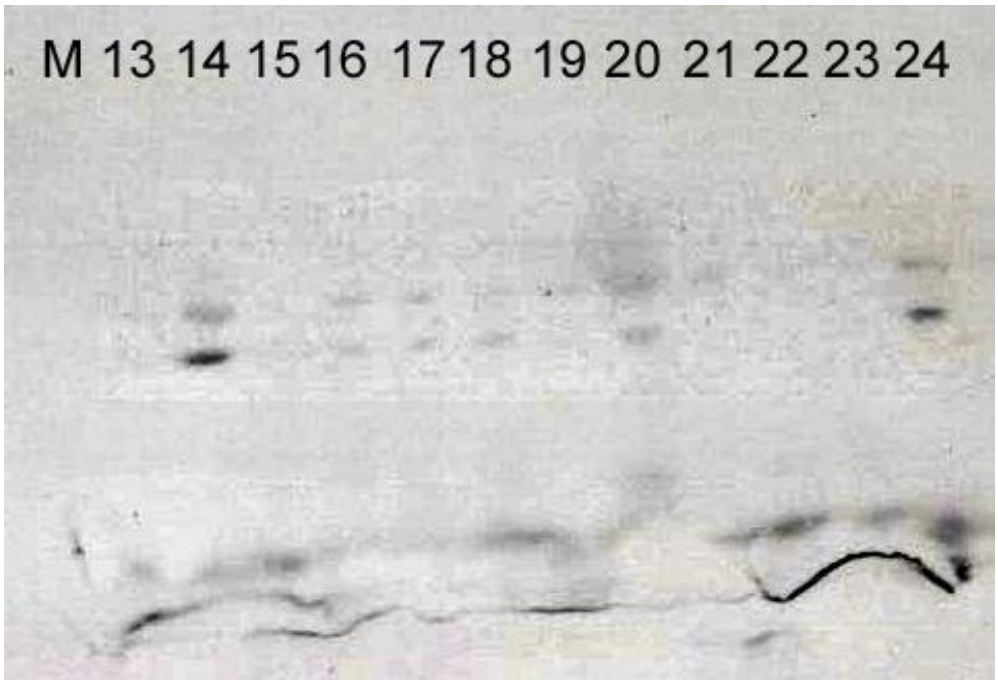

Fig 6. Western blot using anti-β-lactamase antibody on supernatant of initial pellet and pellet of initial pellet fractions from inclusion body purification. Lanes are labeled according to Table 2. 'M' represents molecular weight marker.

To further investigate the possibility of inclusion body formation in cells containing pUC19, we attempted to isolate inclusion body proteins from the cell material. Initially, we spun down the cell lysate at 6000 x g. This step would allow the large macromolecules such as cell wall and cell membrane material to become separated from cytoplasmic proteins. Furthermore, the resuspended precipitate was centrifuged at 14 000 x g and the supernatant was spun down at 25 000 x g. Spinning the resuspended precipitate at 14 000 x g was aimed at isolating protein aggregates that might have been caught in between larger fragments of the cell wall or cell membrane, whereas spinning the supernatant at 25 000 x g was simply to isolate the inclusion bodies from the rest of the cytoplasmic proteins. These angular acceleration values used to purify inclusion bodies were based on the isolation of β-lactamase aggregates (9). However, it is possible that the inclusion body proteins in our strains, if indeed there are any, are of a different type. The assumption that β-lactamase was the source of the inclusions might have led to a major error in our isolation protocol. Since our protocol was based on separation by centrifugation, the value of angular acceleration is an important factor. Angular acceleration is dependent on the density of sedimenting particles, density of the medium, and the viscosity of the medium (7). Therefore, changing these variables would affect the type of protein being isolated and dictate the strategy for purification.

Samples obtained from the isolation protocol were electrophoresed on three sets of gels. One of the gel sets was used to determine the total protein content from each of the different conditions used (Table 2). Initially, an error in calculation caused an incorrect amount of protein to be loaded into each lane of the gel, thus, making it hard to compare the intensities of bands between different samples. However, relative band intensities within each lane could still be compared to determine the extent of protein present for each unique band. Total protein gels showed a distinctive lack of banding pattern observed for the pellet of the initial pellet (Fig. 2, lanes 19-24). This was consistent with our initial prediction that large fragments of the cell wall and membrane would be found in these samples. These fragments do not bind to Coomassie Blue (7), and therefore are not expected to produce a banding pattern on the gel (Fig. 2). Based on our protocol, we expected inclusion body proteins to be isolated into either the pellet of the initial supernatant or the supernatant of the initial pellet. However, there were no consistent bands that would indicate the presence of inclusion bodies for the samples containing pUC19.

If the source of inclusion bodies was β-lactamase, we would expect to see a dark band around 32 kDa throughout all the plasmid containing samples due to its constitutive overexpression (9). However, the bands were not

observed. There were also no consistent bands at approximately 116 kDa, which would correspond to β-galactosidase, throughout the total protein samples containing IPTG. One factor to note is that all DH5α strains have the ability to produce the C-terminal portion of β-galactosidase, which makes up a majority of the functional protein; the N-terminal region consists of 27 amino acids and the C-terminal region consists of 1147 amino acids (7). Nonetheless, only cells induced with IPTG have the potential to over express β-galactosidase and form inclusion bodies containing this protein. Initially we hypothesized that by over expressing the N-terminal region of β-galactosidase, it would lead to the formation of protein aggregates. However, since no distinctive bands were present for samples with pUC19, it seems quite unlikely that inclusion body proteins may have been formed by the plasmid encoded N-terminus of β-galactosidase.

The supernatant of the initial supernatant samples (lanes 1-6 of Fig. 1, 3, 5) produced a smear of proteins throughout each lane on the total protein gel. This smear may have been attributed to the wide range of proteins that are found in the cell. This corresponds with our initial intention of separating cytoplasmic proteins of normal size from potential inclusion body proteins suspected to be located in specific fractions mentioned above. Despite the expectation of separating various proteins into different fractions, gel observations showed the presence of a small band (approximately 6.5 kDa) throughout all the samples, except for the pellet of the initial supernatant samples. This can be used as an indication of the effectiveness of our protocol in separating specific proteins of different size and density into different fractions. The lack of a small band for the pellet of the initial supernatant samples indicates that spinning down the resuspended pellet at 14000 x g was effective in separating out proteins from other non-protein macromolecules. However, the presence of a consistent band throughout the remaining fractions indicates that the remaining centrifugation steps require further optimizing to separate proteins into their respective fractions. The identity of this small band remains unknown, since *E. coli* possess many proteins of similar size.

Western analysis was done on the remaining two gels using either β-galactosidase or β-lactamase antibodies. A thick consistent band observed in lanes containing the initial supernatant-derived samples (Fig. 3) could be speculated to be either the complete β-galactosidase or the C-terminal β-galactosidase. The presence of pUC19 and induction by IPTG appears to increase the expression of either C-terminal β-galactosidase or the complete protein, as there are no indications of β-galactosidase in pUC19 and IPTG negative conditions. However, we are uncertain about the mechanism by which pUC19 would induce higher expression of the C-terminal β-galactosidase portion. No bands of β-galactosidase were observed in lanes containing samples derived from the initial pellet, indicating that 6000 x g was an insufficient speed to precipitate the specific protein. However, as Rinas and Baily (9) have shown, inclusion bodies formed by β-galactosidase can be partially spun down at 6000 x g, and therefore be found in both the supernatant and pellet portions. This suggests that inclusion bodies formed by β-galactosidase are predicted to be heavy or dense enough to be partially fractionated at 6000 x g, with a large proportion found in the supernatant. In contrast, our sample did not show the same outcome, indicating that β-galactosidase was not the likely source of inclusion bodies within our strains. Nevertheless, other factors of centrifugation mentioned above may have also affected the fractionation efficiency.

Initial observations of our western analysis of the β-lactamase gels indicated a similar result to Rinas and Bailey (9). Their gel showed the presence of two distinctive fragments immunoreactive with antibodies against β-lactamase. As they suggest, this outcome may have resulted from complete and truncated forms of β-lactamase. The truncated protein may have been formed by signal sequence cleavage during its exportation from the cytoplasm (9). However, upon further investigation we have determined that the two bands in our result to be different in size by at least 10 kDa. This suggests that the smaller band found in many of the samples in our gel was not the result of signal sequence cleavage. Furthermore, double bands also appeared in samples that did not contain pUC19, which were originally not expected to produce β-lactamase bands. Based on this finding, we hypothesize that the multiple bands observed on our gel may be the result of non-specific binding, instead of altered forms of β-lactamase. In general, results of nonspecific binding may suggest that our procedures of immunoblot are not completely specific for the protein of interest. By far, the most important factor in any such immunoassay is the specificity of the antibody for the antigen. Related to this are the length of time used for antibody incubation and the stringency of wash steps used. Although antibodies are generally highly specific for their antigens, this specificity is not perfect and it is possible that identical or similar epitopes will be found in unrelated proteins, due to the limited structural scaffold upon which antibodies are constructed (2). Since a bacterial cell lysate is likely to possess a very large diversity of proteins, it is quite reasonable that additional proteins to those of interest were detected, especially since the conditions used had not been optimized. This may have been a contributing factor for many anomalous bands present in our western analysis of both β-galactosidase and β-lactamase.

In conclusion, based on our microscopic and biochemical analysis we have found no indication of β-galactosidase and β-lactamase as being the source of the presumed inclusion bodies. Culture conditions such as temperature, pH, and nutrient supply have been found to be major factors contributing to the partitioning of recombinant proteins into

soluble and non-soluble inclusion body fractions (9). Observations originally made K. Lin (unpublished data), indicating the presence of inclusion bodies within DH5α, may have been the result of environmental growth conditions, instead of being plasmid related. Although we were unable to show the existence of inclusion bodies within DH5α harbouring pUC19, optimizing both the isolation technique for suspected inclusion body proteins and reproducing original cell growth conditions, may allow us to reproduce the results previously obtained.

FUTURE EXPERIMENTS

For future experiments, we should initially determine the optimal conditions that would enhance the formation of inclusion bodies within the cell. As suggested in the discussion, we should try varying conditions such as pH, temperature and nutrient supply in order to select for the best protein aggregate forming cells. Furthermore, once the presence of inclusion bodies has been confirmed within DH5α, a total protein gel should be run. Using the estimated size of the protein determined by the gel, we can approximate the appropriate centrifugation acceleration required to precipitate down inclusion bodies formed by the suspected protein of interest. This process would allow us to separate the aggregate proteins more efficiently without getting so much undesired protein containments within the samples. However, it should be noted that the actual size of an inclusion body is much larger than the protein it consists of; therefore, determining the actual size of the inclusion body, from an SDS-PAGE gel, may prove to be difficult. During immunoblotting, it would also be important to run an immunoblotting molecular weight standard and a control lane containing the suspected inclusion body forming proteins of interest. The latter would act as a standard and a positive control to correlate known position of either β-galactosidase or β-lactamase with the bands produced in each of the lanes during a western analysis. In addition, it would be important to perform a control western blot using non-specific IgG antibodies in order to assess the amount of non-specific binding that occurs in a bacterial cell lysate. Thus, allowing us to determine if the smaller bands observed are indeed fragments of suspected proteins contributing to the formation of inclusion bodies, or whether they are the result of non-specific antiserum binding.

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