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Abnormal cell division caused by inclusion bodies in *E. coli*; increased resistance against external stress

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Summary

Inclusion body formation occurs naturally in prokaryotic cells, but is particularly common when heterologous foreign proteins are overexpressed in bacterial systems. The plant disease virus protein CMV 3a (cucumber mosaic virus movement protein) and the 56 kDa *Orientia tsutsugamushi* (OT56) protein (an outer membrane protein), which causes tsutsugamushi disease, were expressed in *Escherichia coli*, and found to form inclusion bodies. Confocal laser scanning microscopy revealed that these inclusion bodies are localized at the cellular poles within *E. coli*. Cells expressing inclusion bodies appeared to be interconnected, and divided abnormally. The clustered cells exhibited biofilm-like characteristics in that the interior cells of the community were protected by the antibiotic resistance of the outer cells. We compared the number of colony-forming units in inclusion body-forming versus non-forming *E. coli* to demonstrate the effects of lysozyme, sonication or antibiotic treatment. *E. coli* clustering provided significantly improved protection against cell disruption/lysis by physical and biochemical stress. This is the first report that shows that abnormal cell division caused by inclusion body formation can cause cellular clustering, resulting in improved resistance to stress *in vitro*.

The fate of newly synthesized cellular proteins is commonly determined by competition between folding, degradation and aggregation. Over-expression of a heterologous protein frequently induces

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protein aggregation that may be amplified by high concentrations of identical nascent chains (Betton et al., 1998). Other factors such as pH, temperature, ionic strength or the inhibition of specific degradative pathways can also induce the production of protein folding intermediates with the potential for aggregate formation (Young et al., 2004). Therefore, various chaperone proteins such as GroES, GroEL or the Dna-series are crucial for the successful progress of newly synthesized proteins through the intermediate steps of the folding system (Maisonneuve et al., 2007). Together, these proteins form a quality control system that monitors folding and refolding, and targets proteases for degradation (McClellan et al., 2005). The GroEL chaperone appears to have multiple roles in the formation of inclusion bodies: it may promote inclusion body formation in bacteria via the clustering of small aggregation nuclei, and also plays an important role in the removal of proteins from inclusion bodies (Villaverde and Carrió, 2003).

Inclusion bodies might have effects at the individual cell or the population level. Inclusion body formation begins when aggregates overwhelm the cell's degradative processes and accumulate to form new subcellular structures (Tanaka et al., 2004; Kovacs et al., 2006). Deposits of inclusion bodies have been observed in periplasmic and cytoplasmic regions in *Escherichia coli* (Lee et al., 2005).

This article focuses on the localization and functional implications of inclusion body formation. In *E. coli*, inclusion bodies seem to be localized in periplasmic regions, at the cellular poles. Their formation is associated with an abnormal phenotype involving cell clustering, the development of biofilm-like properties and increased resistance to external stress.

Materials and methods

Cell culture media and general reagents were purchased from Sigma–Aldrich Chemicals (St. Louis, MO, USA). The cDNA clones of the *Cucumber mosaic virus* or *Orientia tsutsugamushi* were generously provided by Prof. Peter Palukaitis (Scottish Crop Research Institute, UK) and by Prof. Kim (Microbiology Research Center, Korea). All restriction enzymes, T4 DNA ligase and *Taq* DNA polymerase were obtained from TaKaRa Biotechnology (TaKaRa, Japan). *E. coli* strain DH5α and *E. coli BL21*(*DE3*) (Novagen, USA) were utilized as the hosts for subcloning and gene expression, respectively. The pET28a(+) expression vector was obtained from Novagen. pEGFP-N2 was obtained from Clontech. The antibody used was anti-green fluorescent protein (GFP) (FL) from Santa Cruz Biotechnology. The protein electrophoresis marker was the PageRuler^{TM} Prestained Protein Ladder from Fermentas (USA). A HiLoad 16/100 Superdex 200 chromatography and Low Molecular Weight Gel Filtration Calibration Kit was obtained from Amersham Biosciences (Seoul, Korea).

Plasmid constructs

Cloning of GFP-fused proteins

The genes for CMV3a and OT56 were amplified by PCR using CMV cDNA and OT cDNA, respectively, as templates. The PCR products were cloned into subcloned plasmids. The constructed plasmids were designated pET28a(+)-GFP, pET28a(+)-CMV3a-GFP, pET28a(+)-GFP-CMV3a and pET28a(+)-GFP-OT56.

The pET28a-GFP, used as a control vector, was constructed by subcloning the GFP gene from the pEGFP-N2 (Kna^R) vector (NEB, Beverly, MA, USA) into the *Nco I* and *Xho I* sites of the pET28a(+) (Kna^R) expression vector. For the amplification of the GFP gene, the forward primer incorporated an *Nco I* site (underlined): 5′-CATG CCA TGG ATG GTG AGC AAG GGC GAG GAG-3′, and the reverse primer incorporated an *Xho I* site (underlined) 5′-CCG CTC GAG CTT GTA CAG CTC GTC CAT GC-3′.

To construct the pET28a-CMV3a-GFP vector, DNA encoding 279 amino acids of the CMV3a protein was amplified by PCR. The forward primer incorporated an *Nco I* site (underlined): 5′-CCG CCA TGG ATG GCT TTC CAA GGT ACC AGT AG-3′, and the reverse primer incorporated a *BamH I* site (underlined): 5′-CGG GGA TCC AAG ACC GTT AAC CAC CTG C-3′. For the amplification of the GFP gene, the forward primer incorporated a *Sal I* site (underlined): 5′-CATG GTC GAC ATG GTG AGC AAG GGC GAG GAG-3′, and the reverse primer incorporated a *Xho I* site (underlined): 5′-CCG CTC GAG CTT GTA CAG CTC GTC CAT GC-3′.

For the pET28a-GFP-CMV3a vector, the GFP gene was amplified using a forward primer incorporating an *Nco I* site (underlined): 5′-CATG CCA TGG ATG GTG AGC AAG GGC GAG GAG-3′, and a reverse primer incorporating a *BamH I* site (underlined): 5′-CCG GGA TCC CTT GTA CAG CTC GTC CAT GC-3′. DNA encoding 279 amino acids of the CMV3a protein was amplified using a forward primer incorporating a *Sal I* site (underlined): 5′-CCG GTC GAC ATG GCT TTC CAA GGT ACC AGT AG-3′, and a reverse primer