Analysis of the role of amino-terminal acetylation in modulating the cellular distribution and physical properties of the Parkinson's disease associated protein alpha-synuclein

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DECLARATION

No part of this thesis has been submitted in support of an application for any degree or qualification of the University of Kent or any other University or Institute of learning.

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Date:

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TABLE OF CONTENTS

LIST OF FIGURES

Figure.10	Western Blot showing the presence of α-Synuclein-GFP as a monomer in the sample grown with thiamine where expression is at low levels and as both a monomer and dimer in the sample grown without thiamine, where expression is not inhibited and so at a higher level.
Figure.11	OD_{600} Growth Curves for *S.pombe* wild type containing pREP41GFP or pREP41αS-GFP.
Figure.12	Western Blot which shows the presence of α-Synuclein-GFP at the expected MW for a monomer in the sample grown with thiamine where expression is at low levels, but only in the supernatant. Bands of the expected MW's for both monomer and dimer were seen in the supernatant of the sample grown without thiamine where expression is not inhibited and so at a higher level Possible damaged fragments of dimer were seen in both samples grown without thiamine. Dimer and dimer fragments only were seen in the pellet grown without thiamine.
Figure.13	OD_{600} Growth Curves for a naa25Δ *S.pombe* strain containing pREP41GFP or pREP41αS-GFP grown with and without thiamine.
Figure.14	Images of naa25Δ S.pombe containing pREP41GFP grown both with and without thiamine had a low levels of fluorescence. Bright spots indicate the presence of small aggregates that are excluded from the nuclei. Images of naa25Δ S.pombe containing pREP41αS-GFP grown with thiamine, have low levels of fluorescence indicating the presence of low levels of α-Synuclein-GFP which appears to be dispersed through the cytoplasm but excluded from vacuoles. The image of naa25Δ S.pombe containing pREP41αS-GFP grown without thiamine, has higher levels of fluorescence indicating that a higher level of α-Synuclein-GFP was produced. This appears to be dispersed through the cytoplasm, but excluded from vacuoles in some cells. But in other cells bright spots indicate that the α-Synuclein-GFP may be starting to form small aggregates as with GFP alone.

Figure.15	Western Blots that compare the presence of α–Synuclein-GFP as a monomer in the samples grown with thiamine, but seen only in the supernatant. Both monomer, dimer and fragments of dimer were seen in both of the –thiamine supernatants where expression is not inhibited and so at a higher level. Clear bands were only seen at the weight expected for dimers in both of the –thiamine pellets, faint bands were also visible also slightly below this weight possibly due to damaged dimer fragments.
Figure.16	Images of wild type S.pombe pREP41αS-Cerulean3 grown with thiamine to repress vector expression. At low levels of vector protein expression very little fluorescence could be observed and so produced only faint fluorescent images, where the fluorescence appears to be cytoplasmic. Phase images were included to clearly show the cells being observed.
Figure.17	Images of wild type S.pombe pREP41αS-Cerulean3 grown without thiamine. At higher levels of vector protein expression far more fluorescence could be detected. In some cells with slightly lower levels this appears to be cytoplasmic, with a slight concentration at the poles. In others with higher levels it appears to be excluded from vacuoles and concentrated at the separation membrane.
Figure.18	Images of wild type S.pombe pREP41αS-Cerulean3 grown with thiamine. Again low levels of vector protein expression led to very little fluorescence being detected in comparison to the images of induced cells. The fluorescence observed still appears to be cytoplasmic but in one cell did seem to be concentrated at the separation membrane (red arrow). The FM4-64 did not stain all of the cells clearly but some have clearly stained vacuole membranes.
Figure.19	Images of. wild type S.pombe pREP41αS-Cerulean3 grown without thiamine Again higher levels of vector protein expression led to far more fluorescence being detected. In some cells with slightly lower levels this appears to be more cytoplasmic. In others with higher levels it appears to be excluded from vacuoles but present within the nucleus and concentrated at the separation membrane. Two

	cells appear to have aggregates on the membrane (red arrows). The FM4-64 did not stain all of the cells clearly but some have clearly stained vacuole membranes and is excluded from the nucleus.
Figure.20	Images of S.pombe pINT41αS-Cerulean3, grown for 1 day with thiamine, stained with FM4-64. At the very low levels of vector protein expression created by a single copy of the integrated αS-Cer3 vector in the presence of thiamine only extremely low levels of fluorescence could be detected. Despite using image enhancement only very faint cerulean images could be produced. The images obtained using FM4-64 dye and phase images show very misshapen cells, some of which also show multiple septa (red arrows), very thick septa (blue arrow) and unusual shaped vacuoles (green arrow).
Figure.21	Images of S.pombe pINT41αS-Cerulean3, grown for 1 day without thiamine, stained with FM4-64. At the higher levels of vector protein expression created by a single copy of the integrated αS-Cer3 vector without thiamine, compared to growth with thiamine, very low levels of fluorescence could still only be detected. Despite using image enhancement only very faint cerulean images could be produced. The images obtained using FM4-64 dye and phase images show very misshapen cells, some of which also show multiple septa (red arrows) and unusually large vacuoles (green arrow).
Figure.22	Images of S.pombe pINT41αS-Cerulean3, grown for 2 days with thiamine, stained with FM4-64. After 2 days of growth with thiamine very low levels of vector protein expression created by a single copy of the integrated αS-Cer3 vector, meant that only extremely low levels of fluorescence could be detected. Despite using image enhancement only very faint cerulean images could be produced. The images obtained using FM4-64 dye and phase images show very misshapen cells, some of which also show multiple septa (red arrows) and very thick septa (blue arrow). Cell vacuoles can no longer be clearly seen.

Figure.23	Images of S.pombe pINT41αS-Cerulean3, grown for 2 days without thiamine, stained with FM4-64. After 2 days growth without thiamine the higher levels of vector protein expression, compared to cells grown with thiamine, created by a single copy of the integrated αS-Cer3 vector, created only low levels of fluorescence. Despite using image enhancement only very faint cerulean images could be produced. The images obtained using FM4-64 dye and phase images show very misshapen cells, some of which also show multiple septa (red arrows) and very thick septa (blue arrow). Vacuoles can only be seen clearly in a few cells (green arrow). Some cells also appear to be producing bleb like structures (white arrows).
Figure.24	Coomassie stained gel, the two post induction protein samples on the right, show a large increase in protein produced, compared with pre induction.
Figure.25	Images of BL21DE3 αS-Cerulean3 cells and BL21+NatB αS-Cerulean3 cells, Pre induction and 1, 2 and 3 hours post induction. BL21DE3 αS-Cerulean3 cells show only low levels of fluorescence, which appeared to be cytoplasmic. In the image taken at 1 hour post induction, more fluorescence was seen, this still appeared to be predominantly cytoplasmic, but some brighter areas appeared to show the start of some localisation and possible aggregate formation. In the image taken at 2 hours post induction. Some fluorescence still appeared to be cytoplasmic. However, some cells appeared to have bright concentrations of fluorescence, possibly indicating the presence of aggregates or blebbing around the outside of the cells. Other cells appeared to have ribbon like areas of localisation, predominantly but not exclusively, on the inside of the plasma membrane. In the image taken at 3 hours post induction, there was a complete mix of appearances. Some cells still appeared to have cytoplasmic Cerulean3. Some have areas of bright fluorescence or possible aggregates or blebbing around the outside of the cell. Some have ribbon like concentrations within the cell and

	some have a mixture of these appearances. This variation may be due to differing concentrations of plasmid present in different cells. It was not possible to take images of BL21+NatB αS-Cerulean3 cells, Pre induction, as the level of fluorescence was too low. In the image of BL21+NatB αS-Cerulean3 cells taken at 1 hour post induction. Fluorescence appeared to be mainly cytoplasmic. A few cells have areas that seem to have a slightly higher level of fluorescence. The images of BL21+NatB αS-Cerulean3 cells, taken at 2 hours post induction show lots of blebbing around some cells together with some of the ribbon like localisation. In the images of BL21+NatB αS-Cerulean3 cells, taken at 3 hours induction, only a few blebs or aggregates were visible on the outside of the cells. However the ribbon like localisation was seen to be more dominant and more clearly defined. (Scale-1μm)
Figure.26	Images of slices of thin sectioned BL21DE3 αS-Cerulean3 cells taken at 2 hours induction using TEM. The TEM images show clear blebbing on the outside of the cells producing unacetylated α-Synuclein-Cerulean3. The blebs appear to be membrane bound structures being released from the cell surface.
Figure.27	Images of slices of thin sectioned BL21+NatB αS-Cerulean3 cells producing acetylated α-Synuclein-Cerulean3 were seen to be producing lots of smaller blebs. They were also seen to be producing structures with a tubular network. These seemed to originate on the inside of the plasma membrane and build up into the inside of the cell. Some cells appear to be having difficulty dividing, possibly due to the presence of these structures (see arrow on third image). These structures seem to be what creates the ribbon like appearance seen in the 3 hour fluorescent microscopy.
Figure.28	4 TEM images of BL21+NatB αS-Cerulean3 cells, using a darker Osmium tetroxide and Thiocarbohydrazide stain, to enhance the appearance of the internal tubular structures. Red arrows indicate cells that appear to be having difficulty dividing.

Figure.29	Image of BL21+NatB cells containing an empty vector. No immuno staining could be seen indicating an absence of α-Synuclein.
Figure.30	Image of BL21+NatA αS-Cerulean3 cells, showing immuno staining to be concentrated in the areas where the tubular networks were seen in earlier images, in the areas inside the plasma membrane.
Figure.31	Image of BL21+NatB αS-Cerulean3 cells showing immuno staining, which again appears to be concentrated in the areas where the tubular networks were seen previously.
Figure.32	Graph to compare growth rates, of acetylated and unacetylated α-Synuclein and an empty vector using OD_{600}.
Figure.33	Coomassie stained 4-12% Bis-Tris gel and western blot of BL21+NatA αS-Cerulean3 cells and BL21NatB αS-Cerulean3 cell extracts.
Figure.34	Fluorescent growth curve comparing BL21+NatA αS-Cer3 and BL21+NatB αS-Cer3 cells producing acetylated and unacetylated α-Synuclein-Cerulean3, grown with and without the addition of IPTG
Figure.35	Images of BL21+NatB αS-Cerulean3 cells, stained with FM4-64. Blebs can be seen in the Cerulean3 images (Red arrows) (Scale 1μm).
Figure.36	Images of BL21+NatB-GFP cells, stained with FM4-64 (Scale 1μm).
Figure.37	Fluorescent scans of cell cleared culture medium, from cultures of BL21+NatA αS-Cer3, BL21+NatA αS, BL21+NatB GFP, BL21+NatB αS-Cer3 and BL21+NatB αS cells, 90 hours post induction, indicating the presence of fluorescent proteins.
Figure.38	Images of BL21+NatAαS-Cerulean3 cells 24 hours Post Induction, obtained using Phase and Fluorescent light microscopy (stained with FM4-64. Scale 1μm), TEM and Immuno EM.
Figure.39	Images of BL21+NatB αS-Cerulean3 cells 24 hours Post Induction, obtained using Phase and Fluorescent light microscopy (stained with FM4-64. Scale 1μm), TEM and Immuno EM. Red arrows indicate cells that appear to be having difficulty dividing.

Figure.40	Images of BL21+NatA αS cells 24 hours Post Induction, obtained using Phase and Fluorescent light microscopy (stained with FM4-64. Scale 1μm), TEM and Immuno EM.
Figure.41	Images of BL21+NatB αS cells 24 hours Post Induction, obtained using Phase and Fluorescent light microscopy (stained with FM4-64. Scale 1μm), TEM and Immuno EM. Red arrows indicate cells that appear to be having difficulty dividing.
Figure.42	Images of BL21+NatB GFP cells 24 hours Post Induction, obtained using Phase and Fluorescent light microscopy (stained with FM4-64. Scale 1μm), TEM and Immuno EM.
Figure.43	Images of BL21+NatA αS-Cerulean3 cells 90 hours Post Induction, obtained using Phase and Fluorescent light microscopy (stained with FM4-64. Scale 1μm) and TEM.
Figure.44	Images of BL21+NatB αS-Cerulean3 cells 90 hours Post Induction, obtained using Phase and Fluorescent light microscopy (stained with FM4-64. Scale 1μm) and TEM.
Figure.45	Images of BL21+NatA αS cells 90 hours Post Induction, obtained using Phase and Fluorescent light microscopy (stained with FM4-64. Scale 1μm) and TEM. What the spore like structures are is unknown. The white spots are holes where the resin used to fix the cells was unable to penetrate into these structures.
Figure.46	Images of BL21+NatB αS cells 90 hours Post Induction, obtained using Phase and Fluorescent light microscopy (stained with FM4-64. Scale 1μm) and TEM. The images appear to show a large proportion of dead or dying cells.
Figure.47	Images of BL21+NatB GFP cells, 90 hours Post Induction, obtained using Phase and Fluorescent light microscopy (stained with FM4-64) and TEM. What the spore like structures are is unknown. The white spots are holes where the resin used to fix the cells was unable to penetrate into these structures.
Figure.48	Coomassie stained 4-12% Bis-Tris gel and Western Blot analysis of cell cleared culture media, 90 Hours post induction.

Figure.58	Coomassie stained gel for whole cell protein samples from BL21DE3 and BL21+NatB cells with plasmids containing αS-Cerulean3, αS-Cerulean3+cydAB-mCherry and cydAB-mCherry genes, pre induction and 2 hours post induction.
Figure.60	Coomassie stained 4-12% Bis-Tris gel for 5 day post induction protein samples from cell cleared culture medium from cultures of BL21DE3 and BL21+NatB cells containing αS-Cerulean3, αS-Cerulean3+cydAB-mCherry and cydAB-mCherry.
Figure.60	Western Blots using anti α-Synuclein (top) and anti mCherry (bottom) on whole cell protein samples taken 2 hours port induction from cultures of BL21DE3 and BL21+NatB cells containing αS-Cerulean3, αS-Cerulean3+cydAB-mCherry and cydAB-mCherry.
Figure. 61	Plasmid map to show the relative positions of the genes coding for α-Synuclein-Venus-Amino half and α–Synuclein-Venus Carboxyl half within the pETDuet plasmid.
Figure.62	Images of BL21+NatA (left) and BL21+NatB (right) cells containing αS-VC observed by Phase microscopy. No Venus signal was visible, showing that the Carboxyl half of the Venus Protein does not produce fluorescence by itself.
Figure.63	Images of BL21+NatA αS-VN αS-VC cells producing α-Synuclein-Venus Amino half and α-Synuclein-Venus Carboxyl half have Venus fluorescence, showing both blebbing around cells and a ribbon like appearance within many cells. This indicates the presence of α-Synuclein oligomers, both within the cells and within the blebs.
Figure.64	Images of BL21+NatB αS-VN αS-VC cells producing acetylated α-Synuclein-Venus Amino half and acetylated α-Synuclein-Venus Carboxyl half also have Venus fluorescence, showing both blebbing around cells and a ribbon like appearance within cells. This again indicates the presence of α-Synuclein oligomers both within the cells and within the blebs (Scale 1μm).

Figure.65	Coomassie stained 4-12% Bis-Tris gel examining BL21+NatAαS-VC, BL21+NatBαS-VC, BL21+NatAαS-VN αS-VC and BL21+NatBαS-VN αS-VC whole cell proteins, Pre and 3 Hours Post induction. Post induction the αS-VC samples show the production of α-Synuclein-Venus-Carboxyl half (24.1kDa) and the αS-VN αS-VC samples show the production of both α-Synuclein-Venus-Carboxyl half and α-Synuclein-Venus-Amino half (32.1kDa).
Figure.66	Coomassie stained 4-12% Bis-Tris gel and Western Blot, examining protein precipitated from, Cell cleared culture media from BL21+NatAαS-VN αS-VC and BL21+NatBαS-VN αS-VC, cultures at 24, 48, 120 and 144 hours post induction.
Figure.67	Coomassie stained 4-12% Bis-Tris gel showing separate production of α-Synuclein-Venus-Amino half and α-Synuclein-Venus-Carboxyl half in BL21DE3 cells using vectors αS-VN and αS-VC.
Figure.68	Images of BL21DE3 cells containing αS-VN observed by Phase microscopy (left). No Venus signal was visible and so no images possible (right), showing that the Amino half of the Venus Protein does not produce fluorescence by itself.
Figure.69	Venus fluorescent growth curve for BL21DE3 and BL21+NatB cells containing αS-VC, αS-VN αS-VC and αS-VN vectors. A gradual increase in fluorescence was seen with the cells containing αS-VC, and αS-VN vectors alone. A rapid initial increase in fluorescence was seen with both cultures containing αS-VN αS-VC. This rate reduced after 3hours with BL21NatBαS-VN αS-VC cells and a decrease in fluorescence was seen with BL21DE3αS-VN αS-VC cells after 18 hours.
Figure.70	FPLC trace showing protein containing fractions collected, determined by absorbance at 260 and 280nm, from purification of BL21+NatB αS-Cerulean3 Cell Cleared Culture Media.
Figure.71	Coomassie stained 4-12% Bis-Tris gel showing protein content of fractions from purification of BL21+NatB αS-Cerulean3 from cell cleared culture media.

Figure.72	Western Blot using anti α-Synuclein antibody on fractions J2 and J4 (Figure 68) from the purification of BL21+NatB αS-Cerulean3 from cell cleared culture media.

LIST OF TABLES

LIST OF ABBREVIATIONS

BiFC	Bimolecular Fluorescence Complementation
Cer3	Cerulean 3
E.coli	*Escherichia coli*
EDTA	Ethylendiamineteraacetic acid
EM	Electron Microscope
EMMG	Edinburgh minimal media with 20 mM L-Glutamic acid
FM4-64	N-(3-Triethylammoniumpropyl)-4-(6-(4-(Diethylamino) Phenyl) Hexatrienyl) Pyridinium Dibromide
FPLC	Fast Protein Liquid Chromatography
GFP	Green Fluorescent Protein
IPTG	Isopropyl β-D-1-thiogalactopyranoside
LB	Luria-Broth
MCS	Multiple Cloning Site
NAC	Non-Amyloid beta Component
NatA	N-α-terminal acetyltransferase A
NatB	N-□-terminal acetyltransferase B
nmt	No message in Thiaminre
Nt	N-terminal
OD	Optical Density
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PD	Parkinson's Disease
PEI	Polyethylenimine
PRE	paramagnetic Relaxation Effects
S.cerevisiae	*Saccharomyces cerevisiae*
S.pombe	*Schizosaccharomyces pombe*
TAE	Tri-acetate-EDTA
TCA	Trichloroacetic acid
YES	Yeast Extract with supplements
αS	α-Synuclein

ABSTRACT

Alpha-Synuclein (αS) is a small, 140 amino acid, neuronal protein, found predominantly in the presynaptic terminals of neurons. Although the precise cellular function of αS remains unknown, it is known to be critical in the pathology of a number of neurodegenerative disorders including Parkinson's Disease (PD) and other related dementias. A common symptomatic feature of PD and αS associated disease states, is the aggregation of misfolded αS into Lewy bodies. αS is subject to a number of post-translational modifications, including amino-terminal (Nt) acetylation and is known to bind to membranes, both are believed to effect oligomer formation and the accumulation of αS aggregates. In this project I am using different microbial model systems to examine the effect of Nt-acetylation on membrane binding and oligomer formation and their effect on the accumulation of αS aggregates within the cell. The first model system used is the bacteria *E. coli*, in which we are making use of a novel Nt-acetylation system developed within this lab. The second model system being used is the fission yeast *Schizosaccharomyces pombe*, which allows us to use molecular genetic approaches to examine how the post-translational modification of Nt-acetylation effects αS within a eukaryotic cell.

1. CHAPTER I: INTRODUCTION

1.1 α-Synuclein

α-Synuclein is one of the most abundant proteins in cells. It is found predominantly in the presynaptic terminals of neurones and can also be found in red blood cells (Bartels T *et al* 2011), the heart, muscles, and other tissues (Genetics Home Reference: SNCA". U.S. National Library of Medicine. 2013). It is a small 140 amino acid protein, that is normally soluble, but which forms aggregates in several disease states. The SNCA gene codes for α-Synuclein.

α-Synuclein was first identified in β-amyloid plaques in the brain of patients with Alzheimer's disease, but is mostly associated with Parkinson's disease. Parkinson's disease is one of a number of neurodegenerative disorders called α-Synucleinopathies which also includes dementia with Lewy bodies and multiple system atrophy. (Kim WS *et al*. 2014)

α-Synuclein is subject to many different post translational modifications and is also known to interact with lipids, phospholipids and other proteins. It is not fully understood how these interactions affect α-Synuclein structure and function. (Paleologou. *et al*. 2012)

There are several actions proposed for α-Synuclein but the exact function remains unknown. (Snead D. 2014)

1.2 The structure of α-Synuclein

α-Synuclein was long thought to exist primarily as a natively unfolded protein monomer, found mainly in the presynaptic terminals of neurones. The protein is made up of three domains, the N-terminal region (residues 1 to 60) which contains lysine-rich, imperfect 11 amino acid repeats, that are important in membrane binding and α-helix formation. The central non-amyloid beta component (NAC) region (residues 61 to 95) which is predominantly composed of hydrophobic residues, this region is required for α-Synuclein aggregation. The third domain is the C-terminal region (residues 96 to 140) which is acidic and proline rich, this domain is found in a disordered conformation and has been implicated in interactions with proteins, metals and small molecules. (Kim WS. 2014. Pratt MR. 2015).

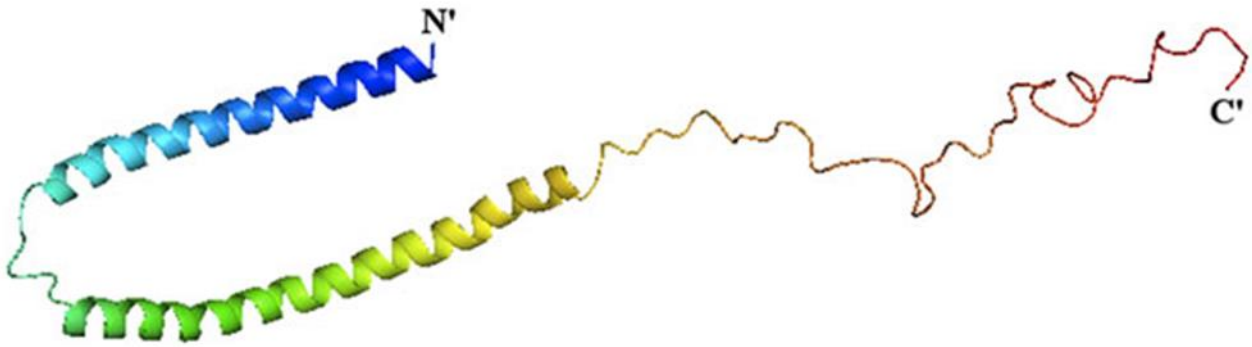


Figure.1. Structure of alpha-Synuclein bound to lipid vesicles. (Ritchie C.M. *et al* 2012)

The N-terminal region has been shown to undergo a change into an amphipathic α-helix on membrane binding. The lipid composition and membrane curvature, can effect this binding, both electrostatic and hydrostatic interactions seem to contribute, with enhanced binding to vesicles with increased curvature and negative charge. Different helical membrane-bound conformations have been observed, including a short helix at the N-terminal with the remainder of this domain unbound, on phospholipid vesicles. An extended helix of approximately 100 residues and a broken-helix conformation in which the extended-helix is broken into two distinct helices separated by a non-helical linker region (residues 39 to 45) have both been observed in the context of detergent micelles and lipid vesicles. (Snead D *et al*.2014)

N-terminal acetylation has also been shown to increase helicity at the N terminus and electrostatic charge and to increase membrane affinity with moderately charged vesicles. (Dikiy I *et al*. 2014) (Maltsev A.S *et al*. 2012).

Evidence is now emerging, that α-Synuclein exists naturally as a tetramer, which may resist aggregation, though some are sceptical of this (Maltsev A.S *et al*. 2012. Fauvet B *et al*. 2012). It is becoming apparent, that tetramers can only be isolated using very gentle, non-denaturing purification methods (Bartels T *et al*. 2011. Luth E.S *et al*. 2015. Wang W, *et al* 2011) and that they may only form with acetylated α-Synuclein. (Trexler A.J *et al*. 2012). This may account for tetramers not being seen with α-Synuclein produced in *E.coli,* which is not acetylated or where heating is used in the protein purification. Different arrangements for tetramer or oligomer formation have also been proposed (Burré J *et al*. 2014. Wang W, *et al* 2011).

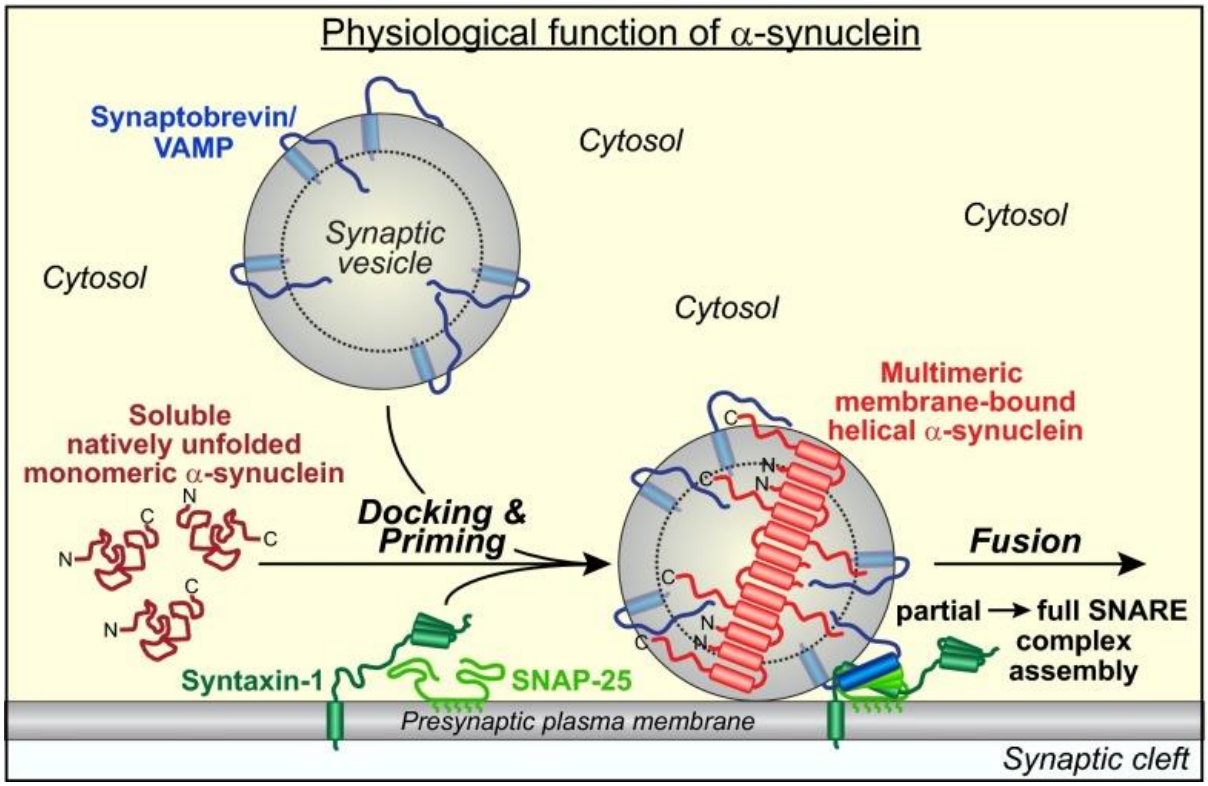


Figure. 2. Scheme of a physiological folding pathway for α-Synuclein. (Burré J *et al*. 2014) showing a proposed oligomer formation.

The oligomer formation (Figure.2.) may be the start of aggregate formation, rather than a progression from physiological tetramers. This is possibly due to the use of recombinant α-Synuclein which is not acetylated and may not therefore be able to form physiological tetramers to start with.

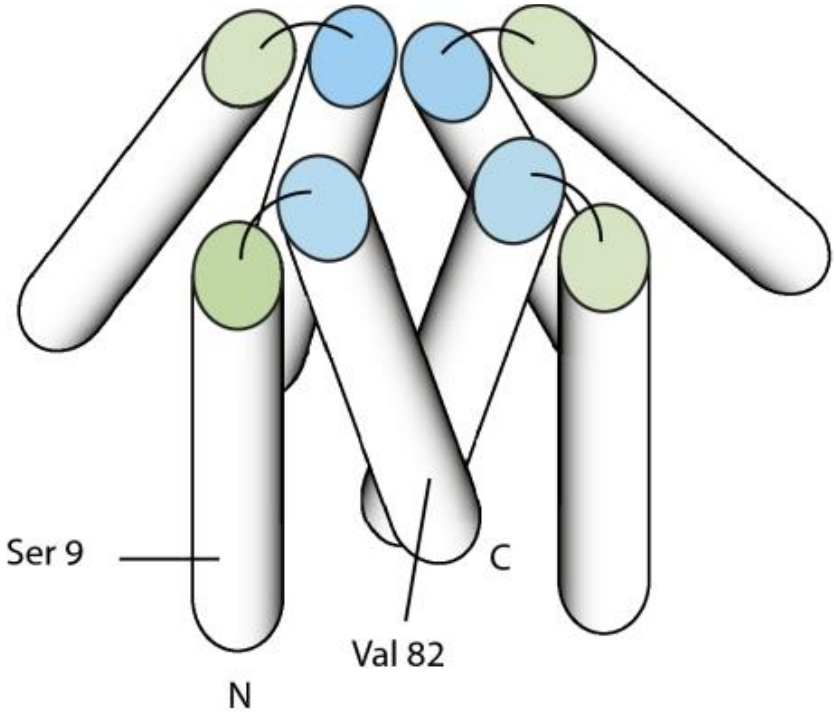


Figure.3. Model for compact α-Synuclein tetramer based on EM reconstruction and PRE. Helices are represented as cylinders. N indicates the N-terminal of the protein, with the first helix (α1, represented by green-ended cylinder) ending at ~residue 43. The second helix (α2, blue ended) starts ~residue 50 and ends at residue 103 (marked C). The remainder of the polypeptide, which is expected to be disordered, is not represented. (Wang W, *et al* 2011).

1.3 The action of α-Synuclein

The exact action of α-Synuclein is unknown, but there are several theories.

As α-Synuclein is predominantly found in the presynaptic terminals of neurons and is known to bind synaptic vesicles it is largely thought to be involved in maintaining a supply of synaptic vesicles in presynaptic terminals and to act as a chaperone in the formation of SNARE complexes, experiments using SNCA knockout mice have also shown an effect on memory and cognitive function. (Kim W.S. *et al*. 2014. Burré J. *et al* 2014).

However α-Synuclein is also found in large amounts in Red Blood Cells (Bartels T *et al*. 2011) and is also thought to potentially interact with other cellular membranes, including the inner nuclear membrane, mitochondrial membranes, the plasma membrane and with lipid rafts and fatty acids. These other potential sites of α-Synuclein action could indicate a more widespread or general function for α-Synuclein

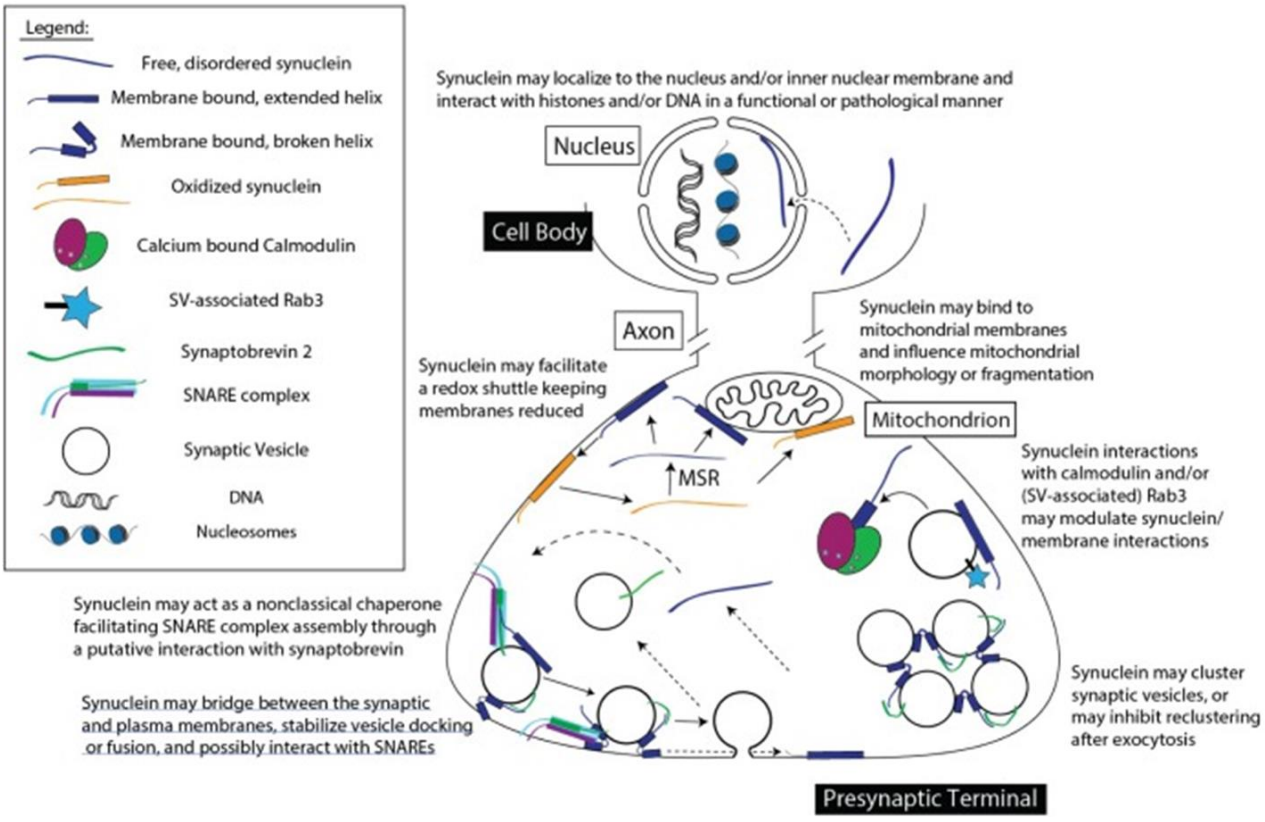


Figure.4. Cellular membranes, targets and pathways potentially involved in the normal, physiological functions of alpha-Synuclein. (Snead D *et al*. 2014)

1.4 Post translational modifications of α-Synuclein

There are several post-translational and co-translational modifications that α-Synuclein can undergo, including phosphorylation, ubiquitination, nitration and N-terminal acetylation. (Kim W.S. *et al* 2014. Pratt. *Et al*. 2015).

Phosphorylation is seen as the most common post-translational modification. In dementia with Lewy Bodies, approximately 90% of insoluble α-Synuclein was found to be phosphorylated at S129, compared to 4% in soluble cytosolic α-Synuclein. Phosphorylation also occurs at S87, Y125, Y133 and Y135. This has been seen to implicate phosphorylated α-Synuclein in aggregate formation and to have led to more research into this modification.

Nt-acetylation has also been investigated, but with varying results. It has been reported that 'Nt-acetylation does not have prominent effects on the biophysical and membrane binding properties of α-Synuclein, *in vitro* and *in vivo*.' (Fauvet B *et al*. 2012). But other papers have reported that Nt-acetylation can have an effect on α-helicity of the N-terminal, membrane interactions (Maltsev A.S. *et al*. 2012. Dikiy I. *et al*. 2014) and oligomer formation. (Trexler A.J. *et al* 2012).

1.5 N-terminal acetylation and use of NatB complex in *E.coli*

Amino terminal-acetylation is one of the most common protein modifications in eukaryotes, but is rarely seen in prokaryotes, occurring on approximately 84% of human proteins and 57% of yeast proteins. (Polevoda B. *et al*.2009). This is a co-translational process in eukaryotes, but is post-translational in prokaryotes. It involves the transfer of an acetyl group from acetyl coenzyme A to the amino-terminal amino acid of a protein. This addition of an acetyl group to the N-terminal residue, neutralizes positive charges which can affect the stability and function of the protein and consequentially it's interactions with other proteins and molecules.

N-terminal acetylation is catalysed by a set of enzyme complexes, the N-terminal-α-acetyltransferases (NATs). Six different NATs have been found in humans - NatA, NatB, NatC, NatD, NatE and NatF. Each of these different enzyme complexes is composed of a catalytic and an auxiliary subunit, and each is substrate specific for target amino-terminal amino acid sequences. The N-terminal amino acids of α-Synuclein are Methionine and Aspartic acid, this sequence is acetylated by the NatB complex.

The NatB complex is composed of a catalytic subunit Naa20 and an auxiliary subunit Naa25, which are found in both yeast and humans but not in *E.coli*. NatB acetylates the N-terminal methionine of substrates starting with Met-Glu-, Met-Asp-, Met-Asn- or Met-Gln- N termini.

In vitro investigations into α-Synuclein have largely used recombinant protein produced and purified from *E.coli*. As *E.coli* do not possess the NatB complex, the recombinant protein produced is un-acetylated and this may in turn have a significant effect on protein stability and interactions.

It is now possible to produce Nt-acetylated recombinant protein in *E.coli*. This is done by simultaneously expressing the recombinant protein with the NatB complex within *E.coli*. (Johnson M. *et al*. 2010).

1.6 Parkinson's Disease

Parkinson's Disease (PD) is a neurodegenerative disorder, caused by the selective loss of dopaminergic neurones in the substantia nigra.

One person in every 500 has Parkinson's, about 127,000 people in the UK, (Parkinson's UK) and an estimated 7 to 10 million people worldwide (Parkinson's Disease Foundation, Inc. USA). The main symptoms are tremor, slowness of movement (bradykinesia) and muscles stiffness (rigidity), though there are many other symptoms that can vary from person to person.

Most people diagnosed with Parkinson's are over 50 with it being most prevalent in people aged over 75, though a small number of people develop early onset or familial Parkinson's. The cause of Parkinson's is unknown and there is no cure.

A defining feature of Parkinson's disease is the presence of Lewy Bodies, cytoplasmic inclusions within the neurones of the substantia nigra, which consist mainly of α-Synuclein. There is no consensus as to when α-Synuclein is most toxic in Parkinson's disease. Some believe it is the soluble protofibrils, oligomers with a β-sheet structure, that are the most toxic whereas others believe it is the matured aggregates (Winderickx J. *et al* 2008).The concentration of α-Synuclein within dopaminergic cells also seems to be linked to toxicity, as duplications and triplications of the SNCA gene are linked to early onset Parkinson's disease.

Familial early onset Parkinson's disease has been linked to several missense mutations in and duplications and triplications of the SNCA gene that codes for α-Synuclein. (Kim W.S. *et al* 2014). Three of these have been the subject of several investigations, A30P, A53T and E46K. (Brandis K.A. *et al* 2006. Fiske M. *et al* 2011).

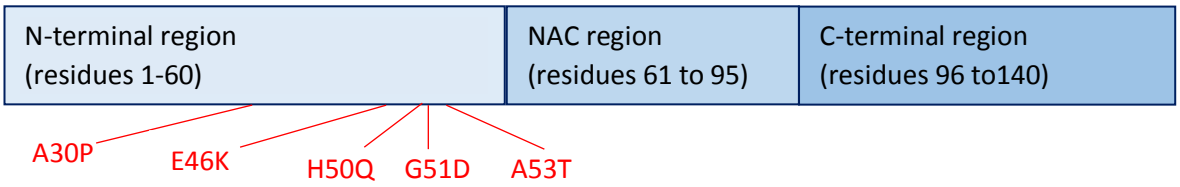


Figure 5. Five SNCA missense mutations identified in dominantly inherited Parkinson's disease.

C-terminal truncations may also play a role in α-Synuclein aggregation, as truncated α-Synuclein has been found in Lewy bodies, from dementia with Lewy bodies brains and other associated neurodegenerative diseases. (Paleologou. *et al*. 2012)

1.7 The use of *S.pombe* and *E.coli* as model organisms together with fluorescent microscopy.

α-synuclein has been widely investigated *in vitro* and *in vivo*, using a variety of cell lines and model organisms, including mice, *Drosophila melanogaster, Caenorhabditis elegans* and *Saccharomyces cerevisiae* (Franssens *V*. *et al* 2013).

Model organisms such as these are all well understood, which are used to study human disease for a number of reasons, including ethical reasons, cost and time. A disease may take several years to develop in a human, but in an organism with a much shorter lifespan such as a mouse effects may be seen far more rapidly. Single celled organisms may not be complex enough to study age related diseases as a direct comparison, but have advantages in studying aspects of diseases such as protein interactions. Many model organisms such as *S. cerevisiae, S. pombe* and *E.coli* are frequently used due to their speed of growth, ease of use and the range of genetic tools that can be used with them. Due to their size, they can also be used for localisation studies using fluorescent microscopy.

Both *S. cerevisiae* and *S. pombe,* have a large number of key cellular processes that are well conserved between them and higher eukaryotes, including humans, though these do vary between the two types of yeast which are very different from each other. A large number of genes known to be involved in human diseases, have also been found to have a yeast orthologue, though this does not include the SNCA gene that codes for α-Synuclein. (Winderickx J. *et al* 2008). To study α-Synuclein in yeast, it is necessary to produce a humanised yeast strain by introducing the SNCA gene to produce α-Synuclein into the yeast, using genetic techniques. *S. cerevisiae* has been used extensively in the study of α-Synuclein, however there has been very little use of *S. pombe.*

S.pombe was first used as a model organism to study α-Synuclein, to investigate protein aggregation with both wild type and A30P, A53T and A30P/A53T mutant α-Synuclein, in this study no membrane binding was seen, as is seen in many studies using *S. cerevisiae*. Cytoplasmic aggregates were seen in cells producing wild type and A53T α-Synuclein (Brandis K.A. *et al* 2006).

A second study using both *S. cerevisiae* and *S.pombe* to investigate wild type and A30P, A53T and E46K mutant α-synuclein, again also found that α-Synuclein did not bind to *S.pombe* plasma membranes, unlike in *S. cerevisiae*. However, this study found that when using a new strain of *S.pombe* the results were the same as in the first study, but with cytoplasmic aggregates also found with the E46K mutant. When this was repeated in the same strain as the first study, wild type, E46K and A53T α-Synuclein was now seen to localise to endomembranes. (Fiske M. *et al* 2011).

Both of these studies show that *S.pombe* can be a useful model organism in investigations into α-Synuclein. The differences between the two studies highlight how the action of α-Synuclein may be very complex, with many unknown factors affecting its action, membrane binding and aggregation. It is also worth noting, that many of the cells in the figures of these papers have a very short appearance, possibly due to them being starved.

E.coli though often used as a model organism, does not appear to have been used as a model organism in its own right, in the study of α-Synuclein. It has been used extensively in α-Synuclein studies to produce recombinant protein for purification and use in, *in vitro* investigations. However, it has not been possible to find any references to studies examining the effect of α-Synuclein on the *E.coli* that it is produced in. *E.coli* provides a very simple model system for gaining insight into mechanisms regulating protein membrane interactions. It has no internal membranes, only membranes at the periplasmic region which is in contrast to eukaryotic systems

Fluorescent microscopy is a powerful tool that can be used with both of these model systems. Fluorescent dyes that bind to specific structures, can be used to see the location of those structures within the cell. Genetic techniques can be used to join the gene for a fluorescent protein to the gene for the protein being studied, enabling the expression of the studied protein attached to a fluorescent protein, within live cells. This allows for the locations at which the studied protein localises to be seen. Different coloured fluorophores can also be used in the same cell enabling co-localisation studies.

1.8 The aim of this project

It was the aim of this project to investigate the membrane interactions, and oligomer and aggregate formation of α-Synuclein, and how these were affected by Nt-acetylation, using both *in vivo* and *in vitro* techniques. This was carried out using two different model organisms, *E.coli* and *S.pombe*, to enable comparisons between both a prokaryotic and a eukaryotic model system. Firstly in *S.pombe* which is a good eukaryote model system used in the study of cell growth and the cell cycle. Secondly in *E.coli*, a commonly used prokaryote system. As *E.coli* would not normally be able to produce acetylated α-Synuclein, a strain producing the NatB complex was used to produce Nt-acetylated α-Synuclein. (Johnson M. *et al* 2010).

2. CHAPTER II: MATERIALS AND METHODS

2.1: Media recipes

LB Medium (1 litre): 5g Yeast extract, 10g Tryptone, 10g Sodium Chloride, 15g Agar (if solid medium required) in 1 litre of distilled water. This was then autoclaved and if antibiotic selection was required 1µl of the required antibiotic (Ampicillin at 50mg/ml in water or Chloramphenicol at 25mg/ml in Ethanol) per ml of medium was added after autoclaving.

YES Medium (1 litre): 5g Yeast extract, 30g Glucose, 15g Agar (if solid medium required) in 1 litre of distilled water. 1 in 50 of Amino Acids are also added prior to autoclaving for liquid medium or just before pouring plates for solid medium.

EMMG Medium (1 litre): 27.3g of Formedium EMM Broth (powder) w/o nitrogen, 3.38g Glutamate, 15g Agar (if solid medium required) in 1 litre of distilled water. This was then autoclaved.

50x Amino Acids (500ml): Uracil, Adenine, Histidine, Leucine. 5.625g of each amino acid is added to 500ml distilled water and then autoclaved. Each amino acid can be omitted as required to produce selective medium.

2.2: Buffer recipes

Phosphate Buffer Saline (PBS 1x): 8.7g Sodium Chloride, 1.82g Dipotassium hydrogenphosphate, 0.23g Potassium dihydrogenphosphate. In 1 litre of distilled water, adjusted to pH7.4

20mMTris Buffer pH7.5: 12.11g Tris base in 5 litres of distilled water, adjusted to pH7.5.

Buffer B: As used in protein purification 58.44g of Sodium Chloride is added to 1litre of 20mMTris buffer pH7.5.

Protein Loading Buffer: 50mM Tris-HCl pH6.8, 2%SDS, 10% Glycerol, 1% β-mercaptoethanol, 12.5mM EDTA. 0.02%bromophenol blue.

Western Blot Detection Buffer: 4ml of 1M Tris, 800µl of 5M Sodium Chloride, 200µl of 1M Magnesium Chloride, made up to 40ml with distilled water.

Stop Buffer: 150mM Sodium Chloride, 10mM EDTA, 1mM Sodium Azide.

IP (protein friendly) Buffer: 7.5ml distilled water, 600µl of 3M Potassium Chloride, 100µl of 10%Triton, 1ml of 0.5M Hepes pH8, 100µl of 0.5M EDTA pH8, 100µl of 0.5M EGTA pH8, 500µl of 1M Sodium 3 glycophosphate, 100µl of PMSF (protease inhibitor added at the last moment).

TAE Buffer: 0.4mM Tris base, 0.4mM glacial acetic acid, 0.01mM EDTA.

2.3: Preparation of competent cells

Strains of *E.coli*, either DH10β, BL21DE3, BL21Nat+A or BL21Nat+B were streaked out onto LB agar plates, containing the appropriate antibiotic if the cells possessed a plasmid and grown at 37ºC overnight. From this a single colony was inoculated into 5ml of LB medium with antibiotic where required and grown with shaking at 120rpm at 37ºC overnight. 0.5ml of this pre culture was then inoculated into 50ml of fresh LB medium with antibiotics as appropriate and grown with shaking at 120rpm at a temperature of 37ºC until an OD_{600} of 0.6 to 0.8 was reached. The cells were then cooled on ice for 10 minutes, before being centrifuged at 3000rpm at 4ºC for 10 minutes. The pelleted cells were then resuspended in 10ml of ice cold 0.1M $CaCl_2$ (filter sterilised)/10%Glycerol solution and kept on ice for a further 15 minutes. The cells were then centrifuged again at 3000prm at 4ºC for 10 minutes. The pelleted cells were then resuspended in 1ml of the 0.1M $CaCl_2$/10%Glycerol solution and 40µl aliquots were frozen in liquid nitrogen, then stored at -80ºC.

2.4: Bacterial transformation

40μl of competent bacterial cells were defrosted on ice, inoculated with an appropriate volume of DNA (1-10μl), and were then kept on ice for 30 minutes. The cells were then heat shocked for 90 seconds at 42ºC, and immediately transferred back onto ice for 2 minutes. 100μl of LB broth medium was then added and the cells incubated at 37ºC for 1 hour. The cells were then plated out onto LB agar with appropriate antibiotics.

2.5: Yeast transformation

S.pombe were grown until a cell count of 10^6 cells per ml was reached. 20ml of the cells were then centrifuged at 3000rpm at 25ºC for 5 minutes. The pelleted cells were then resuspended in 1ml of 0.1M Lithium Acetate pH4.7. The cells were then centrifuged again at 3000rpm at 25ºC for 30 seconds. The pelleted cells were then resuspended in 0.1M Lithium Acetate pH4.7, 100μl per transformation plus 100μl for a No DNA control. The cells were then incubated at 25ºC for 1 hour. 100μl of 70% PEG4000 was then placed into new Eppendorf's, one for each transformation and the No DNA control and 100μl of the cells added to each tube, together with 3ml of the appropriate DNA where required. These were then vortexed to mix well and incubated at 25ºC for an hour, vortexing every 20 minutes. The cells were then heat shocked at 42ºC for 10 minutes, 200μl of EMMG was added and the cell incubated at 25ºC for a further hour. The cells were then centrifuged for 1 minute at 3000rpm and resuspended in 100μl of EMMG, then plated onto EMMG +thiamine plates.

2.6: Small scale preparation of plasmid DNA from bacteria

5ml of LB medium with appropriate antibiotic was inoculated with a single colony from a transformation plate and grown with shaking at 120rpm at 37^{o}C overnight. This was then centrifuged at 3000rpm for 5 minutes and DNA preparation was then carried out using a Qiagen, Qiaprep Spin Miniprep Kit using the manufacturer's reagents and protocol.

2.7: Restriction digests of DNA

Restriction digests of DNA were carried out either for isolating DNA to subsequently be gel purified and ligated to form new constructs (large scale digests) or in order to check newly made clones had bands of the correct sizes when run on agarose gel (small scale digest). Different restriction enzymes were used depending on the restriction sites present, restriction enzymes used were manufactured by Promega and New England Biolabs and were used at the concentrations supplied.

DNA was used where clear bands could be seen in an agarose gel, concentrations were not measured.

A typical small scale digest consisted of:

- 2μl DNA
- 2μl 10x enzyme buffer
- 14/15 μl water
- 1μl Restriction enzyme 1
- 1μl Restriction enzyme 2 (if double digest required)

A Typical large scale digestion consisted of:

- 10μl DNA
- 10μl 10x enzyme buffer
- 76/78 μl water
- 2μl Restriction enzyme 1
- 2μl Restriction enzyme 2 (if double digest required)

2.8: Cloning substrate genes into bacterial expression systems

Ligations of purified insert fragments and vectors were typically carried out as follows:

- 4μl Insert DNA
- 4μl Vector
- 1μl 10x ligase buffer (Promega)
- 1μl T4 DNA ligase (Promega)

PCR products were gel purified and ligated into the pGEM T-Easy vector. An example of this ligation reaction is:

- 3μl Purified PCR product
- 1μl pGEM T-Easy
- 4μl water
- 1μl 10x ligase buffer
- 1μl T4 DNA ligase

All ligations were kept at 4ºC overnight before use in bacterial transformations.

2.9: DNA gel electrophoresis

In order to check newly made clones or for isolating DNA to subsequently be gel purified, restriction digests of DNA were run on agarose gels. 1% agarose gels were made by dissolving 1g of agarose per 100ml of 0.5x TAE buffer. This was then heated until the solution was completely clear and poured into a casting tray, where 1μl of Ethidium Bromide (10μg/ml) per ml of 1% agarose gel was added. The DNA preparation was then run alongside 5μl of Bioline Hyperladder at 50 Volts for 1 hour. DNA bands were then visualised under ultraviolet light.

2.10: Gel purification of DNA

DNA from digests or PCR product was run on an agarose gel as described previously and the appropriate band excised from the gel. The gel piece was weighed and then purified using the Bioline Gene Clean 3 Kit using the manufacturer's reagents and protocol.

2.11: Polymerase Chain Reaction (PCR)

PCR was used to amplify genes encoding for target proteins, allowing them to be cloned into bacterial expression systems. The primers used and their target genes are shown in Table.1.

Primer Name	Primer Sequence	Target Gene
Pci1cydABF	ACatgttagatatagtcgaactgtcgcgc	cydAB
Sal1cydABR	GTCGACgtacagagagtgggtgttacg	cydAB
Sal1mCherryF	GTCGACatggtgagcaagggcgaggagg	mCherry
Not1mCHerryR	gcggccgcTTActtgtacagctcgtccatgcc	mCherry

Table.1. Primers used for PCR during this project.

2.12: Expression of recombinant proteins in *E.coli* and *S.pombe*.

Expression of recombinant protein for live cell imaging and small scale protein analysis: Recombinant protein was expressed in both *E.coli* and *S.pombe* for live cell imaging.

In *S.pombe* a pREP41 vector containing a repressible no message with thiamine (nmt) promoter upstream of the multiple cloning site (MCS) was used, so that recombinant protein expression was suppressed when thiamine was present in the medium used.

Cells were grown in 20ml of EMMG, either with (for very low levels to no expression) or without (moderate levels of expression) 1μl per ml of 0.5mM thiamine.

In *E.coli,* a T7 promoter was located on both the pETDuet and pJC20 plasmids used, this meant that recombinant protein was induced with the addition of 1μl per ml of IPTG (0.1mg/ml stock). *E.coli* cells and the protein produced were examined at time points ranging from pre induction to 90 hours post induction. A single colony from a transformation plate was used, inoculated into 5ml of LB media with appropriate antibiotic and grown with shaking at 120rpm at 37ºC overnight. 100μl of this starter culture was used to inoculate 5ml of LB media with appropriate antibiotic and grown with shaking at 120rpm at 37ºC until an OD_{600} of 0.4 to 0.6 was reached, 1μl per ml of IPTG (0.1mg/ml stock) was then added to induce protein expression where required. Where cells were produced for protein analysis 1ml samples were taken, their OD_{600} measured and then the cells centrifuged at 3000rpm for 5 minutes, pelleted cells were then stored at -20ºC until required.

Expression of recombinant protein for Transmission Electron Microscopy (TEM) and large scale protein purification from growth media: Only *E.coli* was used for TEM and large scale protein purification from growth media. Again a single colony from a transformation plate was used, inoculated into 5ml of LB media with appropriate antibiotic and grown with shaking at 120rpm at 37ºC overnight. 2ml of this starter culture was used to inoculate 200ml of LB media with appropriate antibiotic and grown with shaking at 120rpm at 37ºC until an OD_{600} of 0.4 to 0.6 was reached, 1μl per ml of IPTG (100mg/ml stock) was then added to induce protein expression. For TEM samples 50ml was then placed into a falcon tube and centrifuged at 2500rpm for 10 minutes before being sent for preparation and examination by TEM.

Cells induced for large scale protein purification from cell cleared culture media were grown and induced the same as for TEM, but the induced cells were grown for 4 days, before removal of cells from the culture media.

2.13: Purification of α-Synuclein by Fast Protein Liquid Chromatography (FPLC)

The Protein samples expressed in *E.coli* and concentrated from the growth medium were purified by FPLC. The samples were run through a 5ml HiTrapQ ion exchange column and the bound protein was then eluted from the column using an increasing concentration gradient of Buffer B. Protein containing fractions were determined by absorbance at 260nm and 280nm.

2.14: Preparation of cell extracts and media for protein analysis

Different methods were used to prepare small scale protein samples for analysis.

To prepare protein samples from *S.pombe,* cells were grown to a density of 10^6 cells/ml. All preparation steps were carried out on ice or at 4ºC. 10ml was then centrifuged at 3000rpm for 3 minutes. The pelleted cells were then resuspended in 1ml of Stop Buffer and placed into a ribolyse tube. This was then centrifuged at 13000rpm for 1 minute and the resultant pellet stored at -20ºC. The pellets were then resuspended in 100μl of IP (protein friendly) Buffer and 500μl of glass beads were added. The cells were then broken by being placed in a fast prep ribolyse for 40 seconds at 6.5. A hole was punched through the bottom of the tube and a further 100μl of IP Buffer was added and the whole ribolyse tube placed into a 15ml falcon tube before being centrifuged at 3000rpm for 1 minute. The ribolyse tube was removed and the remaining supernatant and pellet resuspended and placed into an Eppendorf which was centrifuged at 13000rpm for 5 minutes. The resultant supernatant were retained for protein analysis and the pellet was resuspended in 200ml of IP Buffer to produce a further sample for protein analysis.

To prepare whole cell protein samples from *E.coli*, a 1ml sample was taken from the cell suspension to be examined and its OD_{600} measured. The cells were then centrifuged at 3000rpm at 4ºC for 5 minutes and the pelleted cells were frozen at -20ºC. The defrosted pellet was then resuspended in 1xPBS Buffer according to the

OD_{600} measured, e.g. a pellet from a culture with an original OD_{600} of 1.2 would be resuspended in 120μl of 1xPBS.

To prepare small scale protein samples for analysis, from cell cleared culture media, protein was precipitated from the media. A sample of *E.coli* cells growing in LB medium was centrifuged to remove all cells, 400μl of the growth media was then placed to a 2ml tube with 1.6ml of acetone and this was then frozen at -80°C. The thawed precipitated protein was then centrifuged at 13000rpm at 4°C for 30 minutes, the supernatant removed and the pellet dried. The pellet was then resuspended in 40μl of 1xPBS ready for analysis.

To prepare protein samples for FPLC purification and analysis, from growth media, cells growing in 200ml of LB medium were centrifuged at 4600rpm for 10 minutes the supernatant was kept and the pelleted cells discarded. The supernatant was then centrifuged again at 4600rpm for 10 minutes. This supernatant was then placed into dialysis tubing which was placed into changes of 20mM Tris buffer pH7.5 at 4°C with stirring for 7 days. The supernatant was then filtered through a centricon centrifuge filter until concentrated to approximately 7ml ready for purification by FPLC.

2.15: Gel electrophoresis of proteins

Protein samples were analysed by gel electrophoresis using NuPAGE® Novex® 4-12% Bis-Tris Gels from *In vitro*gen. Protein samples were mixed 4 parts sample to 1 part 5x Protein loading buffer and heated at 95°C for 30 minutes with vortexing. 10μl of each sample was run alongside 5μl of Thermo Scientific PageRuler Unstained Protein Ladder #26614, at 150volts for 50 minutes, using the manufacturer's reagents and protocol. Bands were visualised when required using Coomassie Blue (0.2%Coomassie blue, 7.5% Acetic acid, 50% Methanol)

When gels were for use in Western Blotting, Bio-Rad Kaleidoscope Prestained Standards were used.

2.16: Western Blotting

Gels were run as outlined above and protein transferred to a membrane using a Bio-Rad Trans-Blot Electrophoretic Transfer Cell, following the manufacturer's protocols at 10volts for 30 minutes. The membrane was then blocked using Milk solution (1.5g milk powder in 50ml 1x PBS + 100μl tween20) at room temperature for 1 hour. The membrane was then incubated in milk solution with primary antibody diluted 1 in 1000 (anti-GFP or anti-a-Synuclein) at room temperature for 1 hour. The membrane was then rinsed four times in 1xPBS before having milk solution added until the membrane was just covered. 2μl of secondary antibody (anti-mouse or anti-rabbit) added and this was incubated at room temperature for 1 hour. The membrane was then rinsed with 1xPBS and then covered with fresh, Western Blot Detection Buffer for 5 minutes. Enzyme substrate was then added and colour allowed to develop to visualise the bands.

2.17: Preparation of slides for fluorescent microscopy

Lectin slides: During live-cell imaging, yeast cells were cultured in Edinburgh minimal media using 20 mM L-Glutamic acid as a nitrogen source (EMMG). Cells were grown exponentially at 25ºC for 48hr before being mounted (without centrifugation) onto lectin (Sigma L2380; 1 mg/ml) coated coverslip chambers

PEI slides: PEI was diluted in distilled water and 10μl placed onto a coverslip and allowed to dry for 1 minute. 10μl of cells were then added and allowed to dry for 1 minute. The coverslip was then fixed to the slide with double sided tape or nail varnish. This method was used with *E.coli*.

Agarose slides: Agarose slides were used when *E.coli* had to be kept extremely still or for observing misshapen *S.pombe,* which did not stick well to lectin. A spacer slide was prepared by placing two bands of tape around a slide to form a lower space between the tapes. 2% agarose was then made in either distilled water or LB medium and heated until dissolved and appropriate antibiotic then added. LB medium was used when *E.coli* were incubated on the slide. 15μl of hot liquid 2% agarose was then

placed onto the slide being made and the spacer slide placed quickly and firmly on top, so as to form a thin flat disc of agarose between the tapes. The spacer slide was then slid off leaving the agarose disc on the required slide. 10-15μl of the cells to be viewed were then spread across the disc and allowed to dry until sticky, a coverslip was then placed on top and fixed to the slide with nail varnish.

2.18: Obtaining fluorescent images of cells

Samples were visualised using an Olympus IX71 microscope with PlanApo 100x OTIRFM-SP 1.45 NA lens mounted on a PIFOC z-axis focus drive (Physik Instrumente, Karlsruhe, Germany), and illuminated using LED light sources (Cairn Research Ltd, Faversham, UK) with appropriate filters (Chroma, Bellows Falls, VT). An Optosplit device (Cairn Research Ltd) was used to allow simultaneous acquisition of signals from two fluorophores that emitted light of different wavelengths. Samples were visualised using either a QuantEM (Photometrics) EMCCD or Zyla 4.2 (Andor) CMOS camera, and the system was controlled with Metamorph software (Molecular Devices). Each 3D-maximum projection of volume data was calculated from 21 or 31 z-plane images, each 0.2 μm apart, and analysed using Metamorph and Autoquant X software.

Samples were fitted onto an ASI motorised stage (ASI, Eugene, OR) on the above system, with the sample holder, objective lens and environmental chamber held at the required temperature.

2.19: Obtaining images by Transmission Electron Microscopy (TEM) and Immuno EM

E.coli cells were sent to Mr Ian Brown, Microscopy Suite Facility Manager, University of Kent, for preparation and thin sectioning to visualize the interior of the cells. Three different preparation techniques were used including the use of, Osmium tetroxide and Thiocarbohydrazide, which strongly stains membranes, or using an immunogold-staining technique post thin sectioning using 1 in 500 anti-α-Synuclein antibody and a secondary anti-rabbit antibody attached to 15nm gold discs.

TEM: Preparation of samples for thin sectioning

Cells were fixed for 2 hours in 1.5 ml 2.5 % Glutardialdehyde in PBS. Subsequently, the cells were pelleted and washed twice with PBS to remove traces of the fixing solution. The cells were stained for 1 hour in 1% osmium tetroxide and then washed twice with PBS before dehydration. This was accomplished by subjecting the samples to a solvent gradient: 70 % Ethanol for 20 minutes, 100 % Ethanol for 20 minutes, 100 % Ethanol for 30 minutes, twice 100 % Propylene oxide for 30 minutes. The cells were embedded by first washing them in 50 : 50 Agar Low Viscosity Resin : propylene oxide for 60 minutes and then embedding them in 100 % Agar Low Viscosity Resin for 180 minutes constituted for a block with medium hardness. The Samples were placed in 0.5 ml embedding tubes, centrifuged for 5 minutes at 4,000 x g to concentrate the cells to the tip and incubated at 60ºC overnight to polymerise.

Sectioning and visualisation of TEM samples

Specimens were thin sectioned with glass knives on an RMC MT-6000-XL ultramicrotome, collected on formvar-coated copper grids, post-stained with 5% uranyl acetate for 30 minutes at 60ºC and 0.1% lead citrate for 10 minutes at room temperature. Sections were then observed and photographed with a JEOL-1230 transmission electron microscope.

3. CHAPTER III: RESULTS; *Schizosaccharomyces pombe*

3.1: Expression of α-Synuclein with a GFP tag

It was first decided to ensure that human α-Synuclein could be expressed in *S.pombe* and that this could be observed by fluorescent microscopy, by means of a Green Fluorescent Protein (GFP) fluorescent tag. A wild type *S.pombe* strain, $h^{-}Leu^{-}32$ transformed with a pREP41 plasmid containing the gene for α-Synuclein fixed at its 3' end to the cDNA for GFP. pREP41 contains a repressible no message with thiamine (nmt) promoter upstream of the multiple cloning site (MCS), allowing expression levels to be modulated by the addition of thiamine. In this plasmid low levels of protein may still be produced when grown in the presence of thiamine. The same strain of *S.pombe* was also transformed using the same pREP41 plasmid containing only the GFP gene as a negative control.

Cultures of wild type *S.pombe* containing each vector, pREP41GFP (Figures 6 & 7) or pREP41 αS-GFP (Figures 8 & 9), were grown with thiamine to inhibit vector protein production and without thiamine to allow vector protein production, at 0.5μM in EMMG. All four cultures were mounted on slides using Lectin and observed using both phase and fluorescent microscopy.

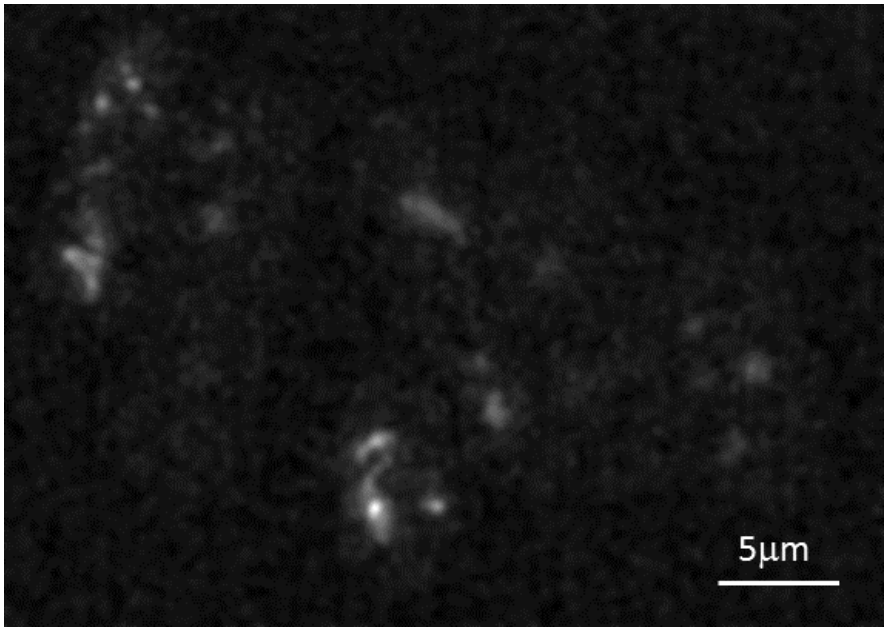


Figure.6. Image of *S.pombe* wild type pREP41GFP grown with thiamine, showing very low levels of cytoplasmic GFP.

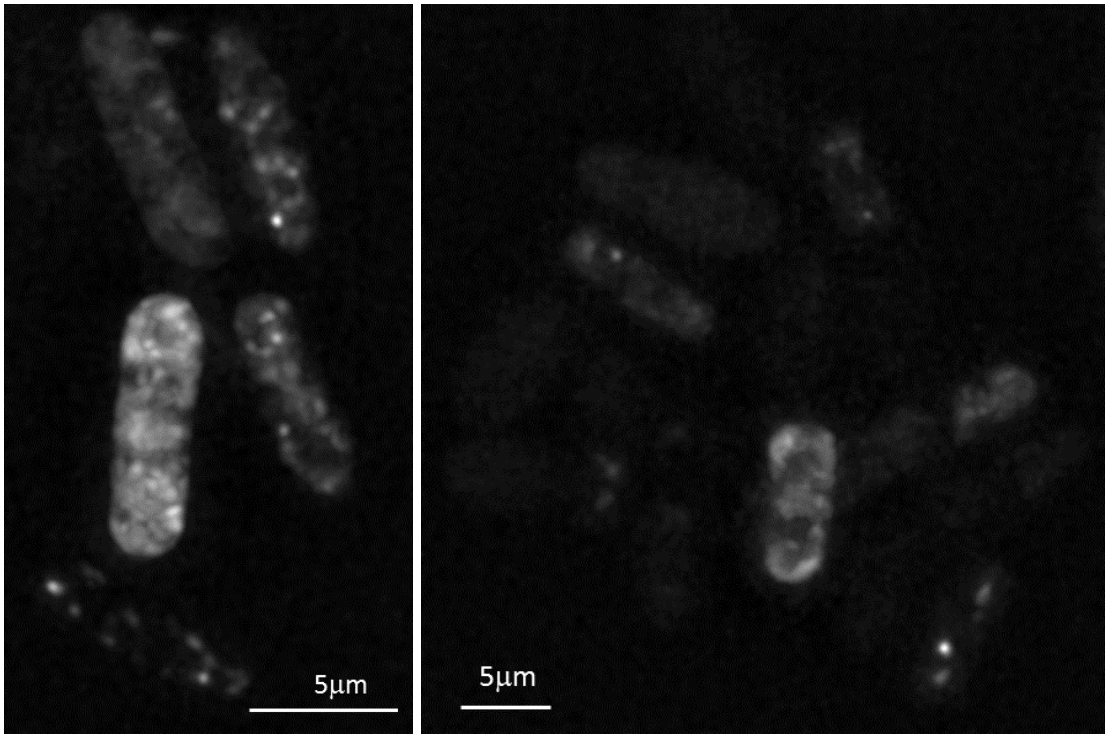


Figure.7. Images of *S.pombe* wild type pREP41GFP grown without thiamine, showing cytoplasmic GFP with some small aggregates.

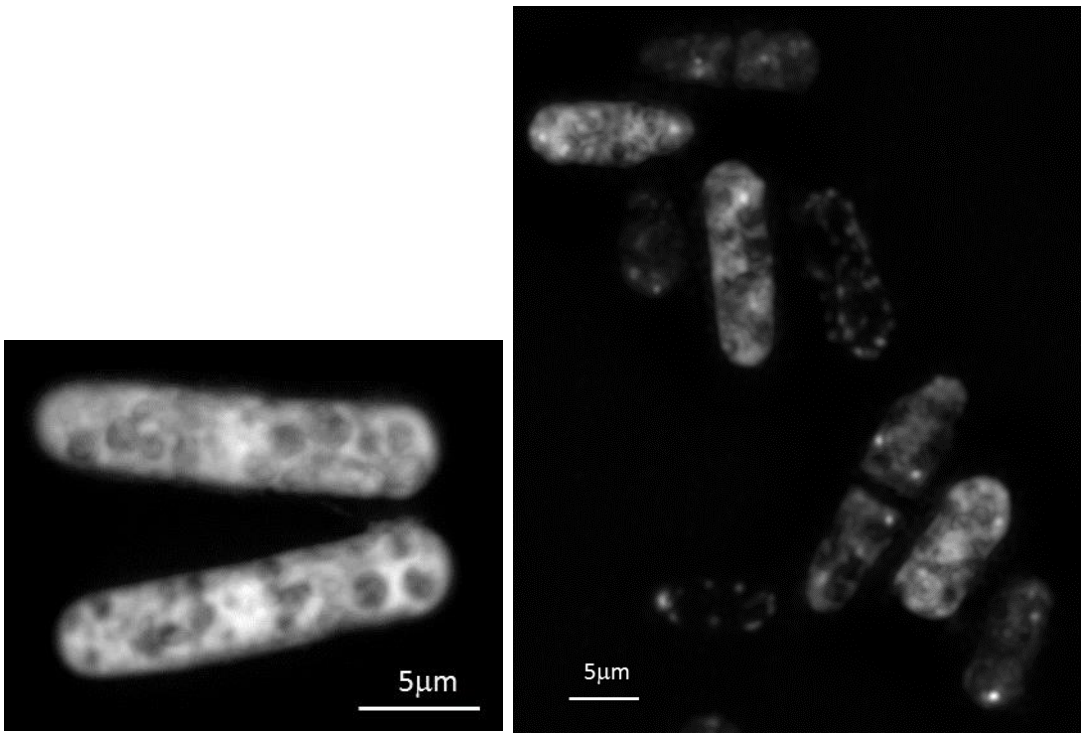


Figure.8. Images of *S.pombe* wild type pREP41αS-GFP grown with thiamine, showing cytoplasmic GFP, though this appears to be excluded from vacuoles, bright spots indicate the possible presence of small aggregates.

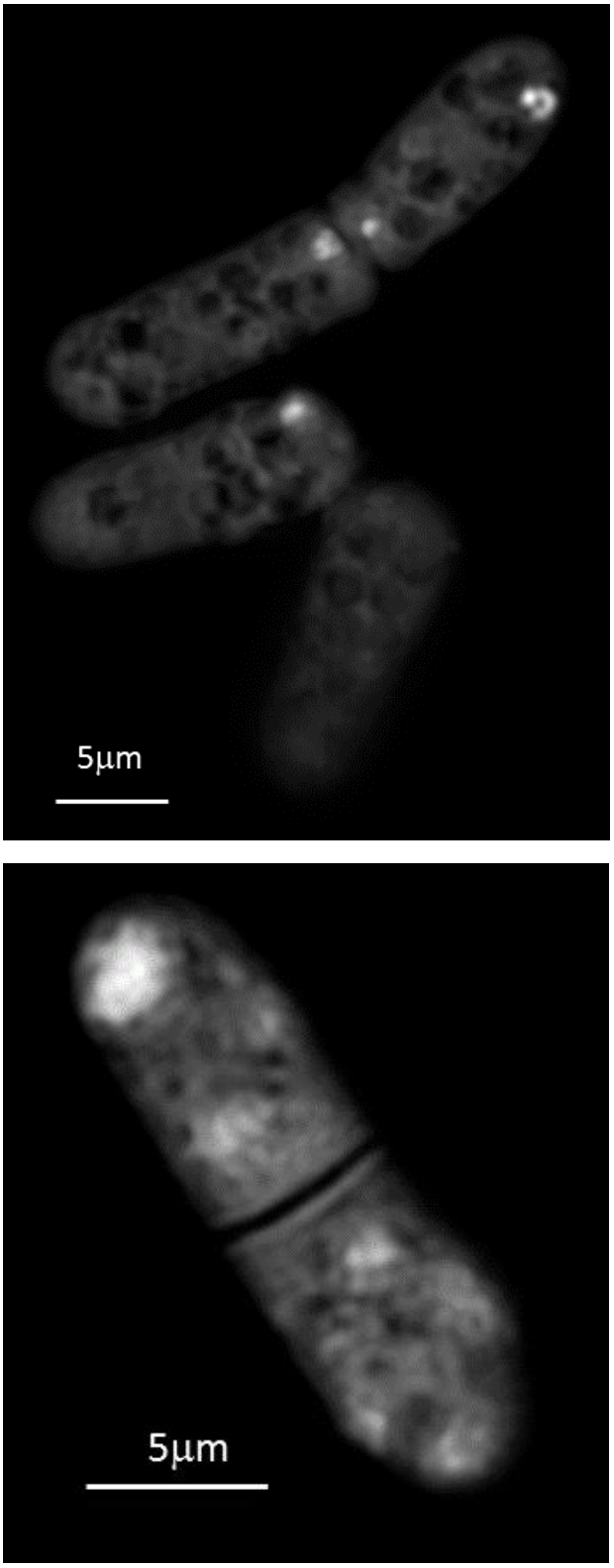


Figure.9. Images of *S.pombe* wild type pREP41αS-GFP grown without thiamine, showing cytoplasmic GFP, which again appears to be excluded from vacuoles, bright spots indicate the possible presence of fewer larger aggregates.

3.2: Western Blot analysis of complete lysed cells

To confirm the presence and examine the levels of α-Synuclein-GFP produced, cultures of *S.pombe* wild type pREP41αS-GFP were grown with and without thiamine until stationary phase was reached, as confirmed by high cell counts.

S.pombe wild type pREP41αS-GFP + thiamine $5.04x10^7$ cells/ml

S.pombe wild type pREP41αS-GFP - thiamine $4.64x10^7$ cells/ml

10ml of each culture was prepared for analysis by Western Blot, keeping the cells and fractions on ice or at 4ºC at all times during handling. During this first protein preparation TCA was used instead of IP (protein friendly) Buffer.

A Western Blot was then carried out on both samples using anti-GFP as the primary antibody and anti-mouse IgG as the secondary antibody (Figure. 10). α-Synuclein has an expected MW of 14.5kDa and GFP an expected MW of 26.9kDa. a-Synuclein-GFP is therefore expected to show a combined MW of 41.4kDa. A dimer of α-Synuclein-GFP would be expected to have a MW of 82.8kDa, a band is seen at about this weight in the sample grown without thiamine.

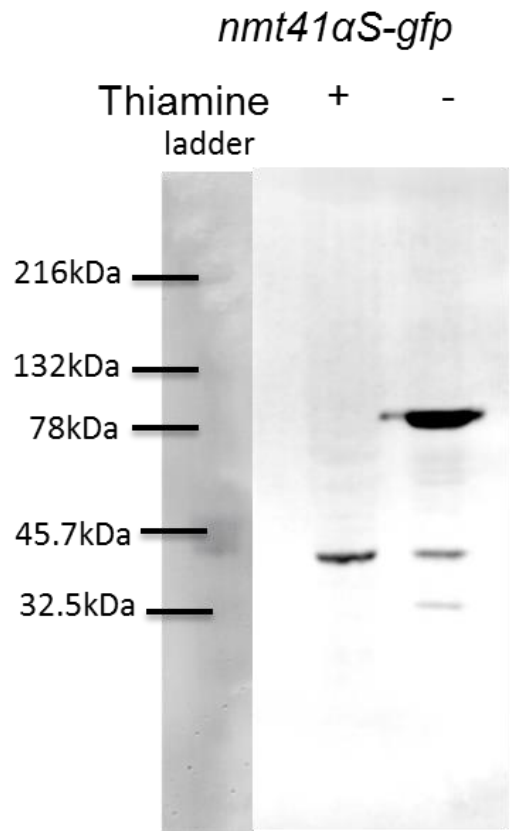


Figure.10. Western Blot showing the presence of α-Synuclein-GFP as a monomer in the sample grown with thiamine where expression is at low levels and as both a monomer and possible dimer in the sample grown without thiamine, where expression is not inhibited and so at a higher level.

3.3: Growth Curves obtained at OD_{600}

The four cultures of *S.pombe* wild type with pREP41GFP or pREP41αS-GFP grown both with and without thiamine were each prepared in EMMG to a density of $5x10^5$ cells/ml. 1ml of each was then placed into 4 wells of a 24 well plate. The plate was then placed into a plate reader set to shake the plate at 25ºC and to take OD_{600} readings every 30 minutes.

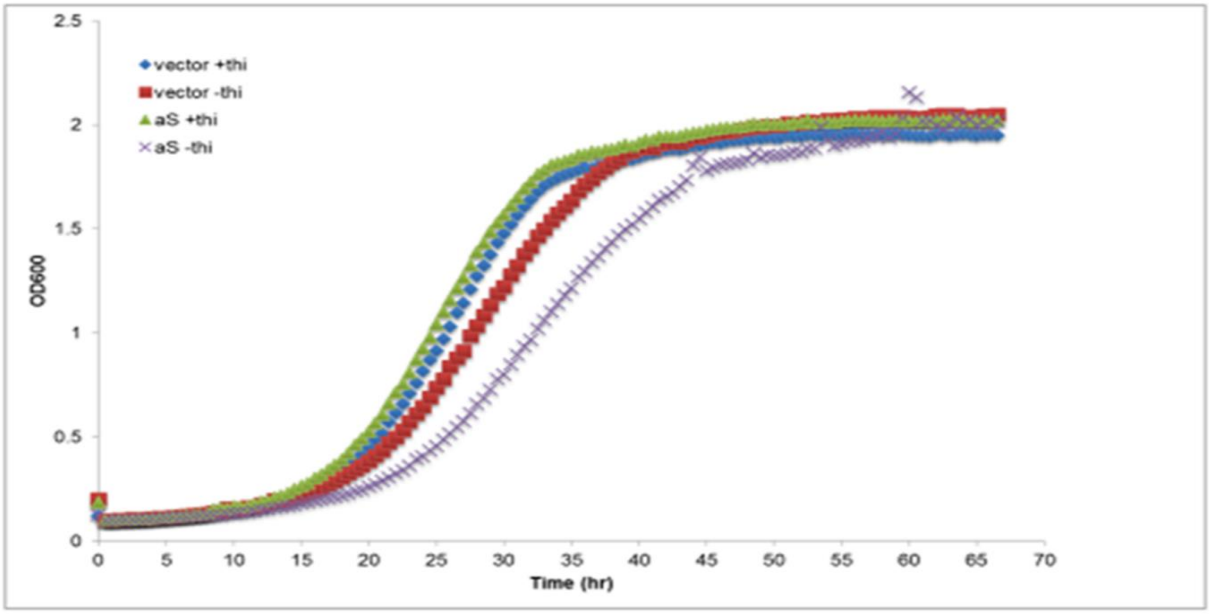


Figure.11. OD_{600} Growth Curves for *S.pombe* wild type containing pREP41GFP or pREP41αS-GFP.

The OD_{600} Growth Curves obtained (Figure. 11) show the two cultures with thiamine have the fastest rate of growth during log phase. The cells producing GFP showed a slightly slower growth rate possibly due to the production of GFP and the cells producing α-Synuclein-GFP show a much slower growth rate during log phase. The change in OD_{600} per hour was calculated for each culture during log phase (25 to 30 hours) (Table.2).

Time	25	30	Change per hour in OD_{600}
GFP +thi	0.913	1.476	0.112
GFP -thi	0.737	1.223	0.097
aS +thi	1.043	1.568	0.105
aS -thi	0.458	0.801	0.068

Table.2. Comparison of OD_{600} growth rates of *S.pombe* containing vectors pREP41GFP or pREP41αS-GFP grown with and without thiamine.

3.4: Western Blot Analysis of Separate Pellet and Supernatant fractions

Having seen evidence of α-Synuclein-GFP dimers in the first Western Blot it was decided to carry out another, using a gentler protein preparation method using protein friendly IP Buffer instead of TCA. It was also decided to look at where the α-Synuclein-GFP was located within the cells by separate examination of the supernatant and pellet obtained following centrifugation of the lysed cells. This was carried out using cultures of *S.pombe* wild type with pREP41GFP or pREP41αS-GFP grown both with and without thiamine.

The Western Blot was carried out on all four samples using anti-GFP as the primary antibody and anti-mouse IgG as the secondary antibody (Figure.12).

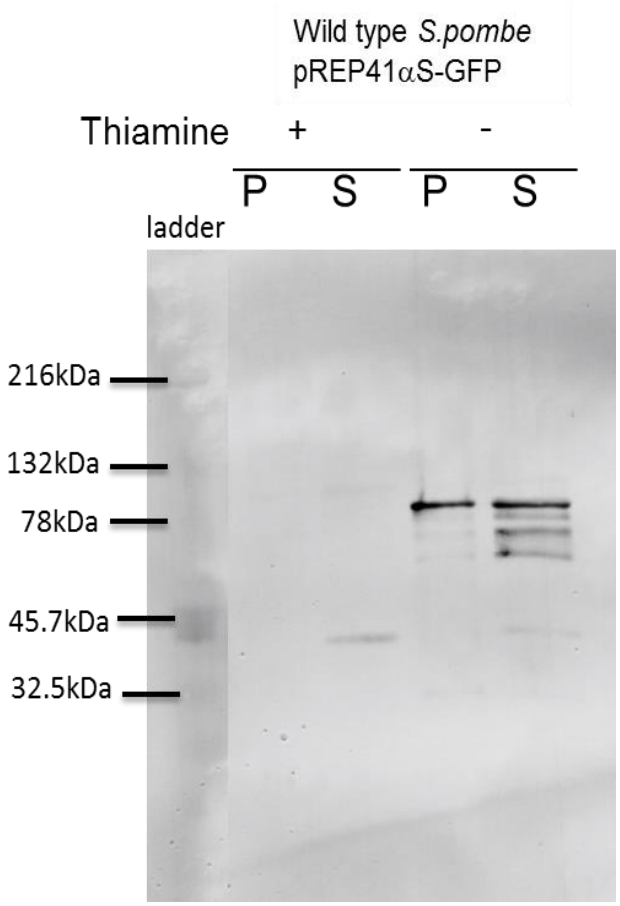


Figure.12. Western Blot which shows the presence of α-Synuclein-GFP at the expected MW for a monomer in the sample grown with thiamine where expression is at low levels, but only in the supernatant. Bands of the expected MW's for both monomer and dimer were seen in the supernatant of the sample grown without thiamine where expression is not inhibited and so at a higher level Possible damaged fragments of dimer were seen in both samples grown without thiamine. Dimer and dimer fragments only were seen in the pellet grown without thiamine.

3.5: Cell length comparisons

It had been observed during Fluorescent microscopy that some of the *S.pombe* wild type with pREP41αS-GFP cells seemed to be longer than would be normal. To investigate whether there was any significant difference in cell length due to the presence of α-Synuclein-GFP and any possible effect on cell cycle and growth. Multiple images were taken of *S.pombe* wild type with pREP41GFP or pREP41αS-GFP grown both with and without thiamine and their cell length measured in pixels on the computer. The average, maximum and minimum cell lengths were calculated for comparison of the different cultures (Table. 3).

Culture	pREP41GFP +thiamine	pREP41GFP -thiamine	pREP41αS-GFP +thiamine	pREP41αS-GFP -thiamine
Total length	2858	3474	5044	2482
No of cells	93	122	183	92
Average length	30.7	28.4	27.5	26.9
Maximum length	42.2	42.1	64.8	47.2
Minimum length	18.5	14.0	14.6	17.0

Table.3. Comparison of cell lengths in *S.pombe* wild type with pREP41GFP or pREP41αS-GFP grown both with and without thiamine, measured in pixels.

Although *S.pombe* wild type with pREP41αS-GFP was found to have a much larger maximum length, the average cell length was found to be midrange for the cultures examined.

3.6: OD_{600} growth curves obtained for a naa25Δ *S.pombe* strain expressing α-Synuclein-GFP

To investigate whether N-terminal acetylation made any difference to the effects of α-Synuclein on cells, the same vectors as used earlier, pREP41GFP and pREP41αS-GFP, where α-Synuclein is C terminally tagged with GFP, were transformed into a naa25Δ *S.pombe* strain. Naa25 encodes the auxiliary subunit of the fission yeast N-α-acetyltransferase complex (NatB). This strain is unable to N-terminally acetylate α-Synuclein. OD_{600} growth curves were obtained for each culture by repeating the procedure followed previously, but allowed to run for a longer time period due to the slow growth of the naa25Δ *S.pombe* strain (Figure. 13)

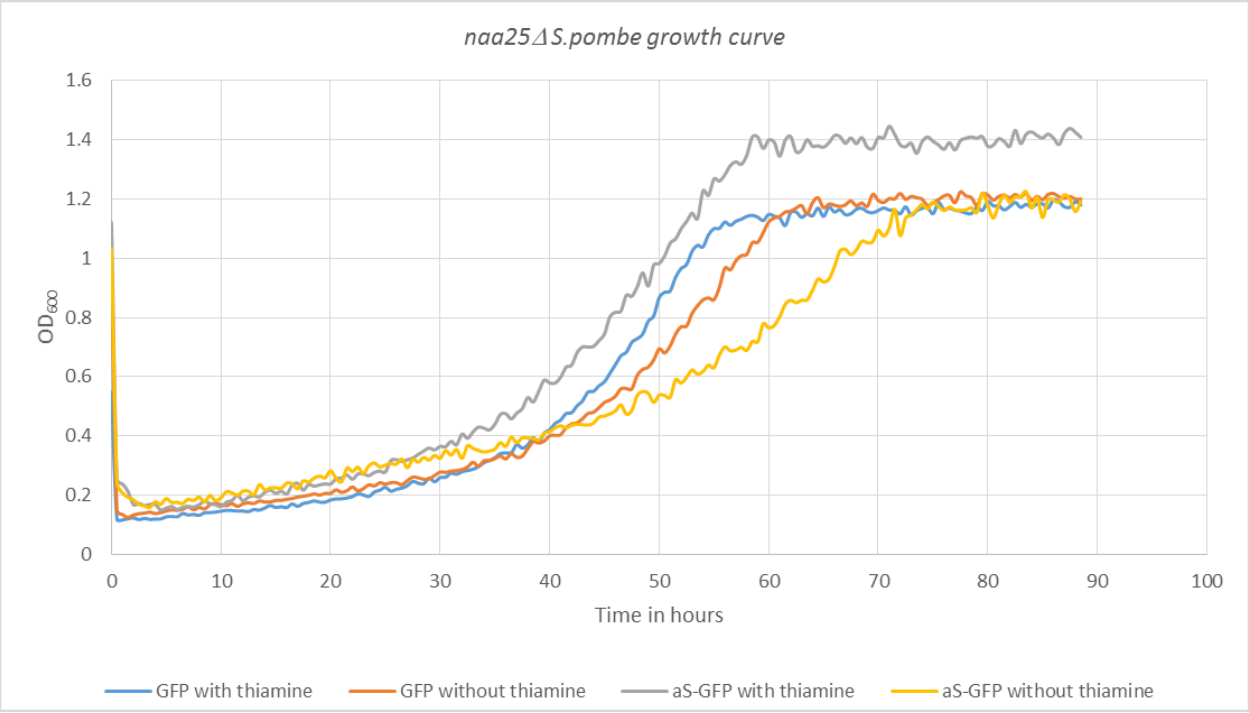


Figure.13. OD_{600} Growth Curves for a naa25Δ *S.pombe* strain containing pREP41GFP or pREP41αS-GFP grown with and without thiamine.

The maximum rate of growth during log phase was calculated for each culture (Table. 4). The two samples with thiamine showed the fastest growth rates. The pREP41GFP culture without thiamine, producing GFP, showed a slightly slower growth rate, possibly due to the production of the extra GFP protein. The pREP41αS-GFP culture without thiamine, producing α-Synuclein-GFP, showed a much slower growth rate, almost half that of the same culture grown with thiamine. The pREP41αS-GFP culture with thiamine also reached a higher overall OD_{600} the reason for this is unknown.

Culture	time points used	OD_{600} at first time point	OD_{600} at second time point	Change per hour
pREP41GFP +thia	45-55	0.583	1.100	0.0517
pREP41GFP -thia	50-60	0.694	1.122	0.0428
pREP41αS-GFP +thi	45-55	0.744	1.267	0.0523
pREP41αS-GFP -thi	50-70	0.537	1.094	0.0278

Table.4. Comparison of OD_{600} growth rates of a naa25Δ *S.pombe* strain containing pREP41GFP or pREP41αS-GFP grown with and without thiamine.

3.7: Fluorescent Microscopy of a naa25Δ *S.pombe* strain expressing GFP or α-Synuclein-GFP

Fluorescent images were taken of the four naa25Δ *S.pombe* strain cultures used to obtain the previous growth curves (Figure 14), to compare the distribution of GFP and α-synuclein-GFP within *S.pombe,* when N-terminal acetylation of α-synuclein is not possible. As mentioned previously these naa25Δ cells are very slow growing, they also tend to grow in unusual deformed shapes and to clump together without any vectors having been transformed into them.

Varying levels of fluorescence were also seen within each field of view this is possibly due to varying levels of plasmid uptake between cells.

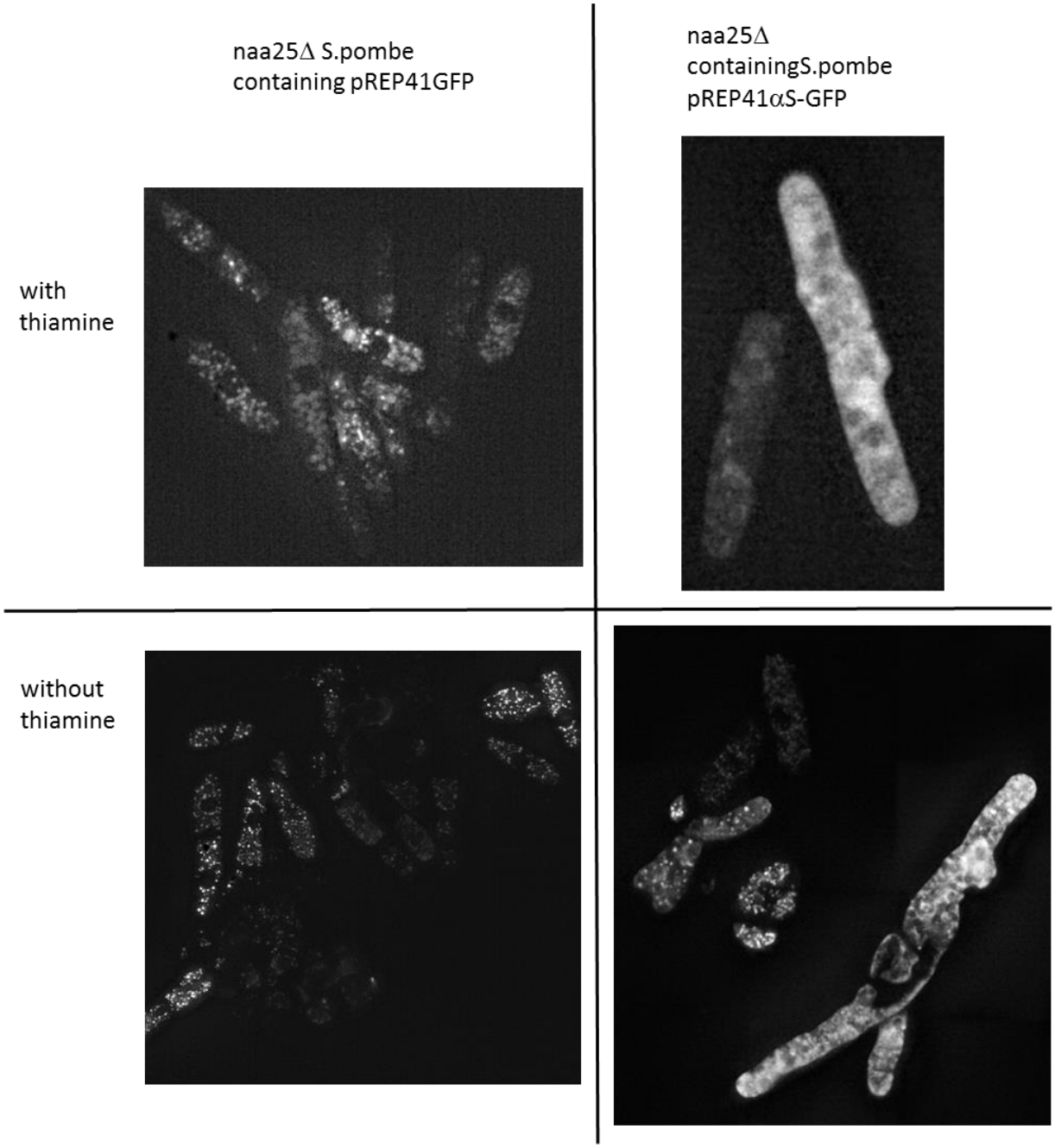


Figure.14. Images of naa25Δ *S.pombe* containing pREP41GFP grown both with and without thiamine had a low levels of fluoresence. Bright spots indicate the presence of small aggregates that are excluded from the nuclei. Images of naa25Δ S.pombe containing pREP41αS-GFP grown with thiamine, have low levels of fluorescence indicating the presence of low levels of α-Synuclein-GFP which appears to be dispersed through the cytoplasm but excluded from vacuoles. The image of naa25Δ S.pombe containing pREP41αS-GFP grown without thiamine, has higher levels of fluoresence indicating that a higher level of α-Synuclein-GFP was produced. This appears to be dispersed through the cytoplasm, but excluded from vacuoles in some cells. But in other cells bright spots indicate that the α-Synuclein-GFP may be starting to form small aggregates as with GFP alone.

3.8: The effect of acetylated and non-acetylated α-Synuclein on growth at different temperatures

It was decided to undertake a separate short investigation into whether the presence of either acetylated or non-acetylated α-Synuclein had any effect on growth at different temperatures.

Six plates were quartered and three had thiamine added to them. Each quarter was streaked with one of the four following colonies. *S.pombe* wild type pREP41GFP, *S.pombe* wild type pREP41αS-GFP, naa25Δ *S.pombe* pREP41GFP and naa25Δ *S.pombe* containing pREP41αS-GFP.One plate with and one without thiamine were incubated together at 25ºC, 28ºC and 35.5ºC. The growth was recorded (Table. 5) and pictures taken. Unfortunately due to computer problems the pictures were lost. No significant difference was seen with or without α-Synuclein present.

3 Days

temperature	*S.pombe* Strain	+thiamine		-thiamine	
		pREP41GFP	pREP41αS-GFP	pREP41GFP	pREP41αS-GFP
25ºC	wild type	lots of growth	lots of growth	lots of growth	lots of growth
	naa25Δ	lots of growth	lots of growth	lots of growth	lots of growth
28ºC	wild type	lots of growth	moderate growth	lots of growth	moderate growth
	naa25Δ	moderate growth	moderate growth	low growth	moderate growth
35.5ºC	wild type	lots of growth	lots of growth	lots of growth	lots of growth
	naa25Δ	very low growth	low growth	very low growth	low growth

6 Days

temperature	*S.pombe* Strain	+thiamine		-thiamine	
		pREP41GFP	pREP41αS-GFP	pREP41GFP	pREP41αS-GFP
25ºC	wild type	lots of growth	lots of growth	lots of growth	lots of growth
	naa25Δ	lots of growth	lots of growth	lots of growth	lots of growth
28ºC	wild type	lots of growth	lots of growth	lots of growth	lots of growth
	naa25Δ	lots of growth	lots of growth	lots of growth	lots of growth
35.5ºC	wild type	lots of growth	lots of growth	lots of growth	lots of growth
	naa25Δ	no growth	very low growth	no growth	very low growth

Table.5. Comparison of observed growth of *S.pombe* producing acetylated and unacetylated α-Synuclein at different temperatures.

3.9: Western Blot analysis of Separate Pellet and Supernatant fractions from a naa25Δ strain

To investigate whether N-terminal acetylation of α-Synuclein causes any differences in where the α-synuclein-GFP is located within *S.pombe* cells a Western Blot was again carried out with separate examination of the supernatant and pellet obtained following centrifugation of the lysed cells, as previously carried out with Wild type *S.pombe* pREP41αS-GFP. This was carried out using cultures of a naa25Δ *S.pombe* strain containing pREP41αS-GFP grown both with and without thiamine. The naa25 deletion means that the cells are unable to produce the naa25 auxiliary subunit of the NatB complex and so is unable to acetylate α-Synuclein. This is shown alongside the previous Western blot for comparison (Figure 15).

Again bands were seen at the approximate MW expected to be seen for monomers (41.4kDa) and dimers (82.8kDa) Bands were also seen again between these weights possibly of damaged dimer fragments.

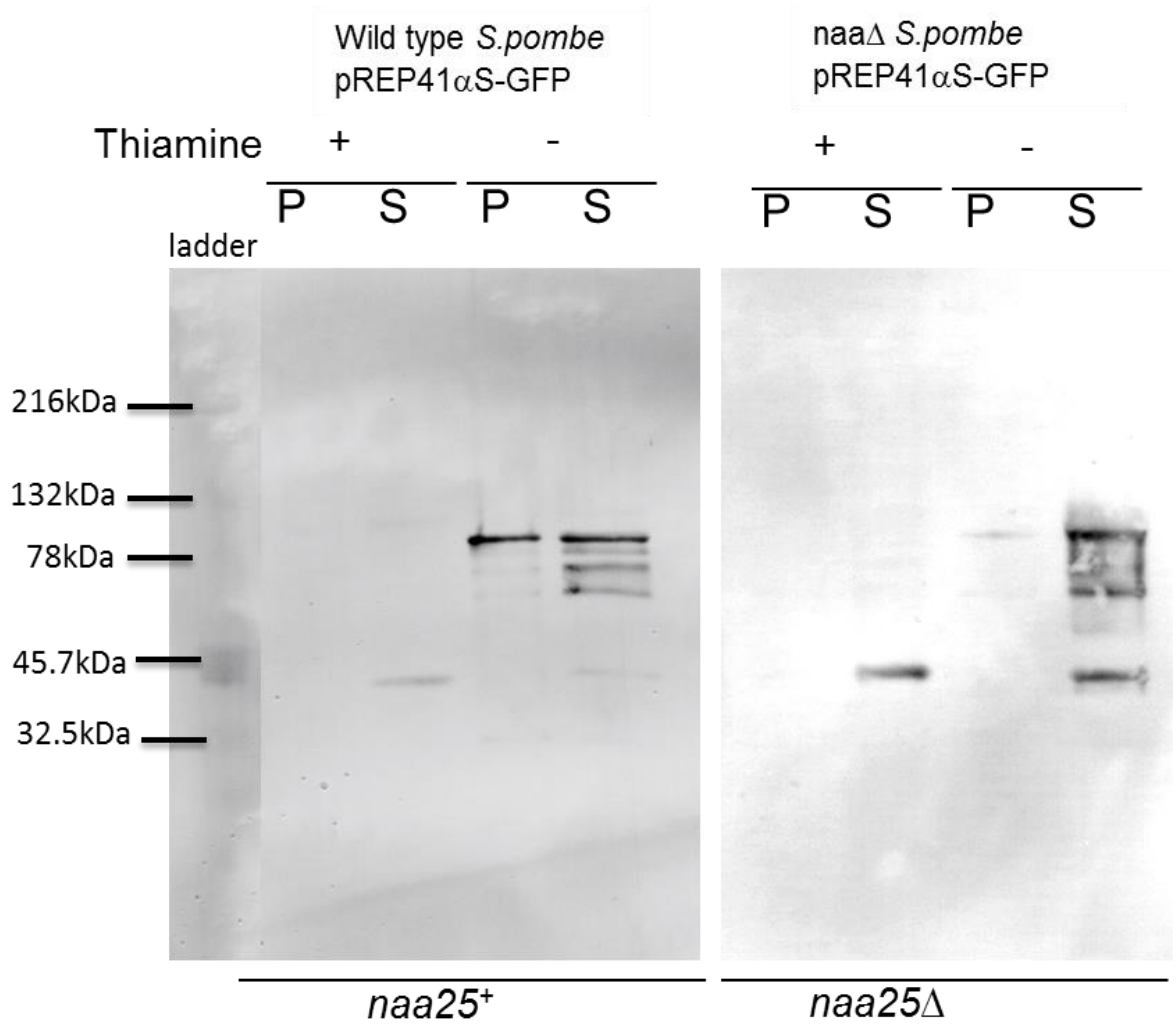


Figure.15. Western Blots that compare the presence of α-Synuclein-GFP as a monomer in the samples grown with thiamine, but seen only in the supernatant. Both monomer, dimer and fragments of dimer were seen in both of the –thiamine supernatants where expression is not inhibited and so at a higher level. Clear bands were only seen at the weight expected for dimers in both of the –thiamine pellets, faint bands were also visible also slightly below this weight possibly due to damaged dimer fragments.

3.10: Fluorescent Microscopy to show α-Synuclein Distribution Using Cerulean3 Fluorescent Protein

In an attempt to improve the resolution of the images taken and for use in combination with other fluorescent proteins, a new vector was produced replacing the cDNA for GFP with the cDNA for Cerulean3 Fluorescent Protein (Cer3) which is a brighter more stable protein. Wild type *S.pombe* cells containing pREP41αS-Cerulean3 were grown with and without thiamine. Slides were then prepared using Lectin and the cells observed and images taken using fluorescent and phase microscopy (Figures 16 & 17).

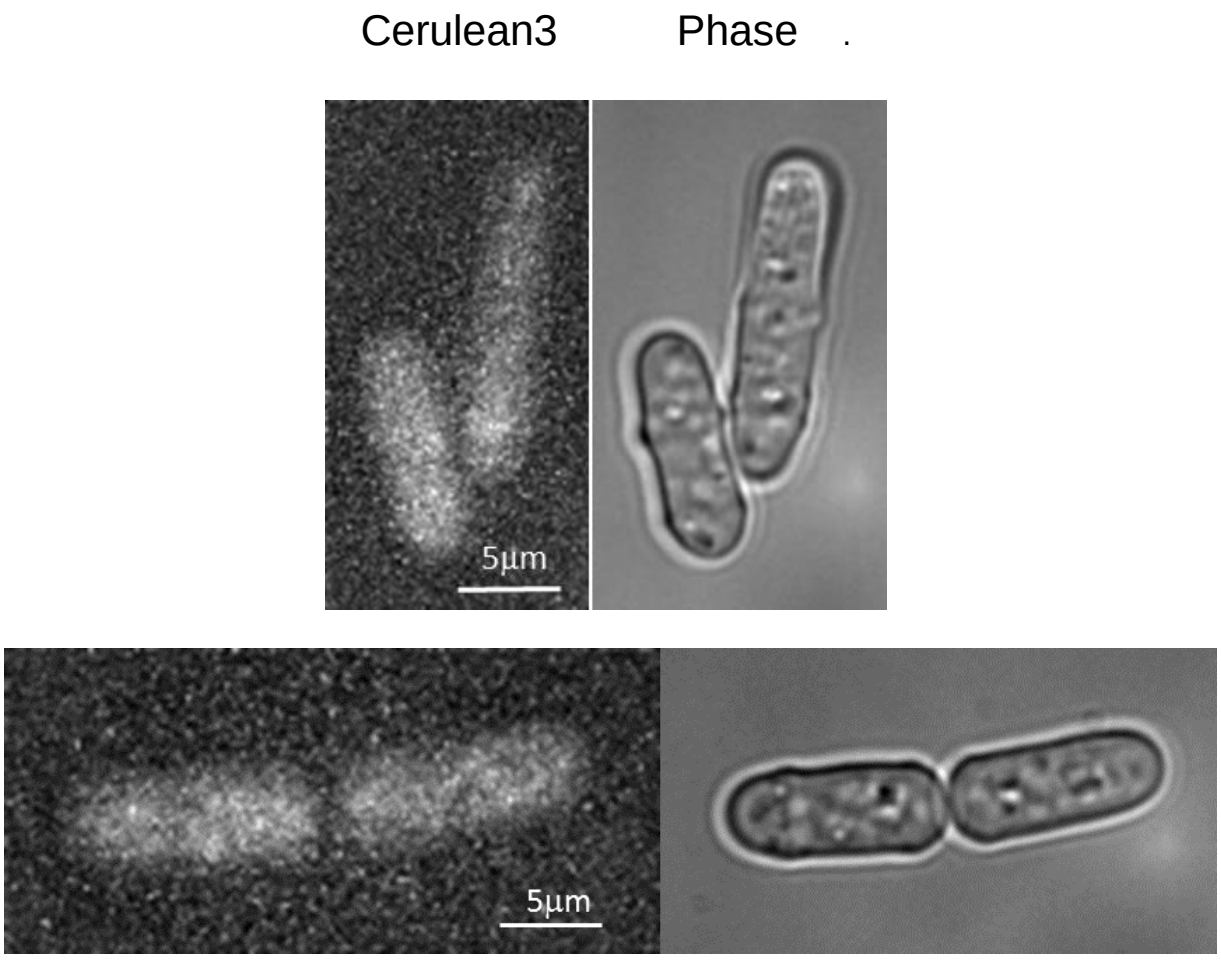


Figure.16. Images of wild type *S.pombe* pREP41αS-Cerulean3 grown with thiamine to repress vector expression. At low levels of vector protein expression very little fluorescence could be observed and so produced only faint fluorescent images, where the fluoresence appears to be cytoplasmic. Phase images were included to clearly show the cells being observed.

Cerulean3 Phase .

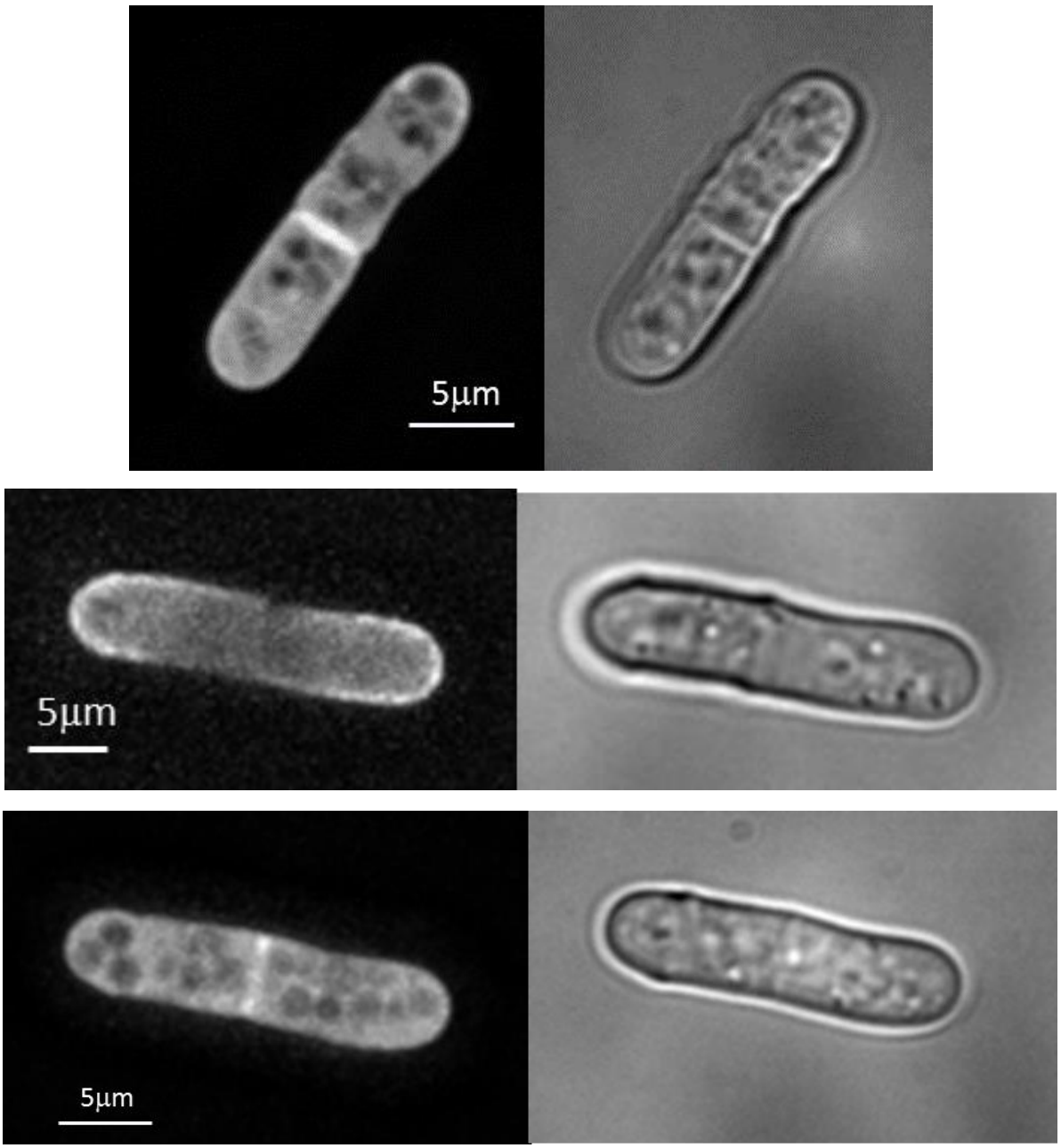


Figure.17. Images of wild type *S.pombe* pREP41αS-Cerulean3 grown without thiamine. At higher levels of vector protein expression far more fluorescence could be detected. In some cells with slightly lower levels this appears to be cytoplasmic, with a slight concentration at the poles. In others with higher levels it appears to be excluded from vacuoles and concentrated at the separation membrane.

3.11: Fluorescent Microscopy to show α-Synuclein Distribution in Relation to Membranes

To compare the distribution of α-Synuclein with the presence of membranes, wild type *S.pombe* pREP41αS-Cerulean3 were grown again with and without thiamine. A 1ml sample of each culture was stained with 1μl of FM4-64, a lipophilic probe which fluoresces red when it associates with plasma membranes. Slides were then prepared using Lectin and the cells observed and images taken using fluorescent and phase microscopy (Figures. 18 & 19) using appropriate filters and Light Emitting Diodes for the aquisition of images of Cerulean3 and Red fluorescent protein.

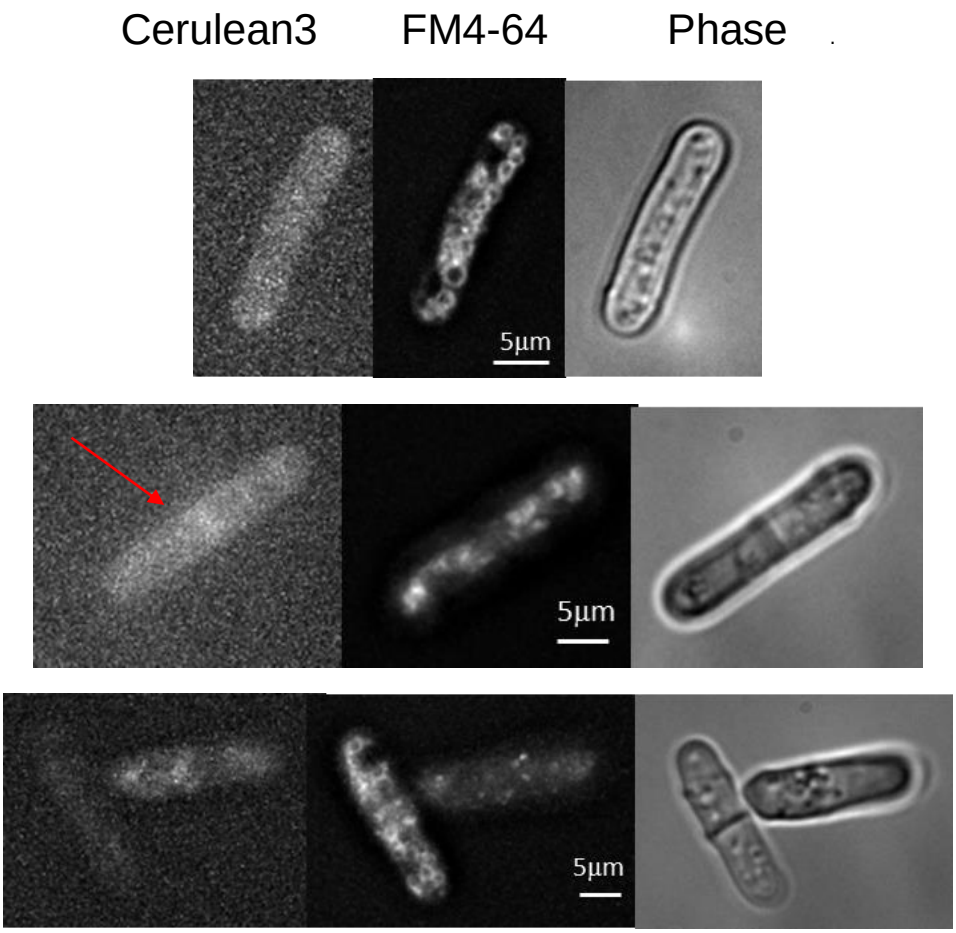


Figure.18. Images of wild type *S.pombe* pREP41αS-Cerulean3 grown with thiamine. Again low levels of vector protein expression led to very little fluorescence being detected in comparison to the images of induced cells. The fluorescence observed still appears to be cytoplasmic but in one cell did seem to be concentrated at the separation membrane (red arrow). The FM4-64 did not stain all of the cells clearlv but some have clearlv stained vacuole membranes.

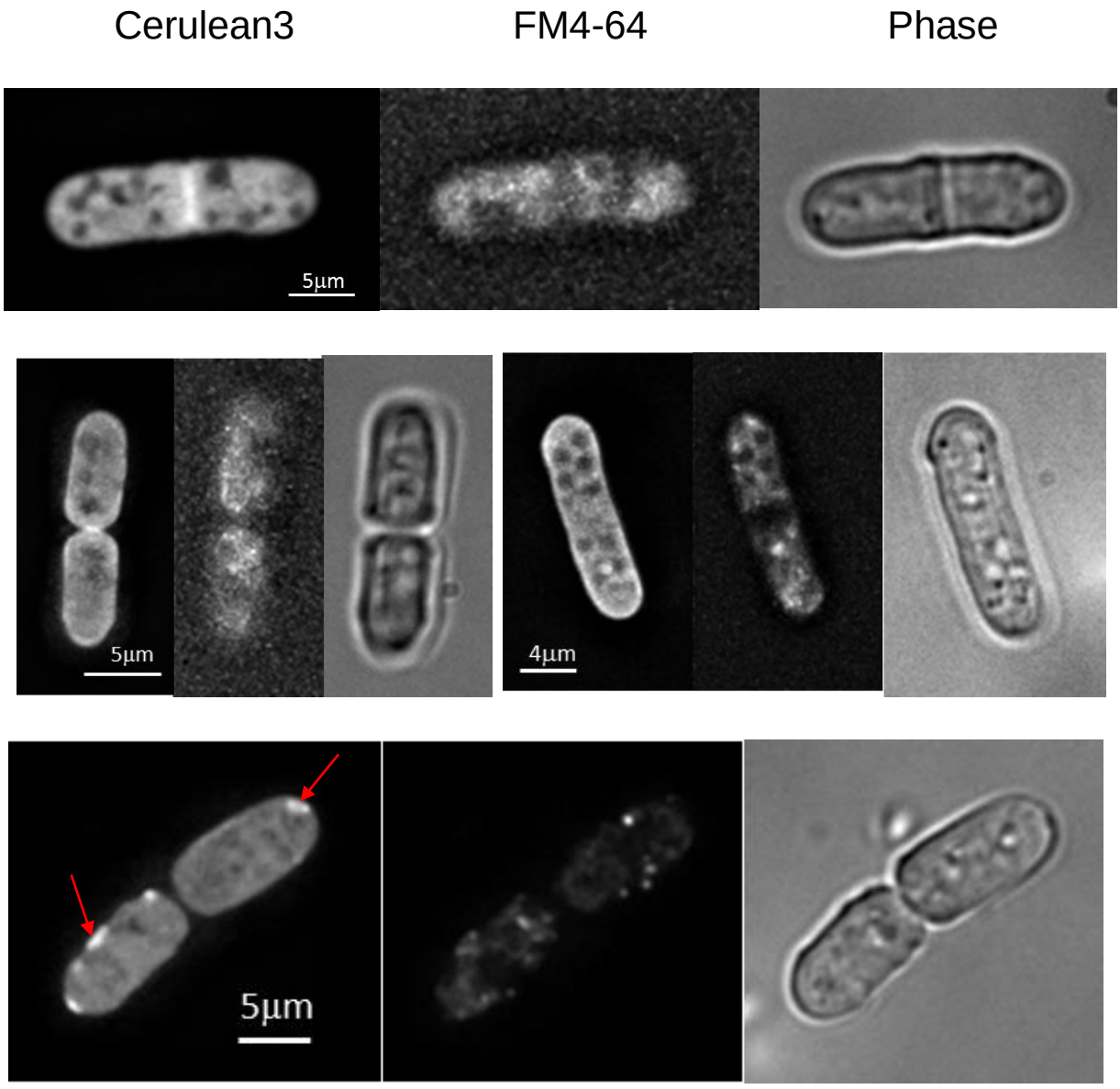


Figure.19. Images of. wild type *S.pombe* pREP41αS-Cerulean3 grown without thiamine Again higher levels of vector protein expression led to far more fluorescence being detected. In some cells with slightly lower levels this appears to be more cytoplasmic. In others with higher levels it appears to be excluded from vacuoles but present within the nucleus and concentrated at the separation membrane. Two cells appear to have aggregates on the membrane (red arrows). The FM4-64 did not stain all of the cells clearly but some have clearly stained vacuole membranes and is excluded from the nucleus.

3.12: Fluorescent Microscopy of *S.pombe* with pINT41αS-Cerulean3 integrated into its genome

To avoid the varying levels of recombinant protein expression seen, when using the pREP41 vector, due to varying levels of plasmid uptake between cells, a pINT41 vector was used to integrate a single copy of the recombinant DNA for α-Synuclein-Cerulean3 into the genome of a wild type *S.pombe* strain with the ability to integrate the pINT vector into its genome. This was integrated into the Leucine gene disrupting it and introduced a new Uracil gene. Cells with the correctly integrated gene were selected as they were able to grow on EMMG + amino acids –Uracil + thiamine, but could no longer grow on EMMG + amino acids –Leucine + thiamine.

The Integration of a single copy of the vector into the genome enables the cells to produce a consistent level of α-Synuclein-Cerulean3 which should negate any effects due to large variations of α-Synuclein-Cerulean3 concentration within the cells of each culture. Different promoters could also be used in the future to more accurately compare the effect of different concentrations of α-Synuclein-Cerlean3 on cells.

Levels of expression of the integrated recombinant protein were very low both with and without the presence of thiamine. However it was apparent that the levels expressed were sufficient to have a large effect on the cell phenotype. Cells were stained with FM4-64 to see more clearly how plasma membranes were being affected within these cells. Images were taken of cells grown for 1 (Figures 20 & 21) and 2 days (Figures 22 & 23) with and without thiamine.

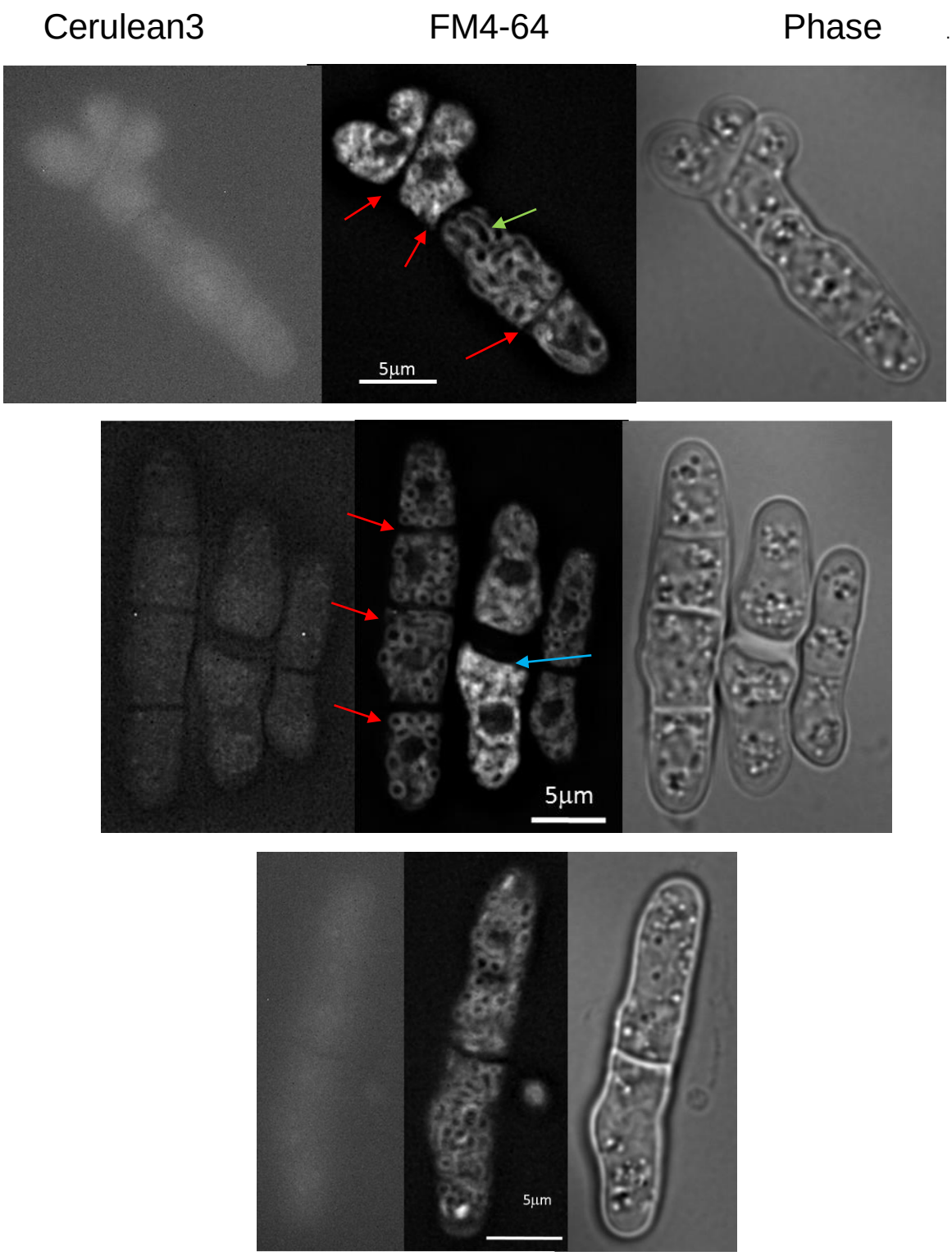


Figure.20. Images of *S.pombe* pINT41αS-Cerulean3, grown for 1 day with thiamine, stained with FM4-64. At the very low levels of vector protein expression created by a single copy of the integrated αS-Cer3 vector in the presence of thiamine only extremely low levels of fluorescence could be detected. Despite using image enhancement only very faint cerulean images could be produced. The images obtained using FM4-64 dye and phase images show very misshapen cells, some of which also show multiple septa (red arrows), very thick septa (blue arrow) and unusual shaped vacuoles (green arrow).

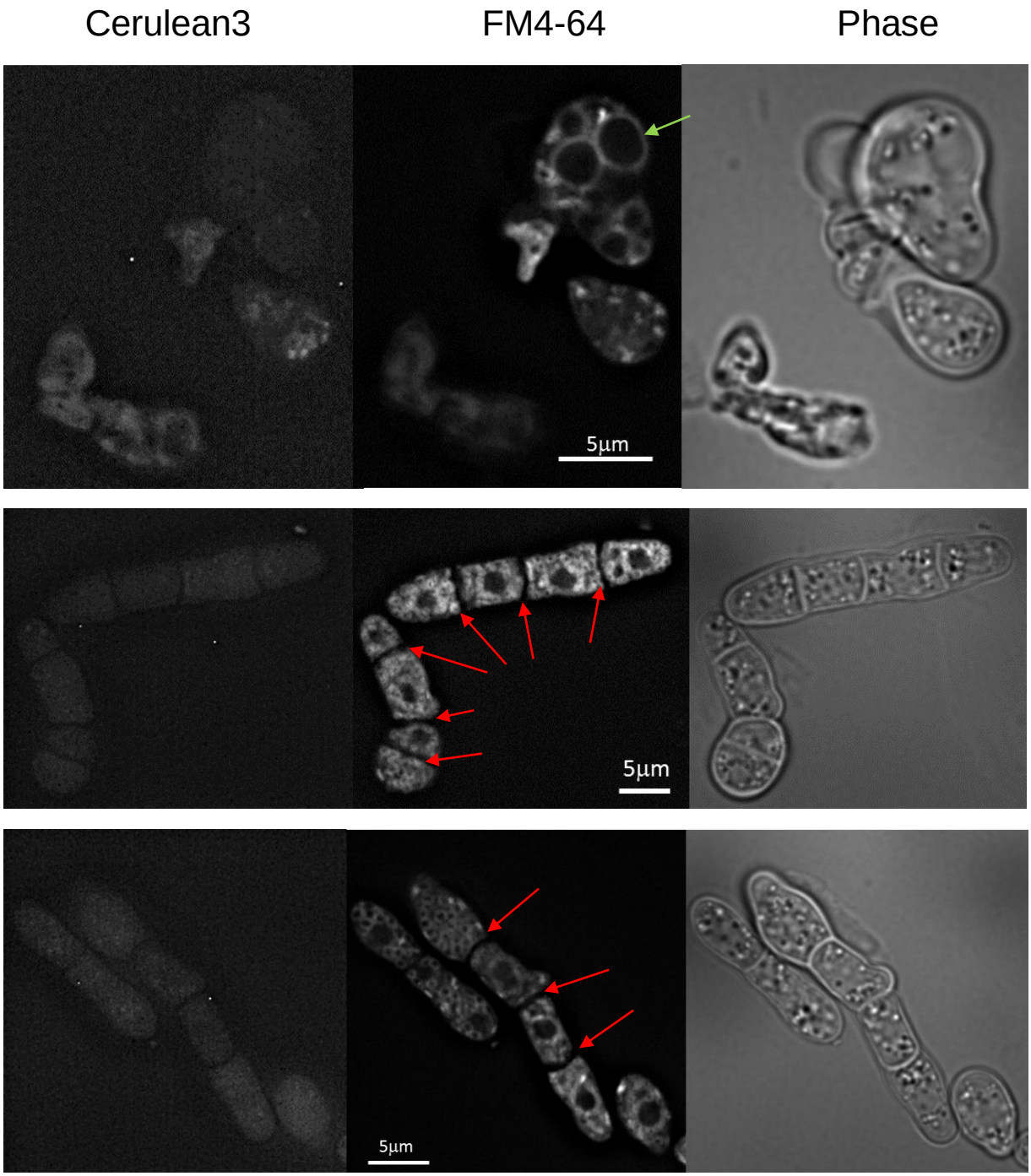


Figure.21. Images of *S.pombe* pINT41αS-Cerulean3, grown for 1 day without thiamine, stained with FM4-64. At the higher levels of vector protein expression created by a single copy of the integrated αS-Cer3 vector without thiamine, compared to growth with thiamine, very low levels of fluorescence could still only be detected. Despite using image enhancement only very faint cerulean images could be produced. The images obtained using FM4-64 dye and phase images show very misshapen cells, some of which also show multiple septa (red arrows) and unusually large vacuoles (green arrow).

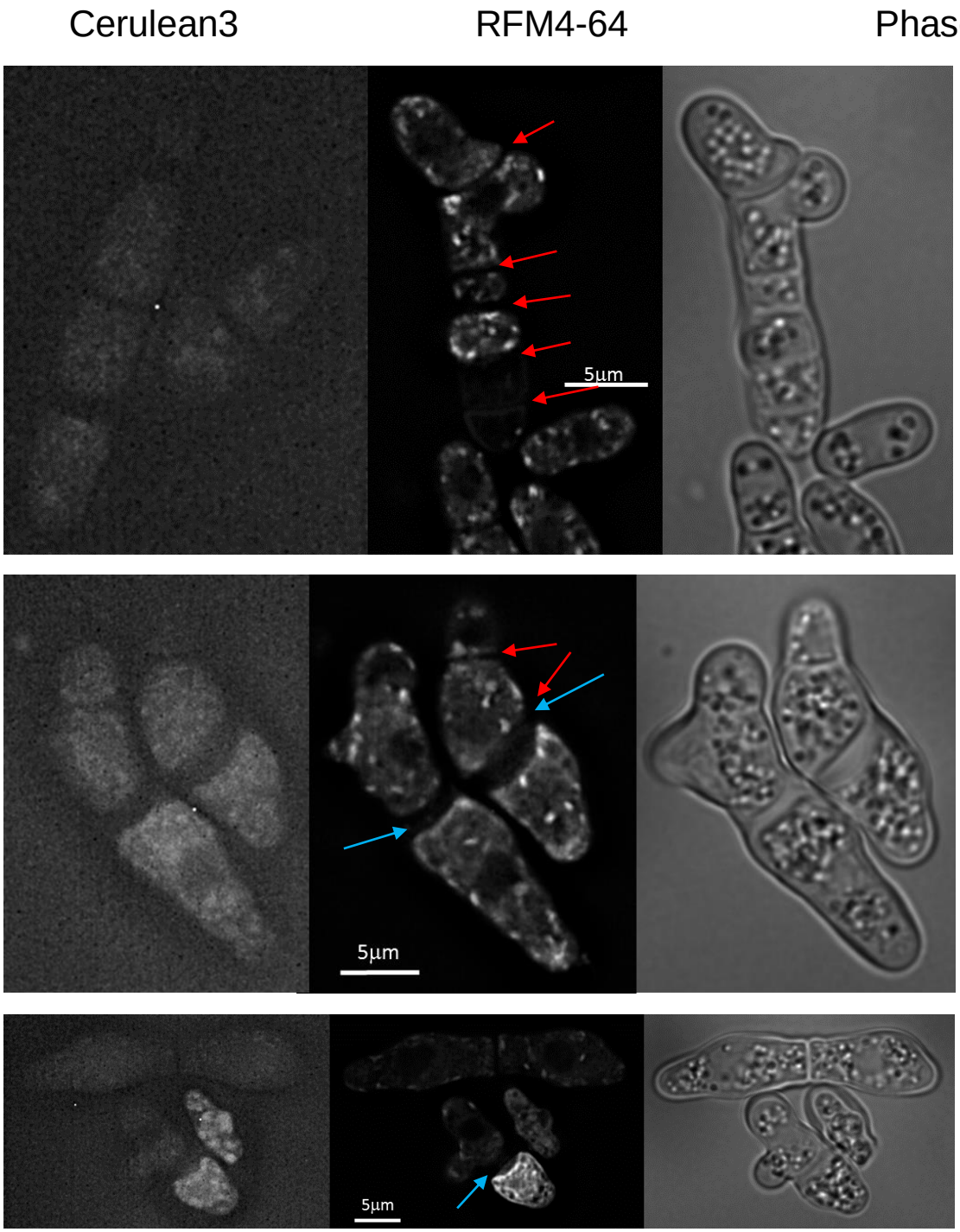


Figure.22. Images of *S.pombe* pINT41αS-Cerulean3, grown for 2 days with thiamine, stained with FM4-64. After 2 days of growth with thiamine very low levels of vector protein expression created by a single copy of the integrated αS-Cer3 vector, meant that only extremely low levels of fluorescence could be detected. Despite using image enhancement only very faint cerulean images could be produced. The images obtained using FM4-64 dye and phase images show very misshapen cells, some of which also show multiple septa (red arrows) and very thick septa (blue arrow). Cell vacuoles can no longer be clearly seen.

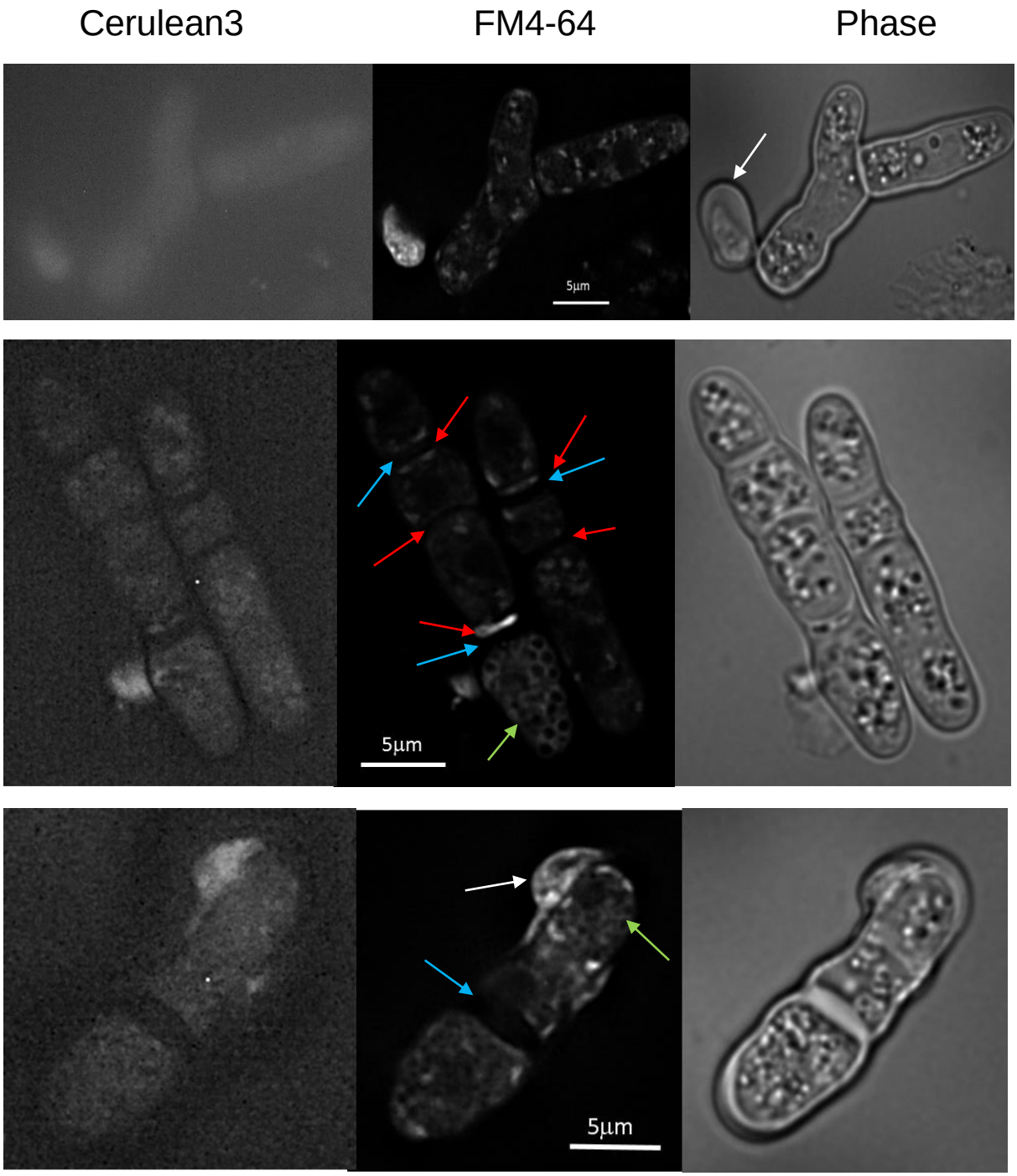


Figure.23. Images of *S.pombe* pINT41αS-Cerulean3, grown for 2 days without thiamine, stained with FM4-64. After 2 days growth without thiamine the higher levels of vector protein expression, compared to cells grown with thiamine, created by a single copy of the integrated αS-Cer3 vector, created only low levels of fluorescence. Despite using image enhancement only very faint cerulean images could be produced. The images obtained using FM4-64 dye and phase images show very misshapen cells, some of which also show multiple septa (red arrows) and very thick septa (blue arrow). Vacuoles can only be seen clearly in a few cells (green arrow). Some cells also appear to be producing bleb like structures (white arrows).

3.13: Examination of numbers of septa seen in wild type *S.pombe* with and without pINT41αS-Cerulean3 integrated into its genome

Cells with pINT41aS-Cerulea3 integrated into their genome appeared to be having difficulty completing their cell cycle, producing septum but then being unable to complete division into two daughter cells, resulting in cells with multiple septa. Due to the large number of cells with multiple septa seen in wild type *S.pombe* with pINT41αS-Cerulean3 integrated into its genome, it was decided to compare cells grown both with and without the integrated gene. Cells were grown in EMMG, both with and without thiamine and images taken of 20 fields of view, after 1 and 2 days incubation at 25ºC with shaking. Cells within each field were counted according to the number of septa within the cells and the percentage of cells with each number of septa was calculated (Table.6).

No of Septa	0	1	2	3	Total No. Of cells
WT *S.pombe,* No vector . 1day +thiamine	100 93.5%	7 6.5%			107
WT *S.pombe,* No vector. 2days +thiamine	136 97.1%	3 2.1%	1 0.8%		140
WT *S.pombe,* No vector. 1day –thiamine	66 85.7%	10 13.0%	1 1.3%		77
WT *S.pombe,* No vector. 2days -thiamine	138 95.2%	7 4.8%			145
WT *S.pombe* pINT41αS-Cerulean3. 1day +thiamine	50 74.0%	27 32.9%	4 4.9%	1 1.2%	82
WT *S.pombe* pINT41αS-Cerulean3. 2days +thiamine	46 57.5%	20 25%	9 11.2%	5 6.3%	80
WT *S.pombe* pINT41αS-Cerulean3. 1day –thiamine	77 74.0%	16 15.4%	10 9.6%	1 1.0%	104
WT *S.pombe* pINT41αS-Cerulean3. 2days - thiamine	90 64.3%	37 26.4%	9 6.4%	4 2.9%	140

Table.6. Septa in wild type *S.pombe* with and without pINT41αS-Cerulean3 integrated into its genome at 1 and 2 days, grown in the presence and absence of thiamine.

4. CHAPTER IV: RESULTS; *Escherichia coli*

4.1: Expression of α-Synuclein in *E.coli*

It was decided to investigate the effect of α-Synuclein upon the physiology of the bacteria *Escherichia coli (E.coli). E.coli* BL21 DE3 cells were transformed using, a pJC20 vector containing the gene for α-Synuclein fused to cDNA encoding the Cerulean3 protein. A broth culture was then induced to produce this protein by the addition of IPTG to a concentration of 0.1mg/ml. Comparison of pre and 2 hours post induction cell extracts (Figure. 24) indicates the induced expression of the fusion protein which migrated at the predicted size of 42.2kDa.

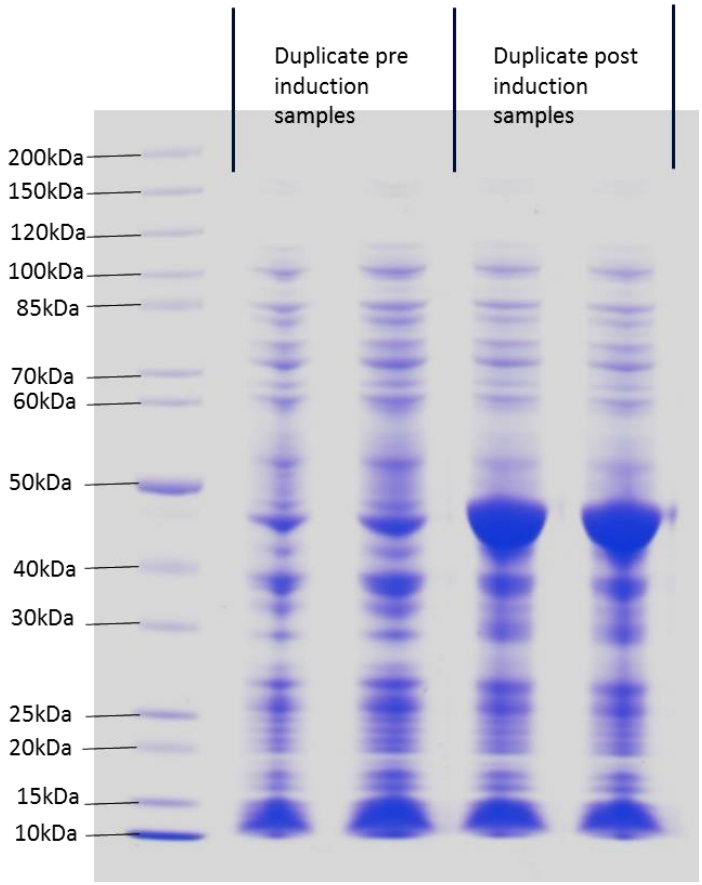


Figure.24 Coomassie stained gel, the two post induction protein samples on the right, show a large increase in protein produced, compared with pre induction.

4.2: Fluorescent microscopy of α-Synuclein in *E.coli* with and without acetylation

Fluorescent microscopy was used to examine *E.coli* cells expressing α-Synuclein-Cerulean3. It was also decided to examine a second culture produced by transforming the same vector pJC20 α-Synuclein-Cerulean3 into *E.coli* BL21+NatB cells. These *E.coli* contain a plasmid expressing the N-terminal acetyltransferase B (NatB) complex, (Johnson M. *et al*. 2010) that facilitates the N-terminal acetylation of the, α-Synuclein, produced within the cell.

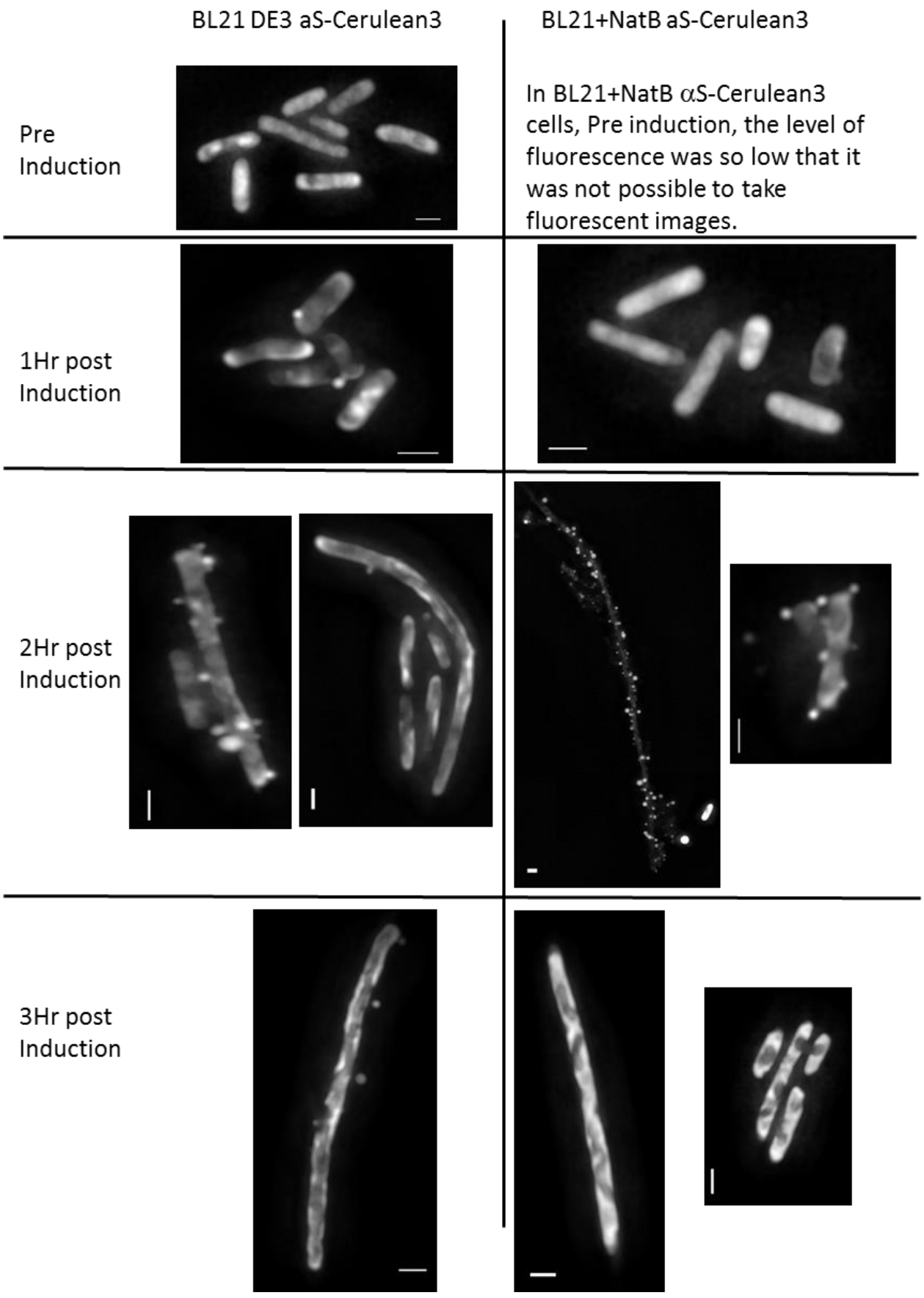
Expression of recombinant protein was induced with IPTG, slides were prepared and images taken at Pre induction and at 1, 2 and 3 hours post induction (Figure 25).

Due to the unusual appearance of these cells, it was decided to further investigate the effects of α-Synuclein in E.coli at higher resolution using negative stain Electron Microscopy.

Figure.25. (Following page) Images of BL21DE3 αS-Cerulean3 cells and BL21+NatB αS-Cerulean3 cells, Pre induction and 1, 2 and 3 hours post induction. BL21DE3 αS-Cerulean3 cells show only low levels of fluorescence, which appeared to be cytoplasmic. In the image taken at 1 hour post induction, more fluorescence was seen, this still appeared to be predominantly cytoplasmic, but some brighter areas appeared to show the start of some localisation and possible aggregate formation. In the image taken at 2 hours post induction. Some fluorescence still appeared to be cytoplasmic. However, some cells appeared to have bright concentrations of fluorescence, possibly indicating the presence of aggregates or blebbing around the outside of the cells. Other cells appeared to have ribbon like areas of localisation, predominantly but not exclusively, on the inside of the plasma membrane. In the image taken at 3 hours post induction, there was a complete mix of appearances. Some cells still appeared to have cytoplasmic Cerulean3. Some have areas of bright fluorescence or possible aggregates or blebbing around the outside of the cell. Some have ribbon like concentrations within the cell and some have a mixture of these appearances. This variation may be due to differing concentrations of plasmid present in different cells.

It was not possible to take images of BL21+NatB αS-Cerulean3 cells, Pre induction, as the level of fluorescence was too low. In the image of BL21+NatB αS-Cerulean3 cells taken at 1 hour post induction. Fluorescence appeared to be mainly cytoplasmic. A few cells have areas that seem to have a slightly higher level of fluorescence. The images of BL21+NatB αS-Cerulean3 cells, taken at 2 hours post induction show lots of blebbing around some cells together with some of the ribbon like localisation. In the images of BL21+NatB αS-Cerulean3 cells, taken at 3 hours induction, only a few blebs or aggregates were visible on the outside of the cells. However the ribbon like localisation was seen to be more dominant and more clearly defined. (Scale-1μm)

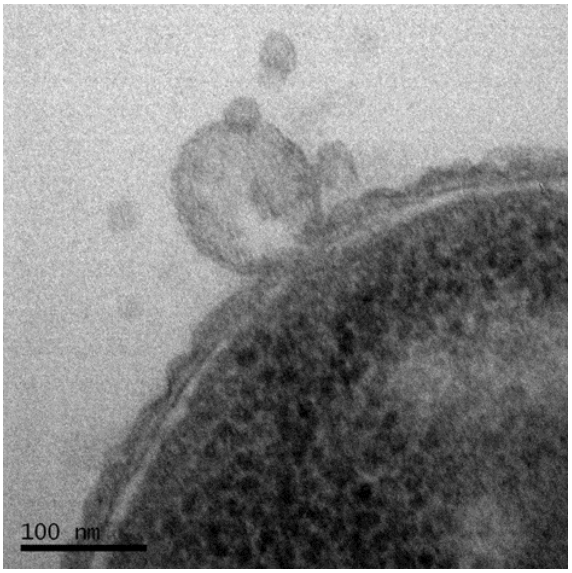
Figure 25.


BL21 DE3 aS-Cerulean3
BL21+NatB aS-Cerulean3
Pre Induction
In BL21+NatB αS-Cerulean3 cells, Pre induction, the level of fluorescence was so low that it was not possible to take fluorescent images.
1Hr post Induction
2Hr post Induction
3Hr post Induction

4.3: Electron microscopy of α-Synuclein in *E.coli* with and without acetylation

The two cultures observed by fluorescent microscopy were observed using Transmission Electron Microscopy (TEM). 50ml cultures were induced during log phase using IPTG for 2 hours, they were then spun at 2500rpm for 10 minutes to produce a pellet that was sent for preparation and observation by TEM.

Figure.26. Images of slices of thin sectioned BL21DE3 αS-Cerulean3 cells taken at 2 hours induction using TEM. The TEM images show clear blebbing on the outside of the cells producing unacetylated α-Synuclein-Cerulean3. The blebs appear to be membrane bound structures being released from the cell surface.



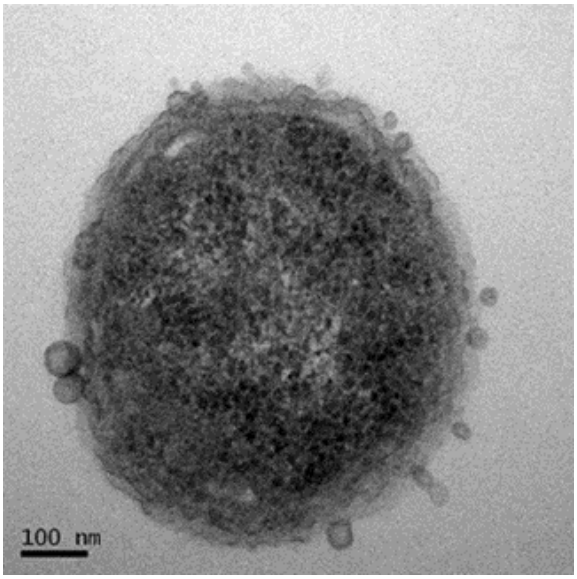
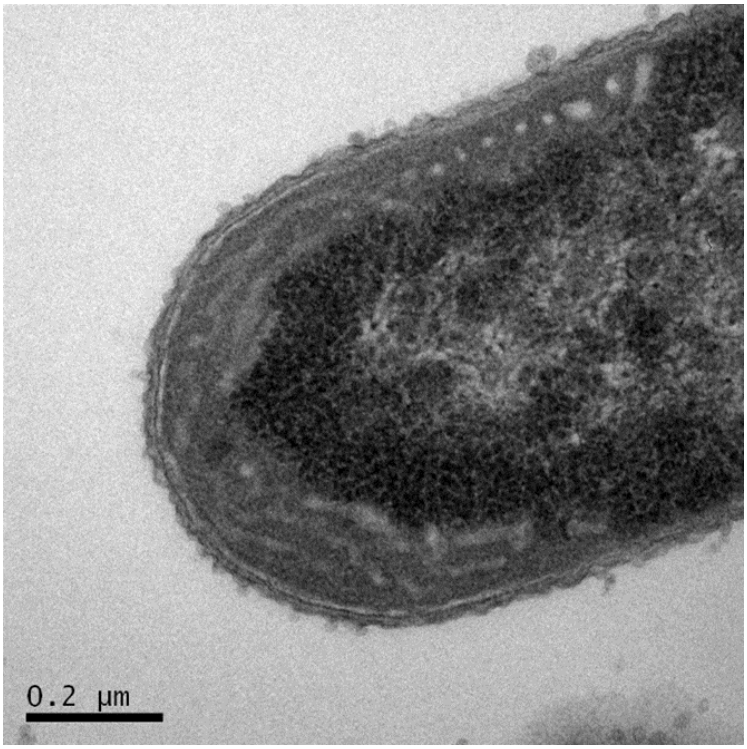
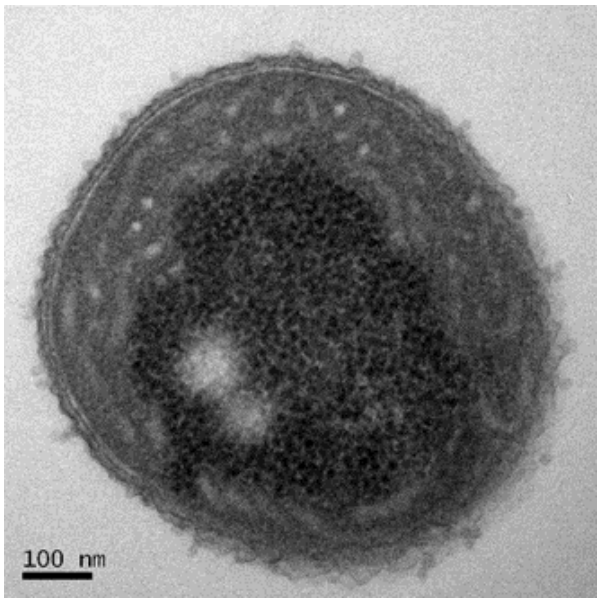
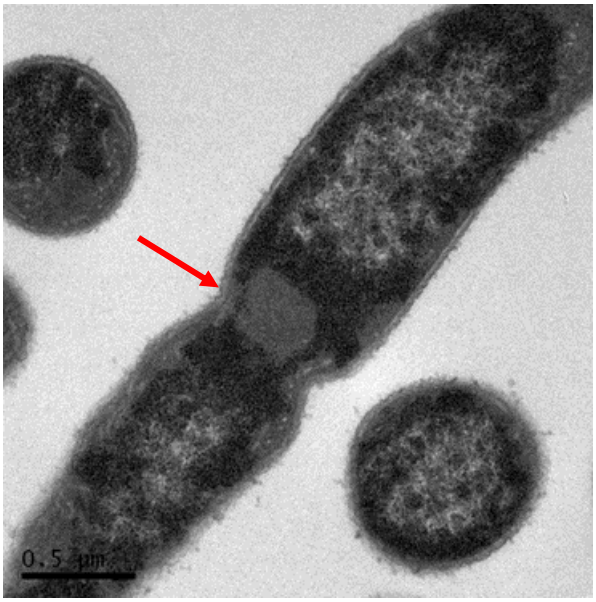


Figure.27. Images of slices of thin sectioned BL21+NatB αS-Cerulean3 cells producing acetylated α-Synuclein-Cerulean3 were seen to be producing lots of smaller blebs. They were also seen to be producing structures with a tubular network. These seemed to originate on the inside of the plasma membrane and build up into the inside of the cell. Some cells appear to be having difficulty dividing, possibly due to the presence of these structures (see arrow on third image). These structures seem to be what creates the ribbon like appearance seen in the 3 hour fluorescent microscopy.



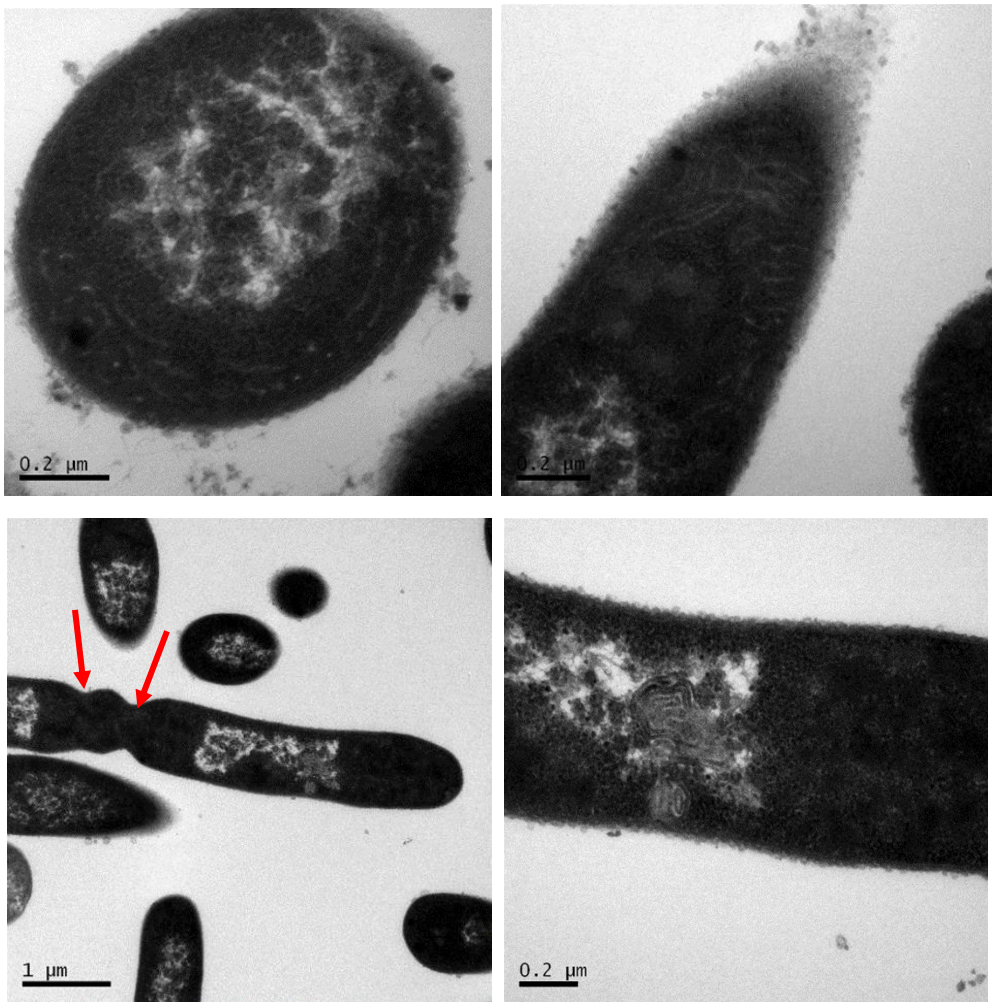




4.4: Electron microscopy of N-terminally acetylated α-Synuclein in *E.coli* using an enhanced membrane preparation.

It was decided to examine BL21+NatB αS-Cerulean3 cells by TEM, using a treatment to enhance membrane staining, with Osmium tetroxide and Thiocarbohydrazide (Figure.28). The structures with a tubular network could be seen more clearly, blebbing around cells and some cells appearing to have separation defects (see arrows on third image), could also be observed. This data is consistent with these structures having significant membrane components.

Figure.28. 4 TEM images of BL21+NatB αS-Cerulean3 cells, using a darker Osmium tetroxide and Thiocarbohydrazide stain, to enhance the appearance of the internal tubular structures. Red arrows indicate cells that appear to be having difficulty dividing.



4.5: Anti α-Synuclein Immuno E.M.

An immunogold-staining technique was employed to confirm the location of α-Synuclein within the cells, to confirm whether it was associated with the α-Synuclein induced structures. Cultures of BL21+NatA αS-Cerulean3, an *E.coli* producing N-terminal acetyltransferase A (NatA), which does not N-terminally acetylate α-Synuclein, BL21+NatB αS-Cerulean3, producing acetylated α-Synuclein and a control of BL21+NatB containing an empty vector, which does not produce α-Synuclein, were examined at 2hours 30 minutes post induction. BL21+NatA αS-Cerulean was used to produce unacetylated α-Synuclein as it is a more directly comparable control to the BL21+NatB αS-Cerulean, as both cell types are producing additional acetylation complexes. During their preparation the cells were treated with a primary antibody, Rabbit polyclonal anti α-Synuclein antibody (abcam ab155038) and then a secondary anti rabbit antibody attached to 15nm gold discs that appear as black spots on the images.

The tubular networks are not seen as clearly as in the initial TEM images, as expected due to the different preparation technique. The black “gold label” spots indicate the presence and location of α-Synuclein (Figures.30 & 31) and an absence of black “gold label” spots indicates an absence of α-Synuclein (Figure 29). They do not show the exact location due to the length of the two antibodies joined together which separate the gold disc and the α-Synuclein protein that they connect to, which can be 15 to 30nm.

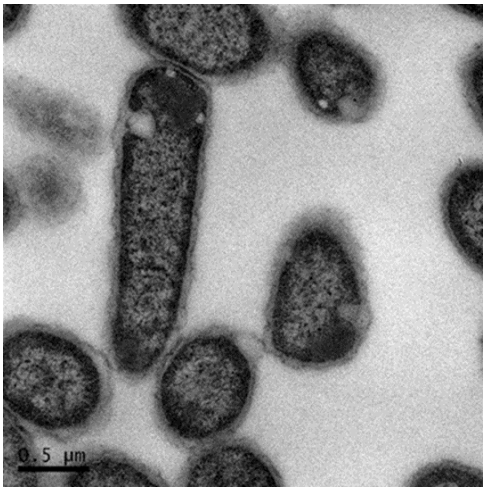


Figure.29. Image of BL21+NatB cells containing an empty vector. No immuno staining could be seen indicating an absence of α-Synuclein.

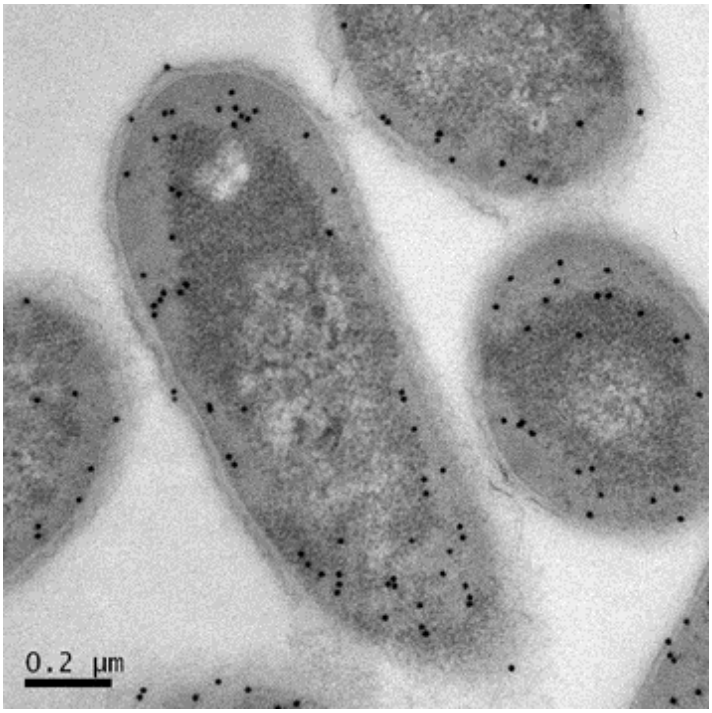


Figure.30. Image of BL21+NatA αS-Cerulean3 cells, showing immuno staining to be concentrated in the areas where the tubular networks were seen in earlier images, in the areas inside the plasma membrane

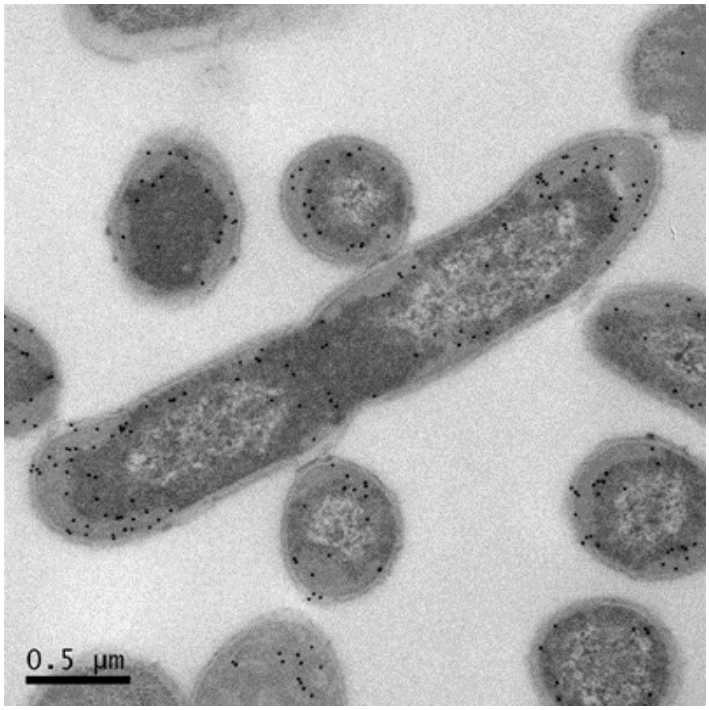


Figure.31. Image of BL21+NatB αS-Cerulean3 cells showing immuno staining, which again appears to be concentrated in the areas where the tubular networks were seen previously.

4.6: Growth curves obtained at OD_{600}.

OD_{600} growth curves were generated for the *E.coli,* BL21NatB αS-Cerulean3 cells, BL21+NatB cells containing an empty vector and BL21+NatA αS-Cerulean3 cells, used in the previous microscopy. A second control BL21+NatA cells containing an empty vector, was also included. This enabled a direct comparison of cells producing α-Synuclein-Cerulean3 or containing an empty vector and both types of cells with and without the ability to Nt-acetylate α-Synuclein. Growth curves were obtained for each of the four cultures, both with and without the addition of IPTG, to induce vector protein production. The cultures were shaken at a temperature of 37ºC with readings taken every 15minutes. Rates of growth were compared by calculating the change in OD_{600} per hour (Table.7).

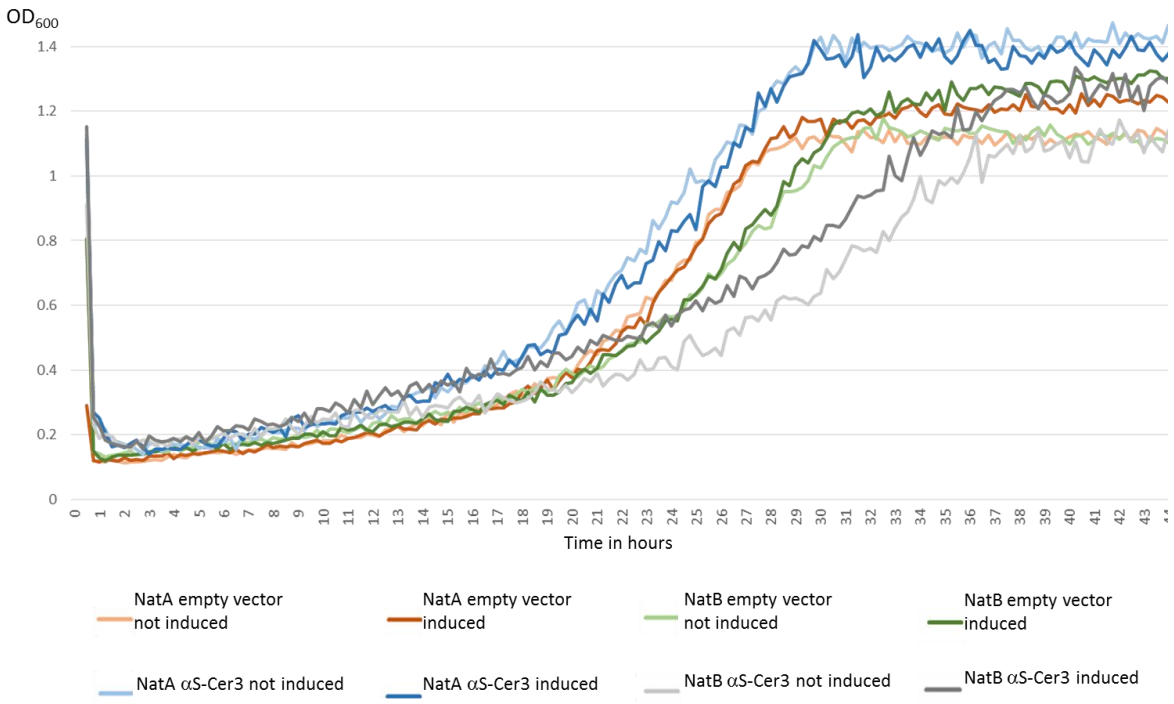


Figure.32. Graph to compare growth rates, of acetylated and unacetylated α-Synuclein and an empty vector using OD_{600}.

Table.7. The rate of growth during log phase, calculated for each culture. For both empty vectors and the unacetylated α-Synuclein the rates were fairly similar. The rate with acetylated α-Synuclein was almost half of that for the other samples.

Sample	Time points used	Tp 1	Tp2	Rate-OD_{600} change/hour
BL21 DE3 NatA empty vector uninduced	23-27	0.641	1.035	0.098
BL21 DE3 NatA empty vector induced	23-27	0.639	1.041	0.100
BL21 DE3 NatB empty vector uninduced	23-30	0.549	1.088	0.077
BL21 DE3 NatB empty vector induced	23-30	0.519	1.156	0.091
BL21 DE3 NatA αS-Cer3 uninduced	23-30	0.837	1.434	0.085
BL21 DE3 NatA αS-Cer3 induced	23-30	0.797	1.363	0.080
BL21 DE3 NatB αS-Cer3 uninduced	23-37	0.437	1.097	0.047
BL21 DE3 NatB αS-Cer3 induced	23-37	0.531	1.267	0.052

Unexpectedly the uninduced BL21 DE3 NatB appears to inhibit growth the most, however at a difference of 0.005 change on OD_{600} per hour this is within the sensitivity of the test

4.7: Comparison of Acetylated and Unacetylated α-Synuclein produced in *E.coli*.

To compare the α-Synuclein protein produced in BL21+NatA αS-Cerulean3 cells and BL21NatB αS-Cerulean3 cells, cell extracts both pre induction and $2^{1}/_{4}$ hours post induction, were examined. Cells were sonicated and supernatant and pellet fractions were separated and run on 4-12% Bis-Tris Gels, which were analysed by either Coomassie stain or anti α-Synuclein Western Blot (Figure 33). This shows that unacetylated α-Synuclein was only found in the supernatant. It also shows that acetylated α-Synuclein is found as a monomer in the supernatant, but that it was present mostly as oligomers. Acetylated α-Synuclein was also found in the pellet as well, as an oligomer.

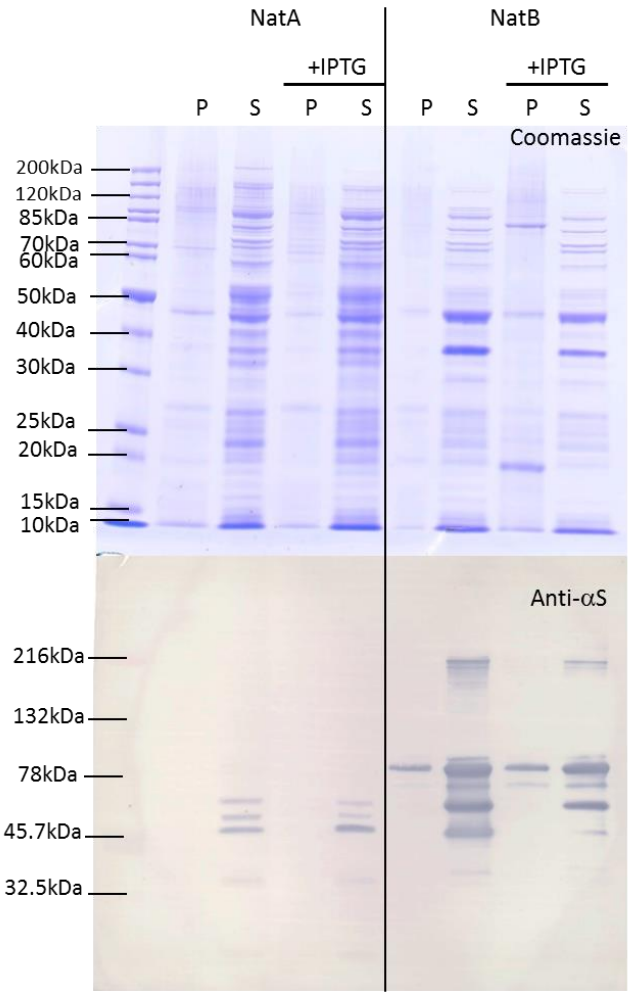


Figure.33. Coomassie stained 4-12% Bis-Tris gel and western blot of BL21+NatA αS-Cerulean3 cells and BL21NatB αS-Cerulean3 cell extracts.

4.8: Fluorescence growth curves of *E.coli* cells expressing α-Synuclein-Cerulean3

To compare the level of toxicity, of acetylated versus unacetylated α-Synuclein-Cerulean3 in the *E.coli* cells in which they were expressed (Figure 41), together with the levels of α-Synuclein-Cerulean3 produced in the *E.coli* cells, as measured by fluorescence (Figure 34). BL21+NatA αS-Cerulean3 cells and BL21NatB αS-Cerulean3 cells were grown both in a fluorescent plate reader, with and without the addition of IPTG, to induce vector protein production. The cultures were shaken at a temperature of 37ºC with readings taken every 15minutes.

Fluorescence was seen at lower levels in the uninduced controls due to the T7 promoter allowing low levels of protein production in the absence of IPTG. The reason for the biphasic increase in fluorescence is not known, but was noticed to have occurred shortly after stationary phase was reached.

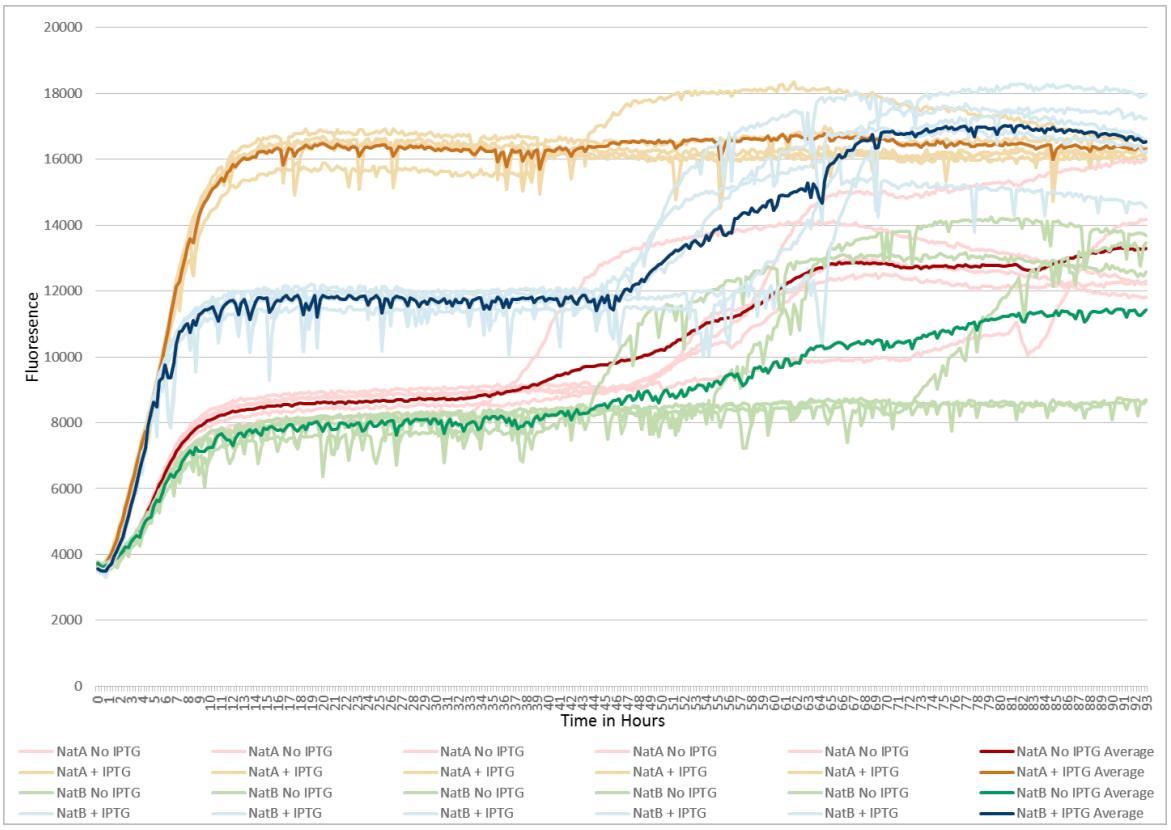


Figure.34. Fluorescent growth curve comparing BL21+NatA αS-Cer3 and BL21+NatB αS-Cer3 cells producing acetylated and unacetylated α-Synuclein-Cerulean3, grown with and without the addition of IPTG

Unexpectedly the acetylated α-Synuclein-Creulean3, which appeared to be the most toxic form to cells when measured by $OD_{600,}$ produced a lower initial level of fluorescence compared to the unacetylated α-Synuclein-Creulean3. However at 50 hours, when according to the OD_{600} readings the cells were in stationary phase, the level α-Synuclein-Creulean3 of fluorescence began to increase again until it reached the same value as that obtained for the unacetylated. Due to these unexpected results, it was decided to further examine these two cultures together with controls producing just GFP and α-Synuclein without a fluorescent marker (a vector obtained from outside of this University), at 24 and 90 hours post induction, by fluorescent microscopy, TEM and immuno EM.

4.9: Fluorescent microscopy of BL21+NatB αS-Cerulean3 and BL21NatB-GFP staining with FM4-64

It was decided to look at the BL21+NatB αS-Cerulean3 culture used previously, together with *E.coli* expressing NatB and GFP, with FM4-64 stain (Figures 35 & 36).

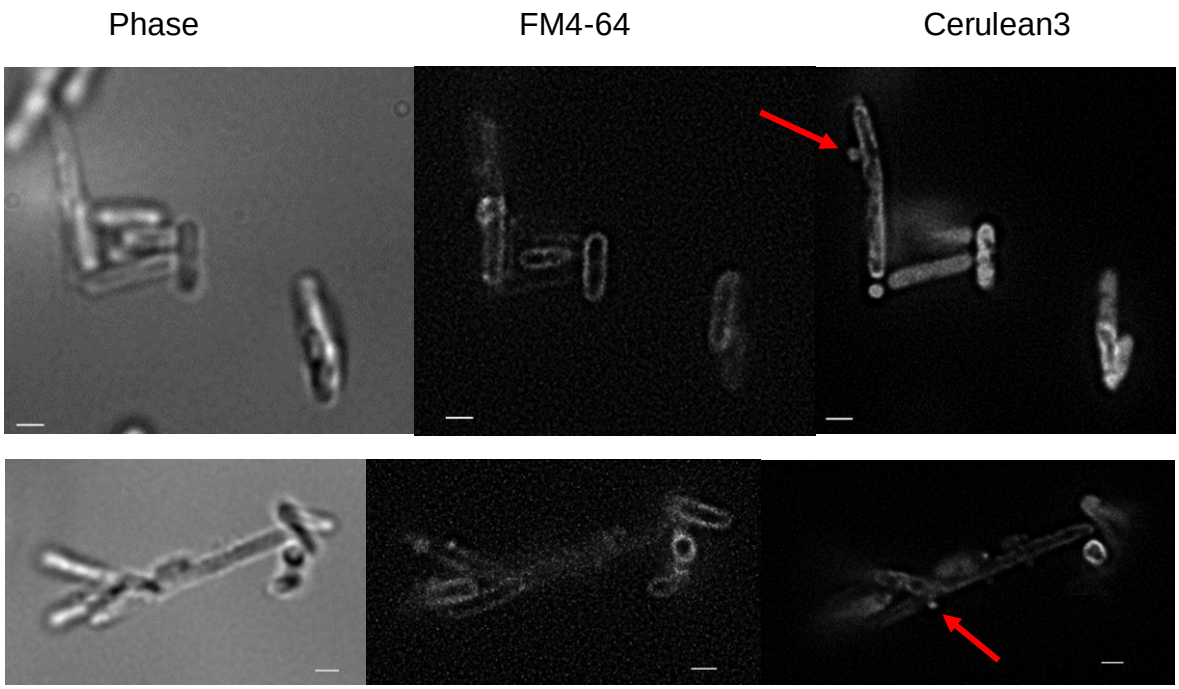


Figure.35. Images of BL21+NatB αS-Cerulean3 cells, stained with FM4-64. Blebs can be seen in the Cerulean3 images (Red arrows) (Scale 1μm).

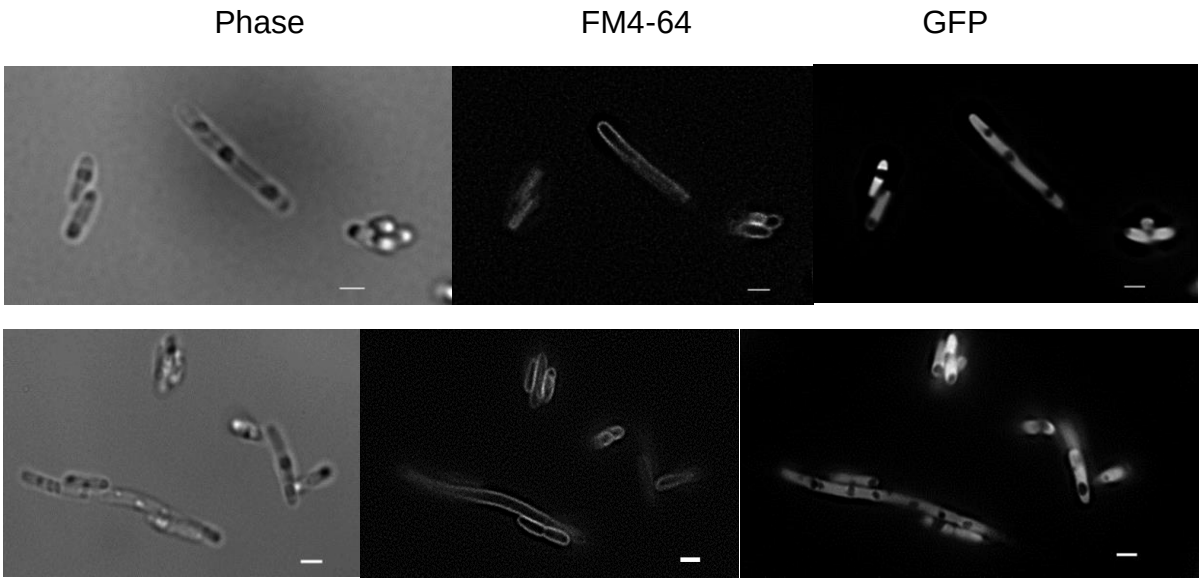


Figure.36. Images of BL21+NatB-GFP cells, stained with FM4-64 (Scale 1μm).

4.10: Simultaneous analysis of BL21+NatA αS-Cerulean3, BL21+NatB αS-Cerulean3, BL21+NatA αS, BL21+NatB αS and BL21+NatB GFP, using Fluorescent Microscopy, TEM, Immunogold TEM and protein analysis.

Due to the unexpected fluorescent curves obtained, it was decided to complete a large scale comparison of *E.coli* producing both acetylated and unacetylated α-Synuclein, both with and without a fluorescent protein marker, together with a control producing NatB and GFP. Previously growth curves had shown that acetylated α-Synuclein-Cerulean3, appeared to be more toxic to cells than unacetylated α-Synuclein-Cerulean3 when measured by OD_{600}, yet produced a lower initial level of fluorescence and possibly correlating lower level of α-Synuclein-Cerulean3, compared to the unacetylated α-Synuclein-Cerulean3. However at 50 hours, when according to the OD_{600} readings the BL21+NatB αS-Cerulean3 cells were in stationary phase, the level of fluorescence began to increase again until it reached the same value as that obtained for the unacetylated.

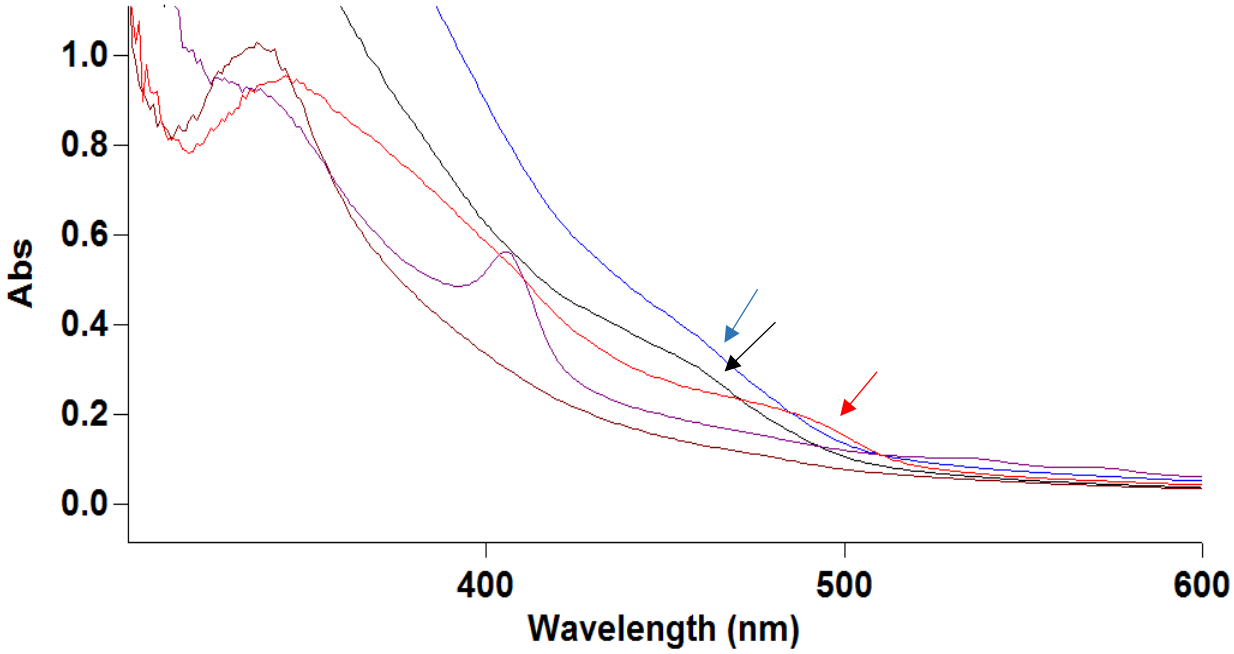
This raised several questions. Were lower levels of acetylated α-Synuclein-Cerulean3 really being produced, was acetylated α-Synuclein-Cerulean3 more toxic than the unacetylated, or was something else causing these results and would it be possible to see differences by microscopic examination.

In an attempt to answer these questions the five different cultures were compared using Fluorescent Microscopy, TEM, anti α-Synuclein Immunogold TEM and protein analysis at 24 and 90 hours post induction.

When the cultures were centrifuged to be sent for preparation for TEM and Immuno EM, The 24hour samples producing αS-Cer3 and GFP produced pellets that could be clearly seen to be fluorescent. The 90 hour samples showed no signs of fluorescence and were thought not to have been induced correctly. All of the 90 hour samples were discarded and prepared again.

The second set of 90 hour samples were set up and observed by fluorescent microscopy at 1 hour post induction to confirm that αS-Cer3 and GFP were being

produced in the appropriate cultures. After 90 hours the samples were centrifuged to produce pellets for preparation for observation by TEM and ImmunoEM. Again the pellets showed no sign of fluorescence, however as induction had been confirmed by the post induction fluorescent microscopy, the cell cleared culture media were kept for examination by fluorescent spectrometry (Figure 37). Protein samples were also prepared for analysis, by precipitating protein from the cell cleared culture medium. For each 90 hour culture, 400μl of supernatant was added to 1.6ml of acetone, to precipitate any protein present, these samples were then frozen at -80ºC for later examination.



Black – BL21+NatA αS-Cerulean3 Purple – BL21+NatA αS Red – BL21+NatB GFP

Blue – BL21+NatB αS-Cerulean3 Brown – BL21+NatB αS

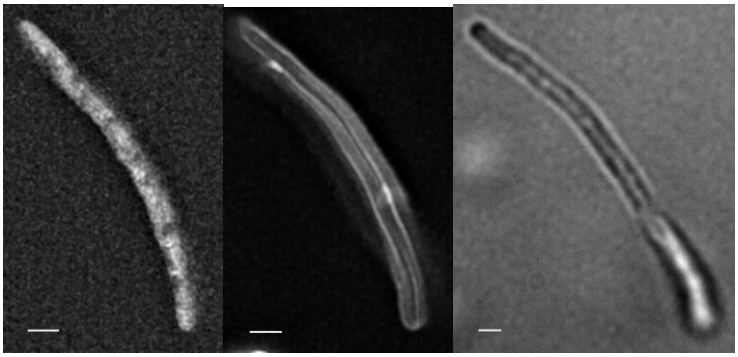
Figure.37. Fluorescent scans of cell cleared culture medium, from cultures of BL21+NatA αS-Cer3, BL21+NatA αS, BL21+NatB GFP, BL21+NatB αS-Cer3 and BL21+NatB αS cells, 90 hours post induction, indicating the presence of fluorescent proteins.

Shoulders were seen in the fluorescent scans, close to the maximum emission wavelengths for Cerulean3 at 475nm and GFP at 508nm, this indicates the presence of Fluorescent Proteins in the 90 hour post induction cell cleared culture medium. The release of proteins into the cell cleared culture medium, explains the lack of fluorescence within the pellets**.**

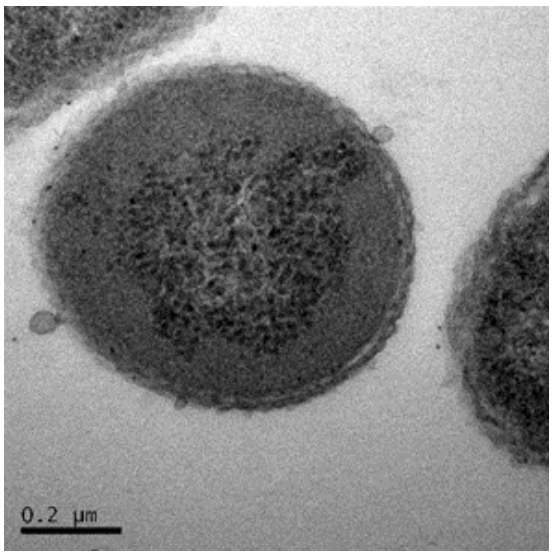
Cells from all five cultures were stained with FM4-64 and examined by phase and appropriate fluorescent microscopy and images taken. Images were also taken using TEM and Immuno EM (Figures 38 to 42). The Immuno EM results were not as expected. The black “gold label” spots present in the BL21+NatA αS-Cerulean3 and BL21+NatB αS-Cerulean3 cells indicate the presence and location of α-Synuclein within them as expected (Figures.38 & 39). However an absence of black “gold label” spots in the BL21+NatA αS, BL21+NatB αS and BL21+NatB GFP cells (Figures 40 to 41) indicates an absence of α-Synuclein, when it was expected to be present in the first two of these cells. In the TEM images some cells again appear to have separation defects (see red arrows). The cells producing α-Synuclein alone were very difficult to fix to the coverslip with Lectin, for fluorescent microscopy.

Figure.38. Images of BL21+NatAαS-Cerulean3 cells 24 hours Post Induction, obtained using Phase and Fluorescent light microscopy (stained with FM4-64. Scale 1μm), TEM and Immuno EM.

Cerulean3 FM4-64 Phase .



TEM



Immuno EM

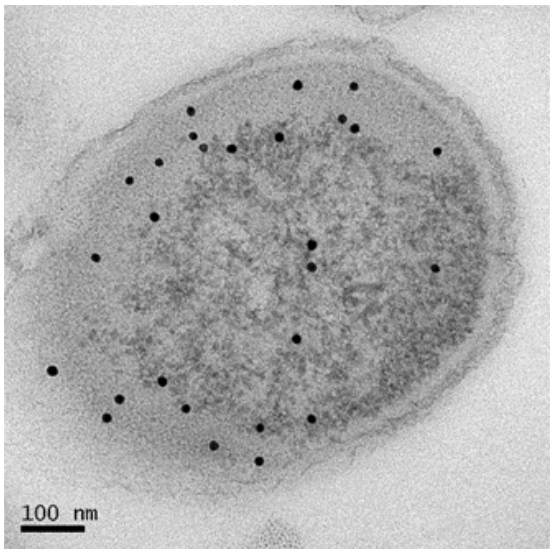
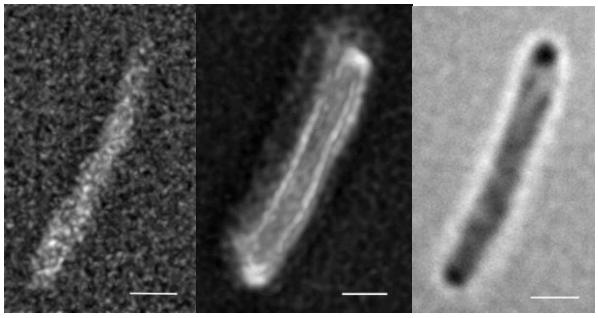
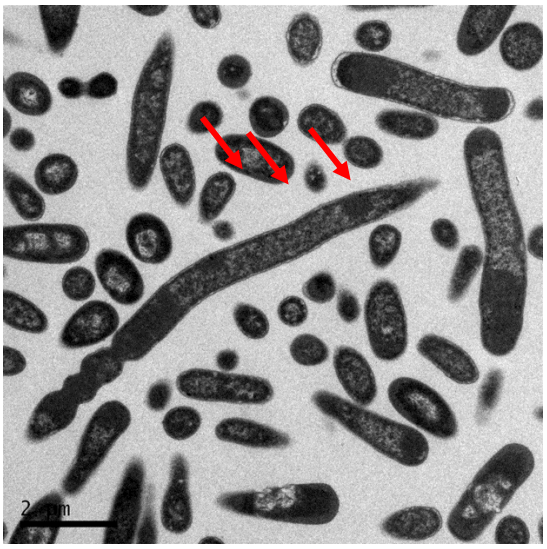


Figure.39. Images of BL21+NatB αS-Cerulean3 cells 24 hours Post Induction, obtained using Phase and Fluorescent light microscopy (stained with FM4-64. Scale 1μm), TEM and Immuno EM. Red arrows indicate cells that appear to be having difficulty dividing.

Cerulean3 FM4-64 Phase



TEM



Immuno EM

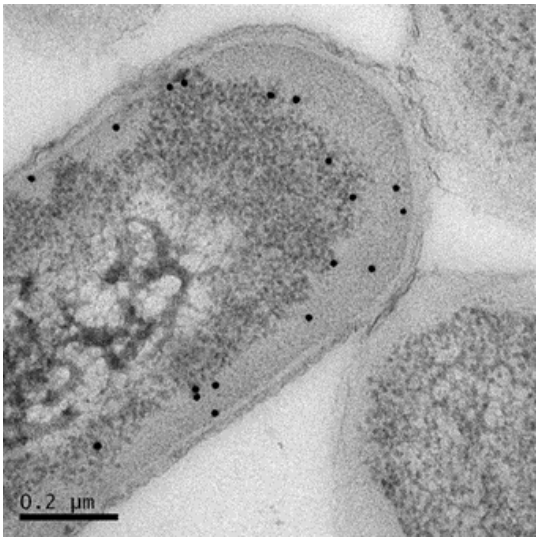
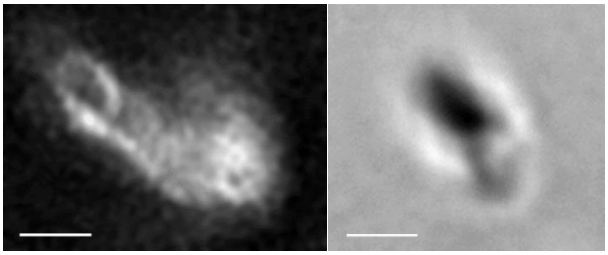
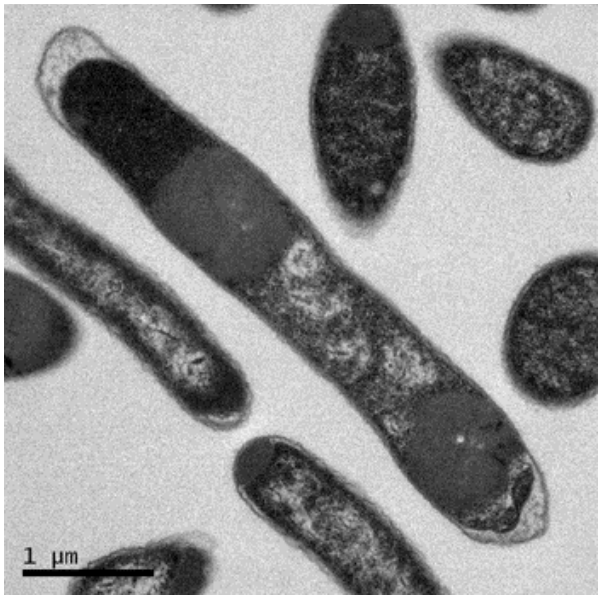


Figure.40. Images of BL21+NatA αS cells 24 hours Post Induction, obtained using Phase and Fluorescent light microscopy (stained with FM4-64. Scale 1μm), TEM and Immuno EM.

FM464 Phase



TEM



Immuno EM

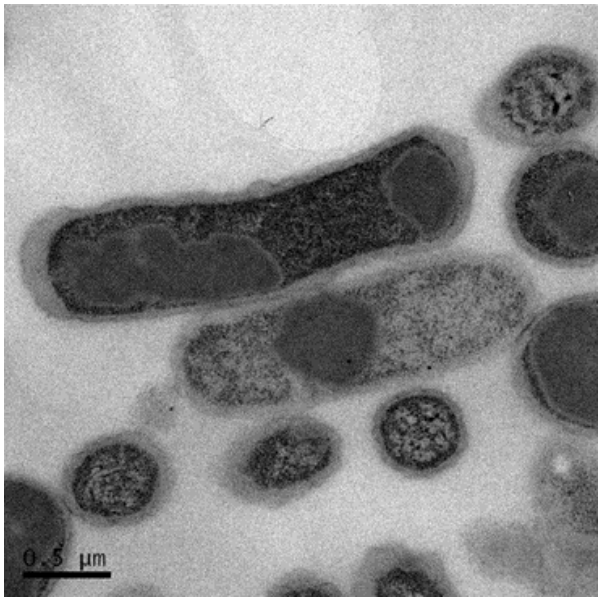
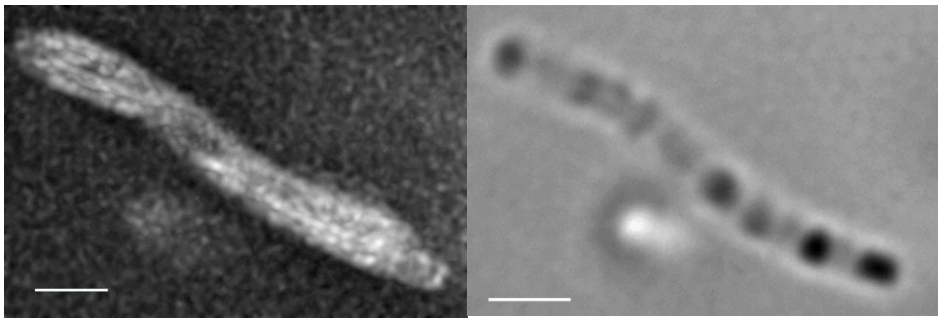
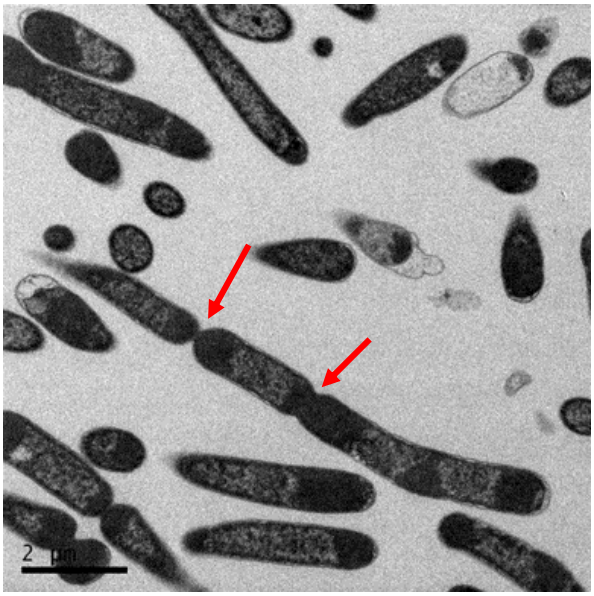


Figure.41. Images of BL21+NatB αS cells 24 hours Post Induction, obtained using Phase and Fluorescent light microscopy (stained with FM4-64. Scale 1μm), TEM and Immuno EM. Red arrows indicate cells that appear to be having difficulty dividing.

FM4-64 Phase



TEM



Immuno EM

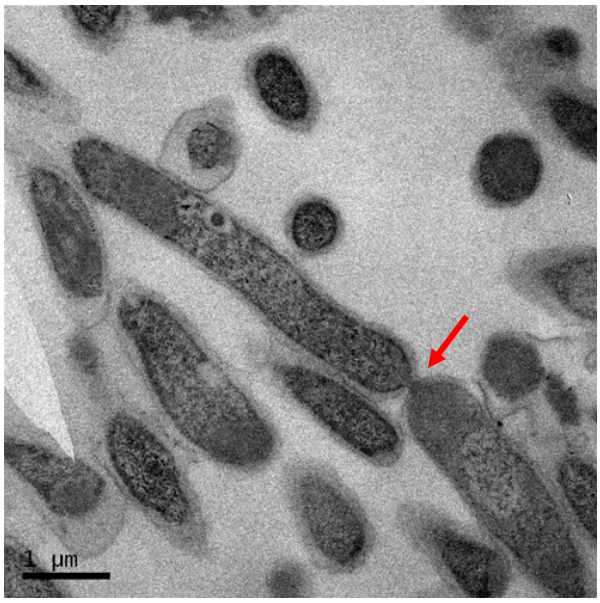
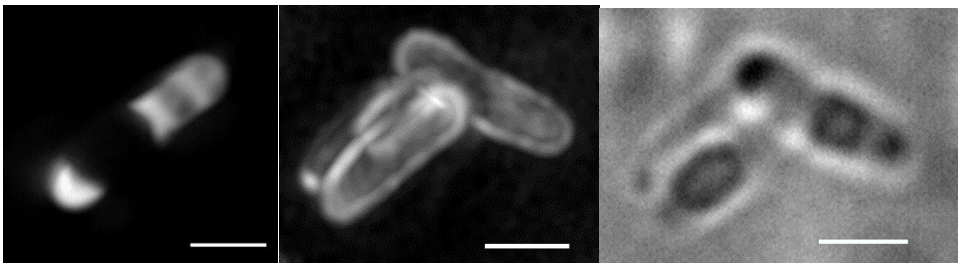
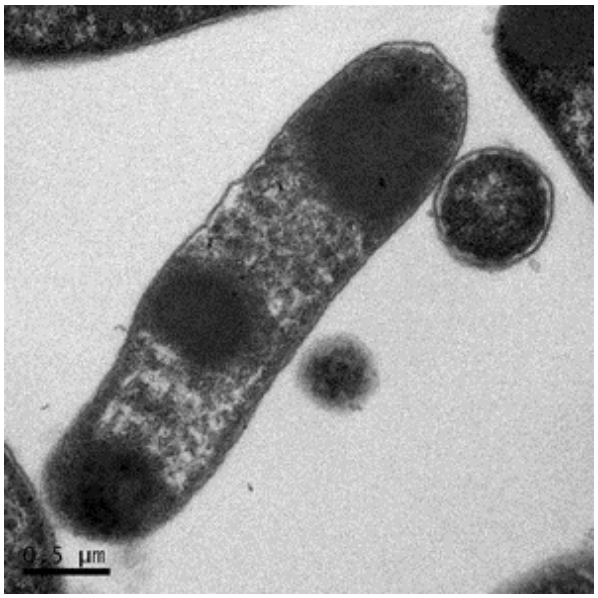


Figure.42. Images of BL21+NatB GFP cells 24 hours Post Induction, obtained using Phase and Fluorescent light microscopy (stained with FM4-64. Scale 1μm), TEM and Immuno EM.

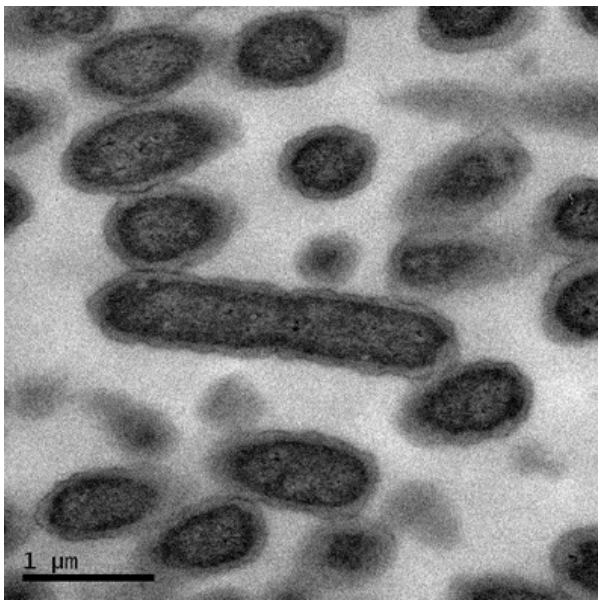
GFP FM4-64 Phase



TEM



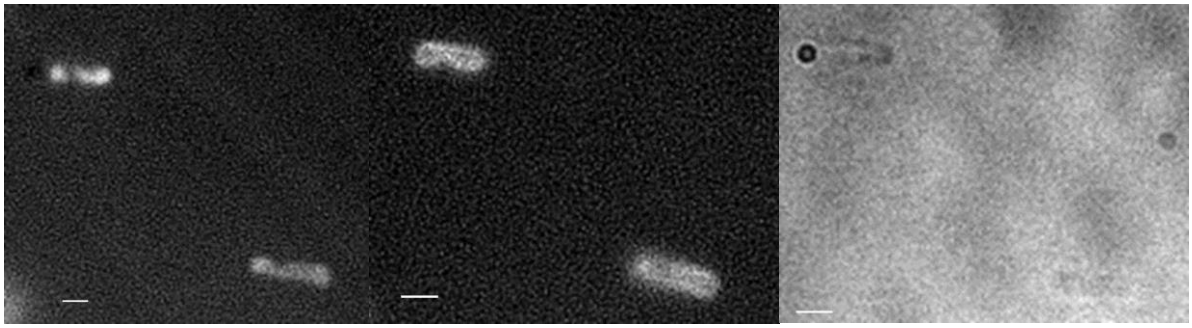
Immuno EM



At 90 hours post induction, the second set of cells, for all five cultures were stained with FM4-64 and examined by phase and appropriate fluorescent microscopy and images taken. Images were also taken using TEM but not by Immuno EM (Figures 43 to 47). Immuno EM images were not taken using the 90 hours post induction cells, as only the cells producing α-Synuclein-Cerulean3 had shown any labelling in the 24 hours post induction samples. A reduced number of the 24 hours post induction cells showed labelling compared to the 2 hours 30 minutes post induction cells, that were initially imaged using Immuno EM, indicating that some of the cells may have lost plasmid. For both only the BL21+NatA αS-Cerulean3 and BL21+NatB αS-Cerulean3 cells all of the cells in 5 fields were counted according to whether they contained gold discs indicating the presence of α-Synuclein. 36% of the BL21+NatA αS-Cerulean3 cells, 39 out of 109 cells and 41% of the BL21+NatB αS-Cerulean3 cells 35 out of 86 cells showed no immune labelling. The fluorescent images were also much fainter, and the BL21+NatA αS and BL21+NatB GFP cells (Figures. 45 & 47) were seen to have an unexpected spherical phenotype. This was also seen in the TEM images.

Figure.43. Images of BL21+NatA αS-Cerulean3 cells 90 hours Post Induction, obtained using Phase and Fluorescent light microscopy (stained with FM4-64. Scale 1μm) and TEM.

Cerulean3 FM4-64 Phase .



TEM

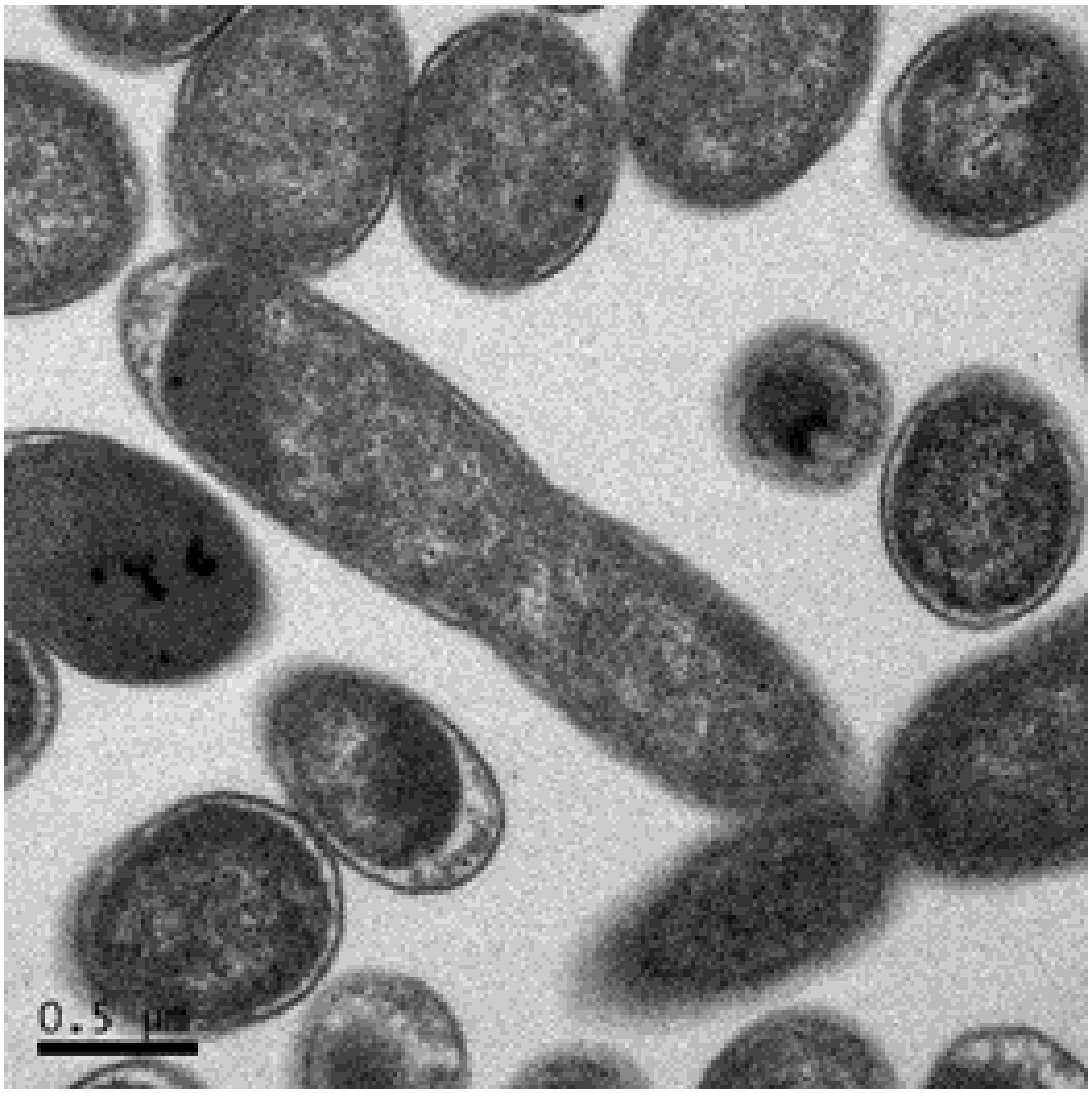
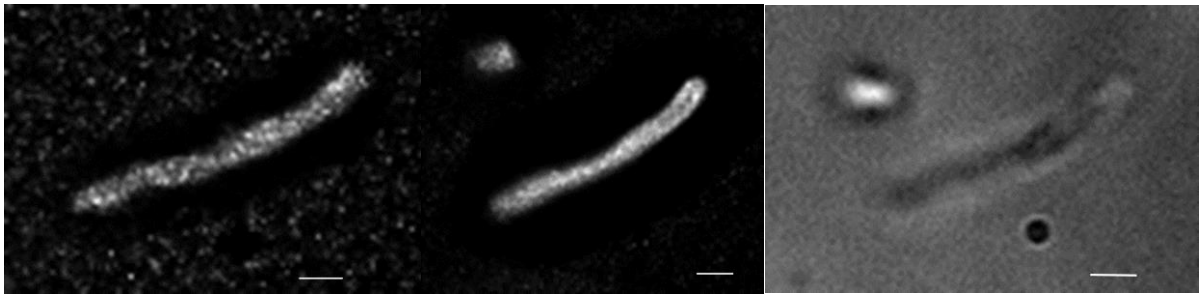


Figure.44. Images of BL21+NatB αS-Cerulean3 cells 90 hours Post Induction, obtained using Phase and Fluorescent light microscopy (stained with FM4-64. Scale 1μm) and TEM.

Cerulean3 FM4-64 Phase .



TEM

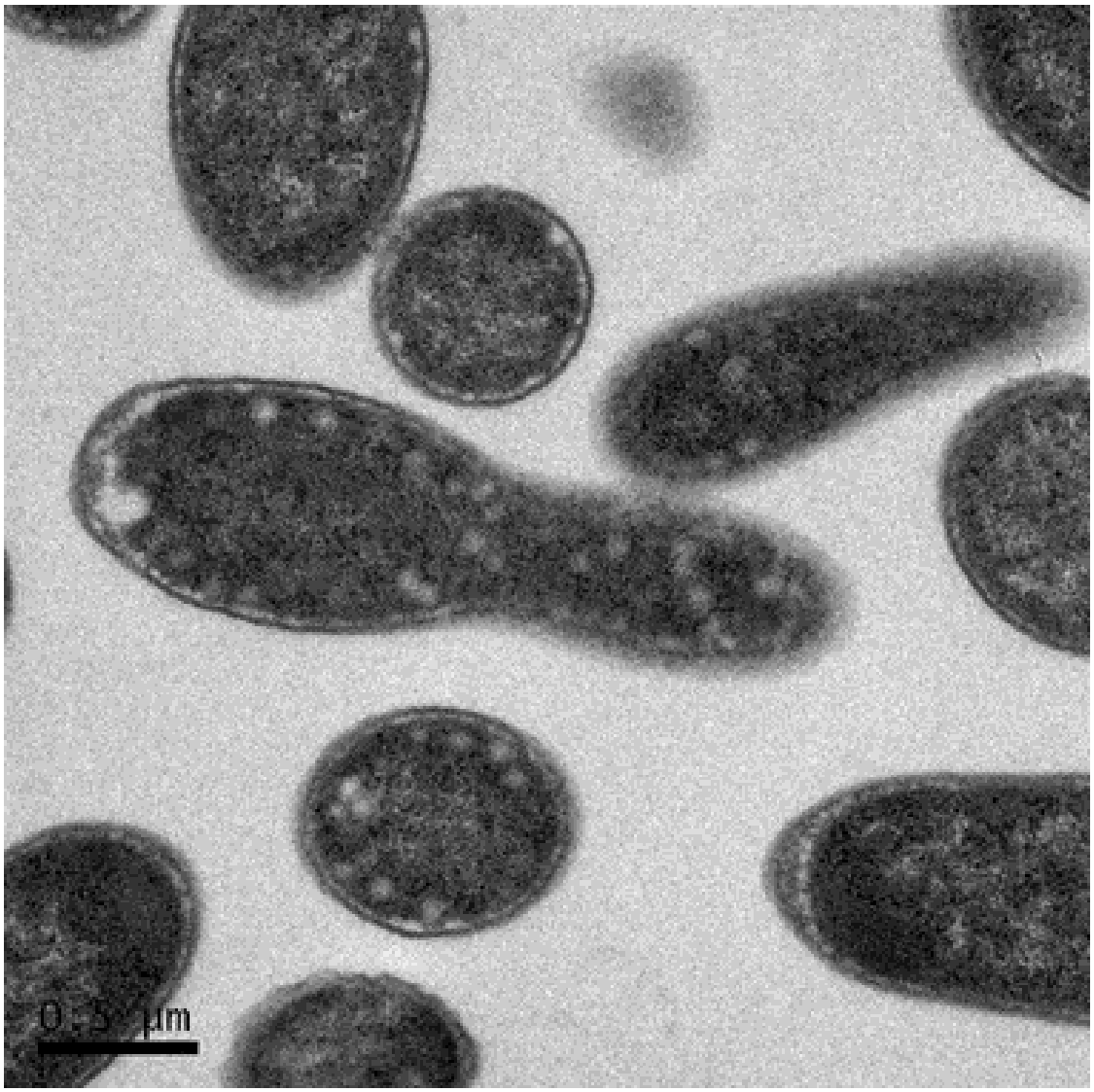
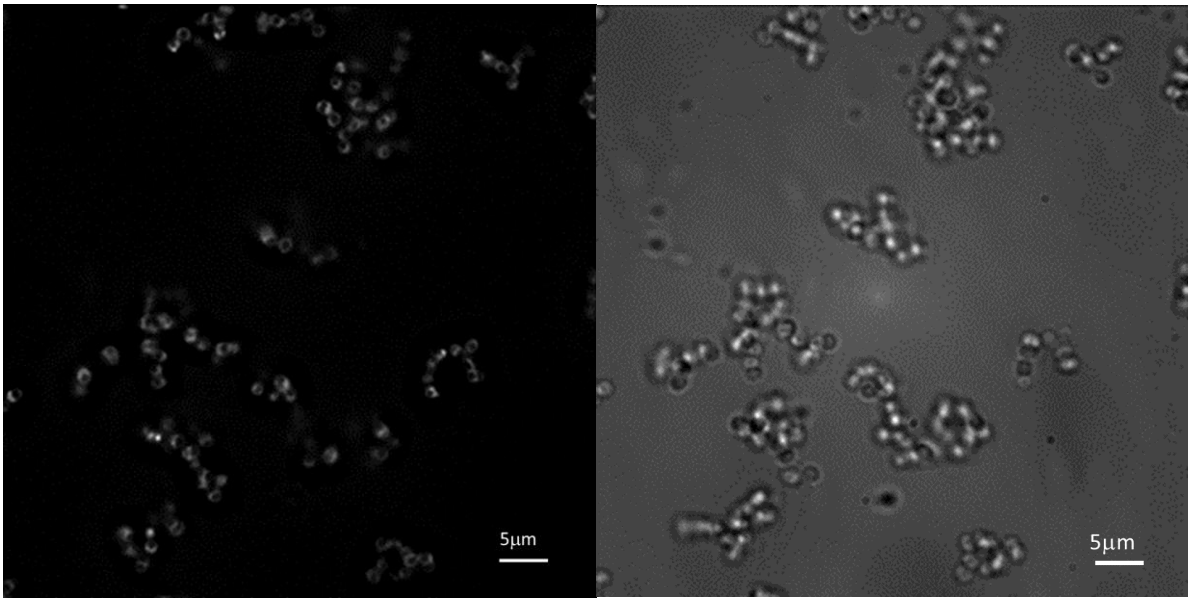


Figure.45. Images of BL21+NatA αS cells 90 hours Post Induction, obtained using Phase and Fluorescent light microscopy (stained with FM4-64. Scale 1μm) and TEM. What the spore like structures are is unknown. The white spots are holes where the resin used to fix the cells was unable to penetrate into these structures.

FM4-64 Phase



TEM

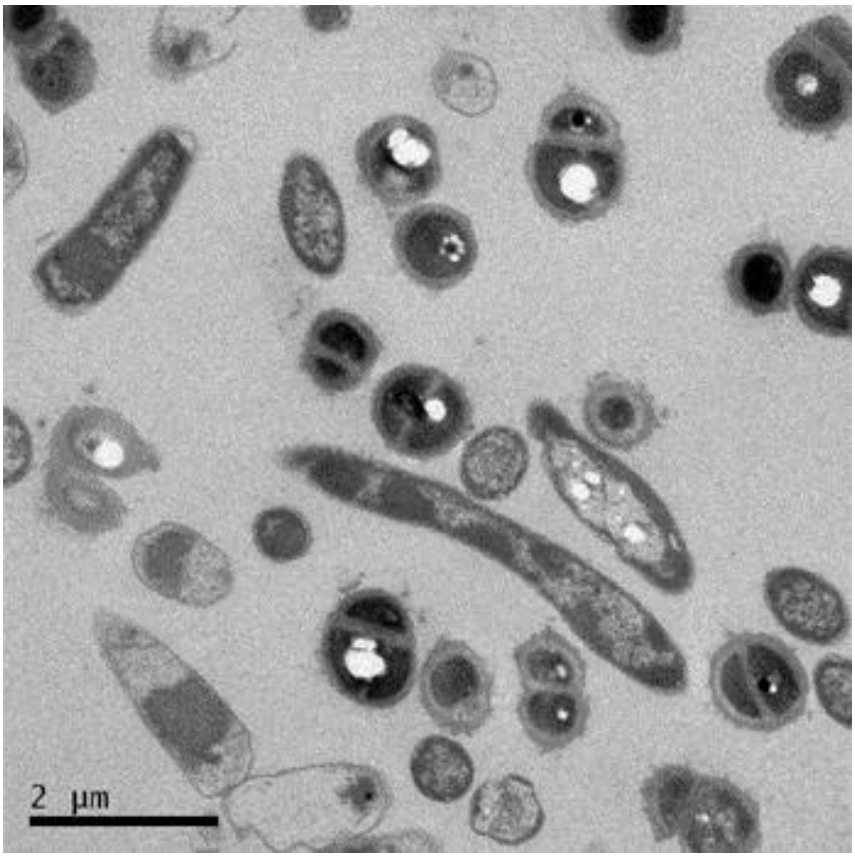
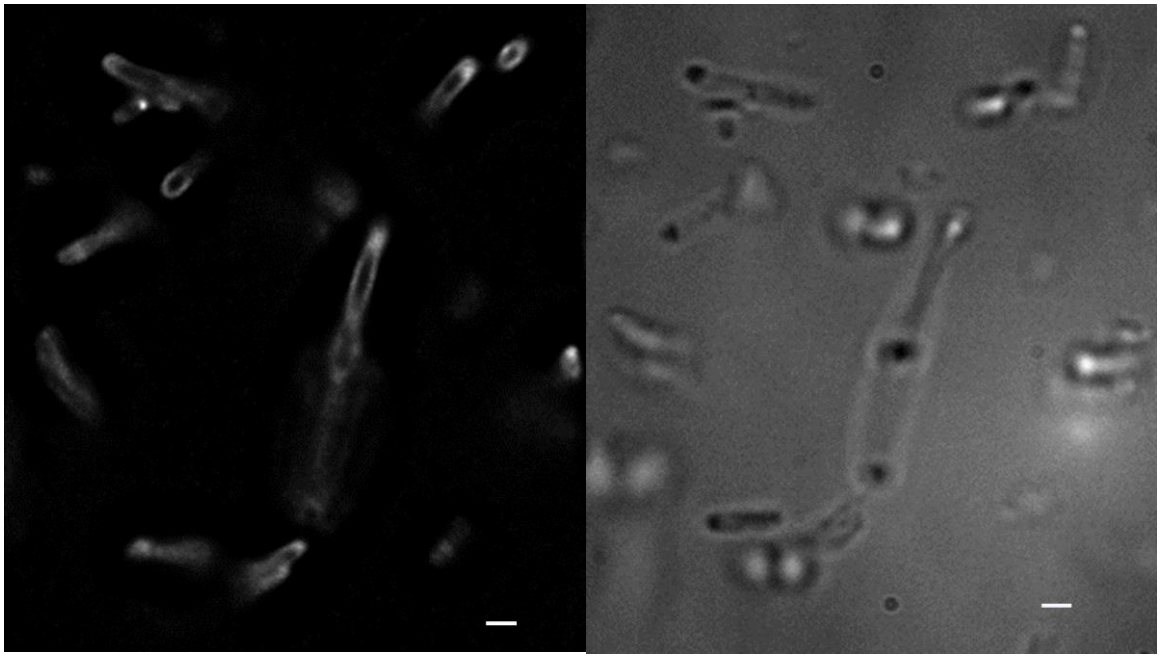


Figure.46. Images of BL21+NatB αS cells 90 hours Post Induction, obtained using Phase and Fluorescent light microscopy (stained with FM4-64. Scale 1μm) and TEM. The images appear to show a large proportion of dead or dying cells.

FM4-64 Phase



TEM

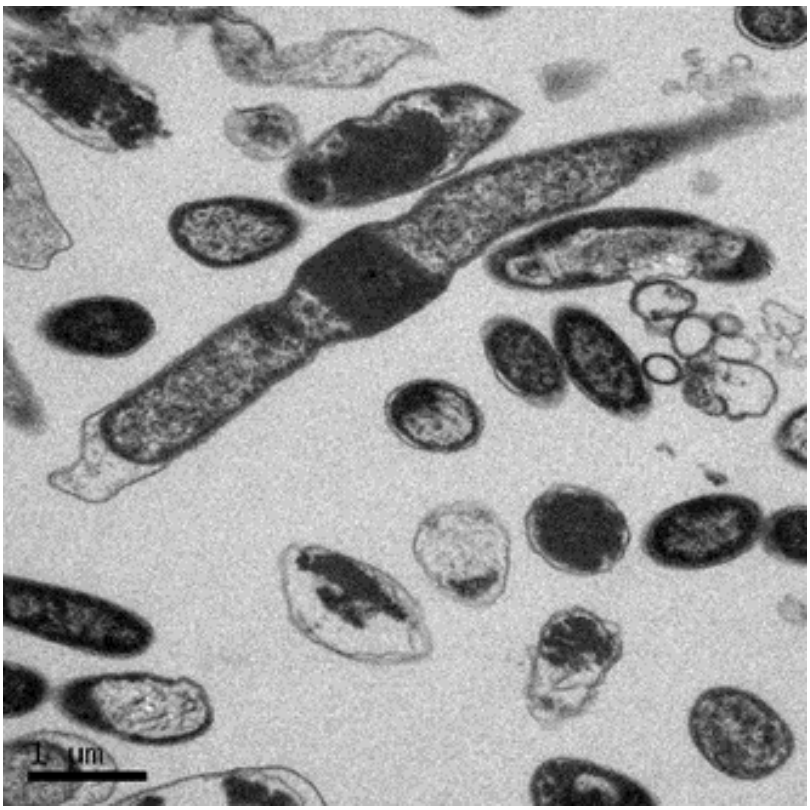
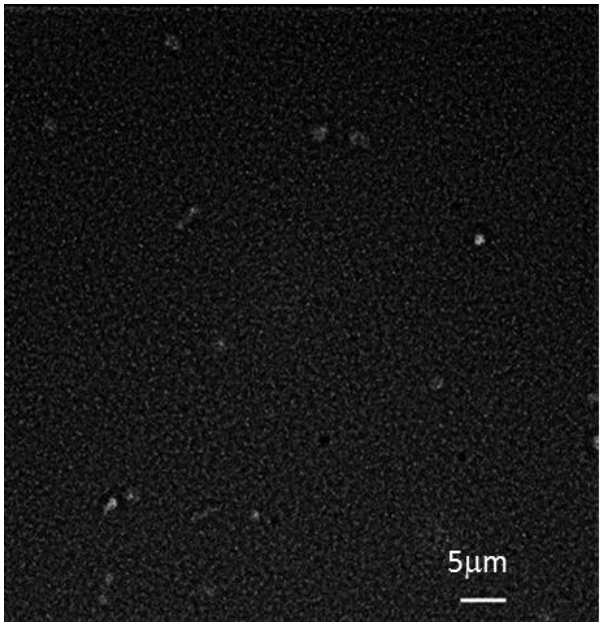
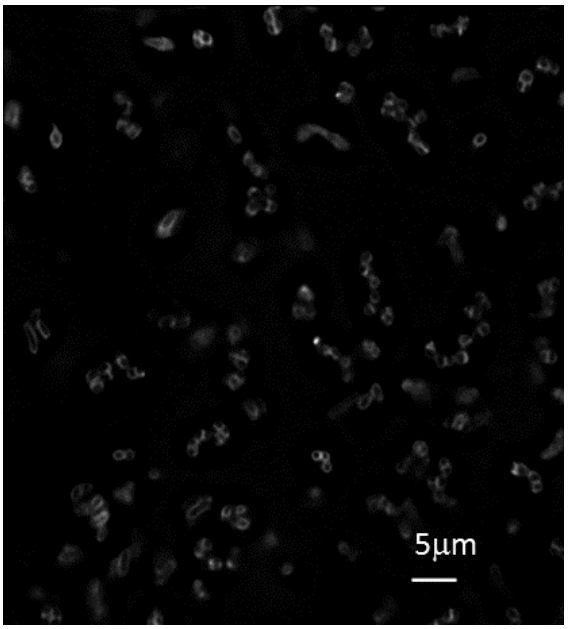


Figure.47. Images of BL21+NatB GFP cells, 90 hours Post Induction, obtained using Phase and Fluorescent light microscopy (stained with FM4-64) and TEM. What the spore like structures are is unknown. The white spots are holes where the resin used to fix the cells was unable to penetrate into these structures

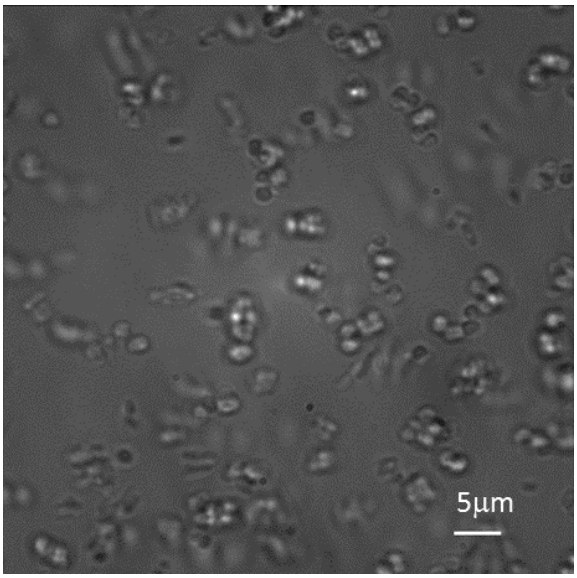
GFP



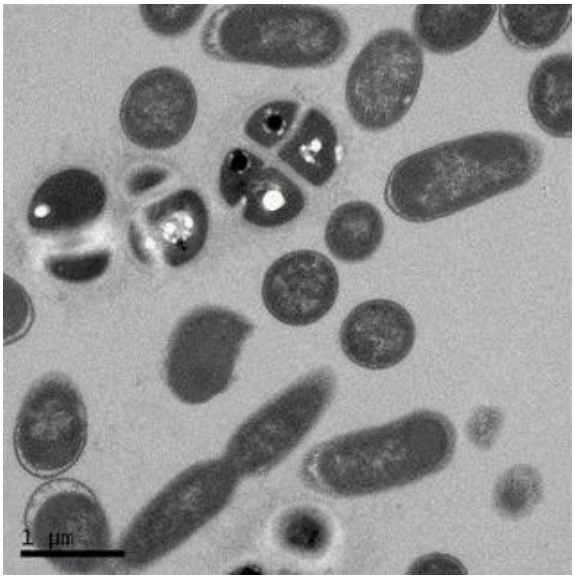
FM4-64



Phase



TEM



The cells expressing α-Synuclein without a fluorescent tag, produced such unexpected images, without blebbing or tubular structures like the as-Cer3 images,

and this together with the lack of labelling in the Immuno EM images, led to a suspicion that this plasmid did not contain the gene for the production of α-Synuclein as it was supposed to.

To investigate this suspicion, the 90 Hour protein samples precipitated from the cell cleared culture media were examined by Coomassie stained Gel and by Western Blot (Figure 48) using anti α-Synuclein antibody at a 1 in 1000 dilution.

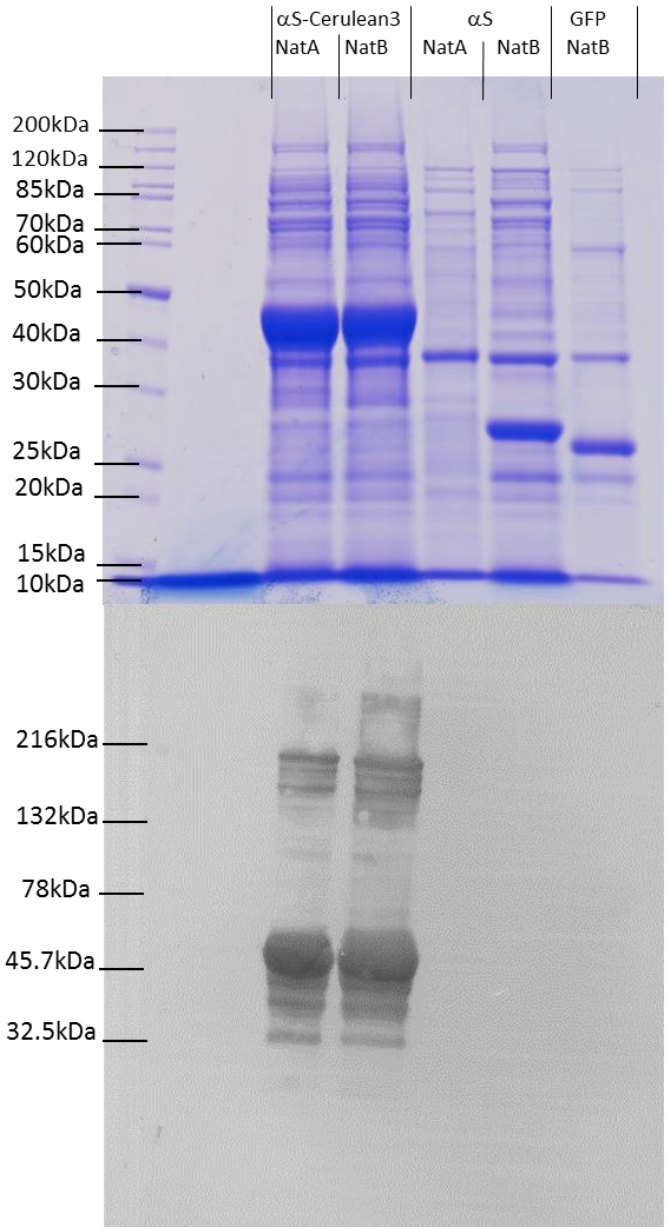


Figure.48. Coomassie stained 4-12% Bis-Tris gel and Western Blot analysis of cell cleared culture media, 90 Hours post induction.

This examination of protein from the 90 hours post induction, cell cleared culture media, further indicated that α-Synuclein was only produced in the BL21+NatA αS-Cerulean3 and BL21+NatB αS-Cerulean3 cells. This again suggests that the αS-plasmid given to this laboratory, did not contain the gene for the production of α-Synuclein.

To finally confirm these suspicions, another matching Coomassie stained gel and Western Blot were performed, (Figure 49) this time using cells taken for protein examination Pre and 90 Hours post induction.

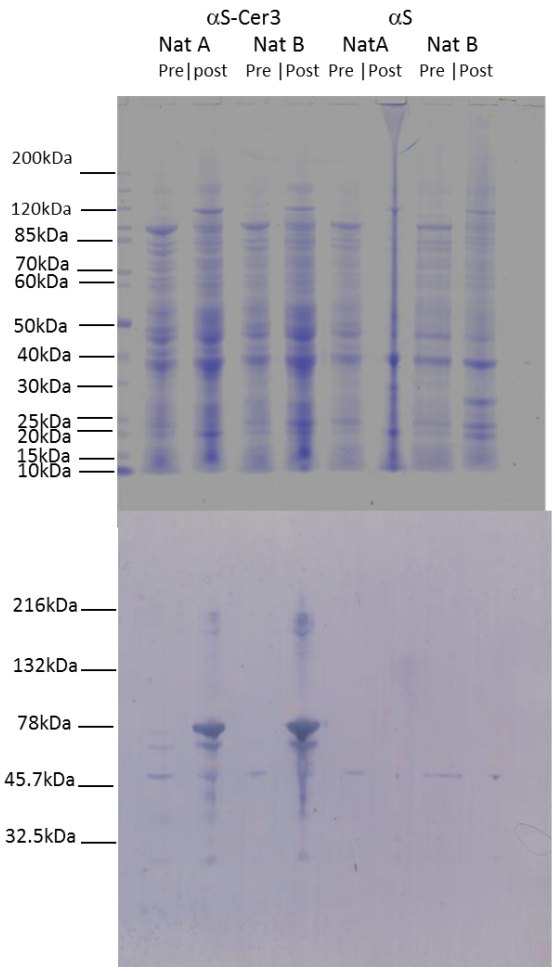


Figure.49. Coomassie stained 4-12% Bis-Tris gel and Western Blot analysis of protein from cells taken, pre and 90 Hours post induction.

This second protein examination further indicated that α-Synuclein was only present in the BL21+NatA αS-Cerulean3 and BL21+NatB αS-Cerulean3 cells. This result was taken to confirm that the αS-plasmid that had been given to the laboratory, did not contain the gene for the production of α-Synuclein.

4.11: Investigation into the role of α-Synuclein in Membrane reorganisation

Having seen on the Electron Micrographs that the blebs produced by cells producing α-Synuclein appear to be membrane bound, it was decide to further investigate this by live cell imaging and fluorescent growth curves.

A pETDuet vector was produced containing the genes for α-Synuclein-Cerulean3 and cydAB-mCherry, a plasma membrane bound protein attached to a mCherry fluorescent protein.

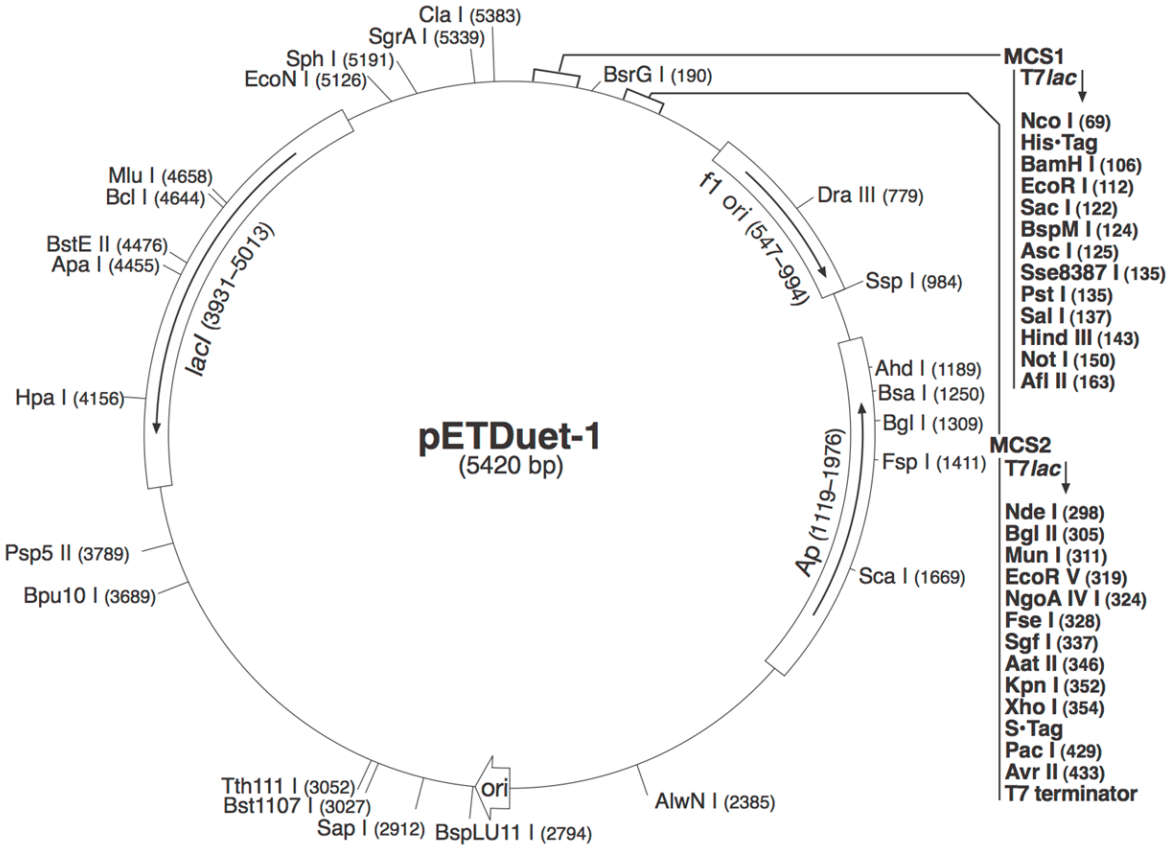


Figure. 50. Plasmid map for the pETDuet plasmid used for the production of just one or simultaneous production of two recombinant proteins.

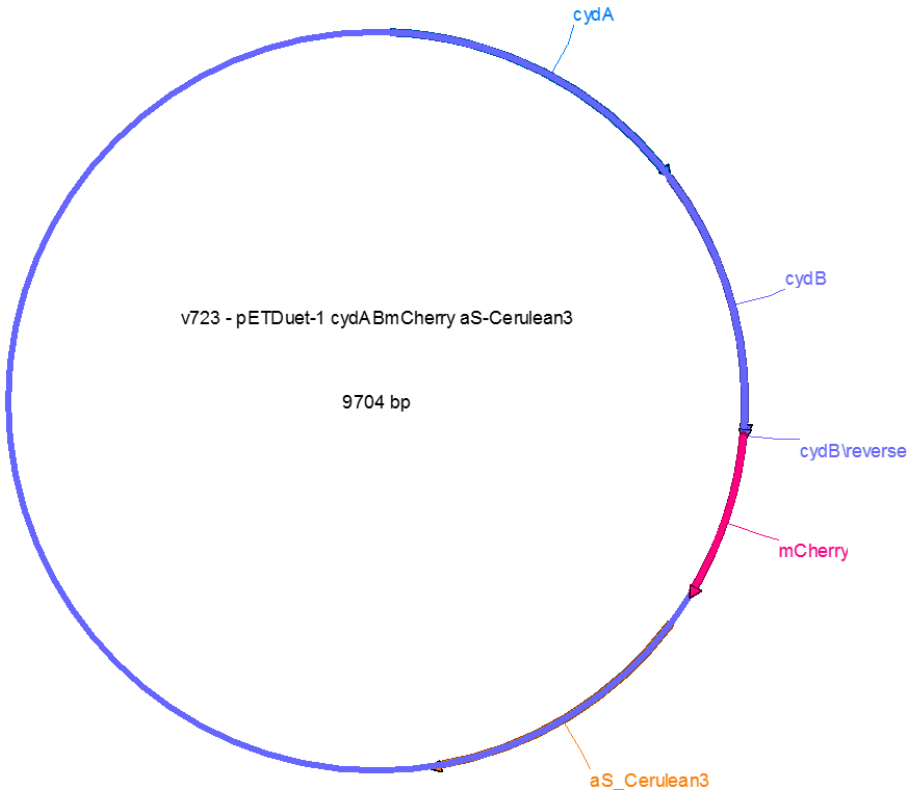


Figure.51. Plasmid map showing the locations of the genes for α-Synuclein-Cerulean3 and cydAB-mCherry proteins,within the pETDuet plasmid.

A control pETDuet vector was produced which only contained the gene for cydAB-mCherry.

Images were taken of BL21DE3 and BL21DE3+NatB cells, with each of these vectors after 0.5 hours incubation in LB and antibiotic and then a further 1 hour incubation on a 1% agarose in LB and antibiotic slide.

In the cells containing pDUETαS-Cerulean3+cydAB-mCherry expressing both α-Synuclein-Cerulean3 and cydAB-mCherry, much higher level of fluorescence were seen with the Cerulean3 than with the mCherry. The reason for this increase in mCherry fluorescence is at present unknown but is possibly due to interactions between the αS-Cer3 and the cell membranes. Many images show both Cerulean3 and mCherry in the same location at membranes and externally, indicating the presence of membrane bound blebs, however other images show different areas of localisation with α-Synuclein appearing to be cytoplasmic within the plasma membrane.

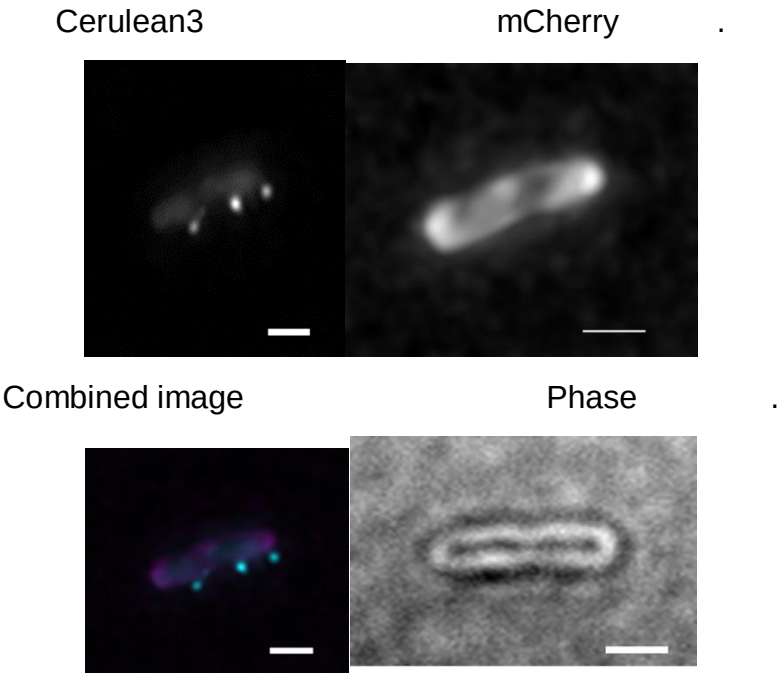


Figure.52. BL21DE3 αS-Cerulean3+cydAB-mCherry cells expressing both α-Synuclein-Cerulean3 and cydAB-mCherry The αS-Cer3 appears to be at lower levels in the cytoplasm and higher levels within blebs, cydAB-mCherry appears to be localised to the cell membrane. (Scale 1μm)

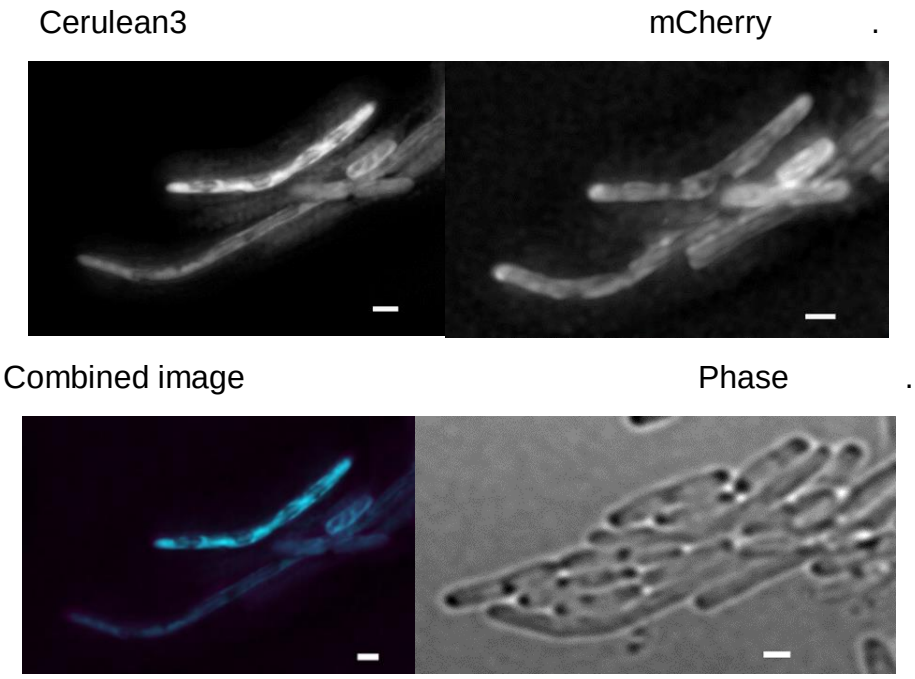


Figure.53. BL21+NatB αS-Cerulean3+cydAB-mCherry cells expressing both α-Synuclein-Cerulean3 and cydAB-mCherry. The aS-Cer3 appears to be at slightly higher levels at cell membranes and at much higher levels in structures giving a stripy appearance to the brightest cell, cydAB-mCherry appears to be at slightly higher levels at cell membranes and poles. (Scale 1μm).

Cerulean3 mCherry Combined image Phase .

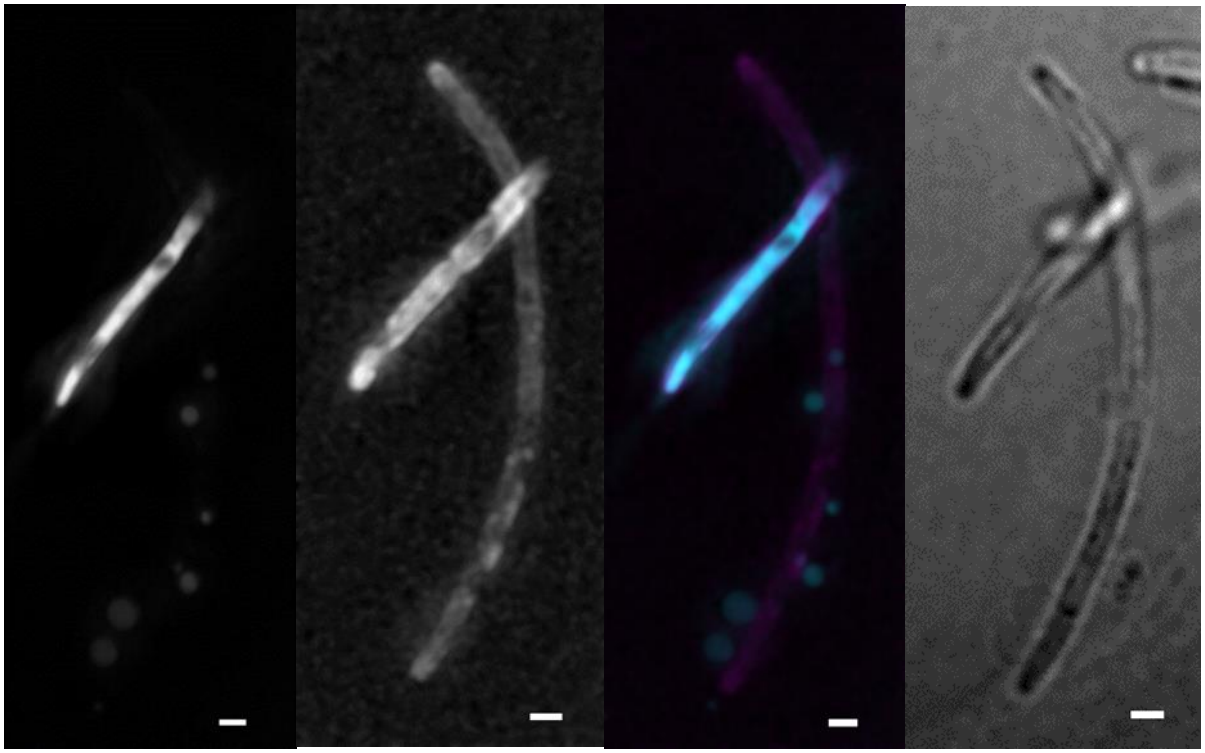


Figure.54. BL2+1NatB αS-Cerulean3+cydAB-mCherry cells expressing both α-Synuclein-Cerulean3 and cydAB-mCherry with the appearance of both ribbons and blebbing (Scale 1μm).

Further images were taken to produce a direct comparison between the three vectors. pJC20αS-Cerulean3 producing α-Synuclein-Cerulean3, pDUETαS-Cerulean3+cydAB-mCherry producing α-Synuclein-Cerulean3 and CydAB-mCherry simultaneously and pDUETcydAB-mCherry producing cydAB-mCherry, in both BL21DE3 cells to produce unacetylated α-Synuclein-Cerulean3 and in BL21+NatB cells to produce acetylated α-Synuclein-Cerulean3.

Six cultures were set up, one for each of the combinations. At an OD_{600} of between 0.51 and 0.63 each was induced by the addition of 1μm per ml of IPTG (100mg/ml stock). They were then incubated at 37°C for 1.5 Hours. The broths were then used to make up slides on 2% agarose in LB broth containing the appropriate antibody and IPTG, and then incubated for a further 0.5 Hour at 37°C.

Keeping all the microscope settings the same throughout, images were taken of Cerulean3 and mCherry signals, so that fluorescent intensities could be compared.

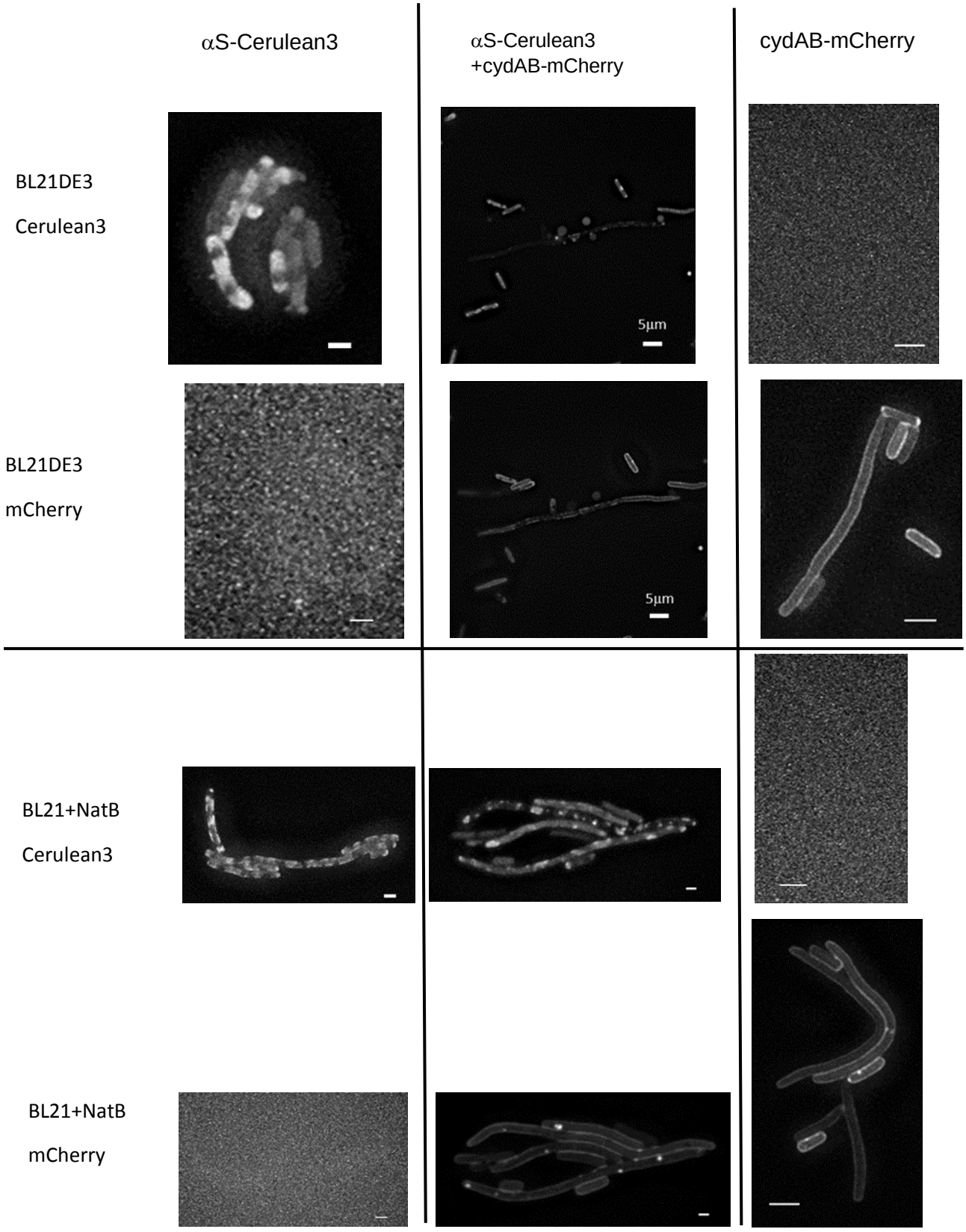


Figure.55. Images of BL21DE3 and BL2+1NatB cells with plasmids containing αS-Cerulean3, αS-Cerulean3+cydAB-mCherry or cydAB-mCherry genes. Cells are compared expressing either α-Synuclein-Cerulean3, cydAB-mCherry or both simultaneously. (Scale 1μm, except where 5μm stated on image).

It was decided to obtain fluorescent growth curves for both Cerulean3 and mCherry simultaneously in the same six samples imaged earlier (Figures 56 & 57).

A new culture of each of the six samples was grown in LB and appropriate antibiotic, until mid-log phase was reached. Each was then diluted according to its OD_{600} to obtain cultures at an OD_{600} of 0.1, and 0.1μl per ml of IPTG (100mg/ml stock) was added. 1ml of each culture was then added to each of 4 wells on a 24 well plate, which was then placed in an automatic plate reader at 37ºC with shaking and fluorescence readings for both Cerulean3 and mCherry were taken every 15 minutes.

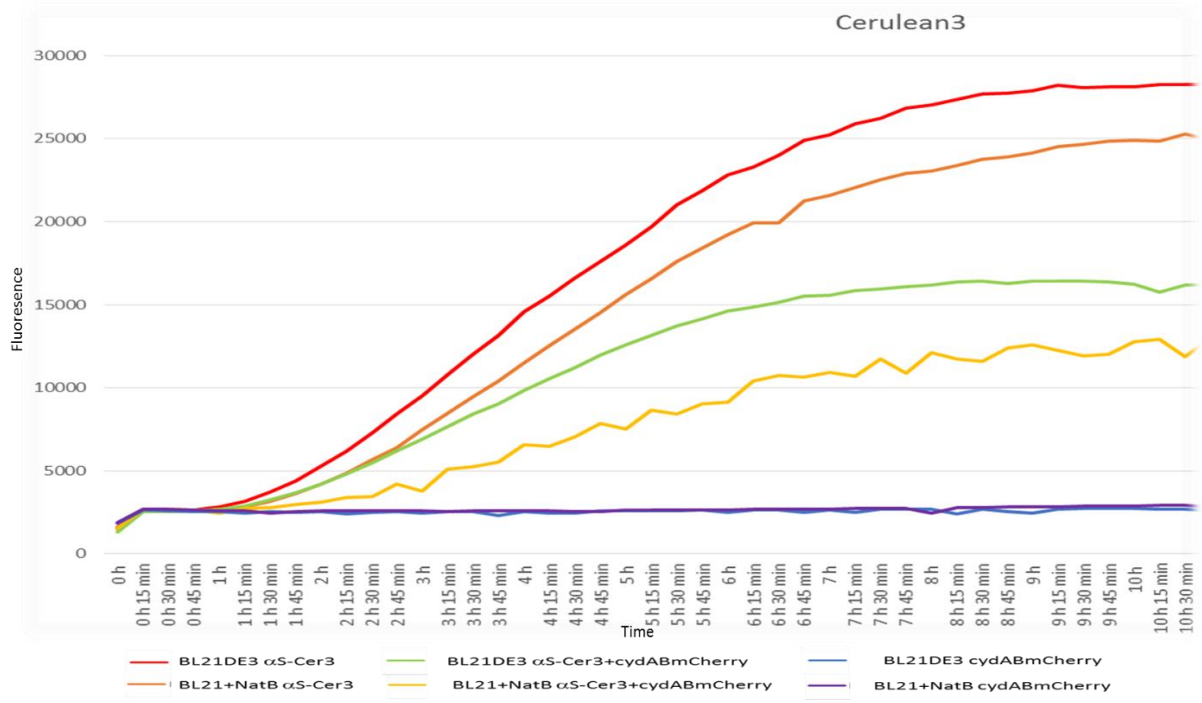


Figure.56. Cerulean3 fluorescent growth curve, for BL21DE3 and BL21+NatB cells with plasmids containing αS-Cerulean3, αS-Cerulean3+cydAB-mCherry or cydAB-mCherry genes.

Cells expressing, cydAB-mCherry showed no increase in Cerulean3 fluorescence, as expected. The cells expressing α-Synuclein-Cerulean3 show the largest increase in Cerulean3 fluorescence, with the unacetylated producing more fluorescence than the acetylated. The cells producing both recombinant proteins simultaneously also showed an increase in fluorescence, of approximately half that measured for the cells producing α-Synuclein-Cerulean3 alone and again the BL21DE3 cells produced more fluorescence than the BL21DE3+NatB cells, possibly due to the extra stress placed upon the cells producing additional NatB complex .

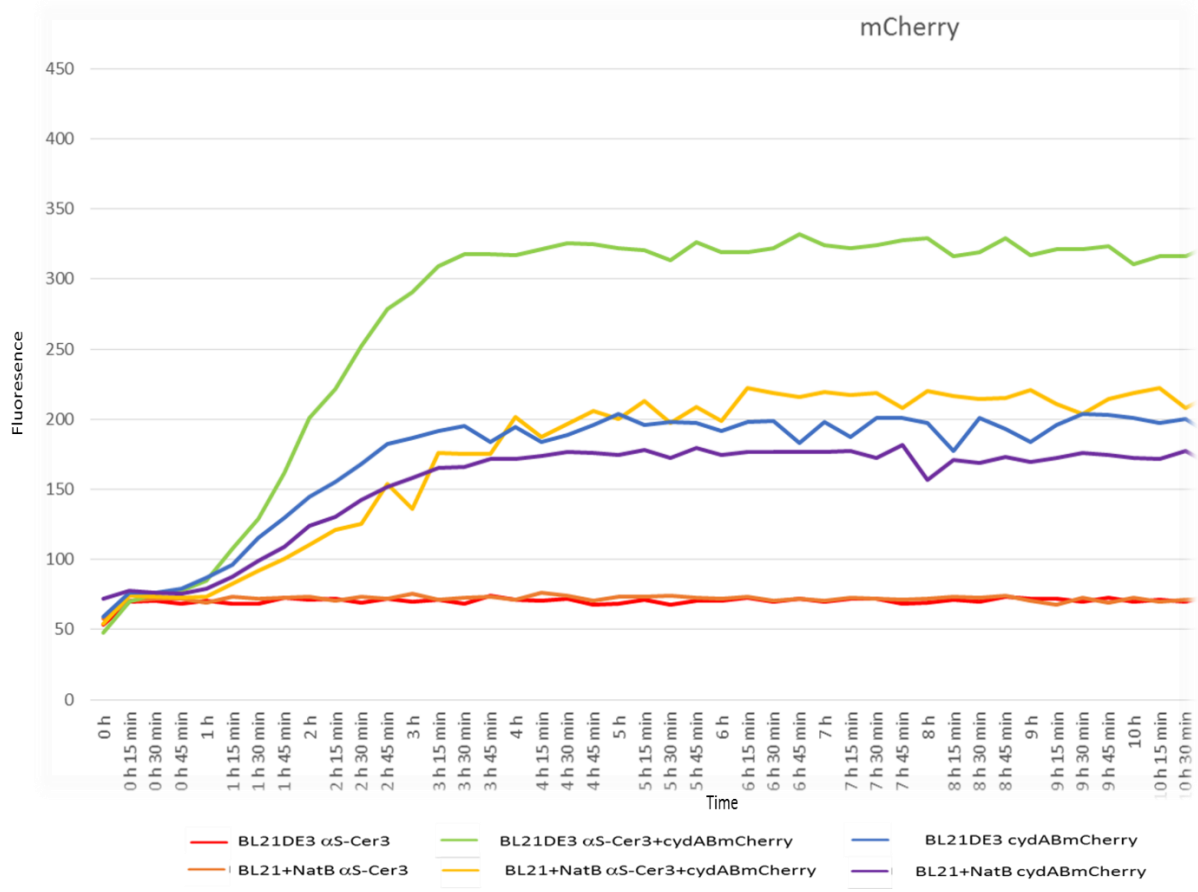


Figure.57. mCherry Fluorescent growth curve, for BL21DE3 and BL21+NatB cells with plasmids containing αS-Cerulean3, αS-Cerulean3+cydAB-mCherry or cydAB-mCherry genes.

The cells expressing α-Synuclein-Cerulean3 show no increase in mCherry fluorescence. Cells expressing just cydAB-mCherry show a clear increase in mCherry fluorescence, as expected, with the unacetylated producing more fluorescence than the acetylated. The cells producing both recombinant proteins simultaneously also showed an increase in mCherry fluorescence. The BL21+NatB αS-Cerulean3+cydAB-mCherry cells showed a slightly higher level of mCherry fluorescence than that measured for the cells producing just cydAB-mCherry. The BL21DE3 αS-Cerulean3+cydAB-mCherry cells showed approximately double the increase in mCherry fluorescence than that measured for the cells producing cydAB-mCherry alone. This was just under double that measured in cells producing acetylated α-Synuclein-Cerulean3 and cydAB-mCherry.

To confirm that the correct recombinant proteins were being produced with each vector, whole cell protein samples from each of the six cultures used for imaging and fluorescent growth curves were examined by Coomassie stained gel (Figure.58) along with 5 days post induction cell cleared culture media samples (Figure.59)

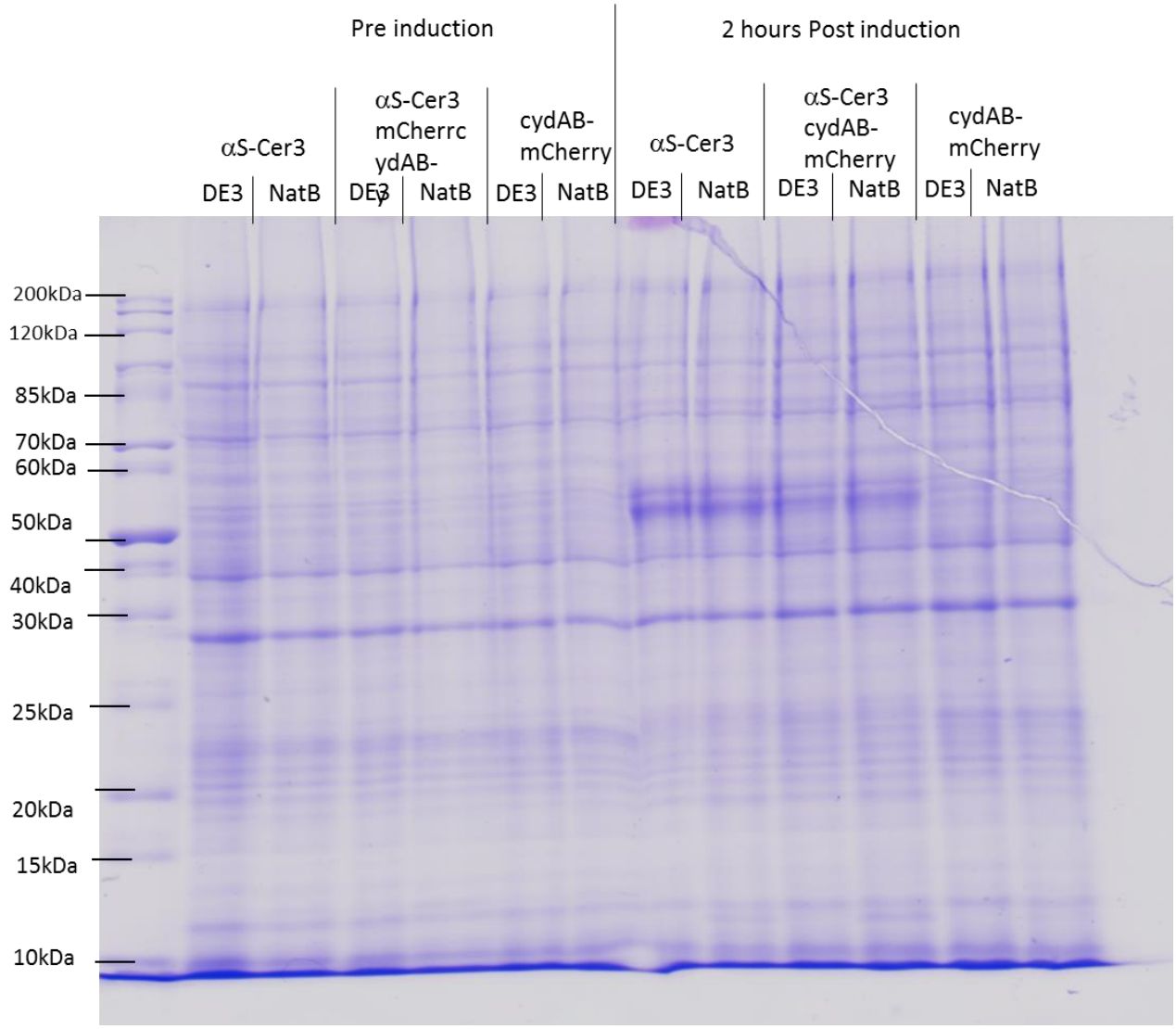


Figure.58. Coomassie stained gel for whole cell protein samples from BL21DE3 and BL21+NatB cells with plasmids containing αS-Cerulean3, αS-Cerulean3+cydAB-mCherry and cydAB-mCherry genes, pre induction and 2 hours post induction.

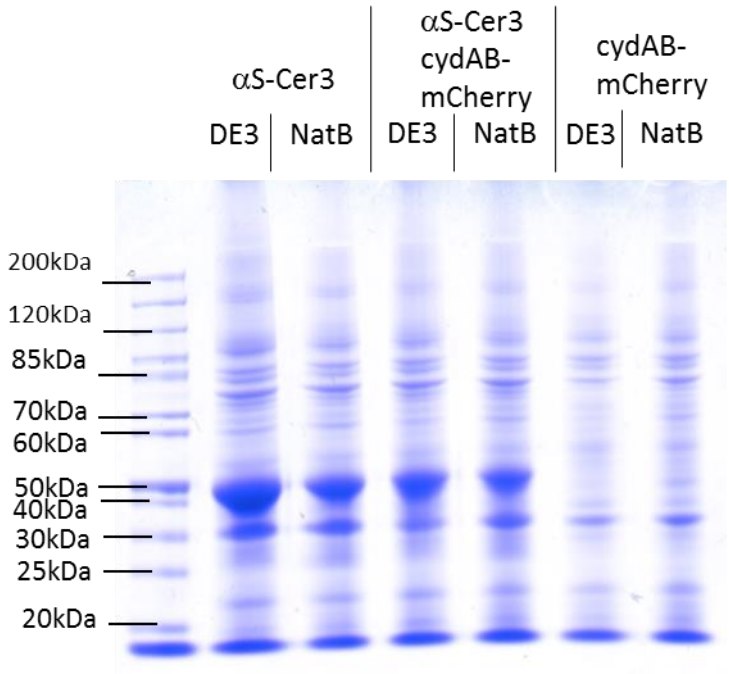


Figure.59. Coomassie stained 4-12% Bis-Tris gel for 5 day post induction protein samples from cell cleared culture medium from cultures of BL21DE3 and BL21+NatB cells containing αS-Cerulean3, αS-Cerulean3+cydAB-mCherry and cydAB-mCherry.

The Coomassie stained gels indicated induction of αS-Cerulean3 but no additional bands could be clearly seen, indicating the induction of cydAB-mCherry. The 2 hours post induction, whole cell protein samples, were used to carry out two Western Blots, one using anti α-Synuclein as the primary antibody and one using anti-mCherry as the primary antibody (Figure.60). Appropriate bands were identified in the samples, confirming that αS-Cerulean3 and cydAB-mCherry were being expressed in the correct cultures.

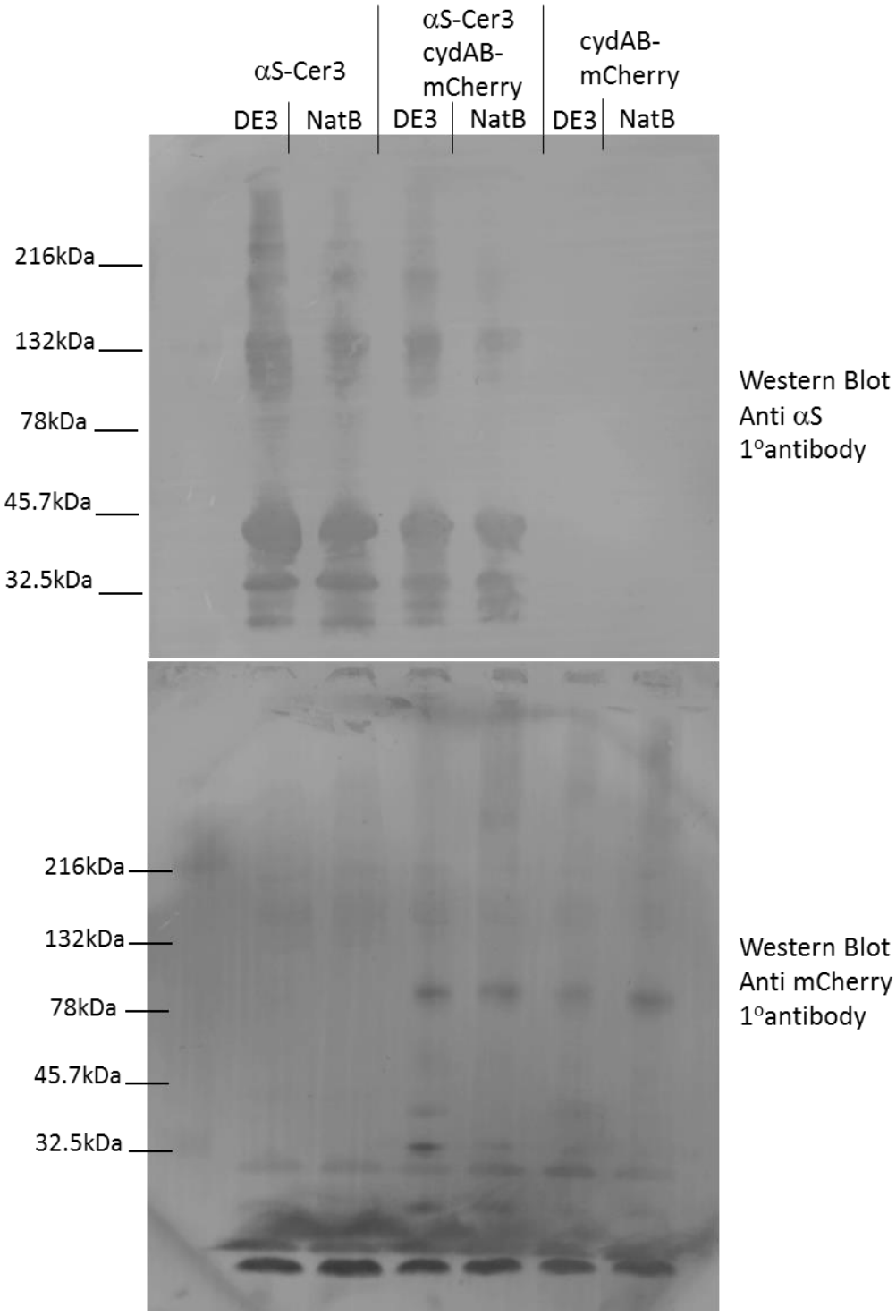


Figure.60. Western Blots using anti α-Synuclein (top) and anti mCherry (bottom) on whole cell protein samples taken 2 hours port induction from cultures of BL21DE3 and BL21+NatB cells containing αS-Cerulean3, αS-Cerulean3+cydAB-mCherry and cydAB-mCherry.

4.12: Investigation of Oligomer formation using Bimolecular Fluorescence Complementation (BiFC)

Having seen the presence of possible dimers and tetramers of α-Synuclein during the comparison of acetylated and unacetylated α-Synuclein produced in *E.coli*, it was decided to investigate oligomer formation using BiFC. The BiFC assay is a useful imaging tool for direct visualization of protein-protein interactions in living cells. It is based on the structural complementation of two nonfluorescent N- and C-terminal fragments of a fluorescent protein, when they are fused to a pair of interacting proteins. (Yutaka Kodama *el al* 2010) Previously BiFC has been used to investigate α-Synuclein oligomer formation (Outeiro *et al*, 2008, Roberts *et al*, 2015) however these investigations used GFP to fluorescently tag both the C and N terminal ends of α-Synuclein. As we are examining the effect of N-terminal acetylation on membrane interactions and oligomer formation we only tagged the C-terminal of α-Synuclein.

Two new vectors were produced for the investigation, to create α-Synuclein fixed to half of a Yellow Fluorescent Protein, Venus. This was done using the pETDuet plasmid, so that either half of the Venus protein attached to α-Synuclein could be produced on its own or both could be produced simultaneously within the same cell. If α-Synuclein forms oligomers with a cell where α-Synuclein is attached to either half of the Venus protein, this should bring the two complementary halves of the Venus protein into close proximity, allowing the production of Venus fluorescence within the cell.

The vectors produced were

1. pDUET-α-Synuclein-Venus-Carboxyl half (αs-VC)

2. pDUET-α-Synuclein-Venus-Amino half, α-Synuclein-Venus Carboxyl half (αS-VN αS-VC)

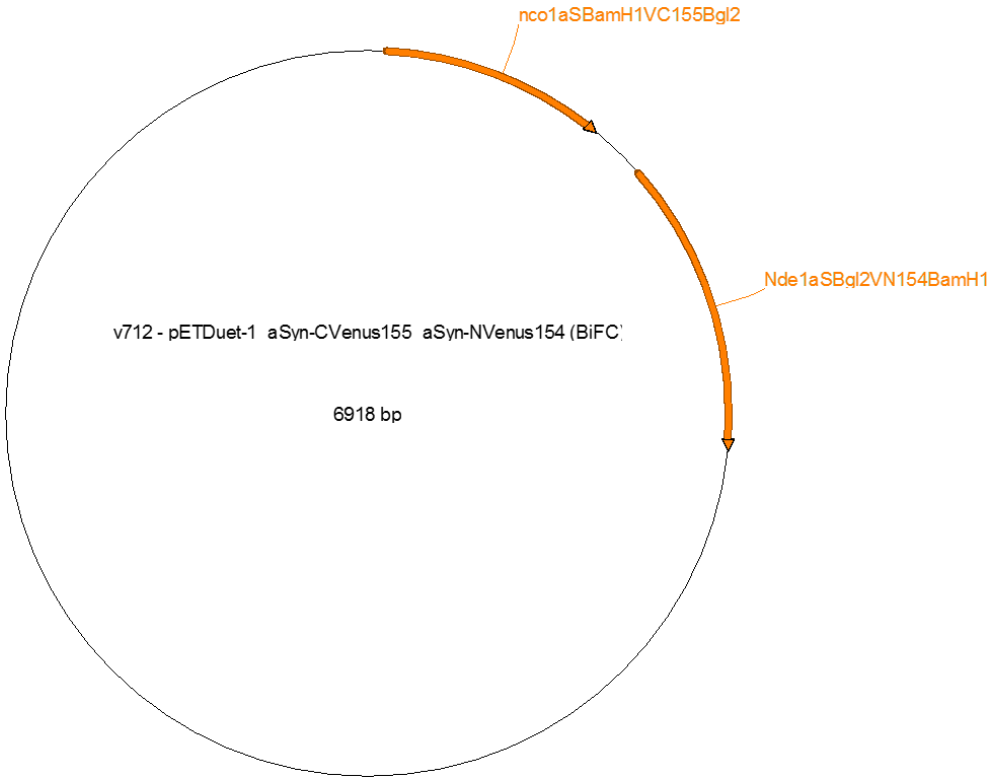


Figure. 61. Plasmid map to show the relative positions of the genes coding for α-Synuclein-Venus-Amino half and α–Synuclein-Venus Carboxyl half within the pETDuet plasmid.

3. pDUET a-Synuclein-Venus-Amino half (αs-VN)

The first two of these plasmids was transformed into BL21+NatA and BL21NatB *E.coli* cells to investigate the effect of acetylation on Oligomer formation. Cells in Log phase were induced with IPTG. Protein samples were taken pre and at 3 hours post induction and cells were examined by fluorescent microscopy at 3 hours post induction (Figures 62 to 64). No fluorescence could be seen in cells containing αS-VC and only phase images could be taken (Figure 62). This confirmed that any oligomers formed with only the Carboxyl half of the Venus protein present did not produce fluorescence unlike those containing the αS-VN αS-VC which produced clear fluorescence (Figures 63 & 64).

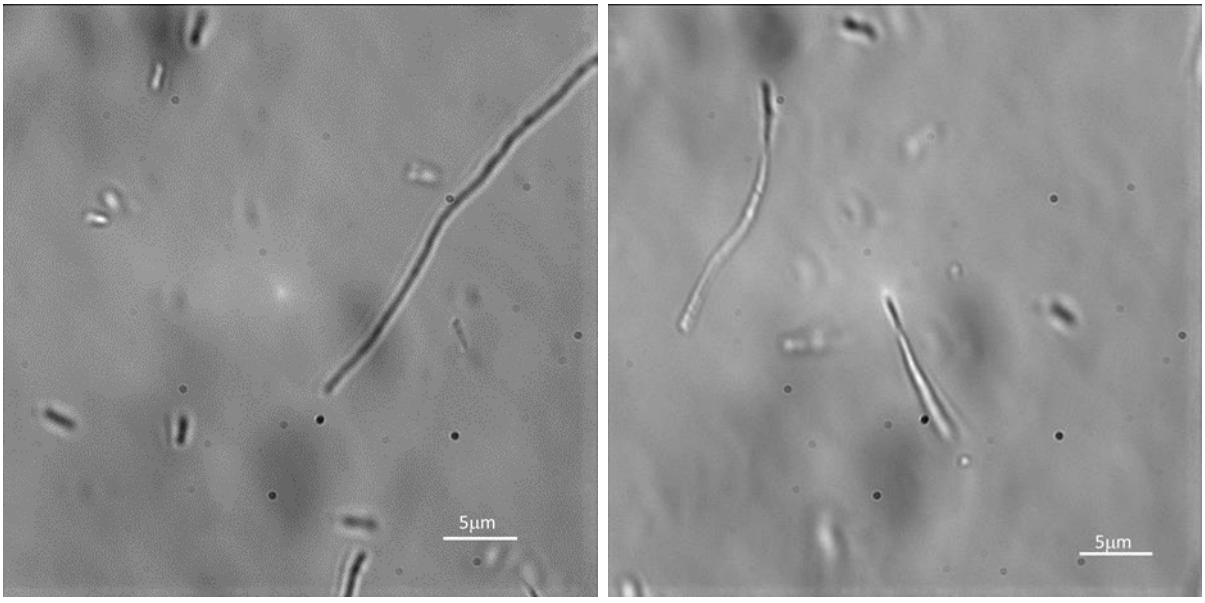
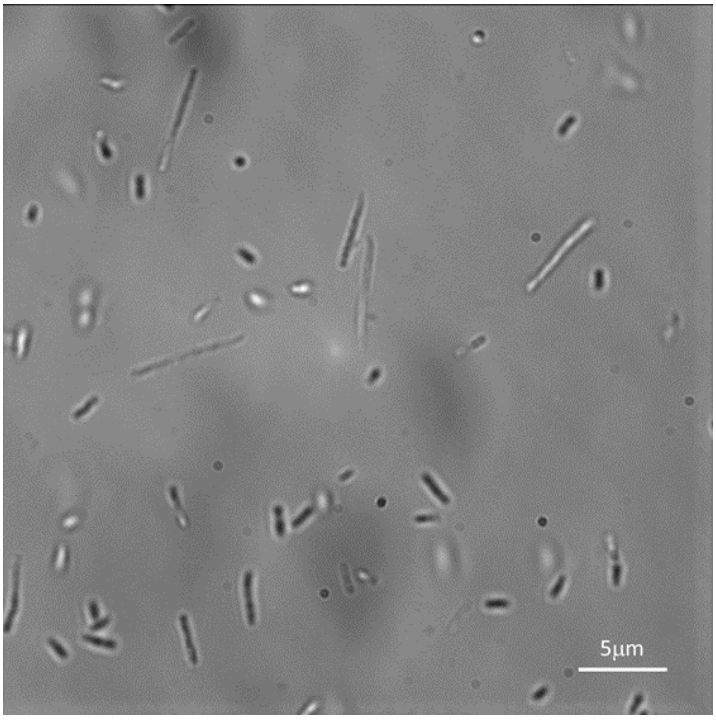


Figure.62. Images of BL21+NatA (left) and BL21+NatB (right) cells containing αS-VC observed by Phase microscopy. No Venus signal was visible, showing that the Carboxyl half of the Venus Protein does not produce fluorescence by itself.

Phase



Venus

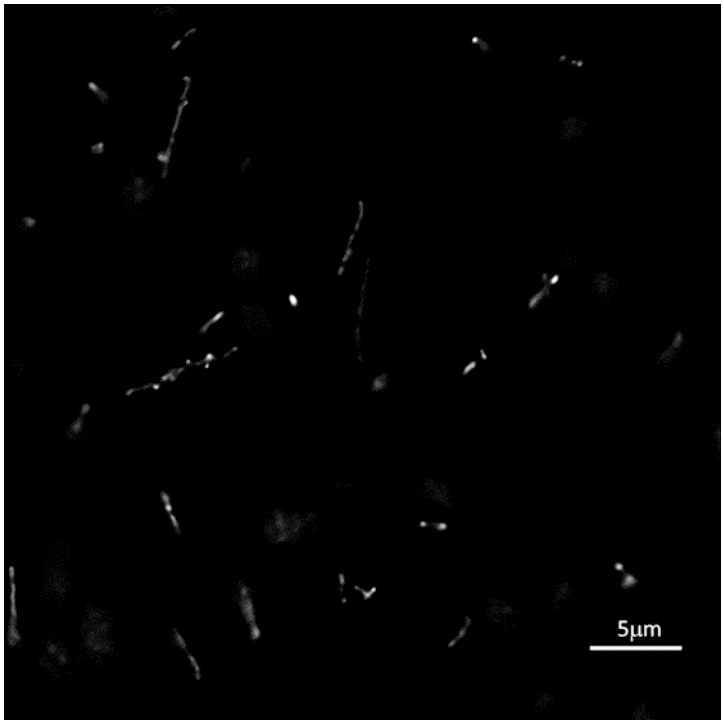


Figure.63. Images of BL21+NatA αS-VN αS-VC cells producing α-Synuclein-Venus Amino half and α-Synuclein-Venus Carboxyl half have Venus fluorescence, showing both blebbing around cells and a ribbon like appearance within many cells. This indicates the presence of α-Synuclein oligomers, both within the cells and within the blebs.

Phase Venus .

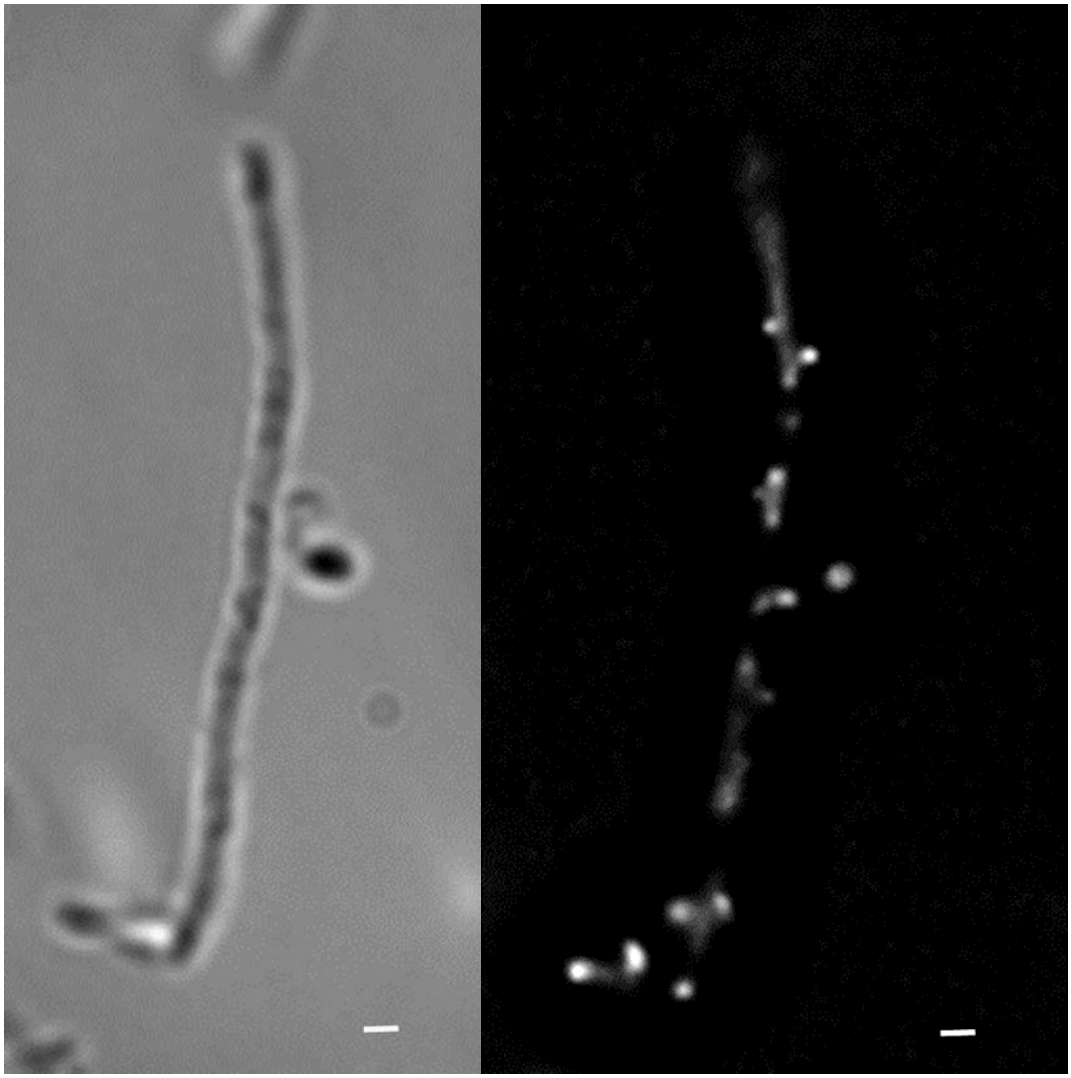


Figure.64. Images of BL21+NatB αS-VN αS-VC cells producing acetylated α-Synuclein-Venus Amino half and acetylated α-Synuclein-Venus Carboxyl half also have Venus fluorescence, showing both blebbing around cells and a ribbon like appearance within cells. This again indicates the presence of α-Synuclein oligomers both within the cells and within the blebs (Scale 1μm).

The whole cell protein samples taken pre and 3 hours post induction were examined on a Coomassie stained gel (Figure 65).

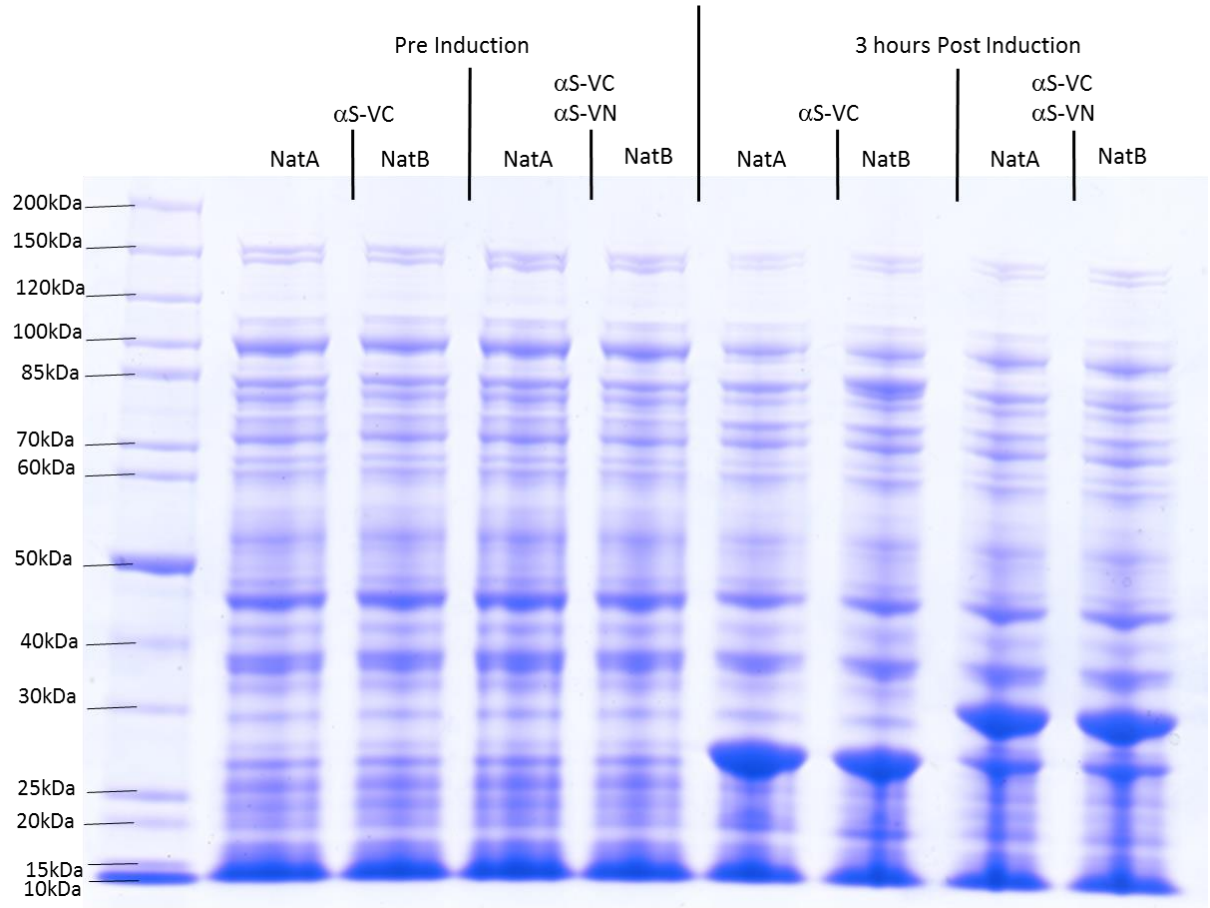


Figure.65. Coomassie stained 4-12% Bis-Tris gel examining BL21+NatAαS-VC, BL21+NatBαS-VC, BL21+NatAαS-VN αS-VC and BL21+NatBαS-VN αS-VC whole cell proteins, Pre and 3 Hours Post induction. Post induction the αS-VC samples show the production of α-Synuclein-Venus-Carboxyl half (24.1kDa) and the αS-VN αS-VC samples show the production of both α-Synuclein-Venus-Carboxyl half and α-Synuclein-Venus-Amino half (32.1kDa).

To investigate the release of protein into the culture medium, precipitated protein samples from Cell cleared culture media, were taken the same as previously, at 24, 48, 120 and 144 hours post induction, from BL21+NatAαS-VN αS-VC and BL21Nat+BαS-VN αS-VC cultures. The protein samples were examined by Coomassie stained gel and Western Blot analysis, (Figure 66). Both show an increasing amount of protein being released into the supernatant, with a higher level being released from the BL21+NatAαS-VN αS-VC cells, possibly including oligomers, at 144 hours.

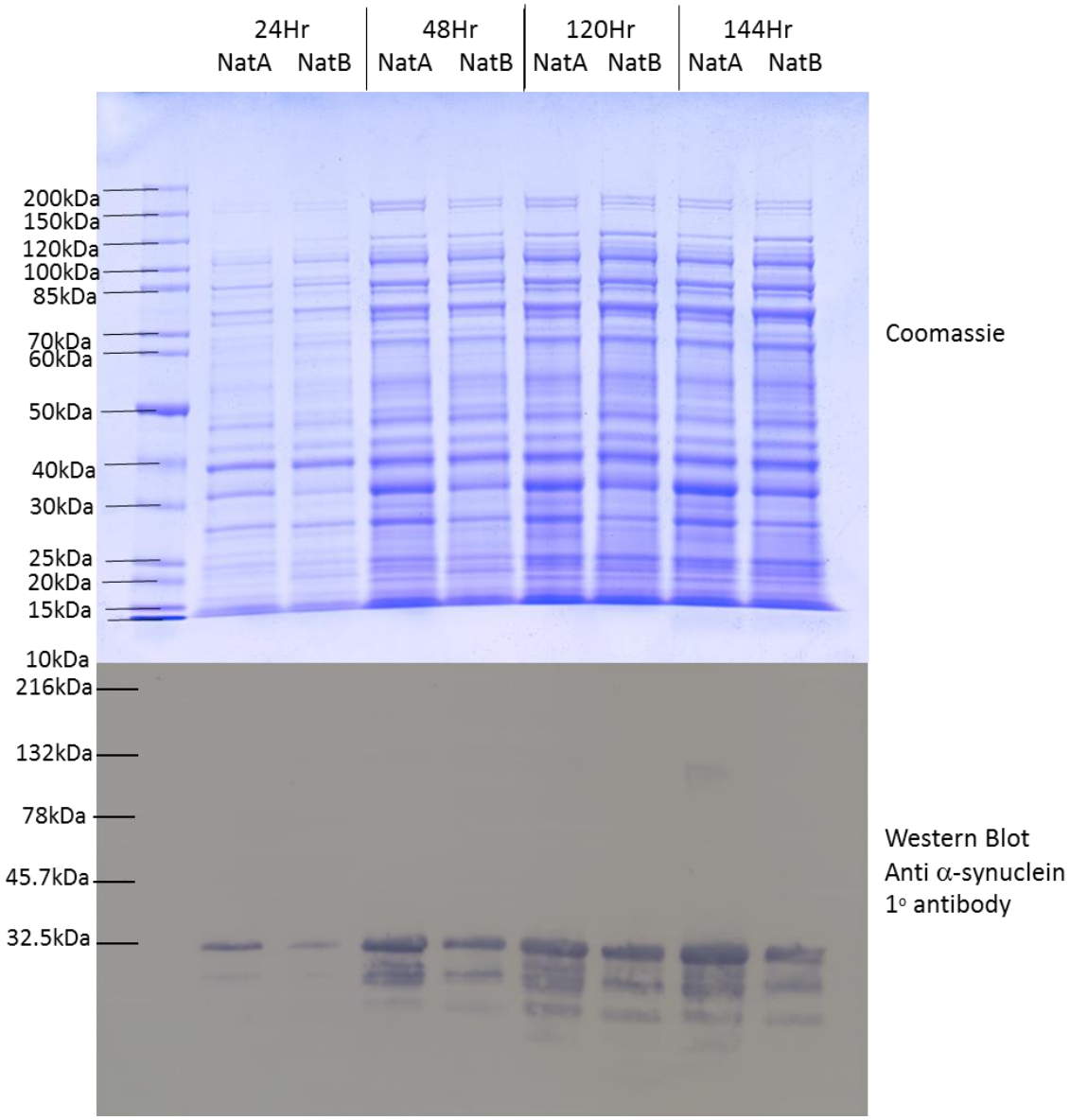


Figure.66. Coomassie stained 4-12% Bis-Tris gel and Western Blot, examining protein precipitated from, Cell cleared culture media from BL21+NatAαS-VN αS-VC and BL21+NatBαS-VN αS-VC, cultures at 24, 48, 120 and 144 hours post induction.

As a further control and for use in obtaining fluorescent growth curves a second single protein vector was produced, α-Synuclein-Venus-Amino half (αS-VN). Two cultures of BL21DE3αS-VC and BL21DE3αS-VN were grown until mid-log phase and induced with IPTG. Whole cell protein samples were taken pre induction and 2 hours post induction. These samples were examined using a Coomassie stained gel to confirm that the two required proteins were produced separately as required (Figure.67).

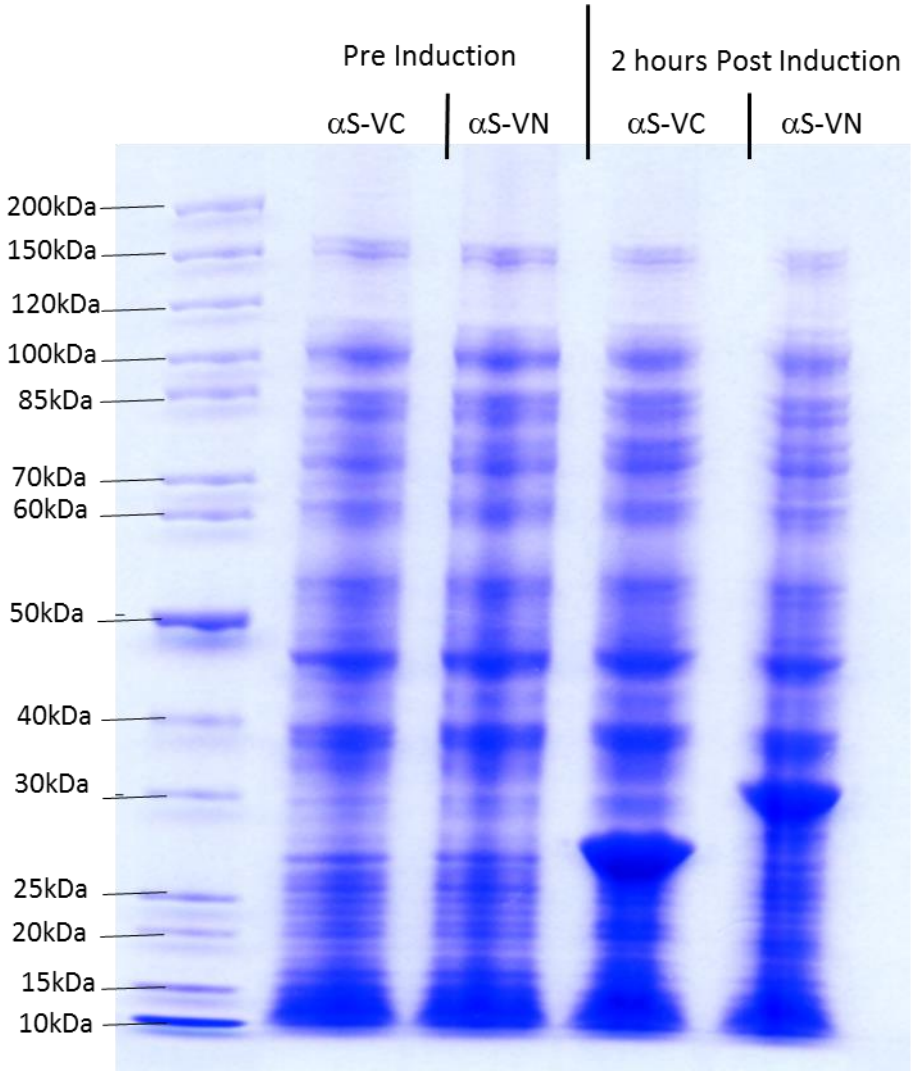


Figure.67. Coomassie stained 4-12% Bis-Tris gel showing separate production of α-Synuclein-Venus-Amino half and α-Synuclein-Venus-Carboxyl half in BL21DE3 cells using vectors αS-VN and αS-VC.

A culture of BL21DE3αS-VN was grown for 2.25 Hours then mounted on agarose and images taken. The BL21DE3αS-VN cells were imaged as a control to confirm that, as with the imaged cells containing αS-VC, no fluorescence could be seen and only phase images could be taken (Figure 68). This confirmed that any oligomers formed with only the Amino half of the Venus protein present, did not produce fluorescence unlike those containing the αS-VN αS-VC which produced clear fluorescence (Figures 63 & 64).

Phase Venus

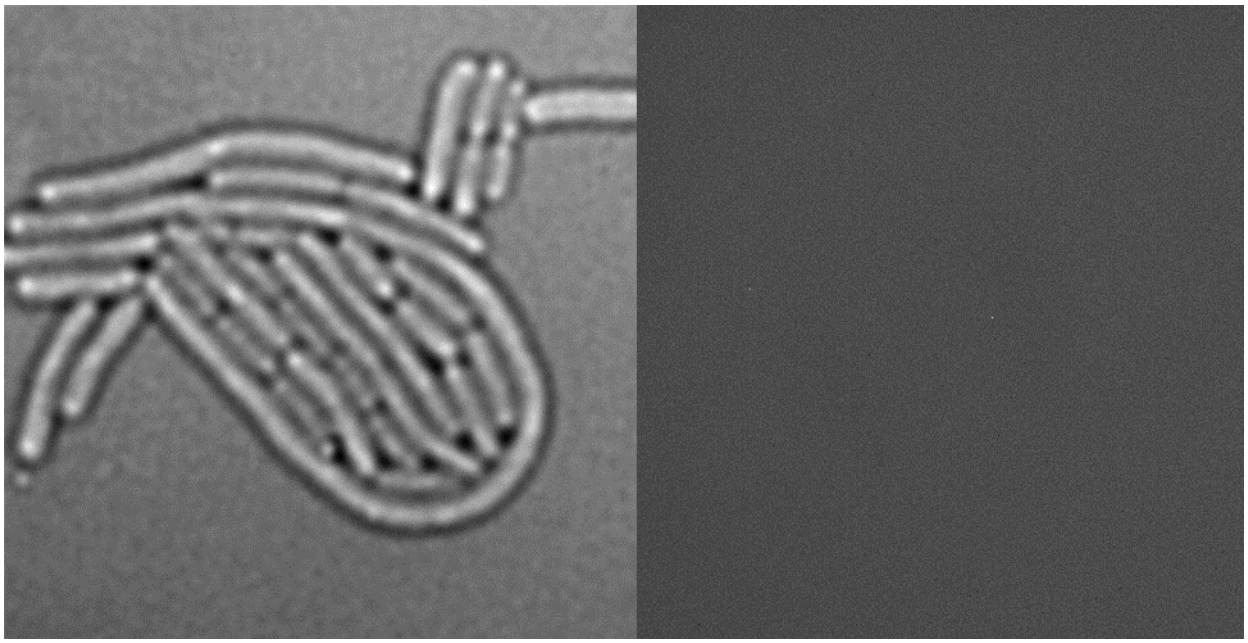


Figure.68. Images of BL21DE3 cells containing αS-VN observed by Phase microscopy (left). No Venus signal was visible and so no images possible (right), showing that the Amino half of the Venus Protein does not produce fluorescence by itself.

A fluorescent growth curve was obtained for BL21DE3 and BL21+NatB cells containing αS-VC, αS-VN αS-VC and αS-VN vectors. Each of the six cultures was grown in LB broth with appropriate antibiotics added, until they reached mid log phase. Each was then diluted according to their OD_{600} to an OD_{600} of 0.1, and 0.1µl per ml IPTG (100mg/ml stock) added. 1ml of each culture was then added to each of 4 wells on a 24 well plate which was then placed in an automatic plate reader at 37°C with shaking, and fluorescence readings for Venus were taken every 15 minutes (Figure.69).

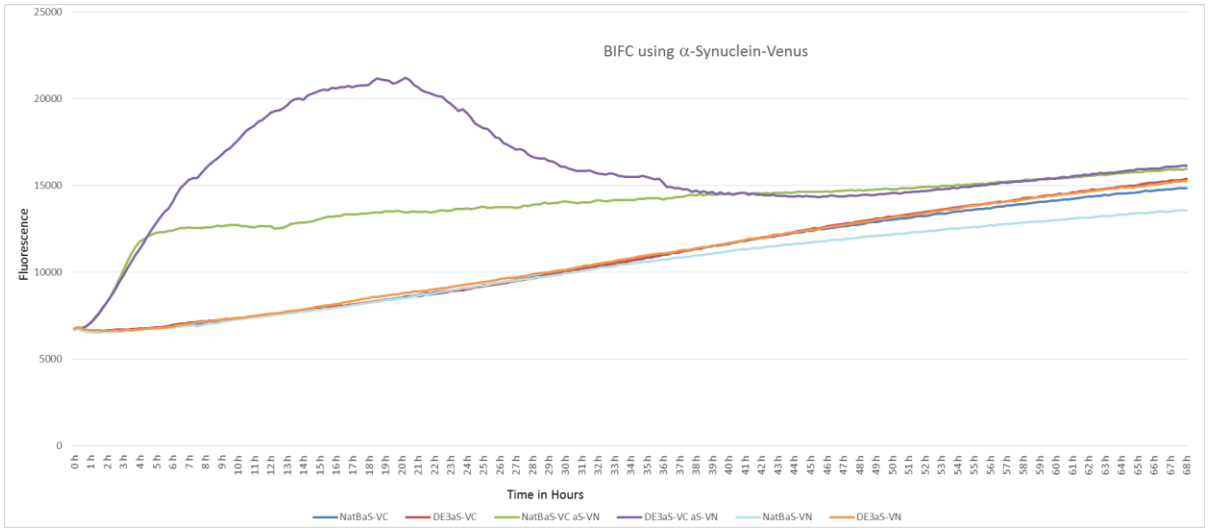


Figure.69. Venus fluorescent growth curve for BL21DE3 and BL21+NatB cells containing αS-VC, αS-VN αS-VC and αS-VN vectors. A gradual increase in fluorescence was seen with the cells containing αS-VC, and αS-VN vectors alone. A rapid initial increase in fluorescence was seen with both cultures containing αS-VN αS-VC. This rate reduced after 3hours with BL21NatBαS-VN αS-VC cells and a decrease in fluorescence was seen with BL21DE3αS-VN αS-VC cells after 18 hours.

Only a small very gradual increase in fluorescence was seen with the cells containing αS-VC, and αS-VN vectors alone. A rapid increase in fluorescence was seen with both cultures containing αS-VN αS-VC. However, at 3 hours this rate of increase in fluorescence decreased sharply, with the BL21NatBαS-VN αS-VC cells, which then only showed a very gradual increase in fluorescence, which was slightly less than that of the cells containing αS-VC, and αS-VN vectors. This was in contrast to the BL21DE3αS-VN αS-VC cells which continued to show a strong increase in fluorescence until approximately 18 hours, at which point the level of fluorescence began to fall until it reached the same level as the BL21+NatBαS-VN αS-VC cells.

4.13: Purification of α-Synuclein-Cerulean3 from cell cleared culture media using dialysis, centrifugation and FPLC

Due to the levels of α-Synuclein in the cell cleared culture media as seen by Coomassie stained gels and Western blots, it was decided to try to purify α-Synuclein-Cerulean3 from 200ml cultures grown in LB with appropriate antibiotics. If successful this could provide a gentle method for purifying larger amounts of acetylated α-Synuclein including oligomers for further *in vitro* investigation. BL21+NatB αS-Cerulean3 cells were grown for 4 days and then centrifuged to obtain cell cleared culture media and then prepared for purification and run on FPLC as in the 'Materials and Methods'. Fractions J1 to J5 (Figure.70) had a bright green colour and were also seen to have the largest amount of protein when fractions were examined by Coomassie stained gel (Figure.71). Fractions J2 and J4 were selected for further examination by Western Blot using anti α-Synuclein as the primary antibody (Figure 72). Monomers were identified by the Western Blot, showing that α-Synuclein-Cerulean3 was successfully purified, however as no dimers or tetramers were seen this purification method may be breaking these up. Further investigations will include looking for gentler methods of purification, which preserve dimers and tetramers.

Unfortunately time restraints meant that mass spectrometry was not performed on this sample. However acetylated α-Synuclein has previously been produced successfully using this protocol, (Maltsev A.S. et al. 2012, Dikiy I. et al. 2014, Trexler A.J. et al 2012).

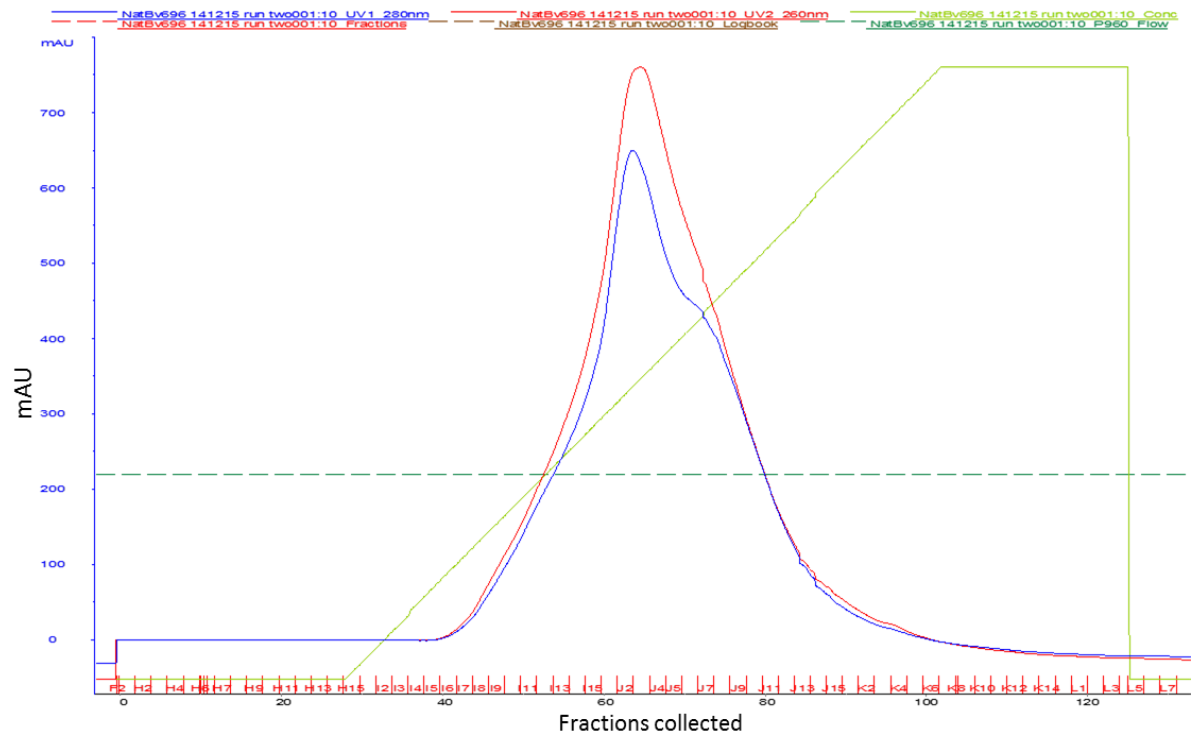


Figure.70. FPLC trace showing protein containing fractions collected, determined by absorbance at 260 and 280nm, from purification of BL21+NatB αS-Cerulean3 Cell Cleared Culture Media.

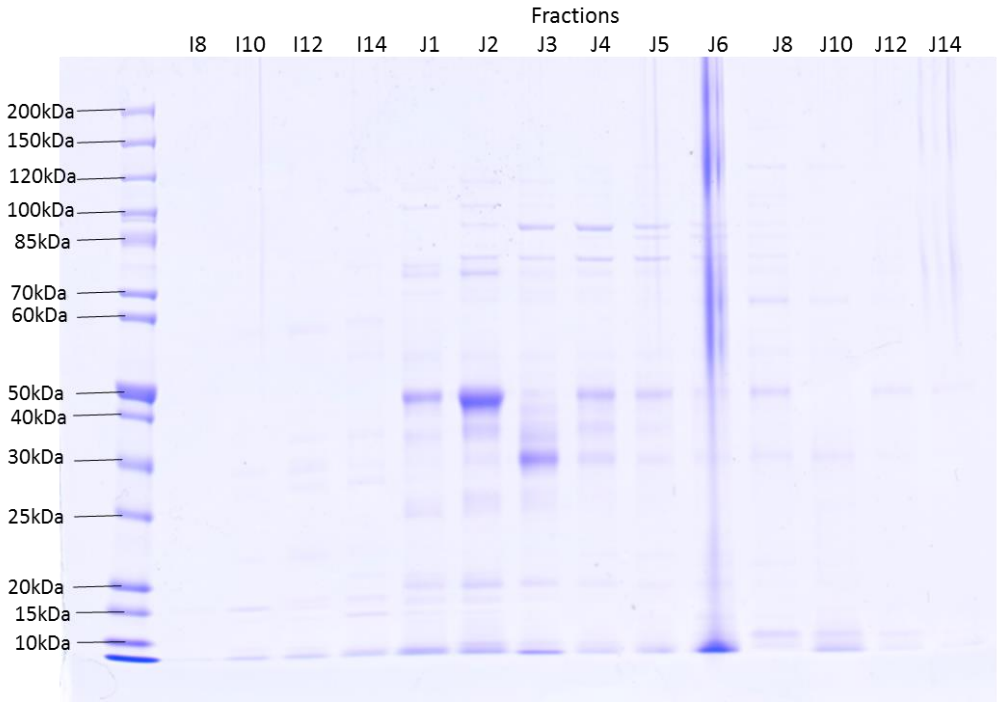


Figure.71. Coomassie stained 4-12% Bis-Tris gel showing protein content of fractions from purification of BL21+NatB αS-Cerulean3 from cell cleared culture media.

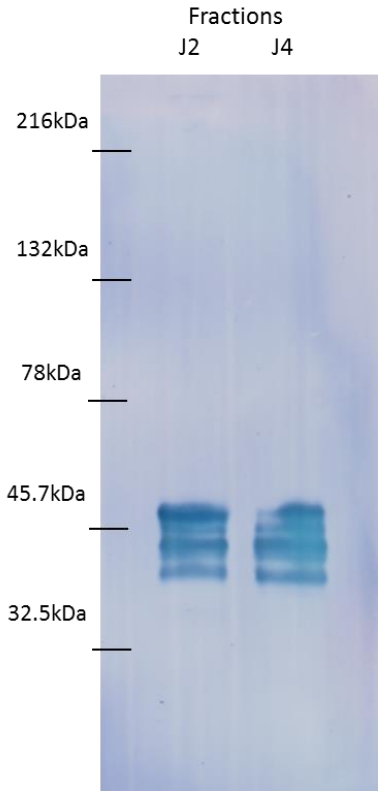


Figure.72. Western Blot using anti α-Synuclein antibody on fractions J2 and J4 (Figure 68) from the purification of BL21+NatB αS-Cerulean3 from cell cleared culture media.

5. CHAPTER V: DISCUSSION

5.1 α-Synuclein in *S.pombe* cells

α-Synuclein was successfully visualised inside *S.pombe* cells using αS-GFP, αs-Cerulean3 and integrated αS-Cerulean3. The images obtained using αS-Cerulean3 were clearer than those obtained using αS-GFP but both show similar distribution of α-Synuclein within the cell. At the levels produced, by the pREP41 vector used during this project, α-Synuclein appears to remain cytoplasmic within *S.pombe* cells, but is excluded from vacuoles. Although some possible aggregates were seen these were few and small (Figures 8, 9, 14 & 19) and very similar to some of the cells expressing just GFP (figures 6 & 7) Localisation was seen in many cell at the separation membrane (Figures 17, 18 & 19) and occasionally at the poles (Figure 20). However as in earlier work in *S.pombe* (Brandis K.A. *et al* 2006, Fiske M. *et al* 2011), no cell membrane binding was seen, as in the many investigations using *S. cerevisiae*. α-Synuclein-GFP fusions have been used in several studies in yeasts previously to show α-Synuclein localisation and aggregation (Brandis K.A. et al 2006, Fiske M. et al 2011, Franssens V. et al 2013, Winderickx J. et al 2008), However the rapid aggregation of the Alzheimer's peptide Aβ when fused to GFP has been shown to cause misfolding of GFP and a resultant lack of fluorescence. Future studies could investigate whether different forms of α-Synuclein oligomerisation effect the levels of fluorescence seen and fluorescent protein folding.

Average cell lengths for wild type cells containing GFP or αS-GFP grown either with or without thiamine were very similar. However in cells containing αS-GFP grown with thiamine, so that only very low levels of α-Synuclein were produced, a few unusually long cells were seen.

The three Western Blots performed on protein samples from *S.pombe* cells all indicated the presence of dimers. In wild type cells where the α-Synuclein produced is acetylated, dimers were found in the pellet sample, indicating that some of the dimers were membrane bound. But only a much smaller dimer band was seen in the pellet protein sample in comparison to that found in the cell supernatant in protein samples obtained from the naa25Δ *S.pombe* strain, where the α-Synuclein produced

remains unacetylated at its amino terminal methionine. This suggests that acetylation may stabilise dimers and membrane binding in *S.pombe*.

The OD_{600} growth curves show that for both wild type and naa25Δ strains of *S.pombe* containing αS-GFP grown without thiamine there is a significantly reduced maximum rate of growth. Due to the very slow growth rate of the naa25Δ strain compared to that of the wild type, before any plasmids are introduced it is not possible to make a direct comparison of the two growth curves.

When αS-Cerulean3 was Integrated into the genome of wild type *S.pombe* it produced a consistently very low level of recombinant protein expression that was barely visible by fluorescent microscopy. However a very extreme phenotype was observed by Phase microscopy, to see more clearly how the cell was being affected FM4-64 stain was used. This showed the phenotype more clearly especially the presence of multiple septa, where the cells appeared to have difficulties completing cell division (Figures. 20 to 23) (Table.5). Also clear, large vacuoles were seen after 1 day of incubation, but were only visible in a few cells after 2 days. This raises the question as to what happened to these structures between 1 and 2 days incubation and why?

5.2 α-Synuclein within *E.coli* cells

α-Synuclein was successfully visualised inside *E.coli* using both fluorescent microscopy, TEM and Immuno EM. The images obtained showed unexpected blebbing and structures within the cell, which indicated interactions between α-Synuclein and the plasma membrane. This is discussed in more detail in section 5.3.

Coomassie stained gels and Western blots again indicated the presence of monomers and dimers (Figures. 48, 49 & 60) and also indicate the presence of tetramers (Figure 33). This is discussed further in section 5.4

An OD_{600} growth curve (Figure 32) obtained from BL21+NatA and BL21+NatB cells, both uninduced and induced to produce acetylated or unacetylated α-Synuclein or empty vector, showed that only the presence of acetylated α-Synuclein caused a significant reduction in the maximum rate of growth as measured by OD_{600}. This was the same for both the induced and uninduced cells, suggesting that only a very low concentration of α-Synuclein needed to be present to cause this effect.

5.3 α-Synuclein membrane interactions within *E.coli* cells

The Blebbing and Ribbon like structures seen in the early fluorescent microscopy of cells producing α-Synuclein, indicated that α-Synuclein may be interacting with the plasma membrane, within *E.coli*. This was backed up by the TEM and immuno EM images, where blebs can be seen clearly coming out from the plasma membrane and the ribbon like structures were seen as tubular structures on the inside of the plasma membrane. This localisation was confirmed by the immuno EM images.

The timing of the appearance of blebs followed by ribbons seemed to be inconsistent. Blebs were only observed within the first few hours post induction, mostly at 2 to 2.5 hour post induction, though reduced numbers were seen before and after this time. Blebbing is seen most often in BL21DE3 and BL21+NatA cell suggesting that blebbing is produced less or over a shorter time span, in the presence of acetylated rather than unacetylated α-Synuclein. As blebbing is only seen relatively soon after induction it may be assumed that it occurs at relatively low α-Synuclein concentrations. At very low concentrations the amount of plasmid taken up by the cells may be having a significant effect on the rate that α-Synuclein is produced and therefore account for these time differences. The age of the culture may also effect the rate at which α-Synuclein is produced, as older cultures may be able to loose plasmid as is indicated by comparing the Immuno EM at 3 and 24 hours.

α-Synuclein membrane interactions within *E.coli* cells were also investigated using BL21DE3 and BL21+NatB cells containing αS-Cerulean3, αS-Cerulean3 cydAB-mCherry and cydAB-mCherry. The initial objective was to see if the higher levels of blebbing seen in BL21DE3 cells producing α-Synuclein, also resulted in an increase in membrane formation, indicated by an increase in mCherry fluorescence. From 1 hour to 3.5 hours post induction the level of mCherry fluorescence within the BL21DE3 αS-Cerulean3 cydAB-mCherry culture increase at a significantly higher rate compared to all the other cultures. These time points also correspond to the times at which images of blebbing were taken. This rapid increase was not seen in BL21DE3 cydAB-mCherry, only when α-Synuclein was also present in the cells.

5.4 α-Synuclein oligomer formation within *E.coli* cells

Using BiFC of split Venus fluorescent protein fused to α-Synuclein to investigate oligomer formation proved very useful. The images obtained with the control vectors confirmed that no fluorescence could be obtained by inducing production of either half of the Venus protein alone. Although this construct was not expressed with a non-interacting protein as a control it has been shown that this Venus protein has a reduced level of self assembly and so a decreased background fluorescence giving an increased signal-to noise ration compared to other fluorescent proteins used in BiFC assays, (Yutaka K. *et al*. 2010) The images obtained for BL21+NatB αS-VN αS-VC and BL21+NatB αS-VN αS-VC cells showed very clear fluorescence within the cells and clear blebbing around them (Figures 63 & 64). As the two halves of the Venus protein only produce fluorescence when in very close proximity to each other, this result indicates that oligomers are being formed.

The fluorescent growth curve obtained using the same six cultures as were imaged, also confirmed a rapid increase in fluorescence for both for BL21DE3 and BL21+NatB cells with plasmids containing αS-VN αS-VC and only a minimal increase in cells where only one half of the Venus protein was present. After 3.5 hours induction there was a change in the rate of increase in fluorescence in the BL21+NatBαS-VN αS-VC cells, as the rate rapidly decreased to almost no change (Figure.69). The BL21DE3αS-VN αS-VC cells in contrast continued to show an increase in fluorescence until approximately 18 hours post induction, achieving an increase in fluorescence of more than double that of the BL21+NatBαS-VN αS-VC cells, before falling again to return to the same level as the BL21+NatBαS-VN αS-VC cells by 39 hours post induction. This indicates that unacetylated α-Synuclein forms more or more stable oligomers than acetylated α-Synuclein.

In the western blot performed on the acetylated α-Synuclein after purification by FPLC only monomer α-Synuclein could be seen, whereas all the other Western Blots of protein from *E.coli* producing α-Synuclein had indicated the presence of dimers and possible tetramers. This suggests that oligomers are broken down during this purification method, future work will investigate alternative methods of protein purification.

5.5 Combined analysis of results

It has become clear from the results obtained in both *S.pombe* and in *E.coli* that only very low concentrations of α-Synuclein need to be present within cells to have a significant effect on the phenotype and behaviour of the cell. From the images taken and the growth curves obtained it appears that the concentration of α-Synuclein at which it causes these effects is exceeded after 3.5 hours post induction, when expressed in *E.coli* with the pDUET vector. The concentration of α-Synuclein that causes extreme phenotype, only appears to be reached in *S.pombe* when a single copy of the pINT vector was integrated into the *S.pombe* genome. When the pREP41 vector was used in *S.pombe*, with unknown levels of uptake into the cell, higher levels of fluorescence were seen and the extreme phenotype was not seen.

Variation in plasmid copy number may also provide one explanation for the differences in the timing of the appearance of blebs, followed by ribbons in *E.coli*. The age of the culture may have a similar effect, as older cultures may be able to lose plasmid as is indicated by comparing the Immuno EM images at 3 and 24 hours. As blebbing is only seen relatively soon after induction it may be assumed that it occurs at relatively low α-Synuclein concentrations. At very low concentrations the amount of plasmid in the cells may have a significant effect on the rate that α-Synuclein is produced and therefore account for these time differences.

The effect of unacetylated α-Synuclein in *E.coli* at 3.5 hours post induction in BL21DE3 αS-Cerulean3 cydAB-mCherry and BL21DE3αS-VN αS-VC cells appears to be related, as the same vector and therefore the same promoter is used in each culture, it may be assumed that the concentrations of α-Synuclein would be similar in each. Beyond this time point, blebs were no longer seen by fluorescent microscopy and only a few smaller blebs were seen by TEM. It is at this time point that the rapid increase in mCherry fluorescence and therefore membrane formation which is possibly related to the blebbing decreases, it is also at this same time point that the rapid increase in Venus fluorescence and possibly related oligomer formation decreases.

The effect of acetylated α-Synuclein in *E.coli* appears to be very different. In BL21+NatB αS-Cerulean3 cydAB-mCherry the increase in mCherry fluorescence was

not seen (Figure.57). This is consistent with the fluorescent images, as blebbing was not seen as often, it seems to occur within a smaller time frame, and the blebs tend to be smaller, this is seen most clearly in the TEM images. The Venus Fluorescent growth curve was also very different between the cells producing acetylated and unacetylated α-Synuclein. Whereas the cells containing unacetylated only show a rapid increase in fluorescence for the first 3.5 hours post induction the cells producing acetylated α-Synuclein show a far more prolonged increase in fluorescence, up to 18 hours post induction (Figure.69). This indicates the formation of more or more stable oligomers. This is also consistent with the protein analysis. Clear indication of the presence of tetramers was only seen in whole cell protein samples examined by Coomassie stained gel and Western Blot (Figure.33) from BL21NatB αS-Cerulean3 cells.

In the OD_{600} growth charts obtained for *E.coli* and *S.pombe,* unexpectedly high final OD_{600} values were obtained for *S.pombe,* producing low levels of unacetylated α-Synuclein (Figure.13) and for *E.coli* producing unacetylated α-Synuclein (Figure 32). These results may be an inaccurate representation of cell growth and possible toxicity, due to the amount and size of blebbing seen with BL21DE3 αS-Cerulean3 and having observed the unusual phenotypes produced in wild type *S.pombe* with the expression of very low levels of α-Synuclein. Future investigations into the toxicity of α-Synuclein within *E.coli* and *S.pombe* may be more accurate if determined by the use of cell counts rather than by measuring OD_{600}.

5.6 Impact of this work

In this project it has been shown that:

A. α-Synuclein affects membrane dynamics.
B. α-Synuclein alone can drive extracellular vesicle formation.
C. These actions are regulated by α-Synuclein Nt-acetylation.

Previous *in vivo* studies of α-Synuclein have used a variety of cell lines and model organisms, including mice, *Drosophila melanogaster, Caenorhabditis elegans* and *Saccharomyces cerevisiae* (Franssens V. *et al* 2013). Only two previous studies using *S.pombe* have been found and none that have used *E.coli* as a model organism, though many have used *E.coli* for the expression of recombinant protein. Both *S.pombe* and *E.coli* have shown themselves to be useful model organisms for the *in vivo* study of α-Synuclein. *E.coli* has proven especially useful in comparing the effects of acetylated and unacetylated α-Synuclein and this work can hopefully be further expanded in *S.pombe*.

Much of the previous work in *S. cerevisiae* has focussed on membrane binding as a precursor to aggregate formation. In *S.pombe* no cell membrane and in E.coli no plasma membrane binding was seen, however in *S.pombe* with pREP41αS-Cerulean3 localisation was seen at the separation membrane and at the poles. This indicates that the membrane binding of α-Synuclein is specific and by comparing different model organisms it may be possible to learn more about the differences in membrane structure and how these effect the binding and aggregate formation of α-Synuclein.

The concentration of α-Synuclein within both *S.pombe* and *E.coli* was found to significantly affect the phenotype and behaviour of the cells. Previous studies in *S.pombe* have looked at the effect of concentration of α-Synuclein, however one used a NMT1 high level expression vector, controlling expression with varying concentrations of thiamine (Fiske M. *et al* 2011). The other used different expression vectors to vary concentration, all without thiamine (Brandis K.A. *et al* 2006). In this project the extreme phenotype changes were only seen at very low levels of

expression, using an integrating vector, where the level of Cerulean3 fluorescence was only just visible. The images of Cerulean3 (Figures 20 to 23) required the brightness and contrast to be increased before inclusion in this thesis. It is possible that the level of α-Synuclein in the previous studies quickly exceeded the concentration at which these changes were seen.

In *in vivo* studies, wild type α-Synuclein is often compared to mutant forms of the protein. If the levels of protein expression are at a higher level than physiological, or even at a moderately high level within a model organism, this may in effect be the comparison of another disease state that does not represent normal α-Synuclein action. Duplications and triplications of the SNCA gene are associated with early onset Parkinson's disease, so it may be assumed that the concentration of α-Synuclein present within cells is of great importance.

5.7 Future work

This project has raised many questions that could be investigated in future work.

Firstly, why are different areas of localisation seen, why does α-Synuclein localise inside the plasma membrane of *E.coli,* as seen by Immuno EM and bind the cell membrane in *S. cerevisiae,* but not in *S.pombe*, what is different between the membranes and why is localisation seen at the separation membrane and poles in *S.pombe.*

The effect of very low concentrations of α-Synuclein appeared to be important. Further work investigating this could use different vectors to produce lower concentrations of α-Synuclein in *S.pombe* and *E.coli*. This could be used to investigate how a very low concentration of α-Synuclein over a longer period of time affects the cells. Would this extend the time period over which blebbing occurs in *E.coli* or the phenotype obtained in *S.pombe*.

Time lapse fluorescent microscopy may help gain a greater understanding of the timings of these effects. This could also be useful in co-localisation studies in *S.pombe* where cells produced unusual phenotypes including multiple septa.

Tetramer formation is another area for future investigation. Purification of α-Synuclein using FPLC appeared to destroy dimers and tetramers. Other methods of purification including purification using a His tag could be investigated to try to produce purified α-Synuclein containing tetramers. It may also be useful to find out why dimers and tetramers were lost during purification.

Finally it would be useful to compare the toxicity of α-Synuclein in *E.coli* BL21DE3 & BL21+NatB by cell counts to see if the OD_{600} growth curves obtained were a true representation of growth or a measurement of blebbing.

6. CHAPTER VI: REFERENCES

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