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Flow cytometric analysis of *E. coli* on agar plates: implications for recombinant protein production

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ABSTRACT

Recombinant protein production in bacterial hosts is a commercially important process in the pharmaceutical industry. Optimisation of such processes is of critical importance for process productivity and reproducibility. In this study, flow cytometry methods were developed to assess characteristics of bacteria during two process steps that are infrequently studied; agar plate culture and liquid culture setup. During storage on agar plates, three discrete populations of varying green fluorescence intensity were observed along with a progressive shift of cells from the high green fluorescence population to an intermediate green fluorescence population, observed to be due formation of amyloid inclusion bodies. The dynamics of cellular fluorescence and scatter properties upon setup of liquid cultures were also assessed. These methods have the potential to improve the development of fermentation set-up, a currently little-understood area.

Keywords

Congo Red; Flow cytometry; Green fluorescent protein; Inclusion bodies; Recombinant protein production.

INTRODUCTION

Recombinant protein production (RPP) in bacterial hosts such as *Escherichia coli* is a mainstay of the worldwide biopharmaceutical bioprocessing industry (Huang et al. 2012, Overton 2013). Bacteria are ideal hosts for the production of relatively simple proteins that do not require extensive post-translational modification; bacterial RPP processes are frequently cheaper, faster and simpler than mammalian cell culture processes and can give rise to large quantities of desired recombinant proteins. Typically, the recombinant gene of interest encoding the desired protein product is carried by the bacteria on a plasmid vector, under the control of a regulated promoter (Terpe 2006).

Analysis of bacterial growth, productivity and aspects of bacterial physiology are essential for understanding and optimising RPP processes at any scale, from initial laboratory screening studies on the millilitre scale to industrial production at the thousands of litre scale (Sevastsyanovich et al. 2010). Typical measurements include biomass (by optical density, dry cell weight or colony forming units), culture pH and dissolved oxygen tension, off-gas composition (oxygen and carbon dioxide) and the quantity of recombinant protein (usually by SDS-polyacrylamide gel electrophoresis analysis of cell pellets). Many of these techniques are time-consuming and are therefore unable to generate data that can be used to direct an RPP process in real time; there is therefore a requirement for the development of more rapid process analytical techniques. One such technique is the quantification of recombinant protein productivity using flow cytometry (FCM) to detect an autofluorescent protein such as green fluorescent protein (GFP) fused to the recombinant protein of interest (Rücker et al. 2001, Jones et al. 2004, Sevastsyanovich et al. 2009). This method is very rapid (data is generated within minutes) and allows monitoring of not only overall RP productivity but also the percentage of bacteria in a culture that are productive. It is also possible to analyse bacteria with a viable but non-culturable (VBNC) phenotype, which will not grow on agar plates even though they may be metabolically active and able to grow, divide and may generate recombinant protein in the RPP culture (Davey 2011). VBNC bacteria are commonly encountered in RPP cultures (Nebe-von-Caron et al. 2000, Sundström et al. 2004), so any RPP process analytical tool must take these bacteria into account.

Since RPP is an industrially-used process, reproducibility is a key factor in the development of effective RPP processes. Setting up liquid cultures for RPP is crucial to this process, whereby bacteria from an agar plate or cell bank are mixed with a volume of liquid growth medium and incubated; the resultant culture is then used to inoculate progressively larger volumes of medium. Physiological variability in the cells used to set up cultures will therefore introduce variability into RPP processes. There are remarkably few studies in this area, especially with respect to single-cell analysis, so research

was undertaken to investigate the physiological state of bacteria used to generate a plasmid-encoded recombinant protein-*gfp* fusion on agar plates during storage, and on transfer from agar plates to liquid culture. We reveal that formation of insoluble RP inclusion bodies inside cells stored on agar plates changes over time and depends upon storage conditions; these factors can influence recombinant protein production in liquid cultures.

MATERIALS AND METHODS

Bacterial strains, plasmids and microbiological methods

Escherichia coli strain BL21* (DE3) (F^- *ompT hsdS*$_B$ ($r_B^- m_B^-$) *gal dcm rne131* (DE3)) was used throughout (Invitrogen, Paisley, UK). The recombinant CheY-GFP fusion protein was encoded by the pET20bhc-CheY::GFP plasmid (Sevastsyanovich 2009, Jones 2007), comprising the *E. coli cheY* gene fused to *gfp* cloned into pET20bhc (Jones et al. 2004, Waldo et al. 1999). Bacteria were transformed with the plasmid using the heat-shock method and transformants selected on nutrient agar (Oxoid) plates supplemented with 100 µg carbenicillin mL^{-1} (Melford, Ipswich, UK; a more stable variant of ampicillin). Following initial growth for 48 hours at 25 °C, transformants were patch-replated to fresh NA-carbenicillin plates, grown for 48 hours at 25 °C and sealed with Nescofilm (Fisher Scientific, Loughborough, UK) before being stored at 4 °C (the 'storage agar plates') and sampled for 16 weeks.

Bacterial growth

Overnight cultures were set up from a single sweep of cells from the storage agar plate into 35 mL of Lennox broth (LB; 5% (w/v) BD Bacto yeast extract, 10% (w/v) BD tryptone, 5% (w/v) NaCl) supplemented with 100 µg carbenicillin mL^{-1} and grown in 250 ml conical flasks at 30 °C with agitation. Recombinant protein production (RPP) cultures were set up in 500 mL conical flasks containing 100 mL of LB supplemented with 0.5 % (w/v) glucose and 100 µg carbenicillin mL^{-1}. Each RPP culture was inoculated with 2 mL of an overnight culture (grown for 14 hours or until the OD_{650} had reached 5, whichever was longer) and incubated with shaking at 25 °C. At an OD_{650} of around 0.5, isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 8 µM to induce production of the CheY-GFP fusion.

Flow cytometry

Bacteria were analysed using a BD Accuri C6 flow cytometer (BD, Oxford, UK). Samples were excited using a 488 nm solid state laser and fluorescence was detected using 533/30 BP and 670 LP filters corresponding to GFP and propidium iodide (PI) or Congo Red (CR) fluorescence respectively. Bacterial samples were taken directly from agar plates, resuspended in phosphate buffered saline (PBS) (Oxoid) and analysed by FCM. Samples were stained with PI to

88 determine viability. A 200 µg PI mL^{-1} stock solution was made up in distilled water and added to samples at a final
89 concentration of 4 µg PI mL^{-1}. Samples were also stained with Congo Red to determine amyloid content and presence of
90 inclusion bodies. A 2 mg Congo Red mL^{-1} stock solution in dimethylsulphoxide (DMSO) was added to samples at a final
91 concentration of 40 µg CR mL^{-1} and incubated under ambient conditions for 30 minutes. Particulate noise was eliminated
92 using a FSC-H threshold. 20000 data points were collected at a maximum rate of 2500 events sec^{-1}. Data was analysed
93 using CFlow (BD). Cells were sorted by FACS using a FACSAria 2 (BD) with similar optical parameters as those used
94 on the C6. Purity sort mode was used, and bacteria were sorted into tubes containing FACSFlow (BD).
95
96 **SDS-PAGE**
97 Proteins were separated according to molecular weight using Tris/Glycine SDS-PAGE with a 15% (w/v) polyacrylamide
98 gel (Sambrook et al. 1987). Bacterial cell pellets were suspended in sample buffer containing β-mercaptoethanol and
99 heated at 100 °C for 10 minutes before being loaded onto the gel. Equal quantities of biomass were loaded into each lane.
100 SDS-PAGE gels were stained with Coomassie Blue and dried, then scanned (Canon Canoscan 9000F) and the density of
101 each protein band quantified using ImageJ (Schneider et al. 2012). Independently, soluble and insoluble bacterial protein
102 fractions were separated using BugBuster (Novagen). Bacterial cell pellets were suspended in a volume of BugBuster
103 equal to that of sample buffer, incubated at room temperature for 10 minutes then fractionated by centrifugation at 16 873
104 g for 20 min. The pelleted insoluble fraction was subsequently washed in PBS to remove any residual soluble protein.
105 Both fractions were then resuspended in a volume of sample buffer equal to the volume of BugBuster used and incubated
106 at 100 °C for 10 minutes. This protocol results in soluble fractions that are twice the volume and hence half the protein
107 concentration of the insoluble; to ensure gels were loaded with samples from an equivalent biomass twice the volume of
108 soluble fractions were loaded on the gel.
109
110 **RESULTS AND DISCUSSION**
111 **Monitoring of bacterial properties over time**
112 *E. coli* BL21*(DE3) was transformed with pETCheY-GFP encoding the *E. coli* chemotaxis protein CheY fused to green
113 fluorescent protein. This fusion protein is liable to form insoluble inclusion bodies during growth in liquid culture
114 (Sevastsyanovich et al. 2009) and is used here as a model 'difficult' recombinant protein. Even though during RPP
115 processes expression of the recombinant *cheY-gfp* is induced by addition of the lactose analogue IPTG (which activates
116 production of the T7 RNA polymerase from the chromosomal DE3 locus, itself activating expression of *cheY::gfp* on the
117 plasmid), pET vectors usually exhibit some expression of the encoded recombinant gene in the absence of inducer caused

118 by incomplete repression of T7 RNA polymerase expression. This allows detection of GFP fluorescence in uninduced
119 bacteria, including those on agar plates.
120
121 At regular intervals up to 16 weeks post-transformation, bacteria were taken from the storage agar plates and analysed by
122 flow cytometry (Figure 1). Figures 1a and 1c show data for cells immediately after transformation, represented as a green
123 fluorescence (FL1-A) histogram and a forward scatter (FSC-A) versus green fluorescence (FL1-A) plot respectively.
124 Figures 1b and 1d show similar data for bacteria after 12 weeks storage on agar plates. On the scatter-versus-fluorescence
125 plots, three distinct populations were visible: P1, highly fluorescent cells generating recombinant CheY-GFP; P2, cells of
126 intermediate green fluorescence; and P3, comprising plasmid-free cells (confirmed by FCM analysis of untransformed
127 BL21*(DE3) cells, data not shown). Representation of fluorescence data on forward scatter versus fluorescence plots
128 (Figs. 1c/d) allows clearer demarcation of populations than fluorescence histograms (Figs. 1a/b), which fail to take into
129 account differences in cell size.
130 *[Location of Figure 1]*
131 The proportion of bacteria on the storage agar plates in each of these three populations was measured over the course of
132 16 weeks storage at 4 °C (Fig. 2a). The proportion of high green fluorescence cells (P1) decreased over time with a
133 concurrent increase in the proportion of cells in population P2 (intermediate green fluorescence). The proportion in
134 plasmid-free population P3 was low throughout. The mean green fluorescence of bacteria in each population remained
135 relatively constant over the course of the 16 weeks (Fig. 2b). The mean forward scatter of bacteria in populations P1 and
136 P2 fluctuated over time (Fig. 2c). Population P1 significantly decreased in mean forward scatter towards the end of the
137 storage period. Population P2 initially increased in mean forward scatter, although this is probably an artefact caused by
138 the very small number of bacteria in this population at the start of the storage period. The mean forward scatter then
139 steadily decreased with storage time, suggesting a decrease in cell size.
140 *[Location of Figure 2]*
141 Population P2 (Fig. 1) was initially thought to comprise either cells which had recently lost the CheY-GFP-encoding
142 plasmid but still contained GFP, or cells containing misfolded CheY-GFP in the form of inclusion bodies. Two
143 approaches were used to investigate population P2. First, populations P1 and P2 were sorted using FACS. Population P1
144 grew far better on nutrient agar (between 6% and 29% of sorted cells being able to grow on plates) than population P2
145 (<0.25 % of sorted cells being able to grow).
146

147 Second, cells taken from agar plates were stained with Congo Red, an amyloid-specific dye that has previously been used
148 to detect bacterial inclusion bodies (Upadhyay et al. 2012). Population P2 was shown to stain positively for Congo Red,
149 while populations P1 and P3 did not (Fig 2d&e). In addition, Congo Red staining of P2 cells decreased their green
150 fluorescence, suggesting FRET between GFP and Congo Red thus close physical association consistent with binding. The
151 percentage of cells staining with Congo Red closely followed the percentage of cells in population P2 over storage. Taken
152 together, these data reveal that population P2 comprises cells that have extremely poor culturability, probably brought
153 about by accumulation of misfolded CheY-GFP in the form of amyloid IBs. Inclusion bodies containing fluorescent
154 proteins have previously been shown to retain some fluorescence (Garcia-Fruitos et al. 2005), hence the intermediate
155 fluorescence of P2. This observation is also consistent with the mean FSC of population P1 being higher than that of P2
156 (Fig. 2c), as bacteria containing CheY-GFP inclusion bodies often display lower FSC (data not shown). Finally,
157 Propidium Iodide (PI) staining revealed relatively low proportions of dead cells in populations P1 and P3 (Fig. 2f).
158 Population P2 was initially composed of mainly dead, PI^+ cells, although the proportion of dead cells decreased over
159 time. The absolute number of PI^+ cells in population P2 increased up to 3 weeks storage, then decreased (data not shown).
160
161 Analysis of cells taken from agar plates after 0, 4 and 12 weeks storage at 4 °C using BugBuster (a detergent used to
162 separate soluble and insoluble protein fractions) did not show any difference in the proportion of CheY-GFP present in
163 the insoluble fraction, despite differences in relative sizes of populations P1 and P2. This reveals that some insoluble
164 CheY-GFP aggregates or IBs were present at all stages of storage on plates. Two broad classes of bacteria inclusion
165 bodies have been identified: classical IBs, which are dense, amyloid in nature and mainly contain inactive protein; and
166 non-classical IBs, which are less dense, less amyloid in character and contain a far higher proportion of active proteins
167 (Upadhyay et al. 2012). Some reports suggest that IBs can exist at some point on a continuum between classical and non-
168 classical IBs (Martínez-Alonso et al. 2009). We propose that bacteria contained some insoluble CheY-GFP at all points
169 throughout storage as determined by BugBuster fractionation, which has previously been shown to be only mildly
170 solubilising (Listwan et al. 2010) and would therefore probably assign even slightly insoluble CheY-GFP to the
171 'insoluble' fraction. However, the form of this insoluble protein fraction changed with extended storage at 4 °C. Initially,
172 CheY-GFP was present in most bacteria as either insoluble aggregates or non-classical IBs, containing a large proportion
173 of functional GFP thus having higher green fluorescence (population P1). Over time, more classical IBs formed
174 stochastically in some bacteria (population P2); these IBs have a lower green fluorescence (in accordance with their
175 higher degree of misfolding; Upadhyay et al. 2012) and are highly amyloid in nature (so bind Congo Red). This process is
176 probably driven by aggregation and amyloid-led nucleation. These amyloid IBs are more toxic than the aggregates

177 present in population P1: initially, this leads to cell death in population P2 (as determined by PI staining); as the cells are
178 stored at 4 °C, cells entering population P2 are mainly alive (PI^-) and therefore have a VBNC phenotype, as witnessed by
179 their lack of growth on agar plates.
180
181 **Comparison of sealed and non-sealed plates**
182 Rates of transfer of cells from the P1 to the P2 population on agar plates were also tested for plates that had not been
183 sealed with gas barrier film. In this case, the proportion of cells in population P1 decreased far faster for unsealed plates
184 than sealed plates (around 35% of cells were in population P1 after 4 weeks compared to 80% for sealed plates); the
185 proportion of cells in population P2 increased correspondingly, as in Fig. 2a. It is difficult to ascribe an exact reason for
186 this difference since sealing plates with film not only alters the composition of the gas that bacterial colonies on agar
187 plates are exposed to, but also changes the rate at which the agar dries during storage. Nonetheless, this provides a
188 potentially useful diagnostic for monitoring the state of bacterial colonies on agar plates in a variety of conditions, and
189 allows improvement of bacterial culturability and depression of amyloid IB formation over time by sealing plates.
190
191 **Transition from agar plates to liquid culture**
192 The ability of bacteria taken from the agar plates to grow in liquid culture and generate recombinant CheY-GFP protein
193 was tested. Previous flow cytometry experiments with liquid cultures of this strain grown in bioreactors identified two
194 populations, GFP^+ and GFP^-. To quantify the shift from populations P1, P2 and P3 observed in agar plate samples to
195 those populations observed in liquid growth samples, bacteria taken from agar plates (freshly transformed, similar to the 0
196 weeks sample above) were grown in 20 mL of LB medium with 100 µg carbenicillin mL^{-1} at 30 °C and FCM was used to
197 measure the fluorescence and scatter properties of the cultures over time (Figure 3). Up to 4 hours following inoculation
198 (Fig. 3a-c), bacteria in population P1 increased in mean forward scatter, whereas bacteria in population P2 maintained
199 their forward scatter and green fluorescence values. This corresponds to lag phase, and suggests that bacteria in
200 population P2 are non-active in liquid culture (reflecting their poor culturability observed above), whereas bacteria in
201 population P1 are metabolically active and increase in size in preparation for cell division (Åkerlund et al. 1995). After 6
202 and 8 hours growth (Fig. 3d&e), corresponding to logarithmic phase, both the forward scatter and green fluorescence of
203 the bacteria in the main population decreased, suggesting that the bacteria are actively dividing; thus overall bacterial size
204 decreases, and the amount of GFP per cell decreases due to dilution caused by rapid cell division. After 24 hours growth
205 (Fig. 3f), the bacteria are in stationary phase and are relatively homogeneous in terms of both green fluorescence (high,

206 due to accumulation of GFP during the logarithmic phase) and forward scatter (low, typical for stationary phase bacteria
207 which tend to have smaller forward scatter values than logarithmic phase bacteria; Åkerlund et al. 1995).
208 *[Location of Figure 3]*
209
210 **Growth and recombinant protein production from the transformants**
211 The ability of bacteria from agar plates stored up to 12 weeks to generate recombinant protein was assessed.
212 Bacteria from the storage agar plates were used to inoculate 35 mL of LB containing 100 µg carbenicillin mL^{-1} and grown
213 for 14 hours. Following growth, flow cytometry showed that more than 96% of cells were GFP^+, irrespective of the
214 proportion of bacteria on the storage agar plate in population P1 (data not shown). It should be noted that as colonies were
215 stored for longer periods of time on the storage agar plates, the time taken to generate an equivalent biomass in liquid
216 cultures increased, consistent with the decreasing size of population P1 (readily culturable cells) on the plates.
217
218 Overnight cultures were then used to set up RPP cultures according to the 'improved' protocol of Sevastyanovich et al.
219 (2009), whereby stress during RPP is minimised to optimise the proportion of recombinant protein generated in a soluble,
220 functional form. Again, green fluorescence was used to rapidly monitor the yield of fluorescent CheY-GFP. The mean
221 green fluorescence of cells in these cultures 24 hours post-induction was lower following 4 weeks storage on agar plates,
222 and lower still after 12 weeks storage (Fig. 4a); however, this may be due to a decrease in the size of the cells as storage
223 time increased, since the forward scatter was seen to decrease with storage (Fig. 4b). The proportion of cells that were
224 GFP^+ remained >95% in all cultures, irrespective of storage time, although the covariance of the green fluorescence
225 increased with storage time (Fig. 4c), demonstrating greater heterogeneity of GFP production in cultures derived from
226 colonies that had been stored for extended periods on agar plates. SDS-PAGE analysis of the proteins within these
227 cultures revealed that storage on agar plates did not significantly affect either the quantity of recombinant CheY-GFP
228 protein per unit biomass, or the percentage of CheY that was in the soluble fraction (data not shown). This supports the
229 conclusion that storage decreases both cell size (indicated by forward scatter) and amount of fluorescent CheY-GFP per
230 cell (indicated by mean green fluorescence), but not the amount of CheY-GFP per unit biomass measured by SDS-PAGE.
231 *[Location of Figure 4]*
232
233 **The importance of rapid analytical methods for bioprocessing**
234 The development of analytical methods for fermentation optimisation is essential for the development of robust
235 production processes, itself a requirement for regulatory approval of the resultant protein product. Ideally, analytical

methods should be rapid, require small volumes of sample and allow measurement of cellular parameters without the requirement for further growth, so to give a 'snapshot' of the current state of the cells. This FCM technique fulfils these three requirements. Time from sampling to data is in the range of one minute, or slightly longer if propidium iodide staining is required for viability assessment (around 10 minutes). A very small sample volume is required, and the technique does not rely upon growth of bacteria to generate data, which eliminates bias introduced by growth-based methods which exclude VBNC cells which are commonly present in RPP cultures due to increased metabolic burden and inclusion body formation (Nebe-von-Caron et al. 2000, Sundström et al. 2004). GFP fusion proteins cannot be used to directly monitor bioprocesses generating biopharmaceuticals, as a GFP fusion protein could not be readily used as a therapeutic, and cleavage of fusion tags adds process complexity and cost. However, we envisage that GFP fusion proteins could be used to plan processes in initial experiments; the GFP fusion could then be replaced by the recombinant protein without GFP for actual production. Comparison of production of recombinant protein with and without GFP fusion tags has previously showed good correlation (Jones et al., 2004, Jones 2007).

Analytical techniques at this stage of recombinant protein production culture setup are rare. As far as we are aware, this is the first published use of flow cytometry to analyse bacteria generating recombinant protein from agar plates. This method could be routinely used for analysis of transformants from agar plates following storage, eliminating variability that originates from inoculating cultures using colonies from agar plates with unknown physiology. The technique can also be used to screen transformants directly following transformation. Indeed, this has been used in our laboratory to select for highly-producing colonies and discard colonies that generate little GFP. In industrial settings, where transformants are cryopreserved into master cell banks and working cell banks, FCM could be readily implemented to monitor cell bank stability over time and variability between individual samples within cell banks. In conclusion, the methods developed in this study could be implemented in a variety of settings in order to better understand, and control, recombinant protein production in bacterial hosts.

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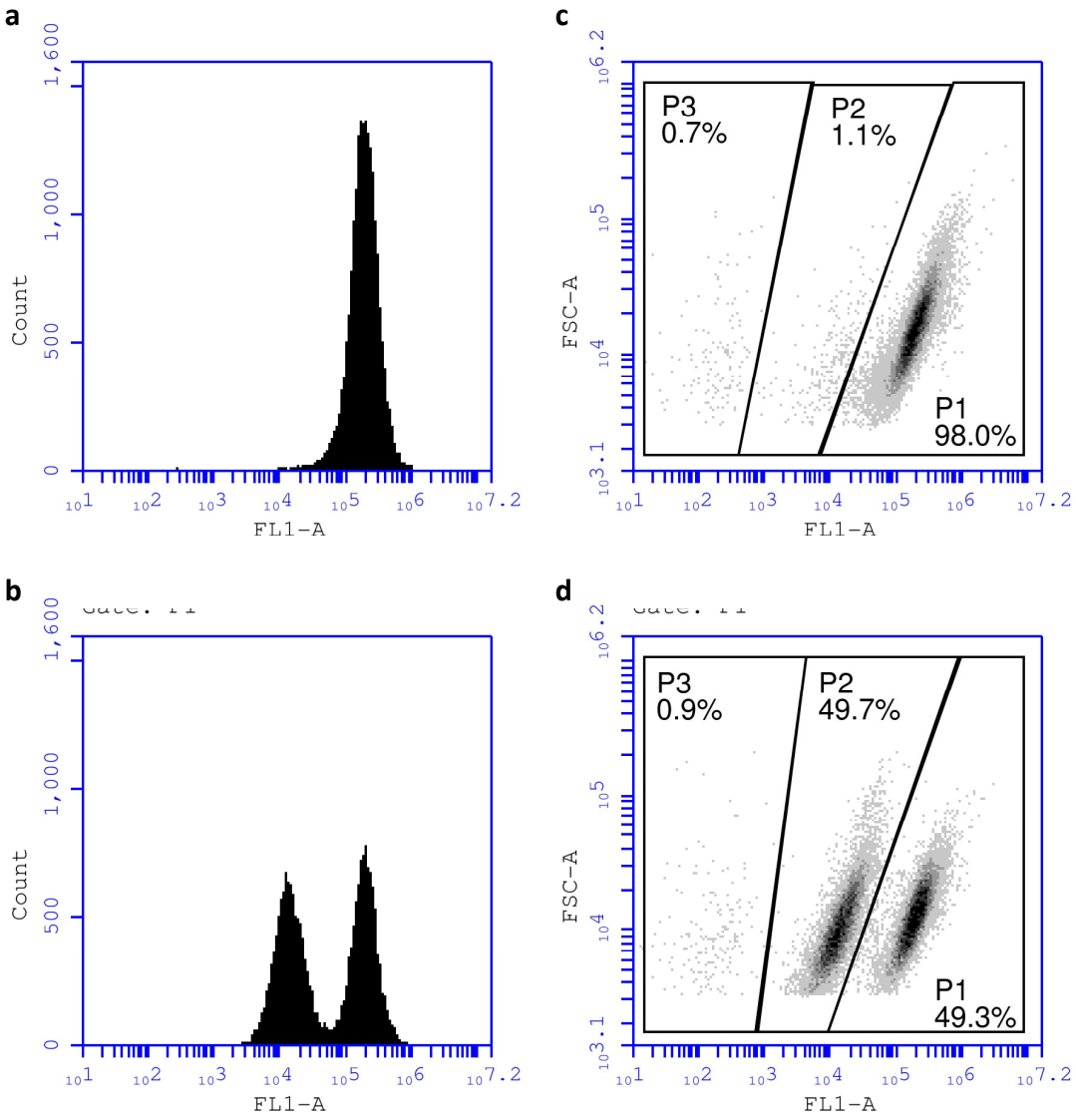
309
310 **FIGURE LEGENDS**
311 **Fig. 1** FCM analysis of bacteria taken directly from storage agar plates and resuspended in PBS after 0 and 12 weeks
312 storage. Histograms of green fluorescence (FL1-A) at (a) 0 and (b) 12 weeks storage, showing two partially overlapping
313 populations. Density plots of forward scatter (FSC-A) versus green fluorescence (FL1-A) with representative gating at (c)
314 0 and (d) 12 weeks storage, showing better separation of populations than histograms.
315
316 **Fig. 2** FCM analysis of bacteria from storage agar plates following different periods of storage. Bacteria were taken from
317 storage agar plates and resuspended in PBS prior to FCM analysis. Data plotted in (a-c) and (f) are the mean of 4
318 independent agar plates, error bars represent ± 1 standard deviation. (a). Distribution of bacteria in populations P1, P2 and
319 P3 over storage time. Populations 1-3 are gated as in Figure 1d. Legend refers to panels (a)-(c) and (f). (b) Mean green
320 fluorescence (FL1-A) of cells in populations P1-P3 over the course of 16 weeks storage. (c) Mean forward scatter (FSC-
321 A) of cells in populations P1 and P2 over the course of 16 weeks storage. Population 3 is not shown due to low
322 abundance. (d) The green (FL1-A, y axis) and red (FL3-A, x axis) fluorescence of cells following 4 weeks storage on agar
323 plates. Populations P1, P2 and P3 are shown by gates. (e) As (d), with the addition of Congo Red stain. Cells in

population P2 have increased red fluorescence caused by Congo Red binding and decreased green fluorescence caused by FRET from GFP to CR, resulting in a shift from the P2 gate to P4. (f) Percentage of cells in each population that stain positively with propidium iodide (PI), indicating loss of membrane integrity and thereby cell death.

Fig. 3 FCM analysis of bacteria immediately after transfer from agar plates to LB medium. A sweep of bacteria were transferred from agar plates to 20 mL of LB containing 100 µg carbenicillin mL^{-1}. Cultures were incubated at 30 °C with shaking. Representative FCM plots are shown from 2 independent replicate cultures after (a) 1, (b) 2, (c) 4, (d) 6, (e) 8 and (f) 24 hours growth. Gates P1-P3 are set throughout corresponding to initial populations for reference and do not correspond to populations in later stages of growth.

Fig. 4 Recombinant protein production from colonies stored on agar plates. After 0, 4 and 12 weeks of storage, colonies were used to set up liquid cultures to generate recombinant CheY-GFP fusion protein. FCM was used to measure the (a) mean green fluorescence (FL1-A) and (b) mean forward scatter (FSC-A) of cells in these cultures 24 hours after induction of protein synthesis by the addition of IPTG. The proportion of GFP^+ cells remained >95% throughout. Data plotted is the mean of two independent cultures ± 1 standard deviation. (c) The covariance of the mean green fluorescence 24 hours after induction increased over storage time, indicating increased heterogeneity.

341
342 Figure 1.
343



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382 Figure 2

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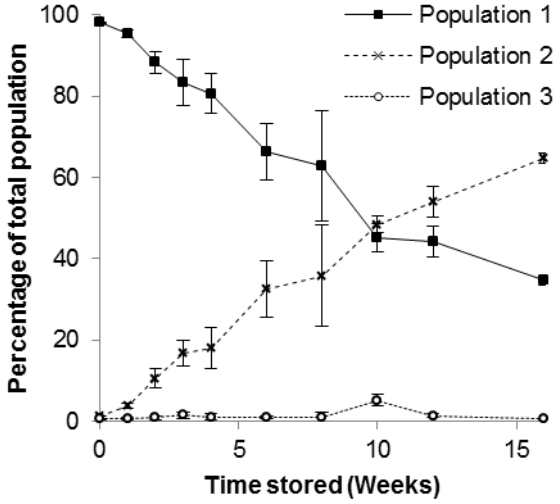

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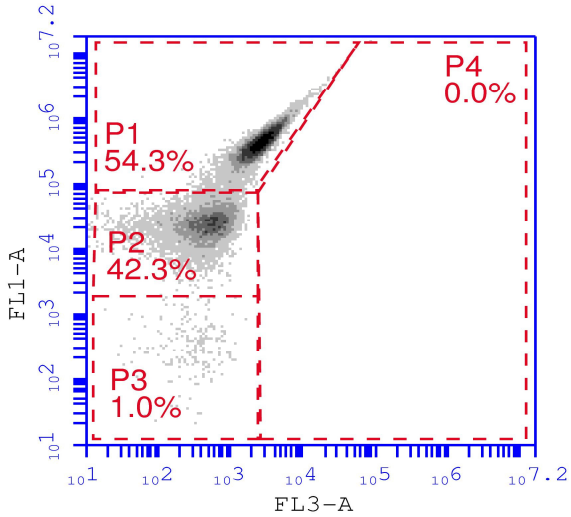

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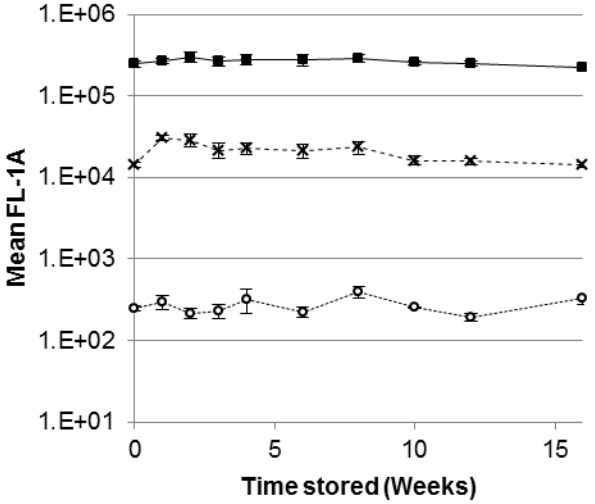

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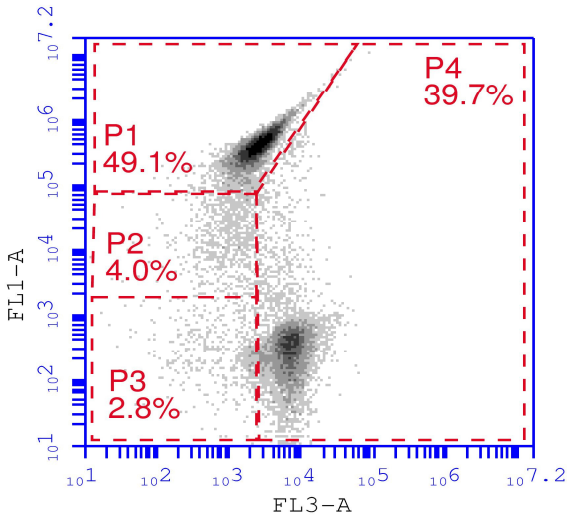

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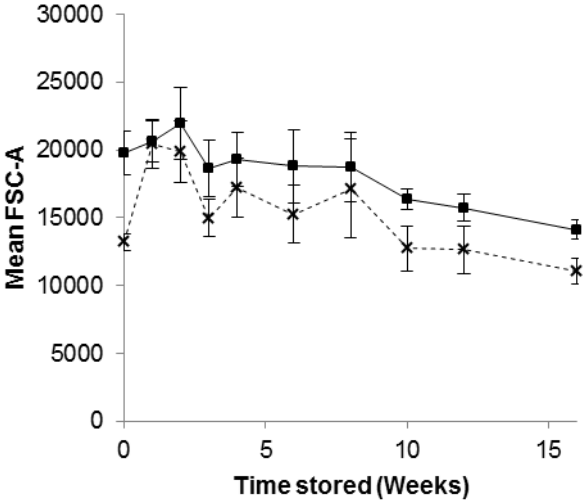

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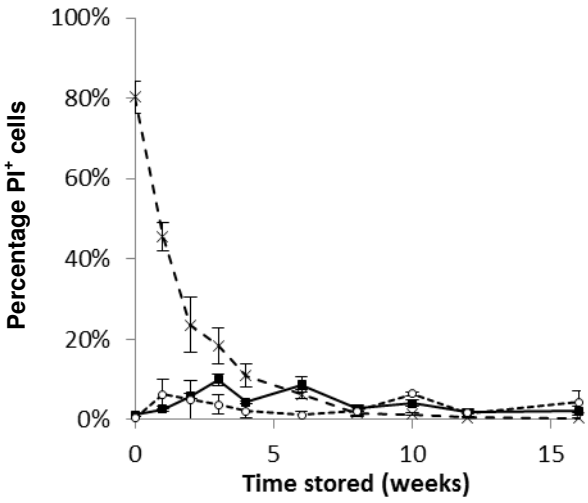

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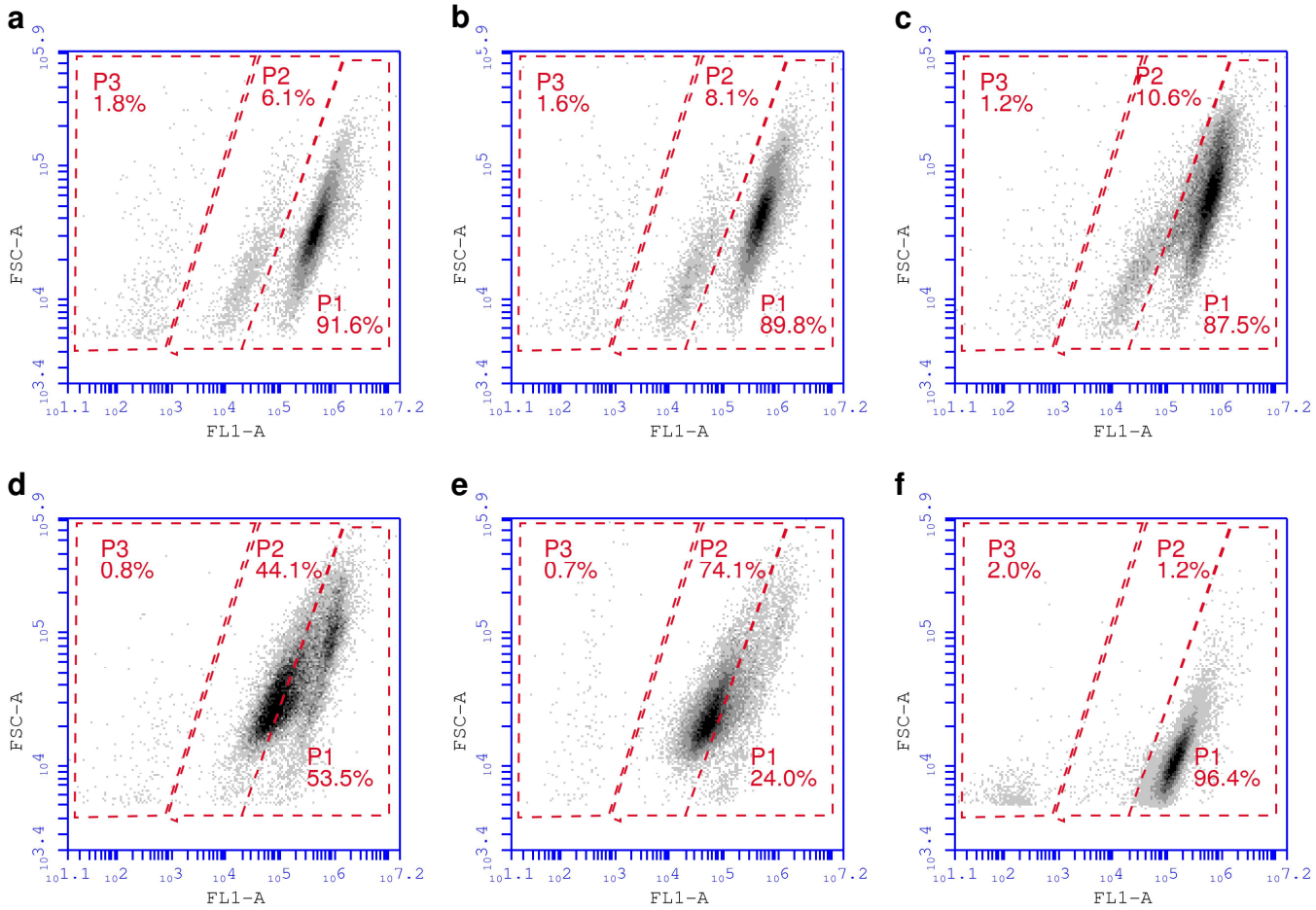
393 Figure 3



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395 Figure 4

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