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Exploitation of GFP fusion proteins and stress avoidance as a generic strategy for the production of high quality recombinant proteins

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Keywords

Recombinant protein production; inclusion bodies; gonococcal I cytochrome *c*₂; heat shock; general stress response; green fluorescent protein; flow cytometry

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Abstract

A C-terminal GFP fusion to a model target protein, *Escherichia coli* CheY, was exploited both as a reporter of the accumulation of soluble recombinant protein, and to develop a generic approach to optimise protein yields. The rapid accumulation of CheY::GFP expressed from a pET20 vector under the control of an IPTG-inducible T7 RNA polymerase resulted not only in the well-documented growth arrest, but also loss of culturability and overgrowth of the productive population by plasmid deficient bacteria. The fluorescence of cultures due to the accumulation of CheY::GFP were optimised by using very low concentrations of IPTG that avoid growth arrest and loss of culturability post-induction. Optimal product yields were obtained with 8 μ M IPTG, a concentration so low that insufficient T7 RNA polymerase accumulated to be detectable by Western blot analysis. The improved protocol was shown to be suitable for process scale-up and intensification. It is also applicable to the accumulation of an untagged heterologous protein, cytochrome *c*₂ from *Neisseria gonorrhoeae*, that requires both secretion and extensive post-translational modification.

Introduction

Many biopharmaceutical projects require the production of recombinant proteins in heterologous bacterial hosts. When the protein itself is the end product, the requirement for rapid, bulk production in high yield drives the design of a successful process. At the other extreme, high quality protein is often required as the starting point for NMR or X-ray crystallographic structure determination, or for understanding the biology underlying a process. Quality rather than quantity or production intensity now becomes the overriding requirement.

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The ability to express almost any gene at a controllable level makes bacterial hosts and plasmids attractive vehicles for generating the desired product. Despite the availability of a plethora of expression systems, detailed knowledge of the genome sequences, molecular biology, physiology and biochemistry of a range of production hosts, many proteins remain difficult to produce at the scale or quality required. Frequently encountered problems include the deposition of the target protein in insoluble inclusion bodies (Villaverde & Carrio, 2003), lysis of the production host due to the physiological stress induced by high-level synthesis of mRNA and the heterologous protein (Gill *et al.*, 2000), and accumulation of multiple fragments of the target protein due to proteolysis (Dürschmid *et al.*, 2008).

The primary cause of many of the problems is the accumulation of incorrectly folded intermediate forms of the target protein. In bacteria like *E. coli*, this is a signal that induces not only the general stress response, but also other overlapping responses (Hoffmann & Rinas, 2004; Gasser *et al.*, 2008). If the correct folding of the target protein is the only problem to be solved, over-expression of chaperones might be sufficient to achieve success, for example, by pre-inducing the RNA polymerase RpoH regulon with a heat shock (Hoffmann & Rinas, 2004), or the co-expression of *groEL*, *dnaK* or other chaperone genes (Nishihara *et al.*, 1998; Chen *et al.*, 2003; Schrodell *et al.*, 2005; Mitsuda & Iwasahi, 2006; Hu *et al.*, 2007; de Marco, 2007). More often, however, failure is due directly to the consequences of the induction of the RpoH-dependent stress response (Vera *et al.*, 2006; Rabhi-Essafi *et al.*, 2007; Lin *et al.*, 2008) especially the disaggregation complex in which DnaK, ClpB and IbpAB remove aggregated recombinant proteins for proteolysis (reviewed by Gasser *et al.*, 2008). We now report results of experiments designed to analyse the physiological cause of failure to accumulate a soluble, cytoplasmic recombinant protein, and the design of a generic strategy to minimise the problem. Although our main model system is based upon the production of the *E. coli* chemotaxis protein CheY, fused with a C-terminal

green fluorescent protein (GFP) tag, we show that a similar approach can be used to accumulate an untagged recombinant protein that requires both secretion to the periplasm and extensive post-translational modification.

Materials and methods

E. coli strain and plasmids

The *Escherichia coli* strains BL21(DE3)* (Invitrogen) and its derivatives, C41 and C43 (Miroux & Walker, 1996) were used for recombinant protein expression work. *E. coli* strain JM109 (Promega) was used to clone the cytochrome *c*₂ gene from *Neisseria gonorrhoeae*. The over-expression of CheY::GFP fusion gene or the gonococcal gene encoding cytochrome *c*₂ from *Neisseria gonorrhoeae* was induced from the IPTG-inducible T7 promoter of the expression vectors pET20bhc-CheY::GFP and pET20bhc-*c*₂, respectively, both of which are derived from a slightly modified version of pET20b (Novagen; Waldo *et al.*, 1999; Jones *et al.*, 2004).

The *cccA* gene (accession number NGO0292) encoding cytochrome *c*₂ was amplified from *Neisseria gonorrhoeae* strain F62 genomic DNA using primers CTACGTCATATGAACACAACCCG and CATAGGGATCCTTAGAAAGGTTTGATTG (incorporating *Nde*I and *Bam*HI restriction sites, respectively, shown in bold type) and PCR SuperMix High Fidelity (Invitrogen) according to manufacturer's instructions. The thermal cycling profile included 1 cycle at 94°C for 3 min., 10x (94°C for 30 s, 40°C for 30 s, 68°C for 1 min.), 27x (94°C for 30 s, 55°C for 30 s, 68°C for 1 min.) and 1x 68°C for 10 min. The 465 bp PCR product was cloned into pGEM-T Easy (Promega), sequenced and the *cccA* fragment was transferred as an *Nde*I - *Bam*HI fragment into pET20bhc vector digested with *Nde*I and *Bam*HI. In experiments to accumulate

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3 97 mature cytochrome *c*₂ from *N. gonorrhoeae*, bacteria were co-transformed with the second
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5 98 plasmid, pST2, that encodes the *E. coli* cytochrome *c* maturation proteins, CcmA-H (Turner
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7 99 *et al.*, 2003).
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12 101 **Growth conditions for the standard protocol**
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15 102 Bacteria were grown aerobically in 100 mL shake flasks with 20 mL working volume or in a
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17 103 3.6 L fermenter (Infors) with a 2.8 L working volume of Luria-Bertani medium supplemented
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19 104 with 2% (w/v) glucose and 100 µg mL⁻¹ carbenicillin for plasmid maintenance. The shake
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21 105 flasks were set up in duplicates or triplicates per culture condition. In fermentations, aeration
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23 106 was maintained at 1 vvm and a stirring speed of 700 rpm. The pH was controlled at 6.3 by the
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25 107 automated addition of 5% (v/v) HCl and 10% (v/v) NH₃ and 0.1% (v/v) silicone antifoam was
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27 108 added in the medium to prevent foaming of the culture during the late stages of the
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29 109 fermentation. The medium was inoculated with 2% (v/v) of seed culture grown aerobically at
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31 110 30°C for approximately 14 hours. Bacteria were grown at 37°C to an OD₆₅₀ of approximately
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33 111 0.5 at which recombinant protein expression was induced with 0.5 mM IPTG. Bacteria were
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35 112 grown at 25°C thereafter to facilitate correct folding of the recombinant protein. Culture
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37 113 samples were taken before induction and at various intervals up to 25 hours post-induction.
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45 115 **Growth conditions for the improved protocol**
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48 116 Bacteria were inoculated and grown in the same medium as that used for the standard
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50 117 protocol. However, bacteria were grown aerobically at 25°C to an OD₆₅₀ of approximately 0.5
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52 118 at which point recombinant protein production was induced with 8 µM IPTG. Bacterial
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54 119 growth was continued at 25°C for up to 70 hours post-induction.
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60 121 **Accumulation of recombinant cytochrome *c*₂ from *Neisseria gonorrhoeae* in *E. coli***

E. coli BL21*(DE3) (Invitrogen) containing pET20bhc-*c*₂ and pST2 was used to produce mature cytochrome *c*₂ in an anaerobic fermenter. The fermentation medium contained 50% Luria-Bertani broth, 40% *E. coli* sulphur-free minimal salts, 10 mM TMAO, 10 mM sodium fumarate, 2% glucose, 0.1% silicone antifoam, 100 µg mL⁻¹ carbenicillin and 30 µg mL⁻¹ chloramphenicol. The fermenter was inoculated with 4% of a seed culture that had been grown for 16 h at 30°C with aeration. The culture in the fermenter was grown at 30°C at 100 rpm stirring speed without aeration; pH was controlled at 6.3 with 5% HCl and 10% ammonia. Protein expression was induced by adding 10 µM IPTG at OD₆₅₀ of approximately 0.5. The bacterial culture samples were taken before induction and at various time points for up to 24 h post-induction.

Plating and replica plating for plasmid retention

To test the effect of recombinant protein production on the culturability of the bacterial host, serial dilutions of the bacterial culture were plated onto non-selective nutrient agar (Oxoid) and incubated at 30°C. The proportion of plasmid-bearing bacteria was estimated by replica plating the resultant colonies on nutrient agar supplemented with carbenicillin (100µg mL⁻¹).

Analysis of recombinant protein accumulation by SDS-PAGE

Proteins were resolved by Tris/Tricine SDS-PAGE using a 15% (w/v) polyacrylamide gel (Sambrook *et al.*, 1989) and stained with 0.2 % (w/v) Coomassie Blue. Total protein was analysed from whole cell samples resuspended in 67 µL of sample buffer per OD₆₅₀ unit so that the biomass per volume for all samples was the same. The samples were heated to 100°C for 10 minutes before loading at the same volume. Recombinant protein yield was estimated by densitometry using the Quantity one software (Bio-Rad).

The yield of soluble and insoluble recombinant protein accumulated was determined from fractionated bacterial samples. Bacterial cell pellets were resuspended in BugBuster lysis reagent (67 μ L of BugBuster per OD₆₅₀ unit) (Novagen) and incubated at room temperature for 10 minutes with gentle shaking. The soluble and insoluble cell fractions were separated by centrifugation at 13,000 rpm at 4°C for 20 min. The separated fractions were resuspended in the same volume of sample buffer as the volume of BugBuster used for lysis and boiled at 100°C for 10 minutes. To ensure that the samples contain equal biomass, two volumes of the soluble and 1 volume of the insoluble cell fractions were loaded and analysed by SDS-PAGE.

Proteins containing covalently attached heme were detected using heme-dependent peroxidase activity (Thomas *et al.*, 1976).

Fluorescence determination

Fluorescence of culture samples taken at various intervals throughout the experiment were measured using a Perkin-Elmer fluorescence spectrophotometer model 203 at settings that allowed accurate readings from a calibration curve. The accumulation of the soluble CheY::GFP was detected using an excitation wavelength of 485 nm and an emission wavelength of 509 nm. The same culture diluted in phosphate buffered saline (PBS; Sambrook *et al.*, 1989) was used for optical density measurement and for fluorescence measurement.

Flow cytometry

The proportion of green fluorescent bacteria overproducing the GFP-tagged recombinant protein in the culture and their physiological state were analysed by flow cytometry (BD FACSAria II: Becton, Dickinson & Co.). Bacteria were diluted in PBS at a final concentration of 10⁵-10⁶ mL⁻¹ and analysed at a data rate of 1000-2000 events sec⁻¹. An eighty-five μ m nozzle was used for the

analysis. The red fluorescent dye propidium iodide (PI, Sigma) was added to the samples to stain dead bacteria. The PI stock solution was made up at 1 mg mL^{-1} in distilled water and used for staining at the working concentration of $5 \text{ } \mu\text{g mL}^{-1}$ (Hewitt *et al.*, 1999). All solutions were passed through $0.2 \text{ } \mu\text{m}$ filter immediately prior to use to remove particles. The backflush cleaning was applied between samples to prevent cross-contamination. The sample was excited with a 488 nm solid state laser (13 mW). The software discriminator was set on the forward scatter to reduce electronic and small particle noise. Forward and side scatter data were collected along with GFP fluorescence (502LB, 530/30BP) and PI fluorescence (610LP, 616/23BP). For each experiment, 100000 data points were collected and analysed using BD FACSDiva software (BD Biosciences).

Western blotting

For Western analysis, culture samples were resuspended in sample buffer as described above (SDS-PAGE section) and loaded onto NuPAGE 4-12% Bis-Tris gel (Invitrogen). The proteins were transferred onto Hybond-ECL nitrocellulose membrane (Amersham) in the Xcell II blot module (Invitrogen). The blots were incubated with T7 RNA polymerase antibodies (Novagen) and then with peroxidase-conjugated anti-mouse IgG (Amersham) according to the manufacturers' instructions. The blots were developed using EZ-ECL Chemiluminescence detection kit (Biological Industries) according to the provided protocol.

Results

Standard protocol for CheY::GFP accumulation

Many laboratories use the commercially available pET plasmids to express a cloned gene in an *E. coli* host under the control of T7 RNA polymerase that is both chromosomally encoded and regulated by an IPTG-inducible promoter. This system was used in initial experiments to

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3 196 produce a 42 kDa CheY::GFP fusion protein (Jones, 2007). It had been observed that, in
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5 197 contrast to N-terminal GFP fusion proteins, optimisation of fermentation conditions for the
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7 198 accumulation of a fluorescent recombinant protein with a carboxy-terminal GFP fusion
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9 199 provides a good prediction of how to generate the correctly folded N-terminal target protein
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11 200 without a fusion tag. Expression of the gene encoding CheY::GFP cloned into plasmid
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13 201 pET20bhc-CheY::GFP was induced with 0.5 mM IPTG at a low biomass density of 0.2 g dry
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15 202 mass L⁻¹, and the temperature was decreased from 37°C to 25°C. Samples of the culture taken
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17 203 before induction and at intervals for 24 h post-induction were analysed for growth,
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19 204 fluorescence, plasmid retention, colony-forming ability, and the accumulation of recombinant
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21 205 protein in both soluble and insoluble cell fractions.
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27 206 The optical density of the culture increased only slowly soon after IPTG addition, but
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29 207 growth resumed after a lag of between 10 to 14 h for up to 24 h post-induction. Plating of
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31 208 serial dilutions of samples taken 2 to 4 h post-induction revealed that only about 1% of the
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33 209 bacteria were able to form colonies on non-selective agar, but high plating efficiency was
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35 210 restored after 24 h (Fig. 1A). In contrast to colonies from samples taken before induction that
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37 211 were pale green due to leaky expression of the recombinant protein, colonies from samples
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39 212 taken 24 h post-induction were white. This was readily shown to be due to overgrowth of the
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41 213 population by plasmid-free bacteria (Fig. 1A). Consistent with these results, SDS-PAGE
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43 214 analysis revealed a rapid burst of CheY::GFP synthesis immediately post-induction (Fig. 1B),
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45 215 but little increase after a further 2 to 4 h. Furthermore, about 80% of the CheY::GFP fusion
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47 216 protein accumulated in inclusion bodies in the insoluble fraction (Fig. 1B), which was almost
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49 217 non-fluorescent, indicating that GFP was incorrectly folded and therefore inactive.
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53 218 Analysis of samples by flow cytometry revealed that the population pre-induction was
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55 219 relatively homogenous but moderately fluorescent, reflecting the leakiness of the pET
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57 220 promoter (Fig. 2A). Fluorescence had increased substantially within 3 h of IPTG addition.
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However, the small population of non-fluorescent bacteria already present in the culture increased from 4% to 12% post-induction. After 25 h, only a minority of the bacteria in the culture were fluorescent due to overgrowth by unproductive, plasmid-free bacteria. Around 20% of the population were permeable to propidium iodide, indicating loss of viability, though these were split equally between fluorescent and non-fluorescent bacteria.

Optimisation of soluble CheY::GFP production by minimising post-induction growth-arrest

Although the consequences of rapid over-expression of cloned genes are well documented, less reported is the loss of colony forming units during recombinant protein production (Sundström *et al.*, 2004). We therefore investigated whether it was possible to optimise the IPTG concentration not on the basis of quantity or speed of recombinant protein production, but on the maximum level of fluorescence under conditions that greatly decreased the general stress response. This involved growing the culture at the same temperature both before and after induction to avoid any stress caused by a change in temperature; and the determination of the concentration of the inducer, IPTG, that would allow maximum GFP fluorescence 24 h post-induction. Based upon these criteria, optimal results were obtained with cultures grown at 25°C and induced with 8 µM IPTG, which had only a slight effect on exponential growth and avoided selection of plasmid-free bacteria (Fig. 1C; D). Flow cytometry analysis showed that fluorescence continued to increase for at least 25 h (Fig. 2B), and much greater homogeneity in the culture: around 98% of bacteria were in the GFP⁺ population both before induction and after 3 and 25 hours post-induction; the number of nonviable, PI⁺ bacteria at 25 h was greatly reduced compared to the original protocol; and the GFP⁻ population actually decreased in size from 2% pre-induction to 0.7% after 25 h (Fig. 2B). Analysis of samples from the culture by SDS-PAGE revealed that about 90% of the CheY::GFP had accumulated

throughout the induction phase in the soluble protein fraction, in contrast to less than 20% soluble product from the standard protocol (Fig. 1E). Furthermore, the yield of recombinant protein was four-fold higher from the improved protocol than from the standard protocol due to the production of a higher yield of biomass (Table 1). Finally it was demonstrated that much higher yields of product could be generated following prolonged expression in fed-batch cultures (Table 1).

Molecular basis for the increased accumulation of CheY::GFP

Aware of the stress on the bacterial host associated with the use of the BL21 / pET system, Miroux and Walker (1996) isolated mutants that were resistant to stress and therefore continued to accumulate recombinant protein far longer than their parent strain. Two of these strains were called C41 and C43, and it was subsequently shown that the basis for the improved performance was a down-mutation of the promoter of the T7 polymerase gene (Wagner *et al.*, 2008). To determine whether the low concentration of IPTG coupled with the low expression temperature might simply limit production of the T7 RNA polymerase and hence explain why the improved protocol was successful, Western blots of samples from both the standard and improved protocols were probed with anti-T7 RNA polymerase antibody (Fig. 3). In contrast to the strong bands of cross-reacting antigen from the standard protocol, so little T7 polymerase was produced using the improved protocol that it was not visible.

We then compared yields of CheY::GFP from strain BL21* using the improved protocol with those from strains C41 and C43 generated using the standard protocol (Fig. 4). The level of fluorescence (per unit volume) from the improved protocol was the same as that from C41 and considerably higher than from C43 (not shown). The specific fluorescence (per unit biomass) at the point of harvest was highest for BL21* using the improved protocol (Fig. 4A) and its cell density was only slightly lower than that for strain C41 (Fig. 4B).

High-level production of a secreted recombinant *c*-type cytochrome using the improved protocol

The model protein, CheY::GFP, is a soluble, cytoplasmic protein. It was therefore of interest to determine whether the improved protocol for CheY::GFP production was sufficiently generic to be exploited in the production of a secreted protein that requires extensive post-translational modification and assembly in the bacterial periplasm. A *Neisseria gonorrhoeae* gene of unknown function predicted to encode a *c*-type cytochrome, which we have designated cytochrome *c*₂, was cloned into the expression plasmid, pET20bhc, and expressed either using the standard protocol (induction with a high concentration of IPTG followed by a decrease in growth temperature from 37°C to 25°C) or the same optimised conditions that were used for CheY::GFP. Under both sets of conditions, two bands of recombinant protein were detected by SDS-PAGE stained for total protein: the upper band was pre-apocytochrome *c* located in the cytoplasm; the lower band stained positively for covalently attached heme, confirming that it was mature cytochrome located in the periplasm. Using the standard protocol, cytochrome rapidly accumulated for a short time, but then production stopped as the culture was overgrown by plasmid deficient bacteria (not shown). Even with the standard protocol, some mature cytochrome was produced, but more than 95% of the product was pre-apoprotein located in cytoplasmic inclusion bodies. In contrast, yields of mature cytochrome *c*₂ from the improved protocol were so high that the resulting *E. coli* culture was slightly orange, and the cytochrome with covalently bound heme accumulated in the soluble, periplasmic fraction of the bacteria (Fig. 5). This demonstrated that the improved protocol had enabled post-translational secretion, periplasmic heme attachment and folding to keep pace with the synthesis of the pre-apoprotein. Furthermore, analysis by SDS-PAGE revealed that

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3 295 only a small percentage of the pre-apoprotein had accumulated in the cytoplasmic fraction, or
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5 296 been deposited into inclusion bodies (Fig. 5A).
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10 298 **Accumulation of other recombinant proteins using the improved protocol**
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12 299 The overproduction of two other recombinant proteins, namely the gonococcal cytochrome *c*
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14 300 peroxidase (CCP; 47 kDa) from *N. gonorrhoeae* (Turner *et al.*, 2003) and a non-*E. coli*
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16 301 protein D-GFP (45 kDa) (Intellectual property; GSK) were produced using both approaches.
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18 302 There was at least an 8-fold increase in the yields of mature CCP with covalently attached
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20 303 heme and of soluble protein D-GFP when the modified approach was used compared with the
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22 304 normal protocol, clearly reflecting the robustness of this approach for improving soluble
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24 305 recombinant protein yields regardless of size, properties or bacterial host origin.
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31 307 **Discussion**
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33 308 The primary cause of failure to produce a correctly-folded recombinant protein in high yield
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35 309 is well understood, namely the accumulation of incorrectly folded intermediates due to rates
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37 310 of protein synthesis overwhelming post-translational modifications such as folding, secretion,
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39 311 folding into membrane-spanning helices or the incorporation of prosthetic groups (reviewed
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41 312 by Gasser *et al.*, 2008). Different, and sometimes opposite, strategies depending on the
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43 313 properties of the target protein are required to solve these problems (Gasser *et al.*, 2008;
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45 314 Hoffmann & Rinas, 2004; Miroux & Walker, 1996; Soriano *et al.*, 2002; Wagner *et al.*,
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47 315 2008). Stress post-induction is especially severe when the IPTG-inducible T7 RNA
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49 316 polymerase system in the *E. coli* BL21 host is used to accumulate high concentrations of
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51 317 recombinant protein. Many genetic strategies have been described to decrease this stress
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53 318 response (Miroux & Walker, 1996; Soriano *et al.*, 2002; Wagner *et al.*, 2008), and a suite of
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55 319 commercially available derivatives of BL21 and pET plasmids have been designed to
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3 320 overcome these problems. Consequently the Holy Grail of recombinant protein production,
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5 321 the availability of generic protocols and hosts for the production of even the most difficult
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8 322 target product, has yet to be achieved: recombinant protein production remains as much an art
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12 324 Although the consequences of rapid over-expression of cloned genes are well
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14 325 documented, less reported is the loss of colony forming units during recombinant protein
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16 326 production (Sundström *et al.*, 2004). We therefore adopted a physiological approach to
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18 327 investigate whether it was possible to optimise the IPTG concentration not on the basis of
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20 328 quantity or speed of recombinant protein production, but on yield of GFP fluorescence under
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22 329 conditions that greatly decreased the stress on the host. This involved growing the culture at
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24 330 the same temperature both before and after induction to avoid any stress caused by a change
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26 331 in temperature; and determination of the concentration of the inducer, IPTG, that would allow
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28 332 optimal yields of GFP fluorescence 24 h post-induction. This approach defined conditions
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30 333 that were suitable for the accumulation of two vastly different types of recombinant protein to
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32 334 levels approaching 30% of the total protein content of the bacteria. Experiments currently in
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34 335 progress are designed to determine whether this approach is applicable to other hosts and
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36 336 expression systems, or limited to expression systems based upon the bacteriophage T7
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38 337 polymerase that, due to the very high rates of transcription post-induction, impose an
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40 338 excessive stress on the host bacterium (Soriano *et al.*, 2002; Sørensen & Mortensen, 2005). It
41
42 339 will be particularly interesting to know whether it can also be beneficial for other, less
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44 340 stressful expression systems.
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For Peer Review

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Table 1

<i>Protocol</i>	<i>Format</i>	<i>Biomass, g dry mass L⁻¹[§]</i>	<i>Percentage recombinant protein, %[†]</i>	<i>Yield of recombinant protein, g L⁻¹[‡]</i>	<i>Specific fluorescence, Fluorescence units/OD₆₅₀</i>	<i>Total fluorescence, units</i>
Standard	Shake flask	2.3	21	0.34	60.8	350
	Batch fermenter	2.4	25	0.42	106.0	627
Improved	Shake flask	6.16	52	2.24	214.0	3240
	Batch fermenter	6.6	29	1.34	269.4	4440
	Fed-batch fermenter	26.6	31	5.8	289.2	19200

[§]Biomass was calculated on the assumption that a culture with an OD₆₅₀ of 1.0 contains 0.4 g dry mass L⁻¹

[†]The percentage of recombinant protein was estimated from SDS-PAGE gels by densitometry

[‡]Recombinant protein yield was estimated based on the assumption that 70% of the bacterial culture dry mass is protein.

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Figure legends

Fig. 1. Optical density, colony forming units, plasmid retention and SDS-PAGE profile for the standard (A, B) and improved (C, D, E) protocols. IPTG was added at time zero. The arrow beside the gels indicates the position of the 42 kDa CheY::GFP protein. BI: before induction; S: soluble fraction; I: insoluble fraction; M: marker proteins.

Fig. 2. Flow cytometry analysis of before, 3 h after and 25 h after IPTG induction for the standard (A) and improved (B) protocols. Note that only the final 25 h samples were stained with propidium iodide (PI). X axes show PI-emitted fluorescence measured at ~630 nm and Y axes show GFP-emitted fluorescence measured at ~510 nm. The numbers in each quadrant show percentage of bacteria in each sub-group.

Fig. 3. Western blot to detect T7 RNA polymerase during CheY::GFP accumulation using the standard (A) and improved (B) protocols. BI: before induction; other time points are post-induction.

Fig. 4. Comparison of CheY::GFP accumulation during expression using the improved and standard protocols and *E. coli* BL21* as the host and in strains C41 and C43 using the Miroux and Walker (1996) protocol. A. Specific fluorescence 3, 7 and 24 h post-induction. B. Growth post-induction, plotted on linear scale to show differences between strains and protocols more clearly. IPTG was added at time zero.

Fig. 5. Production of gonococcal cytochrome *c*₂ using the improved protocol. The SDS-PAGE gels show yields of total protein (A. Coomassie stained gel) and of mature cytochrome

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3 455 c_2 (B. Gel stained for heme-dependent peroxidase activity). The upper arrow indicates the
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6 456 position of the pre-apo-cytochrome located in the cytoplasm; the lower arrow is the mature
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8 457 cytochrome with heme attached post-secretion into the periplasm.
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