Indole generates quiescent and metabolically active *E. coli* cultures

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Abbreviations: a.u., arbitrary units; DAPI, 2-(4-amidinophenyl)-1H -indole-6-carboxamidine; DO, dissolved oxygen; EGFP, enhanced green fluorescent protein; hGM-CSF, human granulocyte macrophage colony stimulating factor; LA, Luria-Bertani agar; LB, Luria-Bertani broth; IPTG, isopropyl β-D-1-thiogalactopyranoside; OD_{600}, optical density at 600 nm; ORF, open reading frame; OUR, oxygen uptake rate; PAGE, polyacrylamide gel electrophoresis, PCR, polymerase chain reaction; PID, proportional integral derivative; RPM, revolutions per minute; scFv, single-chain variable fragment; TB, terrific broth.

Abstract

An inherent problem with bacterial cell factories used to produce recombinant proteins or metabolites is that resources are channeled into unwanted biomass as well as product. Over several years attempts have been made to increase efficiency by unlinking biomass and product generation. One example was the quiescent cell (Q-Cell) expression system that generated non-growing but metabolically active *E. coli* by over-expressing a regulatory RNA (Rcd) in a defined genetic background. Although effective at increasing the efficiency with which resources are converted to product, the technical complexity of the Rcd-based Q-Cell system limited its use. We describe here an alternative method for generating Q-Cells by the direct addition of indole, or related indole derivatives, to the culture medium of an *E. coli* strain carrying defined mutations in the *hns* gene. This simple and effective approach is shown to be functional in both shake-flask and fermenter culture. The method provides a simple way to generate quiescent cells in both shake-flask and fermenter culture. They remain metabolically active and analysis of their performance in the fermenter suggests that they may be particularly suitable for the production of cellular metabolites.

1. Introduction

The Quiescent-Cell (Q-Cell) Expression System is an *E. coli* cell factory where product formation is separated from the accumulation of unwanted biomass. In its original form, bacterial cells were put into a non-growing but metabolically active state (quiescence) by over-expression of a small regulatory RNA, Rcd [1]. Rcd was discovered through its role in the stable maintenance of the multicopy plasmid ColE1 [2] where it is part of a checkpoint linking plasmid dimer formation to the inhibition of cell division. To achieve complete cessation of growth (as opposed to simply slowing the growth rate) it was found necessary to over-express Rcd in an *hns205* mutant of *E. coli*. The role of the *hns* allele in the establishment of quiescence remains unclear but Rcd-induced Q-Cells were observed to contain highly condensed nucleoids and H-NS is known to have an important role in the maintenance of nucleoid structure [3].

To establish quiescence, Rcd was expressed from the λP_R promoter and regulated by the temperature-sensitive *c*I*ts*857 repressor. A temperature shift from 30°C to 42°C induced Rcd expression and, after 2-3 h, there was no further increase in the OD_{600} of the culture. It was observed that the cells remained metabolically active in the quiescent state and plasmid gene expression continued at a high level. Pulse-labelling experiments indicated that chromosomal gene expression was down-regulated, consistent with observations of DAPI-stained cells that suggested the nucleoid was highly condensed [1].

As a test of the synthetic capacity of Q-Cells, their ability to express a single-chain antibody fragment (scFv) in high-density shake flask and fermenter cultures was investigated [4]. The scFv gene was tagged with a *pelB* leader that resulted in the majority of product being secreted into the culture supernatant. In fed-batch fermenter culture, Q-Cells were held at an OD_{600} of 20 for 24 h and extracellular scFv reached a maximum of 150 mg L^{-1}. At an OD_{600} of 50 Q-Cells exhibited a small decline in the specific product formation rate, resulting in an extracellular scFv concentration of 160 mg L^{-1} after 8 h. A conventional (non-quiescent) culture with a similar feed reached an OD_{600} of 80 but, despite the higher density, the extracellular scFv concentration did not exceed 35 mg L^{-1}.

Despite the potential advantages of the Rcd-based Q-Cell system over conventional culture, its relative technical complexity was a disincentive to users. For example, it was necessary to introduce two plasmids into the host strain (one encoding Rcd and the other the product gene) and, to achieve consistent results, the strain had to be reconstructed for each production run. The temperature shift from 30 to 42 °C to induce Rcd production and quiescence meant that product expression occurred at a temperature that was not ideal for protein folding and stability. Furthermore, even if antibiotics were added regularly to maintain selection for the Rcd-expressing plasmid, cultures occasionally escaped from quiescence and resumed normal growth. This was due to a small sub-population of cells lacking the Rcd expression plasmid continuing to grow when the Rcd-producing cells had entered quiescence (Rowe and Summers, unpublished). Finally, the host strain used in the development of Rcd-induced Q-Cells is potentially unstable. The *hns205* allele was created by the integration of a functional Tn*10* transposon into the *hns* gene [5] and the possibility of further transposition of the element makes the host potentially unstable.

Since the original development work with Q-Cells was carried out, a fuller understanding of the normal biological function of Rcd has enabled the development of a second-generation system that avoids the majority of problems described above. In wild-type *E. coli* Rcd is synthesised in response to the accumulation of dimers of plasmid ColE1 [2]. Its expression delays cell division, providing time for Xer-*cer* site-specific recombination to restore the plasmids to the monomeric state [6]. Rcd interacts with tryptophanase to stimulate indole production [7] and indole, in turn, blocks cell division and growth [8]. These discoveries encouraged us to explore whether it might be possible to induce quiescence by the direct addition of indole to the culture medium.

Here we describe the development of an indole-based Q-Cell system and its testing in shake-flask and fermenter culture. The Q-Cell system is covered by US and other patents (US 2009/0004700 A1: Chemical Induction In Quiescence In Bacteria: Jan. 1, 2009). The patents disclose the temperature-independent establishment of a quiescent shake-flask culture of E. coli W3110 *hns205* by the addition of indole or related compounds. An indole-induced quiescent culture is shown to be capable of producing recombinant proteins (cytokine hGM-CSF and LacZ). In this report we present an analysis of the induction of quiescence in shake-flask cultures by indole, isoquinoline or quinolone and demonstrate continued metabolic

activity through the production of EGFP. Moving beyond the patent disclosures we further describe in detail how the system can be scaled up to work in fed-batch fermenter culture and provide an analysis of the productivity of quiescent cultures under these conditions.

2. Materials and Methods

Strains and plasmids. W3110 is an *E. coli* K-12 strain [9]. An *hns*205::Tn*10* derivative of W3110 was constructed by P1 transduction from strain GM230 [5]. The *hns205*::Tn*10* allele was known originally as *osmZ205*::Tn*10*.

W3110 *hns*Δ93 was constructed by recombination with a PCR product containing 2 TGA stop codons immediately after codon 93 (CGT) to stop translation of the *hns* gene at the same place as the insertion of the Tn*10* in *hns205*. In addition the construct replaced *hns* codons 94-136 with a kanamycin resistance gene (*kan*). Oligonucleotide (Sigma-Genosys) primers H1P1 (5'-TTGCTGCCGTTAAATCTGGCACCAAAGCT AAACGTGCTCAG**CGTTGATGA**ATTCCGGGGATCCGTCGACC-3') and H2P2 (5'-GATTTTAAGCAAGTGCAATCTACAAAAGATTATTGCTTGATCAGGAAA TCTGTAGGCTGGAGCTGCTTCG-3') were used with pKD13 [10] as template to generate an 1,403 bp PCR product containing 2 stop codons and a kanamycin resistance cassette, flanked by flippase target sites (FRT) and 44 and 50 bp tails identical to chromosomal sequences adjacent to the target site (*hns* codons 94-136). PCR was performed with *Taq* DNA polymerase (New England Biolabs) and the following cycling conditions: initial denaturing at 94°C for 2 min; 30 cycles of denaturing at 94°C for 30 s, annealing at 59°C for 30 s, and extension at 72°C for 2 min; and a final extension at 72°C for 2 min. W3110 carrying pKD46 [10] was grown in 15 ml LB medium with carbenicillin and 10 mM L-arabinose at 30°C to an OD_{600} of 0.6 before being prepared for electroporation. PCR product digested with *Dpn*I (New England Biolabs) was drop dialysed, column purified (Qiagen), and electroporated into W3110 pKD46. After primary selection (30 μg ml^{-1} kanamycin), transformants were maintained on medium without antibiotic. They were streaked to single colonies once on non-selective agar at 37°C (the non-permissive temperature for pKD46 replication) and then tested for carbenicillin sensitivity to confirm loss of the helper plasmid. The structure of *hns*Δ93 was confirmed by PCR.

Asparaginase expression was from pMALasp, a derivative of vector pMAL-p2X (New England Biolabs) containing the asparaginase-*pelB* fusion from plasmid pPNHAsp [11].

Transformants were selected on LA plates [12] incubated at 37°C. When appropriate, media were supplemented with ampicillin (50 μg ml^{-1}), carbenicillin (100 μg ml^{-1}), kanamycin (30 μg ml^{-1} in shake flask experiments, 10 μg ml^{-1} in fermenter experiments), tetracycline (10 μg ml^{-1} in liquid culture, 12.5 μg ml^{-1} in agar), and IPTG (1 mM). All reagents and antibiotics were obtained from Sigma-Aldrich.

Growth curves of indole-induced quiescence. A single *E. coli* colony was picked from a selective plate and inoculated into 10 ml Luria-Bertani Medium (LB) [13] containing the appropriate antibiotic. This was incubated overnight at 37°C with shaking. Two hundred μl of overnight culture was inoculated into 20 ml LB containing the appropriate antibiotic in 50 ml conical flasks, and grown at 37°C in a shaking water bath to an optical density at 600 nm (OD_{600}) of between 0.2 and 0.3. At this point, 2 ml of culture was inoculated into 18 ml pre-warmed LB containing the appropriate antibiotic and an appropriate volume of indole, quinoline, or isoquinoline stock solution (0.5 M in ethanol) to give the desired final concentration. Growth of the cultures was monitored by sampling at intervals and measuring absorbance at 600 nm. For EGFP yield experiments, pNER31 [14] was introduced into W3110*hns*-205::Tn*10* or W3110*hns*Δ93 by electroporation with a Gene Pulser electroporator (Bio-Rad Laboratories Ltd., Hemel Hempstead, England) in accordance with the manufacturer's instructions.

Fermenter growth conditions. A single colony of W3110*hns*Δ93 pNER31 was picked from a selective plate and inoculated into 10 ml LB medium containing kanamycin (10 μg ml^{-1}) and ampicillin (50 μg ml^{-1}). This was incubated at 37 °C for 16 h at 200 rpm in a rotary shaker to produce the primary inoculum. The primary inoculum was used to inoculate 4 flasks, each containing 50 ml Terrific Broth (TB) [15] containing kanamycin and ampicillin. The cultures were incubated overnight and used to inoculate the fermenter (t = 0). Fermentation was carried out in a 5-litre fermenter (BioFlo 310, New Brunswick Scientific, USA) with a working volume of 2.5 L, equipped with pH, temperature, and dissolved oxygen (DO) control. pH was maintained at 7 by addition of acid or base through automatic feedback control, and temperature was maintained at 37°C. DO was maintained at 40% of air saturation by controlling the agitation rate through a Proportional-Integral-Derivative controller (PID controller). The growth medium was TB supplemented with 10 mM $MgSO_4$ containing kanamycin (10 μg ml^{-1}) and ampicillin (50 μg ml^{-1}), with a continuous feed.

Sigma Antifoam 289 (catalog no. A-5551; Sigma-Aldrich, Poole, England) was added when required. At OD_{600}=8, a feed (12% yeast extract, 12% tryptone, 18% glycerol (all w/v), 10 mM $MgSO_4$, 10 μg ml^{-1} kanamycin, and 250 μg ml^{-1} ampicillin) was started at 18.2 ml h^{-1} and increased exponentially to achieve a specific growth rate of 0.35 h^{-1}. The exponential substrate feed was continued until one hour after starting the feed of indole, after which it was held constant. Indole (0.5 M in ethanol) was added from t = 3-5 h using a pump at a constant feed rate (0.45 ml min^{-1}). This slow addition of indole allowed the culture to stabilize and, since the substrate feed rate was constant at this point, the agitation rate (RPM), pH and exit gas oxygen and carbon dioxide concentration tended to stabilize with time. The onset of indole toxicity could therefore be deduced from a fall in pH and RPM (which was used to control the dissolved oxygen in cascade mode). This was followed by a decline in oxygen uptake and carbon dioxide evolution rate as measured by the exit gas analyzer. Indole addition was stopped at this point. We could thus fine-tune the amount of indole to be added in each bioreactor run; the final indole concentrations varied between 5 and 7 mM. IPTG (1 mM) was added manually to induce EGFP expression. Growth of the cultures was monitored by measuring OD_{600} and 2 ml culture samples were taken at the same time for EGFP fluorescence analysis.

Enhanced green fluorescent protein (EGFP) fluorescence analysis. Cells from 2 ml of culture were harvested by centrifugation (Eppendorf Minispin Plus microfuge, 14,500 rpm, 1 min). The pellet was resuspended in 2 ml of a solution of 1 × PBS. The EGFP fluorescence was quantified using a fluorescence spectrophotometer (Cary Eclipse, Varian, Austria, Data mode: Fluorescence, Scan mode: Emission, X Mode: Wavelength, Start: 498.00 nm, Stop: 648.00 nm, Ex. Wavelength: 488.00 nm, Ex. Slit: 2.5 nm, Em. Slit: 5 nm, Scan rate: 600.00 nm min^{-1}, Data interval: 1.0000 nm, Averaging Time: 0.1000 s, Excitation filter: Auto, Emission filter: Open, PMT voltage: Medium (600 V), Corrected spectra: OFF, Peak Threshold: 50.000).

3. Results

Cellular quiescence can be induced by exogenous indole

To explore the possibility of inducing quiescence by adding indole directly to the growth medium we investigated the effect of 0-5 mM indole on cultures of *E. coli* W3110 and its *hns205* derivative. A single colony of W3110 or W3110 *hns205* was inoculated into 10 ml LB medium (containing tetracycline (10 μg ml^{-1}) for W3110 *hns205* only). The culture was incubated overnight at 37 °C with shaking. Two hundred μl of each overnight culture was inoculated into 20 ml LB (containing tetracycline where appropriate) in a 50 ml conical flask, and incubated at 37 °C in a shaking water bath until the culture reached OD_{600} = 0.2-0.3. Two ml of culture was inoculated into 18 ml pre-warmed LB containing indole (0-5 mM) and tetracycline where appropriate. A 1% ethanol control was included to ensure that the ethanol used as the indole solvent was not affecting growth (cultures with 5 mM indole contain 1% ethanol). Growth of the cultures was monitored over the subsequent 24 h (Fig. 1).

The W3110 no-indole culture and the ethanol control grew normally, reaching stationary phase at OD_{600} = 3.6 and 3.5, respectively (Fig. 1A). The addition of 2 or 3 mM indole had little effect on growth, except that the stationary phase density was slightly reduced. In contrast, 4 and 5 mM indole caused significant growth inhibition. When the experiment was repeated with the *hns205* mutant the culture responded similarly to 2, 4 or 5 mM indole. However, the mutant showed a response to 3 mM indole that was distinct from the wild-type. Growth continued for approx. 7 h but thereafter the culture density remained constant, even though stationary phase had not been reached. This pattern of growth resembles that of quiescent cultures generated by over-expression of the Rcd transcript in an *hns205* strain [1]. The OD_{600} of cultures entering quiescence continues to increase for several hours because, although indole blocks cell division rapidly, there is residual growth in the absence of division, resulting in cell elongation (Supporting Information Online, Fig. S1).

The *hns205* allele contains a functional transposon and its potential instability may be undesirable in commercial applications. A stable alternative to *hns205* was therefore constructed by introducing two TGA stop codons into the *hns* gene of *E. coli* W3110 to terminate translation after amino acid 93 of H-NS. This allele was designated *hns*Δ93. The

response of W3110 *hns205* and W3110 *hns*Δ93 to indole was compared over the range 0-4 mM. In this experiment both strains carried pNER31, a multicopy plasmid that encodes enhanced green fluorescence protein (EGFP) under the control of a P_{lac} promoter. This was introduced so that fluorescent protein production could be used as an indicator of metabolic activity. Overnight cultures of W3110*hns205* pNER31 or W3110 *hns*Δ93 pNER31 were diluted 100-fold into 20 ml LB medium containing carbenicillin (to select for pNER31) and either tetracycline (W3110*hns205*::Tn*10*) or kanamycin (W3110 *hns*Δ93), and incubated at 37 °C until they reached OD_{600} = 0.2-0.3. At this point (*t* = 0) 2 ml of culture was inoculated into a series of flasks containing 18 ml pre-warmed LB medium containing appropriate antibiotics, indole (0-4 mM) and IPTG (1 mM to induce EGFP expression). Subsequent growth was monitored by measuring OD_{600}. Overall the two strains showed similar responses to indole. The *hns205* mutant (Fig. 2A) entered quiescence in response to 2.5 mM indole, while 3 mM indole was required to induce similar growth kinetics for *hns*Δ93 (Fig. 2B).

EGFP fluorescence was measured for both strains over the full range of indole concentrations. To normalise for differences in culture density, fluorescence (measured in arbitrary units: a.u.) was divided by OD_{600} to give the specific product yield. The specific product yield of the W3110*hns-205*::Tn*10* pNER31 indole-free control, and cultures supplemented with 0.5 or 1 mM indole, remained approximately constant throughout the experiment (Fig. 3A). At higher indole concentrations up to 2.5 mM, the specific product yield increased throughout the experiment. The maximum value (936 a.u./OD_{600}, compared to 304 a.u./OD_{600} for the no-indole control) was obtained for the culture that entered quiescence in response to 2.5 mM indole. No protein expression was detected in cultures containing 3 mM indole or above (not shown).

The product yield of W3110 *hns*Δ93 pNER31 cultures was lower than the corresponding *hns205* strain (Fig. 3B). The specific product yield of the untreated control and the 0.5 and 1 mM indole cultures changed little throughout the experiment. For cultures treated with 2-3 mM indole the specific product yield increased throughout the experiment and the maximum yield (465 a.u./OD_{600}, compared to 223 a.u./OD_{600} for the no-indole control) was obtained with the 3 mM indole culture that entered quiescence. At indole concentrations of 3.5 or 4 mM no protein expression was detected.

Q-Cells show no significant lysis and retain metabolic activity

To test whether significant cell lysis was associated with the establishment of quiescence, the culture supernatant was assayed for the intracellular enzyme glucose-6-phosphate dehydrogenase. No detectable activity was found and SDS-PAGE gels of culture supernatant were free of any protein bands that would be expected in the case of significant lysis (data not shown). As a further test, the asparaginase gene was cloned in pMAL-p2X vector along with the *pelB* signal sequence to achieve extracellular expression. Cultures were grown in shake flasks in Terrific Broth (TB) with 0.4% glucose. Quiescence was induced by addition of 3 mM indole and and asparaginase expression was induced with IPTG (1mM). Samples of the culture supernantant were run on SDS-PAGE gels and stained with coomasie to monitor asparaginase expression over time. A continuous increase in extracelluar asparaginase was observed for 8-10 h after induction (Fig. 3C). Protein accumulation continued after growth had stopped, demonstrating the sustained expression capacity of the quiescent culture. The extracellular protein gel did not show any contaminating host proteins, confirming that significant lysis was not taking place. Finally, to demonstrate metabolic activity of quiescent cells, the residual glucose was monitored and a continuous decline was observed in the quiescent culture (Fig. 3D), continuing for several hours after growth had ceased.

Quiescence can be induced by indole-related compounds

In previous (unpublished) studies of the effect of indole-related compounds on *E. coli* we found that 3 mM isoquinoline or quinoline strongly inhibited the growth of W3110. These compounds were therefore tested to see whether they could induce cellular quiescence.

A single colony of W3110 *hns*Δ93 pNER31 was picked from a selective plate, inoculated into 10 ml LB medium containing kanamycin and carbenicillin, and incubated overnight with shaking at 37°C. Two hundred µl of overnight culture was inoculated into 20 ml LB medium containing kanamycin and carbenicillin in a 50 ml conical flask, and incubated at 37°C in a shaking water bath until it reached OD_{600} = 0.2-0.3. Two ml aliquots of this culture were inoculated into flasks containing 18 ml pre-warmed LB medium containing kanamycin,

carbenicillin, IPTG (1 mM to induce EGFP expression) and either quinoline or isoquinoline (0-4 mM). Growth (OD_{600}) and EGFP fluorescence were monitored over the next 24-26 hours.

The growth and specific product yield of isoquinoline-treated cultures is shown in Fig. 4A-B. The culture without isoquinoline grew normally and had reached OD_{600} = 3.1 (stationary phase) after 24 h (Fig. 4A). Increasing concentrations of isoquinoline caused a corresponding inhibition of growth and, in the presence of 2-4 mM isoquinoline, cells entered quiescence after approx. 5 hours. The specific product yield of isoquinoline-treated cultures is shown in Fig. 4B. For all isoquinoline-treated cultures the specific product yield increased throughout the experiment, reaching a maximum of 585 a.u./OD_{600} for the 2 mM isoquinoline-treated culture, compared to 242 a.u./OD_{600} for the no-isoquinoline control (data are the average of three independent experiments).

The growth and specific product yield of quinoline-treated cultures is shown in Fig 4C-D. The control (no quinoline) culture grew normally and reached OD_{600} = 3.2 (stationary phase) after 26 h (Fig. 4C). As the concentration of quinoline was increased, a corresponding reduction in growth was observed. In the presence of 2-4 mM quinoline, growth stopped after approx. 5 hours and the cultures appeared to have entered quiescence. For all quinoline-treated cultures the specific product yield increased throughout the experiment (Fig. 4D). The maximum yield was obtained for the 3.5 mM quinoline-treated culture that reached 783 a.u./OD_{600} after 26 h, compared to 218 a.u./OD_{600} for the no-quinoline control (data are the average of three independent experiments).

Indole addition can induce cellular quiescence in the fermenter

In the next stage of our investigation we explored the establishment of quiescent *E. coli* in fed batch fermenter culture. Potential advantages of establishing quiescence in the bioreactor are the ability to achieve much higher cell density and the possibility of extending the period of metabolic activity by feeding concentrated substrate in fed batch mode. However the amount of indole that would be required to achieve quiescence was not immediately obvious. The high cell density and optimum culture conditions (in terms of dissolved oxygen and pH)

made us suspect that cells might tolerate a higher indole concentration than in shake-flasks, and this was confirmed in preliminary experiments (data not shown).

A series of preliminary experiments was conducted to determine the optimum conditions for indole addition, feed rate and feed composition. Cultures of W3110 *hns*Δ93 pNER31 were grown in a batch bioreactor in TB medium and indole was added to final concentrations in the range 2-5 mM at OD_{600}=5 (mid-log phase). After the addition of 2 or 3 mM indole there was a transient (approx. 30 min) decline in growth. However the cells recovered and reached a final OD_{600} in the range 16-20 which was similar to the OD_{600} of a control (no indole) culture. When the final indole concentration was 4 mM or higher, growth stopped completely and the metabolic activity of the culture (measured by oxygen uptake rate; OUR) declined to zero within 1 h. Apparently the cells had experienced some kind of indole-induced shock which prevented them from entering a metabolically active quiescent state. We concluded that indole should be added slowly to the fermenter to prevent indole shock and maximise the metabolic activity of the quiescent cells.

It was clear from the shake-flask experiments that OD_{600} continued to increase for some time after indole addition. Therefore if indole is added slowly and progressively to the fermenter it is likely to be difficult to assess when sufficient has been added to achieve quiescence. However in the fermenter it is possible to monitor on-line changes in metabolic activity that would indicate the onset of indole toxicity. Since the cells are grown in fed batch mode with a constant feed rate, oxygen uptake (estimated indirectly by the agitation rate) and pH tend to stabilize with time. The oxidant toxicity of indole reduces oxygen uptake and subsequently pH, since the excess carbon flow goes to acetate. It was therefore decided to add indole until the onset of toxicity was signalled by a simultaneous drop in oxygen uptake and pH. This strategy should keep the indole concentration at the higher end of the tolerable range (just below the toxic limit), ensuring complete quiescence. It is interesting to note that when the quiescent state was established in this way it persisted for more than 25 h. There was no need to restart the indole feed, even though the continuous feeding of concentrated substrate increased the culture volume by 50% and concomitantly reduced the indole concentration in the bioreactor.

The optimum feed rate had to be determined experimentally, since in the absence of growth it is impossible to use the concept of biomass yield to determine feeding rates. In the pre-quiescent phase, feeding of complex media (see Materials and Methods) was at an exponential rate, with a doubling every 2 h to achieve a specific growth rate of 0.35 h^{-1}. This feeding rate had been optimised previously to give the maximum specific growth rate, and hence better cellular health, without the production of acetic acid [16]. After the initiation of the indole feed, growth slowed and after 4 hours ceased completely as the cells entered the quiescent state. During this time the metabolic activity of the culture (measured by oxygen uptake and carbon dioxide evolution rate) increased initially and then remained approximately constant. Using this as an online indicator of feed requirement, the exponential growth medium feed was continued for 1 hour from the start of the indole feed and subsequently held constant. This strategy led to a steady rate of acetic acid formation and concomitant drop in pH that was adjusted by base addition. Thus it appears that while quiescent cells are able efficiently to take up substrate and channel it through the glycolytic pathway, the oxidative phosphorylation pathways are not so efficient. To check this, the feed rate was increased in a subsequent run and we observed a higher rate of acetate accumulation (coupled with higher requirement of base to control pH) with no change in the metabolic activity. The long-term viability of the culture was also compromised, possibly because of acetate build up in the culture. On the other hand a lowering of the feed rates did reduce acetate formation but did not improve cellular viability in the long term because the cells became nutrient starved.

A final set of preliminary experiments was conducted to determine the optimum feed composition. It was supposed that once biomass synthesis had stopped, the requirement for complex nitrogen would also be reduced, so feed composition should be changed appropriately to prevent build up of complex nitrogen in the medium. The effect of varying the ratio of glycerol and yeast extract/peptone in the feed was therefore explored. We observed a continuous decline in EGFP yields as the proportion of yeast extract/peptone was lowered, with fluorescence dropping almost to zero when a defined feed of glycerol and ammonia was used. Apparently the amino acid biosynthesis capability of quiescent cells is poor and they rely on an exogenous supply to synthesise proteins.

EGFP production is used to quantify the performance of indole-induced Q-Cells in fed-batch fermenter culture

Having established conditions for the establishment of quiescence in fed-batch fermenter culture, experiments were conducted to investigate the metabolic activity and longevity of quiescent W3110 *hns*Δ93 pNER31. As in the shake-flask experiments, EGFP expressed from plasmid pNER31 was used as an indicator of metabolic activity. To provide a basis for comparison, EGFP expression was first quantified in a control fed-batch culture without indole (Fig. 5A). The culture was induced with 1mM IPTG when OD_{600} reached 30. EGFP expression was monitored over time and a maximum value of 1100 a.u. (arbitrary units) was obtained. Subsequently EGFP fluorescence decreased, despite the continuing increase in the culture density that reached a maximum OD_{600} of 90 after 11 h. To establish quiescence an indole feed was initiated 3 h after the inoculation of the fermenter. Normal growth ceased after a further 2-3 h, with the final culture density typically within the range OD_{600}=25-50. EGFP expression from pNER31 was induced by the addition of IPTG. Fig. 5B shows a run where EGFP was induced approx. 1 h after the cessation of growth (final indole concentration 7.4 mM). EGFP fluorescence from the quiescent cells increased over the next 6 h, reaching approximately 1000 a.u. while the cell concentration remained constant at OD_{600}= 45. Fig. 5C shows the results of a run in which IPTG was added approx. 3h after the cessation of normal growth (final indole concentration 5.7 mM) and EGFP fluorescence increased linearly for 8h, tapering off to a maximum value of approx. 1300 a.u. There was thus no decline in protein expression capacity, even when induction was delayed until 3 h after the cessation of growth, so the retention of protein synthesis capacity in the absence of growth is clearly demonstrated.

4. Discussion

We have developed a new and simple method for generating non-growing but metabolically active quiescent *E. coli* (Q-Cells). Plasmid-encoded EGFP expression has been used to confirm that the cells remain metabolically active although there has been no attempt to optimise protein expression and we do not envisage these cells primarily as recombinant protein factories (see below).

Q-Cells were previously generated by expressing the regulatory transcript Rcd in an *hns205* mutant host [1]. Proof-of-principle experiments established that resources are converted more efficiently into product in Q-Cells than in a bacterial cell factory that continues to grow and divide [4]. However Rcd-induced Q-Cells were not without their problems. Their usefulness was limited by their relative technical complexity and the requirement for a temperature shift to initiate the transition into the quiescent state. When original system was developed, the mechanism of action of Rcd was still unknown. The subsequent discovery that Rcd activates tryptophanase and stimulates indole biosynthesis [7] suggested that it might be possible to establish quiescence more simply. Here we demonstrate that the direct addition of indole to the growth medium of a culture of *E. coli hns205* (or a strain carrying the related allele *hns*Δ*93*) provides a simple method to induce quiescence with no requirement for heat shock. The common requirement of both Rcd- and indole-induced Q-Cells for specific *hns* alleles suggests that quiescence is most likely induced by the same mechanism in both cases.

The *hns*Δ*93* derivative of W3110 lacks the transposon insertion of *hns 205* and, as a stable construct, might be more be appropriate for commercial applications. However, we noted that EGFP expression was lower in *hns*Δ*93* compared to *hns205*. We have no clear explanation for this. It is possibly because translation of the *hns*Δ*93* construct terminates precisely after amino acid 93. In contrast with *hns205*, 93 amino acids of H-NS are translated but the ORF continues into the inserted Tn10 for several amino acids until a termination codon is reached. The truncated H-NS products in the two strains therefore differ at their C-termini.

The concentration of indole required to establish quiescence in a shake-flask culture of *E. coli* lies in the range 2.5-3 mM, depending upon which *hns* allele is present. If this concentration is exceeded, even by as little as 0.5 mM, the cells still enter a non-growing state but are

metabolically inactive. We do not know the reason for this loss of synthetic capacity but it may be a consequence of DNA gyrase inhibition by indole at higher concentrations [17]. It would therefore be important to determine the optimum indole concentration for each combination of host strain and growth medium. When we explored the use of alternative, quiescence-inducing chemicals we found that isoquinoline and quinoline established quiescence with similar kinetics to indole. These cultures maintained their metabolic activity when the minimum concentration for quiescence was exceeded by as much as 1-2 mM. Furthermore, quinoline and isoquinoline-induced quiescent cells showed a higher product yield in shake-flask culture than indole induced quiescent cells (an increase of approx. 2-fold for quinoline over indole). A possible disadvantage to using quinolone and isoquinoline in preference to indole for large-scale applications is cost (approx. 5-fold higher in 2010).

Interestingly we found that the response of *E coli* to indole in fed-batch fermenter culture was quantitatively different from shake-flasks. Significantly higher concentrations (5-8 mM) were required to halt growth in the fermenter but the cells remained metabolically active. Both the production of EGFP and the fact that the substrate levels in the bioreactor did not rise in spite of a constant feed of concentrated medium, provided evidence for the continued metabolic activity of the cells. There was a slow decline in pH (adjusted automatically by the addition of base) demonstrating that a certain amount of acetate formation took place in the quiescent phase. Clearly Q-cells require a substrate feed to maintain metabolic activity and since no biomass is formed, the metabolic flux is potentially diverted to product formation.

When measuring EGFP production in the fermenter, we noted that the rate of increase in Q-Cells was lower than the control culture. However, the maximum specific product yield (a.u. OD^{-1}) was significantly higher than the control culture even when induction was delayed until 3 h after the cessation of growth. The slower rate of increase in EGFP fluorescence after 8 h of quiescence (Fig. 5C) possibly reflects the saturation of the cytoplasm with functional protein. Clearly quiescent cultures allow the uncoupling of growth and product formation for a recombinant protein which otherwise is known to have a tightly growth associated product formation kinetics. From the increased acid production observed at higher feed rates and the maintenance of low residual substrate concentrations we infer that the glycolytic pathway flux remains unaffected by the onset of quiescence. Using the concept of maintenance requirements we could calculate the metabolic activity of the Q-cells in terms of the

maintenance coefficient ‘m’ which represents the grams of substrate consumed for non-growth purposes per gram of biomass per hour. Thus non-growing Q-cells have an ‘m’ of 0.2 h^{-1} which declines to 0.1 h^{-1} in 10 hours of quiescence. This suggests that Q-Cells may be an attractive host for the production of metabolites where high product yields are important and especially those that derive from the end products of the glycolytic pathway, since this pathway remains active in Q-Cells and its flux can be increased simply by increasing feed rates. In recent years, the development of *E. coli* as a vehicle for biosynthesis of heterologous metabolites has seen considerable progress [18]. Nearly all major classes of metabolites have been synthesized and engineered in *E. coli*, including macrolides [19], cyclic peptides [18], terpenes [20], alkaloids [21], and bacterial aromatic polyketides [22]. Since plasmid-borne genes are highly expressed in Q-Cells it should be straightforward to modify Q-Cell metabolism to convert glycolytic pathway metabolites to desired metabolites by introducing plasmids carrying genes encoding the relevant enzymes.

In conclusion, we have developed a simple and direct method for the generation of metabolically active, quiescent *E. coli* in shake-flask or fermenter culture. Quiescence can be achieved by the addition of the natural bacterial signalling molecule, indole, to the culture medium or by the use of the indole related compounds isoquinoline or quinolone. When EGFP expression was used as an indicator of metabolic activity, quinolone-treated cells were most productive but, given the range of potential applications for Q-Cells including biofuels and cellular metabolites, it would be wise to compare the effectiveness of the different quiescence-generating compounds on a case-by-case basis.

Potential conflict of interest: The Q-Cell system described in this report is covered by US, European and other patents (see: US 2009/0004700 A1: Chemical Induction In Quiescence In Bacteria: Jan. 1, 2009). DKS is named as an inventor on a patent covering the Q-Cell system. The IP is owned by Cambridge Enterprise (Cambridge University). DKS stands to benefit from earnings arising from exploitation of Q-Cells under the standard Cambridge University policy for income distribution. All other authors declare no financial or commercial conflict of interest.

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Figure Legends

Figure 1 Induction of *E. coli* quiescence by indole. A: *E. coli* W3110, B: *E. coli* W3110 *hns205*::Tn*10*. Bacteria were cultured in the presence of 0-5 mM indole. A control was included to ensure that ethanol used as the solvent for indole was not affecting the cells. Growth was monitored by measuring OD_{600} of the cultures. Results are representative of three independent experiments. The line in (B) indicates the entry into quiescence of W3110 *hns205*::Tn*10* treated with 3 mM indole.

Figure 2 The induction of quiescence in W3110 carrying *hns* alleles *hns205*::Tn*10* or *hns*Δ93. A: W3110 *hns205*::Tn*10* pNER31. B: W3110 *hns*Δ93 pNER31. Bacteria were cultured in LB medium supplemented with indole (0-4 mM). Growth was monitored by measuring OD_{600} of the cultures. Results are representative of three independent experiments. The results corresponding to 2.5 and 3 mM indole-treated W3110 *hns205*::Tn*10* pNER31 and W3110 *hns*Δ93 pNER31, respectively, are connected by a line to show the establishment of quiescence after approx. 5 h.

Figure 3 Product yields of quiescent cells in shake-flask culture. The specific EGFP yields of W3110*hns-205*::Tn*10* pNER31 **(A)** and W3110*hns*Δ93 pNER31 **(B)** were calculated by dividing EGFP fluorescence by OD_{600}. Data plotted are the mean of three independent assays. The error bars represent standard deviation. (C) SDS-PAGE of 10 μl supernatant samples showing extracellular expression of L-asparaginase (arrowed) from quiescent W3110 *hns*Δ93 pMALasp. Indole (3 mM) and IPTG (1 mM) were added at time t = 0, 4 h after inoculation of the culture. Samples were taken over the period t = 0-10 h, track M contains molecular weight markers. (D) Growth and glucose consumption (green line) of W3110 *hns*Δ93 pMALasp quiescent cells. The control culture (blue line) was grown in TB medium without induction while the quiescent culture (red line) was induced with 3 mM indole and 1 mM IPTG 4 h after inoculation.

Figure 4 The induction of quiescence by isoquinoline and quinolone: growth and specific EGFP yields of W3110*hns*Δ93 pNER31. W3110 *hns*Δ93 pNER31 was cultured in LB medium containing 0-4 mM isoquinoline (A) or quinolone (C). Growth was monitored by measuring OD_{600} of the cultures. Results are representative of three independent experiments. Data points from the 2 mM isoquinoline culture and the 3.5 mM quinoline culture are connected by lines to show entry into quiescence after approx. 5 h. Specific product yield was calculated by dividing EGFP fluorescence intensity by OD_{600} for the isoquinoline (B) or quinolone (D) containing cultures. Data plotted are the mean of three independent assays. The error bars represent standard deviation.

Figure 5 Establishment of quiescence in fed-batch fermenter culture. The culture density (OD_{600}) and EGFP fluorescence were monitored for W3110 *hns*Δ93 pNER31 grown in TB medium supplemented with $MgSO_4$ (10 mM). The results are the means of three samples for each time point. The maximum variation observed was 2.5%. (A) No-indole control. IPTG (1 mM) was added at t = 6.25 h to induce EGFP production. (B) Quiescent culture (8.1 mM indole final concentration). Indole was added from t = 3 h. IPTG was added at t = 7 h; 1 h after growth had stopped. (C) Quiescent culture (5.5 mM indole final concentration). Indole was added from t = 3 h. IPTG was added at t = 8 h; 4 h after growth had stopped.

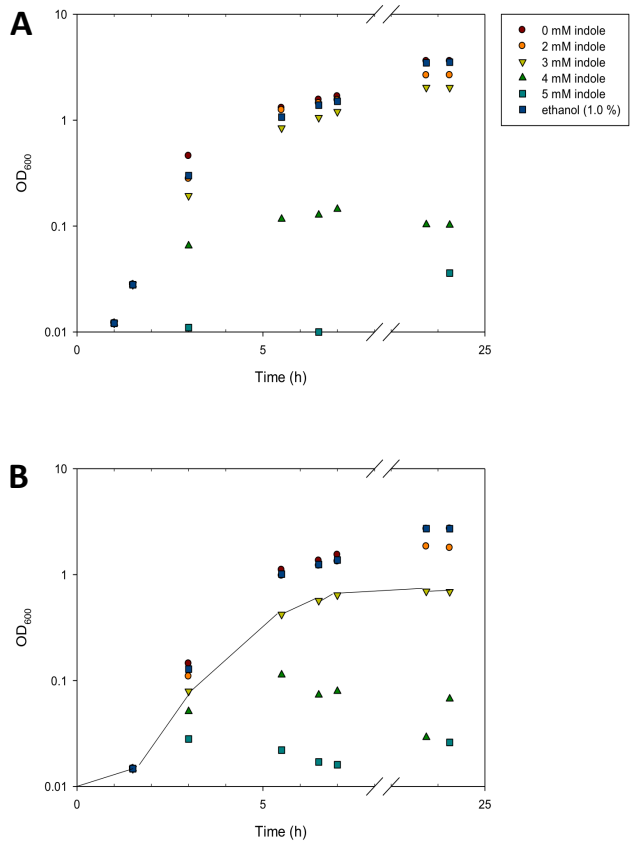
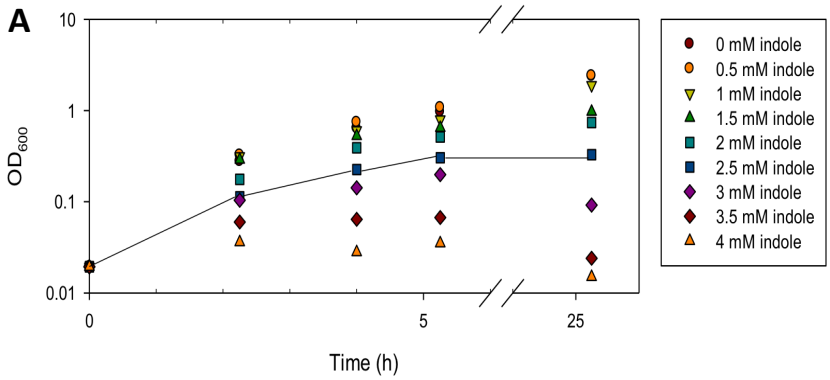

A
B
10
1
0.1
0.01
OD600
0
5
25
Time (h)
0 mM indole
2 mM indole
3 mM indole
4 mM indole
5 mM indole
ethanol (1.0 %)

Figure 1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1
0.1
0.01
OD600
0
5
25
Time (h)
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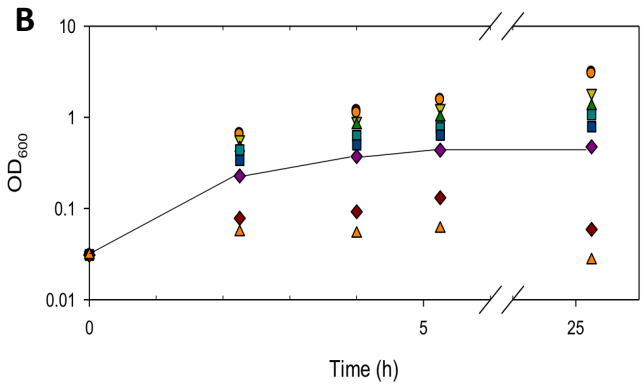
2 mM indole
2.5 mM indole
3 mM indole
3.5 mM indole
4 mM indole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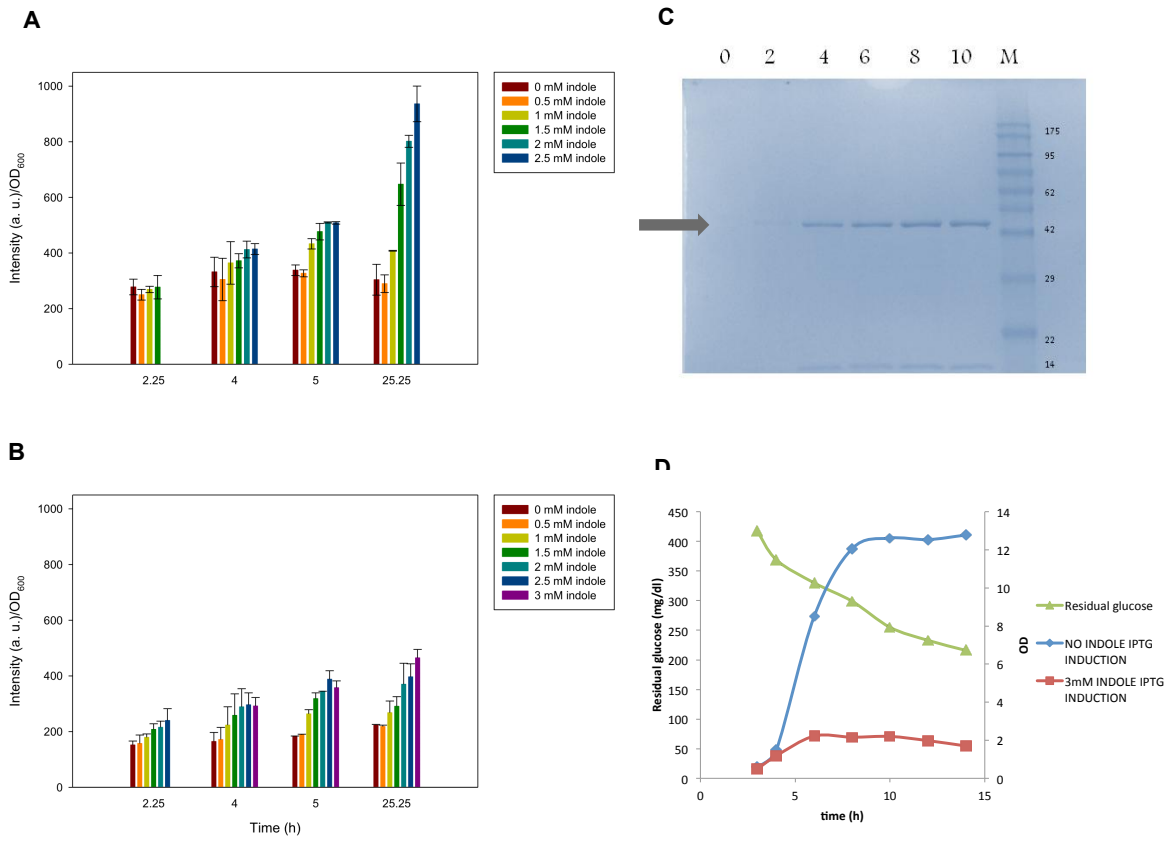
10
1
0.1
0.01
OD600
0
5
25
Time (h)

Figure 2

Figure 3


A
Intensity (a. u.)/OD600
0 mM indole
0.5 mM indole
1 mM indole
1.5 mM indole
2 mM indole
2.5 mM indole
2.25
4
5
25.25
C
0 2 4 6 8 10 M
175
95
62
42
29
22
14
B
Intensity (a. u.)/OD600
0 mM indole
0.5 mM indole
1 mM indole
1.5 mM indole
2 mM indole
2.5 mM indole
3 mM indole
Time (h)
D
Residual glucose (mg/dl)
OD
time (h)
Residual glucose
NO INDOLE IPTG INDUCTION
3mM INDOLE IPTG INDUCTION

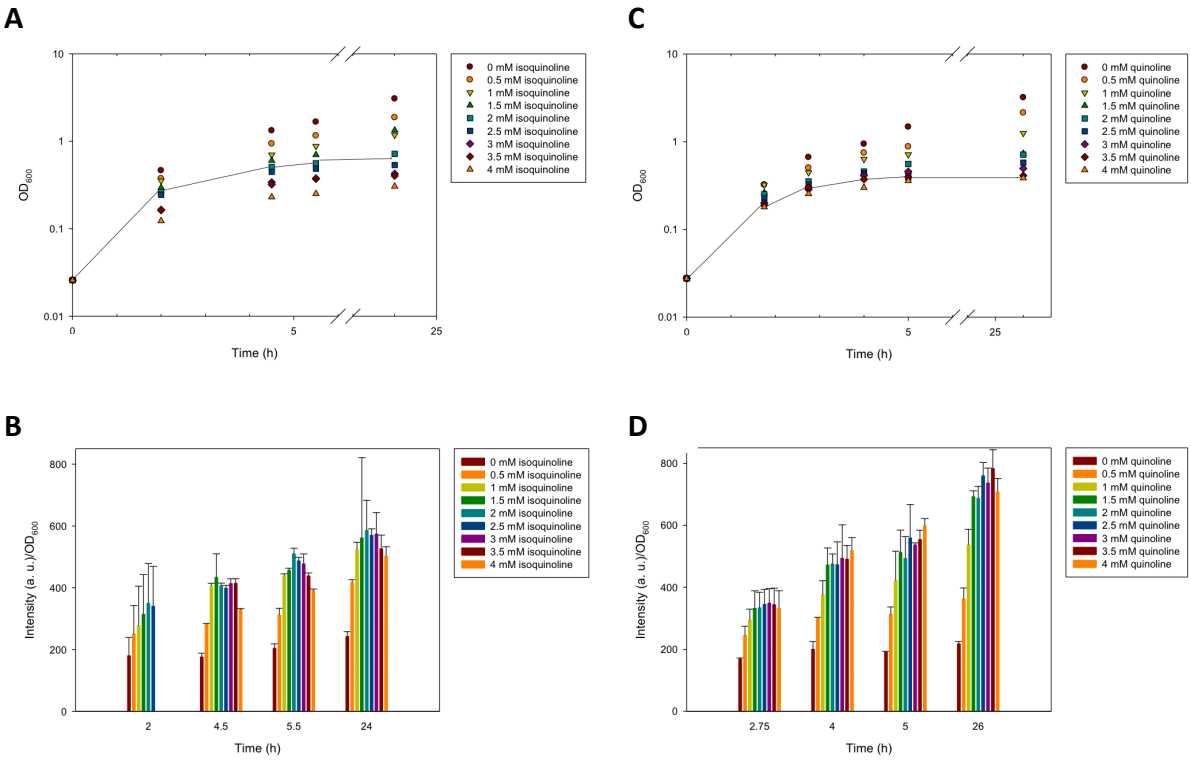

A
C
B
D
OD600
Time (h)
Intensity (a. u.)/OD600
0 mM isoquinoline
0.5 mM isoquinoline
1 mM isoquinoline
1.5 mM isoquinoline
2 mM isoquinoline
2.5 mM isoquinoline
3 mM isoquinoline
3.5 mM isoquinoline
4 mM isoquinoline
0 mM quinoline
0.5 mM quinoline
1 mM quinoline
1.5 mM quinoline
2 mM quinoline
2.5 mM quinoline
3 mM quinoline
3.5 mM quinoline
4 mM quinoline

Figure 4

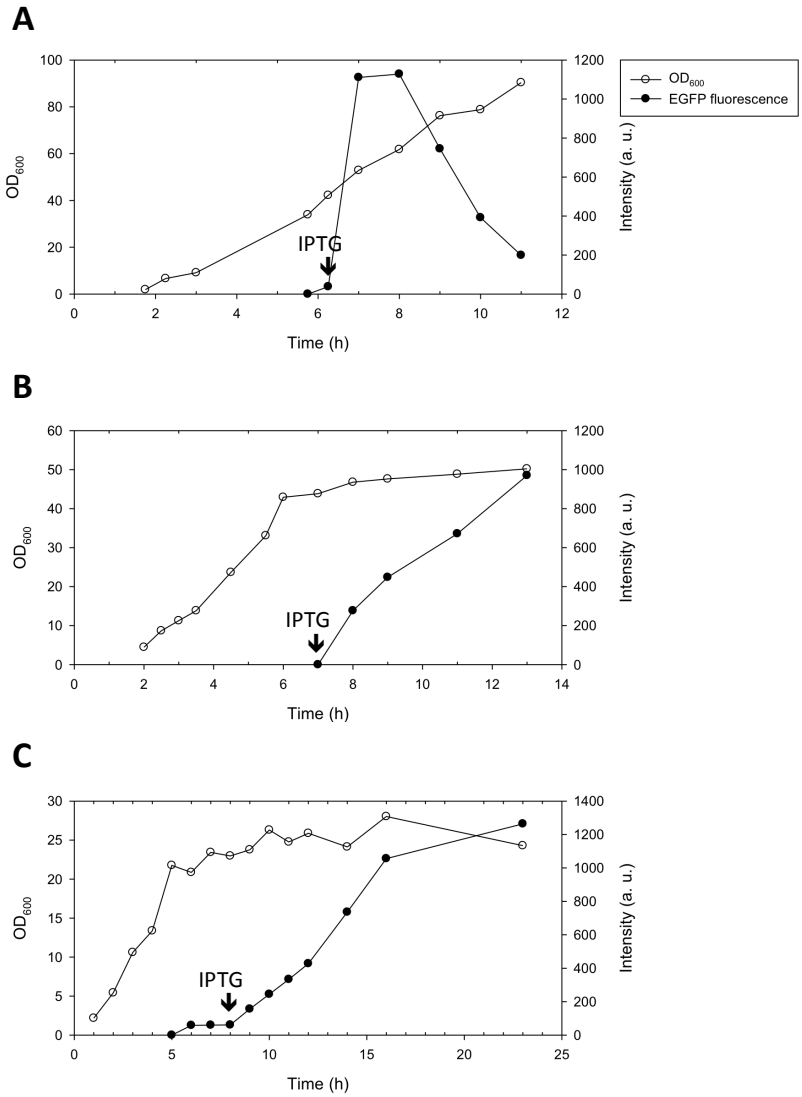
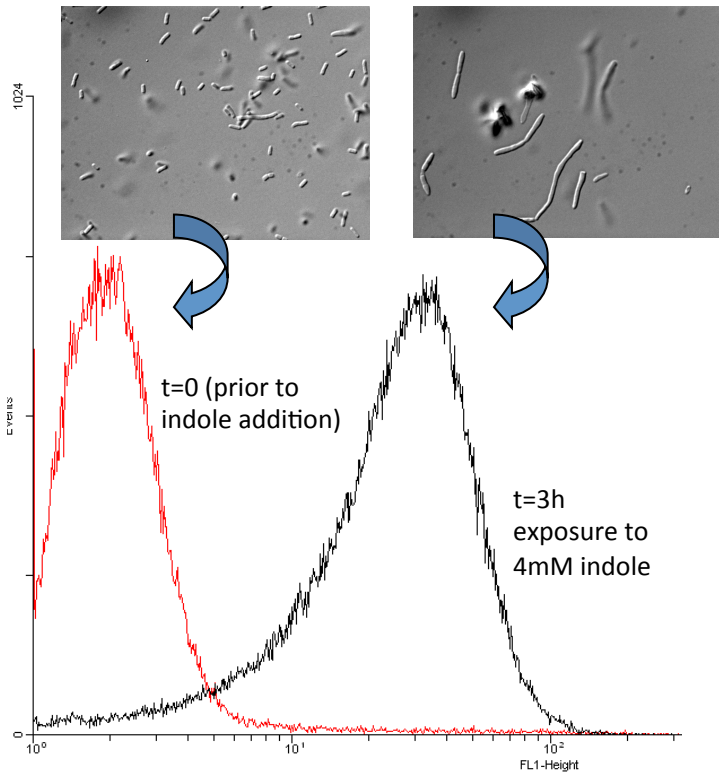


Figure 5



Supporting Information Online. Figure S1: Growth continues after cell division has been blocked by indole. A culture of *E. coli* DS941 growing exponentially in LB was sampled at OD600=0.1 and again after 3h exposure to indole (4 mM). Cell sizes were compared by phase contrast microscopy and by flow cytometry (samples were stained using BacLight green fluorescent stain, Invitrogen).