A quiet revolution in the bacterial cell factory

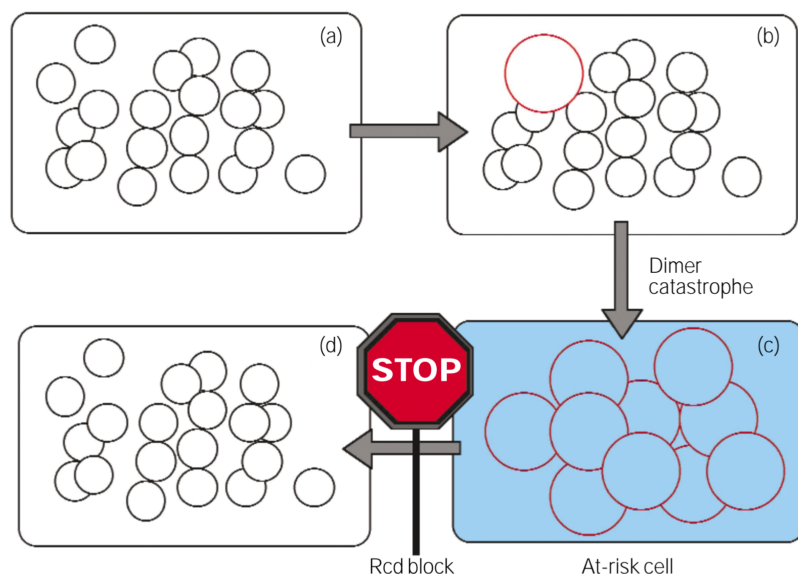
David Summers

David Summers describes the development of a novel Quiescent Cell Expression System which has commercial potential for the production of proteins.

The bacterial cell factory

Some recombinant proteins are very easy to produce in *Escherichia coli*. With the gene expressed from a strong promoter one could virtually culture the cells in a bucket, stir them with a stick and still obtain a good yield. This isn't always true, however, and problems can be associated with poor yield, mis-folding of the polypeptide backbone, precipitation of protein into inclusion bodies and poor biological activity. As genomics gives way to proteomics and the number of proteins requiring expression grows rapidly, the number of problem proteins is increasing too. Sometimes the solution is familiar from economics: export production to another Kingdom where it is cheaper or more efficient. There is no question that non-bacterial cell factories, such as the yeast *Pichia* or insect cells, have their staunch supporters. Mammalian cell culture also provides an attractive alternative to bacteria for human protein expression, especially when post-translational modification such as glycosylation is required. Nevertheless, recombinant protein expression in bacteria remains the most desirable option, not least because of the cheapness and ease of bacterial culture compared to any of its eukaryotic competitors.

Three decades of protein expression in *E. coli* have produced many improvements over the 'bucket and stick' approach. Important advances include the development of promoters such as P_{BAD} and the T7 polymerase-specific promoter which can be effectively regulated. Often it is more important to turn off the promoter tightly than to have a high maximum expression level. Effective repression allows product expression to be delayed to a later stage in the culture, which is particularly important where it harms the bacterial host. Product yield can also be increased by modifying the ribosome-binding site and, where the genetic code is redundant, replacing codons which are rare in bacteria with more common alternatives. Improvements in protein folding have been achieved by expressing the product slowly at a lower temperature, and sometimes by simultaneously over-expressing chaperones which reduce mis-folding and precipitation of the recombinant protein. Ingenious and effective though these modifications are, they are all add-ons to the basic bacterial cell factory. In recent years my research group has tried to take a more radical approach, investigating the possibility of rebuilding the cell factory from the ground up.



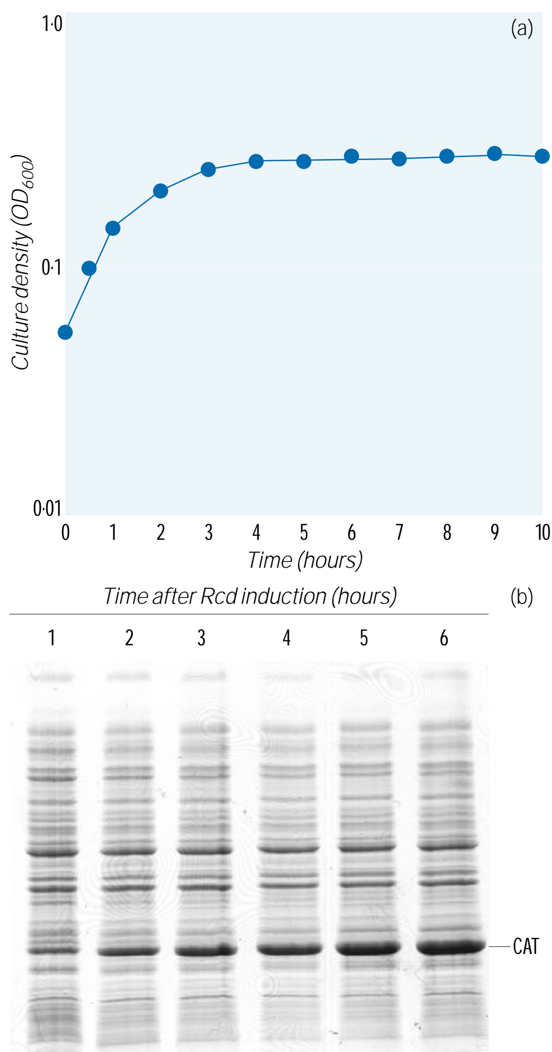
BELOW: **Fig. 1.** How ColE1 survives the dimer catastrophe. The majority of *E. coli* cells carrying plasmid ColE1 contain only plasmid monomers (a). Occasionally a dimer forms by homologous recombination (b) and this dimer out-replicates its monomeric cell-mates. The resulting 'dimer catastrophe' means that some descendants of the cell in which the first dimer formed contain only plasmid dimers (c). The Rcd transcript which is expressed in these at-risk cells prevents their division. Resolution of the dimers to monomers by site-specific recombination at the plasmid *cer* site (d) leads to lifting of the Rcd-imposed block and the restoration of normal growth. COURTESY DAVID SUMMERS

A novel approach to the cell factory

What would be some of the features of an ideal bacterial cell factory? Since in all Kingdoms reproduction is a serious distraction from other activities, it would surely make sense to produce recombinant protein in non-growing cells. In the case of bacteria this would allow the channelling of nutritional resources into product rather than into unwanted biomass. This is not a new idea but it is difficult to stop bacteria growing without limiting resources, and starving your workforce is hardly the way to get the best out of them. Another desirable objective would be to focus cellular resources on product gene expression. It is hardly efficient for the product gene to be one of hundreds all clamouring for the attentions of the transcriptional and translational machinery, and the complex apparatus required for protein folding and export. Ideally, we should give priority to product gene expression. To the microbiologist stopping growth without resource limitation and persuading the cell to concentrate on the expression of a single gene may seem like a tall order. However, in the eukaryotic world this is not at all a radical idea. After all, in the human body the expression of specialist proteins is the province of cells which have ceased division and entered the quiescent G_0 state. This allows them to limit gene expression and concentrate their resources on specialized products. In the development of the *E. coli* Quiescent Cell Protein Expression System (Q-Cells) we have tried to apply the 'best practice' learned from eukaryotes to the bacterial cell factory.

The Q-Cells system has its roots in the early 1990s. Michaela Sharpe, a graduate student in the laboratory, was looking at aspects of plasmid inheritance. She became interested in a 70 nucleotide RNA (known as Rcd) encoded by the multicopy plasmid ColE1. This molecule was expressed when the formation of plasmid multimers threatened to disrupt the proper transmission of the plasmid to daughter cells (Fig. 1). Her work suggested that under normal circumstances Rcd expression delays cell division, but she also found that at higher concentrations it could trap cells permanently in the pre-divisional state.

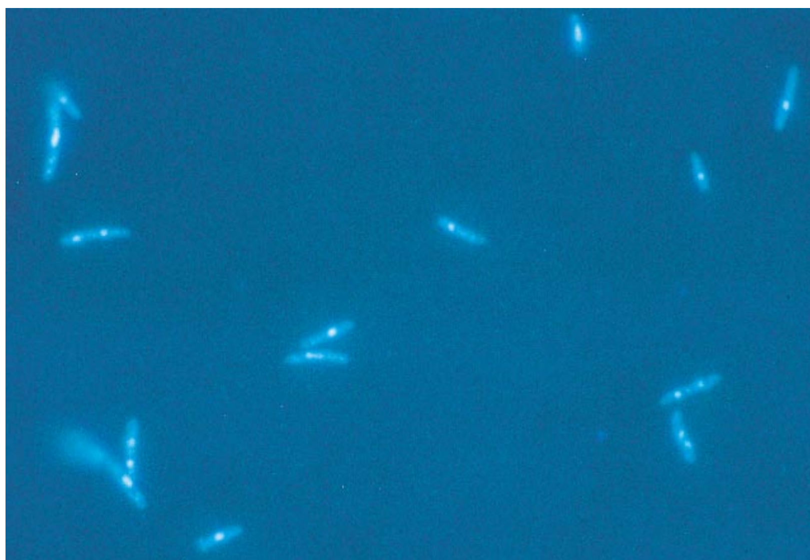
Cells trapped in the pre-divisional stage by Rcd over-expression are not resource-limited, and we hoped they might be useful as cell factories. Michaela Sharpe's work had been carried out with cells grown on solid medium, so before we could go any further we needed to reproduce the effects of Rcd over-expression in broth. This seemed a trivial matter but, to our dismay, Rcd over-expression in broth culture merely reduced the growth rate and the cells eventually struggled into stationary phase. These unhappy bacteria were very poor prospects as cell factories. We wondered why the physical structure of the growth medium should affect the response of the cells to Rcd and began to think about physiological differences between cells grown on different media. If it were possible to modify the physiology of broth-grown cells to resemble that of cells grown on a solid medium, it might be possible to achieve growth-arrest in response to Rcd over-expression. Around this time there was considerable interest in growth-phase-related changes in concentration of global regulators of gene expression such as H-NS, IHF and Fis. It seemed possible that if their concentrations also varied between broth and solid medium, this might be responsible for the different effects of Rcd. Finding that altering the level of Fis in broth-grown cells had no effect on their response to Rcd, we turned our attention to H-NS. It had been reported that the H-NS concentration was higher in slow-growing or stationary-phase cells, so it seemed plausible that increasing H-NS in broth-grown cells might mimic the effect of growth on solid medium. Unfortunately H-NS over-expression makes cells difficult to culture, so Duncan Rowe, who had come to the laboratory as a post-doc to work on protein expression, decided to do a control experiment first and looked at the effect of reducing the H-NS concentration. To our surprise over-expression of Rcd in an *hns205* mutant strain led to a complete cessation of growth within 2 to 3 hours and the cells entered a quiescent state (Fig. 2a). Subsequently we discovered that we had been extremely lucky to use the *hns205* allele, which produces an N-terminal fragment of H-NS. Several other mutations which were tested subsequently, including a null allele, failed to enter quiescence in response to Rcd expression.



LEFT: **Fig. 2.** The establishment of quiescence. (a) A culture of *E. coli hns-205* grows normally in the absence of Rcd expression. However, over-expression of the transcript (at $t=0$) leads to an immediate reduction in growth rate and entry into the quiescent state in 3–4 hours. (b) Expression of plasmid genes continues in quiescent cells. In this case plasmid-encoded CAT protein increases against a background of chromosome-encoded proteins. COURTESY DAVID SUMMERS

Development of the Quiescent Cell Expression System

In our early experiments with cells in broth culture, Rcd was expressed from a plasmid which also carried a chloramphenicol resistance gene. When we analysed the protein composition of cells made quiescent by over-expression of Rcd (called Q-Cells for convenience) we discovered that the CAT protein (the resistance gene product) accumulated to very high levels (Fig. 2b), sometimes as much as 40% of total cell protein. In contrast there was little evidence of chromosomal gene expression in these cells. It seemed that Q-Cells were concentrating on plasmid gene expression. The reason for this odd but useful reallocation of resources became apparent when we examined Q-Cells by DAPI fluorescence microscopy (Fig. 3). DAPI stains DNA and we discovered that the bacterial nucleoid (i.e. the chromosome and associated protein) was highly condensed in Q-Cells. Presumably, condensation of the nucleoid severely inhibits expression of chromosomal genes, in much the same way that heterochromatin formation in eukaryotes results in global repression of transcription. The observation also provided a clue about how the *hns205* allele makes broth-grown cells sensitive to Rcd. H-NS is nucleoid-associated and is likely to be involved in changes in nucleoid structure during chromosome partition. If the N-terminal fragment produced by *hns205* is still nucleoid-associated but functions inappropriately, this could lead to irreversible condensation of the nucleoid.



ABOVE:
Fig. 3. The anatomy of the quiescent cell. DAPI-stained quiescent *E. coli* cells are longer than normal *E. coli* cells and contain highly condensed nucleoids which appear as brightly staining spots within the cells.
COURTESY DAVID SUMMERS

Taking Q-Cells to market

Q-Cells fulfilled many of our criteria for the ideal cell factory, but could they form the basis of a useful protein expression system? We needed to show that the system was sufficiently robust to be exported from the laboratory to the commercial environment. During her tenure of a BBSRC CASE studentship with AstraZeneca, Elisabeth Watson was able to show that the system functioned well at the pilot-plant scale, and was not unduly sensitive to changes in medium composition or culture density. Encouraged by this Drs Duncan Rowe and K.-J. Mukherjee have been working in collaboration with AEA and the Cambridge University Department of Chemical Engineering, and have successfully scaled-up the system into small fermenters.

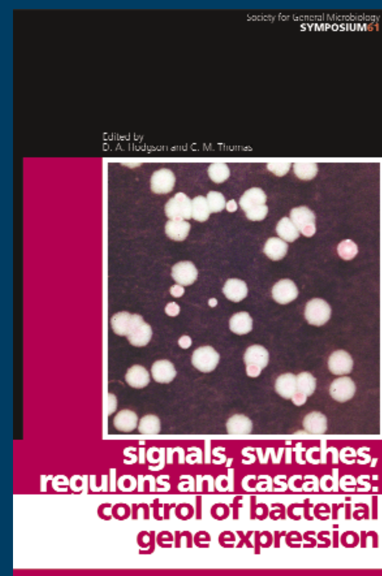
The Q-Cell system has been protected by international patents, and in 1999 a university spin-off company, Cambridge Microbial Technologies, was set up to finance its technical and commercial development. In collaboration with BTG International we have established relationships with several biotechnology companies with whom we are exploring the potential of Q-Cells to express a variety of recombinant proteins, including antibody fragments and cytokines. Basic research in the Cambridge University Department of Genetics into the physiology of Q-Cells and the development of customized vector and promoter systems is currently supported by the BBSRC.

● *Dr David Summers is a Senior Lecturer in the Department of Genetics at Cambridge University, Downing Street, Cambridge CB2 3EH, UK, and a Fellow of Gonville and Caius College.*
Tel. 01223 333991; Fax 01223 333992
email dks11@cam.ac.uk

SGM Symposium Vol. 61 review

Signals, Switches, Regulons and Cascades: Control of Bacterial Gene Expression

SGM Symposium Vol. 61
Edited by D.A. Hodgson & C.M. Thomas
Published by Cambridge University Press (2002)
Non-members: £70.00/US$125.00
Members: £28.00/US$50.00
pp. 294, ISBN: 0-521-81388-3



It is a pleasure to welcome another valuable and well edited SGM symposium book. Several topics are common to the last such SGM volume 16 years back: classical metabolic regulators such as LacI, AraC and CRP/FNR, a diversity of sigma factors, nitrogen regulation (here in relation to nitrogenase), modulated transcription termination, supercoiling; but with new depths of new molecular and structural information. Topics absent from that earlier book include switches via DNA rearrangements, cascades such as the phosphorelay for the initiation of *Bacillus* sporulation, quorum sensing both with homoserine lactone (HML) and peptide autoinducers, antisense RNA's and the control of pathogenicity genes. The contributions tend to focus on details of specific systems, but usually with a preamble, provided with useful references, that sketches the general picture – one suspects this to have been a helpful policy of the Editors.

Gaps? (reviewers, tiresomely, always have to finger a few). Phages only figure in transcription termination and there is little mention of the implications of microarray technology. More important omissions, not just from this volume but perhaps in people's thinking include the possible need for, and problems in attempting, large-scale modelling (alluded to in Neidhardt's introduction) and the adaptive biological roles (or maybe lack thereof – accident/drift?) of the intricate mechanisms so lovingly described here.

Still, a really useful book for everyone.

■ ***Simon Baumberg, University of Leeds***

An order form for Symposium Vol. 61 (and earlier volumes in the series) appears on p. 113.