Going places: forced and natural molecular evolution

Meeting report

Evolution is often considered to be of little significance to biotechnology – of great biological interest, perhaps, but not that relevant so far as exploitation is concerned. How wrong this view is, emerged at a recent workshop sponsored by the UK Biotechnology and Biological Sciences Research Council*. A variety of speakers demonstrated how our knowledge of natural evolutionary processes, and our ability to manipulate nucleotide sequences both *in vitro* and *in vivo*, lead, in some instances, to the development of greatly improved cells and enzymes capable of displaying highly desirable and novel metabolic activities, as well as to novel and potent analytical methods.

When going backwards means progress – forced selection of macromolecules

A common assumption is that evolutionary advances are fairly monotonic – selection acts to preserve variants with improved properties, and those that are less fit do not survive. This turns out to be far from true. Andy Ellington (Indiana University, Bloomington, IN, USA) used several rounds of *in vitro* selection for RNA aptamers that contained random variations in a 10mer sequence contained within a 30mer capable of binding to the human T-cell leukaemia virus type 1 (HTLV-1) *Rex* element. (Aptamers are RNA or DNA molecules that bind with high affinity to a target protein or small molecule. They are obtained by generating pools of random, or partially randomized, nucleic acids, and then carrying out repeated rounds of selective amplification.) The sequence that showed optimal binding contained no fewer than five differences in the 10mer compared with the starting molecule, and was selected from variants that exhibited lower binding activity than their parents. The conclusion is that such selection should recognize the principle of 'reculer pour mieux sauter' – that it may be necessary to explore quite 'unfavourable' parts of the evolutionary 'landscape' in order to attain a greater level of fitness subsequently. Similar methods could be used to select a variety of aptameric inhibitors, [e.g. for protein kinase C (K_d = 7 nM)], and the use of base analogues could greatly extend the serum half-life of RNA-like molecules from less than a second to 20 hours. Many useful diagnostic and therapeutic reagents should emerge from this approach.

Molecular diversity can be explored *in vitro* using a variety of high-throughput methods (Xavier Soberon; University of Mexico, Cuernavaca, Mexico). While classical protein engineering tends only to investigate localized regions of sequence space, random mutations, such as those produced via DNA shuffling, require that huge numbers of clones be screened. Because of the degeneracy of the genetic code, random mutations effectively explore only 30% of sequence space. The highly convoluted nature of the evolutionary landscape is indicated by the non-additive behaviour of individual mutations – two single mutants of β-lactamase each resulted in a ~20-fold improvement in catalytic constant (k_{cat}), while the k_{cat} of the double mutant was 859-fold higher than that of the wild type.

The statistics of random mutations mean that if only screening, rather than selection, is possible, one is forced to study variants with a small number (typically only one) of amino acid changes, because for m substitutions in a given protein of n amino acids, the number of possible variants is:

$$\frac{19^m.n!}{(n-m)!m!}$$

For a protein of 300 amino acids with changes in 1, 2 and 3 amino acids, the possible number of variants are 5700, ~16×10^6 and ~30×10^9, respectively (Jeffrey Moore; California Institute of Technology, Pasadena, CA, USA). Moore and Arnold applied a pseudo-natural diversity-creation and selection process to the evolution of a *p*-nitrobenzyl esterase for deprotection of an intermediate in the synthesis of the antibiotic loracarbef. Screening of four generations of a library generated by error-prone PCR, followed by recombination using DNA shuffling of the most active clones produced, enabled the isolation of mutant enzyme variants that displayed 50–60-fold higher activity (V_{max}) in 15% *N,N*-dimethylformamide. In contrast to a comparable study with subtilisin E, all of the effective amino acid substitutions were found to be located outside the enzyme regions believed to interact with the substrate. This stresses the general importance of the whole enzyme to its activity, and the benefits of inventive and high-throughput screening (HTS) methods in embracing the widest possible range of mutant variations; clearly a rational, step-by-step, site-directed mutagenesis approach designed to produce a similar result could not, with hindsight, have been contemplated in this case. As natural evolution did not, presumably, optimize such enzymes for activity in water-miscible organic solvents, the general scope for the forced evolutionary improvement of existing biocatalysts is clear. While this method will tend not to exploit evolutionary pathways that proceed via variants of lower activity, the use of selection over several generations means that these improvements require only approximately 10000 colonies to be screened.

Natural evolution in the laboratory

A perhaps underexploited potential resource for developing enzymatic processes lies in the latency of the microbial gene pool. John Sutherland (University of Oxford, UK) outlined a case study for the evolution of unnatural pathways for proline (Pro) biosynthesis in *Escherichia coli* Pro auxotrophs. Classically, three models circumscribe such evolutionary developments: retroacquisition, directed forward evolution (which relies on each step providing a selective advantage), and forward evolution through substrate–enzyme ambiguity. *E. coli* has an extensive latent catalytic repertoire that may rely on the flexibility of a number of cellular

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characteristics, such as permeability and regulation of transcription, in addition to evolution of the catalytic moiety *per se*, and all of these features could contribute to elaboration of the novel biosynthetic process.

Many cells are naturally starved of nutrients and are non-growing, and there is a strong selection pressure to acquire the ability to metabolize novel substrates. Thus, the precise nature of mutational processes is a recurring and fundamental theme, both in natural and forced molecular evolution studies; we must understand these processes before we can exploit them in directing biotechnologically useful and efficient *in vivo* evolutionary change. The resurgence of interest in this question probably dates from the famous 1988 paper of Cairns and colleagues[1], in which they showed that the rate of appearance of lactose-utilizing cells in *lac*$^-$ strains was much greater if lactose were present than if it were not; this phenomenon (which was once controversial because of its possible interpretation as a Lamarckian process) is now referred to as adaptive mutation. Barry Hall (University of Rochester, Rochester, NY, USA) described the history of experiments designed to resolve the mechanism(s) at work, and discussed a number of rigorous experimental models in which the role of adaptive mutation in bacterial evolution could be investigated. The three most striking conclusions are that such mutations arise via a mechanism very different from that operating 'normally'; they are specific to the adaptive challenge; and the process is sufficiently powerful to generate mutant phenotypes that would be highly unlikely to occur in growing cells. A detailed analysis of mutations in the *E. coli ebg* (**e**volved **b**eta-**g**alactosidase) operon proved particularly illuminating. Two classes of single-site revertants in *ebgA* (which codes for the α subunit of the enzyme) have been identified; class 1, which permit growth on lactose but not lactulose, and class 2, for which the opposite is true. Although spontaneous class 1 mutants occurred twice as frequently, they never appeared under conditions in which selection was for growth on lactulose. Thus, this study of mutations within the same operon provided a particularly powerful control against many possible counterarguments.

The *ebg* system has also been used for addressing the problem of how to determine rates of adaptive mutation, where multiple mutations are required for rapid growth. Whereas independent mutations in either *ebgA* or *ebgR* (a regulator of the *ebg* operon) enabled slow growth on lactulose with doubling times of 3.2 and 17.3 days, respectively, when both mutations were present together, the cells could grow rapidly with a doubling time of 2.7 hours. In fact, the observed accumulation of lactulose-utilizing mutants was found to be 40 000-times greater than could be accounted for on the basis of classical fluctuation tests, which are designed to determine the rate of spontaneous mutation, but was consistent with the time-dependent adaptive mutation rates measured under the same conditions of prolonged selection.

Evolution and historical accidents

The classical 'frozen accident' theory, initiated by Crick in 1968, argues that once the established universal genetic code had developed, it must have been fixed or frozen in evolution because of the probable catastrophic implications for any cell that then ventured outside the standard framework. This theory could have accommodated differences in the code exhibited by mitochondrial genomes owing to their perceived unique evolutionary status. However, as Mick Tuite (University of Kent, Canterbury, UK) indicated, *Mycoplasma*, some ciliates and, in particular, species of the fungal genus *Candida* display nuclear codon reassignment, raising important questions concerning the flexibility of the code and the nature of the conditions which lead to evolutionary developments. Not the least of these concerns for protein modellers using deduced primary sequence information is the possibility that amino acids may be incorrectly assigned. The *Candida albicans* system involves a change in the CUG codon, resulting in the non-conservative replacement of Leu by Ser. A plausible evolutionary pathway might be via a series of 'ambiguous' intermediates in which the tRNA involved still recognizes Leu, but can only poorly recognize Ser. The production of 'mixed' and variant proteins might then induce a stress response that could permit the selection of cells with such codon reassignments via a coincident stress-response-based thermotolerance.

Although, theoretically, DNA acts as a recording medium for detecting evolutionary changes, much noise is inevitably generated by genetic drift. Many genes have no phenotype under most conditions (John Brookfield; University of Nottingham, UK), and selection is apparently only weakly correlated with function in many cases. Indeed, the recent availability of sequences of entire microbial genomes illustrates how many genes exist for which no function is known (the problem of functional analysis). Martin Kreitman (University of Chicago, IL, USA) showed how biased codon usage and regulatory sequence variation in *Drosophila* appear to evolve under extremely weak selection, but that this can be accounted for by rapid mutational turnover. Conservation of function under these circumstances implies a role for compensatory evolution. An inevitable effect of genetic drift in an asexual lineage is that, even allowing for compensation, the forward mutation rate is typically higher than the reverse rate – a phenomenon known as 'Muller's ratchet'. This has been invoked as a possible 'reason' for the evolutionary advantage of sexual reproduction. Lin Chao (University of Maryland, Baltimore, MD, USA) employed the RNA virus f6 (the genome of which is segmented into three 'chromosomes') in a *Pseudomonas phaseolicola* host to model the advantages of recombination/sex, which amounted to an increase in fitness of 10% in this system.

Biodiversity – natural and artificial

Molecular methods have unequivocally highlighted the range of biodiversity that may be observed in nature. In performing evolutionary tracking, markers of the natural molecular evolution process that provide taxonomic and historical information are, therefore, essential. Thus, microsatellite markers possess many strengths for investigating molecular changes in mammalian population genetics (David Goldstein; Pennsylvania State University, University Park, PA, USA), while the rRNA gene system pioneered by Woese has long represented the premier locus for analysing unicellular organisms (Martin Embley; Natural History Museum, London, UK). Probe methods allow the recognition of uncultured organisms in natural samples, and prove that known cultured species of microorganism

constitute very much less than 1% of those whose genes exist in nature, with exciting consequences for those who wish to exploit this pool of biodiversity in drug discovery programmes. Indeed, 'wild' genes, such as those for disease resistance, continue to be of great importance in the breeding and development of commercial strains of plants of agricultural and horticultural significance (Jonathan Jones; John Innes Centre, Norwich, UK).

The evolution of 'fitness for purpose' via the production of many variants, followed by their selection, as happens in natural evolution, has recently been reflected in the tremendous interest in the artificial 'evolution' of molecules by the methods of combinatorial chemistry (Neil Hales; Zeneca Pharmaceuticals, Macclesfield, UK). As the subject has matured, it has become recognized that particular combinatorial libraries will be more suitable for different drug targets, as molecules are seen to colonize different pharmacological niches. Some of the methods favoured early in the evolution of this field have already been subject to mass extinction, and there will continue to be a balance between random screening and rational drug design. As computational power and methods for its exploitation improve, virtual libraries may increasingly be used for drug design. This is a particularly apt analogue for the studies of 'artificial life', or evolution *in silico*, that have been so helpful in producing the roadmaps of the evolutionary landscapes that we are so slowly beginning to unfold.

Reference

1 Cairns, J., Overbaugh, J. and Miller, S. (1988) *Nature* 335, 142–145

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Animal cell technology

Meeting report

Scientific interactions, like social ones, are oiled by pleasant surroundings, good food and wine, sunshine, and a well thought-out programme of entertainment. This has always been the philosophy of the European Society for Animal Cell Technology (ESACT), and the tradition was continued by Manuel Carrondo and his organizing committee at the 14th ESACT conference*. There were seven sessions, containing a mixture of invited and submitted oral papers, and each session had a complementary poster session. In addition, there were three keynote lectures.

Overview

ESACT exists to promote the use of animal cells, and to organize meetings at which investigators can report on the scientific and economic applications of animal cells, and the tools and products derived from them. As usual, these investigators came to the conference not only from academic institutions, but also from industry and governmental agencies. The meeting included presentations on all aspects of animal cell technology, ranging from the behaviour of the cells used in a process, to the hardware necessary for cell culture, and the purification and product-recovery processes. In addition to the traditional areas of animal cell technology, such as the use of cells to grow virus for vaccine production, the culture of hybridoma cell lines to produce monoclonal antibodies for diagnosis and therapy, and the development of recombinant cell lines, there were presentations on genetic medicine, tissue engineering, biomedical devices and cell-based screening systems. These latter areas include some of the most innovative work in animal cell technology, and are areas in which the society is keen to encourage more presentations.

*The meeting 'Animal Cell Technology: From Vaccines to Medicine' was organized by ESACT, and was held in Vilamoura, Portugal, 20–24 May 1996.

Keynote lectures

The first keynote lecture, outlining the molecular biology of prion diseases, was delivered by Charles Weissmann (University of Zürich, Switzerland). In his excellent and, sadly, highly topical lecture, Professor Weissmann gave an overview of the role of prion protein (PrP) in prion disease in general. He described results obtained with homozygous PrP 'knockout' mice, which demonstrated resistance to scrapie and showed no propagation of prions. Perhaps more surprisingly, heterozygous mice deficient in only one copy of the gene coding for PrP also showed increased resistance to scrapie disease, despite high levels of infectious agent and early signs of the mutant form of PrP in the brain. Interestingly, the introduction of mouse PrP transgenes made the knockout mice susceptible to mouse prions, but not to hamster prions, whereas introduction of the hamster transgene resulted in mice that were much more susceptible to hamster prions than to mouse prions. The conclusion drawn by Professor Weissmann was that normal levels of normal PrP were required for susceptibility to scrapie, and that lack of homology decreased susceptibility to the disease. He also suggested that cattle or sheep devoid of the PrP gene might be viable and resistant to scrapie.

The second keynote lecture, given by Wolf-Dieter Schleuning (Schering AG, Berlin, Germany), concerned the use of animal cell technology in drug discovery and pharmacology. This lecture was given in the memory of Toon van Wezel, a founder member of ESACT and a key contributor in the development of microcarriers – the porous beads often used to support adherent cells in mammalian cell bioreactor cultures. Professor Schleuning gave a fascinating insight into work being undertaken at the Schering Institute, and into the biological sources that have been investigated for the development of new fibrinolytic agents; these include a novel plasminogen activator from the saliva of the vampire bat *Desmodus rofundus*. Another compound under investigation is triabin, which is a novel thrombin inhibitor isolated originally from the saliva of the hematophageous bug *Triatoma pallidipennis*.

In the third keynote lecture (sponsored by Hyclone), Harvey Lodish (Whitehead Institute, Cambridge, MA, USA) discussed the biosynthesis of secretory proteins and cell-surface receptors. This elegant account of protein synthesis, folding and quality control of the final product left me

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