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In silico modelling of directed evolution: Implications for experimental design and stepwise evolution

David C. Wedge [a,c,*], William Rowe [a,c], Douglas B. Kell [a,c], Joshua Knowles [a,b]

[a] Manchester Interdisciplinary Biocentre, University of Manchester, 131 Princess Street, Manchester, M1 7ND, UK
[b] School of Computer Science, University of Manchester, Kilburn Building, Oxford Road, Manchester, M13 9PL, UK
[c] School of Chemistry, University of Manchester, Oxford Road, Manchester, M13, UK

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ABSTRACT

We model the process of directed evolution (DE) *in silico* using genetic algorithms. Making use of the NK fitness landscape model, we analyse the effects of mutation rate, crossover and selection pressure on the performance of DE. A range of values of *K*, the epistatic interaction of the landscape, are considered, and high- and low-throughput modes of evolution are compared. Our findings suggest that for runs of or around ten generations' duration—as is typical in DE—there is little difference between the way in which DE needs to be configured in the high- and low-throughput regimes, nor across different degrees of landscape epistasis. In all cases, a high selection pressure (but not an extreme one) combined with a moderately high mutation rate works best, while crossover provides some benefit but only on the less rugged landscapes. These genetic algorithms were also compared with a "model-based approach" from the literature, which uses sequential fixing of the problem parameters based on fitting a linear model. Overall, we find that purely evolutionary techniques fare better than do model-based approaches across all but the smoothest landscapes.

1. Introduction

Directed evolution (DE) has in recent years emerged as an effective technique for generating and selecting proteins[1] with a variety of uses. The starting point is usually a library containing proteins that already possess the desired function to some extent, although randomly generated proteins have also been used. Through a series of iterative steps, or 'generations', during each of which the proteins are diversified and then screened, the protein library is 'evolved' towards better performance. Proteins have been evolved using DE for a variety of roles including the production of industrial catalysts (Cherry and Fidantsef, 2003; Sylvestre et al., 2006), thermostable enzymes (Yun et al., 2006; Chautard et al., 2007), molecule-specific aptamers (Joyce, 1994) and vaccines (Piatesi et al., 2006).

In this paper we use genetic algorithms (GAs) to model the DE process. This seems like a natural thing to do, given the common roots of evolutionary computation and DE. Both are inspired by Darwinian evolution, proceeding through similar steps of selection, reproduction and variation. Further, variation is typically achieved during DE using error-prone polymerase chain reaction (Leung et al., 1989) or DNA shuffling (Stemmer 1994a, b; Coco et al., 2001). These techniques have similar effects to the mutation and crossover operators commonly used in GAs. It should therefore be possible to simulate the DE process using a GA and an appropriate dataset. However, DE practitioners do not in general apply lessons learned from evolutionary computation in determining experimental parameters, nor *vice versa*. One reason for this may be that the methodology used in GA learning is usually quite different from that used in DE. The former usually runs for hundreds or even thousands of generations whereas the latter usually only runs for a few (rarely more than 10) iterations.

Another difference between GAs and DE is that standard GAs usually incorporate relatively weak selection pressure. Common methods for selecting parents are fitness-proportional (de Jong, 1975) and tournament selection (Goldberg and Deb, 1991). While the severity with which these methods are applied may be varied, both methods typically allow a substantial proportion of offspring to be generated from parents that are somewhat less fit than the 'best' parent. In contrast, DE practitioners usually carry out strict filtering before performing the next mutational step. The filtering step typically allows a small percentage of proteins through to the next stage and in some cases only a single protein is allowed to reproduce. This type of selection is rarely used within GAs although there are a few exceptions (Mühlenbein and Schlierkamp-Voosen, 1993), which have generally been influenced by the related field of evolutionary strategies (Bäck et al., 1991).

* Corresponding author. Tel.: +44 161 3065145.
E-mail addresses: david.wedge@manchester.ac.uk, wedge@manchester.ac.uk (D.C. Wedge).

[1] DE has been performed on DNA and RNA as well as on proteins. References to 'proteins' in this paper refer to the molecules being manipulated by DE, whether they are proteins, DNA or RNA.

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One of the questions we hope to answer in this study is whether such high selection pressure is likely to be beneficial within DE.

There are a number of questions that are frequently asked within the GA community that are also asked by DE practitioners. Most of these are part of experimental design within DE but in the context of GAs are described as 'control parameters' or 'hyper-parameters'. Some examples are the choice of mutation rate, screen size and the use of recombination. The effect of each of these choices is examined in this study. Early work in DE applied low mutation rates, of the order of one mutation per protein, with the assumption that evolution was unlikely to make beneficial mutations at higher mutation rates (Arnold, 1996). However, more recently, improved results have been achieved with much higher mutation rates—between 3 and 30 per protein (Zaccolo and Gherardi, 1999; Daugherty et al., 2000; Reetz, 2004; Drummond et al., 2005). The choice between high- or low-throughput screening is related to the question of optimal mutation rate: it has been suggested that higher mutation rates are appropriate when a larger screen size is used, but that lower mutation rates are necessary when using a small screen (Arnold, 1996; Voigt et al., 2001). Recombination has been shown to be useful in a number of studies (Stemmer, 1994a, b; Moore et al., 1997; Zhang et al., 2002; Rowe et al., 2003) and examining the value of recombination within a range of algorithms is a further aim of this study.

DE may be contrasted (Arnold, 1996) with rational protein design. Ideally, the rational approach involves the creation of a realistic model of the mechanism by which a protein interacts with the system under study. In some areas, such as protein folding, substantial progress has been made in understanding mechanisms, although simplifying approximations have to be made in order to produce computationally tractable models (Cowperthwaite and Meyers, 2007). Unfortunately, our knowledge of protein folding is not easily translated into predictions of functional performance (Alviso et al., 2007) because most reaction mechanisms are insufficiently well understood and/or too complex to model with reasonable accuracy (Arnold, 2001).

Given the limited applicability of rational approaches, methods based upon search algorithms have flourished. These algorithms owe much to machine learning and statistics and fall into two classes. The first is a fully evolutionary approach, which has been described as 'blind' (Arnold, 1998) to draw attention to the analogy with the process of natural evolution, popularised as the 'blind watchmaker' (Dawkins, 1986). This approach is close to that used in GAs: search is guided by the processes of reproduction, variation and selection only.

The second is a model-based approach, in which a mathematical model of the relationship between protein sequence and function is constructed. This model may then be used to predict promising sequences. As far as the authors are aware, all existing model-based approaches that have been used in DE are stepwise. Each iteration involves the mutation of local sites (single bases or a small number of bases). Once the 'optimum' amino acid or acids have been identified at a particular site, this site is then fixed. A number of approaches fall into this general category, including Reetz's CAST method (Reetz et al., 2006), Fox's ProSAR (Fox et al., 2003, 2007) and Iwakura's quasi-additive adaptive walking (QAW) (Iwakura et al., 2006). These methods select bases at each position independently. However, covariation analysis suggests that there are significant correlations between residue frequencies within evolved proteins (Pritchard et al., 2001). It is apparent that stepwise approaches generate different sequences from those generated by evolutionary methods.

Stepwise approaches are usually considered to be a branch of DE. However, they have similarities to the rational design of proteins. Both attempt to identify active sites and hence reaction mechanisms and to use this information to select which proteins to screen. Experiments that use a stepwise algorithm might be better described as 'semi-rational' to distinguish them from both fully evolutionary experiments and from fully 'rational' approaches.

The task of identifying mechanisms is clearly an important one. However, the stepwise approach has a potential flaw: fixing bases after each step runs the risk of becoming trapped in a local optimum (Arnold, 1996), particularly in situations where there are epistatic interactions between amino acids and hence many local optima. A second drawback of semi-rational approaches is that they require the sequencing of proteins, which can be time-consuming and expensive. If semi-rational methods demonstrate superior performance to evolutionary methods the extra financial and time costs may be justified. The final task that we hope to perform through *in silico* simulations is a comparison between the capacities of evolutionary and stepwise methods, to ascertain whether the latter are likely to result in more rapid progress, and if so under what conditions.

To summarise, the aim of this study is to answer the following questions:

- How important is selection pressure in DE?
- What are the optimal mutation rates in DE?
- Does recombination play a useful role in DE?
- Are different approaches appropriate for high-throughput and low-throughput scenarios?
- In what situations is a full evolutionary approach superior to a stepwise approach?

A small number of previous studies have modelled DE or similar methods. Moore and Maranas (2000) constructed quantitative models to predict the probability of producing a specific nucleotide sequence from a number of cycles of error-prone PCR or DNA shuffling. This approach has been generalised to allow for variable amplification efficiency and initial population size (Pritchard et al., 2005). Fox demonstrated ProSAR models on NK-landscapes with low K (Fox et al., 2003; Fox, 2005). Corne et al. (2002) compared the performances of GAs using a range of mutation rates and of one with a variable mutation rate in the presence of strong selection pressure, as used during DE, using the MAX-ONES function as a test problem. They found that high rates of mutation (between $2/L$ and $4/L$) were beneficial, with a variable rate resulting in further improvements in performance. One aim of this study is to ascertain whether such high mutation rates are also usefully applied to the landscapes commonly encountered during DE.

2. Protein landscapes and NK-landscapes

We believe that knowledge obtained by examining the processes of variation and selection *in silico* using synthetic models can inform *in vitro* experimental design, but only if the *in silico* simulations have control parameters and a dataset that closely match those of the *in vitro* experiment. Producing a GA with control parameters that closely match the experimental design used in DE is fairly straightforward. Choosing a dataset with properties that mimic those of proteins is more difficult. In particular it is important that the relationships between data are similar to those between proteins. Proteins are known to display epistatic behaviour. In this study we use Kaufmann's NK-landscapes as a model of the protein 'landscape' (Kauffman, 1989). In this model, each genotype is a binary string of length N. The fitness of the genotype is calculated as the average fitness of each allele within the gene. However, each allele's fitness is affected by

the values of K other alleles. In some NK models epistatically linked alleles must be adjacent. However, we use a model in which the alleles may be anywhere within the bit-string, so incorporating the long-range interactions that have been observed in proteins (Reetz, 2004). The value of K may be varied, thereby giving rise to non-epistatic, smooth landscapes or more epistatic, rugged landscapes.

NK-landscapes represent only a subset of all possible epistatically linked landscapes (Heckendorn and Whitley, 1997; Heckendorn et al., 1998). NK_P-landscapes are a superset of NK-landscapes, in which a proportion, P, of allele combinations make no contribution to the overall fitness (Barnett, 1998). By increasing the value of P, the 'neutrality' of landscapes can be increased. However, it has been shown that varying the value of P does not affect the 'juxtapositional complexity' (Smith and Smith, 1998), with the result that P will have little effect on the relative performance of different GAs. For this reason, NK_P-landscapes are not considered in this study. For the interested reader, a number of further extensions to the NK-model have been suggested (Smith and Smith, 2000; Geard et al., 2002; Aguirre and Tanaka, 2004).

There is a lack of agreement concerning the extent to which epistasis affects the linkage between protein sequences and behaviour. It has been observed that the effects on enzyme activity due to mutations of dihydrofolate reductase were approximately additive (Aita et al., 2001; Iwakura et al., 2006). Govindarajan et al. (2003) found that the enzymatic activity of a series of subtilisin variants showed little epistatic interaction. However, the criterion for the identification of epistasis was very strict (p-value <0.001). Presumably weaker but still significant epistatic interactions occurred between a much larger number of pairs of sites.

Other authors have found that the protein landscape is highly epistatic (Kauffman and Weinberger, 1989; Schaeffer et al., 2003; Bershtein et al., 2006). Further, some protein landscapes are highly anisotropic. Hayashi et al. (2006) evolved a defective phage with the aim of increasing infectivity and found that most of the landscape was smooth and easily searched but that the most important areas, i.e. those containing the most highly infectious phages, were much more rugged. They suggested that different approaches might be required to search the smoother and more rugged parts of the landscape, such as the use of a lower mutation rate for the more rugged sections.

The studies that identified low epistasis considered only naturally occurring mutations whereas those that observed higher epistasis included synthetic variants. The low level of epistasis within naturally occurring proteins has been explained by the observation that they are likely to occur in additive or neutral areas of the protein landscape in which mutations are unlikely to be fatally deleterious (Govindarajan et al., 2003). However, DE often aims to produce proteins with properties that have *not* been selected for by natural evolution and should therefore jump out of a local optimum (or neutral network) (Arnold, 2001). This will require higher mutation rates which may enable the protein to move away from the local optimum and hence discover improved functional performance (Eigen and Schuster, 1978). However, this movement is likely to produce proteins in more epistatic regions of sequence space (Bershtein et al., 2006).

Given the lack of certainty concerning the level of epistasis in protein landscapes, we have performed a series of simulations on landscapes with varying levels of epistasis, with values of K varying between 0 (totally smooth) and 10 (very rugged). By using a variety of mutation rates we test whether different mutation rates are optimal for smooth and rugged landscapes.

NK-landscapes have been used previously as a model of protein activity landscapes. Aita and Husimi (1998) used adaptive walks on NK-landscapes to identify the optimum search strategy. At each generation this involved random mutations followed by selection of the best sequence. They found that the use of small screen sizes was effective when searching smooth landscapes but that it could lead to stagnation within rough landscapes, i.e. entrapment within local optima. Kauffman and Macready (1995) assessed the efficacy of mutation, recombination and 'pooling' strategies in generating proteins with enhanced fitness using the NK-model. Pooling strategies involve the creation of separate pools of proteins in which each pool has certain amino acids fixed. At each iteration the best pool is selected and sub-pools are created by fixing the amino acids at further positions. According to our definition, pooling is therefore a stepwise algorithm. Kauffman and Macready (1995) observed that fixing bases through the selection of a single 'pool' could be detrimental. They showed that the pooling approach could be improved through the introduction of a hill-climbing procedure involving recombination and/or mutation after the selection of each pool or sub-pool. Fox et al. (2003) reached the opposite conclusion when comparing their ProSAR algorithm with an evolutionary algorithm in their performances when searching NK-landscapes, finding that ProSAR gave better results for $K \leqslant 3$. The contrasting results may be explained by the fact that the two studies used different algorithms. Kauffman and Macready (1995) generate libraries using the mutation and recombination operators and select the best variant as the starting point for the next step. Fox et al. (2003) on the other hand do not use the standard mutation and recombination operators but generate libraries using a biased random selection of each allele. Muñoz and Deem (2008) used an NK-model to demonstrate that it is better to search a large library sparsely than a small library thoroughly, using GAs that ran for 100 generations. This is related to the setting of mutation rates: for a fixed screen size, a high mutation rate implies searching a large library sparsely while a low mutation rate results in thorough search of a small library.

One aim of this study is to re-evaluate the lessons learnt in previous studies. Like Aita and Husimi (1998), we compare the effects of large and small screen sizes. We extend their study through the introduction of recombination and by allowing more than one protein to contribute to producing the next generation. We attempt to cast light on the debate between evolutionary and stepwise methods by using algorithms that are very similar to the stepwise and evolutionary algorithms used in DE experiments. Finally, we attempt to identify the optimum mutation rate when only a small number of generations are available.

Although NK-landscapes have been widely used as models of protein landscapes, care must be taken when interpreting the results. The 'No Free Lunch Theorem' (NFL) demonstrates that all search algorithms are equally effective when averaged over the space of all possible problems (Wolpert and Macready, 1997), at least for single-objective optimisation problems. One consequence of this is that no single algorithm is optimal for all possible problems: an algorithm that has been optimised to solve one problem may be sub-optimal for other problems. The implication of NFL is that optimising an algorithm to search NK-landscapes does not guarantee optimal behaviour on protein landscapes. However, NFL may be partially circumvented by considering landscapes that are similar to each other. There is considerable evidence that NK-landscapes have similar structural features to protein landscapes and that search algorithms perform similarly when faced with these, respectively, real and synthetic landscapes (Kauffman, 1993). We believe that conclusions drawn from this theoretical study may therefore be used to guide the selection of experimental parameters, even though they do not guarantee optimal results.

3. Method

This research is composed of two parts. The main part is the simulation of DE using GAs with a variety of control parameters. These control parameters (screen size, selection pressure, mutation rate, use of crossover) were chosen to cover the range of values used in 'real-world' DE experiments, as described below. All simulations were run on a number of landscapes each with a different ruggedness (K value). The second part of the study involved the simulation of a stepwise algorithm searching on the same landscapes, in order to allow comparisons between the fully evolutionary and semi-rational approaches.

In order to maximise the relevance of our simulations we have chosen ranges of experimental parameters that reflect the values used in 'real' DE experiments. A large number of different techniques are available for generating protein libraries from parent proteins. A recent review (Lutz and Patrick, 2004) listed 12 'new technologies for generating molecular diversity', which generate between 100 and 3,000,000 proteins during each generation. Table 1 summarises the parameters used in a number of studies over the last 15 years. It may be seen that screen sizes are typically in the thousands with a few studies using smaller libraries containing hundreds of proteins while a small number use larger libraries containing hundreds of thousands or even millions of proteins.

When running our GAs we chose two different library sizes that roughly span the range used in DE experiments. For our low-throughput simulation we used a library size of 120. This is close to the smallest libraries that have been used experimentally and is the same size as the library used by Fox et al. (2003) when demonstrating ProSAR, thereby making possible direct comparisons with this technique. For the high-throughput simulation we used a library size of 40,000. This value is representative of large screen sizes as indicated by Table 1 and simulations may be run *in silico* within a reasonable timescale with this screen size. The upper limit on library sizes is increasing each year as a result of technological advances. However, parallel increases in computer speeds mean that running larger simulations that reflect this change should become viable in the future.

Proteins were represented in the simulation by binary strings of length N, with $N = 100$ for the high-throughput regime and 40 for the low-throughput regime. For the high-throughput regime, K took values from the set {0,2,5,7,10}. The low-throughput regime was applied to landscapes with $K = \{0,1,2,3,4,5,6,7,8,9,10\}$.

From Table 1 it is seen that the number of proteins that are successful in passing through a screen, i.e. proceed to the next generation of reproduction, is generally small and often just one protein is allowed to generate the next generation. In the evolutionary computing community selection pressures are usually kept relatively low. Low selection pressures are used in order to avoid premature convergence as a result of loss of diversity which can occur on runs of a large number of generations (hundreds or thousands) (Reeves and Rowe, 2002). In DE, in contrast, very strong selection pressure is usually applied in order to obtain a significant improvement in performance within a small number of generations. One aim of this study is to assess whether such selection pressure is beneficial. Selection pressure is varied using a (μ,λ) algorithm (Beyer, 2001), in which μ and λ are integers, with $\mu<\lambda$. In the first generation λ individuals are generated and the best μ are chosen to reproduce the next generation. From these μ sequences, parents are selected at random to reproduce λ 'offspring'. The selection and reproduction routines are repeated in subsequent generations. The value of λ is set to a fixed value (40,000 for high-throughput and 120 for low-throughput simulations), representing a constant screen size. Selection pressure is varied by changing the value of μ: lower μ indicates stronger selection pressure, i.e. more strict filtering of parents. The range of μ values used is given in Table 2.

The (μ,λ) algorithm is very close to the procedure usually followed during DE. In order to provide a benchmark against which to compare the results with this algorithm we also ran a standard GA using 'tournament selection'. In this algorithm all parents had the opportunity to produce offspring. Four parents were chosen at random and the parent with the highest fitness was selected to reproduce. During recombination the second parent was chosen in the same way. The resulting offspring passed into the next generation. The procedure was repeated until the new generation had the same population size as the previous

Table 1
Experimental parameters used during DE.

Reference	Screen size	Number selected	Number of generations	Mutation rate	Recombination used?
Chen and Arnold (1993)	~1300	1	3	Unknown	No
You and Arnold (1994)	~1000	1	7	Unknown	No
Moore and Arnold (1996)	1000–7500	1	4	1.5–4.5	No
Moore et al. (1997)	~1000	~5	6	0.6	Yes
Reetz et al. (1997)	~2000	1	4	1–2	No
Zhang et al. (1997)	10,000	20–40	7	~1	Yes
Yano et al. (1998)	4,000,000	100	5	15	No
Oue et al. (1999)	~50,000,000	~100	8	Unknown	No
Zaccolo and Gherardi (1999)	~40,000	1	3	8.2–27.2	No
Zhao and Arnold (1999)	~5000	1–5	5	2–3	Yes
Daugherty et al. (2000)	~1,000,000	~10,000	4–5	1.7–22.5	No
Liebeton et al. (2000)	800	1	5	~1	No
Reetz (2000)	2000	1	5	1, 2	No
Yano and Kagamiyama (2001)	2,000,000–6,000,000	~200	50	~4	Yes
Kaper et al. (2002)	2048		3	Unknown	Yes
Oh et al. (2002)	~10,000	10	2	Unknown	Yes
Bessler et al. (2003)	7200	16	2	1	Yes
Bulter et al. (2003)	~2000	1–12	10	Unknown	Yes
Ikebukuro et al. (2005)	10	5	7	1	Yes
Johannes et al. (2005)	~8000	1	3	1–2	No
Hayashi et al. (2006)	10–1,000,000	1	20	2.4	No
Piatesi et al. (2006)	10,000,000–30,000,000	Unknown	4	<2, 2–4, >4	No
Reetz et al. (2006)	~3000	1	6	2–3	No
Fox et al. (2007)	~14,000	5–10	18	~1	Yes

Table 2
Algorithms applied to the NK-landscapes.

High throughput	Low throughput
Standard GA (population 40,000)	Standard GA (population 120)
GA with $(\mu,\lambda) = (4000, 40{,}000)$	GA with $(\mu,\lambda) = (12, 120)$
GA with $(\mu,\lambda) = (400, 40{,}000)$	GA with $(\mu,\lambda) = (6, 120)$
GA with $(\mu,\lambda) = (40, 40{,}000)$	GA with $(\mu,\lambda) = (3, 120)$
GA with $(\mu,\lambda) = (4, 40{,}000)$	GA with $(\mu,\lambda) = (1, 120)$
GA with $(\mu,\lambda) = (2, 40{,}000)$	
GA with $(\mu,\lambda) = (1, 40{,}000)$	Stepwise (population 120)

generation. This may be contrasted with the (μ,λ) algorithm in which offspring are over-produced and then culled. Crossover was applied with a probability of 0.6 and mutation at a rate of $1/L$, where L is the length of the bit-strings (equal in value to N). These parameters are known to perform well over a range of fitness landscapes (Bäck, 1996). The number of proteins in the GA 'population' was generally equal to the screen size used in the (μ,λ) algorithm, i.e. 120 during low-throughput and 40,000 during high-throughput simulation. In order to evaluate the improvement in performance due to the (μ,λ) and stepwise algorithms, standard GAs were also run in the low-throughput regime with a range of larger population sizes, up to 4000.

In most evolutionary computing applications, GAs are run to 'convergence', i.e. until no more improvement is observed and most of the population diversity has been lost as a result of repeated selection. However, due to time and money limitations, DE is rarely run for more than 10 generations (see Table 1). In order to model DE experiments realistically our simulations therefore ran for 10 generations.

From Table 1 it may be seen that a wide range of mutation rates have been applied during DE experiments reported in the literature, ranging from less than $1/L$ up to $27/L$. Approximately half of the studies identified used some from of recombination; the others relied entirely on mutation to produce variation. The range of values used within this study has been chosen to reflect the variation in experimental design. All of our experiments were performed both with and without crossover and with a range of different mutation rates from the set {0.1,0.2,0.4,0.6,0.8,1,2,3,4,5, 10,15,20,25,30}.

The performance of a stepwise algorithm was compared with the fully evolutionary algorithms. This algorithm was based on ProSAR and uses partial least squares (PLS) models (Wold, 1966) to determine which areas of the protein landscape to search. PLS creates a linear mathematical model relating the genotype alleles (0 or 1) to the overall fitness. Coefficients are set by simultaneously regressing onto the dependent and independent variables, thereby avoiding the overfitting which tends to occur when basic regression is applied to a problem with a large number of input variables. ProSAR was primarily designed to operate on small libraries. Furthermore, the computational requirements of PLS make it impractical for large libraries. For these reasons we have only applied it within the low-throughput methodology. In the first generation, sequences were generated randomly. A PLS model was constructed and the 4 positions that had the greatest influence on fitness, i.e. those with the largest PLS coefficients, were set to their optimum values (0 or 1). In subsequent generations only those positions that had not been fixed were allowed to vary. In each generation 4 more positions were fixed, so that at the end of 10 generations all 40 positions had been determined.

For each NK pair 100 landscapes were independently generated. For each algorithm and set of mutation/crossover rates, independent runs were performed on each landscape and the results were averaged across all 100 runs. The best fitnesses achieved and optimal mutation rates were recorded for each algorithm. Overall, over 1,80,000 runs were performed.

The crossover and mutation operators are described using pseudo-code in the Appendix A, as are the standard GA, (μ,λ) and stepwise algorithms used within this study.

4. Results

4.1. High throughput

Figs. 1 and 2 show the performance of a standard GA with and without crossover, respectively, for landscapes of different ruggedness. The smoothness of these and later plots reflects the precision attainable in these analyses. Table 3 gives the best fitness achieved on each landscape. It indicates that crossover enhances the performance on smoother landscapes but is detrimental for more rugged landscapes. In order to give an impression of the scale of the improvements, the best fitnesses achieved using random selection of sequences is also included in Table 3.

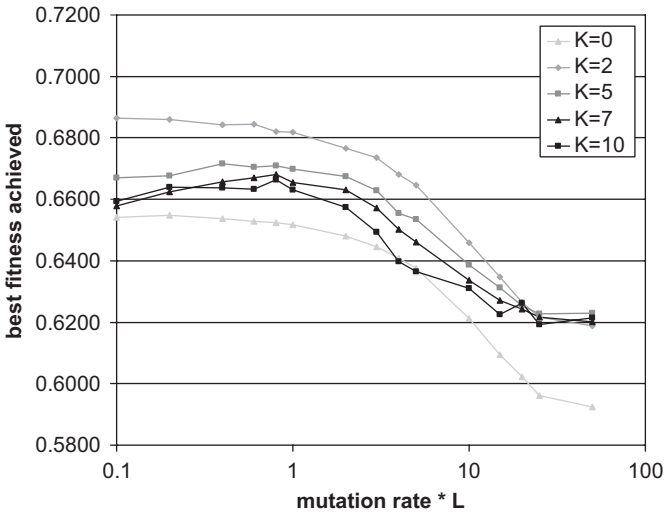

Fig. 1. Best fitnesses achieved by a standard GA with crossover.

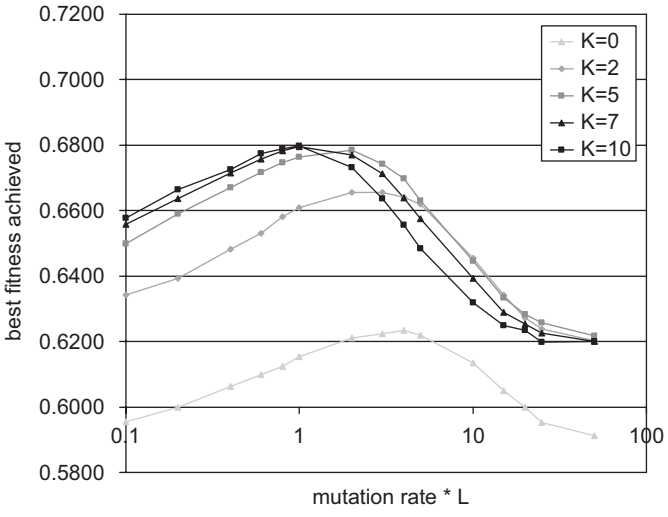

Fig. 2. Best fitnesses achieved by a standard GA without crossover.

The introduction of stronger selection pressure via the (μ,λ) algorithm improves performance considerably: the improvement over random selection is approximately doubled when the (μ,λ) algorithm is used for all except the completely smooth landscape ($K=0$). Figs. 3 and 4 show the best fitness achieved on each landscape with a moderate increase in selection pressure ($\mu=4000$) with and without crossover, respectively.

Table 4 gives the best fitnesses achieved and the corresponding mutation rates. Apart from the overall improvement in fitnesses compared to the standard GA, there are 3 points to note:

- The optimum mutation rates are similar for the (μ,λ) algorithm and the standard GA algorithm.
- The advantage of incorporating crossover is found to apply up to a greater level of ruggedness ($K\leqslant 5$ rather than $K\leqslant 2$) when greater selection pressure is introduced.
- Introduction of crossover reduces the sensitivity to mutation rate. When mutation is the only operator it is very important that the mutation rate is set to its optimum value, but when crossover is present mutation acts as a secondary operator (Maynard Smith, 1978; Ochoa et al., 1999).

A comparison across different landscapes suggest that the maximum fitness attained is similar for all landscapes with $K>0$.

Table 3
Best fitnesses and best mutation rates achieved with a standard GA (high throughput).

K	Random	Mutation only		With crossover	
		Best fitness	Best mutation rate	Best fitness	Best mutation rate
0	0.5804	0.6234	4/L	**0.6547**	0.2/L
2	0.6034	0.6655	3/L	**0.6865**	0.1/L
5	0.6229	**0.6785**	2/L	0.6715	0.4/L
7	0.6246	**0.6795**	1/L	0.6681	0.8/L
10	0.6290	**0.6798**	1/L	0.6663	0.8/L

Bold entries indicate whether the mutation-only or with-crossover algorithm is superior. GAs have a population of 40,000 and are run for 10 generations. 'Random' is the best fitness achieved from 4,00,000 randomly generated sequences.

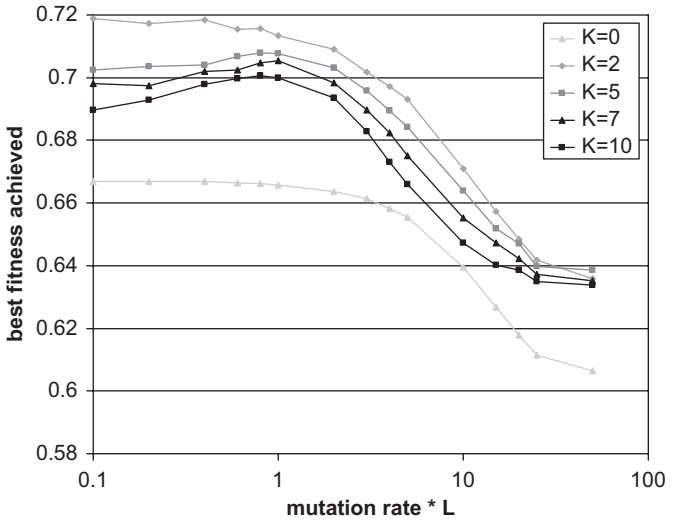

Fig. 3. Best fitnesses achieved by a (μ,λ) GA with crossover, with $\mu=4000$ and $\lambda=40{,}000$.

The *global* optimum for different landscapes has been observed to be independent of K in one study (Smith and Smith, 1998). More recent work suggests that the global optimum increases with K, but search algorithms may not reflect this, due to the increased difficulty of searching more rugged landscapes (Skellett et al., 2005). The average maximum global fitness for a $K=0$ landscape is predicted to be $\frac{2}{3}$ and it appears that this is attained when crossover is used.

The consequences of further increasing the selection pressure were investigated by decreasing μ. The best fitnesses achieved on

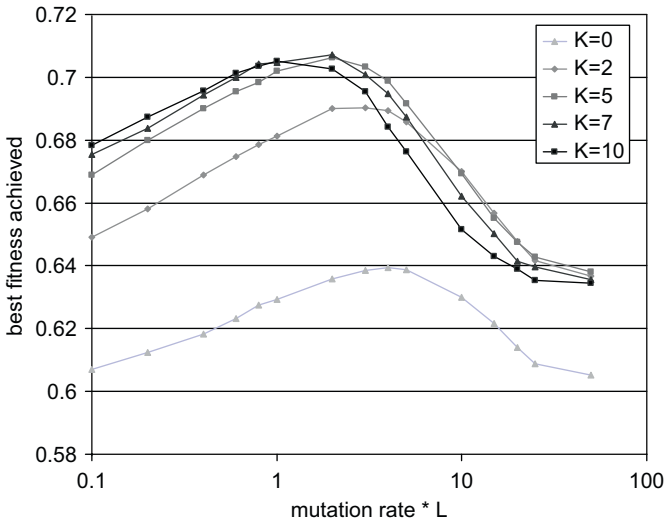

Fig. 4. Best fitnesses achieved by a (μ,λ) GA without crossover, with $\mu=4000$ and $\lambda=40{,}000$.

Table 4
Best fitnesses and best mutation rates achieved with a (μ,λ) GA, with $\mu=4000$ and $\lambda=40{,}000$.

K	Mutation only		With crossover	
	Best fitness	Best mutation rate	Best fitness	Best mutation rate
0	0.6394	4/L	**0.6670**	0.1/L
2	0.6904	3/L	**0.7188**	0.1/L
5	0.7063	2/L	**0.7080**	0.8/L
7	**0.7072**	2/L	0.7053	1/L
10	**0.7050**	1/L	0.7006	0.8/L

Bold entries indicate whether the mutation-only or with-crossover algorithm gives the higher mean fitness.

Table 5
Best fitnesses and best mutation rates achieved with (μ,λ) GAs with varying selection pressure (high throughput, $K=5$).

(μ,λ)	Mutation only		With crossover	
	Best fitness	Best mutation rate	Best fitness	Best mutation rate
(4000, 40,000)	0.7063	2/L	**0.7080**	0.8/L
(400, 40,000)	0.7464	3/L	**0.7478**	2/L
(40, 40,000)	0.7490	4/L	**0.7495**	4/L
(4, 40,000)	**0.7494**	3/L	0.7488	3/L
(2, 40,000)	0.7426	4/L	**0.7434**	3/L
(1, 40,000)	**0.7457**	4/L		

Bold entries indicate whether the mutation-only or with-crossover algorithm gives the higher mean fitness.

the $K=5$ landscape are given in Table 5. It is clear from these results that

- Performance peaks at a selection pressure of between 1000 and 10,000, i.e. $\mu = 40$–4, with selection pressure defined as λ/μ. Both higher and lower selection pressures give lower fitnesses.
- There is a fairly constant optimum mutation rate of approximately $3/L$.
- The introduction of crossover gives a slight edge to the performance of the (μ,λ) algorithm.

Very similar results are achieved by applying strong selection pressure to landscapes with $K=0$, 2, 7 and 10. For all of these landscapes a selection pressure of 1000 was found to be optimal or near-optimal. Crossover was seen to be beneficial for the smoother landscapes ($K \leqslant 5$) but detrimental for the more rugged landscapes.

The differences between the mean fitness values were shown to be statistically significance using a series of 2-way analysis of variance (ANOVA) tests. For each value of μ and each landscape, p-values were obtained by conditioning on mutation rate and presence/absence of crossover. All p-values were below 0.05 indicating that the difference in mean values is significant at this level.

4.2. Low throughput

Selection pressure was seen to have a similar effect in the low-throughput regime. Tables 6 and 7 show the best fitnesses achieved on various NK-landscapes with and without the use of the crossover operator and with varying selection pressure. The following observations may be made:

- As the ruggedness of the landscape increases there seems to be a slight decrease in the optimum selection pressure. However, a selection pressure of about 40, i.e. a (μ,λ) algorithm with $\mu=3$ and $\lambda=120$, performs well across a wide range of landscapes.
- For smoother landscapes (K up to approximately 6), crossover appears to play a useful role. For more rugged landscapes the mutation only algorithm performs better.

Table 6
Best fitnesses achieved with standard and (μ,λ) GAs with crossover (low throughput).

K	Standard GA	(12,120)	(6,120)	(3,120)	(2,120)
0	0.6482	0.6680	0.6686	**0.6686**	0.6671
1	0.6744	0.7040	0.7052	**0.7053**	0.6996
2	0.6875	0.7270	0.7286	**0.7308**	0.7178
3	0.6928	0.7329	**0.7376**	0.7355	0.7287
4	0.6981	0.7341	**0.7397**	0.7374	0.7303
5	0.6984	0.7370	0.7350	**0.7382**	0.7313
6	0.6962	0.7305	**0.7337**	0.7323	0.7231
7	0.6935	0.7248	0.7259	0.7269	0.7189
8	0.6944	0.7172	0.7201	0.7225	0.7148
9	0.6904	0.7170	0.7156	0.7178	0.7092
10	0.6874	0.7112	0.7136	0.7135	0.7071

Shading indicates the optimum selection pressure for each landscape. Bold entries indicate that crossover is advantageous.

Table 7
Best fitnesses achieved with standard and (μ,λ) GAs without crossover (low throughput).

K	Standard GA	(12,120)	(6,120)	(3,120)	(2,120)	(1,120)
0	0.6392	0.6613	0.6657	0.6672	0.6659	0.7000
1	0.6659	0.6972	0.7019	0.7038	0.6978	0.6987
2	0.6791	0.7174	0.7240	0.7266	0.7177	0.7162
3	0.6853	0.7272	0.7351	0.7364	0.7256	0.7227
4	0.6901	0.7319	0.7374	0.73492	0.7296	0.7264
5	0.6911	0.7299	0.7355	0.7337	0.7267	0.7260
6	0.6968	0.7263	0.7280	0.7300	0.7232	0.7231
7	0.6902	0.7218	**0.7272**	0.7252	0.7180	0.7157
8	0.6872	0.7187	0.7200	**0.7227**	0.7150	0.7169
9	0.6890	0.7147	**0.7179**	0.7170	0.7119	0.7094
10	0.6862	0.7089	0.7139	**0.7152**	0.7085	0.7039

Shading indicates the optimum selection pressure for each landscape. Bold entries indicate that crossover is disadvantageous.

Tables 8 and 9 show the optimum mutation rates for each algorithm. It is clear that the optimum mutation rate increases with selection pressure. However, when the optimal selection pressure is present, i.e. $(\mu,\lambda)=(3,120)$, a mutation rate of $2/L$ gives good results across a range of landscapes both with and without the use of crossover.

Again ANOVA tests indicated that the mutation rates and presence/absence of crossover had a statistically significant effect (p-value <0.05) on the mean fitness achieved on each landscape.

4.3. Stepwise algorithm

The best fitnesses achieved with a stepwise approach are given in Table 10. Fig. 5 shows the variation in best fitness for stepwise and a variety of evolutionary methods. In order to make comparisons across different landscapes, all fitnesses f have been normalised to f_{norm} using Eq. (1), in which $f(GA,1N)$ and $f(GA,100N)$ are the average fitnesses achieved using standard GAs with screen sizes of, respectively, 1 and $100N$.

$$f_{norm} = \frac{f - f(GA,1N)}{f(GA,100N) - f(GA,1N)} \quad (1)$$

Table 8
Best mutation rates for standard and (μ,λ) GAs with crossover (low throughput).

K	Standard GA	(12,120)	(6,120)	(3,120)	(2,120)
0	2	0.6	1	1	2
1	2	0.8	2	3	2
2	1	1	2	1	2
3	0.8	2	2	1	2
4	0.8	0.8	2	2	3
5	0.6	1	2	2	3
6	0.8	1	2	2	2
7	0.8	2	2	2	2
8	0.6	2	2	2	3
9	0.6	1	1	3	3
10	0.6	1	2	1	2

Table 9
Best mutation rates for standard and (μ,λ) GAs without crossover (low throughput).

K	Standard GA	(12,120)	(6,120)	(3,120)	(2,120)	(1,120)
0	2	2	2	2	2	2
1	2	2	2	2	3	3
2	2	2	2	3	3	3
3	2	2	2	2	2	2
4	2	2	2	2	3	3
5	1	2	2	1	2	3
6	1	2	2	2	2	3
7	1	1	2	2	2	3
8	1	2	2	3	3	2
9	1	2	2	2	3	3
10	1	0.8	2	2	2	3

This technique has been borrowed from a paper by Fox et al. (2003) introducing ProSAR and facilitates comparison with the results reported in that paper. In Fig. 5, the reader's attention is drawn to the results using screen sizes of 3N. It is clear that the stepwise method performs better than a standard GA with this size for relatively smooth landscapes ($K \leqslant 6$). However, for $K = 7$ the performance of the standard GA is similar to that of the stepwise algorithm and for higher K the GA has superior performance.

The (μ,λ) GA, applied with crossover and the optimum selection pressure of (3,120) also gives much better performance than the standard GA. However, this increase in performance is seen to carry over to more rugged landscapes. Even when $K = 10$, this algorithm performs better than a standard GA with 10 times the screen size (30N rather than 3N), whereas the stepwise algorithm performs worse than a standard GA with a screen size of N.

4.4. Hitting times

The results have been presented so far as mean fitness levels achieved within a fixed number of generations. This gives an indication of the expected improvement in outcome for a fixed experimental set-up. An alternative approach is to measure the amount of experimental effort required to reach a target fitness value. The results are provided here for a selected problem in order to illustrate the savings possible through good parameter choice. They are presented as 'hitting times', the number of generations required to achieve the target fitness.

Table 10
Best fitnesses achieved with the stepwise algorithm (low throughput).

K	Best fitness
0	0.6688
1	0.706776
2	0.727978
3	0.72855
4	0.723386
5	0.716273
6	0.708067
7	0.694728
8	0.685902
9	0.669519
10	0.666328

A landscape of medium ruggedness ($N = 40$, $K = 5$) was selected and 4 different algorithms were applied: a standard GA, a (μ,λ) GA with modest selection pressure ($\mu = 12$), a (μ,λ) GA with strong selection pressure ($\mu = 3$) and a stepwise algorithm. All GAs used crossover. The target fitness was the mean fitness achieved by the standard GA (0.6942). One hundred runs were performed using each algorithm.

When using the standard algorithm, 55 runs achieved the target and the average hitting time was 8.13 generations. Weak (μ,λ) selection pressure increased the number of successful runs to 86 and reduced the hitting time to 6.49. Strong selection pressure gave 90 successful runs, with an average hitting time of 5.66. It is clear that the application of appropriate selection pressure can considerably reduce the experimental outlay required to achieve satisfactory results. The stepwise algorithm had less impact on hitting times, yielding 69 successful runs, with an average hitting time of 8.31 generations.

5. Discussion

The primary aim of this study has been to identify effective DE experimental parameters through the use of *in silico* simulations. Our results show that experimental design is of crucial importance, with some methods giving much faster evolution than others. To summarise our findings:

- Strong selection pressure, implemented via filtering of the 'parents' allowed to reproduce, increases the chances of finding beneficial mutants greatly.
- However, it is possible to apply too much selection pressure: many DE experiments use just one parent in each generation, but it appears to be most effective to select 3 parents in the low-throughput regime and approximately 40, (equivalent to 1 in 1000) in the high-throughput regime.
- Moderately high mutation rates (above $1/L$) are beneficial in the presence of strong selection pressure. A mutation rate of $2/L$ performs well across a wide range of landscapes.
- The introduction of recombination makes the setting of an optimal mutation rate less critical. Recombination is beneficial for relatively smooth landscapes but may be detrimental for more rugged landscapes.
- The stepwise algorithm gives a boost in performance similar to that of the (μ,λ) algorithm for smooth landscapes ($K \leqslant 2$).

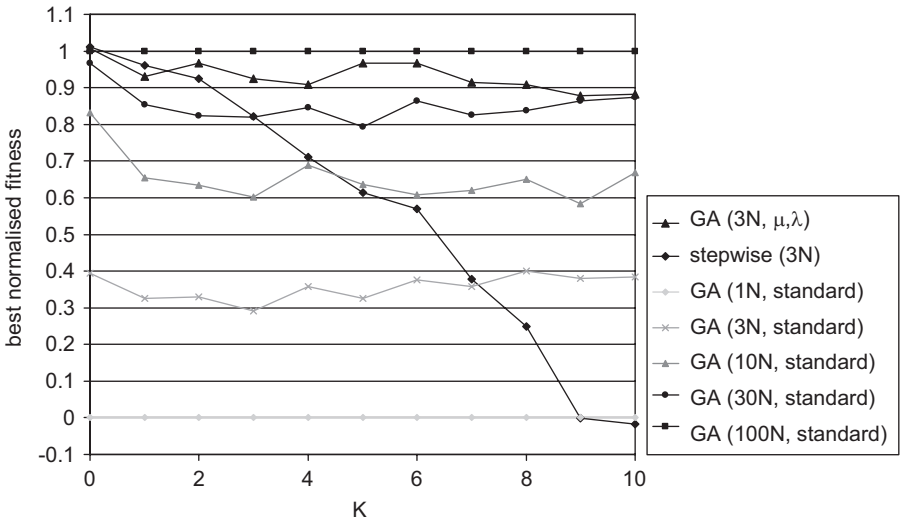

Fig. 5. Normalised best fitnesses achieved by various GA algorithms and a stepwise algorithm (low throughput). All included GAs used crossover and the (μ,λ) GA had $\mu = 3$ and $\lambda = 120$. Screen sizes are indicated in brackets, as a multiple of N, where $N = 40$.

However, this advantage falls off rapidly as the ruggedness increases, with catastrophic consequences for the most rugged landscapes.
- The pattern of results was very similar for the high- and low-throughput models. In particular, the use of high mutation rates (above $1/L$) was found to be beneficial in low-throughput as well as high-throughput mode.
- An illustrative experiment indicates that the experimental settings that produce sequences with the highest fitness are also likely to give the quickest results. This finding suggests that the lessons learnt from this study may be used to achieve a reduction in experimental costs as well as an improvement in screening efficiency.

These findings on NK-landscapes could be used to guide the choice of parameters in DE experiments but we have yet to show that, in practice, these predictions are valid. It is our intention to do this, making use of on-chip technology (Knight et al., 2008), with which it would be possible to run a variety of GAs, like those used here, and compare their performance directly in raising an aptamer to a target ligand.

As well as providing a guide to the experimental design of evolutionary methods, this study casts light on the debate between stepwise and fully evolutionary methods within DE. The primary distinction between these two methods is in the selection of mutants to be screened. This issue is of the utmost importance in DE. As Reetz has stated:

Efficient directed evolution is not a matter of generating huge libraries that then require considerable efforts in screening for the desired property. The goal is to create a maximum in structural diversity while minimizing the size of the libraries. (Reetz, 2004)

The belief that the selection of mutants to be screened is crucial to the success of DE has been a driving force behind the development of model-based methods. However, our investigations suggest that such methods may not be useful and may even be detrimental. Looked at from the opposite point of view they show the power and resilience of evolutionary methods. Of course, the poor performance of stepwise methods compared to evolutionary methods does not show that all model-based approaches are flawed but the authors believe that it indicates a significant problem with certain types of modelling. There are two likely sources of the problems observed with the stepwise approach used here. The first is that it fixed amino acids at each iteration, without allowing backtracking. The absence of backtracking is likely to cause particular problems in the presence of epistasis. Epistasis results in local optima and a procedure that does not include backtracking is likely to become trapped on one of these optima (Liebeton et al., 2000; Reetz et al., 2006). The second likely source of inaccuracies is the use of a linear model to approximate non-linear interactions. Again this will cause problems when the contributions of individual amino acids are non-additive.

The presence of appropriate selection pressure has been shown to greatly enhance the evolutionary process. Stepwise methods also have strong selection pressure, since library mutants are guided towards the regions of the protein space which the model indicates are best. Evolutionary methods concentrate on promising areas of protein space in a similar way. However, the selection of mutants occurs via the selection of parent proteins rather than through the accumulation of 'knowledge'. It seems likely that the presence of strong selection pressure is essential during DE, given the small number of iterations that are generally available (Bäck, 1996). Whether evolutionary or model-based methods are superior depends upon whether this selection pressure is selecting the best areas on which to concentrate the search procedure. Our results show that fully evolutionary methods with strong selection pressure are robust, giving good performance across a range of problem landscapes. The model-based approach used here appears to be more fragile, performing well for smooth landscapes but less well for medium and rugged landscapes.

While we have shown that model-based approaches should be used with care within the evolutionary process itself, we believe that they have two useful roles. The first is in choosing sites to be targeted for mutation. The incorporation of computational methods into this choice has been shown to be beneficial (Wong et al., 2007). However, the implication of our results is that simultaneous mutations at multiple sites are likely to be more successful than stepwise mutations at individual sites. The second use of models is in trying to describe a protein landscape *after* it has been explored using evolutionary methods.

6. Conclusions and future work

We have shown that simulating the DE process *in silico* can give clear answers to questions concerning preferred experimental design. Based on our simulations, the following tentative recommendations may be made:

- Strong selection pressure is highly advantageous during DE. However it is possible to have too much selection pressure: selecting a single parent at each generation is likely to be detrimental.
- A high mutation rate—$2/L$ to $3/L$—combines well with strong selection pressure.
- Recombination is valuable in the presence of low or medium epistasis but detrimental in highly epistatic protein landscapes ($K \geqslant 5$).

These conclusions seem to be quite general. The advantages of strong selection pressure and fairly high mutation rates have been shown to operate on landscapes with highly varied levels of ruggedness, operating in either high- or low-throughput mode.

A model-based approach has been seen to perform poorly compared to an evolutionary approach on landscapes of medium and high ruggedness. It is likely that a different type of modelling, particularly one incorporating non-linearities, could well perform better. However, fitting the type of model to the observed protein landscape may be non-trivial. The two main issues to be considered are the selection of the model to be used and the way in which selection of library mutants is performed. The model used here is linear in nature and is therefore likely to perform well only when the contributions of individual amino acids are roughly additive. The problem may have been exacerbated by the selection procedure, which fixed a number of bases at each iteration. We would not recommend the fixing of individual amino acids based on model predictions, since this is likely to lead to stagnation at a local optimum.

While care needs to be taken when using a model to select a mutant library, there are two situations in which modelling is undoubtedly useful. The first is that where the mechanism of the target protein function is well understood. In this situation a model could introduce constraints that restrict the search space to those proteins that are known to fulfil the desired function well. A strategy such as this could combine 'rational' design with evolutionary methods (Wong et al., 2007; Xiong et al., 2007). The second role for modelling is in improving our understanding of mechanism *after* performing DE. At the end of a DE run, a large number of proteins will have been sequenced and an assessment made of their ability to perform some desired function. From this information it is possible to construct models that indicate

reactive sites and epistatic links. This information could support particular mechanisms of action.

In future work we intend to investigate the extent to which predictions for optimal experimental design settings derived from *in silico* investigations may be applied to *in vitro* experiments. We also intend to explore further the uses (and limitations) of mathematical models within two areas of the DE process: library generation and post-evolution explanation. The focus of our attention is likely to be on non-linear modelling techniques such as Random Forests (Breiman, 2001) and Genetic Programming (Koza, 1992; Kell, 2002).

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Appendix A. Algorithms used

The mutation operator was applied to each protein using the following procedure:

1. Select a protein using tournament selection.
2. Set current bit = 1.
3. Generate a random number between 0.0 and 1.0.
4. If the random number is greater than the mutation rate, 'flip' the current bit.
5. Set current bit = current bit+1.
6. If current bit >bit length, exit, else go to 3.

Uniform crossover was used. This operator used the following procedure:

1. Select 2 parents using tournament selection.
2. Set current bit = 1.
3. Generate a random number between 0.0 and 1.0.
4. If the random number is greater than 0.5 assign the offspring the bit at the current bit position from parent 1, else assign the corresponding bit from parent 2.
5. Set current bit = current bit+1.
6. If current bit >bit length, exit, else go to 3.

The standard GA had the following control parameters:
Mutation rate $= 1/L$.
Crossover rate = 0.6.
Selection method = 4-fold tournament.
It used the following procedure:

1. Generate a population of p sequences randomly.
2. Set generation_no = 1.
3. Set current_sequence = 1 and new_population to null.
4. Select parent(s) from the population using 4-fold tournament selection
5. Generate offspring from parents using crossover/mutation operators
6. Add offspring to new_population.
7. Set current_sequence = current_sequence+1
8. If current_sequence $\leqslant p$ go to 4.
9. Set population = new_population
10. generation_no = generation_no +1.
11. If generation_no >10, exit, else, go to 3.

The (μ,λ) algorithm used the following procedure:

1. Generate a population of ë sequences randomly.
2. Set generation_no = 1.
3. Select the best μ sequences from the current population as parents.
4. Set current_sequence = 1 and new_population to null.
5. Select parent(s) randomly.
6. Generate offspring from parents using crossover/mutation operators
7. Add offspring to new_population.
8. Set current_sequence = current_sequence+1
9. If current_sequence $\leqslant \lambda$ go to 5.
10. Set population = new_population
11. generation_no = generation_no +1.
12. If generation_no >10, exit, else, go to 3.

The stepwise algorithm used the following procedure

1. Generate a population of λ sequences randomly.
2. Create a PLS model.
3. Fix the 4 positions that have the highest PLS coefficients.
4. If all positions are fixed, exit.
5. Vary the unfixed positions randomly.
6. Go to 2.

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