Evolutionary Engineering of Industrially Important Microbial Phenotypes

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The tremendous complexity of dynamic interactions in cellular systems often impedes practical applications of metabolic engineering that are largely based on available molecular or functional knowledge. In contrast, evolutionary engineering follows nature's 'engineering' principle by variation and selection. Thus, it is a complementary strategy that offers compelling scientific and applied advantages for strain development and process optimization, provided a desired phenotype is amenable to direct or indirect selection. In addition to simple empirical strain development by random mutation and direct selection on plates, evolutionary engineering also encompasses recombination and continuous evolution of large populations over many generations. Two distinct evolutionary engineering applications are likely to gain more relevance in the future: first, as an integral component in metabolic engineering of strains with improved phenotypes, and second, to elucidate the molecular basis of desired phenotypes for subsequent transfer to other hosts. The latter will profit from the broader availability of recently developed methodologies for global response analysis at the genetic and metabolic level. These methodologies facilitate identification of the molecular basis of evolved phenotypes. It is anticipated that, together with novel analytical techniques, bioinformatics, and computer modeling of cellular functions and activities, evolutionary engineering is likely to find its place in the metabolic engineer's toolbox for research and strain development. This review presents evolutionary engineering of whole cells as an emerging methodology that draws on the latest advances from a wide range of scientific and technical disciplines.

Keywords. Adaptation, Directed evolution, Evolutionary engineering, Metabolic engineering, Selection

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List of Abbreviations

BOICS	Brown and Oliver interactive chemostat selection
bp	base pair
DNA	desoxyribonucleic acid
EMS	ethyl methane sulfonate
IS	insertion element
kb	kilo base pairs
MS	mass spectrometry
NTG	nitroso-methyl guanidine
PCR	polymerase chain reaction
PTS	phosphotransferase system
mRNA	messenger ribonucleic acid
UV	ultra violet

1 Introduction

Research programs attempting to improve industrial properties of microorganisms were initially focused on strain selection after classical mutagenesis but the advent of recombinant DNA technology has dramatically expanded our capabilities and affected most contemporary research. In the area of cellular functions, rational applications of recombinant DNA technology are referred to today as *metabolic engineering* [1] and several successful approaches are reviewed in other contributions of this volume and elsewhere [1–3]. However, the complex nature of the highly interactive and elaborate informational and biochem-

ical networks that govern cellular function presents major challenges to any metabolic engineering attempt and, in fact, has hampered successful industrial implementation in many cases. Although algorithms and modeling frameworks are being developed to improve identification of effective genetic changes, the extensive molecular and mechanistic information that is required to guide *constructive* metabolic engineering approaches remains a main drawback to rational, deductive strategies. An additional problem arises from the difficulty of predicting secondary responses or side-effects due to lack of knowledge of inter-related regulatory and metabolic processes in a cell. Experimental experience in both academic and industrial labs has shown that secondary responses to genetic modifications often occur in pathways or reactions that are seemingly unrelated to the target, thereby confounding the rational strategies [1, 4, 5].

Very similar problems were associated with rational protein engineering, and so it is both stimulating and instructive to consider recent developments in this related field. Much like current constructive metabolic engineering, previous strategies in protein engineering mainly attempted a rational design via defined, site-directed changes based on structural and mechanistic information [6]. Because such fundamental information is often not available, commercial applications were limited. Moreover, many rational attempts to alter protein properties failed because either the chosen target amino acids were not appropriate or the introduced substitutions exerted unanticipated influences on structure or function. Today, novel high-throughput techniques and discovery approaches including biodiversity screening, genomic sequencing, phage display, in vitro screening methods, and directed evolution are rapidly replacing or complementing rational design in industrial biocatalysis [7, 8].

One of the most promising strategies in protein engineering is directed evolution, which has been successfully employed to improve existing protein functions several thousand-fold and also to tailor completely new, artificial enzyme properties (but, so far, not de novo functions) that are not found in the natural environment [9, 10]. Such capabilities are also useful for metabolic engineering. Directed evolution is generally understood as the use of repeated cycles of creating genetic diversity and sifting pools of variants by immediate selection or screening to recover only those with a desired functional property (Fig. 1). For a general introduction to the field see [11]. A major technological advance in evolutionary protein engineering was the introduction of in vitro recombination by 'hybrid PCR', for example by DNA shuffling, because multiple, related starting points can be used rather than a single gene [9]. The power of recombination arises from the possibility of removing neutral or deleterious mutations as well as preserving useful mutations, which may improve the desired property in a synergistic fashion when combined. The generated libraries of chimeric genes are searched either by selection, in which a protein is linked to host survival, or, if that is not feasible, by direct screening, which is basically selection at the single variant level [12]. This evolutionary concept has already been extended from single proteins to entire pathways [11] and the next frontiers are the shuffling of entire viral or even microbial genomes and directed evolution of novel pathways [13, 14].

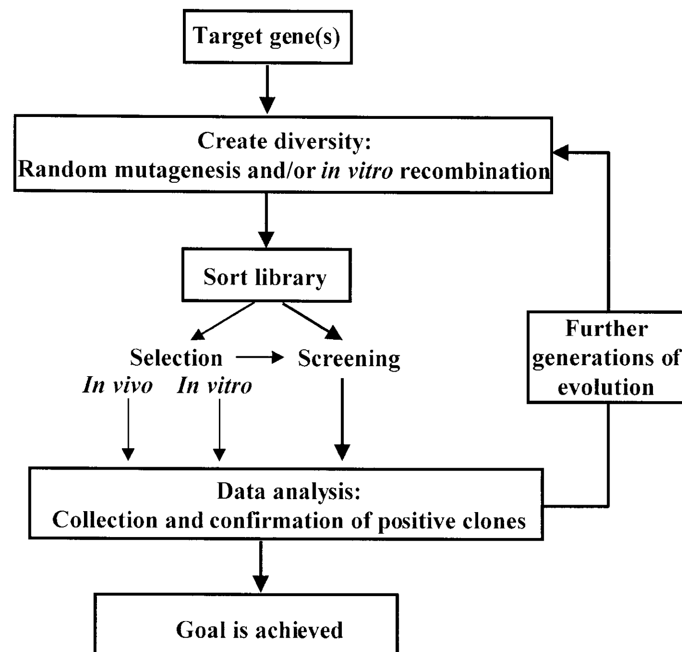

Fig. 1. Flow chart for directed enzyme evolution. Reproduced with permission from Zhao et al. [146]

Obviously, engineering of proteins shares many features with engineering of whole cells and so it is quite instructive to consider the suitability of evolutionary methods for metabolic engineering. In discussing evolutionary approaches it is helpful to employ the concept of *fitness landscapes* [15–17], which are topological representations of biological fitness in a given environment. Each genotype (or protein sequence) is associated with a fitness value (the phenotype) and the distribution of these functional values over the sequence space of all genotypes constitutes a fitness landscape. In natural evolution, fitness applies principally to the reproductive success of a species, and thus is rarely assigned to single genes. When referring to well-defined, *desired* characteristics of proteins or cells, the term *local* fitness landscape is frequently used to indicate that a particular fitness landscape is projected onto the sequence space. Thus, fitness is generally used in a much more restricted sense in applied evolutionary approaches. As a practical matter, sequence spaces are extraordinarily large, because the number of all possible sequences N is an exponential function of the number of information units λ (i.e., 4 nucleotides for DNA and 20 amino acids for proteins) and the length of the sequence (v), according to

$$N = \lambda^{v} \tag{1}$$

Thus, even a single protein with 230 amino acids spans a sequence space of 10^{300} points [8, 18], which is not fully accessible by any experimental method. Cells are several order of magnitude more complex than proteins, and so the sequence

spaces of even very modest genetic changes are dauntingly large. Fortunately, evolution proceeds not by exploring all possible variants but by incorporating single mutations, selecting the fittest of those, and then expanding the population and incorporating additional alterations [15, 19]. Therefore, most applied evolutionary strategies assume the existence of an evolutionary path that yields detectably improved fitness for each mutation that is required for a desired phenotypic change. Thus, it resembles natural evolution which is, in effect, a method of searching among an enormous number of possibilities for small, step-wise improvements that allow organisms to survive better and reproduce in their environments.

The basic concept of directed evolution is also evident in classical, empirical strain development by classical, random mutagenesis and direct selection on plates. This approach has a long history of success in industrial strain development, in particular in the absence of extensive genetic or physiological information. The best example of this is probably the greater than 4000-fold improvement of penicillin titers via empirical strain improvement [20–22]. Empirical procedures are particularly well suited for relieving feedback inhibition in biosynthetic pathways because simple and direct selection schemes can be applied, for instance resistance to toxic analogs of metabolic intermediates (antimetabolites). Unfortunately, most desired phenotypes cannot be selected by simply increasing resistance towards a challenging agent. Analytical screening for desired phenotypes in random variants is not an alternative, because it does not provide access to any significant fraction of most local cellular fitness landscapes. Another disadvantage of extensive passage through cycles of mutagenesis and selection is the concomitant accumulation of unfavorable mutations, which eventually leads to highly specialized but crippled strains, a commonly observed phenomenon. This cost to asexual evolution of small populations is known as Müller's ratchet [23], the underlying principle for reductive evolution of resident genomes such as endosymbionts or cellular organelles [24].

These problems of step-wise directed evolution with whole cells can potentially be solved by two strategies that are also at work in nature: recombination and *continuous* selection in large populations for many generations. In the first strategy, recombination of genetic elements and subsequent selection is used to combine beneficial mutations from different variants in one strain and to reduce the mutational load by eliminating deleterious mutations, thereby potentially avoiding Müller's ratchet. Consequently, additional beneficial mutations need not be 'rediscovered' in a selected strain to become incorporated in future generations. The most powerful tool to navigate fitness landscapes in protein engineering, in vitro recombination [18], is presently restricted to subgenomic elements that can be amplified by PCR, and thus is not applicable to entire microbial genomes. Although microorganisms are naturally capable of in vivo recombination, this process has rarely been exploited for directed evolution of biotechnologically relevant phenotypes.

In the second strategy, continuous in vivo evolution of entire populations circumvents passage through the single variant level after each mutation-selection cycle. This is possible because microorganisms are self-replicating, unlike proteins, so that the phenotype is coupled to the genotype (at least as a first approximation). Due to their small size, microbial laboratory populations are

large, exceeding 10^{11} individuals per liter (solutions with less than 5×10^9 cells per liter appear completely clear to the human eye), so that continuous evolution can be far more effective than step-wise procedures. The steady interplay between selection by the artificially posed conditions and mixed populations of continuously occurring genetic variants gives such continuous evolution its direction – potentially towards a desired phenotype, provided a pertinent selection scheme can be devised.

Due to the immense size of sequence spaces, evolutionary paths to improved variants may go astray or reach suboptimal solutions. This is intuitively recognized, since most evolutionary strategies are initiated with a phenotype that is already close to the desired one and thus may be considered more as engineering than as design strategies. Unlike step-wise evolutionary protein engineering, successful evolution of improved cells cannot be expected to lead to fully developed processes or products, but rather to constitute an important intermediate step in an engineering strategy. In industrial practice, strain developmental problems are often solved by synergistic application of metabolic engineering and empirical mutagenesis/selection. Thus, it can be anticipated that even more elaborate evolutionary methods will likewise be most powerful if used in combination with, or as the basis for, metabolic engineering to create synergistic effects for process improvement. I will refer to such applications of evolutionary techniques to microbial properties in a biotechnological context as *evolutionary engineering*, a term introduced by Butler et al. [25]. A prerequisite for any such evolutionary engineering is a selection scheme that directly or indirectly favors a desired phenotype.

A comprehensive understanding of microbial evolution combined with the ability to apply its principles to experimental systems are prerequisites to creating or optimizing microbial phenotypes with scientific or applied value by evolutionary engineering. Thus, without attempting to review comprehensively the literature on microbial evolution, this review highlights key concepts in designing and running evolutionary engineering programs. Furthermore, recent studies that employ evolutionary strategies to generate desired, heritable microbial phenotypes are reviewed and discussed. Applications of empirical mutagenesis/screening were recently reviewed [20–22], and so are not covered here. Finally, novel analytical procedures that may facilitate identification of the molecular basis of evolved phenotypes and thus impact evolutionary engineering will be briefly discussed.

2 Mutagenesis and Recombination

Mutations are a double-edged sword – the ultimate source of all genetic variation upon which any evolutionary process depends, yet the vast majority either have no apparent effect or are harmful, and so the rate of mutagenesis has to be appropriately tuned to design an efficient evolutionary process. Spontaneous mutations in microbial populations occur much less frequently than in viruses – generally at about 0.003 point mutations per genome (independent of its size) and round of replication [26]. Notable exceptions are the so-called hyper-

mutable genes in pathogenic organisms that are prone to mutation through various specific mechanisms [27]. At first glance, accelerated generation of variation, or an increase in the population size for that matter, thus appears to be advantageous for practical application of continuous evolution. In asexual populations, however, higher mutation rates need not accelerate the pace of evolutionary adaptation [28], which is the underlying principle of selection for new or improved phenotypes. Examples are populations in which two different lineages of beneficial mutations interfere with one another's spread. Because the two mutations cannot be combined into the same lineage without recombination, such clonal interference imposes a speed limit on adaptive evolution. In small or initially well-adapted populations that spend long times waiting for beneficial mutations, on the other hand, an increase in the mutation rate may effectively accelerate the evolutionary process. Mutability is genetically determined like any other property, hence mutability itself can be affected by environmental (Sects. 2.1 and 2.2) or genetic (Sects. 2.3 and 2.4) manipulations, including recombination (Sect. 2.5).

2.1 Physiologically Enhanced Spontaneous Mutagenesis

Spontaneous alterations in the inheritable genetic sequence may result from a multitude of causes and mechanisms that can be grouped into three categories – (i) small local changes, (ii) DNA rearrangements, and (iii) horizontal DNA transfer, as illustrated in Table 1 [29, 30]. While the overall rate of spontaneous mutagenesis is usually rather stable and low [26], it may rise considerably under certain circumstances and modulation of environmental conditions provides a convenient means to accelerate this rate. For example, the global rate of mutagenesis in a population increases during adverse environmental conditions, for instance metabolic stress or stationary phase [29, 31]. Such environmental stimuli induce enzyme systems, mostly DNA polymerases that are designed to generate mutations, such as the SOS DNA repair system. Unlike the replicative DNA polymerases, which faithfully copy DNA sequences, these polymerases introduce errors at high rates, thereby increasing the genetic diversity and adaptation potential of the endangered population. Less well recognized is the fact that glucose repression may also reduce spontaneous mutagenesis, as the rate at which spontaneous *E. coli* mutants occur is several-fold lower on glucose than, for example, on glycerol [32]. While such environmental factors can accelerate the rate of mutagenesis, they will inevitably also influence the process of selection.

2.2 Chemical or Radiation Induced Mutagenesis

Induction of mutagenesis by chemicals or radiation treatment is frequently used because it is technically simple and widely applicable to almost any organism [29]. Most chemical mutagens preferentially introduce certain types of mutations such as exchange of specific nucleotides or frame-shifts, but many, including ethyl methane sulfonate (EMS), can also induce deletions of considerable

Table 1. Classification of mutations, their origins, and potential effects

Type of change	Length	Source of mutation	Effects[a]
Small local changes			
Substitution Insertion Deletion Duplication	1 bp 1 to several bp 1 to several bp 1 to several bp	Spontaneous mutagenesis Replication infidelities	Gene silencing Gene expression Cryptic gene activation Altered protein specificities
DNA rearrangements			
Inversion Duplication Insertion Deletion Excision	Several bp up to several kb	Homologous recombination Mobile genetic elements (i. e. IS elements, transposons)	Gene silencing Gene expression Cryptic gene activation Gene dosage Gene organization Gene mobilization Domain fusion Domain swapping
DNA acquisition			
Horizontal DNA transfer	Several kb up to hundreds of kb	Transformation Conjugation Transduction (phage-mediated)	Increase of total genetic information content Gene silencing

[a] A particular source of mutation is not necessarily capable of causing all listed effects.

length. For example, about 13% of the EMS-induced mutations in *Caenorhabditis elegans* are reported to be DNA rearrangements, and most of these are deletions with an average size of 1300 bp and a broad size range [33]. The use of nitroso-methyl guanidine (NTG), on the other hand, typically results in closely linked mutations in one clone due to its specificity for mutating DNA at the replication fork. Another factor that needs to be borne in mind is the phenomenon of biological mutagen specificity, whereby a given mutagenic treatment preferentially mutates certain parts of the genome [21]. Thus, for repetitive uses, it is advisable to change mutagens periodically, to take advantage of their presumably different mechanisms of action. The preferred mutagens for most applications are far UV, EMS, and NTG, because they induce a great variety of molecular alterations with no apparent specificity for genomic subregions [34].

For efficient evolutionary engineering, mutagenic treatment with an optimum dose of mutagen is particularly critical when performing successive rounds of mutagenesis and selection [34]. While the primary requirement is to increase the proportion of mutants in the surviving population, the optimum dose yields the highest proportion of desirable mutants. Although the optimum dose may be difficult to estimate for complex or difficult-to-detect phenotypes, related but easily scorable phenotypes may be used to help determine the optimum range. Any mutagenic treatment will give a dose response curve similar to

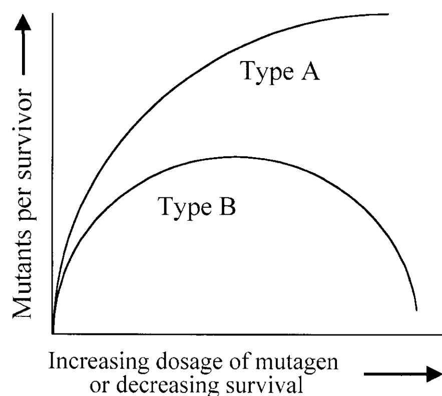

Fig. 2. Typical mutation kinetics curves. Reproduced from Rowlands [34]

either curve A or B in Fig. 2, wherein the type of curve appears to depend on the scored phenotype rather than on the mutagenic treatment used. While suboptimal mutagen doses will obviously create less diversity, overdoses of mutagens will simply kill the cells. Moreover, dosages even slightly above the optimum will increase the frequency at which neutral or potentially harmful mutations also become incorporated into the selected mutants. This is because advantageous adaptive mutations that occur in the background of neutral or weakly counter-selected mutations allow these undesired mutations to hitchhike along [35].

2.3 Mutator Strains

A fascinating option for accelerating continuous evolution is the use of so-called mutator strains, which are characterized by frequencies of spontaneous mutagenesis that are orders of magnitude higher than usual. In many cases, such mutations promote more rapid adaptive evolution, and mutator strains were shown to outcompete quickly the wild-type in glucose-limited environments [36]. In fact, mutations in mutator genes occur frequently in populations that are propagated over extended periods under identical conditions [37]. Intuitively, such mutations appear advantageous for evolutionary adaptation but their frequent occurrence in adapted populations is more likely circumstantial, resulting from numerous opportunities for the mutator mutation to hitchhike along with beneficial mutations to which they are genetically linked under these conditions [28]. Thus, mutators do not necessarily accelerate the pace of evolutionary adaptation, as was discussed more generally for spontaneous mutations before. Nevertheless, mutator genotypes can be very valuable in well-designed continuous evolution strategies, such as when evolving populations would be expected to spend most of their time waiting for beneficial mutations (e.g., [38]), as may be the case with already well-adapted strains.

A negative aspect of using such highly mutating strains is the potential accumulation of deleterious mutations that may reduce overall fitness [39] and their

inherent phenotypic instability. Consequently, mutator genotypes have more frequently been used as convenient tools to introduce mutations into plasmid- or phage-encoded recombinant proteins, which can simply be separated from the background of accumulated harmful and neutral genomic mutations [40, 41]. A potentially very useful strategy for accelerated continuous evolution of particular genes is based upon propagating a phagemid population in a mutator strain. In one study using a β-lactamase, which confers resistance to the antibiotic cefotaxime, up to 1000-fold more resistant variants were obtained after a few weeks of selection in media with increasing cefotaxime concentration [42]. Briefly, a mutator strain was co-infected with a helper phage and a phagemid that carries the β-lactamase gene. After selecting the population for increased resistance to cefotaxime, live cells were heat-inactivated and the evolved phagemid population of about 10^6 variants was used to infect a fresh mutator host. This procedure ensured that only mutations within the phagemid genome are transferred into the next evolutionary cycle.

Many genes that cause a mutator phenotype are involved in repair or error avoidance systems, and bacterial mutator genes were recently reviewed by Miller [43]. For example, mutations in the *E. coli dnaQ* gene, which encodes the exonuclease activity-providing ε subunit of DNA polymerase III, impair the proofreading activity and hence lead to a very strong mutator phenotype. Similarly, mutations in components involved in the mismatch repair system also cause a strong mutator phenotype. Mutator genes in the eukaryote *S. cerevisiae* include the MMS2 gene (involved in postreplication repair) [44] and the POL30 gene, which is involved in mutation suppression [45]. The mutations caused by mutator phenotypes are mostly base transitions and frameshifts, but may also include deletions. At least for *E. coli*, such mutator strains can either be generated by defined genetic manipulations or by direct selection on a single plate [46].

2.4 Tagged Mutagenesis

All heretofore mentioned mutagenesis procedures have a serious disadvantage in that it is difficult to locate the modification, unless phenotypic characterization and a known gene-function relationship provide a clear lead. The use of tagged mutagenesis is one approach to facilitating the transfer of an evolved phenotype by metabolic engineering to others strains or organisms. For this purpose, a broad range of transposable elements is available, including genetically engineered mini-transposons [47]. These DNA elements catalyze their own movement, or transposition, to a location within a chromosome or, in certain cases, preferentially within extrachromosomal elements [48]. In addition to gene disruption, such transposable elements may also be used for random gene overexpression if equipped with suitable outward-oriented promoters. Most transposons, however, exhibit some degree of target preference and their capability for multiple insertions within one strain is usually limited.

An alternate strategy for mutagenesis and gene tagging is based on random insertion of unique, short DNA fragments ('signatures'), which is normally used for parallel identification of important, habitat-specific genes by negative selec-

tion [49]. Because insertional inactivation of genes may also improve fitness in evolutionary engineering, this strategy can be used for positive selection and rapid identification of genes that are disadvantageous under the given conditions. While this procedure is normally performed with pools of up to a few hundred mutants at a time, hybridization to a high-density array (DNA Chip) of signature tags provides an interesting option for genome-wide selection and identification of relevant genes [50] (see also Sect. 6). Additionally, random insertion-duplication mutagenesis can be used when efficient transformation systems are available [51].

2.5 In Vivo Recombination

Although generally perceived of as clonal, prokaryotes show a wide range of population structures that range from almost strictly clonal (e.g., *Salmonella*) to fully sexual (e.g., certain *Neisseria*) [52]. Akin to directed evolution of proteins, it would be of utmost importance to enhance recombination between different variants with improved phenotypes. To exploit the potential of homologous recombination for evolutionary engineering, DNA exchange within a population may be mediated by the well-known natural mechanisms of horizontal DNA transfer: conjugation, transduction, and transformation. An applied example of this approach is strain improvement of starter cultures in the dairy industry using naturally occurring conjugative plasmids [22]. The use of natural or artificial (e.g., plasmid- or virus-based expression libraries) horizontal DNA transfer and non-homologous recombination, on the other hand, also allows random DNA transfer from other organisms or previously selected variants into a host prior to selection. Thus, appropriate selection will enrich for clones bearing DNA segments that confer a selective advantage and, upon continuation of selection, additional fitness-increasing mutations can occur in this background.

In contrast to the haploid prokaryotes, the use of eukaryotic microorganisms that may exist in haploid, diploid, or even polyploid form, such as *Saccharomyces cerevisiae*, offers the potential for breeding independently improved variants, for instance by creating a diploid cell from two haploids. The offspring from this chimeric diploid cell may than be selected for improved combinations of both haploid variants. This very powerful approach for evolutionary engineering has often been used in industrial strain development of fungal production processes. For example, desired qualities such as robustness, high growth rates, or sporulation have been reintroduced into high yielding, but crippled production strains [34]. It is a pertinent question to ask whether, given the choice, haploid or diploid strains should be used in an evolutionary experiment. It is interesting to note in this context that the frequency at which adaptive mutations are fixed in diploid populations of *S. cerevisiae* was found to be 1.6-fold higher than the frequency in isogenic haploid populations [53]. Although it was argued that diploidy would slow down adaptation under many conditions [54], it appears to be advantageous in asexual populations when the number of favorable mutations per generation is very small – a situation that is not unlikely to occur in evolutionary engineering.

As opposed to the random recombinatorial approaches discussed above, a major benefit to complementing evolutionary engineering with rational design using genetic engineering resides in the potential to jump into new, rationally selected regions of the fitness landscape. Such designs may be based on knowledge of genes or proteins that are anticipated to be relevant for a particular phenotype and this insight would then be used to preselect genes for random expression in selection experiments. Such hypotheses about the relevance of components may be rather vague as hundreds to thousands of genes could be propagated in evolving populations. In practice, rational evolutionary design can be achieved either with multiple heterologous variants of one or more chosen genes or with entire expression libraries of heterologous organisms with desired features. An example of such a rational design is the improvement of recombinant plasmid stability by random cloning of DNA fragments from stable endogenous plasmids [55]. If transfer of large numbers of genes or of entire genomic segments is anticipated, artificial bacterial or yeast chromosomes that allow stable propagation of DNA segments up to several hundred kb in length may replace plasmid-based expression systems.

3 Selection

Natural evolution is thought to be responsible for the extraordinary variety and complexity of the biosphere, and today's life forms are the variants that are presently most fit variants to cope with their particular environments and ecosystems. In the simplest form of directed evolution, a person that differentially removes certain phenotypes from the population establishes relative fitness by screening of individual variants [21, 22]. The obvious advantage of selection by screening is the flexibility that basically any cellular function can be used, provided that a suitable assay is available. Such screening applications profit significantly from recent advances in high-throughput procedures such as robotic (sub-) microliter liquid handling, 384- and 1536-well microtiter plates, digital camera-equipped picking robots, and analytical procedures such as parallel photocells that can rapidly access the various microtiter plate formats. These technical advances are also, in part, responsible for the success of directed evolution strategies in protein engineering. Two general problems pertain to such step-wise evolution approaches: the size of local fitness landscapes for complex cellular phenotypes that require multiple, often unlinked genetic modifications and the strong dependence of phenotypes on environmental conditions. Thus, a critical question is if interesting phenotypes that are identified in multi-well screening procedures translate into the conditions of production processes.

The power of continuous evolution resides in its efficiency and the possibility to select under process-relevant conditions. To avoid unanticipated solutions, the selection procedure should reflect the characteristics of the industrial process, for example aeration, carbon limitation or abundance, fluctuating or constant substrate supply, complexity and concentration of the nutrient sources, pH, osmolarity, mechanical stress, liquid or solid media, cell density, etc. In cer-

tain cases, however, pleiotrophic effects of evolutionary adaptation to a particular environment may also increase competitiveness in an alternative environment (see, for example, [56–58]). It needs to be borne in mind that fitness in continuous evolution is a function of competition among the variants that are present under the given conditions, and this property is not under the direct control of the experimenter. Any property that increases the relative number of a variant or the ability of one variant to limit the number of offspring left by other variants under the imposed conditions would improve competitive fitness. Such competitive fitness in a population is not necessarily identical with fitness in the biotechnological sense, which usually refers to improved properties at the single cell level.

3.1 Natural Evolution

The genome of each organism contains not only information for its functioning in the current environment, but the potential to evolve novel functions that will allow it to thrive in alternative environments [19]. To improve understanding of this process and the selective constraints, microorganisms with their short generation times are perfect research subjects, because thousands of generations can thus be studied in simple laboratory environments. At their most basic level, the 'rules' of evolution are remarkably simple: species evolve by means of random variation (via mutation, recombination, or other operators); this is followed by natural selection in which the fittest tend to survive and reproduce, propagating their genetic material to future generations. In addition to horizontal DNA transfer, novel catabolic or metabolic functions are often acquired by mutational activation of cryptic genes, which constitute a versatile genetic repertoire that enhances the adaptive potential of a species [59]. Such cryptic genes are phenotypically silent DNA sequences, which are not normally expressed under any conditions, and are assumed to have played important roles in natural evolution. Another important group of genes in this context are the so-called evolution genes, whose main function in DNA repair appears to be acting for the benefit of evolution itself by generating and modulating spontaneous mutagenesis [30, 31]. Different from mutator genes, however, the rate of mutagenesis that is introduced by these evolution genes is subject to cellular control.

Evolutionary adaptation of species to changing environments occurs in all but the simplest cultivation systems. In fact, our so-called wild-type laboratory strains are the product of an evolutionary domestication process, perhaps most pronounced for *S. cerevisiae*, which has been exploited for baking and alcohol production by virtually every human society. The phenomenon of evolutionary adaptation to laboratory environments has long been recognized and is known as *periodic selection*, referring to the periodic appearance and subsequent exponential take-over of the population by variants with a selection advantage over the currently present cells [60–62]. The kinetics of such population take-overs can be monitored by tracking the replacement of the resident population via markers that have no impact on the fitness of the cells under the cultivation conditions used. This will reveal repeated (periodic) fluctuations in the level of the

independent, or neutral, marker. Because these mutations are completely neutral, gain-of-function reversions for such phenotypes, e.g., resistance to a phage or a chemical or utilization of a substrate (other than the one actually used during selection), occur at a constant rate that equals the mutation rate and thus these phenotypes should increase linearly in a population of constant size. In contrast, variants with fitness affecting mutations will substitute the population at a rate that is a function of population structure as well as strength and direction of the selection.

In a culture inoculated from a single clone, a new advantageous mutation is most likely to occur in the much larger population that does not have the neutral mutation, as illustrated schematically in Fig. 3. The adaptive mutant then replaces the currently existing population (including the fraction of neutral mutants) at the log linear rate of selection. The neutral mutation will continue to occur at the same linear frequency in the adaptive mutant, until another advantageous mutation occurs, again in the still predominant population without the neutral marker phenotype. Thus, the abundance of the neutral marker phenotype drops again and the cycle is repeated. Extensive experimental evidence for this phenomenon is given in the excellent review of Dykhuizen [61]. Periodic selection and hitchhiking in bacterial populations are also discussed on theoreti-

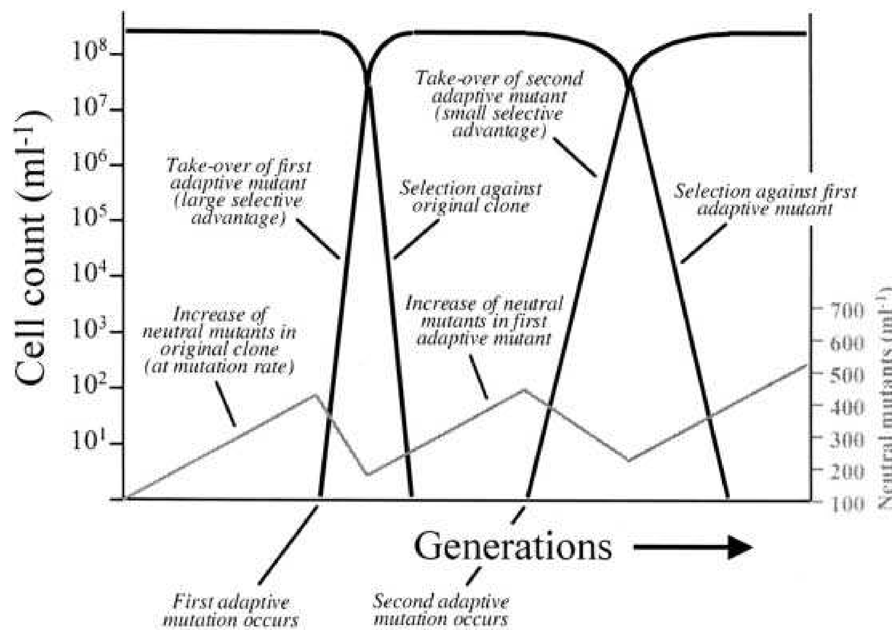

Fig. 3. Schematic representation of the population dynamics during adaptive evolution of an asexual population. The *gray line* at the bottom represents the abundance of neutral mutants (at a linear scale). The other lines indicate periodic selection of two consecutively evolving advantageous mutants (at a logarithmic scale). This was inspired by a similar drawing by Dykhuizen [61]

cal grounds by Berg [60], who developed a stochastic theory to describe the dynamics of large asexual populations.

In addition to monitoring mutant take-overs, such neutral markers are particularly valuable for quantifying differences in fitness between evolved clones. In studies on natural evolution, differences in fitness may depend on subtle variations at one or more loci so that the overall fitness is often difficult to identify. For this purpose, competition experiments are performed using two strains that are distinguished by different neutral markers [61]. By following the relative numbers of two competing strains during a growth experiment, the differential growth rate (s) per unit time (t) can be determined from a plot of $ln(x_i/x_j)$ vs time, where x_i and x_j denote the cell densities of the two strains. Competitive fitness of one strain over another is then quantified by the selection coefficient s_{ij} according to

$$\ln[x_i(t)/x_j(t)] = \ln[x_i(0)/x_j(0)] + s_{ij}t. \quad (2)$$

3.2
Solid Media

Selection on solid media is frequently used because large numbers of mutants can conveniently be screened by visual inspection of growth as such, a zone around the colony as a consequence of a diffusing product, or a color change due to a coupled reaction. Generally, useful results are obtained only when expected differences in fitness are large and the advantageous types are rare. In empirical strain development, plate selection procedures are frequently used for removal of specific feedback inhibition loops in biosynthetic production pathways by selecting for resistance to an antimetabolite of the regulatory substance. The parent strain cannot grow in the presence of this antimetabolite, but any mutant capable of growing must not be feedback inhibited any more [21]. Another example of positive selection for increased tolerance of toxic compounds is the selection for increased antibiotic resistance based on overexpression of inactivating proteins [63].

An advantage of step-wise plate selection is its direct read-out on the progress of evolutionary adaptation, in particular when it is unclear a priori to what extent improvement is possible (see, for example, [64]). However, this mode of selection is likely to be inefficient for complex phenotypes that require multiple mutations. Moreover, the ultimate destination of most strains are some sort of bioreactor, and the importance of mimicking the most relevant production system conditions during selection cannot be overemphasized. From this perspective, plate-based selection assays have an inherent danger of selecting for phenotypes that are not reproducible in liquid media.

3.3
Batch

In liquid media, fitter variants in a particular environment evolve over time and eventually replace the parental population as a consequence of adaptation by selection, which is often studied in batch cultures. An important characteristic of

selection in batch culture are dramatic changes in environmental conditions from feast to famine, so that the cells are subjected to alternating periods of growth and stasis upon serial transfer.

A particularly intriguing set of asexual evolution experiments in batch culture was performed by Lenski and coworkers and encompassed the fitness analysis in 12 independent *E. coli* populations founded from a single ancestor [65–67]. Daily serial transfer propagated these populations for 1500 days (about 10,000 generations) in the simple, unstructured environment of glucose-supplemented minimal medium in shaking flasks. After 10,000 generations, the average fitness of the derived clonal variants was increased by about 50% relative to the common ancestor, based on competition experiments in the same batch culture environment. The primary reason for this improvement was attributed to reduced lag phases and higher maximum growth rates. Experiments with alternative carbon substrates also revealed higher fitness on substrates with similar uptake systems, which suggests enhanced transport as an important target of evolution [66]. Although these phenotypic changes were consistent in the 12 independently evolved populations, their genetic diversity – as determined by analysis of restriction fragment length polymorphism with seven insertion sequences as probes – was large [65]. Over time, the evolved genomes became increasingly different from their ancestor and each other, to the extent that almost every individual within a population had a different fingerprint after 10,000 generations. Point mutations were rather rare in the evolved populations, meaning that the accumulated genomic, and possibly phenotypic, changes were mostly a consequence of chromosomal rearrangements. Certain pivotal mutations were apparently shared among all members of a given population, and these constitute prime candidates for phenotypically relevant mutations.

Thus, evolution of adaptive performance is remarkably reproducible, although the phenotypic adaptation may be achieved by greatly different genotypes. While probably only a handful of mutations were relevant for the investigated phenotype, at least some of the other genetic alterations would certainly gain importance under different environmental conditions. Consequently, the history of evolved strains from continuous evolution experiments is very important, as identical selections will inevitably lead to different variants. Another very important observation that pertains to applications of evolution procedures is the hyperbolic rate of change in competitive fitness, as about half of the phenotypic improvement occurred within the first 2000 generations (of 10,000 generations) (Fig. 4). Thus, the rate of fitness gains in microbial populations appears to decelerate significantly over time.

3.4 Microcolonization

A particular problem in selecting for variants with improved secretory capacity in liquid media is the absence of a physical link between the clones in a population and their secreted products. This may lead to interactions between individual clones, such as cross feeding or inactivation of selective agents by few clones within a population. Faced with this problem, a group at Genencor developed an

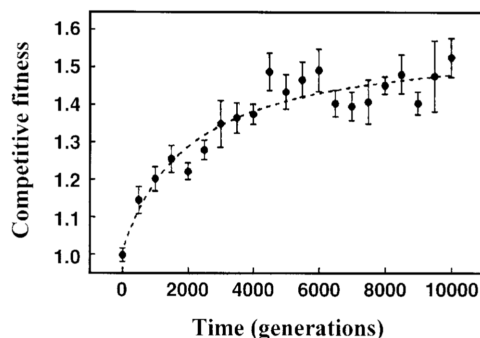

Fig. 4. Change in competitive fitness during 10,000 generations of experimental evolution with *E. coli*. Fitness is expressed relative to the common ancestor. Each point is the grand mean averaged over twelve replicate populations. *Error bars* are the 95% confidence intervals. The *dashed curve* indicates the best fit of a hyperbolic model to the data from Lenski and Travisano [67]. Figure reproduced with permission from Lenski et al. [66]

innovative strategy that enabled the efficient enrichment of better protein secretors from large populations by growing the cells in hollow fibers. The 0.5-μl interior compartments of the fibers act as miniature cultivation vessels [68]. Under these microcolonization conditions, each colony grows in its own microenvironment and cross feeding between neighboring colonies is effectively eliminated. When bovine serum albumin is the sole nitrogen source, clones that secreted either more protease or a better protease variant grew faster than the parent did. After four rounds of selection in such microcolonies, the population was sufficiently enriched with variants exhibiting increased secretion to allow for detailed characterization of individual mutants [68]. Because each hollow-fiber cartridge provides about 3×10^5 such 0.5-μl compartments, this technique is applicable to populations that are too large to be analyzed by screening in microtiter plates. In addition, this procedure can simply be repeated with enriched populations for several rounds such that a bio-panning effect is achieved, which is not possible by selection on solid media. Given its apparent technical simplicity, this approach should also be applicable to other secreted products, provided that a positive selection method can be conceived.

3.5 Chemostat

During growth in batch culture, a population typically passes through the distinct phases of lag, exponential, transition, and stationary growth. Thus, evolutionary events may arise from advantages in any of these phases. In contrast, continuous culture systems provide a constant environment that is also frequently used for studying evolution [61, 69]. Under continuous culture conditions, the removal of cells from the growth chamber by outflow is random and thus becomes a selective function with the growth rate as the main factor determining survival. The most frequently used continuous culture system is the

chemostat, which, in physiological steady state, maintains a constant cell density by the continuous influx of a growth limiting nutrient. These well-defined environmental conditions allow for independent variation of growth parameters such as the rate of growth or the concentration of a limiting nutrient. Bioreactors for continuous culture in biotechnological research are usually equipped with sophisticated (and expensive) instrumentation. However, this expense is not necessarily required for evolutionary experiments and the choice of smaller scale chemostats with a simpler design allows performing continuous evolution experiments at reasonable costs in parallel [70].

Continuous cultures that extend for fewer than 20 generations allow for quantitative physiological investigations in a defined steady state. Experiments of longer duration become the study of evolution in action. In continuously operating production processes, the danger of genetic drift resulting from spontaneous mutations poses significant challenges. This is of practical relevance because recombinant organisms are usually engineered to maximize product formation, often at the expense of growth rates or overall fitness. Mutations that increase growth rate will be advantageous and eventually take over the population, thereby likely reducing product formation. However, if used properly, direct control of physiological culture parameters in continuous cultures is a valuable tool that can be employed to modulate selective pressure in favor of a desired phenotype. The influence of these parameters on the competition between different species was reviewed by Harder et al. [36]. When the limiting substrate in a chemostat is the carbon source, the culture is characterized by high efficiency in converting carbon to biomass. When growth is limited by nutrients other than the carbon source, the carbon flux into the cell is generally less tightly controlled, leading to profound effects on cellular energetics [71]. The specific effects of nitrogen, phosphate, potassium, sulfur, and other limitations are reviewed by Dawson [72]. In such cases, various metabolic by-products (e.g., acetate or lactate) or extra- and intracellular polymers are often overproduced, as compared to carbon-limited operation. Consequently, the choice of limiting nutrient will profoundly influence the selection pressure in a chemostat.

During prolonged cultivation in carbon substrate-limited chemostats, two general types of evolutionary events that confer selective advantages to emerging mutants prevail – increased maximum specific growth rates and reduction in the value of the Monod constant K_s for the limiting nutrient [69, 73]. However, any mutation that increases the residence in a chemostat will be favorable, including adherence to bioreactor walls. An important phenomenon concerning the clone-specific metabolism in such evolving cultures is cometabolism, which manifests itself as a physiological and often morphological polymorphism within the population [57, 74]. A particularly well-studied example is *E. coli* cultures in glucose-limited chemostats. A single clone evolved over the period of 773 generations at a dilution rate of 0.2 h^{-1} to form a polymorphic population in which several distinct mutant strains coexisted [74]. In this miniature ecosystem, the largest fraction consisted of efficient glucose scavengers with a metabolite secretion phenotype, and the smaller fraction consisted of mutants that thrived on the secreted, incompletely oxidized metabolites acetate and, to a

lesser extent, glycerol [75]. Such an acetate-cross feeding polymorphism is reproducible in long-term populations of *E. coli*, occurring in 6 out of 12 independently studied glucose-limited chemostat populations [76]. In all cases, it was associated with semi-constitutive overexpression of acetyl-CoA synthetase, which allowed for enhanced uptake of low levels of exogenous acetate. Such a polymorphic coevolution potentially complicates selection strategies as the whole population may express a desired phenotype that is not exhibited by any single variant within the population.

Another potential drawback of continuous asexual evolution in continuous culture is the strictly sequential appearance and fixation of adaptive mutations. Consequently, a newly appearing variant may compete only with its immediate one or few predecessors, if historically older variants were previously counter-selected. Thus, new variants could in fact exhibit lower fitness compared to more distant predecessors. Such a result was seen with haploid and diploid *S. cerevisiae* cultures that were grown in glucose-limited chemostats for up to 300 generations [77]. As expected, the relative fitness of clones isolated later was always higher than that of the clones isolated immediately preceding the adaptive shift. This was shown by pair-wise competition experiments in which the frequency of the strains was monitored by newly introduced neutral markers. In several cases, however, the relative fitness of clones carrying multiple adaptive mutations were lower than the fitness of clones isolated earlier in the experiment. Thus, combinations of adaptive mutations may result in maladapted clones, as compared to their progenitor, which may have never directly competed with the later occurring variants. During selection in batch culture for 10,000 generations, in contrast, a steady, although hyperbolic improvement in fitness compared to the ancestral strain was observed, as is illustrated in Fig. 4 [66].

The discussions in the previous two paragraphs warrant a note of caution for the use of continuous culture selections in evolutionary engineering of useful phenotypes. Fitness of a particular variant in continuous culture is not only a function of its capability to thrive under the given chemical and physical conditions – usually the phenotype desired by the applied scientist – but is inevitably linked to the presence of and, possibly, interaction with other variants. Thus, fitness in continuous culture is determined by the ability to compete with all other variants that are present at a given time under the applied conditions. This is not necessarily identical with the improvement of a biotechnologically desired phenotype. Because there may not be one optimal phenotype for any set of variants and environmental conditions [60], a population could be cycling through periodic selection indefinitely without actually achieving a long-term improvement in fitness (or a desired phenotype). To ensure that evolutionary adaptation during continuous selection proceeds indeed in the desired direction, it is of utmost importance to monitor evolutionary progress at the single clone level. Additionally, it is probably good advice to inoculate occasionally a new selection culture with the best clone(s) from different stages of the previous selection culture(s), so as to avoid or at least minimize potential evolution of both co-metabolism and unfavorable combinations of adaptive mutations.

3.6
Other Continuous Culture Devices

Variations of conventional chemostats that enable alternative modes of operation for continuous culture have been introduced and exploited. One example is auxostats that modulate the rate of feeding to control a state variable in continuous culture [78]. These devices can be operated under difficult or unstable conditions and thus overcome some of the disadvantages associated with chemostat cultures [78, 79]. Generally, auxostats permit growth near the maximum growth rate without the danger of washout that is inherent to chemostat operation. At high dilution rates, selection rates are remarkable because the effects of small differences between growth rate and washout are magnified. As the culture calls for increased feeding to maintain a constant value of the control variable, there is an accompanying decrease in residence time, which causes slower growing variants to washout. Probably the best known auxostat is the turbidostat, which maintains a constant cell density (turbidity) of an exponentially growing culture using an optical sensor for feedback control of nutrient inflow [80]. A major problem for long-term turbidostat cultivation is microbial adhesion to surfaces, including the optical sensor, as this confounds the turbidity determination. However, the choices of feedback parameters for auxostats are quite broad, including pH, concentrations of dissolved oxygen, nutrients, or metabolic (by-) products in the culture broth, and the concentrations of CO_2, O_2, or volatile compounds in the effluent gas, as well as combinations thereof [78].

Growth in auxostats is usually limited by the availability of a nutrient but may likewise be limited by toxic or inhibitory substances in the growth environment or by some other environmental stress. Generally, variants that are tolerant of toxic agents evolve quickly, and the selective pressure must be increased to further increase the tolerance level and/or to suppress adaptations in which a few members of the population consume or inactivate all the toxin. In the latter case, the selection pressure would effectively be relieved for the rest of the population [81]. To optimize adjustment of the selection pressure, the stress should be increased automatically, preferably via feedback control utilizing a growth parameter that can be measured on-line. Upon periodic mutant take-over, the environmental stress is thus gradually increased in a procedure that is referred to as interactive continuous selection. In principle, any growth parameter could be used for automatic feedback control, provided an appropriate sensor and control design is available.

A particularly ingenious automatic feedback system for interactive continuous selection was devised by Brown and Oliver [82], who used the CO_2 concentration in the effluent gas of a continuous culture to maintain selective pressure for tolerance to increasing concentrations of ethanol in a process that is also referred to as Brown and Oliver interactive continuous selection (BOICS). Specific applications of BOICS are reviewed in Sect. 5.1. Using a model-based approach, guidelines for appropriate BOICS controller design were recently presented that will likely pave the way to a broader application of this very useful selection technique [83]. Comparing the outcome of selection for inhibitor-tolerant mutants in chemostat, turbidostat, and BOICS, it was argued that only the latter se-

lects specifically for variants that are tolerant to extreme concentrations of the inhibitor [84]. Chemostats, in contrast, select for tolerant mutants that can sustain a given growth rate, whereas turbidostats select for tolerant mutants that exhibit increased growth rates under the given nutritional conditions and inhibitor concentrations.

3.7
Fitness Landscapes and Effective Means of Conquering Fitness Peaks

All possible genotypes represent the sequence space, whereas the functional values of the associated phenotypes (or phenotypic characteristics) commonly called fitness, define a fitness landscape. We can conceive of evolution as carrying out adaptive walks towards peaks in more or less mountainous fitness landscapes of sequence spaces, such as among possible DNA or protein sequences. This walk is guided by incremental increases in competitive fitness to drive the distribution of a population towards regions of higher fitness. Although this general view is widely accepted as a fact, quantitative population genetics of adaptive evolution is still a matter of debate [85, 86].

The concept of fitness landscapes as introduced by Wright [16, 17] provides an important contribution to evolutionary theory and is a very useful concept for the discussion of evolutionary processes. Such fitness landscapes are not fixed in structure but deform in response to changes in the abiotic environment and in response to coevolution [15]. In coevolutionary processes, the fitness of one organism depends upon characteristics of another organism with which it interacts, while all simultaneously adopt and change. Although evolutionary engineering is usually initiated with a single strain, coevolution can occur in evolving populations as shown for example in Sect. 3.5. The movement of a population over the fitness landscape depends on the topology of the landscape and on whether the population is sexual or asexual. Local protein-fitness landscapes in directed evolution are usually assumed to be 'Fujiyama-like' (i. e., they increase more or less monotonically towards a fitness optimum) because the protein under investigation has already some characteristics of the desired kind [18]. In contrast, most local fitness landscapes of cellular phenotypes are rugged or, if an organism does not exhibit a desired characteristic (for example utilization of a nutrient), are mostly plain (that is empty of function) with isolated peaks of fitness. For a more comprehensive treatise of this subject, the interested reader is referred to the excellent and provocative book of Kauffman [15].

In general, natural selection tends to drive a population to the nearest peak, which is not necessarily a global optimum. Because there are usually many molecular solutions that enable individuals to surmount environmental challenges, there will be many fitness peaks, the majority of which represent local optima. Depending on whether a population occupies a single niche at high density or is dispersed sparsely over a wide range, it reaches a state of either near-stasis (which most likely represents a local fitness optimum) or gradually improving adaptation, respectively. As microbial laboratory populations are usually of the former type, adapted populations in evolutionary engineering may be stuck with a suboptimal solution to cope with its environment because natural selec-

tion opposes passage through a 'valley' of maladapted intermediate states. This theory is, at least partially, supported by Lenski's 10,000-generation experiment, in which resulting populations have seemingly reached distinct fitness peaks of unequal height [66]. In this context, two questions are of immediate applied interest. First, how much time is required for a population to attain a local optimum (or how can this time span be reduced) and, second, how can populations be treated so that they arrive at a global optimum?

The answer to the first question is appropriate tuning of the rate of mutagenesis to minimize the time of selection. Various approaches to that end are covered in Sect. 2. Moreover, it may be advantageous for efficient evolutionary engineering to modify slightly the selection scheme at appropriate intervals. This is because adaptation to the selection conditions usually involves first a modest number of mutations that exert large positive effects that are followed by a greater number of mutations of smaller effect, as was shown both experimentally (e.g., [66]) and on theoretical grounds [85, 87] (Fig. 5). Clearly, it is of utmost importance for any evolutionary engineering experiment to monitor the progress of evolution. Slight modifications in selection schemes may also avoid evolution of overly specialized variants that exhibit the desired phenotype only under the exact conditions of the selection. The answer to the second question is recombination, so that a population does not necessarily need to reinvent novel properties, as they could simply be transferred from different organisms or previously selected variants. Selection is then used to choose the most appropriate from different molecular incarnations of this property and to incorporate it optimally into the host strain.

While the above discussion concerned crossing of valleys between different but related fitness peaks, another problem is the distance between the starting point in sequence space and the nearest fitness peak. This poses the practical

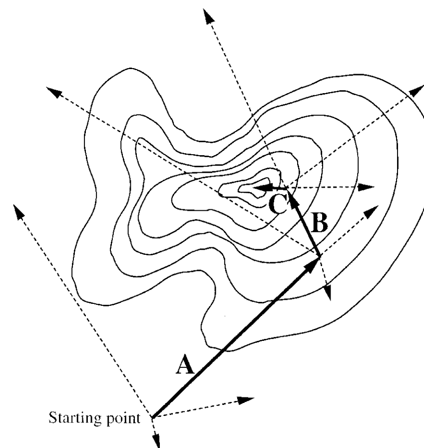

Fig. 5. An evolutionary walk to the optimum in a three-dimensional fitness landscape. The *arrows* represent random mutations having different magnitudes (length) and directions (effect on fitness). *Solid and dashed arrows* illustrate beneficial (A to C) and ineffective/detrimental mutations, respectively

difficulty of achieving multiple mutations to yield any improvement in the desired phenotype, in particular for evolutionary engineering of novel phenotypes. Consequently, there may not be a gradually ascending slope to the nearest fitness peak for guiding the evolutionary walk. A practical example is the requirement of three novel enzyme activities to convert a non-metabolizable nutrient source into a common biosynthetic intermediate. In this case, there is no increase in fitness if only one or two of these enzymes become available. Therefore, even in the most advantageous scenario where the required enzymes are already present in the form of cryptic genes, chances for simultaneous appearance of three independent deregulatory mutations in one variant are very low (6.4×10^{19} for the case of three independent point mutations in a genome with 4000 kb). In such cases, evolutionary approaches are likely to fail unless extremely large populations or rationally selected pathway intermediates are used (see also Sect. 4). Nature approaches this problem by recombination and horizontal DNA transfer (see Sect. 2.5), which allows 'jumping' closer to a fitness peak. For certain phenotypes, such DNA sequences may have to be provided by the experimenter.

Naturally it would be desirable to predict the success of selection schemes. Although, in many cases, this may not be possible with any confidence, some general guidelines may be given. The chances of selecting a phenotype of interest in a particular organism are good when (i) a phenotype can be detected in at least rudimentary form, (ii) a fairly close relative of the organism in question exhibits the phenotype, (iii) a related phenotype such as activity toward an analog of a novel substrate can be detected, or (iv) important aspects of the phenotype are susceptible to recombinant approaches because they are encoded on transferable genetic elements such as a few genes or operons.

3.8
Screening of Desired Variants from Evolved Populations

According to the quasispecies concept, the result of evolution is not a single variant, but rather a distribution of related variants that occupy a distinct region in sequence space [12]. Consequently, populations evolved from continuous selections are often heterogeneous, and representative, often large, numbers of individual clones from such populations must be examined to identify the most suitable individuals. The most important prerequisite for screening is efficient spatial separation and access to an assay system that allows characterization of the desired phenotype. To this end, several methodologies with different levels of automation and throughput are presently available [20].

The highest throughput can be achieved by the combination of flow cytometry and cell sorting. This is a rapid method for the analysis of *single* cells as they flow in a liquid medium through the focus of a laser beam surrounded by an array of detectors. By simultaneous use of different fluorescent stains, flow cytometry can yield multiparametric data sets which are, however, often difficult to interpret [88]. These are then used to discriminate between different types of cells, a procedure that is suitable for rapid enrichment of certain types of cells from large populations. An important and potentially very useful contribution to flu-

orescence-based screening comes from green fluorescent protein and its recombinant derivatives, which can also be exploited as expression markers at the single cell level.

Most analytical methodologies, however, cannot function at the single cell level. This means that variants have to be characterized as cultures, which requires laborious segregation, isolation, and cultivation of individual clones. In the simplest case, a desired phenotype is defined by growth under certain conditions, so it can be directly assessed by visually inspecting the ability to grow on plate or in liquid media. However, desired growth phenotypes frequently cannot be determined by a simple yes or no experiment, but are based on improved tolerance of certain unfavorable process conditions, in which case survival becomes a statistical process. In such cases, the survival rate is usually estimated by comparing colony-forming units on solid media. Alternatively, survival can also be assessed by measuring the most probable number of viable cells, based on the potential of various dilutions of the culture to serve as an inoculum for liquid media [89]. In practice, three to five serial dilutions are performed in parallel and used as inocula in a procedure that readily lends itself to automation in microtiter plates [90]. A great deal of ingenuity has also gone into the design of protocols that couple a desired function with activation of a marker gene, which than effects a color change if used with appropriate chromogenic substrates [8].

Additionally, a variety of analytical equipment and techniques that allow the examination of small- (and micro-) scale microbial cultures and their products have become available. Examples include near infrared and Fourier transform infrared spectroscopy, which offer the ability for in situ detection of specific compounds in fermentation broth [22]. However, sensitivity and the required sample volumes pose serious obstacles that still have to be overcome. Another alternative is offered by sensitive pyrolysis mass spectroscopy, which was demonstrated to be suitable for quantitative analysis of antibiotics in 5-μl aliquots of fermentation broth when combined with multivariate calibration and artificial neural networks [91]. The authors concluded that a throughput of about 12,000 isolates per month could be expected. Furthermore, standard chromatographic methods such as gas chromatography or high-performance liquid chromatography, possibly in combination with mass spectroscopy (MS) for detection, can provide simultaneous quantitative detection of many metabolic products.

Given the availability of analytical procedures, throughput is now largely limited by the ability to cultivate cells in suitably miniaturized vessels that provide process-relevant environmental conditions. Although many microbes are, in principle, amenable to growth in microtiter plates, investigation of their phenotypes in the standard 200-μl working volume plates is often limited to qualitative information because aeration and/or mixing tend to be limiting [92]. An interesting alternative is a recently developed miniaturized microbial growth system that consists of special 96-well plates equipped with deep (2-ml) wells and a spongy silicone/cotton wool sandwich cover that adequately prevents both cross contamination and excessive evaporation during vigorous aeration [93]. It was shown that aeration in these deep-well microtiter plates was comparable to that in baffled shake flasks and allowed the attaining of cell densities of up to 9 g dry

weight per liter. Such cultivation systems in combination with appropriate analytical tools will enable quantitative physiological characterization of larger numbers of clones.

Data from such characterization studies may then also be used for metabolic flux analysis, a method of estimating the rates of intracellular reactions. This modern offspring of quantitative physiology combines data on uptake and secretion rates, biosynthetic requirements, quasi-steady state mass balances on intracellular metabolites, and assumptions about metabolic stoichiometry to compute the intracellular flux distribution [94]. In addition, ^{13}C-labeling experiments are now increasingly used to avoid or validate critical assumptions [95]. Currently, labor and expense prevent the direct application of such methodologies in screening processes, but less complex approaches may offer the possibility of examining intracellular flux responses at reduced resolution in a smaller-scale screen [96]. For example, using a recently introduced nuclear magnetic resonance methodology based on isotopic imprinting of amino acids by their precursors, the active central carbon pathways and the ratios of their fluxes can be directly determined from two-dimensional nuclear magnetic resonance analysis of ^{13}C-labeled biomass [97]. This metabolic flux ratio analysis was recently demonstrated to provide valuable insights into intracellular carbon metabolism of different *E. coli* strains under various environmental conditions, including shake flask cultures [98]. Further increases in throughput can be expected from the use of MS-based procedures for labeling pattern analysis [96, 99, 100]. The interest in metabolic flux analysis resides in its analytical power at the metabolic level and its potential to provide insights for strain improvement, genetic manipulation, and process optimization. Thus, the growing field of metabolic flux analysis together with functional genomics [101] and computational models of cellular metabolism [102, 103] will likely become important tools in directing screening work, possibly by identifying easy to determine physiological variables that are indicative of a desired phenotype.

4 Evolutionary Engineering of Simple Cellular Subsystems

Evolutionary selection principles have been used to approach biotechnological problems of various complexities (Table 2). In the simplest case, conceptually, a desired phenotype is based on a ‘single property’ and is thus susceptible to straightforward gain-of-function selection. In such cases, the behavior of a relatively simple cellular subsystem (e.g., transport of a nutrient) can be directly linked to fitness in the selection scheme. In the definition employed here, simple cellular subsystems have only a small, defined number of involved components and, more importantly, their interaction with other aspects of cellular metabolism are not limiting for the property under investigation. For practical reasons, complex cell systems in industrial strain development such as entire biosynthetic pathways are often separated into simpler subsystems. This can be achieved, for example, by selecting for properties that render individual enzymes of such pathways insensitive to toxic structural analogs of pathway intermediates [20, 22]. In the absence of complete knowledge of what components are

Table 2. Recent examples of evolutionary engineering

Evolved phenotype	Selection system	Reference
Novel catabolic activities		
Utilization of carbon substrates (coryneform bacteria)	Plates (with limiting amount of yeast extract)	[114]
Utilization of pentoses (*E. coli*)	Plates (non-growing cells)	[111]
Novel esterase activities (*P. putida*)	Plates (non-growing cells)	[38]
Galactitol dehydrogenase (*Rhodobacter*)	Chemostat (glucose-limited, excess galactitol)	[115]
PTS-independent glucose uptake	Chemostat	[106]
Improved enzyme properties		
Secretability	Microcolonies	[68]
Thermostability	Thermophilic hosts	[8]
Functionality (*E. coli* mutator strain)	Batch (increasing antibiotic concentrations)	[42]
Improved plasmid functions		
Stability (Gram positives, yeast)	Chemostats (antibiotic and auxotrophic marker selection)	[55, 81, 125, 126]
Stable host-plasmid combinations (*E. coli*)	Chemostat	[128]
Improved stress resistance		
Acetate tolerance (yeast)	Turbidostats	[118]
Organic solvent tolerance (mutator strains)		[119]
Ethanol tolerance (yeast)	BOICS	[82]
Antibiotic resistance (*Streptomyces*)	BOICS	[25]
Multiple stress resistance (yeast)	Chemostats and batches (with stress challenges)	[90]
Membrane protein overexpression (*E. coli*)	Plate	[124]
Periplasmic protein production (*E. coli*)	Chemostat	[57, 137]
Improved production properties		
Endo-enzyme overexpression	Chemostats	[109, 110]
Antibiotic production (*Streptomyces*)	BOICS	[25]
Nucleoside secretion (*E. coli*)	Chemostat (phosphate-limited, added biosynthetic inhibitors)	[121]
Protein secretion (*Streptomyces*)	Chemostats (different selection schemes)	[125]
Biomass yield (yeast, *E. coli*)	Chemostat (carbon-limited)	[57, 133, 134]
Adhesive cells (*Streptococcus*)	Chemostat	[108]
Altered mycelial morphology (fungi, actinomycetes)	Chemostats	[125, 129, 131, 132]

involved, however, a priori classification of phenotypic properties according to their complexity is difficult.

A particularly well-studied example of a simple subsystem in evolutionary research is utilization of lactose, which consists of three essential components: (i) porin-mediated diffusion through the cell wall, (ii) active uptake via a permease, and (iii) intracellular hydrolysis into glucose and galactose by β-galactosidase. Assuming that central metabolism will utilize these cleavage products, the lac-

tose flux should be directly proportional to the growth rate in lactose-limited media, and this is indeed the case [104]. In lactose-limited chemostats, periodic selection of *E. coli* predictably generates lactose-constitutive variants [69]. Further beneficial mutations reduce the K_s value of the permease; this is in agreement with the calculated control coefficients for the three components under these conditions [105].

Excluding classical mutagenesis and selection on solid media, there are several reports on evolutionary engineering of simple cellular subsystems with an applied background. For example, experiments were performed with an *E. coli* strain that produced an aromatic compound and carried a deletion of the phosphotransferase system (PTS) for glucose uptake. Spontaneous glucose revertants were selected that apparently utilized a non-PTS system for glucose uptake [106]. One variant was identified that exhibited improved production of aromatic compounds, presumably because the use of a non-PTS uptake system for glucose uptake saves at least some intracellular phosphoenolpyruvate (which is otherwise converted to pyruvate during PTS transport of glucose), increasing its availability for biosynthesis of aromatics. Interestingly, using the same approach in a similar host but following the rational strategy of cloning a heterologous, non-PTS system for glucose uptake did not improve production of aromatics [107]. This example illustrates the advantage of evolutionary engineering for optimally accommodating a metabolic component into the complex system of cellular metabolism. Selection procedures have also been used to improve more specialized desirable properties such as improved downstream processing characteristics or resistance to phage infection. Although usually undesired, adhesive phenotypes can be selected for the use in certain types of bioreactors that require attachment of cells [108].

The isolation of mutants overproducing endo-enzymes that directly influence growth fitness has often been achieved using chemostat selection (e.g., [109, 110]) or other means [111]. A successful example of the conceptually more difficult improvement of exo-enzyme production involves the enrichment of more efficiently secreted protease variants by using bovine serum albumin as the sole nitrogen source in a selection procedure based on microcolonies (compare with Sect. 3.4) [68]. Specifically, (rare) protease variants with up to fivefold increased secretion levels were isolated after mutagenesis and four rounds of selection by growth in hollow fibers. While this strategy was successfully applied to select for better protein secretion, it could also potentially be used to select for host strains that exhibit an improved secretion phenotype. In several cases, evolutionary engineering of thermostable enzyme variants was successfully achieved by expression in thermophilic organisms and selection of transformants for recombinant activity-dependent survival at elevated temperatures (for a review see [8]). This powerful concept may also be extended to microbes capable of growing under other adverse environmental conditions, including extremes of pH and salinity.

Acquisition of novel catabolic activities has been deliberately studied since the early 1960s and is of particular applied relevance for bioremediation of waste or by-products from manufacturing processes and improving the ability to use cheaper raw materials in the production of commodity chemicals. Most

of these studies are either conducted with well-characterized laboratory strains [111, 112] or based on the analysis of naturally evolving species in the environment that can degrade pollutants of human origin [112, 113]. When multi-step catabolic pathways are required to degrade a pollutant, the most important mechanism for expanding the metabolic capabilities appears to be incorporation of existing genetic material via horizontal DNA transfer. However, less complex alterations for acquisition of new activities can also be achieved by test tube evolution with a single strain. Such evolutionary gain-of-function selections revealed the general principle that new metabolic functions are often established by 'borrowing' enzyme or transport activities from preexisting pathways [111, 114]. Two types of mutations are found to account for most newly evolved pathways: (i) the initial events are almost always activation of cryptic genes or regulatory mutations of genes normally used in other metabolic pathways, and (ii) subsequent mutations in structural genes that alter properties such as substrate specificity. To select for mutants that can use or degrade new compounds, microorganisms are placed in media containing these non-metabolizable nutrient sources. Typically, cells are provided with a limiting concentration of a normal nutrient to support some growth in liquid or on solid media, because the desired mutants are often not obtained by direct selection [114]. Moreover, it may not be possible to select directly for a desired phenotype in one step when multiple mutations are required. In such cases, it is worthwhile to attempt selection on structural analogs of the novel substrate or intermediates of the anticipated catabolic pathway.

Successful evolution of novel catabolic functions has been demonstrated in a number of bacteria [112]. Using a plasmid-based mutator gene, novel esterase activities were selected in *Pseudomonas putida* [38]. Another application is selection of the 'new' catalytic activity of a galactitol dehydrogenase by cultivating *Rhodobacter sphearoides* in a chemostat with a limiting concentration of a normal substrate and an excess of the non-metabolizable galactitol [115]. After about 50 days, a spontaneous several-fold increase in cell density indicated an adaptive mutation that enabled utilization of galactitol. Biochemical characterization of the resulting galactitol dehydrogenase showed it to be a previously unrecognized enzyme in the wild-type. Evolution of this 'new' enzyme was presumably based upon activation of a cryptic gene (compare with Sect. 3.1). After up to 60 days in stationary phase, mutants capable of utilizing several novel carbon substrates were obtained from industrially important coryneform bacteria that were plated on mineral media with a very low concentration of yeast extract and a high concentration of the carbon source of interest [114]. Alternatively, selection may also be achieved without an initial growth promoting substrate, as evidenced by the isolation of ribose-positive *E. coli* mutants after 12–20 days of incubation in a minimal medium containing ribose as the sole carbon source [111]. The latter two cases of evolutionary adaptation presumably take advantage of the increased rate of mutagenesis and population dynamics during prolonged nutritional stress in stationary phase [29, 116, 117].

Clearly, evolutionary engineering of simple cellular subsystems is complementary but also competing with directed in vitro evolution, provided sequence information on the involved components is available.

5 Evolutionary Engineering of Complex Cellular Subsystems

5.1 Resistance to Environmental Stress

Although modern process equipment enables tight control of many environmental factors, industrial microorganisms often have to cope with adverse conditions that are inherent to an industrial process, for instance high concentrations of toxic or inhibitory products. In many cases, evolutionary procedures have been used to improve performance by adapting strains to such process conditions. For example, moderately acetate-tolerant baker's yeast variants were selected in turbidostats to improve the dough raising power in acetate containing sourbread [118]. Similarly, improved organic solvent resistant bacteria were selected by using mutator strains [119]. Also, to maintain the extraordinary resistance to high concentrations of acetate in industrial acetic acid bacteria that are used for the production of vinegar, these cultures are continuously propagated in acetate fermentations [120]. To avoid problems of over- or under-addition of toxic agents in the selection of mutants tolerant of extreme environmental stresses, the selection pressure is best adjusted automatically in response to periodic mutant take-overs via feedback control of the culture conditions in a process known as interactive chemostat selection (see also Sect. 3.6). In a particular interactive chemostat procedure using CO_2 output as a measure of the culture condition (BOICS), ethanol-tolerant yeast mutants were successfully isolated [82]. BOICS was also used to obtain *Streptomyces griseus* mutants that exhibited greatly increased resistance to the antibiotic streptomycin [25]. Associated with increased resistance, the best mutant produced 10 to 20 times more streptomycin when grown in the medium used for BOICS. The strategy apparently implemented by BOICS uses the mean specific growth rate of the culture as a measure of its health and CO_2 output is used as a measurable surrogate for growth rate to control the environmental conditions [84].

Resistance to inhibitors added to liquid media may also be used to select for variants that secrete desired metabolites, as exemplified by chemostat selection of *E. coli* mutants secreting thymidine, cytosine, uracil, guanine, and thymine [121]. Since it was not possible to favor directly secretion of the desired compound, thymidine, a chemostat population was challenged with increasing concentrations of two inhibitors of the pyrimidine biosynthesis pathway. Phosphate limitation successfully prevented growth disadvantages due to squandering of critical resources under carbon limitation. Thymidine-secreting mutants were then detected on the basis of cross feeding of an auxotrophic *thyA* mutant in a plate assay. Interestingly, the isolated mutants also secreted other nucleosides and nucleobases, so that the underlying principle of this design may be generally applicable to select metabolite-secreting mutants.

Another biotechnologically desirable characteristic of process organisms is robustness or resistance to the multiple stresses that frequently occur in large-scale processes or in food applications. However, increased tolerance of multiple stresses is likely to be a complex phenotype that would be difficult to engineer

rationally. A recent study compares selection procedures to select for improved multiple stress resistant phenotypes from chemically mutagenized *S. cerevisiae* [90]. Specifically, glucose-limited chemostats with either permanent or transient stress challenges as well as repeated cycles of mutation and selection against various stresses in batch culture were investigated. Evolution of stress resistance was followed by monitoring the relative tolerance to four stresses: ethanol, rapid freezing, oxidation (H_2O_2), and high temperature. The analyzed samples were either from population aliquots that originated at various stages of the selection processes or, in selected cases, from 24 representative clones that were picked from plates. The most appropriate strategy for obtaining multiple stress resistant variants appeared to be selection in chemostats with transient stress challenges, after which the population was allowed to recover for several generations. Several clones from this heterogeneous population exhibited five- to tenfold improved resistance to three out of the four stresses. Two to three cycles of transient exposure to stresses prior to growth in batch culture, on the other hand, selected for variants with higher resistance (up to 150-fold) but to only two out of four stresses.

5.2 Resistance to Metabolic Stress

Generally, overproduction of antibiotics, vitamins, or fine chemicals constitutes a metabolic and energetic burden for the cell, and hence is frequently counter-selected in production processes if not maintained by strong selective pressure [112]. However, even in the presence of marker gene-based selection pressure, a complex phenotype such as vitamin production may be counter-selected during moderately extended cultivation [122].

Another biotechnologically relevant stress stems from toxic effects of recombinant protein overexpression that impair growth of the host cell. While *E. coli* is a powerful vehicle for the overproduction of many heterologous proteins, certain proteins cannot be expressed at all or only at very low levels. Foremost among those are membrane proteins that are difficult to overexpress in both microbial and eukaryotic hosts [64]. This problem may be partly related to the observation that laboratory strains are generally not well suited for protein overproduction, as they have been selected for maximum growth [123]. In a very interesting study, Miroux and Walker [124] provided a solution by selecting *E. coli* mutants that proved to be superior to the parental strain for overexpression of problematic globular and membrane proteins. The plate-based selection procedure was initiated with a strain carrying an inducible expression plasmid for the least toxic of seven tested membrane proteins. After growth and a short induction phase in liquid medium, transformants were diluted on plates containing both ampicillin and IPTG for plasmid maintenance and induction, respectively. Two (minor) sub-populations with different colony sizes survived, one of which had apparently lost the capacity to express the recombinant protein, while the other expressed appreciable amounts of the membrane protein. An isolate of the latter population, morphologically characterized by a small colony size, was found to be a suitable host for overexpression of many previously problematic

proteins. Because the toxicity of overexpression for certain proteins persisted in the isolated mutant, a second round of selection was conducted on this mutant after transformation with an expression plasmid for one of the remaining problematic proteins. One of the mutants obtained from this second selection proved to be a better producer for some but not all of the problematic proteins, even compared to the previously isolated mutant. Both mutant phenotypes were stable propagated and are apparently caused by genomic mutations that were hypothesized to reduce the level or activity of T7 RNA polymerase, and so prevent uncoupling of transcription and translation [64, 124].

5.3 Plasmid Stability

Structural and segregational stability of plasmids is a prerequisite for development of efficient processes and, moreover, important for validation of pharmaceutical manufacturing processes. Segregational instability occurs when a plasmid-bearing host fails to pass the plasmid on to a daughter cell(s), and a variety of (often unknown) factors contribute to segregational stability. To improve plasmid retention in Gram-positive bacteria, selective chemostats have successfully been employed to alter both host [81] and plasmid [55] factors. In both cases, cultures hosting segregationally unstable plasmids were grown for up to 100 generations in carbon-limited chemostats at a high dilution rate (of about 0.5 h^{-1}) under selective pressure from supplemented antibiotics. Variants of a normally unstable recombinant *Bacillus* strain exhibiting about 30-fold improved plasmid retention were enriched by this procedure [81]. In this case, the stability characteristics resided in the host rather than on the plasmid. The improved strains had growth rates comparable to that of the original, plasmid-free host and were consequently better competitors. Using a recombinatorial approach, Seegers et al. [55] selected stable plasmids in lactobacilli from a large background population of recombinant plasmids with different stabilities. After shotgun cloning of DNA fragments from a stable lactococcal plasmid into an unstable expression vector, three classes of mutations were selected and subsequently identified. The first class mutations in the selection plasmid itself increased copy number, thereby rendering the plasmid more stable. The other two classes were based on the insertion of two different stability-promoting sequences in the selection plasmid.

In another evolutionary approach, expression and secretion of a recombinant protein in the Gram-positive bacterium *S. lividans* was increased 60- to 100-fold, most likely by improving plasmid stability in combination with other host properties [125]. Improved strains were selected from four consecutive chemostat processes run at a dilution rate of 0.12 h^{-1} under different selection regimes. In the first step, after about 100 generations under ammonium limitation and glucose excess, variants with about fivefold improved recombinant protein secretion were isolated. In the second step, cultivation under maltose limitation for another 100 generations was supposed to lead to increased segregational plasmid stability and clones with 30-fold higher protein secretion relative to the original strain were isolated. Finally, two more rounds of selection with increas-

ingly selective antibiotic concentrations for about 33 generations each were performed, leading to clones that exhibited about 60- to 100-fold increased recombinant protein secretion, as compared to the original strain.

A critical factor for successful selection of segregationally stable host-vector combinations is the selection pressure applied. While the above positive selections for antibiotic resistant cells were successful, a similar experiment that used a negative selection for plasmid-bearing clones of *S. cerevisiae* with an auxotrophic marker did not enrich for more stable clones over a period of 420 generations [126]. Although a large variety of clones with altered recombinant plasmid stability evolved over time, it appeared to be mainly a result of non-specific periodic selection. Moreover, the best clones exhibited only about a 30% improvement in stability. This apparent absence of selection pressure for stable clones may have been caused by cross feeding of the plasmid-free population with the auxotrophic nutrient that was synthesized by the plasmid-bearing population. This is a common phenomenon in recombinant yeast cultures [127]. Similarly, during selection for plasmid retention with chloramphenicol, the selection procedure also promoted a higher rate of chloramphenicol degradation, which, in turn, resulted in a progressive increase of the chloramphenicol-sensitive, plasmid-free population [81]. However, in this case the selection pressure was monitored and could be gradually increased simply by raising the antibiotic concentration.

Although generally considered to impose a burden and thus to reduce fitness, plasmid retention may become beneficial for coevolved hosts by unexpected means. After propagation of a plasmid-carrying *E. coli* strain for 500 generations, a host phenotype evolved that, relative to its progenitor, exhibited a competitive advantage from plasmid maintenance in the absence of selection pressure [128]. Although the mutation within the host genome remained unknown, it was shown that the plasmid-encoded tetracycline resistance, but not the chloramphenicol resistance, was required to express this beneficial effect. These results indicate that the co-evolved host phenotype acquired some new (unknown) benefit from the expression of a plasmid-encoded function. This also suggests a general strategy for stabilizing plasmids in biotechnological applications by evolutionary association of plasmids with their hosts. Thus, antibiotic selection could be avoided in industrial processes without the danger of phenotypic instabilities due to plasmid loss.

5.4 Mycelial Morphology

Mycelial morphology is an important process variable in fermentations with filamentous fungi. This is particularly true for the commercial production of the Quorn myco-protein, a meat substitute with a texture that is based on the morphology of the mycelium. Continuous-flow production of this material by the fungus *Fusarium graminearum* is prematurely terminated if highly branched mutants appear in the process. From a series of glucose-limited chemostats, it was possible to isolate mutants in which the appearance of such highly branched mutants was significantly delayed, compared to the parental strain [129]. A more

detailed analysis of periodic selection within the evolving population during continuous production of Quorn revealed that pH oscillations or a consistently low pH are complementary conditions that delay the appearance of the undesired, highly branched mutants, without affecting the normal morphology of the mycelium [130].

For other applications, mycelium formation is undesired and may be reduced by appropriate selection procedures. This was achieved, for example, in the bacterium *S. lividans* by extended growth in chemostat cultures under ammonium limitation and glucose excess [125]. After about 70 generations, selected variants showed an altered growth behavior that was characterized by repression of aerial mycelium and spore formation on solid media. Similar results were obtained with different fungi [131, 132].

5.5 General Physiological Properties

While novel reactions and pathways can often be efficiently installed in microorganisms by metabolic engineering [1], general physiological properties such as specific growth rate, overall metabolic activity, energetic efficiency, competitive fitness, and robustness in industrial environments remain mostly the property of the chosen host organism. It would, therefore, be advantageous if host organisms could be tailored for the specific requirements of different industrial processes. One such industrial example is (*R*)-lactate production with *Lactobacillus* by BASF [112]. In this case, an improved, fast growing mutant was isolated from semi-continuous fermentation in production scale because lactate production is linked to growth.

High yields of biomass represent a general host property that is desired in many applications, and has been achieved by evolutionary strategies. Comparing an *S. cerevisiae* mutant isolated after 450 generations in a strictly glucose-limited chemostat at a dilution rate of 0.2 h^{-1} with its ancestor, Brown et al. [133] found the evolved strain to exhibit significantly greater transport capacity and also enhanced metabolic efficiency in processing of glucose under these conditions. The evolved strain had acquired the remarkable capability to grow at a biomass yield of 0.6 (g/g), compared to 0.3 (g/g) for the parent. This improved growth phenotype under strict glucose limitation apparently did not compromise the performance under non-limiting conditions in batch cultures. In fact, the overall yield of cells on glucose was increased in batch culture as well. The two- to eightfold faster glucose uptake of the evolved strain, compared to the parent, was correlated with elevated expression of the two high-affinity hexose transporters, *HXT6* and *HXT7*, which, in turn, was caused by multiple tandem duplications of both genes [133]. Although the genetic basis for the enhanced glucose transport has been unraveled, these genetic alterations are probably not responsible for the biotechnologically relevant phenotype of more efficient biomass production. Inoculated from the same parent, three *S. cerevisiae* mutants were isolated from independent glucose-limited chemostat cultures after 250 generations and all of them produced about threefold greater biomass concentrations in steady state [134]. Reduced ethanol fermentation and in-

creased oxidative metabolism apparently achieved this improvement in metabolic efficiency. Analysis of total cellular mRNA levels revealed significant changes in the transcription levels of several hundred genes compared to the parent, but a remarkable similarity in the expression patterns of the three independently evolved strains [134]. Consistent with the observed physiology, many genes with altered transcription levels in all three strains were involved in glycolysis, tricarboxylic acid cycle, and the respiratory chain. These results indicate that increased fitness was acquired by altering regulation of central carbon metabolism, because only about five to six mutations were expected to contribute to the changes. Possibly as a consequence of the evolutionary principle that different populations may evolve under identical conditions, a different outcome was seen in an earlier but apparently identical selection experiment for 260 generations [135]. In this case, the biomass yields of isolated yeast clones fluctuated with the progress of evolution and clones from later generations exhibited significantly reduced yields under the selection conditions, whereas the yields in batch culture were not affected.

In an effort to select for variants that would perform well under the typical industrial fed-batch condition of slow growth, an *E. coli* mutant was isolated after 217 generations from a glycerol-limited chemostat that was operated at the very low dilution rate of 0.05 h^{-1} [57]. Like the yeast strain described above, this mutant was found to exhibit an increased biomass yield. Additionally, other general physiological properties such as the specific growth rate and resistance to a variety of stresses were found to be improved. Unexpectedly, the mutant also exhibited high metabolic activity in the absence of growth, which indicated impaired stationary phase regulation [136]. Some of these improvements were also evident with carbon sources other than the one used during selection, indicating that not only substrate-specific features but also general physiological properties were altered. In subsequent studies, these improved phenotypic properties were shown to be exploitable for biotechnological applications, including periplasmic secretion of recombinant protein [137] and production of low molecular weight biochemicals [136]. Moreover, the isolated mutant was shown to be significantly less impacted by periplasmic expression of the recombinant protein, as evidenced by the significantly higher segregational stability of the expression plasmid during growth in non-selective media (Fig. 6). Consistent with the total cellular mRNA data obtained from the metabolically more efficient yeast strains, several proteins involved in central carbon metabolism were found at significantly higher levels on two-dimensional protein gels from the isolated *E. coli* mutant [138].

The above examples clearly illustrate that it is feasible to select for generally improved microbial phenotypes for industrial applications. Dictated by economic pressure, it is, however, often impractical to switch host strains in advanced stages of process development. Thus, it would be highly desirable to develop production hosts for the specific requirements of bioprocesses by metabolically engineering them to have desirable physiological properties, which necessitates elucidation of the genetic basis of these often complex phenotypes. In the case of the *E. coli* mutant, this has partly been achieved by identifying two genes, *rspAB*, which, when overexpressed in wild-type *E. coli*, partly mimic the

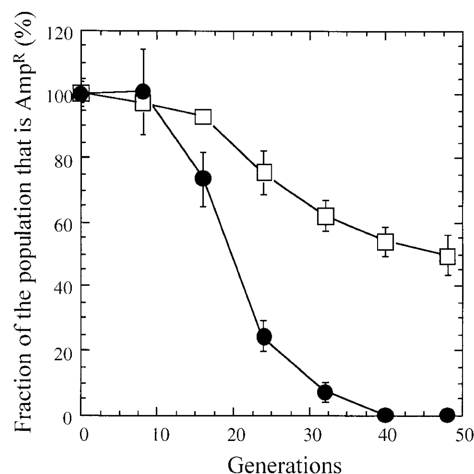

Fig. 6. Fraction of ampicillin-resistant clones of *E. coli* MG1655 (*circles*) and a chemostat-selected descendant (*squares*) from serial batch cultivations in ampicillin-free minimal medium. Both strains harbor the expression vector pCSS4-p for periplasmic production of the recombinant α-amylase of *B. stearothermophilus*. Reproduced with permission from Weikert et al. [137]

mutant phenotype [139]. Specifically, co-overexpression of RspAB was found to improve the formation of recombinant β-galactosidase in batch and fed-batch culture of *E. coli*. Although the exact functions of the corresponding gene products are not fully elucidated, they are reported to be involved in the degradation of the metabolic by-product (or signaling molecule) homoserine lactone [140].

6 Outlook

The use of evolutionary principles will undoubtedly play a major role in twenty-first century biotechnology [141]. The capabilities of directed in vitro evolution will eventually extent beyond improving existing properties of proteins or short pathways to the engineering of de novo functions, new pathways, and perhaps even entire genomes [12, 13]. However, the problem of phenotypic complexity will shift the limitations even more to the available screening or selection procedures [11]. For two primary reasons, evolutionary engineering of whole cells offers an interesting alternative. First, through the use of continuous evolution using large populations, evolutionary engineering can navigate rugged fitness landscapes much more efficiently than can step-wise screening or selection procedures. Second, cellular phenotypes depend strongly on the environment and appropriate process conditions may be simpler to establish in bioreactor systems than in Petri dish- or microtiter plate-based screening or selection systems. Moreover, for complex microbial phenotypes with many, often unknown molecular components, there is currently no alternative to evolutionary engineering. Although such applications were not covered here, evolutionary studies with mi-

crobes are also likely to provide important input to medicine, for example by suppressing the emergence of novel pathogens through environmental controls, reducing virulence reacquisition of live vaccines, or avoiding the evolution of drug resistant variants [19].

The greatest limitation for evolutionary engineering of industrially useful cellular phenotypes resides in the contradictory selection demands for such phenotypes. In highly engineered production strains, for example, it may not be possible to devise a selection scheme for two useful but potentially incompatible phenotypes such as overproduction of a metabolite *and* high efficiency of growth. In such cases, both direct evolution and evolutionary engineering approaches are envisioned to become components in effective metabolic engineering, as illustrated in Fig. 7. Upon successful evolutionary engineering towards one desired phenotype, this strain is used either as the host for further rational improvements by metabolic engineering or the desired property is transferred to a production host. The latter is essentially *inverse metabolic engineering*, a concept introduced by Bailey et al. [4]. Here a desired phenotype is first identified and/or constructed and, upon determination of the genetic or environmental basis, it is endowed on another strain or organism.

Until very recently, searching for the genetic or molecular basis of complex phenotypes would have been a hopeless venture because multiple, random genetic changes at the genome level could not be identified. To a large extent, this

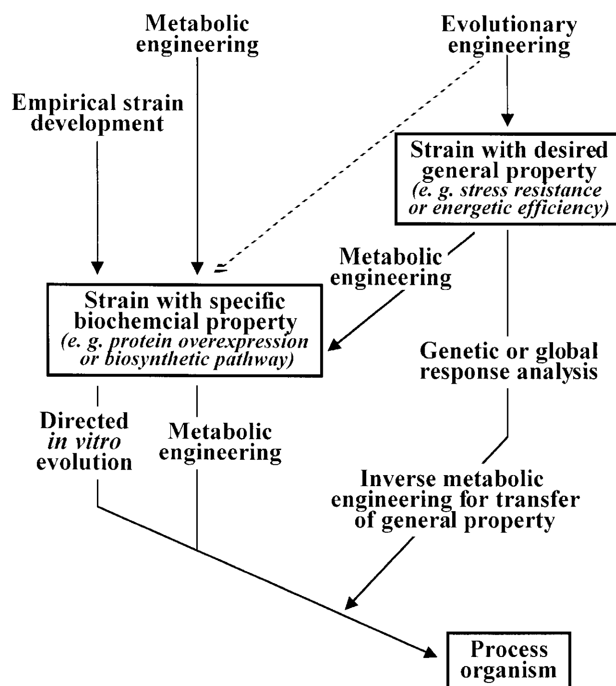

Fig. 7. Flow chart for future biotechnological strain development. The *dashed arrow* indicates a less likely but possible route

may have been the primary reason why, with few exceptions [134, 139], this road has remained almost untrodden in biotechnological research. However, recent technological advances are rapidly changing this situation and inverse metabolic engineering is likely to gain more relevance in the near future. Mass sequencing and functional genomics are currently the most effective approaches for increasing such knowledge at the molecular level of different organisms. Several methods that provide access to global cellular responses can now routinely be used for the identification of the molecular bases for useful phenotypes. One example is simultaneous and comprehensive analysis of gene expression at the protein level by two-dimensional protein gel electrophoresis in combination with genomic sequence information and mass spectrometric spot identification. This is often referred to as *proteome* analysis [142]. Similarly, genome-wide mRNA levels can be monitored by so-called *transcriptome* analysis, which is based upon extraction of total mRNA that is then hybridized to arrays of oligonucleotides or open reading frames arranged on DNA chips or membranes [143]. Successful identification of the molecular basis for evolved phenotypes through these technologies includes proteome analysis of *E. coli* variants [138, 144] and transcriptome analysis of improved yeast variants [134].

An alternative application of DNA chips in evolutionary engineering is the rapid identification of beneficial or detrimental genes with respect to a particular phenotype in selection experiments. Briefly, hybridizing PCR-amplified DNA from positively selected clones to a genomic DNA chip of this organism can reveal enrichment or depletion of clones from an overexpression library as a consequence of a selection procedure [145]. Similar to, but more rapid than, the signature-tagged mutagenesis introduced in Sect. 2.4, this strategy provides access to genes that confer a selective advantage or disadvantage upon overexpression.

Supported by complementary information on global responses at both the metabolite [101] and the flux level [94, 96, 98] (see also Sect. 3.8), these methodologies will pave the road to efficient revelations of the molecular and functional bases of phenotypic variations, even for multifactorial changes. Such global cellular response analyses provide detailed comparative information on many aspects of cellular metabolism, and thus can provide leads to genes that are likely to be involved in a particular phenotype. However, global response analysis cannot directly reveal the mutation(s) that will cause the desired phenotype. Consequently, endowing useful phenotypes on other hosts by inverse metabolic engineering requires intellectual and/or computational interpretation of the results, followed by formulation of hypotheses that would then have to be verified experimentally. Genetic methods that provide more direct access to genomic alterations include genome sequencing, single nucleotide polymorphism, and restriction fragment length polymorphism mapping. Recent developments that make these genetic methods and global response analyses widely available are also expected to stimulate activities in evolutionary engineering.

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