Progress in Drug Research, Vol. 65
(Frank Petersen and René Amstutz, Eds.)

Strain improvement for production of pharmaceuticals and other microbial metabolites by fermentation

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Abstract

Microbes have been good to us. They have given us thousands of valuable products with novel structures and activities. In nature, they only produce tiny amounts of these secondary metabolic products as a matter of survival. Thus, these metabolites are not overproduced in nature, but they must be overproduced in the pharmaceutical industry. Genetic manipulations are used in industry to obtain strains that produce hundreds or thousands of times more than that produced by the originally isolated strain. These strain improvement programs traditionally employ mutagenesis followed by screening or selection; this is known as 'brute-force' technology. Today, they are supplemented by modern strategic technologies developed *via* advances in molecular biology, recombinant DNA technology, and genetics. The progress in strain improvement has increased fermentation productivity and decreased costs tremendously. These genetic programs also serve other goals such as the elimination of undesirable products or analogs, discovery of new antibiotics, and deciphering of biosynthetic pathways.

1 Introduction

Microbes tend not to overproduce their metabolites and usually produce valuable products only in amounts that they need for their own benefit. In strain improvement programs of industry, a strain producing a high titer is usually the desired goal. Genetics has contributed to the production of microbial products for many years. The tremendous increases in fermentation productivity and the resulting decreases in costs have come about mainly by mutagenesis and screening/selection for higher producing microbial strains and the application of recombinant DNA technology.

Strain improvement encompasses creation of strains with (i) efficient assimilation of inexpensive and complex raw materials; (ii) alteration of product ratios and elimination of byproducts; (iii) product excretion; (iv) tolerance to high product concentrations; (v) short fermentation times; and (vi) overproduction of native products or foreign products after genetic recombination [1].

The contributions of microbial genetics to industrial microbiology began in the 1940s when the fermentative production of penicillin became an international necessity. The early studies in basic genetics concentrated heavily on the production of mutants and their properties. The ease with which 'permanent' characteristics of microorganisms could be changed by mutation and the simplicity of the mutation techniques had tremendous

appeal to microbiologists. Mutation has been the major factor involved in the hundred to thousand-fold increases obtained in production of microbial metabolites. The ability to modify genetically a microbial culture to higher productivity has been the most important factor in keeping the fermentation industry in a healthy state.

2 Mutagenesis

Microorganisms generate new genetic characters ('genotypes') by two means: (i) mutation and (ii) genetic recombination techniques such as protoplast fusion, transformation, conjugation and recombinant DNA technology, including metabolic engineering.

In mutagenesis, a gene is modified either unintentionally ('spontaneous mutation') or intentionally ('induced mutation'). Although the change is usually detrimental and eliminated by selection, some mutations are beneficial to the microorganism. Even if not beneficial to the organism, but beneficial to humans, the mutation can be detected by screening and preserved indefinitely. Mutation has been mainly used to improve the productivity of industrial cultures [2, 3], although it has also been used to shift the proportion of metabolites produced in a fermentation broth to a more favorable distribution, elucidate the pathways of secondary metabolism, yield new compounds, and other functions.

The most useful mutagens include nitrosoguanidine (NTG), 4-nitroquinolone-1-oxide, methylmethane sulfonate (MMS), ethylmethane sulfonate (EMS), hydroxylamine (HA) and ultraviolet light (UV). The most common method used to obtain high yielding mutants is that of treating a population with a mutagenic agent until a certain 'desired' kill is obtained, plating out the survivors and testing each resulting colony or a randomly selected group of colonies for product formation in flasks. The optimum level of kill for increased production of antibiotics is thought to be in the range of 70–95% [4], although some industrial programs use much higher levels, e.g., up to 99.99%. Although a successful mutation and screening procedure decreases the average production ability of all the survivors, more importantly, it increases frequency of improved mutants. Although single cells or spores are preferred for mutagenesis

[5–8], non-spore-forming filamentous organisms can also be handled. Here mycelia are mutagenized, protoplasts are formed and regeneration is carried out on solid medium [9]. Sonication is sometimes used to break up mycelia after mutagenesis and before screening for improved mutants [10–12].

2.1 Increasing metabolite production

Genetics has led to tremendous increases in fermentation productivity and decreased costs mainly by mutagenesis and screening for higher producing microbial strains. Overproduction of microbial metabolites is effected by (i) increasing precursor pools, (ii) adding, modifying or deleting regulatory genes, (iii) altering promoter, terminator and/or regulatory sequences, (iv) increasing copy number of genes encoding enzymes catalyzing bottleneck reactions, or (v) removing competing unnecessary pathways [13].

The first superior penicillin-producing mutant, *Penicillium chrysogenum* X-1612, was isolated afer X-ray mutagenesis in the mid-20th Century. This heralded the beginning of a long and successful relationship between mutational genetics and industrial microbiology [14]. Improvement of penicillin production by conventional strain improvement resulted both from enhanced gene expression and from gene amplification [15, 16]. Increased levels of mRNA corresponding to the three enzymes of penicillin G biosynthesis have been found in high-penicillin producing strains of *P. chrysogenum* as compared to wild-type strains [17]. High-producing strains contain an amplified region which is at least 35 kb. A 106 kb region amplified 5 to 6 times as tandem repeats was detected in a high-producing strain whereas Fleming's original strain of *Penicillium notatum* contained only a single copy [18].

Impressive titers of industrial metabolites have been achieved in all mutagenesis programs. The production titer of tetracycline as far back as 1979 was over 20 g/l [19], mainly due to strain improvement. Later, titers of 30–35 g/l were reached for chlortetracycline and tetracycline. Penicillin production titer is 70 g/l and that of cephalosporin C over 30 g/l [20]. Tylosin titer has been reported to be over 15 g/l [21] and that of salinomycin 60 g/l [22].

2.1.1 Mutants with altered morphology or color

Randomly obtained mutants as well as morphological mutants are often improved in production ability but the mechanisms are relatively unknown. Probably many of these mutations involve regulatory genes, especially since regulatory mutants obtained in basic genetic studies are sometimes found to be altered in colonial morphology. Morphological mutants were crucial in the strain improvement of *Streptomycees coeruleorubidus*, the producer of the anthracycline antitumor agent daunomycin (now daunorubicin) [23]. Screening for 'bald' mutants (suppressed in aerial mycelium development) followed by screening for white mutants (asporogenous aerial mycelium) led to a 15-fold improvement. Morphological variation was also useful in improving production of another anthracycline, beromycin [24]. The parent culture, producing about 100 mg/l, was streaked on three different agar media of which only one revealed different types of colonies. The best type had reddish orange colonies, no aerial mycelia, orange submerged mycelia, no melanin and produced between 400 mg/l and 900 mg/l. Further streaking out from submerged cultures and testing colonies of the last-mentioned type increased production to 1,000–1,100 mg/l. Additional work on medium development increased production to 3,350 mg/l [25]. An improved teicoplanin-producing strain of *Actinoplanes teichomyceticus* was obtained which produced pink instead of brown mycelia [26].

2.1.2 Nutritional ('auxotrophic') mutants

The end product of a biosynthetic pathway to a metabolite often exercises strict control over the amount of an intermediate accumulated by an auxotrophic mutant of that pathway. Only at a growth-limiting concentration of the end product would a large accumulation of the substrate of the deficient enzyme occur. This principle of decreasing the concentration of an inhibitory or repressive end product to bypass feedback inhibition or repression is best accomplished by the use of auxotrophic mutants. Production of secondary products is markedly affected by auxotrophic mutation, even when the auxotrophs are grown in nutritionally complete or even complex media. Although the change in product formation is usu-

ally in the negative direction, higher-producing auxotrophs are obtained from producers of antibiotics. For example, some leucine auxotrophs made more bacitracin than their prototrophic parent [27]. When several metabolites are produced by a single branched pathway, mutation in one branch of the pathway often leads to overproduction of the product of the other branch. In the case of branched pathways leading to a primary metabolite and a secondary metabolite, auxotrophic mutants requiring the primary metabolite sometimes overproduce the secondary metabolite [28].

Reversion of an auxotroph to prototrophy sometimes leads to new prototrophs possessing higher biosynthetic enzyme activity than present in the original 'grandparent' prototroph. Such increased enzyme activity is probably the result of a structural gene mutation producing a more active enzyme or an enzyme less subject to feedback inhibition. A revertant of an aspartate auxotroph of *Streptomyces fradiae* was found to be an improved producer of tylosin [29]. The auxotroph, lacking aspartate aminotransferase, produced no tylosin whereas the revertant produced 4-fold more than the grandparent strain. Similar examples have been described by Polsinelli and colleagues [30] dealing with actinomycin overproduction and by Saburova and co-workers [31] with respect to daunorubicin.

2.1.3 Revertants of non-producing mutants

Non-producing mutants sometimes produce increased amounts of secondary metabolites when mutated back to production. This has been observed in the cases of chlortetracycline [32] and aurodox [33].

2.1.4 Mutants resistant to antimetabolites

It is possible to select regulatory mutants, which overproduce end products of pathways, by the use of toxic metabolite analogs. Such antimetabolite-resistant mutants often possess enzymes that are insensitive to feedback inhibition, or enzyme-forming systems resistant to feedback repression.

Mutants of *Streptomyces clavuligerus* resistant to thialysine were found to have an aspartokinase resistant to covalent feedback inhibition by threonine and lysine, and 20% of these were hyperproducers (1.5 to 4-fold) of

cephalosporins [34]. Production of desferrioxamine, a commercial siderophore used for diseases associated with iron overload and pathological iron deposition, by *Streptomyces pilosus*, was markedly increased by mutagenesis and selection for resistance to thialysine [35]. Monensin A and B production by *Streptomyces cinnamonensis* was increased over 7-fold *via* deregulation of valine feedback inhibition by mutation to resistance to 2-ketobutyrate in the presence of valine or isoleucine [36]. Valine is the precursor of the fatty acid moiety of teicoplanin $A_{2\text{-}2}$ and its addition to the medium increases both total teicoplanin titer and the proportion of the desirable $A_{2\text{-}2}$ produced [37]. A mutant of *A. teichomyceticus* resistant to valine hydroxamate produced 50% more total teicoplanins and 50% more $A_{2\text{-}2}$ [38].

A variation of the antimetabolite selection techniques is possible when a precursor is toxic to the producing organism. The principle here is that the mutant most capable of detoxifying the precursor by incorporating it into the antibiotic will be the best grower in the presence of the precursor. Barrios-Gonzalez and co-workers [39] obtained phenylacetate-resistant cultures of *P. chrysogenum* which showed enhanced production of penicillin. Rifamycin B production was increased from 6 g/l to 19 g/l by sequential mutation to resistance to tryptophan (feedback inhibitor), *p*-hydroxybenzoate (analog of precursor) and propionate (precursor) [40]. When the produced secondary metabolite is itself a growth inhibitor of the producing culture, as in the case of certain antibiotics, the metabolite can sometimes be used to select resistant mutants which are improved producers. This has been reported with aurodox [33], nocardicin [41], leucomycin [42], oxytetracycline [43] and neomycin [43a].

Certain streptomycin resistance mutations cause increased production of unrelated antibiotics [44]. Selection for streptomycin-resistant mutants in wild-type *Streptomyces coelicolor* A3(2) led to 58% of the mutants producing 5- to 15-fold or more actinorhodin [45, 46]; similar results were obtained with *Streptomyces lividans*. Resistance to gentamicin and to paromomycin was reported to be even more effective than resistance to streptomycin [47]. Mutants resistant to combinations of two or all three of the antibiotics showed increased production up to 48-fold over wild-type [48]. The frequency of improved mutants varied between 5% and 18% of the total number of resistant mutants obtained. Selection of antibiotic-resistant mutants has been successfully used to increase titer of *Streptomyces* cultures even with high producers [49]. Starting with a strain of *Streptomy-*

ces albus producing 10 g/l of salinomycin, spontaneous Str^r, Gen^r or Rif^r mutants gave improved producers at a high rate (8–12%). The best strains made 12–15 g/l when 40 of each type were examined. Double mutants were generated by making spontaneous Gen^r mutants from one of the Str^r mutants; 7% were improved, the best producing 18 g/l. Triple mutants were made with rifamycin using the double mutant as parent; 8% were improved. The best made 23 g/l. It grew as well as the original parent and produced more abundant aerial mycelia and spores.

Nutritional repression can often be decreased by mutation to antimetabolite resistance. Examples of selective agents are 2-deoxyglucose (2-DOG) for enzymes and pathways controlled by carbon source regulation, methylammonium for those regulated by nitrogen source repression, and arsenate for phosphate regulation. Mutants of *P. chrysogenum* resistant to 2-DOG were found to be improved producers of penicillin [50]. A mutant of *Streptomyces aureofaciens* which used phosphate less efficiently for growth showed improved (60%) tetracycline production [51]. Mutants of *Streptomyces griseus* that make candicidin in the presence of levels of phosphate (that normally inhibit secondary metabolism) were found to be hyperproducers of the antibiotic even under low phosphate conditions [52].

2.1.5 Agar zone mutants

Fermentation performance on an agar plate is often related to production in submerged liquid culture and the method has application as a means of detecting superior mutants. 'Zone mutants' have proven useful for improved production of cephalosporin C [53], chlortetracycline [22, 54], mycobacillin [55] and penicillin [56]. A widely used modification involves the production of antibiotics by confluent growth atop separate plugs of agar followed by placement of these plugs on a seeded assay plate and measurement of the resultant clear zones. Use of this 'agar piece method' resulted in improvement of kasugamycin production from 0.5 g/l to 8 g/l [57], a 500-fold increase in bialaphos production [10], and improved cephalosporin C production [58]. Agar-piece screening of antibiotic production in the presence of inhibitory levels of phosphate (15 mM) led to isolation of six markedly improved and stable *Streptomyces hygroscopicus* strains producing the macrolide antifungal complex '165' [59].

2.2 Elimination of undesirable products or analogs

Since many organisms produce secondary metabolites as mixtures of a chemical family or of several chemical families, mutation has been used to eliminate undesirable products in such fermentations. As an example, streaking out of a natural single colony isolate from *S. aureofaciens* (producing the polyether narasin and the broad-spectrum antibiotic enteromycin) on galactose led to yellow and white sectoring [60]. The effect was specific for galactose. Of the four colony types obtained, one produced only narasin and two produced only enteromycin.

S. griseus subsp. *cryophilus* makes four R_3-sulfated and four R_3-unsulfated carbapenems. To completely eliminate the R_3 sulfated forms, that are less active than the unsulfated forms, sulfate transport mutants were obtained. These were of two types: (i) auxotrophs for thiosulfate or cysteine; and (ii) selenate-resistant mutants. Each type produced completely unsulfated forms and titers were equivalent to the total titer of the parent [61].

Eight avermectins are produced by *Streptomyces avermitilis* of which only a small number are desirable. A non-methylating mutant produced only four of the compounds and a mutant who failed to make the 25-isopropyl substituent (from valine) produced a different mixture of components. By protoplast fusion, a hybrid strain was obtained which made only two components, B2a and B1a [62]. Random PCR mutagenesis into gene *aveC* yielded a mutant which produced an avermectin B1:B2 ratio of 2.5, much improved over the 0.6 ratio of the parent *S. avermitilis* strain [63]. Gene shuffling further improved the ratio to about 15 [64].

Mutation was used to eliminate the undesirable polyketides sulochrin and asterric acid from broths of the lovastatin producer, *Aspergillus terreus* [65]. Mutants have also been employed to eliminate undesirable coproducts from the monensin fermentation [66].

2.3 Production of novel antibiotics

The medically useful demethyltetracycline and doxorubicin were discovered by simple mutation of the cultures producing tetracycline and daunomycin, respectively. Later, the technique of mutational biosynthesis ('mutasynthesis') was devised [67]. In this process, a mutant blocked in

secondary metabolism is fed analogs of the moiety whose biosynthesis is blocked. If successful, the mutant (called an 'idiotroph') produces a new antibiotic derivative [68]. Since then, mutational biosynthesis has been used for the discovery of many new secondary metabolites [69–71]. The most well-known is the commercial antihelmintic agent doramectin, production of which employed a mutant of the avermectin producer *S. avermitilis* [72].

New anthracyclines and aglycones have been isolated from blocked mutants of the daunorubicin and doxorubicin producers [73, 74]. By adding carminomycinone or 13-dihydrocarminomycinone to an idiotroph of *Streptomyces galilaeus* (the producer of aclacinomycin), the aglycones were glycosylated to form a new trisaccharide anthracycline, trisarubicionol, by mutational biosynthesis [75]. New macrolide antibiotics have been produced from blocked mutants of the tylosin-producer, *S. fradiae* [76]. Four new hybrid macrolide antibiotics were obtained by feeding erythronolide B to a blocked mutant of the oleandomycin producer, *Streptomyces antibioticus* [77]. A blocked-mutant of the mycinamicin producer, *Micromonospora polytrota*, was fed various rosaramicin precursors and converted them into new rosaramicins [78].

3 Deciphering biosynthetic pathways

The use of mutants for the elucidation of metabolic pathways been exploited for the biosynthesis of tetracyclines [79], novobiocin [80], erythromycin [81, 82], neomycin [83], tylosin [84], other aminoglycosides [85–89], rosaramicin [90], daunorubicin [73], other anthracyclines [91, 92], actinomycin [93], carbapenems [94, 95], ansamycins [96, 97], patulin [98] and phenazines [99].

4 Recombination

In contrast to the extensive use of mutagenesis and screening/selection in industry, employment of genetic recombination was meagre for many years. This lack of interest was prevalent, despite early claims of success [100, 101], mainly due to the total absence or the extremely low frequency

of genetic recombination in industrial microorganisms (in streptomycetes, it was usually 10^{-6} or even less). Other problems were evident with the β-lactam producing fungi. Although *Aspergillus* had sexual and parasexual reproduction, the most interesting genera from the commercial sense, *Cephalosporium* and *Penicillium*, only had parasexual reproduction that rarely resulted in recombination.

Recombination was erroneously looked upon as an alternative to mutation instead of a method which would complement mutagenesis programs. The most balanced and efficient strain development strategy would not emphasize one to the exclusion of the other; it would contain both mutagenesis-screening and recombination-screening components. In such a program, strains at different stages of a mutational line, or from lines developed from different ancestors, would be recombined. Such strains would no doubt differ in many genes and by crossing them, genotypes could be generated which would never occur as strictly mutational descendants of either parent.

Recombination has also been of importance in the mapping of production genes. Studies on the genetic maps of overproducing organisms such as actinomycetes are rather recent. The model for such investigations was the genetic map of *S. coelicolor* [102] which was found to be very similar to those of other *Streptomyces* species, such as *Streptomyces bikiniensis*, *Streptomyces olivaceous*, *Streptomyces glaucescens* and *Streptomyces rimosus*. The problem of low frequency of recombination was mainly solved by use of protoplast fusion. After 1980, there was a heightened interest in the application of genetic recombination to the production of important microbial products such as antibiotics. Today, frequencies of recombination have increased to even greater than 10^{-1} in some cases [103], and strain improvement programs routinely include protoplast fusion between different mutant lines. Conventional strain improvement increased clavulanate production 10-fold over the wild-type *S. clavuligerus* NRRL 3585. Then, protoplast fusion of arginine and cysteine auxotrophs yielded a fusant (CKD 1386) producing 30-fold more clavulanic acid than the wild-type [104]. Recombination is especially useful when combined with conventional mutation programs to solve the problem of 'sickly' organisms produced as a result of accumulated genetic damage over a series of mutagenized generations. For example, a cross *via* protoplast fusion was carried out with strains of *Acremonium chrysogenum*

(formerly *Cephalosporium acremonium*) from a commercial strain improvement program. A low-titer, rapidly-growing, spore-forming strain which required methionine to optimally produce cephalosporin C was crossed with a high-titer, slow-growing, asporogenous strain which could use the less expensive inorganic sulfate. The progeny included a recombinant which grew rapidly, sporulated, produced cephalosporin C from sulfate and made 40% more antibiotic than the high-titer parent [105]. Protoplast fusion was used to modify the characteristics of an improved penicillin-producing strain of *P. chrysogenum* which showed poor sporulation and poor seed growth. Backcrossing with a low-producing (12 g/l) strain yielded a high-producing (18 g/l) strain with better sporulation and better growth in seed medium [106]. Another application of protoplast fusion is the recombination of improved producers from a single mutagenesis treatment. By recombination, one could combine the yield-increase mutations and obtain an even more superior producer before carrying out further mutagenesis. Two improved cephamycin-C producing strains from *Nocardia* were fused and among the recombinants were two cultures which produced 10–15% more antibiotic than the best parent [107]. Genetic recombination allows the discovery of new antibiotics by fusing producers of different or even the same antibiotics. Protoplast fusion between non-producing mutants of a streptomycin producer (*S. griseus*) and an istamycin producer (*Streptomyces tenjimariensis*) led to a hybrid strain producing a new antibiotic [108]. A recombinant obtained from two different rifamycin-producing strains of *Nocardia mediterranei* produced two new rifamycins (16,17-dihydrorifamycin S and 16,17-dihydro-17-hydroxy-rifamycin S) [109]. However, according to Hopwood [110], these examples may reflect the different expression of genes from parent A in the cytoplasm of parent B rather than the formation of hybrid antibiotics. Interspecific protoplast fusion between S. griseus and five other species (*Streptomyces cyaneus*, *Streptomyce exfoliatus*, *Streptomyces griseoruber*, *Streptomyces purpureus* and *Streptomyces rochei*) yielded recombinants of which 60% produced no antibiotics and 24% produced antibiotics different from the parent strains [111]. New antibiotics can also be created by changing the order of the genes of an individual pathway in its native host [112]. A new antibiotic, indolizomycin, was produced by protoplast fusion between non-antibiotic producing mutants of *S. griseus* and *S. tenjimariensis* [113].

4.1 Transformation and transposition

Virtually all antibiotic-producing species of *Streptomyces* contain plasmid DNA. Some are sex plasmids and constitute an essential part of the sexual recombination process and others contain either structural genes or genes somehow influencing the expression of the chromosomal structural genes of antibiotic biosynthesis. Very few antibiotic biosynthesis processes are encoded by plasmid-borne genes. However, production of methylenomycin A is encoded by genes present on plasmid SCP1 in *S. coelicolor*. For many years, plasmid SCP1 was never observed nor isolated as a circular DNA molecule. The reason was that it was a giant linear plasmid. It was initially difficult to separate such giant linear plasmids from chromosomal DNA but this was later accomplished by pulsed field gel electrophoresis or orthogonal field alteration gel electrophoresis (OFAGE) [114]. When the plasmid was transferred to other streptomycetes, the recipients produced the antibiotic.

Cloning a 34 kb fragment from *S. rimosus via* a cosmid into *S. lividans* and *S. albus* resulted in oxytetracycline production by the recipients [115]. Contrary to earlier reports, all the oxytetracycline genes were clustered together on the *S. rimosus* chromosomal map. The biosynthetic genes were flanked by two resistance genes [116].

Plasmids have also been used to devise bioconconversions. 3-0-Acetyl-4"-*0*-isovaleryltylosin (AIV) is useful in veterinary medicine against tylosin-resistant *Staphylococcus aureus*. It is made by first producing tylosin with *S. fradiae* and then using *Streptomyces thermotolerans* (producer of carbomycin) to bioconvert tylosin into AIV. A new strain capable of carrying out a direct fermentation to AIV was constructed by transforming *S. fradiae* with *S. thermotolerans* plasmids containing acyl transferase genes [117].

Some products of unicellular bacteria are plasmid-encoded. These include aerobactin, a hydroxamate siderophore and virulence factor produced by *E. coli* [118] and other Gram-negative bacteria (*Enterobacter aerogenes*, *Enterobacter cloacae*, *Vibrio mimicus*, *Klebsiella*, *Salmonella*, and *Shigella*). Aerobactin is synthesized by a plasmid-borne five-gene cluster which is negatively regulated by iron [119], and also *via* chromosomal genes [120]. A microcin, an antimetabolite of methionine which is produced by *E. coli* and acts as a competitive inhibitor of homoserine-O-tran-

succinylase, is encoded by a plasmid which occurs at 20 copies per genome equivalent [121].

Instability in *Streptomyces* is brought about by environmentally stimulated macrolesions, e.g., deletions, transpositions, rearrangements and DNA amplification. They occur spontaneously or are induced by environmental stresses such as intercalating dyes, protoplast formation and regeneration, and interspecific protoplast fusion. Streptomycetes are the only prokaryotes known to be subject to spontaneous DNA amplification, sometimes amounting to several hundred tandem copies, accounting for over 10% of total DNA, in the absence of selection. Amplification seems to be coupled to DNA deletion and may involve insertion sequence (IS)-like elements [5]. Ethidium bromide cures plasmids in streptomycetes but also increases the frequency of deletion mutations especially in areas of the chromosome which are already unstable [122].

Transposable elements, i.e., DNA sequences encoding a transposase enzyme [123], that move from one replicon to another without host recombination functions or extensive homology with the site of integration, have been extremely useful for the following reasons: (i) they usually provide stable, nonreverting mutants; (ii) they can be used to determine the order of genes in an operon; (iii) it is easy to select for mutants since transposons contain antibiotic- or mercury-resistance markers; (iv) they provide portable regions of homology for chromosomal mobilization; (v) they provide markers for non-selectable genes and allow the cloning of such genes which can then be used as hybridization probes to fish out the wild type gene from a genomic library; and (vi) they often have unique restriction sites, thus are good markers for isolating defined deletion derivatives or locating the precise position of a gene by heteroduplex mapping.

In the daptomycin producer *Streptomyces roseosporus*, some Tn *5099* transposition mutants produced 57–66% more daptomycin than the parent whereas others produced less or the same as the parent [124, 125]. Transposition increased the rate-limiting step of tylosin biosynthesis in *S. fradiae*, i.e., the conversion of macrocin to tylosin. Transposing a second copy of *tylF* into a neutral site on the *S. fradiae* chromosome increased its gene product, macrocin O-methyltransferase, and tylosin production, while decreasing the concentration of the final intermediate (macrocin). Tylosin production was increased by up to 60% and total macrolide titer

was unchanged [126]. Transposon mutagenesis eliminated production of the troublesome toxic oligomycin by the avermectin-producing *S. avermitilis* [127].

5 Genetic engineering

One of the first indications that recombinant DNA technology could be applied to production of antibiotics and other secondary metabolites was that it could be carried out in streptomycetes [128]. Plasmids were constructed from plasmid SLP 1.2 of *S. lividans* and plasmid SCP2* from *S. coelicolor*. In mating of plasmid-negative *S. lividans*, 'pocks' (circular zones of sporulation inhibition associated with plasmid transfer in the lawn of streptomycete growth arising from a regenerated protoplast population) were seen. This was due to looping out of a piece of *S. coelicolor* DNA which became a series of small *S. lividans* plasmids (SLP 1.1 to 1.6) which were good cloning vehicles. Genetic engineering of actinomycetes was limited for a number of years by (i) restriction barriers hindering DNA introduction and (ii) inhibition of secondary metabolism by self-replicating plasmid-cloning vectors [129], but these problems were mainly overcome. Early reviews on cloning and expressing antibiotic production genes in *Streptomyces* were by Martin and Gil [130] and Liras [131].

An interesting possibility is the transfer of operons from one streptomycete to another in the hope that the structural genes might be better able to express themselves in another species. Clustering facilitates transfer of an entire pathway in a single manipulation. Studies revealed that many antibiotic biosynthesis genes were arranged in clusters including undecylprodigiosin, actinorhodin, chloramphenicol, rifamycin, cephamycin, erythromycin, tetracyclines and tylosin. Thus, the entire undecylprodigiosin pathway (*red* pathway) of *S. coelicolor* was transferred on a 37 kb fragment into *Streptomyces parvulus* and the antibiotic was produced [132]. Similarly, the entire cephamycin C pathway was cloned and expressed from a cephamycin-producing strain of *Streptomyces cattleya*. When the 29 kb DNA fragment was cloned into the non-β-lactam producer, *S. lividans*, one transformant (out of 30,000) made cephamycin [133]. When the fragment was introduced into another cephamycin producer, *Streptomyces lactamgens*, a 2 to 3-fold improvement in titer was obtained.

Cloning has been very important in understanding the biosynthesis of β-lactam antibiotics [134], its genetics, and improvement of the processes. The cloning of gene *pcbAB* from *P. chrysogenum* [135], *A. chrysogenum* [136], and *Nocardia lactamdurans* contributed greatly to the elucidation of the biosynthetic pathways. Gene *pcbC* (encoding isopenicillin N synthase) was cloned from *P. chrysogenum* [137], *A. chrysogenum*, *Aspergillus nidulans* [138], *S. clavuligerus* [139], *S. griseus* [140], *Streptomyces lipmannii* and *Streptomyces jumonjinensis* [138, 141] in order to provide pure enzyme for structural studies. Cloning multiple copies of this gene into *A. chrysogenum* yielded an improved cephalosporin C-producing strain [142].

Penicillin acyltransferase acts on IPN in *P. chrysogenum* to produce penicillin G. Its gene *penDE* (also known as *iat*, *aat* and *acyA* in *A. nidulans*) was cloned from *P. chrysogenum* into *A. chrysogenum* leading to production of penicillin G (in the presence of exogenous phenylacetic acid) along with cephalosporin C [136]. Without cloning, *A. chrysogenum* cannot produce penicillin G.

Transformation of early strain *P. chrysogenum* Wis54-1255 with individual genes, pairs of genes, and the entire three genes of the penicillin pathway showed that the major increases occurred when all three genes were overexpressed [143]. The best transformant contained three extra copies of *pcbAB*, one extra copy of *pcbC* and two extra copies of *penDE*, and produced 299% of control shake flask production and 276% of control productivity in continuous culture.

When an industrial production strain of *A. chrysogenum* was transformed with a plasmid containing genes *pcbC* and *cefEF* from an early strain of the *A. chrysogenum* mutant line, a transformant producing 50% more cephalosporin C than the production strain, as well as less penicillin N, was obtained. Production in pilot plant (150 liter) fermentors was further improved by 15% [144]. One copy of *cefEF* had been integrated into chromosome III whereas the native gene is on chromosome II.

An industrial strain improvement program based on genetic transformation showed that the best genes to increase cephalosporin C production in *A. chrysogenum* were *cefEF*, encoding expandase-hydroxylase, and *cefG*, encoding acetyltransferase [145]. The increased gene dosage raised production of cephalosporin C and decreased the concentration of intermediates deacetylcephalosporin and deacetoxycephalosporin C.

A. chrysogenum produces cephalosporin C but also excretes the intermediate DAOC at 1–2% of the cephalosporin C level. This undesirable situation was modified by genetically engineering the strain with two extra copies of the expandase-hydroxylase gene. The new strain excreted only half as much of this intermediate with no effect on cephalosporin C production [146].

Cloning of the benzylpenicillin acylase gene of *E. coli* on multicopy plasmids resulted in a 45-fold increase as compared to enzyme production by the uninduced wild-type. Interestingly, the cloned enzyme is constitutive [147]. Cloning of additional penicillin V amidase genes into wild-type *Fusarium oxysporium* increased enzyme titer by 130-fold [148]. These two enzymes are valuable for converting penicillin G into the valuable intermediate for making semi-synthetic penicillins, i.e., 6-aminopenicillanic acid (6-APA).

In another example, cephalosporin C was directly converted to 7-ACA by using an *E. coli* strain containing the D-amino acid oxidase gene from *Trigonopsis variabilis* and the glutaryl-7-aminocephalosporanic acid acylase gene from *Pseudomonas* sp. [149].

6 New strategies

In recent years, new techniques which markedly increase the options available to improve the production of microbial metabolites, have been added to the 'toolbox' including metabolic engineering, genomics, transcriptome analysis, proteomics, directed evolution, and whole genome shuffling, among others. These are described below.

'*Metabolic engineering*' is the directed improvement of product formation or cellular properties through the modification of specific biochemical reactions or introduction of new ones with the use of recombinant DNA technology [150, 151]. Its essence is the combination of analytical methods to quantify fluxes and the control of fluxes with molecular biological techniques to implement suggested genetic modifications. Metabolic control analysis has revealed that the overall flux through a metabolic pathway depends on several steps, not just a single rate-limiting reaction [152]. This strategy has been succesfully applied to antibiotic production [153–155], including complex polyketides [156, 157].

Progress in metabolic engineering to improve production of microbial metabolites has involved three major strategies: (i) manipulation of structural genes; (ii) manipulation of regulatory genes; and (iii) engineering of well-known pathways.

(i) *Manipulation of structural genes*. Amplification of an entire pathway resulted in a 2.3-fold increase in cephamycin C production [133]. Amplification of a pathway segment yielded a 7-fold increase in formation of daunorubicin formation [158], a 30% increase in tetracenomycin C production [159], and 3 to 4-fold more spinosyn [160]. Perturbing central metabolism by deleting either of the genes encoding the first two enzymes in the pentose phosphate pathway led to increased actinorhodin production in *S. lividans* [161].

(ii) *Manipulation of regulatory genes*. The gene *afsR2* is a global regulatory gene originally discovered in *S. lividans* with positive control action on actinorhodin and undecylprodigiosin formation; it is also present in *S. coelicolor* [162]. Incorporation of multiple copies of *afsR2* from *S. lividans* into *S. avermitilis* wildtype increased avermectin production by 2.3-fold [163]. Another regulatory gene appears to be an 8 kb DNA fragment of *S. avermitilis* which stimulates actinorhodin and undecylprodigiosin formation in *S. lividans*, as well as avermectin production in wild-type *S. avermitilis*, in an improved strain, and in a semi-industrial strain of *S. avermitilis* [164]. Amplification of other pathway-specific regulators resulted in a 5-fold enhancement of spiramycin titer in *Streptomyces ambofaciens* [165] and a 1.6-fold increase in mithramycin production in *Streptomyces argillaceus* [166].

Disruption of negative pathway specific regulatory genes improved production of avermectin by 1.5 to 3.5-fold [164], lovastatin by 7 to 10-fold [167], and also improved formation of methylenomycin [163] and mitomycin C [168]. Also, disruption of a negative global regulator resulted in production increases for actinorhodin and undecylprodigiosin [169]. Gene *nysF*, encoding a putative 4'-phosphopantetheinyl transferase in the nystatin producer, *Streptomyces noursei*, was first thought to be involved in a biosynthetic step but was actually found to be a negative regulatory gene [170]. Knocking out *nysF* increased production by 60%.

(iii) *Engineering of pathways*. By increasing the expression of rate-limiting enzymes, tylosin production was enhanced [171, 172] and so was penicillin. In this latter case, overexpression of the *acvA* gene by replacing

the normal promoter with the ethanol dehydrogenase promoter increased penicillin production in *A. nidulans* by 30-fold [173].

Eliminating the accumulation and excretion of pathway intermediate penicillin N raised cephalosporin C production by 15%. Weak acetyltransferase promoter activity appears to be the cause of the undesirable accumulation of intermediate DAC in broths of *A. chrysogenum*. Cloning of gene *cefG* (encoding DAC acetyltransferase) increased cephalosporin C titers in a dose-dependent manner [173a, 173b]. Cloning of the gene with its own promoter had no effect on the low level of DAC acetyltransferase normally observed in *A. chrysogenum* [174]. However, use of foreign promoters (the *gpd* promoter from *A. nidulans*, the *bla* promoter from *Aspergillus niger*, or the *pbcC* promoter from *P. chrysogenum*) had a major effect on the level of *cefG* transcripts, DAC acetyltransferase protein level and activity, and antibiotic production; cephalosporin C production rose 2- to 3-fold. Of the cephalosporins produced, the undesirable DAC decreased from 80% of the total down to 30–39%, whereas cephalosporin C increased by a similar amount.

Transformation of *P. chrysogenum* with the *S. lipmanii cefD* gene and the *S. clavuligerus cefE* gene allowed production of the intermediate deacetoxycephalosporin C (DAOC) [175] at titers of 2.5 g/l. DAOC is a valuable intermediate in the commercial production of semi-synthetic cephalosporins.

Metabolic engineering of *P. chrysogenum* allowed production of valuable cephalosporin intermediates (adipyl-7-ADCA and adipyl-7-ACA) [176]. Disruption and one-step replacement of the *cefEF* gene of an industrial cephalosporin C production strain of *A. chrysogenum* yielded strains accumulating up to 20 g/l of penicillin N. Cloning and expression of the *cefE* gene from *S. clavuligerus* into those high producing strains yielded recombinant strains producing high titers of DAOC [177]. Production levels were nearly equivalent (80%) to the total β-lactams biosynthesized by the parental strain.

Deletion of a gene leading to a side-product eliminated oligomycin production from the producer of avermectins [127]. Genetically increasing oxygen availability resulted in a 60% improvement of erythromycin titer [178, 179]. Enhancement of precursor uptake gave a 4-fold increase in production of deoxyerythonolide B and 8,8a-deoxyoleoandolide [180]).

'*Inverse Metabolic Engineering*' [181, 182] (also known as 'reverse engineering') involves construction or calculation of a desired phenotype, identifi-

cation of the molecular basis of that desirable property, and incorporation of that phenotype into another strain or other species by genetic manipulations and funtional genomics [183]. Once the differences are found, they can be introduced into other organisms or their dosages increased in the improved strain to further improve it. The techniques available are (i) genomic DNA sequencing; (ii) DNA sequencing of selected genes; (iii) transcriptional profiling; (iv) proteome analysis; (v) metabolite profiling; and (vi) comparative flux analysis. This strategy was used to increase erythromycin production by *Aeromicrobium erythreum* [184]. Plasmid insertion mutagenesis yielded 26 mutants (out of 3,049 isolates) with >50% increased production; seven were chosen for reverse engineering. Three were identical *mutB* mutants encoding the α subunit of methylmalonyl-CoA mutase. The other four mutants were in *cobA*, encoding Cob(I)alamin adenosyl transferase. The *mutB* mutant produced about 500 mg/l of erythromycin compared to ca 200 mg/l made by the parent. The *cobA* mutant produced 22% more than the parent. Feeding of nutrients such as leucine and propionate, which increase pools of propionyl-CoA and methylmalonyl-CoA, to the mutants increased production. Methionine also increased production by the *cobA* strain. The probable reason for improved production by methylmalonate-CoA mutase knockouts is that its substrate is a branchpoint intermediate and the favored direction is the production of succinyl-CoA, thus diverting methylmalonyl-CoA away from the erythromycin pathway. Since coenzyme B_{12} is its cofactor, *cobA* mutants would also favor flux into the erythromycin pathway. As expected, leucine, propionate and methionine, which are known precursors of methylmalonyl-CoA *via* propionyl-CoA, stimulated production in the mutants.

'*Genome-based strain reconstruction*' allows one to construct a strain superior to the production strain because it only contains mutations crucial to hyperproduction, but not other unknown mutations which accumulate by brute-force mutagenesis and screening. Although this approach has been done with a primary metabolite production process (lysine) [185], it should also be useful for secondary metabolites.

During the last few years, an expanded view of the cell has been possible due to the impressive advances in all the 'omics' techniques (genomics, proteomics, metabolomics) and high-throughput technologies for measuring different classes of key intracellular molecules. '*Systems Biology*' has recently emerged as a term and a scientific field to describe an approach

that considers genome-scale and cell-wide measurements in elucidating process and mechanisms [186].

Integrating transcriptional and metabolite profiles from 21 strains of *A. terreus* producing different levels of lovastatin and another 19 strains with altered (+)-geodin levels led to an improvement in lovastatin production of over 50% [187]. This approach, named '*Association Analysis*', served to reduce the complexity of profiling data sets in order to identify those genes whose expression is most tightly linked to metabolite production. Such applications are applicable to all industrially useful organisms for which genome data are limited.

Gene expression analysis of wild type and improved production strains of *Saccharopolyspora erythraea* and *S. fradiae* using microarrays of the sequenced *S. coelicolor* revealed that regulation of antibiotic biosynthetic enzymes as well as enzymes involved in precursor metabolism were altered in those mutated strains [188]. Comparison of *S. erythraea* wild type and an improved erythromycin producer was done and results revealed that the *S. erythraea* overproducer expressed the entire erythromycin gene cluster several days longer than the wild-type. It seems that the *eryA* gene and protein expression differences observed for the overproducer could account over 50% of the total erythromycin titer increase. A different situation was found with tylosin production. The overproducing *S. fradiae* strain produced tylosin for the same length of time but the rate of antibiotic production was 2-fold higher in the overproducer. The tylosin cluster was expressed over the same period of time; however, there were two genes: *aco* (encoding acyl-CoA dehydrogenase) and *icmA* (encoding isobutyryl-CoA mutase) that were expressed to a greater extent than in the wild type strain. The induction of these two genes could increase the flux of metabolites from fatty acids to tylosin precursors in the overproducer.

'*Directed Evolution*' (also known as applied molecular evolution or directed molecular evolution) is a fast and inexpensive way of finding variants of existing enzymes that work better than naturally occurring enzymes under specific conditions [189–191]. It has been used to improve the properties of rate-limiting enzymes of natural product biosynthesis. The process involves evolutionary design methods using random mutagenesis, gene recombination and high throughput screening [192]. A key limitation of these strategies is that they introduce random 'noise' mutations into the gene at every cycle and hence improvements are limited to

small steps. This strategy has been successfully used in various applications [193].

'*Molecular Breeding*™' techniques come closer to mimicking natural recombination by allowing *in vitro* homologous recombination [194]. DNA shuffling not only recombines DNA fragments but also introduces point mutations at a very low controlled rate [195, 196]. Unlike site directed mutagenesis, this method of pooling and recombining parts of similar genes from different species or strains has yielded remarkable improvements in enzymes in a very short amount of time [197]. A step forward in this technique was breeding a population with high genetic variability as a starting point to generate diversity (DNA Family Shuffling). Innovations that expand the formats for generating diversity by recombination include formats similar to DNA shuffling and others with few or no requirements for parental gene homology [198, 199]. These random redesign techniques are being currently used to increased biological activity of protein pharmaceuticals and biological molecules [197, 198] as well as novel vaccines [200, 201].

'*Whole genome shuffling*' is a novel technique that combines the advantage of multi-parental crossing allowed by DNA shuffling with the recombination of entire genomes. This method was successfully applied to improve tylosin production in *S. fradiae* [202]. Historically, twenty cycles of classical strain improvement at Eli Lilly and Co. carried out over 20 years employing about one million assays improved production 6-fold. In contrast, two rounds of this recursive recombination technique with seven early strains each were sufficient to achieve similar results in one year and involved only 24,000 assays.

'*Combinatorial biosynthesis*' is not used to increase titer but for discovery of new and modified drugs [203, 204]. In this strategy, recombinant DNA techniques are utilized to introduce genes coding for antibiotic synthases into producers of other antibiotics or into non-producing strains to obtain modified or hybrid antibiotics. In the first demonstration of this technology, gene transfer from a streptomycete strain producing the isochromanequinone antibiotic actinorhodin into strains producing granaticin, dihydrogranaticin and mederomycin (which are also isochromanequinones) led to the discovery of two new antibiotic derivatives, mederrhodin A and dihydrogranatirhodin [205]. Since then, hundreds of new polyketides have been made by combinatorial biosynthesis [206–228]. Some of these novel

polyketides contain sugars at normally unglycosylated positions [229] or as new sugar moieties [230, 231]. New anthracyclines [232–237] and peptide antibiotics [238] have been made by combinatorial biosynthesis. Manipulations include [239]: (i) deletion of one of the domains of a particular module; (ii) addition of a copy of the thioesterase domain to the end of an earlier module resulting in a shortened polyketide; (iii) replacement of an AT domain of a polyketide synthase (PKS) with an AT domain from another PKS, resulting in addition of a methyl group at a particular site or removal of a methyl group; (iv) addition of a reductive domain(s) to a particular module, thus changing a keto group to a double bond or to a methylene group; (v) use of synthetic diketides delivered as N-acetylcysteamine thioesters to load onto the active site of the ketosynthase (KS) in module 2 and to be taken all the way to a novel final product; (vi) replacement of the loading module of one PKS with the loading module of another PKS, thus changing the starter unit from propionate to acetate, for example; and (vii) replacement of the hydroxylase or glycosylase enzymes from one pathway to another, thus modifying the ring structure with respect to OH groups and/or sugars.

Progress in strain development will depend, not only on all the technologies mentioned above, but also on the development of mathematical methods that facilitate the elucidation of mechanisms and identification of genetic targets for modification. The availability of the complete genome sequences of *S. coelicolor*, producer of actinorhodin and three other antibiotics [240], and *S. avermitilis*, producer of avermectins [241], the published and ongoing sequencing projects involving hundreds of microbial genomes, as well as the ability to rapidly identify clusters of genes encoding biosynthesis of bioactive products and to predict their structures based on gene sequences [242] will contribute to an acceleration of strain improvement programs.

Closing comments

Microorganisms produce many compounds of industrial interest. These may be very large materials such as proteins, nucleic acids, carbohydrate polymers, or even cells, or they can be smaller molecules which can be essential for vegetative growth (primary metabolites) or inessential (sec-

ondary metabolites). The power of the microbial culture in the competitive world of commercial synthesis can be appreciated by the fact that even simple molecules are made by fermentation rather than by chemical synthesis. Most natural products are so complex that they probably will never be made commercially by chemical synthesis. Strains isolated from nature produce only tiny amounts of product. The reason is that they need small amounts of these compounds for their own competitive benefit (survival) in nature; they do not overproduce these metabolites. Regulatory mechanisms have evolved in microorganisms that enable a strain to avoid excessive production of its metabolites. Thus, strain improvement programs are absolutely required for commercial application. Their goal is to isolate cultures exhibiting desired phenotypes. Most commonly, the ability of a strain to improve titer is what is desired, although the spectrum of improvements can also include other traits. The tremendous increases in fermentation productivity and the resulting decreases in costs have come about mainly by mutagenesis and screening/selection. In recent years, recombinant DNA technology has contributed greatly. The promise of the future is *via* extensive use of new genetic techniques such as (i) metabolic engineering accomplishing quantification and control of metabolic fluxes and including inverse metabolic engineering, (ii) transcript expression analyses, (iii) directed evolution, (iv) molecular breeding, and (v) combinatorial biosynthesis. These efforts will facilitate not only the isolation of improved strains but also the elucidation and identification of new genetic targets to be used in strain improvement programs.

References

1 Parekh S (2000) Strain improvement. In: J Lederberg (ed): *Encyclopedia of microbiology*, vol. 4, 2nd ed. Academic Press, San Diego, 428–443

2 Vinci VA, Byng G (1999) Strain improvement by nonrecombinant methods. In: AL Demain, JE Davies (eds): *Manual of industrial microbiology and biotechnology*, 2nd ed. ASM Press, Washington, DC, 103–113

3 Parekh S, Vinci VA, Strobel RJ (2000) Improvement of microbial strains and fermentation processes. *Appl Microbiol Biotechnol* 54: 287–301

4 Simpson IN, Caten CE (1979) Induced quantitative variation for penicillin titre in clonal populations of *Aspergillus nidulans*. *J Gen Microbiol* 110: 1–12

5 Baltz RH (1986) Mutagenesis in *Streptomyces*. In: AL Demain, NA Solomon (eds): *Manual of industrial microbiology and biotechnology*. ASM, Washington, DC, 184–190

6 Baltz RH (1995) Gene expression in recombinant *Streptomyces*. In: A Smith (ed): *Gene expression in recombinant microorganisms*. Marcel Dekker, New York, 309–381

7 Baltz RH (1998) Genetic manipulation of antibiotic producing *Streptomyces*. *Trends Microbiol* 6: 76–83

8 Baltz RH (1999) Mutagenesis. In: MC Flickinger, SW Drew (eds): *Encyclopedia of bioprocessing technology: fermentation, biocatalysis, and separation*. Wiley, New York, 1819–1822

9 Keller U (1983) Highly efficient mutagenesis of *Claviceps purpurea* by using protoplasts. *Appl Environ Microbiol* 46: 580–584

10 Takebe H, Imai S, Ogawa H, Satoh A, Tanaka H (1989) Breeding of bialaphos producing strains from a biochemical engineering viewpoint. *J Ferm Bioeng* 67: 226–232

11 Kim KS, Cho NY, Pai HS, Ryu DDY (1983) Mutagenesis of *Micromonospora rosaria* by using protoplasts and mycelial fragments. *Appl Environ Microbiol* 46: 689–693

12 Kurzatkowski W, Kurylowicz W, Solecka J, Penyige A (1986) Improvement of *Streptomyces* strains by the regeneration of protoplasts. In: G Szabo, S Biro, M Goodfellow (eds): *Biological, biochemical and biomedical aspects of actinomycetes. Part A*. Academiai Kiado, Budapest, 289–292

13 Strohl WR (2001) Biochemical engineering of natural product biosynthesis pathways. *Metab Eng* 3: 4–14

14 Hersbach GJM, van der Beck CP, van Dijck PWM (1984) The penicillins: properties, biosynthesis, and fermentation. In: EJ Vandamme (ed): *Biotechnology of industrial antibiotics*. Marcel Dekker, New York, 45–140

15 Smith DJ, Bull JH, Edwards J, Turner G (1989) Amplification of the isopenicillin N synthetase gene in a strain of *Penicillium chrysogenum* producing high levels of penicillin. *Mol Gen Genet* 216: 492–497

16 Barredo JL, Diez B, Alvarez E, Martin JF (1989) Large amplification of a 35-kb DNA fragment carrying two penicillin biosynthetic genes in high penicillin producing strains of *P. chrysogenum*. *Curr Genet* 16: 453–459

17 Smith DJ, Burnham MKR, Bull JH, Hodgson JE, Ward JM, Browne P, Brown J, Barton B, Earl AJ, Turner G (1990) β-Lactam antibiotic biosynthesis genes have been conserved in clusters in prokaryotes and eukaryotes. *EMBO J* 9: 741–747

18 Fierro F, Barredo JL, Diez B, Gutierrez S, Fernandez FJ, Martin JF (1995) The penicillin gene cluster is amplified in tandem repeats linked by conserved hexanucleotide sequences. *Proc Natl Acad Sci USA* 92: 6200–6204

19 Podojil M, Blumauerova M, Culik K, Vanek Z (1984) The tetracycyclines: properties, biosynthesis and fermentation. In: EJ Vandamme (ed): *Biotechnology of industrial antibiotics*. Marcel Dekker, New York, 259–279

20 Demain AL, Elander RP (1999) The β-lactam antibiotics: past, present, and future. *Ant v Leeuwenhoek* 75: 5–19

21 Chen W-Q, Yu ZN, Zheng Y-H (2004) Expression of *Vitreoscilla* hemoglobin gene in *Streptomyces fradiae* and its effect on cell growth and synthesis of tylosin. *Chin J Antibiot* 29: 516–520

22 Liu C-M (1982) Microbial aspects of polyether antibiotics: activity, production and biosynthesis. In: JW Westley (ed): *Polyether antibiotics. Naturally occurring acid ionophores. Vol. 1. Biology*. Marcel Dekker, New York, 43–102

23 Blumauerova M, Pokorny V, Stastna J, Hostalek Z, Vanek Z (1978) Developmental mutants of *Streptomyces coeruleorubidis*, a producer of anthracyclines: isolation and preliminary characterization. *Folia Microbiol* 23: 177–182

24 Blumaerova M, Podojil M, Gauze G., Maksikmova TS, Panos J, Vanek Z (1980) Spontaneous variability of *Streptomyces glomeratus*, a producer of anthracycline antibiotics beromycins. *Folia Microbiol* 25: 207–212

25 Blumaerova M, Podojil M, Gauze GF, Maksikmova TS, Panos J, Vanek Z (1980) Effect of cultivation conditions on the activity of the beromycin producer *Streptomyces glomeratus* 3980 and its spontaneous variants. *Folia Microbiol* 25: 213–218

26 Lee JC, Park HR, Park DJ, Son KH, Yoon KH, Kim YB, Kim CJ (2003) Production of teicoplanin by a mutant of *Actinoplanes teicomyceticus*. *Biotechnol Lett* 25: 537–540

27 Haavik HI, Froyshov O (1982) On the role of L-leucine in the control of bacitracin formation by *Bacillus licheniformis*. In: H Kleinkauf, H von Doehren (eds): *Peptide antibiotics: biosynthesis and functions*. Walter de Gruyter, Berlin, 155–159

28 Godfrey O (1973) Isolation of regulatory mutants of the aspartic and pyruvic acid families and their effect on antibiotic production in *Streptomyces lipmanii*. *Antimicrob Agents Chemother* 4: 73–79

29 Lee SH, Lee KJ (1995) Threonine dehydratases in different strains of *Streptomyces fradiae*. *J Biotechnol* 43: 95–102

30 Polsinelli M, Albertini A, Cassani G, Ciferri O (1965) Relation of biochemical mutations to actinomycin synthesis in *Streptomyces antibioticus*. *J Gen Microbiol* 39: 239–246

31 Saburova TP, Lapchinskaya OA, Sinyagina OP, Trutneva EM, Lvova NA (1977) Auxotrophic mutants in selection of *Actinomyces coeruleorubidus*, an organism producing rubomycin. *Antibiotiki* 22: 1095–1100

32 Dulaney EL, Dulaney DD (1967) Mutant populations of *Streptomyces viridifaciens*. *Trans NY Acad Sci II* 29: 782–799

33 Unowsky J, Hoppe DC (1978) Increased production of the antibiotic aurodox (X–5108) by aurodox-resistant mutants. *J Antibiot* 31: 662–666

34 Mendelovitz S, Aharonowitz Y (1983) β-Lactam antibiotic production by *Streptomyces clavuligerus* mutants impaired in regulation of aspartokinase. *J Gen Microbiol* 129: 2063–2069

35 Smith A (1987) Enzyme regulation of desferrioxamine biosynthesis: a basis for a rational approach to process development. In: M Alacevic, D Hranueli, Z Toman (eds): *Fifth International Symposium on the Genetics of Industrial Microorganisms*, 1986. Pliva, Zagreb, 513–527

36 Pospisil S, Kopecky J, Prikrylova V, Spizek J (1999) Overproduction of 2-ketoisovalerate and monensin production by regulatory mutants of *Streptomyces cinnamonensis* resistant to 2-ketobutyrate and amino acids. *FEMS Microbiol Lett* 172: 197–204

37 Wang MR, Ding H, Hu YJ (1996) The action of arginine and valine in the biosynthesis of teicoplanin. *Chin J Antibiot* 21 (Suppl): 77–80

38 Jin ZH, Wang MR, Cen PL (2002a) Production of teichoplanin by valine-analogue resistant mutant strains of *Actinoplanes teichomyceticus*. *Appl Microbiol Biotechnol* 58: 63–66

39 Barrios-Gonzalez J, Montenegro E, Martin JF (1993) Penicillin production by mutants resistant to phenylacetic acid. *J Ferm Bioeng* 7: 455–458

40 Jin ZH, Lin JP, Xu ZN, Cen PL (2002b) Improvement of industry-applied rifamycin B-producing strain, *Amycolatopsis mediterranei*, by rational screening. *J Gen Appl Microbiol* 48: 329–334

41 Elander RP, Aoki H (1982) β-Lactam producing microorganisms – their biology and fermentation behavior. In: RB Morin, M Gorman (eds): *Chemistry and biology of* β*-lactam antibiotics*, vol. 3. Academic Press, New York, 83–153

42 Higashide E (1984) The macrolides; properties, biosynthesis, and fermentation. In: EJ Vandamme (ed): *Biotechnology of industrial antibiotics*. Marcel Dekker, New York, 451–509

43 Gravius B, Glocker D, Pigac J, Pandza K, Hranueli D, Cullum J (1994) The 387 kb linear plasmid pPZG101 of *Streptomyces rimosus* and its interactions with the chromosome. *Microbiology* 140: 2271–2277

43a Crameri R, Davies JE (1986) Increased production of aminoglycosides associated with amplified antibiotic resistance genes. *J Antibiot* 39: 128–135

44 Hosoya Y, Okamoto S, Muramatsu H, Ochi K (1998) Acquisition of certain streptomycin resistant (*str*) mutations enhances antibiotic production in bacteria. *Antimicrob Agents Chemother* 42: 2041–2047

45 Hesketh A, Ochi K (1997) A novel method for improving *Streptomyces coelicolor* A3(2) for production of actinorhodin by introduction of rpsL (encoding ribosomal protein S12) mutations conferring resistance to streptomycin. *J Antibiot* 50: 532–535

46 Okamoto S, Lezhava A, Hosaka T, Okamoto-Hosoya Y, Ochi K (2003) Enhanced expression of S-adenosylmethionine synthetase causes overproduction of actinorhodin in *Streptomyces coelicolor* A3(2). *J Bacteriol* 185: 601–609

47 Okamoto-Hosoya Y, Sato T, Ochi K (2000) Resistance to paromomycin is conferred by rpsL mutations, accompanied by an enhanced antibiotic production in *Streptomyces coelicolor* A3(2). *J Antibiot* 53: 1424–1427

48 Hu H, Ochi K (2001) Novel approach for improving the production of antibiotic-producing strains by inducing combined resistant mutations. *Appl Environ Microbiol* 67: 1885–1892

49 Tamehiro N, Hosaka T, Xu J, Hu H, Otake N, Ochi K (2003) Innovative approach for improvement of an antibiotic-overproducing industrial strain of *Strepromyces albus*. *Appl Environ Microbiol* 69: 6412–6417

50 Chang LT, McGrory EL, Elander RP (1980) Penicillin production by glucose-derepressed mutants of *Penicillium chrysogenum*. *J Indust Microbiol* 6: 165–169

51 Colombo AL, Crespi-Pevellino N, Grein A, Minghetti A, Spalla CJ (1981) Metabolic and genetic aspects of the relationship between growth and tetracycline production in *Streptomyces* aureofaciens. *Biotechnol Lett* 3: 71–76

52 Martin JF, Naharro G, Liras P, Villanueva JR (1979) Isolation of mutants deregulated in phosphate control of candicidin biosynthesis. *J Antibiot* 32: 600–606

53 Elander RP (1969) Applications of microbial genetics to industrial fermentations. In: D Perlman (ed): *Fermentation Advances*. Academic Press, New York, 89–114

54 Katagiri I (1954) Study on chlortetracycline. Improvement of chlortetracycline-producing strain by several kinds of methods. *J Antibiot* 7: 45–52

55 Bannerjee AB, Bose SK (1964) A rapid method for isolating mutants of *Bacillus subtilis* producing increased or decreased amounts of the antibiotic, mycobacillin. *J Appl Bacteriol* 27: 93–95

56 Ditchburn P, Giddings B, MacDonald KD (1974) Rapid screening for the isolation of mutants of *Aspergillus nidulans* with increased penicillin yields. *J Appl Bacteriol* 37: 515–523

57 Ichikawa T, Date M, Ishikura T, Ozaki A (1971) Improvement of kasugamycin-producing strain by agar piece method and the prototroph method. *Folia Microbiol* 16: 218–224

58 Chang LT, Elander RP (1979) Rational selection for improved cephalosporin C productivity in strains of *Acremonium chrysogenum*. *Devel Indust Microbiol* 20: 367–379

59 Gesheva V (1994) Isolation of spontaneous *Streptomyces hygroscopicus* 111-81 phosphate-deregulated mutants hyperproducing its antibiotic complex. *Biotechnol Lett* 16: 443–448

60 Nakatsukasa WM, Mabe JA (1978) Galactose induced colonial dissociation in *Streptomyces aureofaciens*. *J Antibiot* 31: 805–808

61 Kitano K, Nozaki Y, Imada A (1985) Selective accumulation of unsulfated carbapenem antibiotics by sulfate transport-negative mutants of *Streptomyces griseus* subsp. *cryophilus* C-19393. *Agric Biol Chem* 49: 677–684

62 Omura S, Ikeda H, Tanaka H (1991) Selective production of specific components of avermectins in *Streptomyces avermitilis*. *J Antibiot* 44: 560–563

63 Stutzman-Engwall K, Conlon S, Fedechko R, Kaczmarek F, McArthur H, Krebber A, Chen Y, Minshull J, Raillard SA, Gustafsson C (2003). Engineering the *aveC* gene to enhance the ratio of doramectin to its CHC-B2 analogue produced in *Streptomyces avermitilis*. *Biotechnol Bioeng* 82: 359–369

64 del Cardayre SB (2003) Delivering industrial microorganisms through gene, pathway, and genome shuffling. *Abstr S75, SIM Ann Mtg Prog & Abstr* 73

65 Vinci VA, Hoerner TD, Coffman AD, Schimmel TG, Dabora RL, Kirpekar AC, Ruby CL, Stieber RW (1991) Mutants of a lovastatin-hyperproducing *Aspergillus terreus* deficient in the production of sulochrin. *J Indust Microbiol* 8: 113–120

66 Pospisil S, Peterkova M, Krumphanzl V, Vanek Z (1984) Regulatory mutants of *Streptomyces cinnamonensis* producing monensin A. *FEMS Microbiol Lett* 24: 209–213

67 Shier WT, Rinehart KL Jr, Gottlieb D (1969) Preparation of four new antibiotics from a mutant of *Streptomyces fradiae*. *Proc Natl Acad Sci USA* 63: 198–204

68 Nagaoka K, Demain AL (1975) Mutational biosynthesis of a new antibiotic, streptomutin A, by an idiotroph of *Streptomyces griseus*. *J Antibiot* 28: 627–635

69 Lemke JR, Demain AL (1976) Preliminary studies on streptomutin A. *Eur J Appl Microbiol* 2: 91–94

70 Daum SJ, Lemke JR (1979) Mutational biosynthesis of new antibiotics. *Ann Rev Microbiol* 33: 241–265

71 Kitamura S, Kase H, Odakura Y, Iida T, Shirahata K, Nakayama K (1982) 2-Hydroxysagamicin: A new antibiotic produced by mutational biosynthesis of *Micromonospora sagamiensis*. *J Antibiot* 35: 94–97

72 Cropp TA, Wilson DJ, Reynolds KA (2000) Identification of a cyclohexylcarbonyl CoA biosynthetic gene cluster and application in the production of doramectin. *Nature Biotechnol* 18: 980–983

73 McGuire J, Thomas MC, Pandey RC, Toussaint M, White RJ (1981) Biosynthesis of daunorubicin glycosides: analysis with blocked mutants. In: M Moo-Young (ed): *Advances in biotechnology. Vol. III. Fermentation products*. Pergammon Press, New York, 117–122

74 Cassinelli G, Di Matteo F, Forenza S, Ripamonti M, Rivola G, Arcamone F, Di Marco A, Cassaza AM, Soranzo C, Pratesi G (1980) New anthracycline glycosides from *Micromonospora*. II. Isolation, characterization and biological properties. *J Antibiot* 33: 1468–1473

75 Yoshimoto A, Matsuzawa Y, Matsuhashi Y, Oki T, Takeuchi T, Umezawa H (1981) Trisarubicinol, new antitumor anthracycline antibiotic. *J Antibiot* 34: 1492–1494

76 Kirst HA, Wild GM, Baltz RH, Seno ET, Hamill RL, Paschal JW, Dorman DE (1983) Elucidation of structure of novel macrolide antibiotics produced by mutant strains of *Streptomyces fradiae*. *J Antibiot* 36: 376–382

77 Spagnoli R, Cappalletti L, Toscano L (1983) Biological conversion of erythronolide B,

an intermediate of erythromycin biogenesis, into new 'hybrid' macrolide antibiotics. *J Antibiot* 36: 365–375

78 Lee BK, Puar MS, Patel M, Bartner P, Lotvin J, Munayyer H, Waitz JA (1983) Multistep bioconversion of 20-deoxo-20-dihydro-12, 13-deepoxy-12,13-dehydrorosaranolide to 22-hydroxy-23-o-mycinosyl-20-deoxo-20-dihydro-12,13-deepoxy-rosaramicin. *J Antibiot* 36: 742–743

79 McCormick JRD (1965) Biosynthesis of the tetracyclines. In: Z Vanek, Z Hostalek (eds): *Biogenesis of antibiotic substances*. Publishing House of the Czechoslovak Academy of Science, Prague, 73–91

80 Kominek LA (1972) Biosynthesis of novobiocin by *Streptomyces niveus*. *Antimicrob Agents Chemother* 1: 123–134

81 Martin JR, Perun TJ, Girolami RL (1966) Studies on the biosynthesis of erythromycins. I. Isolation of an intermediate glycoside, 3-alpha-L-mycarosylerythronolide B. *Biochemistry* 5: 2852–2856

82 Martin JR, Rosenbrook WR (1967) Studies on the biosynthesis of erythromycins. II. Isolation and structure of a biosynthetic intermediate, 6-deoxyerythronolide B. *Biochemistry* 6: 435–440

83 Pearce CJ, Akhtar M, Barnett JEG, Mercier D, Sepulchre AM, Gero S (1978) Sub-unit assembly in the biosynthesis of neomycin. The synthesis of 5-O-β-D-ribofuranosyl and 4-O-β-D-ribofuranosyl-2,6-dideoxystreptamines. *J Antibiot* 31: 74–81

84 Baltz RH, Seno ET, Stonesifer J, Wild GM (1983) Biosynthesis of the macrolide antibiotic tylosin: a preferred pathway from tylactone to tylosin. *J Antibiot* 36: 131–141

85 Takeda K, Aihara K, Furumai T, Ito Y (1978) An approach to the biosynthetic pathway of butirosins and the related antibiotics. *J Antibiot* 31: 250–253

86 Fujiwara T, Takahashi Y, Matsumoto K, Kondo E (1980) Isolation of an intermediate of 2-deoxystreptamine biosynthesis from a mutant of *Bacillus circulans*. *J Antibiot* 33: 824–829

87 Kase H, Odakura Y, Nakayama K (1982) Sagamycin and the related aminoglycosider: fermentation and biosynthesis. I. Biosynthetic studies with the blocked mutants of *Micromonospora sagamiensis*. *J Antibiot* 35: 1–9

88 Hanssen R, Kirby R (1983) The induction by N-methyl-N'-nitro-nitrosoguanidine of multiple closely linked mutations in *Streptomyces bikiniensis* ISP5235 affecting streptomycin resistance and streptomycin biosynthesis. *FEMS Microbiol Lett* 17: 317–320

89 Penzikova GA, Levitov MM (1970) A study on transamidinase activity with respect to streptomycin biosynthesis. *Biologiya* 39: 337–342

90 Vaughn RV, Lotvin J, Puar MS, Patel M, Kershner A, Kalyanpur MG, Marquez J, Waitz JA (1982) Isolation and characterization of two 16-membered lactones: 20-deoxorosaramicin and 20-deoxo-12,13-desepoxy-12,13-dehydrorosaramicin aglycones, from a mutant strain of *Micromonospora rosaria*. *J Antibiot* 35: 251–253

91 Motamedi H, Wendt-Pientowski E, Hutchinson CR (1986) Isolation of tetracenomycin C non-producing *Streptomyces glaucescens* mutants. *J Bacteriol* 167: 575–580

92 Yue S, Motamedi H, Wendt-Pienskowski E, Hutchinson CR (1986) Anthracycline metabolites of tetracenomycin-non-producing *Streptomyces glaucescens*. *J Bacteriol* 167: 581–586

93 Troost T, Katz E (1979) Phenoxazinone biosynthesis: accumulation of a precursor, 4-methyl-3-hydroxyanthranilic acid, by mutants of *Streptomyces parvulus*. *J Gen Microbiol* 1: 121–132

94 Nozaki Y, Kitano K, Imada I (1984) Blocked mutants in the biosynthesis of carbapenem antibiotics from *Streptomyces griseus* subsp. *cryophilus*. *Agric Biol Chem* 48: 37–44

95 Kojima I, Fukagawa Y, Okabe M, Ishikura T, Shibamoto N (1988) Mutagenesis of OA-6129 carbapenem-producing blocked mutants and the biosynthesis of carbapenems. *J Antibiot* 41: 899–907

96 Kibby JJ, McDonald IA, Rickards RW (1980) 3-Amino-5-hydroxybenzoic acid as a key intermediate in ansamycin and maytansinoid biosynthesis. *J Chem Soc Chem Comm* 768–769

97 Ghisalba O, Fuhrer H, Richter W, Moss S (1981) A genetic approach to the biosynthesis of the rifamycin-chromophore in *Nocardia mediterranei*. III. Isolation and identification of an early ansamycin-precursor containing the seven-carbon amino starter-unit and three initial acetate/propionate-units of the ansa chain. *J Antibiot* 34: 58–63

98 Gaucher GM, Lam KS, Grootwassink JWD, Neway J, Deo YM (1981) The initiation and longevity of patulin biosynthesis. *Devel Indust Microbiol* 22: 219–232

99 Byng GS, Eustice DC, Jensen RA (1979) Biosynthesis of phenazine pigments in mutant and wild-type cultures of *Pseudomonas aeruginosa*. *J Bacteriol* 138: 846–852

100 Jarai M (1961) Genetic recombination in *Streptomyces aureofaciens*. *Acta Microbiol Acad Sci Hung* 8: 73–79

101 Mindlin SZ (1969) Genetic recombination in the actinomycete breeding. In: G Sermonti, M Alacevic (eds): *Genetics and breeding of Streptomyces*. Yugoslav Academy of Sciences and Arts, Zagreb, 147–159

102 Kieser HM, Kieser T, Hopwood DA (1992) A combined genetic and physical map of the *Streptomyces coelicolor* A3(2) chromosome. *J Bacteriol* 174: 5496–5507

103 Ryu DDY, Kim KS, Cho NY, Pai HS (1983) Genetic recombination in *Micromonospora rosaria* by protoplast fusion. *Appl Environ Microbiol* 45: 1854–1858

104 Choi KP, Kim KH, Kim JW (1997) Strain improvement of clavulanic acid producing *Streptomyces clavuligerus*. *Abstr. 12P9, 10th Internat Symp Biol Actinomycetes (Beijing)*

105 Hamlyn PF, Ball C (1979) Recombination studies with *Cephalosporium acremonium*. In: OK Sebek, AI Laskin (eds): *Genetics of industrial microorganisms*. ASM, Washington, DC, 185–191

106 Lein J (1986) The Panlabs penicillin strain improvement program. In: Z Vanek, Z Hostalek (eds): *Overproduction of microbial metabolites; strain improvement and process control strategies*. Butterworth Publishers, Boston, 105–139

107 Wesseling AC, Lago B (1981) Strain improvement by genetic recombination of cephamycin producers, *Nocardia lactamdurans* and *Streptomyces griseus*. *Devel Indust Microbiol* 22: 641–651

108 Yamashita F, Hotta K, Kurasawa S, Okami Y, Umezawa H (1982) Antibiotic formation by interspecific protoplast fusion in streptomycetes and emergence of drug resistance by protoplast regeneration. *Abstr P-II-20, 4th Internatl Symp Genet Indust Microorgs, (Kyoto):* 108

109 Traxler P, Schupp T, Wehrli W (1982) 16,17-dihydrorifamycin S and 16,17-dihydro-17-hydroxyrifamycin S, two novel rifamycins from a recombinant strain C5/42 of *Nocardia mediterranei*. *J Antibiot* 35: 594–601

110 Hopwood DA (1983) Actinomycete genetics and antibiotic production. In: LC Vining (ed): *Biochemistry and genetic regulation of commercially important antibiotics*. Addison Wesley, Reading, MA, 1–23

111 Okanishi M, Suzuki N, Furuta T (1996) Variety of hybrid characters among recombinants

obtained by interspecific protoplast fusion in streptomycetes. *Biosci Biotechnol Biochem* 60: 1233–1238

112 Hershberger CL (1996) Metabolic engineering of polyketide biosynthesis. *Curr Opin Biotechnol* 7: 560–562

113 Gomi S, Ikeda D, Nakamura H, Naganawa H, Yamashita F, Hotta K, Kondo S, Okami Y, Umezawa H, Iitaka Y (1984) Isolation and structure of a new antibiotic, indolizomycin, produced by a strain SK2-52 obtained by interspecies fusion treatment. *J Antibiot* 37: 1491–1494

114 Kinashi H, Shimaji M (1987) Detection of giant linear plasmids in antibiotic producing strains of *Streptomyces* by the OFAGE technique. *J Antibiot* 40: 913–916

115 Binnie C, Warren M, Butler MJ (1989) Cloning and heterologous expression in *Streptomyces lividans* of *Streptomyces rimosus* genes involved in oxytetracycline biosynthesis. *J Bacteriol* 171: 887–895

116 Butler MJ, Friend EJ, Hunter IS, Kaczmarek FS, Sugden DA, Warren M (1989) Molecular cloning of resistance genes and architecture of a linked gene cluster involved in biosynthesis of oxytetracycline by *Streptomyces rimosus*. *Molec Gen Genet* 215: 231–238

117 Arisawa A, Kawamura N, Narita T, Kojima I, Okamura K, Tsunekawa H, Yoshioka T, Okamoto R (1996) Direct fermentative production of acyltylosins by genetically-engineered strains of *Streptomyces fradiae*. *J Antibiot* 49: 349–354

118 McDougall S, Neilands JB (1984) Plasmid- and chromosome-coded aerobactin synthesis in enteric bacteria: insertion sequences flank operon in plasmid-mediated systems. *J Bacteriol* 159: 300–305

119 Roberts M, Leavitt RW, Carbonetti NH, Ford S, Cooper RA, Williams PH (1986) RNA-DNA hybridization analysis of transcription of the plasmid ColV-K30 aerobactin gene cluster. *J Bacteriol* 167: 467–472

120 Moon YH, Tanabe T, Funahashi T, Shiuchi K, Nakao H, Yamamoto S (2004) Identification and characterization of two contiguous operons required for aerobactin transport and biosynthesis in *Vibrio mimicus*. *Microbiol Immunol* 48: 389–398

121 Perez-Diaz JC, Clowes RC (1980) Physical characterization of plasmids determining synthesis of a microcin which inhibits methionine synthesis in *Escherichia coli*. *J Bacteriol* 141: 1015–1023

122 Crameri R, Davies JE, Huetter R (1986) Plasmid curing and generation of mutations induced with ethidium bromide in Streptomycetes. *J Gen Microbiol* 132: 819–824

123 Berg DE, Berg CM (1983) The prokaryotic transposable element Tn5. *Bio/Technology* 1: 417–435

124 McHenney MA, Baltz RH (1996) Gene transfer and transposition mutagenesis in *Streptomyces roseosporus*: mapping of insertions that influence daptomycin or pigment production. *Microbiology* 142: 2363–2373

125 Baltz RH, McHenney MA, Cantwell CA, Queener SW, Solenberg PJ (1997) Applications of transposition mutagenesis in antibiotic producing streptomyces. *Ant v Leeuwenhoek* 71: 179–187

126 Solenberg PJ, Cantwell CA, Tietz AJ, McGilvray D, Queener SW, Baltz RH (1996) Transposition mutagenesis in *Streptomyces fradiae*: identification of a neutral site for the stable insertion of DNA by transposon exchange. *Gene* 16: 67–72

127 Ikeda H, Takada Y, Pang CH, Tanaka H, Omura S (1993) Transposon mutagenesis by Tn4560 and applications with avermectin-producing *Streptomyces avermitilis*. *J Bacteriol* 175: 2077–2082

128 Thompson CJ, Ward JM, Hopwood DH (1982) Cloning of antibiotic resistance and nutritional genes in streptomycetes. *J Bacteriol* 151: 668–677
129 Baltz RH, Hosted TJ (1996) Molecular genetic methods for improving secondary-metabolite production in actinomycetes. *Trends Biotechnol* 14: 245–250
130 Martin JF, Gil JA (l984) Cloning and expression of antibiotic production genes. *Bio/Technology* 2: 63–72
131 Liras P (1988) Cloning of antibiotic biosynthetic genes. In: JA Thompson (ed): *Use of recombinant DNA techniques for improvement of fermentation organisms*. CRC Press, Boca Raton, 217–253
132 Coco EA, Narva KE, Feitelson JS (1991) New classes of *Streptomyces coelicolor* A3(2) mutants blocked in undecylprodigiosin (Red) biosynthesis. *Mol Gen Genet* 227: 28–32
133 Chen CW, Lin HF, Kuo CL, Tsai HL, Tsai JFY (1988) Cloning and expression of a DNA sequence conferring cephamycin C production. *Bio/Technology* 6: 1222–1224
134 Demain AL, Elander RP (1999) The β-lactam antibiotics: past, present, and future. *Ant v Leeuwenhoek* 75: 5–19
135 Diez B, Gutierrez S, Barredo JL, van Solinger P, van der Voort LHM, Martin JF (1990) The cluster of penicillin biosynthetic genes. Identification and characterization of the *pcbAB* gene encoding the γ-aminoadipyl-cysteinyl-valine synthetase and linkage to the *pcbC* and *pcbDE* genes. *J Biol Chem* 265: 16358–16365
136 Gutierrez S, Diez B, Alvarez E, Barredo JL, Martin JF (1991) Expression of the *pen*DE gene of *Penicillium chrysogenum* encoding isopenicillin N acyltransferase in *Cephalosporium acremonium*: production of benzyl penicillin by the transformants. *Mol Gen Genet* 225: 56–64
137 Carr LG, Skatrud PL, Sheetz ME, Queener SW, Ingolia TD (1986) Cloning and expression of the isopenicillin N synthetase gene from *Penicillium chrysogenum*. *Gene* 48: 257–266
138 Weigel BJ, Burgett SG, Chen VJ, Skatrud PL, Frolik CA, Queener SW, Ingolia TD (1988) Cloning and expression in *Escherichia coli* of isopenicillin N synthetase genes from *Streptomyces lipmanii* and *Aspergillus nidulans*. *J Bacteriol* 170: 3817–3826
139 Leskiw BK, Aharonowitz Y, Mevarech M, Wolfe S, Vining LC, Westlake DWS, Jensen SE (1988) Cloning and nucleotide sequence determination of the isopenicillin N synthetase gene from *Streptomyces clavuligerus*. *Gene* 62: 187–196
140 Garcia-Dominguez M, Liras P, Martin JF (1991) Cloning and characterization of the isopenicillin N synthase gene of *Streptomyces griseus* NRRL 3851 and studies of expression and complementation of the cephamycin pathway in *Streptomyces clavuligerus*. *Antimicrob Agents Chemother* 35: 44–52
141 Shiffman D, Mevarech M, Jensen SE, Cohen G, Aharonowitz Y (1988) Cloning and comparative analysis of the gene encoding isopenicillin N synthase in *Streptomyces*. *Mol Gen Genet* 214: 562–569
142 Skatrud PL, Fisher DL, Ingolia TD, Queener SW (1987) Improved transformation of *Cephalosporium acremonium*. In: M Alacevic, D Hranueli (eds): *Genetics of industrial microorganisms, part B*. Pliva, Zagreb, 111–119
143 Theilgaard HA, van den Berg MA, Mulder CA, Bovenberg RAL, Nielsen J (2001) Quantitative analysis of *Penicillium chrysogenum* Wis54-1255 transformants overexpressing the penicillin biosynthetic genes. *Biotechnol Bioeng* 72: 379–388
144 Skatrud PL, Tietz AJ, Ingolia TD, Cantwell CA, Fisher DL, Chapman JL, Queener SW (1989) Use of recombinant DNA to improve production of cephalosporin C by *Cephalosporium acremonium*. *Bio/Technology* 7: 477–485

145 Rodriguez-Saiz M, Lembo M, Bertetti L, Muraca R, Velasc, J, Malcangi A, Luis de la Fuente J, Barredo JL (2004) Strain improvement for cephalosporin production by *Acremonium chrysogenum* using geneticin as a suitable transformation marker. *FEMS Microbiol Lett* 235: 43–49
146 Basch J, Chiang S-JD (1998) Genetic engineering approach to reduce undesirable byproducts in cephalosporin C fermentation. *J Indust Microbiol Biotechnol* 20: 344–353
147 Mayer H, Collins J, Wagner F (1980) Cloning of the penicillin G-acylase gene of *Escherichia coli* ATCC 11105 on multicopy plasmids. In: HH Weetall, GP Royer (eds): *Enzyme engineering*, vol. 5. Plenum, New York, 61–69
148 Elander RP (1995) Genetic engineering applications in the development of selected industrial enzymes and therapeutic proteins. In: R Sankaran, KS Manja (eds): *Microbes for better living*. Defense Food Research Laboratory, Mysore, India, 619–628
149 Luo H, Yu H, Li Q, Shen Z (2004) Cloning and co-expression of D-amino acid oxidase and glutaryl-7-aminocephalosporanic acid acylase genes in *Escherichia coli*. *Enzyme Microb Technol* 35: 514–580
150 Stephanopoulos G (1999) Metabolic fluxes and metabolic engineering. *Metab Eng* 1: 1–11
151 Nielsen J (2001) Metabolic engineering. *Appl Microbiol Biotechnol* 55: 263–283
152 Kacser H, Acerenza L (1993) A universal method for achieving increases in metabolite production. *Eur J Biochem* 216: 361–367
153 Khetan A, Hu WS (1999) Metabolic engineering of antibiotic biosynthetic pathways. In: AL Demain, JE Davies (eds): *Manual of industrial microbiology and biotechnology*. ASM Press, Washington, DC, 717–724
154 Khetan A, Hu W-S (1999) Metabolic engineering of antibiotic biosynthesis for process improvement. In: SY Lee, ET Papoutsakis (eds): *Metabolic engineering*. Marcel Dekker, New York, 177–202
155 Thykaer J, Nielsen J (2003) Metabolic engineering of β-lactam production. *Metab Eng* 5: 56–69
156 Pfeifer BA, Admiraal SJ, Gramajo H, Cane DE, Khosla C (2001) Biosynthesis of complex polyketides in a metabolically engineered strain of *Escherichia coli*. *Science* 291: 1790–1792
157 Khosla C, Keasling JD (2003) Metabolic engineering for drug discovery and development. *Nature Rev/Drug Disc* 2: 1019–1025
158 Otten SL, Stutzman-Engwall J, Hutchinson CR (1990) Cloning and expression of daunorubicin biosynthesis genes from *Streptomyces peucetius* and *S. peucetius* subsp. *caisius*. *J Bacteriol* 172: 3427–3434
159 Decker H, Summers RG, Hutchinson CR (1994) Overproduction of the acyl carrier protein component of a type II polyketide synthase stimulates production of tetracenomycin biosynthetic intermediates in *Streptomyces glaucescens*. *J Antibiot* 47: 54–63
160 Madduri K, Waldron C, Matsushima P, Broughton MC, Crawford K, Merlo DJ, Baltz RH (2001) Genes for the biosynthesis of spinosyns: applications for yield improvement in *Saccharopolyspora spinosa*. *J Indust Microbiol Biotechnol* 27: 399–402
161 Butler MJ, Bruheim P, Jovetic S, Marinelli F, Postma PW, Bibb MJ (2002) Engineering of primary carbon metabolism for improved antibiotic production in *Streptomyces lividans*. *Appl Environ Microbiol* 68: 4731–4739
162 Voegtli M, Chang PC, Cohen SN (1994) *afsR2*: a previously undetected gene encoding

a 63-amino acid protein that stimulates antibiotic production in *Streptomyces lividans*. *Mol Microbiol* 14: 643–653

163 Lee JY, Hwang YS, Kim SS, Kim ES, Choi CY (2000) Effect of a global regulatory gene, afsR2, from *Streptomyces lividans* on avermectin production in *Streptomyces avermitilis*. *J Biosci Bioeng* 89: 606–608

164 Hwang YS, Kim ES, Biro S, Choi CY (2003) Cloning and analysis of a DNA fragment stimulating avermectin production in various *Streptomyces avermitilis* strains. *Appl Environ Microbiol* 69: 1263–1269

165 Geistlich M, Losick R, Turner JR, Rao RN (1992) Characterization of a novel regulatory gene governing the expression of a polyketide synthase gene in *Streptomyces ambofaciens*. *Mol Microbiol* 6: 2019–2029

166 Lombo F, Brana AF, Mendez C, Salas JA (1999) The mithramycin gene cluster of *Streptomyces argillaceus* contains a positive regulatory gene and two repeated DNA sequences that are located at both ends of the cluster. *J Bacteriol* 181: 642–647

167 Kennedy J, Auclair K, Kendrew SG, Park C, Vederas JC, Hutchinson CR (1999) Modulation of polyketide synthase activity by accessory proteins during lovastatin biosynthesis. *Science* 284: 1368–1372

168 Mao Y, Varoglu M, Sherman DH (1999) Molecular characterization and analysis of the biosynthetic gene cluster for the antitumor antibiotic mitomycin C from *Streptomyces lavendulae* NRRL 2564. *Chem Biol* 6: 251–263

169 Brian P, Riggle PJ, Santos RA, Champness WC (1996) Global negative regulation of *Streptomyces coelicolor* antibiotic synthesis mediated by an *absA*-encoded putative transduction system. *J Bacteriol* 178: 3221–3231

170 Volokhan O, Skletta H, Sekurova ON, Ellingsen TE, Zotchev SB (2005) An unexpected role for the putative 4′-phosphopantetheinyl transferase-encoding gene *nysF* in the regulation of nystatin biosynthesis in *Streptomyces noursei* ATCC 11455. *FEMS Microbiol Lett* 249: 57–64

171 Cox KL, Fishman SE, Larson JL, Stanzak R, Reynolds PA, Yeh WK, Van Frank RM, Birmingham VA, Hershberger CL, Seno ET (1987) Cloning and characterization of genes involved in tylosin biosynthesis. In: M Alacevic, D Hranueli, Z Toman (eds): *Genetics of industrial microorganisms, part B*. Pliva, Zagreb, 337–346

172 Fishman SE, Cox K, Larson JL, Reynolds PA, Seno ET, Yeh WK, Van Frank R, Hershberger CL (1987) Cloning genes for the biosynthesis of a macrolide antibiotic. *Proc Natl Acad Sci USA* 84: 8248–8252

173 Kennedy J, Turner G (1996) δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine synthetase is a rate limiting enzyme for penicillin production in *Aspergillus nidulans*. *Mol Gen Genet* 253: 189–197

173a Matsuda A, Sugiura H, Matsuyama K, Matsumoto H, Ichikawa S, Komatsu KI (1992) Molecular cloning of acetyl coenzyme A: deacetylcephalosporin C O-acetyltransferase cDNA from *Acremonium chrysogenum*: sequence and expression of catalytic activity in yeast. *Biochem Biophys Res Commun* 182: 995–1001

173b Mathison L, Soliday C, Stepan T, Aldrich T, Rambosek J (1993) Cloning, characterization, and use in strain improvement of the *Cephalosporium acremonium* gene cefG encoding acetyl transferase. *Curr Genet* 23: 33–41

174 Gutierrez S, Velasco J, Marcos AT, Fernandez FJ, Fierro F, Barredo JL, Diez B, Martin JF (1997) Expression of the *cefG* gene is limiting for cephalosporin biosynthesis in *Acremonium chrysogenum*. *Appl Microbiol Biotechnol* 48: 606–614

175 Cantwell C, Beckmann R, Whiteman P, Queener SW, Abraham EP (1992) Isolation of deacetoxycephalosporin C from fermentation broths of *Penicillium chrysogenum* transformants: construction of a new fungal biosynthetic pathway. *Proc R Soc Lond (Biol)* 248: 283–289

176 Crawford L, Stepan AM, Mcada PC, Rambosek JA, Conder MJ, Vinci VA, Reeves CD (1995) Production of cephalosporin intermediates by feeding adipic acid to recombinant *Penicillium chrysogenum* strains expressing ring expansion activity. *Bio/Technology* 13: 58–62

177 Velasco J, Adrio JL, Moreno MA, Diez B, Soler G, Barredo JL (2000) Environmentally safe production of 7-aminodeacetoxycephalosporanic acid (7-ADCA) using recombinant strains of *Acremonium chrysogenum*. *Nature Biotechnol* 18: 857–861

178 Brunker P, Minas W, Kallio PT, Bailey JE (1998). Genetic engineering of an industrial strain of *Saccharopolyspora erythrea* for stable expression of the *Vitreoscilla* hemoglobin gene (*vhb*). *Microbiology* 144: 2441–2448

179 Minas W, Brunker P, Kallio PT, Bailey JE (1998) Improved erythromycin production in a genetically engineered industrial strain of *Saccharopolyspora erythraea*. *Biotechnol Prog* 1: 561–566

180 Lombo F, Pfeifer B, Leaf T, Ou S, Kim YS, Cane DE, Licari P, Khosla C (2001) Enhancing the atom economy of polyketide biosynthetic processes through metabolic engineering. *Biotechnol Prog* 17: 612–617

181 Bailey JE, Sburlati A, Hatzimanikatis V, Lee K, Renner WA, Tsai PS (1996) Inverse metabolic engineering: a strategy for directed genetic engineering of useful phenotypes. *Biotechnol Bioeng* 52: 109–121

182 Khosla C, Bailey JE (1988) Heterologous expression of a bacterial haemoglobin improves the growth properties of recombinant *Escherichia coli*. *Nature* 331: 633–635

183 Bro C, Nielsen J (2004) Impact of 'ome' analyses on inverse metabolic engineering. *Metab Eng* 6: 204–211

184 Reeves AR, Cernota WH, Brikun IA, Wesley RK, Weber JM (2004) Engineering precursor flow for increased erythromycin production in *Aeromicrobium erythreum*. *Metab Eng* 6: 300–312

185 Ohnishi J, Mitsuhashi S, Hayashi M, Ando S, Yokoi H, Ochiai K, Ikeda M (2002) A novel methodology employing *Corynebacterium glutamicum* genome information to generate a new L-lysine-producing mutant. *Appl Microbiol Biotechnol* 58: 217–223

186 Stephanopoulos G, Alper H, Moxley J (2004) Exploiting biological complexity for strain improvement through systems biology. *Nature Biotechnol* 22: 1261–1267

187 Askenazi M, Driggers EM, Holtzman DA, Norman TC, Iverson S, Zimmer DP, Boers ME, Blomquist PR, Martinez EJ, Monreal AW et al (2003) Integrating transcriptional and metabolite profiles to direct the engineering of lovastatin-producing fungal strains. *Nature Biotechnol* 21: 150–156

188 Lum AM, Huang J, Hutchinson CR, Kao CM (2004) Reverse engineering of industrial pharmaceutical-producing actinomycete strains using DNA microarrays. *Metab Eng* 6: 186–196

189 Skandalis A, Encell LP, Loeb LA (1997) Creating novel enzymes by applied molecular evolution. *Chem Biol* 4: 889–898

190 Kuchner O, Arnold FH (1997) Directed evolution of enzyme catalysis. *Trends Biotechnol* 15: 523–530

191 Arnold FH (1998) Design by directed evolution. *Acc Chem Res* 31: 125–131

192 Arnold FH (2001) Combinatorial and computational challenges for biocatalyst design. *Nature* 409: 253–257
193 Zhao H, Chockalingam K, Chen Z (2002) Directed evolution of enzymes and pathways for industrial biocatalysis. *Curr Opin Biotechnol* 13: 104–110
194 Ness JE, Del Cardayre SB, Minshull J, Stemmer WP (2000) Molecular breeding: the natural approach to protein design. *Adv Prot Chem* 55: 261–292
195 Stemmer WP (1994) Rapid evolution of a protein *in vitro* by DNA shuffling. *Nature* 370: 389–391
196 Zhao H, Arnold FH (1997) Optimization of DNA shuffling for high fidelity recombination. *Nucleic Acids Res* 25: 1307–1308
197 Patten PA, Howard RJ, Stemmer W (1997) Applications of DNA shuffling to pharmaceuticals and vaccines. *Curr Opin Biotechnol* 8: 724–733
198 Kurtzman AL, Govindarajan S, Vahle K, Jones JT, Heinrichs V, Patten PA (2001) Advances in directed protein evolution by recursive genetic recombination: applications to therapeutic proteins. *Curr Opin Biotechnol* 12: 361–370
199 Lutz S, Ostermeier M, Moore GL, Maranas CD, Benkovic SP (2001) Creating multiple-crossover DNA libraries independent of sequence identity. *Proc Natl Acad Sci USA* 98: 11248–11253
200 Marshall SH (2002) DNA shuffling: induced molecular breeding to produce new generation long-lasting vaccines. *Biotechnol Adv* 20: 229–238
201 Locher CP, Soong NW, Whalen RG, Punnonen J (2004) Development of novel vaccines using DNA shuffling and screening strategies. *Curr Opin Mol Ther* 6: 34–39
202 Zhang YX, Perry K, Vinci VA, Powell K, Stemmer WPC, del Cardayre SB (2002) Genome shuffling leads to rapid phenotypic improvement in bacteria. *Nature* 415: 644–646
203 Hutchinson C (1998) Combinatorial biosynthesis for new drug discovery. *Curr Opin Microbiol* 1: 319–329
204 Reeves CD (2003) The enzymology of combinatorial biosynthesis. *Crit Rev Biotechnol* 23: 95–147
205 Hopwood DA, Malpartida F, Kieser HM, Ikeda H, Duncan J, Fujii I, Rudd BAM, Floss, HG, Omura S (1985) Production of 'hybrid' antibiotics by genetic engineering. *Nature* 314: 642–644
206 Rodriguez E, McDaniel R (2001) Combinatorial biosynthesis of antimicrobials and other natural products. *Curr Opin Microbiol* 4: 526–534
207 Donadio S, Sosio M (2003) Strategies for combinatorial biosynthesis with modular polyketide synthases. *Comb Chem High Throughput Screen* 6: 489–500
208 Kantola J, Kunnari T, Mantsala P, Ylihonko K (2003) Expanding the scope of aromatic polyketides by combinatorial biosynthesis. *Comb Chem High Throughput Screen* 6: 501–512
209 McAlpine JB, Tuan JS, Brown DP, Grebner KB, Whittern DN, Buko A, Katz L (1987) New antibiotics from genetically engineered actinomycetes. I. 2-Norerythromycins, isolation and structural determinations. *J Antibiot* 40: 1115–1122
210 Epp JK, Huber MLB, Turner JR, Goodson T, Schoner BE (1989) Production of hybrid macrolide antibiotic in *Streptomyces ambofaciens* and *Streptomyces lividans* by introduction of a cloned carbomycin biosynthetic gene from *Streptomyces thermotolerans*. *Gene* 85: 293–301
211 Weber JM, Leung JO, Swanson SJ, Idler KB, McAlpine JB (1991) An erythromycin deriva-

tive produced by targeted gene disruption in *Saccharopolyspora erythraea*. *Science* 252: 114–117

212 Donadio S, Staver MJ. McAlpine JB, Swanson SJ, Katz L (1991) Modular organization of genes required for complex polyketide biosynthesis. *Science* 252: 675–679

213 Donadio S, McAlpine JB, Sheldon PA, Jackson MA, Katz L (1993) An erythromycin analog produced by reprogramming of polyketide synthesis. *Proc Natl Acad Sci USA* 90: 7119–7123

214 Hara O, Hutchinson CR (1992) A macrolide 3-O-acyltransferase gene from the mide-camycin-producing species *Streptomyces mycarofaciens*. *J Bacteriol* 174: 5141–5144

215 Katz L, Donadio S (1993) Polyketide synthesis: prospects for hybrid antibiotics. *Annu Rev Microbiol* 47: 875–912

216 Hopwood DA (1993) Genetic engineering of *Streptomyces* to create hybrid antibiotics. *Curr Opin Biotechnol* 4: 531–537

217 McDaniel R, Ebert-Khosla S, Hopwood D, Khosla C (1993) Engineered biosynthesis of novel polyketides. *Science* 262: 1546–1550

218 McDaniel R, Ebert-Khosla S, Hopwood D, Khosla C (1993) Engineered biosynthesis of novel polyketides: manipulation and analysis of an aromatic polyketide synthase with unproven catalytic specificities. *J Am Chem Soc* 115: 11671–11675

219 McDaniel R, Thamchaipenet A, Gustafsson C, Fu H, Betlach M, Betlach M, Ashley G (1999) Multiple genetic modifications of the erythromycin polyketide synthase to produce a library of novel 'unnatural' natural products. *Proc Natl Acad Sci USA* 96: 1846–1851

220 Khosla C, McDaniel R, Ebert-Khosla S, Torres R. Sherman DH, Bibb MJ, Hopwood DA (1993) Genetic construction and functional analysis of hybrid polyketide synthases containing heterologous acyl carrier proteins. *J Bacteriol* 175: 2197–2204

221 Decker H, Hutchinson CR (1993) Transcriptional analysis of the *Streptomyces glaucescens* tetracenomycin biosynthesis gene cluster. *J Bacteriol* 175: 3887–3892

222 Hutchinson CR, Fujii I (1995) Polyketide synthase gene manipulation: a structure-function approach in engineering novel antibiotics. *Annu Rev Microbiol* 49: 201–238

223 Tsoi CJ, Khosla C (1995) Combinatorial biosynthesis of unnatural natural products – the polyketide example. *Chem Biol* 2: 355–362

224 Kao CM, Luo GL, Katz L, Cane DE, Khosla C (1995) Manipulation of macrolide ring size by directed mutagenesis of a modular polyketide synthase. *J Am Chem Soc* 117: 9105–9106

225 Pacey MS, Dirlam JP, Geldart RW, Leadlay PF, McArthur HA, McCormick EL, Monday RA, O'Connell TN, Staunton J, Winchester TJ (1998) Novel erythromycins from a recombinant *Saccharopolyspora erythraea* strain NRRL 2338pIG1. I. Fermentation, isolation and biological activity. *J Antibiot* 51: 1029–1034

226 Wohlert SE, Blanco G, Lombo F, Fernandez E, Brana AF, Reich S, Udvarnoki G, Mendez C, Decker H, Frevert J et al (1998) Novel hybrid tetracenomycins through combinatorial biosynthesis using a glycosyltransferase encoded by the elm-genes in cosmid 16F4 and which shows a very broad sugar substrate specificity. *J Amer Chem Soc* 120: 10596–10601

227 Xue Q, Hutchinson CR, Santi DV (1999) A multi-plasmid approach to preparing large libraries of polyketides. *Proc Natl Acad Sci USA* 96: 11740–11745

228 Pfeifer BA, Khosla C (2001) Biosynthesis of polyketides in heterologous hosts. *Microbiol Molec Biol Rev* 65: 106–118

229 Trefzer A, Blanco G, Remsing L, Kunzel E, Rix U, Lipata F, Brana AF, Mendez C, Rohr J, Bechtold A et al (2002) Rationally designed glycosylated premithramycins: hybrid aromatic polyketides using genes fom three different biosynthetic pathways. *J AmChem Soc* 124: 6056–6062

230 Zhao L, Ahlert J, Xue Y, Thorson JS, Sherman DH, Liu H-W (1999) Engineering a methymycin/pikromycin-calicheamicin hybrid: construction of two new macrolides carrying a designed sugar moiety. *J Am Chem Soc* 121: 9881–9882

231 Mendez C, Salas JA (2001) Altering the glycosylation pattern of bioactive compounds. *Trends Biotechnol* 19: 449–456

232 Bartel PL, Zhu CB, Lampel JS, Dosch DC, Connors NC, Strohl WR, Beale JM Jr, Floss HG (1990) Biosynthesis of anthraquinones by interspecies cloning of actinorhodin biosynthesis genes in streptomycetes; clarification of actinorhodin gene functions. *J Bacteriol* 172: 4816–4826

233 Strohl WR, Bartel PL, Li Y, Connors NC, Woodman RH (1991) Expression of polyketide biosynthesis and regulatory genes in heterologous streptomycetes. *J Indust Microbiol* 7: 163–174

234 Hwang CK, Kim HS, Hong YS, Kim YH, Hong SK, Kim SJ, Lee JJ (1995) Expresssion of *Streptomyces* peucetius genes for doxorubicin resistance and aklavinone 11-hydroxylase in *Streptomyces galilaeus* ATCC 31133 and production of a hybrid aclacinomycin. *Antimicrob Agents Chemother* 39: 1616–1620

235 Niemi J, Mantsala P (1995) Nucleotide sequences and expression of genes from *Streptomyces purpurescens* that cause the production of new anthracyclines in *Streptomyces galilaeus*. *J Bacteriol* 177: 2942–2945

236 Ylihonko K, Hakala J, Kunari T, Mantsala P (1996) Production of hybrid anthracycline antibiotics by heterologous expression of *Streptomyces nogalater* nogalamycin biosynthesis genes. *Microbiology* 142: 1965–1972

237 Kim HS, Hong YS, Kim YH, Yoo OJ, Lee JJ (1996) New anthracycline metabolites produced by the aklavinone 11-hydroxylase gene in *Streptomyces galilaeus* ATCC 31133. *J Antibiot* 49: 355–360

238 Stachelhaus T, Schneider A, Marahiel MA (1995) Rational design of peptide antibiotics by targeted replacement of bacterial and fungal domains. *Science* 269: 69–72

239 Staunton J (1998) Combinatorial biosynthesis of erythromycin and complex polyketides. *Curr Opin Chem Biol* 2: 339–345

240 Hopwood DA (2003) The *Streptomyces* genome – be prepared! *Nature Biotechnol* 21: 505–506

241 Ikeda H, Ishikawa J, Hanamoto A, Shinose K, Kikuchi H, Shiba T, Sakaki Y, Hattori M, Omura S (2003) Complete genome sequence and comparative analysis of the industrial microorganism *Streptomyces avermitilis*. *Nature Biotechnol* 21: 526–531

242 Zazopoulos E, Huang K, Staffa A, Liu W, Bachmann BO, Nonaka K, Ahlert J, Thorson JS, Shen B, Farnet CM (2003) A genomics-guided approach for discovering and expressing cryptic metabolic pathways. *Nature Biotechnol* 21: 187–190