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Recombinant organisms for production of industrial products

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A revolution in industrial microbiology was sparked by the discoveries of ther double-stranded structure of DNA and the development of recombinant DNA technology. Traditional industrial microbiology was merged with molecular biology to yield improved recombinant processes for the industrial production of primary and secondary metabolites, protein biopharmaceuticals and industrial enzymes. Novel genetic techniques such as metabolic engineering, combinatorial biosynthesis and molecular breeding techniques and their modifications are contributing greatly to the development of improved industrial processes. In addition, functional genomics, proteomics and metabolomics are being exploited for the discovery of novel valuable small molecules for medicine as well as enzymes for catalysis. The sequencing of industrial microbal genomes is being carried out which bodes well for future process improvement and discovery of new industrial products.

Genetic Recombination in Industrial Microorganisms

In contrast to the extensive use of mutation in industry, employment of genetic recombination was meager at first. This lack of interest was prevalent despite early claims of success,[1,2] mainly due to the absence or the extremely low frequency of genetic recombination in industrial microorganisms (in streptomycetes, it was usually 10^{-6} or even less). 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 was also of importance in the mapping of production genes. The model for such investigations was the genetic map of *Streptomyces coelicolor*[3] which was found to be very similar to those of other Streptomyces species, such as *Streptomyces bikiniensis*, *Streptomyces olivaceous*, *Streptomyces glaucescens* and *Streptomyces rimosus*.

Recombination in microorganisms occurs through three parasexual processes: conjugation, transduction, and transformation. Internal genetic rearrangements can also occur via translocatable DNA segments (insertion sequences or transposons). Conjugation involves transfer of DNA via cell-to-cell contact. Transduction occurs from host cell to recipient cell via mediation by bacteriophage. Transformation involves uptake and expression of naked DNA by competent cells. Competence occurs naturally but can also be induced by changes in the physical and chemical environment. In the laboratory, it can be induced by cold calcium chloride treatment, protoplasting, electroporation and heat shock.

As mentioned above, genetic recombination was virtually ignored in industry, mainly due to the low frequency of recombination. However, use of protoplast fusion changed the situation markedly. 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,[4] and strain improvement programs routinely include protoplast fusion between different mutant lines. The power of recombination was demonstrated by Yoneda[5] who recombined individual mutations, each of which increased α-amylase production by two- to seven-fold in *Bacillus subtilis*. A strain constructed by genetic transformation, which contained all five mutations, produced 250-fold more α-amylase than the starting strain.

Primary Metabolites

Primary metabolites are microbial products made during the exponential phase of growth whose synthesis is an integral part of the normal growth process. They include intermediates and end products of anabolic metabolism, which are used by the cell as building blocks for essential macromolecules (e.g., amino acids, nucleotides) or are converted to coenzymes (e.g., vitamins). Other primary metabolites (e.g., citric acid, acetic acid and ethanol) result from catabolic metabolism; they are not used for building cellular constituents but their production, which is related to energy production and substrate utilization, is essential for growth. Industrially, the

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Table 1. Novel genetic technologies successfully applied to improvement of primary metabolite production

Genetic technologies	Metabolites
Genome-based strain reconstruction	Amino acids, vitamins, organic acids, alcohols, carotenoids.
Metabolic engineering including reverse (inverse) metabolic engineering	Amino acids, vitamins, organic acids, ethanol, 1,3-propanediol, carotenoids, 5'-inosinic acid.
Genome-wide transcript expression analysis	Riboflavin
Molecular breeding (whole genome shuffling)	Lactic acid, ethanol

most important primary metabolites are amino acids, nucleotides, vitamins, solvents and organic acids.

Recent approaches utilize the techniques of modern genetic engineering to develop strains overproducing primary metabolites. This rationale for strain construction aims at assembling the appropriate characteristics by means of in vitro recombinant DNA techniques. This is particularly valuable in organisms with complex regulatory systems, where deregulation would involve many genetic alterations.

Production of a particular primary metabolite by deregulated organisms may inevitably be limited by the inherent capacity of the particular organism to make the appropriate biosynthetic enzymes, i.e., even in the absence of repressive mechanisms, there may not be enough of the enzyme made to obtain high productivity. There are some ways to overcome this: (1) to increase the number of copies of structural genes coding for these enzymes by genetic engineering, and (2) to increase the frequency of transcription, which is related to the frequency of binding of RNA polymerase to the promoter region. Increasing gene copies can be achieved by incorporating the biosynthetic genes in vitro into a plasmid which, when placed in a cell, will replicate into multiple copies. Increasing the frequency of transcription involves constructing a hybrid plasmid, which contains the structural genes of the biosynthetic enzymes but lacks the regulatory sequences (promoter and operator) normally associated with them. The ideal plasmid for metabolite synthesis would contain a regulatory region with a constitutive phenotype, preferably not subject to nutritional repression.

One of the major problems in using strains in which the desired characteristics are encoded by a plasmid is the difficulty in maintaining the plasmids during fermentation. One solution is to use antibiotic pressure during fermentation so that only organisms resistant to the antibiotic (due to the presence of a plasmid-borne resistance gene) can survive. Combinations of deregulation and plasmid amplification can yield a synergistic effect.

Novel genetic technologies are important for the development of effective overproducers. Those which have proven to be very useful for increasing production of primary metabolites are shown in **Table 1**. Ongoing sequencing projects involving hundreds of genomes, the availability of sequences corresponding to model organisms, new DNA microarray and proteomic tools, as well as the new techniques for mutagenesis and recombination described above, will no doubt accelerate strain improvement programs.

Genome-based strain reconstruction achieves the construction of a superior strain which only contains mutations crucial to hyperproduction, but not other unknown mutations which accumulate by brute-force mutagenesis and screening.[6]

The directed improvement of product formation or cellular properties via modification of specific biochemical reactions or introduction of new ones with the use of recombinant DNA technology is known as metabolic engineering.[7,8] Analytical methods are combined to quantify fluxes and to control them with molecular biological techniques in order to implement suggested genetic modifications. Different means of analyzing flux are (1) kinetic based models, (2) control theories, (3) tracer experiments, (4) NMR magnetization transfer, (5) metabolite balancing, (6) enzyme analysis and (7) genetic analysis.[9] The overall flux through a metabolic pathway depends on several steps, not just a single rate-limiting reaction. Amino acid production is one of the fields with many examples of this approach.[10] Other processes improved by this technique include vitamins, organic acids, ethanol, 1,3-propanediol and carotenoids.

Reverse (inverse) metabolic engineering is another approach that involves (1) choosing a strain which has a favorable cellular phenotype, (2) evaluating and determining genetic and/or environmental factors that confer that phenotype, and (3) transferring that phenotype to a second strain via direct modifications of the identified genetic and/or environmental factors.[11,12]

Molecular breeding techniques come closer to mimicking natural recombination by allowing in vitro homologous recombination.[13] DNA shuffling not only recombines DNA fragments but also introduces point mutations at a very low controlled rate.[14] Unlike site directed mutagenesis, this method of pooling and recombining parts of similar genes from different species or strains has yielded remarkable improvements in a very short amount of time.[15] Whole genome shuffling is a novel technique for strain improvement combining the advantage of multiparental crossing allowed by DNA shuffling with the recombination of entire genomes.[16,17]

Amino acids. Genetic engineering has made an impact on the production of amino acids by using the following strategies: (1) amplification of a rate-limiting enzyme of pathway; (2) amplification of the first enzyme after a branchpoint; (3) cloning of a gene encoding an enzyme with more or less feedback regulation; (4) introduction of a gene encoding an enzyme with a functional or energetic advantage as replacement for the normal enzyme; and (5) amplification of the first enzyme leading from central metabolism to increase carbon flow into the pathway followed by sequential removal of bottlenecks caused by accumulation of intermediates.

Transport mutations have also become useful, i.e., a mutation decreasing amino acid uptake allows for improved excretion and

lower intracellular feedback control. This has been especially useful in production of tryptophan and threonine. In cases where excretion is carrier-mediated, increase in activity of these carrier enzymes increases production of the amino acid.

Among the amino acids, L-glutamate (MSG) and L-lysine, mostly used as feed and food additives, respectively, represent the largest products in this category. Produced by fermentation are 1.5 million tons of L-glutamate and 850,000 tons of L-lysine-HCl. The total amino acid market was about 4.5 billion dollars in 2004.[18] Top fermentation titers reported in the literature are shown in **Table 2**.

Table 2. Top titers of amino acid fermentations

Amino Acid	Titer (g l^{-1})
L-alanine	114
L-arginine	96
L-glutamic acid	130
L-glutamine	49
L-histidine	42
L-hydroxyproline	41
L-isoleucine	40
L-leucine	34
L-lysine HCl	170
L-phenylalanine	51
L-proline	100
L-serine	65
L-threonine	100
L-tryptophan	60
L-tyrosine	55
L-valine	105

Strains of Corynebacterium, Brevibacterium and Serratia[19] have been routinely used for the commercial production of amino acids. Plasmid vector systems for cloning in *Corynebacterium glutamicum* were established and amino acid production by *C. glutamicum* and related strains has been improved by gene cloning.[20] Extensive research has been done on sequencing the genome of *C. glutamicum* by Kyowa Hakko scientists, by scientists at the Ajinomoto Co., of Japan, and also by a collaboration of German workers.[21,22] The genome of the closely related glutamate-overproducing species, *Corynebacterium efficiens*, has also been sequenced.[23]

L-Glutamate. Glutamate was the first amino acid to be produced by fermentation, because of its use as MSG in flavoring. High titers have been obtained over the years. Introduction of the Vitreoscilla hemoglobin gene *vgb* into *C. glutamicum* strains increased cell growth and glutamic acid and glutamine production via increased oxygen uptake.[24] Workers at Ajinomoto Co., Inc., increased glutamate production from glucose by 9% by suppressing CO_2 liberation in the pyruvate dehydrogenase reaction.[25] They did this by cloning the *xfp* gene encoding phosphoketolase from *Bifidobacterium animalis* into *C. glutamicum* and overexpressing it.

L-Lysine. Metabolic engineering has been used in *C. glutamicum* to improve L-lysine production.[10] Metabolic flux studies of wild-type *C. glutamicum* and four improved lysine-producing mutants available from the ATCC showed that yield increased from 1.2% to 24.9% relative to the glucose flux. Lysine production by *C. glutamicum* mutants has been increased by several different approaches including:

(a) deletion of PEP carboxykinase, deletion of glucose-6-phosphate dehydrogenase, disruption of malate quinone oxidoreductase, and lowering of citrate synthase activity by mutation,[26]

(b) improving the availability of the precursor pyruvate by eliminating pyruvate dehydrogenase activity,[27]

(c) overexpression of pyruvate carboxylase or DAP dehydrogenase genes,

(d) overexpression of gene NCg10855 encoding a methyltransferase or the *amtA-ocd-soxA* operon encoding an ammonium uptake system, a putative ornithine cyclodeaminase and an uncharacterized protein.[28]

L-Threonine. Overproduction of L-threonine has been achieved with the use of several microorganisms. In *Serratia marcescens*, construction of a high threonine producer was achieved by transductional crosses which combined several feedback control mutations in a single organism.[29] It produced 25 g l^{-1} of threonine. Another six regulatory mutations derived by resistance to amino acid analogues were combined into a single strain of *S. marcescens* by transduction. These mutations led to desensitization and derepression of the key enzymes in threonine synthesis. The resultant transductant produced 40 g l^{-1} of threonine.[30] These *S. marcescens* overproducing strains were further improved by recombinant DNA technology. A mutant overproducing PEP carboxylase made 63 g l^{-1} threonine, a 21% increase.[31] An *Escherichia coli* strain was developed via mutation and genetic engineering and optimized by inactivation of threonine dehydratase (TD) resulting in a process yielding 100 g l^{-1} of L-threonine in 36 hours of fermentation.[32]

In *C. glutamicum* ssp. *lactofermentum*, L-threonine production reached 58 g l^{-1} when a strain producing both threonine and lysine (an isoleucine auxotroph resistant to thialysine, α-amino-β-hydroxyvaleric acid and S-methylcysteine sulfoxide) was transformed with a recombinant plasmid carrying its own *hom* (encoding homoserine dehydrogenase), *thrB* (encoding homoserine kinase) and *thrC* (encoding L-threonine synthase) genes.[33] A recombinant strain of *E. coli* (made by mutating to isoleucine auxotrophy, cloning in extra copies of the *thrABC* operon, inactivating the threonine-degrading gene *tdh*, and mutating to resistance to high concentrations of L-threonine and L-homoserine) produced 80 g l^{-1} L-threonine in 1.5 days at a yield of 50%.[34] Cloning extra copies of threonine export genes into *E. coli* led to increased threonine production.[35]

L-Valine. Production by mutant strain VAL1 of *C. glutamicum* amounted to 105 g l^{-1}.[36] The mutant was constructed by overexpressing biosynthetic enzymes via a plasmid, eliminating *ilvA* encoding threonine dehydratase, and deleting two genes encoding enzymes of pantothenate biosynthesis. The culture was grown with limitation of isoleucine and pantothenate.

L-Isoleucine. Feedback regulation in *C. glutamicum* was eliminated[37] by replacing the native threonine dehydratase gene *ilvA*

with the feedback resistant gene from *E. coli*. By introducting additional copies of genes encoding branched amino and biosynthetic enzymes, lysine- or threonine-producing strains were converted into L-isoleucine producers with improved titers.[38,39] Amplification of the wild-type threonine dehydratase gene *ilvA* in a threonine-producing strain of *Corynebacterium lactofermentum* led to isoleucine production.[40]

L-Alanine. Lee et al.[41] introduced into an *E. coli* double mutant [lacking genes encoding a protein of the pyruvate dehydrogenase complex (*aceF*) and lactate dehydrogenase (*ldhA*)] a plasmid containing the *Bacilus sphaericus* alanine dehydrogenase gene (*alaD*). The strain produced L-alanine in 27 hours with a yield on glucose of 0.63 g g^{-1} and a maximum productivity of 2 g $l^{-1}h^{-1}$.

L-Proline. A mutant of *S. marcescens* resistant to 3,4-dehydroproline, thiazolidine-4-carboxylate and azetidine-2-carboxylate and unable to utilize proline produced 50 to 55 g l^{-1} L-proline.[42] Cloning of a gene bearing the dehydroproline-resistance locus on a plasmid yielded a recombinant strain of *S. marcescens* producing 75 g l^{-1}.[43] Further development work increased production to over 100 g l^{-1}.[44]

L-Hydroxyproline. Introduction of the proline 4-hydroxylase gene from Dactylosporangium sp. into recombinant *E. coli* producing L-proline at 1.2 g l^{-1} led to a new strain producing 25 g l^{-1} of hydroxyproline (trans-4-hydroxy-L-proline).[45] When proline was added, hydroxyproline reached 41 g l^{-1}, with a yield of 87% from proline.

Aromatic amino acids. Overproduction of aromatic amino acids and derivatives has also been achieved by metabolic engineering.[46] An engineered strain of *C. glutamicum* producing 50 g l^{-1} of L-tryptophan was further modified by cloning in additional copies of its own transketolase gene in order to increase the level of the erythrose-4-phosphate precursor of aromatic biosynthesis.[47] A low copy number plasmid increased production to 58 g l^{-1}, whereas a high copy number plasmid decreased production. Early use of a 6-fluorotryptophan- and 8-azaguanine-resistant mutant of a recombinant *E. coli* strain yielded more than 50 g l^{-1} tryptophan from added anthranilic acid and glucose. Work on *C. glutamicum* in which the first enzyme (3-deoxy D-arabinoheptulosonate 7-phosphate synthase) entering the aromatic pathway was amplified, yielded 50 g l^{-1} of L-tryptophan without adding anthranilic acid.[48] By further engineering central metabolism to raise the availability of PEP and E4P (via a PEP-carboxylase-deficient mutant with only 25% remaining activity and amplification of *tkt*-encoding transketolase), *C. glutamicum* produced 58 g l^{-1} L-tryptophan in a fed batch sucrose process. Using a plasmid containing tryptophan synthase plus induction with 3-indole acrylate, recombinant *E. coli* was able to carry out a bioconversion producing 180 g l^{-1} of L-tryptophan from indole plus L-serine in 8 hours.[49]

Production of L-phenylalanine amounted to 28 g l^{-1} when a plasmid was cloned into *E. coli* containing a feedback inhibition-resistant version of the CM-prephenate dehydratase (PD) gene, a feedback inhibition-resistant DAHPS and the O_RP_R and O_LP_L operator-promoter system of lambda phage. Control of plasmid expression was by temperature manipulation.[50] Further process development of genetically engineered *E. coli* strains brought phenylalanine titers up to 46 g l^{-1}.[51] Independently, genetic engineering based on cloning *aroF* and feedback resistant *pheA* genes created an *E. coli* strain producing 50 g l^{-1}.[52]

L-Tyrosine overproduction was obtained by amplification of *aro* II (encoding desensitized type II-DS) and *csm* in a tryptophan-producer of *C. glutamicum*. The manipulation shifted production from 18 g l^{-1} L-tryptophan to 26 g l^{-1} L-tyrosine.[53]

Vitamins. *Biotin.* Biotin has been made traditionally by chemical synthesis but recombinant microbes have approached a competitive economic position. Cloning of a biotin operon (*bioABFCD*) on a multicopy plasmid allowed *E. coli* to produce 10,000 times more biotin than did the wild type strain.[54] Sequential mutation of *S. marcescens* to resistance to the biotin antimetabolite acidomycin (=actithiazic acid) led to mutant strain SB412 which produced 20 mg l^{-1} of biotin.[55] Further improvements were made by mutating selected strains to ethionine-resistance (strain ET2, 25 mg l^{-1}), then mutating ET2 to S-2-aminoethylcysteine resistance (strain ETA23, 33 mg l^{-1}) and finally cloning in the resistant *bio* operon yielding a strain able to produce 500 mg l^{-1} in a fed-batch fermentor along with 600 mg/l of biotin vitamers. Later advances led to production by recombinant *S. marcescens* of 600 mg l^{-1} of biotin.[56]

Riboflavin. A process for riboflavin production by *Corynebacterium ammoniagenes* (previously *Brevibacterium ammoniagenes*) was developed by cloning and overexpressing the organism's own riboflavin biosynthesis genes[57] and its own promoter sequences. The resulting culture produced 15 g l^{-1} riboflavin in three days. Genetic engineering of a *B. subtilis* strain already containing purine analog-resistance mutations led to improved production of riboflavin.[58] The industrial strain of *B. subtilis* was produced by inserting multiple copies of the *rib* operon at two different sites in the chromosome, making purine analog-resistance mutations to increase guanosine triphosphate (GTP; a precursor) production and a riboflavin analog (roseflavin)-resistance mutation in *ribC* that deregulated the entire pathway.[59] Resulting production was over 25 g l^{-1}. A genome-wide transcript expression analysis called *massive parallel signature sequencing*[60] was successfully used to discover new targets for futher improvement of riboflavin production by the fungus *A. gossypii*.[61] The authors identified 53 genes of known function, some of which could clearly be related to riboflavin production. This approach also allowed the finding of sites within the genome with high transcriptional activity during riboflavin biosynthesis that are suitable integration loci for the target genes found.

Vitamin C. Vitamin C (ascorbic acid) has traditionally been made in a five-step chemical process by first converting glucose to 2-keto-L-gulonic acid (2-KGA) with a yield of 50% and then converting the 2-KGA by acid or base to ascorbic acid. A novel process for vitamin C synthesis involved the use of a genetically engineered *Erwinia herbicola* strain containing a gene from Corynebacterium sp. The engineered organism converted glucose into 1 g l^{-1} of 2-KGA.[62] A better process was devised independently which converted 40 g l^{-1} glucose into 20 g l^{-1} of 2-KGA.[63] This process involved cloning and expressing the gene encoding 2,5-diketo-D-gluconate reductase from Corynebacterium sp. into *Erwinia citreus*. Another process used a recombinant strain

of *Gluconobacter oxydans* containing genes encoding L-sorbose dehydrogenase and L-sorbosone dehydrogenase from *G. oxydans* T-100. The new strain was an improved producer of 2-KGA.[64] Further mutation to suppress the L-idonate pathway and to improve the promoter led to production of 130 g l^{-1} of 2-KGA from 150 g l^{-1} sorbitol.

Organic acids. Microbial production of organic acids is an excellent approach for obtaining building-block chemicals from renewable carbon sources.[65] Production of some organic acids started decades ago and titers have been improved by classical mutation and screening/selection techniques as well as by metabolic engineering.[66] Metabolic engineering of *E. coli* and *C. glutamicum* has yielded high levels of acetate, pyruvate, succinate and lactate.[67]

Acetic acid. Titers of acetic acid reached 53 g l^{-1} with genetically engineered *E. coli*[68] and 83 g l^{-1} with a *Clostridium thermoaceticum* mutant.[69] Cloning of the aldehyde dehydrogenase gene from *Acetobacter polyoxogenes* on a plasmid vector into *Acetobacter aceti* subsp. *xylinum* increased the rate of acetic acid production by over 100% (1.8 g $l^{-1}h^{-1}$ to 4 g $l^{-1}h^{-1}$) and titer by 40% (68 g l^{-1} to 97 g l^{-1}).[70]

Lactic acid. A transgenic wine yeast genetically engineered to contain six copies of the bovine L-lactate dehydrogenase gene produced L-(+)-lactate at 122 g l^{-1}.[71] Whole genome shuffling was used to improve the acid-tolerance of a commercial lactic acid-producing Lactobacillus sp.[72] Further approaches using the recursive protoplast fusion technique yielded strains of *Lactobacillus rhamnosus* ATCC 11443 with improved glucose tolerance (above 160–200 g l^{-1} glucose as initial concentration), while simultaneously enhancing L-lactic acid production by 71% as compared to the wild type. Shuffling of a mutant strain of *Lactobacillus delbrueckii* NCIM 2025 and the amylase-producing non-fastidious strain of *Bacillus amyloliquefaciens* ATCC 23842 produced a fusant that could utilize liquefied cassava bagasse starch directly with minimal nutrient supplementation for lactic acid production. A titer of 40 g l^{-1} of lactic acid was achieved which amounted to a 96% conversion of starch to lactic acid.[73]

A recombinant *E. coli* strain was constructed that produced optically active pure D-lactic acid from glucose at virtually the theoretical maximum yield, e.g., two molecules from one molecule of glucose.[74] The organism was engineered by eliminating genes of competing pathways encoding fumarate reductase, alcohol/aldehyde dehydrogenase and pyruvate formate lyase, and by a mutation in the acetate kinase gene. D-Lactic acid has also been produced at 61 g l^{-1} by a recombinant strain of *S. cerevisiae* containing the D-lactic dehydrogenase gene from *Leuconostoc mesenteroides*.[75]

Whereas lactobacilli make more lactic acid than *Rhizopus oryzae,* they require yeast extract and produce mixed isomers. However, *R. oryzae* produces L-(+) lactic acid exclusively. The fungus yields lactic acid at 60–80% of added glucose, the remainder going to ethanol. By increasing lactic dehydrogenase levels via plasmid transformation with *ldhA*, more lactate could be made from pyruvate.[76] Production was increased to 78 g l^{-1} whereas undesirable coproduct ethanol was reduced from 10.6 to 8.7 g l^{-1} and fumaric acid from 0.2 to 0.05 g l^{-1}. Recombinant *Saccharomyces cerevisiae* containing six copies of bovine L-lactate dehydrogenase produced 122 g l^{-1} from cane sugar with optical purity of 99.9% or higher.[77] *Kluyveromyces lactis*, also with the bovine enzyme and a deleted pyruvate decarboxylase gene, produced 109 g l^{-1}.[78]

Succinic acid. Metabolic engineering of *Mannheimia succiniciproducens* led to a strain producing 52 g l^{-1} of succinic acid at a yield of 1.16 mol mol^{-1} glucose and a productivity of 1.8 g $l^{-1}h^{-1}$ in fed-batch culture.[79] Reduced formation of acetic, formic and lactic acids was accomplished by disruption of genes *ldhA, pflB, pta* and *ackA*. A metabolically engineered succinate-producing strain of *E. coli* with inactivated genes encoding succinate dehydrogenase, pyruvate oxidase, acetate kinase-phosphotransacetylase, glucose phosphotransferase and the repressor of the *aceBAK* operon, and overexpression of the gene encoding PEP carboxylase, yielded 58 g l^{-1} succnate in a 59 hour fed-batch fermentation under aerobic conditions.[80] The average succinate yield was 0.94 mol mol^{-1} of glucose, the average productivity was 1.08 g $l^{-1}h^{-1}$; and the average specific activity was 90 mg $g^{-1}h^{-1}$. Sanchez et al.[81] used metabolic engineering to create an *E. coli* strain which had three deactivated genes of the central metabolic pathway, i.e., *adhE, ldhA* and *act-pta*, and an inactivated *iclR* gene which resulted in activation of the glyoxylate pathway. The strain produced 40 g l^{-1} of succinate. A titer of 99 g l^{-1} has been reached with recombinant *E. coli* with a productivity of 1.3 g $l^{-1}h^{-1}$.[82]

Shikimic acid. Shikimic acid is the starting point for chemical synthesis of Tamiflu, an antiviral agent. Metabolic engineering of *E. coli* yielded an overproducer making 84 g l^{-1} of shikimic acid with a 0.33 molar yield from glucose.[83] 50–90 g l^{-1} of shikimic acid was produced in 30% (mol mol^{-1}) yield from glucose with an *E. coli aroL* and *aroK* mutant which overexpressed *aroF, aroB* and *aroE*, and *tktA* and *ppsA* of central metabolism.[83,84]

Other acids. Metabolic engineering of *Clostridium tyrobutyricum* created a fermentation strain yielding 80 g l^{-1} butyric acid and a yield on glucose of 0.45 g g^{-1}.[85]

Whereas *S. cerevisiae* nomally produces 2 g l^{-1} of malic acid from fumaric acid, a recombinant strain containing a cloned fumarase gene was able to produce 125 g l^{-1} with a yield of almost 90%.[86]

An oxidative bioconversion of saturated and unsaturated linear aliphatic 12–22 carbon substrates to their terminal dicarboxylic acids was developed by gene disruption and gene amplification.[87] Product concentrations reached 200 g l^{-1} and problematic side-reactions such as unsaturation, hydroxylation and chain-shortening did not occur.

Alcohols. *Ethanol. E. coli* was converted into a good ethanol producer (43 g l^{-1}) by recombinant DNA technology.[88] Alcohol dehydrogenase II and pyruvate decarboxylase genes from *Zymomonas mobilis* were inserted in *E. coli* and became the dominant system for NAD regeneration. Ethanol represented over 95% of the fermentation products in the genetically-engineered strain. By cloning and expressing the same two genes in *Klebsiella oxytoca*, the recombinant was able to convert crystalline cellulose to ethanol in high yield when fungal cellulase was added.[89] Maximum theoretical yield was 81–86% and titers as high as 47 g l^{-1} of ethanol were produced from 100 g l^{-1} of cellulose.

Most recombinant strains of *E. coli,* Zymomonas and Saccharomyces convert corn fiber hydrolysate to 21–35 g l^{-1} with yields of 0.41–0.50 ethanol per g of sugar consumed.[90] For a recombinant *E. coli* strain making 35 g l^{-1}, time was 55 hours and yield was 0.46 g ethanol per g of available sugar, which is 90% of the attainable maximum.

A *S. cerevisiae* fusant library obtained by genome shuffling was screened for growth at 35, 40, 45, 50 and 55°C on agar plates containing different concentrations of ethanol.[91] After three rounds of genome shuffling, a strain (F34) was obtained which was able to grow on plate culture up to 55°C, completely utilized 20% (w/v) glucose at 45–48°C, produced 99 g l^{-1} ethanol and tolerated 25% (v/v) ethanol stress.

Three rounds of shuffling allowed isolation of a strain with a substantial improvement in multiple stress tolerance to ethanol, glucose, and heat. Its cycle of fermentation was not only shortened, but ethanol yield was increased by up to 11% as compared with the control in very-high-gravity (VHG) fermentations.[92] Tolerance of *Candida krusei* GL560 to acetic acid was also improved by genome shuffling.[93] A mutant, S4-3, which was isolated and selected after four rounds, had a higher viability in different media containing acetic acid than did the parent strain GL560. The mutant also improved its multiple stress tolerance to ethanol, H_2O_2, heat and freeze-thawing.

1,3-propanediol. A strain of *Clostridium butyricum* converts glycerol to 1,3-propanediol (PDO) at a yield of 0.55 g g^{-1} glycerol consumed.[94] In a two-stage continuous fermentation, a titer of 41 to 46 g l^{-1} was achieved with a maximum productivity of 3.4 g l^{-1} h^{-1}. Recent metabolic engineering triumphs have included the development of an *E. coli* culture that grows on glucose and produces PDO at 135 g l^{-1}, with a yield of 51% and a rate of 3.5 g l^{-1} h^{-1}.[95] To do this, eight new genes were introduced to convert dihydroxyacetone phosphate (DHAP) into PDO. These included yeast genes converting dihydroxyacetone to glycerol and *Klebsiella pneumoniae* genes converting glycerol to PDO. Production was further improved in the recombinant by modifying eighteen *E. coli* genes, including regulatory genes.[95] PDO is the monomer used to chemically synthesize industrial polymers such as polyurethanes and the polyester fiber Sorono™ by DuPont. This new bioplastic is polytrimethylene terephthalate (3GT polyester) made by reacting terephthalic acid with PDO.[96] PDO is also used as a polyglycol-like lubricant and as a solvent.

D-Mannitol. D-Mannitol is a naturally occurring polyol, widely used in the food, chemical and pharmaceutical industries. About 40,000 tons are produced annually. It is considered to be a low-calorie sweetener. It is produced mainly by catalytic hydrogenation of glucose/fructose mixtures but 75% of the product is sorbitol, not mannitol. For this reason, microbial processes are being considered. A whole cell bioconversion of D-fructose to D-mannitol was developed by metabolic engineering of *E. coli*.[97] The *mdh* gene encoding mannitol dehydrogenase from *Leuconostoc pseudomesenteroides* and the *fdh* gene encoding formate dehydrogenase from *Mycobacterium vaccae* were co-expressed in *E. coli* along with the *glf* gene encoding the glucose facilitator protein of *Z. mobilis*. The process yielded 75–91 g l^{-1} of D-mannitol, a specific productivity of 3.1–4.1 g $g^{-1}h^{-1}$ and a molar yield of 84–92% with no byproducts. An improved bioconversion process was developed with a recombinant *E. coli* strain in the presence of added glucose isomerase yielding 145 g l^{-1} of D-mannitol from 180 g l^{-1} glucose.[98]

Sorbitol. Sorbitol, also called D-glucitol, is 60% as sweet as sucrose and has use in the food, pharmaceutical and other industries. Its worldwide production is estimated to be higher than 500,000 tons per year and it is made chemically by catalytic hydrogenation of D-glucose. Metabolic engineering of *Lactobacillus plantarum* for high sorbitol production was successfully achieved by a simple two-step strategy overexpressing the two sorbitol-6-phosphate dehydrogenase genes (srlD1 and srlD2) identified in the genome sequence.[99]

Other compounds. *Nucleosides.* Genetic engineering of the inosine monophosphate (IMP) dehydrogenase gene in a *B. subtilis* strain producing 7 g l^{-1} of the desirable guanosine and 19 g l^{-1} of the undesirable inosine changed production to that of 20 g l^{-1} guanosine and 5 g l^{-1} inosine.[100] Guanosine is used to produce guanosine monophosphate (GMP) a potent flavoring agent.

Carotenoids. Carotenoids were overproduced by introducing carotenoid gene clusters from *Erwinia uredovora* into *E. coli* and overexpressing the *E. coli* deoxyxylulose phosphate synthase, the key enzyme of the non-mevalonate isoprenoid biosynthetic pathway.[101] Lycopene accumulated to 1.3 mg g^{-1} dry cell weight and zeaxanthin to 0.6 mg g^{-1}.

Glucosamine. Glucosamine is used for osteoarthritis and is made by acid hydrolysis of chitin from shellfish waste. Since many patients have shellfish allergies, a microbial source is desirable. Metabolic engineering of *E. coli* yielded a process producing 17 g l^{-1} of glucosamine.[102] In this strain, glucosamine synthase was overexpressed, glucosamine degradative genes were inactivated, and the inhibition of glucosamine synthase by glucosamine was decreased by mutational modification of the enzyme via error-prone PCR. Overexpression of a heterologous glucosamine-6-P-N acetyltransferase yielded a strain making 110 g l^{-1} of N-acetylglucosamine which is easily converted to glucosamine by mild acid hydrolysis.

Solvents. Cloning of its *ace* (acetone) operon genes *adc* (encoding acetoacetate decarboxylase), and *ctfA* and *ctfB* (two genes encoding coenzyme A transferase) on a plasmid containing the *adc* promoter into *Clostridium acetobutylicum* resulted in a 95% increased production. The increases were 95% in production of acetone, 37% for butanol, 90% for ethanol, 50% for solvent yield from glucose and a 22-fold lower production of undesirable acids.[103] Introduction of the acetone operon from *C. acetobutylicum* into *E. coli* led to high acetone production by the latter.[104]

Secondary Metabolites

These compounds have a major effect on the health, nutrition and economics of our society. The best-known are the antibiotics. This remarkable group of compounds form a heterogeneous assemblage of biologically active molecules with different structures and modes of action. They attack virtually every type of

microbial activity such as DNA, RNA, and protein synthesis, membrane function, electron transport, sporulation, germination and many others. Other secondary metabolites are pesticides, pigments, toxins, effectors of ecological competition and symbiosis, pheromones, enzyme inhibitors, immunomodulating agents, receptor antagonists and agonists, pesticides, antitumor agents, immunosuppressives, cholesterol-lowering agents, plant protectants and growth promotants of animals and plants. To obtain/develop overproducing strains, the technologies already described for the production of primary metabolites (see section 2) have been applied.

Antibiotics. Antibiotics have tremendous economic importance. More than 350 agents have reached the world market as antimicrobials.

β-*lactams.* Of great importance in chemotherapy of bacterial infections are the β-lactam antibiotics, namely penicillin G, cephalosporin C, cephamycin C and the semi-synthetic penicillins and cephalosporins.

Penicillin G. Protoplast fusion was used to modify the characteristics of an improved penicillin-producing strain of *Penicillium chrysogenum* which showed poor sporulation and poor seed growth. Backcrossing with a low-producing strain yielded a higher-producing strain with better sporulation and better growth in seed medium.[105]

Metabolic engineering of the penicillin-producing *P. chrysogenum* strains showed that increases could result by introducing extra copies of biosynthetic genes and by increasing copy number and high transcription levels of the whole cluster. Penicillin production was increased by overexpressing the gene encoding phenylacetic acid-activating CoA ligase from *Pseudomonas putida.* Overexpression of gene *acvA* in *A. nidulans*, by replacing the normal promoter with the ethanol dehydrogenase promoter,[106] increased penicillin production up to 30-fold.

Cephalosporin C. Protoplast fusion was carried out with strains of *Acremonium chrysogenum* (formerly *Cephalosporium acremonium*) obtained 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 parent.[107]

Cloning multiple copies of cyclase into *A. chrysogenum* yielded an improved cephalosporin C-producing strain.[108] When an industrial production strain of *A. chrysogenum* 394-4 was transformed with a plasmid containing the *pcbC* and the *cefEF* gene from an early strain of the 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%.[109] One copy of the *cefEF* had been integrated into chromosome III whereas the native gene is on chromosome II. Cephalosporin C production by *A. chrysogenum* was further improved by introducing extra copies of the the acetyl-transferase gene (*cefG*) along with additional copies of the expandase/hydroxylase gene (*cefEF*).

Cephamycin C. Another application of protoplast fusion is the recombination of improved producers from a single mutagenesis treatment. By recombination, one can 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 lactamdurans* were fused and among the recombinants were two cultures which produced 10–15% more antibiotic than the best parent.[110] Overexpression of *ccaR*, a positive regulatory gene in *Streptomyces clavuligerus*[111] led to a two- to three-fold increase in antibiotic production-Cephamycin C production by *Nocardia lactamdurans* has also been improved by overxpression of *lat,* encoding lysine-aminotransferase.[112]

Semisynthetic cephalosporins. Semisynthetic cephalosporins are made from 7-aminocephalosporanic acid (7-ACA) or 7-aminodeacetoxycepalosporanic acid (7-ADCA). Chemical methods had traditionally been used to produce 7-ACA and 7-ADCA using expensive and toxic reagents and yielding sideproducts harmful to the environment. These processes are being replaced by safer microbiological processes. Transformation of *P. chrysogenum* with the *Streptomyces lipmanii cefD* and *S. clavuligerus cefE* genes allowed production of the intermediate deacetoxycephalosporin C (DAOC)[113] at titers of 2.5 g l^{-1}, along with penicillin V. DAOC is another valuable intermediate in the commercial production of semi-synthetic cephalosporins. Also, cloning of *cefE* from *S. clavuligerus* or *cefEF* and *cefG* from *A. chrysogenum* into *P. chrysogenum* grown with adipic acid as side-chain precursor[114] resulted in formation of adipyl-6-aminopenicillanic acid (adipyl-6-APA) and adipyl-7-ADCA in the case of *cef*E, and adipyl-6-APA, adipyl-7ADCA, adipyl-7-DAC, and 7-ACA in the case of *cefEF* and *cefG*. Feeding adipic acid as side-chain precursor and then enzymatically removing the adipoyl side chain leads to production of 7-ADCA.

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^{-1} 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.[115] Production levels were nearly equivalent (80%) to the total β-lactams biosynthesized by the parental strain. Luo et al.[116] constructed a strain of *E. coli* containing the D-amino acid oxidase gene from *Trigonopsis variabilis* and the glutaryl-7-aminocephalosporanic acid acylase gene from Pseudomonas sp. The recombinant *E. coli* was able to convert cephalosporin C directly to 7-ACA.

Clavulanic acid. Although clavulanic acid is a β-lactam compound, it has only low antibacterial activity and instead, it is used widely as an inhibitor of β-lactamase, an enzyme strongly incvolved in antibiotic resistance. 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.[117]

Clavulanic acid biosynthesis begins with a condensation of L-arginine and D-glyceraldehyde-3-phosphate (G3P). The supply of G3P is limited, so inactivation of two G-3-P dehydrogenases, encoded by *gap1* and *gap2* by targeted gene disruption,

Table 3. Genetic techniques used to increase secondary metabolite production

Genetic techniques	Metabolites
Protoplast fusion	Penicillin G, cephalosporin C, cephamycin C, clavulanic acid, indolizomycin, rifamycins
Metabolic engineering	Antibiotics (penicillin G, cephalosporin C, cephamycin C, clavulanic acid, semisynthetic cephalosporins), antitumor agents (anthracyclines, glycopeptolides, anthracenones), avermectins, xanthan gum, artemisinin
Transposition	Daptomycin, tylosin
Association analysis	Lovastatin
Combinatorial biosynthesis	Erythromycins, tetracenomycins, tylosin, spiramycins, surfactins
Whole genome shuffling	Epothilones, spinosad
Genome mining	Echinosporamicin-type antibiotics, antifungal compounds (ECO-02301) and others

doubled clavulanic acid production.[118] Also, increased dosage of biosynthetic genes *ceas* and cs2[119] or overexpression of positive regulatory genes increased production two- to three-fold.[120,121]

Non-β-lactam antibiotics. Non-β-lactam antibiotics have also been genetically engineered. Daptomycin. Some *Streptomyces roseosporus* Tn *5099* transposition mutants produce 57–66% more daptomycin than the parent.[122]

Transposition increased the rate-limiting step of tylosin biosynthesis in *Streptomyces 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 up to 60%, while decreasing the concentration of the final intermediate (macrocin).[123]

Need for new antibiotics. Despite the success of the antibiotic field, new antibiotics are sorely needed. About 30 years ago, the difficulty and high cost of isolating novel antibiotic structures and agents with new modes of action for such uses became apparent and the field entered a phase of decline. However, efforts must continue to discover and develop new antibiotics, in order to protect the health of the world's population. This is coming about by the combination of complementary technologies such as natural product discovery via high throughput screening with new genetic technologies. The most promising genetic techniques are shown in **Table 3**.

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 (*Streptomyces griseus*) and an istamycin producer (*Streptomyces tenjimariensis*) led to a hybrid strain producing a new antibiotic.[124] Another new antibiotic, indolizomycin, was produced by protoplast fusion between non-antibiotic producing mutants of *S. griseus* and *S. tenjimariensis*.[125]

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).[126] 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.[127]

The production of antibiotics in heterologous hosts via combinatorial biosynthesis is becoming very popular in antibiotic production and discovery.[128] New derivatives of antibiotics have been obtained after the biosynthetic paths were elucidated and the biosynthetic genes isolated.[129] Techniques used are (1) targeted gene disruption in which single genes are inactivated; (2) tailoring by introducing a single gene or a few genes from another pathway; and (3) by combination of (1) and (2) above. Over 200 new polyketides have been made by combinatorial biosynthesis.[130,131]

Mutation in the genes encoding synthases of erythromycin biosynthesis in *Saccharopolyspora erythrea* or introducing heterologous DNA in a blocked mutant has led to production of new erythromycins. These products are the 2-norerythromycins,[132] 6-deoxyerythromycins,[133] Δ6,7-anhydroerythromycin C[134] and 5-6-dideoxy-3α-mycarosyl-5-oxyerythronolide B.[135] Two amino acid substitutions were made in the NAD(P)H binding motif of the enoyl reductase domain encoded by *eryAII*. By substituting the acyltransferase (AT) and β-carbon processing domains of 6-deoxyerythronolide B synthase [DEBS; the polyketide synthase (PKS) of the erythromycin macrolide ring] with counterparts from the rapamycin PKS, a library of over 60 new macrolide aglycones was produced.[136]

Novel erythromycins with fatty acids attached to C-13 were produced using a recombinant *S. erythraea* culture with the loading domain of the erythromycin PKS replaced by the one from the avermectin producer, *Streptomyces avermitilis*.[137] One such compound containing a cycopentyl group at C13 instead of an ethyl group was much more active against *Pasteurella multocida* and *E. coli*, although less active against *S. aureus* than erythromycins A and B.

A three plasmid system for heterologous expression of 6-deoxyerythronolide B synthase was used for combinatorial biosynthesis.[138] A library of modified polyketide lactones was produced by using 14 modified versions of each plasmid and inserting them in combinations into *Streptomyces lividans*. The modified versions were produced by replacing distinct gene segments in the erythromycin PKS with those from the rapamycin producer. Triple transformants able to produce 43 different polyketides were obtained.

Combinatorial biosynthesis has also been used to construct macrolides with new sugar moieties.[139,140] Methymycin and pikromycin, produced by a gene cluster of *Streptomyces venezuelae* and normally containing the sugar desosamine, were modified by cloning of a gene from the calicheamicin producer, *Micromonospora echinospora* subsp. *calichensis*. The gene encodes TDP-glycero-hexulose aminotransferase. Transfer of a 12.6 kb DNA fragment from the tetracenomycin C-producing *S. glaucescens* to *S. lividans* resulted in tetracenomycin C production by the latter. The fragment contains 12 genes of biosynthesis and resistance. Novel hybrid tetracenomycins were produced by

introducing a 25 kb cosmid from the elloramycin biosynthetic pathway of *S. olivaceus* into the polyketide synthase (PKS)-deleted mutant of the urdamycin producer, *S. fradiae*, and into the mithramycin producer, *Streptomyces argillaceus*. The cosmid contains a glycosyltransferase gene whose enzyme has broad substrate specificity and thus produces hybrid products containing different D-and -L sugars.

3-*O*-Acetyl-4''-*O*-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 novel direct fermentation organism was constructed by transforming *S. fradiae* with *S. thermotolerans* plasmids containing acyl transferase genes.[141]

Introduction of a gene encoding a modifying enzyme has been used to generate analogs of the macrolide spiramycin. Thus, spiramycin-4''-O-isovaleryl ester was produced by cloning the *carE* gene from *S. thermotolerans* (carbomycin producer) into S*treptomyces ambofaciens* (spiramycin producer).[142] These are both 16-membered macrolides but one of the two sugars in carbomycin is isovalerylmycarose and the corresponding sugar in spiramycin is mycarose. Furthermore, spiramycin was converted into its 3-O-acetyl- and 3-O-propionyl esters by *S. lividans* transformed with the *mdmB* gene from *Streptomyces mycorofaciens*, the producer of midecamycin.[143]

Cloning of the *actI*, *actIV* and *actVII* genes from *S. coelicolor* into the 2-hydroxyaklavinone producer, *Streptomyces galilaeus* 31671, yielded novel hybrid metabolites, desoxyerythrolaccin and 1-*O*-methyl-desoxyerythrolaccin.[144] Cloning of these genes into other streptomycetes resulted in the production of the novel metabolite aloesaponarin II.[145]

New peptide antibiotics (modified surfactins) were produced by combinatorial biosynthesis.[146] Genes encoding activating domains for phenylalanine, ornthine and leucine from the gramicidin S producer *Bacillus brevis* and for cysteine and valine from the ACV and penicillin producer *P. chrysogenum* were transferred into a mutant of the surfactin producer *B. subtilis* which had a disrupted *leu7* gene. The five new surfactins were slightly less hemolytic than surfactin.

The use of microbial genomics for improving the discovery of new antibiotics has been reviewed by Van Lanen and Shen.[147] The efforts include mining of whole genome sequences, genome scanning, heterologous expression and discovery of novel chemistry. 294 microbes had been sequenced and annotated at that time. The number of genes expected to be part of secondary metabolic sequences is much greater than the number of known secondary metabolites.[148]

Genomics will provide a huge group of new targets against which natural products can be screened.[149] The human genome has 30,000–35,000 genes, less than 50% having a putative function.[150] These genes have the potential to produce over 100,000 proteins. Estimates of the number of proteins acting as useful targets range from 600–10,000.

Genome mining utilizes DNA sequencing and bioinformatics to rapidly identify clusters of genes in antibiotic-producing cultures encoding biosynthesis of new bioactive products and to predict structure based on gene sequences.[151] More than 450 natural product clusters were identified in this 2003 study.

Bacterial pathogens contain about 2,700 genes of which current antibiotics target less than 25.[152] A genomic comparison of the pathogenic *Haemophilus influenzae* with a nonpathogenic *E. coli* revealed 40 potential drug targets in the former.[153] Similarly, a comparison of genomes of *Helicobacter pylori* with *E. coli* and *H. influenzae* revealed 594 *H. pylori*-specific genes of which 196 were known, 123 of the known genes being involved in known host-pathogen interactions and 73 targets of novel potential.[154]

Antitumor agents. In their review on the use of microbes to prescreen potential antitumor compounds, Newman and Shapiro[155] concluded that microorganisms have played a cucial role in identifying compounds with therapeutic benefit against cancer. Most of the important compounds used for chemotherapy of tumors are microbially-produced antibiotics. Approved antitumor agents from microorganisms are actinomycin D (dactinomycin), anthracyclines [including daunorubicin, doxorubicin (adriamycin), epirubicin, pirirubicin, idarubicin, valrubicin, amrubicin], glycopeptolides (bleomycin, phleomycin), the mitosane mitomycin C, anthracenones (mithramycin, streptozotocin, pentostatin) and the enediyne calcheamycin attached to a monoclonal antibody (Mylotarg®).

Anthracyclines. A new anthracycline, 11-hydroxyaclacino mycin A, was produced by cloning the doxorubicin resistance gene and the aklavinone 11-hydroxylase gene *dnrF* from the doxorubicin producer, *Streptomyces peucetius* subsp. *caesius*, into the aclacinomycin A producer. The hybrid molecule showed greater activity against leukemia and melanoma than aclacinomycin A. Additional new anthracyclines have been made by (1) introducing DNA from *Streptomyces purpurascens* into *Streptomyces galilaeus*, both of which normally produce known anthracyclines, (2) cloning DNA from the nogalomycin producer, *Streptomyces nogalater*, into *S. lividans* and into an aclacinomycin-negative mutant of *S. galilaeus*, (3) cloning of the *actI*, *actIV* and *actVII* genes from *S. coelicolor* into the 2-hydroxyaklavinone producer, *S. galilaeus* 31671 yielding the novel hybrid metabolites, desoxyerythrolaccin and 1-O-methyl-desoxyerythrolaccin, and (4) blocking deoxysugar biosynthesis in a *S. peucetius* strain and cloning of the *avrE* gene of the avermectin-producing *S. avermitilis* or the *eryBIV* genes from the erythromycin producer, *Streptomyces erythraea*.

Epothilones. Epothilones are highly promising prospective anticancer agents that are produced by the myxobacterium, *Sorangium cellulosum*. Using the classical UV-mutation method plus selection pressure, the production capacity was increased 2.5 times that of the starting strain.[156] The mutants with higher production and different phenotypes were further subjected to recursive protoplast fusion and the fusants products were screened under multi-selection pressure. Production of epothilone B by one fusant was increased about 130 times compared to the starting strain.

Cholesterol-lowering agents. Integrating transcriptional and metabolite profiles from 21 strains of *Aspergillus terreus* producing different levels of lovastatin and another 19 strains with altered

Table 4. Top levels of recombinant proteins made by different biological systems*

System	Protein (g/L)
Bacteria	14
Yeasts	30
Mammalian cells	27
Insect cells	11

(+)-geodin levels led to an improvement in lovastatin production of over 50%.[157] This approach, named association analysis, served to reduce the complexity of profiling data sets to identify those genes whose expression was most tightly linked to metabolite production. Improvement of lovastatin titers was achieved by increasing dosage of lovastatin biosynthetic genes and of regulatory genes for secondary metabolism, i.e., *lovF, creA, fadA, ganA, gnaI, gna3* and *gpa1*. Of these, gene *lovF* was found to be the important one. They fused its promoter to the *ble* gene encoding resistance to phleomycin. The *lovF::ble* transcriptional fusion protein was used as a reporter-based system to select improved mutants.

Antihelminthics. A *S. avermitilis* culture, isolated by Omura and coworkers at the Kitasato Institute in Japan, was found by Merck scientists to produce a family of secondary metabolites having both antihelminthic and insecticidal activities. These killed the intestinal nematode, *Nematosporoides dubius*, in mice and were named avermectins. They are disaccharide derivatives of macrocyclic lactones with exceptional activity against parasites, i.e., at least ten times higher than any synthetic antihelminthic agent known. Despite their macrolide structure, avermectins lack antibiotic activity, do not inhibit protein synthesis nor are they ionophores; instead they interfere with neurotransmission in many invertebrates. They have activity against both nematode and arthropod parasites in sheep, cattle, dogs, horses and swine.

S. avermitilis has gene *afsR2* which was originally discovered as a global regulatory gene in *S. lividans* with positive control action on actinorhodin and undecylprodigiosin formation. Incorporation of multiple copies of *afsR2* from *S. lividans* into wildtype *S. avermitilis* increased avermectin production by 2.3-fold.[158] Another regulatory gene appears to be an 8 kb DNA fragment of *S. avermitilis* which stimulates actinorhodin and undecylprodigiosin formation in *S. lividans.* It also stimulated avermectin production in wild-type *S. avermitilis* by 2.5-fold, in an improved strain by 1-fold, and by 0.4-fold in a semi-industrial strain.[159] Transposon mutagenesis eliminated production of the troublesome toxic oligomycin by the avermectin-producing *S. avermitilis*.[160]

A semi-synthetic derivative, 22,23-dihydroavermectin B1 (Ivermectin) is 1,000 times more active than thiobenzole and is a commercial veterinary product. Ivermectin is made by hydrogenation at C22-C23 of avermectin B1a and B1b with rhodium chloride acting as catalyst. By genetic engineering of *S. avermitilis* in which certain PKS genes were replaced by genes from the PKS of *S. venezuelae* (the pikromycin producer), ivermectin could be made directly by fermentation, thus avoiding semisynthesis.[161]

Biopharmaceuticals

Recombinant proteins. By means of genetic engineering, desired proteins have been produced in great quantities to meet the copious demands of industry.[162] Hence, most biopharmaceuticals produced today are recombinant. Protein quality, functionality, production speed and yield are the most important factors to consider when choosing the right expression system for recombinant protein production. Top yields for different systems are shown in **Table 4**.

Between 100 and 200 therapeutic proteins have been approved in Europe and the USA. Non-glycosylated proteins are usually made in *E. coli* or yeasts and they constitute 55% of the therapeutic protein market (39% by *E. coli,* 1% by other bacteria and 15% by the yeasts).[163] N-glycosylated proteins are usually made in mammalian cells which mimic human glycosylation. Chinese hamster ovary (CHO) cells provide about 35% of the therapeutic protein market but the process is very expensive and the glycoproteins made are not exactly the human type, and in some cases, they must be modified. Ten percent of the market is supplied by other mammalian systems such as NSO (mouse myeloma) cells. Insect cells are also used. Although yeasts, molds and insect cells are generally unable to provide mammalian glycosylation, the popular methylotrophic yeast, *Pichia pastoris*, has been genetically engineered to produce a human type of glycosylation.[164]

Bacteria. Bacterial systems are used to make somatostatin, insulin, bovine growth hormone for veterinary applications, α-1 antitrypsin, interleukin-2, tumor necrosis factor, β-interferon, and γ-interferon. Avecia Biologics has achieved a titer of 14 g l^{-1} of recombinant protein using *E. coli*.[165] Bacilli have yields as high as 3 g l^{-1}. An improved Gram-negative host for recombinant protein production has been developed using *Ralstonia eutropha*.[166] The system appears superior to *E. coli* with respect to inclusion body formation. Organophosphohydrolase, a protein prone to inclusion body formation with a production of less than 100 mg l^{-1} in *E. coli*, was produced at 10 g l^{-1} in *R. eutropha. Staphylococcus carnosus* can produce 2 g l^{-1} of secreted mammalian protein.

Yeasts. High recombinant protein yields can be obtained in the methylotrophic yeast, *P. pastoris*, e.g., as high as 15 g l^{-1}.[167] Claims have been made that *P. pastoris* can make 20–30 g l^{-1} of recombinant proteins.[168] Some other proteins were made at or over 10 g l^{-1} by *P. pastoris*, e.g., tumor necrosis factor, gelatin, intracellular tetanus toxin fragment C and serum albumin. Heterologous gene expression in another methylotroph *Hansenula polymorpha* yielded 13.5 g l^{-1} of phytase. Another utilized yeast is *S. cerevisiae*. Glucose oxidase from *Aspergillus niger* is produced by *S. cerevisiae* at 9 g l^{-1}. Recombinant products on the market which are made in *S. cerevisiae* are insulin, hepatitis B surface antigen, urate oxidase, glucagons, granulocyte macrophage colony stimulating factor (GM-CSF), hirudin, and platelet-derived growth factor.

Mammalian cells. CHO cells constitute the preferred system for producing monoclonal antibodies and some other recombinant proteins. Other cell types include (1) various mouse myelomas such as NSO murine myeloma cells,[169] (2) baby hamster kidney (BHK) cells for production of cattle foot-and-mouth

disease vaccine, (3) green monkey kidney cells for polio vaccine[170] and (iv) human cell lines such as human embryonic kidney (HEK) cells. NSO is a non-secreting subclone of the NS-1 mouse melanoma cell line.

Monoclonal antibody production in NSO animal cells reached over 2.5 g l^{-1} in fed-batch processes.[171] Animal-free, protein-free and even chemically-defined media with good support of production have been developed.[172] Protein production by CHO cells went from 5–50 mg l^{-1} in 1985 to 50–500 mg l^{-1} in 1995, to 5 g l^{-1} in 2005.[173] A number of mammalian processes are producing 3–5 g l^{-1} of recombinant protein[174] and, in some cases, protein titers have reached 10 g l^{-1} in industry[175] including antibodies.[176] A rather new system is that of a human cell line known as PER. C6 of Crucell Holland BV, which, in cooperation with DSM Biologics, was reported to produce 15 g l^{-1},[177] and then later, 27 g l^{-1} of a monoclonal antibody.[178] Protein production of over 20 g l^{-1} has been achieved in serum-free medium but production of 2–3 g l^{-1} in such media is more usual.

Insect cells. Insect cells are able to carry out more complex post-translational modifications than can be accomplished with fungi. They also have the best machinery for the folding of mammalian proteins and are therefore quite suitable for making soluble protein of mammalian origin.[179] The most commonly used vector system for recombinant protein expression in insects is the baculovirus, especially the nuclear polyhedrosis virus (*Autographa californica*) which contains circular double-stranded DNA, is naturally pathogenic for lepidopteran cells, and can be grown easily in vitro. The usual host is the fall armyworm (*Spodoptera frugiperda*) in suspension culture. A larval culture can be used which is much cheaper than mammalian cell culture. Recombinant insect cell cultures have yielded over 200 proteins encoded by genes from viruses, bacteria, fungi, plants and animals.[180] Current thought is that the baculovirus insect cell system can produce 11 g l^{-1} of recombinant protein.

Enzymes

Genes encoding many microbial enzymes have been cloned and the enzymes expressed at levels hundreds of times higher than those naturally produced. Over 60% of the enzymes used in the detergent, food and starch processing industry are recombinant proteins.[181] Recombinant DNA technology has been used beneficially in the enzyme industry in the following ways:[182,183]

(a) to produce in industrial organisms enzymes obtained from microbes which are difficult to grow or modify genetically;

(b) to increase enzyme productivity by use of multiple gene copies, strong promoters, and efficient signal sequences;

(c) to produce in a safe host useful enzymes obtained from a pathogenic or toxin-producing microorganism; and

(d) to improve the stability, activity or specificity of an enzyme by protein engineering.

The industrial enzyme business adopted rDNA methods eagerly to increase production levels and to produce enzymes from industrially-unknown microorganisms in industrial organisms such as Aspergillus spp., Trichoderma spp., *K. lactis*, *S. cerevisiae*, *Yarrowia lipolytica* and *Bacillus licheniformis*.

Filamentous fungi can produce recombinant proteins at levels as high as 4.6 g l^{-1}. They include *Aspergillus niger, Aspergillus oryzae, Aspergillus awamori, Chrysosporium lucknowense* and *A. chrysogenum*. Recombinant molds are one of the main sources of enzymes for industrial applications.

There are three fungal recombinant lipases currently used in the food industry. They are from *Rhizomucor miehi, Thermomyces lanuginosus* and *Fusarium oxysporum*, and are produced in *A. oryzae*. Lipases are employed in leather processing where they are combined with proteases, peroxidases and oxidases. In order to be effective in detergents, lipases need to remain functional under harsh conditions such as the presence of surfactants and oxidants, temperatures above 45°C, pH values of about 10, and should be alkalophilic. Washing powders have been improved in activity and low temperature operation by application of recombinant DNA technology and site-directed mutagenesis to proteases and lipases.[183,184] In 1994, Novo Nordisk introduced Lipolase, the first commercial recombinant lipase for use in a detergent by cloning the *Humicola lanuginose* lipase gene into the *A. oryzae* genome. Such lipases are used for laundry cleaning, interesterification of lipids, and esterification of glucosides producing glycolipids which have applications as biodegradable non-ionic surfactants for detergents, skin care products, contact lenses and as food emulsifiers.

The α-amylase gene from *B. amyloliquefaciens* was cloned using multicopy plasmid pUB110 in *B. subtilis*.[185] Production was 2,500-fold greater then that in wild-type *B. subtilis* and five-fold that of the *B. amyloliquefaciens* donor. An exoglucanase from the cellulolytic *Cellulomonas fimi* was overproduced after cloning in *E. coli* to a level of over 20% of cell protein.[186] The endo-β-glucanase components of the cellulase complexes from Thermomonospora and *Clostridium thermocellum* were cloned in *E. coli* as was the cellobiohydrolase I gene of *Trichoderma reesei*.[187] *P. pastoris,* the methanol-utilizing yeast, was engineered to produce *S. cerevisiae* invertase and to excrete it into the medium at 100 mg l^{-1}.[188] Self-cloning of the xylanase gene in *S. lividans* resulted in six-fold overproduction of the enzyme.[189]

Many enzymes are made by filamentous organisms which are slow-growing and difficult to handle in fermentors. Transfer of these genes to rapidly-growing unicellular bacteria provide an economical move achieving rapid growth and more reproducible production. Other advantages are more rapid nutrient uptake due to a greater surface/volume ratio, better oxygen transfer, better mixing and thus more reliable control of pO2, pCO2 and pH, and a better organism for mutagenesis.

Aspartase production was increased 30-fold by cloning in *E. coli*.[190] Captopril esterase of *P. putida,* used in preparing the chiral captopril sidechain, was cloned in *E. coli* with a 38-fold increase in activity.[191] A 1,000-fold increase in phytase production was achieved in *A. niger* by use of recombinant technology.[192] Cloning of the benzylpenicillin acylase gene of *E. coli* on multicopy[50] plasmids resulted in a 45-fold increase as compared to uninduced wild-type production. Interestingly, the cloned enzyme is constitutive.[193]

The properties of many enzymes have been altered by genetic means. "Brute force" mutagenesis and random screening of

microorganisms over the years led to changes in pH optimum, thermostability, feedback inhibition, carbon source inhibition, substrate specificity, Vmax, K_m and K_i. This information was later exploited by the more rational techniques of protein engineering. Single changes in amino acid sequences have yielded similar types of changes in a large variety of enzymes. Today, it is no longer necessary to settle for an enzyme's natural properties; these can be altered to suit the needs of the investigator or the process. For example, a protease from *Bacillus stearothermophilus* was increased in heat tolerance from 86°C to 100°C, being made resistant to boiling. The enzyme was developed by site directed mutagenesis.[194] Only eight amino acids had to be modified. Temperature stability at 100°C was increased 340-fold without a decrease in activity at lower temperatures. All eight mutations were far from the enzyme's active site.

Molecular breeding techniques, e.g., DNA shuffling, are being currently used to generate enzymes with improved properties such as: activity and stability at different pH values and temperatures,[195] increased or modified enantioselectivity,[196] altered substrate specificity,[197] stability in organic solvents,[198] novel substrate specificity and activity,[199] increased biological activity of protein pharmaceuticals and biological molecules[200] as well as novel vaccines.[201,202] Proteins from directed evolution work were already on the market by 2000.[203] These were green fluorescent protein of Clontech[204] and Novo Nordisk's LipoPrime® lipase.

Other Compounds

Rapamycin. Rapamycin, a 31-member ring macrolide immuno suppressant produced by *Streptomyces hygroscopicus,* has many applications in clinical medicine. One round of genome shuffling using seven mutants of *S. hygroscopicus* with different features and rapamycin productivities, generated an improved rapamycin-producing strain.[205] An interspecies fusion of protoplasts of *S. hygroscopicus* D7-804 and *S. erythreus* ZJU325 also yielded an improved strain.

Artemisinin. A leading antimalarial today is artemisinin obtained from extracts of the Wormwood tree, *Artemisia annua*.[206] Artemisinin is a sesquiterpene lactone, active against both drug-resistant and cerebral malaria-causing strains. It still is isolated from this plant but it is a very expensive process and there is an extreme shortage. Martin et al.[207] used metabolic engineering of *E. coli* to produce 100 mg l^{-1} in 12 hours. The genes came from a truncated mevalonic acid pathway in *S. cerevisiae*. A genetically-engineered strain of *S. cerevisiae* can produce its precursor artemisinic acid.[208] Using this yeast in a fed-batch process in chemically-defined medium with galactose as carbon source, addition of methionine and controlled aeration and agitation speed, 2.5 g l^{-1} of artemisinic acid was produced.[209]

Spinosad. Spinosad (spinosyns A and D) is a mixture of secondary metabolites produced by *Saccharopolyspora spinosa* which is used in agriculture as a potent insect control agent with exceptional safety with respect to non-target organisms. After four rounds of genome shuffling with 10 mutant strains, generated by nitrosoguanidine and ultraviolet irradiation, a markedly improved strain was isolated.[210]

Xanthan gum. Microbially-produced xanthan gum is not only an acceptable food-thickener but is one of the most promising agents for enhanced oil recovery in the petroleum industry. Recombinant DNA manipulation of *Xanthomonas campestris* increased titers of xanthan by two-fold and increased pyruvate content by over 45%.[211,212] Formation amounted to 0.6 g g^{-1} of sucrose utilized.[213] Ten–twenty thousand tons of xanthan are produced annually for use in the oil, pharmaceutical, cosmetic, paper, paint and textile industries.[214]

Closing Remarks

Discovery of new bioactive compounds, as well as the development of new and more efficient bioprocesses, is coming about by the use of recombinant strains. The combination of complementary technologies such as mutation and genetic recombination have led to remarkable improvements in the productivity of many primary and secondary metabolites as well as protein biopharmaceuticals and enzymes. New genetic approaches for the development of overproducing strains are continuously emerging. Among those that have proven to be very successful are metabolic engineering, combinatorial biosynthesis and molecular breeding techniques.

More exciting advances are still to come as the complete sequencing of industrially important microbial genomes takes place. Functional genomics, proteomics and metabolomics are now being exploited for the discovery of novel valuable small molecules for medicine and enzymes for catalysis.

References

1. Jarai M. Genetic recombination in *Streptomyces aureofaciens*. Acta Microbiol Acad Sci Hungary 1961; 8:73-9.
2. Mindlin SZ. Genetic recombination in the actinomycete breeding. In: Sermonti G, Alacevic M, eds. Genetics and Breeding of Streptomyces. Zagreb: Yugoslav Academy of Sciences and Arts 1969; 147-59.
3. Kieser HM, Kieser T, Hopwood DA. A combined genetic and physical map of the *Streptomyces coelicolor* A3(2) chromosome. J Bacteriol 1992; 174:5496-507.
4. Ryu DDY, Kim KS, Cho NY, Pai HS. Genetic recombination in *Micromonospora rosaria* by protoplast fusion. Appl Environ Microbiol 1983; 45:1854-8.
5. Yoneda Y. Increased production of extracellular enzymes by the synergistic effect of genes introduced into *Bacillus subtilis* by stepwise transformation. Appl Environ Microbiol 1980; 39:274-6.
6. Ohnishi J, Mitsuhashi S, Hayashi M, Ando S, Yokoi H, Ochiai K, et al. A novel methodology employing *Corynebacterium glutamicum* genome information to generate a new L-lysine-producing mutant. Appl Microbiol Biotechnol 2002; 58:217-23.
7. Stephanopoulos G. Metabolic fluxes and metabolic engineering. Metab Eng 1999; 1:1-11.
8. Nielsen J. Metabolic engineering. Appl Microbiol Biotechnol 2001; 55:263-83.
9. Eggeling L, Sahm H, de Graaf AA. Quantifying and directing metabolic flux: application to amino acid overproduction. Adv Biochem Eng Biotechnol 1996; 54:1-30.
10. Sahm H, Eggeling L, de Graaf AA. Pathway analysis and metabolic engineering in *Corynebacterium glutamicum.* Biol Chem 2000; 381:899-910.
11. Santos CSS, Stephanopoulos G. Combinatorial engineering of microbes for optimizing cellular phenotype. Curr Opin Chem Biol 2008; 12:168-76.
12. Bailey JE, Sburlati A, Hatzimanikatis V, Lee K, Renner WA, Tsai PS. Inverse metabolic engineering: a strategy for directed genetic engineering of useful phenotypes. Biotechnol Bioeng 1996; 52:109-21.
13. Ness JE, del Cardayre SB, Minshull J, Stemmer WP. Molecular breeding: the natural approach to protein design. Adv Prot Chem 2000; 55:261-92.
14. ZhaoH, Arnold FH. Optimization of DNA shuffling for high fidelity recombination. Nucl Acids Res 1997; 25:1307-8.

15. Patten PA, Howard RJ, Stemmer WP. Applications of DNA shuffling to pharmaceuticals and vaccines. Curr Opin Biotechnol 1997; 8:724-33.
16. Zhang YX, Perry K, Vinci VA, Powell K, Stemmer WP, del Cardayre SB. Genome shuffling leads to rapid phenotypic improvement in bacteria. Nature 2002; 415:644-6.
17. Hou L. Novel methods of genome shuffling in *Saccharomyces cerevisiae*. Biotechnol Lett 2009; 31:671-7.
18. Leuchtenberger W, Huthmacher K, Drauz K. Biotechnological production of amino acids and derivatives: current status and prospects. Appl Microbiol Biotechnol 2005; 69:1-8.
19. Demain AL. The business of biotechnology. Indust Biotechnol 2007; 3:269-83.
20. Kirchner O, Tauch AJ. Tools for genetic engineering in the amino acid-producing bacterium *Corynebacterium glutamicum*. J Biotechnol 2003; 104:287-99.
21. Ikeda M, Nakagawa S. The *Corynebacterium glutamicum* genome: features and impacts on biotechnological processes. Appl Microbiol Biotechnol 2003; 62:99-109.
22. Kalinowski J, Bathe B, Bartels D, Bischoff N, Bott M, Burkowski A, et al. The complete *Corynebacterium glutamicum* ATCC 13032 genome sequence and its impact on the production of L-aspartate-derived amino acids and vitamins. J Biotechnol 2003; 104:5-25.
23. Nishio Y, Nakamura Y, Kawarabayashi Y, Usuda Y, Kimura E, Sugimoto S, et al. Comparative complete genome sequence analysis of the amino acid replacements responsible for the thermostability of *Corynebacterium efficiens.* Genome Res 2003; 13:1572-9.
24. Liu Q, Zhang J, Wei XX, Ouyang SP, Wu Q, Chen GQ. Microbial production of L-glutamate and L-glutamine by recombinant *Corynebacterium glutamicum* harboring Vitreoscilla hemoglobin gene *vgb*. Appl Microbiol Biotechnol 2008; 77:1297-304.
25. Chinen A, Kozlov YI, Hara Y, Izui H, Yasueda H. Innovative metabolic pathway design for efficient L-glutamate production by suppressing CO_2 emission. J Biosci Bioeng 2007; 103:262-9.
26. Radmacher E, Eggeling L. The three tricarboxylate synthase activities of *Corynebacterium glutamicum* and increase of L-lysine synthesis. Appl Microbiol Biotechnol 2007; 76:587-95.
27. Blombach B, Schreiner ME, Moch M, Oldiges M, Eikmanns BJ. Effect of pyruvate dehydrogenase complex deficiency on L-lysine production with *Corynebacterium glutamicum*. Appl Microbiol Biotechnol 2007; 76:615-23.
28. Sindelar G, Wendisch VF. Improving lysine production by *Corynebacterium glutamicum* through DNA microarray-based identification of novel target genes. Appl Microbiol Biotechnol 2007; 76:677-89.
29. Komatsubara S, Kisumi M, Chibata I. Transductional construction of a threonine-producing strain of *Serratia marcescens.* Appl Environ Microbiol 1979; 38:1045-51.
30. Komatsubara S, Kisumi M, Chibata I. Transductional construction of a threonine-hyperproducing strain of *Serratia marcescens*: lack of feedback controls of three aspartokinases and two homoserine dehydrogenases. Appl Environ Microbiol 1983; 45:1445-52.
31. Sugita T, Komatsubara S. Construction of a threonine-hyperproducing strain of *Serratia marcescens* by amplifying the phosphoenolpyruvate carboxylase gene. Appl Microbiol Biotechnol 1989; 30:290-3.
32. Debabov VG. The threonine story. Adv Biochem Eng/Biotechnol 2003; 79:113-36.
33. Ishida M, Kawashima H, Sato K, Hashiguchi K, Ito H, Enei H, et al. Factors improving L-threonine production by a three L-threonine biosynthetic genes-amplified recombinant strain of *Brevibacterium lactofermentum.* Biosci Biotechnol Biochem 1994; 58:768-70.
34. Eggeling L, Sahm H. Amino acid production: principles of metabolic engineering. In: Lee SY, Papoutsakis ET, eds. Metabolic Engineering. New York: Marcel Dekker 1999; 153-76.
35. Kruse D, Kraemer R, Eggeling L, Rieping M, Pfefferle W, Tchieu JH, et al. Influence of threonine exporters on threonine production in *Escherichia coli*. Appl Microb Biotechnol 2002; 59:205-10.
36. Lange C, Rittmann D, Wendisch VF, Bott M, Sahm H. Global expression profiling and physiological characterization of *Corynebacterium glutamicum* grown in the presence of L-valine. Appl Environ Microbiol 2003; 69:2521-32.
37. Guillouet S, Rodal AA, An G-H, Lessard PA, Sinskey AJ. Expression of the *Escherichia coli* catabolic threonine dehydratase in *Corynebacterium glutamicum* and its effect on isoleucine production. Appl Environ Microbiol 1999; 65:3100-7.
38. Morbach S, Sahm H, Eggeling L. L-Isoleucine production with *Corynebacterium glutamicum*: further flux increase and limitation of export. Appl Environ Microbiol 1996; 62:4345-51.
39. Hashiguchi K, Takesada H, Suzuki E, Matsui H. Construction of an L-isoleucine overproducing strain of *Escherichia coli* K-12. Biosci Biotechnol Biochem 1999; 63:672-9.
40. Colon GE, Nguyen TT, Jetten MSM, Sinskey A, Stephanopoulos G. Production of isoleucine by overexpression of ilvA in *Corynebacterium lactofermentum* threonine producer. Appl Microbiol Biotechnol 1995; 43:482-8.
41. Lee M, Smith GM, Eiteman MA, Altman E. Aerobic production of alanine by *Escherichia coli aceF IdhA* mutants expressing the *Bacillus sphaericus alaD* gene. Appl Microbiol Biotechnol 2004; 65:56-60.
42. Sugiura M, Takagi T, Kisumu M. Proline production by regulatory mutants of *Serratia marcescens*. Appl Microbiol Biotechnol 1985; 21:213-39.
43. Sugiura M, Imai Y, Takagi T, Kisumi M. Improvement of a proline-producing strain of *Serratia marcescens* by subcloning of a mutant allele of the proline gene. J Biotechnol 1985; 3:47-58.
44. Masuda M, Takamatu S, Nishimura N, Komatsubara S, Tosa T. Improvement of culture conditions for L-proline production by a recombinant strain of *Serratia marcescens.* Appl Biochem Biotechnol 1993; 43:189-97.
45. Shibasaki T, Hashimoto S, Mori H, Ozaki A. Construction of a novel hydroxyproline-producing recombinant *Escherichia coli* by introducing a proline 4-hydroxylase gene. J Biosci Bioeng 2000; 90:522-5.
46. Bongaerts J, Kraemer M, Mueller U, Raeven L, Wubbolts M. Metabolic engineering for microbial production of aromatic amino acids and derived compounds. Metab Eng 2001; 3:289-300.
47. Ikeda M, Katsumata R. Hyperproduction of tryptophan by *Corynebacterium glutamicum* with the modified pentose pathway. Appl Environ Microbiol 1999; 65:2497-502.
48. Ikeda M, Nakanishi K, Kino K, Katsumata R. Fermentative production of tryptophan by a stable recombinant strain of *Corynebacterium glutamicum* with a modified serine-biosynthetic pathway. Biosci Biotechnol Biochem 1994; 58:674-8.
49. Yukawa H, Kurusu Y, Shimazu M, Yamagata H, Teresawa M. Stabilization of an *Escherichia coli* plasmid by a mini-F fragment of DNA. J Indust Microbiol 1988; 2:323-8.
50. Sugimoto S, Yabuta M, Kato N, Seki T, Yoshida T, Taguchi H. Hyperproduction of phenylalanine by *Escherichia coli*: application of a temperature-controllable expression vector carrying the repressor-promotor system of bacteriophage lambda. J Biotechnol 1987; 5:237-53.
51. Konstantinov KB, Yoshida T. Knowledge-based control of fermentation processes. Biotechnol Bioeng 1992; 39:479-86.
52. Backman K, O'Connor MJ, Maruya A, Rudd E, McKay D, Balakrishnan R, et al. Genetic engineering of metabolic pathways applied to the production of phenylalanine. Ann NY Acad Sci 1990; 589:16-24.
53. Ikeda M, Katsumata R. Metabolic engineering to produce tyrosine or phenylalanine in a tryptophan-producing *Corynebacterium glutamicum* strain. Appl Environ Microbiol 1992; 58:781-5.
54. Levy-Schil S, Debussche L, Rigault S, Soubrier F, Bacchette F, Lagneaux D, et al. Biotin biosyntheric pathway in a recombinant strain of *Escherichia coli* overexpressing bio genes: evidence for a limiting step upstream from KAPA. Appl Microbiol Biotechnol 1993; 38:755-62.
55. Sakurai N, Imai Y, Masuda M, Komatsubara S, Tosa T. Improvement of a D-biotin-hyperproducing recombinant strain of *Serratia marcescens.* J Biotechnol 1994; 36:63-73.
56. Masuda M, Takahashi K, Sakurai N, Yanagiya K, Komatsubara S, Tosa T. Further improvement of D-biotin production by a recombinant strain of *Serratia marcescens*. Proc Biochem 1995; 30:553-62.
57. Koizumi S, Yonetani Y, Maruyama A, Teshiba S. Production of riboflavin by metabolically engineered *Corynebacterium ammoniagenes*. Appl Microbiol Biotechnol 2000; 51:674-9.
58. Perkins JB, Pero J. Biosynthesis of riboflavin, biotin, folic acid, and cobalamin. In: Sonenshein AL, Hoch JA, Losick R, eds. *Bacillus subtilis* and Other Gram Positive Bacteria: Biochemistry, Physiology and Molecular Genetics. Washington, DC: ASM Press 1993; 319:34.
59. Perkins JB, Sloma A, Hermann T, Theriault K, Zachgo E, Erdenberger T, et al. Genetic engineering of *Bacillus subtilis* for the commercial production of riboflavin. J Indust Microbiol Biotechnol 1999; 22:8-18.
60. Brenner S, Johnson M, Bridgham J, Golda G, Lloyd DH, Johnson D, et al. Gene expression analysis by massively parallel signature sequencing (MPSS) on microbead arrays. Nat Biotechnol 2000; 18:630-4.
61. Karos M, Vilarino C, Bollschweiler C, Revuelta JL. A genome wide transcription analysis of a fungal rivoflavin overproducer. J Biotechnol 2004; 113:69-76.
62. Anderson S, Marks CB, Lazarus R, Miller J, Stafford K, Seymour J, et al. Production of 2-keto-L-gulonate: an intermediate in L-ascorbate synthesis by a genetically modified *Erwinia herbicola*. Science 1985; 230:144-9.
63. Grindley JF, Payton MA, van de Pol H, Hardy KG. Conversion of glucose to 2-keto-L-gulonate, an intermediate in L-ascorbate synthesis by a recombinant strain of *Erwinia citreus*. Appl Environ Microbiol 1988; 54:1770-5.
64. Saito Y, Ishii Y, Hayashi H, Imao Y, Akashi T, Yoshikawa K, et al. Cloning of genes coding for L-sorbose and L-sorbosone dehydrogenases from *Gluconobacter oxydans* and microbial production of 2-keto-gulonate, a precursor of L-ascorbic acid, in a recombinant *G. oxydans* strain. Appl Environ Microbiol 1997; 63:454-60.
65. Sauer M, Porro D, Mattanovich D, Branduardi P. Microbial production of organic acids: expanding the markets. Trends Biotechnol 2008; 26:100-8.
66. Sanchez S, Demain AL. Metabolic regulation and overproduction of primary metabolites. Microb Biotechnol 2008; 1:283-319.

67. Wendisch VF, Bott M, Eikmanns BJ. Metabolic engineering of *Escherichia coli* and *Corynebacterium glutamicum* fot biotechnological production of organic acids and amino acids. Curr Opin Microbiol 2006; 9:268-74.
68. Causey TB, Zhou S, Shanmugam KT, Ingram LO. Engineering the metabolism of *Escherichia coli* W3110 for the conversion of sugar to redox-neutral and oxidized products: homoacetate production. Proc Natl Acad Sci USA 2003; 100:825-32.
69. Parekh SR, Cheryan M. High concentrations of acetate with a mutant strain of *C. thermoaceticum*. Biotechnol Lett 1994; 16:139-42.
70. Fukaya M, Tayama K, Tamaki T, Tagami H, Okumura H, Kawamura Y, et al. Cloning of the membrane-bound aldehyde dehydrogenase gene of *Acetobacter polyoxogenes* and improvement of acetic acid production by use of the cloned gene. Appl Environ Microbiol 1989; 55:171-6.
71. Saitoh S, Ishida N, Onishi T, Tokuhiro K, Nagamori E, Kitamoto K, et al. Genetically engineered wine yeast produces a high concentration of L-lactic acid of extremely high optical purity. Appl Environ Microbiol 2005; 71:2789-92.
72. Patnaik R, Louie S, Gavrilovic V, Perry K, Stemmer WPC, Ryan CM, et al. Genome shuffling of Lactobacillus for improved acid tolerance. Nat Biotechnol 2002; 20:707-12.
73. John RP, Gangadharan D, Madhavan Nampoothiri K. Genome shuffling of *Lactobacillus delbrueckii* mutant and *Bacillus amyloliquuefaciens* through protoplastic fusion for L-latic acid production from starchy wastes. Bioresour Technol 2008; 99:8008-15.
74. Zhou S, Yomano LP, Shanmugam KT, Ingram LO. Fermentation of 10% (w/v) sugar to D(-)-lactate by engineered *Escherichia coli* B. Biotechnol Lett 2003; 27:1891-6.
75. Ishida N, Suzuki T, Tokuhiro K, Nagamori E, Onishi T, Saitoh S, et al. D-Lactic acid production by metabolically engineered *Saccharomyces cerevisiae*. J Biosci Bioeng 2006; 101:172-7.
76. Skory CD. Lactic acid production by *Rhizopus oryzae* transformants with modified lactate dehydrogenase activity. Appl Microbiol Biotechnol 2004; 64:237-42.
77. Saito S, Ishida N, Onishi T, Tokuhiro K, Nagamori E, Kitamoto K, et al. Genetically engineered wine yeast produces a high concentration of L-lactic acid of extremely high optical purity. Appl Environ Microbiol 2005; 71:2789-92.
78. Porro D, Bianchi MM, Brambilla L, Menghini R, Bolzani D, Carrera V, et al. Replacement of a metabolic pathway for large scale production of lactic acid from engineered yeasts. Appl Environ Microbiol 1999; 65:4211-5.
79. Lee SJ, Song H, Lee SY. Genome-based metabolic engineering of *Mannheimia succiniciproducens* for succinic acid production. Appl Environ Microbiol 2006; 72:1939-48.
80. Lin H, Bennett GN, San KY. Fed-batch culture of a metabolically engineered *Escherichia coli* strain designed for high-level succinate production and yield under aerobic conditions. Biotechnol Bioeng 2005; 90:775-9.
81. Sanchez AM, Bennett GN, San KY. Novel pathway engineering design of the anaerobic central metabolic pathway in *Escherichia coli*. Metab Eng 2005; 7:229-39.
82. Vemuri GN, Eiteman MA, Altman E. Succinate production in dual-phase *Escherichia coli* fermentations depends on the time of transition from aerobic to anaerobic conditions. J Ind Microbiol Biotechnol 2002; 28:325-32.
83, Chandran SS, Yi J, Draths KM, Von Daeniken R, Weber W, Frost JW. Phosphoenolpyruvate availability and the biosynthesis of shikimic acid. Biotechnol Prog 2003; 19:808-14.
84. Kraemer M, Bongaerts J, Bovenberg R, Kremer S, Mueller U, Orf S, et al. Metabolic engineering for microbial production of shikimic acid. Metab Eng 2003; 5:277-83.
85. Liu X, Yang ST. Metabolic engineering of *Clostridium tyrobutyricum* for butyric acid fermentation. Proc 229th ACS National Meeting San Diego 2005; Abstract 70.
86. Neufeld RJ, Peleg Y, Rokem JS, Pines O, Goldberg I. L-Malic acid formation by immobilized *Saccharomyces cerevisiae* amplified for fumarase. Enzyme Microb Technol 1991; 13:991-6.
87. Picataggio S, Rohver T, Deander K, Lanning D, Reynolds R, Mielenz J, et al. Metabolic engineering of *Candida tropicalis* for the production of long-chain dicarboxylic acids. Nat Biotechnol 1992; 10:894-8.
88. Ingram LO, Conway T, Clark DP, Sewell GW, Preston JF. Genetic engineering of ethanol production in *Escherichia coli*. Appl Environ Microbiol 1987; 53:2420-5.
89. Doran JB, Ingram LO. Fermentation of crystalline cellulose to ethanol by *Klebsiella oxytoca* containing chromosomally integrated *Zymomonas mobilis* genes. Biotechnol Prog 1993; 9:533-8.
90. Dien BS, Nichols NN, O'Bryan PJ, Bothast RJ. Development of new ethanologenic *Escherichia coli* strains for fermentation of lignocellulosic biomass. Appl Biochem Biotechnol 2000; 84-86:181-96.
91. Shi DJ, Wang CL, Wang KM. Genome shuffling to improve thermotolerance, ethanol tolerance and ethanol productivity of *Saccharomyces cerevisiae*. J Ind Microbiol Biotechnol 2009; 36:139-47.
92. Hou L. Improved production of ethanol by novel gene shuffling in *Saccharomyces cerevisiae*. Appl Biochem Biotechnol 2010; 160:1084-93.
93. Wei P, Li Z, He P, Lin Y, Jiang N. Genome shuffling in the ethanologenic yeast *Candida krusei* to improve acetic acid tolerance. Biotechnol Appl Biochem 2008; 49:113-20.
94. Papanikolaou S, Ruiz-Sanchez P, Pariset B, Blanchard F, Fick M. High production of 1,3-propanediol from industrial glycerol by a newly isolated *Clostridium butyricum* strain. J Biotechnol 2000; 77:191-208.
95. Sanford K, Valle F, Ghirnikar R. Pathway engineering through rational design. Tutorial: Designing and building cell factories for biobased production. Gen Eng News 2004; 24:44-5.
96. Nakamura C, Whited G. Metabolic engineering for the microbial production of 1,3 propanediol. Curr Opin Biotechnol 2003; 14:454-9.
97. Kaup B, Bringer-Meyer S, Sahm H. Metabolic engineering of *Escherichia coli*: construction of an efficient biocatalyst for D-mannitol formation in a whole-cell biotransformation. Appl Microbiol Biotechnol 2004; 64:333-9.
98. Kaup B, Bringer-Meyer S, Sahm H. D-Mannitol formation from D-glucose in a whole-cell biotransformation with recombinant *Escherichia coli*. Appl Microbiol Biotechnol 2005; 69:397-403.
99. Ladero V, Ramos A, Wiersma A, Goffin P, Schanck A, Kleerbezem M. High-level; production of the low-calorie sugar sorbitol by *Lactobacillus plantarum* through metyabolic engineering. Appl Environ Microbiol 2007; 73:1864-72.
100. Miyagawa K, Kimura H, Nakahama K, Kikuchi M, Doi M, Akiyama S, et al. Cloning of the *Bacillus subtilis* IMP dehydrogenase gene and its application to increased production of guanosine. Nat Biotechnol 1986; 4:225-8.
101. Matthews PD, Wurtzel ET. Metabolic engineering of carotenoid accumulation in *Escherichia coli* by modulation of the isoprenoid precursor pool with expression of deoxyxylulose phosphate synthase. Appl Microbiol Biotechnol 2000; 53:396-400.
102. Deng M-D, Severson DK, Grund AD, Wassink SL, Burlingame RP, Berry A, et al. Metabolic engineering of *Escherichia coli* for industrial production of glucosamine and N-acetylglucosamine. Metab Eng 2005; 7:201-14.
103. Mermelstein LD, Papoutsakis ET, Petersen DJ, Bennett GN. Metabolic engineering of *Clostridium acetobutylicum* ATCC 824 for increased solvent production by enhancement of acetone formation enzyme activities using a synthetic operon. Biotechnol Bioeng 1993; 42:1053-60.
104. Bermejo LL, Welker NE, Papoutsakis ET. Expression of *Clostridium acetobutylicum* ATCC 824 genes in *Escherichia coli* for acetone production and acetate detoxification. Appl Environ Microbiol 1998; 64:1079-85.
105. Lein J. The Panlabs penicillin strain improvement program. In: Vanek Z, Hostalek Z, eds. Over production of Microbial Metabolites; Strain Improvement and Process Control Strategies. Boston, MA: Butterworth Publishers 1986; 105-39.
106. Kennedy J, Turner G. γ-(L-α-aminoadipyl)-L-cysteinyl-D-valine synthetase is a rate limiting enzyme for penicillin production in *Aspergillus nidulans*. Mol Gen Genet 1996; 253:189-97.
107. Hamlyn PF, Ball C. Recombination studies with *Cephalosporium acremonium*. In: Sebek OK, Laskin AI, eds. Genetics of Industrial Microorganisms. Washington, DC: American Society for Microbiology 1979; 185-91.
108. Skatrud PL, Fisher DL, Ingolia TD, Queener SW. Improved transformation of *Cephalosporium acremonium*. In: Alacevic M, Hranueli D, Toman Z, eds. Genetics of Industrial Microorganisms, Part B. Zagreb: Pliva 1987; 111-9.
109. Skatrud PL, Tietz AJ, Ingolia TD, Cantwell CA, Fisher DL, Chapman JL, et al. Use of recombinant DNA to improve production of cephalosporin C by *Cephalosporium acremonium*. Nat Biotechnol 1989; 7:477-85.
110. Wesseling AC, Lago B. Strain improvement by genetic recombination of cephamycin producers, *Nocardia lactamdurans* and *Streptomyces griseus*. Devel Indust Microbiol 1981; 22:641-51.
111. Bignell DRD, Tahlan K, Colvin KR, Jensen SE, Leskiw BK. Expression of *ccaR*, encoding the positive activator of cephamycin C and clavulanic acid production in *Streptomyces clavuligerus*, is dependent on *bldG*. Antimicrob Agents Chemother 2005; 49:1529-41.
112. Chary VK, de la Fuente JL, Leitao AL, Liras P, Martin JF. Overexpression of the *lat* gene in *Nocardia lactamdurans* from strong heterologous promoters results in very high levels of lysine-6-aminotransferase and up two-fold increase in cephamycin C production. Appl Microbiol Biotechnol 2000; 53:282-8.
113. Cantwell C, Beckmann R, Whiteman P, Queener SW, Abraham EP. Isolation of deacetoxycephalosporin C from fermentation broths of *Penicillium chrysogenum* transformants: construction of a new fungal biosynthetic pathway. Proc R Soc Lond (Biol) 1992; 248:283-9.
114. Crawford L, Stepan AM, Mcada PC, Rambosek JA, Conder MJ, Vinci VA, et al. Production of cephalosporin intermediates by feeding adipic acid to recombinant *Penicillium chrysogenum* strains expressing ring expansion activity. Nat Biotechnol 1995; 13:58-62.
115. Velasco J, Adrio JL, Moreno MA, Diez B, Soler G, Barredo JL. Environmentally safe production of 7-aminodeacetoxycephalosporanic acid (7ADCA) using recombinant strains of *Acremonium chrysogenum*. Nat Biotechnol 2000; 18:857-61.
116. Luo H, Yu H, Qiang L, Shen Z. Cloning and co-expression of D-amino acid oxidase and glutaryl-7-aminocephalosporanic acid acylase genes in *Escherichia coli* Enzyme Microb Technol 2004; 35:514-8.

117. Choi KP, Kim KH, Kim JW. Strain improvement of clavulanic acid producing *Streptomyces clavuligerus*. Proc 10th Internat Symp Biol Actinomycetes (ISBA) (Beijing) 1997; 12:9.
118. Li R, Townsend CA. Rational strain improvement for enhanced clavulanic acid production by genetic engineering of the glycolytic pathway in *Streptomyces clavuligerus.* Metab Eng 2006; 8:240-52.
119. Perez-Redondo RA, Rodriguez-Garcia A, Martin JF, Liras P. Deletion of the *pyc* gene blocks clavulanic acid biosynthesis except in glycerol-containing medium: evidence for two different genes in formation of the C3 unit. J Bacteriol 1999; 181:6922-8.
120. Paradkar AS, Aiodoo KA, Jensen SE. A pathway-specific transcriptional activator regulates late steps of clavuklanic acid biosynthesis in *Streptomyces clavuligerus*. Mol Microbiol 1998; 27:831-43.
121. Perez-Llarena FJ, Liras P, Rodriguez-Garcia A, Martin JF. A regulatory gene (*ccaR*) required for cephamycin and clavulanic acid production in *Streptomyces clavuligerus*: amplification results overproduction of both β-lactam compounds. J Bacteriol 1997; 179:2053-9.
122 Baltz RH, McHenney MA, Cantwell CA, Queener SW, Solenberg PJ. Applications of transposition mutagenesis in antibiotic producing streptomyces. Ant v Leeuwenhoek 1997; 71:179-87.
123. Solenberg PJ, Cantwell CA, Tietz AJ, McGilvray D, Queener SW, Baltz RH. Transposition mutagenesis in *Streptomyces fradiae*: identification of a neutral site for the stable insertion of DNA by transposon exchange. Gene 1996; 16:67-72.
124. Yamashita F, Hotta K, Kurasawa S, Okami Y, Umezawa H. Antibiotic formation by interspecific protoplast fusion in streptomycetes and emergence of drug resistance by protoplast regeneration. Proc 4th Internat Symp Genet Indust Microorgs (Kyoto) 1982; 20:108.
125. Gomi S, Ikeda D, Nakamura H, Naganawa H, Yamashita F, Hotta K, et al. Isolation and structure of a new antibiotic, indolizomycin, produced by a strain SK2-52 obtained by interspecies fusion treatment. J Antibiot 1984; 37:1491-4.
126 Traxler P, Schupp T, Wehrli W. 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 1982; 35:594-601.
127. Okanishi M, Suzuki N, Furuta T. Variety of hybrid characters among recombinants obtained by interspecific protoplast fusion in streptomycetes. Biosci Biotechnol Biochem 1996; 60:1233-8.
128. Galm U, Shen B. Expressionof biosynthetic gene clusters in heterologous hosts for natural product production and combinatorial biosynthesis. Exp Opin Drug Disc 2006; 1:409-37.
129. Mendez C, Salas JA. On the generation of novel anticancer drugs by recombinant DNA technology: the use of combinatorial biosynthesis to produce novel drugs. Comb Chem High Throughput Screen 2003; 6:513-26.
130. Rodriguez E, McDaniel R. Combinatorial biosynthesis of antimicrobials and other natural products. Curr Opin Microbiol 2001; 4:526-34.
131. Trefzer A, Blanco G, Remsing L, Kunzel E, Rix U, Lipata F, et al. Rationally designed glycosylated premithramycins: hybrid aromatic polyketides using genes from three different biosynthetic pathways. J Amer Chem Soc 2002; 124:6056-62.
132. McAlpine JB, Yuan JS, Brown DP, Grebner KD, Whittern DN, Buko A, et al. New antibiotics from genetically engineered actinomycetes. 1.2-Norerythromycins, isolation and structural determination. J Antibiot 1987; 40:1115-22.
133. Weber JM, Leung JO, Swanson SJ, Idler KB, McAlpine JB. An erythromycin derivative produced by targeted gene disruption in *Saccharopolyspora erythrea*. Science 1991; 252:114-7.
134. Donadio S, McAlpine JB, Sheldon PJ, Jackson M, Katz L. An erythromycin analog produced by reprogramming of polyketide synthesis. Proc Natl Acad Sci USA 1993; 90:7119-23.
135. Donadio S, Staver MJ, McAlpine JB, Swanson SJ, Katz L. Modular organization of genes required for complex polyketide biosynthesis. Science 1991; 252:675-9.
136. McDaniel R, Thamchaipenet A, Gustafsson C, Fu H, Betlach M, Betlach M, et al. Multiple genetic modifications of the erythromycin polyketide synthase to produce a library of novel "unnatural" natural products. Proc Natl Acad Sci USA 1999; 96:1846-51.
137. Pacey MS, Dirlam JP, Geldart RW, Leadlay PF, McArthur HAI, McCormick EL, et al. Novel erythromycins from a recombinant *Saccharopolyspora erythraea* strain NRRL 23338 pIGI I. Fermentation, isolation and biological activity. J Antibiot 1998; 51:1029-34.
138. Xue Q, Ashley G, Hutchinson CR, Santi DV. A multi-plasmid approach to preparing large libraries of polyketides. Proc Natl Acad Sci 1999; 96:11740-5.
139. Zhou L, Ahlert J, Xue Y, Thorson JS, Sherman DH, Liu H-W. Engineering a methymycin/pikromycin-calicheamicin hybrid: construction of two new macrolides carrying a designed sugar moiety. J Amer Chem Soc 1999; 121:9881-2.
140. Mendez C, Salas JA. Altering the glycosylation pattwern of bioactive compounds. Trends Biotechnol 2001; 19:449-56.
141. Arisawa A, Kawamura N, Narita T, Kojima I, Okamura K, Tsunekawa H, et al. Direct fermentative production of acyltylosins by genetically-engineered strains of *Streptomyces fradiae*. J Antibiot 1996; 49:349-54.
142. Epp JK, Huber MLB, Turner JR, Goodson T, Schoner BE. Production of a hybrid macrolide in *Streptomyces ambofaciens* and *Streptomyces lividans* by introduction of a cloned carbomycin biosynthetic gene from *Streptomyces thermotolerans*. Gene 1989; 85:293-301.
143. Hara O, Hutchinson CR. A macrolide 3-O-acyltransferase gene from the midecamycin-producing species *Streptomyces mycarofaciens*. J Bacteriol 1992; 174:5141-4.
144. Strohl WR, Bartel PL, Connors NC, Woodman RH. Expression of polyketide biosynthesis and regulatory genes in heterologous streptomycetes. J Indust Microbiol 1991; 7:163-74.
145. Bartel PL, Zhu CB, Lampel JS, Dosch DC, Connors NC, Strohl WR, et al. Biosynthesis of anthraquinones by interspecies cloning of actinorhodin biosynthesis genes in streptomycetes: clarification of actinorhodin gene functions. J Bacteriol 1990; 172:4816-26.
146. Stachelhaus T, Schneider A, Marahiel M. Rational design of peptide antibiotics by targeted replacement of bacterial and fungal domains. Science 1995; 269:69-72.
147. Van Lanen SG, Shen B. Microbial genomics for the improvement of natural product discovery. Curr Opin Microbiol 2006; 9:252-60.
148. Jenke-Kodama H, Sandmann A, Müller R, Dittmann E. Evolutionary implications of bacterial polyketide synthases. Mol Biol Evol 2005; 22:2027-39.
149. Mills SD. When will the genomics investment pay off for antibacterial discovery? Biochem Pharmacol 2006; 71:1096-102.
150. Knowles J, Gromo G. Target selection in drug discovery. Nat Revs/Drug Disc 2003; 2:63-9.
151. Zazopoulos E, Hwang K, Staffa A, Liu W, Bachmann BO, Nonaka K, et al. A genomics-guided approach for discovering and expressing cryptic metabolic pathways. Nat Biotechnol 2003; 21:187-90.
152. Glass JI, Belanger AE, Robertson GT. *Streptococcus pneumonia* as a genomics platform for broad spectrum antibiotic discovery. Curr Opin Microbiol 2002; 5:338-42.
153. Huynen MA, Diaz L, Bork P. Differential genome display. Trends Genet 1997; 13:389-90.
154. Huynen M, Dandekar T, Bork P. Differential genome analysis applied to the species-specific features of *Helicobacter pylori*. FEBS Lett 1998; 426:1-5.
155. Newman DJ, Shapiro S. Microbial prescreens for anticancer activity. SIM News 2008; 58:132-50.
156. Gong GL, Sun X, Liu XL, Hu W, Cao WR, Liu H, et al. Mutation and high-throughput screening method for improving the production of epothilones of Sorangium. J Ind Microbiol Biotechnol 2007; 34:615-23.
157. Askenazi M, Driggers EM, Holtzman DA, Norman TC, Iverson S, Zimmer DP, et al. Integrating transcriptional and metabolite profiles to direct the engineering of lovastatin-producing fungal strains. Nat Biotechnol 2003; 21:150-6.
158. Lee JY, Hwang YS, Kim SS, Kim ES, Choi CY. Effect of a global regulatory gene, *afsR2*, from *Streptomyces lividans* on avermectin production in *Streptomyces avermitilis*. Biosci Bioeng 2000; 89:606-8.
159. Hwang YS, Kim E-S, Biró S, Choi C-Y. Cloning and analysis of a DNA fragment stimulating avermectin production in various *Streptomyces avermitilis* strains. Appl Environ Microbiol 2003; 69:1263-9.
160. Ikeda H, Takada Y, Pang C-H, Tanaka H, Omura S. Transposon mutagenesis by Tn4560 and applications with avermectin-producing *Streptomyces avermitilis*. J Bacteriol 1993; 175:2077-82.
161. Zhang X, Chen Z, Li M, Wen Y, Song Y, Li J. Construction of ivermectin producer by domain swaps of avermectin polyketide synthase in *Streptomyces avermitilis*. Appl Microbiol Biotechnol 2006; 72:986-94.
162. Demain AL, Vaishnav P. Production of recombinant proteins by microbes and higher organisms. Biotechnol Adv 2009; 27:297-306.
163. Rayder RA. Expression systems for process and product improvement. BioProc Internat 2008; 6:4-9.
164. Choi BK, Bobrowicz P, Davidson RC, Hamilton SR, Kung DH, Li H, et al. Use of combinatorial genetic libraries to humanize N-linked glycosylation in the yeast *Pichia pastoris.* Proc Natl Acad Sci USA 2003; 100:5022-7.
165. Morrow KJ Jr. Grappling with biologic manufacturing concerns. Gen Eng Biotechnol News 2009; 29:54-5.
166. Barnard GC, Henderson GE, Srinivasan S, Gerngross TU. High level recombinant protein expression in *Ralstonia eutropha* using T7 RNA polymerase based amplification. Protein Expr Purif 2004; 38:264-71.
167. Werten MWT, van den Bosch TJ, Wind RD, Mooibroek H, De Wolf FA. High-yield secretion of recombinant gelatins by *Pichia pastoris.* Yeast 1999; 15:1087-96.
168. Morrow KJ Jr. Strategic protein production. Gen Eng Biotechnol News 2007; 27:50-4.
169. Andersen DC, Krummen L, Recombinant protein expression for therapeutic applications. Curr Opin Biotechnol 2002; 13:117-23.
170. Wrotnowski C. Animal cell culture; novel systems for research and production. Gen Eng News 1998; 18:13-37.
171. Zhang J, Robinson D. Development of animal-free, protein-free and chemically-defined media for NS0 cell culture. Cytotechnology 2005; 48:59-74.
172. Decaria P, Smith A, Whitford W. Many considerations in selecting bioproduction culture media. BioProc Internat 2009; 7:44-51.
173. Scott C, Montgomery SA, Rosin LJ. Genetic engineering leads to microbial, animal cell and transgenic expression systems. BIO Internat Conv 2007; 27-34.
174. Morrow KJ Jr. Improving protein production processes. Gen Eng & Biotechnol News 2007; 27:44-7.
175. Ryll T. Antibody production using mammalian cell culture-How high can we push productivity? Proc SIM Ann Mtg Prog Abstr, San Diego 2008; 146:101.

176. Meyer HP, Biass J, Jungo C, Klein J, Wenger J, Mommers R. An emerging star for therapeutic and catalytic protein production. BioProc Internat 2008; 6:10-21.
177. CocoMartin JM, Harmsen MM. A review of therapeutic protein expression by mammalian cells. BioProcess Internat 2008; 6:28-33.
178. Jarvis LM. A technology bet. DSM's pharma product unit leverages its biotech strength to survive in a tough environment. Chem Eng News 2008; 86:30-1.
179. Agathos SN. Production scale insect cell culture. Biotechnol Adv 1991; 9:51-68.
180. Knight P. Baculovirus vectors for making proteins in insect cells. ASM News 1991; 57:567-70.
181. Cowan D. Industrial enzyme technology. Trends Biotechnol 1996; 14:177-8.
182. Falch E. Industrial enzymes-developments in production and application. Biotech Adv 1991; 9:643-58.
183. Vaishnav P, Demain AL. Industrial biotechnology overview. In: Schaechter M, Lederberg J, eds. Encyclopedia of Microbiology, 3rd edition, Oxford: Elsevier 2009; 335-48.
184. Wackett LP. Bacterial biocatalysis: stealing a page from nature's book. Nat Biotechnol 1997; 15:415-6.
185. Palva I. Molecular cloning of α-amylase gene from *Bacillus amyloliquefaciens* and its expression in *Bacillus subtilis*. Gene 1982; 19:81-7.
186. O'Neill GP, Kilburn DG, Warren RAJ, Miller RC Jr. Overproduction from a cellulase gene with a high guanosine-plus-cytosine content in *Escherichia coli*. Appl Environ Microbiol 1986; 52:737-43.
187. Shoemaker S, Schweickart V, Ladner M, Gelfand D, Kwok S, Myambo K, et al. Molecular cloning of exocellobiohydrolase I derived from *Trichoderma reesei* strain L27. Bio/Technology 1983; 1:691-6.
188 Van Brunt J. Fungi: the perfect hosts? Bio/Technology 1986; 4:1057-62.
189. Mondou F, Shareck F, Morosoli R, Kleupfel D. Cloning of the xylanase gene of *Streptomyces lividans*. Gene 1986; 49:323-9.
190. Komatsubara S, Taniguchi T, Kisumi M. Overproduction of aspartase of *Escherichia coli* K-12 by molecular cloning. J Biotechnol 1986; 3:281-91.
191. Elander RP. Genetic engineering applications in the development of selected industrial enzymes and therapeutic proteins. In: Sankaran R, Manja KS, eds. Microbes for Better Living. Mysore, India: Defense Food Research Laboratory 1995; 619-28.
192. Van Hartinsveldt W, van Zeijl CM, Harteeld GM, Gouka RJ, Suykerbuyk M, Luiten RG, et al. Cloning, characterization and overexpression of the phytase-encoding gene (phyA) of *Aspergillus niger*. Gene 1993; 127:87-94.
193 Mayer H, Collins J, Wagner F. Cloning of the penicillin G-acylase gene of *Escherichia coli* ATCC 11105 on multicopy plasmids. In: Weetall HH, Royer GP, eds. Enzyme Engineering, vol. 5. New York: Plenum 1980; 61-9.
194. Van den Burg B, Vriend G, Veltman O, Venema G, Eijsink VGH. Engineering an enzyme to resist boiling. Proc Natl Acad Sci USA 1998; 95:2056-60.
195. Ness JE, Welch M, Giver L, Bueno M, Cherry JR, Borchert TV, et al. DNA shuffling of subgenomic sequences of subtilisin. Nat Biotechnol 1999; 17: 893-6.
196. Jaeger KE, Reetz MT. Directed evolution of enantioselective enzymes for organic chemistry. Curr Opin Chem Biol 2000; 4:68-73.
197. Suenaga H, Mitsokua M, Ura Y, Watanabe T, Furukawa K. Directed evolution of biphenyl dioxygenase: emergence of enhanced degradation capacity for benzene, toluene and alkylbenzenes. J Bacteriol 2001; 183:5441-4.
198. Song JK, Rhee JS. Enhancement of stability and activity of phospholipase A(1) in organic solvents by directed evolution. Biochim Biophys Acta 2001; 1547:370-8.
199. Raillard S, Krebber A, Chen Y, Ness JE, Bermudez E, Trinidad R, et al. Novel enzyme activities and functional plasticity revealed by recombining highly homologous enzymes. Chem Biol 2001; 8:891-8.
200. Kurtzman AL, Govindarajan S, Vahle K, Jones JT, Heinrichs V, Patten PA. Advances in directed protein evolution by recursive genetic recombination: applications to therapeutic proteins. Curr Opin Biotechnol 2001; 12:361-70.
201. Marshall SH. DNA shuffling: induced molecular breeding to produce new generation long-lasting vaccines. Biotechnol Adv 2002; 20:229-38.
202. Locher CP, Soong NW, Whalen RG, Punnonen J. Development of novel vaccines using DNA shuffling and screening strategies. Curr Opin Mol Ther 2004; 6:34-9.
203. Tobin MB, Gustafsson C, Huisman GW. Directed evolution: the 'rational' basis for 'irrational' design. Curr Opin Struct Biol 2000; 10:421-7.
204. Crameri A, Whitehorn A, Stemmer WPC. Improved green fluorescent protein by molecular evolution using DNA shuffling. Nat Biotechnol 1996; 14:315-9.
205. Chen X, Wei P, Fan L, Yang D, Zhu X, Shen W, et al. Generation of high-yield rapamycin-producing strains through protoplast-related techniques. Appl Microbiol Biotechnol 2009; 83:507-12.
206. Weathers PJ, Elkholy S, Wobbe KK. Artemisinin: the biosynthetic pathway and its regulation in *Artemisia annua*, a terpenoid-rich species. In Vitro Cell & Devel Biol Plant 2006; 42:309-17.
207. Martin VJJ, Pitera DJ, Withers ST, Newman JD, Keasling JD. Engineering a metabolic pathway in *Escherichia coli* for production of terpenoids. Nat Biotechnol 2003; 21:796-802.
208. Ro D-K, Paradise EM, Ouellet M, Fisher KJ, Newman KL, Ndubgu JM, et al. Production of the antimalarial drug precursor artemisinic acid in engineered yeast. Nature 2006; 440:940-3.
209. Lenihan JR, Tsuruta H, Diola D, Renninger NS, Regentin R. Developing an industrial artemisinic acid fermentation process to support the cost-effective production of antimalarial artemisinin-based combination therapies. Biotechnol Prog 2008; 24:1026-32.
210. Jin ZH, Xu B, Lin SZ, Jin QC, Cen PL. Enhanced production of spinosad in *Saccharopolyspora spinosa* by genome shuffling. Appl Biochem Biotechnol 2009; (ahead of print).
211. Bigelas R. Industrial products of biotechnology: application of gene technology. In: Jacobson GK, Jolly SO, eds. Biotechnology, vol. 7b. Weinheim: VCH 1989; 230-59.
212. Tseng YH, Ting WY, Chou HC, Yang BY, Chun CC. Increase of xanthan production by cloning xps genes into wild-type *Xanthomonas campestris*. Lett Appl Microbiol 1992; 14:43-6.
213. Letisse F, Chevallereau P, Simon JL, Lindley ND. Kinetic analysis of growth and xanthan gum production with *Xanthomonas campestris* on sucrose, using sequentially consumed nitrogen sources. Appl Microbiol Biotechnol 2001; 55:417-22.
214. Becker A, Katzen F, Puehler A, Ielpi L. Xanthan gum biosynthesis and application: a biochemical/genetic perspective. Appl Microbiol Biotechnol 1998; 50:145-52.