OVERPRODUCTION STRATEGIES FOR MICROBIAL SECONDARY METABOLITES: A REVIEW

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ABSTRACT

The formation of secondary metabolites is regulated by nutrients, growth rate, feedback control, enzyme inactivation, and enzyme induction. Regulation is influenced by unique low molecular mass compounds, transfer RNA, sigma factors and gene products formed during post-exponential development. The synthases of secondary metabolism are often coded by clustered genes on chromosomal DNA and infrequently on plasmid DNA. Strategies for overproduction of microbial products can be based on microbial response (Elicitors, quorum sensing), genetic engineering, metabolic engineering and ribosome engineering. Also molecular genetic improvement methods include amplification of SM biosynthetic genes, inactivation of competing pathways, disruption or amplification of regulatory genes, manipulation of secretory mechanisms, expression of a convenient heterologous protein, combinatorial biosynthesis.

Key words: Secondary metabolites, Overproduction, Microbial, Strategies, Genetic.

INTRODUCTION

Secondary metabolites are organic compounds that are not directly involved in the normal growth, development or reproduction of an organism (Anon, 2008). Secondary metabolites often play an important role in defense systems of different organisms (Stamp N, 2003). Humans use secondary metabolites as medicines, flavorings, and recreational drugs. Microbial secondary metabolites include antibiotics, pigments, toxins, effectors of ecological competition and symbiosis, pheromones, enzyme inhibitors, immunomodulating agents, receptor antagonists and agonists, pesticides, antitumor agents and growth promoters of animals and plants. They have a major effect on the health, nutrition and economics of our society. This paper discusses in detail the regulation of secondary metabolites, strategies for overproduction of such metabolites with the aim of highlighting strategies based on genetically modification methods.

\textbf{Regulation of secondary metabolites production}

The formation of secondary metabolites is regulated by nutrients, growth rate, feedback control, enzyme inactivation, and enzyme induction. Regulation is influenced by unique low molecular mass compounds, transfer RNA, sigma factors and gene products formed during post-exponential development. The synthesis of secondary metabolites are often encoded by clustered genes on chromosomal DNA and infrequently on plasmid DNA. Unlike primary metabolism, the pathways of
secondary metabolism are still not understood to a great degree and thus provide opportunities for basic investigations of enzymology, control and differentiation. Secondary metabolism is brought on by exhaustion of anutrient, biosynthesis or addition of an inducer, and/or by agrowth rate decrease. These events generate signals which affect a cascade of regulatory events resulting in chemical differentiation (secondary metabolism) and morphological differentiation (morphogenesis). The signal is often a low molecular weight inducer which acts by negative control, i.e. by binding to and inactivating a regulatory protein (repressor protein/receptor protein) which normally prevents secondary metabolism and morphogenesis during rapid growth and nutrient sufficiency. Nutrient/growth rate/inducer signals presumably activate a “master gene” which either acts at the level of translation by encoding a rare tRNA, or by encoding a positive transcription factor. Such master genes control both secondary metabolism and morphogenesis. At a second level of regulatory hierarchy genes could exist which control one branch of the cascade, i.e. either secondary metabolism or morphogenesis but not both. In the secondary metabolism branch, genes at a third level could control formation of particular groups of secondary metabolites. At a fourth level there may be genes which control smaller groups, and finally, fifth level genes could control individual biosynthetic pathways; these are usually positively acting but some act negatively. There are also several levels of hierarchy on the morphogenesis branch. The second level could include genes which control aerial mycelium formation in filamentous organisms plus all the sporulation genes lower in the cascade. Each third level locus could control a particular stage of sporulation. Some of these loci code for sigma factors. Feedback regulation also is involved in secondary metabolite control (Demain AL, 1998).

1. STRATEGIES FOR OVER-PRODUCTION OF MICROBIAL PRODUCTS

1.1. Microbial Response (Quorum Sensing, Elicitation)

1.1.1. Elicitors

Environmental abiotic and biotic stress factors have been proved to effect variety of responses in microbes. Elicitors, as stress factors, induce or enhance the biosynthesis of secondary metabolites added to a biological system (Raina S et al, 2011). They are classified into various groups based on their nature and origin: physical or chemical, biotic or abiotic. Initial studies on elicitation of secondary metabolites were carried out on plant cells and extended, over the years, to bacteria, animal cell cultures and filamentous fungi. Abiotic stress (abiotic elicitors) imposed by pH improves pigment production by Monascus purpureus and antibiotic production by Streptomyces spp. Traditionally carbohydrates have been used as carbon sources in fermentation processes. They have also been used widely in small amounts (mg L\(^{-1}\)) as elicitor molecules in bacterial and fungal fermentations for overproduction of commercially important secondary metabolites. In one approach to improve production, the effect of carbohydrate-biotic elicitors (oligosaccharides, oligomannuronate, oligoguluronate and mannan-oligosaccharides) on a variety of fungal systems: Penicillium spp., Ganoderma spp., Corylopsis spp., and bacterial cultures: Streptomyces spp., Bacillus spp. for production of antibiotics, enzymes, pigments and changes in morphology was investigated (Raina S et al, 2011).

1.1.2. Quorum Sensing

Quorum sensing is the communication between cells through the release of chemical signals when cell density reaches a threshold concentration (critical mass. Under these conditions, they sense the presence of other microbes. This process, investigated for more than 30 years, was first discovered in Gram-negative bacteria, and then in Gram-positive bacteria and dimorphic fungi (Raina S et al, 2011). The quorum sensing signals differ in

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1. Morphogenesis (from the Greek morphê shape and genesis creation, literally, ”beginning of the shape”) is the biological process that causes an organism to develop its shape. It is one of three fundamental aspects of developmental biology along with the control of cell growth and cellular differentiation (Anon, 2012).
different microbial systems; examples are acylhomoserine lactones, modified or unmodified peptides, complex γ -butyrolactone molecules and their derivatives. A number of physiological activities of microbes (e.g. symbiosis, competence, conjugation, sporulation, biofilm formation, virulence, motility and the production of various secondary metabolites) are regulated through the quorum-sensing. There is great potential for the use of this communication process for industrial exploitation. Filamentous fungi are a main microbial source for production of pharmaceutical and biotechnological products. However, until recently, very little was reported in the literature regarding quorum sensing phenomena in these fungi. Scientists explored, for the first time, the possibility of overproduction of fungal metabolites in response to the supplementation of liquid cultures by variety of quorum sensing molecules. *Bacillus licheniformis* widely present in the environment. Its metabolic diversity has resulted in its use for production of enzymes, antibiotics and fine chemicals. Bacteriocin produced by *B. licheniformis* is a polypeptide antibiotic active against Gram positive and some Gram negative bacteria. Bacteriocin is also used as animal feed additive (Raina S et al, 2011). Sclerotiorin synthesized by *Penicillium sclerotiorum* a phospholipase A2 inhibitor has been classified as an octaketide. Sclerotiorin has also been studied for its cholesterol ester transfer protein (CETP) inhibitory activity and recently, the extracts from *Penicillium sclerotiorum* have been studied for their activity against methicillin resistant *Staphylococcus aureus* (MRSA) (Raina S et al, 2011).

Precursors often stimulate production of secondary metabolites either by increasing the amount of a limiting precursor, by inducing a biosynthetic enzyme (synthase) or both. These are usually amino acids but other small molecules also function as inducers. The most well-known are the auto-inducers which include butyrolactones (butanolides) of the actinomycetes, N-acylhomoserine lactones of Gram-negative bacteria, oligopeptides of Gram-positive bacteria, and B-factor (3’- [1-butyolphosphoryl] adenosine) of *Amycolatopsis mediterranei*.

The actinomycete butanolides exert their effects via receptor proteins which normally repress chemical and morphological differentiation (secondary metabolism and differentiation into aerial mycelia and spores respectively) but, when reacted with butanolide, can no longer function. Homoserine lactones of Gram-negative bacteria function at high cell density and are structurally related to the butanolides. They turn on plant and animal virulence, light emission, plasmid transfer, and production of pigments, cyanide and β-lactam antibiotics. They are made by enzymes homologous to LuxI, excreted by the cell, enter other cells at high density, bind to a LuxR homologue, the complex then binding to DNA upstream of genes controlled by “quorum sensing” and turning on their expression. Quorum sensing also operates in the case of the peptide pheromones of the Gram-positive bacteria. Here, secretion is accomplished by an ATP binding cassette (ABC transporter), the secreted pheromone being recognized by a sensor component of a two-component signal transduction system (Demain AL, 1998). The pheromone often induces its own synthesis as well as those proteins involved in protein/peptide antibiotic (including bacteriocins and lantibiotics) production, virulence and genetic competence. The B-factor of *A. Mediterranei* is an inducer of ansamycin (rifamycin) formation (Demain AL, 1998).

2. GENETIC ENGINEERING (STRAIN IMPROVEMENT)

Improvement of the productivity of commercially viable microbial strains is an important field in microbiology, especially since wildtype strains isolated from nature usually produce only a low level (1–100 g/ml) of antibiotics. Therefore, a great deal of effort and resources have been committed to improving antibiotic-producing strains to meet commercial requirements. Although classical methods are still effective even without using genomic information or genetic tools to obtain highly productive strains, these methods are always time and resource consuming. One of the current topics is to use microorganisms for bioremediation. Environmental protection efforts have been focused
on the development of more effective processes for the treatment of toxic wastes. Soil bacteria have a wide range of metabolic abilities that make them useful tools for mineralization of toxic compounds (Ochi K et al 2004).

2.1. Overproduction of Primary Metabolites

Overproduction Of Primary Metabolites based on genetic engineering is regulated by feedback inhibition by the end product of a particular pathway is suppressed by generation of auxotrophs (i.e. mutation to cause accumulation of metabolite of interest), mutants resistant to antimetabolites through modification of enzyme structure at allosteric site, modification of operator or regulator gene to express the enzyme constitutively (Barrios Gonzalez J et al, 2003).

2.2. Overproduction of Secondary Metabolites

Overproduction Of Secondary Metabolites based on genetic engineering is regulated by the structural genes (directly participating in their biosynthesis), regulatory genes, antibiotic resistance gene (immunizing responsible for their own metabolites) and genes involved in primary metabolism (affecting the biosynthesis of secondary metabolites). Improvement strain advantages include increasing yields of the desired metabolite, removal of unwanted co-metabolites, improving utilization of inexpensive carbon and nitrogen sources, alteration of cellular morphology to a form better suited for separation of the mycelium from the product and/or for improved oxygen transfer in the fermenter (Barrios Gonzalez J et al, 2003). Genetic engineering methods are divided into two groupsnamely: Classical genetic methods and Molecular genetic improvement methods.

2.3. Classical Genetic Methods

1. mutation and random selection
2. mutation and rational selection
3. Genetic recombination methods

Mutation: Mutant generation of the existing wild strains is the most practiced strategy for enhancing the yield of primary and secondary metabolites. Mutant generation has improved the yield of certain antibiotics by 15-400 times in comparison to wild strains.

2.3.1. Mutation and Random Selection

Relied on mutation, followed by random screening, then careful fermentation tests are performed and new improved mutants are selected. Physical mutagens such as UV-light or chemical mutagens such as N-methyl-N’–nitro-N-nitrosoguanidine and ethyl ethanesulphonate are used in these methods. Advantages of Classical genetic methods are simplicity, no need to sophisticated equipment, minimal specialized technical manipulation, effectiveness (rapid titer increases) the only drawback is labour intensive.

2.3.2. Mutation and Rational Selection (Directed Selection Techniques)

Selection for a particular characteristic of the desired genotype, different from the one of final interest, but easier to detect. Eliminate all undesirable genotypes, allowing very high numbers of isolates to be tested easily. Design of these methods requires: some basic understanding of the product metabolism and pathway regulation. For example addition a toxic precursor of penicillin to the agar medium of penicillin producing microorganisms prevents the growth of sensitive strains and only resistant mutants with more penicillin production propagated. The other example, addition of rose Bengal and thymol to the medium of carotenoid producing yeast exposed to visible light has been used to select carotenoid over producing strains (Barrios Gonzalez J et al, 2003).

2.3.3. Recombination Methods

Recombination by protoplast fusion between related species of fungi with genetic development (high levels of a SM) and new isolate (low levels of a new SM) results in high productivity of the newly identified SM from the two strains.

2.4. Molecular Genetic Improvement Methods

Requirement knowledge and tools to perform molecular genetic improvement include identification of biosynthetic pathway, adequate vectors and effective transformation protocols. The main strategies being used in molecular genetic improvement of SM producing strains are as follow:
2.4.1. Amplification of SMBiosynthetic Genes (Targeted duplication or amplification of SM production gene)

This strategy is divided into two approaches:

- Targeted gene duplication (or amplification):
  Identify a neutral site in the chromosome where genes can be inserted without altering the fermentation properties of the strain. Then the neutral site is cloned and incorporated into the vector with the antibiotic gene. After transformation, the gene is inserted into the chromosomal neutral site by homologous recombination. Example for neutral site cloning: targeted duplication of the tylF gene that encodes the rate limiting O3methylation of macrocin in the tylosin biosynthesis in an industrial production strain of *streptomyces radjiae*. Transformants that contained two copies of the tylF gene produced 60% more tylosin than the parental strain were developed (Barrios Gonzalez J et al, 2003).

- Whole pathway amplification

2.4.2. Inactivation of Competing Pathways

Block a pathway that competes for a common intermediate key precursors such as cofactors, reducing power and energy supply are being used to perform this strategy. Improved strains by this strategy could be able to channel the precursors to the SM biosynthesis. Transposon mutagenesis in actinomycetes, gene disruption, or inserting an antisense synthetic gene are used to perform such strategy. For example α3aminoadipic acid isa precursor of penicillin biosynthesis, also acts as branching point to lysine synthesis. Disruption of gene lys2 connects α3aminoadipic acid towards lysine has generated auxotrophs of amino acid with 100% increase in penicillin yields (Barrios Gonzalez J et al, 2003).

2.4.3. Disruption or Amplification of Regulatory Genes

Regulation at a molecular level is more complicated than identifying the biosynthetic pathway and cloning the corresponding genes. For example Amplification of a regulatory gene (ccaR1) has led to 3 fold overproduction of β-lactam compounds (*streptomyces clavuligerus*). Also disruption of negatively acting regulatory gene mmy of methylenoomycin biosynthesis has led to 17 fold overproduction of actinorhodine. In another investigation, Introduction of a single copy of the positively acting gene actII has led to 35 fold overproduction of actinorhodine (*streptomyces coelicolor*) (Barrios Gonzalez J et al, 2003).

2.4.4. Manipulation of Secretory Mechanisms

Several protein hyperproducing yeast strains have been constructed by increasing specific genes of secretion path (such as *askar2* and *pdi1* genes) or by disruption of genes like *pmr1* gene (Barrios Gonzalez J et al, 2003).

2.4.5. Expression of a Convenient Heterologous Protein

Incorporation a new enzymae in the strain (heterologous gene) that will lead to the formation of a new related product of industrial interest (Barrios Gonzalez J et al, 2003).

2.4.6. Combinatorial Biosynthesis

Development of novel antibiotics, by using non-conventional compounds as substrate for the biosynthetic enzymes of the microorganism. These enzymes can be modified or mutated in such a way as to increase their affinity for those unnatural substrates. Different activity modules of enzymes like polyketid synthases can be rearranged by genetic engineering to obtain a microbial strain that synthesizes an antibiotic with novel characteristics. For example expression of glycosyltransferase genes from *A. orientalis* in *Streptomyces toyoensis* (producer of the non-glycosylated hepta-peptid) generate novel products.

\[\text{ccaR is a regulatory gene that located within the cephameycin gene cluster of } S.\text{ clavuligerus, is linked to a gene (bfp) encoding a protein similar to a } b\text{-lactamase-inhibitory protein. Expression of ccaR is required for cephamecin and clavulanic acid biosynthesis in } S.\text{ clavuligerus (Pérez-Llarena F J et al 1997)}.\]

\[\text{In cell biology and protein biochemistry, heterologous expression means that a protein is experimentally put into a cell that does not normally make (i.e., express) that protein (Anon, 2011).}\]
monoglycosilated derivative (Barrios Gonzalez J et al, 2003).

3. METABOLIC ENGINEERING

Since the advent of recombinant DNA technology, genetic engineering of cells, particularly microorganisms, has been successfully practiced for the development of strains capable of overproducing recombinant proteins and small molecule chemicals. For the latter, strategies beyond simple genetic engineering are often required as they are synthesized through multiple intracellular reactions, which are further complicated by various factors including cofactor balance and regulatory circuits. Metabolic engineering can be defined as purposeful modification of cellular metabolism using recombinant DNA and other molecular biological techniques. Metabolic engineering considers metabolic and cellular system as an entirety and accordingly allows manipulation of the system with consideration of the efficiency of overall bioprocess, which distinguishes itself from simple genetic engineering. Furthermore, metabolic engineering is advantageous in several aspects, compared to simple genetic engineering or random mutagenesis, since it allows defined engineering of the cell, thus avoiding unnecessary changes to the cell and allowing further engineering if necessary. Many drugs and drug precursors found in natural organisms are rather difficult to synthesize chemically and to extract in large amounts. Metabolic engineering is playing an increasingly important role in the production of these drugs and drug precursors. This is typically achieved by establishing new metabolic pathways leading to the product formation, and enforcing or removing the existing metabolic pathways toward enhanced product formation. Recent advances in system biology and synthetic biology are allowing us to perform metabolic engineering at the whole cell level, thus enabling optimal design of a microorganism for the efficient production of drugs and drug precursors. Among the many successful examples of metabolic engineering, recent reports on the efficient production of L-valine, L-threonine, lycopene, antimalarial drug precursor, and benzylisoquinoline alkaloids represent how metabolic engineering can be performed to achieve desired goals (Yup Lee S et al, 2009).

Homofermentative lactic acid bacteria have a relatively simple metabolism completely focused on the rapid conversion of sugar to lactic acid. The general habitat of lactic acid bacteria are nutritious, high-sugar-containing environments. Under these conditions, the lactic acid bacteria have developed a typical metabolism that allows rapid sugar conversion and is devoid of most biosynthetic activities. Under normal food fermentation conditions, the main product of metabolism is lactic acid, but other products are formed as by-products, such as acetic acid, acetaldehyde, ethanol, and diacetyl, all contributing to the specific flavor of fermented products. The main function of this sugar metabolism is to generate the energy necessary for rapid growth and for maintenance of intracellular pH during acidification of the environment. The biosynthetic capacity of these food microorganisms is very limited. The building blocks for growth generally originate from hydrolysis of food protein. The bacteria possess an elaborate proteolytic system centered on the complete breakdown of protein fragments into free amino acids. The amino acids are subsequently taken up and used for cell protein synthesis or for modification reactions in the biosynthesis of other nitrogen compounds, such as vitamins and nucleotides. There is almost no overlap between the energy (carbon) metabolism and the biosynthesis (nitrogen) metabolism in lactic acid bacteria. This makes them ideal as targets for metabolic engineering. Either metabolism can be changed dramatically without influencing the other as long as energy generation or biosynthesis of cell material is undisturbed.

3.1. Production of Exopolysaccharides

Although exopolysaccharide (EPS) production is a result of biosynthetic pathway, and not an energy generating pathway, it is closely linked to the general glycolysis reactions. The sugar moieties in the polysaccharide repeating unit are supplied by sequential addition reactions of ‘activated’ sugar–nucleotides building blocks by specific glycosyltransferases. These sugar–nucleotides, such
as UDP–glucose, UDP–galactose and TDP–rhamnose, are all synthesized from glucose-1-phosphate as a general precursor. The conversion of the glycolytic intermediate glucose-6-phosphate to glucose-1-phosphate, catalyzed by the enzyme phosphoglucomutase (PGM), and the synthesis of UDP–glucose from glucose-1-phosphate, catalyzed by GalU, could very well be controlling points in EPS production. Preliminary studies in laboratory have already shown that overexpression of either the pgm or the galU gene results in increased accumulation of the UDP–glucose and UDP–galactose, respectively, in cells of *L. lactis*. Interestingly, EPS production by *L. lactis* is much lower when growing on fructose than on glucose or lactose as the energy source. This could be a result of the low activity of fructose biphosphatase, which is essential for growth (and EPS production) on fructose but not on the other sugars. Overexpression of the fbp gene in *L. lactis*, via the NICE system, led to increased intracellular levels of nucleotide sugars, accelerated growth and higher levels of EPS during growth on fructose.

### 3.2. Nitrogen Metabolism

Lactic acid bacteria that form the inherent flora of fermented foods usually have an intricate machinery for breakdown of protein. This has been most extensively studied in the dairy lactic acid bacteria such as *L. lactis* and several *Lactobacillus* spp. This proteolysis provides the lactic acid bacteria with the essential free amino acids for growth and, as a result, these bacteria have a very limited capacity for the biosynthesis of amino acids. Some remnants of these reactions remain in specific strains, most evident as amino acid converting reactions resulting in the generation of flavor components, for example, methanethiol as the product of methione metabolism. Some metabolic engineering on the level of increased proteolysis and/or flavor production has been undertaken in lactic acid bacteria (Figure 1). Because most of the fermentable substrates are rich in vitamins, nucleotides and minerals, the resident lactic acid bacteria generally have a limited biosynthetic capacity for these compounds. *Lactobacillus* is especially known for its inability to synthesize vitamins, such as folic acid, vitamin B12, panthotenic acid, and so on. In fact, these bacteria are used in the biological assays for these vitamins because the growth of these bacteria is strictly dependent on the presence of small amounts of these compounds. Still, some lactic acid bacteria are able to produce vitamins and it is interesting to speculate how production by these lactic acid bacteria can be enhanced.

### 3.3. Proteolysis

Proteolysis is an essential process for growth of lactic acid bacteria in milk. Already several years ago, increased growth rate of *L. lactis* in milk was observed upon overproduction of the cell-wall-associated proteinase PrtP. It was also reported that in the whole process of protein breakdown to peptides and subsequently to free amino acids, the uptake of larger peptides from the external medium to the inside of the cell, dictated by the oligopeptide transporter (OPP), was a crucial step in growth of *L. lactis* in milk. This was evident from the inability of OPP-negative mutants to grow on culture medium with casein as the sole source of amino acids. The complete breakdown of the oligopeptides to single amino acids, in lactic acid bacteria, is a result of the simultaneous action of a whole set of intracellular peptidases. These peptidases have overlapping substrate specificity and none of them are individually essential for growth on these peptides. This was elegantly shown where the genes coding for the intracellular peptidases were disrupted individually and in combinations of two, three, four and five different peptidase disruptions. Only when three or more peptidases were disrupted simultaneously, the growth of *L. lactis* in milk was clearly affected. The proteolysis and subsequent amino acid conversion by lactic acid bacteria is an essential process in flavor formation in cheese during the ripening process. Metabolic engineering of lactic acid bacteria, on the level of proteolysis, has been attempted in numerous occasions to improve flavor development in cheese. The most promising results, however, have not been gained by increased activity of the enzymes involved, but by increased release of some relevant enzymes into the culture medium. By directly controlling lysis of the lactic acid bacteria, resulting in release of intracellular peptidases and/or...
amino acid converting enzymes, increased flavor formation has been observed. The most effective example of accelerating the cheese ripening process by metabolic engineering, so far, has been the nisin-induced expression of bacteriophage lyssin and holin in *L. lactis*, resulting in complete lysis of the cells, complete release of peptidases and other enzymes and a sharp increase in production of free amino acids and flavor compounds in cheese.

![Figure 1](image)

**Figure 1**

*Overview of metabolic engineering on the level of proteolysis and biosynthesis of nitrogen compounds.*

*hol/lys, the holin and lysin from bacteriophage origin; OPP, oligopeptide transfer; PepX, different peptidases; PrtP, cell-wall proteinase*

### 3.4. Vitamin Production

As mentioned before, lactic acid bacteria have a very limited biosynthetic capability for the production of vitamins; however, there are certain exceptions. The yogurt bacterium *Streptococcus thermophilus* has been observed to produce folic acid which, in fact, stimulates the growth of the other yogurt bacterium, *Lactobacillus bulgaricus*. *L. lactis* also produces substantial amounts of folic acid during fermentation. Many of the genes coding for the pathway of folic acid biosynthesis have been identified in the genome of this bacterium. Also, genes for riboflavin (vitamin B2) and biotin (vitamin B6) biosynthesis have been identified in *L. lactis*. This would make it possible to engineer the production of these vitamins in these food-grade bacteria, just as recently reported for *B. subtilis*. Vitamin production processes by lactic acid bacteria would have huge advantages over the current used processes (by *Bacillus* or *Pseudomonas*) as they could also be implemented for *in situ* production processes, such as food fermentations (Hugenholtz J and Kleerebezem M, 1999).

### 4. RIBOSOME ENGINEERING

The discovery of microorganisms capable of tolerating, or growing on, high concentrations of organic solvents provides a potentially interesting avenue for development of genetically engineered organisms for treating hazardous wastes. Thus, strain improvement is crucially important to fully exploit the cell’s ability. Sinceresearchers found a dramatic activation of antibiotic production by a certain ribosomal mutation (a mutation in *rpsL* gene encoding theribosomal protein S12), they had an...
idea that bacterial gene expression may be changed dramatically by modulating their ribosomal proteins or rRNA, eventually leading to activation of inactive (silent) genes. In bacteria, the ribosome plays a special role for their own gene expression by synthesis of a bacterial alarmone, ppGpp. One of the most important adaptation systems for bacteria is the stringent response, which leads to the repression of stable RNA synthesis in response to nutrient limitation. The stringent response depends on the transient increase of hyperphosphorylated guanosine nucleotide ppGpp, which is synthesized from GDP and ATP by the relA gene product (ppGpp synthetase) in response to binding of uncharged tRNA to the ribosomal A site. Since bacterial secondary metabolism is often triggered by ppGpp when cells enter into stationary phase, it is important to take the stringent response into consideration in activating or enhancing the bacterial secondary metabolism.

4.1. Antibiotic Overproduction by rpsL (Ribosomal Protein S12) Mutations
4.1.1. Activation of Actinorhodin Production
Members of the genus Streptomyces produce a wide variety of secondary metabolites that include about half of the known microbial antibiotics. Advances in understanding the regulation of secondary metabolism in this genus have come from the studies of antibiotic production in *Streptomyces coelicolor* A3 and its close relative *Streptomyces lividans*. *S. coelicolor* produces at least four antibiotics, including the blue-pigmented polyketide antibiotic actinorhodin (Act). *S. lividans* normally does not produce Act, although the strain has a complete set of Act biosynthetic genes. However, Act production in this organism can be activated by the introduction of certain regulatory genes or by cultivation under specific conditions. A strain of *S. lividans*, TK24, has been found to produce a large amount of Act under normal culture conditions (Figure 2). Genetic analyses revealed that a streptomycin-resistant mutation, str-6, in TK24 is responsible for activation of Act synthesis and that str-6 is a point mutation in the rpsL gene encoding ribosomal protein S12, changing Lys-88 to Glu (K88E mutation). It was also shown that introduction of streptomycin-resistant mutations improves Act production in wild-type *S. coelicolor* (Figure 2) and circumvents the detrimental effects on Act production in certain developmental mutants (*relA*, *relC*, and *brcA*) of *S. coelicolor*.

**Figure 2.**
*Activation of antibiotic production by rpsL (encoding ribosomal protein S12) mutations in Streptomyces lividans 66 and Streptomyces coelicolor A3(2).* Blue color represents an antibiotic, actinorhodin. K88E means a mutation at lysine-88 altering glutamate.
These streptomycin-resistant mutations result in the alteration of the Lys-88 to Glu (K88E) or Arg(K88R) and Arg-86 to His (R86H) in the rpsL gene. In addition to these streptomycin-resistant rpsL mutations, apalromomycin-resistant rpsL mutation (P91S) also can activate Act production in S. coelicolor. These findings indicate that the antibiotic production (secondary metabolism) in Streptomycetes is significantly controlled by the translational machinery, that is, the "ribosome." Much progress has been made in elucidating the organization of antibiotic biosynthesis gene clusters in several Streptomyces species, and a number of pathway-specific regulatory genes have been identified, which are required for the activation of their cognate biosynthetic genes. In the Act biosynthetic gene cluster, actII-ORF4 plays such a pathway-specific regulatory role, and the expression level of this gene directly determines the productivity of Act. Western blot analysis using anti-ActII-ORF4 antibody showed that the expression of ActII-ORF4 protein was strongly enhanced in the Act-high-producing rpsL mutant strains. Furthermore, RT-PCR experiments revealed that the increase of this regulatory protein can be attributed to the enhanced expression of actII-ORF4 mRNA. Thus, certain rpsL mutations enhance expression of the actII-ORF4 gene, leading to massive production of Act.

4.2. Antibiotic Overproduction by rpoB (RNA Polymerase) Mutations

Antibiotic biosynthesis pathways and their genetic regulatory cascades comprise one of the most attractive fields in Streptomyces genetics and are important in considering strain improvement. Onset of the morphological differentiation and the secondary metabolism, including antibiotic production, are thought to be coupled and influenced by a variety of physiological and environmental factors. Antibiotic production in Streptomyces is generally growth phase dependent. Thus, the signal molecule for growth rate control, ppGpp, is suggested to play a central role in triggering the onset of antibiotic production in Streptomyces. Namely, the ribosomes play an essential role in adjusting gene expression levels by synthesizing ppGpp in response to nutrient limitation. There is a positive correlation between ppGpp and antibiotic biosynthesis: disruption of the ppGpp synthetase gene, relA, or a deletion mutation (designated as relC) in the ribosomal L11 protein gene has been shown to lead to a deficiency in ppGpp accumulation after amino acid depletion (so-called "relaxed" phenotype) accompanied by impairment in antibiotic production. The expression level of many genes is regulated by ppGpp, either positively or negatively. Many genetic studies in E. coli suggested that RNA polymerase (RNAP) is the target for ppGpp regulation. Genetic analysis reveals that four major functional domains exist in the RNAP β-subunit (Figure 3). The ppGpp sensitivity domain is close to another important domain of the RNAP β-subunit, the rifampicin (Rif)-binding domain.
The crystal structure clearly revealed that Rif-cluster I is involved in the *E. coli* RNAP active center. Therefore it is reasonable to consider that certain mutations in the Rif-binding domain could affect the activity of RNAP and then may affect the function of the adjacent ppGpp-binding domain. Researchers postulated that the impaired ability to produce antibiotic due to *relA* or *relC* mutation may be circumvented by introducing certain Rif-resistant (rif) mutations into the RNAP-subunit. This hypothesis is based on the notion that the mutated RNAPs may behave like "stringent" RNAP without ppGpp binding. The results from *rel* mutants of *S. coelicolor* A3(2) and *S. lividans* strongly supported this hypothesis. The Rif-resistant isolates from the *rel* mutants regained the ability to produce the colored antibiotic actinorhodin, and various types of point mutation were mapped in the so-called Rif-cluster I in the rpoB gene that encodes the RNAP β-subunit (Figure 3). More impressively, gene expression analysis revealed that the restoration of actinorhodin production in the relrif double mutant strains is accompanied by increased expression of the pathway-specific regulatory gene *actII-ORF4*, which normally decreased in the *rel* mutants. Accompanying the restoration of antibiotic production, the relrif mutants also exhibited a lower rate of RNA synthesis compared to the parental strain when grown in a nutritionally rich medium. Since the dependence of *S. coelicolor* A3 on ppGpp to initiate antibiotic production can apparently be bypassed by certain mutations in the RNAP, the mutant RNAP may function by mimicking the ppGpp-bound form (Figure 4). This proposal can be supported by the fact that the mutant RNAP behaved like "stringent" RNAP with respect to RNA synthesis, as demonstrated using cells growing in a nutritionally rich medium (Ochi K et al. 2004).
Figure 4

Hypothesis for ppGpp-independent antibiotic (actinohordin) production. ActII-ORF4 is the gene encoding a pathway specific regulatory protein Act-ORF4. β$^{rif}$ represents mutated β-subunit. relA and relC are mutations that block the synthesis of ppGpp.

4.3. Effect of str and rpoB Mutations in Various Bacteria

4.3.1. Antibiotic Overproduction

Members of the genera Streptomyces, Bacillus, and Pseudomonas are soil bacteria that produce a high number of agriculturally and medically important antibiotics. The development of rational approaches to improve the production of antibiotics from these organisms is therefore of considerable industrial and economic importance. The impairment in antibiotic production resulting from a relA or relC mutation (that causes a failure to synthesize ppGpp) could be completely restored by introducing mutations conferring resistance to streptomycin (str). It is apparent that acquisition of certain str mutations allows antibiotic production to be initiated without the requirement for ppGpp. This offers a possible strategy for improving antibiotic productivity. Indeed, in addition to actinorhodin production by S. coelicolor and S. lividans, introduction of a str mutation was effective in enhancing antibiotic production by various bacteria (Ochi K et al 2004).

4.3.2. Enzyme Overproduction

Introduction of drug-resistant mutations has also been verified to be effective in improving enzyme productivity. Several str mutants of B. subtilis were shown to produce an increased amount (20–30%) of α-amylase and protease. It is shown that rpoB mutations are also effective for overproduction (1.5-fold to 2-fold) of extracellular enzymes such as amylase and protease. Thus these methods may be applicable for overproduction of other enzymes produced by various microorganisms, especially at late growth phase (Ochi K et al 2004).

4.4. Future Prospects for Ribosome Engineering

Researchers demonstrated that a cell’s function can be altered dramatically by modulating the ribosome using a drug-resistance mutation technique. Their approach is characterized by focusing on ribosome malfunction at late growth phase (i.e., stationary phase). In summary, their novel breeding approach is based on two different aspects: modulation of the translational apparatus by induction of str and gen mutations, and modulation of the transcriptional apparatus by induction of a rif mutation (Figure 5). Modulation
of these two mechanisms may function cooperatively to increase antibiotic productivity. Introduction of mutations conferring resistance to fusidic acid (fus) or thiostrepton (tsp), also causes activation of antibiotic production as well as str mutation. Moreover, these fus and tsp mutations were found to give rise to an aberrant protein synthesis activity, as did the str mutant ribosome. Resistance to fusidic acid and thiostrepton is known to come frequently from a mutation in elongation factor G and ribosomal protein L11, respectively. However, no mutations were found within the genes encoding elongation factor G or ribosomal protein L11. It is therefore highly likely that these fus and tsp mutations are located on the genes encoding rRNAs. This is important because it implies the existence of a new way to modulate ribosomal function, in addition to ribosomal protein mutations (Ochi K et al 2004).

**Figure 5.**

*Scheme of “ribosome engineering” to activate cell’s ability*

**5. GENETIC TECHNIQUES USED TO INCREASE SECONDARY METABOLITE PRODUCTION**

Genetic techniques used to increase secondary metabolite production are shown in table 1. These compounds have a major effect on the health, nutrition and economics of our society. The best-known are the antibiotics. These remarkable groups 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 (Adrio JL and Demain AL, 2010).

Table 1

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<tr>
<th>Genetic techniques used to increase secondary metabolite production</th>
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<tr>
<td>Genetic techniques</td>
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<tr>
<td>Protoplast fusion</td>
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<td>Metabolic engineering</td>
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<td>Transposition</td>
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CONCLUSIONS

The combination of complementary technologies such as mutation and genetic recombination has 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 successful are metabolic engineering, ribosome engineering, combinatorial biosynthesis and molecular breeding techniques. Functional genomics, proteomics and metabolomics are now being exploited for the discovery of novel valuable small molecules for medicine and enzymes for catalysis (Adrio JL and Demain AL, 2010).

REFERENCES


