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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, immune modulating agents, receptor antagonists and agonists, pesticides, antitumor agentsand growth promoters of animals and plants. They have a majoreffect 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 modificationmethods.

Regulation of secondary metabolites production

The formation of secondary metabolites is regulated by nutrients, growth rate, feedback control, enzymeinactivation, and enzyme induction. Regulation is influencedby unique low molecular mass compounds, transfer RNA, sigmafactors and gene products formed during post-exponentialdevelopment.Thesynthesis of secondary metabolites areoftencoded by clustered genes on chromosomal DNA and infrequently on plasmid DNA. Unlike primary metabolism, thepathways of secondary metabolism are still not understood to a

great degree and thus provide opportunities for basicinvestigations of enzymology, control and differentiation. Secondary metabolism is brought on by exhaustion of anutrient, biosynthesis or addition of an inducer, and/or by a growth rate decrease. These events generate signals which affecta cascade of regulatory events resulting in chemical differentiation (secondary metabolism) and morphologicaldifferentiation (morphogenesis[1]). The signal is often a lowmolecular weight inducer which acts by negative control, i.e.by binding to and inactivating a regulatory protein (repressorprotein/receptorprotein) which normally prevents secondary metabolism and morphogenesis during rapid growth andnutrient sufficiency. Nutrient/growth rate/inducer signalspresumably 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 bothsecondary metabolism and morphogenesis. At a second levelof regulatory hierarchy genes could exist which control onebranch of the cascade, i.e. either secondary metabolism ormorphogenesis but not both. In the secondary metabolismbranch, genes at a third level could control formation ofparticular groups of secondary metabolites. At a fourth levelthere 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 controlaerial mycelium formation in filamentous organisms plus allthe sporulation genes lower in the cascade. Each third levellocus could control a particular stage of sporulation. Some ofthese loci code for sigma factors. Feedback regulation also isinvolved in secondary metabolite control (Demain AL, 1998).

[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).

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 ofresponses in microbes. Elicitors, as stress factors, induce or enhance the biosynthesis ofsecondary metabolites added to a biological system (Raina S et al, 2011). They are classified into variousgroups based on their nature and origin: physical or chemical, biotic or abiotic.Initial studies on elicitation of secondary metabolites werecarried out on plant cells and extended, over the years, to bacteria, animal cell cultures and filamentous fungi. Abiotic stress (abiotic elicitors) imposed bypH improves pigment production by *Monascuspurpureus* and antibiotic production by Streptomyces *spp*. Traditionallycarbohydrates have been used as carbon sources in fermentation processes. They havealso been used widely in small amounts (mg L^{-1}) as elicitor molecules in bacterial andfungal fermentations for overproduction of commercially important secondarymetabolites.In one approach to improve production, the effect of carbohydratebiotic elicitors(oligosaccharides, oligomannuronate, oligoguluronate and mannan- oligosaccharides) on variety of fungal systems: Penicillium*spp.*,Ganoderma*spp*., Corylopsis*spp*. and bacterialcultures: 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 celldensity reaches a threshold concentration (critical mass. Under these conditions, they sense the presence of other microbes. This process, investigated for more than30 years, was first discovered in Gram-negative bacteria, and then in Gram-positivebacteria and dimorphic fungi (Raina S et al, 2011). The quorum sensing signals differ in

different microbialsystems; examples are acyl-homoserine lactones, modified or unmodified peptides,complex γ -butyrolactone molecules and their derivatives.

A numberofphysiologicalactivities of microbes(e.g. symbiosis, competence, conjugation, sporulation, biofilmformation, virulence, motility and the production of various secondary metabolites) are regulated through the quorum-sensing.Thereis great potential for the use of this communication process for industrial exploitation.Filamentous fungi are a main microbial source for production of pharmaceutical andbiotechnological products. However, until recently, very little was reported in theliterature regarding quorum sensing phenomena in these fungi. Scientistsexplored, for thefirst time, the possibility of overproduction of fungal metabolites inresponse to the supplementation of liquid cultures by variety of quorum sensingmolecules.*Bacillus licheniformis*is widely present in the environment. Its metabolic diversity hasresulted in its use for production of enzymes, antibiotics and fine chemicals. bacteriocin produced by *B. licheniformis*is a polypeptide antibiotic active against Gram positiveand some Gram-negative bacteria. bacteriocinis also used as animal feed additive (Raina S et al, 2011). Sclerotiorin synthesized by *Penicilliumsclerotiorum*is a phospholipase A2 inhibitorand has been classified as an octaketide. Sclerotiorin has also been studied for itscholesterol ester transfer protein (CETP) inhibitory activity and recently, the extracts from *Penicilliumsclerotiorum* 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 byincreasing the amount of a limiting precursor, by inducing a biosynthetic enzyme (synthase) or both. These are usually amino acids but other small molecules alsofunction as inducers. The most well-known are the auto-inducers which include butyrolactones (butanolides) of the actinomycetes, N-acyl-homoserine lactones of Gram-negativebacteria, oligopeptides of Gram-positive bacteria, and B-factor (3'-[1-butylphosphoryl] adenosine) of *Amycolatopsismediterranei*.

Theactinomycetebutanolides exert their effects via receptor proteins which normally repress chemicaland morphological differentiation (secondary metabolism and differentiation intoaerial mycelia and spores respectively) but, when reacted with butanolide, can no longer function. Homoserine lactones of Gram-negative bacteria function athigh cell density and are structurally related to the butanolides. They turn on plantand animal virulence, light emission, plasmid transfer, and production of pigments, cyanide and β-lactam antibiotics. They are made by enzymes homologous to Lux1, excreted by the cell, enter other cells at high density, bind to a Lux Rhomologue, 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 thepeptide pheromones of the Gram-positive bacteria. Here, secretion is accomplishedby an ATP binding cassette (ABC transporter), the secreted pheromone beingrecognized 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 involvedin protein/peptide antibiotic (including bacteriocins and lantibiotics) production,virulence and genetic competence. The B-factor of *A. Mediterranei*is an inducer ofansamycin (rifamycin) formation (Demain AL, 1998).

2. GENETICENGINEERING (STRAINIMPROVMENT)

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 highlyproductive 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 Metabolitesbased on genetic engineering is regulated byfeed back 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 bythe 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

I. mutation and random selection
II. mutation and rational selection
III. 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 orchemicalmutagens such as N-methyl-N'–nitro-N-nitrosoguanidineand ethyl ethanesulphonate are used in these methods.Advantagesof 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. MutationandRational 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. RecombinationMethods

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 ImprovementMethods

Requirementknowledge and tools to perform molecular genetic improvementinclude,identification of biosynthetic pathway, adequate vectors and effective transformation protocols**.**The main strategies being used in molecular genetic improvement of SMproducing strains are as follow:

2.4.1. Amplification ofSMBiosynthetic 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 *tyl*F gene that encodes the rate limiting O-methylation of macrocin in the tylosin biosynthesis in an industrial production strain of *streptomycesfradiae*. Transformants that contained two copies of the *tyl*Fgene produced 60% more tylosin than the parental strain were developed (Barrios Gonzalez J et al, 2003).

- Whole pathway amplification

2.4.2. Inactivation ofCompeting 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. Transposonmutagenesis in actinomycetes, gene disruption, or inserting an antisense synthetic gene are used to perform suchstrategy.For example α-aminoadipic acidisa precursor of penicillin biosynthesis, also acts as branching point to lysine synthesis. Disruption of gene lys2 connects α-aminoadipic 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 ofRegulatoryGenes

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 (ccaR[1]) hasled to 3 fold overproduction of β-lactam compounds(in *streptomycesclavuligerus*). 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 act II has led to 35 fold over production actinorhodine (in*streptomycescoeli color*) (Barrios Gonzalez J et al, 2003).

2.4.4. Manipulation of Secretory Mechanisms

Several protein hyper producing yeast strains have been constructed by increasing specific genes of secretion path (such as*kar*2 and *pdi*1 genes) or by disruption of genes like *pmr*1gene (Barrios Gonzalez J et al, 2003).

2.4.5. Expression of a Convenient Heterologous Protein[2]

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. CombinatorialBiosynthesis

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 insuch 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 toyo*caensis (producer of the non-glycosylated hepta-peptid) generate novel monoglycosilated derivative (Barrios Gonzalez J et al, 2003).

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[1] *ccaR*is A regulatory gene that located within the cephamycin gene cluster of *Streptomyces clavuligerus*, is linked to a gene (*blp*) encoding a protein similar to a b-lactamase-inhibitory protein. Expression of *ccaR*is required for cephamycin and clavulanic acid biosynthesis in *S. Clavuligerus*(Pérez-Llarena F J et al 1997).

[2] 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).

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 relativelysimple metabolism completely focused on the rapid conversionof sugar to lactic acid. The general habitat of lacticacid bacteria are nutritious, high-sugar-containing environments.Under these conditions, the lactic acid bacteria havedeveloped a typical metabolism that allowrapidsugar conversion and is devoid of most biosynthetic activities.Under normal food fermentation conditions, the mainproduct of metabolism is lactic acid, but other products areformed 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 sugarmetabolism is to generate the energy necessary for rapidgrowth and for maintenance of intracellular pH during acidificationof theenvironment.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 centred on the complete breakdown of protein fragments into free aminoacids. The amino acids are subsequently taken up andused for cell-protein synthesis or for modification reactionsin the biosynthesis of other nitrogen compounds, such asvitamins and nucleotides.There is almost no overlap between the energy (carbon)metabolism and the biosynthesis (nitrogen) metabolism inlactic acid bacteria. This makes them ideal as targets formetabolic engineering. Either metabolism can be changeddramatically without influencing the other as long as energygeneration or biosynthesis of cell material is undisturbed.

3.1. Production of Exopolysaccharides

Although exopolysaccharide (EPS) production is a result of abiosynthetic pathway, and not an energy generating pathway,it is closely linked to the general glycolysis reactions. The sugarmoieties in the polysaccharide repeating unit are supplied bysequential addition reactions of ‘activated’ sugar–nucleotidebuilding blocks by specific glycosyltranferases. Thesesugar–nucleotides, such as UDP–glucose, UDP–galactose and TDP–rhamnose, are all synthesized from glucose-1-

phosphate as a general precursor. The conversion of theglycolytic 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 controllingpoints in EPS production. Preliminary studies in laboratoryhave already shown that overexpression of either the*pgm*or the *galU*gene results in increased accumulation of theUDP–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 forgrowth (and EPS-production) on fructose but not on theother sugars. Overexpression of the *fbp*gene in *L. lactis*, viathe NICE-system, led to increased intracellular levels ofnucleotide sugars, accelerated growth and higher levels ofEPS during growth on fructose.

3.2. Nitrogen Metabolism

Lactic acid bacteria that form the inherent flora infermented foods usually have an intricate machinery forbreakdown ofprotein. This has been most extensivelystudied in the dairy lactic acid bacteria such as *L. lactis* and several *Lactobacillus* spp. This proteolysis provides the lactic acid bacteriawith the essential free amino acids for growth and, as a result, these bacteria have a very limited capacity for thebiosynthesis of amino acids. Some remnants of these reactions remain in specific strains, most evident as amino acidconverting reactions resulting in the generation of flavor components, for example, methanethiol as the product of methione metabolism. Some metabolic engineering on thelevel of increased proteolysis and/or flavor production hasbeen undertaken in lactic acid bacteria (Figure1).Becausemost of the fermentable substrates are rich invitamins, nucleotides and minerals, the resident lacticacid bacteria generally have a limited biosynthetic capacityfor these compounds. *Lactobacillus* is especially knownfor its inability to synthesize vitamins, such as folic acid, vitamin B12, panthotenic acid, and so on. In fact, these bacteriaare used in the biological assays for these vitaminsbecause the growth ofthese bacteria is strictly dependent on the presence ofsmall amounts of these compounds.Still, some lactic acid bacteria are able to produce vitaminsand it isinteresting to speculate how production by these lacticacid bacteria can be enhanced.

3.3. Proteolysis

Proteolysis is an essential process for growth of lactic acidbacteria in milk. Already several years ago, increased growthrate of *L. lactis*in milk was observed upon overproduction ofthe cell-wall-associated proteinase PrtP. It was alsoreported that in the whole process of protein breakdown topeptides and subsequently to free amino acids, the uptake oflarger peptides from the external medium to the inside of thecell, dictated by the oligopeptide transporter (OPP), was acrucial step in growth of*L. lactis*in milk. This was evidentfrom the inability ofOPP-negative mutants to grow onculture 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 simultaneousaction of a whole set of intracellular peptidases.These peptidases have overlapping substrate specificityand none of them are individually essential for growth onthese peptides. This was elegantly shown where the genes coding for the intracellularpeptidases were disrupted individually and incombinations of two, three, four and five different peptidase-disruptions. Only when three or more peptidaseswere disrupted simultaneously, the growth of *L. lactis*in milk was clearly effected. The proteolysis and subsequent amino acid conversion bylactic acid bacteria is an essential process in flavor formation in cheese during the ripening process. Metabolicengineering of lactic acid bacteria, on the level of proteolysis, has been attempted in numerous occasions to improve flavor development in cheese. The most promisingresults, however, have not been gained by increasedactivity of the enzymes involved, but by increased releaseof some relevant enzymes into the culture medium. By directly controlling lysis of the lactic acid bacteria, resultingin release of intracellular peptidases and/or amino acidconverting enzymes, increased flavor formation has beenobserved. The most effective example of

acceleratingthe cheese ripening process by metabolic engineering, sofar, has been the nisin-induced expression of bacteriophagelysin and holin in *L. lactis,* resulting in complete lysis of the cells, complete release of peptidases and ofother enzymes and a sharp increase in production of freeamino acids and flavor compounds in cheese.

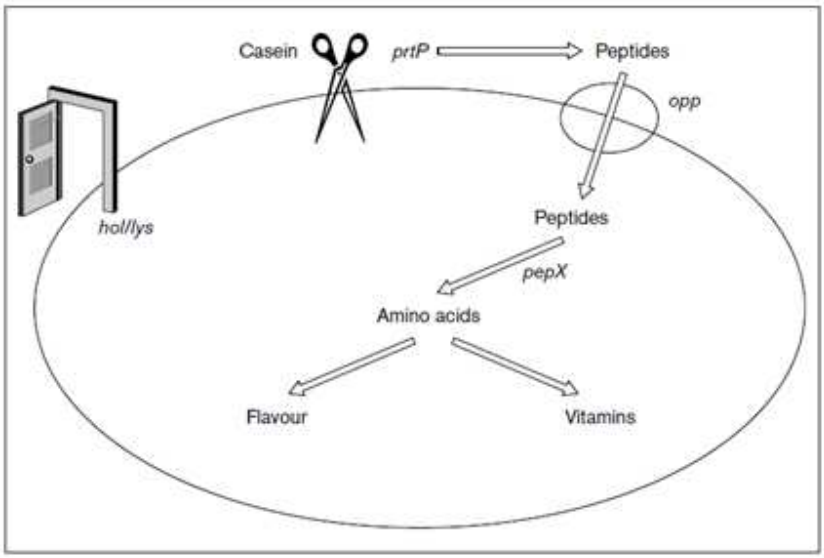

Figure 1
Overview of metabolic engineering on the level of proteolysis and biosynthesis of nitrogen compounds. hol/lys, the holin and lysin from bacteriophage origin; OPP, oligopeptide transfer; PepX, different peptidases; PrtP, cell- wall proteinase

3.4. Vitamin Production

As mentioned before, lactic acid bacteria have a very limitedbiosynthetic capability for the production of vitamins; however, there are certain exceptions. The yogurt bacterium *Streptococcus thermophilus* has been observed to producefolic acid which, in fact, stimulates the growth of theother 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 acidbiosynthesis 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 productionof these vitamins in these food-grade bacteria, just asrecently reported for *B. subtilis*. Vitamin productionprocesses by lactic acid bacteria would have huge advantagesover 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 potentiallyinteresting avenue for development of genetically engineeredorganisms for treating hazardous wastes. Thus, strain improvement iscrucially 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 the ribosomal protein S12), they had an idea that bacterialgene expression may be changed dramatically by modulating the ribosomal proteins

or rRNA, eventually leading to activation of inactive(silent) genes.In bacteria, the ribosome plays a special role for their own geneexpression by synthesis of a bacterial alarm one, ppGpp. One of the most important adaptation systems for bacteria is the stringentresponse, which leads to the repression of stable RNA synthesisin response to nutrient limitation. The stringentresponse depends on the transient increase of hyperphosphorylated guanosinenucleotideppGpp, which is synthesized from GDP and ATPby the *relA* gene product (ppGppsynthetase) in response to binding ofunchargedtRNA to the ribosomal A site. Since bacterial secondarymetabolism is often triggered by ppGpp when cells enter into stationaryphase, it is important to take the stringent response into considerationin activating or enhancing the bacterial secondary metabolism.

4.1. Antibiotic Overproduction by rpsL (RibosomalProtein S12) Mutations

4.1.1. Activation ofActinorhodin Production

Members of the genus Streptomyces produce a wide variety of secondarymetabolites that include about half of the known microbialantibiotics. Advances in understanding the regulation of secondarymetabolism 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 acomplete set of Act biosynthetic genes. However, Act production inthis organism can be activated by the introduction of certain regulatorygenes or by cultivation under specific conditions. A strain of *S. lividans*, TK24 has been found to produce a largeamount of Act under normal culture conditions (Figure2). Genetic analyses revealed that a streptomycin-resistant mutation,str-6, in TK24 is responsible for activation of Act synthesis andthat str-6 is a point mutation in the rpsL gene encoding ribosomal protein S12, changing Lys-88 to Glu (K88E mutation). It was alsoshown that introduction of streptomycin-resistant mutationsimproves Act production in wild-type *S. coelicolor* (Figure2) and circumvents the detrimental effects on Act production in certain developmental mutants (*relA, relC, and brgA*) of *S. coeliolor*.

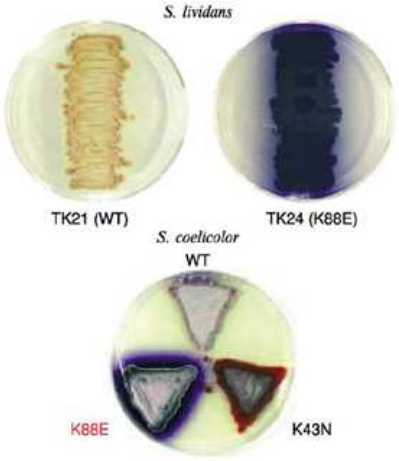

Figure 2.

Activation of antibiotic production by rpsL (encoding ribosomal protein S12) mutations in Streptomyces lividans 66 and Streptomyces coelicolorA3(2). Blue color represents an antibiotic, actinorhodin. K88E means a mutation at lysine-88 altering glutamate.

These streptomycin-resistant mutations result in the alterationof the Lys-88 to Glu (K88E) or Arg(K88R) and Arg-86 to His (R86H) intherpsL gene. In addition to these streptomycin-resistant *rpsL*mutations, aparomomycin-resistant *rpsL* mutation (P91S) also can activateAct production in *S. coelicolor*.Thesefindings indicate that the antibiotic production (secondary metabolism) in streptomycetes is significantly controlled by the translationalmachinery, that is, the ‘‘ribosome.’’Much progress has been made in elucidating the organization ofantibiotic 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 biosyntheticgenes. In the Act biosynthetic gene cluster, actII-ORF4 plays such a pathway-specific regulatory role, and the expressionlevel of this gene directly determines the productivity ofAct. Western blot analysisusing anti-ActII-ORF4 antibody showed that the expression of ActIIORF4protein was strongly enhanced in the Act-high-producing *rpsL* mutant strains. Furthermore, RT-PCR experiments revealed that the increase ofthis regulatory protein can be attributed to the enhanced expressionof actII-ORF4 mRNA. Thus, certain*rpsL* mutations enhance expression of the actII-ORF4 gene, leading tomassive production of Act.

4.2. Antibiotic Overproduction by rpoB (RNA Polymerase) Mutations

Antibiotic biosynthesis pathways and their genetic regulatory cascadescomprise one of the most attractive fields in Streptomyces geneticsand are important in considering strain improvement. Onset of themorphological differentiation and the secondary metabolism, including antibiotic production, are thought to be coupled and influenced bya variety of physiological and environmental factors. Antibiotic production in Streptomycetes 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 ofantibiotic production in Streptomyces. Namely, the ribosomes play anessential 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 (socalled‘‘relaxed’’ phenotype) accompanied by impairment in antibioticproduction. The expression level ofmany 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 fourmajor functional domains exist in the RNAP β-subunit (Figure 3). TheppGpp-sensitivity domain is close to another important domain of theRNAP β -subunit, the rifampicin (Rif)-binding domain.

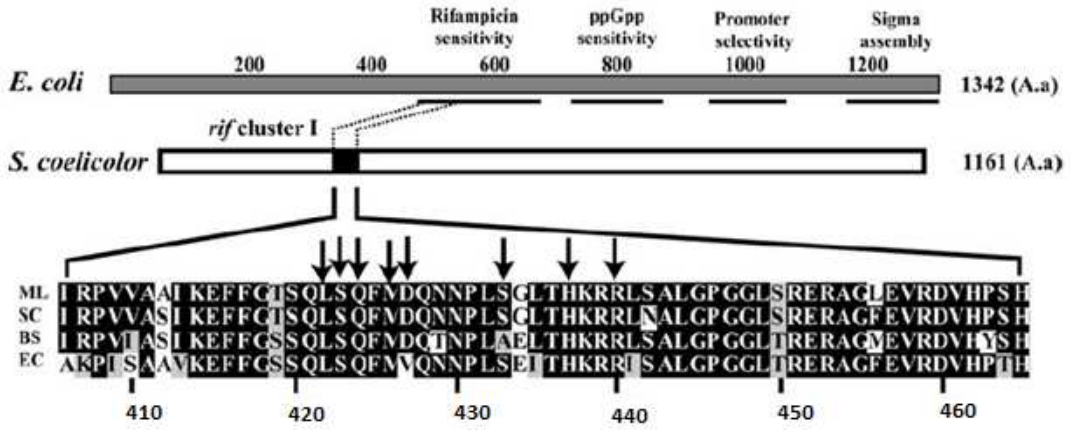

Figure 3.

Functional map of E.coli RNA polymerase β-subunit and location of rif cluster* I *within RNA polymerase β-subunit in relation to the previously suggested ppGpp-binding site in E.coli. Positions of the rif mutations found in the present study are designated by arrows. Numbering begins at the start codon of the open reading frame. ML, Mycobacterium leprae; SC, Streptomyces coelicolorA3(2); BS, Bacillus subtilis; EC, Escherichia coli.

The crystal structure clearly revealed that Rif-cluster I isinvolved in the *E. coli* RNAP active center. Therefore it is reasonableto consider that certain mutations in the Rif-binding domain couldaffect the activity of RNAP and then may affect the function of the adjacent ppGpp-binding domain. Researcher spostulated that the impaired ability to produce antibiotic due to the *relA* or *relC* mutation may be circumvented by introducing certainRif-resistant (rif) mutations into the RNAP-subunit. This hypothesisis based on a notion that the mutated RNAPs may behave like ‘‘stringent’’RNAP without ppGpp binding. The results from *rel* mutants of *S. coelicolor A3* 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 inthe so-called Rif-cluster I in the rpoB gene that encodes the RNAP β-subunit (Figure3). More impressively, gene expression analysis revealedthat the restoration of actinorhodin production in the relrif double mutant strains is accompanied by increased expression ofthe 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 ofRNA synthesis compared to the parental strain when grown in anutritionally rich medium. Since the dependence of *S. coelicolorA3* on ppGpp to initiate antibiotic production can apparently bebypassed by certain mutations in the RNAP, the mutant RNAP mayfunction by mimicking the ppGpp-bound form (Figure4). This proposalcan be supported by the fact that the mutant RNAP behaved like ‘‘stringent’’ RNAP with respect to RNA synthesis, as demonstratedusing cells growing in a nutritionally rich medium (Ochi K et al 2004).

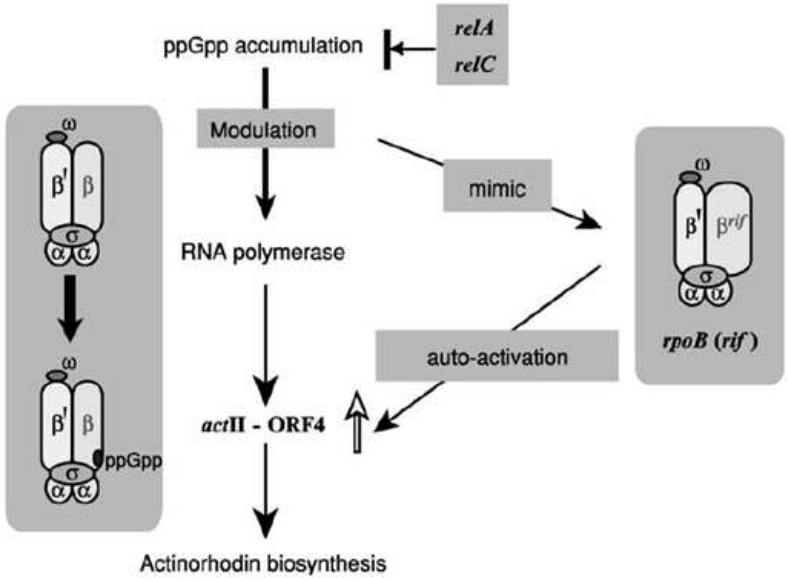

Figure 4

Hypothesis for ppGpp-independent antibiotic (actinohordin) production. ActII-ORF4 is the gene encodding 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 Pseudomonasare soil bacteria that produce a high number of agriculturally andmedically important antibiotics. The development of rational approachesto improve the production of antibiotics from these organismsis therefore of considerable industrial and economic importance.Theimpairment in antibiotic production resulting from a relA or relC mutation(that causes a failure to synthesize ppGpp) could be completelyrestored by introducing mutations conferring resistance to streptomycin(str). It is apparent that acquisition of certain strmutations allows antibiotic production to be initiated without therequirement for ppGpp. This offers a possible strategy for improvingthe antibiotic productivity.Indeed, in addition to actinorhodinproductionby S. coelicolor and S. lividans, introduction of a strmutationwas 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 beeffective 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 alsoeffective for overproduction (1.5-fold to 2-fold) of extracellular enzymessuch as amylase and protease. Thus these methods may beapplicable for overproduction of other enzymes produced by variousmicroorganisms, especially at late growth phase(Ochi K et al 2004).

4.4. Future ProspectsforRibosomeEngineering

Researchersdemonstrated that a cell's function can be altereddramatically by modulating the ribosome using a drug-resistance mutationtechnique. Their approach is characterized by focusing on ribosomalfunction at late growth phase (i.e., stationary phase). In summary, their novel breeding approach is based on twodifferent aspects, modulation of the translational apparatus by inductionof str and gen mutations, and modulation of the transcriptionalapparatus by induction of a rif mutation (Figure 5). Modulation

ofthese two mechanisms may function cooperatively to increase antibioticproductivity. Introduction of mutations conferring resistance to fusidic acid (fus) or thiostrept on (tsp), also causes activation of antibiotic production as well as str mutation. Moreover, these fus and tsp mutations were found to give rise to anaberrant protein synthesis activity, as did the str mutant ribosome. Resistance to fusidic acid and thiostrept on is known to come frequently from a mutationin elongation factor G and ribosomal protein L11, respectively.However, no mutations were found within the genes encoding elongationfactor 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 wayto modulate ribosomal function, in addition to ribosomal protein mutations (Ochi K et al 2004).

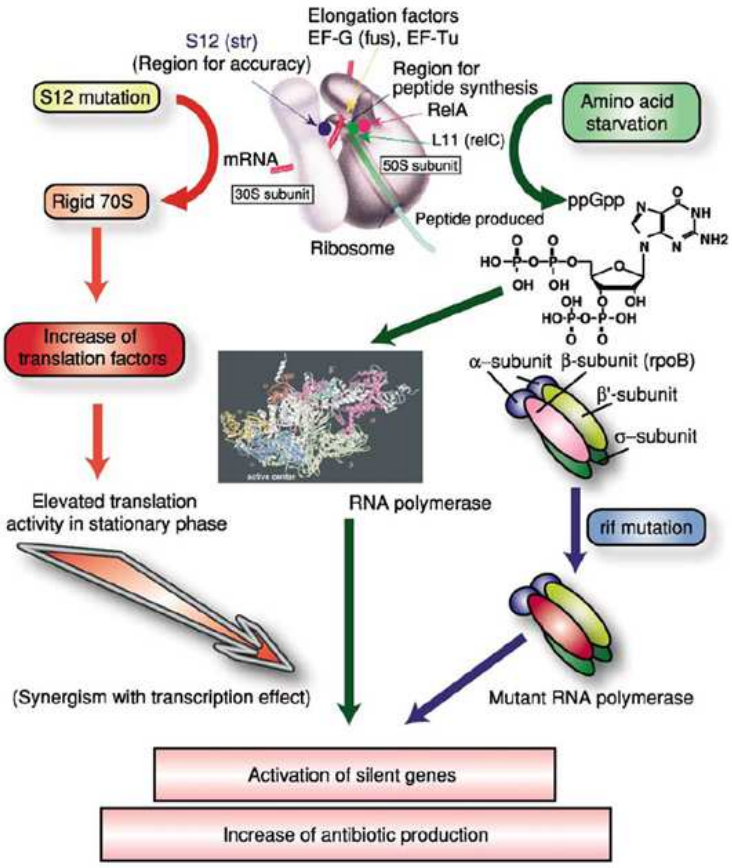

Figure 5.
Scheme of ''ribosome engineering'' to activate cell's ability

5. GENETICTECHNIQUES USED TO INCREASE SECONDARY METABOLITE PRODUCTION

Genetic techniques used to increase secondary metabolite productionare 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, immune modulating agents, receptor antagonists and agonists, pesticides, antitumor agents, immune suppressives, cholesterol-lowering agents, plant protectants and growth promotants of animals and plants (Adrio JL and Demain AL, 2010).

Table1

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

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).

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