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REVIEW ARTICLE

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Induction of microbial secondary metabolism

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Summary 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 γ-butyrolactones (butanolides) of the actinomycetes, N-acylhomoserine lactones of Gram-negative bacteria, oligopeptides of Gram-positive bacteria, and B-factor (3'-[1-butylphosphoryl] 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 complexed with the 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 Lux1, 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 casette (ABC transporter), the secreted pheromone being recognized by a sensor component of a two-component signal transduction system. 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.

Key words *Amycolatopsis mediterranei* · *Streptomyces* spp. · Butanolides · Quorum sensing · Secondary metabolism

Introduction

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. They often have unusual structures and their formation 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. 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 exhausion of a nutrient, biosynthesis or addition of an inducer, and/or by a growth rate decrease. These events generate signals which effect 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. This contribution focuses on the role of low molecular weight compounds which act to induce secondary metabolism.

Stimulation by precursors

In a number of secondary metabolite pathways, primary metabolites increase production of the final product. These effectors are often precursors and one has to determine whether the effect is merely due to an increase in precursor supply and/or includes induction of one or more synthases of the biosynthetic pathway. Stimulatory precursors that are also inducers include tryptophan for dimethylallyltryptophan synthetase in ergot alkaloid biosynthesis [33], leucine for bacitracin synthetase [19], methionine for ACV synthetase, cyclase and expandase in the cephalosporin pathway of *Cephalosporium acremonium* [68], lysine for lysine aminotransferase in the cephamycin pathway of *Streptomyces clavuligerus* [51], valine for valine dehydrogenase of the tylosin process in *Streptomyces fradiae* [41] and phenylacetate for the phenylacetate uptake system in penicillin G formation by *Penicillium chrysogenum* [15]. Branched amino acids stimulate nikkomycin synthesis [67] and phenylalanine induces benzodiazapene alkaloid formation [37] by unknown mechanisms. Precursor stimulation is also observed with glycine, phenylalanine, tyrosine and arginine in vancomycin fermentation [39]. Stimulation by something other than a precursor molecule is evident in the production of the polyketide, jadomycin B by *Streptomyces venezuelae*. Here, production is induced by ethanol or by one hour of heat shock, both of which are known to induce heat-shock proteins [11]. Jadomycin B synthesis in *S. venezuelae* is regulated by the product of the negatively-acting gene *jadR2* [65]. In the wildtype, jadomycin B formation requires carbon and nitrogen limitation in addition to inducible stresses such as ethanol addition, heat shock or phage infection. In *jadR2* disrupted mutants, the stress is not required; however ethanol still stimulates production.

γ-Butyrolactones of actinomycetes

Autoinducers include the γ-butyrolactones (butanolides) of the actinomycetes, the N-acylhomoserine lactones (HSLs) of Gram-negative bacteria, the oligopeptides of Gram-positive bacteria, and B-factor [3'-(1-butylphosphoryl)adenosine] of rifamycin production in *Amycolatopsis mediterranei* [29]. They function in development, sporulation, light emission, virulence, production of antibiotics, pigments and cyanide, plasmid-driven conjugation and competence for genetic transformation [21].

Of great importance in actinomycete fermentations is the inducing effect of endogenous γ-butyrolactones, e.g. A-factor (2-S-isocapryloyl-3R-hydroxymethyl-γ-butyrolactone). A-factor induces both morphological and chemical differentiation in *Streptomyces griseus* and *Streptomyces bikiniensis*, bringing on formation of aerial mycelia, conidia, streptomycin synthases and streptomycin. Conidia can actually form on agar without A-factor but aerial mycelia cannot [57]. The spores form on branches morphologically similar to aerial hyphae but they do not emerge from the colony surface.

In *S. griseus*, A-factor is produced just prior to streptomycin production and disappears before streptomycin is at its maximum level [22]. It induces at least ten proteins at the transcriptional level. One of these is streptomycin 6-phosphotransferase, an enzyme which functions both in streptomycin biosynthesis and in resistance. In an A-factor-deficient mutant, there is a failure of transcription of the entire streptomycin gene cluster [38]. Many other actinomycetes produce A-factor, or related γ-butyrolactones, which differ in the length of the side-chain. In those strains which produce antibiotics other than streptomycin, the γ-butyrolactones induce formation of the particular antibiotics that are produced, as well as morphological differentiation [18]. A group of five different γ-butyrolactones, termed virginiae butanolides (VBs), are produced by the virginiamycin producer, *Streptomyces virginiae* [32] and by other streptomycetes. Addition of synthetic autoregulator VB-C to *S. virginiae* at 8 or 11.5 h stimulates virginiamycin production by about 200% [66]. Pristinamycin production by *Streptomyces pristinaespiralis* is under control of A-factor type induction [45]. Commercial lactones are active but A-factor is 250 times more active. An active lactone produced extracellularly has been isolated from a production culture; its structure is not yet known. IM-2, the γ-butyrolactone of *Streptomyces* sp. FRI-5, previously known to be the autoinducer of blue pigment formation [64], is also involved in antibiotic production [20]. It turns off D-cycloserine production and turns on formation of blue pigment and four nucleoside antibiotics including showdomycin and minimycin. Although *Streptomyces coelicolor* does not produce A-factor, it does form up to six autoregulatory factors, at least one being a γ-butyrolactone [1, 6, 12].

The γ-butyrolactones exert their effects via receptor proteins which are repressors of chemical and morphological

differentiation. In *S. virginiae*, the butanolide binding protein BarA has an M_r of 26,000 [43]. In *S. griseus*, an A-factor-binding protein has been isolated [40] which is present in the cell at about 40 copies per genome [22].

A-factor receptor protein ArpA is encoded by *arpA* in *S. griseus* [44] that encodes a deduced protein of 29.1 kDa containing 276 amino acid residues which acts as a homodimer. Its N terminus contains an α-helix-turn-α-helix whose binding to DNA leads to repression of streptomycin production, of streptomycin resistance, and of formation of aerial mycelium and spores. The IM-2 binding protein FarA from *Streptomyces* sp. strain FR1-5 is a dimer composed of two 27 kDa subunits [52]. Butyrolactone binding proteins FarA, ArpA, BarA and that of *S. griseus* IFO 13350 are all proteins of about 26–29 kDa, possess helix-turn-helix DNA-binding motifs in their N termini but are very specific for their own autoinducer molecule [61, 63]. For example, VBs show no induction of streptomycin production by *S. griseus* and A-factor has no effect on virginiamycin production by *S. virginiae*.

When A-factor binds to its receptor, the protein is apparently removed from its DNA-binding site, allowing formation of aerial mycelia, conidia and streptomycin synthases. Mutants of *S. griseus* lacking A-factor-binding protein sporulate and produce streptomycin earlier than their parent and produce 10 times more streptomycin [5]. Early synthesis is also brought on by adding A-factor to the wild-type or cloning and expressing the structural gene of A-factor biosynthesis, *afsA*. It was shown a number of years ago [10] that A-factor, when present, stimulates the formation of transcripts of *aphD* (encoding the streptomycin biosynthetic and resistance enzyme, streptomycin-6-phosphotransferase), *strR* (encoding the regulatory gene of streptomycin biosynthesis) and *strB* (encoding the amidotransferase of the biosynthetic scheme). Of the promoters of these three genes, only that of *strR* is controlled by A-factor [59], which suggests that *strR* and/or its promoter controls expression of *aphD* and *strB*. A-factor, by removal of its binding protein from DNA, leads to the formation of a positively-acting transcription factor which binds to the *strR* regulatory region activating its expression [60]. A-factor seems to work as follows: in the presence of A-factor, the binding protein no longer can bind to the promoter of gene X. Protein X, which is thus derepressed, is an upstream positive transcription factor which binds to DNA 300–400 bp upstream of the transcriptional start point of *strR* and turns on transcription of *strR* and *aphD* [22]. Protein StrR is the positively-acting regulatory protein controlling streptomycin production, resistance and sporulation [7]. It activates the entire streptomycin biosynthetic pathway including *strB*, probably by an anti-termination mechanism [10]. It is assumed that most of the *strB* mRNA is prematurely terminated and that StrR suppresses the termination. The *aphD* gene, which encodes the streptomycin resistance determinant, is transcribed by read-through from the *strR* promoter.

Thirty six genes have been identified in *S. griseus* (and *Streptomyces glaucescens*) as being involved in biosynthesis, regulation, resistance, and export of streptomycin (or 5' hydroxystreptomycin) [47]. A-factor action works via *strR*, the gene encoding the regulatory protein, StrR. The protein is produced only in the presence of A-factor and has a molecular mass of 38 kDa in *S. griseus* and 46 kDa in *S. glaucescens*. The proteins exhibit 62.5% amino acid identity. Both have a helix-turn-helix DNA-binding motif and are positively-acting regulatory proteins. StrR controls expression of *strB1* which encodes the synthase, amidinotransferase. StrR interacts with an operator upstream of the promoter strB1p [50]. StrR biding sites have the consensus sequence GTTcGActG$(N)_{11}$CagTcGAAc and are often found in the genes of the streptomycin and hydroxystreptomycin clusters.

Gene *afsA* is the structural gene for A-factor formation and codes for an enzyme of 32.6 kDa. Virginiae butanolide A is biosynthesized by *Streptomyces antibioticus* from 2 moles of malonyl-CoA (from acetate), 1 mole of dihydroxyacetone-3-phosphate (from glycerol) and 1 mole of isovaleric acid (the starting unit) [54]. Extrapolating the pathway to that of A-factor biosynthesis, the precursors would be 3 moles of malonyl-CoA, 1 mole of a glycerol derivative, possibly dihydroxyacetone-3-phosphate, and 1 mole of a polyketide β-ketoacid such as isobutyryl-CoA. Horinouchi and Beppu [22] speculate that AfsA is the enzyme responsible for the condensation of the glycerol derivative and the polyketide β-ketoacid.

Homoserine lactones of Gram-negative bacteria

The inducer of carbapenem antibiotic biosynthesis in *Erwinia carotovora* is a modified HSL, i.e., N-(β-ketocaproyl)-L-homoserine lactone (KHL), which was previously known as VA1, the autoinducer of *luxR*, involved in bioluminescence in *Vibrio fischeri* [2]. HSLs function by "quorum sensing", i.e., when they reach a particular extracellular concentration due to high cell density, they act in bioluminescence, antibiotic biosynthesis, animal pathogenicity, plant pathogenicity, extracellular enzyme synthesis and conjugal transfer of the Ti plasmid [27]. Mutants of *E. carotovora* which can not produce the simple β-lactam antibiotic 1-carbapen-2-em-3-carboxylic acid are of two types. Group 1 non-producers form KHL which turn on antibiotic production by group 2 non-producers. Many other bacteria produce autoinducers, e.g. *Citrobacter freundii, Enterobacter agglomerans, Vibrio harveyi, Yersinia enterocolitica, Proteus mirabilis*, etc. In the cases of *Pseudomonas aeruginosa, E. carotovora, Erwinia herbicola*, and *Serratia marcescens*, the inducer has been shown to be KHL. HSLs are similar in structure to A-factor. HSLs also induce exoenzymes which are plant and animal virulence determinants in *E. carotovora* and *P. aeruginosa* respectively [28, 46]. N-(β-ketooctanoyl) L-HSL induces conjugal transfer of the Ti plasmid in *Agrobacterium tumefaciens* [26, 69], and acts as a starvation signal in *Escherichia coli* [25]. An HSL

is required for production of σ^s in *E. coli*, a sigma factor involved in stationary phase expression [24, 25]. Production of virulence factors (e.g., elastase, rhamnolipid) by the opportunistic human pathogen *P. aeruginosa*, cyanide production by *P. aeruginosa* and violacein formation by *Chromobacterium violaceum* are under control of the HSL induction system [36, 42, 62]. The Gram-negative autoinducers differ in sidechain length and in substitution at the β-carbon of the acyl sidechain [16]. They are made by proteins homologous to Lux1. These signals of high cell density are secreted, enter other cells and bind to a LuxR homologue, the complex then binding to DNA upstream of genes sensitive to quorum sensing [17].

Quorum-sensing peptides of Gram-positive unicellular bacteria

Quorum-sensing functions in Gram-positive unicellular bacteria include genetic competence (*Bacillus subtilis* and *Streptococcus pneumoniae*), virulence (*Staphylococcus aureus*) and protein/peptide antibiosis via lantibiotic and non-lantibiotic bacteriocin production [30]. The signal molecule (pheromone) is a secreted peptide that has been processed post-translationally and is sometimes further modified. Secretion is accomplished by an ATP-binding cassette (ABC) transporter. The secreted pheromone is recognized by the sensor component of a two-component signal transduction system. The pheromone also induces its own synthesis.

Bacteriocins are antibacterial proteins produced by bacteria which possess narrow specificities, i.e., they have bactericidal activity against microorganisms closely related to the producing microorganisms. Whereas bacteriocins of Gram-negative bacteria range in size from peptides to complex proteins of over 10^6 Da, often containing carbohydrates or lipids, Gram-positive bacteria produce small bacteriocins (usually less than 10 kDa) with broader specificity. They include unmodified peptides (lactococcins, pediocins, etc.) and modified peptides called lantibiotics (nisin [*Streptococcus lactis*], subtilin [*B. subtilis*], epidermin [*Staphylococcus epidermidis*], etc.). The lantibiotics are small, containing 32 to 34 residues, and have a broader spectrum of activity than most bacteriocins, acting by depolarizing energized cytoplasmic membranes of sensitive bacteria. They are of three types: (i) elongated, amphiphilic and pore-forming (e.g., nisin), (ii) globular phospholipase inhibitors (e.g., duramycins) and (iii) inhibitors of bacterial cell wall biosynthesis (e.g., actagardine) [53]. Lantibiotics are produced on ribosomes, unlike most antibiotics.

All bacteriocins of lactic acid bacteria are regulated temporally and production depends on two-component regulatory systems. IF, the autoinducer of *Lactobacillus sake* LTH673 which produces sakacin P, is found extracellularly and is a non-bacteriocin peptide of 19 amino acid residues; it presumably functions as the quorum-sensing signal in this organism [13]. Bacteriocin production in *Lactobacillus acidophilus* [4] and *Lactobacillus plantarum* [9] is induced by non-antibacterial proteins formed by these organisms. In *L. plantarum*, the gene encoding the inducer is part of an operon which also contains genes for a multicomponent regulatory signal transduction system including a histidine kinase and two response regulators. The gene encoding the bacteriocin is located near this operon.

Although lantibiotics are produced on ribosomes, they contain unusual post-translationally modified amino acids such as D-alanine, lanthionine, methyllanthionine, dehydrobutyrine and dehydroalanine. Serine and threonine, after incorporation into the peptide, are dehydrated to dehydroalanine and dehydrobutyrine, respectively. The latter two compounds can add to the SH group of a cysteine residue to form meso-lanthionine and β-methyllanthionine, respectively. Nisin is produced by *Lactococcus lactis* and is used commercially in food processing, being active against growth of Gram-positive bacteria and against the outgrowth of spores. The genes for the above antibiotics have been cloned and sequenced [3, 55]. Comparison of the gene sequences of nisin, subtilin and epidermin leads to the tentative conclusion that they evolved from a common ancestor. Once thought to be plasmid-borne, the nisin biosynthetic genes of *L. lactis* have been shown to reside on a novel chromosomal conjugative transposon, 70 kb in size [23]. Biosynthesis of nisin is directed by a gene cluster located on a 10 kbp DNA fragment of the nisin-sucrose transposon Tn*5276* [34]. It contains genes *nisA*, *nisB*, *nisT*, *nisC*, *nisI*, *nisP*, *nisR*, *nisK*, *nisF*, *nisE* and *nisG* in that order. The *nisA* gene encodes a translation product of 57 residues which includes a 23 residue leader peptide which is later eliminated to form the precursor of pre-nisin. The mRNA transcript may be a polycistronic message including sequences encoding the processing enzymes. Regulation of the formation of the precursor protein is a function of *nisK* and *nisR*, a two-component signal transduction system. This system ensures production during growth and not during stationary phase. Gene *nisK* encodes the histidine kinase which recognizes external nisin and phosphorylates protein NisR. Phosphorylated NisR activates the promoter of the nisin biosynthesis operon. Thus external nisin induces its own synthesis [14, 35, 48].

Genes *nisB* and *nisC* appear to encode the enzymes involved in the modifications to form the mature precursor, pre-nisin. Gene *nisT* presumably encodes an ABC transport protein involved in the movement of the mature precursor out of the cell. Gene *nisP* encodes the peptidase that cleaves the leader sequence after secretion of the pre-nisin into the medium [58]. Gene *nisI* is concerned with immunity of the producer organism to nisin. Its product appears to be an extracellular membrane-anchored lipoprotein. Expression of *nisI* in *L. lactis* increases resistance to nisin. Gene *nisA*, the structural gene, must be expressed along with *nisI* to attain nisin resistance [34]. Genes *nisFEG* encode a second putative ABC transporter involved in self-protection [56].

In addition to nisin A, the strain also produces nisin Z which differs from A by having asparagine at residue 27 instead of histidine. The cluster structure for nisin Z is *nisZBTCIPRKFEG*, split into two operons of *nisZBTCIPRK* and *nisFEG*, with two homologous promoters [49]. Mature nisin Z is formed throughout the growth phase but is adsorbed on the cell surface during early exponential growth until the pH drops below 5.5. Transcription of the two operons requires external nisin Z [48]. Promoters preceding *nisZ* and *nisF* (or *nisA*) are inducible by nisins and regulated by *nisRK* [8]. However *nisR* itself is not influenced by nisins.

In the biosynthesis of the similar lantibiotic, subtilin, by *B. subtilis*, regulation occurs by two genes, *spaR* and *spaK*. Protein SpaK has the sequence of a sensor histidine kinase of signal transduction systems with two membrane-spanning regions. Protein SpaR has the sequence of a cytoplasmic response regulator acting in a positive way after possible phosphorylation [31].

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