Mining and Polishing of the Treasure Trove in the Bacterial Genus Streptomyces

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The complex morphogenesis of the bacterial genus Streptomyces has made this genus a model prokaryote for study of multicellular differentiation, and its ability to produce a wide variety of secondary metabolites has made it an excellent supplier of biologically active substances, including antibiotics. This review summarizes our study of these two characteristics of Streptomyces, focusing on the A-factor regulatory cascade and work derived from the A-factor study. A microbial hormone, A-factor (2-isocapryloyl-3R-hydroxymethyl-γ-butyrolactone), triggers morphological differentiation and secondary metabolism in Streptomyces griseus. The key steps in the A-factor regulatory cascade, including afsA, encoding the key enzyme for A-factor biosynthesis, arpA, encoding the A-factor receptor, and adpA, encoding a transcriptional activator, are elucidated. The target genes of the regulatory cascade include genes of various functions required for morphological development and secondary metabolite formation. The biosynthesis gene clusters for grixazone and hexahydroxyperylenequinone are examples. The former contains the enzymes for novel benzene ring formation and phenoxazinone formation, and the latter contains enzymes belonging to a type III polyketide synthase and a cytochrome P-450. Enzymes of various catalytic functions in Streptomyces are useful as members of an artificial gene cluster constructed in Escherichia coli for fermentative production of plant-specific flavonoids, including isoflavones and unnatural compounds.

Key words: Streptomyces; A-factor; serine/threonine kinase; secondary metabolism; combinatorial biosynthesis

The genus Streptomyces comprises Gram-positive, soil-dwelling, filamentous bacteria. It shows complex morphological differentiation resembling that of filamentous fungi, which makes this genus a model prokaryote for the study of multicellular differentiation. On agar medium, one or more substrate hyphae formed from a germinating spore branch frequently and grow rapidly by cell wall extension at the hyphal tips. Subsequently aerial hyphae emerge by reuse of material assimilated into the substrate mycelium, such as DNA, proteins, and storage compounds. Many cells in substrate hyphae thus lyse and die. When apical growth of aerial hyphae stops, in contrast to substrate mycelium, septa are formed at regular intervals along the hyphae to form many unigenomic compartments within a sheath composed of elongated hollow or grooved elements, finer fibrillar elements, and amorphous material. The sporulation septa consist of two membrane layers separated by a double layer of cell-wall material, which permits the eventual separation of adjacent spores. Spore chains usually consist of many tens of spores. The aerial spores thus formed are resistant to heat-treatment and lysozyme-digestion. Streptomyces strains have hence been called “boundary organisms” between prokaryotes and eukaryotes.

Another characteristic of the genus Streptomyces is the ability to produce a wide variety of secondary metabolites, including antibiotics and biologically active substances. Secondary metabolism is sometimes termed “physiological” differentiation because it occurs during the idiophase after the main period of rapid vegetative growth and assimilative metabolism. Because of the ability to produce a wide variety not only of secondary metabolites, such as antibiotics, immunosuppressants, and enzyme inhibitors, but also of industrially important enzymes, such as glucose isomerase and transglutaminase, Streptomyces has contributed much to the world-leading fermentation industry in Japan. In addition, a variety of enzymes for secondary metabolite formation have become members of artificial gene clusters for the biosynthesis of novel compounds by so-called combinatorial biosynthesis.

Morphological development and secondary metabolism are simultaneously under the control of various nutritional environments, such as carbon, nitrogen, and phosphorous nutrients, and trace elements. Chemical
signaling molecules having a γ-butyrolactone ring also control both morphological and physiological differentiation of *Streptomyces* spp. The pioneer work of Khokhlov et al. on an autoregulatory factor (A-factor, 2-isocapryloyl-3R-hydroxymethyl-γ-butyrolactone; see Fig. 1 for chemical structure), which induces both sporulation and streptomycin biosynthesis in a mutant of *Streptomyces griseus*, revealed an exact link between secondary metabolism and morphological differentiation. Subsequent studies from several laboratories have indicated that A-factor and its derivatives in various *Streptomyces* spp. are actually autoregulators or microbial hormones that switch on morphological differentiation or secondary metabolism, or both.2–6) Studies of A-factor over more than 25 years at the Laboratory of Fermentation and Microbiology, The University of Tokyo have established that it can be termed a microbial hormone that switches on morphological and physiological differentiation with specific timing by monitoring the physiological conditions of the cell.7–9) In addition to hormonal control by A-factor, serine/threonine kinases also control secondary metabolism and morphogenesis. A representative of the serine/threonine kinases is AfsK, which activates the DNA-binding activity of a transcriptional factor AfsR by phosphorylating its threonine residue.10,11) Cyclic-AMP that is biosynthesized by a eukaryotic-type adenylcyclase12) and a calmodulin-like protein 13) are also involved in morphological differentiation. Therefore, *Streptomyces* members are boundary organisms from the viewpoint not only of their morphogenesis but also of gene regulatory systems.

This review article summarizes the study of the biology and chemistry of *Streptomyces* at this laboratory, on the occasion of an award I have received from the Japan Society for Bioscience, Biotechnology, and Agrochemistry. I summarize study of (i) the A-factor regulatory cascade that leads to aerial mycelium formation and secondary metabolite formation in *S. griseus*, (ii) the AfsK/AfsR/AfsS system, which controls both secondary metabolism and morphogenesis, and (iii) the catalytic properties of certain enzymes involved in secondary metabolism, and (iv) application of *Streptomyces* enzymes in fermentative production of natural and unnatural flavonoids by combinatorial biosynthesis.

### I. The A-Factor Regulatory Cascade

The strain we have studied is a historical one, *S. griseus*IFO13350 (= ISP5236; *Streptomyces griseus* subsp. griseus, Waksman and Henrici 1948), which was discovered by Selman A. Waksman as a streptomycin-producer. The strain Khokhlov studied appears to be the same as the Waksman’s strain.2,3) Figure 1 illustrates the A-factor regulatory cascade we so far have identified7,8) for the historical strain that won the Nobel Prize for Waksman. In *S. griseus*, A-factor is gradually accumulated in a growth-dependent manner by the activity of AfsA, which catalyzes γ-ketoacyl transfer between a glycerol derivative and a γ-keto acid. When the concentration of A-factor reaches a critical level at or near the middle of the exponential growth, it binds the A-factor receptor protein (ArpA), which has bound and repressed the promoter of a gene encoding a key transcriptional factor (AdpA), and dissociates ArpA from the promoter, leading to transcription and trans-
lation of *adpA*. AdpA then activates a variety of genes of various functions that are required for secondary metabolism and morphological development. One of the targets of AdpA is *strR*, encoding the pathway-specific transcriptional factor for the streptomycin biosynthesis genes. Thus, the A-factor signal is transferred to the streptomycin biosynthesis genes, starting with AfsA, then to ArpA to AdpA to *StrR*, and finally to all the streptomycin biosynthetic genes, causing the onset of streptomycin biosynthesis. Khokhlov’s discovery forty years ago of a mysterious link between A-factor and streptomycin production has thus been explained at the molecular level.

1. Properties of A-factor and its homologs in actinomycetes

A-factor was originally discovered by Khokhlov and coworkers\(^1\) in the culture broth of *S. griseus* as a factor that induced streptomycin production and aerial mycelium formation in a mutant strain of *S. griseus*. The genetic study of A-factor biosynthesis by Hara and Beppu\(^14,15\) in this laboratory confirmed their observations, and revealed that chemically synthesized, optically active A-factor, provided by Mori\(^16\) in the same department, restores all the phenotypic defects of A-factor-deficient mutant strain HH1 at concentrations as low as 10\(^{-9}\) M. Subsequent studies showed that A-factor also controls the production of a yellow pigment and a melanin-like pigment, in addition to streptomycin.\(^2\)

Study of the yellow pigment, grixazone, and the melanin-like pigment, hexahydroxyperylenequinone, has led to fruitful discoveries of novel enzymes responsible for the biosynthesis of these pigments, as described below.

A-factor-deficient mutants, like mutant HH1, were readily obtained by treatment of the wild-type strain by UV irradiation or incubation at 32 °C.\(^14\) The extreme instability of A-factor productivity was later explained in terms of the location of *afsA*, a gene encoding a key A-factor biosynthesis enzyme, AfsA,\(^17,18\) which is located in the vicinity of one end of the linear chromosome.\(^19\) The ends of the *Streptomyces* chromosomes are deleted at high frequency due to homologous recombination between a long repeat sequence on both ends of the linear chromosome. The genome sequence of *S. griseus* in a total of 8,545,929 bp (Y. Ohnishi et al., manuscript in preparation) shows that *afsA* is located 272 kb from one end of the chromosome.

A-factor homologs having a \(\gamma\)-butyrolactone structure have been found in various *Streptomyces* species, such as *S. bikinniensis*, *S. coelicolor* A3(2), *S. cyaneofuscatus*, *S. lavendulae*, *S. virginiae*, and *S. viridochromogenes* (reviewed in Refs. 3 and 7). Virginiae butanolides (VBs) controlling virginiamycin production in *S. virginiae*,\(^20\) IM-2 controlling pigment production in *S. lavendulae*,\(^21\) and SCB1 controlling actinorhodin and undecylprodigiosin production in *S. coelicolor* A3(2)\(^22\) are examples. These strains contain homologs of *afsA*, encoding a key A-factor biosynthesis enzyme, and *arpA*, encoding the A-factor receptor, which implies that the mechanism of regulation by these \(\gamma\)-butyrolactones is the same as that for the A-factor regulatory system including *afsA* and *arpA*.

The \(\gamma\)-butyrolactone-type autoregulators produced at a portion of a hypha in response to certain environmental stimuli can move freely within the individual hypha and spread into neighboring hyphae. Due to filamentous growth, *Streptomyces* have developed diffusible \(\gamma\)-butyrolactone regulatory systems that facilitate communication between the cells at a distance within an individual hypha and between different hyphae. The filamentous mycelia of *Streptomyces* are close enough to communicate with one another. The signaling system between physically separate individual cells in the same mycelium can be termed hormonal regulation, as opposed to the quorum sensing found in Gram-negative single-cell bacteria growing in liquid culture.\(^23,24\) However, A-factor is also important in cell-to-cell communication between neighboring mycelia, similarly to the quorum sensing system. Since a given *Streptomyces* strain contains its own \(\gamma\)-butyrolactone and its receptor with strict ligand specificity, this system also facilitates discrimination of signals originating from neighboring living things, thus allowing the cell to recognize the neighbor as a member of the same species or not. This system is also advantageous to survival in the ecosystem; A-factor produced by a cell is accepted by several hyphae and causes rapid sporulation of a whole population, which is advantageous, as compared with piecemeal sporulation of individual hyphae induced by environmental stimuli such as nutritional limitation.

The A-factor and receptor system in *S. griseus* acts as an all-or-nothing switch (i.e., a crucial switch) for both morphological and physiological differentiation. CprA and CprB, both of which are A-factor receptor homologs, act as tuners for these processes in *S. coelicolor* A3(2).\(^25\) On the other hand, the VB system in *S. virginiae* also controls the timing of antibiotic production but not morphological development. These observations imply that *Streptomyces* has evolved the \(\gamma\)-butyrolactone regulatory system to control different steps in the regulatory hierarchy for healthy growth, as an all-or-nothing switch for some phenotypes and simply as a tuner for other phenotypes. That may be why a *Streptomyces* strain contains redundant \(\gamma\)-butyrolactone regulatory systems.

2. Biosynthesis of A-factor

Concerning the A-factor biosynthesis enzymes, we proposed that AfsA was a key A-factor biosynthesis enzyme, because (i) *afsA* mutants lost A-factor productivity,\(^14,17\) (ii) introduction of *afsA* into A-factor non-producing *Streptomyces* strains caused overproduction of A-factor with a gene dosage effect,\(^17\) and (iii) introduction of *afsA* into *Escherichia coli* caused the host to produce a substance having A-factor activity.\(^26\)
Concerning the biosynthesis pathway, Sakuda et al. proposed a route on the basis of feeding experiments in virginiae butanolide-producing Streptomyces antibioticus (pathways A and B in Fig. 2). According to their proposal, the skeleton is formed by coupling between a 3-carbon (C₃) unit derived from glycerol and a C₁₀/C₁₂-keto acid derivative. Structure modeling of AfsA by S. Nakamura (unpublished data) in this department showed that, like C₁₂-hydroxyacyl acyl carrier protein (ACP) dehydratase, AfsA has a tunnel that can accept an acyl chain of acyl-ACP. The presence of such a tunnel in AfsA supported the idea that AfsA is involved in A-factor biosynthesis at a step of condensation of a C₃ compound and a C₁₀ fatty acid derivative containing a β-ketoacyl chain. On the basis of this information, Kato has elucidated the A-factor biosynthesis pathway.

In vivo A-factor synthesis by E. coli carrying afsA alone. E. coli carrying afsA produces two new substances that are absent in the broth of E. coli without afsA, with their m/z 241 and 213 and the same MS/MS fragmentation pattern as A-factor. Because the culture broth shows A-factor activity and because E. coli does not produce branched fatty acids, we assume that these substances are A-factor analogs with a C₁₀ straight side chain (m/z 241) and a C₈ straight side chain (m/z 213).

In vitro synthesis of A-factor. Since the fatty acids of Streptomyces consist primarily of branched-chain fatty acids that are synthesized from isobutyryl- and methylbutyryl-coenzyme A (CoA), the C₁₀ branched side chain of A-factor is probably synthesized by condensation of isobutyryl-CoA and three acetate units. Kato et al. revealed that AfsA catalyzes acyl transfer between 8-methyl-3-oxononanoyl-N-acetylcysteamine (NAC), a mimic of the corresponding β-ketoacyl-ACP, and the hydroxyl group of dihydroxyacetone phosphate (DHAP) (Fig. 2). The fatty acid ester produced is non-enzymatically converted to a butenolide phosphate by intramolecular condensation. The butenolide phosphate is then reduced by the bprA product encoded just downstream of afsA. The stereoselectivity, the R-form, at position 3 is determined by this reduction step. The phosphate group on the resulting butanolide is finally removed by a phosphatase, resulting in the formation of A-factor. Kato also confirmed that the 8-methyl-3-oxononanoyl-DHAP ester produced by the action of AfsA is converted to A-factor in an alternative way (pathway A) proposed by Sakuda et al. The phosphate group on the ester is first removed by a phosphatase and the dephosphorylated ester is converted non-enzymatically to a butenolide, which is then reduced by a reductase different from BprA, resulting in A-factor. Because the introduction of afsA alone into Escherichia coli causes the host to produce a substance having A-factor activity, the reductases and phosphatases are not specific to the A-factor biosynthesis, but are commonly present in bacteria. AfsA is thus the key enzyme for the biosynthesis of γ-butyrolactones.

Fig. 2. The A-Factor Biosynthesis Pathway.
AfsA catalyzes the condensation of DHAP and a β-keto acid derivative. After this condensation step, there are two biosynthesis routes to A-factor. Pathway A: A phosphate group is removed by a phosphatase, followed by non-enzymatic intramolecular aldol condensation. A C=C double bond is reduced by an NAD(P)H-dependent reductase, resulting in A-factor formation. Pathway B: Non-enzymatic intramolecular condensation of the ester, yielding a butenolide, is followed by a C=C double bond reduction step catalyzed by BprA. The last dephosphorylation step yields A-factor.
position. Due to the absence of such reductases in *S. griseus*, position 6 of A-factor remains as a keto group. On the other hand, VBs in *S. virginiae* and SCB1 in *S. coelicolor* A3(2) have a hydroxyl group at position 6. BarS1 in *S. virginiae* is an NADPH-dependent reductase that reduces the 6-oxo group of the penultimate intermediate in VB biosynthesis.36

**Regulation of A-factor biosynthesis.** A-factor is accumulated in a growth-dependent manner and reaches a maximum, 25 to 30 ng/ml (about 100 nM), at or near the middle of exponential growth. How is A-factor biosynthesis in such an extremely small amount controlled during growth? The transcription of *afsA*, starting at two points, is almost constant throughout growth.28 Therefore, it is most likely that the time-course of A-factor production at this extremely low concentration is due to the availability of the substrates for *AfS*. The concentration of DHAP, derived from glycolysis, depends on the physiological state. 8-Methyl-3-oxononanoyl-CoA, which is synthesized by condensation of three acetate units with the starter substrate isobutyryl-CoA, is an intermediate in primary fatty acid biosynthesis, and is leaked from the pathway. Therefore, the intracellular pool of the β-keto acid derivatives must be extremely small. Both glycolysis and fatty acid synthesis are active during exponential growth, and this is reflected on the time-course of A-factor production. A-factor that is synthesized from DHAP and 8-methyl-3-oxononanoyl-ACP via the β-ketoacyl transfer activity of *AfS* functions as a transmitter that monitors the physiological conditions of Gram-negative bacteria; *N*-acylhomoserine lactones are also active during exponential growth, and this is reflected on the time-course of A-factor production. A-factor appears to be a dimer with an extremely small amount of *AfS* and *AfS* functions as a transmitter that monitors the physiological state of the cell through the primary metabolites, leading to activation of the genes required for secondary metabolism and morphological differentiation. Biosynthesis of γ-butyrolactones is analogous to that of *N*-acylhomoserine lactones in Gram-negative bacteria; *N*-acylhomoserine lactones are biosynthesized from *S*-adenosylmethionine derived from the amino acid biosynthesis pathway and diverse intermediates in fatty acid biosynthesis.29

3. ArpA, the A-factor receptor protein

*Identification of ArpA.* Miyake32,33 detected A-factor-binding activity in the cytoplasmic fraction of *S. griseus* using [3H]A-factor, and determined its repressor function as to streptomycin production and aerial mycelium formation by genetic studies. Onaka34,35 then cloned an A-factor receptor gene named *arpA* and determined the DNA-binding activity of ArpA and a consensus ArpA-binding sequence. The consensus ArpA-binding sequence determined was a 22-bp palindromic site with the sequence 5′-GG(T/C)CGGT(A/T)(T/C)G(T/G)-3′ as one half of the palindrome. ArpA binds this site in the absence of A-factor, and the exogenous addition of A-factor to the ArpA-DNA complex induces immediate release of ArpA from the DNA. These observations led to the idea that ArpA acts as a repressor-type regulator for secondary metabolism and morphological differentiation by preventing the expression of a certain key gene(s) during the early growth phase. A-factor, produced in a growth-dependent manner, releases ArpA from the DNA, thus switching on the expression of key genes, leading to the simultaneous onset of secondary metabolism and morphogenesis at a certain time during growth. Kato and Miyahisa36 later identified *adpA* as the sole target of ArpA.

Site-directed mutagenesis of the helix-turn-helix DNA-binding motif of ArpA yielded a mutant ArpA protein (Val41Ala) that lacks DNA-binding ability but still retains the A-factor-binding ability.37 Conversely, mutant Pro115Ser lacks the A-factor-binding ability but retains DNA-binding ability.38 Mutant Trp119Ala also lacks A-factor-binding ability but retains DNA-binding ability, indicating that Trp-119 is essential for A-factor binding.37 These findings show that ArpA contains two independently functional domains, a DNA-binding and an A-factor-binding domains.

**Crystallography of γ-butyrolactone receptors.** We attempted to analyze ArpA by X-ray crystallography. ArpA readily aggregated and our repeated attempts to obtain a crystal of ArpA failed. Next we tried to crystallize CprB, an ArpA homolog in *S. coelicolor* A3(2).25 CprB, consisting of 215 amino acids, shows about 30% identity in amino acid sequence to ArpA. Although the ligand of CprB is still unknown, it recognizes and binds the same nucleotide sequence as ArpA.37 In addition, CprB serves as a negative regulator for both morphological differentiation and secondary metabolism in *S. coelicolor* A3(2), as ArpA does in *S. griseus*. The crystal structures of two different forms were determined at 2.4 Å resolution by Natsume,39 and they turned out to be a dimer with an Ω shape (Fig. 3). The DNA-binding domain is composed of three N-terminal helices, α1, α2, and α3. Of the three, α2 and α3 form a typical helix-turn-helix motif. Consistently with the observation that γ-butyrolactone receptors recognize the same nucleotide sequence, the residues on helix α3 are completely conserved among the receptors.

**Ligand-binding pocket.** A large cavity is present in the regulatory domain, which appears to be a ligand-binding pocket 5 Å in diameter and 20 Å long. Trp-127, corresponding to Trp-119 of ArpA, which has been found to be essential for A-factor binding by site-directed mutagenesis,37 participates in forming the pocket. The pocket is completely embedded in the molecule and a flexible loop covers the entrance to it, serving as a lid for it.

**How A-factor dissociates receptor from DNA.** A database search for structural comparison revealed that the overall structure of CprB is similar to those of the TetR family proteins, TetR and QacR. Hence we can predict how γ-butyrolactones dissociate their cognate receptors from DNA upon binding the ligands, on the basis of the mechanism of the conformational changes of TetR upon tetracycline binding.40,41 Ligand binding induces the relocation of a long helix α4 that links the
ligand binding pocket with the DNA-binding domain. As a result of the relocation of the DNA-binding domain, ArpA dissociates from the DNA.

4. AdpA, a key transcriptional activator in the A-factor regulatory cascade

Identification of AdpA. A study by Vujaklija\textsuperscript{42,43} on the transcriptional organization of part of the streptomycin biosynthetic gene cluster showed that one mRNA species covering a regulatory gene (\textit{strR}) and the streptomycin-6-phosphotransferase (\textit{aphD}) gene is dependent on A-factor. Intensive work by Ohnishi\textsuperscript{44} led to identification of an A-factor-responsive protein (AdpA) able to bind the upstream activation sequence, about 270 bp upstream of the transcriptional start point of \textit{strR}. AdpA, encoding a 405-amino-acid protein with a helix-turn-helix DNA-binding motif at the central portion, showed sequence similarity to transcriptional regulators belonging to the AraC/XylS family. The $-35$ and $-10$ regions of \textit{adpA} contained a 22 bp palindrome, cagccAGGAACGGACC$^*$GCCCGTGATCCG (underlining indicates the $-35$ and $-10$ promoter elements, and $^*$ indicates a dyad axis), which showed similarity to the consensus sequence of the AraA-binding site, (A/C)(A/G)(T/A)ACCC(A/G)CC$^*$GG(T/C)CGGT(A/T)\textendash(T/C)G(T/G). As expected, ArpA bound the promoter region of \textit{adpA} in the absence of A-factor, but not in the presence of A-factor. In addition, exogenous addition of A-factor to the AraPA-DNA complex immediately dissociated ArpA from the DNA. Thus the promoter of \textit{adpA} turned out to be a target of ArpA. Consistent with this, S1 nuclease mapping showed that \textit{adpA} is transcribed only in the presence of A-factor and that \textit{strR} is transcribed only in the presence of intact \textit{adpA}. Furthermore, \textit{adpA} disruptants produced no streptomycin, and overexpression of \textit{adpA} caused the wild-type \textit{S. griseus} strain to produce streptomycin at an earlier growth stage and in larger amounts.

\textit{adpA} as a single target of ArpA. Because ArpA acts as a repressor of aerial mycelium formation and secondary metabolism, an \textit{arpA} disruptant forms aerial hyphae and spores earlier than the wild-type strain and overproduces streptomycin and other secondary metabolites. On the other hand, mutant KM2, expressing a mutant ArpA (Trp119Ala), neither produces secondary metabolites nor forms aerial hyphae, since this A-factor-insensitive mutant ArpA always binds to and represses the \textit{adpA} promoter. Trp-119 of ArpA is essential for A-factor binding, and replacement of this Trp residue with Ala abolishes its A-factor-binding ability, resulting in...
the formation of a mutant ArpA that binds the target DNA irrespective of the presence of A-factor.\textsuperscript{37} When adpA under the control of a foreign, constitutively expressed promoter is introduced into mutant KM2, all the phenotypes that we can observe are restored.\textsuperscript{36} Hence we can conclude that the only significant target of ArpA is adpA.

\textit{Autorepression of adpA.} The intracellular concentration of AdpA must be important for ordered gene expression for healthy growth. Consistent with this idea, Kato\textsuperscript{45} found that AdpA represses its own transcription by forming a DNA loop via two molecules of AdpA dimer that bind the operator sites in the adpA promoter. Thus AdpA self-controls the intracellular concentration at an appropriate level by cooperatively binding to the two operator sites, allowing effective regulation to result from small alterations in the AdpA concentration and serving as a fine sensor for the concentration of AdpA.

\textit{AdpA regulon.} For isolation of targets of AdpA by gel mobility shift assay in a combination with immunoprecipitation and PCR, Yamazaki\textsuperscript{46} collected more than 60 DNA fragments that were specifically bound by AdpA. The presence of many genes, all of which are simultaneously activated by AdpA at a specific point in the growth phase, means that the signal from A-factor is greatly amplified at this regulatory step via AdpA as an amplifier (Fig. 1). The targets of AdpA required for morphological differentiation are the following: \textit{adsA}, encoding an extracytoplasmic function (ECF) \textit{σ}-factor;\textsuperscript{46} \textit{amfR}, encoding a transcriptional factor that activates the \textit{amf} operon;\textsuperscript{47,48} extracellular proteases, including a metalloendopeptidase,\textsuperscript{49} two trypsin-type proteases,\textsuperscript{50} and three chymotrypsin-type proteases;\textsuperscript{51} a \textit{Streptomyces} subtilisin inhibitor (SSI) gene;\textsuperscript{52} and \textit{ssgA}, which is essential for spore septum formation.\textsuperscript{53} The A-factor-dependent proteases in conjunction with the SSI are supposed to control aerial mycelium formation, since \textit{Streptomyces} forms aerial hyphae by reuse of material assimilated in substrate hyphae by “apoptosis” or “cannibalism.” The \textit{amf} operon is for production of a hydrophobin, AmfS, that is essential for the ejection of aerial hyphae into the air.\textsuperscript{54,55} For secondary metabolism, AdpA indirectly activates \textit{griR}, a pathway-specific transcriptional activator for grizxone production (T. Higashi \textit{et al.}, manuscript in preparation) and a gene encoding a transcriptional factor probably for biosynthesis of a polyketide compound,\textsuperscript{56} in addition to \textit{strR} for streptomycin production.\textsuperscript{57}

\textit{Open complex formation.} For activation of target genes, a dimer of AdpA binds various positions, for example more than 200 bp upstream and 25 bp downstream of their transcriptional start points.\textsuperscript{57–59} In addition, AdpA binds a single site for activation of certain genes and two or three sites for others. For transcriptional activation, some genes require simultaneous binding of a dimer of AdpA to multiple sites.\textsuperscript{48,49} Despite the differences in binding position and number of binding sites, AdpA recruits RNA polymerase to the specific promoter region of the target genes and facilitates isomerization of the RNA polymerase–DNA complex into an open complex for transcriptional initiation,\textsuperscript{48,56} as found for other transcriptional activators.

\textit{Consensus AdpA-binding sequence.} Alignment of more than 10 AdpA-binding sequences, which were determined by DNase I footprinting, did not predict an apparent consensus sequence for AdpA binding. Yamazaki\textsuperscript{56} performed interference assays on several AdpA-binding sites to determine the nucleotides directly associated with amino acids of AdpA and deduced a consensus AdpA-binding sequence, \textit{5'}'-TGGCSNGW-WY-3'} (S: G or C; W: A or T; Y: T or C; N: any nucleotide). The AdpA-binding sites so far identified all contain a sequence similar to this consensus sequence. Among the consensus sequence, the 4th C is essential for AdpA binding, and replacement of this C residue with other nucleotides results in abolishment of AdpA binding. In addition to the consensus sequence of 10 nucleotides, additional eight nucleotides 3' to this sequence also contribute to the affinity of AdpA, although there is no sequence conservation in this region.

5. \textit{How A-factor triggers streptomycin production} \textit{strR}, encoding the pathway-specific transcriptional activator for all the streptomycin biosynthesis genes, is a member of the AdpA regulon. The signal relay from A-factor to the streptomycin biosynthetic genes is from \textit{afsA} to \textit{arpA} to \textit{adpA} to \textit{strR} to the streptomycin production genes. A-factor produced by the action of \textit{AfsA} in a growth-dependent manner binds ArpA that has bound and repressed the promoter of \textit{adpA} at an early growth stage. When the concentration of A-factor reaches a critical level, it binds the DNA-bound ArpA and dissociates ArpA from the DNA, thus causing transcription of \textit{adpA}. Two AdpA dimers then bind the upstream activation sequences of \textit{strR}, approximately at nucleotide positions −270 and −50 with respect to the transcriptional start point of \textit{strR}, and activate its transcription, as shown by Tomono and Tsai.\textsuperscript{57} AdpA assists RNA polymerase in forming an open complex at an appropriate position for transcriptional initiation of \textit{strR}, as determined by potassium permanganate footprinting. The pathway-specific transcriptional activator \textit{StrR} induces transcription of all the streptomycin biosynthetic genes by binding multiple sites in the gene cluster, thus leading to biosynthesis of streptomycin from glucose. The major streptomycin resistance determinant, \textit{aphD}, located downstream of \textit{strR}, encoding streptomycin-6-phosphotransferase, is also transcribed by read-through from the A-factor-dependent \textit{strR} promoter.\textsuperscript{42} The co-transcription of \textit{strR} and \textit{aphD} accounts for the prompt induction of streptomycin resistance by A-factor and achieves a rapid increase in self-resistance just before induction of streptomycin biosynthesis.
II. The AfsK/AfsR/AfsS Regulatory System

1. Involvement of serine/threonine kinases in secondary metabolism

During my experiments 25 years ago, in which DNA fragments of *S. coelicolor* A3(2) were shotgun cloned in *S. lividans* TK21, I found a colony that produced a thick blue pigment in large amounts. The pigment contained actinorhodin and undecylprodigiosin. These pigments are not produced by strain TK21 on routine agar medium, which implied that a gene introduced in *S. lividans* awakened ‘‘sleeping’’ antibiotic production genes. The cloned gene, *afsR*, encoded a protein of 993 amino acids whose N-terminal portion showed similarity to the pathway-specific transcriptional activators of *Streptomyces*, also known as SARP-family proteins (streptomycete antibiotic regulatory proteins). Subsequent studies by Hong and Matsumoto revealed that AfsR is phosphorylated on a threonine residue by a serine/threonine kinase AfsK, on sensing some stimulus. KbpA binds a non-phosphorylated form of AfsK and inhibits the autophosphorylation of AfsK, modulating the AfsK kinase activity by protein–protein interaction. The two phosphorylated AfsR monomers bind the same face of the DNA covering exactly the promoter region of *afsS* and dimerize upon binding. The AfsR dimer then recruits RNA polymerase (RNAP) on the opposite face of the DNA and forms a complex of DNA-(AfsR):RNAP competent for transcriptional initiation. The *afsS* product of 63 amino acids contains three repeats of 12 amino acids. AfsS activates secondary metabolism in an as yet unknown way. Reproduced in modified form from Lee et al.

![Fig. 4. The AfsK-AfsR-AfsS Regulatory System in S. coelicolor A3(2).](image)

**Fig. 4.** The AfsK-AfsR-AfsS Regulatory System in *S. coelicolor* A3(2).

External and internal stimuli are sensed by serine/threonine kinases, AfsK, AfsL, and PkaG, and transferred to AfsR by means of phosphorylation of its Thr residue. These kinases activate their kinase activity by autophosphorylation of a single Thr residue, Thr-168 for AfsK, on sensing some stimulus. KbpA binds a non-phosphorylated form of AfsK and inhibits the autophosphorylation of AfsK, modulating the AfsK kinase activity by protein–protein interaction. The two phosphorylated AfsR monomers bind the same face of the DNA covering exactly the promoter region of *afsS* and dimerize upon binding. The AfsR dimer then recruits RNA polymerase (RNAP) on the opposite face of the DNA and forms a complex of DNA-(AfsR):RNAP competent for transcriptional initiation. The *afsS* product of 63 amino acids contains three repeats of 12 amino acids. AfsS activates secondary metabolism in an as yet unknown way. Reproduced in modified form from Lee et al.

**II. The AfsK/AfsR/AfsS Regulatory System**

1. Involvement of serine/threonine kinases in secondary metabolism

2. Mechanism of transcriptional activation by the AfsR family proteins

As described above, the N-terminal portion of AfsR findings show that AfsR serves as an integrator of intracellular and extracellular signals that are sensed by multiple serine/threonine kinases.

Lee identified *afsS* as a target of AfsR; the phosphorylated form of AfsR binds two direct repeat of 9 nucleotides, 5'-CGTTTCATCG-3' and 5'-CGTTTATCT-3', covering exactly the promoter region of *afsS*, and enhances its transcription. Because the promoter sequence is in many cases the site to which repressors bind, the mechanism by which AfsR activates *afsS* transcription involves an interesting aspect (see below). AfsS is a 63-amino-acid protein containing three repeats of the sequence Thr-X$_2$-Asp-Asn-His-Met-Pro-X$_2$-Pro-Ala (X: non-conserved amino acids). Although the introduction of multicopies of *afsS* enhances production of actinorhodin and undecylprodigiosin by enhancing the transcription of *actII-ORF4* and *redD* respectively, the way AfsS stimulates the transcription of these pathway-specific transcriptional activator genes remains to be elucidated. The *afsK/afsR/afsS* gene set is found in several *Streptomyces* spp., which suggests that this type of signal transduction via protein serine/threonine phosphorylation is common in this genus. The AfsK-AfsR-AfsS signal relay exhibits more apparent phenotypic expression for secondary metabolism in *S. coelicolor* A3(2) and for aerial mycelium formation in *S. griseus*.67)
belongs to the SARP family. Some SARP family members, such as ActII-ORF4 for actinorhodin production in *S. coelicolor* A3(2)\(^{60}\) and DnrI for daunorubicin production in *Streptomyces peucetius*,\(^ {60}\) bind direct repeats covering the promoter elements of the respective targets, as does AfsR. Therefore, the manner of DNA-binding of AfsR and the mechanism of transcriptional activation by AfsR should hold true for SARP family members. We constructed AfsR\(\Delta C\) (from Met-1 to Ala-270), containing an OmpR-type DNA-binding domain, and found that, like the full-length AfsR, it binds direct repeats, one of which overlaps with the sequence corresponding to the −35 region of the *afsS* promoter, a target of AfsR. Tanaka and Takano found that two AfsR\(\Delta C\) monomers cooperatively form a ternary complex of DNA-(AfsR\(\Delta C\))\(_2\)-RNA polymerase (RNAP), although RNAP alone cannot bind the *afsS* promoter (Fig. 4). The DNA-(AfsR\(\Delta C\))\(_2\)-RNAP complex is capable of transcriptional initiation both *in vitro* and *in vivo*. Mutant *afsS* promoters, having the distance between the AfsR-binding repeats and the −10 promoter element, still recruit RNAP and form an (AfsR)\(_2\)-DNA-RNAP complex. However, deletion or addition of even a single nucleotide between the −10 element and the repeat sequence on the 3′ side, leaving the AfsR-binding site intact, abolishes the ability of the ternary complex to initiate transcription (A. Tanaka et al., manuscript in preparation). We assume that the RNAP in the (AfsR)\(_2\)-DNA-RNAP complex formed on the intact *afsS* promoter is capable of making a contact with the −10 element, thus initiating the transcription. On the other hand, the RNAP in the ternary complex formed on the mutant *afsS* promoter cannot topologically make a stable contact with the −10 element. The same domain structure of AfsR\(\Delta C\) as SARP and the same organization of their target sites suggest that SARP family members activate the transcription of their targets by recruiting RNAP to the respective promoter sites and forming a ternary DNA-(SARP)\(_2\)-RNAP complex competent for transcriptional initiation.

### III. Catalytic Properties of Enzymes Involved in Secondary Metabolism

A-factor switches on production of almost all secondary metabolites in *S. griseus*. Study of the A-factor regulatory cascade has inevitably driven us to identify the genes for secondary metabolite formation and to elucidate their catalytic functions. During the early stage of my study on the A-factor regulation, I noticed that the production of a yellow pigment and a brown melanin-like pigment was dependent on A-factor. Intensive study by Suzuki et al.\(^ {70,71}\) of the yellow pigment, named grixazone, has revealed its biosynthesis pathway, which includes steps for novel, simple benzene ring formation and for phenoxazinone formation. Study by Funa et al.\(^ {72,73}\) of the brown pigment has led us to the discovery of a novel melanin biosynthesis pathway that includes a type III polyketide synthase (PKS). I will briefly summarize the research on the A-factor-dependent pigments.

#### 1. Grixazone biosynthesis pathway

The A-factor-dependent yellow pigment is produced under phosphate depletion, indicating that production of the pigment is also dependent on the phosphate concentration of the medium.\(^ {74}\) Therefore, the biosynthesis gene cluster of the pigment makes a good target to study the regulation of secondary metabolite formation not only by A-factor but also by phosphate. The whole gene cluster consists of 13 genes, among which the promoter of *griE*, the pathway-specific transcriptional activator, receives the signals of A-factor and the concentration of phosphate (our unpublished data).

**Novel benzene ring biosynthesis.** A combination of gene disruption and identification of compounds accumulated in disruptants showed that 3-amino-4-hydroxybenzoic acid (3,4-AHBA, Fig. 5) is an intermediate of grixazone. The shikimate pathway, including seven enzymatic steps for production of chorismate via shikimate from phosphoenolpyruvate and erythrose-4-phosphate, is common in various organisms for the biosynthesis not only of aromatic amino acids but also of most biogenic benzene derivatives. However, 3,4-AHBA turned out to be a benzene derivative that is biosynthesized by a novel, simple enzyme system.\(^ {71}\) Two genes, *griG* and *griH*, were found to be responsible for the biosynthesis of 3,4-AHBA; the two genes confer the *in vivo* production of 3,4-AHBA even on *Escherichia coli*. *In vitro* analysis showed that Grif catalyzes aldol condensation between two primary metabolites, L-aspartate-4-semialdehyde and dihydroxyacetone phosphate, to form a C\(_7\) product, 2-amino-4,5-dihydroxy-6-one-heptanoic acid-7-phosphate, which is subsequently converted to 3,4-AHBA by GriH. This pathway is independent of the shikimate pathway, representing a novel, simple enzyme system responsible for the synthesis of a benzene ring from the C\(_7\) and C\(_8\) primary metabolites. GriH homologs are found in the genomes of bacteria and higher plants, such as *Arabidopsis thaliana* and *Oryza sativa*, suggesting the possibility that the benzene ring biosynthetic pathway involving GriH homologs is distributed widely in nature.

**Biosynthesis of a phenoxazinone chromophore.** This pigment contains a phenoxazinone chromophore, suggesting that the gene cluster for grixazone biosynthesis should contain the genes encoding the enzymes responsible for its synthesis. Although several secondary metabolites containing a phenoxazinone skeleton, including actinomycin D, were isolated, no enzymes responsible for the biosynthesis of a phenoxazinone were known. *griE* and *griF* were identified by Suzuki et al.\(^ {70}\) GriE activates GriF by transferring copper ions to GriF, as is observed for a *Streptomyces* melanogenes strain in which MelC1 copper chaperon transfers two Cu ions to MelC2 tyrosinase. Despite the
significant similarity of GriF to the tyrosinases, GriF shows no monophenolase activity, but oxidizes various o-aminophenols as substrates preferable to catechol-type substrates. Deletion of the griEF locus on the chromosome resulted in accumulation of 3-amino-4-hydroxybenzaldehyde (3,4-AHBAL), which suggested that the substrate of GriF was 3,4-AHBAL. 3,4-AHBA appears to be reduced by the action of GriC and GriD in an ATP-dependent way to form 3,4-AHBAL, which is then coupled by GriEF in the presence of N-acetylcysteine, resulting in grixazone A. GriE is a copper chaperon for GriF. Grixazone A is oxidized by an oxidase to yield grixazone B.

Fig. 5. The Grixazone Biosynthesis Pathway.

Top: A-factor and the phosphate concentration of the medium control the transcription of the whole grixazone biosynthesis gene cluster, which consists of 13 genes, from griA to griS, by transferring their signals to the promoter of griR, the pathway-specific transcriptional activator gene. GriR then activates the two transcriptional units, griCDEFG and griJIH, covering all the biosynthesis enzymes. griAB appears to encode an exporter, perhaps serving as a resistance determinant. Bottom: The grixazone biosynthesis route. Chemically rationalized routes for the reactions for GriIH, GriCD, and GriEF are shown. Gri catalyzes aldol condensation between the aldehyde carbon of aspartate-4-semialdehyde and the hydroxylated carbon of dihydroxyacetone phosphate to yield the condensed product, which is then converted to 3,4-AHBAL by GriH. The catalysis pathway for GriH in the inset is our proposal. The carboxyl group of 3,4-AHBA is reduced in an ATP-dependent way by GriCD to form 3,4-AHBAL, which is then coupled by GriEF in the presence of N-acetylcysteine, resulting in grixazone A. GriE is a copper chaperon for GriF. Grixazone A is oxidized by an oxidase to yield grixazone B.
of the ω-quinone imine (Fig. 5). GriF is thus a novel ω-aminophenol oxidase that is responsible for the formation of the phenoxazinone chromophore in the grixazone biosynthesis pathway.

We have identified the whole biosynthesis pathway of grixazone, which includes the novel benzene ring and phenoxazinone chromophore formation. **griA** and **griB** are perhaps involved in the transport of grixazone. The biosynthesis gene cluster contains **griR**, the pathway-specific transcriptional activator, to which the A-factor signal and the phosphate signal are transferred (our unpublished data). **griS** and **griT** in the cluster are probably regulators. Their functions remain to be elucidated.

2. **Type III polyketide synthases in bacteria**

**Type III PKSs in Streptomyces.** **rppA** encoding a chalcone synthase-like protein was cloned from *S. griseus* as a gene that conferred melanin-like pigment production on *Streptomyces* and *E. coli*. The pigment production was A-factor dependent in *S. griseus*. Funa *et al.* identified the catalytic function of RppA and found that it selects malonyl-CoA as the starter, carries out four successive extensions and releases the resulting pentaketide to cyclize to THN. The THN formed is oxidatively biaryl coupled to yield HPQ by the action of P-450mel. HPQ readily autopolymerizes to form HPQ melanin. **griA** A homodimer of RppA catalyzes the four successive extensions of malonyl-CoA, starting with malonyl-CoA, and releases the resulting pentaketide to cyclize to THN. The THN formed is oxidatively biaryl coupled to yield HPQ by the action of P-450mel. HPQ readily autopolymerizes to form HPQ melanin. A vivid contrast in THN melanin biosynthesis between streptomycetes and fungi is that the THN synthesized by the action of a type III PKS is used directly for condensation in the former, whereas the THN synthesized by the action of type I PKSs is first reduced, and the resulting 1,8-dihydroxynaphthalene is then condensed in the latter. In various streptomycetes, on the other hand, the THN synthesized by RppA is monooxygenated by the action of MomA, belonging to the “cupin” superfamily, to form flaviolin. The flaviolin is further modified to give spore pigments in many streptomycetes.

**Phenolic lipid syntheses by type III PKS in Azotobacter.** Our discovery of RppA has established the idea that type III PKSs are distributed widely in bacteria. Cysts of the Gram-negative, nitrogen-fixing soil bacterium *Azotobacter vinelandii* are resting cells surrounded by a protective coat that confers resistance to various chemical and physical stresses. The major chemical components of the cyst coat are alkylresorcinols, amphiphilic molecules possessing an aromatic ring with a long aliphatic carbon chain. The genome database predicted the presence of an operon consisting of four genes, two of which encode a type III PKS, named **arsB** and **arsC**. In *vitro* experiments by Funa and Ozawa revealed that ArsB and ArsC, sharing 71% amino acid sequence identity, are an alkylresorcinol synthase and an alkylpyrone synthase respectively. The THN auto-oxidizes to form flaviolin, a red-brown pigment. The THN formed by RppA serves as an intermediate in the biosynthesis pathway not only for melanins but also for various secondary metabolites containing a naphthoquinone ring. For example, the THN formed by the action of RppA is converted to 1,4,6,7,9,12-hexahydroxynaphthalene-3,10-quinone (HPQ) by oxidative biaryl coupling catalyzed by P-450mel. HPQ readily autopolymerizes to form HPQ melanin. A vivid contrast in THN melanin biosynthesis between streptomycetes and fungi is that the THN synthesized by the action of a type III PKS is used directly for condensation in the former, whereas the THN synthesized by the action of type I PKSs is first reduced, and the resulting 1,8-dihydroxynaphthalene is then condensed in the latter. In various streptomycetes, on the other hand, the THN synthesized by RppA is monooxygenated by the action of MomA, belonging to the “cupin” superfamily, to form flaviolin. The flaviolin is further modified to give spore pigments in many streptomycetes.

**Fig. 6.** Type III Polyketide Synthases.

A, A homodimer of RppA catalyzes the four successive extensions of malonyl-CoA, starting with malonyl-CoA, and releases the resulting pentaketide to cyclize to THN. The THN formed is oxidatively biaryl coupled to yield HPQ by the action of P-450mel. HPQ readily autopolymerizes to form HPQ melanin. B, ArsB and ArsC accept the acyl-CoA as the starter substrate and cyclize the resultant tetraketide intermediates to yield an alkylresorcinol and an alkylpyrone respectively.
mation by a type III PKS has been found also in *S. griseus* and the fungus *Neurospora crassa* (our unpublished data).

IV. Combinatorial Biosynthesis of Flavonoids and Isoflavone

The presence of a chalcone synthase-like enzyme in *Streptomyces*, the key enzyme for the biosynthesis of a variety of flavonoid compounds in plants, gave me the idea that we might be able to produce plant-specific flavonoids by assembling genes required for their biosynthesis in bacteria. Once we produce flavonoids in bacteria, we should be able to modify the flavonoids further to yield various flavonoids, including unnatural compounds and isoflavones, by well-established techniques of fermentation and gene manipulation.78) Flavonoid-derived compounds have received much attention due to their use as health-promoting components of the human diet for their cancer chemopreventive, antioxidant, and antiasthmatic activities.

1. Fermentative production of natural and unnatural flavonoids and stilbenes

Natural flavonoids. In plants, phenylalanine ammonia-lyase (PAL), which deaminates phenylalanine to yield cinnamic acid, is the first enzyme in the general phenylpropanoid pathway (Fig. 7). Cinnamic acid is hydroxylated by cinnamate 4-hydroxylase (C4H) to 4-coumaric acid, which is then activated to 4-coumaroyl-CoA by the action of 4-coumarate:CoA ligase (4CL). Chalcone synthase (CHS), which belongs to the type III polyketide synthase family, catalyzes the stepwise condensation of three acetate units from malonyl-CoA to yield naringenin chalcone, the precursor of a large number of flavonoids. Naringenin chalcone is converted to (2S)-naringenin by the action of chalcone isomerase (CHI). Naringenin is further modified to flavones, flavonols, and isoflavone, for example.

During the course of assembling the genes, Kaneko et al.79) found from *S. coelicolor A3(2)* a cinnamate/coumarate:CoA ligase (ScCCL) that attaches CoA to both cinnamate and coumarate at almost the same efficiency. When this gene is used as a member of the artificial gene cluster designed for the fermentative production of naringenin, one would expect that naringenin is produced from tyrosine and pinocembrin is produced from phenylalanine. The gene cluster we constructed a high-copy-number plasmid involves *PAL* from the yeast *Rhodotorula rubra*, ScCCL from *S. coelicolor A3(2)*, *CHS* from a licorice plant *Glycyrrhiza echinata*, and *CHI* from the *Pueraria* plant, all of which are under the control of the T7 promoter and a synthetic ribosome-binding sequence. Because the intracellular pool of malonyl-CoA is extremely small, we also constructed a plasmid in which the two subunit genes of acetyl-CoA carboxylase, catalyzing the conversion of acetyl-CoA to malonyl-CoA, from *Corynebacterium glutamicum*, are under the control of the T7 promoter and the ribosome-binding sequence. Under cultural conditions under which the *E. coli* cells harboring the two plasmids at a cell density of 50 g/l were incubated in the presence of 3 mM tyrosine or phenylalanine, the yields of naringenin and pinocembrin reached about 60 mg/l (Fig. 7).80,81) Further introduction of the flavone synthase gene from *Petroselinum crispum* under the control of the T7 promoter caused the *E. coli* cells to produce flavones: 13 mg/l of apigenin from tyrosine and 9.4 mg/l of chrysin from phenylalanine. Introduction into the *E. coli* cells of the flavanones 3β-hydroxylase and flavonol synthase genes from the plant *Citrus* led to production of flavonols: 15.1 mg/l of kaempferol from tyrosine and 1.1 mg/l of galangin from phenylalanine.82)

In addition to scale, cost, fermentation period, one of the advantages in production of plant-derived polyketide compounds in *E. coli* is that a single species of flavonoids and stilbenes is produced in a buffer (minimal medium) containing substrates, glucose, and an inducer of the promoters, which allows one easily to purify polyketide compounds from the buffer. Hence the *E. coli* cell containing artificial biosynthesis gene clusters is called an enzyme bag. It converts the substrate into a target polyketide in a buffer.

Unnatural flavonoids and stilbenes. We divided the flavonoid biosynthesis pathway into three steps: substrate synthesis, polyketide synthesis, and a modification step. For the substrate synthesis step, various carboxylic acids are converted to the corresponding CoA esters by CoA ligases. The CoA esters can be replaced by chemically synthesized *N*-acylcysteamine (NAC) derivatives. For the polyketide synthesis step, one can use CHSs showing different times of condensation and different modes of cyclization. Instead of CHSs, stilbene synthases, showing a different mode of cyclization from those by CHSs, can also be used for this step. For the final modification step, flavone synthases, flavonol synthases, and other flavonoid- and stilbene-modifying enzymes of various origins are used. When one uses *x* kinds of CoA substrates, *y* kinds of chalcone and stilbene synthases, and *z* kinds of modification enzymes, it is to be expected that (*x* × *y* × *z*) kinds of unnatural flavonoids and stilbenes can be produced. In fact, Funa and Katsuyama have produced more than 60 unnatural flavonoid compounds so far (manuscript submitted).

2. One-pot synthesis of isoflavone from tyrosine

Isoflavonoids, synthesized predominantly in soybeans and other leguminous plants, have several beneficial effects on human health, such as chemopreventive actions against cancer, osteoporosis, cardiovascular disease. Genistein, a common precursor of the biosynthesis of isoflavonoids, has attracted much attention due to its phytoestrogen activity. Katsuyama et al.83) constructed a system in which *E. coli* cells producing naringenin and yeast *Saccharomyces cerevisiae* cells
A. Flavonoid biosynthesis in E. coli

![Flavonoid biosynthesis diagram]

B. Isoflavone biosynthesis by co-incubating E. coli and S. cerevisiae

![Isoflavone biosynthesis diagram]

**Fig. 7.** Combinatorial Biosynthesis of Flavonoids and Isoflavone.

**A.** In plants, flavanones [naringenin (R = OH) and pinocembrin (R = H)] synthesized mainly from tyrosine (R = OH) through four steps catalyzed by PAL, 4CL, CHS, and CHI, are the key intermediates of a variety of flavonoid compounds. For example, flavanones are modified to flavones by flavone synthase (FNS), and to flavonols by flavonones 3β-hydroxylase (F3H)/flavonol synthase (FLS). Plasmid 4GS contains the four enzyme genes under the control of the T7 promoter and a synthetic ribosome-binding sequence for the production of naringenin from tyrosine and of pinocembrin from phenylalanine. To increase the intracellular pool of malonyl-CoA, acetyl-CoA carboxylase (ACC) genes, disR1 and accBC, from C. glutamicum, are placed on a plasmid. The plasmids carrying FNS and F3H/FLS are for modification of flavanones into flavones and flavonols respectively. **B.** The E. coli cells producing naringenin are co-incubated with the S. cerevisiae cells carrying IFS in the presence of tyrosine. The naringenin produced by E. coli is excreted from the cell, incorporated into the yeast cell, modified into an isoflavone (genistein), and finally excreted into the medium.

V. Future Perspectives

Elucidation of the regulation by γ-butyrolactones and serine/threonine kinases is important not only for the biology of the filamentous bacterial genus *Streptomyces* but also for the practical use of this genus in the production of useful secondary metabolites. Sequencing of the *S. griseus* genome has been completed in this laboratory (Y. Ohnishi *et al.*, manuscript in preparation). Comprehensive study employing DNA microarray techniques, proteomics, and metabolomics should enable us to depict overall, precise pictures of the A-factor regulatory cascade and the network of protein phosphorylation. Comparative genomics by use of the genome sequences of *S. coelicolor* A3(2) and *S. avermitilis* helps us to understand the bacterium *S. griseus* and to mine and polish its treasure. Even between *S. griseus* and *S. coelicolor* A3(2), the signaling pathways for early developmental events and the genes for secondary metabolite formation are different. Some genes for secondary metabolite formation are species-specific, but many genes differ from strain to strain. Mining and polishing useful genes for practical purposes is important, and at the same time, awakening of “sleeping” genes by various approaches is also important. Genome projects for actinomycetes, such as Actinoplanes, Kitasatospora, and Micromonospora, showing more complex morphology than *Streptomyces*, in Japan should contribute further to our understanding of the boundary microorganisms.
In addition to \(\gamma\)-butyrolactones, possible autoregulatory factors in \textit{Streptomyces} species that are involved in secondary metabolism and morphological differentiation have been reported, although most of these are not precisely defined as autoregulators due to a shortage of genetic studies.\(^2\)\(^3\)\(^7\) The following two compounds can be termed regulators: B-factor (3'-butylyphosphoryl AMP) at a few nm concentration controls rifamycin biosynthesis in \textit{Nocardia mediterranei},\(^8\)\(^9\) and PI factor [pimarcin inducer; 2,3-diamino-2,3-bis(2-hydroxymethyl)-1,4-butanediol] at a few hundred nm induces the production of a macrolide, pimarcin, in \textit{Streptomyces natalensis}.\(^8\)\(^5\) It is conceivable that during evolution \textit{Streptomyces} spp. have employed various chemical substances as autoregulators to control their own secondary metabolism and morphogenesis. The autoregulators must have evolved concomitantly with their specific receptors. Future studies might reveal chemical substances as novel autoregulators, together with their specific receptors.

A \textit{Streptomyces} cell is a useful enzyme bag for the production of complex compounds by means of metabolic engineering and combinatorial biosynthesis, because it contains transport systems for a variety of the substances to be produced. During our experiments on the production of intermediates for medicines by bioconversion, we noticed that successful dioxygenation of benzoic acid derivatives by a hydroxylating enzyme bioconversion, we noticed that successful dioxygenation of benzoic acid derivatives by a hydroxylating enzyme in \textit{E. coli} due to lack of a transport system.\(^8\)\(^7\) Our success in fermentative production of flavonoid compounds in \textit{E. coli} indicates one of the future directions in applied microbiology. \textit{Streptomyces} should become a useful host for production of more complex, non-bacterial compounds by a similar procedure. For efficient production, it is important to feed back knowledge of the regulation and secondary metabolism to the architecture of artificial gene clusters and the mode of assembling genes.

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