Review

Hormonal control by A-factor of morphological development and secondary metabolism in *Streptomyces*

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(Contributed by Teruhiko BEPPU, M.J.A.)

Abstract: *Streptomyces griseus*, a well-known industrial producer of streptomycin, is a member of the genus *Streptomyces*, which shows a complex life cycle resembling that of fungi. A-factor, a C₁₃ γ -butyrolactone compound, was discovered as a self-regulatory factor or a bacterial hormone to induce morphological differentiation and production of secondary metabolites, including streptomycin, in this organism. Accumulating evidence has revealed an A-factor-triggered signal cascade, which is composed of several key steps or components. These include: (i) AfsA catalyzing a crucial step of A-factor biosynthesis, (ii) the A-factor-specific receptor (ArpA), which acts as a transcriptional repressor for *adpA*, (iii) *adpA*, a sole target of ArpA, which encodes a global transcriptional activator AdpA, and (iv) a variety of members of the AdpA regulon, a set of the genes regulated by AdpA. A-factor is biosynthesized via five reaction steps, in which AfsA catalyzes acyl transfer between a β -ketoacyl-acyl carrier protein and the hydroxyl group of dihydroxyacetone phosphate. The receptor ArpA, belonging to the TetR family, is a homodimer, each subunit of which contains a helix-turn-helix DNA-binding motif and an A-factor-binding pocket. The three-dimensional structure and conformational change upon binding A-factor are elucidated, on the basis of X-ray crystallography of CprB, an ArpA homologue. AdpA, belonging to the AraC/XylS transcriptional activator family, binds operators upstream from the promoters of a variety of the target genes and activates their transcription, thus forming the AdpA regulon. Members of the AdpA regulon includes the pathway-specific transcriptional activator gene *strR* that activates the whole streptomycin biosynthesis gene cluster, in addition to a number of genes that direct the multiple cellular functions required for cellular differentiation in a concerted manner. A variety of A-factor homologues as well as homologues of *afsA/arpA* are distributed widely among *Streptomyces*, indicating the significant role of this type of molecular signaling in the ecosystem and evolutionary processes.

Keywords: A-factor, *Streptomyces*, morphological differentiation, secondary metabolism, A-factor receptor, streptomycin biosynthesis

Introduction

The Gram-positive, soil-inhabiting, filamentous bacterial genus *Streptomyces* shows a characteristic life cycle with complex morphological de-

velopment resembling that of filamentous fungi. *Streptomyces* is also characterized by its ability to produce a wide variety of secondary metabolites, including a number of antibiotics and other biologically active substances. For example, even a single species of *Streptomyces griseus* is known to produce not only streptomycin but also about 180 other secondary metabolites. In fact, *Streptomyces* is one of the most important microbial resources for discovering novel functions useful in a variety of biotechnology, such as medicinal, agricultural, and industrial applications. *Streptomyces* is therefore called a “natural reservoir of structurally complex

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compounds on the earth." Lines of evidence have shown that the morphological differentiation and secondary metabolism in *Streptomyces* are closely correlated with each other as a facet of the cellular differentiation processes, and therefore this genus has been one of the model organisms to study molecular mechanisms for multicellular differentiation in prokaryotes. Among them, *Streptomyces coelicolor* A3(2) has been the *de facto* standard strain owing to the excellent genetic systems developed by D. A. Hopwood, which greatly facilitate the detailed molecular genetic analyses and result in large accumulation of genetic information.¹⁾ An additional species is *S. griseus*, whose streptomycin production and aerial mycelium formation are simultaneously triggered by a diffusible self-regulatory substance called A-factor, a representative of the chemical signaling γ -butyrolactone compounds that control the cellular differentiation in *Streptomyces*.²⁻⁴⁾

A-factor, a 13-carbon (C_{13}) γ -butyrolactone compound, was originally discovered by a Russian scientist, A. S. Khokhlov, in the 1960s as a diffusible self-regulatory factor in *S. griseus*, which simultaneously induces sporulation and streptomycin production in the same organism.⁵⁾ The pioneer work of Khokhlov, however, had long been neglected, partly because isolation of the mutants deficient in the diffusible factor with an extremely low effective concentration (10^{-9} M) was technically difficult and such mutants were easily overlooked in the routine genetic work, but more probably because an unconscious barrier existed even in science during the cold war era. In 1980, O. Hara and T. Beppu studied marked genetic instability of the industrial streptomycin-producing strain of *S. griseus* and found that a characteristic pleiotropic mutant deficient in both streptomycin production and sporulation appeared at abnormally high frequencies exceeding about 10%. Cross-feeding experiments between colonies of the mutant and the parental strain revealed that both the defects in this group of mutants were simultaneously restored by a highly diffusible substance secreted by the parental strain. This finding quickly revived the Khokhlov's famous but ignored works in their mind and led them to the rediscovery of A-factor.⁶⁾ Subsequent study has established the molecular mechanisms of the A-factor regulatory cascade consisted of a specific receptor and a set of transcriptional acti-

vators to control secondary metabolism and morphological differentiation in *S. griseus*.^{2,4)}

A-factor homologues with a γ -butyrolactone ring have been found as similar self-regulatory factors for secondary metabolism in a number of *Streptomyces* species and its related genera, which has thus opened a novel paradigm of the characteristic regulatory system for cellular differentiation in prokaryotes.²⁾ On the other hand, similar diffusible factors, *N*-acylhomoserine lactones also having a γ -butyrolactone ring, were found as autoinducers for diverged cellular functions such as bioluminescence and toxin production in several Gram-negative bacteria, which have now been recognized as signaling molecules involved in the so-called quorum sensing for cell-cell communication in various species belonging to Proteobacteria.^{7,8)} In spite of the apparent similarity in chemical structure between these two groups of signaling molecules, their biosynthetic pathways, as well as the genes involved in regulation by the respective signals, are distinctly different, indicating that these two regulatory systems have evolved from different ancestors. It has become more and more evident that bacterial species have characteristic chemical signaling systems, which enable their cells to communicate and cross-talk with one another to control their functions in the ecosystems.

In this review, we describe general characteristics of the γ -butyrolactones regulatory systems in *Streptomyces* and their molecular architecture to exert the pleiotropic cellular functions, focusing mainly on the A-factor regulatory cascade in *S. griseus*. Wide distribution of homologous γ -butyrolactone signals and their specific receptors among various *Streptomyces* species is also discussed in relation to their significant role in the ecosystems and evolutionary processes.

1. General characteristics of the A-factor regulatory system in *S. griseus*

Cellular differentiation in *S. griseus*. *S. griseus* growing on solid media shows a typical life cycle of *Streptomyces* as shown in Fig. 1. A spore germinates to form a branched, multinucleoid substrate mycelium growing on the surface of and into solid media. Partial apoptotic lysis of substrate mycelium is accompanied by the appearance of aerial mycelia or hyphae growing into the air. After septa have been formed at regular intervals along

Fig. 1. Life cycle of *S. griseus*. A transmission electron micrograph of a matured spore and scanning electron micrographs of substrate and aerial hyphae are also shown. Sm, streptomycin; GX, grizaxone. Reproduced from Ohnishi and Horinouchi.⁶⁵⁾

the hyphae, long chains of uninucleoid spores are formed. The primary decision point for morphological differentiation in this life cycle is the step to form aerial mycelium, and genetic blocks at this point generate a bald (*bld*) phenotype of colonies.¹⁾ Biosynthesis of not only streptomycin but also other secondary metabolites, such as grizaxone (a yellow pigment)^{9),10)} and a melanin-like pigment named hexahydroxyperylenequinone,^{11),12)} also starts at this step, suggesting that the same switch is involved in the initiation of secondary metabolism in this species. A-factor (2-isocaprolyl-3*R*-hydroxymethyl- γ -butyrolactone; for the structure see Fig. 1) is a chemical signal responsible for this step, which clearly indicates the close correlation between secondary metabolism and morphological differentiation as facets of global cellular differentiation in *Streptomyces*. Secondary metabolism is sometimes regarded as “chemical expression” of physiological differentiation.

A-factor as a bacterial hormone. A-factor is a self-regulatory factor involved in the definitive step of cellular differentiation, which is produced by *S. griseus* into culture media and essentially required for aerial mycelium formation and secondary metabolism in the same organism.^{5),6)} An A-factor-deficient mutant strain can neither form aerial

hyphae nor produce streptomycin, and exogenous supply of culture filtrate of the wild-type *S. griseus* strain or chemically synthesized, optically active (3*R*)-A-factor at a concentration of 10^{-9} M restores simultaneously both the deficient phenotypes of the mutant. These characteristics of A-factor — (i) an intrinsic chemical signal essentially involved in the physiological processes of the producing organism, (ii) a very low effective concentration, and (iii) pleiotropic activity to control diverged multiple cellular functions — may allow one to regard it as a bacterial hormone. The assumption directly led to the idea that a specific receptor might be involved in the regulation by A-factor, which was confirmed as described below. Ligand specificity of the putative receptor was thought to explain the narrow activity of A-factor restricted only to *S. griseus* among various species of *Streptomyces*. In fact, several γ -butyrolactone homologues have been found as self-regulatory factors for secondary metabolism in some *Streptomyces* species, all of which show strict species specificities.^{13)–15)} An extremely low effective concentration of A-factor facilitates to exert its activity between different portions of hyphae or colonies at a distance to induce rapid and simultaneous sporulation and production of antibiotics and other secondary metabolites. Such a

Fig. 2. The A-factor regulatory cascade leading to morphological development and secondary metabolite production. Sm, streptomycin; GX, grixazone; PK, a polyketide compound. See text for details.

system may be useful for a species to respond to environmental changes and to compete with other species in the hustle and bustle in the ecosystem.

The A-factor regulatory cascade. Our studies on A-factor since its rediscovery in 1982 until now have established a model of the signal transduction cascade shown in Fig. 2.^{3,4)} Key genes and components constituting this system are: (i) A-factor and its biosynthesis gene *afsA* encoding a key enzyme, AfsA, (ii) the A-factor-specific receptor ArpA (A-factor receptor protein), (iii) a global transcriptional activator AdpA (A-factor-dependent protein), and (iv) the AdpA regulon, a

set of the genes controlled by AdpA. An overall view of the cascade is as follows.

A-factor is synthesized via five steps, in which AfsA catalyzes a crucial coupling reaction between a C₃ compound and a C₁₀ acyl derivative. The A-factor receptor ArpA has dual abilities to bind A-factor as a specific ligand and to bind a specific DNA sequence in the promoter of *adpA* as a transcriptional repressor. Binding of A-factor to DNA-bound ArpA causes its immediate dissociation from the promoter to initiate transcription and translation of *adpA*. The gene product AdpA then activates a number of genes belonging to the AdpA regulon by

binding the consensus sequences located upstream of their promoters in most cases, which are required for secondary metabolism and morphological differentiation. In the case of streptomycin biosynthesis, *strR*, a member of the AdpA regulon located within the streptomycin biosynthetic gene cluster, is activated, and StrR in turn induces transcription of all the genes within the cluster.

In this signaling system, a single signal pathway starting from the receptor ArpA is divided into diverged multiple targets involved in various cellular functions required for secondary metabolism and morphological differentiation. This characteristic feature is based primarily on the fact that the target of ArpA is limited only to a single global transcriptional activator gene *adpA*, which possesses highly multiple target genes to be controlled. It makes a sharp contrast to the feature of most γ -butyrolactone signals in other *Streptomyces* species; a pair of *afsA/arpA* homologues in most *Streptomyces* species is linked to a biosynthesis gene cluster for a certain secondary metabolite and it controls only production of the secondary metabolite (see below). In the following sections characteristics of these regulatory components and genes in this signal cascade are described.

2. The A-factor biosynthesis pathway

Properties of *afsA*. The genetic instability of streptomycin production and sporulation in *S. griseus* was a start of the work on A-factor.⁶⁾ The A-factor-deficient mutants of *S. griseus* were readily derived from the wild-type strain at about 10% frequency by culturing at higher temperatures of 32–37 °C and in the presence of acridine orange.¹⁶⁾ Mutant HH1 was an example obtained by incubation at 35 °C. *afsA* was cloned as a gene that restored the deficiency of the mutant.¹⁷⁾ This gene appeared to encode an A-factor biosynthesis enzyme, because it conferred A-factor production on A-factor-nonproducing *Streptomyces* species with a marked gene dosage effect¹⁸⁾ and caused *E. coli* to produce substance(s) with A-factor activity.¹⁹⁾

The extreme genetic instability of the A-factor productivity was explained in terms of the location of the *afsA*, which is mapped in the vicinity of one end of the linear chromosome.²⁰⁾ It is known that the chromosomes of most *Streptomyces* species are linear and both terminal regions contain long inverted repeats (terminal inverted repeats; TIRs)

ranging from a few kb to 1 Mb in length. The ends of *Streptomyces* chromosomes undergo large deletion, insertion, inversion, as well as exchange events at high frequencies due not only to homologous recombination between the TIRs but also to transposable elements located at the termini or some other mechanisms.²¹⁾ In fact, Southern hybridization with *afsA* as the probe revealed a deletion of the entire *afsA* sequence in mutant HH1.¹⁷⁾ The genome sequence of *S. griseus*, which has been recently determined, has mapped *afsA* 272 kb from one end of the chromosome (manuscript in preparation). A gene *bprA* (butenolide phosphate reductase), which encodes a reductase involved in the A-factor biosynthesis, locates just downstream of *afsA*, forming an *afsA-bprA* operon.²²⁾

AfsA as the key enzyme for A-factor biosynthesis. Although A-factor and a variety of its homologues are distributed widely as self-regulatory factors for cellular differentiation in *Streptomyces*, a definitive route or enzymes for the biosynthesis of γ -butyrolactones had not been determined until recently. S. Sakuda *et al.*²³⁾ studied a γ -butyrolactone, named virginiae butanolide, which controls virginiamycin production in *S. virginiae*, by feeding experiments and proposed a route in which a C₃ glycerol derivative and a C₁₀ β -keto acid derivative were coupled. However, the real substrates and the catalyzing enzymes were still unknown.

Expression of the *afsA* gene alone in *E. coli* caused the host to produce a substance(s) having A-factor activity in the culture broth, but the substance was predicted to be slightly but distinctly different from A-factor, judged from the retention time in HPLC analysis.¹⁸⁾ Mass spectrometry analysis revealed that the substances were A-factor analogues having a straight chain of C₁₀ and C₈, which we assume are produced by coupling between a C₃ unit and a β -keto acid of C₁₀ or C₈ derived from fatty acid synthesis in *E. coli*.²²⁾ Because the fatty acids in *E. coli* are all straight, not branched, the A-factor analogues must have a straight acyl chain.

In vitro A-factor biosynthesis was achieved by using a cell-free lysate of an AfsA-disrupted strain of *S. griseus* and a histidine-tagged AfsA protein expressed in and purified from *E. coli* cells with chemically synthesized C₁₀ *S*-(8-methyl-3-oxononano-yl)-*N*-acetylcysteamine, a mimic of the activated

Fig. 3. The whole A-factor biosynthesis pathway. The major pathway in *S. griseus* is hatched.

form of the expected β -keto acid bound to acyl-carrier protein (ACP) (Fig. 3).²²⁾ Detailed analysis of the *in vitro* A-factor synthesis established the whole A-factor biosynthesis pathway as follows. AfsA catalyzes a new type of acyl transfer between 8-methyl-3-oxononanoyl-ACP (Fig. 3; **3**) and the hydroxyl group of dihydroxyacetone phosphate (DHAP) (**2**), forming a fatty acid ester (**4**). The ester (**4**) is non-enzymatically converted to a butenolide phosphate (**5**) by intramolecular aldol condensation, which is then reduced to a butenolide phosphate (**6**) in the presence of NADPH. The BprA product showing similarity to oxidoreductases, which is encoded by *bprA* just downstream of *afsA*, was tested as a reductase responsible for this reduction, on the assumption that functionally related genes are in most cases encoded as a gene cluster. This assumption turned out to be true and BprA actually reduced the butenolide phosphate (**5**) to yield the butenolide phosphate (**6**) with the *R*-form stereospecificity at position 3. The phosphate group on the resulting butenolide (**6**) is finally removed by a phosphatase, resulting in the formation of A-factor (**1**). There is an alternative pathway, in which the phosphate group on the fatty acid ester (**4**) is first removed by a phosphatase and the dephosphorylated ester (**7**) is converted non-enzymatically to a butenolide (**8**), which is then reduced by a reductase different from BprA,

resulting in A-factor (**1**). The phosphatase and reductase in the latter route are not specific to A-factor biosynthesis but generally present in bacteria. This is why *afsA* alone causes *E. coli* to produce substances having A-factor activity. Because of the operon structure of *afsA-bprA*, we assume that the former route is major. A *bprA* homologue is also located downstream of *scbA*, an *afsA* orthologue in *S. coelicolor* A3(2).

Structure modeling of AfsA by S. Nakamura (unpublished data) showed that the protein molecule has a tunnel which could accept an acyl chain of the substrate acyl-ACP, as observed for β -hydroxyacyl-ACP dehydratase involved in the bacterial type II fatty acid biosynthesis.²⁴⁾ The predicted tunnel in AfsA may accommodate the C₁₀ substrate β -ketoacyl-ACP in an extended form and orient it to couple with the hydroxyl group of DHAP.

Structural variety of γ -butyrolactones. A-factor homologues having a γ -butyrolactone have been found in various *Streptomyces* species, such as *S. bikiniensis*, *S. coelicolor* A3(2), *S. cyaneofuscatus*, *S. lavendulae*, *S. virginiae* and *S. viridochromogenes*.^{2),25)} The γ -butyrolactones are divided into three major types: a 6-keto type (e.g., A-factor in *S. griseus*), a (6*R*)-hydroxy type [e.g., SCB1, SCB2 and SCB3 in *S. coelicolor* A3(2)] and a (6*S*)-hydroxy type [e.g., virginiae butanolides (VB)-A,

Fig. 4. A-factor and its homologues in *Streptomyces*. These include A-factor from *S. griseus*, controlling both secondary metabolism and morphological differentiation; IM-2 from *S. lavendulae*, controlling pigment and antibiotic production; SCBs from *S. coelicolor* A3(2), controlling antibiotic production; and virginiae butanolides (VBs) from *S. virginiae*, controlling virginiamycin production. Two differences in chemical structure among the γ -butyrolactones are the length and branching of the acyl chains and the reduction state, either a keto or a hydroxyl group, at position 6.

-B, -C, -D and -E in *S. virginiae*] (Fig. 4). On the basis of the A-factor biosynthesis pathway, the difference in side chain can be ascribed to the variety of the β -ketoacyl-ACPs, one of the substrates used by AfsA and its homologues. The branching of the side chain reflects the fact that the fatty acids in the cell membrane of *Streptomyces* consist primarily of branched-chain fatty acids that are synthesized from isobutyryl- and methylbutyryl-CoA. The difference at position 6, either a keto or a hydroxyl group, can be ascribed to the existence and stereoselectivity of a 6-keto-reductase that reduces this position. BarS1 of *S. virginiae* is such a reductase responsible for the final step of VB biosynthesis.²⁶⁾ Due to the absence of such a reductase in *S. griseus*, position 6 of A-factor remains as a keto group. It is thus apparent that the structural variety of the γ -butyrolactones results from the substrate specificity of AfsA homologues, yielding the variety in length and branching of the acyl chain, and the presence and stereoselectivity of the BarS1-type 6-keto-reductase, yielding the variety in reduced state and stereospecificity at position 6.

Regulation of A-factor biosynthesis. In the wild-type *S. griseus* strain, A-factor is accumulated in a growth-dependent manner and reaches its maximum, 25 to 30 ng/ml (about 100 nM), at or near the middle of exponential growth.²⁷⁾ The

transcription of *afsA* is constant during early growth and appeared to increase during the stationary phase.²¹⁾ Why is A-factor produced in such an extremely small amount? A possible explanation is that production of A-factor is limited by the availability of the intracellular substrates for AfsA, especially by a small amount of β -ketoacyl-ACP leaked from the fatty acid biosynthetic pathway. The C₁₀ β -ketoacyl-ACP as one of the substrates of AfsA is an intermediate in fatty acid biosynthesis and the amount of the ACP intermediate “escaped” from the pathway must be extremely small. However, *afsA* confers A-factor production on various *Streptomyces* with a marked gene dosage effect¹⁸⁾ and an *adpA* disruptant produces almost ten times more A-factor than the wild-type strain.²⁸⁾ These observations suggest the presence of a direct or indirect regulatory feedback loop(s) between *afsA* and *adpA* in the A-factor signal cascade, although AdpA does not bind any regions upstream of *afsA*. On the other hand, disruption of the *arpA* homologues causes loss of the production of respective γ -butyrolactones in several *Streptomyces* species as observed for *barA* and VBs in *S. virginiae*,²⁹⁾ but not in *S. griseus*. The result indicates the presence of another regulatory loop between the receptors and biosynthesis of the respective ligands. Details of the regulatory mechanisms for the biosynthesis of γ -butyrolactones still remain to be elucidated.

3. ArpA, the A-factor receptor protein

Properties of ArpA. The extremely low effective concentration of A-factor and its pleiotropic regulatory function suggest the involvement of a specific receptor. By using the chemically synthesized optically active 3*R*-form of tritium-labeled A-factor, a specific binding protein was identified in the cytoplasmic fraction of *S. griseus*.³⁰⁾ The protein, named ArpA, binds A-factor in the molar ratio of 1:1 with a dissociation constant, K_d , of 0.7 nM. The small K_d value is consistent with the effective concentration of A-factor *in vivo* (10^{-9} M) and comparable to those of eukaryotic hormone receptors. Involvement of the binding protein in the A-factor signal transduction as an A-factor receptor was suggested by properties of a mutant, *S. griseus* 2247, deficient in the A-factor binding activity, in which both streptomycin production and sporulation were markedly higher than those of the parental strain.³¹⁾ Although a receptor-negative mutant had initially been expected to show loss of these phenotypes, the observed positive effects of the mutation apparently implied that the binding protein did work as a receptor in a negative manner or as a repressor responding to A-factor. The assumption turned out to be true; ArpA binds the promoter region of the target gene (later identified as *adpA*) and represses its transcription in the early growth stage when the concentration of A-factor is still low. When A-factor reaches a critical concentration, it binds the ArpA that has bound the promoter of *adpA* and dissociates the ArpA from the DNA to switch on expression of the necessary functions for cellular differentiation. Intracellular localization of AfsA suggests that A-factor diffuses freely through the membrane to induce signal transduction.

ArpA was purified from a cell-lysate of *S. griseus*, and on the basis of its partial amino acid sequence, the *arpA* gene, encoding a 276-amino-acid protein with a calculated M_r of 29.1 kDa, was cloned.¹³⁾ The A-factor-binding activity was observed with a homodimer of ArpA. The N-terminal portion of the protein contains a helix-turn-helix DNA-binding motif, which suggests that it exerts its regulatory function for the various phenotypes by directly binding to a certain DNA sequence(s) in a key gene(s). Oligonucleotides specifically bound by ArpA were selected from a pool of chemically

synthesized random-sequence oligonucleotides by rounds of a binding/immuno-precipitation/amplification procedure or SELEX by using histidine-tagged ArpA and anti-ArpA antibody. The fragments recovered by SELEX were found to contain a 22 bp palindromic binding site with a consensus sequence, 5'-GG(T/C)CGGT(A/T)(T/C)G(T/G)-3', as one-half of the palindrome.³²⁾ The palindromic binding site suggests that one subunit of ArpA dimer binds one half of the palindrome and the other subunit binds the other half of the palindrome. *In vitro* experiments with the consensus binding site showed that ArpA binds the target site in the absence of A-factor, and does not bind the target in the presence of A-factor at concentrations more than 32 nM.³²⁾ In addition, exogenous addition of A-factor to the ArpA-DNA complex caused immediate dissociation of ArpA from the DNA.

X-ray crystallography of a γ -butyrolactone receptor. Site-directed mutagenesis of the helix-turn-helix DNA-binding motif of ArpA yielded a mutant ArpA protein (Val41Ala) that lacked the DNA-binding ability but still retained the A-factor-binding ability.³³⁾ Conversely, mutant Trp119Ala lacked the A-factor-binding ability but retained the DNA-binding ability, indicating that Trp-119 is essential for A-factor-binding. On the other hand, mutant Pro115Ser retained the A-factor-binding ability but lacked the DNA-binding ability.³⁴⁾ These findings showed that ArpA contains two independently functional domains, a DNA-binding domain and an A-factor-binding domain (or regulatory domain).

Since all attempts to crystallize ArpA had failed due to its property to aggregate very readily in solution, CprB,³⁵⁾ an ArpA homologue in *S. coelicolor* A3(2) consisting of 215 amino acids with about 30% identity, was chosen for structural analysis. Although the ligand of CprB is still unknown, it recognizes and binds the same nucleotide sequence as ArpA.³³⁾ CprB was crystallized in three different forms, Ia, Ib, and II. The structures of two forms (Ia and Ib) of the three were determined at 2.4 Å resolution, which turned out to be a dimer with an "Ω" shape (Fig. 5).³⁶⁾ The two subunits interact with six hydrogen bonds and three water-mediated hydrogen bonds. In addition, there is a disulfide bond via Cys-159 between the subunits. This disulfide bridge is specific to CprB, because typical γ -butyrolactone receptors contain

Fig. 5. Overall structure of CprB, an ArpA homologue. Each subunit of the CprB dimer contains a C-terminal ligand-binding pocket and an N-terminal helix-turn-helix DNA-binding motif. A model structure of the CprB-DNA complex, provided by R. Natsume,³⁶⁾ is shown. One subunit of the CprB dimer is drawn by a ribbon model and the other subunit is drawn by a tube model. Helices $\alpha 1$, $\alpha 2$, $\alpha 3$, and $\alpha 4$ are indicated in red, orange, yellow, and green. The ligand-binding pocket is 5 Å diameter and 20 Å long, which is enough to accommodate the entire molecule of a γ -butyrolactone in an extended form, shown by a ball model. When the ligand is embedded in the pocket, a long helix-4 is relocated to move the DNA-binding domain (DBD) outside, as shown, resulting in dissociation of the receptor from the DNA, shown by a stick model. Reproduced in a modified form from Horinouchi.⁴⁾

no Cys residue at this position. The DNA-binding domain is composed of three N-terminal helices, $\alpha 1$, $\alpha 2$, and $\alpha 3$. Of the three, $\alpha 2$ and $\alpha 3$ form a typical helix-turn-helix motif. The residues on helix $\alpha 3$ are completely conserved among the several γ -butyrolactone receptors, which is consistent with the observations that some γ -butyrolactone receptors, such as CprA, CprB and AprA, recognize and bind the same nucleotide sequence.³³⁾

A large cavity is present in the regulatory domain, which seems to be a ligand-binding pocket with 5 Å diameter and 20 Å long. Trp-127, corresponding to Trp-119 of ArpA essential for A-factor binding, participates in forming the pocket. The docking study suggests that a γ -butyrolactone molecule is completely accommodated in the pocket in an extended manner and Trp-127 causes a hydrophobic interaction with the alkyl chain of a γ -butyrolactone molecule. The hydrophobic inter-

action between Trp-127 and the alkyl chain of the ligand would stabilize the ligand binding.

A database search for structural comparison revealed that CprB has an overall structure similar to the TetR family proteins, TetR and QacR. TetR, controlling the tetracycline resistance gene in *Escherichia coli*, uses tetracycline- Mg^{2+} as its effector ligand and QacR, controlling a multi-drug resistance gene in *Staphylococcus aureus*, uses multiple lipophilic drugs, such as rhodamine, ethidium, crystal violet, and malachite green. The conformational changes of TetR and QacR caused by the respective ligands cause the shift of a helix $\alpha 6$, which in turn induces the relocation of a long helix $\alpha 4$ and the DNA-binding domain through van der Waals interactions between them. As a result of the relocation of the DNA-binding domain, QacR/TetR dissociates from the promoter region of the target genes. The crystal structure of form Ib CprB is closely related to that of QacR in complex with its target DNA,³⁷⁾ and form Ia to that of TetR. In addition, in the case of CprB, ligand binding causes the concomitant shifts of two helices, corresponding to $\alpha 4$ and $\alpha 6$ in the TetR family members, and the DNA-binding domain. According to the mechanism of conformational changes of TetR upon tetracycline binding,^{38),39)} it is therefore reasonable to assume that binding of A-factor induces the relocation of the DNA-binding domain via the long helix $\alpha 4$, which in turn causes dissociation of the receptor from the promoter region of the target gene(s) to initiate their transcription.

4. AdpA, a transcriptional activator for streptomycin biosynthesis

AdpA as an A-factor-dependent protein that binds an upstream activation sequence of *strR*. All the 27 genes involved in streptomycin biosynthesis consist of a big cluster on the chromosome of *S. griseus*, and a positive regulatory gene *strR* for streptomycin biosynthesis is located amid the cluster (Fig. 6).^{40),41)} Concerning A-factor as a trigger of streptomycin biosynthesis, a question we raised was by what protein the A-factor signal was received and transmitted to the streptomycin biosynthesis genes. Analyses conducted by D. Vujaklija *et al.*⁴²⁾ on transcription of part of the streptomycin biosynthesis genes by S1 nuclease mapping showed that transcription of one mRNA species starting from the promoter of *strR* was

Fig. 6. The A-factor signal relay to the streptomycin biosynthesis genes. Construction and transcriptional organization of the gene cluster for streptomycin biosynthesis, including the pathway-specific transcriptional activator *strR*, are shown. The A-factor signal relay starts with A-factor and then transmitted to ArpA to AdpA to StrR, and finally to the nine transcriptional units covering the whole gene cluster. StrR is required for the transcriptional activation of all the nine transcriptional units by directly binding to the promoters (indicated by solid lines) and by a still unknown mechanism (indicated by dotted lines).

dependent on A-factor. Subcloning experiments indicated that the region 371 to 241 bp upstream of the transcriptional start point of *strR* is essential for its A-factor dependence. These findings implied that a regulatory protein recognizes a specific upstream activation sequence (UAS) for the *strR* promoter to respond to A-factor. Further subcloning shortened the UAS sequence to be 293 to 242 bp from the transcriptional start point of *strR*. Gel mobility shift assay with the 52 bp UAS sequence for *strR* led to detection of a specific DNA-binding protein in a cell-lysate of the parental strain, but not of an A-factor-deficient mutant of *S. griseus*, suggesting that the binding protein was produced in response to A-factor.⁴³⁾ We therefore assumed that the A-factor signal from the receptor ArpA is transmitted to the UAS upstream from the *strR* promoter within the streptomycin biosynthetic gene cluster via this A-factor-dependent protein, named AdpA. Since *strR* was later found to be the pathway-specific transcriptional activator for the streptomycin biosynthesis genes,^{41),44),45)} we postulated that the A-factor signal might be transmitted via AdpA to the UAS for *strR* to induce its transcription and the StrR protein, in turn, activates the transcription of the streptomycin biosyn-

thesis genes. Our postulation turned out to be true, as described below.

Transcriptional activation of the whole streptomycin biosynthesis genes by AdpA via *strR*. After purification of AdpA and determination of its partial amino acid sequences, the *adpA* gene was cloned by PCR.⁴⁵⁾ 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 *adpA* contained a 22 bp palindrome, caggcAGGAACGGACC*GCGCGGTACGCT (the underlines indicate the -35 and -10 promoter elements; * indicates a dyad axis),⁴⁵⁾ which showed similarity to the consensus sequence of the ArpA-binding site, (A/C)C(A/G)(T/A)ACCC(A/G)CC*GG(T/C)CGGT(A/T)(T/C)G(T/G).³²⁾ As expected, ArpA bound the promoter region of *adpA* in the absence of A-factor but did not in the presence of A-factor. In addition, exogenous addition of A-factor to the ArpA-DNA complex immediately dissociated ArpA from the DNA. Thus, the promoter of *adpA* turned out to be a target of ArpA. Consistent with this, S1 nuclease mapping showed that *adpA* was transcribed only in the presence of

A-factor and *strR* was transcribed only in the presence of intact *adpA*. Furthermore, *adpA* disruptants produced no streptomycin and overexpression of *adpA* caused the wild-type *S. griseus* strain to produce streptomycin at an earlier growth stage in a larger amount.

All the above-described data revealed a missing link between ArpA and streptomycin biosynthesis genes; the A-factor signal must be relayed from ArpA to AdpA to StrR, and finally to the streptomycin biosynthesis genes (Fig. 6).⁴¹⁾ Subsequent detailed analysis of transcriptional activation of *strR* by AdpA showed that an AdpA dimer binds two sites in front of the *strR* promoter; one is the above-described UAS for *strR* at nucleotide position –270, with respect to the transcriptional start point of *strR*, and the other is approximately at nucleotide position –50. One subunit of AdpA consists of an N-terminal dimerization domain and a C-terminal DNA-binding domain, which are probably connected via a flexible linker. For transcriptional activation, the two AdpA-binding sites should be occupied by an AdpA dimer, speculatively because the two AdpA dimers form a complex, as a result of which a DNA loop via the AdpA complex is formed. StrR as the pathway-specific transcriptional activator then activates all nine polycistronic transcriptional units covering the whole streptomycin biosynthesis genes in a total of 27 genes, triggering biosynthesis of streptomycin from glucose.⁴¹⁾

It is noted that the major streptomycin resistance determinant, *aphD*, encoding streptomycin-6-phosphotransferase is located just downstream of *strR* and co-transcribed with *strR* by read-through from the AdpA-dependent *strR* promoter.⁴²⁾ The co-transcription of *strR* and *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.

5. The A-factor regulatory cascade

AdpA regulon. Although AdpA was discovered as a transcriptional activator for *strR* encoding a key regulator for streptomycin biosynthesis, an *adpA*-disruptant lost the abilities to produce not only streptomycin but also a yellow pigment, griseofavin,^{9),46)} and even to form aerial mycelium. These observations implied that AdpA controls multiple, unlinked genes necessary for physiological

and morphological differentiation. By SELEX using gel mobility shift assay in the selection step, multiple DNA sequences from the *S. griseus* chromosome bound by AdpA were isolated, such as the upstream sequence of an A-factor-dependent extracytoplasmic function (ECF) sigma factor (σ^{AdsA}) that is essential for aerial mycelium formation.⁴⁷⁾ Repeated experiments yielded more than 60 DNA fragments specifically bound by AdpA. From this library various genes involved in secondary metabolism and morphological differentiation have been identified to be targets of AdpA (Fig. 2).^{3),4)}

The AdpA target genes contain one or more AdpA-binding sites in the vicinity of their promoters, and some genes require simultaneous binding of a dimer of AdpA to multiple sites as observed with the *strR* promoter. All the target sites contain a consensus AdpA-binding sequence, 5'-TGGCSNGWWY-3' (S: G or C; W: A or T; Y: T or C; N: any nucleotide). The binding sites are, for example, 200 bp upstream and 25 bp downstream from the transcriptional start points. Despite the differences in binding position with respect to the promoter and in number of binding sites, AdpA recruits RNA polymerase to the promoter of the target genes and facilitates isomerization of the RNA polymerase-DNA complex into an open complex competent for transcriptional initiation.^{41),48)}

Members of the AdpA regulon and their functions. A number of genes have been identified as targets of AdpA by not only the binding assay mentioned above but also by genetic analysis. These genes consist of the AdpA regulon, whose functions are simultaneously activated by AdpA at a specific point in the growth phase (Fig. 2). It means that the signal from ArpA is greatly amplified and diverged at this regulatory step via AdpA to control a large number of genes involved in morphological differentiation and secondary metabolism. Members of the AdpA regulon are as follows.

(i) *Genes for morphological differentiation:* *adsA* encoding an ECF σ factor (σ^{AdsA}),⁴⁷⁾ *amfR* encoding a transcriptional activator for the *amf* operon,^{49),50)} *ssgA* encoding a small acidic protein essential for spore septum formation,⁵¹⁾ genes encoding extracellular proteases including a metalloendopeptidase,⁵²⁾ two trypsin-type proteases,⁵³⁾ and three chymotrypsin-type proteases,⁵⁴⁾ and a gene for *Streptomyces* subtilisin inhibitor (SSI).⁵⁵⁾

σ^{AdpA} belongs to an ECF subfamily of the σ^{70} class, which are known as environmentally responsive transcriptional regulators to control a variety of functions in various bacterial species.⁵⁶⁾ *S. griseus* presumably uses this σ factor to transcribe specific genes for morphological development in response to A-factor. It is conceivable that one or more σ factors function downstream of σ^{AdpA} as in the sigma cascade for endospore development in *Bacillus subtilis*. The *amf* operon is an orthologue of the *ram* gene cluster in *S. coelicolor* A3(2),⁵⁷⁾ which directs production of a morphogenetic surfactant, a lantibiotic-type 22-amino-acid peptide containing unusual amino acids with a thioether-cross linked loop structure. AmfT is responsible for processing and modification of AmfS, a 43-amino-acid peptide, to form the morphogenetic peptide, which is secreted into medium and acts on substrate hyphae for upward growth of aerial hyphae into the air.⁵⁸⁾ SsgA is a 136-amino-acid protein with a strong negative charge, which is detected in *S. griseus* only in the presence of A-factor.⁵⁹⁾ Extracellular proteases and a protease inhibitor are probably involved in the apoptotic lysis of cellular proteins in substrate hyphae to supply materials for generation of aerial hyphae.

(ii) *Genes for secondary metabolism: strR*, the pathway-specific transcriptional activator for streptomycin biosynthesis⁴¹⁾ and a gene encoding a transcriptional factor probably for biosynthesis of a polyketide compound.⁴⁸⁾ *griR*, the pathway-specific transcriptional activator for grixazone biosynthesis, is also indirectly activated by AdpA.⁴⁶⁾ In addition to these genes, many gene clusters for biosynthesis of a certain secondary metabolite in *S. griseus* are activated by AdpA, as revealed by our DNA microarray analysis on the basis of the whole genome sequence of *S. griseus* (manuscript in preparation).

The genome of *S. griseus* consists of 8,545,929 bp, which contains about 7,140 open reading frames (manuscript in preparation). Preliminary DNA microarray analysis has shown that more than 640 genes are activated by AdpA and about 370 genes are relatively down-regulated in the ΔadpA background. The number of genes whose expression is switched-on or affected by *adpA* (actually, A-factor itself) is amazing, which convinces one to believe that A-factor is a true microbial hormone.

Autoregulation of *adpA*. AdpA was found to repress its own transcription by cooperative binding to the promoter region containing multiple operator sites (Fig. 7).⁶⁰⁾ Three operator sites exist in the region upstream of *adpA*, site 1 approximately at nucleotide position -100, site 2 at the promoter elements, and site 3 at position +80. Site 2 therefore serves as the operators for both ArpA and AdpA. AdpA bound to a strong binding site 1 increases the affinity for AdpA of a weak site 2, probably by forming a DNA loop *via* the two molecules of AdpA dimer, thus preventing RNA polymerase from access to the promoter. AdpA bound to site 3 with rather weak affinity represses the *adpA* promoter activity independently of sites 1 and 2, perhaps preventing RNA polymerase from chain elongation. Consistent with this model, the *in vivo* transcription of *adpA* containing mutated site 1 or site 3 was greatly increased along with a marked enhancement in streptomycin production. The cooperative binding of AdpA to the multiple operator sites allows effective regulation to result from small alterations in the AdpA concentration and serves as a fine sensor of the AdpA concentration. Highly self-controlled transcription of *adpA* may be required for ordered expression of a number of genes of the AdpA regulon to control complex processes of physiological and morphological differentiation in *S. griseus*.

***adpA* as the only significant target of ArpA.** Although all the results described above allow us to construct a model of the A-factor regulatory cascade shown in Fig. 2, the possibility that ArpA targets additional genes other than *adpA* was still not excluded. However, the following observations support the idea that there is no significant target gene of ArpA other than *adpA*. As already mentioned, sporulation and streptomycin production in an *arpA*-disruptant are markedly enhanced due to the absence of ArpA acting as a repressor for these phenotypes. On the other hand, a mutant strain KM2 expressing a mutant ArpA (Trp119Ala) failed to produce streptomycin or to form spores, since this amino acid substitution abolishes A-factor-binding ability of ArpA, resulting in permanent repression of the target gene irrespective of the presence of A-factor.³³⁾ When *adpA* under the control of a foreign constitutively expressed promoter was introduced into mutant strain KM2, all these defects in the mutant were

Fig. 7. A model for strict control of *adpA* transcription by the A-factor receptor ArpA in the early stage of growth and by AdpA itself after the middle of exponential growth phase. (A) Architecture of the *adpA* promoter and operators. The number on the sequence indicate the nucleotide positions with respect to the transcriptional start point +1 of *adpA*. The -35 and -10 promoter elements of *adpA* overlap with the ArpA-binding site and one (site 2) of the AdpA-binding sites (sites 1 to 3). (B) Control of the *adpA* transcription by ArpA and AdpA. *Stage 1*. In the early growth stage when the concentration of A-factor is still low, ArpA sits exactly on the promoter elements and represses its transcription to avoid production of AdpA. *Stage 2*. When the concentration of A-factor (shown by a triangle) reaches a critical level, it binds the DNA-bound ArpA and dissociates ArpA from the DNA, thus allowing RNA polymerase to bind the *adpA* promoter and initiate transcription. *Stage 3*. When the intracellular concentration of AdpA is low, it probably binds site 1 having the highest affinity to AdpA among the three AdpA-binding sites. However, the binding of AdpA only to site 1 exerts no effect on the *adpA* transcription. *Stage 4*. When the intracellular concentration of AdpA exceeds an appropriate level required for ordered gene expression in a concerted manner, the AdpA dimer bound to site 1 recruits another AdpA dimer to a rather weak binding site 2, thus forming a DNA loop in a way that RNA polymerase cannot gain access to the promoter. A third AdpA dimer also binds to a weak binding site 3, located downstream of +1, inhibits elongation of the *adpA* transcript that has escaped from the repression by the AdpA dimers that bind sites 1 and 2.

restored.²⁹⁾ This result would not be obtained if ArpA has additional targets other than *adpA*, because introduction of the constitutively expressed *adpA* is not sufficient to restore the phenotypes directed by the putative additional target gene(s), which might still be repressed by the A-factor-insensitive mutant ArpA. Thus complete restora-

tion of the defects only by constitutively expressed *adpA* implied that *adpA* is the sole significant target of ArpA.

6. Distribution and evolution of γ -butyrolactone regulatory systems

Distribution of γ -butyrolactone regulatory

γ -Butyrolactone receptors γ -Butyrolactone synthases

Fig. 8. Comparison of the phylogenetic positions of several ArpA homologues (γ -butyrolactone receptors) and AfsA homologues (γ -butyrolactone synthases) from the same *Streptomyces* species. Phylogenetic trees were constructed by the maximum likelihood method. Reproduced in a modified form from Nishida *et al.*⁶³⁾

systems in other *Streptomyces*. A variety of A-factor homologues have been recognized as auto-regulatory factors for secondary metabolism and morphological differentiation in various *Streptomyces* species. Genes homologous to those involved in the A-factor signaling system in *S. griseus*, especially the *afsA/arpA* homologues, are distributed more widely among a number of *Streptomyces* species and a few other species of Actinobacteria. On the basis of amino acid sequence similarity, at least ten *afsA* homologues have been listed.²⁵⁾ *barX* for production of VBs in *S. virginiae*¹⁴⁾ and *scbA* for SCB1 in *S. coelicolor* A3(2)⁶¹⁾ are examples. Although there was controversial discussion about the function of AfsA homologues,²⁵⁾ recent results on the *in vitro* biosynthesis of a key intermediate (4, Fig. 3) of A-factor by AfsA strongly suggest that these homologues also direct similar γ -butyrolactone synthases.²²⁾ On the other hand, nine A-factor receptor (ArpA) homologues, such as BarA in *S. virginiae*,¹⁴⁾ FarA in *S. lavendulae*⁶²⁾ and ScbR

in *S. coelicolor* A3(2),⁶¹⁾ have been reported as a γ -butyrolactone receptor in the respective strains. Furthermore, Takano²⁵⁾ listed at least 33 candidates as probable γ -butyrolactone receptors. The receptors so far examined show strict ligand specificity for their cognate γ -butyrolactone ligands,^{13)–15)} while they share the consensus amino acid sequence for DNA binding.³³⁾

In various *Streptomyces* species, genes of the *afsA* and *arpA* homologues are present in a topological pair on the chromosome as is found for *barX/barA*, *farX/farA*, and *scbA/scbR*, and most of the pairs locate within a biosynthetic gene cluster for a certain secondary metabolite. The construction might reflect the function of the γ -butyrolactone signals in these species, which directly control the respective pathway-specific transcriptional activator genes or some other regulatory genes within the cluster, thus controlling production of only the secondary metabolite directed from the gene cluster.

Among the pairs without a topological linkage, *afsA/arpA* in *S. griseus* is unique as to the location of *afsA* near the terminal region of the linear chromosome, which causes marked genetic instability of the γ -butyrolactone signaling system in this species.²⁰⁾ Perhaps, the definitive genetic determinant that switches the cellular stage from vegetative growth to differentiation is in a dynamic balance of rapid loss and acquisition in population under the selective pressure in the environment. This may be beneficial to modulate the ability of the whole population to respond to environmental conditions in such a manner that a decrease in the differentiation ability in population results in an increase in the growing capacity, thus facilitating competition with other species in a nutritionally rich environment.

Evolution of γ -butyrolactone regulatory systems. Phylogenetic analysis of the γ -butyrolactone receptors suggests that the ancestral ArpA protein had existed as a DNA-binding protein, not as a γ -butyrolactone receptor, before the appearance of a γ -butyrolactone receptor in the course of the bacterial evolution.⁶³⁾ Once a *Streptomyces* strain acquired a γ -butyrolactone as a chemical signaling molecule during the evolution, the pre-existing ArpA ancestor employed it as a ligand to modulate its own DNA-binding activity.

When the phylogenetic positions of the *afsA* and *arpA* homologues existing as a pair in a given *Streptomyces* species are compared, each in the pair is located at a greatly different position in the phylogenetic tree (Fig. 8).⁶³⁾ For example, the γ -butyrolactone synthase AfsA in *S. griseus* is the most closely related to ScbA in *S. coelicolor* A3(2), although the γ -butyrolactone receptor ArpA in *S. griseus* is the most closely related to BarA in *S. virginiae*. Even if an *afsA* homologue and an *arpA* homologue locate adjacently on the chromosome as observed in many *Streptomyces* species, their evolutionary history of the individuals was found to be different. These facts indicate that the evolutionary processes of the AfsA homologues and the ArpA homologues are independent of each other. This is quite different from the evolutionary relationship of the inducer/receptor or LuxI/LuxR pair in quorum sensing systems in the Gram-negative bacteria, in which LuxI and LuxR in a given strain have evolved concomitantly.⁶⁴⁾ Noteworthy is that a receptor in a given *Streptomyces*

species strictly recognizes the specific γ -butyrolactones produced by the same species, indicating the tight correlation between the ligand specificity of ArpA homologues and the product specificity of AfsA homologues, which has been established during the evolutionary processes. It implies that the regulation of cellular functions via the diffusible γ -butyrolactone signals may be effective as the survival strategy of this group of bacteria in the ecosystem.

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Profile

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Profile

Teruhiko Beppu was born in 1934 in Tokyo. He is currently a member of Japan Academy since 2004 as well as Professor Emeritus of the University of Tokyo and Professor of Advanced Research Institute for the Sciences and Humanities in Nihon University. He started his research careers at the University of Tokyo, where he obtained PhD in 1957, and worked as Professor of Fermentation and Microbiology at Department of Agricultural Chemistry during 1977–1994. His research activity during this period covered rather wide fields from basic and applied microbiology to enzymology and eukaryotic cell biology, the accomplishments of which are exemplified by (i) the first cloning and expression of calf prochymosin cDNA for production as a milk-coagulant in cheese industry, (ii) discovery of unique bioactive microbial products, such as trichostatin (a histone deacetylase inhibitor) and leptomycin (an inhibitor of nuclear pore transport), and (iii) rediscovery of A-factor and elucidation of its mechanism to control cellular differentiation in the streptomycin-producing bacterium, *Streptomyces griseus*. In the last topic, Prof. S.

Horinouchi, the coauthor of this review article, had been a close coworker since almost its beginning. In 1994, TB moved to College of Bioresource Sciences, Nihon University, and started a pioneering work on microbial symbiosis, which was supported as a 21st Century Center of Excellence Research Program of the Ministry of Education, Culture, Sports, Science and Technology.

According to these achievements and efforts, he received several awards such as the Award of the Society of Agricultural Chemistry in 1986, the Charles Tom Award of the Society of Industrial Microbiology, U.S.A. in 1995, the Purple Ribbon Medal in 1996, and the Japan Academy Prize in 1998. He was also involved in several social activities such as Chairman of the Japan Bioindustry Association during 2003–2007.