Appl Microbiol Biotechnol (2002) 59:419–425
DOI 10.1007/s00253-002-1045-1

MINI-REVIEW

T. Umeyama · P.-C. Lee · S. Horinouchi

Protein serine/threonine kinases in signal transduction for secondary metabolism and morphogenesis in *Streptomyces*

Received: 18 March 2002 / Revised: 3 May 2002 / Accepted: 3 May 2002 / Published online: 25 June 2002
© Springer-Verlag 2002

Abstract A number of proteins in the Gram-positive bacterial genus *Streptomyces* are phosphorylated on their serine/threonine and tyrosine residues in response to developmental phases. AfsR is one of these proteins and acts as a transcriptional factor in both the regulation of secondary metabolism in *Streptomyces coelicolor* A3(2) and morphological differentiation in *Streptomyces griseus*. In *S. coelicolor* A3(2), AfsR is phosphorylated on its serine and threonine residues by more than three protein kinases whose kinase activity is enhanced by means of autophosphorylation on their serine and threonine residues. The degree of autophosphorylation of AfsK is regulated by KbpA which, by binding directly to the kinase domain of AfsK, inhibits its autophosphorylation. Phosphorylation of AfsR enhances its DNA-binding activity and causes it to bind the promoter elements, including –35, of *afsS*, thus resulting in activation of *afsS* transcription. ATPase activity of AfsR is essential for this transcriptional activation, probably because the energy available from ATP hydrolysis is required for the isomerization of the closed complex between AfsR and RNA polymerase to a transcriptionally competent open complex. *afsS*, encoding a 63-amino-acid protein, then activates transcription of *act*II-ORF4, a pathway-specific transcriptional activator in the actinorhodin biosynthetic gene cluster, in an as yet unknown way. Distribution of the *afsK-afsR* systems in a wide variety of *Streptomyces* species and the presence of many phosphorylated proteins in a given *Streptomyces* strain suggest that the signal transduction via not only two-component regulatory systems but also serine/threonine kinases generally regulates secondary metabolism and morphogenesis in this genus.

T. Umeyama · P.-C. Lee · S. Horinouchi (✉)
Department of Biotechnology,
Graduate School of Agriculture and Life Sciences,
The University of Tokyo, Bunkyo-ku, Tokyo 113–8657, Japan
e-mail: asuhori@mail.ecc.u-tokyo.ac.jp
Tel.: +81-3-58415123, Fax: +81-3-58418021

Present address:
T. Umeyama, National Institute of Infectious Diseases,
Shinjuku-ku, Tokyo 162–8640, Japan

Introduction

Protein kinases, especially serine/threonine and tyrosine kinases, control cellular responses to a plethora of environmental cues via signal transduction networks and response mechanisms in eukaryotes. It has long been thought that histidine and aspartate are the phosphorylated residues in the typical two-component regulatory systems of prokaryotes. However, recent studies together with genome analyses of prokaryotes have shown that serine/threonine and tyrosine kinases are also present in prokaryotes (Kennelly and Potts 1996; Zhang 1996). Possibly, all prokaryotes, at one time, contained the genetic information necessary to construct protein phosphorylation-dephosphorylation networks that target serine, threonine, and/or tyrosine residues on proteins. The genus *Streptomyces* consists of Gram-positive, filamentous, soil-inhabiting bacteria and is characterized by complex morphological differentiation resembling that of filamentous fungi and by the ability to produce a wide variety of secondary metabolites including antibiotics, immunosuppressors, and other biologically active substances. It is conceivable that *Streptomyces* has conserved signal transduction systems via "eukaryotic"-type protein phosphorylation for the growth as multi-cellular filamentous hyphae, by which distant cells in an individual hypha simultaneously respond to environmental cues in soil. Like morphological differentiation, secondary metabolism is also influenced by environmental conditions, implying these processes may be controlled by the same signal transduction pathways.

A number of proteins in *Streptomyces* have been found to be phosphorylated: in *S. griseus* (Hong et al. 1993), *S. coelicolor* A3(2) (Hong and Horinouchi 1998), *S. collinus* (Mikulik et al. 1998), *S. albus* (Dobrova et al. 1990), and *S. fradiae* (Elizarov et al. 2000; Elizarov and Danilenko 2001). Eukaryotic protein kinase inhibitors,

such as K-252a, staurosporine, and herbimycin, inhibited phosphorylation of these proteins and impaired aerial mycelium formation and secondary metabolism in *S. griseus* and *S. coelicolor* A3(2) (Hong et al. 1993; Hong and Horinouchi 1998). Mikulik et al. (2001) reported that 11 ribosomal proteins in *S. collinus* are phosphorylated at serine and/or threonine residues. The peptidyl transferase activity of phosphorylated ribosomes was higher than that of unphosphorylated ones, suggesting that protein phosphorylation on ribosomes controls the protein synthesis rate. In addition to protein phosphorylation on serine and threonine residues, growth-phase dependent tyrosine phosphorylation has been observed for *S. griseus* (Kang et al. 1999) and several other *Streptomyces* strains (Waters et al. 1994). Recent completion of the genome project of *S. coelicolor* A3(2) (http://www.sanger.ac.uk/Projects/S_coelicolor/) has revealed the presence of about 30 proteins having a kinase catalytic domain similar to those of the typical eukaryotic serine/threonine kinases. All these observations clearly show that *Streptomyces* possesses several protein kinases of eukaryotic type, some of which regulate growth, morphogenesis, and secondary metabolism.

Of the protein serine/threonine kinases in *Streptomyces*, AfsK is the first example that has been shown to influence secondary metabolism (Matsumoto et al. 1994). Identification of AfsR as one of the substrates of AfsK made the AfsK-AfsR system the first example of a functionally relevant serine/threonine phosphorylation system not only in the genus *Streptomyces* but also in the world of prokaryotes. Subsequent studies from this laboratory have shown that the AfsK-AfsR system is widely distributed in *Streptomyces* and influences secondary metabolism and morphogenesis. Furthermore, we have revealed that AfsR serves as a transcriptional activator for *afsS*, which encodes a small protein and activates the pathway-specific transcriptional activator, *act*II-ORF4, in the actinorhodin biosynthetic gene cluster (Lee et al. 2002). Thus, part of the signal transduction by AfsK-AfsR has been elucidated. In this mini-review, we summarize the AfsK-AfsR signal transduction leading to secondary metabolite formation in *S. coelicolor* A3(2).

AfsK, a serine/threonine kinase

Phosphorylation of AfsR by AfsK

During our study on a pleiotropic regulatory gene *afsR* we found, downstream of that gene, an open reading frame coding for a protein consisting of 799 amino acids and showing significant sequence similarity to eukaryotic protein serine/threonine kinases (Matsumoto et al. 1994) (Fig. 1). All 11 major subdomains conserved in serine/threonine-specific and tyrosine-specific protein kinases (Hanks et al. 1988) were conserved in the NH_2-terminal portion of AfsK. AfsK produced in *Escherichia coli* was capable of autophosphorylation on serine and threonine residues and phosphorylation of AfsR on serine and threonine residues. The catalytic domain alone (Met-1 to Arg-311) also showed activities to autophosphorylate and phosphorylate AfsR. The K44A mutant enzyme, in which one of the active-sites, Lys-44, was replaced by alanine by site-directed mutagenesis, lost the ability to autophosphorylate or to phosphorylate AfsR (Umeyama and Horinouchi 2001).

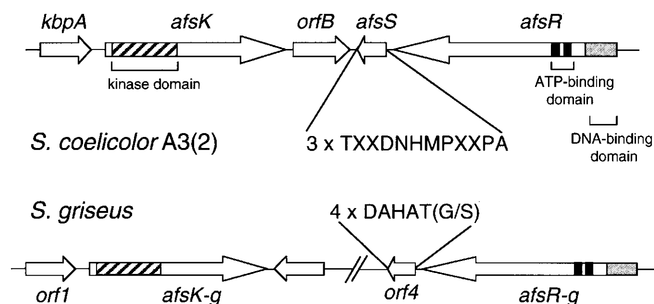

Fig. 1 Gene organizations in the neighbor of *afsK* and *afsR* in *Streptomyces coelicolor* A3(2) and *S. griseus*. AfsR contains a DNA-binding domain in the NH_2-terminal portion (*gray box*) and a nucleotide-binding motif in the mid-region (*black box*). AfsK has the kinase catalytic domain in its NH_2-terminal region (*diagonal box*). *afsS*, encoding a small protein, is located downstream of *afsR*. Three repeats of 12 amino acids in *S. coelicolor* A3(2) AfsS and four repeats of 6 amino acids in *S. griseus* AfsS are shown

Nádvorník et al. (1999) pointed out that AfsK has seven tandem repeats of 11 or 12 amino acids in the COOH-terminal region with similarity to the tryptophan-docking (WD) motif known to stabilize a symmetrical three-dimensional structure called a propeller structure. The tandem repeats are separated from the kinase catalytic domain by an Ala/Pro-rich region which, with the seven-repeat WD motifs, may contribute to membrane anchoring. In fact, AfsK appears to bind loosely to the inner side of the membrane, because it is recovered from the membrane fraction by mild treatment with detergents during purification (Matsumoto et al. 1994).

Involvement of *afsK* in secondary metabolism

As described below, disruption of *afsR* in *S. coelicolor* A3(2) resulted in severe reduction of actinorhodin production; *afsK* disruptants produced a smaller amount of actinorhodin than the parental strain (Matsumoto et al. 1994). However, the *afsK* mutations did not affect morphogenesis to a noticeable extent, suggesting that *afsK*, like *afsR*, is concerned only with secondary metabolism in *S. coelicolor* A3(2). The difference in the degree of reduction of actinorhodin production between the *afsR* and *afsK* disruptants can be ascribed to the presence of additional kinases able to phosphorylate AfsR, as described below.

KbpA, an inhibitor of AfsK autophosphorylation

We focused on an open reading frame named *kbpA* that is located just upstream of *afsK* in both *S. coelicolor*

A3(2) (Umeyama and Horinouchi 2001) and *S. griseus* (Umeyama et al. 1999) (Fig. 1), on the assumption that functionally related genes are usually organized as an operon. The *kbpA* products (KbpA, AfsK-binding protein) in both strains show 47% identity to each other. Overexpression of KbpA in *S. coelicolor* A3(2), by means of placing *kbpA* under the control of the thiostrepton-inducible promoter *tipA* in a high-copy-number plasmid, reduced actinorhodin production. On the other hand, complete deletion of the *kbpA*-coding region caused overproduction of actinorhodin on various media. These observations suggest that KbpA acts as a repressor for actinorhodin production.

In vitro experiments showed that KbpA directly binds the catalytic domain of the unphosphorylated form of AfsK and inhibits the autophosphorylation of that protein (Umeyama and Horinouchi 2001) but it does not bind the autophosphorylated form. This is consistent with the in vivo observations that KbpA serves as a repressor for actinorhodin production. It is thus concluded that KbpA inhibits the autophosphorylation of AfsK by means of protein–protein interaction. *kbpA* is transcribed throughout growth, and its transcription is enhanced when production of actinorhodin and undecylprodigiosin has already started. KbpA appears to put a brake on the unlimited production of the pigments that has been commenced by the AfsK-AfsR system, playing a role in a negative-feedback system.

AfsR, a pleiotropic regulator

afsR was cloned from *S. coelicolor* A3(2) as a gene that "awakened" the sleeping genes for the biosynthesis of actinorhodin, undecylprodigiosin, and A-factor in *S. lividans*, in which the biosynthetic genes for these metabolites are silent (Horinouchi et al. 1983; Horinouchi and Beppu 1984). The cloned gene phenotypically complemented an *afsB* mutation of *S. coelicolor* A3(2) (Horinouchi et al. 1983). *afsB* of *S. coelicolor* A3(2) was thought to be a regulatory gene for antibiotic production because *afsB* mutants failed to produce these metabolites (Hara et al. 1983). Although the cloned *afsR* gene had been assumed to represent *afsB*, Stein and Cohen (1989) showed that the *afsR* product is a bypass function with regard to *afsB* complementation. *afsR* encodes a 993-amino-acid protein with a molecular mass of 106 kDa (Horinouchi et al. 1990). Disruption of *afsR* on the *S. coelicolor* A3(2) chromosome results in significant, but not complete, loss of production of actinorhodin, undecylprodigiosin and calcium-dependent antibiotic (CDA). *afsR* is therefore called a global regulatory gene for secondary metabolite production. *afsR* disruptants normally develop spores on any medium.

We found that AfsR was phosphorylated by incubation with [γ-^{32}P]ATP and a cell lysate of *S. coelicolor* A3(2), indicating the presence of a kinase to phosphorylate AfsR (Hong et al. 1991). Subsequently, we found that AfsK, encoded by a region near *afsR* (Fig. 1), phosphorylates serine and threonine residues of AfsR (Matsumoto et al. 1994). The NH_2-terminal portion of AfsR shows similarity to the pathway-specific transcriptional regulators of *Streptomyces*, also known as SARP-family proteins (streptomycete antibiotic regulatory proteins), such as ActII-ORF4 (Fernández-Moreno et al. 1991) and RedD (Narva and Feitelson 1990), both of which contain no typical helix-turn-helix motifs found in many bacterial DNA-binding proteins. SARPs induce transcription of their respective gene clusters through DNA-binding to specific nucleotide sequences (Wietzorrek and Bibb 1997). Despite partial similarity of AfsR to SARPs, AfsR could not substitute for ActII-ORF4 or RedD, but might function independently of these regulatory proteins to influence antibiotic production (Floriano and Bibb 1996). AfsR was therefore thought to bind some specific DNA sequences and activate their transcription, as a result of which production of secondary metabolites in *S. coelicolor* A3(2) was enhanced. In fact, introduction of multicopies of *afsR* into *S. lividans* (Horinouchi et al. 1989) and *S. coelicolor* A3(2) (Floriano and Bibb 1996) increased transcription of *act*II-ORF4, thereby enhancing the yield of actinorhodin. As described below, the true target(s) of AfsR as a DNA-binding protein is *afsS*, not *act*II-ORF4.

The *afsR* product contains Walker's A- and B-type nucleotide-binding consensus sequences in the mid-region of the protein (Fig. 1). Mutations at either one of the two nucleotide-binding sequences, which were generated by site-directed mutagenesis, resulted in reduced actinorhodin production in both *S. lividans* and *S. coelicolor* A3(2). Thus, the nucleotide-binding motifs in AfsR turned out to be important for its regulatory function (Horinouchi et al. 1990). The nucleotide-binding domain of AfsR showed similarity in amino acid sequence to ATPase domains of transcriptional factors. As expected, AfsR showed distinct ATPase and GTPase activities (Lee et al. 2002). The phosphorylation of AfsR modulated its ATPase activity by changing the sensitivity to one of the products, ADP, although the initial rates of ATP hydrolysis by the phosphorylated- and unphosphorylated-AfsR were the same. By using the promoter of *afsS* or the target of AfsR, we have revealed the essential role of the ATPase activity of AfsR in transcriptional activation, as shown below.

AfsS, a target of AfsR

Involvement of *afsS* in antibiotic biosynthesis

In *S. coelicolor* A3(2), a small open reading frame encoding a 63-amino-acid protein, named AfsS, is located immediately 3' to *afsR* (Fig. 1). AfsS contains three repeats of Thr-Xaa_2-Asp-Asn-His-Met-Pro-Xaa_2-Pro-Ala (Xaa represents a non-conserved amino acid) over its amino acid sequences (Fig. 1). In *S. griseus*, a small open reading frame coding for a protein of 58 amino acids is also present just downstream of *afsR*, which shows no homol-

ogy with AfsS (Umeyama et al. 1999). Despite no homology between the two products, AfsS in *S. griseus* contains four repeats of Asp-Ala-His-Ala-Thr-Gly/Ser. We assumed that AfsS might be functionally related to the *afsK-afsR* system, as functionally related genes are usually organized together as an operon in prokaryotes. Consistent with this idea, overexpression of *afsS* on a plasmid led to overproduction of actinorhodin and undecylprodigiosin in *S. lividans* (Matsumoto et al. 1995). The stimulatory effect of *afsS* on actinorhodin production was observed in *S. coelicolor* A3(2) and even in an *afsR* null mutant, indicating that *afsS* stimulated actinorhodin production in the absence of the intact *afsR* function (Lee et al. 2002). Floriano and Bibb (1996) observed that *afsS* enhanced actinorhodin production by stimulating transcription of *act*II-ORF4 in an as yet unknown way. In addition, Vögtli et al. (1994) also reported that overexpression of *afsR2*, the *afsS* counterpart in *S. lividans*, stimulated actinorhodin production by activating transcription of biosynthetic and regulatory genes in the *act* and *red* clusters in *S. lividans* in both *afsR*$^+$ and *afsR*$^-$ genetic backgrounds. However, *afsR2* was not transcribed in Δ*afsR* backgrounds (Kim et al. 2001), suggesting the dependence of *afsR2* transcription on *afsR*. *afsS* disruptants produced a reduced amount of actinorhodin, compared to the parental strain, although *afsR* disruptants produced almost no detectable amount of actinorhodin (Floriano and Bibb 1996; Lee et al. 2002). All these results strongly suggest the involvement of *afsS* in the regulation of secondary metabolism, possibly at a step downstream of *afsR* in the hierarchy of regulation.

Binding of AfsR to the *afsS* promoter

Transcription of *afsS* depends on AfsR, since no detectable transcription of *afsS* occurred in *afsR* mutants (Lee et al. 2002). Furthermore, *afsS* transcription was delayed in *afsK* mutants. On the other hand, *afsR* transcription was not affected by *afsS* mutations. Thus, *afsS* transcription is positively controlled by the AfsK-AfsR system. As expected from the presence of an OmpR-like DNA-binding fold in AfsR (Wietzorrek and Bibb 1997), AfsR binds a sequence, from positions –15 to –40 with respect to the transcriptional start point of *afsS*, of the sense strand and a sequence, from positions –20 to –42, of the anti-sense strand (Lee et al. 2002). The transcriptional start point of *afsS* is 143 nucleotides upstream of the start codon, with a probable –35 sequence, TTCAGC, and a –10 sequence, CACTGT. These promoter elements are somewhat deviated from the *Streptomyces* promoter sequences of housekeeping genes [TTGACA for –35 and TAGRRT (R: A or G) for –10] (Strohl 1992). Furthermore, the space between the two elements is 20 bp, whereas the standard spacing of *Streptomyces* promoters are 17–18 bp.

The binding site of AfsR, as a transcriptional activator, overlaps the –35 element of the *afsS* promoter. Although the –35 and –10 sequences are in many cases the sites to which repressors bind, several transcriptional activators bind the promoter of target genes. Examples are MerR mediating mercury resistance (Ansari et al. 1992) and SoxR mediating a global response to superoxide-generating agents (Hidalgo and Demple 1997). These transcriptional factors are believed to optimize the spacing between the –35 and –10 elements by introducing a bend and untwisting of the DNA. The standard spacing between the two elements in *E. coli* and related species is 17±1 bp, but MerR and SoxR bind the targets with a space of 19 bp. As described above, the spacing between the probable –35 and –10 sequences of *afsS* is 20 bp, which is larger than those of the standard *Streptomyces* promoters. We therefore speculate that AfsR binding to the *afsS* promoter induces a DNA bend to optimize the spacing for RNA polymerase to recognize and bind the promoter sequence.

Binding of AfsR to the *afsS* promoter was found to be greatly enhanced by phosphorylation, which clearly shows the role of phosphorylation of AfsR by AfsK in DNA-binding (Lee et al. 2002). However, the ATPase activity of AfsR did not affect its DNA-binding activity, since AfsRΔC (Met-1 to Arg-311), which lacked the nucleotide-binding motif, and AfsRΔATPase, which lacked ATPase activity due to site-directed mutations at four amino acids in the nucleotide-binding motif, still showed the same DNA-binding activity as AfsR. AfsR thus appears to be modular, in that it is composed of physically separable DNA-binding and ATPase domains that can function independently of one another, as found in many eukaryotic enhancer-binding proteins and some prokaryotic transcriptional factors including NtrC (nitrogen regulatory protein) and NifA (nitrogen fixation) (Frankel and Kim 1991; North et al. 1993). The enhancement of DNA-binding activity of AfsR by phosphorylation is analogous to that of NtrC, although NtrC, a response regulator of a two-component system found in enteric bacteria, accepts a phosphate at Asp-54 in the receiver domain from its sensor autokinase NtrB. Phosphorylation of NtrC at the Asp residue induces dimerization of the receiver module (Fiedler and Weiss 1995) and accelerates tetramerization on DNA binding, which facilitates strong co-operative binding to DNA, essential for transcriptional activation (Weiss et al. 1992; Porter et al. 1993; Wyman et al. 1997).

ATPase activity of AfsR is essential for transcriptional activation of afsS

What is the role of the ATPase activity of AfsR? *afsR* on a plasmid restored actinorhodin production in Δ*afsR* mutants. *afsR*ΔATPase on the same plasmid, however, failed to restore the actinorhodin production, indicating that the ATPase activity of AfsR is essential for transcriptional activation of *afsS*. Multiple copies of *afsR*ΔATPase slightly reduced actinorhodin production in the *afsR*$^+$ strain, probably because AfsRΔATPase, having the ability to bind the promoter of *afsS* but not to ac-

tivate transcription of that gene, exerts a dominant-negative effect. By analogy with the role of the ATPase activity of NtrC (Wedel and Kustu 1995), we speculate that the energy available from ATP hydrolysis is required for the isomerization of a closed complex consisting of RNA polymerase and AfsR into a transcriptionally competent open complex. The energy supplied by the intrinsic low ATPase activity of unphosphorylated AfsR is supposedly insufficient to overcome the activation energy barrier to open complex formation. NtrC interacts with the σ^{54} RNA polymerase complex. AfsR may interact with the RNA polymerase with a certain σ factor other than σ^{HrdB} used for the housekeeping genes, as the *afsS* promoter sequence is slightly different from those of the housekeeping genes and would not be predicted to bind σ^{HrdB}.

Repeated sequences in AfsS

How AfsS stimulates transcription of *act*II-ORF4 remains unknown. AfsS is also thought to stimulate transcription of *redD*, a pathway-specific transcriptional activator, as multicopies of *afsS* enhance the yield of undecylprodigiosin. The presence of three repeats of the 12-amino-acid sequence in the 63-amino-acid AfsS protein prompts us to speculate that it interacts with some protein via the repeats, thereby exerting their regulatory function. An *afsR* homologue in *Streptomyces noursei* is located adjacent to *ssmA* encoding a 55-amino-acid protein with two repeats of Pro-Xaa-Asp-Asn-His-Thr-Pro-Ile-Xaa-Pro (Sekurova et al. 1999), which is very similar to the repeated sequence in AfsS. Like *afsS*, *ssmA* also enhances actinorhodin production in *S. lividans*. On the other hand, the four repeats in AfsS of *S. griseus*, which is involved in aerial mycelium formation in response to glucose (Umeyama et al. 1999), have the sequence Asp-Ala-His-Ala-Thr-Gly/Ser. We speculate that the difference in the phenotype, secondary metabolism or morphological development, influenced by the small proteins in two species, reflects the difference in the putative proteins which recognize and bind the repeats; the putative protein in *S. coelicolor* A3(2) is involved in secondary metabolism and that in *S. griseus* is involved in aerial mycelium formation.

PkaG and AfsL, serine/threonine kinases able to phosphorylate AfsR

An additional protein serine/threonine kinase able to phosphorylate AfsR had been predicted from the finding that *afsK* null mutants still contained the activity to phosphorylate AfsR in vitro on serine and threonine residues (Matsumoto et al. 1994). We chose two genes in the database of the *S. coelicolor* A3(2) genome, *pkaG* encoding a 592-amino-acid protein and *SCD10.09* encoding a 580-amino-acid protein (tentatively named AfsL), both of which show high similarity in the kinase catalytic domain to that of AfsK, and tested for their ability to phosphorylate AfsR. Both proteins, produced in *E. coli*, were capable of autophosphorylation on serine and threonine residues and phosphorylation of AfsR on serine and threonine residues (our unpublished results). Furthermore, disruption of the chromosomal *pkaG* gene resulted in reduced production of actinorhodin, suggesting the involvement of this gene in secondary metabolism. AfsR is thus a substrate for multiple kinases, although the serine and threonine residues phosphorylated by each of these kinases can not yet be identified.

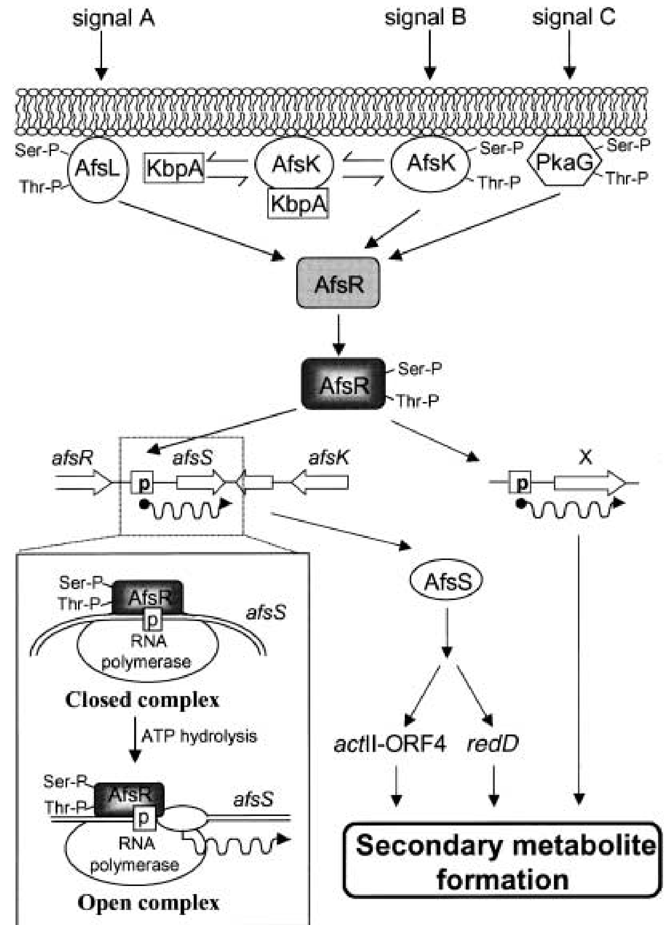

Fig. 2 A hypothetical scheme for the regulation of secondary metabolism by the KbpA-AfsK-AfsR-AfsS system in *S. coelicolor* A3(2). See text for details

A model of the AfsK-AfsR regulatory system

The data on the AfsK-AfsR regulatory system in *S. coelicolor* A3(2) have revealed part of the signal transduction cascade leading to actinorhodin production. Since *afsR* also enhances the yields of undecylprodigiosin, CDA, and A-factor, this system globally controls secondary metabolite formation. On the basis of the results obtained so far and our unpublished data, we present a hypothetical model for the regulation of antibiotic production by the KbpA-AfsK-AfsR-AfsS system (Fig. 2). By analogy with eu-

karyotic signal transduction, we speculate that AfsK, which is attached to the inner side of the membrane, autophosphorylates its serine and threonine residues on sensing a certain external stimulus and transfers the signal to AfsR in the cytoplasm by means of phosphorylation. It is unclear whether the phosphorylated form of AfsK is still attached to the membrane. Additional kinases, PkaG and AfsL, also autophosphorylate and phosphorylate AfsR, resulting in enhanced DNA-binding activity. The stimuli that activate PkaG and AfsL are not known. The phosphorylated form of AfsR activates the transcription of *afsS*. AfsS thus induced activates the transcription of *act*II-ORF4 and *redD*, resulting in production of actinorhodin and undecylprodigiosin.

We assume that phosphorylated AfsR activates not only *afsS* but also some other gene(s) involved in the stimulation of actinorhodin production, because *afsR* enhances the yield of actinorhodin in the absence of the intact *afsS* function. Consistent with this idea, Δ*afsR* mutants produce almost no actinorhodin whereas Δ*afsS* mutants still produce a detectable amount.

In *S. griseus*, *afsK* and *afsR* play a regulatory role in morphological differentiation (Umeyama et al. 1999). Disruption of either *afsK* or *afsR* in *S. griseus* resulted in a failure of aerial mycelium formation on medium containing glucose at concentrations higher than 1%. The disruptants formed spores normally and produced streptomycin and a yellow pigment on medium containing glucose at a lower concentration. This means that the AfsK-AfsR system in *S. griseus* is essential for morphogenesis in the presence of glucose at high concentrations. Perhaps, a glucose-sensor transfers a glucose signal to AfsK and causes it to autophosphorylate and activate its own kinase activity. A possible interaction of the AfsK-AfsR system and a glucose repression system remains to be elucidated.

Concluding remarks

Antibiotic production by *S. coelicolor* A3(2) is influenced by apparently independent systems, such as *bld* genes (Chater 1993), ppGpp (Martínez-Costa et al. 1996; Chakraburtty and Bibb 1997), two-component phosphorylation systems (Ishizuka et al. 1992; Chang et al. 1996; Anderson et al. 2001), γ-butyrolactone-type regulators (Onaka et al. 1998; Takano et al. 2001), cAMP (Süsstrunk et al. 1998), and the AfsK/AfsR phosphorylation system. These systems are supposedly accompanied by machinery that senses nutritional conditions, such as carbon, nitrogen and phosphate, and environmental conditions, such as temperature and a territorial dispute with other bacteria. The stimuli sensed are then transferred via these systems to a pathway-specific transcriptional activator in antibiotic biosynthetic gene cluster, for example, *act*II-ORF4 for actinorhodin biosynthesis. More members in the respective regulatory systems will be revealed by the combination of the genome sequence and DNA-array methodology.

Protein dephosphorylation is no less important than protein phosphorylation. However, little is known about eukaryotic-type phosphatases in *Streptomyces*, although SppA with dual substrate specificity, which is involved in vegetative growth (Umeyama et al. 2000), and PtpA, a low-molecular-weight protein tyrosine phosphatase (Li and Strohl 1996; Umeyama et al. 1996), have been reported. It is easily conceivable that protein phosphatases control the degree of phosphorylation of proteins at specific growth phases, thus playing important roles in morphological development and secondary metabolism.

Acknowledgements The work from this laboratory was supported by the Nissan Science Foundation, by the Asahi Glass Foundation, by the "Research for the Future" Program of the Japanese Society for the Promotion of Science, and by the Bio Design Program of the Ministry of Agriculture, Forestry, and Fisheries of Japan (BDP-02-VI-2–1).

References

Anderson TB, Brian P, Champness WC (2001) Genetic and transcriptional analysis of *absA*, an antibiotic gene cluster-linked two-component system that regulates multiple antibiotics in *Streptomyces coelicolor*. Mol Microbiol 39:553–566

Ansari AZ, Chael ML, O'Halloran TV (1992) Allosteric underwinding of DNA is a critical step in positive control of transcription by Hg-MerR. Nature 355:87–89

Chakraburtty R, Bibb M (1997) The ppGpp synthetase gene (*relA*) of *Streptomyces coelicolor* A3(2) plays a conditional role in antibiotic production and morphological differentiation. J Bacteriol 179:5854–5861

Chang HM, Chen MY, Shieh YT, Bibb MJ, Chen CW (1996) The *cutRS* signal transduction system of *Streptomyces lividans* represses the biosynthesis of the polyketide antibiotic actinorhodin. Mol Microbiol 21:1075–1085

Chater KF (1993) Genetics of differentiation in *Streptomyces*. Annu Rev Microbiol 47:685–713

Dobrova Z, Jiresova M, Petrik T, Rysavy P, Naprstek J, Janecek J (1990) Protein phosphorylation in *Streptomyces albus*. FEMS Microbiol Lett 59:145–148

Elizarov SM, Danilenko VN (2001) Multiple phosphorylation of membrane-associated calcium-dependent protein serine/threonine kinase in *Streptomyces fradiae*. FEMS Microbiol Lett 202:135–138

Elizarov SM, Mironov VA, Danilenko VN (2000) Calcium-induced alterations in the functioning of protein serine/threonine and tyrosine kinases in *Streptomyces fradiae* cells. IUBMB Life 50:139–143

Fernández-Moreno MA, Caballero JL, Hopwood DA, Malpartida F (1991) The *act* cluster contains regulatory and antibiotic export genes, direct targets for translational control by the *bldA* tRNA gene of *Streptomyces*. Cell 66:769–780

Fiedler U, Weiss V (1995) A common switch in activation of the response regulators NtrC and PhoB: phosphorylation induces dimerization of the receiver modules. EMBO J 14:3696–3705

Floriano B, Bibb M (1996) *afsR* is a pleiotropic but conditionally required regulatory gene for antibiotic production in *Streptomyces coelicolor* A3(2). Mol Microbiol 21:385–396

Frankel AD, Kim PS (1991) Modular structure of transcriptional factors: implications for gene regulation. Cell 65:717–719

Hanks SK, Quinn AM, Hunter T (1988) The protein kinase family: conserved features and deduced phylogeny of the catalytic domains. Science 241:42–52

Hara O, Horinouchi S, Uozumi T, Beppu T (1983) Genetic analysis of A-factor synthesis in *Streptomyces coelicolor* A3(2) and *Streptomyces griseus*. J Gen Microbiol 129:2939–2944

Hidalgo E, Demple B (1997) Spacing of promoter elements regulates the basal expression of the *soxS* gene and converts SoxR from a transcriptional activator into a repressor. EMBO J 16:1056–1065

Hong S-K, Horinouchi S (1998) Effects of protein kinase inhibitors on *in vitro* protein phosphorylation and on secondary metabolism and morphogenesis in *Streptomyces coelicolor* A3(2). J Microbiol Biotechnol 8:325–332

Hong S-K, Kito M, Beppu T, Horinouchi S (1991) Phosphorylation of the *afsR* product, a global regulatory protein for secondary-metabolite formation in *Streptomyces coelicolor* A3(2). J Bacteriol 173:2311–2318

Hong S-K, Matsumoto A, Horinouchi S, Beppu T (1993) Effects of protein kinase inhibitors on *in vitro* protein phosphorylation and cellular differentiation of *Streptomyces griseus*. Mol Gen Genet 236:347–354

Horinouchi S, Beppu T (1984) Production in large quantities of actinorhodin and undecylprodigiosin induced by *afsB* in *Streptomyces lividans*. Agric Biol Chem 48:2131–2133

Horinouchi S, Hara O, Beppu T (1983) Cloning of a pleiotropic gene that positively controls biosynthesis of A-factor, actinorhodin, and prodigiosin in *Streptomyces coelicolor* A3(2) and *Streptomyces lividans*. J Bacteriol 155:1238–1248

Horinouchi S, Malpartida F, Hopwood DA, Beppu T (1989) *afsB* stimulates transcription of the actinorhodin biosynthetic pathway in *Streptomyces coelicolor* A3(2) and *Streptomyces lividans*. Mol Gen Genet 215:355–357

Horinouchi S, Kito M, Nishiyama M, Furuya K, Hong S-K, Miyake K, Beppu T (1990) Primary structure of AfsR, a global regulatory protein for secondary metabolite formation in *Streptomyces coelicolor* A3(2). Gene 95:49–56

Ishizuka H, Horinouchi S, Kieser HM, Hopwood DA, Beppu T (1992) A putative two-component regulatory system involved in secondary metabolism in *Streptomyces* spp. J Bacteriol 174:7585–7594

Kang D-K, Li X-M, Ochi K, Horinouchi S (1999) Possible involvement of cAMP in aerial mycelium formation and secondary metabolism in *Streptomyces griseus*. Microbiology 145: 1161–1172

Kennelly PJ, Potts M (1996) Fancy meeting you here! A fresh look at “prokaryotic” protein phosphorylation. J Bacteriol 178:4759–4764

Kim E-S, Hong H-J, Choi C-Y, Cohen SN (2001) Modulation of actinorhodin biosynthesis in *Streptomyces lividans* by glucose repression of *afsR2* gene transcription. J Bacteriol 183:2198–2203

Lee P-C, Umeyama T, Horinouchi S (2002) *afsS* is a target of AfsR, a transcriptional factor with ATPase activity that globally controls secondary metabolism in *Streptomyces coelicolor* A3(2). Mol Microbiol 43:1413–1430

Li Y, Strohl WR (1996) Cloning, purification and properties of a phosphotyrosine phosphatase from *Streptomyces coelicolor* A3(2). J Bacteriol 178:136-142

Martínez-Costa OH, Arias P, Romero NM, Parro V, Mellado RP, Malpartida F (1996) A *relA/spoT* homologous gene from *Streptomyces coelicolor* A3(2) controls antibiotic biosynthetic genes. J Biol Chem 271:10627–10634

Matsumoto A, Hong S-K, Ishizuka H, Horinouchi S, Beppu T (1994) Phosphorylation of the AfsR protein involved in secondary metabolism in *Streptomyces* species by a eukaryotic-type protein kinase. Gene 146:47–56

Matsumoto A, Ishizuka H, Beppu T, Horinouchi S (1995) Involvement of a small ORF downstream of the *afsR* gene in the regulation of secondary metabolism in *Streptomyces coelicolor* A3(2). Actinomycetologica 9:37–43

Mikulik K, Dobrova Z, Janecek J, Khanh-Hoang Q (1998) Pattern of phosphoproteins during cell differentiation in *Streptomyces collinus*. FEMS Microbiol Lett 158:147–151

Mikulik K, Suchan P, Bobek J (2001) Changes in ribosome function induced by protein kinase associated with ribosomes of *Streptomyces collinus* producing kirromycin. Biochem Biophys Res Commun 289:434–443

Nádvorník R, Vomastek T, Janecek J, Techniková Z, Branny P (1999) Pkg2, a novel transmembrane protein Ser/Thr kinase of *Streptomyces granaticolor*. J Bacteriol 181:15–23

Narva KE, Feitelson JS (1990) Nucleotide sequence and transcriptional analysis of the *redD* locus of *Streptomyces coelicolor* A3(2). J Bacteriol 172:326–333

North AK, Klose KE, Stedman KM, Kustu S (1993) Prokaryotic enhancer-binding proteins reflect eukaryote-like modularity: the puzzle of nitrogen regulatory protein C. J Bacteriol 175:4267–4273

Onaka H, Nakagawa T, Horinouchi S (1998) Involvement of two A-factor receptor homologues in *Streptomyces coelicolor* A3(2) in the regulation of secondary metabolism and morphogenesis. Mol Microbiol 28:743–753

Porter SC, North AK, Wedel AB, Kustu S (1993) Oligomerization of NTRC at the *glnA* enhancer is required for transcriptional activation. Genes Dev 7:2258–2273

Sekurova O, Sletta H, Ellingsen TE, Valla S, Zotchev S (1999) Molecular cloning and analysis of a pleiotropic regulatory gene locus from the nystatin producer *Streptomyces noursei* ATCC11455. FEMS Microbiol Lett 177:297–304

Stein D, Cohen SN (1989) A cloned regulatory gene of *Streptomyces lividans* can suppress the pigment deficiency phenotype of different developmental mutants. J Bacteriol 171:2258–2261

Strohl WR (1992) Compilation and analysis of DNA sequences associated with apparent streptomycete promoters. Nucleic Acids Res 20:961–974

Süsstrunk U, Pidoux J, Taubert S, Ullmann A, Thompson CJ (1998) Pleiotropic effects of cAMP on germination, antibiotic biosynthesis and morphological development in *Streptomyces coelicolor*. Mol Microbiol 30:33–46

Takano E, Chakraburtty R, Nihira T, Yamada Y, Bibb MJ (2001) A complex role of the γ-butyrolactone SCB1 in regulating antibiotic production in *Streptomyces coelicolor* A3(2). Mol Microbiol 41:1015–1028

Umeyama T, Horinouchi S (2001) Autophosphorylation of a bacterial serine/threonine kinase, AfsK, is inhibited by KbpA, an AfsK-binding protein. J Bacteriol 183:5506–5512

Umeyama T, Lee P-C, Ueda K, Horinouchi S (1999) An AfsK/AfsR system involved in the response of aerial mycelium formation to glucose in *Streptomyces griseus*. Microbiology 145:2281–2292

Umeyama T, Naruoka A, Horinouchi S (2000) Genetic and biochemical characterization of a protein phosphatase with dual substrate specificity in *Streptomyces coelicolor* A3(2). Gene 258:55–62

Umeyama T, Tanabe Y, Aigle BD, Horinouchi S (1996) Expression of the *Streptomyces coelicolor* A3(2) *ptpA* gene encoding a phosphotyrosine protein phosphatase leads to overproduction of secondary metabolites in *S. lividans*. FEMS Microbiol Lett 144:177–184

Vögtli M, Chang PC, Cohen SN (1994) *afsR2*: a previously undetected gene encoding a 63-amino-acid protein that stimulates antibiotic production in *Streptomyces lividans*. Mol Microbiol 14:643–653

Waters B, Vujaklija D, Gold MR, Davies J (1994) Protein tyrosine phosphorylation in streptomycetes. FEMS Microbiol Lett 120:187–190

Wedel A, Kustu S (1995) The bacterial enhancer-binding protein NTRC is a molecular machine: ATP hydrolysis is coupled to transcriptional activation. Genes Dev 9:2042–2052

Weiss V, Claverie-Martin F, Magasanik B (1992) Phosphorylation of nitrogen regulator I of *Escherichia coli* induces strong cooperative binding to DNA essential for activation of transcription. Proc Natl Acad Sci USA 89:5088–5092

Wietzorrek A, Bibb M (1997) A novel family of proteins that regulates antibiotic production in streptomycetes appears to contain an OmpR-like DNA-binding fold. Mol Microbiol 25:1181–1184

Wyman C, Rombel I, North AK, Bustamante C, Kustu S (1997) Unusual oligomerization required for activity of NtrC, a bacterial enhancer-binding protein. Science 275:1658–1661

Zhang C-C (1996) Bacterial signalling involving eukaryotic-type protein kinases. Mol Microbiol 20:9–15