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Autophosphorylation of a Bacterial Serine/Threonine Kinase, AfsK, Is Inhibited by KbpA, an AfsK-Binding Protein

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A protein serine/threonine kinase, AfsK, and its target protein AfsR globally control physiological and morphological differentiation in the bacterial genus *Streptomyces*. A protein (KbpA) of 252 amino acids encoded by an open reading frame in a region upstream of *afsK* in *Streptomyces coelicolor* A3(2) was identified as an AfsK-interacting protein. The interaction site of AfsK was in the N-terminal portion containing the kinase catalytic domain. KbpA bound a nonphosphorylated form of AfsK and inhibited its autophosphorylation at serine and threonine residues. KbpA in the reaction mixture containing AfsK and AfsR also inhibited the phosphorylation of AfsR by AfsK, presumably because KbpA inhibited the conversion from the inactive, nonphosphorylated form of AfsK to the active, phosphorylated form. *kbpA* was transcribed throughout growth, and the transcription was enhanced when production of actinorhodin had already started. KbpA thus appeared to play an inhibitory role in a negative feedback system in the AfsK-AfsR regulatory pathway. Consistent with these in vitro observations, *kbpA* served as a repressor for actinorhodin production in *S. coelicolor* A3(2); disruption of *kbpA* greatly enhanced actinorhodin production, and overexpression of *kbpA* reduced the production.

In the gram-positive, soil-living, filamentous bacteria *Streptomyces coelicolor* A3(2) and *Streptomyces griseus*, a serine/threonine kinase, AfsK, and its target protein AfsR control secondary metabolism and morphological development, respectively (17, 25). Disruption of either one of the two genes reduced actinorhodin production in *S. coelicolor* A3(2) (9, 17). In *S. griseus*, disruption of either *afsK* or *afsR* resulted in the failure of aerial mycelium formation on medium containing glucose at concentrations higher than 1% (25). Even under the usual culture conditions in the laboratory, the AfsK-AfsR system in *S. coelicolor* A3(2) contributes considerably to pigment production because of reduced production of the pigment by *afsK* and *afsR* mutants. The wide distribution of *afsK* and *afsR* in various *Streptomyces* spp. (9, 17) shows their general and important roles in the regulation of secondary metabolism and morphogenesis in this genus. Biochemical and genetic studies of AfsK-AfsR have led us to assume that AfsK on the inner side of the membrane activates its own kinase activity by autophosphorylating its serine and threonine residues on sensing some external stimuli and then phosphorylates serine and threonine residues of AfsR; the phosphorylated AfsR serves as a transcriptional factor for many genes required for antibiotic production and morphogenesis (2, 6). Wietzorrek and Bibb (28) pointed out the possibility that AfsR is a DNA-binding protein. The genome project for *S. coelicolor* A3(2) has predicted the presence of more than 20 AfsK homologues with a serine/threonine kinase catalytic domain (www.sanger.ac.uk/Projects/S coelicolor/), and some AfsK homologues have been cloned from this species (21, 26) and other *Streptomyces* spp. (20, 27). Although some of these kinases are perhaps capable of autophosphorylation, it is not known what their targets are and how their autophosphorylation is controlled.

In *S. griseus*, the AfsK-AfsR system is involved in aerial mycelium and spore formation in response to glucose in the medium (25). However, transcription of neither *afsK* nor *afsR* depended on the concentration of glucose. These observations led us to assume that autophosphorylation of AfsK and phosphorylation of AfsR by AfsK may be controlled by some mechanism other than one at the transcriptional level. We focused on an open reading frame (ORF) that is located upstream of *afsK* and conserved in *S. coelicolor* A3(2) and *S. griseus*. Because the AfsK-AfsR system in *S. coelicolor* A3(2) could be assessed by monitoring actinorhodin (blue pigment) production, we studied the possible relationship between the conserved ORF and the AfsK-AfsR system in *S. coelicolor* A3(2). The ORF product was found to bind specifically to the nonphosphorylated form of AfsK and inhibit its autophosphorylation. Consistent with this in vitro observation, gene disruption and overexpression experiments suggested a role of this ORF as a repressor for actinorhodin production. We here describe control by this ORF product, named KbpA (for AfsK-binding protein A), of AfsK autophosphorylation by means of protein-protein interaction.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. *Escherichia coli* JM109 and pUC19 (29) were used for DNA manipulation. *E. coli* AD494(DE3) and pET32a(+), purchased from Novagen, were used for expression of proteins fused to thioredoxin (TRX). *E. coli* BL21 and pGEX5X-1, purchased from Amersham Pharmacia Biotech, were used for expression of proteins fused to glutathione *S*-transferase (GST). *E. coli* BL21 *trxB*(DE3), purchased from Novagen, was used for preparation of ^{32}P-labeled AfsK fusion proteins. *E. coli*

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JM110 *dam dcm*, deficient in the methylases, was purchased from Takara Shuzo. A high-copy-number plasmid, pIJ6021, containing a thiostrepton-inducible promoter was obtained from M. J. Bibb (24). *S. coelicolor* A3(2) was routinely cultured at 30°C on Trypto-Soya broth (TSB) (Nissui), which was supplemented with 10 μg of thiostrepton or kanamycin per ml when necessary.

Construction of plasmids. DNA manipulations in *E. coli* were as described by Maniatis et al. (16), and those in *Streptomyces* were as described by Hopwood et al. (8). The *kbpA* coding sequence was amplified with the two primers 5′-GCC GAATTC*CATA**TG***GCCGAAAACGGGGCATTC-3′ (the underlined and italic letters indicate *Eco*RI and *Nde*I sites, respectively; the boldface letters indicate the start codon of *kbpA*) and 5′-GGCAAGCTT*CTCGAG***TCA**GCGTCGCAGC AGCGCGAAC-3′ (the underlined and italic letters indicate *Hin*dIII and *Xho*I sites, respectively; the boldface letters indicate the stop codon of *kbpA*) and cloned between the *Eco*RI and *Hin*dIII sites of pUC19, resulting in pUC19-*kbpA*. After the *kbpA* coding sequence had been checked for errors in amplification, it was inserted between the *Eco*RI and *Hin*dIII sites of pET32a(+) to construct pTRX-KbpA. Similarly, *kbpA* as an *Eco*RI-*Xho*I fragment was inserted in pGEX5X-1 to generate pGST-KbpA. The *kbpA* sequence as an *Nde*I-*Hin*dIII fragment was also inserted in pIJ6021 to construct pIJ6021-*kbpA*.

For placing the *afsR* coding sequence in pGEX5X-1, a procedure similar to that used for constructing pGST-AfsR-g (25) was employed. The pGST-AfsR thus constructed directed the synthesis of an AfsR protein fused to GST. For construction of pTRX-AfsK and pGST-AfsK, directing the synthesis of TRX-AfsK and GST-AfsK, respectively, the *afsK* sequence was divided into two. The sequence for the N-terminal portion was amplified by PCR with the primers 5′-GCCGAATTC**ATG**GTGGATCAGCTGACGCAG-3′ (the underlined and boldface letters indicate an *Eco*RI site and the initiation codon of *afsK* [originally GTG], respectively) and 5′-GGCAAGCTT**TCA***GCGGCCGC*CGGCCGTGGT GGCGGGC-3′ (the underlined and italic letters indicate *Hin*dIII and *Not*I sites, respectively; the boldface letters indicate an artificial stop codon), and the *Eco*RI-*Hin*dIII fragment was inserted in pUC19 to construct pUC19-AfsKΔC, containing the region from Met-1 to Arg-311. The sequence for the C-terminal portion was amplified with 5′-GCCGAATTC*GGCGGCCGC*GGCCACGGCC ACGGCC-3′ and 5′-GGCAAGCTT*CTCGAG***TCA**CGTCGTACGGGCGGTC CCCGTG-3′ (the italic and boldface letters indicate an *Xho*I site and the stop codon of *afsK*, respectively; the underlined letters indicate restriction sites used for cloning) and inserted between the *Eco*RI and *Hin*dIII sites of pUC19, resulting in pUC19-AfsKΔN, containing the region from Gly-309 to the stop codon of *afsK*. The *Eco*RI-*Not*I fragment from pUC19-AfsKΔC, the *Not*I-*Hin*dIII fragment from pUC19-AfsKΔN, and the *Eco*RI-*Hin*dIII fragment from pET32a(+) were connected by three-fragment ligation to construct pTRX-AfsK, which would direct the synthesis of TRX-His_6-S tag-AfsK. The *Eco*RI-*Not*I fragment from pUC19-AfsKΔC, the *Not*I-*Xho*I fragment from pUC19-AfsKΔN, and the *Eco*RI-*Xho*I fragment from pGEX5X-1 were similarly connected to construct pGST-AfsK. pTRX-KΔC_{wt}, which would direct the synthesis of the kinase domain (Met-1 to Arg-311) of AfsK, was constructed by inserting the *Eco*RI-*Hin*dIII fragment from pUC19AfsKΔC in pET32a(+). For site-directed mutagenesis to replace Lys-44 with Ala, 5′-CGGCGCGTGGCGATC**GC**GACG GTGCGC-3′ (the nucleotides in boldface were originally AA) as a mutant primer and the Mutan-Super Express Km kit (Takara Shuzo) were used according to supplier's manual. The *Eco*RI-*Hin*dIII fragment containing the mutation was inserted in pET32a(+) to construct pTRX-KΔC_{K44A} with essentially the same construction as pTRX-KΔC_{wt}.

Production and preparation of GST- and TRX-fused proteins. An overnight culture (1 ml) of *E. coli* harboring each of the expression plasmids was inoculated in 9 ml of L broth. After cultivation at 30°C for 2 h, isopropyl-β-D-thiogalactopyranoside (IPTG) was added at a final concentration of 1 mM and the culture was continued at 30°C for 3 h, except for *E. coli* harboring pGST-AfsK. GST-AfsK appeared to readily form inclusion bodies and was cultured at 20°C for 8 h. The *E. coli* cells were harvested by centrifugation, suspended in 0.5 ml of buffer A (10 mM Tris-HCl [pH 7.2] and 10% glycerol), and sonicated to prepare soluble fractions by centrifugation at 24,000 × *g* for 30 min. TRX and TRX-KbpA were purified with an Ni-nitrilotriacetic acid Spin kit (Qiagen) for GST pull-down assays. GST, GST-KbpA, and GST-AfsR were purified with a MicroSpin GST purification module (Amersham Pharmacia Biotech) for in vitro phosphorylation assay. TRX-AfsK, TRX-KΔC_{wt}, and TRX-KΔC_{K44A} were mainly produced as inclusion bodies, and these were solubilized with 6 M urea, purified with the Ni-bound resin, and finally refolded into active forms by gradual dialysis against buffer A, as described previously (25). Protein concentrations were measured with the Bio-Rad protein assay kit using bovine serum albumin as the standard.

AfsK-KbpA interaction assay. The soluble fraction (10 μg of protein) containing GST-AfsK was incubated with 10 μl of glutathione-Sepharose 4B (Amersham Pharmacia Biotech) at 4°C for 1 h in 0.5 ml of pull-down assay buffer (PDA buffer) (10 mM Tris-HCl [pH 7.2], 100 mM NaCl, 1 mM $MgCl_2$, 0.025% β-mercaptoethanol, 1% Triton X-100, and 10% glycerol). Glutathione-Sepharose was collected by centrifugation, and the pellet was washed twice with 1 ml of PDA buffer. The pellet was suspended in 0.5 ml of PDA buffer, and 5 μg of purified TRX-KbpA was added. After incubation at 4°C for 1 h, the Sepharose was collected by centrifugation and washed three times with 1 ml of PDA buffer. The pellet was suspended in 15 μl of sodium dodecyl sulfate (SDS) loading buffer (58 mM Tris-HCl [pH 6.8], 1.7% SDS, 6% glycerol, 100 mM dithiothreitol, and 0.002% bromophenol blue) and boiled for 5 min to release GST complexes from the Sepharose. A portion (10 μl) of the supernatant obtained by centrifugation of the boiled sample was subjected to SDS–12.5% polyacrylamide gel electrophoresis (PAGE). After the proteins had been transferred to a nitrocellulose membrane, TRX-KbpA was detected by Western blotting with S protein horseradish peroxidase (HRP) conjugate (Novagen) and the ECL Western blotting reagents (Amersham Pharmacia Biotech). The membrane was reprobed with anti-GST antibody HRP conjugate (Santa Cruz Biotechnology) to detect the GST-fused proteins. As negative controls, the soluble fractions containing GST and GST-AfsR, instead of GST-AfsK, were used. TRX itself, instead of TRX-KbpA, was also used as a negative control. The interactions of TRX-KΔC_{wt} and TRX-KΔC_{K44A} with GST-KbpA were similarly assayed, but with the proteins purified with the Ni or GST resins, as described above.

For preparation of ^{32}P-TRX-KΔC_{wt} and TRX-KΔC_{K44A}, *E. coli* BL21 *trxB*(DE3) harboring pTRX-KΔC_{wt} or pTRX-KΔC_{K44A} was cultured in the presence of 100 μCi of phosphorus-32 (Amersham Pharmacia Biotech). The *E. coli* cells were collected and disrupted with the BugBuster protein extraction reagent containing Benzonase nuclease (Novagen). The TRX proteins were solubilized and purified as described above.

In vitro phosphorylation assay. The standard reaction mixture, containing 15 pmol of TRX-AfsK in 10 mM Tris-HCl (pH 7.2)–5 mM $MnCl_2$–10 mM $MgCl_2$–0.1 mM ATP–10 μCi of [γ-^{32}P]ATP–1 mM dithiothreitol, was incubated at 30°C for 5 min. For phosphorylation of GST-AfsR by TRX-AfsK, 30 pmol of GST-AfsR was added. Autophosphorylation of TRX-AfsK and GST-AfsR phosphorylation by TRX-AfsK were examined in the presence of 300 pmol of GST-KbpA. In this case, the reaction mixture without ATP and $MgCl_2$ was placed on ice for 1 h to allow formation of the complex between TRX-AfsK and GST-KbpA. The reaction was started by adding ATP and $MgCl_2$ and continued at 30°C for 5 min. After separation of the proteins by SDS-PAGE, the gel was placed on a Fuji BAS2000 image analyzer. Phosphoamino acid analysis by one-dimensional electrophoresis on a cellulose thin-layer plate was carried out with hydrolyzed samples, as described previously (4, 12).

Disruption of chromosomal *kbpA*. A 1,014-bp region upstream of *kbpA* was amplified by PCR with primers 5′-GGCGAATTCGGGGTCGTCACGGTGCT GAACTTC-3′ (the underlining indicates an *Eco*RI site) and 5′-GCCAAGCTT *TCTAGA***CAT**GCCGTCAAAGTAACCGC-3′ (the underlined and italic letters indicate *Hin*dIII and *Xba*I sites, respectively; the boldface letters indicate the start codon of *kbpA*). A 1,154-bp region downstream of *kbpA* was amplified with 5′-GGCGAATTC*TCTAGA***TGA**CCCGGCGGCCACGGCG-3′ (the underlined and italic letters indicate *Eco*RI and *Xba*I sites, respectively; the boldface letters indicate the stop codon of *kbpA*) and 5′-GCCAAGCTT*GGATCC*GCGGCCG CCGGCCGTGGTGGCGGGCTTG-3′ (the underlined and italic letters indicate *Hin*dIII and *Bam*HI sites, respectively). The regions upstream and downstream of *kbpA* were each cloned between the *Eco*RI and *Hin*dIII sites of pUC19. The two regions in the pUC19 plasmids were then connected by use of the *Xba*I sites, generating pDisKbpA, which contained a 2,192-bp insertion with complete deletion of the *kbpA* sequence. The thiostrepton resistance (*tsr*) gene, obtained as a *Bam*HI-*Hin*dIII fragment from pKU209 (11), was inserted between the *Bam*HI and *Hin*dIII sites of pDisKbpA. The circular form of the resultant plasmid prepared from *E. coli* JM110 was alkali denatured (22) and introduced by protoplast transformation into *S. coelicolor* A3(2) M130 to isolate mutants in which the whole plasmid was integrated in the chromosome by homologous recombination. Thiostrepton-resistant colonies were incubated on TSB for 7 days in the absence of thiostrepton, and spores were recovered. Of the isolated spores, thiostrepton-sensitive colonies were selected to obtain mutants containing the deletion of *kbpA* due to double crossover. Correct deletion of the *kbpA* sequence was checked by Southern hybridization with the 0.7-kb *Eco*RI-*Hin*dIII fragment containing *kbpA* on pUC19-*kbpA* and the 2.3-kb *Eco*RI-*Sph*I fragment containing *kbpA* and part of *afsK* (Fig. 1B).

Transcriptional analysis of *kbpA*. RNA was purified from mycelium grown on TSB agar medium (13). For S1 nuclease mapping of *kbpA*, a 342-bp fragment was prepared by PCR with 5′-AGCATTTCGCTGAGGCAGTCGAGCAGTTTC-3′ and 5′-TCCTGGAAGGTCCAGCCGAACAGCTCGCCG-3′ (corresponding to positions −217 to +125, taking the transcriptional start point of *kbpA* as +1, which was later determined) and used as the ^{32}P-labeled probe, as described

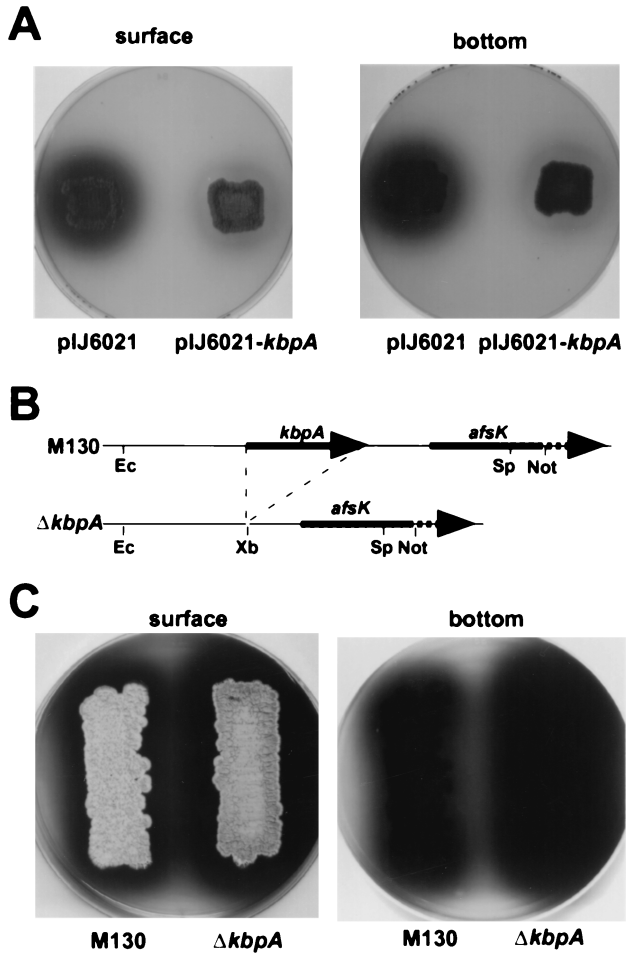

FIG. 1. Effects of overexpression and disruption of *kbpA* in *S. coelicolor* A3(2) M130. (A) Strain M130 harboring pIJ6021 produces the blue pigment actinorhodin, whereas that harboring *kbpA* on pIJ6021 produces a much smaller amount of the pigment. (B) Schematic representation of the chromosomal *kbpA* disruption. The *kbpA* coding region is completely deleted so that this deletion does not affect the promoter in front of *kbpA* or *afsK*. Abbreviations: Ec, *Eco*RI; Not, *Not*I; Sp, *Sph*I; Xb, *Xba*I. (C) The *kbpA* disruptant produces a larger amount of actinorhodin than strain M130.

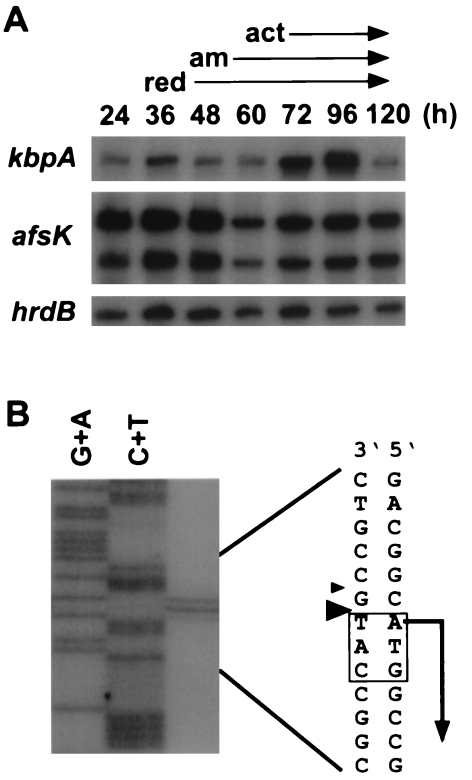

FIG. 2. Transcription of *kbpA*. (A) Low-resolution S1 mapping of *kbpA*, *afsK*, and *hrdB*. *kbpA* and *hrdB* contain a single promoter, and *afsK* contains two promoters. On agar medium, production of the red pigment undecylprodigiosin (red) and the blue pigment actinorhodin (act) started at 48 and 72 h, respectively. Aerial mycelium (am) was formed at 60 h. (B) High-resolution S1 mapping of *kbpA*. In this particular experiment, the strongest signal of the S1-protected fragments corresponds to the second residue of the translational start codon ATG, since S1-protected fragments run slower than the chemically cleaved fragments (23). It is reasonable, however, to assume that transcription of *kbpA* starts at the first nucleotide of the ATG.

previously (13). Transcription of *afsK* and *hrdB* was determined as described previously (25). For reverse transcription-PCR, two primers containing the start codons of *kbpA* and *afsK* were used: 5′-**ATG**GCCGAAAACGGGGCATTCGA GAAG-3′ (the boldface letters indicate the start codon of *kbpA*) and 5′-ATCG TGCTGCGTCAGCTGATCCACC**AC**-3′ (the boldface letters indicate the start codon of *afsK*).

Nucleotide sequence accession number. The nucleotide sequence of *kbpA* has been deposited in the DDBJ, EMBL, and GenBank databases under accession number D45382.

RESULTS

Repression of actinorhodin production by *kbpA*. The ORF products (KbpAs) that are encoded by a region upstream of *afsK* in *S. coelicolor* A3(2) and *S. griseus* showed 47% identity to each other. The KbpA of 252 amino acids in *S. coelicolor* A3(2) also showed 36 and 33% identity to SgaA, which is involved in suppression of the growth disturbance caused by high osmolality in *S. griseus* (1), and to DnrV, which is located in the doxorubicin biosynthetic gene cluster in *Streptomyces peucetius* (15), respectively. The functions of these products, however, are not understood. In the database of the *S. coelicolor* A3(2) genome, there are six KbpA homologues (5H1.9.C, 9B10.0C, 2G4.09, F1.07, 4C6.24C, and 4G10.18C). Since in prokaryotes, genes for the same biological function comprise a cluster in most cases, we examined a possible role of *kbpA* in actinorhodin production in *S. coelicolor* A3(2). Overexpression of *kbpA* in strain M130 by means of placing *kbpA* under the control of the thiostrepton-inducible promoter *tip* in a high-copy-number plasmid, pIJ6021, severely reduced actinorhodin production on TSB agar (Fig. 1A). The reduction was independent of the nutritional conditions, because strain M130 harboring pIJ6021-*kbpA* produced a much smaller amount of actinorhodin on media containing various carbon and nitrogen sources. The degree of reduction was almost the same as that in *afsK* disruptants (data not shown). On the other hand, complete deletion of the *kbpA* coding region (Fig. 1B) caused overproduction of actinorhodin on various media (Fig. 1C). This mutant also produced undecylprodigiosin at an earlier stage and accumulated it in a larger amount than strain M130 (data not shown). The Δ*kbpA* mutant produced spores normally, which showed that the mutation did not affect morpho-

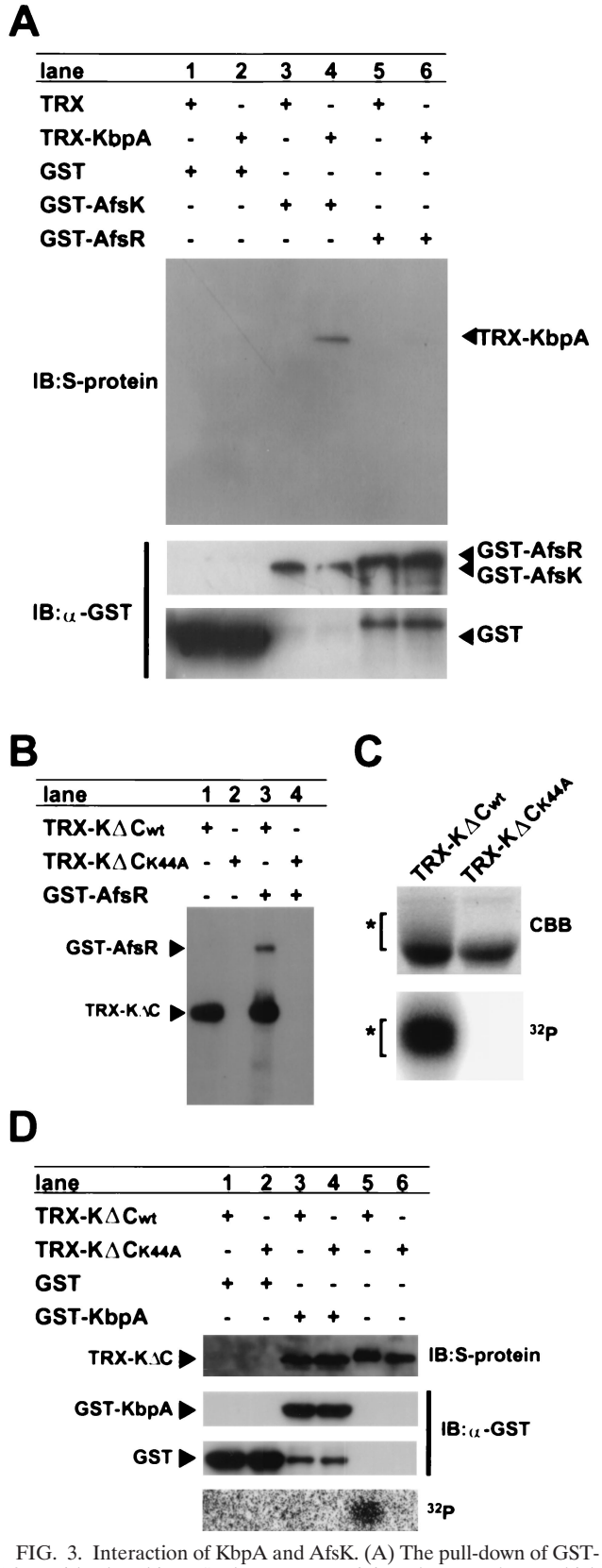

FIG. 3. Interaction of KbpA and AfsK. (A) The pull-down of GST-AfsK with glutathione-Sepharose coprecipitated TRX-KbpA, which was detected with S protein by Western blotting (immunoblotting [IB]) (lane 4). TRX-KbpA was not recovered when GST or GST-AfsR was used. The pull-down of GST-AfsR (132 kDa), GST-AfsK (110 kDa), and GST (28 kDa) with glutathione-Sepharose was apparent by Western blotting with the antibody for GST (α-GST). The small protein found in lanes 5 and 6 is a degradation product derived from GST-AfsR. (B) Autophosphorylation of TRX-KΔC_{wt} (51 kDa) and phosphorylation of GST-AfsR by TRX-KΔC_{wt}. TRX-KΔC_{wt} (5 μg) was incubated at 30°C for 10 min in the presence of [γ-^{32}P]ATP, subjected to SDS-PAGE, and analyzed by autoradiography. GST-AfsR (3 μg) in the reaction mixture was also phosphorylated. Neither autophosphorylation nor phosphorylation of GST-AfsR occurred for TRX-KΔC_{K44A}. (C) SDS-PAGE of the TRX-KΔC proteins labeled in vivo. Coomassie brilliant blue (CBB) staining revealed smeared bands for TRX-KΔC_{wt}, as indicated by an asterisk, which represent phosphorylated forms of TRX-KΔC_{wt}, as found by autoradiography. (D) The pull-down of GST-KbpA (54 kDa) with glutathione-Sepharose coprecipitated TRX-KΔC_{wt} (lane 3) and TRX-KΔC_{K44A} (lane 4) without recovering smeared, phosphorylated forms of TRX-KΔC_{wt}. The small protein recovered by anti-GST antibody in lanes 3 and 4 is a degradation product. GST itself did not pull down the TRX-KΔC proteins (lanes 1 and 2). TRX-KΔC_{wt} gave smeared bands (lane 5), but TRX-KΔC_{K44A} did not (lane 6).

logical differentiation. The AfsK-AfsR system in *S. coelicolor* A3(2) caused no detectable effect on morphogenesis (7, 17). We thus concluded that *kbpA* acted as a repressor for actinorhodin production, irrespective of the culture conditions.

Transcription of *kbpA*. Because of the involvement of *kbpA* in actinorhodin production, we determined the course of its transcription, in relation to actinorhodin production, using RNA from mycelium grown on agar medium. *afsK* was constantly transcribed from two promoters throughout growth. Constant transcription from two promoters was also observed for the *afsK* gene of *S. griseus* (2). *hrdB* mRNA, encoding the major sigma factor of RNA polymerase, is known to be transcribed constantly. *kbpA* was transcribed from a single promoter throughout growth, but transcription was enhanced when production of both undecylprodigiosin and actinorhodin and aerial mycelium formation had already started (Fig. 2A). It thus appeared that under usual culture conditions, KbpA repressed secondary metabolism when these pigments had been produced and accumulated. High-resolution S1 mapping revealed the transcriptional start point to be the first A of the start codon (Fig. 2B). A sequence, TACTTT, similar to the −10 consensus sequence was present at an appropriate position from the transcriptional start point, but no sequence similar to the −35 consensus sequence was present. Transcription of leaderless mRNA is not uncommon in *Streptomyces*. For example, the 23S rRNA methylase gene mediating erythromycin resistance in *Streptomyces erythraeus* (3) and *afsA*, which probably encodes an A-factor biosynthetic enzyme in *S. griseus* (10), are transcribed from leaderless transcripts.

kbpA and *afsK* are separated by 219 nucleotides, which suggested that the genes were transcribed independently. Reverse transcription-PCR with primers containing the start codons of *kbpA* and *afsK* yielded only a weak signal representing a 1,004-bp DNA fragment after 30 cycles of amplification (data not shown). We thus concluded that *kbpA* and *afsK* were transcribed mainly from their own promoter and that very little *kbpA* transcript leaked into *afsK*.

Direct interaction of AfsK with KbpA. For examining possible KbpA-AfsK interaction, we produced TRX-KbpA with the structure of TRX-His_6-S tag-KbpA (419 amino acids, 45 kDa), detectable with S protein HRP conjugate, and GST-

AfsK (1,029 amino acids, 110 kDa), precipitable with glutathione-Sepharose. The S tag consisted of 15 amino acids derived from RNase S protein. TRX-KbpA was produced in the soluble fraction of *E. coli* and purified with His-bind resin. GST-AfsK was produced in a soluble fraction of *E. coli* when the cells were cultured at 20°C to avoid formation of inclusion bodies. After the soluble fraction (10 μg of protein) containing GST-AfsK had been incubated with 5 μg of purified TRX-KbpA, the GST complexes were pulled down with glutathione-Sepharose and separated by SDS-PAGE. TRX complexes and GST complexes were detected by Western blotting with the S protein HRP conjugate and the antibody for GST, respectively (Fig. 3A). This pull-down assay recovered TRX-KbpA (Fig. 3A, lane 4), indicating that GST-AfsK formed a complex with TRX-KbpA. The lack of recovery of proteins reactive with the antibody in the control experiments with TRX instead of TRX-KbpA or with GST instead of GST-AfsK showed that the complex was formed via parts of KbpA and AfsK. In addition, KbpA did not interact with AfsR, since the same experiment with GST-AfsR (1,223 amino acids, 132 kDa) instead of GST-AfsK did not recover TRX-KbpA.

During the pull-down assay, we noticed that the amount of TRX-KbpA recovered as a complex of TRX-KbpA and GST-AfsK appeared to depend on the degree of phosphorylation of GST-AfsK. The amount of TRX-KbpA recovered and detected by Western blotting was decreased as the amount of the phosphorylated form of GST-AfsK was increased. The degree of autophosphorylation of GST-AfsK in *E. coli* could be estimated by the intensity of smeared, slow-moving bands on SDS-PAGE. We examined this observation in detail by reducing the size of AfsK and by using a K44A mutant that lost the ability to autophosphorylate because of the mutation at one of the active-site residues, Lys-44. The TRX-KΔC proteins (478 amino acids, 51 kDa) contained the kinase domain (Met-1 to Arg-311). In vitro phosphorylation assay of TRX-KΔC_{wt} revealed autophosphorylation and phosphorylation of AfsR (Fig. 3B). Phosphoamino acid analysis showed that both contained phosphorylated serine and threonine residues (data not shown), as does the native AfsK-AfsR phosphorylation system. No phosphorylation occurred for TRX-KΔC_{K44A}.

To confirm that the smeared bands observed for TRX-KΔC_{wt} represented the phosphorylated forms, we purified the TRX-KΔC proteins from recombinant *E. coli* cells grown in the presence of ^{32}P-labeled inorganic phosphate. TRX-KΔC_{wt} showed a smeared pattern on SDS-PAGE, but TRX-KΔC_{K44A} did not (Fig. 3C). Autoradiography of the gel indicated the presence of ^{32}P in the smeared bands of TRX-KΔC_{wt} and the absence of ^{32}P in TRX-KΔC_{K44A}.

We did pull-down assays to examine the interaction between GST-KbpA and TRX-KΔC_{wt} or TRX-KΔC_{K44A} (Fig. 3D). TRX-KΔC proteins labeled in vivo were used. After incubation, the GST complexes were precipitated with glutathione-Sepharose and separated by SDS-PAGE. TRX complexes and GST complexes were detected by Western blotting with S protein and the antibody for GST, respectively. Both TRX-KΔC_{wt} and TRX-KΔC_{K44A} were coprecipitated with GST-KbpA (Fig. 3D, lanes 3 and 4). Only a sharp band, with no smeared bands, was detected for TRX-KΔC_{wt}, although the TRX-KΔC_{wt} preparation contained smeared bands (lane 5). These observations suggested that GST-KbpA interacted only with the nonphosphorylated form of AfsK. In fact, the TRX-KΔC_{wt} that was coprecipitated with GST-KbpA contained no radioactivity.

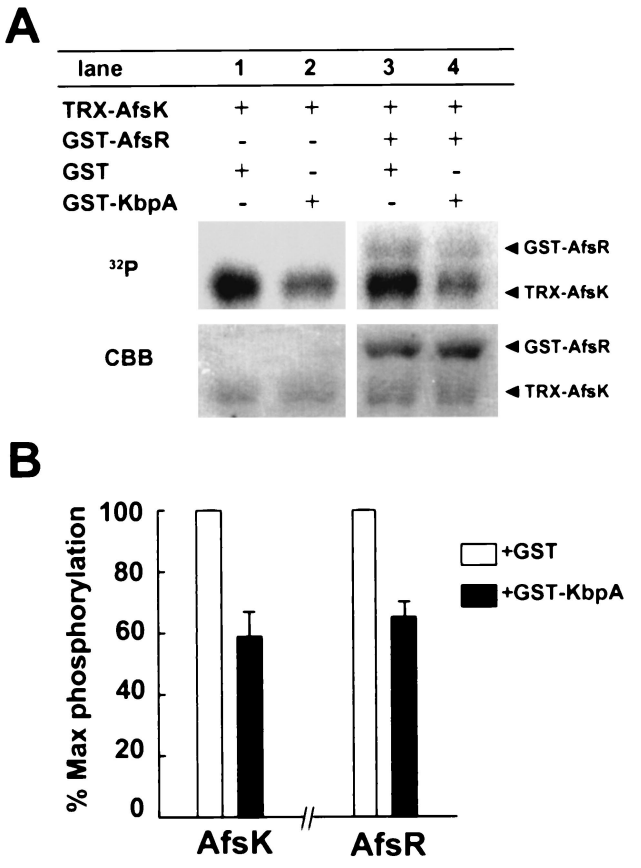

FIG. 4. Inhibition of AfsK autophosphorylation by KbpA. (A) Incubation of TRX-AfsK (15 pmol) in the presence of [γ-^{32}P]ATP and GST (300 pmol) yielded the autophosphorylated form (lane 1). The presence of GST-KbpA (300 pmol) instead of GST inhibited the autophosphorylation. Phosphorylation of GST-AfsR (30 pmol) was also inhibited by GST-KbpA but not by GST (lane 4). The amounts of the proteins contained in the reaction mixture were monitored by staining the gel with Coomassie brilliant blue (CBB). (B) The gels were analyzed on an image analyzer, and the inhibition of autophosphorylation of TRX-AfsK and phosphorylation of GST-AfsR by TRX-AfsK was plotted. The degrees of inhibition are the means of values obtained from three independent experiments. Error bars indicate standard deviations.

Inhibition of AfsK autophosphorylation by KbpA. Because KbpA was found to bind the kinase domain in the nonphosphorylated form of AfsK, we examined the effect of KbpA binding to AfsK on autophosphorylation. Incubation of TRX-AfsK (966 amino acids, 102 kDa) in the presence of [γ-^{32}P]ATP yielded the autophosphorylated form (Fig. 4A), because the TRX-AfsK preparation contained a large population of the nonphosphorylated form. GST-KbpA inhibited this phosphorylation by about 40% when analyzed with an image analyzer (Fig. 4B). The inhibition of autophosphorylation to a small extent may be attributed to the equilibrium of association and dissociation between AfsK and KbpA and rapid autophosphorylation of dissociated AfsK. Coincubation of TRX-AfsK and GST-AfsR yielded a phosphorylated form of GST-AfsR,

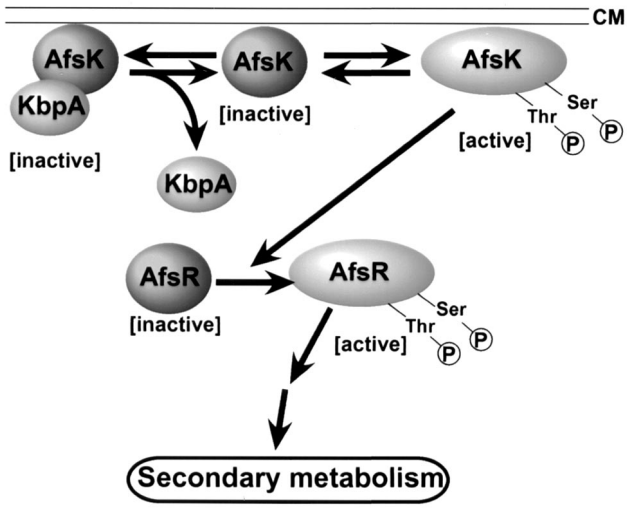

FIG. 5. Model of inhibition of AfsK autophosphorylation by KbpA.

in addition to the autophosphorylated form of TRX-AfsK. The presence of GST-KbpA inhibited the phosphorylation of AfsR by AfsK, because GST-KbpA reduced the amount of the phosphorylated, active form of TRX-AfsK by inhibiting the autophosphorylation. The small extent of inhibition can be attributed to the autophosphorylated, active AfsA that was inevitably present in a small amount in the AfsK preparation. GST-KbpA also inhibited autophosphorylation of TRX-KΔC_{wt} to a similar extent (data not shown). It is thus apparent that KbpA binds the kinase domain of AfsK and inhibits its autophosphorylation.

DISCUSSION

The in vitro experiments have shown that KbpA directly binds the nonphosphorylated form of AfsK and inhibits the autophosphorylation of AfsK. This is consistent with the in vivo observations that KbpA serves as a repressor for actinorhodin production. Also consistent with this are the observations that a mutant AfsK unable to autophosphorylate did not complement the reduced actinorhodin production by an *afsK* mutant (unpublished results). The kinase domain at the N-terminal portion of AfsK interacts with KbpA. Recovery of only nonphosphorylated forms of TRX-KΔC_{wt} and TRX-KΔC_{K44A} by the pull-down assay with GST-KbpA suggests that the presence of even a single phosphorylated site at either serine or threonine residue prevents KbpA from binding to AfsK, although the numbers and the exact positions of serine and threonine residues to be autophosphorylated are still unknown. We thus assume that the population of the phosphorylated, active form of AfsK is modulated by the amount of KbpA, as a result of which the degree of phosphorylation of AfsR is controlled (Fig. 5). At a later stage of growth under usual culture conditions on agar medium, KbpA binds the nonphosphorylated form of AfsK, which has not yet autophosphorylated or which has been dephosphorylated by protein phosphatases, and inhibits its autophosphorylation at serine and threonine residues. It is unclear whether the AfsK-KbpA complex is associated with the membrane. The nonphosphorylated AfsK is inactive and unable to activate the positive regulator AfsR by phosphorylating it at serine and threonine residues, as a result of which secondary metabolism, including actinorhodin production, is repressed. Thus, KbpA serves as an inhibitor in a negative feedback system in the AfsK-AfsR regulatory pathway. The Δ*kbpA* mutation, allowing AfsK activation by autophosphorylation, would result in accumulation of a larger amount of the phosphorylated form of AfsR and in overproduction of actinorhodin and undecylprodigiosin, as is observed in strains overexpressing *afsR*. A larger amount of KbpA would result in a decrease of the amount of the active form of AfsR and a reduction of the pigment production, as is found in *afsR* and *afsK* mutants. The hydropathy plot of KbpA excludes the possibility that it is a membrane protein. Therefore, KbpA seems not to control the localization of AfsK inside the hyphae, unlike the many kinase-anchoring proteins that determine the specificities and activities of kinase-mediated signaling pathways in eukaryotes (5, 14, 18). 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 (17). A seven-repeat sequence in the C-terminal portion may be a motif to anchor a membrane protein, as pointed out by Nadvornik et al. (20).

kbpA is transcribed throughout growth, and its transcription is enhanced when production of actinorhodin and undecylprodigiosin has already started. Since *afsK* is transcribed constantly throughout growth, KbpA appears to put a brake on the unlimited production of the pigments that has been commenced by the AfsK-AfsR system. AfsK-AfsR seems to operate independently of the pathway-specific regulatory proteins to mainly control pigment production, such as ActII-ORF4 for actinorhodin and RedD for undecylprodigiosin (6), suggesting an inhibitory role of KbpA in a negative feedback system in the AfsK-AfsR regulatory pathway.

The genome project for *S. coelicolor* A3(2) revealed the presence of six KbpA homologues and more than 20 AfsK homologues containing a catalytic domain of protein serine/threonine kinases. AfsR is phosphorylated not only by AfsK but also by an additional kinase (17). We have recently found that two other kinases are capable of phosphorylation of AfsR at serine and threonine residues (unpublished results). By analogy with the eukaryotic systems, these AfsK homologues recognize their respective signals and transfer them to AfsR by means of phosphorylation. It is unclear whether KbpA and the six KbpA homologues discriminate and bind these kinases and modulate their activity. Functionally unknown KbpA homologues found in *Streptomyces* and various bacteria may be elucidated through an approach based on the assumption that KbpA homologues serve as a modulator by protein-protein interaction. Among KbpA homologues in various bacteria, PA1672, encoded by the region just downstream of *sty1* encoding a serine/threonine kinase in *Pseudomonas aeruginosa* (19) (www.pseudomonas.com/), may serve as a *Sty*I-binding protein.

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