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Effects of Protein Kinase Inhibitors on *In Vitro* Protein Phosphorylation and on Secondary Metabolism and Morphogenesis in *Streptomyces coelicolor* A3(2)

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Abstract *In vitro* phosphorylation experiments with a cell extract of *Streptomyces coelicolor* A3(2) M130 in the presence of γ-[^{32}P]ATP revealed the presence of multiple phosphorylated proteins, including the AfsR/AfsK kinases which control the biosynthesis of A-factor, actinorhodin, and undecylprodigiosin. Phosphorylation of AfsR by a cell extract as an AfsK source was significantly inhibited by Ser/Thr protein kinase inhibitors, staurosporine and K-252a, at concentrations giving 50% inhibition (IC_{50}) of 1 μM and 0.1 μM, respectively. Further *in vitro* experiments with the cell extracts showed that phosphorylation of multiple proteins was inhibited by various protein kinase inhibitors with different inhibitory profiles. Manganese and calcium ions in the reaction mixture also modulate phosphorylation of multiple proteins. Manganese at 10 mM greatly enhanced the phosphorylation and partially circumvented the inhibition caused by staurosporine and K-252a. A calcium-activated protein kinase(s) was little affected by these inhibitors. Herbimycin and radicicol, which are known as tyrosine kinase inhibitors, did not show any significant inhibition of AfsR phosphorylation. Consistent with the *in vitro* effect of the kinase inhibitors, they inhibited aerial mycelium formation and pigmented antibiotic production on solid media. On the contrary, when assayed in liquid culture, the amount of actinorhodin produced was increased by staurosporine and K-252a and greatly decreased by manganese. All of these data clearly show that the genus *Streptomyces* possesses several protein kinases of eukaryotic types which are involved in the regulatory network for morphogenesis and secondary metabolism.

Key Words: Protein kinase inhibitors, protein phosphorylation, secondary metabolism, morphogenesis, *Streptomyces coelicolor* A3(2)

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Streptomycetes have unique features of cell differentiation and produce various secondary metabolites during their life cycle. Many observations suggest that protein phosphorylation is involved in the regulation of secondary metabolism and cell differentiation in *Streptomyces*. For example, the AfsR/AfsK system globally controls secondary metabolite formation [6, 10, 11, 12, 14, 23]. Introduction of one of these genes on a low-copy number plasmid into *Streptomyces lividans* induced marked production of the pigmented antibiotics actinorhodin and undecylprodigiosin whose biosynthetic genes are sleeping under the usual cultural conditions in this strain [9, 13]. AfsR was found to be phosphorylated by a membrane-associated phosphokinase, named AfsK, which autophosphorylated on Ser and Tyr residues [6, 23]. AfsK phosphorylates AfsR at both Ser and Thr residues, and an additional protein kinase in the cell is also able to phosphorylate AfsR at the same residues [23]. The *afsK* gene is able to reverse the aerial mycelium-negative phenotype of an A-factor-deficient mutant strain, *Streptomyces griseus* HH1, and induces sporulation without affecting A-factor productivity or streptomycin production. Disruption of an *afsK* homologue in *S. griseus* failed to form aerial mycelium formation on glucose-containing medium (our unpublished results), which suggests that a Ser/Thr kinase similar to *S. coelicolor* A3(2) AfsK plays a regulatory role in aerial mycelium formation in *S. griseus* [32]. In addition to the AfsR/AfsK systems, the *ptpA* gene coding for a phosphotyrosine protein phosphatase, in *S. coelicolor* A3(2) M130 conferred a similar effect when it was introduced into *S. lividans* [35]. It is therefore apparent that various protein kinases and phosphatases similar to those found in higher organisms control secondary metabolite formation or morphogenesis in *Streptomyces*.

Various compounds such as staurosporine [25, 26, 30, 31], K-252a [17, 18, 30, 36], herbimycin [33, 34],

radicicol [24, 25], synthetic rabbit sequence [3], and H-7 [5] have been listed as inhibitors of various types of eukaryotic protein kinases, which are tightly involved in signal transduction processes in mammalian and yeast cells. In general, eukaryotic protein kinases are classified into five groups depending on effectors: cyclic AMP-dependent kinases: cyclic GMP-dependent kinases, and Ca^{2+}-activated kinases which are subdivided into Ca^{2+}/calmodulin dependent kinase, myosin light chain kinases, and Ca^{2+}/phospholopid dependent kinase (protein kinase C) [4]. The function of these phosphorylating proteins, which can transfer the γ-phosphate of ATP to Ser/Thr and Tyr residues of a substrate protein, have been studied with protein kinase inhibitors in detail. In constrast to the profound effects of the inhibitors of these kinases on eukaryotic regulatory systems both *in vitro* and *in vivo*, only few reports have been published with regards to bacterial signal transduction systems. This paper reports our discorvery that AfsR phosphorylation by AfsK and phosphorylation of several proteins by still unknown kinases in the cell extracts of *S. coelicolor* A3(2) M130 are significantly inhibited by eukaryotic protein kinases inhibitors. We also investigated effects of trace elements on the phosphorylation. *In vivo* effects of these inhibitors on the morphology and antibiotic biosynthesis in the same strain and the difference of regulatory pattern previously reported in *S. griseus* are also described in this paper.

MATERIALS AND METHODS

Materials and Bacterial Culture Conditions

Protein kinase inhibitors used and their sources were: staurosporine and K-252a (obtained from Dr. Y. Matsuda of Kyowa Medex Co., Tokyo), protein kinase inhibitor H-7 [5] (Seikagaku Kogyo Ltd., Tokyo), synthetic rabbit sequence [3] (Sigma Chemical Co., U.S.A.), radicicol [25], and herbimycin [33] (Y. Uehara of National Institute of Health, Japan). [γ-^{32}P]ATP (5,000 Ci/mol) and nucleotides were purchased from Amersham International and all the chemicals used were from Sigma Chemical Co. *S. coelicolor* A3(2) M130 was obtained from D. A. Hopwood, John Inns Institute [1]. R2YE medium without agar was used for liquid culture, and R2YE agar medium [8] was used for regeneration of protoplasts.

Preparation of Crude Extracts as a Source of Protein Kinases

S. coelicolor A3(2) M130 was cultivated at 30°C for 2.5 days on a reciprocal shaker, and the mycelium was harvested. After the mycelium had been washed with 20 mM Tris-HCl (pH 7.0) buffer containing 1 mM EDTA, 1 mM dithiothreitol, and 10% (v/v) glycerol, they were suspended in the same buffer and disrupted by French pressing. Cell debris was removed by centrifugation at 40,000 × g for 1 h, and the supernatant was used as a kinase source for phosphorylation of AfsR. Protein concentrations were measured by the method of Lowry *et al.* [21].

Phosphorylation Protocol

The purified AfsR protein (3 pmoles) was phosphorylated with the crude extract (3 μg protein) of *S. coelicolor* A3(2) M130 as a kinase preparation in the reaction mixture containing 50 mM Tris-HCl (pH 7.4), 50 mM NaCl, 10 mM $MgCl_2 \cdot 6H_2O$, 1 mM EDTA, 1 mM dithiothreitol, and 10 μ Ci of [γ-^{32}P]ATP in a total volume of 10 μl, mainly according to the method described for PhoR-PhoB phosphorylation [22]. When necessary, protein kinase inhibitors and trace elements at various concentrations were added to the reaction mixture. After 5 min of incubation at room temperature, the reaction was terminated by the addition of 4 × SDS sample buffer containing 250 mM Tris-HCl (pH 6.8), 40% glycerol, 8% SDS, 20% 2-mercaptoethanol, and 0.1% bromphenol blue. The samples were boiled for 2 min and phosphorylated proteins were separated by 0.1% SDS-10% polyacrylamide gel electrophoresis [20]. After the gel had been stained, destained, and dried, it was placed for autoradiography at -80°C with a Du Pont Cronex intensifying screen. The degree of phosphorylation of AfsR was estimated more accurately by counting the radioactivity incorporated into AfsR in an Aloka Liquid Scintillation Spectrophotometer Model LSC-670 after a gel slice had been cut out.

Similarly, the effects of protein kinase inhibitors on *in vitro* phosphorylation of the cellular proteins of *S. coelicolor* A3(2) M130 were examined. The conditions were the same as for the AfsR phosphorylation described above, except that AfsR was omitted.

Quantitation of Actinorhodin

A stationary culture of *S. coelicolor* A3(2) M130 was transferred to 100 ml of R2YE medium in a 500-ml Sakaguchi flask which contained different concentrations of protein kinase inhibitors or trace elements, and incubated at 30°C on a reciprocal shaker. Portions (5 ml) of the culture broth taken out at intervals were scanned with a Jasco UVIDEC-610 scanning spectrophotometer. The amounts of actinorhodin were calculated from the A 615 nm values at pH 12, as described previously [9, 12].

Mycelial Growth and Pigment Production

We checked *in vivo* influences of protein kinase inhibitors and trace elements on *S. coelicolor* A3(2) M 130. Spores or protoplasts [8] prepared from strain M130 were grown on solid R2YE medium with paper discs containing different concentrations of protein kinase

inhibitors or trace elements. Aerial mycelium formation, sporulation, and pigment production were examined after 3 to 7 days of growth at 28°C and further examined by scanning electron microscopy.

Scanning Electron Microscopy

Agar pieces with mycelium and spores were cut and fixed under 1% osmium tetroxide vapor for 15 h. These fixed specimens were dehydrated and dried with a freeze-dryer for another 15 h. After being coated with platinum-gold under vacuum by sputter coating for 5 min, mycelium and spores were observed under a Hitachi Model S4000 scanning electron microscope.

RESULTS

Effects of Protein Kinase Inhibitors on Phosphorylation of AfsR

To study the effects of these inhibitors on the AfsR phosphorylation by the membrane-bound phosphokinase, AfsK, we added the inhibitors to the reaction mixture containing [γ-^{32}P]ATP, the purified AfsR protein, and the cell extract of *S. coelicolor* A3(2) M130 as a source of AfsK. The ^{32}P-labeled AfsR protein was detected by autoradiography (Fig. 1). For quantification of the inhibitory effects, the radioactivity incorporated into AfsR was counted by liquid scintillation counting of the gel slices containing the AfsR signal. Staurosporine and K-252a, which inhibit most of protein kinases rather nonspecifically probably due to their modes of action (i.e., competition with ATP) [30], markedly inhibited the phosphorylation of AfsR. The concentrations giving 50% inhibition (IC_{50}) were estimated to be lower than 1 μM for staurosporine and 0.1 μM for K-252a (lanes 2-7). Phosphorylation of another protein, C3 derived from the cell extract of *S. coelicolor* A3(2) M130, was also inhibited by these agents at slightly higher concentrations. On the other hand, a protein kinase inhibitor H-7 which is known as an inhibitor of cyclic nucleotide-dependent kinases and protein kinase C [5], and the synthetic peptide (thr-thr-tyr-ala-asp-phe-ile-ala-ser-gly-arg-thr-gly-arg-arg-asn-ala-ile-his-asp), which is designed to mimic a partial sequence of a rabbit inhibitor protein for a cyclic AMP-dependent kinase [3], did not show any significant inhibition on the AfsR phosphorylation (lanes 8-11). Herbimycin [33, 34] and radicicol [24], which are weak inhibitors for tyrosine protein kinases, also did not show any significant inhibition of the AfsR phosphorylation (lanes 12-15). This is consistent with the fact that AfsR is phosphorylated on its Ser and Thr residues.

We also found that the inhibition of AfsR phosphorylation by staurosporine and K-252a was circumvented by the presence of 10 mM manganese to some extent (Fig. 2). Careful observations on this effect revealed that the addition of manganese alone caused elevated phosphorylation of not only AfsR but also several other proteins such as C1, C3, C4, and C5 (lane 2). Addition of 30 mM calcium also stimulated the phosphorylation of C2, C3, and C5 (lane 3), which were almost undetectable in the control reaction (lane 1). Although the phosphorylation of C3 and C5 were too weak to present an apparant difference, the extent of

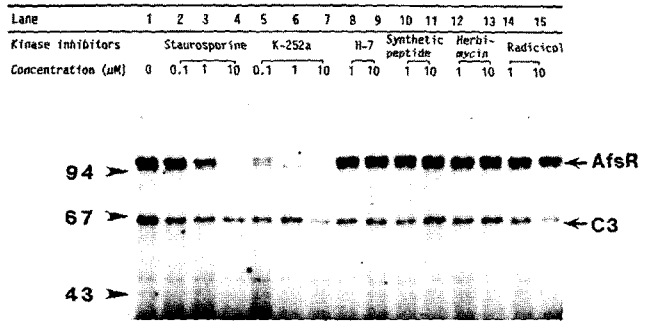

Fig. 1. Effects of protein kinase inhibitors on the phosphorylation of AfsR.

The reaction mixtures containing 3 pmole of AfsR, 3 μg of the crude-extract from *S. coelicolor* A3(2), 10 μCi of [γ-^{32}P]ATP, and various amounts of protein kinase inhibitors in a total volume of 10 μl were incubated for 5 min at room temperature. The mixtures were then seperated by SDS-polyacrylamide gel electrophoresis and its autoradiogram was obtained. The ^{32}P-labeled AfsR bands are indicated by an arrow, and band C3 is also designated for explanation.

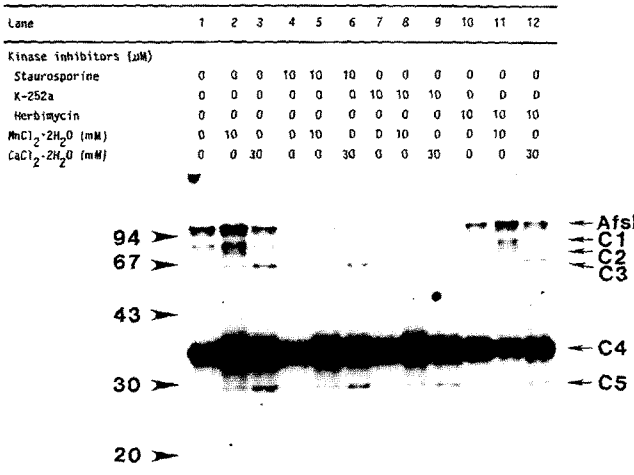

Fig. 2. Autoradiograms showing the phosphorylation of AfsR and cellular proteins of *S. coelicolor* A3(2) in the presence of protein kinase inhibitors together with manganese or calcium ions.

All the reaction mixtures contained 3 pmoles of AfsR, 5 μg of the *S. coelicolor* A3(2) crude-extract, 10 μCi of [γ-^{32}P]ATP, and various chemicals as indicated. The reaction was continued for 5 min at room temperature. The AfsR bands are indicated, and bands from C1 to C5 are shown for explanation.

inhibitory effects by staurosporine, K-252a, or herbimycin on the calcium-stimulated phosphorylation was negligible compare to those on manganese-stimulated phosphorylation as reported in *S. griseus*. For example, inhibition of the AfsR phosphorylation by staurosporine and K-252a was not restored by calcium, unlike manganese.

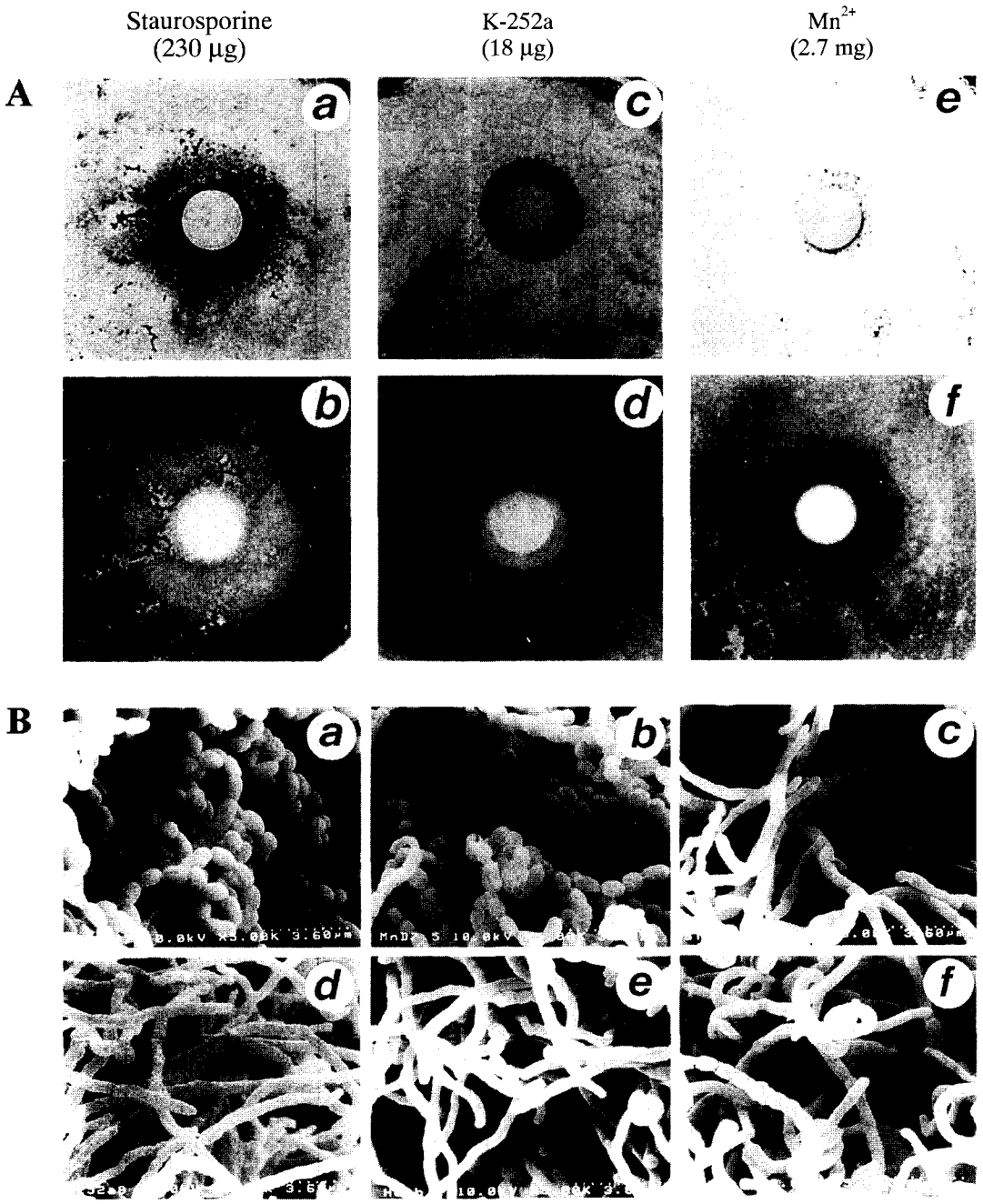

Fig. 3. Effects of protein kinase inhibitors and manganese on morphogenesis and pigmented antibiotic production of *S. coelicolor* A3(2) M130.

Photographs of the plates (A) and scanning electron microscopic observations (B). A. keep upper, photographs of the surface of the plate; bottom, photographs of the reverse side of the plate. Each paper disc contains the indicated chemicals of indicated amount. (B) a, *S. coelicolor* A3(2) M130 as a control; b, taken from the doughnut zone caused by Mn^{2+} (A-e), where abundant spores are formed; c, taken from the inhibition zone caused by staurosporine (A-a), where no aerial mycelium, but substrate mycelium with no detectable change in shape and in size, is formed; d, taken from the inhibition zone caused by K-252a (A-c), where no aerial mycelium is formed; e, taken from the area close to the paper disc containing 100 μg of herbimycin, where aerial mycelium formation is delayed by about 2 days; and f, taken from the area close to the paper disc containing 100 μg of radicicol, where aerial mycelium formation is delayed by about 1 day.

Effects of Protein Kinase Inhibitors on Morphogenesis and Antibiotic Production in *S. coelicolor* A3(2)

Because of the distinct inhibition by protein kinase inhibitors of the *in vitro* phosphorylation of cellular proteins in *S. coelicolor* A3(2) M130, we next examined *in vivo* effects of these inhibitors as well as manganese on morphology and antibiotic production. We put a paper disc containing each of the inhibitors or metals on R2YE agar plates seeded with spores and observed mycelial morphology.

When staurosporine was applied to strain M130, the formation of aerial mycelium and spores around the disc was completely inhibited (Fig. 3A-a). No growth inhibition, even in the area close to the disc, was observed, indicating that staurosporine inhibited cellular differentiation without affecting vegetative growth. The substrate mycelium grown in medium containing a high concentration of staurosporine was normal, as observed by scanning electron microscopy, but a few small sprouts or buds which seemed to differentiate into aerial mycelia were seen (Fig. 3B-c). In addition to the aerial mycelium formation, the production of the pigmented antibiotics, actinorhodin and undecylprodigiosin, were inhibited by staurosporine (Fig. 3A-b). No growth inhibition by staurosporine in these experiments shows that this drug causes almost no effect on germination of spores.

K-252a (23-230 μg/disc) caused formation of a growth inhibition zone around the disc indicating its killing effect at a high concentration (Fig. 3A-c). A ring was formed outside the clear zone where only substrate mycelium was formed with no pigment production. This implied that K-252a at a certain concentration inhibited aerial mycelium formation and pigmentation without exerting a killing effect. In fact, a smaller amount of K-252a (18 μg/disc) caused appearance of a distinct zone around the disc where aerial mycelium formation and pigmentation were completely inhibited (Figs. 3A-c, d). Herbimycin and radicicol (100 μg each) caused no significant effect on pigmentation (data not shown). However, delayed aerial mycelium formation by one or two days and relatively scarce sporulation were observed, especially with herbimycin (Figs. 3B-e, f).

When manganese was applied, a ring showing much abundant sporulation was formed (Figs. 3A-e, f). The thickness of the mycelium lawn in this zone was apparently increased due to the abundant formation of aerial mycelium. Although an electron micrograph showingonly a local viable field (Fig. 3B-b) does not present an apparant difference from that for strain M130 grown normally (Fig. 3B-a), the size of spores are smaller, but the shape of spores and probably the length of spore chains are the same. Manganese may cause more fragment aerial mycelium and smaller and denser spore formation.

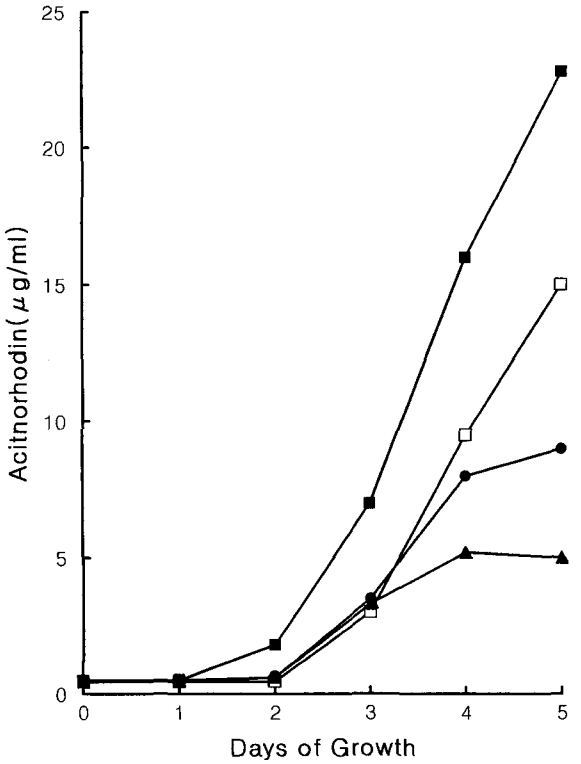

Fig. 4. Time course of actinorhodin production by *S. coelicolor* A3(2) M130 in the presence of protein kinase inhibitor or manganese.

Portions sampled as described in Materials and Methods were scanned with the spectrophotometer and the calculated amounts of actinorhodin from the value of OD_{615} at pH 12 are plotted as a function of time. ● — ●, control; □ — □, staurosporine (1 μM) containing culture; ■ — ■, K-252a (1 μM) containing culture; ▲ — ▲, $MnCl_2$ (1 mM) containing culture.

Although protein kinase inhibitors showed significant inhibitory effects on pigment production of strain M130 on solid media, enhancing effects were also observed when they were applied in liquid culture (Fig. 4). Staurosporine and K-252a enhanced actinorhodin production by two- and three-folds, respectively, and manganese markedly decreased its production in liquid culture.

DISCUSSION

Protein phosphorylations play very important roles in prokaryotic and eukaryotic systems. In general, Ser/Thr/Tyr kinases are major components tightly involved in the signal transduction processes in mammalian and yeast cells. The regulatory function of these phosphoproteins have been intensively studied with the protein kinase inhibitors so far known to be specific to eukaryotic protein kinases. We previously reported the effects of protein kinase inhibitors on the protein phosphorylation and morphogenesis of *S. griseus* [7]. In this study, we

found that staurosporine and K-252a exert a strong inhibitory effect on not only the phosphorylation of AfsR but also several cellular proteins in *S. coelicolor* A3(2). The different inhibitory profiles of their phosphorylation by kinase inhibitors predict unambiguously the presence of multiple protein kinases in the genus *Streptomyces* in general. Consistent with the *in vitro* inhibition, these drugs at concentrations showing no killing effects inhibit morphogenesis, especially aerial mycelium formation, in *S. coelicolor* A3(2). The involvement of protein phosphorylation sensitive to the above drugs reminds us of a similarity between *Streptomyces* and the eukaryotic organism.

Manganese and calcium were found to modulate the *in vitro* phosphorylation of several proteins in the presence and absence of the inhibitors. Although the effective concentrations of these cations seem to be far higher than the physiological concentrations, it is possible that these trace elements are required for activities of the several protein kinases in *Streptomyces*. For example, we assume that a system similar to the eukaryotic Ca^{2+}/calmodulin system or the Ca^{2+}/phospholipid system [4, 19, 27, 28] may be present in *Streptomyces*. During the course of the purification of AfsK from *S. coelicolor* A3(2), we observed that partially purified AfsK showed marked reactivation by 10 mM Mn^{2+} (unpublished data). It seems possible that staurosporine and K-252a exert their inhibitory effects by competing with not only ATP but also Mn^{2+}, which are required for corresponding kinases.

Although the effects of these inhibitors on the *in vitro* protein phosphorylation of *S. coelicolor* A3(2) were very similar to those of *S. griseus*, the number of detectable phosphoproteins was fewer in *S. coelicolor* A3(2) than in *S. griseus*. Multiple protein phosphorylations were very sensitive to staurosporine and K-252a in both strains, but protein phosphorylation sensitive to herbimycin, radicicol, H-7, and synthetic peptide was not detected in *S. coelicolor* A3(2). Radicicol and herbimycin have been known as specific tyrosine kinase inhibitors and have the ability to suppress transformation by diverse oncogenes such as *Src*, *Ras* and *Mos*. Recently it was suggested that the anti-transformation effects of radicicol may be mediated, at least in part, by the association of radicicol with HSP90 and the consequent dissociation of the Raf/HSP90 complex leading to the attenuation of the Ras/MAP kinase signal transduction pathway [30]. When we applied herbimycin and radicicol into *in vitro* protein phosphorylation experiments, we could observe two distint herbimycin-sensitive bands only in *S. griseus* [7]. This fact supports the idea radicicol acts in a different way from herbimycin in signal transduction pathway.

Similar inhibitory effects as in *S. griseus* by staurosporine and K-252a on the aerial mycelium formation of *S. coelicolor* A3(2) were observed without growth inhibition (Figs. 3A-a, c and 3B-c, d). At a higher dosage (230 μg/disc) of K-252a, an inner zone showing normal sporulation and yellow pigmentation appeared around the disc, which was surrounded by a ring showing complete inhibition of aerial mycelium formation in *S. griseus*, but in *S. coelicolor* A3(2), complete inhibition of morphogenesis, even in the area close to the disc, was observed. Pigment antibiotic production was also completely inhibited on solid media, which coincides with the inhibitory effect of staurosporine and K-252a on yellow pigment production in *S. griseus* (Fig. 3A-b, d). When manganese was applied to *S. coelicolor* A3(2), abundant sporulation and pigment production were induced, just like the case of *S. griseus* (Figs. 3A-e, f and 3B-b).

Actinorhodin production by *S. coelicolor* A3(2) in liquid culture was increased by several times by staurosporine and K-252a at a final concentration of 1 μM and decreased by half at a final concentration of 1 M $MnCl_2$ (Fig. 4). These results contradict the results obtained from solid culture. In general, the physiological conditions are quite different between the liquid and solid cultures. For example, most *Streptomyces* can sporulate on solid culture but not in liquid culture. Therefore, we suppose that the physiological regulatory mechanism for secondary metabolism and morphogenesis operates in different ways depending on physiological conditions. When a similar experiment was performed in liquid culture of *S. griseus*, the streptomycin production was completely inhibited by staurosporine and K-252a and stimulated by $MnCl_2$ (unpublished data). This fact implies that various regulatory components involved in morphogenesis and secondary metabolism are working together in a concerted manner in *Streptomyces* strains.

Staurosporine does not show any inhibitory effect on the phosphotransfer between OmpR and EnvZ of *Escherichia coli* which compose typical bacterial two-component systems with His/Asp kinases (T. Mizuno, personal communication). AfsR shows no similarity in amino acid sequence to the regulators in the prokaryotic two-component regulatory systems so far known, whereas it shows significant homology with several putative regulatory proteins of the antibiotic biosynthetic gene clusters in other *Streptomyces* spp., such as *actII*-ORF4, *dnrR1*-ORF1, and *redD*-ORF1 (C. R. Hutchinson, personal communication). This fact suggests that the staurosporine-sensitive systems of AfsR-AfsK type generally control the differentiation processes in *Streptomyces*. However, it should also be noted that other two-component regulatory sytems, AfsQ1-AfsQ2 [15] and AbsA1-AbsA2 [2], associated with secondary metabolism in *S. coelicolor* A3(2), show great similarity in amino acid sequence to the typical prokaryotic two-component systems such as OmpR-EnvZ and PhoB-PhoR, which appear to be insensitive to staurosporine [16, 29].

Identification of each protein kinase involved in the differentiation process of *Streptomyces* will present a new aspect in the study on *Streptomyces* biology. Protein kinase inhibitors as tools have brought out many fruitful accomplishments in the study of eukaryotic cellular differentiation including that of animal cells. The development of new protein kinase inhibitors specific to prokaryotic- or eukaryotic-type protein kinases will be able to offer valuable methods of understanding the regulatory network concerned with physiological and morphological differentiation.

Acknowledgments

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