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EXPERIMENTAL ARTICLES

Ca^{2+}-Dependent Modulation of Antibiotic Resistance in *Streptomyces lividans* 66 and *Streptomyces coelicolor* A3(2)

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Abstract—The level of resistance to antibiotics of various chemical structure in actinobacteria of the genus *Streptomyces* is shown to be regulated by Ca^{2+} ions. The inhibitors of Ca^{2+}/calmodulin and Ca^{2+}/phospholipid-dependent serine/threonine protein kinases (STPK) are found to reduce antibiotic resistance of actinobacteria. The effect of Ca^{2+}-dependent phosphorylation on the activity of the enzymatic aminoglycoside phosphotransferase system protecting actinobacteria from aminoglycoside antibiotics was studied. It is shown that inhibitors of Ca^{2+}/calmodulin and Ca^{2+}/phospholipid-dependent STPK reduced the Ca^{2+}-induced kanamycin resistance in *Streptomyces lividans* cells transformed by a hybrid plasmid which contained the aminoglycoside phosphotransferase VIII (APHVIII) gene. In *S. coelicolor* A3(2) cells, the protein kinase PK25 responsible for APHVIII phosphorylation in vitro was identified. It is suggested that STPK play a major role in the regulation of antibiotic resistance in actinobacteria.

Key words: serine/threonine protein kinases, multiple drug resistance, actinobacteria, aminoglycoside phosphotransferase VIII.

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Biochemical and physiological investigations have shown that calcium ions in bacterial cells are involved in the regulation of various processes, such as the cellular cycle, pathogenesis, motility and chemotaxis, stabilization and integrity maintenance of the outer lipopolysaccharide layer and bacterial cell wall, and stabilization of bacterial proteins and alteration of their enzymatic activity, as well as activation or inhibition of protein phosphorylation by protein kinases [1, 2]. Ca^{2+} ions control secretory processes and regulate porin expression in the outer membrane in response to fluctuations in the osmotic environment [1]. The recent reviews [1, 2] summarize data on the role of Ca^{2+} in many processes occurring in actinobacterial cells, including cell differentiation, intercellular communication, and signal transduction. Thus, investigation of Ca^{2+}-dependent regulatory proteins is required for the study of the role of Ca^{2+} in bacterial cells.

Actinomycetes of the genus *Streptomyces* are soil gram-positive mycelial bacteria, producers of a number of bioactive compounds, including about 70% of all antibiotics used in medical and veterinary practice [3]. Actinomycetes that produce antibiotics of various chemical nature have developed protective mechanisms against them. A gene cluster responsible for the antibiotic biosynthesis usually contains one or multiple antibiotic resistance genes [4]. Antibiotic resistance of antibiotic producers and clinical isolates of pathogenic bacteria depends on many mechanisms. For instance, the mechanism that underlies resistance to aminoglycosides results from their enzymatic inactivation by aminoglycoside phosphotransferases [5].

Genetic determination of antibiotic resistance was confirmed by the isolation of *S. coelicolor* A3(2) mutants sensitive to various antibiotics, as well as by mapping of the resistance genes of this microorganism [6]. One of these genes, *aphVIII*, encoding aminoglycoside phosphotransferase VIII in *S. rimosus*, was cloned into *S. lividans* [7]. Due to its structural and functional properties, APHVIII fits into a new group of aminoglycoside kinases [8], whose members differ from the analogous enzymes of aminoglycoside antibiotic producers.

It is well known that actinomycetes are resistant to many antibiotics, including their own [6]. Recently, it was shown that 480 actinomycete strains isolated from soils exhibited multiple resistance to various antibiotics, including new ones which were recently introduced into clinical use [9]. The term "resistome" has been proposed for the phenomenon of multiple drug resistance of actinomycetes. This term designates a complex of all the resistance genes, expressed and unexpressed (those which remain silent under laboratory conditions), both in pathogenic and nonpathogenic bacteria

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[10]. Taking into account the crucial importance of multiple drug resistance for clinical practice [11], as well as the role of actinobacteria as a reservoir of resistance genes, resistome may become the key object of the study aimed at this issue.

Analysis of available genome databases of *Streptomyces* species enabled us to suggest that streptomycetes can be used as an object for detection and characterization of new mechanisms of antibiotic resistance. Considering the important role of Ca^{2+} as a secondary messenger of bacterial cells [1, 2], it was of particular interest to study the Ca^{2+}-dependent pathways of signal transduction.

The aim of the present work was to study the effect of Ca^{2+} on the regulation of antibiotic resistance controlled by the serine/threonine protein kinases of the studied *Streptomyces* strains.

MATERIALS AND METHODS

Strains, media, and cultivation conditions. The main objects of investigation were *Streptomyces lividans* 66 and *Streptomyces coelicolor* A3(2) [12, 13] (Russian National Collection of Industrial Microorganisms, VKPM, Moscow), closely related species whose genomes have been thoroughly studied. The cultures were grown on complete agarized YSP medium [12]. To determine antibiotic resistance patterns, the strains were grown on agarized MG medium (pH 7.5) containing the following (%): malt extract (Sigma), 0.5; yeast extract (Difco), 0.4; NaCl, 0.05; $MgSO_4$, 0.05; K_2HPO_4, 0.05; $FeSO_4$, 0.0001; KNO_3, 0.1; and glucose, 2.

To determine the protein phosphorylation level, *S. coelicolor* A3(2) were grown at 28°C for 48 h (early stationary phase) in a liquid nutrient medium (pH 7.0) containing the following (%): glucose, 4; bacto peptone, 0.2; yeast extract, 0.2; ammonium sulfate, 0.2; $MgSO_4$, 0.05; and K_2HPO_4, 0.05. The cells were grown with or without 20 mM Ca^{2+}. The mycelium was harvested by centrifugation at 3000 *g* for 30 min.

The cells of *Escherichia coli* BL21 (Novagen) were grown at 37°C according to Mierendorf et al. [14]. The plasmid construction and the *aphVIII* construct expression in *E. coli* were performed as described in [8]. The construct contained an additional encoding sequence for the His_{10} decapeptide adjacent to the N-terminal region of the APHVIII molecule.

Determination of antibiotic resistance levels. The test was based on determination of the growth inhibition zones around paper disks containing antibiotics or antibiotics and inhibitors/STPK promoters, on a lawn of *Streptomyces* strains on the agarized medium.

The spore suspension obtained by washing-off streptomycete cells from the bacterial lawn formed on complete agarized medium was filtered through cotton-wool filters and mixed with agarized MG medium (0.7%) with or without Ca^{2+} (10 mM). Petri dishes containing the agarized MG medium (2%) were inoculated with the mixture aliquots (1×10^7 spores per dish). Paper disks containing antibiotics or the tested agents were then placed on the agar surface. The cultures were incubated at 28°C for 24 h. The antibiotics or the tested agents were added in subinhibitory concentrations; however, their contents were usually less than 50% of the minimum inhibitory concentration.

Modifiers of the activities of STPK and calcium-binding proteins. We used the following commercial preparations manufactured by Sigma and Calbiochem: *bis*-indolyl-maleimide-1 (100 nmol/disk), *bis*-indolyl-maleimide-5 (100 nmol/disk), verapamil (100 nmol/disk), prenylamine (100 nmol/disk), chlorpromazine (100 nmol/disk), dibutyryl-cAMP (1 μmol/disk), forskolin (1 μmol/disks), and dichlorobenzimidazole riboside (1 μmol/disk).

Antibiotics. In our experiments, we used paper disks containing the following antibiotics: streptomycin (10 μg/disk), tobramycin (10 μg/disk), gentamycin (10 μg/disks), kanamycin (30 μg/disk), amikacin (30 μg/disk), erythromycin (15 μg/disk), oleandomycin (15 μg/disk), clindamycin (2 μg/disk), azithromycin (15 μg/disk), lincomycin (15 μg/disk), tetracycline (30 μg/disk), chloramphenicol (30 μg/disk), rifampicin (5 μg/disk), and oligomycin (5 μg/disk).

Databases. The obtained results were summarized using the *S. coelicolor* genome database (http://www.sanger.ac.uk/Projects/S_coelicolor/).

Obtaining of cell extracts. The washed actinobacterial mycelium was resuspended in the buffer A (50 mM Tris–HCl, 125 mM NaCl, 1 mM $CaCl_2$, 5 mM $MgCl_2$, 10% glycerol, 1 mM of phenilmethylsulfonyl fluoride (PMSF), 0.5 mM DTT, 150 mM β-glycerophosphate, 100 mM NaF, 1 μg/ml of leupeptin, and 1 μg/ml of pepstatin; pH 7.8). The cells were then treated twice with UZDN-1 and U-42 sonicators (Russia) at 25 kHz for 5 min. The obtained suspensions were incubated in the presence of pancreatic RNase (25 μg/ml) and DNase (25 μg/ml) at 4°C for 15 min; the cell debris was removed by centrifugation at 20000 *g* for 30 min. The supernatants were used to isolate STPK.

The *E. coli* cells suspended in the buffer (pH 7.8) containing 20 mM Tris–HCl, 1 mM EDTA, and 1 mM PMSF were sonicated twice in an ultrasonic cell disruptor at 15 kHz for 1 min. The insoluble residue was removed by centrifugation at 20000 *g* for 20 min. Analysis of the soluble proteins contained in the supernatant was carried out by SDS PAGE according to Laemmli [15]. The presence of an additional polypeptide with a molecular weight of 31.5 kDa (His_{10}–APHVIII) in the cloned cells indicated the *aphVIII* gene expression. Clones with a high content of soluble APHVIII were used for the isolation of this enzyme in its native form. In this case, *E. coli* cells were resuspended in buffer B (50 mM Na phosphate, 300 mM NaCl, 1 mM PMSF, 10% glycerol; pH 8.0) and sonicated twice, as described above. The obtained lysate was incubated in

the presence of nucleases; the cell debris was removed by centrifugation. Then, His_{10}–APHVIII was isolated from the supernatant.

Isolation of protein kinases. For affinity isolation of protein kinases, proteins of the cell extracts in buffer C (20 mM of triethanolamine–HCl, 200 mM NaCl, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 3 mM DTT, 0.5 mM PMSF, 10% glycerol; pH 7.8) were adsorbed on blue Sepharose CL-6B (Pharmacia Biotech). The resin was washed with buffer C. This buffer, which was additionally supplemented with 15M NaCl and 20 mM ATP, was also used to elute the adsorbed proteins. The activity of APHVIII modifying protein kinases in the eluate fractions was analyzed at a final concentration of labeled ATP of 0.5 mM (0.005 Bq/pmol); APHVIII (20 μg/ml) was used as a substrate. The fractions containing APHVIII STPK were mixed and purified from salt and ATP using Sephadex C-20.

Purification of APHVIII in the native form. The fraction of salt-soluble *E. coli* proteins in buffer B was layered over a 0.5-ml column with Ni-NTA agarose (Qiagen); the resin was intensively washed with the same buffer at pH 6.0. The adsorbed protein was eluted by passing a gradient of the same buffer (10 ml, pH 6.0–4.3) through the column. The eluate was analyzed by SDS-PAGE; protein fractions with a molecular mass of 31.5 kDa were mixed together and chromatographed using a 10-ml column packed with Sephadex G-75 and calibrated using the standard set of protein markers for gel filtration chromatography (Pharmacia, United States). The peak value of the native APHVIII distribution in the eluate was observed at a molecular weight of 34 kDa.

Analysis of protein phosphorylation in vitro. Phosphorylation of His_{10}–APHVIII with partly purified protein kinases was carried out at 28°C for 10 min in the reaction mixture in the presence of 2 μg of proteins from the kinase preparation, 40 μg His_{10}–APHVIII, and 0.25 mM [γ-^{32}P]ATP (0.15 Bq/pmol) (Fosfor, Russia) in 50 μl of the buffer (pH 7.8) containing the following: Tris–HCl, 50 mM; NaCl, 125 mM; $MgCl_2$, 5 mM; glycerol, 10%; DTT, 0.5 mM; and $CaC1_2$, 1 mM. The reaction was started by addition of protein and terminated by addition of 17 μl of electrophoresis buffer [15] (with concentrations of all its components increased fourfold) followed by 5-min heating at 95°C. The maximum level of His_{10}–APHVIII phosphorylation was determined by decreasing its concentration in the mixture to 0.5 μg and increasing the incubation time threefold. Then, the specimens were prepared for SDS-PAGE in 12.5% gels, as described above, or APHVIII labeling analysis was carried out. For this purpose, His_{10}–APHVIII was isolated on Ni-NTA agarose; the content of the labeled protein was then determined.

Phosphoamino acid analysis. Analysis of phosphoamino acids produced by the APHVIII phosphorylation was performed in HCl hydrolysates of labeled polypeptides after the samples were electrophoretically separated on cellulose plates for thin-layer chromatography on plastic support (Merck, Germany), as described earlier [16]. Labeled amino acids were identified by their mobility relative to the reference unlabeled phosphoserine, phosphothreonine, and phosphotyrosine visualized by ninhydrin staining.

Analysis of protein kinases in the gel. STPK renaturation in the gel after SDS-PAGE was performed according to Kameshita and Fujisawa [17]. After renaturation, the gels containing electrophoresed kinases were incubated in the presence of 50 μCi/ml [γ-^{32}P]ATP (Fosfor, Russia) in 10 ml of the buffer for in vitro protein phosphorylation. After removal of the unreacted label, the gels were dried and autoradiographed. The kinases separated in substrate-containing gels were treated in a similar way. In this case, before polymerization, the solution was supplemented with 1 mg/ml of the APHVIII protein.

Cloning of PK25 protein kinase. The homologue of the protein kinase PK25 gene was isolated from *S. lividans* and cloned. For cloning, the following oligonucleotides have been isolated: 5'-ATCCGAATTCCGTTACCGGCTCCATGAGCGGC-3' (homologous to the N-terminal region of the catalytic PK25 domain and containing a *Eco*RI restriction site) and 5'-CCGCAAGCTTCATCCGCTGGGCCGACGCCG-3' (homologous to the C-terminal region of the catalytic domain and containing a *Hin*dIII restrictase site). After digestion and purification by agarose gel electrophoresis, the PK25 encoding PCR fragments amplified with these primers were ligated into the pET32a expression cloning vector (Novagen). Strain *E. coli* DH5a was then transformed with the obtained products. Screening of recombinant clones was performed using the standard T7prom and T7term primers. Plasmid DNAs were isolated from the selected transformants and subjected to restriction analysis to reveal the insertion. Then, DNA sequencing was performed.

RESULTS

Effect of Ca^{2+} Ions on the Antibiotic Resistance of S. coelicolor A3(2) and S. lividans 66

In a series of experiments, the resistance of growing cultures of *S. coelicolor* A3(2) and *S. lividans* 66 to various antibiotics was investigated. Analysis was carried out in the presence or absence of Ca^{2+} ions in the media (Table 1). The results obtained indicate that, using the response of actinomycetes to addition of Ca^{2+} as a criterion, the antibiotics under study may be divided into two groups. The first group includes aminoglycosides, macrolides, tetracyclines, rifampicin, and oligomycin; the resistance of actinomycetes to these antibiotics increased in the presence of Ca^{2+} ions. The second group includes lincomycin, clindamycin, and chloramphenicol; the resistance of the cultures to these antibiotics was not affected by addition of Ca^{2+} ions into the medium. On the whole, the results obtained indicate

Table 1. Modulation of antibiotic resistance in *S. coelicolor* and *S. lividans* by Ca^{2+} ions

Antibiotic	Concentration, μg/disk	*S. coelicolor* growth inhibition zone, diameter, mm*		*S. lividans* growth inhibition zone, diameter, mm*	
		without Ca^{2+}	with Ca^{2+}	without Ca^{2+}	with Ca^{2+}
Streptomycin	10	24 ± 1	18 ± 1	21 ± 1	8 ± 1
Tobramycin	10	22 ± 1	20 ± 1	20 ± 1	17 ± 1
Gentamycin	10	23 ± 1	21 ± 1	21 ± 1	18 ± 1
Kanamycin	30	29 ± 1	28 ± 1	25 ± 1	21 ± 1
Erythromycin	15	22 ± 1	18 ± 1	16 ± 1	14 ± 1
Oleandomycin	15	15 ± 1	12 ± 1	17 ± 1	11 ± 1
Clindamycin	2	8 ± 1	8 ± 1	8 ± 1	8 ± 1
Azithromycin	15	12 ± 1	9 ± 1	12 ± 1	10 ± 1
Lincomycin	15	9 ± 1	9 ± 1	9 ± 1	9 ± 1
Tetracycline	30	16 ± 1	11 ± 1	14 ± 1	11 ± 1
Chloramphenicol	30	11 ± 1	11 ± 1	11 ± 1	11 ± 1
Rifampicin	5	15 ± 1	12 ± 1	14 ± 1	13 ± 1
Oligomycin	5	18 ± 1	14 ± 1	19 ± 1	14 ± 1

* The presented results are averages of three independent measurements.

that Ca^{2+} effectively modulates the resistance of the studied actinomycetes to antibiotics of various chemical structure. The sensitivity of both strains to aminoglycosides in the presence of Ca^{2+} decreased, while the resistance of *S. lividans* 66 to aminoglycoside antibiotics was modulated by Ca^{2+} ions to a greater degree than that of *S. coelicolor* A3(2).

The Effect of the Protein Kinase Inhibitor Bis-Indolyl-maleimide 1 on Antibiotic Resistance of S. coelicolor A3(2) and S. livida 66

We have previously demonstrated that, in the case of *S. rimosus*, its kanamycin resistance increased due to phosphorylation of APHVIII by two endogenous STPK [18], one of which was Ca^{2+}-dependent. In this work, we analyzed the relationships between protein phosphorylation by STPK and the phenomenon of multiple antibiotics resistance. A highly selective inhibitor of Ca^{2+}/phospholipid-dependent protein kinase C (PKC), *bis*-indolyl-maleimide 1, was used as an analysis tool [19]. Its mechanism of action is based on the competitive (with respect to ATP) interaction with the ATP-binding PKC site. Its inactive analogue, *bis*-indolyl-maleimide 5, which is structurally similar to other indolyl maleimides, was used as a negative control. Analysis was carried out in the presence or absence of Ca^{2+} by application of paper disks containing both an antibiotic (at subinhibitory concentrations) and the PKC inhibitor (Table 2). The results indicated that *bis*-indolyl-maleimide 1 reliably decreased the resistance of strain *S. lividans* 66 to streptomycin, gentamycin, kanamycin, amikacin, tobramycin, and oligomycin; however, the strain resistance to macrolide antibiotics, clindamycin, and lincomycin did not change. In the presence of Ca^{2+}, its effect on the strain resistance to streptomycin, tobramycin, and gentamycin was more pronounced than in the absence of Ca^{2+}. In the case of kanamycin, Ca^{2+} reduced the effect of *bis*-indolyl-maleimide 1. The negative analogue, *bis*-indolyl-maleimide 5, did not affect strain *S. lividans* 66 resistance to antibiotics irrespective of the presence of Ca^{2+} ions. It has been suggested [18] that an increase in the strain sensitivity to antibiotics in the presence of an STPK inhibitor can be attributed to inactivation of specific kinases required to activate the proteins responsible for antibiotic resistance. Taking into account the high selectivity of the effect that *bis*-indolyl-maleimide 1 exerts on PKC-like enzymes, one can assume that these enzymes are responsible for the regulation of *S. lividans* 66 resistance to a wide spectrum of antibiotics.

The Effect of Inhibitors of Serine/threonine Protein Kinases on the Resistance to Kanamycin of Strain S. lividans aphVIII

In our subsequent experiments we studied the effect of bacterial signal transduction system components on the activity of the components of the enzymatic system for antibiotic inactivation. An *S. lividans* mutant which contained an *S. rimosus* gene (which enhances the cell resistance to aminoglycoside antibiotics kanamycin and neomycin) on the multicopy plasmid pSU23 *aphVIII* was used as a test object [7].

The effect of the known modulators of specific STPK, as well as the effect of compounds that modulate intracellular concentrations of calcium ion or ATP, on *S. lividans aphVIII* sensitivity to kanamycin was studied (Table 3).

Table 2. Effect of *bis*-indolyl-maleimide 1 on the antibiotic resistance of *S. lividans*

Antibiotic	*S. lividans* growth inhibition zone, diameter, mm*			
	without Ca^{2+}		with Ca^{2+}	
	Antibiotic	Antibiotic + *bis*-1	Antibiotic	Antibiotic + *bis*-1
Streptomycin	21 ± 1	23 ± 1	8 ± 1	12 ± 1
Tobramycin	20 ± 1	22 ± 1	17 ± 1	20 ± 1
Gentamycin	21 ± 1	23 ± 1	18 ± 1	24 ± 1
Kanamycin	25 ± 1	29 ± 1	21 ± 1	24 ± 1
Amikacin	28 ± 1	30 ± 1	26 ± 1	28 ± 1
Erythromycin	16 ± 1	18 ± 1	14 ± 1	15 ± 1
Oleandomycin	17 ± 1	18 ± 1	11 ± 1	12 ± 1
Clindamycin	8 ± 1	9 ± 1	8 ± 1	8 ± 1
Azithromycin	12 ± 1	13 ± 1	10 ± 1	10 ± 1
Lincomycin	9 ± 1	9 ± 1	9 ± 1	9 ± 1
Tetracycline	14 ± 1	16 ± 1	11 ± 1	12 ± 1
Chloramphenicol	11 ± 1	13 ± 1	11 ± 1	12 ± 1
Rifampicin	14 ± 1	15 ± 1	13 ± 1	17 ± 1
Oligomycin	19 ± 1	22 ± 1	14 ± 1	17 ± 1

* The presented results are averages of three independent measurements.
** The amount of *bis*-indolyl-maleimide 1 was 100 nmol/disk so that the growth inhibition zone of *S. lividans* was not evident.

Table 3. Effect of STPK modulators on kanamycin resistance of *S. lividans APHVIII*

Inhibitor	Mechanism of action	Amount, nmol/disk	Growth inhibition zone, diameter, mm*	
			Km, 30 μg/disk	Km, 30 μg/disk + inhibitor
Bis-indolyl-maleimide 1	ATP-competitive STPK inhibitor	100	7.5 ± 0.5	15 ± 0.5
Verapamil	Ca^{2+} channel blocker	100	7.5 ± 0.5	11 ± 0.5
Prenylamine	Calmodulin inhibitor	100	7.5 ± 0.5	12 ± 0.5
Chlorpromazine	Calmodulin inhibitor	100	7.5 ± 0.5	11 ± 0.5
Dibutyryl-cAMP	cAMP activator	1000	7.5 ± 0.5	8 ± 0.5
Forskolin	Adenylate cyclase activator	1000	7.5 ± 0.5	9 ± 0.5
DBIR	Inhibitor of casein kinase II	1000	7.5 ± 0.5	7 ± 0.5
Oligomycin	Inhibitor of F_0F_1 ATP synthase	1	7.5 ± 0.5	12 ± 0.5

* The presented results are an average of three independent measurements. Km stands for kanamycin.

The experiment carried out in the presence of the subinhibitory concentrations of the tested compounds (100 nmol/disk) revealed that the PKC inhibitor *bis*-indolyl-maleimide 1 had the most intense effect on decreasing *S. lividans aphVIII* resistance to kanamycin. The calmodulin inhibitors prenylamine lactate and chlorpromazine, the Ca^{2+} channel blocker verapamil, and the inhibitor of Ca^{2+}-dependent F_0F_1 ATP synthase, oligomycin, have reliably decreased kanamycin resistance of bacterial cells. At the same time, the adenylate cyclase activator forskolin, the casein type II STPK inhibitor 5,6-dichlorobenzyl imidazole riboside (DBIR), as well as the activator of cAMP-dependent STPK dibutyryl-cAMP (which do not depend on Ca^{2+}), added in the concentrations of 1 μmol/disk did not exert a significant effect on the cell sensitivity to kanamycin (Table 3). A decrease in the *S. lividans aphVIII* resistance to kanamycin in the presence of Ca^{2+}-dependent protein phosphorylation inhibitors, as well as of the inhibitors of ATP synthesis, indicates the possible involvement of Ca^{2+}-dependent STPK in the post-translation regulation of the kanamycin-

Table 4. Ca^{2+}-dependent phosphorylation of APHVIII STPK of *S. coelicolor* A3(2)

Specimen examined	Addition of Ca^{2+} to the incubation mixture	Phosphorylation of APHVIII by kinases, %
PK from the culture grown in the presence of Ca^{2+}	+	100*
PK from the culture grown in the presence of Ca^{2+}	–	39.7 ± 3
PK from the culture grown without Ca^{2+}	+	49.3 ± 4
PK from the culture grown without Ca^{2+}	–	21.1 ± 3

* 100% activity is defined as 1.02 pmol P_i transferred from ATP to 1 μg APHVIII min^{-1} under standard conditions. The presented results are averages of three independent measurements.

inactivating enzyme APHVIII. The following experiments were carried out to validate this hypothesis.

Ca^{2+}-*Dependent in vitro Phosphorylation of S. coelicolor A3(2) Proteins and the APHVIII Enzyme*

Preliminary experiments have demonstrated that the efficiency of the endogenous proteins and APHVIII phosphorylation by kinases in the extract and the total preparation of *S. lividans* 66 kinases was insignificant (the rate of the APHVIII phosphorylation was less than 0.05 pmol P_i transferred from ATP to 1 μg APHVIII min^{-1}). This is due to high interference activity of ATPase in these bacterial extracts, which results in rapid ATP hydrolysis. Subsequent experiments were therefore carried out using *S. coelicolor* A3(2) protein kinase preparations. It was also taken into account that 34 STPK of this actinobacterium had been found by chromosome sequencing, which facilitated successful identification of kinases capable of APHVIII phosphorylation.

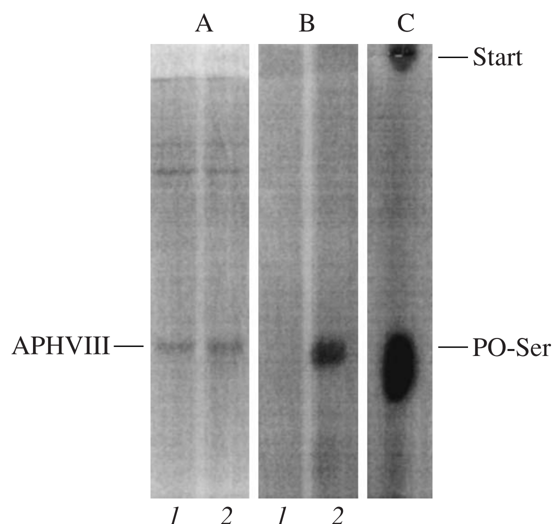

Fig. 1. Coomassie staining (A) and autoradiography (B) of the electrophoretogram of the APHVIII protein phosphorylated in vitro by *S. coelicolor* protein kinases in the absence of Ca^{2+} (*1*) and in the presence of Ca^{2+} (*2*), and autoradiography of muriatic hydrolysate [^{32}P] APHVIII (C). The arrows in the electrophoretograms point to the locations of His_{10}–APHVIII and phosphoserine.

We used a total preparation of *S. coelicolor* A3(2) protein kinases isolated from stationary phase cultures grown in the presence of Ca^{2+} after its incubation with labeled ATP. A total of nine labeled phosphoproteins with molecular masses of 95, 81, 65, 49, 41, 37, 35, and 32 kDa, as well as the 27/25-kDa doublet were detected. The maximum level of the 81-, 65-, 41-, 37-, and 32-kDa protein labeling was observed upon addition of calcium to the working mixture for analysis. The capacity of the above proteins for autophosphorylation was evaluated. The *S. coelicolor* A3(2) kinases separated in polyacrylamide gel were renatured; their autophosphorylation in the gel in the presence of labeled ATP was examined. Phosphorylation of 81-, 65-, 49-, 41-, 37-, and 32-kDa proteins was demonstrated. Phosphorylation of 81-, 41-, and 32- kDa proteins was stimulated by Ca^{2+}. It is obvious that autophosphorylation of the proteins separated in polyacrylamide gel occurs if these proteins have an active catalytic kinase domain. The more restricted set of proteins phosphorylated in the gel (as compared to their number in the cell extract) may be due to the fact that only some kinases were renatured after electrophoretic separation, as well as to the fact that some phosphoproteins in the extract were STPK substrates, rather than STPK.

Detection of active Ca^{2+}-dependent STPK in *S. coelicolor* A3(2) allowed us to hypothesize that some of them are able to phosphorylate APHVIII, affecting its activity. To evaluate the level of in vitro APHVIII phosphorylation, the recombinant His_{10}–APHVIII (277 residues, M_r 30625) was produced in *E. coli.* The cells were cultivated in the presence or absence of Ca^{2+}; the protein was then extracted and purified by chromatography on His-binding Ni-NTA resin. The purified His_{10}–APHVIII was incubated in the presence or absence of Ca^{2+} with [γ-^{32}ATP] and the total preparation of *S. coelicolor* A3(2) protein kinases. Kinases and ATP were removed from the phosphorylated protein on His-binding resin. Radioactive labeling was assessed (Table 4), and the protein was then isolated by SDS-PAGE with subsequent staining by autoradiography (Fig. 1). In all cases, one major 31.5-kDa phosphoprotein was detected (Fig. 1). The labeling of the protein under study was quite efficient in the presence of Ca^{2+} in the working mixture which contained protein kinases isolated from the cultures grown in the presence of cal-

cium (Figs. 1A and 1B, Table 4). Growth on the medium without Ca^{2+} resulted in a halving of the activity of the APHVIII-phosphorylating STPK in *S. coelicolor* A3(2) cells. The removal of Ca^{2+} from the working mixture for assessing of the STPK activity resulted in a 2.5-fold decrease in their APHVIII phosphorylating activity (Table 4).

Thus, it was established that endogenous STPK of *S. coelicolor* A3(2) phosphorylate His_{10}–APHVIII and that this phosphorylation is induced by calcium ions. Incubation of His_{10}–APHVIII in the presence of 0.1–0.5 mM [γ-^{32}P]ATP demonstrated that its labeling level peaked at an ATP concentration of 0.25 mM and then remained almost unchanged. The results of phosphoamino acid analysis of the highly phosphorylated [^{32}P]His_{10}–APHVIII indicated that its hydrolysate contained a labeled amino acid whose electrophoretic mobility was the same as in the case of the reference phosphoserine (Fig. 1C). Thus, the studied polypeptide was modified on serine residues, which proves that STPK are involved in this protein phosphorylation in *S. coelicolor* A3(2).

To identify these STPK, their total preparation was separated in the gel that contained His_{10}–APHVIII polymerized into it beforehand; the kinases were then renatured, and, after the incubation with labeled ATP, phosphorylation of His_{10}–APHVIII by these kinases was assessed. To lower the level of labeling of the kinases, their preparations were preincubated at 7°C for 12 h in the presence of 20 mM of “cold” ATP. The results obtained indicated that, after re-electrophoresis, a labeled 31.5-kDa component corresponding to His_{10}–APHVIII was detected only in the 41-kDa kinase domain (Fig. 2). Hence, it was demonstrated that the 41-kDa STPK is able to phosphorylate APHVIII enzyme. According to the results of the bioinformation analysis of *S. coelicolor* A3(2) genome, the 41-kDa kinase is similar in molecular mass to the Ca^{2+}-dependent protein kinase encoded by the *SCO4778* gene (42 kDa) and can be identified as the protein kinase PK25 of *S. coelicolor* A3(2).

Then, using oligonucleotides complementary to the N- and C-terminal sequences of the *SCO 4778* gene product and their restriction profiles, we isolated the fragment of the *S. lividans* 66 chromosomal DNA contained between them, sequenced it, and found that this fragment contained a gene that encodes an STPK with an amino acid sequence identical to that of *S. coelicolor* A3(2) protein kinase PK25. We believe that this kinase is responsible for modification of APHVIII protein in *S. lividans* cells.

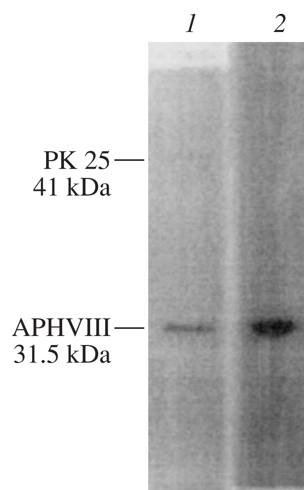

Fig. 2. Phosphorylation of APHVIII by protein kinase PK25: *1*, Coomassie staining; *2*, autoradiogram. Protein kinases were separated in the gel that contained APHVIII which was polymerized into it beforehand. Then, STPK were renatured, and the gels were incubated in the presence of [γ-^{32}P]ATP. The labeled component of the gel was extracted and re-electrophoresed.

DISCUSSION

Ca^{2+}-dependent protein phosphorylation is a common trait of actinobacteria belonging to the genus*Streptomyces* (Table 5). Some of these proteins are represented by Ca^{2+}-dependent serine/threonine protein kinases (Table 5). In this work, we demonstrated that Ca^{2+}-dependent protein kinase PK25 of *S. coelicolor* is able to phosphorylate APHVIII enzyme in vitro. The results obtained, as well as the results of our previous investigation [18] suggest that Ca^{2+}-modulated antibiotic resistance of *S. lividans* 66 and *S. coelicolor* A3(2)

Table 5. Ca^{2+}-dependent phosphorylation of *Streptomyces* proteins

Streptomyces species	In vitro phosphorylated proteins (Pp), kDa	In vitro autophosphorylated kinases, kDa	Source
S. lividans	Pp35, Pp42, Pp55, Pp61, Pp66, Pp74, Ppl00, Pp205		[21]
S. avermitilis	Pp24, Pp37.5, Pp41, Pp72, Pp85	Pp85	[22]
S. rimosus	Pp31, Pp55, Pp74	Pp55, Pp74	[18]
S. fradiae	Ppl8, Pp25, Pp31.5, Pp58, Pp62, Pp65, Pp88, Ppl27	Pp65, Ppl27	[16]
S. coelicolor	Pp32, Pp37, Pp41, Pp65, Pp81	Pp32, Pp41(pk25), Pp81	Present work, [23]

can also be due to phosphorylation of certain enzymes. The enhanced aminoglycoside resistance of *S. lividans*, dependent on Ca^{2+}, as well as its suppression by the inhibitors of specific Ca^{2+}-dependent STPK, confirm the above suggestion. Thus, utilization of the inhibitors of specific STPK may enable manipulation of the resistome activity and increase (in the case of producer strains) or decrease (in the case of clinical strains) microbial antibiotic resistance.

The results of the *S. coelicolor* A3(2) genome analysis using the known databases (http://www.sanger.ac.uk/) allowed detection of several dozen DNA sequences which may represent the genes responsible for bacterial cell resistance to various known antibiotics. It is noteworthy that DNA sequences (indirectly controlled by Ca^{2+} [20]), similar to the genes encoding human multiple drug resistance, *MDR1* and *MDR2*, were detected in bacterial genomes.

Many genes remain phenotypically silent under various growth conditions. For instance, in the *S. coelicolor* A3(2) genome, six genes encoding proteins involved in the enzymatic inactivation of aminoglycoside antibiotics were detected. *S. coelicolor* A3(2) cells were found to be resistant to aminoglycosides and macrolides at concentrations of 0.5 μg/ml and 10–50 μg/ml, respectively [6].

In this work, we demonstrated that Ca^{2+} ions (at a concentration of 10 mM) enhance the resistance of *S. lividans* 66 and *S. coelicolor* A3(2) to aminoglycosides, macrolides, tetracyclines, rifampicin, and oligomycin. Calmodulin inhibitors (and, therefore, Ca^{2+}/calmodulin-dependent enzymes, including respective STPK) and the ATP-competitive STPK inhibitor *bis*-indolyl-maleimide 1 decrease the *aphVIII*-encoded resistance to the aminoglycoside antibiotic kanamycin. We have established that Ca^{2+}-dependent protein kinase PK25 of *S. coelicolor* is capable of in vitro phosphorylation of the APHVIII enzyme. Along these lines, one can assume that the antibiotic resistance of *S. lividans* 66 and *S. coelicolor* A3(2), regulated by Ca^{2+} ions, is also controlled by the protein kinase of the signal transduction cascade and results from the phosphorylation of antibiotic resistance enzymes.

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