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EXPERIMENTAL ARTICLES

# Dynamics of Serine/Threonine Protein Kinase Activity during the Growth of the Wild-Type *Streptomyces avermitilis* Strain and Its Chloramphenicol-Resistant Mutant

**S. M. Elizarov*, V. A. Mironov**, and V. N. Danilenko****

**Bach Institute of Biochemistry, Russian Academy of Sciences, Leninskii pr. 33, Moscow, 117071 Russia*

***All-Russia Research Institute of Biosynthesis of Protein Substances, Moscow, Russia*

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**Abstract**—The dynamics of serine/threonine protein kinase activity during the growth of the wild-type *Streptomyces avermitilis* strain 964 and its chloramphenicol-resistant ($Cml^r$) pleiotropic mutant with an enhanced production of avermectins was studied by measuring the transfer of radiolabeled phosphate from [$\gamma$-$^{32}$P]ATP to the serine and threonine residues of proteins in cell-free extracts. In both of the strains studied, radiolabeled phosphate was found to incorporate into polypeptides with molecular masses of 32, 35, 41, 68, 75, 79, 83, and 137 kDa; however, the degree and the dynamics of phosphorylation of particular peptides were different in these strains. The differences revealed could not be accounted for by the interference of ATPases or phosphoprotein phosphatases. The data obtained may be interpreted as evidence that $Cml^r$ mutation activates the protein kinase signalling system of *S. avermitilis* cells in the early stationary growth phase and thus enhances the production of avermectins and leads to some other physiological changes in the mutant strain.

*Key words*: phosphorylation of proteins, serine/threonine protein kinase, streptomycetes, chloramphenicol resistance, avermectin

Actinomycetes of the genus *Streptomyces* are gram-positive soil filamentous bacteria with a relatively complex morphological differentiation characterized by the formation of the spore-bearing substrate and aerial mycelia [1]. The most profound metabolic alteration associated with the morphological differentiation of streptomycetes is an enhanced biosynthesis of secondary metabolites, including antibiotics [1]. An important role in the regulation of morphological differentiation and synthesis of secondary metabolites in *Streptomyces* is played by various signal-transducing systems, such as the phosphorylation of proteins by protein kinases [2], tyrosine and serine/threonine protein kinases in particular [3]. Urabe and Ogawara have succeeded in the cloning, sequencing, and expression of two protein kinase–encoding genes from *S. coelicolor* A3(2) [4]. There is evidence that Ser/Thr protein kinases are also present in other *Streptomyces* strains [5–8], including *S. avermitilis* [9, 10]. At the same time, little is known about the relationship between the genes, their mutations, and the respective changes in the phosphorylation of particular proteins, as well as about the physiological and morphological changes in *Streptomyces* cells associated with the phosphorylation of the serine and threonine residues of proteins. In view of this, it would be of interest to perform a comparative investigation of protein kinases in mutant actinomycetes with alterations in morphological differentiation and the regulation of the biosynthesis of antibiotics.

Earlier, we reported the detection of Ser/Thr protein kinase activity in cell-free extracts of *S. avermitilis* 964 and its $Cml^r$ mutant with an enhanced production of the macrolide antibiotic avermectin and characterized the multiple endogenous target proteins of this kinase phosphorylated at their Ser and Thr residues [10].

In the present work, we performed a detailed comparative analysis of the intensity of phosphorylation of these target proteins by endogenous Ser/Thr protein kinase in cell-free extracts of *S. avermitilis* 964 and its $Cml^r$ mutant in the course of their growth from the early exponential to the stationary phase.

## MATERIALS AND METHODS

**Strains.** The two strains used in this work, *S. avermitilis* 964 (ATCC 31272) and its $Cml^r$ mutant 12, were obtained from the All-Russia Collection of Industrial Microorganisms (VKIM) of the State Scientific Research Institute of Genetics and Selection of Industrial Microorganisms. Mutant 12, derived from the wild-type strain 964, was resistant to 20 μg/ml chloramphenicol and produced avermectin in amounts of up to 2 mg/ml, as compared with 25 μg/ml produced by the wild-type strain [10, 11].

**Cultivation conditions.** Strains were maintained at 4–6°C on a solid medium containing (%) Difco malt extract, 1; Difco yeast extract, 0.4; $CaCl_2$, 0.1; and agar, 2.

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Material for inoculation was prepared as follows. The suspension of spores that was washed off of agar medium with distilled water was placed in 750-ml flasks with 50 ml of a medium containing (%) glucose, 2; soybean meal, 1.5; yeast extract, 0.5; and NaCl, 0.3 (pH 7). The culture for inoculation was grown on a shaker (240 rpm) at 28°C for 42–44 h and introduced, in an amount of 2.5 ml, into 750-ml flasks with 50 ml of a medium containing (%) glucose, 4; yeast extract, 0.8; and NaCl, 0.3 (pH 7). Cultures were grown on a shaker (240 rpm) at 28°C with regular sampling for the preparation of cell-free extracts as described below.

**Preparation of cell-free extracts.** Culture samples were filtered, and residue cells were washed with 1.5% Tris buffer (pH 7.3) containing 1% NaCl and then thrice with 50 ml of standard buffer of the following composition: 10 mM triethanolamine, 10 mM KCl, 125 mM NaCl, 5 mM $MgCl_2$, 6 mM 2-mercaptoethanol, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 10% glycerol (pH 7.8). The biomass was frozen in acetone cooled by dry ice and ground with two weight portions of alumina powder in a porcelain mortar to a pastelike consistency. The paste was suspended in an equal volume of standard buffer supplemented with DNase and RNase at a concentration of 20–25 μg/ml each, and alumina powder was removed by low-speed centrifugation. The supernatant was cleared by centrifugation at 15000 *g* for 30 min in a Spinco L5/50 centrifuge (Beckman, the United States) and immediately used for the protein kinase assay.

**Ser/Thr protein kinase activity** was assayed at 28°C in standard buffer supplemented with 0.1 M NaF, 0.15 M β-glycerophosphate, 1 μg/ml leupeptin and pepstatin, and 0.25 mM [$\gamma$-$^{32}$P]ATP (5000 cpm/pmol). The reaction mixture volume was 50–100 μl. The reaction was started by adding 15 μg of cell-free extract proteins and allowed to proceed for 15 min. Then the reaction was stopped by adding 17–33 μl of 4-fold-strength electrophoresis buffer [12], and the mixture was heated at 95°C for 10–15 min. After cooling, the mixture was dialyzed against single-strength electrophoresis buffer and subjected to electrophoresis.

**Electrophoresis** was carried out in 10% polyacrylamide gel slabs in the presence of sodium dodecyl sulfate [12] by applying 20–30 μg of proteins into sample wells. Rabbit muscle myosin (205 kDa), *Escherichia coli* β-galactosidase (116 kDa), phosphorylase *b* (97.4 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (36 kDa), bovine erythrocyte carbonic anhydrase (29 kDa), and bovine milk α–lactalbumin (14 kDa) were used as molecular weight markers. Protein bands were visualized by placing developed gel slabs in a solution of Coomassie R-250 in 50% trichloroacetic acid for 30 min and then destaining them in 7% acetic acid. After drying, gel slabs were autographed by exposing them with Kodak X-OMAT AR film (Germany) for 5 days. The radioactivity of the excised gel pieces with protein bands was determined by the Cherenkov method.

**Analytical procedures.** The concentration of proteins was determined by the Shaffner–Weissmann method [13]. The ATPase activity of extracts was assayed as described previously [14]. The dry weight of the biomass was determined by weighing the mycelium dried to a constant weight at 105°C on filter paper. The glucose concentration was measured by the Bertrand method. The concentration of avermectin was determined by the densitometry of thin-layer chromatograms [15].

**Reagents.** ATP was purchased from Merck (Germany), pronase was from Calbiochem (the United States), PMSF, EDTA, RNase, and sodium dodecyl sulfate from Serva (Germany), mercaptoethanol and Coomassie Brilliant Blue R-250 from Loba-Chemie (Austria), DNase from Worthington (the United States), Tris and β-glycerophosphate from Fluka (Germany), leupeptin and pepstatin from Sigma (the United States), and [$\gamma$-$^{32}$P]ATP was obtained from Obninsk (Russia).

## RESULTS AND DISCUSSION

*S. avermitilis* produces eight macrolide compounds, known as avermectins and possessing antihelmintic and insecticide activities [16]. The mutant strain *S. avermitilis* 12 was derived from the parent strain ATCC 31272 (VKIM 964) through a one-step selection of chloramphenicol resistance mutation [11]. Cml$^r$ mutation led to a 40-fold increase in avermectin production and a decrease in the level of the morphological variability of strain 12. As shown in [17], five minor morphotypes of colonies of strain 964 amounted from 6 to 31% of the total number of colonies of this strain grown on agar media, whereas such morphotypes amounted to less than 10% in the case of the mutant strain 12 [17]. Therefore, Cml$^r$ mutation has a pleiotropic character and causes global changes in cell differentiation and the regulation of secondary metabolism, avermectin production in particular. It is likely that chloramphenicol resistance mutation triggers some mechanisms in the global regulation system of *S. avermitilis*.

The functional role of the antibiotic resistance genes of *Streptomyces* is yet unknown [11]. To gain some insight into this problem, we investigated biochemical mechanisms that may be responsible for the enhanced production of avermectins and the reduced morphological variability of the Cml$^r$ mutant of *S. avermitilis*. In the previous work [10], we revealed protein kinase activity in cell-free extracts of *S. avermitilis* strains and optimized a scheme for the in vitro assay of Ser/Thr protein kinases. In the present work, this scheme was applied to comparatively study proteins phosphorylated by endogenous protein kinase(s) in cell-free extracts of the wild-type and mutant strains of *S. avermitilis*. The design of the experiment was as follows:

cell-free extract proteins were incubated in the assay mixture for protein kinases and then analyzed by electrophoresis and autoradiography, as well as by measuring the radioactivity of excised gel pieces with separated protein bands.

The results of the electrophoretic analysis of proteins in cell-free extracts of wild-type and mutant cells grown for 12 to 64 h are shown in Fig. 1. It can be seen that some polypeptides (up to eight in number) were phosphorylated by endogenous protein kinases in the course of incubation of cell-free extracts in the assay mixture. The molecular masses of phosphorylated polypeptides were estimated to be 137, 83, 79, 75, 68, 41, 35, and 32 kDa. The degree of phosphorylation of the 79-kDa polypeptide was ten times higher and that of the 35- and 32-kDa polypeptides and four times higher than of other polypeptides. In some samples with a high level of phosphorylation, two minor phosphorylated polypeptides with molecular masses of 90 and 62 kDa were detected. The intensity of phosphorylation as a whole and that of particular proteins varied as the strains grew.

In strain 964, the total intensity of phosphorylation was maximum by the 14th to 15th h of growth, then decreased to comprise 1/15 of the maximum value by the 27th h of growth, and virtually did not change in the next 37 hours. At the same time, the degree of phosphorylation of 41-, 35-, and 32-kDa polypeptides was maximum after 12–13 h of growth. By the 14th to 15th h, the degree of phosphorylation of the 41-kDa polypeptide decreased by an order of magnitude, and that of 35- and 32-kDa polypeptides decreased threefold. By the 18th h, the degree of phosphorylation of the 41-kDa polypeptide fell to zero, and that of 35- and 32-kDa polypeptides exhibited a further fourfold decrease. Within 24–27 h of growth, the degree of phosphorylation of the latter polypeptides increased two- to threefold, whereas that of the other polypeptides continued to diminish.

In the mutant strain 12, the degree of phosphorylation of all polypeptides was maximum by the 13th to 14th h of growth. By the 21st to 24th h, the degree of phosphorylation of all polypeptides decreased by about 30 times, except 35- and 32-kDa polypeptides, whose degree of phosphorylation comprised 20% of the maximum value by the 15th to 18th h of growth. The second maximum of phosphorylation of all polypeptides was observed by the 41st h of growth. The degrees of phosphorylation of 32-, 35-, 41-, 68-, and 137-kDa polypeptides in the first and second maxima were close or equal, whereas those of 75-, 79-, and 83-kDa polypeptides in the second maximum were 50–70% less than in the first maximum.

It should be emphasized that the range and the relative intensity of protein bands visualized by staining with Coomassie R-250 were almost the same in all of the cell-free extracts studied (Fig. 2).

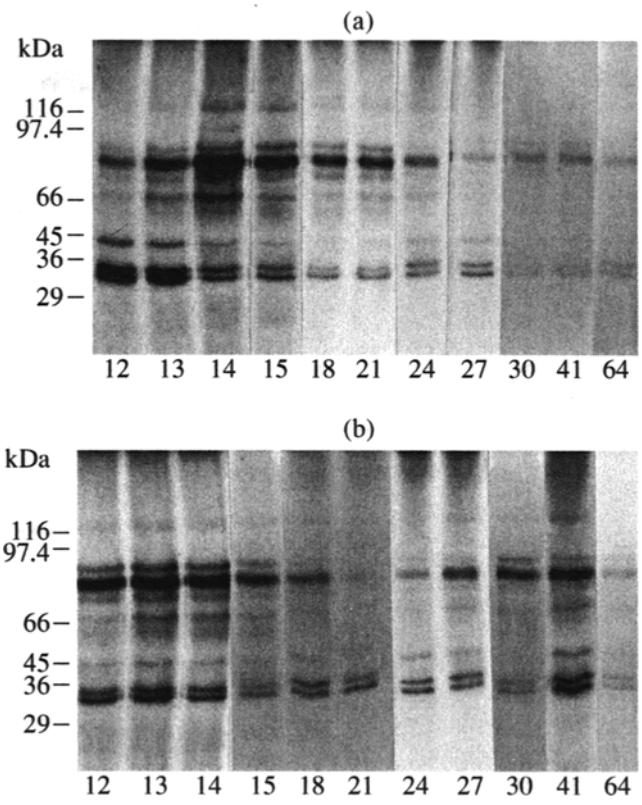


**Fig. 1.** Autoradiograms of the in vitro phosphorylated proteins of (a) wild-type strain 964 and (b) mutant strain 12 of *S. avermitilis*. Cultivation times in hours are indicated below the respective tracks. Samples were applied in amounts of 5 μg protein per well. Arrows show the position of molecular weight markers. X-ray film was exposed to developed gels for 5 days.

Thus, the inference may be drawn that both wild-type and mutant cells of *S. avermitilis* contain Ser/Thr protein kinases phosphorylating at least eight endogenous proteins. Variations in the degree of their phosphorylation could be explained by growth-dependent changes in the activity of protein kinases or by the interfering effects of phosphoprotein phosphatases and ATPases possibly present in the cell-free extracts. Although the protein kinase assay mixture used in our experiments contained nonspecific inhibitors of phosphatases, we thought it reasonable to additionally check the possibility of the interfering action of phosphatases. For this purpose, cell-free extracts of 15-h-old cells of both strains incubated in the protein kinase assay mixture were supplemented with a 100-fold excess of unlabeled ATP and equal amounts of cell-free extracts of 15-, 21-, and 30-h-old cells. The mixtures were incubated at 28°C for the next 20 min and analyzed by electrophoresis. As is evident from Fig. 3, none of the extracts tested exhibited noticeable phosphoprotein phosphatase activity. The ATPase activity of cell-free extracts of 18-, 24-, and 30-h-old cells was, respectively, 1.25, 0.82, and 0.71 pmol ATP/(min mg protein) (strain 964) and 1.05, 0.9, and 0.77 pmol

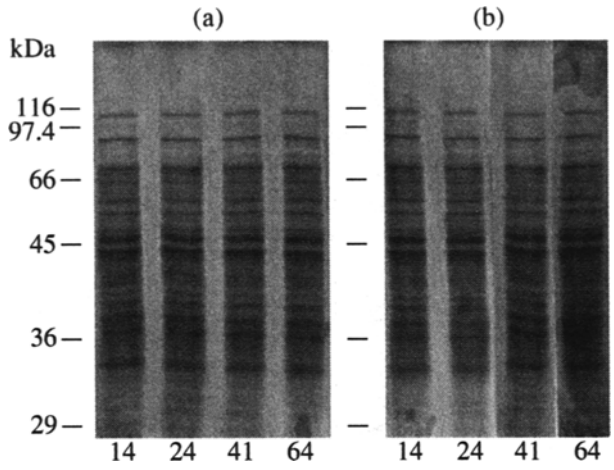


**Fig. 2.** Electrophoretograms of the in vitro phosphorylated proteins of (a) strain 964 and (b) strain 12 of *S. avermitilis* stained with Coomassie Brilliant Blue R-250. Cultivation times in hours are indicated below the respective tracks. Samples were applied in amounts of 5 μg protein per well. Arrows show the position of molecular weight markers.

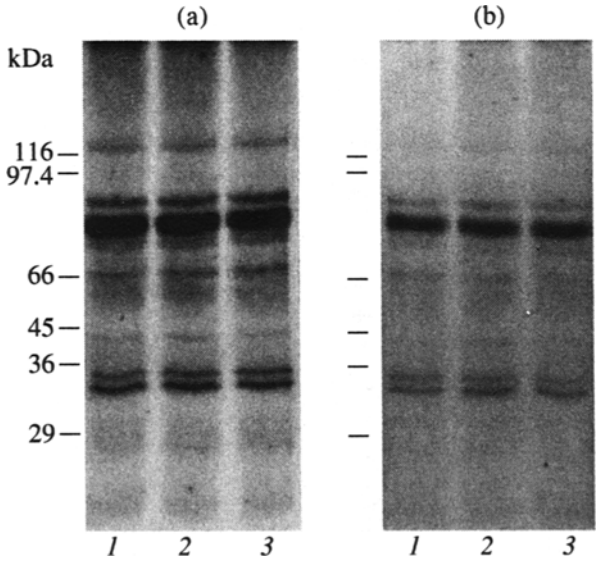


**Fig. 3.** Autoradiograms of the proteins of 15-h-old cells of (a) strain 964 and (b) strain 12 incubated in the protein kinase assay mixture with [$\gamma$-$^{32}$P]ATP and then with 2.5 mM of unlabeled ATP and equal amounts of cell-free extracts of (*1*) 15-h, (*2*) 21-h, and (*3*) 30-h cultures. Samples were applied in amounts of 10 μg protein per well. Arrows show the position of molecular weight markers. X-ray film was exposed to developed gels for 5 days.

ATP/(min mg protein) (strain 12). This implies that the ATPase activity of cell-free extracts could not essentially affect the results of the measurement of the ATP : protein phosphotransferase reaction in the assay mixture containing only 2.5 × $10^4$ pmole of ATP and no more than 15 μg of proteins. It should also be noted that these control measurements were carried out with cell-free extracts in which changes in the intensity of protein phosphorylation were especially drastic. Therefore, we can conclude that the changes observed in the activity of endogenous protein kinases are not due to the interfering effect of ATPases or phosphoprotein phosphatases.

The data presented suggest that Cml$^r$ mutation activates the protein kinase signal-transducing system of *S. avermitilis* in the early stationary growth phase. This may be due to changes in the activity of enzymes responsible for the reversible phosphorylation of proteins or in the biosynthesis of target proteins.

Taking into account evidence available in the literature that the physiological parameters of growth may affect the antibiotic-producing activity of *S. avermitilis* strains [18], we performed analogous experiments with strains 964 and 12 (Fig. 4). As seen from this figure, the growth rate of strain 12 in the exponential phase was higher than that of strain 964. In both strains, the biosynthesis of avermectin began in the stationary phase. In the mutant strain 12, however, it began earlier and proceeded at a higher rate.

The comparison of the electrophoretograms of phosphorylated proteins (Fig. 1) and the growth kinetics of the strains (Fig. 4) shows that there is a correlation between the intensity of protein phosphorylation, the growth rate, and the intensity of avermectin production. Indeed, the maximum drop in the intensity of protein phosphorylation was observed in the late exponential phase, after 21 h of growth of strain 12 and after 27–30 h of growth of strain 964; this corresponded to a higher growth rate of strain 12 and its earlier transition to the stationary growth phase. Furthermore, the second maximum in the intensity of protein phosphorylation in strain 12, observed in the 41st h of growth, corresponded to the beginning of the intense synthesis of avermectin in this strain (since the 48th h of growth). Taking into account the fact that the phosphorylated protein patterns of cell-free extracts of 41-h-old cells of strain 12 and of early-exponential-phase cells of strains 12 and 964 are very similar, the inference can be drawn that the same proteins are involved in the regulation of the growth and synthesis of secondary metabolites in these strains.

Earlier, the dynamics of growth and the intensity of protein phosphorylation were studied in *S. lividans* [5], *S. coelicolor* [6], *S. lincolnensis* [7], *S. granaticolor* [8], and *S. avermitilis* [9]. In most of these species, the phosphorylation of proteins with molecular masses of 98–100, 41–45, and 33–35 kDa was observed in the early exponential phase (20–24 h of growth), which is considered the key phase determining the subsequent synthesis of secondary metabolites [19]. It is believed that information about environmental conditions gained by actinomycetes at this growth phase is transduced via a network of signalling systems and triggers various biosynthetic pathways [11]. The most important of these systems is probably protein phosphorylation by specific protein kinases [3]. Taken together, the experimental data presented in this and in the afore-

mentioned works indicate that the phosphorylation of proteins with molecular masses of 98–100, 41–45, and 33–35 kDa is typical of actinomycetes of the genus *Streptomyces* and may play a significant role in their life cycle.

Let us consider in more detail the experimental results obtained by Licha *et al.* [9], who performed a comparative investigation of the phosphorylation of proteins in cell-free extracts of the wild-type *S. avermitilis* strain and its avermectin-superproducing mutant RX2. The authors showed the incorporation of radiolabeled phosphate into nine proteins, some of which had molecular masses close to those determined by us (33–35, 67, 78–79, and 98–100 kDa). In the mutant strain RX2, the 35-kDa protein exhibited two phosphorylation maxima, one of which corresponded to the exponential growth phase (12 h of growth) and the other of which corresponded to the early stationary phase (48 h of growth). Between these phases (in the 24th h of growth), there was a drastic drop in the intensity of phosphorylation of this protein (such a drop was absent in the wild-type strain). These data, which are similar to those presented here, indicate that the second rise in the intensity of protein phosphorylation in the avermectin-superproducing mutant strains has a universal character.

Cml mutation, whose frequency is about $3 \times 10^6$, makes *S. avermitilis* strain 12 resistant to 20 μg/ml chloramphenicol. This mutation is pleiotropic; i.e., it decreases the morphological variability of the mutant strain and augments the production of avermectin by about 40 times. As was suggested earlier [11], Cml$^r$ mutation may be localized in the regulatory gene responsible for the expression of silent genes in actinomycetes and for their physiological (regulatory) instability. The differences in the degree of protein phosphorylation in the wild-type and mutant cells from late growth phases, which exhibit different levels of secondary metabolism, avermectin biosynthesis in particular, are unequivocal evidence that Cml$^r$ mutation is pleiotropic and that it can affect the protein kinase regulatory system.

It is known that actinomycetes possess multiple resistance to antibiotics produced by other microorganisms [20] and that antibiotic-resistance determinants can be silent [11]. The nucleotide sequence of the kanamycin-resistance silent gene *aph* VIII of *S. rimosus* turned out to be highly homologous to the four eukaryotic Ser/Thr protein kinase genes [21]. Based on these results, we may suggest that Cml$^r$ mutation in *S. avermitilis* activates the protein kinase signalling system, which is involved in the recognition and adaptation of actinomycetes to antagonistic microorganisms. The presence in the medium of an antibiotic produced by an antagonistic microorganism may activate the signalling system involved in the survival and maintenance of the actinomycete population under stress conditions.

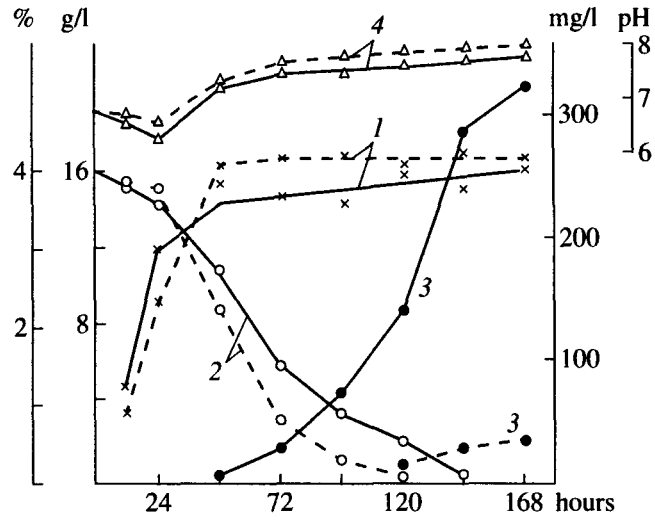


**Fig. 4.** Cultivation parameters of *S. avermitilis* 964 (dashed line) and *S. avermitilis* 12 (solid line): (*1*) biomass, g dry wt./l; (*2*) glucose concentration in the medium, %; (*3*) avermectin concentration in the medium, mg/l; and (*4*) pH.

Thus, the antibiotic-resistance genes of actinomycetes, the Cml$^r$ gene of *S. avermitilis* in particular, may function as signalling systems informing about the presence in the neighborhood of other, competing or antagonistic, microorganisms. On the other hand, they may be involved in cell differentiation and the modulation of secondary metabolism.

## REFERENCES

1. Chater, K.F., Genetic Regulation of Secondary Metabolic Pathways in *Streptomyces, Secondary Metabolites: Their Function and Evolution*, Chadwick, D.G. and Whelan, J., Eds., Washington, 1992, pp. 144–156.
2. Horinouchi, S., *Streptomyces* Genes Involved in Aerial Mycelium Formation, *FEMS Microbiol. Lett.*, 1996, vol. 141, pp. 1–9.
3. Zhang, C.C., Bacterial Signalling Involving Eukaryotic-Type Protein Kinases, *Mol. Microbiol.*, 1996, vol. 20, pp. 9–15.
4. Urabe, H. and Ogawara, H., Cloning, Sequencing, and Expression of Serine/Threonine Kinase–Encoding Genes from *Streptomyces coelicolor* A3(2), *Gene*, 1995, vol. 153, pp. 99–104.
5. Elizarov, C.M., Michurina, T.A., and Danilenko, V.N., Serine/Threonine-Type Protein Kinase Activity in Cell-Free Extracts of *Streptomyces lividans, Antibiot. Khimioter.*, 1998, vol. 43, pp. 3–8.
6. Stowe, D., Atkinson, T., and Mann, N.H., Protein Kinase Activities in Cell Free Extracts of *Streptomyces coelicolor* A3(2), *Biochimie*, 1989, vol. 71, pp. 1101–1105.
7. Janecek, J., Dobrova, Z., Naprstek, J., *et al.*, Evidence for Protein Phosphorylation in *Streptomyces lincolnensis, Folia Microbiol.*, 1995, vol. 40, pp. 249–252.

8. Janecek, J., Moravec, V., Dobrova, Z., *et al.*, Protein Phosphorylation in Submerged Spores and Vegetative Mycelium of *Streptomyces granaticolor, FEMS Microbiol. Lett.*, 1995, vol. 133, pp. 91–94.
9. Licha, I., Janecek, J., and Vanek, Z., Protein Kinase Activity in Cell Free Extracts of *Streptomyces avermitilis*: Comparing Wild Type and Overproducing Mutant, *Biotechnol. Lett.*, 1995, vol. 17, pp. 41–44.
10. Elizarov, S.M., Mironov, V.A., and Danilenko, V.N., Serine/Threonine-Type Protein Kinase Activity in Cell-Free Extracts of *Streptomyces avermitilis*: Comparing Wild Type and Overproducing Mutant, *Prikl. Biokhim. Mikrobiol.*, 1999, vol. 35, pp. 647–653.
11. Danilenko, V.N. and Akopyants, K.E., Genomic Instability and Silent Genes of Actinomycetes, *Biotechnologiya*, 1995, vols. 7, 8, pp. 104–112.
12. Laemmli, U.K., Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4, *Nature* (London), 1970, vol. 227, pp. 680–685.
13. Shaffner, W. and Weissmann, C., A Rapid, Sensitive, and Specific Method for the Determination of Protein in Dilute Solution, *Anal. Biochem.*, 1973, vol. 56, pp. 502–514.
14. Elizarov, S.M., Zabazarnykh, M.Yu., Musolyamov, A.Kh., *et al.*, Protein Kinase Activity Strongly Associated with Bovine Tryptophanyl-tRNA Synthetase, *Mol. Biol.*, 1997, vol. 31, pp. 253–262.
15. Malanikova, M. and Malanik, V., Use of Thin-Layer Chromatography for the Testing of Avermectins Produced by *Streptomyces avermitilis* Strains, *J. Chromatogr.*, 1990, vol. 513, pp. 404–407.
16. Hikeola, H. and Omura, S., Avermectin Biosynthesis, *Chem. Rev.*, 1997, vol. 97, pp. 2591–2609.
17. Danilenko, V.N., Mironov, V.A., Sergeeva, A.V., *et al.*, A Method for Avermectin Production, Avermectin-producing *Streptomyces avermitilis* Strain, *Russian Inventor's Certificate No 2098483*, 1997.
18. Burg, R.W., Miller, B.M., Baker, E.E., *et al.*, Avermectins, New Family of Potent Antihelmintic Agents: Producing Organism and Fermentation, *Antimicrob. Agents Chemother.*, 1979, vol. 15, pp. 361–367.
19. Horinouchi, S. and Beppu, T., Regulation of Secondary Metabolism and Morphogenesis in *Streptomyces, Gene*, 1992, vol. 115, pp. 167–172.
20. Cullum, J., Flett, F., and Piedl, W., Genetic Instability, Deletions, and DNA Amplifications in *Streptomyces* Species, *Genetics and Molecular Biology of Industrial Microorganisms*, Hershberger, L., Queener, S.W., and Hegeman, G., Eds., Washington, 1989, pp. 127–132.
21. Akopyants, K.E., Sizova, I.A., Michurina, T.A., and Danilenko, V.N., Nucleotide Sequencing of the Silent Aminoglycoside Phosphotransferase Gene of *Streptomyces rimosus* R3, *Antibiot. Khimioter.*, 1997, vol. 42, pp. 3–13.