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SHORT COMMUNICATIONS

Aminoglycoside Phosphotransferase AphSR2 from *Streptomyces rimosus* ATCC 10970: Dependence of Antibiotic Resistance on Serine-Threonine Protein Kinases PkSR1 and PkSR2

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Abstract—*Streptomyces rimosus* ATCC 10970 contains 14 genes annotated as aminoglycoside phosphotransferases in its genome: *aphSR1–aphSR14*. We have previously shown that the *aphVIII* (*aphSR5*) and *aph*(*3''*)-*Id* (*aphSR3*) genes, when cloning in *E. coli*, cause resistance to kanamycin, neomycin, paromomycin, and streptomycin. It was found for Aph(3')-VIII that antibiotic resistance increased after phosphorylation at the Ser146 motif in the active site of the enzyme by serine-threonine protein kinases (STPKs). The *aphSR2* gene, when cloning in *E. coli*, causes resistance to neomycin and hygromycin. In this work, in order to assess the possibility of influence of STPK genes on increasing resistance to aminoglycoside antibiotics, we performed a combined cloning into *E. coli* at pET32a of the *aphSR2* gene and the STPK genes (*pkSR1* and *pkSR2*) localized in one cluster of the *S. rimosus* ATCC 10970 genome. We detected that, in the construction *E. coli/aphSR2/pkSR1*, there is a 2-fold increase in resistance to neomycin. The presented data are the second example of the STPK effect on the modulation of the level of resistance to aminoglycoside antibiotics in bacteria of the genus *Streptomyces*.

Keywords: Streptomyces rimosus, aminoglycoside phosphotransferase (aph), serine-threonine protein kinase, spectrum and level of resistance to antibiotics

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The ability of bacteria to acquire antibiotic resistance has been known since the discovery of antimicrobial agents [1]. The concept of "resistance," which stands for a pool of resistance genes specific to a particular bacterial community, has been established in recent decades. The Antibiotic Resistance Genes Database (ARDB) currently numbers 23137 resistance genes. The downregulation of resistance genes makes it possible to use the existing antibiotics in fighting against antibiotic-resistant bacteria [2].

Bacterial resistance to aminoglycoside antibiotics was first discovered in 1952. A hypothesis for explaining the origin of resistance genes was put forward in the early 1970s, in particular, of aminoglycoside transferases from soil-dwelling antibiotic-producing bacteria [3]. In 1983, aminoglycoside-3'-phosphotransferase genes were identified and sequenced in plasmids and mobile elements in clinical strains of gram-negative and gram-positive bacteria [4].

It is known that antibiotic resistance genes could have originated from bacterial antibiotic producers that belong to the *Streptomyces* genus. The genome annotation revealed four to 14 aminoglycoside phosphotransferase genes in Actinobacteria. However, the functions of genes annotated as *aph* in the sequenced genomes (https://www.ncbi.nlm.nih.gov/genome/) are still to be elucidated [5].

When sequencing the genome of the strain *Streptomyces rimosus* subsp. *rimosus* ATCC 10970 (oxytetracycline producer) [6], 14 *aph* genes were annotated, which we designated as *aphSR1–aphSR14*. We conducted a comparative and phylogenetic analysis of amino acid sequences for the products of 14 *aph* genes with previously known *aph* genes from clinical isolates and aminoglycoside antibiotic-producing strains from seven subfamilies. It was revealed that AphSR5 (AphVIII) belongs to the Aph(3') subfamily, AphSR3 belongs to the Aph(3'') subfamily, and AphSR2 is located on the same branch with Aph(7'')-Ia.

We previously identified and characterized two aminoglycoside phosphotransferases in *S. rimosus* ATCC 10970 with a high-level aminoglycoside resistance. AphSR5 (Aph(3')-VIII) leads to resistance to kanamycin, neomycin, and paromomycin; an import-

ant feature of AphVIII from *S. rimosus* is the dependence of its enzymatic activity on the degree of phosphorylation by serine-threonine proteinkinases; the 3D structure of AphVIII (code PDB 4H05) was obtained [7–10]. Cloning of the *aphSR3* gene (*aph*(*3''*)-*Id*) in *E. coli* revealed that this gene causes resistance to streptomycin [11].

The object of this study is AphSR2 aminoglycoside phosphotransferase. According to the phylogenetic analysis, AphSR2 is located on the same branch with Aph(7")-Ia, but the tree node was not well supported (the bootstrap value was <60%); it was however possible to classify them with the Aph(7") subfamily. At the same time, the comparative analysis of AphSR2 using the SAS software program (https://www.ebi.ac.uk/thornton-srv/databases/sas/) with the known 3D structures showed similarity between 3D structures of AphSR2 and Rv3168 transferase (PDB ID—3ATS) of the strain *Mycobacterium tuberculosis* H37Rv (31.2% identity).

An analysis of amino acid sequence alignment conducted in the Clustal Omega software program (https://www.ebi.ac.uk/Tools/msa/clustalo/) showed that the sequence of AphSR2 shares a significantly greater number of common conserved amino acid residues with the sequence of Rv3168 than with aminoglycoside phosphotransferase Aph(7")-Ia (Fig. 1a).

Using the NCBI and UniProt databases (http://www.uniprot.org/), it was found that the *aphSR2* gene is located in the same cluster with the genes of two serine-threonine protein kinases (STPKs), which we designated as follows (according to the numbers of the gene loci): SRIM_07563—*pkSR1*, SRIM_07568—*pkSR2*. The transcription of the *pkSR1* gene occurs in the same direction as that of the *aphSR2* gene (Fig. 1b).

We previously classified STPKs of gram-positive bacteria on the basis of an analysis of the signature of nine variable amino acid residues, the side chains of which are exposed to the adenine binding region. According to the results of this classification, all STPKs were divided into 20 groups. According to the proposed classification of STPKs, PkSR1 belongs to the group IIa, and PkSR2 belongs to group IIb [12, 13]. The orthologs for these STPKs are kinases from *Streptomyces coelicolor* Pk13 (BLAST identity 89%) and Pk12 (BLAST identity 93%), respectively [14]. The discussed kinases from *S. coelicolor* are located on the chromosome in the same way as kinases from *S. rimosus*—adjacent to each other, but facing in the opposite directions. None of the 11 described and well-studied STPKs from *M. tuberculosis* are orthologs of the considered STPKs of *S. rimosus*.

In this work, we have studied experimentally the effect of the *pkSR1* and *pkSR2* STPK genes on the resistance of *E. coli* BL21(DE3), which contains the *aphSR2* gene, to aminoglycoside antibiotics by combined cloning of these genes in the pET32a expression vector.

The PkSR1 protein consists of 573 amino acid residues; the domain structure of the protein consists of the catalytic domain (19–288 a.a.) and the PASTA domain (503–569 a.a.). The PkSR2 protein consists of 516 amino acid residues; the domain structure of the PkSR2 protein comprises the catalytic domain (5–265 a.a.).

At the first stage, the catalytic domains of protein kinases were cloned in *E. coli*. The *pkSR1* and *pkSR2* genes were amplified from the genomic DNA of the *S. rimosus* strain using PCR (PCK-100 kit manufactured by Dialat Ltd. with the PTC-0150 instrument (MJ Research, Inc.)) using oligonucleotides PkSR1-N (5'-tcgcggatcccgctaccagctccgtgatct-3') and PkSR1-C (5'-tcgcggatcccgctaccggctcacgcgccg-3') for the *pkSR1* gene and oligonucleotides PkSR2-N (5'-tcgcggatcccgctaccggctcacgcgccg-3') and PkSR2-C (5'-ccgcaagcttgcgcatctcctccgcggtctg-3') for the *pkSR2* gene. The fragments were cloned at the restriction sites of *Bam*HI and *Hind*III endonucleases into the pET28a expression vector (selection marker Km). Then, the resulting hybrid plasmid pET28a:*pkSR1* and 28a:*pkSR2* was treated with *Xba*I and *Xho*I restriction endonucleases and inserted at the specified restriction sites into the pET32a expression vector (selection marker ampicillin). The pET32aM:*pkSR1* and pET32aM:*pkSR2*By hybrid plasmids were obtained by cloning.

The *aphSR2* gene was then cloned into pET32aM:*pkSR1* and pET32aM:*pkSR2* plasmids at the *Xba*I endonuclease restriction site. As a control, the *aphSR2* gene was cloned into the pET32a plasmid at the *Xba*I endonuclease restriction site. The *aphSR2* gene was amplified from the pET16b:*aphSR2* plasmid DNA using the oligonucleotides T7prom (5'-ttaatacgactcactatagg-3') and AphSR2C-XbaI (5'-agcctctagatcactccgtgaaggccgcc-3'). Screening of clones was performed with PCR using the oligonucleotides T7prom and AphC-XbaI, which made it possible to select clones with the desired orientation. The plasmids were designated as pET32aM:*aphSR2/pkSR1*, pET32aM:*aphSR2/pkSR2* and pET32a:*aphSR2*.

To study the expression of the *aphSR2*, *pkSR1*, and *pkSR2* genes in *E. coli*, the hybrid plasmids were transformed to competent *E. coli* cells of the strain BL21(DE3) (F^-, dcm, ompT, hsdS($r_B^-m_B^-$), gal λ (DE3)) (Novagen) and grown in liquid LB medium at 37°C to an optical density of 0.6 (~2 h), and the expression was then induced by adding isopropyl β-thiogalactopyranoside (IPTG) to a final concentration of 1.3 mM. Next, the cells were cultured at 28°C for 18 h, precipitated by centrifugation (5000 rpm, 10 min, 4°C), and suspended in the Sample buffer and analyzed by 12.5% SDS-PAGE electrophoresis according to the Laemmli protocol. The protein fractions of *E. coli* strains containing the pET32a plasmid

(a)

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AphSR2      MLR-----SSDVRTAAPPDGHTLGSLLRRY----GAGEPLSCVPVAEGLLNHGYRLATTH   51
APH(7")-Ia  ------------------------------------------------------MTQ   3
Rv3168      MANEPAIGAIDRLQRSSRDVTTLPAVISRWLSSVLPGGAAPEVTVESGVDSTGMSSET--   58

AphSR2      GRYFLKHHLDGDQAAI-------ARQHRATRRLGALGLPVAPPLADADG-----------   93
APH(7")-Ia  ESLLLLDRIDSDDSYASLRNDQEFWEPLARRALEELGLPVPPVLRVPGESTNPVLVGEPD   63
Rv3168      --IILTARWQQDGRSI-------QQKLVARVAPAAEDVPVFPTYRLDHQFEVIRLVGEL-   108
               :*  : : *             :  *       .:** *

AphSR2      ------------------------------------RTVTVLGG----RCYALHPWVE   111
APH(7")-Ia  PVIKLFGEHWCGPESLASESEAYAVLADAPVPVPRLLGRGELRPGTGAWPWPYLVMS---   120
Rv3168      -------------------------TDVPVPRVRWIETTGDVLGT-----PFFLMDYVE   137
                                                  *         : :

AphSR2      GRHRDGA------------ALTRHQSRCLGALLGQVHTALEQVITPDTAGPGVPYEHAGA   159
APH(7")-Ia  ---------RMTGTTWRSAMDGTTDRNALLALARELGRVLGRLHRVPLTGNTVLTPHSEV   171
Rv3168      GVVPPDVMPYTFGDNWFADAPAERQRQLQDATV----AALATLHSIPNAQNTFSFLTQGR   193
                                  : .   *       .*  :     :   .

AphSR2      D----PAR---TFEMIDELLALARRSRPRSSFDELAEHRLLERRALLEREAHRRPGADRV   212
APH(7")-Ia  FPELLRERRAATVEDHRGW--------------GYLSPRLLDRLEDWLPDVDT-LLAGRE   216
Rv3168      TSDTTLHR---HFNWVRSWYDFA--------VEGIGRSPLLERTFEWLQSHWPDDAAARE   242
              *    .:                              **:*      .       * *

AphSR2      PATGWVHGDFHPLNLLYRDA--EPAAIVDWDRLAVQPRAEEAVRAA--AIFFVQPA-GTL   267
APH(7")-Ia  PR--FVHGDLHGTNIFVDLAATEVTGIVDFTDVYAGDSRYSLVQLHLN--AFRGDREILA   272
Rv3168      PV--LLWGDARVGNVLYRDF--QPVAVLDWEMVALGPRELDVAWMIFAHRVFQELA-GLA   297
            *    : ** :  *::      : ..::*:  :       . .         *

AphSR2      DLPKVGAY------AGAYRRASGAGAAELAAAVHRVWWERL-NDFWMLDWRYRLGDRRAD   320
APH(7")-Ia  ALLDGAQWKRTEDFA-----------RELLAFTFLHDFEVFE-------------ETPLD   308
Rv3168      TLPGLPEVMREDDVRATYQALTGVELGDL------HWFYVYSGVMWACVF-MRTGARRVH   350
             *                            :*        :                .

AphSR2      P---QFPAAAALAVWWTREYGAVRAAFTE--     346
APH(7")-Ia  LSGFTDPEELAQFLWGPPDTAP-----GA--     332
Rv3168      FGEIEKPDDV-ESLFYH--AGLMKHLLGEEH     378
              *     ::     .
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Fig. 1. Characterization of the *aphSR2* and *pkSR1*, *pkSR2* gene cluster from the strain *S. rimosus* ATCC 10970 and the encoded proteins. (a) Comparison of the amino acid sequence of AphSR2 with sequences of APH(7")-Ia and Rv3168 (residues conserved between all three sequences are highlighted in black; between two, in gray); (b) transcription organization of the *aphSR2* and *pkSR1*, *pkSR2* gene cluster of the strain *S. rimosus* ATCC 10970 (*hp*—hypothetical protein, *citB*—putative two-component system response transcriptional regulator, *pdha*—pyruvate dehydrogenase E1); (c) electrophoresis of soluble fraction of proteins from the strain *E. coli* BL21(DE3), containing plasmids: (*1*) pET32a, (*2*) pET32a:*aphSR2*; (*3*) pET32a:*pkSR1*, (*4*) pET32a:*aphSR2* + *pkSR1*, (*5*) pET32a:*pkSR2*, (*6*) pET32a:*aphSR2* + *pkSR2*. M—molecular weight protein marker SM0441 (Fermentas, Lithuania).

without the insertion were used as controls. The electropherogram analysis revealed the expression of proteins with molecular weight of 33, 32, and 41 kDa, which corresponds to the estimated molecular weights of the catalytic domains of the PkSR1 and PkSR2 protein kinases and the AphSR2 protein molecular weight (Fig. 1c).

At the next stage, the spectrum of resistance to aminoglycoside antibiotics was tested using standard disks of *E. coli* BL21(DE3) strains containing all recombinant plasmids. The spectrum of resistance was tested using paper disks with aminoglycoside antibiotics: kanamycin (30 μg/disk), neomycin (30 μg/disk), amikacin (30 μg/disk), streptomycin

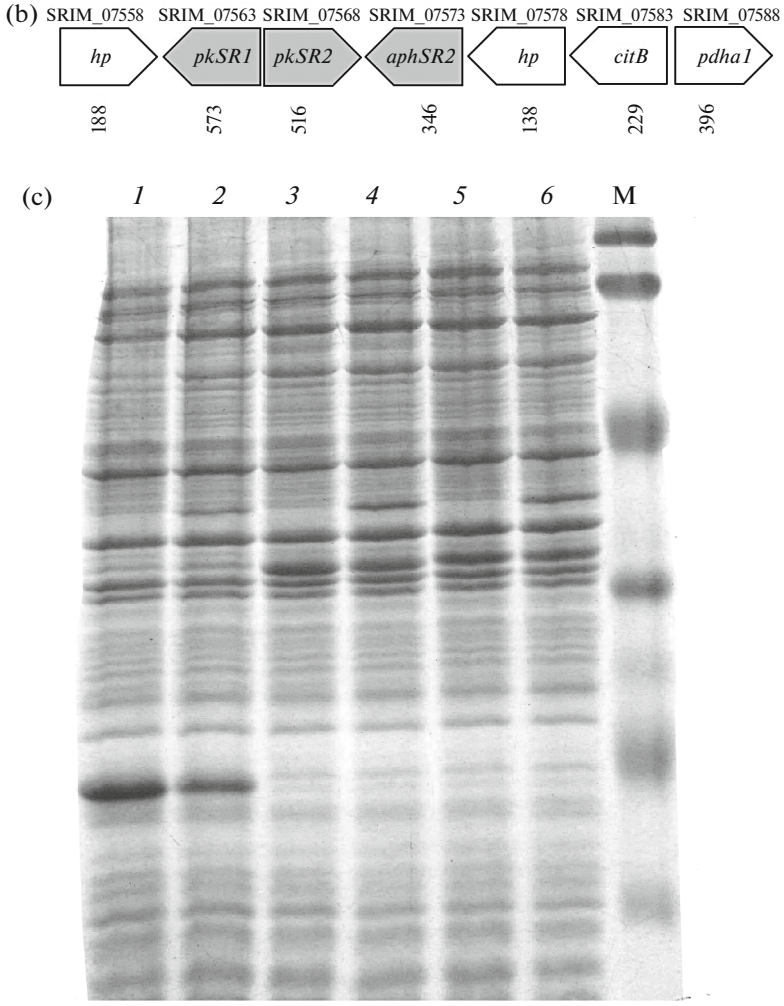

Fig. 1. (Contd.)

(10 μg/disk), gentamycin (10 μg/disk), tobramycin (10 μg/disk), sisomycin (10 μg/disk), netilmycin (10 μg/disk), isepamycin (30 μg/disk), and hygromycin (100 μg/disk). The results were evaluated after incubation for 16–18 h at 37°C.

The investigation of the growth inhibition zone surrounding the antibiotic disks showed that the *aphSR2* gene causes resistance to neomycin and hygromycin in *E. coli* BL21(DE3) (Table 1). The combination of the *aphSR2* and *pkSR1* genes in one vector increased the level of *E. coli* resistance to neomycin, and the combination of the *aphSR2* and *pkSR2* genes did not change the resistance. The combination of the *aphSR2* and STPK (*pkSR1* or *pkSR2*) genes had no effect on hygromycin resistance. The effect of STPK genes on the resistance of *E. coli* BL21(DE3) was investigated as a control; these genes had no effect on resistance to neomycin, but increased susceptibility to hygromycin. The results suggest that the *aphSR2* gene may be a candidate hygromycin resistance gene from *S. rimosus*. According to the published data, *aph*(*7″*)-*Ia* causes resistance to hygromycin B [15] and *rv3168* causes resistance to kanamycin [16]. In contrast to *rv3168* and *aph*(*7″*)-*Ia*, *aphSR2* influenced the resistance of *E. coli* cells to neomycin and hygromycin.

Next, the level of resistance to neomycin and hygromycin was tested using the analysis of the minimum-inhibitory concentrations (MIC). For the analysis, we used clones of the *E. coli* BL21(DE3) transformants containing recombinant plasmids pET32a, pET32a:*aphSR2*, and pET32aM:*aphSR2/pkSR1*. Individual colonies were collected and transferred to tubes containing 2 mL of LB broth; an overnight culture was grown to an optical density of $OD_{625} = 0.3$ and then diluted with LB medium to obtain the final density of 10^5–10^6 CFU/mL. Cell culture (100 μL) (IPTG, 100 μM) was added to each of the series of tubes containing twofold dilutions of neomycin in 2 mL of LB medium in order to induce the expression of the *aphSR2* and *pkSR1* genes. After incubation of the cultures at room temperature (~25°C) and 250 rpm for 18 h, the MIC values were estimated as the lowest

Table 1. Change in the level of resistance to aminoglycoside antibiotics upon combined expression of the *aphSR2*, *pkSR1*, and *pkSR2* genes in *E. coli* BL21(DE3)

Tested antibiotic*	Constructs						
	BL21(DE3)	BL21(DE3) pET32a	BL21(DE3) pET32a:*aphSR2*	BL21(DE3) pET32a:*aphSR2* + *pkSR1*	BL21(DE3) pET32a:*pkSR1*	BL21(DE3) pET32a:*aphSR2* + *pkSR2*	BL21(DE3) pET32a:*pkSR2*
Neomycin (30 μg/disk)	19 ± 0.61	19 ± 0.47	16 ± 0.59	12.5 ± 0.71	19 ± 0.61	16 ± 0.54	19 ± 0.55
Kanamycin (30 μg/disk)	21 ± 0.50	21 ± 0.55	21 ± 0.80	21 ± 0.56	21 ± 0.51	21 ± 0.55	21 ± 0.73
Hygromycin (100 μg/disk)	15 ± 0.53	15 ± 0.59	12 ± 0.47	14 ± 0.44	17 ± 0.55	16.5 ± 0.58	18 ± 0.53

Average results for three independent measurements are given; the data are shown as the mean ± σ (sigma, the standard deviation). The significance of differences between the samples was estimated using the Student's *t*-test. The level of significance of differences between the samples was 0.05 (Statistica v6). * Susceptibility to antibiotics tested by disk diffusion.

concentration of neomycin or hygromycin which resulted in the complete growth inhibition (assessed by spectrophotometry at OD_{625}).

In experiments on MIC estimation using a method of serial dilution, the concentration of neomycin was 8 μg/mL for the control strain *E. coli* BL21(DE3); upregulation of the recombinant protein expression increased MIC to 16 μg/mL; combined *pkSR1* and *aphSR2* gene expression caused an increase in MIC to 32 μg/mL. Thus, it was found that, upon combined cloning in *E. coli*, *aphSR2* causes resistance to neomycin, showing the modulation of the antibiotic resistance level by *pkSR1.* The MIC of hygromycin for the control strain of *E. coli* BL21(DE3) was 100 μg/mL, indicating that the strain of *E. coli* is resistant to hygromycin, which complicates the MIC measurement [17].

Thus, AphSR2 is the second aminoglycoside phosphotransferase of *Streptomyces*, in particular, *S. rimosus*, for which the data show that the level of resistance increases under the influence of STPKs, being a cumulative result of their combined expression in *E. coli*. The results confirm the hypothesis on the role of signal-transmitting systems of soil-dwelling bacteria in the modulation of expression of antibiotic resistance genes in Actinobacteria [18].

The tasks for subsequent work include the isolation of recombinant proteins AphSR2, PkSR1, and PkSR2 and an investigation in vitro of the level and sites of phosphorylation in the studied aminoglycoside phosphotransferase and the effect of phosphorylation on enzymatic activity.

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COMPLIANCE WITH ETHICAL STANDARDS

The authors declare that they have no conflict of interest. This article does not contain any studies involving animals or human participants performed by any of the authors.

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