New Test System for Serine/Threonine Protein Kinase Inhibitors Screening: *E. coli* APHVIII/Pk25 design.

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ABSTRACT An efficient test system for serine/threonine protein kinase inhibitors screening has been developed based on the *E. coli* protein system APHVIII/Pk25. Phosphorylation of aminoglycoside phosphotransferase VIII (APHVIII) by protein kinases enhances resistance of the bacterial cell to aminoglycoside antibiotics, e.g. kanamycin. Addition of protein kinase inhibitors prevents phosphorylation and increases cell sensitivity to kanamycin. We have obtained modifications of APHVIII in which phosphorylatable Ser146 was encompassed into the canonical autophosphorylation sequence of *Streptomyces coelicolor* Pk25 protein kinase. Mutant and wild-type *aphVIII* were cloned into *E. coli* with the catalytic domain of *pk25*. As a result of the expression of these genes, accumulation of corresponding proteins was clearly observed. Extracted from bacterial lysates, Pk25 demonstrated its ability to autophosphorylate. It was shown that variants of *E. coli* containing both *aphVIII* and *pk25* were more resistant to kanamycin than those carrying only *aphVIII*. Protein kinase inhibitors of the indolylmaleimide class actively inhibited Pk25 and reduced cell resistance to kanamycin. Modeling of APHVIII and Pk25 3D structures showed that pSer146 is an analog of phosphoserine in the ribose pocket of protein kinase A. Pk25 conformation was similar to that of PknB of *Mycobacterium tuberculosis*. Potential indolylmaleimide inhibitors were docked into the ATP-binding pocket of Pk25. The designed test system can be used for the primary selection of ATP-competitive small molecule protein kinase inhibitors.

KEYWORDS serine/threonine protein kinases, indolylmaleimides, protein kinase inhibitors screening, bacterial test system, *Streptomyces*

Serine/threonine protein kinases (STPK) are a group of universal regulators of the cellular metabolism in eukaryotes [1-3]. They play leading roles in the regulation of apoptosis, proliferation and differentiation, cellular transport, etc. It has been shown that kinase malfunction can be associated with various human diseases, such as diabetes [4], schizophrenia [5], cardiovascular disorders [6,7], and immune dysfunctions [8]. Over the past decades, the search for new targeted modulators (inhibitors) of protein kinases, that are considered as potential drugs, has been intensive [9-11].

Eukaryotic type serine/threonine protein kinases were found in bacteria, including human pathogens [12]. It was shown that STPKs participate in virulence, bacterial biofilm formation, tolerance, and persistence of pathogenic microorganisms. STPKs were shown to play the key role in the virulence of *Streptococcus pneumonia*, *Mycobacterium tuberculosis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and certain other pathogens [12-14]. It is established that STPKs participate in the modulation of antibiotic resistance in *M. tuberculosis* [15]. Intensive STPK inhibitors screening is currently under way [16-19].

We have developed a test system [20] for prescreening of STPK inhibitors based on the newly constructed strain *Streptomyces lividans* TK24 (66) APHVIII+. The key part of this test system is type VIII aminoglycoside phosphotransferase (APHVIII), an enzyme that inactivates aminoglycoside antibiotics. Gene *aphVIII*, which is isolated from *Streptomyces rimosus*, was cloned into *S. lividans* TK24 (66) and expressed. Importantly, the activity of APHVIII-S. rimosus is dependent on phosphorylation by endogenous STPKs [21]. Phosphorylation of APHVIII confers *Streptomyces* cells resistance to aminoglycoside antibiotics, while inhibitors of STPK render cells more sensitive to aminoglycosides [20]. Alteration of the cellular sensitivity to aminoglycoside antibiotics in the presence of STPK inhibitors allows to perform primary screening of these inhibitors. Upon annotation of the genome of the *S. coelicolor* A3(2) strain (NC_003888), which is close to *S. lividans* TK24...
34 STPKs were identified. At least one of them – Pk25 (NCBI Reference Sequence: Protein NP_628936.1) – is able to phosphorylate APH-VIII [22]. In order to rule out a nonspecific action of STPK inhibitors on the other STPKs of \textit{S. lividans} TK24 (66) that are presumably able to phosphorylate APHVIII, the catalytic domain of Pk25 kinase and APHVIII were hosted in \textit{Escherichia coli}. The genome of \textit{E. coli} does not contain its own eukaryotic STPKs, which makes the test system more sensitive and allows the screening of inhibitors that are specific to Pk25 and its homologous enzymes [23].

**EXPERIMENTAL PROCEDURES**

**Strains:** \textit{S. coelicolor} A3(2) (Russian Collection of Pathogenic Microorganisms, Moscow), \textit{S. lividans} TK24 (66) APHVIII+ (GenBank ACEY01000000), \textit{E. coli} DH5\textalpha:\ F\textsuperscript{−}Φ80 ΔlacZΔM15, Δ(lacZYA-argF) U169 (Promega); BL21(DE3): F\textsuperscript{−} dcm ompT, hsdS(r\textsubscript{B} m\textsubscript{B} g\textsubscript{B}) gal(DE3) (Novagen).

**Plasmids:** pET16b, pET22b and pET32a (Novagen).

**Media:** \textit{S. coelicolor} A3(2) and \textit{S. lividans} TK24 (66) APHVIII+ strains were grown on YSP and YEME media [24]. \textit{E. coli} strains were grown on Luria broth (L-broth), NZCYM, M9 supplemented with 1.5% glycerol (1 L): 6 g Na\textsubscript{2}HPO\textsubscript{4}, 3 g KH\textsubscript{2}PO\textsubscript{4}, 0.5 g NaCl, 1 g NH\textsubscript{4}Cl, pH 7.4, 2 mL 1 M MgSO\textsubscript{4}, 15 mL glycerol. Ampicillin (100 μg/ml) was added to the media to ensure the survival of plasmid-containing cells. Protein expression was induced by IPTG (1 mM).

**Molecular cloning:** Total DNA was isolated from \textit{S. coelicolor} A3(2) and \textit{S. lividans} TK24 (66) APHVIII+ strains according to [24]. Isolation of plasmid DNA, preparation of competent \textit{E. coli} cells, transformation and analysis of recombinant plasmids was performed according to standard protocols [25]. DNA amplification by PCR was carried out by the “Amplification” kit (Dialat Ltd.) on the Tertsik TP4-PCR01 amplification (DNA-teknologiya). The temperature cycle was designed according to primer length and composition. Oligonucleotides were purchased from Syntol (see Table 1). The DNA sequence was determined according to Sanger. Nucleotide sequences were compared with BLAST (http://www.ncbi.nlm.nih.gov/blast).

**Protein electrophoresis** was carried out in 12% polyacrylamide gels under denaturing conditions, as described earlier [21]. Bacterial cells containing constructed plasmids were grown on a NZCYM liquid medium supplemented with ampicillin (150 μg/ml) at 34°C up to an optical density equal to 0.6 (~1.5 h). Expression of the Pk25 catalytic domain was induced by the addition of IPTG (final concentration 1 mM). Cells were grown at 28°C for 4 h, harvested and re-suspended in a buffer containing 62.5 mM TrisHCl, pH 6.8; 5% glycerol; 2% β-mercaptoethanol; 0.1% SDS; and Bromophenol Blue. Cells were lysed by boiling for 10 min in the above-mentioned buffer and subjected to electrophoresis in polyacrylamide gels. Approximately 25 μg of the protein was loaded into each well in the gel. Electrophograms were scanned by laser densitometer Ultrascan 2205 LKB. A protein fraction of \textit{E. coli} BL21(DE3) cells transfected with the empty vector was used as control.

**Isolation of the catalytic domain of Pk25 cloned into \textit{E. coli} cells.** Cells were destroyed by sonication in a buffer containing 20 mM TrisHCl, pH 7.8, 10 mM 2-mercaptoethanol, 300 mM NaCl, and 1 mM PMSF, or in the same buffer supplemented with 8 M urea. Cell debris and other undissolved material was removed by centrifugation at 20,000g in 20 min. Fractions of soluble proteins were loaded onto a Ni-NTA agarose column (Qiagen) which was washed by the above-described buffer containing 50 mM imidazole pH 6.0. The protein was eluted by the buffer with imidazole concentration increasing from 0.05 M up to 0.5 M [20]. Protein fractions were analyzed by SDS-PAGE.

**Analysis of autophosphorylation of isolated protein by catalytic domain of Pk25 in situ** was performed after separation of the protein under denaturing conditions. Re-naturation of the kinase in gel was carried out according to Kashemita and Fujisawa [26]. Gels containing the protein were intensively washed in 50 mM TrisHCl, pH 7.8, with 25% 2-propanol and 8 M urea in order to remove SDS. Following that, protein re-naturation was carried out by washing gels in buffer A: 50 mM TrisHCl, pH 7.8 and B: 50 mM TrisHCl, pH 7.8, 100 mM NaCl, 6 mM β-mercaptoethanol, 5 mM MgCl\textsubscript{2}, and 1 mM CaCl\textsubscript{2}. After re-naturation, the gels were incubated in the presence of 50 μCi/ml [γ\textsuperscript{32}P]ATP (7000 Ci/mM, Phosphor, Russian Federation) in the buffer for the analysis of kinase activity [21]. The gels were fixed and stained in 40% TCA, washed in 5% acetic acid, dried and autoradiographed by exposure to a Kodak X-Omat AR film.

**Cloning into expression vectors** pET32a, pET22b, and pET16b. Gene pk25 of the \textit{S. coelicolor} A3(2) strain and the gene of \textit{S. lividans} TK24 (66) were cloned into \textit{E. coli} in pET32a plasmid at EcoRI and HindIII (primers Pk25EN and Pk25C) (Table 1). The gene of the catalytic domain of pk25 of the \textit{S. coelicolor} A3(2) strain was cloned into \textit{E. coli} in pET22b plasmid at NdeI and HindIII (primers Pk25CN and Pk25CC). The modified gene aphVII was cloned into \textit{E. coli} in pET16b plasmid at NdeI and XhoI (primers AphN and AphC). The gene of the catalytic domain of pk25 of the \textit{S. coelicolor} A3(2) strain was cloned into \textit{E. coli} in pET16b + aphVII146-S with the non-modified phosphorylation site of APHVIII, pET16b + aphVII146-1, pET16b + aphVII146-2, and pET16b + aphVII146-3 with modified phospho-
rlylation sites at the BamHI (primers Pk25NBgl and Pk25CBgl).

Cloning of the nucleotide sequence of the pk25 catalytic domain was performed in the pET22b vector at NdeI–HindIII restriction sites (primer Pk25CN homologous to the N-terminal region of the catalytic domain, and primer Pk25CC homologous to the C-terminal region of the catalytic domain). The DNA sequence of the catalytic domain was amplified using total DNA of S. coelicolor as a template. The PCR product was purified from the agarose gel, then sequenced and cloned into the pET22b vector at the NdeI and HindIII.

E. coli DH5α cells were transformed by the resulting ligase mix, and screening of recombinant clones was carried out by PCR using standard primers T7prom and T7term. Plasmid DNA was isolated from selected transformants, and the obtained recombinant plasmids were sequenced and subjected to restriction analysis to verify the presence of the insert. Then, the plasmids were used for the transformation of E. coli BL21 (DE3) cells.

Site-directed mutagenesis of Ser146 in aminoglycoside phosphotransferase APHVIII was carried out according to Nelson [27]. In order to obtain the mutant variant 1 (amino acid substitutions Ser146Thr, Glu144Thr, Asp148Ser), the primers APH 146-1(+) and APH 146-1(−) (Table 1) were used. The primers APH146-2(+) and APH146-2(−) were used to obtain the mutant variant 2 (Glu144Thr, Asp148Ser, Glu150Ser). The mutant variant 3 represents substitution Ser146Thr, which was introduced using the APH146-T(+) and APH146-T(−) primers.

AphN and AphC corresponding to the 5′- and 3′-terminal fragments of the aphVIII gene were used as flanking primers.

The obtained mutant PCR fragments were sequenced to verify the nucleotide substitutions and cloned at the NdeI and BamHI restriction sites into the high-copy number plasmid pET16b containing T7 phage transcriptional and translational regulatory elements in the same reading frame with the ATG codone of the gene of interest. This plasmid was used for the transformation of E. coli DH5α cells, and screening of the recombinant clones was carried out by PCR using T7prom and T7term primers. The selected transformants were used for purification of plasmid DNA, which was re-sequenced to ensure the substitutions.

Determination of resistance to kanamycin in selected transformants of E. coli BL21 (DE3). E. coli BL21 (DE3) clones containing genes of either native or modified aphVIII or aphVIII and pk25 in the pET16b vector were used for the analysis. The clones resistant to ampicillin (100 μg/ml) were transferred on the plates with the LB-medium containing various concentrations

Table 1. Primers which were used in the present work*

| Primer      | Restriction site | Primer structure, 5′–3′         |
|-------------|------------------|---------------------------------|
| Pk25EN      | EcoRI            | ATCCGAATTATGGCACGGAAGATCGGCAG   |
| Pk25C       | HindIII          | CCCGAAAGCTTTGGTGCGTGCGAGCGCC    |
| Pk25CN      | NdeI             | TCGCATATGCGTACCAGGCACGCGGC     |
| Pk25CC      | HindIII          | CCCGAAGCTTCATCCGGTGCGACGCC     |
| Pk25NBgl    | Bgl II           | TTATAGCTTATAGGAGATACATGTACGGGCT |
| Pk25CBgl    | Bgl II           | CCG CAG ATC TAT CCG CTG GCC CGA |
| T7prom      | —                | TTAATACGACTCATAATG          |
| T7term      | —                | CTAGTATTATGCTCAGGG          |
| APH 146-1(+) | —                | GCTGTCGCTACAGGGAGGAGGTAGCTTAGAG |
| APH 146-1(−) | —                | GTCCAGATCTCCAGCGTCATGTAGCGACG |
| APH 146-2(+) | —                | GCTGTCGCTACAGGGAGGAGGTAGCTTAGAG |
| APH 146-2(−) | —                | CTGTCGCTACAGGGAGGAGGTAGCTTAGAG |
| APH 146-T(+) | —                | GTCGTCGCTACAGGGAGGAGGTAGCTTAGAG |
| APH 146-T(−) | —                | CTCCAGATCAGGACTCCCCTCAGGACG|
| AphN        | NdeI             | TTATAGCTTATAGGAGATACATGTACGGGCT |
| AphC        | BamHI            | TTTTTGATTCGTCAAAGAAGATCGTCAGCG |

**Restriction sites are given in **bold**, nucleotide substitutions are underlined.
of aminoglycoside antibiotic kanamycin and IPTG as the inductor. The growth of colonies was monitored after 25 hours of incubation at 37°C.

**Determination of activity of protein kinase inhibitors in the bacterial test system.** Inhibitor activity was determined using paper discs. The paper discs soaked with antibiotic or an antibiotic/inhibitor mixture were placed on the surface of agar plates, and the size of the zone of bacterial growth suppression was measured. Test system: *E. coli* BL21(DE3)APHVIII/Pk25. Bacteria grown on agar plates supplemented with ampicillin were washed off into a liquid LB-medium and grown overnight at 37°C. Then the cells were pelleted (4,000 rpm, 10 min) and re-suspended in a liquid M9 medium. Bacterial suspension was mixed up in a 1:1 ratio with a melted M9-agar medium containing ampicillin and IPTG as the inductor. The mixture was poured onto Petri dishes with a M9-agar medium containing ampicillin and IPTG. Ampicillin is necessary for maintaining the plasmid in the *E.coli* cells. Paper discs containing kanamycin or kanamycin plus the kinase inhibitor were placed on the surface of the agar plates and incubated for 16 hours at 37°C.

**Modeling of Pk25 catalytic domain structure.** X-ray structures of kinase from *M. tuberculosis* PknB (PDB entries PDB [28]: 1MRU [29], 10Y [30], 2FUM [31], 3F61 [32], 3F69 [32]) were used as templates for the model building. Amino acid sequences of the template protein were extracted directly from structural files; the sequence of *S. coelicolor* A3(2) was taken from GenBank (access code 21223157 [33]). The catalytic domain was annotated according to homology. Alignment of amino acid sequences was carried out using the ClustalX 2.0.11 [34] software. The modeling was performed with the Modeller 9v5 [35] program. Thirty-five models of the catalytic domain were generated, and each of them was optimized by simulated annealing. The best model was selected according to the DOPE scoring function (Modeller software) and PROCHECK validation score [36]. Further optimization was performed in SYBYL 8.0 [37]; all hydrogen atoms were added to the models, and the energy was minimized in the Tripos force field [38] by the Powell method.

**Modeling of the APHVIII catalytic domain structure** was based on the X-ray structure of its closest homologue APH(3’)-Ila (PDB entry 1ND4 [39], 36% identity of amino acid sequence) in complex with kanamycin. The modeling method is similar to the one described above: the only difference was that 50 models were generated in each case.

**Docking of inhibitors** into the Pk25 model was performed using the Autodock 4.1 [40] software. The structures of the inhibitors were drawn by SYBYL 8.0 and optimized with the MMFF94 force field [41]. Docking preparation was carried out in MGLTools 1.5.4 [42] according to standard recommendations. Generation of the grids and docking were performed using default parameters, while the position of the docking grid was chosen to include all essential amino acid residues of the ATP-binding site. During docking of each ligand, 100 runs of the genetic algorithm were performed. Docking results were grouped into clusters using a threshold value of RMSD equal to 2.0 Å. The results were analyzed in MGLTools 1.5.4.

**RESULTS**

**Cloning and comparison of full-size genes of serine/threonine protein kinase pK25 from *S. coelicolor* and pK25 *S. lividans.*

According to genome sequencing data, the gene of the pk25 kinase from *S. lividans* TK24 (ACEY01000000) and the gene of pK25 kinase from *S. coelicolor* share 99.8% homology and differ only in 6 base pairs, including an insertion of C at position 664. The presence of this insertion leads to the shift of the reading frame in the catalytic domain sequence. In order to compare the kinases of interest from our collection of *S. coelicolor* A3(2) and *S. lividans* TK24 (66) strains, we cloned and sequenced the genes of these kinases. To isolate pK25 kinase genes from the genomes of *S. coelicolor* and *S. lividans,* two oligonucleotides containing HindIII and EcoRI were synthesized (pK25EN, homologous to the N-terminal part of the gene; and Pk25C, complementary to the C-terminal part of the gene, Table 1). Amplification from total DNA of *S. coelicolor* and *S. lividans* yielded the needed DNA fragments, which were cloned into the pET32a vector at the HindIII and EcoRI and introduced into *E. coli* DH5a cells. Sequencing verified that the obtained DNA fragments contained the pK25 gene. Comparison of the genes from *S. coelicolor* and *S. lividans* revealed nucleotide substitutions at positions 123, 237, 279, 435, and 963 starting from the first ATG of the structural region of the *S. lividans* pK25 gene. These nucleotide substitutions do not lead to amino acid substitutions in the corresponding proteins. Therefore, the amino acid sequences of the full-size pK25 genes from *S. coelicolor* and *S. lividans* were shown to be identical.

**Cloning and expression of the *S. coelicolor* Pk25 catalytic domain nucleotide sequence into pET22b vector.**

Two oligonucleotides (Pk25CN and Pk25CC) containing NdeI and HindIII were synthesized for the cloning (Table 1). The fragment obtained during amplification was digested with the relevant restriction endonucleases and cloned into the pET22b vector that was introduced into *E. coli* DH5a cells. After resequencing, the obtained vectors were introduced into the *E. coli* BL21(DE3) strain used for protein overexpression.
In order to study the expression of the catalytic domain of Pk25 kinase in *E. coli* cells, we set up gel electrophoresis of a fraction of soluble cellular proteins under denaturing conditions. A protein fraction from *E. coli* BL21(DE3) transformed with an empty pET22b vector were used as a control. Electrophoresis showed that *E. coli* cells transformed with the plasmid carrying the gene of the pk25 catalytic domain containing an additional protein fraction of 28 kDa (Fig 1a) as compared with the control cells. This value coincides with the calculated molecular weight (27.8 kDa) of the catalytic domain of Pk25 kinase from the *S. coelicolor* A3(2) strain. Scanning revealed that the additional fraction represents about 3.5% of the total cellular protein.

**Modeling of the Pk25 catalytic domain 3D structure.**

The key structural features of Pk25 are revealed by the model, which is very close to the structure of the template protein PknB *M. tuberculosis* due to the close similarity of the sequences (Fig. 2a). The net charge of the catalytic domain is equal to -3. The most obvious differences between the model and the template are observed in the region of helix C (an insertion of four amino acid residues in Pk25), in the loop between fragments β4 and β5 (deletion of four amino acid residues in Pk25), and in the region of the η3 helix (deletion of five amino acid residues in Pk25). Conformation of the activation loop differs from that in the template due to its flexibility. However, the mentioned differences do not affect the orientation of amino acid residues in the ATP-binding site. There are five amino acid residues in the ATP-binding pocket of Pk25 that differ from the corresponding residues of PknB (Fig. 2b): Val72Ile, Ile90Met, Tyr94Leu, Met145Leu, and Met155Thr (numeration of the PknB sequence is used). The former three substitutions are relatively conserved and should not seriously affect the interaction with the inhibitor. Substitution Tyr94Leu is located in the hinge region, so ligands interact with the backbone of this residue but not with its sidechain. The latter two substitutions are not conservative and, therefore, should influence the interaction with the inhibitor. In particular, these substitutions increase the accessible volume of the binding pocket. Finally, an additional hydroxy group appears in this region due to the introduction of a threonine residue at position 155.

**Analysis of autophosphorylation of the Pk25 activation loop.**

A protein fraction of the Pk25 catalytic domain was isolated from lysed *E. coli* cells by means of chromatography on a His-binding resin under either native conditions or in the presence of 8M urea. After electrophoresis, we were able to analyze its intracellular localization and autophosphorylation *in situ*. The catalytic domain of Pk25 was absent in the fraction of salt-soluble cellular proteins. This recombinant protein is present in the fraction of insoluble cellular proteins and can be dissolved in the presence of urea, as was done. After separation by electrophoresis, the molecular mass of the studied fragment was determined as equal to 28 kDa, which is very close to the calculated value. In the presence of [γ-32P] ATP, the protein accumulates labeled phosphate (Fig. 1b). Separation of the protein components by gel electrophoresis excludes any possible interfering influences of other proteins. Over all, the obtained data indicate that the isolated catalytic domain of Pk25 kinase is an enzy-
matically active protein and undergoes autophosphorylation. Localization of the Pk25 catalytic domain in the fraction of insoluble cellular proteins does not contradict the possibility of its autophosphorylation during expression and, likewise, does not exclude its activity towards both soluble and insoluble proteins. These results are in agreement with the data obtained on the catalytic domains of *Streptomyces* and *Mycobacterium* protein kinases [43-45]. It was shown that phosphorylation in the activation loop of STPK occurs at a Ser residue. Analysis of the substrate specificity of STPK from *M. tuberculosis* showed that the highest phosphorylation efficiency was observed at the regions that are similar to the autophosphorylation sites of the kinase [46].

**Modeling of the aminoglycoside phosphotransferase VIII 3D structure**

The model of the APHVIII catalytic domain structure closely resembles the template protein APH(3')-IIa (Fig. 3a). The two structures are most similar in the sites of the ATP and kanamycin binding, as well as in the activation loop. The small insertions in the APHVIII sequence are located in the structurally nonconserved regions of the loops, and between various elements of the secondary structure they are unlikely to seriously affect the 3D pattern of the polypeptide chain. The physico-chemical properties and conformations of the crucial amino acid residues that form the ATP and kanamycin binding sites also correspond within the template and
The structure of APHVIII146-1 almost fully matches that of wild-type protein. Differences are observed in the activation loop and are unlikely to affect the global conformation of the protein (Fig. 3b).

The phosphorylation site of APHVII (Ser146) is an homologue of phosphoserine in the ribose pocket of PKA-type serine/threonine kinases [47]. A molecular dynamics simulation of unphosphorylated APHVIII in complex with kanamycin (with bound ATP and two Mg\textsuperscript{2+} ions) revealed pronounced alterations of the enzyme’s structure, such as weakening of the contact between the N- and C-terminal domains. [48, 49].

Modification of Ser146 region of aminoglycoside phosphotransferase APHVII – the site of phosphorylation by Pk25 kinase

The autophosphorylation site in the Pk25 activation loop was determined through a comparison with the corresponding region of \textit{M. tuberculosis} PknB [50]:

PknB DFG I ARAIAD SGN\textsuperscript{146}VTQ\textsuperscript{146}TAAGTATQYL\textsuperscript{146}SPE

Pk25 DFGV AQV\textsuperscript{146}A TTT\textsuperscript{146}TESGS\textsuperscript{146}FVG\textsuperscript{146}SPE\textsuperscript{146}YTAPE

To optimize the test system \textit{E.coli} APHVIII/Pk25, we modified the potential phosphorylation site AVAEG\textsubscript{146}S\textsubscript{146}VDLED in the APHVIII activation loop. The objective was to make site Ser146 APHVIII more structurally similar to the Pk25 autophosphorylation site TTT\textsuperscript{146}TESGS\textsuperscript{146}FVG. To achieve that, we modified the amino acid residues in the vicinity of APHVIII Ser146 (Table 2), introducing the underlined amino acid substitutions. The obtained mutant variants of the gene \textit{aphVIII}146-1, \textit{aphVIII}146-2, \textit{aphVIII}146-3, and \textit{aphVIII}146-4 were ligated into the pET16b vector at the NdeI-BamHI and introduced into \textit{E.coli} DH5a. After resequencing, the plasmids pET16baphVIII\textsubscript{146}m1 (Fig. 4a) were used for the transformation of \textit{E. coli} BL21(DE3).

Expression of all variants of APHVIII in \textit{E.coli} was checked by gel electrophoresis of soluble cellular proteins under denaturing conditions.

\textit{E.coli} cells containing pET16baphVIII plasmid expressed a 31 kDa protein, which corresponded to the calculated molecular mass of APHVIII equal to 31.5 kDa.

Creation of a construct containing genes of aminoglycoside phosphotransferase \textit{aphVIII} and protein kinase \textit{pk25}

Amplification of \textit{pk25} was performed with the primers Pk25\textit{NBgl} and Pk25\textit{CBgl} (Table 1) from the DNA of the plasmid vector pET22b-pk25. The Pk25\textit{NBgl} primer contained a ribosome-binding site (RBS) and an ATG codone for the catalytic domain of protein kinase Pk25. The nucleotide sequence of \textit{pk25} was amplified and digested by BglII and then ligated into the pET16baphVIII\textsubscript{146}m1 containing the previously described variants of \textit{aphVIII} at the BamHI. At the first stage, the presence of the insert was checked by amplification with T7 primers. At the second stage, the clones were selected by amplification with \textit{AphN} and Pk25\textit{CC} primers (table 1) according to the presence and the size of the insert. After resequencing, pET16APC plasmids (Fig. 4b) were used for the transformation of \textit{E. coli} BL21(DE3). Expression of genes \textit{aphVIII} and \textit{pk25} after induction was checked by gel electrophoresis. In the first four variants (Fig. 5) (lanes 2-5), additional fractions of APHVIII are observed. Lane 6 contains an additional fraction of \textit{pk25}, while lanes 7-10 contain both a 31.5 kDa fraction and a 28 kDa fraction, which are APHVIII and Pk25, respectively. Mass-spectrometry was used to verify the protein fraction on lane 7. It was shown that the heavier fraction contains APHVIII (AAY27879.1 aminoglycoside-O-phosphotransferase VIII). The other fraction contains the catalytic domain of Pk25 (1100219).

| Native enzymes and modified species of APHVIII | Phosphorylation sites and their modifications* |
|-----------------------------------------------|-----------------------------------------------|
| Pk25                                         | ATTLTESGSFVG                                  |
| APHVIII                                      | AVAEG\textsubscript{146}S\textsubscript{146}VDLED |
| APHVIII146-1                                 | AVA\textsuperscript{146}G\textsuperscript{146}T\textsuperscript{146}VSLED |
| APHVIII146-2                                 | AVA\textsuperscript{146}G\textsuperscript{146}T\textsuperscript{146}VS\textsuperscript{146}D |
| APHVIII146-3                                 | AVAEG\textsuperscript{146}T\textsuperscript{146}VDLED |
| APHVIII146-4                                 | AVAEGA\textsuperscript{146}VDLED |

*Amino acid substitutions are underlined.
NP_628936.1 serine/threonine protein kinase *S. coeli*-color A3(2)).

**Analysis of kanamycin resistance in *E.coli* BL21(DE3) variants containing different modifications of *aphVIII* and their combinations with *pk25***.

Resistance of all constructs to aminoglycoside antibiotic kanamycin was studied (Table 3). The BL21(DE3) strain containing the plasmid pET16b encoding gene *aphVIII* was resistant to kanamycin (325 μg/ml).

The substitutions contained in the APHVIII146-1 variant led to a 48% decrease in resistance. In APH-VIII146-2, resistance underwent a 54% decrease. The level of resistance in APHVIII146-3 containing the Ser(146)Thr substitution remained unchanged. In the case of the Ser(146)Ala substitution (APHVIII146-4), which causes the full inactivation of phosphorylation at Ser146, a 70% decrease in kanamycin-resistance was observed. The level of activity of APHVIII146-4 in *vivo* corresponds to the obtained data - the mutant variants exhibited a level of activity equal to 30% of that in wild type [51]. All constructs containing APHVIII with *pk25* showed a higher level of activity. We observed a 91% increase in kanamycin resistance in the case of APHVIII146-4/Pk25, an 83% increase in the case of APHVIII146-2/Pk25, and a 23% increase in the case of the initial construct APHVIII/Pk25, as well as in that with the Ser(146)/Thr substitution.

**Docking of indolylmaleimide inhibitors into the Pk25 model**

The constructed test system *S. lividans* APHVIII+ has been used earlier for the screening of various chemical substances such as benzodiazines, benzophtalazines, cyclopentendions, indolylmaleimides, pyrazoles, thiazoles, thiazoltetrazines, etc. (unpublished data). A number of indolylmaleimide compounds that exhibit inhibitory activity towards protein kinases were identified. In order to propose the binding mechanisms of these substances, we carried out a molecular docking study. The results suggest that the inhibitors selected in the *S. lividans* TK24 (66) APHVIII/STPK test system (LCTA-1385, LCTA-1398, LCTA-1425 [20], bis-indolylmaleimide-1 [52]) can potentially interact with the ATP binding site of Pk25.

Docking of these inhibitors into the Pk25 model reveals conservative interactions between the maleimide...
moiety and the backbone of the kinase (Fig. 2c). The inhibitors bis-indolylmaleimide-1 (Bis-I) and LCTA-1425 form two hydrogen bonds – one between the carbonyl oxygen atom of the maleimide moiety and the amide hydrogen of Val96, and another one between the imide hydrogen atom of the maleimide moiety and the carbonyl oxygen of Glu94. Inhibitors LCTA-1385 and LCTA-1398 were shown to form only the former hydrogen bond, since the hydrogen atom of the maleimide moiety is substituted in their molecules. Therefore, the estimated energy of interaction between Pk25 and the latter two inhibitors is 1 kcal/mol less than that for the first two compounds. However, in both cases the interaction between the kinase and inhibitor is considered favorable.

Choice and validation of E.coli APHVIII/Pk25-based test system

Earlier we constructed and validated [20, 53, 54] a test system based on S. lividans TK24 (66) APHVIII/STPK [20]. The effect registered in this system was based on the cumulative action of the antibiotic kanamycin and a STPK modulator in a sub-inhibiting concentration [20] that resulted in the appearance of or increase in a zone of growth inhibition of the indicator culture. The size of the no-growth zone allowed to roughly estimate the efficiency of the STPK inhibitor [20]. That is why the range of changes of the resistance level to kanamycin, which is determined by various constructions of APHVII, is of crucial importance. Based on this consideration, APHVIII146-1 is more favorable; so, in fur-

Table 3. Kanamycin resistance of E. coli BL21(DE3) strain containing various APHVIII species.

| Name   | Modified constructs APHVIII | Kanamycin resistance of E. coli, μg/ml |
|--------|------------------------------|---------------------------------------|
|        |                              | APHVIII             | APHVIII+Pk25          |
| 146-S  | AAVAEG S₁₄₆ VDLED            | 325±5                 | 400±10                 |
| 146-1  | AVATG T₃₆ VSLED             | 170±10                | 325±5                 |
| 146-2  | AVATG S₁₄₆ VSLSD            | 150±10                | 275±10                |
| 146-3  | AAVAEG T₃₆ VDLED            | 325±5                 | 400±10                |
| 146-4  | AAVAEG A₁₄₆ VDLED           | 100±5                 | -                     |
ther studies we used *E. coli* APHVIII146-1/Pk25 cells. For test system validation, we employed the previously described indolylmaleimide STPK inhibitors LCTA-1385, LCTA-1398, LCTA-1425 [20], and Bis-1 [52] (Table 4). The standard concentration of kanamycin was 5 mg/disc, causing the appearance of a 10-mm zone of growth inhibition. All investigated compounds lowered kanamycin resistance. Substances from the indolylmaleimide library (LCTA-1033, LCTA-1196, Bis-5) that did not exhibit inhibitory activity in *S. lividans* TK24 (66) APHVIII/STPK [20] showed no effect in the *E. coli* APHVIII146-1/Pk25-based test system that confirms the relevance of the latter.

**DISCUSSION**

**Perspectives of application of *E. coli* APHVIII146-1/Pk25-based test system in screening for STPK inhibitors**

The proposed test system can be used in prescreening for ATP-competitive low molecular weight inhibitors [2] that are able to diffuse in an agar-based medium, permeate through the *E. coli* cell wall, and interact with the adenine binding site of Pk25. The selectivity of the inhibitors depends on their affinity to adenine binding pocket of the kinase. Functional similarity of the amino acid sequences leads to the similarity of the three-dimensional structures. This is also applicable in the case of the ligand binding sites that are responsible for the selectivity of inhibitors [55, 56].

The alignment of the amino acid sequence of the Pk25 catalytic domain with the catalytic domains of other bacterial STPKs, including those from pathogenic microorganisms performed in Genomic BLAST (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi), revealed 13 proteins that share more than 35% identity. Among them are STPKs from *Mycobacterium*, *Staphylococcus*, *Streptococcus*, and *Pseudomonas*. Comparison of Pk25 with human STPKs revealed 19 proteins with more than 30% identity, including kinases from the SAD, BR, NUAK (SNF), DAPK3, PNCK, CAMKII, CAMKI, Zip, PKA, HUNK, PAK2, Mark-PAR1, SIK2, and OPK NimA families.

STPK inhibitors compete with ATP for the binding site and interact with the adenine binding pocket of the ATP binding site, which contains conservative and variable amino acid residues. We classified STPKs of gram-positive bacteria [23, 57] based on the physico-chemical properties of the sidechains of 9 variable amino acids in

### Table 4. Dependence of *E. coli* APHVIII146-1/Pk25 kanamycin resistance on various STPK inhibitors.

| Known indolylmaleimide inhibitors of STPKs. | Structure | Subinhibiting concentration, nmol/disc | Inhibition zone in *E. coli* APHVIII146-1/Pk25 test-system In the presence of kanamycin and inhibitor, mm |
|--------------------------------------------|-----------|----------------------------------------|--------------------------------------------------|
| Bis-1                                      | ![Bis-1](image1) | 700                                    | 13.0                                              |
| LCTA-1425                                  | ![LCTA-1425](image2) | 125                                    | 12.0                                              |
| LCTA-1398                                  | ![LCTA-1398](image3) | 250                                    | 13.0                                              |
| LCTA-1385                                  | ![LCTA-1385](image4) | 125                                    | 12.0                                              |

*Note.* Kanamycin-caused inhibition (5 mg/disc) zone size is 10 mm. Kanamycin resistance of *E. coli strain* containing APHVIII146-1 and Pk25 was determined by means of the paper disc method as described in “Experimental procedures”. Indolylmaleimides of LCTA series, provided by Prof. M.N. Preobrazhenskaya, were described earlier [20].
Table 5. Ligand-binding amino acid residues of adenine-binding pocket of bacterial and human serine-threonine protein kinases.

| Protein kinase                        | Functions of kinases in pathogenesis and physiology                                                                 | Amino acids of the binding pocket | Effect of substitution |
|--------------------------------------|---------------------------------------------------------------------------------------------------------------------|-----------------------------------|-----------------------|
| **Bacterial STPKs**                  |                                                                                                                     |                                   |                       |
| Pk25 *S. coelicolor*                 | Modulation of resistance of aph-gene                                                                             | LVAVMLVL                          | T                     |
| PknA *M. tuberculosis*               | Synthesis of cell wall                                                                                            | IVAILMLVL                          | T                     |
| PknJ *M. tuberculosis*               | Persistence in the host                                                                                            | LVAIMFVL                           | S                     |
| Stk1 *Streptococcus agalactiae*      | Gerulation of intracellular segregation of GBS and virulence                                                      | IVAIMYVL                           | T                     |
| StkP *S. pneumonia*                  | A fragment of signal pathway involved in lung and blood invasion                                                 | IVAIMYVL                           | T                     |
| SP-STK *S. pyogenes*                 | Cell division, colony morphology, virulence                                                                      | IVAIMYVL                           | T                     |
| PpkA *P. aeruginosa*                 | Regulation of expression of virulence factors                                                                     | LVAMYL                              | S                     |
| PknB/Stk1 *Staphylococcus aureus subsp. aureus* | Regulation of purine biosynthesis, autolysis, core metabolism pathways                                              | LVAIMYL                            | E                     |
| PknB *M. tuberculosis*               | Cell division, inhibition of lysosome fusion                                                                     | LVAIMYVM                           | M                     |
| **Homo sapiens STPK**                |                                                                                                                     |                                   |                       |
| PKA *H. sapiens*                     | Allergy, myocardial disorders                                                                                     | LVALMYVL                           | T                     |
| CaMK ID *H. sapiens*                 | Type II diabetes                                                                                                  | LVAMIL                               | S                     |
| Pac2 *H. sapiens*                    | UV-caused epidermis diseases                                                                                      | IVAIMYLL                           | T                     |
| BR kin1 *H. sapiens*                 | Regulation of cellular homeostasis                                                                                | LVAMLHV                            | A                     |
| NUAK SNF1-11 *H. sapiens*            | Modulation of TNF-alpha in cancer cells                                                                          | LVAIMYAL                           | A                     |
| CaMKII *H. sapiens*                  | Induction of long-termed synaptic memory                                                                         | LVAIMYAL                           | A                     |

Note. Table represents serinen-threonine kinases containing no more than four amino acid substitutions in ligand-binding sequence LVAVMLVLVT Pk25. Non-essential amino-acid substitutions are marked (–), while essential substitutions are marked (=), substitutions in the gatekeeper region are marked (§). Selectivity of inhibitors is determined by their affinity to the 9 amino acid motifs of the adenin-binding pocket of STPKs.

the adenine binding pocket of the catalytic domain. In this work, we used this classification to select STPKs of pathogenic bacteria that can be inhibited by the compounds selected in the proposed *E. coli* APHVIII/Pk25 test system. Table 5 contains 9 of the 13 potential ligand binding motifs of STPKs from pathogenic bacteria and 6 of the 19 motifs of human STPKs. The selection was based on the presence of no more than 4 out of 9 amino acid substitutions in variable positions of the adenine-binding pocket of Pk25 *S. lividans* (*S. coelicolor*) LVAVMLVLVT. Substitutions of nonpolar amino acids by polar ones at the first four positions, as well as in position 8 (double underlined), were considered essential. All substitutions (except for the introduction of a similar amino acid) in position 9 (double underlined), and any substitutions in position 5 gatekeeper (underlined), were also considered essential. Substitutions in the hinge region (position 7 and in position 6) are less essential. Nonessential substitutions were underlined. The presence of two out of four nonessential substitutions does not alter the interaction mode of inhibitors with the adenine binding site of the protein kinase. Therefore, Pk25 can serve as a tool for the selection of inhibitors for 5 of the 13 STPKs from pathogenic bacteria and 3 of the 19 human STPKs.
The principle of structural similarity of the adenine binding pockets is a more reliable criterion than homology of the sequences of the whole catalytic domain.

In conclusion, the proposed test system can be used in prescreening for inhibitors of STPKs from some pathogenic microorganisms such as PknA, PknJ from M. tuberculosis, StkP from S. pneumonia, SP- STK from S. pyogenes, as well as some human STPKs, e.g. PKA, CaMkinase1, and Pac2.

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