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# $F_oF_1$-ATP Synthase of *Streptomyces fradiae* ATCC 19609: Structural, Biochemical, and Functional Characterization

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**Abstract**—The patterns of protein phosphorylation in inverted membrane vesicles from the strain *Streptomyces fradiae* ATCC 19609 were investigated to elucidate the mechanisms of regulation of bacterial membrane bound $F_oF_1$-ATP synthase. We found for the first time by two-dimensional gel electrophoresis and mass spectrometry that the β- and b-subunits of the $F_oF_1$-ATP synthase complex undergo phosphorylation; 20 proteins with known functions were identified. All eight subunits of $F_oF_1$-ATP synthase, i.e. α, β, γ, δ, ε, a, b, and c, were cloned into *Escherichia coli* and expressed as recombinant proteins. Using a crude preparation of serine/threonine protein kinases, we demonstrated the phosphorylation of recombinant γ-, β-, α- and ε-subunits. The β-subunit was phosphorylated both as a recombinant protein and in vesicles. Differential phosphorylation of membrane-bound and recombinant proteins can be attributed to different pools of protein kinases in each preparation; in addition, certain steps of $F_oF_1$-ATP synthase assembly and function might be accompanied by individual phosphorylation patterns. The structure of the operon containing all subunits and regulatory protein I was identified. The phylogenetic similarity of $F_oF_1$-ATP synthase from *Streptomyces fradiae* ATCC 19609 with the respective proteins in saprophytic and pathogenic (including *Mycobacterium tuberculosis*) bacteria was investigated. Thus, bacterial serine/threonine protein kinases are important for the regulation of $F_oF_1$-ATP synthase. From the practical standpoint, our results provide a basis for designing targeted antibacterial drugs.

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*Key words*: $F_oF_1$-ATP synthase, Ser/Thr protein kinase, inverted membrane vesicles, *Streptomycetes*

The search of novel drugs for various human diseases is a vital problem of modern biology and medicine. Many studies aimed at creating innovative drugs are based on the data on molecular targets of the new generation of drugs. One of the currently popular biotargets is $F_oF_1$-ATP synthase, a universal molecular machinery that performs ATP synthesis (and, under certain conditions, hydrolysis) in eukaryotic mitochondria, plant chloroplasts, and bacteria. $F_oF_1$-ATP synthase is a multisubunit enzyme consisting of the soluble $F_1$-part catalyzing ATP synthesis from ADP and inorganic phosphate due to rotational movement of the central core of the molecule and the associated $F_o$-part submerged in the membrane of the molecule [1, 2]. The $F_1$-part of bacterial $F_oF_1$-ATP synthase comprises five subunits: α, β, γ, δ, and ε. The $F_o$-part contains three subunits: a, b, and c. This complex is present in eukaryotic mitochondria and bacterial cell wall [3-5].

$F_oF_1$-ATP synthase is involved in maintaining pH level in cells, as well as in the regulation of endocytosis, proliferation, and apoptosis [6, 7]. Drugs developed in recent years can influence the function of $F_oF_1$-ATP synthase of different organisms, in first place human beings and pathogenic bacteria [8, 9]. The activity of $F_oF_1$-ATP synthase can be inhibited not only via the interaction with its own subunits, but also with coupled biotargets in subbacterial inverted membrane vesicles. A novel preparation targeted at the $F_oF_1$-ATP synthase of *Mycobacterium*

*Abbreviations*: a.a., amino acid residue; Bis-I, bis-indolylmaleimide I; bp, base pairs; DTT, dithiothreitol; KN-62, inhibitor of $Ca^{2+}$-dependent protein kinases; PMSF, phenylmethylsulfonyl fluoride; STPK, serine/threonine protein kinases; TCA, trichloroacetic acid.

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*tuberculosis* was developed: the compound bedaquiline, which inhibits mycobacterial $F_oF_1$-ATP synthase and demonstrates high and selective activity *in vitro* and *in vivo* [10, 11]. The difference of actinobacterial $F_oF_1$-ATP synthases from the enzymes of other bacteria, with all their great structural similarity, is the dependence of their activity on $Ca^{2+}$ [12, 13] and sensitivity to oligomycin A [14-17]. The main biotarget is the c-subunit of $F_oF_1$-ATP synthase and its homolog in eukaryotic cells [18]. Antibiotics of the oligomycin family and other human and bacterial $F_oF_1$-ATP synthase inhibitors are considered as potential drugs [19]; however, their advancement as pharmaceuticals is limited by their high toxicity. Russian scientists have obtained for the first time more than 20 semisynthetic oligomycin A derivatives with lower toxicity [20, 21].

The literature gives some indications of phosphorylation of the δ- and c-subunits in eukaryotic mitochondria [22, 23], the β-subunit in the mitochondria of human skeletal muscle [24] and chloroplasts [25], and the β-subunit in *M. tuberculosis* [26]. Phosphorylation of the components of the complex supposedly influences mitochondrial membrane permeability (subunit c) and the structure of the $F_oF_1$-ATP synthase complex (subunit β) and perhaps PDGF-dependent (subunit δ). Previously, we established the phenomenon of enhanced resistance of bacterial cells and $F_oF_1$-ATP synthase as a component of membrane vesicles to oligomycin A because of appearance of an oligomycin A-insensitive form of the $F_oF_1$-complex in cells and assessed the sensitivity of $F_oF_1$-ATP synthases from three streptomycete strains *in vivo* and *in vitro* to inhibitors (oligomycin A, etc.). We showed that the fraction of membrane vesicles of the actinobacterium *Streptomyces fradiae* ATCC 19609 contains at least two active serine/threonine protein kinases (STPK) capable of membrane protein phosphorylation, and their activity is regulated by $Ca^{2+}$ [17]. The $F_oF_1$-ATP synthase operon was constructed and the subunits of proteins encoded by this operon were compared phylogenetically with analogous proteins from different groups of saprophytic and pathogenic bacteria.

The main objective of this work was to study the phosphorylation of proteins of the $F_oF_1$-ATP synthase complex from *S. fradiae* ATCC 19609 using two approaches: (1) as a component of membrane vesicles including membrane-bound STPK and (2) via the phosphorylation of recombinant proteins of all eight subunits obtained in *Escherichia coli* using the total preparation of STPK from *S. fradiae* ATCC 19609.

## MATERIALS AND METHODS

**Bacterial strains, vectors, media, and cultivation conditions.** The strains used in the work were *S. fradiae* ATCC 19609 (the genome was sequenced at the Laboratory of Genetics of Microorganisms, Vavilov Institute of General Genetics, Russian Academy of Sciences; the genome sequence is deposited in GenBank with access number JNAD00000000) and *E. coli* DH5α (F⁻, Φ80 ΔlacZΔM15 Δ(lacZYA-argF) U169) from Promega (USA) [27] and BL21(DE3) (F⁻ *dcm ompT* *hsdS*($r_B^-m_B^-$) *gal* λ (DE3)), and the pET32a plasmid from Novagen (USA) [28]. The strain *S. fradiae* ATCC 19609 was grown in a liquid YEME medium with 25% sucrose (w/v) [29] at 28°C for 24 h (late logarithmic growth); mycelium was harvested by centrifugation at 3000*g* for 30 min. *Escherichia coli* cells were grown in Luria medium (L-broth). Solid media contained 2% agar (w/v) [30]. Ampicillin (100 μg/ml) was added for selective growth of plasmid-carrying cells.

**DNA manipulations.** The total DNA of the strain *S. fradiae* ATCC 19609 was isolated by the method described by Kieser et al. [29]. Plasmid DNA isolation, preparation of a competent *E. coli* culture, transformation, and analysis of recombinant plasmids were carried out by the standard methods [30]. PCR with the total DNA of the strain *S. fradiae* ATCC 19609 was performed with a PCK-100 kit (Dialat Ltd., Russia) using PTC-0150 (MJ Research, USA). Ten oligonucleotides constructed for amplification of the fragments of DNA from all subunits using NCBI/Primer-BLAST (www.ncbi.nlm.nih.gov/tools/primer-blast/) were homologous to the flanking regions of the a-, c-, b-, δ-, α-, γ-, β-, and ε-subunits of $F_oF_1$-ATP synthases present in the NCBI database (http://www.ncbi.nlm.nih.gov/) from the strains of *S. coelicolor*, *S. lividans*, and *S. avermitilis*. The oligonucleotides for cloning purposes were constructed based on sequencing the amplified subunits (Table 1). The amplified subunits were cloned into pET32a expression vector at the sites of restriction endonucleases *Eco*RI and *Hind*III. The resulting recombinant clones were screened by PCR using S·Tag and T7term standard primers.

**Study of $F_oF_1$-ATP synthase gene expression in *E. coli*.** The genes of subunits of $F_oF_1$-ATP synthase from the strain *S. fradiae* ATCC 19609 were expressed in *E. coli* BL21(DE3) cells. For the study of protein expression and for biomass production, *E. coli* cells carrying the constructed plasmids were grown in liquid medium (L-broth) with ampicillin on a shaker at 34°C until the optical density of 0.6 (~2 h); then expression was induced by adding isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. The cultivation was carried out at 28°C for 4 h; the biomass was harvested and suspended in buffer of the following composition: 62.5 mM Tris-HCl (pH 6.8), 5% glycerol (w/v), 2% mercaptoethanol (w/v), 0.1% SDS, bromophenol blue. Then the cells were destroyed by boiling for 10 min. The soluble protein fraction was analyzed by SDS-PAGE. The soluble protein fraction of the strain *E. coli* BL21(DE3) carrying the pET32a plasmid without an insert was analyzed as a control. Recombinant proteins were isolated from extracts

**Table 1.** Oligonucleotides used in the work

| Oligonucleotide | Oligonucleotide structure (5′–3′)* | Restriction endonuclease |
|---|---|---|
| ATPAN | CCATGCGCCACGCTGAAGG | – |
| ATPAC | TGCTCAGTGGTGCTCGGC | – |
| ATPCN | CGGTGGCCAACCCCCAC | – |
| ATPCC | AGGCCGATGACGAGCTCGGGGA | – |
| ATPBN | GGCGGCCGGCCTGATCCGC | – |
| ATPBC | GGCCAGCTCGTCGGCGAGC | – |
| ATPIN | ATGCCGTCCAATGACGTCCG | – |
| ATPIC | CTCCTTCAGCGTGGCGCATG | – |
| Del(+) | CGGCAGCGCGAGGAGATCAT | – |
| Del(–) | GACCTCCTCGCGCGAGGCCG | – |
| Alp(+) | TAGCCTGGAGTCGGGACTC | – |
| Alp(–) | GTGACGGATCGGATGCGACG | – |
| Gam(+) | GCACCACCGGCAAGATGGAC | – |
| Gam(–) | GGAGATGGTGCGGACCAGGC | – |
| Beta(+) | AATCAGCGAGATCGTCGGTGGC | – |
| Beta(–) | GACGTGCAGCTCAGCAGCCA | – |
| Eps(+) | GGCGTTCTTCATGTGCGGTGGC | – |
| Eps(–) | CCGCACACAGTCAGAGCGAG | – |
| ATPFAN | ATCC**GAATTC**GTGAGTGCTGACCCGACAACG | *Eco*RI |
| ATPFAC | CCGC**AAGCTT**GTGGTGCTCTGCGAGAGCG | *Hind*III |
| ATPFCN | ATCC**GAATTC**ATGTCCCAGACCCTTGCTGC | *Eco*RI |
| ATPFCC | CCGC**AAGCTT**ACGAACGGCATGACGAGGCC | *Hind*III |
| ATPFBN | ATCC**GAATTC**GTGAACGTTCTGGTTCACCT | *Eco*RI |
| ATPFBC | CCGC**AAGCTT**GTCGGCCGGCCTCGGCCTTC | *Hind*III |
| DelN | ATCC**GAATTC**ATGAACGGAGCGAGCCGCG | *Eco*RI |
| DelC | CCGC**AAGCTT**GCCGGCCATCCGCCGGGA | *Hind*III |
| AlpN | ATCC**GAATTC**ATGGCGGAGCTCACGATCCG | *Eco*RI |
| AlpC | CCGC**AAGCTT**CTTGCCGGCGGCCGGAACGT | *Hind*III |
| GamN | ATCC**GAATTC**ATGGGTGCCCAGATCCGGGT | *Eco*RI |
| GamC | CCGC**AAGCTT**CCTGTCACTCCCCGCGGTCG | *Hind*III |
| BetaN | ATCC**GAATTC**ATGACCACCACTGTTGAGCCG | *Eco*RI |
| BetaC | CCGC**AAGCTT**CAGAATAACGGGCGTGGATCC | *Hind*III |
| EpsN | ATCC**GAATTC**TTGGCTGCTGAGCTGCACGT | *Eco*RI |
| EpsC | CCGC**AAGCTT**GCGCTTGCTCGCGGCCGCG | *Hind*III |

* Recognition sites for *Eco*RI (GAATTC) and *Hind*III (AAGCTT) restriction endonucleases are in bold.

under native conditions on His-binding Ni-NTA columns (Qiagen, Germany) according to the QIAexpress protocol.

**Membrane vesicle preparations for proteomic analysis.** The mycelium of *S. fradiae* ATCC 19609 was washed three times in 0.1 M Tris-HCl buffer (pH 7.5). The cells were suspended in 10 volumes of buffer containing 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 10% glycerol (w/v), 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.5 mM dithiothreitol (DTT), and protease inhibitor cocktail (Promega, USA), with the addition of lysozyme to final concentration of 1 mg/ml. The resulting suspension was incubated at 37°C for 30 min under continuous stirring. Protoplasts were broken by sonication in a Vibra Cell™ Ultrasonic Processor (Sonics, USA) at 20 kHz three times by 30 s, with a 15-s interval between the treat-

ments at 4°C. Cell debris was precipitated by centrifugation for 20 min at 4°C and 10,000*g*. Vesicles were isolated as follows: supernatant proteins were centrifuged for 10 h at 4°C and 100,000*g*, suspended in the same buffer (without lysozyme), and centrifuged for 16 h at 4°C and 150,000*g*. The precipitates were dissolved in six volumes of the buffer (without lysozyme). Protein concentration was assayed with a Qubit 2.0 fluorimeter (Invitrogen, USA). Membrane vesicles were frozen in liquid nitrogen and stored at −70°C.

**Proteins of membrane vesicles were phosphorylated** for 10 min at 25°C in buffer containing 25 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 5 mM $MnCl_2$, 1 mM DTT, 1 mM EDTA, and 0.1 mM PMSF. The reaction was started by adding ATP to final concentration of 100 μM, containing 10-20 μCi [$\gamma$-$^{32}$P]ATP (5000 Ci/mmol; 186 PBq/mol) (Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences), in the presence of 100 μg of vesicular protein. Proteins were precipitated with five volumes of cold acetone and kept for 2 h at −20°C; the precipitates were harvested by centrifugation for 20 min at 4°C and 20,000*g*. Slightly dried precipitates were dissolved in buffer containing 8.5 M urea, 2% Triton X-100 (w/v), 2.2% ampholytes (w/v), and 5% β-mercaptoethanol (w/v); insoluble aggregates were removed by centrifugation, and the supernatant was used for further separation by two-dimensional electrophoresis.

**Two-dimensional gel electrophoresis (SDS-PAGE)** was carried out according to the method of O'Farrell [31] with minor modifications. The samples for mass spectrometry were prepared by the method recommended by Promega (USA) (In Gel Digest Protocol); mass spectra were obtained by the method recommended by Bruker Daltonics (USA) with a Bruker Daltonics analytic mass spectrometer (UltrafleXtreme MALDI TOF/TOF Ms) (Bruker Daltonics GmbH, Germany) at the Department of Proteomic Research, Orekhovich Research Institute of Biomedical Chemistry, Russian Academy of Medical Sciences. Proteins were identified using Mascot software (www.matrixscience.com). The search was carried out in the NCBI database.

**Membrane vesicles for phosphorylation of $F_oF_1$-ATP synthase subunits.** The strain *S. fradiae* ATCC 19609 was grown in basic liquid nutrient medium prepared as described previously [32, 33] to the late exponential growth phase; the mycelium was harvested by centrifugation and suspended in buffer containing 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 10% glycerol (w/v), 1 mM PMSF, 0.5 mM DTT, 1 mg/ml lysozyme, and 1 μg/ml DNase; protoplasts were obtained by incubation at 37°C for 30 min. The protoplasts were broken by sonication in a domestic ultrasonic disintegrator UZDN-1, U-42 at frequency of 30 kHz for 3 min; cell debris was removed by 20-min centrifugation at 4°C and 10,000*g*. Membrane vesicles were precipitated by 120-min centrifugation at 4°C and 180,000*g*, washed with the above buffer, and stored in liquid nitrogen.

**Phosphorylation of $F_oF_1$-ATP synthase subunits.** The subunits were phosphorylated for 10 min at 28°C in a buffer containing 50 mM Tris-HCl, 100 mM NaCl, 5 mM $MgCl_2$, 5 mM β-mercaptoethanol, 0.1 mM PMSF, 0.01% Tween-20, 10% glycerol (w/v) (pH 7.8), and 0.5 mM [$\gamma$-$^{32}$P]ATP (5000 pulses/min per pmol; Fosfor, Russia), in the presence of 100 μg/ml protein.

**Total STPK preparation from *S. fradiae* ATCC 19609** was isolated from bacterial extracts by chromatography on Cibacron Sepharose [34].

**ATPase activity in the membrane vesicles of *Streptomyces fradiae* 19609** was analyzed by the release of $^{32}P_i$ after its separation from [$\gamma$-$^{32}$P]ATP bound with Norit A activated carbon as described previously [17]. In all cases, the average values of three independent measurements with root mean square deviations are presented.

**Bioinformatic methods of analysis.** The nucleotide and amino acid sequences of the subunits of bacterial $F_oF_1$-ATP synthases were searched in the nucleotide and amino acid databases available at the NCBI website (http://www.ncbi.nlm.nih.gov/). The sequences were analyzed with BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The subunits were aligned using ClustalW [34] and TCOFFEE (http://igs-server.cnrs-mrs.fr/Tcoffee/tcoffee_cgi/index.cgi) [35]. The alignment was visualized with GeneDoc (http://www.nrbsc.org/gfx/genedoc/) [36]. The percentage of identity of amino acid sequences was calculated using Blastp software [37]. Phylogenetic trees were constructed using MEGA v. 5.1 [38] with the neighbor-joining algorithm [39]. The reliability of topology of the phylogenetic tree was estimated by bootstrap analysis (1000 replicas) [40]. Potential phosphorylation sites were mapped with NetPhos 2.0 (http://www.cbs.dtu.dk/services/NetPhos/) and NetPhosK (http://www.cbs.dtu.dk/services/NetPhosK/).

## RESULTS

**Analysis of ATPase activity of *S. fradiae* ATCC 19609 in membrane vesicles.** Vesicles containing $F_oF_1$-ATP synthase were isolated from *S. fradiae* ATCC 19609; ATPase activity was determined by the cleavage of the γ-phosphoryl residue from [$\gamma$-$^{32}$P]ATP in the presence and absence of STPK inhibitors. The vesicles were preincubated with ATP and with the ATPase and STPK inhibitors, and their ATPase activity was determined in the presence or absence of oligomycin A. The activity of $F_oF_1$-ATP synthase was shown to be 157 ± 19 U/mg protein in the presence of 10 mM $CaCl_2$ and 104 ± 7 U/mg protein of membrane vesicles in the presence of 10 mM $MgCl_2$ (1 activity unit is equal to 1 nmol of phosphate released during 1 min in the presence of 1 mg of protein under standard conditions). The differences in the efficiency of divalent

cations may be accounted for by the differences in the atomic structure of their complex with the nucleotide substrate, influencing conformational changes in the catalytic center of the enzyme; earlier, they were found in ATPases from various *Streptomyces* species [12, 13]. The effects of increasing concentrations of the selective F-type ATPase inhibitor, oligomycin A, on ATPase activity were analyzed; it was shown that the $IC_{50}$ values of oligomycin A for $Ca^{2+}$- and $Mg^{2+}$-dependent ATPase activity in membrane vesicle preparations were 165 ± 23 and 110 ± 11 nM, respectively. Separate experiments showed that dicyclohexylcarbodiimide (the inhibitor affecting the $F_o$-part at a concentration of 0.05 mM) reduces ATPase activity of the studied strain to 7-10% of the initial level. Then the vesicles of *S. fradiae* were preincubated with STPK inhibitors, and their ATPase activity was determined in the presence and absence of oligomycin A. The results presented in Table 2 show that the $Ca^{2+}$-dependent protease inhibitor KN-62 (5 μM) and the ATP-competitive kinase inhibitor bis-indolyl-maleimide I (Bis I) (1 μM) reduce the activity by 25 and 23%, respectively. At the same time, the determined ATPase activity can be almost completely ascribed to $F_oF_1$-ATP synthase, because its specific inhibitors dicyclohexylcarbodiimide and oligomycin A suppress the ATPase activity of membrane vesicles by 83 and 56%, respectively. KN-62 and Bis I enhance the sensitivity of $F_oF_1$-ATP synthase to oligomycin A by 4-6% when added separately and nearly twofold when added together (Table 2). These data suggest that endogenous membrane-bound STPKs are involved in $F_oF_1$-ATP synthase activation and protection from antibiotic and confirm the preliminary data of the same analysis of the phosphorylation and potential regulation of activity of $F_oF_1$-ATP synthase in *S. fradiae* vesicles that we published previously [17].

**Recombinant proteins of subunits of $F_oF_1$-ATP synthase from *S. fradiae* ATCC 19609.** The genes of $F_oF_1$-ATP synthase subunits were isolated from genomic DNA by PCR. The following oligonucleotides were used for DNA amplification: ATPAN and ATPAC for the a-subunit, ATPCN and ATPCC for the c-subunit, ATPBN and ATPBC for the b-subunit, Del(+) and Del(−) for the δ-subunit, Alp(+) and Alp(−) for the α-subunit, Gam(+) and Gam(−) for the γ-subunit, Beta(+) and Beta(−) for the β-subunit, and Eps(+) and Eps(−) for the ε-subunit (Table 1). The start codons of translation and the termination codons were determined by sequencing all of the amplified DNA fragments. The oligonucleotides synthesized for cloning all subunits were homologous to the *N*- and *C*-terminal regions of the subunit genes, respectively, and contained the *Eco*RI and *Hind*III restriction sites (Table 1). The amplified DNA fragments were sequenced and cloned into pET32a expression vector, the linker containing His-Tag and S-tag for protein isolation and purification and the Trx·Tag sequence (109 bp) in the *N*-terminal region. IPTG induction for 1, 2, and 4 h was used to study protein expression. The maximal level of gene expression for all subunits was achieved with 4-h induction. The cloning of δ-, α-, γ-, β-, ε-, a-, c-, and b-subunits in *E. coli* cells carrying the plasmids pET32aAtpH, pET32aAtpA, pET32aAtpG, pET32aAtpD, pET32aAtpC, pET32aAtpB, pET32aAtpE, and pET32aAtpF revealed the presence of additional protein fractions with respective molecular masses of 51, 80, 56, 75, 36, 53, 30, and 43 kDa (Fig. 1). These values correspond to the calculated molecular masses of proteins of the respective subunits plus the protein molecular mass of the whole linker of the plasmid pET32a containing thioredoxin. The recombinant proteins of all subunits were purified on His-bindings Ni-NTA columns and produced in preparative amounts for further study of their phosphorylation.

**Phosphorylation of recombinant subunits of $F_oF_1$-ATP synthase from *S. fradiae* ATCC 19609 by total STPK preparation.** The phosphorylation of recombinant sub-

**Table 2.** Effects of oligomycin A and STPK inhibitors on the activity of ATPase from *S. fradiae* 19609

| Inhibitor | Inhibitor concentration, μM | ATPase activity, U/mg protein* | ATPase activity, % of the control |
|---|---|---|---|
| No inhibitor | – | 163 ± 21 | 100 |
| Oligomycin A | 0.25 | 72 ± 12 | 44 |
| KN-62 | 5.0 | 102 ± 15 | 75 |
| Bis I | 1.0 | 125 ± 10 | 77 |
| Oligomycin A + KN-62 | 0.25 + 5.0 | 63 ± 9 | 38 |
| Oligomycin A + Bis I | 0.25 + 1.0 | 66 ± 8 | 40 |
| Oligomycin A + KN-62 + Bis I | 0.25 + 5.0 + 1.0 | 26 ± 7 | 16 |

* Results of three independent measurements ± S. D. are presented.

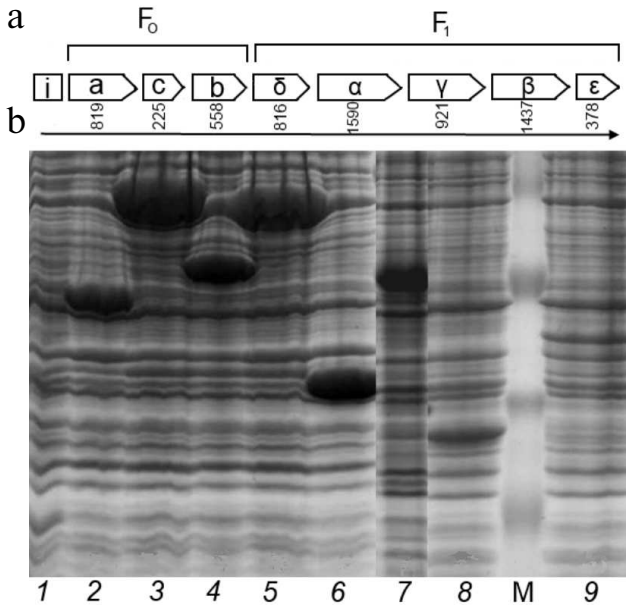


**Fig. 1.** a) Structure of the operon of $F_oF_1$-ATP synthase from the strain *S. fradiae* 19609: I-protein, $F_o$-part (subunits a, c, and b), and $F_1$-part (subunits δ, α, γ, β, ε). b) Electrophoregram of the soluble protein fraction of the strain *E. coli* BL21(DE3). *Escherichia coli* clones containing plasmids: *1*) pET32a (control); *2*) pET32aAtpH (subunit δ); *3*) pET32aAtpA (subunit α); *4*) pET32aAtpG (subunit γ); *5*) pET32aAtpD (subunit β); *6*) pET32aAtpC (subunit ε); *7*) pET32aAtpB (subunit a); *8*) pET32aAtpE (subunit c); *9*) pET32aAtpF (subunit b); M) protein marker SM0441 (Fermentas, Lithuania).

units of $F_oF_1$-ATP synthase was analyzed in the presence of total protein kinase preparation from *S. fradiae* 19609 obtained by affinity chromatography on Cibacron Sepharose. To avoid misrepresentation of results because of endogenous phosphorylation in the preparation of kinases, the latter were preincubated with unlabeled ATP immediately before being added to the working mixture for analysis. Then ATP was removed by gel filtration. The analytic mixture included the labeled ATP and $F_oF_1$-ATP synthase subunits isolated on His Bind resin. The inclusion of radioactive phosphate in the subunits was detected by autoradiography. Figure 2 shows that labeled phosphate is included in the proteins with molecular weights of 75 and 56 kDa, which are thioredoxin-fused β- and γ-subunits of $F_oF_1$-ATP synthase, respectively. Specific labeling of γ-subunit is higher by approximately an order of magnitude compared to that of β-subunit. There is a minor inclusion of the label in proteins with molecular weights of 80 and 36 kDa (α- and ε-subunits). The inclusion of labeled phosphate in other subunits of the $F_o$- and $F_1$-parts of $F_oF_1$-ATP synthase (a, b, c, and δ) was not observed. The absence of inclusion indicates that it is just $F_oF_1$-ATP synthase subunits, but not thioredoxin, that are modified in the recombinant hybrid proteins fused with thioredoxin of α-, β-, γ-, and ε-subunits. This is the first evidence of the fact that α-, β-, γ-, and ε-subunits of the actinomycete complex can be phosphorylated by endogenous STPKs.

**Identification of phosphorylated proteins in the membrane vesicle fraction of *S. fradiae* ATCC 19609.** The vesicles isolated from the culture of *S. fradiae* ATCC 19609 were incubated in the presence of [γ-$^{32}$P]ATP. Phosphorylated proteins separated by 2D electrophoresis are shown in Fig. 3. Mass spectrometry revealed two proteins identical to the β-subunit of the $F_1$-part and the b-subunit of the $F_o$-part of $F_oF_1$-ATP synthase. The other 18 identified proteins can be divided into seven functional groups (Table 3). The functional group of "energy conversion and fatty acid metabolism", in addition to β- and b-subunits, contains proteins probably associated with the function of $F_oF_1$-ATP synthase. The absence of γ-subunit in the preparations might be accounted for by its selective loss when preparing for electrophoresis.

**Determination of operon structure of $F_oF_1$-ATP synthase from strain *S. fradiae* ATCC 19609.** The sequencing of amplified DNA fragments of all subunits showed that the operon of $F_oF_1$-ATP synthase from strain *S. fradiae* 19609 has a standard structure: subunits a, c, and b (the $F_o$-part) and subunits δ, α, γ, β, and ε (the $F_1$-part) (Table 4). Comparative analysis showed that the nucleotide sequences of all subunits of $F_oF_1$-ATP synthase from *S. fradiae* ATCC 19609 have high homology with the genes of the respective subunits from strains of *S. coelicolor*, *S. lividans*, and *S. avermitilis*. The nucleotide sequences of the genes of all subunits of $F_oF_1$-ATP synthase from *S. fradiae* ATCC 19609 are registered in GenBank (KC169996, KC169997, KC169998, KC169999, KC170000, KC170001, KC170002, and KC170003). The operon of $F_oF_1$-ATP synthase from the strain *S. fradiae* ATCC1 9609 is shown in Fig. 1a. The whole genome of *S. fradiae* 19609 was sequenced at the Laboratory of Genetics of Microorganisms (Vavilov

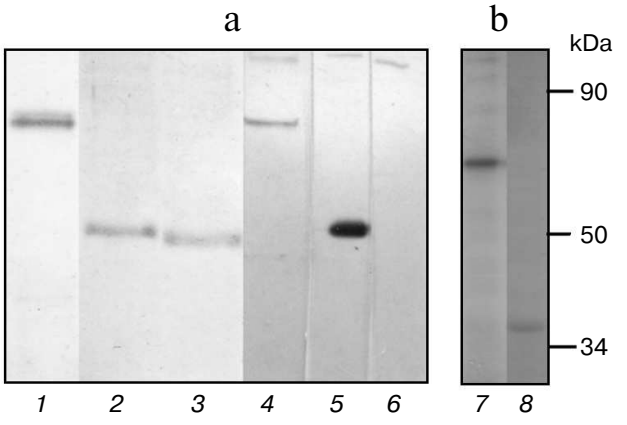


**Fig. 2.** a) Electrophoregram of the gels after electrophoresis of recombinant subunits α (*1*), γ (*2*), δ (*3*). b) Autoradiograph of phosphorylated subunits α (*4*), γ (*5*), δ (*6*), ε (*7*), and β (*8*) of the $F_oF_1$-ATP synthase from *S. fradiae* ATCC 19609.

**Table 3.** Major proteins with established functions identified in the membrane vesicle fraction of *Streptomyces fradiae* 19609 presented in Fig. 3

| Functional group | No.* | Function | Gene | Contig number/ localization** | Molecular mass, kDa | p*I* |
|---|---|---|---|---|---|---|
| 1. Energy conversion and fatty acid metabolism | *1* | $F_oF_1$-ATP synthase β-subunit | *atpD* | 0001 / 207730...209184 | 52.38 | 4.91 |
| | *2* | $F_oF_1$-ATP synthase b-subunit | *atpF* | 0001 / 203744...204301 | 20.23 | 5.16 |
| | *3* | ABC transporter ATP-binding protein | *SCO4240* | 0068 / 16582...17502 | 31.78 | 5.54 |
| | *4* | crotonyl-CoA reductase | *SCO6473* | 0008 / 3364...4992 | 49.27 | 6.88 |
| 2. Carbohydrate metabolism and transport | *5* | isocitrate dehydrogenase | *SCO7000* | 0023 / 44255...46474 | 79.31 | 5.04 |
| | *6* | glycerophosphodiesterase | *SCO1565* | 0015 / 122785...123618 | 30.64 | 7.21 |
| | *7* | glucose-6-phosphate isomerase | *pgi* | 0010 / 68231...69883 | 60.06 | 5.97 |
| | *8* | phosphoenolpyruvate phosphotransferase | *SCO1391* | 0045 / 53716...55386 | 57.36 | 4.89 |
| 3. Repair | *9* | NAD-dependent DNA ligase | *ligA* | 0001 / 334323...336530 | 80.83 | 5.16 |
| 4. Translation, ribosomal structures, and biogenesis | *10* | elongation factor Tu | *SCO1321* | 0026 / 32423...33616 | 43.87 | 5.15 |
| | *11* | 50S ribosomal protein L29 | *SCO4710* | 0026 / 46129...46518 | 13.44 | 4.61 |
| | *12* | amino acid adenylation protein | *SCO2198* | 0004 / 62044...68697 | 232.26 | 5.56 |
| 5. Transport and metabolism of amino acids, biosynthesis of antibiotics | *13* | 1-pyrroline-5-carboxylate dehydrogenase | *SCO5520* | 0102 / 2059...3690 | 58.49 | 5.72 |
| | *14* | sensory transduction protein eryC1 | *SACTE_2966* | 0080 / 4231...5403 | 41.33 | 6.20 |
| 6. Posttranslational modifications and folding | *15* | SAM-dependent methyl-transferase | *SCO0995* | 0001 / 197491...198315 | 30.25 | 5.10 |
| | *16* | GCN5-family acetyltrans-ferase | *SCO0995* | 0039 / 23628...24401 | 28.01 | 5.69 |
| | *17* | SCO4595 oxidoreductase | *SCO4595* | 0010 / 4454...15731 | 44,52 | 6.41 |
| | *18* | GroEL chaperon | *groEL* | 0009 / 98884...100506 | 56.80 | 4.90 |
| 7. Transport and metabolism of inorganic ions | *19* | metal ABC transporter ATPase | *SCO2505* | 0009 / 130411...132894 | 87.07 | 6.74 |
| | *20* | Fe-S cluster for SufB protein assembly | *SCO1925* | 0010 / 48435...49856 | 54.45 | 5.00 |

* Protein numbers indicated in Fig. 3a.
** Localization of nucleotide sequences in the NCBI database for *S. fradiae* 19609.

Institute of General Genetics, Russian Academy of Sciences), and the amino acid sequences were deposited in GenBank (JNAD00000000). The $F_oF_1$-ATP synthase genes were annotated; the nucleotide sequences of all subunits coincided with those sequenced previously.

**Comparative analysis of subunits of $F_oF_1$-ATP synthases from *S. fradiae* 19609, *S. lividans* TK24, and *S. avermitilis* MA-4680.** The genetically best-studied strains *S. lividans* TK24 and *S. avermitilis* MA-4680 were chosen for this experiment. The alignment of amino acid sequences of eight subunits of $F_oF_1$-ATP synthase from strain *S. fradiae* 19609 with subunits from strain *S. lividans* TK24 (oligomycin A sensitive) and strain *S. avermitilis* MA-4680 producing oligomycin A (oligomycin A resistant) showed that subunits a, c, b, α, and β from strain 19609 have high similarity to the analogous subunits from strain TK24, while subunits δ and γ from strain 19609 have high similarity to subunits from strain MA-4680. The sequence of subunit ε is similar in all three strains. The results of comparative analysis of the operon of $F_oF_1$-ATP synthase from *S. fradiae* 19609 with operons of $F_oF_1$-ATP synthases from *S. lividans* TK24 and *S. avermitilis* MA-4680 are given in Table 5.

Comparative analysis of nucleotide sequences located upstream and downstream of the $F_oF_1$-ATP synthase genes on the chromosome showed the presence of I-pro-

tein; the identity (similarity) with the respective amino acid sequences of strains *S. lividans* TK24 and *S. avermitilis* MA-4680 was 64 (77) and 64 (80%), respectively; the nucleotide sequence homology was no more than 35-38%. The neighborhoods of the genes of $F_oF_1$-ATP synthase from *S. fradiae* 19609 completely coincide with the neighborhoods typical of most strains of the genus *Streptomyces* (with the exception of *S. avermitilis* strains): at the 5′-end of the chromosome, immediately before the operon, there are genes of tyrosine phosphatase, serine hydroxymethyl transferase, and transferase; after the operon, there are genes of secreted protein and chitinase C. In *S. avermitilis* strains, the genes for the synthesis of oligomycin A ($F_oF_1$-ATP synthase inhibitor) are located before the operon.

**Phylogenetic analysis of subunits of the $F_oF_1$-ATP synthase operon from strains of genus *Streptomyces*.** Phylogenetic relationships between the $F_1$-part subunits of the $F_oF_1$-ATP synthase operon were established by aligning the amino acid sequences of α-, β-, δ-, and γ-subunits for different representatives of the genus *Streptomyces* and by assessing sequence identity. Four phylogenetic trees were constructed for *Streptomyces* bacteria based on the alignment of amino acid sequences of four subunits (α, β, δ, and γ) of the $F_1$-part of the $F_oF_1$-ATP synthase operon (Fig. 4).

As Fig. 4 shows, the closest neighbors of *S. fradiae* on three trees constructed for subunits β, δ, and γ are *S. violaceusniger*, *S. bingchenggensis*, and *S. cattleya*, in contrast to the tree constructed for subunit α. On the α-subunit tree, the closest neighbors of *S. fradiae* are *S. coelicolor*, *S. avermitilis*, and *S. scabiei*. This suggests that subunits of the $F_1$-part of the $F_oF_1$-ATP synthase operon evolved separately.

**Comparative analysis of subunits of the $F_oF_1$-ATP synthase operon from strains of genus *Streptomyces*.** The alignment of amino acid sequences showed a high degree of conservatism for subunits α and β. The identity of these subunits was 88-99%. Subunits δ and γ were less conservative; their identity was 75-92%. Conservative regions in these subunits cover almost the entire amino acid sequence (Figs. S1-S4 in the Supplement to this paper on the website of the journal (http://protein.bio.msu.ru/biokhimiya), in black). However, the sequence of the γ-subunit (Fig. S4 in the Supplement) of the completely sequenced genome of *S. bingchenggensis* is shorter by 56 a.a. than its homologs from other streptomycete species. It is unknown why this subunit has lost some the amino acids in its *N*-terminal part. Inserts of different length were found in the γ-subunit of plants and the bacterium *M. tuberculosis*. These variations are considered as adaptations of the subunit to the function of ATP synthesis in different organisms [9].

**Comparative analysis of subunits of the $F_oF_1$-ATP synthase operon in actinobacteria.** The analytical methods of bioinformatics were used to compare the eight subunits

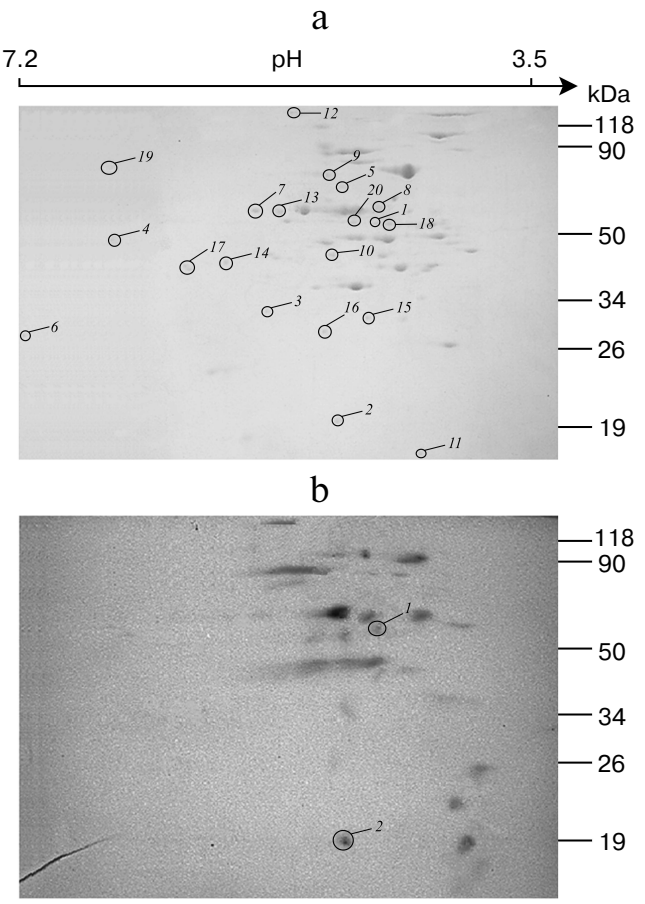


**Fig. 3.** 2D SDS-PAGE of proteins of membrane vesicles from *S. fradiae* 19609. a) Electrophoregram of Coomassie G250 stained gel: *1-20* are the proteins identified by mass spectrometry and presented in Table 3. b) Autoradiogram. Mass-spectrometric identification of the marked polypeptides: *1*) $F_1$ part of $F_oF_1$-ATP synthase, subunit β; *2*) $F_o$ part of $F_oF_1$-ATP synthase, subunit b.

of $F_oF_1$-ATP synthases from different actinobacteria. The results of this analysis showed similarity of operon structure for $F_oF_1$-ATP synthases from all actinobacteria. The alignment of amino acid sequences of subunits α, β, δ, and γ from different species of the type *Actinobacteria* (Figs. S5-S8 of the Supplement) showed that subunits α and β are conservative nearly along the entire length (>70% identity). The most variable regions are observed in subunits α and β only in the *C*-terminal part of the sequence; in subunit β, the first 40 a.a. are variable too.

Subunits δ and γ are less conservative (identity of subunits γ and δ is within the range of 78-33 and 71-30%, respectively). Subunit γ has a region of elongated deletions approximately in the middle of the amino acid sequence. In subunit δ, the region of deletions is located in the *N*-terminal part of the sequence, while identical regions are represented by single conservative amino acid residues located mainly in the center and at the end of the sequence.

It is interesting to note that the amino acid sequences of a-, β-, and γ-subunits from the strains of probiotic anaerobic actinobacteria of the genus *Bifidobacterium* contain additional amino acid regions, so-called inversions: 222-226 a.a. in subunit a; 53-56 and 119-121 a.a in subunit β; and 205-211 a.a in subunit γ. Investigation of the significance of these differences for the function of $F_oF_1$-ATP synthase in human microbiota under anaerobic conditions is a subject for further research.

**Phylogenetic analysis of $F_oF_1$-ATP synthases from actinobacteria.** Four phylogenetic trees were constructed based on alignment of the amino acid sequences of the four subunits α, β, δ, and γ of the $F_1$-part of the $F_oF_1$-ATP synthase operon from different species of actinobacteria (Fig. 5). All the trees reflect evolutionary divergence of the species. The identity percentage of each subunit is indicated for the bacterial genera closest to the genus *Streptomyces* (Fig. 5). The findings show that subunits α and β have been most conservative during evolution (as demonstrated above), because the identity percentage of these subunits is higher compared to subunits δ and γ.

**Comparative analysis of subunits of $F_oF_1$-ATP synthase complex from *S. fradiae* ATCC 19609 and ATPases from pathogenic microorganisms.** The strain *S. fradiae* 19609 is considered as a convenient test system for studying inhibitors of bacterial $F_oF_1$-ATP synthase, including

**Table 4.** Operon structure of $F_oF_1$-ATP synthase from *S. fradiae* ATCC 19609

| *S. fradiae* $F_oF_1$-ATP synthase subunits | Gene length, bp | Protein length, a.a. | Molecular mass, kDa | p*I* |
|---|---|---|---|---|
| $F_o$ a AtpB | 819 | 272 | 30.24 | 8.95 |
| $F_o$ c AtpE | 225 | 74 | 7.41 | 4.78 |
| $F_o$ b AtpF | 558 | 185 | 20.23 | 5.16 |
| $F_o$ δ AtpH | 816 | 271 | 28.94 | 5.54 |
| $F_1$ α AtpA | 1590 | 529 | 57.21 | 5.01 |
| $F_1$ γ AtpG | 921 | 306 | 32.70 | 6.07 |
| $F_1$ β AtpD | 1437 | 478 | 52.38 | 4.91 |
| $F_1$ ε AtpC | 378 | 125 | 13.07 | 5.03 |

**Table 5.** Comparative analysis of the operon of $F_oF_1$-ATP synthase from *S. fradiae* ATCC 19609 with operons of $F_oF_1$-ATP synthases from *S. lividans* TK24 and *S. avermitilis* MA-4680

| *S. fradiae* $F_oF_1$-ATP synthase subunits | Homology with $F_oF_1$-ATP synthase from *S. fradiae* ATCC 19609, % | | | |
|---|---|---|---|---|
| | *S. lividans* TK24 | | *S. avermitilis* MA-4680 | |
| | identity | similarity | identity | similarity |
| $F_o$ a AtpB | 78.8 | 91.2 | 76.1 | 88.3 |
| $F_o$ c AtpE | 92.1 | 96.0 | 85.1 | 90.0 |
| $F_o$ b AtpF | 84.0 | 89.0 | 80.0 | 85.0 |
| $F_o$ δ AtpH | 68.8 | 88.6 | 75.5 | 93.8 |
| $F_1$ α AtpA | 98.7 | 99.8 | 94.3 | 98.1 |
| $F_1$ γ AtpG | 76.1 | 89.0 | 80.0 | 85.0 |
| $F_1$ β AtpD | 89.3 | 96.0 | 86.0 | 93.0 |
| $F_1$ ε AtpC | 83.0 | 92.0 | 84.0 | 92.0 |

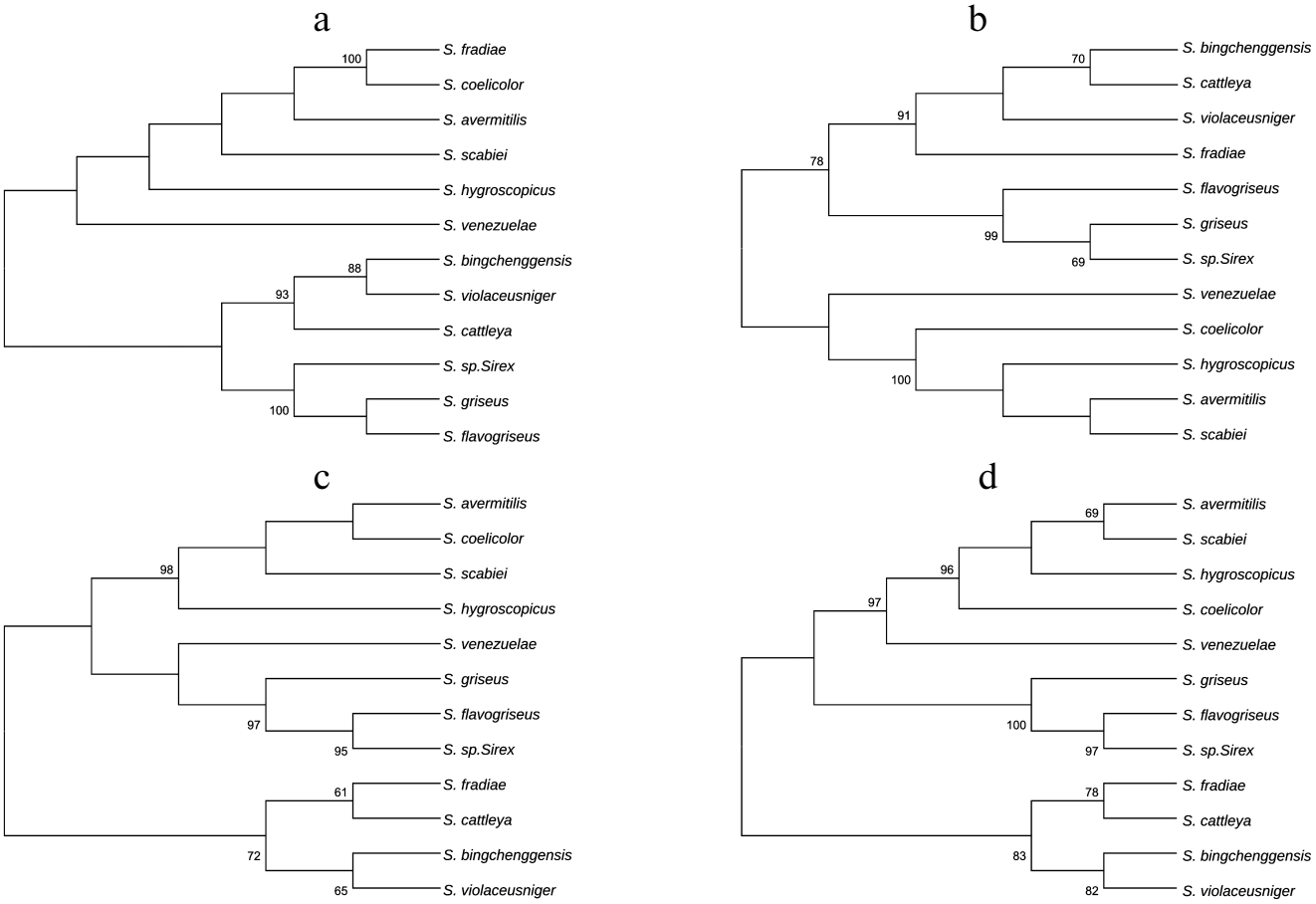


**Fig. 4.** Phylogenetic trees of representatives of the *Streptomyces* genus based on comparison of four subunits of the $F_1$-part of the $F_oF_1$-ATP synthase operon: a) α-subunit; b) β-subunit; c) δ-subunit; d) γ-subunit. The trees were constructed using the MEGA 5 software package by alignment of amino acid sequences using the neighbor-joining (NJ) algorithm and the *p*-distance model. The numerals show the stability of branches calculated for bootstrap NJ analysis (1000 replicas). The branches reproduced in less than 60% bootstrap replicas are marked.

oligomycin derivatives [17, 20, 21]. Therefore, it was interesting to examine the similarity between the proteins of this operon and the respective proteins of some pathogenic bacteria. We aligned the amino acid sequences of eight subunits of $F_oF_1$-ATP synthases from *S. fradiae* with the subunits of $F_oF_1$-ATPases from *M. tuberculosis* (type (division) *Actinobacteria*) and *C. difficile* (type (division) *Firmicutes*). These alignments showed that: (1) subunits α and β remained rather conservative and are aligned along the entire length at 52-70% identity; (2) subunits δ and γ are less conservative, have low homology (25-48%), and are aligned not by the entire length; (3) in subunit ε from *S. fradiae* (the full length of subunit ε is 121 a.a. on average), only a 46-a.a. region was aligned with the analogous region from *M. tuberculosis* and *C. difficile*, with alignment identity of 43 and 35%, respectively; and (4) subunits a, b, and c from *S. fradiae* either are very poorly aligned with the homologs from *M. tuberculosis* and *C. difficile* (~26% identity) and not along the entire length, or there are no similar regions at all; e.g. all other conditions being equal, Blastp did not align whatsoever the amino acid sequence of subunit b from *S. fradiae* with the amino acid sequence of the same subunit from *C. difficile*.

Oligomycin A is known to bind only to certain amino acids in the c-subunit. Table 6 shows the comparison of oligomycin-binding domains of the c-subunit of $F_oF_1$-ATP synthase in oligomycin sensitive and resistant organisms, demonstrating that *S. fradiae* ATCC19609 has the highest similarity with *Homo sapiens sapiens* by these amino acids.

## DISCUSSION

We show for the first time that the recombinant proteins of the γ-, β-, α-, and ε-subunits of the $F_1$-part of the ATPase complex from the bacterium *S. fradiae* ATCC 19609 are phosphorylated by the STPK complex as a component of the cell extract. Two-dimensional gel electrophoresis and mass spectrometry (MALDI-TOF) shows

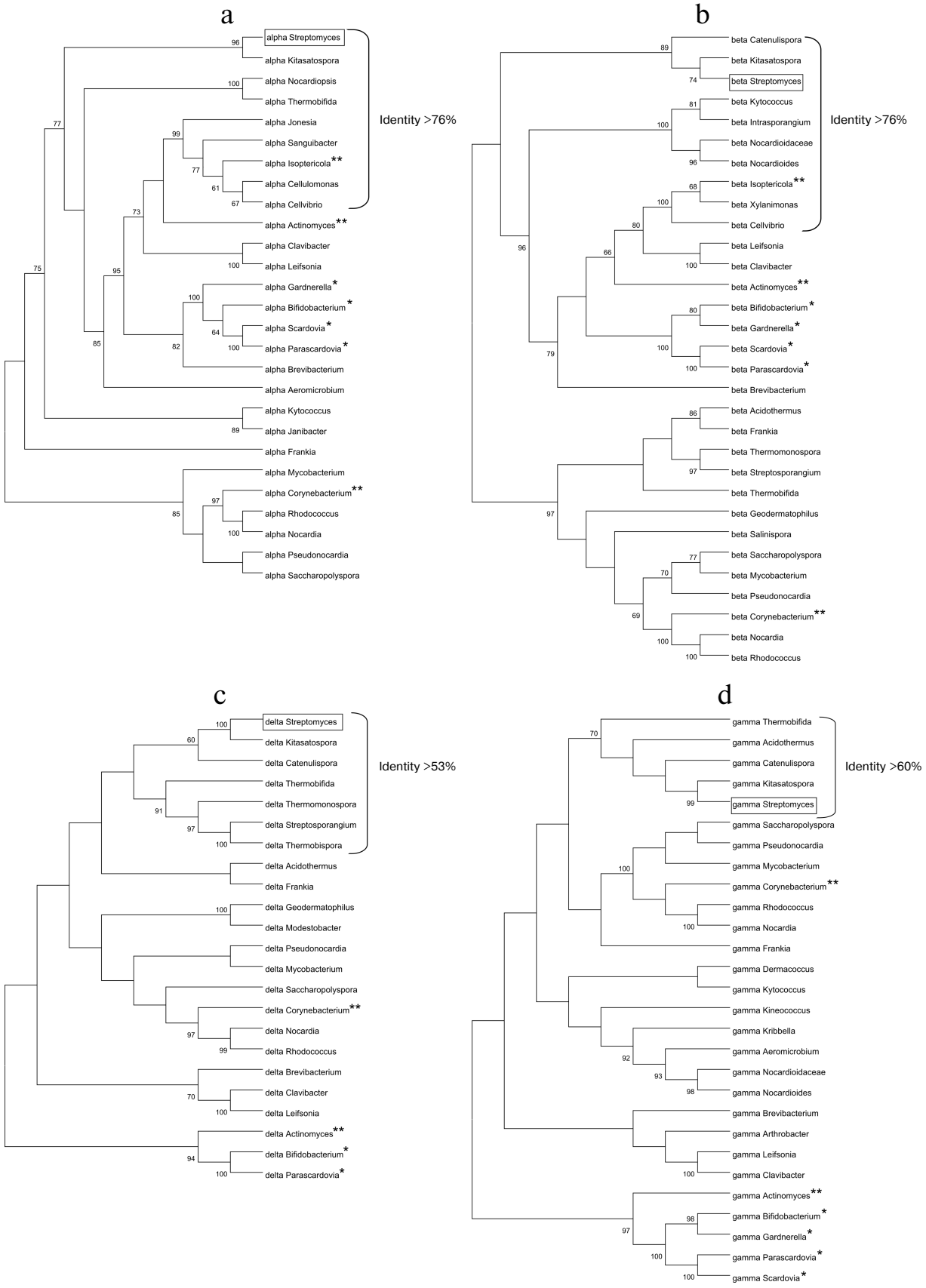


**Fig. 5.** Phylogenetic trees based on the comparison of amino acid sequences of four subunits of the $F_1$-part of the $F_oF_1$-ATP synthase operon of actinobacteria: a) α-subunit; b) β-subunit; c) δ-subunit; d) γ-subunit.

**Table 6.** Comparison of oligomycin-binding domains of the $F_oF_1$-ATP synthase c-subunit in oligomycin sensitive and resistant organisms (the table is taken from Ko et al. [22], with modifications)

| Microorganism | Oligomycin-binding domain in the c-subunit of $F_oF_1$-ATP synthase | | |
|---|---|---|---|
| | first a.a. | amino acid sequence*** | last a.a. |
| *S. fradiae** | 54 | **IL**GFA**F**CE**ALALIGL** | 68 |
| *S. lividans** | 54 | **IL**GFA**F**CE**ALALIGL** | 68 |
| *S. avermitilis*** | 58 | **IL**GF**V**LCE**ALALIGL** | 72 |
| *M. tuberculosis*** | 54 | F**I**TVGLVE**AAYFINL** | 68 |
| *C. difficile*** | 54 | **LL**GV**A**IAESSAIYG**L** | 68 |
| *H. sapiens** | 51 | **IL**GFALSEAMG**LFCL** | 65 |

* Oligomycin A sensitive organisms.
** Oligomycin A resistant organisms.
*** Amino acids involved in oligomycin A binding are in bold.

for the first time the phosphorylation of the β- and b-subunits of the $F_oF_1$-ATP synthase complex in the membrane vesicle fraction. Phosphorylation of the β-subunit is observed in both types of experiments. The phosphorylation of the β-subunit was found in eukaryotic mitochondria [22-24] and plant chloroplasts [25], as well as in *M. tuberculosis* [26]. The ability to phosphorylate γ- and α-subunits in one type and b-subunit in the other type of experiments might be indicative of the importance of phosphorylation of γ- and α-subunits for the assembly of $F_oF_1$-ATP synthase complex and for its functioning at the stage of ATP synthesis or hydrolysis. Differences in phosphorylation conditions between different experiments *in vitro* and *in vivo* might also be significant.

Ser-Thr protein kinases are present and have been characterized in many bacteria; they have diverse functions including viability and virulence in pathogenic bacteria [41, 42]. The sequenced genome of *S. fradiae* 19609 (JNAD00000000) was shown to contain 30 STPKs; some are membrane-bound and can participate in the phosphorylation of $F_oF_1$-ATP synthase complex and associated proteins involved in cell energy supply and regulation of the function of this complex. In recent years, the $F_oF_1$-ATP synthase complex and the coupled proteins from bacteria and human mitochondria have become objects of keener interest as biotargets for drug production [9, 43, 44], as well as key objects of research, since the normal function of this complex is of practical importance for human health and longevity [45-47]. The universal nature of $F_oF_1$-ATP synthase in all living organisms, including bacteria and human beings, and its role as a biological nanomotor, require detailed studies of the function of this complex in all organisms. More profound understanding of the mechanisms of bacterial $F_oF_1$-ATP synthase functioning, the role of STPKs in this process, the differences of its structure and work from the human $F_oF_1$-ATP synthase can contribute to the development of novel drugs. Previously we showed that strain *S. fradiae* 19609 is supersensitive to oligomycin A and its semisynthetic derivatives, as well as more sensitive to a number of compounds of other chemical classes [17, 20]. It is important to note the fact that the sensitivity of *S. fradiae* 19609 and *S. lividans* TK24 to oligomycin A increases during inhibition of STPKs [17, 48]. This opens new possibilities for the development of anti-actinobacterial drugs with synergistic effects based on $F_oF_1$-ATP synthase inhibitors and STPKs. The $F_oF_1$-ATP synthase from *S. fradiae* 19609, due to similarity of its c-subunit (proton transport system) with the c-subunit of human $F_oF_1$-ATP synthase, as well as the high sensitivity of this strain to oligomycin A and its derivatives, can be a convenient model for studying human $F_oF_1$-ATP synthase.

These findings provide for scientific substantiation and expansion of prospects for creating a highly sensitive test system based on strain *S. fradiae* 19609 for selection of inhibitors of human and bacterial ATP synthases using approaches that we have developed [49, 50].

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