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The influence of an innovative antibacterial drug of the thiadiazinone class on the virulence factors of bacteria of the phylum *Pseudomonadota*, which chronically infect patients with cystic fibrosis

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Abstract

Introduction. Infections of the lower respiratory tract by bacteria of the *Pseudomonadota* phylum: *Pseudomonas aeruginosa, Burkholderia* spp., *Achromobacter* spp. are critical to the quality and life expectancy of patients with cystic fibrosis (CF). When the infection is chronic, eradication of bacteria with existing antibacterial drugs is practically impossible. To explore alternative drugs, trials are needed on bacteria isolated from CF patients and characterized using genomic approaches.

The objective of our study was a comparative analysis of virulence factors of 6 isolates of bacteria of the *Pseudomonadota* phylum and testing the efficacy of the innovative drug Fluorothiazinone (FT) in suppressing the pathogenicity of bacteria *in vitro*.

Materials and methods. Isolates of *A. ruhlandii* ST36, *A. xylosoxidans* ST555, *B. cepacia* ST2140, *B. gladioli* ST2141, *P. aeruginosa* ST859 and ST198 were examined using whole-genome sequencing and bioinformatics analysis to search for resistance and virulence determinants. The FT drug was tested for its effect on bacteria in *in vitro* experiments on cytotoxicity on HeLa cells, motility and biofilm formation.

Results. Genomic studies have confirmed the arsenal of resistance determinants, especially the efflux systems of bacteria isolated from patients with CF, and the diversity of virulence factors, among which we identified factors in the categories of motility, signals of quorum-sensing systems, secretion systems, exotoxins, as the most essential for the adaptation of bacteria to conditions of the lower respiratory tract. *In vitro* tests of the FT drug showed its effectiveness in suppressing cytotoxicity (2.6–4.0 times), motility (2.0–3.6 times) and the process of biofilm formation (2.0–7.7 times).

Conclusion. For the first time, the effectiveness of the innovative antibacterial drug Fluorothiazinone has been shown against bacteria of the *Pseudomonadota* phylum, isolated from chronically infected patients with CF, with the described potential of virulence factors.

Keywords: *microbial adhesion factors, Pseudomonadota, Cystic Fibrosis, WGS, virulence, Fluorothiazinon, anti-virulence*

Ethics approval. The study was conducted with the informed consent of the patients or their legal representatives. The research protocol was approved by the Ethics Committee of the N.F. Gamaleya National Research Center for Epidemiology and Microbiology (protocol No. 59, September 8, 2023).

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Оригинальное исследование
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Влияние инновационного антибактериального препарата класса тиадиазинонов на факторы вирулентности бактерий филума *Pseudomonadota*, хронически инфицирующих больных муковисцидозом

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Аннотация

Введение. Инфекции нижних дыхательных путей бактериями филума *Pseudomonadota*: *Pseudomonas aeruginosa, Burkholderia* spp., *Achromobacter* spp. критичны в отношении качества и продолжительности жизни больных муковисцидозом (МВ). При хронизации инфекции эрадикация бактерий существующими антибактериальными препаратами практически невозможна. Для исследования препаратов альтернативного действия необходимы испытания, проведённые на бактериях, выделенных от пациентов с МВ и охарактеризованных с помощью геномных подходов.

Целями нашего исследования были сравнительный анализ факторов вирулентности 6 изолятов бактерий филума *Pseudomonadota* и проверка эффективности инновационного препарата фтортиазинон (ФТ) в подавлении патогенности бактерий *in vitro*.

Материалы и методы. *И*золяты *A. ruhlandii* ST36, *A. xylosoxidans* ST555, *B. cepacia* ST2140, *B. gladioli* ST2141, *P. aeruginosa* ST859 и ST198 исследовали с помощью полногеномного секвенирования и биоинформационного анализа для поиска детерминант резистентности и вирулентности. ФТ испытали по действию на бактерии в экспериментах *in vitro* по цитотоксичности на клетках HeLa, подвижности и формированию биоплёнок.

Результаты. Геномные исследования подтвердили арсенал детерминант резистентности, особенно систем эффлюкса бактерий, полученных от пациентов с МВ, и разнообразие факторов вирулентности, среди которых мы выделили факторы в категориях: подвижность, сигналы систем quorum-sensing, системы секреции, экзотоксины как наиболее существенные для адаптации бактерий к условиям нижних дыхательных путей. *И*спытания ФТ *in vitro* показали его эффективность в подавлении цитотоксичности (в 2,6–4,0 раза), подвижности (в 2,0–3,6 раза) и процесса формирования биоплёнок (в 2,0–7,7 раза).

Заключение. Впервые показано эффективное действие инновационного антибактериального препарата ФТ на бактерии филума *Pseudomonadota,* выделенные от хронически инфицированных пациентов с МВ, с описанным потенциалом факторов вирулентности.

Ключевые слова: *факторы адгезии микроорганизмов, Pseudomonadota, муковисцидоз, полногеномное секвенирование, факторы вирулентности, фтортиазинон, антивирулентность*

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Конфликт интересов. Авторы декларируют отсутствие явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи.

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Introduction

Cystic fibrosis (CF) is one of the most common autosomal recessive diseases in which mutations in the gene for the transmembrane regulator of the chlorine channel cause impaired mucociliary clearance and the development of chronic colonization of the respiratory tract by bacteria of the *Pseudomonadota* phylum. Microorganisms of this phylum – *Pseudomonas aeruginosa*, *Burkholderia spp.* and *Achromobacter spp.* – are characterized by high natural resistance to antimicrobial drugs. Despite the use of aggressive antibiotic therapy, a progressive decline in lung function and early mortality are observed in CF patients with age. According to the latest edition of the Russian CF patient registry, the proportion of patients chronically infected with the listed bacteria is 33.6% for *P. aeruginosa*, 7.6% for *Achromobacter spp.* and 5.5% for *Burkholderia spp.* [1].

Penetrating the lower respiratory tract by aspiration, bacteria move along the surface of epitheliocytes using flagella and pili/fimbriae; these same structures serve as adhesins when attaching to cells to initiate biofilm formation [2]. Afterwards, several migration pathways of bacteria of these genera are possible: translocation through intercellular contacts and transepithelial migration [3]. In the latter case, invasion is first accomplished through the secretion systems of types 3 and 6 (T3SS, T6SS). The bacteria that have emerged from the epitheliocytes then cross the basal membrane and reach the connective tissue cells. On this pathway, they are protected by exotoxins that affect collagen and interfere with its antimicrobial activity [4]. *P. aeruginosa*, *Achromobacter spp.* and *Burkholderia spp.* can carry out invasion into macrophages, neutrophils and dendritic cells, thus being able to be protected from external influences in 4 types of eukaryotic cells. Bacteria not only survive inside cells, but can also disrupt their normal functioning, leading to pyroptosis/apoptosis or necrosis [5]. Cell death, bacterial escape and further multiplication cause an inflammatory response that damages lung tissue [3]. All the above mentioned stages in the life cycle of pathogens are coordinated by Quorum-Sensing (QS) signaling [6]. Survival mechanisms help these bacteria to compete with each other and with other representatives of the lung microbiome, so microbial diversity in such infections becomes minimal [7].

The eradication of such successful pathogens requires new approaches, one of which was used in the development of an innovative antibacterial drug of the thiadiazinone class (fluorothiazinone, FT) at the N.F. Gamaleya National Research Center for Epidemiology and Microbiology. T3SS effectors [8], as well as, presumably, highly conserved ATPases of the flagellar apparatus and T3SS, became a target for the effect of FT, which suppresses the pathogenicity of bacteria but does not kill them [8, 9], which ensures the absence of resistance development to such a drug. The efficacy of FT *in vitro* and in animal models was shown against a number of Gram-negative bacteria: *Salmonella enterica*, *P. aeruginosa*, *Escherichia coli*, *Acinetobacter baumannii* and *Klebsiella pneumoniae* [10]. The drug inhibited the obligate intracellular pathogen *Chlamydia trachomatis* [11], proving the possibility of FT penetration into eukaryotic cells.

Earlier, in the study of *P. aeruginosa* isolates from sputum and tracheal aspirate of CF patients, we showed that the cytotoxicity testing conditions selected for cultures isolated in nosocomial infections [9] were optimal only for isolates of genotypes ST235 and ST313 [12], characteristic of nosocomial *P. aeruginosa* belonging to the ExoU-lineage, named after the effector T3SS [13]. The other isolates were characterized by slow growth *in vitro* due to changes in cell physiology during chronic lung infection. Adaptation of experimental conditions to the peculiarities of *Pseudomonadota* of CF patients was one of the objectives of the study.

Considering the diversity of bacterial virulence factors used in adaptation and persistence in the respiratory tract of CF patients, we studied the genomic characteristics of isolates selected to evaluate the effect of FT and compared factors in the categories: motility, QS signaling, secretion systems, and exotoxins in selected representatives of *P. aeruginosa*, *Burkholderia spp.* and *Achromobacter spp.*

The objectives of our study were to comparatively analyze the virulence factors of 6 isolates of *Pseudomonadota* phylum bacteria infecting the lower respiratory tracts of CF patients and to test the efficacy of an innovative FT drug in suppressing the pathogenicity of bacteria *in vitro*.

Materials and methods

Materials

Six cultures of *Pseudomonadota* phylum bacteria were isolated from the sputum of chronically infected CF patients (Table 1). The study was conducted under the conditions of obtaining voluntary informed consent from patients or their legal representatives. The study protocol was approved by the Biomedical Ethics Committee of N.F. Gamaleya National Research Center for Epidemiology and Microbiology (protocol No. 59 of 08.09.2023).

FT is a novel antibacterial drug of thiadiazinone class C19H17F2N3O4S: N-(2,4-difluorophenyl)-4(3-ethoxy-4-hydroxybenzyl)-5-oxo-5,6-dihydro-

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Table 1. Isolates of the *Pseudomonadota* phylum used in the study

Specie	Isolate	Accession number	Sequence type	Genome size, Mb	Genes	Protein-coding
P. aeruginosa	GIMC5045:PA33P25	JAVMRC000000000	ST859	6,4	5973	5841
P. aeruginosa	GIMC5047:PA33P30	JAVMRD000000000	ST198	6,4	6023	5842
B. cepacia	SCCH90:Bcn202840	JAQOTY000000000	ST2140	8,4	7704	7512
B. gladioli	SCCH61:Bgd92-3601	JAQOTZ000000000	ST2141	8,2	9105	8385
A. ruhlandii	SCCH137:Ach2231057	JAQZZN000000000	ST36	6,3	5884	5679
A. xylosoxidans	SCCH131:Ach223717	JAPZVF000000000	ST555	6,4	5912	5806

4H-[1,3,4]-thiadiazine-2-carboxamide[1]. A stock solution of FT was prepared from the substance with a concentration of 5.0 mM in 0.3M CH_3COO-Na, pH 7.0 ± 0.2.

Cytotoxicity was studied on HeLa cervical carcinoma cells (ATCC CCL2, 22603).

Bacteria cultivation

Bacteria were grown for 18 h at 37°C in LB broth to a concentration of 109 microbial cells/mL (OD_{600}).

Genome analysis

The protocol [14] was used for DNA extraction from isolates, supplemented by polysaccharide purification using CTAB (cetyltrimethylammonium bromide).

DNA libraries were prepared using the protocols Nextera DNA Flex Library Prep (Illumina) and KAPA HyperPlus Kit (Roche). Sequencing was performed on a NextSeq 500/550 instrument (Illumina) using a Mid Output 300 cycles cartridge.

Genomes were assembled using CLC Genomic Workbench v. 21.0.1 (Qiagen) and SPAdes v. 3.13.0[2]. Rapid Annotations Subsystems Technology (RAST) [15] and NCBI Prokaryotic Genome Annotation Pipeline [16] were used for annotation. Results were deposited in GenBank (bioproject PRJNA561493) under the numbers shown in Table 1.

Genomes were analyzed using BV-BRC resources[3] [17]. Virulence factors were investigated using VFDB[4] [18] and BlastKOALA[5] [19]. Plasmids were searched using PlasmidFinder 2.1[6]. CARD[7] [20], BV-BRC [17] and BlastKOALA [19] were used to identify resistance determinants.

Investigation of the effect of FT in vitro

Bacterial cytotoxicity was determined according to the method [9], with modifications. A monolayer of HeLa cells grown in IMDM (Iscove's Modified Dulbecco's Medium) supplemented with 10% FBS (fetal bovine serum) and 2 mM L-glutamine in 96-well plates was washed and IMDM containing 1% FBS was added. HeLa cells were infected with bacterial cultures at an initial multiplicity of infection (MOI) of 5. Plates were incubated for 18 h in the presence of FT (60 μg/mL). 0.3M CH_3COONa, pH 7.0 ± 0.2, was used as a control. Cells were precipitated by centrifugation for 20 min at 1500 rpm. In the supernatants, the activity of released lactate dehydrogenase (LDH) was determined using the CytoTox 96® Non-Radioactive Cytotoxicity Assay Kit (Promega) according to the manufacturer's protocol. The percentage of LDH release was calculated relative to uninfected control (0% LDH release) and HeLa cells lysed with Triton X-100 (100% LDH release).

The ability of FT to inhibit the swimming motility of isolates was evaluated on Petri dishes with 0.3% semi-liquid agar [21]. Bacterial cultures were incubated with FT (100 μg/mL) for 3 h at 37°C, then 2 μL of the suspension was added to the thickness of semi-liquid agar containing FT (100 μg/mL) and incubated for 48 h at 37°C. The degree of bacterial motility was determined by the diameter of radial migration in agar.

The following approach was used to study the effect of FT on bacterial biofilm formation. Static biofilms were formed on the abiotic surface according to the protocol [22] with changes in incubation conditions. FT (100 μg/mL) was added to overnight bacterial cultures at a concentration of 10^7 microbial cells/mL (OD_{600}) and incubated in the wells of the plate for 48 h without changing the medium, then 125 μL of 0.1% crystal violet (CV) solution was added one at a time to stain the biofilms. The dye bound to the biofilms was extracted with 100

[1] Clinical studies: RCT No. 389 dated 08/03/2018 (completed); RKI 169 dated March 14, 2022 (ongoing). Application for registration with the Ministry of Health of the Russian Federation (incoming No. 4253550 dated May 30, 2023). Status under review.

[2] St. Petersburg genome assembler, Russia, URL: http://cab.spbu.ru/software/spades/

[3] Bacterial and Viral Bioinformatics Resource Center, URL: https://www.bv-brc.org

[4] Virulence Factor Database, http://www.mgc.ac.cn/VFs

[5] KEGG Orthology And Links Annotation, URL: https://www.kegg.jp/blastkoala

[6] URL: https://cge.food.dtu.dk/services/PlasmidFinder

[7] Comprehensive Antibiotic Resistance Database, URL: https://card.mcmaster.ca

μl of 96% ethanol and OD_{540} was determined on a Multiskan EX instrument (Thermo Labsystems). Qualitative studies of biofilms were performed under a Nikon Eclipse 50i microscope (Nikon) at 20× magnification.

Each FT experiment was repeated 3 times.

Statistical processing of the analysis results and visualization were performed using Prism-GraphPad (GraphPad Software). The criterion of statistical reliability of the difference between the obtained data was considered to be the error value $p < 0.05$.

Results

Genome analysis

Six isolates of the *Pseudomonadota* phylum represented 3 genera. *A. ruhlandii* and *A. xylosoxidans*, selected for the study, were representatives of the genus most common in CF patients in Russia. The choice of *B. cepacia* and *B. gladioli* was determined by the emergence of new species of *Burkholderia spp.* infecting CF patients against the background of decreasing spread of *B. cenocepacia* of epidemic genotype ST709 [23]. *P. aeruginosa* of different genotypes belonging to the ExoS lineage, according to E.A. Ozer et al. [13], were taken into the study as more common in infections of CF patients compared to the ExoU phenotype [12, 24].

The genomes of the isolates were represented by one chromosome in *Achromobacter* and *Pseudomonas* and 3 chromosomes in *Burkholderia*. The 48 Kb conjugative plasmid was present only in the genome of *A. ruhlandii* (IncP1), but did not include resistance determinants. The size of *Burkholderia* genomes was one-third larger than *Achromobacter* and *Pseudomonas* genomes (**Table 1**). In all genomes, 92-98% of the identified genes encoded proteins.

When assessing the resistance potential of the studied isolates, the number of efflux systems encoded by genomes was noteworthy: 12 in *P. aeruginosa*, 16 in *Achromobacter spp.* each, 27 in *B. cepacia*, 38 in *B. gladioli*, which creates additional opportunities to counteract the applied antibiotic therapy.

Investigating the virulence factors of isolates, we focused on 4 main groups necessary for bacterial adaptation to lower respiratory tract conditions: secretion systems, motility, toxins, and QS signaling.

The secretion systems (**Table 2**) Sec, SRT, Tat, and T2SS are represented by all components in the genomes of the isolates. T3SS is found in all isolates except *B. cepacia*; T6SS is found in 5 isolates, and in *A. ruhlandii* it contains only genes of secreted substrate Hcp and inner membrane protein *IcmF*; finally, complete T1SS is found in *B. gladioli*, and in the other isolates it is represented only by outer membrane protein *TolC*.

The main motility apparatus, the flagella apparatus, is present in the genomes of all isolates. The identified hfp pili/fimbriae differed in composition among the isolates. As shown in **Table 3**, pili responsible for twitching motility and chemosensory activity were found only in the genomes of *P. aeruginosa*. Type IV pili are present in pseudomonads and *Burkholderia spp.* but differ in the list of components, and in *Achromobacter* they are represented only by the prepilin *pilD* peptidase. Type IVb pili are present in all isolates. Chaperone-usher pili, which encode 2 different operons: *fimACD* and *cupE1-6*, are present in *P. aeruginosa* and *B. cepacia* with a complete set of components.

Table 2. Classes of bacterial secretion systems represented in the genomes of the studied isolates

Classes of bacterial protein secretion systems	*P. aeruginosa* GIMC5045: PA33P25	*P. aeruginosa* GIMC5047: PA33P30	*B. cepacia* SCCH90: Bcn202840	*B. gladioli* SCCH61: Bgd92-3601	*A. ruhlandii* SCCH137: Ach2231057	*A. xylosoxidans* SCCH131: Ach223717
Sec	+	+	+	+	+	+
SRT	+	+	+	+	+	+
Tat	+	+	+	+	+	+
T1SS	+ (TolC)	+ (TolC)	+ (TolC)	+ (TolC, HlyB, HlyD)	+ (TolC)	+ (TolC)
T2SS	+	+	+	+	+	+
T3SS	+	+		+	+	+
T4SS					+ VirD4	+ VirB5, VirB6
T6SS	+	+	+	+	+ Hcp, IcmF	+

Note. VirD4 — ATPase; VirB5 — surface/pilus protein; VirB6, IcmF — inner membrane protein; Hcp — secreted substrate.

Table 3. Pilus systems in the genomes of the studied isolates

Pilus systems	*P. aeruginosa* GIMC5045:PA33P25	*P. aeruginosa* GIMC5047:PA33P30	*B. cepacia* SCCH90:Bcn202840	*B. gladioli* SCCH61:Bgd92-3601	*A. ruhlandii* SCCH137:Ach2231057	*A. xylosoxidans* SCCH131:Ach2237117
Twitching motility pili	*pilGHIJKRSTUKRS*	*pilGHIJKRSTUKRS*	–	–	–	–
Chemosensory pili	*chpABCDE*	*chpABCDE*	–	–	–	–
Type IV pili	*pilABCDFQPONMZVWXY1Y2E*	*pilABCDFQPNMZVWXY1Y2E*	*pilABCDEQW*	*pilABCDEW*	*pilD*	*pilD*
Type IVb pili	*flp, cpaABCEF*	*flp, cpaABCEF*	*flp, cpaABCEF*	*flp, cpaABCEF*	*flp, cpaABCEF*	*flp, cpaABCEF*
Chaperone-Usher Pathway (CUP) pili	*fimACD, cupE*	*fimACD, cupE*	*fimACD, cupE*	*fimAD*	*fimCD, cupE*	*fimCD, cupE*
Positive phototactic motility proteins	–	–	–	–	*pixH*	–

Note. *pilD* — prepilin peptidase; *pixH* — response regulator.

In *B. gladioli*, the *fimACD* operon lacks a chaperone gene and the *cupE* operon is not detected. In *Achromobacter* genomes, the full *cupE1-6* operon is present, while the *fimACD* operon lacks the gene encoding pilin.

The QS system as the most important means of bacterial communication is represented in the analyzed genomes in all its diversity. QS of AI-1 type (AutoInductor), whose signaling molecules are homoserinlactone derivatives, was found in the genomes of *P. aeruginosa* (2 each) and *Burkholderia* (1 each). An AI-1-regulated operon of rhamnolipid biosynthesis is also present in these genomes. Rhamnolipids are included in the QS system, are used by bacterial cells to reduce surface tension, and are important for motility, biofilm formation, and absorption of hydrophobic substrates [25].

The second system is named DSF from signaling molecules which are diffusible signaling factors. For *Burkholderia spp.* this BDSF is cys-2-dodecenoic acid. Another name for it is *rpfF/R/B/G* – by genes. *Achromobacter* genomes have 2 DSF systems each, *B. cepacia* has 1 DSF. The genomes of *P. aeruginosa* and *B. gladioli* have only rpfB genes.

The third system, PQS (pseudomonas quinolone signal), whose signaling molecule is 2-heptyl-3-hydroxyl-4-quinolone ($C_{16}H_{21}NO_2$), in the complete set: pqsABCDHE, *phnAB*, is present only in *P. aeruginosa*. The genome of *B. cepacia* contains pqsE, a gene for a protein that responds to quinolone signaling, and phnAB, encoding an anthrenylate synthesis protein, a precursor of PQS [26]. *Achromobacter* and *B. gladioli* have only *phnAB*.

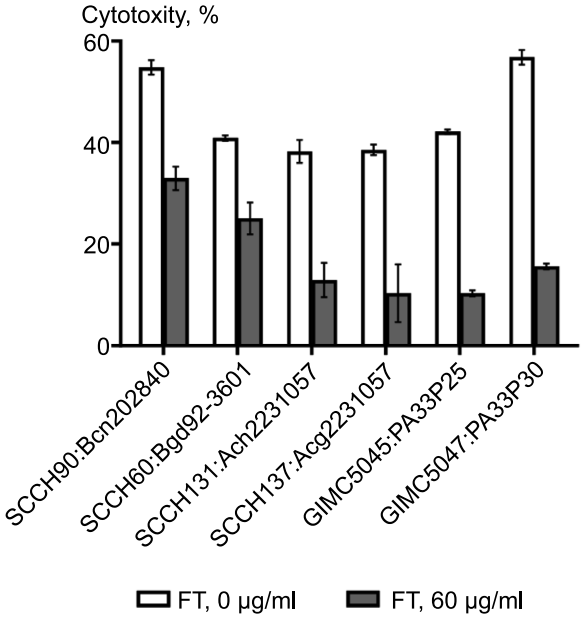

Fig. 1. Effect of FT on the cytotoxicity of bacterial cells against HeLa cells.

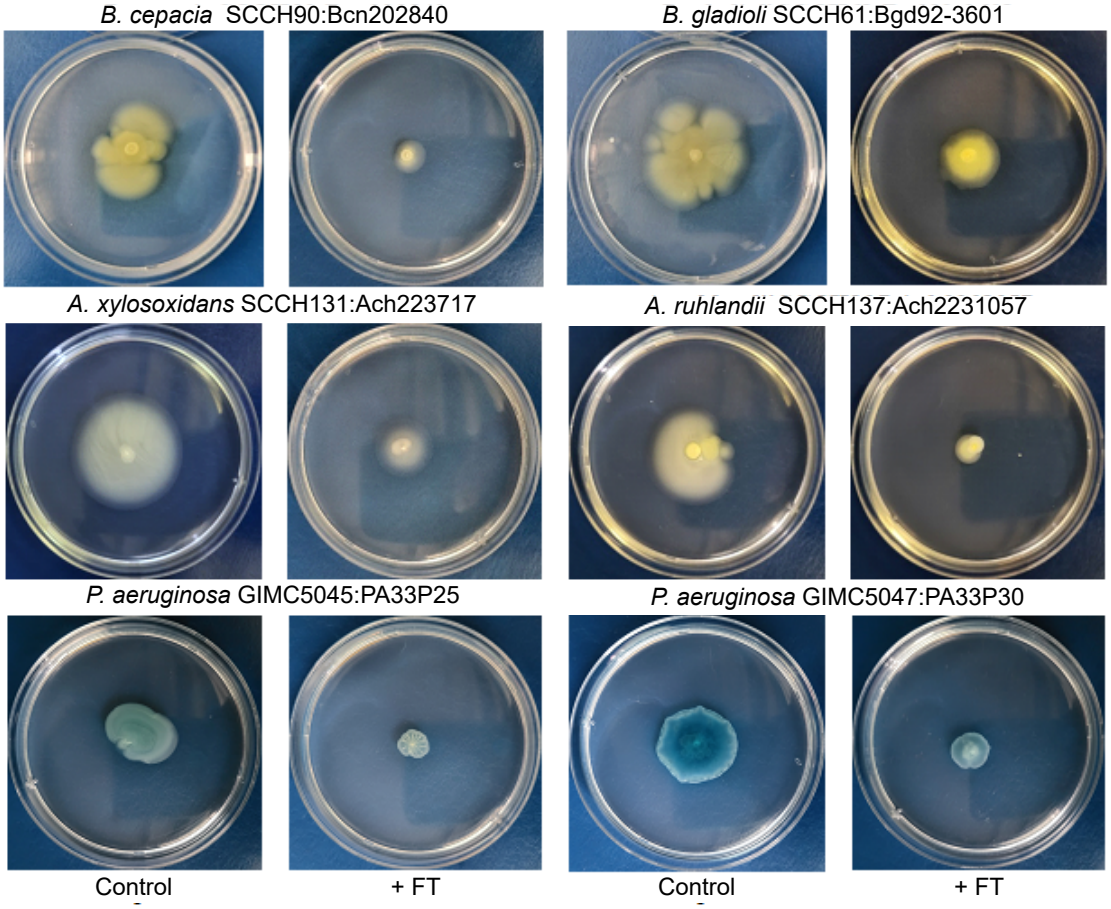

Fig. 2. Effect of FT on swimming mobility of bacterial cells.

The toxins that the studied isolates are capable of producing can be divided into 4 groups. The first one is T3SS toxins acting inside the eukaryotic cell. In the genome of *P. aeruginosa* GIMC5045:PA33P25 they are represented by 5 genes: *toxA*, *exoS*, *exoT*, *exoY*, *zot*. The *toxA* gene encodes an ADP-ribosyltransferase. The *zot* gene is a homolog of cholera toxin acting on zonula occludens (the main one of the tight contacts proteins of the intestinal epithelium), the second isolate of *P. aeruginosa* has 4 genes of this group. The T3SS effector gene was also found in the genomes of *Achromobacter* – *axoU*. The second group contains genes of toxins that damage the membrane of eukaryotic cells. The genes of phospholipase C and hemolysin III are present in all genomes, the gene *tlyC* (pore-forming toxin) is absent in *Achromobacter*, and the genome of *B. cepacia* contains another gene of this group, *tlh*, encoding thermolabile hemolysin. In the third group of nonspecific toxins, only *P. aeruginosa* has *hcnABC* (hydrogen cyanide synthase) genes. The fourth group of toxins damaging the extracellular matrix is found only in *B. cepacia* and is represented by the *colA* gene of microbial collagenase.

Effect of FT in vitro

The effect of FT on the cytotoxicity of isolates from CF patients was investigated by selecting the time of contact of bacterial cells with HeLa cells,

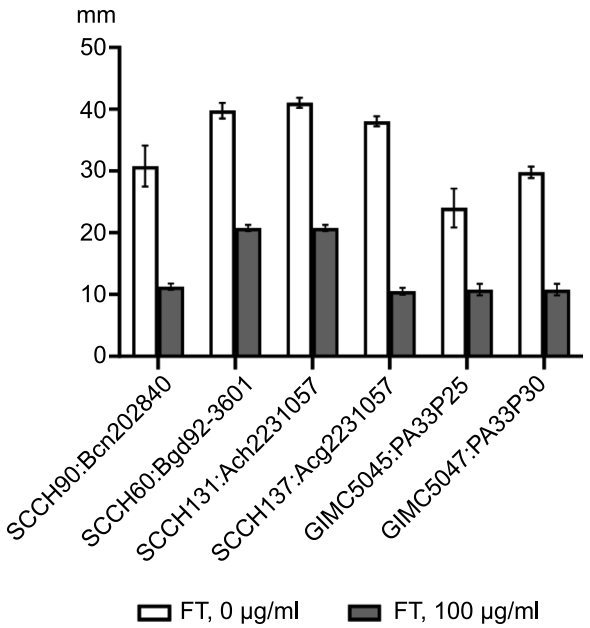

Fig. 3. Changes in the diameter of bacterial swimming mobility zones.

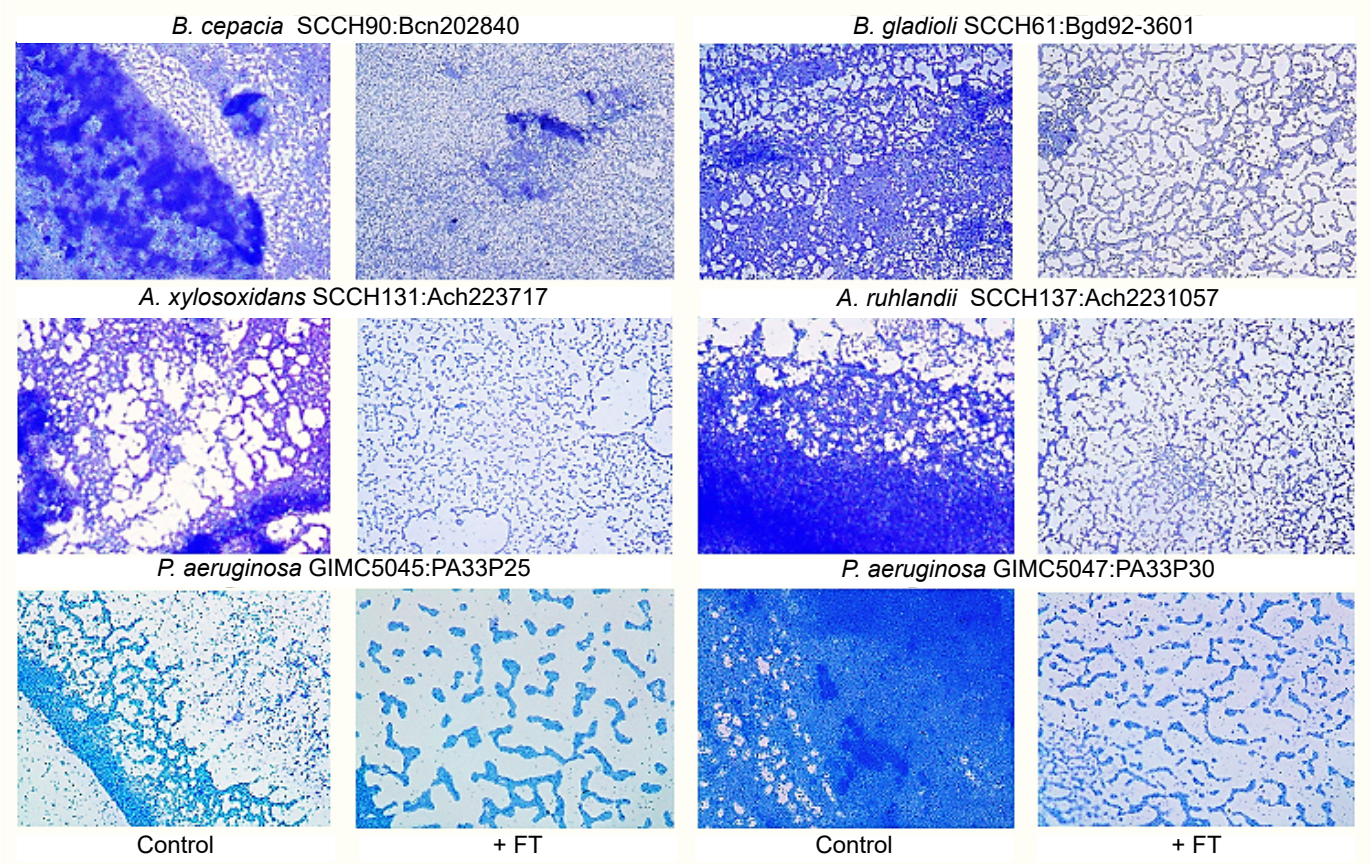

Fig. 4. Effect of FT on the formation of bacterial biofilms.
Microphotographs of the formed biofilm fragments (the densest fragments) are shown.

taking into account the slow growth of such bacteria in culture. While at a contact time of 4 h at doses of 10 and 50 MOI the cytotoxicity of isolates was 16–26 and 27–43%, respectively, after 20 h of contact the toxicity rose to 70-100%. A dose of 5 MOI and a contact time of 18 h were determined as optimal for studying the effect of FT. Under the influence of FT there was a decrease in cytotoxicity for all isolates: for *P. aeruginosa* isolates – by 3.6 and 4.0 times, for *B. cepacia* – by 2.6, for *B. gladioli* – by 3.2, for *A. xylosoxidans* – by 3.0, for *A. ruhlandii* – by 3.7 (**Fig. 1**).

Comparison of the swimming mobility of isolates showed a significant reduction of the bacterial movement zone under the influence of FT: for *P. aeruginosa* – 2.2 and 2.8 times, for *B. cepacia* – 2.7 times, for *B. gladioli* – 2.0 times, for *A. xylosoxidans* – 2.0 times, for *A. ruhlandii* – 3.6 times (**Fig. 2**). The mobility of *P. aeruginosa* isolates in the control was lower than other bacteria, however, the difference with samples incubated with FT was statistically significant ($p < 0.05$).

The process of biofilm formation differed among isolates of 3 genera (**Fig. 3**). *Burkholderia* and *Achromobacter* were characterized by a fairly rapid development of dense biofilm structures over the entire well area, while for *Pseudomonas* biofilm formation was slower and was mainly concentrated at the edges of the well, where a dense ring was formed (**Fig. 4**). The effect of FT was strongest for *B. cepacia*. The biofilm biomass decreased 7.7-fold in the presence of the antibacterial agent. For the other isolates the decrease in biomass was lower but significant: for *A. xylosoxidans* and *A. ruhlandii* – 3-fold, for *B. gladioli* – 2.3-fold, for *P. aeruginosa* – 2.4-fold and 2.0-fold (**Fig. 5**).

Discussion

Respiratory tract infections with *P. aeruginosa*, *Achromobacter spp.* and *Burkholderia spp.* are the most frequent and most dangerous for CF patients. Multiple natural resistance of the *B. cepacia* complex is already postulated and warnings about it are stated in the EUCAST antibiotic susceptibility testing guidelines[8]. The antibiogram for *Achromobacter spp.* is also a definite challenge for laboratories, as EUCAST thresholds are only given for 3 substances, even in the 2024 guidelines. For *P. aeruginosa*, the problem is that sensitive *in vitro*

[8] Antimicrobial susceptibility testing of Burkholderia cepacia complex (BCC). 2013. URL: https://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/General_documents/BCC_susceptibility_testing_130719.pdf

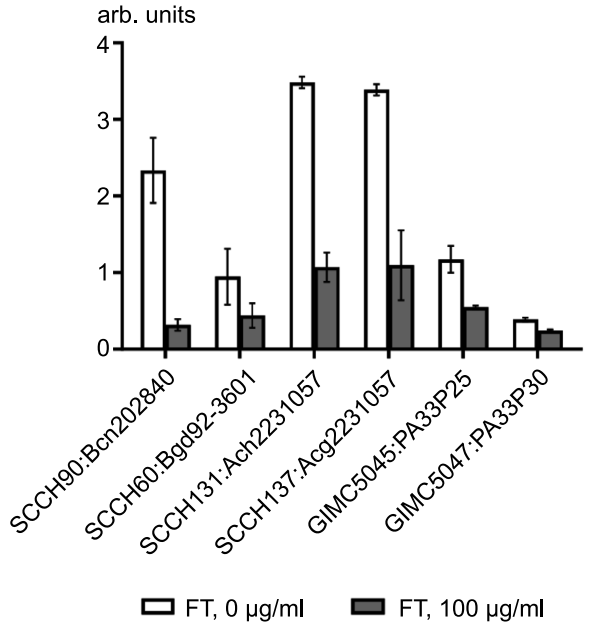

Fig. 5. Evaluation of biofilm biomass accumulation by the degree of crystal violet staining based on optical density (λ = 540 nm).

isolates cannot be eradicated with selected drugs. Our genomic studies have shown that the potential for resistance in *P. aeruginosa* and other bacteria studied may be the efflux systems present in genomes in large numbers. We should not forget about biofilms, the formation of which is successfully coordinated by QS signals, the presence and diversity of which we confirmed for all genomes studied. *In vitro* experiments showed that all 6 isolates formed dense biofilms. It is these structures that help bacteria avoid the effects of antibiotics in human lungs. However, the innovative FT drug was effective against all isolates tested.

Cytotoxicity of representatives of three genera is a serious problem for lung tissues of CF patients. The spectrum of exotoxins that can be produced by the studied isolates is quite wide. It should be noted that in the study of the proteomes of *P. aeruginosa*, *Achromobacter spp.* and *Burkholderia spp.* there are still many surprises and discoveries awaiting us, since at present it is possible to annotate a little more than half of the translation products that encode the genomes we have sequenced. The leader in annotation is *P. aeruginosa* (57.8%), with *B. gladioli* in last place at 38.2%. The databases of annotation resources lack, for example, the sequences of genes encoding the *Achromobacter spp.* T3SS effector, so we performed an additional search for the axoU gene, finding it in both *Achromobacter* genomes, named the hypothetical protein gene. For *B. gladioli*, the search for T3SS effectors is ongoing. S.K. Yadav et al. found the ortholog of the T3SS effector in *B. gladioli* strain NGJ1, showing the presence of a secretion signal at the N-terminus of a polypeptide annotated as a prophage protein, and demonstrated its calcium-dependent secretion mediated by T3SS [27]. The frame of such a protein is also present in the genome sequenced by us as part of the prophage. During *in vitro* experiments, all 6 isolates tested showed cytotoxicity against HeLa cells. The cytotoxicity of *B. cepacia* was at the level of the most effective *P. aeruginosa* isolate in the absence of T3SS, as shown by our genomic studies. It should be noted that the first publication mentioning the absence of T3SS in *B. cepacia* dates back to 2001. [28]. It is possible that another nanomachine, T6SS, is involved in the delivery of *B. cepacia* toxins, especially since in other bacteria T6SS and T3SS work in coordination [29].

Genomic studies have demonstrated an arsenal of factors responsible for motility of the studied bacteria of the *Pseudomonadota* phylum. The main one for swimming motility is flagella, the genes of which apparatus are present in all genomes. We observed this type of motility in the isolates tested, more pronounced in *B. gladioli* and *Achromobacter* under experimental conditions.

Conclusion

Thus, genomic studies and *in vitro* analysis of isolates allowed us to describe the virulence factors of 6 bacteria isolated from chronically infected patients and to demonstrate the possibility of their realization by all isolates for important processes in the development and chronicity of infection: cytotoxicity, motility and biofilm formation.

The innovative antibacterial agent FT inhibited the three processes in all isolates *in vitro*. The efficacy of FT against isolates from CF patients has been shown for the first time. These experiments will serve as a basis for further preclinical trials of the drug against a new nosology, including animal models. Ongoing comprehensive studies of FT itself have demonstrated accumulation of the drug administered intragastrically to rats in various animal organs, including the lungs [10]. Thus, the evidence base for the efficacy of FT *in vivo* and *in vitro* is constantly expanding, which gives hope that a new helper in the prevention and treatment of respiratory tract infections in CF patients may emerge.

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