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THESIS

RESISTANCE FACTORS OF BACTERIAL NOSOCOMIAL INFECTIONS CAUSATIVE AGENTS AS BACKGROUND FOR THE MODERN ANTIMICROBIALS DEVELOPMENT

091 – Biology

09 -– Biology

Applying for the Doctor of Philosophy degree

The dissertation contains the results of own research. The use of ideas, results and texts of other authors are linked to the corresponding source.

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ANNOTATION

Wu Lin. Resistance factors of bacterial nosocomial infections causative agents as background for the modern antimicrobials development – qualification scientific work on manuscript rights.

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The relevance of the research. The era of antibiotics, which began with the discovery of penicillin in the XX century, may soon end and humanity will face a challenge to overcome which will have to find new solutions. This challenge is now the "era of antibiotic resistance", caused not only by evolutionary mechanisms of protection of pathogens, but also by many factors of human activity.

Particular importance are methods of combating infectious agents when they are in treatment centers and a large number of people can both become infected and be a source of their spread. Such infections are nosocomial (hospital-acquired infection) and are defined by World Organization of Health (WHO) as infections that can infect the patient during treatment in hospital or other health care facilities. The sources of infection in hospitals are not only other patients and staff, but also surfaces, instruments, medical manipulations and operations, which is cause to problems in ensuring proper conditions. However, one of the important factors in the treatment of nosocomial infections is their resistance to many antibiotics used in hospitals at the same time, and as a result “superbug” arise, for which there is no effective counteraction. WHO defines a list of such relevant "superbugs", and almost half of them are included in the already established acronym ESCAPE – ***E****nterococcus faecium,* ***S****taphylococcus aureus,* ***K****lebsiella pneumoniae,* ***A****cinetobacter baumannii,* ***P****seudomonas aeruginosa* and ***E****nterobacter spp.*

It was found that the natural and induced variability of microorganisms that cause inflammatory processes can lead to increased resistance to the action of previously effective antiseptics due to the acquisition of resistance. In addition, the selection of resistant forms of microbial pathogens, can cause low efficiency of

therapy, severe disease, long-term treatment or, in some cases, the inability to overcome the infection at all. The mentioned and many other sources, citing WHO and the Center for Disease Control (CDC, USA), state that the need to develop effective antimicrobials against these and other nosocomial infections is a "need of the hour".

Obviously, the solution of the problem of overcoming nosocomial infections has many dimensions, including organizational, educational, medical and so on. But these long-term strategies do not remove the urgent task of finding effective antimicrobials or new combinations to treat these severe, often combined infections, right today. This work currently involves a large number of scientists and practitioners, with different approaches to the solvation of the problem. One of the most effective method is undoubtedly the identification of the most vulnerable sites of infectious agents and their application as targets for new drugs. Such vulnerable points of microbial pathogens mainly determine their resistance and pathogenicity, and therefore these factors should be considered more closely to find the target.

Therefore, the systematization of scientific data and results in this regard, the analysis and evaluation of the main factors of pathogenicity and resistance of nosocomial and other infections are relevant to determine modern approaches to the development of the latest antimicrobial agents.

The goal of the work was to justify systemic approaches to the development of modern antimicrobials based on the analysis of resistance factors of bacterial pathogens of nosocomial infections.

To achieve the goal, it was necessary to solve the following problems:

- on the basis of theoretical analysis and research, determine the factors and mechanisms of resistance of microbial pathogens, which can be chosen as targets for the action of antiseptics;

- to establish the molecular-genetic characteristics of individual representatives of bacterial pathogens that can be used to counter their distribution and to select strategies for antimicrobial therapy;

- to propose a method of screening antimicrobial substances for selected pathogenesis targets of pathogenic bacteria, which can be applied in research practice;

- to show the prospects of creating effective antiseptics based on microbial products - antibiotics and enzymes of the streptomycete *Streptomyces albus*;

- to develop recommendations for the implementation of methods of monitoring the resistance of nosocomial infections and the principles of modern antimicrobials development.

Scientific novelty of the obtained results.

The following scientific results were obtained for the first time in the dissertation:

- on the basis of genome sequencing and gene annotation of the strain *Bacteroides thetaiotaomicron* DSMZ 2079 isolated from the patient's blood, the presence of a one- and two-component system for recognizing environmental signals and responding to them was shown, the presence of four homologs of the self-transmitted conjugative transposon CTnDOT, which provides the extension of resistance to tetracycline and erythromycin. The *rpoB* and *tuf* genes (in the JHR92_RS03155 and JHR92_RS03195 loci, respectively), which determine the strain's resistance to antibiotics, were identified;

- based on the results of the analysis of the 16S rRNA sequences of the isolated clinical strain *Pseudomonas oryzihabitans* JN 873340 and its comparison with 29 other strains of the species from GenBank, a phylogenetic tree was built and the possibility of identifying their geographical origin by two hypervariable regions V4 and V5 was shown;

- the minimum inhibitory concentrations of the new streptofungin antibiotic against *C. albicans* ATCC 10231 (10 μg/ml), *B. subtilis* ATCC 6633 (200 μg/ml) and *P. aeruginosa* ATCC 9027 (500 μg/ml) were established, as well as the absence of toxicity in a wide range of concentrations (from 2.5 to 500 μg/ml), which determines its potential as an active pharmaceutical ingredient;

Practical significance of the obtained results. The practical significance of the dissertation consists in solving the scientific and practical problem of developing antimicrobial agents that do not cause the resistance development of microbial pathogens.

The developed method for screening inhibitors of sortase A, which is distinguished by the use of cheaper substrates and, accordingly, the possibility of wide application, has been implemented in the practice of the Environmental Comprehensive laboratory of the School of Tropical Medicine of Hainan Medical University, China. (Act of implementation dated September 4, 2023).

Developed "Recommendations for the implementation of methods of resistance control of nosocomial infections and principles of development of modern antimicrobial drugs", approved (08.25.23) at the Shupyk National Healthcare University of Ukraine, for use in practice.

Proposed as a result of the dissertation research the principles and approaches for the creation of preparations based on biologically active substances with different mechanisms of action were used in the production program of LLC "PHARMA INTERNATIONAL GROUP 2" (Kyiv) in the development of functional cosmetics (Instructions for use dated 20.07.2023 r.).

The results of the work were implemented in the teaching of the courses "General microbiology and virology" and "Fundamentals of pharmaceutical production" for students of the specialty 162 "Biotechnology and bioengineering" at the department of industrial biotechnology and biopharmacy of KPI named after Igor Sikorskyi (Act of implementation dated 20.09.2023), as well as for students of the specialty "Tropical medicine" within the framework of lectures and laboratory (practical) classes in the disciplines "Medical immunology", "Pathobiology", "Medical microbiology", "Environmental microbiology ", "Hygienic microbiology", "Environment and health" at Hainan Medical University (China) (Act of implementation dated 01.09.2023).

The main provisions of the work are presented in 10 scientific papers: 4 scientific articles included in the Scopus scientometric database, including 2 of them

in publications assigned to the 2nd quartile (Q2) in accordance with the SCImago Journal and Country Rank classification (equal to two publications that are counted in accordance with the first paragraph of paragraph 11 of the Resolution of the Cabinet of Ministers of Ukraine dated March 6, 2019 No. 167); 4 articles in specialized foreign periodicals; 2 abstracts at all-Ukrainian and international conferences.

Key words: infections, pathogens, antimicrobials, toxicity, resistance, enzymes, antibiotic

АНОТАЦІЯ

У Лінь. Фактори резистентності бактерійних збудників нозокоміальних інфекцій як засади для розробки сучасних протимікробних препаратів – кваліфікаційна наукова праця на правах рукопису.

Дисертація на здобуття наукового ступеня доктора філософії за спеціальністю 091 – Біологія. Національний технічний університет України «Київський політехнічний інститут імені Ігоря Сікорського», Київ, 2023.

Актуальність теми дослідження. Ера антибіотиків, яка почалася з відкриттям пеніциліну в XX столітті, може незабаром закінчитися, і перед людством постане виклик, для подолання якого доведеться шукати нові рішення. Цим викликом нині є «ера антибіотикорезистентності», спричинена не лише еволюційними механізмами захисту патогенів, а й багатьма факторами діяльності людини.

Особливе значення набувають методи боротьби зі збудниками інфекцій, що знаходяться в лікувальних закладах і велика кількість людей може як інфікуватися, так і бути джерелом їх поширення. Такі інфекції є нозокоміальними (внутрішньолікарняними інфекціями) і визначаються Всесвітньою організацією охорони здоров’я (ВООЗ) як інфекції, якими може інфікуватися пацієнт під час лікування в клініці чи іншому закладі охорони здоров’я. Джерелами інфекції в лікарнях є не тільки інші пацієнти та персонал, а й поверхні, інструменти, медичні маніпуляції та операції, що пов'язано з

проблемами забезпечення належних умов. Проте одним із важливих факторів у лікуванні внутрішньолікарняних інфекцій є їх резистентність до багатьох антибіотиків, що застосовуються одночасно в лікарнях, і в результаті виникають «супербактерії», ефективної протидії яким немає. ВООЗ визначає перелік таких релевантних «супербактерій», і майже половина з них включена у вже усталену абревіатуру ESCAPE – ***E****nterococcus faecium,* ***S****taphylococcus aureus,* ***K****lebsiella pneumoniae,* ***A****cinetobacter baumannii,* ***P****seudomonas aeruginosa та* ***E****nterobacter spp.* [1, 7-9].

Встановлено, що природна та індукована мінливість мікроорганізмів, що викликають запальні процеси, призводить до підвищення їх стійкості за рахунок набуття резистентності – здатності протистояти дії раніше ефективних антисептиків. Крім того, відбувається селекція резистентних форм мікробних збудників, що зумовлює низьку ефективність терапії, тяжкий перебіг захворювання, тривале лікування, а в окремих випадках і взагалі неможливість подолати інфекцію. Вищезазначені та багато інших джерел із посиланням на ВООЗ та Центр контролю захворювань (CDC, США) стверджують, що необхідність розробки ефективних антимікробних засобів проти цих та інших інфекцій є «вимогою часу».

Очевидно, що вирішення проблеми подолання внутрішньолікарняних інфекцій має багато вимірів, зокрема організаційний, освітній, медичний тощо. Але ці довгострокові стратегії не знімають термінового завдання пошуку ефективних протимікробних препаратів або нових комбінацій для лікування цих важких, часто комбінованих інфекцій прямо сьогодні. У цих розробках наразі задіяна велика кількість науковців і практиків, кожен з яких обирає власний підхід, одним із найефективніших з яких, безсумнівно, є виявлення найбільш уразливих ділянок інфекційних агентів та їх використання як мішеней для нових ліків. Такі вразливі точки мікробних патогенів в основному визначають їх стійкість і патогенність, і тому ці фактори потрібно розглядати більш детально, щоб знайти відповідні мішені.

Тому актуальним є систематизація наукових даних і результатів у цьому відношенні, аналіз та оцінка основних факторів патогенності та резистентності нозокоміальних та інших інфекцій для визначення сучасних підходів до розробки новітніх протимікробних засобів.

Метою роботи було обгрунтування системних підходів до розробки сучасних протимікробних препаратів на основі аналізу факторів резистентності бактерійних збудників нозокоміальних інфекцій.

Для досягнення поставленої мети необхідно було розв′язати такі **задачі**:

- на основі теоретичного аналізу та досліджень визначити фактори і механізмів резистентності мікробних патогенів, які можуть бути обрані мішенями для дії антисептиків;

- встановити молекулярно-генетичні характеристики окремих представників бактерійних збудників інфекцій, що можуть бути використані для контрою їх поширення та вибору стратегій антимікробної терапії;

- запропонувати метод скринінгу антимікробних субстанцій щодо обраних мішеней патогенезу бактерійних збудників, що може бути застосований у дослідницькій практиці;

- показати перспективи створення ефективних антисептиків на основі мікробних продуктів – антибіотиків та ензимів стрептоміцету *Streptomyces albus*;

- розробити рекомендації до впровадження методів контролю резистентності нозокоміальних інфекцій та принципів розробки сучасних протимікробних препаратів.

Наукова новизна отриманих результатів. У дисертації *вперше* одержані такі наукові результати:

- на основі сиквенування геному та анотації генів виділеного з крові пацієнта штаму *Bacteroides thetaiotaomicron* DSMZ 2079 показано наявність одно- та двокомпонентної системи розпізнавання сигналів навколишнього середовища та відповіді на них, наявність чотирьох гомологів самопередаючого кон’югативного транспозону CTnDOT, який забезпечує

розширення стійкості до тетрацикліну та еритроміцину. Ідентифіковано гени *rpoB* та *tuf* (в локусах JHR92_RS03155 та JHR92_RS03195, відповідно), що визначають резистентність штаму до антибіотиків;

- за результатами аналізу послідовностями 16S рРНК виділеного клінічного штаму *Pseudomonas oryzihabitans* JN 873340 та його порівняння з 29 іншими штамами виду з GenBank побудоване філогенетичне дерево та показана можливість ідентифікувати їх географічне походження за двома гіперваріабельними ділянками V4 та V5;

- встановлені мінімальні інгібуючі концентрації нового антибіотика стрептофунгіну щодо *C. albicans* ATCC 10231 (10 мкг/мл), *B. subtilis* ATCC 6633 (200 мкг/мл) та *P. aeruginosa* ATCC 9027 (500 мкг/мл), а також відсутність токсичності в широкому діапазоні концентрацій (від 2,5 до 500 мкг/мл), що визначає його потенціал як активного фармацевтичного інгредієнту;

Практичне значення одержаних результатів. Практичне значення дисертаційної роботи полягає у вирішенні науково-практичної проблеми розробок антимікробних засобів, що не спричиняють розвиток резистентності мікробних патогенів.

Розроблений метод скринінгу інгібіторів сортази А, що відрізняється використанням більш дешевих субстратів та відповідно можливістю широкого застосування, впроваджено у практику Екологічної комплексної лабораторії факультету тропічної медицини Хайнанського медичного університету, Китай. (Акт впровадження від 04.09.2023 р.).

Розроблені «Рекомендації до впровадження методів моніторингу резистентності нозокоміальних інфекцій та принципів розробки сучасних протимікробних препаратів», що затверджені (25.08.23 р.) в Національному університет охорони здоров'я України імені П. Л. Шупика, для використання у практиці.

Запропоновані в результаті виконання дисертаційного дослідження принципи та підходи щодо створення препаратів на основі біологічно активних субстанцій з різним механізмом дії використані у виробничій

програмі ТОВ «ФАРМА ІНТЕРНЕШНЛ ГРУП 2» (м. Київ) при розробці засобів функціональної косметики (Довідка від 20.07.2023 р.):

Результати роботи впроваджено у викладання курсів “Загальна мікробіологіч та вірусологія” та “Основи фармацевтичних виробництв” для студентів спеціальності 162 «Біотехнології та біоінженерія» на кафедрі промислової біотехнології та біофармації КПІ ім. Ігоря Сікорського (Акт впровадження від 20.09.2023 р.), а також для студентів спеціальності «Тропічна медицина» в рамках лекційних та лабораторних (практичних) занять з дисциплін «Медична імунологія», «Патобіологія», «Медична мікробіологія», «Екологічна мікробіологія», «Гігієнічна мікробіологія», «Довкілля та здоров’я» в Хайнанському Медичному унверситеті (Китай) (Акт впровадження від 01.09.2023 р).

Основні положення роботи викладено у 10 наукових працях: 4 наукових статтях, які входять до наукометричної бази Scopus, в т.ч. 2 з них у видання, віднесених до 2-го квартилю (Q2) відповідно до класифікації SCImago Journal and Country Rank (прирівнюється до двох публікацій, які зараховуються відповідно до абзацу першого пункту 11 постановою Кабінету Міністрів України від 6 березня 2019 р. № 167); 4 статті у фахових закордоних періодичних виданнях; 2 тез на всеукраїнських та міжнародних конференціях.

Ключові слова: інфекції, патогени, антимікробні засоби, токсичність, резистентність, ензими, антибіотики

LIST OF PUBLICATIONS OF THE ACQUIRER ARTICLES IN PROFESSIONAL AND INTERNATIONAL JOURNALS

1. **WU Lin**, LI Li-hua, WU Li-xian. Phylogenetic analysis of *Pseudomonas oryzihabitans* of different geographical populations based on partial sequences of 16S rRNA gene (in Chinese). China Tropical Medicine. 2012; 12(12):1453-1456. DOI:10.13604/j.cnki.46-1064/r.2012.12.018. (is included in the international scientometric databases: CNKI, EBSCO, WPRIM, CA, CABI, Global Health and

others). *The acquirer carried out a review of the literature, the formation of conclusions and preparation for publication.*

2. WU Zhi-Cheng, **WU Lin**. Clinical distribution and drug resistance change of respiratory nosocomial infections of *Acinetobacter baumannii* (in Chinese). Journal of Hainan Medical University. 2013; 19(2):271-274. http://www.cqvip.com/QK/90826X/201302/44859186.html (is included in the international scientometric databases: CNKI, CA, CABI, DOAJ, EBSCO and others). *The acquirer conducted research and analysis of the results.*

3. **Lin Wu**, Huijun Li, Tianle Tang. A Novel Yeast Surface Display Method for Large-Scale Screen Inhibitors of Sortase A. Bioengineering. 2017; 4,6. DOI:10.3390/bioengineering4010006 (**Q2**) (is included in the international scientometric databases: PubMed/Medline, PMC, **Scopus**, Science Citation Index Expanded and others). *The acquirer conducted research and analysis of the results.*

4. **L Wu**, ZC Wu, TS Todosiichuk, OM Korneva. Nosocomial infections: pathogenicity, resistance and novel antimicrobials. Innov Biosyst Bioeng. 2021; 5(2):73–84. DOI: 10.20535/ibb.2021.5.2.228970 (**categ. A)** (is included in the international scientometric databases: PubMed/Medline, PMC, **Scopus** and others). *The acquirer carried out a review of the literature, analysis of literary data, formation of conclusions and preparation for publication.*

5. ZC Wu, **L Wu**, M Zhang, W Zhou. Genome sequence and annotation of *Bacteroides sp aff. Thetaiotaomicron* strain isolated from blood. Infection, Genetics and Evolution. 2021; 91: 104816. PMID: 33771725. DOI: 10.1016/j.meegid.2021.104816. (**Q2**) (is included in the international scientometric databases: Directory of Open Access Journal(DOA), Embase, **Scopus**, Zoological Record and others). *The acquirer conducted research and analysis of the results.*

6. Tetiana S. Todosiichuk, Serhii O. Soloviov, **Lin Wu**, Iryna V. Dzyublyk, Olena P. Trokhimenko, Magdalena Dudek, Artem Symchuk, Volodymyr Vasylenko. Directions in the development of modern and promising antimicrobial agents. BIOLOGIJA. 2022; 68 (4): 218–229. DOI:10.6001/biologija.v68i4.4838 (is included in the international scientometric databases: DOAJ; ROAD; HINARI;

Chemical Abstracts Service and others). *The acquirer carried out a review of the literature, analysis of literary data, formation of conclusions.*

7. Klochko V, Todosiichuk T, **Lin W**, Kobzysta O, Bobyr V. Antimicrobial and Cytotoxic Characteristics of Antibiotic Streptofungin. Innov Biosyst Bioeng [Internet]. 2023Aug.22;7(2):13-21. DOI:10.20535/ibb.2023.7.2.286158 (**categ. A**) (is included in the international scientometric databases: **Scopus**, DOAJ, ROAD and others). *The acquirer conducted a review of the literature and preparation for publication.*

8. Yu Zhou, **Lin Wu**，Yifan Zhou，Dengqing Si, Riwen Lin, Tianle Tang. Isolation and characterization of Vibrio sinaloensis from Penaeus vannamei Boone in Hainan province. Agricultural Biotech. 2017; 6(3): 55-58. DOI: CNKI:SUN:AGBT.0.2017-03-014. (is included in the international scientometric databases: CNKI, EBSCO, WPRIM and others). *The acquirer conducted a review of the literature and preparation for conclusions.*

ABSTRACTS OF REPORTS

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CONTENT

LIST OF CONVENTIONAL ABBREVIATIONS

MDR – multi-drug resistant bacteria

PDR – pan-drug resistant

MRSA – Methicillin-resistant *Staphylococcus aureus*

PCR – polymerase chain reaction

rRNA – ribosomal ribonucleic acid

bp – base pairs

NGS – The next generation sequencing

PE – paired-end

HCS – HiSeq Control Software

NCBI – National Center for Biotechnology Information

GO – Gene Ontology

KEGG – Kyoto Encyclopedia of Genes and Genomes

COG – Clusters of Orthologous Groups

Mb – megabases

GC – guanine-cytosine

tRNA – transfer ribonucleic acid

ncRNA – non-coding ribonucleic acid

CAZy – the Carbohydrate-Active enzymes Database

GHs – glycoside hydrolases

GTs – glycosyl transferases

PLs – polysaccharide lyases

CEs – carbohydrate esterases

AA – auxiliary enzymes

CAMP – cationic antimicrobial peptide

ECF – extracytoplasmic function

CTns – conjugative transposons

RGI – resistance gene

LB medium – lysogeny broth medium

MD medium – minimal dextrose medium

YPD medium – yeast extract peptone dextrose medium

BMGY medium – buffered glycerol- complex medium

BMMY medium – buffered methanol- complex medium

YNB medium – yeast nitrogen base medium

IPTG – isopropyl β-d-thiogalactoside

SDS-PAGE – SDS-polyacrylamide gels

LPXTG – leucine, proline, any amino acid, threonine and glycine

EGFP – enhanced green fluorescence

FRET – Fluorescence resonance energy transfer (FRET) assay

MDBK – Madin-Darby epithelial cells

A549 – epithelial cells obtained from human lungs

MIC – minimum inhibitory concentration

API – active pharmaceutical ingredients

LA – lytic activity

TEM – transmission electron microscopy

INTRODUCTION

The relevance of the research. The era of antibiotics, which began with the discovery of penicillin in the XX century, may soon end and humanity will face a challenge to overcome which will have to find new solutions. This challenge is now the "era of antibiotic resistance", caused not only by evolutionary mechanisms of protection of pathogens, but also by many factors of human activity [1-3].

Particular importance are methods of combating infectious agents when they are in treatment centers and a large number of people can both become infected and be a source of their spread. Such infections are nosocomial (hospital-acquired infection) and are defined by World Organization of Health (WHO) as infections that can be infected the patient during care in a hospital or other health care facility [3-5]. The sources of infection in hospitals are not only other patients and staff, but also surfaces, instruments, medical manipulations and operations, which is due to problems in ensuring proper conditions. However, one of the important factors in the treatment of nosocomial infections is their resistance to many antibiotics used in hospitals at the same time, and as a result “superbug” arise, for which there is no effective counteraction [4-6]. WHO defines a list of such relevant "superbugs", and almost half of them are included in the already established acronym ESCAPE – ***E****nterococcus faecium,* ***S****taphylococcus aureus,* ***K****lebsiella pneumoniae,* ***A****cinetobacter baumannii,* ***P****seudomonas aeruginosa* and ***E****nterobacter spp.* [1, 5, 7-9].

It was found that the natural and induced variability of microorganisms that cause inflammatory processes can lead to increased resistance to the action of previously effective antiseptics due to the acquisition of resistance. In addition, the selection of resistant forms of microbial pathogens, can cause low efficiency of therapy, severe disease, long-term treatment or, in some cases, the inability to overcome the infection at all [4, 9, 10]. The above and many other sources, citing WHO and the Center for Disease Control (CDC, USA), state that the need to develop effective antimicrobials against these and other nosocomial infections is a "need of the hour" [3, 4, 5].

Obviously, the solution of the problem of overcoming nosocomial infections has many dimensions, including organizational, educational, medical and so on. But these long-term strategies do not remove the urgent task of finding effective antimicrobials or new combinations to treat these severe, often combined infections, right today. This work currently involves a large number of scientists and practitioners, with different approaches to the solvation of the problem. One of the most effective method is undoubtedly the identification of the most vulnerable sites of infectious agents and their application as targets for new drugs [11-13]. Such vulnerable points of microbial pathogens mainly determine their resistance and pathogenicity, and therefore these factors can be considered more closely to find the target.

Therefore, the systematization of scientific data and results in this regard, the analysis and evaluation of the main factors of pathogenicity and resistance of nosocomial and other infections are relevant to determine modern approaches to the development of the latest antimicrobial agents.

Interaction of work with scientific programs, plans, topics. The dissertation was completed as part of the research project "Creating a line of innovative biologically active products for medicine, food industry and agriculture" (state registration number 0112U002390) at the Department of Industrial Biotechnology and Biopharmacy of KPI named after Igor Sikorsky and within the framework of the General project of Hainan Provincial Natural Science Foundation (№ 817324), The Program of Hainan Association for Science and Technology Plans to Youth R & D Innovation (№ 201513) in the laboratory of environmental ecology and research on health problems of Hainan Medical University, Haikou (China).

The goal of the work was to justify systemic approaches to the development of modern antimicrobials based on the analysis of resistance factors of bacterial pathogens of nosocomial infections.

To achieve the goal, it was necessary to solve the following problems:

- on the basis of theoretical analysis and research, determine the factors and mechanisms of resistance of microbial pathogens, which can be chosen as targets for the action of antiseptics;

- to establish the molecular-genetic characteristics of individual representatives of bacterial pathogens that can be used to counter their distribution and to select strategies for antimicrobial therapy;

- to propose a method of screening antimicrobial substances for selected pathogenesis targets of pathogenic bacteria, which can be applied in research practice;

- to show the prospects of creating effective antiseptics based on microbial products - antibiotics and enzymes of the streptomycete *Streptomyces albus*;

- to develop recommendations for the implementation of methods of monitoring the resistance of nosocomial infections and the principles of modern antimicrobials development.

The object of research is the problem of resistance of pathogens of nosocomial infections and directions for the development of the novel antiseptics.

The subject of research are the factors and mechanisms of resistance of microbial pathogens, as well as promising substances for the development of modern antiseptics.

Research methods. microbiological, molecular genetic, bioinformatics, biochemical, physical research methods and statistical methods for processing research results were used to fulfill the tasks set in the work.

Scientific novelty of the obtained results.

The following scientific results were obtained for the first time in the dissertation:

- on the basis of genome sequencing and gene annotation of the strain *Bacteroides thetaiotaomicron* DSMZ 2079 isolated from the patient's blood, the presence of a one- and two-component system for recognizing environmental signals and responding to them was shown, the presence of four homologs of the self-transmitted conjugative transposon CTnDOT, which provides the extension of

resistance to tetracycline and erythromycin. The *rpoB* and *tuf* genes (in the JHR92_RS03155 and JHR92_RS03195 loci, respectively), which determine the strain's resistance to antibiotics, were identified;

- based on the results of the analysis of the 16S rRNA sequences of the isolated clinical strain *Pseudomonas oryzihabitans* JN 873340 and its comparison with 29 other strains of the species from GenBank, a phylogenetic tree was built and the possibility of identifying their geographical origin by two hypervariable regions V4 and V5 was shown;

- the minimum inhibitory concentrations of the new streptofungin antibiotic against *C. albicans* ATCC 10231 (10 μg/ml), *B. subtilis* ATCC 6633 (200 μg/ml) and *P. aeruginosa* ATCC 9027 (500 μg/ml) were established, as well as the absence of toxicity in a wide range of concentrations (from 2.5 to 500 μg/ml), which determines its potential as an active pharmaceutical ingredient;

Practical significance of the obtained results. The practical significance of the dissertation consists in solving the scientific and practical problem of developing antimicrobial agents that do not cause the resistance development of microbial pathogens.

The developed method for screening inhibitors of sortase A, which is distinguished by the use of cheaper substrates and, accordingly, the possibility of wide application, has been implemented in the practice of the Environmental Comprehensive laboratory of the School of Tropical Medicine of Hainan Medical University, China. (Act of implementation dated September 4, 2023).

Developed "Recommendations for the implementation of methods of resistance control of nosocomial infections and principles of development of modern antimicrobial drugs", approved (08.25.23) at the Shupyk National Healthcare University of Ukraine, for use in practice.

Proposed as a result of the dissertation research the principles and approaches for the creation of preparations based on biologically active substances with different mechanisms of action were used in the production program of LLC "PHARMA

INTERNATIONAL GROUP 2" (Kyiv) in the development of functional cosmetics (Instructions for use dated 20.07.2023 r.).

The results of the work were implemented in the teaching of the courses "General microbiology and virology" and "Fundamentals of pharmaceutical production" for students of the specialty 162 "Biotechnology and bioengineering" at the department of industrial biotechnology and biopharmacy of KPI named after Igor Sikorskyi (Act of implementation dated 20.09.2023), as well as for students of the specialty "Tropical medicine" within the framework of lectures and laboratory (practical) classes in the disciplines "Medical immunology", "Pathobiology", "Medical microbiology", "Environmental microbiology ", "Hygienic microbiology", "Environment and health" at Hainan Medical University (China) (Act of implementation dated 01.09.2023).

Personal contribution of the dissertation student. The results of the work presented in the dissertation were obtained by the author personally or with her direct participation. Planning of the work and discussion of the results was carried out jointly with the academic supervisors.

The author personally selected and critically analyzed a large volume of modern literature on the topic of the conducted scientific research, carried out bioinformatic analysis of nucleotide sequences from databases, isolated total DNA, separated proteins by electrophoresis, statistically processed and analyzed the obtained results, prepared abstracts for the conferences, developed recommendations for introduction of methods of controlling the resistance of nosocomial infections and the principles of development of modern antimicrobial drugs; the results presented in the paper are formalized. Numbers of studies were conducted in cooperation with specialists who are co-authors of the acquirer's publications submitted for defense.

Approbation of the results of the dissertation. The materials of the dissertation were presented and discussed at the following scientific and practical conferences: XIV All-Ukrainian Scientific and Practical Conference "Biotechnology of the 21st Century" (May 20, 2020, Kyiv, Ukraine), I International

Scientific and Practical Internet Conf. "Problems and achievements of modern biotechnology" (March 25, 2021, Kharkiv, Ukraine), VII International scientific and practical conference "New achievements of biotechnology" (September 21-22, 2023, Kyiv, Ukraine, certificate of participation).

The main provisions of the work are presented in 10 scientific papers: 4 scientific articles included in the Scopus scientometric database, including 2 of them in publications assigned to the 2nd quartile (Q2) in accordance with the SCImago Journal and Country Rank classification (equal to two publications that are counted in accordance with the first paragraph of paragraph 11 of the Resolution of the Cabinet of Ministers of Ukraine dated March 6, 2019 No. 167); 4 articles in specialized foreign periodicals; 2 abstracts at all-Ukrainian and international conferences.

LIST OF PUBLICATIONS OF THE ACQUIRER ARTICLES IN PROFESSIONAL AND INTERNATIONAL JOURNALS

1. **WU Lin**, LI Li-hua, WU Li-xian. Phylogenetic analysis of *Pseudomonas oryzihabitans* of different geographical populations based on partial sequences of 16S rRNA gene (in Chinese). China Tropical Medicine. 2012; 12(12):1453-1456. DOI:10.13604/j.cnki.46-1064/r.2012.12.018. (is included in the international scientometric databases: CNKI, EBSCO, WPRIM, CA, CABI, Global Health and others). *The acquirer carried out a review of the literature, the formation of conclusions and preparation for publication.*

2. WU Zhi-Cheng, **WU Lin**. Clinical distribution and drug resistance change of respiratory nosocomial infections of *Acinetobacter baumannii* (in Chinese). Journal of Hainan Medical University. 2013; 19(2):271-274. http://www.cqvip.com/QK/90826X/201302/44859186.html (is included in the international scientometric databases: CNKI, CA, CABI, DOAJ, EBSCO and others). *The acquirer conducted research and analysis of the results.*

3. **Lin Wu**, Huijun Li, Tianle Tang. A Novel Yeast Surface Display Method for Large-Scale Screen Inhibitors of Sortase A. Bioengineering. 2017; 4,6. DOI:10.3390/bioengineering4010006 (**Q2**) (is included in the international scientometric databases: PubMed/Medline, PMC, **Scopus**, Science Citation Index Expanded and others). *The acquirer conducted research and analysis of the results.*

4. **L Wu**, ZC Wu, TS Todosiichuk, OM Korneva. Nosocomial infections: pathogenicity, resistance and novel antimicrobials. Innov Biosyst Bioeng. 2021; 5(2):73–84. DOI: 10.20535/ibb.2021.5.2.228970 (**categ. A)** (is included in the international scientometric databases: PubMed/Medline, PMC, **Scopus** and others). *The acquirer carried out a review of the literature, analysis of literary data, formation of conclusions and preparation for publication.*

5. ZC Wu, **L Wu**, M Zhang, W Zhou. Genome sequence and annotation of *Bacteroides sp aff. Thetaiotaomicron* strain isolated from blood. Infection, Genetics and Evolution. 2021; 91: 104816. PMID: 33771725. DOI: 10.1016/j.meegid.2021.104816. (**Q2**) (is included in the international scientometric databases: Directory of Open Access Journal(DOA), Embase, **Scopus**, Zoological Record and others). *The acquirer conducted research and analysis of the results.*

6. Tetiana S. Todosiichuk, Serhii O. Soloviov, **Lin Wu**, Iryna V. Dzyublyk, Olena P. Trokhimenko, Magdalena Dudek, Artem Symchuk, Volodymyr Vasylenko. Directions in the development of modern and promising antimicrobial agents. BIOLOGIJA. 2022; 68 (4): 218–229. DOI:10.6001/biologija.v68i4.4838 (is included in the international scientometric databases: DOAJ; ROAD; HINARI; Chemical Abstracts Service and others). *The acquirer carried out a review of the literature, analysis of literary data, formation of conclusions.*

7. Klochko V, Todosiichuk T, **Lin W**, Kobzysta O, Bobyr V. Antimicrobial and Cytotoxic Characteristics of Antibiotic Streptofungin. Innov Biosyst Bioeng [Internet]. 2023Aug.22;7(2):13-21. DOI:10.20535/ibb.2023.7.2.286158 (**categ. A**) (is included in the international scientometric databases: **Scopus**, DOAJ, ROAD and others). *The acquirer conducted a review of the literature and preparation for publication.*

8. Yu Zhou, **Lin Wu**，Yifan Zhou，Dengqing Si, Riwen Lin, Tianle Tang. Isolation and characterization of Vibrio sinaloensis from Penaeus vannamei Boone

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Scope and structure of the dissertation. The dissertation is presented on 144 pages of printed text (110 pages of main content). The work consists of the following sections: introduction; literature review; research materials and methods; 3 sections of own research and their discussion; conclusions; references; addition. The list of used sources includes 210 sources, placed on 21 pages. The work is illustrated with 14 tables and 19 figures.

CHAPTER 1. LITERATURE REVIEW

1.1 Nosocomial infections: pathogenicity, resistance and promising antimicrobials

1.1.1 Factors and mechanisms of bacterial pathogenicity

Pathogens cause pathogenicity to the host mainly through four steps, including adhesion, colonization, invasion and toxin production, among which adhesin play essential roles in binding to host epithelial and endothelial cells, interactions with host mucosal layers and components of the extracellular matrix (ECM) that surround host cells, and in biofilm formation [14-16]. It can not only be an inherent component of a pathogen that causes damage to host cells and/or tissues (e.g., exotoxins), but also a molecule or structure (e.g., capsule, biofilm) that enables the pathogen to evade or modulate host defense systems to its replicative advantage. Moreover, it enhances the ability of a pathogen to resist host fluid flow, attach to specific target cells, and potentially invade those target cells.

Bacteria have evolved an abundance of mechanisms to engage with host cells and manipulate their cellular signaling programs to facilitate colonization [17].

Adhesion of bacteria to host surfaces is a crucial aspect of host colonization as it prevents the mechanical clearing of pathogens and confers a selective advantage towards bacteria of the endogenous flora. Bacteria have evolved a very large arsenal of molecular strategies allowing them to target and adhere to host cells. Depending on the biochemical identity of the adhesive structure, its role during colonization may vary: it may be to enable initial, weak, and nonspecific adhesion, by establishing hydrophobic interactions with the host surface, thereby overcoming the electrostatic repulsion between bacterial and host surface. Other adhesins engage in highly specific interactions with host surface receptors, giving rise to high-affinity, stable interactions.

Pili, which are polymeric hair-like organelles protruding from the surface of bacteria, represent a first class of structures involved in the binding of bacteria to host cells [14]. The base of these structures, initially discovered in gram-negative

bacteria, is anchored to the bacterial outer membrane, whereas the tip is usually an adherence factor conferring the binding specificity of these structures. The most important pili kinds are Type I pili and Type IV pili. Type I pili at the surface of gram-negative bacteria, which have binding specificity to d-mannosylated receptors, such as the uroplakins of the bladder [18]. Type IV pili constitute another class of polymeric adhesive surface structure expressed by different gram-positive bacteria [19]. Type IV pili can retract through the bacterial cell wall, while the pilus tip remains attached to its target surface, allowing the so-called "twitching motility", a flagella-independent mode of motility important for efficient colonization of host surfaces [20].

In the last decade, pili structures have also been observed in gram-positive bacteria. Two types of pili have been described so far in these species. The first class consists in "sortase-assembled pili", in which successive pilin subunits are linked by isopeptide bonds after translocation across the bacterial membrane. This linkage is catalyzed by bacterial transpeptidases called «sortases» allowing the formation of completely covalent polymers that are eventually linked to the pentapeptide crossbridge found within the lipid II component of the peptidoglycan layer [21-23]. The second class consists in "type IV-like pili", which are like type IV pili of Gram-negative bacteria, even though the lack of outer membranes and the thick peptidoglycan structures of Gram-positive bacteria imply differences in the assembly mechanisms of these filaments [19].

In addition to pili, a wide range of bacterial surface factors with adhesive properties have been described. These adhesins recognize various classes of host molecules including transmembrane proteins such as integrins or cadherins, or components of the extracellular matrix such as collagen, fibronectin, laminin or elastin [14, 24,25]. Some of these adhesins, after allowing the binding of bacteria to host cell surfaces, are also triggering the internalization of bacteria inside host cells.

In parallel to these canonical mechanisms of bacterial adhesion, the EPEC (Entero-Pathogenic *E. coli*) and EHEC (Entero-Hemorragic *E. coli*) pathogens, which are responsible respectively for diarrheal disease in children, and severe

foodborne infections, use a very particular mechanism to create an intimate contact with host cells: they inject an effector, called Tir, that inserts into the host cell plasma membrane and serves as an “exogenous” receptor for the bacterial surface protein intimin [26].

Over time, we can discover more adhesion factors and mechanisms. Adhesion represents a crucial step for extracellular bacteria that facilitates their persistence in the host. We will learn more about intracellular bacteria， which is first essential step that precedes their internalization within host cells.

Professional phagocytes, such as macrophages or M-cells of the intestinal Peyer's patches, represent a frontline defense against pathogens. Although these cells are playing a key role in coordinating the innate and adaptative immune response to limit the colonization of pathogens in the host, they also constitute entry portals for pathogens. Many bacteria can also induce their internalization into non-professional phagocytes. Translocation through non phagocytic cells of the intestinal epithelium is another key mechanism used by pathogens to reach the lamina propria and to cause infections. Two main mechanisms of entry are involved in this case, namely the zipper and the trigger mechanisms [27].

In the case of their internalization mechanism, engagement of bacterial proteins with host membrane proteins normally involved in cellular adhesion such as cadherins or integrins, leads to the recruitment of various host factors involved in the strengthening of cell–cell or cell-matrix contacts. These proteases can not only degrade the immune molecule's action and destroy tissue structure to facilitate the spread of bacteria but also exert more invasive effects by activating or inhibiting proteases in the human body to activate receptors [28]. For example, the alkaline protease of *P. aeruginosa* can hydrolyze complement components C1q and C3, as well as a variety of cytokines and chemokines, blocking the effects of immune factors on bacteria.

A significant factor of the virulence and pathogenicity of pathogens of nosocomial infections is their ability to secrete toxins. Typical exotoxins are *S. aureus* hemolysins, endogenous toxic compounds are synthesized by *Shigella*

dysenteriae, and *Clostridium botulinum* pathogenic clostridia are capable of synthesizing neurotoxins that are partially bound to the cell. Both cellular proteins and nucleic acids can be targets for bacterial toxins. Some of them, such as lethal distending toxins (CDTs), like DNAse I, can cleave DNA during its replication, inhibiting cell division.

Genetic determinism of toxin synthesis has been shown in *P. aeruginosa*, as well as the expression of genes responsible for their oversynthesis (genes toxA, lasB and exoS) has also been established [29]. It is obvious that the very process of gene expression may be a target for the action of new antimicrobials, which has been noted in the study of metabolism of *S. aureus* [30].

1.1.2 Resistance development and mechanisms

The selection pressure caused using of tons of antibiotics over the past 75 years since antibiotics were introduced has made almost all disease-causing bacteria resistant to antibiotics commonly used to treat them. Nearly 1000 resistance-related β-lactamases that inactivate these antibiotics have been identified, a ten times increase since before 1990 [31]. The distribution of resistance genes, such as *Enterobacteriaceae*-producing extended-spectrum β-lactamase (ESBL), New Delhi metallo-β-lactamase 1 (NDM-1), and *K. pneumoniae* carbapenemase (KPC), indicates the ease with which resistance can spread. Findings of a study done in New Delhi showed NDM-1-producing bacteria (including *Shigella boydii* and *Vibrio cholera*) in two (4%) of 50 drinking water samples and 51 (30%) of 171 seepage samples suggesting the possibility of acquiring resistance outside health-care facilities [32].

Quinolone antibiotics are synthetic and so do not arise in nature, yet 30 years after their widespread introduction resistance is epidemic [33]. More specifically, whole genome studies suggest that quinolone resistance was a crucial factor in the evolution of hospital methicillin-resistant *S. aureus* (MRSA) [34], which indicates it is a long way to understand present epidemics of resistant healthcare-associated infections [35].

In health-care settings, the spread of a resistant clone can be rapid and have severe consequences for vulnerable hosts. The proportion of Enterobacteriaceae that were resistant to carbapenems increased from 0% in 2001 to 1.4% in 2010, with most of the increase recorded in *Klebsiella* sp. [36]. Healthcare associated infections are also increasingly recognized in low- and middle-income countries. Findings of a recent review showed that pooled prevalence of healthcare-associated infections in resource-limited settings (15.5 per 100 patients) was twice the average prevalence in Europe (7.1 per 100 patients) [37]. Incidence of infections acquired in intensive care units in developing countries (pooled density 47.9 per 1000 patient-days) was three times the rate in the USA (13.6 per 1000 patient-days).

Increasing rates of resistance to colistin and polymyxin B in Gram-negative organisms are being reported from countries around the world, including South Korea [38], Italy [39], Greece [40], and Saudi Arabia [41]. Moreover, there is some evidence of cross-resistance to colistin and host antimicrobial peptides that are part of the body's immune response [42].

Antibiotics are a subset of antimicrobials that inhibit essential functions in bacteria. Antibiotics are natural products or derivatives of natural products and are used widely to treat and prevent bacterial infections in humans and other animals. Most antibiotic-resistant infections are thought to occur in hospitals, where they increase the risks associated with medical treatments and undermine the ability of hospitals to provide safe places to heal [43, 44]. Bacterial antibiotic resistance (AR) is already making routine surgeries and hospital visits increasingly risky. The epidemic is particularly problematic in long-term acute care facilities, where over 25% of healthcare-associated infections are caused by antibiotic-resistant bacteria. Resistant bacterial populations spread when antibiotics exert selective pressures that favor resistance. Antibiotics can also eliminate susceptible microbial populations, reducing competition and expanding the resources available to resistant bacteria [45, 46]. Additionally, AR is spreading rapidly because once a resistance gene evolves in one bacterium, it can spread to other cells and other bacterial species [47-49]. To

tackle the rising problem of AR, we must understand how bacteria acquire and transmit resistant genes in clinical settings.

Mechanisms for the manifestation of virulence and pathogenicity of pathogens include their genetic evolutionary natural changes, as well as caused by artificial factors. However, in any case, such signs of resilience are passed on as survival benefits [50-53]. It should also be noted that such artificial factors of increasing resistance include not only medical practice, but also the widespread use of antibiotics in various fields - primarily in food and agriculture [54, 55]. Another factor in the rapid transfer of acquired antibiotic resistance is the coexistence of pathogens of different species in one association (in the wound, in the hospital in general), which is carried out by horizontal or lateral gene transfer (HGT) [56, 57].

Plasmids, bacteriophages, and extracellular DNA are the three primary drivers of HGT through the processes of conjugation, transduction, and natural transformation, respectively. The capacity for natural transformation is more sporadically distributed, yet it predates diversification of the bacterial Gram-positive and Gram-negative clades [58]. Gene transfer by each of the three mechanisms is favored between closely related organisms, but can occur between phylogenetically distant organisms [59]. Reservoirs of antibiotic-resistant organisms in hospitals have been well documented [60, 61], as have transmission routes between these reservoirs [62, 63], but the rates of horizontal transfer in clinical environments and the impacts of HGT on disease frequency remain unknown or speculative.

The development of drug resistance, which is based on mutations in chromosomal genes or the acquisition of drug resistance plasmids, is another component of pathogen resistance [64, 65]. Known families of microorganisms are naturally resistant to certain antibiotics: in their genome there are genes that control this characteristic. The highest level of mutations is observed in the genes mutS, mutL, mutH, mutT, mutY, mutM and uvrD, which are included in the MMR system [66-69]. The consequence of such mutations is an increase in genetic recombination and an overall increase in various mechanisms of resistance. For the genus *Acinetobacter*, for example, resistance to penicillin is a taxonomic trait. Polyresistant

to antibiotics and representatives of pseudomonads, non-clostridial anaerobes and some other microorganisms. Such bacteria are essentially natural repositories of drug resistance genes.

Adaptive resistance can be the result of numerous environmental factors and lead to the invention of effective defense mechanisms by pathogens [69-71]. Perhaps the most effective of them - the formation of biofilms. And the possibility of such existence of pathogens both on wound surfaces, and on catheters, endoscopes, etc. makes them important factors of their pathogenicity and targets for fight against causative agents of nosocomial infections. Increased resistance of pathogens in the form of biofilms to antibiotics is due to the following reasons [72-74]:

• inactivation of antibiotics by extracellular polymers or enzymes;

• slowing down the metabolism and, accordingly, reducing the growth rate of microorganisms in the conditions of limiting nutrients in the biofilm, due to which the antibacterial drug diffuses from the biofilm faster than it has time to act on it;

• expression of possible genes for antibiotic resistance;

• appearance of persister microorganisms in the biofilm under the action of antibiotics.

The most well-known mechanism of protection of pathogens from antimicrobial substances is enzymatic inactivation of antibiotics, which is realized through their synthesis of hydrolytic and redox enzymes, as well as transferases [75-77]. One such well-known enzyme is β-lactamase, which provides resistance of microorganisms to β-lactam antibiotics due to direct cleavage of the beta-lactam ring of these drugs. Other enzymes are able not to break down but to modify the active part of the antibiotic molecule, as is the case with enzymatic inactivation of aminoglycosides and chloramphenicol. Changing the permeability of the cell wall for an antibiotic or inhibiting its transport into bacterial cells, for example, underlies resistance to tetracycline. Structural changes in bacterial ribosomes are accompanied by increased resistance to aminoglycosides and macrolides, and changes in the structure of RNA synthetases – to rifampicin [78-82].

1.1.3 Antimicrobials for the «superbugs»

The traditional way to solve the problem of nosocomial infections is to search for new antimicrobial substances and create complex drugs that combine several antimicrobial substances with different mechanisms of action. Among the new classes of antiseptics developed by pharmaceutical companies, new peptides are attracting special attention; drugs that block fatty acid synthesis or early stages of protein synthesis in the microbial cell, as well as β-lactamase inhibitors that do not have their own antibacterial activity [83]. Thus, a new synthetic low molecular weight boron-containing drug (AN3365) blocks protein synthesis in gram-negative bacteria by inhibiting the synthesis of aminoacyl-t-RNA.

Another promising direction in the search for new antibiotic compounds is the selection of microbial producers from exotic and non-studied ecotopes. One of them is new antibiotics hexalactin and hexamycin, related to ansamycins, which include the currently used rifampicin [84-86]. The OSMAC (one strain many compounds) approach led to the discovery of three new *S. leeuwenhoekii* compounds of the rare class of 22-membered macrolactone polyketides, hexalactins A-C. Similarly, *S. leeuwenhoekii* was found to produce four new ansamycin-type compounds called hexamycins, which inhibit the development of *S. aureus* ATCC 25923 (minimum inhibitory concentration 0.05-0.13 μg/ml) and inhibit a number of methicillin-resistant isolates [86].

Isolated from marine sediments in California strain *Streptomyces* sp. CNH365 showed significant activity against the anthrax pathogen *B. anthracis* and methicillin-resistant staphylococcus, and the resulting antibiotic – polyketide antibiotic with a 14-membered macrolide ring, enolized β-diketone and lactone was named anthramycin [87, 88]. Polyketide 13 was obtained and structurally characterized polycarbonic compound of endophytic actinomycete *S. sundarbansensis* isolated from *Algerian algae Fucus* sp., shows selective activity against gram-positive microorganisms resistant to methicillin [89, 90].

In addition to finding new compounds among microbial producers, screening sensitivity-based techniques are proposed: when the intracellular level of the target

affected by the desired antibiotic is reduced by the action of the corresponding antisense-RNA, test strains become more sensitive to this antibiotic. Thus, it is possible to detect compounds that under normal conditions do not inhibit the growth of test strains. This method has identified a new class of antibiotics, which includes platensimycin, which is produced by *S. platensis* [91].

Such approaches to the development of new antiseptics to combat nosocomial infections are one way to solve the problem. However, more promising is the development of antiseptics aimed at the selected target in pathogen cells, which is potentially the least associated with the possibility of developing resistance. Therefore, the choice of such targets is a fundamental and decisive factor in the development of new antiseptics.

The development of drugs aimed at inhibiting the quorum sensing (QS) systems of pathogens as the main target, avoids the rapid development of resistance, as such substances do not have bactericidal or bacteriostatic action on pathogenic bacteria. Such drugs lead to the suppression of pathogenicity and are called "poisons of pathogenicity" [92, 93]. Inhibition of QS systems can be achieved in several ways. One of the strategies is to inhibit the synthesis of precursor molecules of autoinducers or autoinducers themselves (acylhomoserine lactones (AGL), peptides, amino acids and similar amine compounds). Second, drugs may be targeted by inhibiting the binding of autoinducers to the corresponding receptor proteins. Considerable attention is paid to such natural QS antagonists as furane derivatives, the role of which has already been proven in the suppression of QS in *P. aeruginosa* and *E. coli*. [94].

Among other promising compounds that can solve the problem of fighting nosocomial infections - enzybiotics, which now include substances with a specific mechanism of action (bacteriocins, cathelicins, lysines, bacteriophages, immunobiotics) [95-98] The authors identify the benefits and broad prospects of such drugs, which significantly increase the effectiveness of antimicrobial action without causing the emergence of resistant forms of pathogens. Part of the development focuses on the destruction of the biofilm of pathogens as an important

factor in their stability. It is shown that the combination of antimicrobial enzyme and fluoroquinolone antibiotic causes a synergistic effect against *S. aureus*, which is based on the breakdown of the biofilm layer by the enzyme and the subsequent bactericidal action of the antibiotic [99]. A similar mechanism is used in the development of a new drug «Dispersin» that acts on biofilms by destroying the cementitious substance of the biofilm matrix – poly-N-acetyl-glucosamine [100].

In the study of the combinative action of antibiotics and lytic enzymes, the effectiveness of their joint use in the treatment of superficial wounds of various etiologies and internal infections has been shown [101]. Therefore, the synergistic effect of enzymes and antibiotics will significantly reduce the effective dose of the latter, and consequently – reduce the cost of the drug and the development of pathogen resistance [102].

The experience of using bacteriophages as a basis for antimicrobial drugs already has a history but continues to be a promising way to combat resistant microbial pathogens [103]. Preparations of bacteriophages as antimicrobial agents have advantages because they do not affect the normal human microflora, do not cause resistance to pathogens, but their activity depends on the effectiveness of their replication. The development of this direction is the use of bacteriophage enzymes as an antimicrobial substance [104]. This solution provides high selectivity of the antimicrobial effect, while the enzymes (unlike the bacteriophages themselves) have no effect on the environment and the transfer of genetic information to microorganisms. An example of modern antibacterial and antifungal drugs is the development of a preparate of this profile, which differs from analogues in the content of bacteriophages with impaired replication function. Such drugs do not have these defects of live bacteriophage preparations and their enzymes.

The control of pathogenic microorganisms, which has been going on since the discovery of antibiotics, has led to the emergence of super pathogens capable of withstanding high concentrations of antibiotics and their combinations. It is said that it is possible to return to the pre-antibiotic era, when there were no means to combat infectious diseases; a future is possible, however, when there really will be no such

means, because human activities will lead to the emergence of resistant forms of pathogens. Obviously, the control strategy must be radically changed and focused not on killing the pathogens themselves but on influencing the factors of their pathogenicity and virulence. Numerous attempts to find such approaches can be seen in recent decades. They are generally targeted at inhibiting host-pathogen contact, cell adhesion, or immune system response regulation, which opens up the possibility of developing medications that can prevent disease progression even after the pathogen has entered the body. The development of new antimicrobials should be based on an in-depth study of the pathways and mechanisms of pathogen virulence, and therefore the choice of targets that are associated with non-vital cell processes and are least protected by pathogen cells. The development of such new generation drugs has the prospect of long-term effectiveness in overcoming the current problem of pathogen resistance and can provide a chance for the host organism with a low rate of evolution to successfully resist infectious diseases [105].

1.2 Streptomycetes – the producers of antimicrobial substances

The genus *Streptomyces* of the order *Actinomycetales*, currently produces a huge number of secondary metabolites which belong to different compound classes and are characterized by various biological activities, including antibacterial, antifungal, antitumor, immunosuppressive, etc. Streptomyces is a known to produce 70-80% of natural bioactive substances with pharmaceutical, agrochemical, biochemical, etc. applications [106-107]. New metabolites with different biological activity are constantly being isolated from the *Streptomyces* strains.

1.2.1 Antibiotic compounds

Antibiotics isolated from actinobacteria can be grouped according to the main structural classes, which include ansamycins (ritamycin), macrolides (erythromycin), aminoglycosides (streptomycin, kanamycin, tobramycin and neomycin), tetracyclines, anthracyclines, cyclophexycin [106].

Antibiotics produced by *Streptomyces* can also be divided according to the direction of action into those disrupting cell wall biosynthesis (vancomycin, cycloserine), translation (streptomycin kanamycin, erythromycin), RNA transcription (rifampicin), DNA replication and synthesis (novobiocin and metronidazole), synthesis of membrane structures (polymyxins), or those inhibiting bacterial growth in general [108].

Among the secondary metabolites produced by representatives of *Streptomyces*, a large class is represented by antibiotics which block protein synthesis at various levels. One of the first representatives of this group is streptomycin, an antibiotic belonging to the group of aminoglycosides, which binds to the bacterial 30S subunit, inhibits the translation of peptidyl-tRNA from the A-site to the P-site, and causes mRNA miscoding, which in turn leads to the death of the bacterium, unable to synthesize normal vital proteins [109]. Among all representatives of the genus *Streptomyces*, *S. atroverins* and *S. griseus* are the most active producers of streptomycin [110-112].

Antibiotics such as neomycin, kanamycin, and tobramycin, which are aminoglycoside by nature, have a similar mechanism of action. Neomycin, which is a complex substance (neomycin A, B, C), is synthesized by many representatives of the *Streptomyces*, with *S. fradia* being considered the most productive culture [113]. The difference between its action and other antimicrobial substances is that it does not only inhibit protein synthesis by binding to the bacterial 30S subunit, but it also inhibits bacterial DNA polymerase [114]. Similar to streptomycin, kanamycin binds to the 30S subunit, which leads to disruption of protein synthesis. Like neomycin, it is a complex mixture of kanamycin A, B and C. This complex is synthesized by *S. kanamyceticus* and *S. venerzuelae* [115, 116].

Tobramycin, produced by *S. tenebrarius*, has a more complex mechanism of action, which consists in binding to the 30S and 50S subunits of the ribosome, leading to the inability of forming the 70S complex and subsequentl disruption of protein translation [117]. The disadvantage of using this antibiotic is that its effect can be inhibited by Krebs cycle metabolites, such as glyoxylate, which protects the

bacterial cell by diverting carbon flow from the Krebs cycle, disrupting cellular respiration, and inhibiting tobramycin uptake [118].

The aforementioned aminoglycoside antibiotics synthesized by different cultures of *Streptomyces* have a broad spectrum of action, as they are active against a variety of gram-positive and gram-negative microorganisms [119]. They are particularly effective against members of the *Enterobacteriaceae* family, including *E.coli*, *E.cloacae* and *E.aerogenes*, *Morganella spp*., *Proteus spp*., *Klebsiella spp*., *Serratia spp*., *Providencia spp*., *Enterobacter spp*., *Shigella spp*., *Salmonella spp*., *Citrobacter spp*. They are active against the plague pathogen (*Y.pestis*) and tularemia (*F.tularensis*). This class also has sufficient effectiveness in inhibiting *Staphylococcus spp.*, *Streptococcus spp*. and *Enterococcus spp*.

Another class of antibiotic substances that block protein synthesis is tetracyclines, which include tetracycline, chlortetracycline, and oxytetracycline. At low concentrations, tetracyclines have a bacteriostatic effect by contributing to the inhibition of protein synthesis by binding to the 30S subunit of the bacterial ribosome, inhibiting the attachment of tRNA to the ribosome and amino acids to tRNA [120, 121]. In higher concentrations, this group of antibiotics has a bactericidal effect. Among *Streptomyces*, *S. aureofaciens* and *S. rimosus* have the highest activity of antibiotic synthesis of this group, with the former species producing mainly tetracycline and chlortetracycline, and the latter – oxytetracycline [122]. Although this group of antibiotics has moderate broad-spectrum antimicrobial activity against a large number of gram-positive and gram-negative bacteria, their overall effectiveness has declined recently due to the spread of antibiotic resistance [123].

Representatives of the macrolide class, such as erythromycin and oleandomycin, are considered to be more effective than tetracyclines. Synthesized by *S. werraensis* and *S. erythreus*, erythromycin exhibits bacteriostatic activity by binding to the 50S subunit of the bacterial rRNA complex, blocking protein synthesis and subsequent protein modification processes [124]. In addition, this compound prevents aminoacyl translocation and transfer of tRNA bound in the A-

site of the rRNA complex to the P-site, inhibiting the addition of subsequent amino acids [127].

Oleandomycin, produced by *S. antibioticus*, binds to the 50S subunit of the ribosome, like erythromycin, but unlike it, has a different structure, which affects negatively the interaction of the antibiotic with the rRNA complex and explains its lower efficacy [128]. The mentioned macrolide antimicrobials inhibit both gram-positive and gram-negative strains, including *Staphylococcus spp., Streptococcus spp., Neisseria gonorrhoeae, Haemophilus influenzae, Brucella spp., Legionella spp., Chlamydia spp.* and *Treponema spp.*

Novobiocin, also known as albomycin or katomycin, is an aminocoumarin antibiotic produced by the actinomycete *S. spheroids*. Novobiocin is a potent inhibitor of bacterial DNA gyrase and acts by interacting with the GyrB subunit of the enzyme involved in energy conversion [129, 130]. The antibiotic is highly effective against gram-positive microorganisms such as *Staphylococcus spp*. and *Streptococcus spp*.

A special representative of antibiotic compounds is cycloserine, a cyclic analog of D-alanine isolated from *S. orchidaceus, S. garyphalus* and *S. lavendulae*, which acts as an antibiotic and inhibits the biosynthesis of the bacterial cell wall [131, 132]. Its effect is the inhibition of two important enzymes: alanine racemase and D-Ala-D-Ala ligase. If both enzymes are inhibited, new D-alanine residues cannot be formed, and previously synthesized ones cannot be connected to each other [132]. As a result, peptidoglycan synthesis is inhibited. The antibiotic is an effective antibacterial agent against G^+ and G^- microorganisms, but its use is limited due to serious negative side effects [133].

Studies of the characteristics of actinomycetes have shown that these microorganisms are capable of producing more than one antibiotic, for example, *S. griseus* synthesizes bafilomycin (an ATPase inhibitor), frigocyclinone, and streptomycin. At the same time, the same antibiotic can be synthesized by different strains under different conditions and in different concentrations, for example, tetracycline was isolated from *S. achromogenes* and *S. rimosus* [106].

Sequencing of *Streptomyces* genomes from various environments revealed that most genomes contain 20 or more biosynthetic clusters of secondary metabolites, and most of them have never been isolated and characterized, which is why the search for and identification of new antibiotic metabolites of these actinomycetes has recently begun [134]. For example, in 2019, Sottorf *et al.* isolated a strain of *Streptomyces griseus subsp. griseus* DSM 40236, which synthesizes such compounds as gancidin W, emicin E, netropsin, actifenol, 6-beta-deoxy-5-hydroxytetracycline among its secondary metabolites. Moreover, the spectrum of action of these substances covers both gram-positive and gram-negative bacteria, which is due to the presence of 6-beta-deoxy-5-hydroxytetracycline, gancidin W, phenatic acid, and netropsin.

1.2.2 Lytic enzyme complexes

Among the enzymes synthesized by *Streptomyces*, there are several enzymes that are considered bacteriolytic. These compounds are divided into glycosidases (N-acetylmuramidase) and peptidases or proteinases.

The first class of enzymes, glycosidases, disrupt bacterial cell walls by acting on a linear sequence of N-acetyl-D-glucosamine and N-acetylmuramic acid residues. The microorganism *S. globisporus* produces two types of N-acetylmuramidase: M1 and M2 [135]. The M1 enzyme shows greater lytic specificity for cell walls of mutant strains of *Streptococcus spp*. than the M2 enzyme. They differ from each other in amino acid composition and methods of lytic action. The hydrolyzing action of M1 does not depend on the presence of acetyl groups on muramic acid residues, while the action of M2 is inhibited by the presence of these groups [135]. N-acetylmuramidase M1 has broad-spectrum bacteriological activity and is particularly effective for the lysis of lysozyme-resistant bacteria such as *Streptococcus* and *Lactobacillus* [136]. In addition to the above-mentioned *S. globisporus*, the enzyme is also found in other streptomycetes, including *S. griseus*, *S. erythraeus*, *S. levoris*, and *S. rutgersensis* [135-138].

The second class is peptidases, the enzymatic activity of which based on hydrolyzing peptide bonds between peptidoglycan monomers, which destroys the bacterial cell wall. The filtrate of *S. griseus* culture contains a complex of peptidases with broad endo- and exopeptidase activity [139]. The main advantage of *Streptomyces peptidases* is that most peptidases are thermostable and act in a wide pH range [140]. To date, peptidases are obtained as by-products in the biosynthesis of antibiotics in the logarithmic growth phase from the fermentation broths of *S. fradiae, S. griseus,* and *S. rimosus* [108].

1.2.3. Antimicrobial metabolites of *Streptomyces albus*

Streptomyces albus (*S.recifensis var. lyticus*), being a representative of the genus *Streptomyces*, synthesizes a significant number of secondary metabolites. The *S. albus* genome sequence contains 26 clusters of secondary metabolic synthesis genes, including salinomycin, paulomycin A and B, and a complex of enzymes [141]. In addition, many *S. albus* cultures, such as *S. albus* J1074, are used as a host for heterologous expression of several natural product gene clusters, including mycinomycin, stefimycin, triocoralin, etc. [142, 143].

The representative of ionophore polyester antibiotic substances synthesized by *S. albus* is salinomycin, which has high antimicrobial activity against Gram-positive bacteria, particularly methicillin-resistant cultures of *S. aureus, S. epidermidis* and *M. tuberculosis*, and is not effective against Gram-negative bacteria [144, 145]. The mechanism of action of ionophore antibiotics is to block intracellular protein transport, and the antibacterial properties of salinomycin are also due to their ability to transport metal cations across cellular and subcellular membranes [146]. To date, salinomycin is used in the study of antitumor substances and as a basis for the creation of new semi-synthetic antibiotics [147].

S. albus, particularly the strain *S. albus* J1074 and *S. albus G*, are the producers of paulomycin and four of its derivatives, consisting of quinone ring A, acetylated D-alose, paulomycose, etherified 2-methylbutyric acid and polysaccharide, inhibiting protein synthesis [148, 149]. The isolated compounds exhibit significant

antibiotic activity against gram-positive bacteria (*S. aureus* and *B. cereus*) and are ineffective against gram-negative microorganisms [150].

Moenomycin A is a member of the moenomycin phosphoglycolipid family of antibiotics that functions by binding bacterial transglycosylases, immutable enzymes that catalyze the elongation of the cell wall glycan chain to form a stable peptidoglycan layer. Moenomycins mimic the action of these enzymes and thus compete with the enzyme's natural substrate, inhibiting cell wall formation [151]. In general, the antibiotic is particularly effective against gram-positive bacteria at minimal inhibitory concentrations, and at higher concentrations it is also effective against gram-negative bacteria. Myenomycin is produced by at least four cultures of streptomycetes – *S. ghanaensis, S. bambergiensis, S. ederensis, S. geysiriensis*, as well as *S. albus* J1074 [142, 152].

Another antimicrobial compound isolated from *S. albus* is staphylimycin B, an antibiotic substance of the anthracycline family (aromatic polyketides) [143]. Staffimycin B is active against gram-positive bacteria and can be used in a variety of environments to kill or control such bacteria, especially when fighting *Streptococcus pneumoniae* [141]. This antibiotic has a complex mechanism of cytotoxic action by inhibiting the synthesis of peptidoglycans at different levels of their synthesis [143].

Recently, there has been renewed interest in the further development of this expression platform, in particular due to its rapid growth and naturally minimized genome, as well as in the further search for new *S. albus* producing strains and new metabolites by changing the genome and culture conditions [107]. The study of the ability to synthesize antibiotic substances by different cultures of *S. albus* continues, particularly, two substances with antimicrobial activity were isolated from the strain *S. albus* UN44 in 2019 [153]: bis-(2-ethylhexyl)-phthalate and 3-O-methylcyclopic acid, which are derivatives of phthalic aldehyde and are effective against microorganisms such as *B. subtilis, C. cefyr* and *M. flavus*.

In addition to antibiotics, *S. albus* synthesizes a complex of enzymes (proteinase, peptidase, glucosaminidase and muramidase), the action of which is

aimed at disrupting the structure of the cell wall and proteins. These enzymes have a similar mechanism of action as other members of this genus, and their activity was found against *S. aureus, B. cereus, C. gravis, P. aeruginosa, E. coli, P. rettgeri, S. thermophiles, S. sonnei, S. typhi, K. pneumonia* [154]. Moreover, the level of lysis of gram-positive bacteria is higher than that of gram-negative cells. Since *S. albus* is a representative of the genus *Streptomyces*, its genome contains a large number of secondary metabolites, in particular those with antimicrobial activity, culture studies, selection of nutrient media, cultivation conditions and mutant strains of this producer are being continued in order to isolate new antimicrobial highly effective substances.

Microbial pathogens have developed a variety of mechanisms to counteract antimicrobial agents and are constantly developing them, increasing their resistance and pathogenicity. It is on these mechanisms that the action of the novel antiseptics should be directed, which at the same time should not contribute to the emergence of additional resistance to pathogens. Analysis of numerical research results shows that antimicrobial agents that inhibit autoinducers, quorum sensing-systems of pathogens, biofilms, as well as the synthesis of enzymes that destroy antibiotics will be promising. It is important to develop multicomponent drugs with different mechanisms of action that will enhance the overall effect by destroying the protection of pathogens at different points in the process. It is interesting to use bacteriophages and antibiotics in such agents, as well as to search for substances with highly specific action on critical points of signal transmission of protective reactions of microorganisms. In concern of this, it is important to study new aspects of pathogenicity and resistance of pathogens of nosocomial infections, their analysis and consideration in the development of novel antiseptics.

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CHAPTER 2. MATERIALS AND METHODS

The experimental study was carried out in the laboratory of the Department of Industrial Biotechnology and Biopharmacy of the National Technical University of Ukraine "Igor Sikorsky Kyiv Polytechnic Institute" and Ecological Complex Laboratory of the Faculty of Tropical Medicine of Hainan Medical University (China). During the research, the bioethical norms of National Chinese and international legislation were observed, including the provisions of the IV European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (ETS 123 (1986)).

2.1 Materials

2.1.1. Isolated microbial pathogen culture

Clinical strains of *Acinetobacter baumannii* were isolate from the patient's sputum samples in the Affiliated Hospital of Hainan Medical College (China) from January 1, 2009 to December 30, 2011. The sputum samples were sent for examination and separation according to the requirements of the National guide to clinical laboratory procedures (3rd Edition) [155]. Of the total 5372 all bacterial infections, 206 strains of *A. baumannii* (excluding the repeated strains isolated from the same patient in one week) were detected and use for the investigation in relation of it antibiotic resistance and clinical characteristics.

Clinical strain of *Pseudomonas oryzihabitans* was isolate from the patient in the Affiliated Hospital of Hainan Medical College (China) in 2010. Its phenotypic characteristics and identification performed using the Vitek-2 bacterial identification system (French Bio-Merieux).

Sample of *Bacteroides* for examining was isolated from the blood of a 57-year-old male patient diagnosed with descending colon cancer and experiencing postoperative symptoms of fever in 2014. Blood culture analysis revealed the presence of bacteria. Partial dehiscence of the abdominal wall incision after surgery may be the cause of the intestinal flora entering the bloodstream. The sample was

cultivated in standard media and transferred to the laboratory of GENEWIZ (Jiangsu, China) for sequencing and bioinformation analysis.

2.1.2. Strains and media

The selected strains of *Streptomyces albus* culture from the museum of the Department of Industrial biotechnology and biopharmacy Igor Sikorsky Kyiv Polytechnic Institute were used in the work: strain 2435 (CMIM S-668), strain 2435/M (IMV Ac-5001), strain UN44 (IMV Ac-5030) and strains 4S, US101, AE6, 105, 80/5 (work museum of the Department). This culture synthesizes a complex of biologically active substances, which includes glycosidases, lytic endopeptidases, muramidases, non-lytic proteinases, amylases, both complex of antifungal and antibacterial antibiotics – streptofungin [153, 156].

To determine the antagonistic activity of *Streptomyces albus* producer strains and the minimal inhibitory concentration (MIC) of the obtained antibiotic used the test-strains from the museum of the antibiotic department of the Zabolotny Institute of Microbiology and Virology of the NAS of Ukraine: *Staphylococcus aureus* ATCC 6538, *Candida albicans* ATCC 10231, *Candida utilis* LIA-01, *Bacillus subtilis* ATCC 6633, *Proteus vulgaris* ATCC 6896, *Pseudomonas aeruginosa* ATCC 9027, *Escherichia coli* ATCC8739.

To study the antimicrobial activity (lytic, bacteriostatic, combined action) of the developed pharmaceutical compositions of antiseptics, the following test cultures from the Museum of the Laboratory of Medical Microbiology with the Museum of Human Pathogenic Microorganisms of the State University "Institute of Epidemiology and Infectious Diseases" L.V. Gromashevsky National Academy of Sciences of Ukraine" were used.: *Pseudomonas aeruginosa* ATCC 27853, ATCC 9027, *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Candida albicans* ATCC 10231.

The *S. aureus* strain (ATCC6538)) (Huankai Microbial, Guangdong, China) was cultivated in LB medium. The *E. coli* Top10 (Invitrogen, Carlsbad, CA, USA) was used for vector manipulation and propagation. The *E. coli* DE3(BL21)/pTRX-

srtA [157], was constructed in Laboratory of Biology, South China University of Technology, China and cultivated in LB medium, which was added ampicillin at 100 μg/mL as a marker. The *P. pastoris*GS115 strain (Invitrogen, Carlsbad, CA, USA) and the yeast-displayed vector pKFS [158] were used to display the LPETG-EGFPs. *P. pastoris* GS115 was cultivated in MD (1.34% (w/v) yeast nitrogen base, 6×10^{-5}% biotin, 1% (w/v) dextrose), YPD (1% yeast extract, 2% peptone, 2% dextrose), BMGY (1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 1.34% YNB, 4×10^{-5}% Biotin, 1% glycerol) and BMMY (1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 1.34% YNB, 4×10^{-5}% Biotin, 1% methanol) medium.

Yeast Culture *Pichia pastoris*

A single transformant was inoculated and kept according to the procedure [158]. The products were assayed by flow cytometry (Beckman-Coulter, Fullerton, CA, USA) with a total number of events of 5000 cells for each sample, and this data was analyzed using EXP032 software. The same products were determined by fluorescence spectrophotometry at excitation and emission wavelengths of 488 nm and 513 nm. Flow cytometry results were compared to the fluorescence spectrophotometry data in order to investigate the sensitivity and efficiency of the method.

2.2 Methods

2.2.1 Identification of clinical strains and Drug sensitivity test

The samples of the separated culture *A. baumannii* were treated according to the China National guide to clinical laboratory procedures (3rd Edition). Vitek-2 compact system (French Bio-Merieux) used for the identification and drug sensitivity analysis.

2.2.2 Polymerase chain reaction amplification of 16S rRNA gene from *Pseudomonas oryzihabitans*

16S rRNA gene amplification primers F8 (5'-AGAGTTTG ATCCTGGCTCAG-3') and R1492 ('-GGTTACCTTGTTACGACTT-3') were designed according to the [159] and synthesized by Shanghai Biotech company (China). 50μl PCR reaction system contains: sample 1 μl, rTaq DNA- polymerase (5 U/μL) 0.2μl, 10×PCR buffer (containing $Mg2^{+}$) 5μL, 1dNTP Mixture (25 mmol/L) 4μL, upstream primer (10 pmol/mL) 0.5 μL, downstream primer (10 pmol/mL) 0.5 μL, ddH_2O 38.8 μL. The PCR reaction conditions were: 95 ° C for 10 min; 94 ° C for 40 s, 55 ° C for 40 s, 72 ° C for 1 min, 30 cycles; 72 ° C for 10 min. The PCR product was observed by agarose gel electrophoresis. The PCR product amplification was purified and sent to Shenzhen Huada Gene (China) for sequencing.

2.2.3 Sequence analysis and establishment of phylogenetic tree of *Pseudomonas oryzihabitans*

The 16S rRNA gene sequences of the Hainan clinical isolate strain *Pseudomonas oryzihabitans* and other currently known *Pseudomonas oryzihabitans* strains in GenBank were analyzed by Blast. As the comparative was used 29 strains of *Pseudomonas oryzihabitans* from GenBank (Table 2.1) with gene sequence length >1200 bp. The phylogenetic tree was constructed using the MEGA 4.1 software Neighbor-joining method, and the Bootstrap method (repeated number 1000) was used.

Table 2.1

Reference sequence of *Pseudomonas oryzihabitans*

Strain	GenBank accession number	Gene sequence length, bp	Isolation place	Host
XA2-11	JF496264	1409	China	Soil surface

C-G-PYD10	HM755668	1330	Korea	Snow crab in the Far East
C-D-R2A7	HM755627	1303	Korea	Snow crab in the Far East
C-S-PYD4	HM755611	1227	Korea	Snow crab in the Far East
C-S-MA6	HM755547	1309	Korea	Snow crab in the Far East
C-G-NA8	HM755542	1382	Korea	Snow crab in the Far East
KPE62106H	HQ009876	1380	Korea	Japanese Ling
BBAL-03d	FJ217181	1435	Korea	Type b
LP11	HM038118	1414	China	Suaeda salsa
LKS06	HQ331135	1410	China	Solanum nigrum
KCB005	FJ824120	1366	Australia	deep sea
WB2003S	AY850170	1240	France	patient
WB2000S	AY850169	1240	France	Aquatic environment
LMG7040	GQ250598	1527	Belgium	unknown
1P03PE	EU977742	1414	United States	Spacecraft clean room
1P03PD	EU977741	1404	United States	Spacecraft clean room
1P03PC	EU977740	1423	United States	Spacecraft clean room
1P03PA	EU977738	1426	United States	Spacecraft clean room
1P3AnC2	EU977734	1430	United States	Spacecraft clean room
1P03AnA	EU977717	1203	United States	Spacecraft clean room
1P02MD	EU977605	1436	United States	Spacecraft clean room

1P03ME	EU977590	1445	United States	Spacecraft clean room
1P03MB	EU977587	1442	United States	Spacecraft clean room
1P03MA	EU977586	1436	United States	Spacecraft clean room
54-1	AB675634	1499	Japan	banana
KNUC338	EU239166	1228	Korea	plant
KNUC365	EU239123	1223	Korea	plant
IAM1568	D84004	1527	Japan	paddy
IAM1568T	AM262973	1466	Germany	soil

2.2.4 Sequence analysis of *Bacteroides thetaiotaomicron*

The next generation sequencing (NGS) library was prepared according to the protocol of the Bioinformatics Laboratory GENEWIZ, Inc. (www.genewiz.com). For each sample, 100 ng genomic DNA was randomly fragmented to <500 bp by sonication [160, 177]. The fragments were treated with End Prep Enzyme Mix for end repairing, 5′phosphate amplification and dA-tailing in one reaction followed by a TA cloning to add adaptors to both ends. Afterward, a size selection of Adaptor Ligated DNA was performed, and fragments of ~470 bp (with the approximate insert size of 350 bp) were recovered. Each sample was then amplified by polymerase chain reaction (PCR) for 8 cycles using P5 and P7 primers with both carrying sequences, which can bind to the flowcell to perform bridge PCR and P7 primer carrying a six-base index allowing multiplexing. The PCR products were cleaned up and validated using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA), and quantified by Qubit 3.0 Fluorometer (Invitrogen, Carlsbad, CA, USA). Libraries with different indices were multiplexed and loaded on an Illumina HiSeq instrument according to manufacturer’s instructions (Illumina, San Diego, CA, USA). Sequencing was carried out using a 2 × 150 paired-end (PE) configuration. Image analysis and base calling were carried out using HiSeq Control

Software (HCS) + OLB +GAPipeline− 1.6 (Illumina) on the HiSeq platform. The reads were qualified followed by assembling with Velvet software and gap-filling with SSPACE and GapFiller [161]. The Prodigal (for prokaryotic genomes) [162, 177] and Augustus (for eukaryotic genomes) gene-prediction software was applied for finding coding genes in bacteria. Transfer RNAs (tRNAs) were detected in the genome using the tRNAscan-SE program with default parameter settings. Ribosomal RNA (rRNA) were identified by using RNAmmer. The coding genes were annotated according to National Center for Biotechnology Information (NCBI) database by BLAST searches and the pathways annotation using KEGG (Kyoto Encyclopedia of Genes and Genomes) database. The genes-encoding proteins were classified based on COG resources (Clusters of Orthologous Groups).

2.2.5 Bioinformation analysis of the *Bacteroides thetaiotaomicron*

The software used for bioinformation analysis is shown in Table 2.2. We presented identification and visualization of genomic islands of the tested strain. The results were obtained using an online service IslandViewer 4 (2021). To determine the microorganism isolated from the patient's blood, we used the ANI Average nucleotide identification using an online calculator ANI Calculator (2021), based on an OrthoANI: An improved algorithm and software for calculating average nucleotide identity [177].

Table 2.2

Software analysis list

Analysis content	Software	Functional description	Version
quality control	cutadapt	Filter low quality data and remove linker sequences	1.9.1
assembly	kmergenie	Estimated genome size	1.7039
	velvet	Build contig	1.2.10
	SSPACE	Building scaffold	v3.0

	GapFiller	Fill up gap	v1–10
gene finding	prodigal	Gene prediction	v2.6.3
ncRNA analysis	cmscan	Aligning genomic sequences to the Rfam library	1.1.2
gene annotation	diamond	Nr comment	0.8.15
	blast	Kegg comment	2.2.28+
	blast2go	Go Comment	v2.5
	hmmscan	Cog comment	3.1b2
repeat analysis	RepeatModeler	Repeat sequence analysis	1.0.8
	RepeatMasker		4.0.6

2.2.6 Optimization of expression condition of Sortase A

SrtA was induced according to a previously documented procedure [157] from the overexpression *E. coli* DE3(BL21) harboring plasmid pTRX-srtA. *E.coli* DE3/ pTRX-srtA were induced expressed in the gradual induction temperature (22 °C, 28 °C, 37 °C and 40 °C), induction time (4 h, 5 h, 6 h and 7 h) and isopropyl β-d-thiogalactoside (IPTG) concentration (0.25 mmol/L, 0.5 mmol/L, 1 mmol/L, and 2 mmol/L). Other conditions were the same as in the previously documented procedure. The induction cells were detected by SDS-polyacrylamide gels (SDS-PAGE). SrtA was gained after purification by affinity chromatography, preserved by vacuum freeze drying at −20 °C.

2.2.7. Construction of pKFS/LPETG vector and transformation

To allow expression of LPETG-EGFP, its coding sequence was amplified using oligonucleotides P1: 5'-CCCACGCGTATGCAAGCTTTGCCTGAAACTGGTGAAGAAGGAGGAATTGGAATTGCTC-3' and P2: 5'-CGCGAATTCTTACTTGTACAGCTCGTCCATG-3', resulting plasmid vector was named pKFS-LPETG. MluI and EcoRI were used to linearize *P.pastoris* vector

(Fig. 2.1). The constructed pKFS-LPETG and pKFS were linearized and transformed into *P.pastoris* GS115 using the lithium acetate method [163]. The transformants GS115/ pKFS-LPETG and GS115/ pKFS were obtained by MD selective medium.

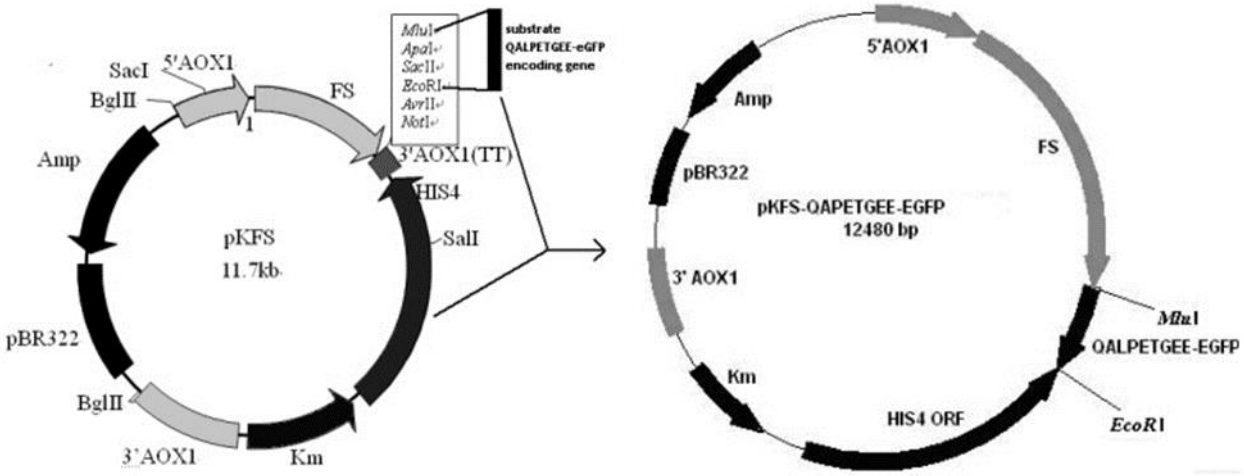


Fig. 2.1 Construction of plasmid pKFS-LPETG.

2.2.8 Fluorescence assay

SrtA activity was detected by quantifying the fluorescence intensity base on the cleavage of the yeast-surface-displayed LPETG protein. Reactions were performed in a total volume of 5 mL and the culture and berberine chloride or control treatment was added. A 150 μL volume of GS115/pKFS-LPETG-EGFP (OD600 = 1.0) or the same amount of GS115/pKFS (as a negative control) was added to each aliquot, 5 μM SrtA, and this was transferred to an Eppendorf tube. The mixture was tested every 30 min for 2 h by fluorescence spectrophotometry. Then, decreasing concentrations starting from 1 μg/mL to 30 μg/mL of berberine chloride (a well-known sortase inhibitor was purchased from Kaitong chemical Co., Ltd., Tianjin, China) was added and the effect was investigated according to the activity of SrtA, and the results were compared to the substrates of Dabcyl-QALPETGEE-Edans [164]. In this way it was possible to compare any differences in binding of the cells to SrtA due to differences in inhibitor concentrations treatment and cell growth.

2.2.9 Method of antagonistic activity determination

On Petri dishes with Gause medium to the culture of *Streptomyces albus* (a circle with a diameter of 1-2 cm), test-strains were seeded radially with strokes, using their suspension at a concentration of 10^9 cells/ml in a physiological solution. The seeded cups were incubated for 24 hours at a temperature of 37°C. After that, the growth inhibition zones of the test-strains were measured from the edge of the producer colony to the beginning of the growth of the test-strain.

Samples of antibiotic streptofungin were obtained according to the following scheme: cultivation of the producer was performed in 750 ml rolling flasks with 150 ml of nutrient medium based on glucose and soya flour, for 72 h at 28±1°C and stirring at 180 rpm [153, 156]. Nutrient medium, (g/l): glucose – 6.0; soybean flour – 8.0; NaCl – 14.0; $CaCl_2$ – 4.5; $MgSO_4 \times 7H_2O$ – 5.8; $MnCl_2$ – 0.04; K_2HPO_4 – 1.5; H_2O – up to 1 l. After completion of the biosynthesis process, and antibiotic was extracted with chloroform in a ratio of 1:1, and the resulting extract was dried by the vacuum method.

2.2.10 Method of determining of the minimal inhibitory concentration (MIC). A series of antibiotic dilutions was made using its 1% (10 mg/ml) solution. A series of Petri dishes was prepared, in each of which 10 ml (total volume) of pre-melted and cooled to 40°C MPA (Meet-peptone agar) medium was added, containing the appropriate amount of the antibiotic itself (serial dilution method). The following final antibiotic concentrations were obtained in Petri dishes: 1, 5, 10, 20, 50, 100, 200, 500 μg/ml. The cups were dried at room temperature for 2 hours and a suspension of test-cultures (concentration 5×10^8 cells/ml) was applied to the surface of the medium with a bacteriological loop. The sown cups were incubated in a thermostat for 24 hours at a temperature of 37°C. MPA medium without antibiotic used as reference. The maximum dilution of the antibiotic where there is no growth of the test-strain is the MIC.

Antibiotic concentration was determined by the Bouguer-Lambert-Bere formula [156, 165].

$$C = \frac{1}{E_{1см}^{1\%} \cdot b} \cdot D \cdot P$$

where C – antibiotic concentration, mg/ml;

$E_{1ml}^{1\%}$ – 2.1 (extinction of a 1% alcohol solution of an antibiotic, the optical path length is 1 cm);

b – thickness of the substance layer in the cuvette (1 cm);

D – optical density of the antibiotic solution at a λ=275 nm;

P – total dilution of the sample.

2.2.11 Method of determining the cytotoxicity of antibiotic preparation [156, 166]. Cells of the MDBK lines (Madin-Darby epithelial cells obtained from bull kidneys) and A549 (epithelial cells obtained from human lungs) were previously grown in a CO_2 incubator at 37°C on a standard Minimum Essential Medium (MEM Eagle, Sigma-Aldrich) nutrient medium with growth factors in 96-well plates for a day to form a monolayer. The nutrient substrate was carefully deleted, and the cells were washed twice with phosphate-salt buffer (FSB, pH 7.2, Sigma #P4417) and left in 200 μl of FSB for introduction of the antibiotic sample.

The antibiotic solution was prepared in 96% ethanol at the rate of 10 mg/ml. From this solution, appropriate volumes were added to MDBK and A549 cells, so that the final concentration of it was 2.5–500 μg/ml. Cells treated in this way were kept in a CO_2 incubator for an hour. After that, the cells were repeatedly washed twice from the antibiotic in FSB and left in 200 μl of FSB for introduction of resazurin.

A stock solution of resazurin (sodium resazurinate 85.6%, pure, China) was prepared at the FSB at a rate of 0.15 mg/ml. The solution was filtered through a filter with a pore diameter of 0.2 μm and stored in an opaque bottle at 4°C. Before use, the resazurin solution was warmed to room temperature and 20 μl was added for

every 100 µl of FSB in the wells of the plate. The plate with cells and resazurin was placed in a CO_2 incubator for 2-3 hours. After that, the optical density of the solutions in the wells of the plate was determined at 538 nm using a plate photocolorimeter Multiskan FC Microplate Photometer (Thermo Scientific, USA).

The obtained data were processed statistically using the Statistica v.10 program (StatSoft Inc., USA). The reliability of the differences between the average values of the effectiveness indicators of different concentrations of the substance under study was established using the method of variance analysis in accordance with the t-criterion. The samples were compared using two methods, which differ in their mathematical approach and therefore complement each other: the LSD method (method of least significant differences) and the Tukey-HSD (method of true significant differences). Differences between mean values were considered significant at $p<0.05$.

2.2.12 Method of electron microscopy

Cell morphology analyses were carried out by mounting cells onto 400 mesh copper grids, counterstaining with 2% uranyl acetate and examining in transmission electron microscopy (TEM) JEM-1400 (JEOL, Ltd., Japan) at 80 kV.[167]

2.2.13 Methods of biosynthesis, preparing and analyzing the activity of developed antimicrobial drugs

Samples of lytic enzyme cytal from *Streptomyces albus* was obtained according to the following scheme: cultivation of the producer was performed in 750 cm^3 rolling flasks with 150 ml of nutrient medium based on glucose and soya flour, for 48-72 h at 28±1°C and stirring at 180 rpm [153, 156]. Nutrient medium, (g/l): glucose – 6.0; soybean flour – 8.0; NaCl – 14.0; $CaCl_2$ – 4.5; $MgSO_4 \times 7H_2O$ – 5.8; $MnCl_2$ – 0.04; K_2HPO_4 – 1.5; H2O – up to 1 l. After completion of the biosynthesis process, the biomass was separated by centrifugation and the supernatant was sterilizing, concentrating, purify from macromolecular compounds by ultrafiltration and was dried by the vacuum method.

The lytic activity (LA) of the experimental samples was determined by the turbidimetric method according the lysis ability of *S. aureus* suspension and was expressed in IU/ cm^3. For 1 IU unit of LA was taken the amount of enzyme that reduces the optical density of the test-culture suspension by 0.001 per 1 min in 1 cm^3 of the reaction mix [156]. To 4 cm^3 of the test-culture suspension was added 0.2 ml of sample and incubated for 15 min at 37 °C. 0.2 cm^3 of distilled water was added to the control and incubated under the same conditions. The level of LA was determined by the difference in optical density of the suspension before and after incubation. The optical density was determined on a photocolorimeter KFK-3 at λ = 540 nm in a cuvette 0.5 cm on a background of distilled water.

The pharmaceutical composition was prepared as follows: streptofungin is dissolved in ethyl alcohol, cytal was dissolved in distilled water with stirring at a temperature of 25-30 °C. The molding base was prepared separately by adding glycerol, proxanol-268, polyethylene glycol (PEG)-600, PEG-1000 and polyethylene oxide (PEO)-400 to distilled water, after which the resulting mixture is heated to 40-45 °C while stirring until complete dissolution and obtaining a homogeneous mass. Solutions of active substances, anesthetic and dimexide were injected into the molten base cooled to 30-35°C, after which the resulting mass is cooled with stirring to 25°C until a homogeneous mass is obtained, which is packaged in dark glass vials.

2.2.14 Statistical methods

The special statistical methods that relate to individual research processes was described before. The other measurement data in all data were expressed as mean±standard deviation. The differences between groups were analyzed by variance. The mean of the two groups were compared by Student t-criterion. $P<0.05$ was considered statistically significant. Statistical processing was performed using SPSS13 software.

CHAPTER 3. RESEARCH AND CONTROL OF THE DISTRIBUTION AND RESISTANCE FACTORS OF THE BACTERIAL NOSOCOMIAL INFECTIONS CAUSATIVE AGENTS

3.1 Clinical distribution and drug resistance change of respiratory nosocomial infections *Acinetobacter baumannii*

Acinetobacter baumanii is the most common opportunistic pathogen in the genus Acinetobacter, usually a commensal bacterium. It is clinically mixed infection, with wide distribution, strong viability and strong transmission. In recent years, the clinical isolation rate and infection pathogenic rate of *A. baumannii* have increased year by year, and it can cause tissue infection and organ infection in the elderly to be more likely to occur in the hospital respiratory tract *A. baumannii* infection [168]. With the widespread use of broad-spectrum antibiotics, immunosuppressants, and the use of interventional medical treatments, the number of nosocomial infections has increased year by year. At the same time, the drug resistance spectrum of *A.baumannii* has also changed. Many countries have multi-drug resistant bacteria (MDR) or "pan-drug resistant" (PDR) *A. baumannii* [169]. It poses a serious challenge to clinical treatment, also known as Gram-negative MRSA. In order to understand the clinical characteristics of *A. baumannii* and the resistance to common antibiotics in recent times, the results are important for controlling the infection of *A. baumannii* and guiding clinical drug use.

During 2009, 2010 and 2011 years in the Affiliated Hospital of Hainan Medical College (China) were detected the bacteria from sputum of 1562, 1823 and 1987 patients, respectively, of which 206 identified strains of *A. baumannii* were mainly distributed in department of intensive care unit (25.30%), neurology (16.87%), respiratory medicine (15.66%) and neurosurgery (12.06%).

The infection rates of *A. baumannii* male and female patients in 2009, 2010 and 2011 are shown in Table 3.1. The results showed no statistical significance.

Table 3.1

Infection rate of male and female patients of *A. baumannii*

Year	Number of hospital infections (example)	Female *A. baumannii* infection rate (%)	Male *A. baumannii* infection rate (%)	Statistical significance (χ^2, P)
2009	1562	3.4（18/533）	3.3（34/1029）	χ^2=0.01, P＞0.05
2010	1823	4.0（24/601）	3.8（47/1222）	χ^2=0.02, P＞0.05
2011	1987	4.2（29/689）	4.3（56/1298）	χ^2=0.01, P＞0.05

Notes: (*A. baumannii* strains infection number/ hospital infections number)

The infection rates of this patients with different ages of *A. baumannii* infection in 2009, 2010 and 2011 are shown in Table 3.2. The results showed that the majority of patients over 60 years old and occupied for around 50% of *A. baumannii* infections.

Table 3.2

Infection rate of *A. baumannii* patients of different ages patients

Year	≤1 year old (example)	2-18 years old (example)	19-60 years old (example)	≥ 60 years old (example)	Statistical significance (χ^2, P)
2009	10/273	7/389	9/423	23/477	χ^2=11.49, P＜0.01
2010	12/317	9/453	15/522	35/531	χ^2=16.19, P＜0.01
2011	14/361	10/489	15/548	44/589	χ^2=24.43, P＜0.01

Notes: (*A. baumannii* strains infection number/ hospital infections number)

The high rate of detection of *A. baumannii* strains in hospitalized patients over 60 years old in the hospital with respiratory tract sputum for three consecutive years (about 50%) is consistent with the literature [170]. With the increase of age, the respiratory mucosa become weaker. The weakening of the defensive function allows the *A. baumannii*, which is fixed in the oropharynx or exogenous, to enter the lower respiratory tract and become infected.

The infection rates of *A. baumannii* in different seasons in 2009, 2010 and 2011 are shown in Table 3.3. The results showed that *A. baumannii* had infections throughout the year, and the detection rate in autumn was higher than that in the other three seasons.

Table 3.3

Infection rate of *A. baumannii* in different seasons

Year	Spring (example)	Summer (example)	Autumn (example)	Winter (example)	Statistical significance (χ^2, P)
2009	8/397	13/369	24/423	7/373	χ^2=11.84, P<0.01
2010	10/469	15/432	30/477	16/445	χ^2=11.51, P<0.01
2011	15/508	17/465	35/516	16/498	χ^2=12.13, P<0.01

Notes: (*A. baumannii* strains infection number/ hospital infections number)

The detection rate of *A. baumannii* isolates from the respiratory tract specimens of the autumn patients was significantly higher than that of the other three seasons, which was slightly different from the high detection rate in summer and autumn reported by Cai Lihong et al. [171] in summer and autumn. This is not only because of warm and humid in autumn. The climate may be more suitable for the growth and reproduction of *A. baumannii*, and it is also related to factors such as the difficulty of adapting seasonal climate change in the elderly. The study also showed

that *A. baumannii* has no difference in different gender groups, but it's still needed the further verification by amplifying the number of specimens.

The resistance rate of *A. baumannii* concerning the 11 antimicrobials during the investigated period shown in Table 3.4. The resistance to the Cefepime and Cefotaxime was not significantly changed in 3 years; Levofloxacin, Ceftazidime, Cotrimoxazole, aminoglycosides (Gentamicin, Tobramycin) resistance rate decreased; the resistance rate to Piperacillin/ Tazobactam, Imipenem and Meropenem increased year by year, as shown.

Table 3.4

Resistance of *A. baumannii* clinical strains to antimicrobial agents

Antibacterial drugs	2009 year (52 strains)		2010 year (71 strains)		2011 year (83 strains)		Total (n=206 strains)	
	Number of cases	Resistance rate, %	Number of cases	Resistance rate, %	Number of cases	Resistance rate, %	Number of cases	Resistance rate,%
Levofloxacin	39	75.0	36	50.7	17	20.4	92	55.4
Cefepime	12	23.1	18	25.4	21	25.3	51	24.8
Cefotaxime	40	76.9	56	78.9	62	74.6	156	77.7
Ceftazidime	28	53.8	30	42.3	25	30.1	83	42.5
Gentamicin	41	78.8	35	49.3	33	39.8	109	57.5
Piperacillin/ tazobactam	4	7.7	11	15.5	17	20.4	32	17.1
Imipenem	3	5.7	6	8.5	12	14.5	21	11.5
Meropenem	6	11.5	12	16.9	16	19.3	34	17.1
Cotrimoxazole	37	71.2	41	57.7	31	37.3	109	56.5
Tobramycin	12	23.1	12	16.9	11	13.6	35	18.0
Amikacin	29	55.8	30	42.3	25	30.1	84	43.3

Resistance rate in 2009 was higher and number of *A. baumannii* infections decreased significantly in 2010 and 2011. The multi-reasons are as followed. Firstly, the administrative intervention measures were implemented, and the "Regulations on the Use of Antibacterial Drugs" were formulated in accordance with the actual situation of the hospital, and strict disinfection, isolation and aseptic operation was carried out, the probability of nosocomial infection is greatly reduced. Secondly, aminoglycosides have ototoxicity and nephrotoxicity, which restricts the use of some patients [172]. Sulfonamides have a long clinical application time, and the drugs with high drug resistance are replaced, and the drugs with frequent use are replaced according to the results of drug susceptibility. Among them, the resistance of Piperacillin/ Tazobactam, Imipenem, and Meropenem was increased year by year. It could be caused by the several main reasons. It may be the application of β-lactam drugs, Carbapenems. Acinetobacter is highly susceptible to drug resistance by plasmid binding and hospital epidemics. Among the investigating, the Imipenem is the drug of choice for the infection of *A. baumannii* in this hospital.

The infection rate had no relation with sex, which were more frequent in autumn, geriatric patients and department of Intensive care units. The higher resistance *A. baumannii* shown to Cefotaxime (77.7%), Cotrimoxazole (56.5%), Gentamicin (57.5%) and the lowest to Imipenem (11.5%). The infection of *Acinetobacter baumannii* with acute respiratory tract infection is closely related to season, age and department in Affiliated Hospital of Hainan Medical College. Age above 60 years old and stay at the department of Intensive care units are significantly higher probability of *A. baumannii* infection.

Spectrum antibiotics such as aminoglycosides, quinolones, and third-generation cephalosporins are essential medicines in China. They are widely used in clinical practice, leading to reduced drug sensitivity and drug resistance problems. Therefore, the clinical selection of antibiotics should be strict: have the indications for medication, avoid large-scale use of the same class of drugs, reduce the selective

pressure of antibacterial drugs. Establish a hospital infection and regularly provide a scientific basis for controlling infection and clinical treatment through the distribution and drug resistance trends of the bacteria.

3.2 Genetic analysis of *Pseudomonas oryzihabitans* from different geographical populations

Pseudomonas oryzihabitans is a group of non-fermentative, aerobic Gram-negative bacilli. The bacteria are widely found in humid environments such as water, soil, and decaying plants, and can cause various diseases such as bacteremia, pneumonia, meningitis, soft tissue infection and peritonitis [173, 174]. In recent years, microbes have undergone great changes. The original normal flora infects people with low immune function can also cause disease, which has attracted the attention of medics. There are few reports on the bacteria, mainly distributed in China, South Korea, Japan, the United States, Germany and Belgium.

Because of this the isolated clinical strain of *Pseudomonas oryzihabitans* 6676 was investigated for the establishment the possibility to identify the strain from different geographical populations, as well as representative of the genus of the another species of pathogens like *Pseudomonas aeruginosa.*

After PCR amplification using strain genomic DNA as a template, agarose gel electrophoresis showed that the fragment was about 1425 bp (Fig. 3.1). The Hainan clinical isolate *Pseudomonas oryzihabitans* 6676 was recovered, purified, and sequenced, and the GenBank accession number was JN873340.

The similarity analysis between the sequencing results of *Pseudomonas oryzihabitans* 6676 and other 16S rRNA gene sequences of *Pseudomonas oryzihabitans* in GenBank was carried out with Blast, and the similarity with *Pseudomonas oryzihabitans* strains was more than 99%, indicating that the obtained strain is indeed *Pseudomonas oryzihabitans*.

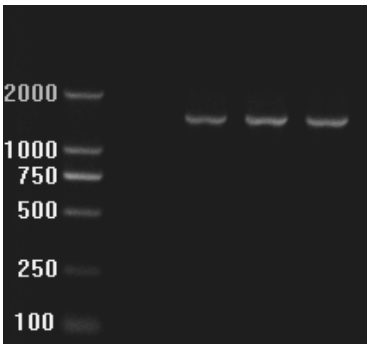


Fig. 3.1 16S rRNA PCR product of the Hainan clinical strain *Pseudomonas oryzihabitans* 6676

29 reference strains of *Pseudomonas oryzihabitans* with a gene sequence length of about 1200-1500 bp and *Pseudomonas oryzihabitans* 6676 used for the phylogenetic tree constructed by MEGA 4.1 software using Clustal X software (Fig. 3. 2).

The results show that all strains can be divided into three clusters, one cluster (I) consisting of Asian strains and European strains, a cluster II consisting of a Oceania strain, and a cluster III consists of a small number of Asian strains and American strains.

The 16S rRNA gene sequence analysis of 30 strains showed that some highly variable regions in each cluster can be used to distinguish *Pseudomonas oryzihabitans* from different regions. Such area as the variable region V4 cluster I has two genotypes, cluster II has one genotype and cluster III has two genotypes . variable region V5 cluster I has only one genotype, cluster II has one genotype, cluster III has one genotype (Table 3.5).

The 16S rRNA gene set is conserved and variegated. Conservativeness can reflect the genetic relationship of the species and provide clues for phylogeny and reconstruction. The hypervariable properties reveal the characteristic nucleotide sequence of the species and are the molecular biology of genus identification. The study about the use of 16S rRNA in the identification of *Pseudomonas oryzihabitans* has not been reported, but never use for its distinguishing types. The molecular level of the study was only found in Taiwanese scientists Liu PY-F [175] used pulse gel

electrophoresis to determine the clinical isolates of *Pseudomonas oryzihabitans* genotypes.

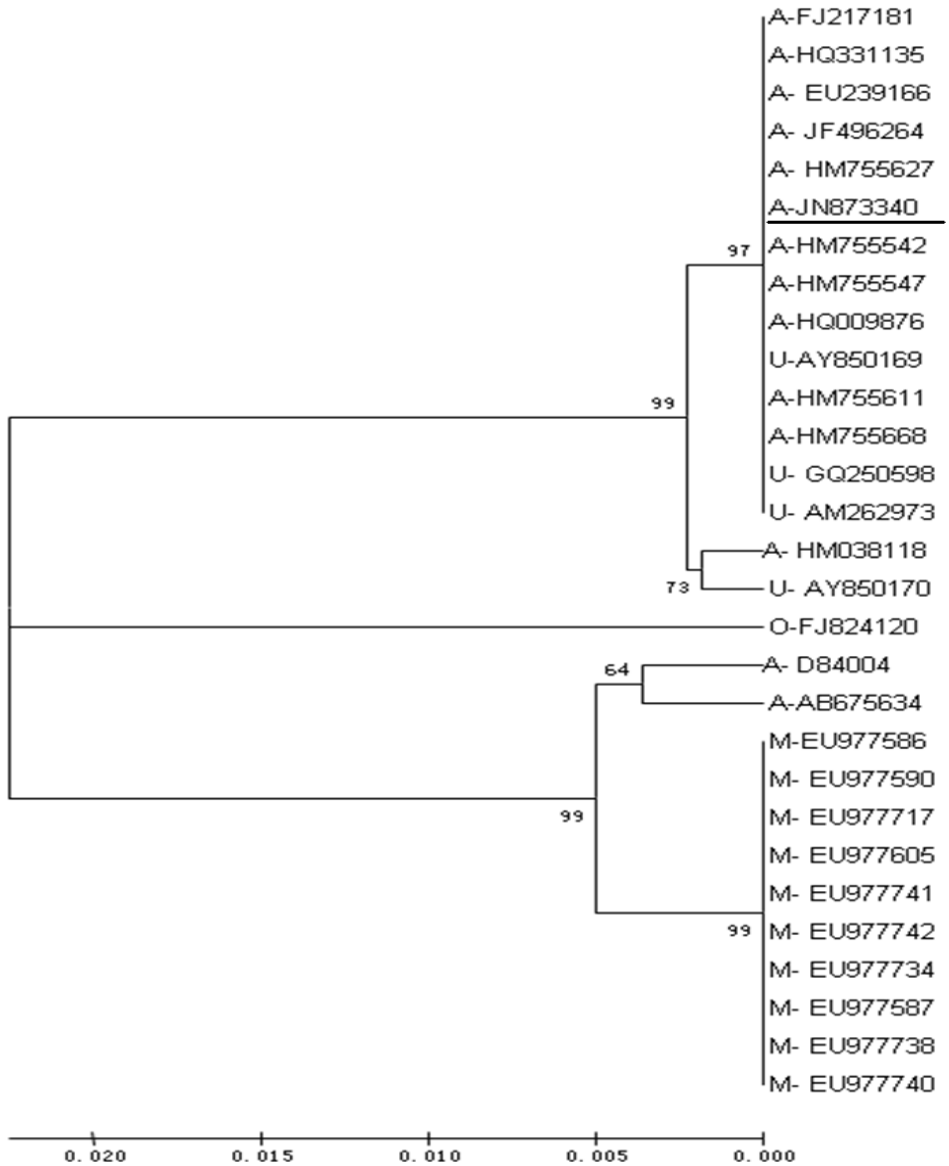


Fig. 3.2 The 16S rRNA phylogenetic analysis of *Pseudomonas oryzihabitans* in different geographical population (A - Asian strains, O - Oceania strain, U - European strain, M - American strain)

Table 3.5

Comparison of partial sequences of V4 and V5 hypervariable area of *Pseudomonas oryzihabitans*

Strain	GenBank accession number	Cluster	V4 area, 598-621	V5 area, 734-754
LKS06	HQ331135	I	GCTCATAGCGAATACCTGTGAGTT	TGATAAGTTGGATGTGAAAT
WB2003S	AY850170	I	· · · T· C· · · · · · · · · · · · · · · · ·	· · · · · · · · · · · · · · · · · · · ·
IAM1568T	AM262973	I	· ·	· · · · · · · · · · · · · · · · · · · ·
XA2-11	JF496264	I	· ·	· · · · · · · · · · · · · · · · · · · ·
C-HN728	JN873340	I	· ·	· · · · · · · · · · · · · · · · · · · ·
C-D-R2A7	HM755627	I	· ·	· · · · · · · · · · · · · · · · · · · ·
WB2000S	AY850169	I	· ·	· · · · · · · · · · · · · · · · · · · ·
LP11	HM038118	I	· · · T· C· · · · · · · · · · · · · · · · ·	· · · · · · · · · · · · · · · · · · · ·
KCB005	FJ824120	II	· TAGTA· CTT· · · · · G· TGCTAC·	CAG· · · · · · · · · A· · · · · · ·
1P03MA	EU977586	III	· · AGC· · · · · · · · · · · · · GTT· · ·	· · TT· · · · · · · · · · · · · · · G
IAM1568	D84004	III	· · AGTA· · · · · · · · · · · TGCT· · ·	· · TT· · · · · · · · · · · · · · · G
ATCC 11775T	X80725		· AGT· A· · TT· · · · · · · T· · CTCA	· · TT· · · · CA· · · · · · · · · ·

We can see (see Tabl. 3.5) Cluster I was consisted by different Korean strains (HM755668, HM755627, HM755611, HM755547, HM755542, HQ009876, FJ217181, EU239166), respectively from Yeticrab in the Far East; B-type Louse; Chinese strains (JF496264, HM038118, HQ331135, JN873340) are derived from soil, various plants and patients respectively; Belgian strain (GQ250598), unknown source; German strain (AM262973), derived from soil; The French strains (AY850169, AY850170) were derived from the aquatic environment and patients. Most of the *Pseudomonas oryzihabitans* strains showed no significant variability due to different hosts; only French strains showed greater variability due to their different hosts [176]. From this, it can be inferred that the degree of genetic variation of *Pseudomonas oryzihabitans* parasitic in different organisms is diverse during

evolution. Cluster II could be seen that the Australian strain is an independent branch and has a distant genetic relationship with strains in Asia, Europe and America. In Cluster III the American strain is relatively close to the Japanese strain. Therefore, it can be considered that most of the *Pseudomonas oryzihabitans* can be distinguished according to their geographical origin, and the source of *Pseudomonas oryzihabitans* can be roughly judged according to the difference of 16S rRNA gene sequences of different hosts.

This study further analyzed the 16S rRNA gene sequences of selected strains and found that some highly variable regions in each cluster may have key sequences that distinguish strains from different regions. From the perspective of genetic evolution, the bacteria are recognized and classified and identified at the molecular level, which not only ensures timeliness and extensiveness, but also quickly and reliably identifies the species of bacteria, which will be more in clinical and scientific research. Widely and quickly applied, it also provides reference for the identification of *Pseudomonas oryzihabitans* and the diagnosis and treatment of its diseases.

3.3 Genome sequence and annotation of *Bacteroides thetaiotaomicron*

The human gut is colonized with an estimated 50 trillion different microorganisms, which is about 1.3 times higher than the total number of cells in the body [177, 178]. Among others, representatives of normal colonic microbiota are gram-negative rods and obligate anaerobes of the genus Bacteroides. Microbial organisms are assigned many useful functions for the host, including anaerobic digestion of biodegradable material to provide energy, immune system training, and preventing the growth of harmful species. Bacteroides are involved in carbohydrate metabolism, protein utilization, and biotransformation of bile acids. *B. thetaiotaomicron* was reported to be the second most common member of the human intestinal microbiota, behind *Bacteroides fragilis* [179]. The 6.26 Mb genome of *B. thetaiotaomicron* sequenced in 2003 includes a large number of genes encoding polysaccharide-degrading enzymes and proteins [180].

B. thetaiotaomicron is considered to be commensal or symbiotic [181]. However, it has the potential to cause opportunistic infections, which are responsible for a variety of purulent-inflammatory diseases of soft tissues. Metabolizing polysaccharides, *B. thetaiotaomicron* creates a breeding ground for many microorganisms in the gastrointestinal tract. Bacteroides can initiate the so-called anaerobic infections. Representatives of the *Bacteroides, Prevotella*, and *Porphyromonas* genera are combined in a single pathocomplex [182] and can cause chronic sinusitis, chronic middle ear inflammation, oral infections, various abscesses, and necrotic pneumonia [177, 183]. *Bacteroides* are determined as relatively pathogenic due to its inability to produce endotoxin, but the progression of disease caused by *Bacteroides* is often affected by capsule polysaccharides, to which patients produce antibodies. Moreover, *Bacteroides* are often reasons for intravascular blood coagulation resulting in severe phlebitis (inflammation of veins, thrombosis), and, in more severe cases, they can cause endocarditis (inflammation of the inner layer of the heart, usually the valves), skin and genitals mucous membranes ulcers, peritonitis, and bacteremia. Bacteremia is defined as the presence of bacteria in the bloodstream, which may occur during certain tissue infections or as a result of healthcare-associated procedures. As such can be the usage of implanted urogenital or intravenous catheters, dental procedures, gastrointestinal tract or genitourinary system treatment, wound care, surgery or other procedures. Bacteremia can initiate metastatic infections, including endocarditis, especially in patients with heart valves disorders.

At present, there are relatively few studies performed on the pathogenic mechanism of anaerobic bacteria. By sequencing the genetic structure, it is possible to quickly predict the function of a gene and, thus, the subsequent pathogenic mechanism.

Comparative analysis of nucleotide sequence of a 16S ribosomal RNA (rRNA) with the National Center for Biotechnology Information (NCBI) database, revealed similarities with *Bacterium* NLAE-zl-P700 16S and *Bacteroides thetaiotaomicron strain* 1203–27,010 16S. Thus, this strain is considered in this

study as *Bacteroides thetaiotaomicron* related species. According to the Non-Redundant (NR) Database, which is compiled by NCBI as a protein database for BLAST searches, the discussed strain contains 2202 genes associated with *Bacteroides thetaiotaomicron*. We registered the results in the (NCBI) database. Our strain was named *Bacteroides thetaiotaomicron* DSMZ 2079. Genome features of this strain are shown in Table 3.6.

Table 3.6

Genome features of sample associated with *B. thetaiotaomicron* strain

Features	Values
Genome size (bp)	6.271.157
Number of genes	4320
Total gene length (bp)	33.038
Min length (bp) of the coding gene	60
Max length (bp) of the coding gene	6759
GC content	43.94%
N50 (bp)	1467
rRNA (n)	13
tRNA (n)	66
Other ncRNA (n)	26

*bp – base pairs; GC – guanine-cytosine; rRNA – ribosomal ribonucleic acid; tRNA – transfer ribonucleic acid; ncRNA – non-coding ribonucleic acid (or RNA genes)

As mentioned before, the genome of *B. thetaiotaomicron* strain VPI-5482 (ATCC 29148) was sequenced in 2003. It was originally isolated from the feces of a healthy adult patient [180]. The number of nucleotides in the genome of this strain was 6.293.399 bp, number of protein genes - 4816, number of RNA genes - 86. For comparison, the genome of another typical intestinal bacteria *B. fragilis* contains

5.241.700 bp of nucleotides, including 4236 protein genes and 91 RNA genes. *Bacteroides fragilis* NCTC 9343 strain was sequenced in 2005 [177, 184].

Strain associated with *Bacteroides thetaiotaomicron* isolated from the blood of a patient with descending colon cancer had a genome size and gene count slightly smaller as compared to *B. thetaiotaomicron* strain VPI-5482 and larger than those in *Bacteroides fragilis* strain NCTC 9343. The isolate under consideration may belong to a new bacterial species of the genus *Bacteroides*, and this assumption requires confirmation.

The analysis of *B. thetaiotaomicron* associated enzymes was performed using the Carbohydrate-Active enZYmes Database (CAZy) (Carbohydrate, 2020). It is a directory of enzymes involved in carbohydrate degradation, synthesis, and modification, which describes its catalytic structure and functions. CAZy classifies related enzymes as glycoside hydrolases (GHs), glycosyltransferases (GTs), polysaccharide lyases (PLs), carbohydrate esterases (CEs), auxiliary enzymes (AA), and others. The CAZy database annotation results are presented in Fig. 3.3

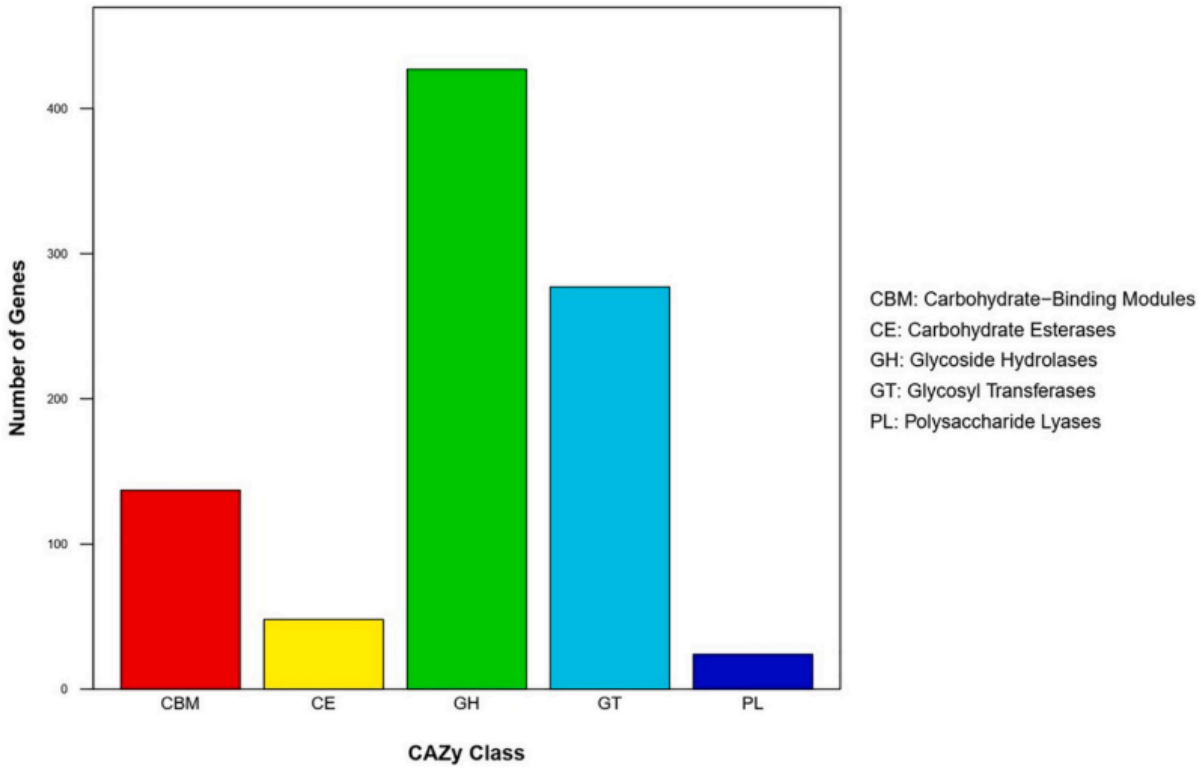


Fig. 3.3 The CAZy database annotation results for *Bacteroides sp. aff. thetaiotaomicron*

As seen from Fig. 3.3, the metabolic pathways associated with the carbohydrate metabolism involve 427 glycosyl hydrolases, 277 glycosyltransferases, 137 carbohydrate-binding modules, 48 carbohydrate esterases, and 24 polysaccharide lyases. Glycoside hydrolases (EC: 3.2.1) catalyze the glycolytic breakdown of O-glycosidic bonds in the molecules of carbohydrates resulting in its splitting in two smaller molecules [178, 185]. The set of glycosyl hydrolases in microorganisms depends on their environmental function: glycoside genes are often duplicated, i.e. can be represented as several paralogues, eliminated, i.e. the set of genes may differ even in strains of the same species [186], and subjected to horizontal transfer, which implies that phylogenetic glycoside trees are generally very different from the host trees. Glycosyltransferase is an enzyme (EC 2.4) that transfers residues of monosaccharides from carbohydrates donor to an acceptor molecule.

Thus, the variety of enzymes available in strain associated with *Bacteroides thetaiotaomicron* (*Bacteroides thetaiotaomicron* DSMZ 2079) enables the hydrolysis of glycosidic bonds and is capable of metabolizing a variety of polysaccharides.

The KEGG database (Kyoto Encyclopedia of Genes and Genomes) is a directory of gene functions and genomic information, which integrates information on genomics, biochemistry and systemic functional omics, and draws them according to different types of biological processes. The corresponding bio-path maps help researchers to study genes and expressions as a whole network. By annotating the genes on the KEGG biopath database, it is possible to define the biological pathways these genes participate in. [178]

KEGG suggests the division of biological metabolic pathway into six categories: Cellular Processes, Environmental Information Processing, Genetic Information Processing, Human Diseases, Metabolism, and Organismal Systems. The number of genes of the strain associated with *Bacteroides thetaiotaomicron* in each biological metabolic pathway in the secondary classification is displayed in Fig. 3.4.

Strain associated with *Bacteroides thetaiotaomicron* revealed the presence of genes associated with pathway 199. The majority relates the following pathways: biosynthesis of amino acids – 120, amino sugar and nucleotide sugar metabolism – 98, carbohydrates metabolism – 89, twocomponent system – 88, galactose metabolism – 79, other glycans degradation – 75, purine metabolism – 60, starch and sucrose metabolism – 60, ribosomes – 54, pyrimidine metabolism – 50, sphingolipid metabolism – 46, beta-lactam resistance – 42, *quorum sensing* – 41, alanine, aspartate and glutamate metabolism – 41, *cationic antimicrobial peptide (CAMP) resistance* – 39, pentose and glucuronate interconversions – 38, pyruvate metabolism – 37, glycolysis / gluconeogenesis – 36, oxidative phosphorylation – 36, ABC transporters – 36, fructose and mannose metabolism – 34, carbon fixation pathways in prokaryotes – 34, lysosome – 33, and bacterial secretion system – 31 [177].

Thus, the genome of the studied strain comprises a variety of genes encoding enzymes associated with the pathway that breaks down complex sugars (glycosylhydrolases, cell-surface carbohydrate-binding proteins) and synthesizes capsule polysaccharides (i.e., lysoziltranspherase). Besides, the genome contains a large number of encoding genes involved in environmental sensing, signal transduction (two-component systems; extracytoplasmic function (ECF)-type sigma-factors) and the DNA mobilization (transposases, conjugative transposons).

Examined strain *Bacteroides thetaiotaomicron* DSMZ 2079 has a significantly extended community of ECF-type sigma factors (as well as *B. thetaiotaomicro*n, VPI-5482) [180]. Considering environmental sensing functions of these factors, their use by *Bacteroides thetaiotaomicron* DSMZ 2079 to regulate the expression of its complicated polysaccharide-utilizing apparatus, is the feature that grants an advantage over the less empowered inhabitants of microbiota. Another significant feature of the studied strain to recognize and respond to environmental signals is a rich representation of one and twocomponent signal transduction systems.

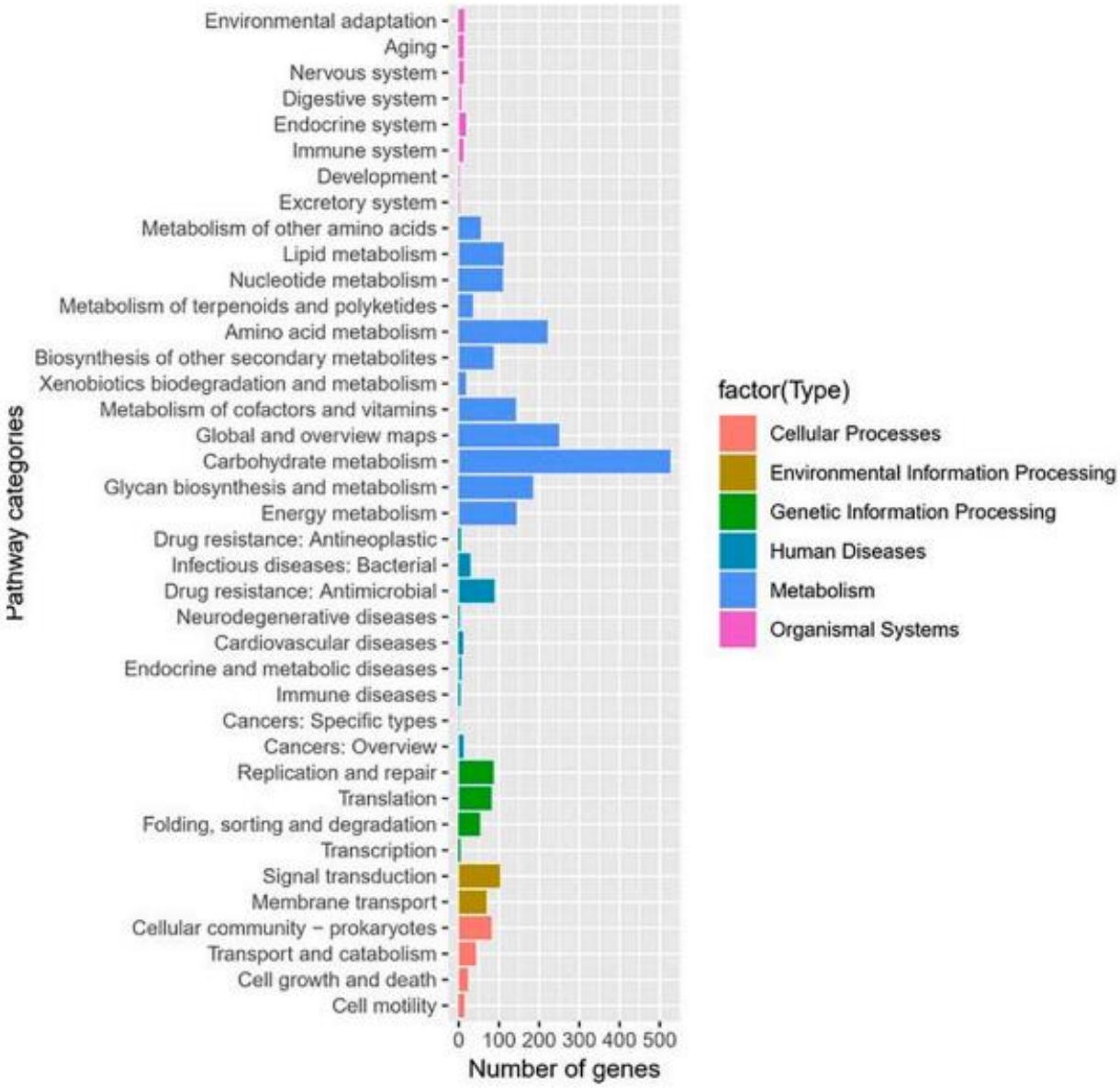


Fig. 3.4. Genes enrichment in strain associated with *Bacteroides thetaiotaomicron* according to the KEGG pathway database.

Bacteroides thetaiotaomicron DSMZ 2079 includes several types of mobile genetic elements: a 33-kb plasmid, transposases, and four homologs of the self-transmitting conjugative transposon CTnDOT. CTnDOT ensures the expansion of tetracycline and erythromycin resistance among *Bacteroides spp*. [187]. The presence of four conjugative transposons (CTns) in combination with a wide host range of CTnDOT implies that they may facilitate horizontal DNA transfer between *Bacteroides thetaiotaomicron* DSMZ 2079 and other bacterial components of the distal intestine, thereby contributing to their microevolution. [177]

The Cluster of Orthologous Groups (COG) database is a dataset of proteins created and maintained by NCBI. It is constructed based on the evolutionary relationships of the encoded protein systems of bacteria, algae, and eukaryotes genomes. A certain protein sequence can be annotated into a COG, and each cluster of COGs is composed of orthologous sequences, so that the function of the sequence can be predicted. The statistical results of the COGs function classification are given in Fig. 3.5

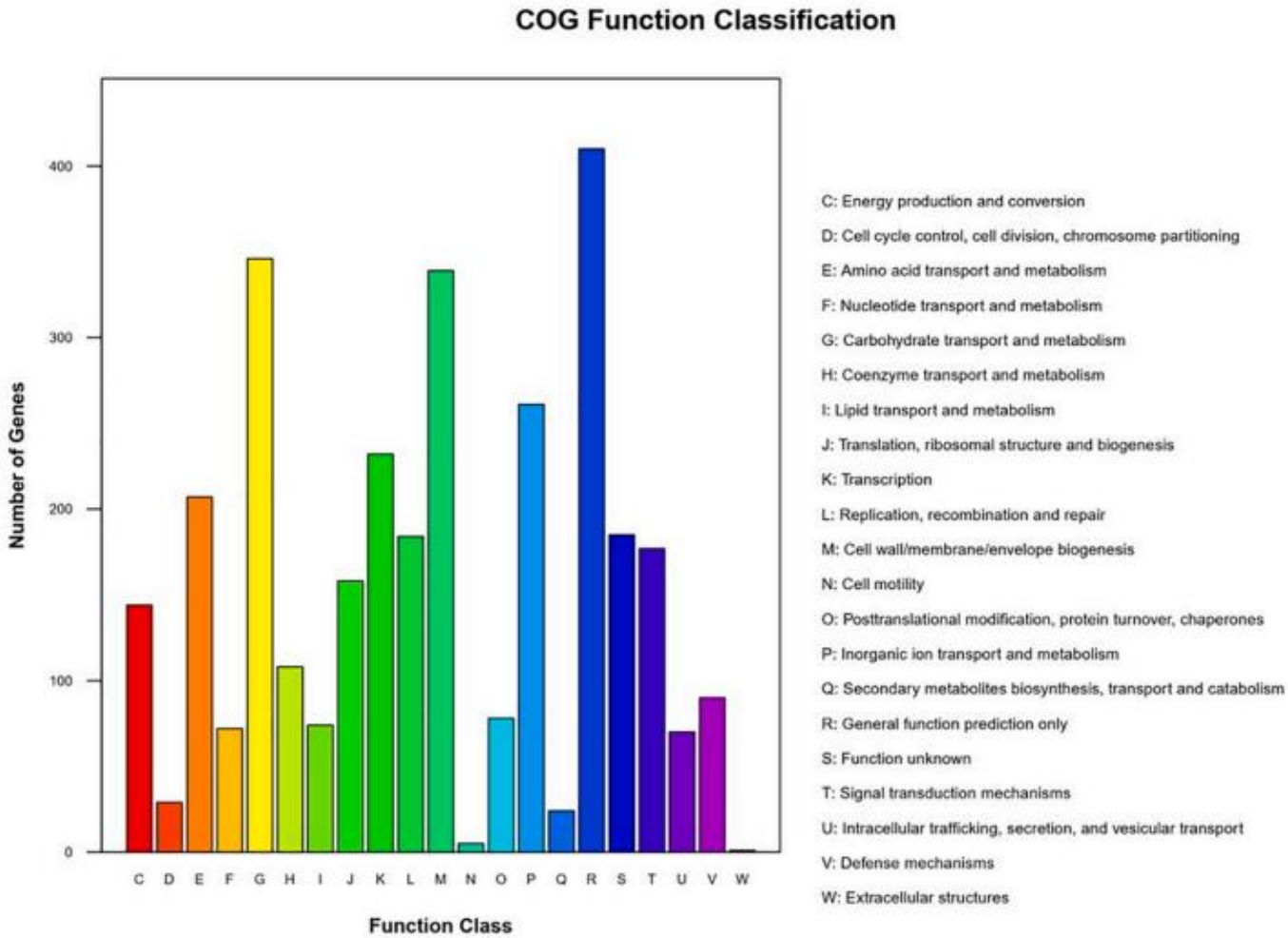


Fig. 3.5. Annotation of genes in *Bacteroides sp. aff. thetaiotaomicron* in the COG database

At the presented visualization of genomic islands of *Bacteroides thetaiotaomicron* DSMZ 2079 we discovered two genomic islands that contain information about the resistance gene (RGI) (Fig. 3.6, Table 3.7).

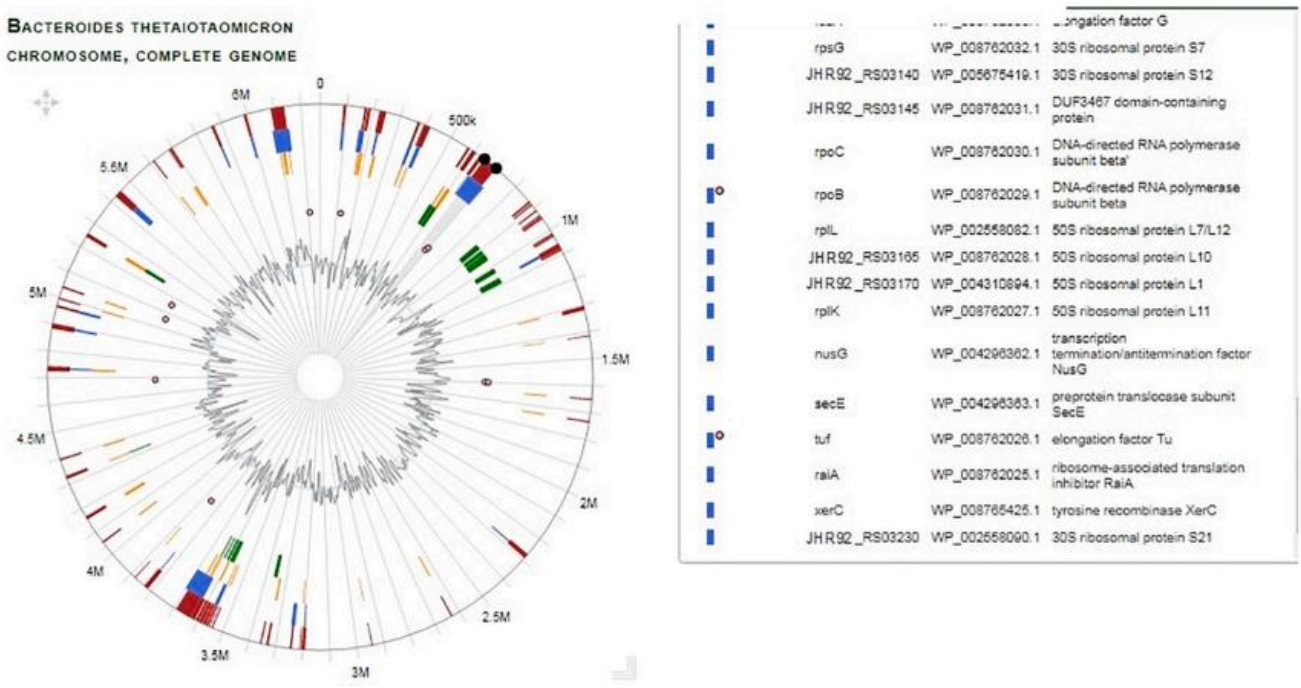


Fig. 3.6 Visualization of genomic islands of tested strain *Bacteroides thetaiotaomicron* DSMZ 2079 with designation of resistance genes (RGI).

Table 3.7 demonstrats the results of identification of genomic islands to determine the gene that causes the resistance of microorganisms to adverse factors, including antibiotics. We have found two genes encoding the resistance of microorganisms [177].

To determine the belonging of the studied strain to the genus *Bacteroides thetaiotaomycron*, we compared the studied strain with the reference one. The data given in Table 3.8 showed high convergence of the results.

Table 3.7

Identification of genomic islands of *Bacteroides thetaiotaomicron* DSMZ 2079

Gene ID	Locus	Gene start	Gene end	Strand	Product	External Annotations
rpoB	JHR92_RS03155	689,682	693,494	-1	DNA-directed RNA polymerase subunit beta	Resistance gene(RGI)
tuf	JHR92_RS03195	696,667	697,851	-1	elongation factor Tu	Resistance gene(RGI)

Table 3.8

OrthoANIu results for tested strain *Bacteroides thetaiotaomicron* DSMZ (Genome A) *Bacteroides thetaiotaomicron* VPI-5482 (Genome B)

Metric	Value
OrthoANIu value (%)	99.99
Genome A length (bp)	6,270,960
Genome B length (bp)	6,259,740
Average aligned length (bp)	4,875,122
Genome A coverage (%)	77.74
Genome B coverage (%)	77.88

Bacteroides are classified as obligately anaerobic components that build a normal microbiota of the gut. Generally considered as mutually beneficial for human colon, they can cause significant pathology if penetrated the bloodstream, which among others, implies bacteremia. Bacterial invasion into the bloodstream is mainly healthcarerelated and can be initiated by surgery procedures in the large intestine.

An increased risk of gram-negative bacteremia is observed in immunocompromised patients or those with chronic diseases. The inability of *Bacteroides* to synthesize endotoxic make them conditionally pathogenic and less common reason for bacteriemia progression compared *Enterobacter*. However, they are covered with a polysaccharide capsule, to which patients produce antibodies. An immune response to bacteria invasion can result in sepsis (blood infection) and septic shock. Bacteria employs blood to spread through the body like in case of endocarditis. Therefore, investigating the genome of these bacteria is of high importance for developing treatment tactics for severe effects of bacteremia. Such research is currently highly insufficient.

Conclusion for the Chapter 3

1. Of the total 3529 cases of infection and 206 strains of *Acinetobacter baumannii* were detected. The infection of *Acinetobacter baumannii* with acute respiratory tract infection is closely related to season, age in Affiliated Hospital Of Hainan Medical College (China). Age above 60 years old and stay at the ICU are significantly higher probability of *Acinetobacter baumannii* infection. Susceptibility results show that resistance rates to the third generation of cephalosporin step up.

2. 16S rRNA gene of the Hainan clinical strain *Pseudomonas oryzihabitans* 6676 was amplified, sequenced, compared with 16S rRNA sequences of others 29 *Pseudomonas oryzihabitans* strains from GeneBank from different geographical population, and created a phylogenetic tree. The phylogenetic tree analysis showed that according to different geographical population, most of *Pseudomonas oryzihabitans* strains could divide into three branches. Furthermore, sequencing analysis showed the key sequences in variable region might be existed to discriminate from different geography. Different geographical *Pseudomonas oryzihabitans* had genotype and phenotype diversity, most of which were identified by 16S rRNA sequence.

3.Based on genome sequencing and gene annotation of *Bacteroides thetaiotaomicron* DSMZ 2079 strain isolated from the blood of a patient, the presence of a one- and two-component system for recognizing environmental signals and responding to them, the presence of several types of mobile genetic elements - a 33 kb plasmid, transposases and four homologues of the self-transmitting con 'jugative transposon CTnDOT, which provides the expansion of resistance to tetracycline and erythromycin. The *rpoB* and *tuf* genes were identified (in the JHR92_RS03155 and JHR92_RS03195 loci, respectively), which determine the strain's resistance, including to antibiotics and adhesion factors.

The results presented in Chapter 3 were published in these papers:

WU Lin, LI Li-hua, WU Li-xian. Phylogenetic analysis of *Pseudomonas oryzihabitans* of different geographical populations based on partial sequences of 16S rRNA gene (in Chinese). China Tropical Medicine. 2012; 12(12):1453-1456. DOI:10.13604/j.cnki.46-1064/r.2012.12.018

WU Zhi-Cheng, **WU Lin**. Clinical distribution and drug resistance change of respiratory nosocomial infections of *Acinetobacter baumannii* (in Chinese). Journal of Hainan Medical University. 2013; 19(2):271-274. http://www.cqvip.com/QK/90826X/201302/44859186.html

ZC Wu, **L Wu**, M Zhang, W Zhou. Genome sequence and annotation of Bacteroides sp aff. Thetaiotaomicron strain isolated from blood. Infection, Genetics and Evolution. 2021; 91: 104816. PMID: 33771725. DOI: 10.1016/j.meegid.2021.104816**. (Scopus, Q2)**

CHAPTER 4. SCREENING AND CHARACTERIZATION OF NEW ANTIMICROBIAL SUBSTANCES

4.1 Development the method of large-scale screen inhibitors of Sortase A

Sortase A (SrtA) is a membrane-bound cysteine transpeptidase that is in charge of catalyzing the covalent anchoring of surface proteins to the Gram-positive bacterial cell wall. These surface proteins play critical roles in bacterial adhesion and invasion of host tissues, biofilm formation, and immune evasion by inhibition of opsonization and phagocytosis [189]. Thus, SrtA constituted an ideal target for the development of new anti-virulence agents, as SrtA is required for the Gram-positive pathogenesis of many different bacterial infections. Proteins destined for cell wall anchoring are directed for secretion by their N-terminal signal sequence and the Sec pathway [190], which consists of a pentapeptide motif (LPXTG (leucine, proline, any amino acid, threonine and glycine)) at the carboxyl terminus [191]. Secretion of the LPXTG proteins are recognized by the membrane-bound SrtA and cleaved between the Thr and Gly residues, and then anchored to the cell wall [188, 191].

Several methods had been developed for the identification and characterization of new SrtA inhibitors. These include the screening of natural products or small compound libraries [192,193], as well as virtual screening [194 ,195]. Both approaches have been successfully used and have identified several SrtA inhibitors to date. High Throughput Screening (HTS) has been widely used in the search of new and potent SrtA inhibitors, as it allows the identification of active molecules among thousands of screened compounds. The activity of SrtA is measured by monitoring the cleavage of a fluorescence resonance energy transfer substrate, such as peptide Abz-LPETG-Dnp. However, the use of substrates of fluorescent peptides was frustrated largely by the high price of substrates.

It has been reported that certain natural SrtA inhibitors (berberine chloride, psammaplin A1, etc.) can inhibit *Staphylococcus aureus* cell adhesion to fibronectin (LPXTG protein) via fibronection-binding protein, resulting in success in reducing

the infection rate [189, 196]. In contrast to prokaryotic display technique, the yeast display technique has stark advantages, including (1) producing a soluble and functional protein; and (2) obtaining results at high densities without obtaining unwanted specific proteins via centrifugation [197]. These advantages are very helpful upon harvesting fusion proteins which are extremely difficult to purify and so are expensive to purchase [188, 198]. Thus, the yeast display system is expected to be of great interest for further biotechnological applications, and it is suitable to display SrtA substrates to reduce costs.

To overcome these obstacles, we would like to work out a new high throughput technique of high efficiency and low cost for the screening of inhibitors of SrtA. Firstly, we optimize SrtA expression conditions including induction time, induction temperature and induction concentration of IPTG. Then, the enhanced green fluorescence (EGFP) change of its substrates on the surface of *Pichia pastoris* over time was detected by flow cytometry and fluorescence spectrophotometry. Finally, using berberine chloride for positive control, the yeast strain displaying the LPXEG motif was mixed and interacted with SrtA or/and inhibitors to investigate the method of screening inhibitors of SrtA.

4.1.1 Optimization of pTRX-srtA vector's expression in *E. coli* BL21 (DE3)

The expression levels of pTRX-srtA vector under different induction times (Fig. 4.1A), IPTG concentrations and induction temperatures was examined by SDS-PAGE (Fig.4.1 B). The results indicated that the expression of pTRX-srtA vector was significantly influenced by the induction temperature, time, and IPTG concentration. The optimal induction time was determined to be 6 h, induction temperature was 28 °C and IPTG concentration was 1.0 mmol/L. Prolonged induction times (from 4 h to 6 h) resulted in an increase in the yield of SrtA, while shorter induction times (from 6 h to 7 h) resulted in a reduction. As IPTG-induction concentration was increased, the yield of SrtA was increased. It increased slowly until IPTG-induction concentration exceeded 1.0 mmol/L. When the SrtA was induced at the temperature of 22 °C, 28 °C, 37 °C and 40 °C, the expression gave a

sharp band under the condition of 28 °C. Under the optimized expression conditions, the expressed protein accumulated up to about 90% of total bacterial protein after purification using affinity chromatography (in Fig. 4.1C). These results demonstrated that the pTRX-srtA vector was efficiently expressed in *E. coli* BL21 (DE3) under the optimized expression conditions. The purity product was collected by centrifugation and then dried by vacuum freezing in a dryer to obtain the solid powder of the neutral SrtA.

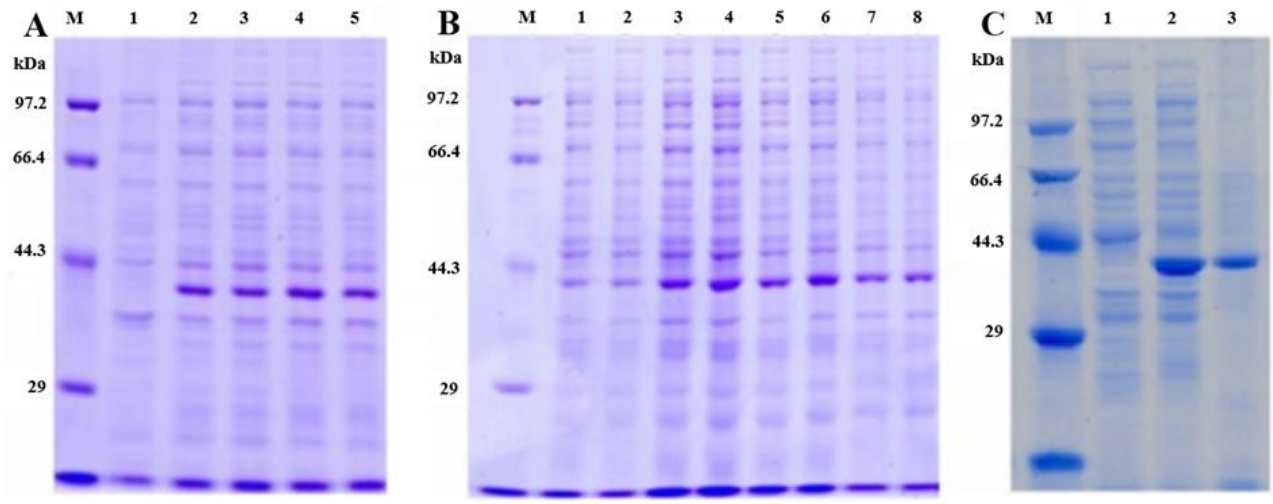


Fig. 4.1 Expression of pTRX-srtA in *E. coli* BL21 (DE3):

A) Effects of induce time on pTRX-srtA expression. M: Protein molecular mass standards; 1: Lysate of pTRX-srtA transfectant without induction; 2–5: Lysate of pTRX-srtA transfectant induced with 1.0 mmol/L IPTG at 37 ◦C for 4 h, 5 h, 6 h and 7 h, respectively;

B) Effects of IPTG concentration and induction temperature on pTRX-srtA expression. M: Protein molecular mass standards; 1–4: Lysate of pTRX-srtA transfectant induced at 37 ◦C for 6 h with different IPTG concentration of 0.25 mmol/L, 0.5 mmol/L, 1.0 mmol/L, and 2.0 mmol/L, respectively; 5–8: Lysate of pTRX-srtA transfectant induced with 1 mmol/L IPTG for 6 h, at the temperature of 22 ◦C, 28 ◦C, 37 ◦C and 40 ◦C;

C) Purification of pTRX-srtA proteins. M: Protein molecular mass standards; 1: Lysate of pTRX-srtA transfectant without induction; 2: unpurification of pTRX-srtA transfectant with 1.0 mmol/L IPTG at 28 ◦C for 6 h; 3: purification of pTRX-srtA transfectant with 1.0 mmol/L IPTG at 28 ◦ C for 6 h

4.1.2 Analysis of the EGFP protein expressed on cell surface of *P. pastoris*

Flow cytometry histograms were depicted in Fig. 4.2. The mean fluorescence signal of LPXTG-EGFP proteins was measured at several different times, 0 h (yellow), 96 h (red), 120 h (blue) and 144 h (pink), as the control of GS115/pKFS cells (gray) had poor fluorescence signal at the same time.

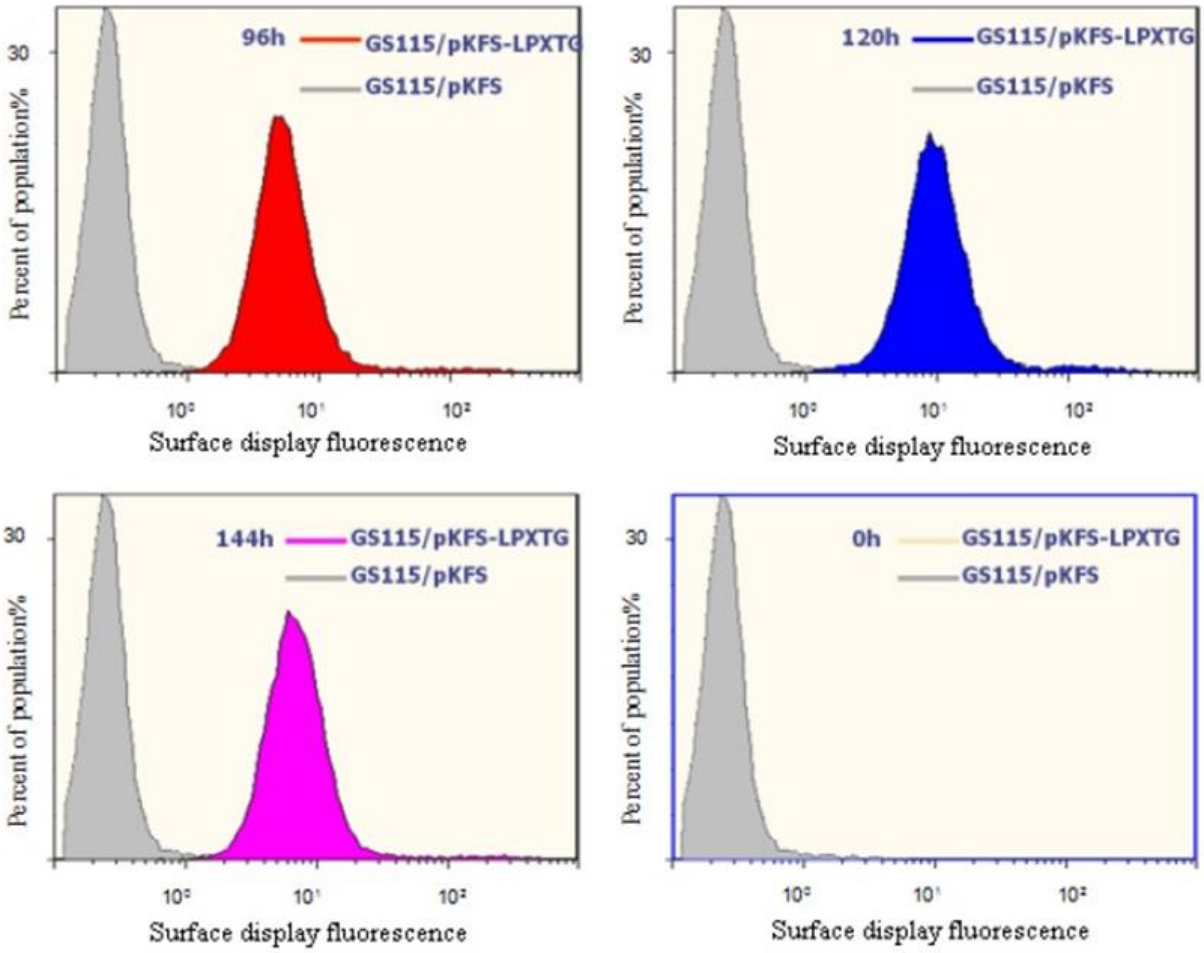


Fig. 4.2 Detection of LPXTG-EGFP displayed on yeast surface: Flow cytometry detection of LPXTG-EGFP displayed on yeast surface.

The time courses of LPXTG-EGFP proteins were detected by fluorescence spectrophotometry (in Fig. 4.3), and depicted the mean fluorescence signal of the yeast-displayed LPXTG-EGFP proteins. The green fluorescence signal that emitted from GS115/pKFS-LPETG was clearly detected by flow cytometry and

fluorescence spectrophotometry, while hardly any green fluorescence signal emitted from GS115/pKFS. Therefore, the results showed that the fusion proteins were expressed at a high level, and the total number of EGFP proteins increased up to 120 h and then slightly decreased up to 168 h. Furthermore, it is shown that both methods could be used to detect SrtA activity. Compared with other methods, the biggest advantage of spectrophotometry is its affordability.

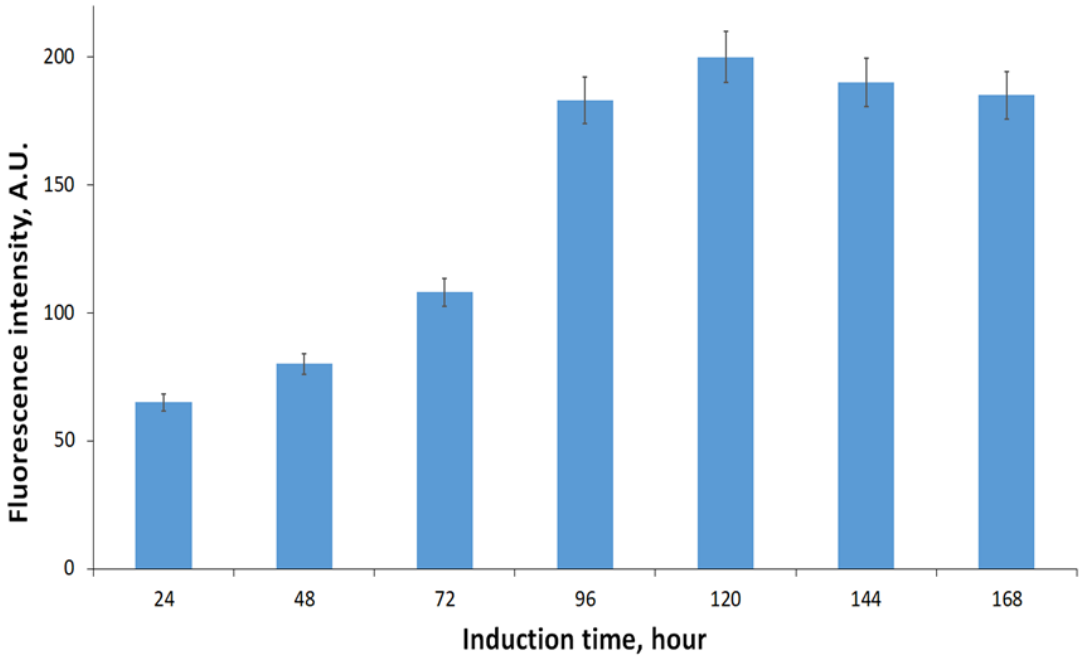


Fig. 4.3 Detection of LPXTG-EGFP displayed on yeast surface: Fluorescence spectrophotometry detection of LPXTG-EGFP displayed on yeast surface

4.1.3 Detection of Sortase A activity

The SrtA activity assay is based on the fact that the fluorescence intensity will change as the LPETG-EGFP on the yeast surface are cleaved by SrtA. An inhibitor would affect the SrtA in such a way that they will keep the -EGFP on the yeast surface and will not be cleaved by SrtA. By investigating treated and untreated samples over time and by investigating the differences in cleaving, the idea is that it would be possible to study an inhibition.

The results of the fluorescence spectrophotometry assay in Fig. 4.4 show that the fluorescence changes are linear by the time that the yeast surface display of substrate LPETG-EGFP interacted with SrtA. Berberine chloride was added in the

reactions using different concentrations to investigate the potential role of the inhibitor.

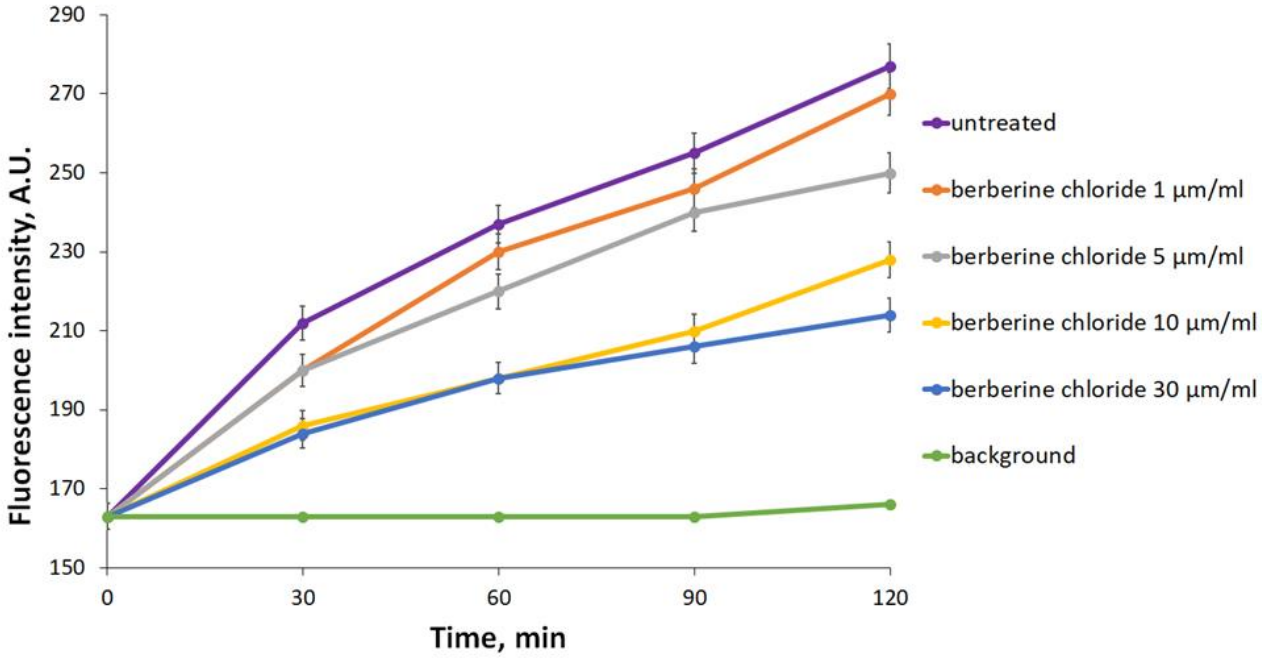


Fig 4.4. Fluorescence spectrophotometry detection of SrtA activity: Fluorescence spectrophotometery detection of SrtA interaction with LPXTG-EGFP in time

Also, a high linearity is seen in the results and a general trend in the difference between the control and the sample with inhibitor was recorded. Before investigating the inhibition of SrtA, it is crucial that a successful assay for the untreated sample is developed. The fluorescence intensity of Dabcyl-QALPXTGEE-Edans of the positive control group was diminished by fluorescence spectrophotometry investigation with 350 nm for excitation and 495 nm for recordings shown in Fig.4.5. All the results indicated that the control and the sample with inhibitor increased with respect to increased time of growth which was fitted for the screening of inhibitors. However, the positive control was more robust.

So, the emergence of Gram-positive bacteria with intermediate or full resistance to antibiotics has become a major public health threat [199]. The bacteria need to be pathogenic to develop an infection, and they need to interact with the host. Gram-positive bacteria mediate this interaction via a special mechanism

involving anchoring of proteins present in the cell membrane to the peptidoglycan [200].

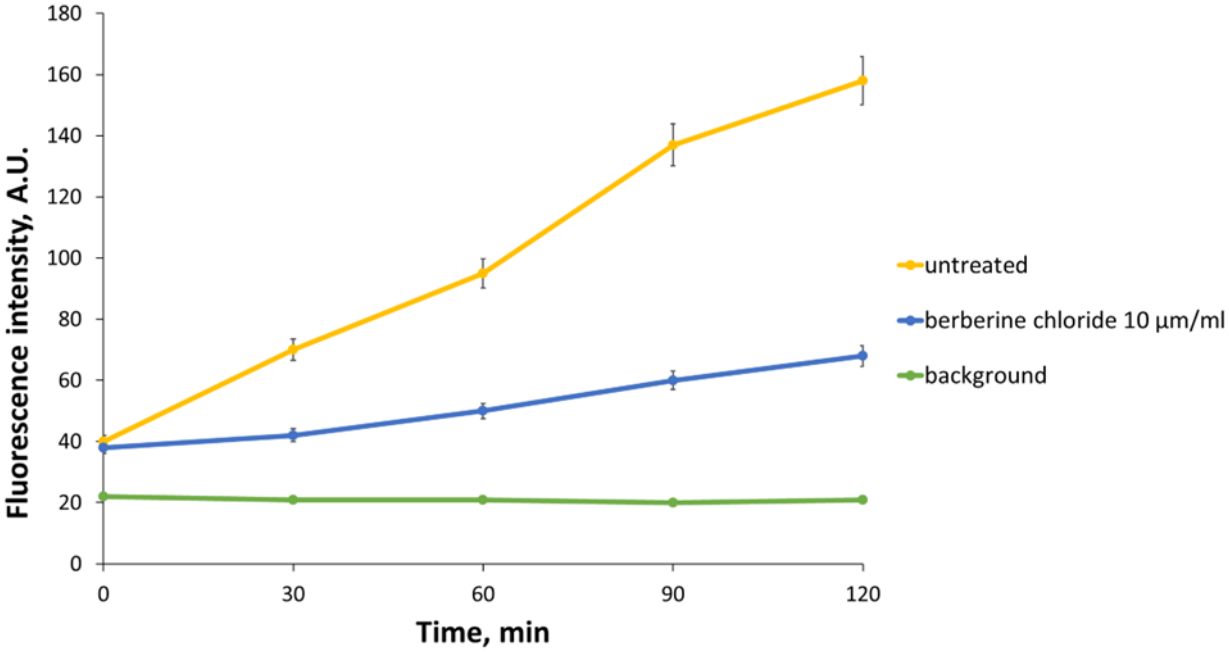


Fig. 4.5. Fluorescence spectrophotometry detection of SrtA activity: Fluorescence spectrophotometry detection of SrtA interaction with Dabcyl-QALPXTGEE-Edans in time

These proteins are believed to be essential for the survival of the bacteria during the infection [201]. A housekeeping enzyme called SrtA is responsible for this mechanism and might be a suitable target for inhibition to prevent the bacterial infection developing [188, 202].

To be able to investigate the activity of SrtA and its potential inhibitors, different approaches were investigated, including FRET assay, labeling of bacterial cells, a fibronectin-binding protein assay, and so on. FRET assays [192,193] have been created and these assays turned out to be a good starting point to study inhibition, but due to the complex manipulation and the high costs of synthesized substrates the assay does not seem to be convenient or economical enough for the investigation of inhibitors. Both the labeling of bacterial cells [196] and the fibronectin-binding protein assay provided an advantage for investigating the SrtA in its natural environment. Unfortunately, although the protocol was adjusted in a

number of different ways, the results of the assays indicated that the assay does not seem to be robust and sensitive enough for the investigation of inhibitors.

In this study, to optimize the induction condition of SrtA expression in *E. coli* DE3(BL21) and catalyze the LPXTG motif of the substrate which displays on the surface of *P. pastoris* for detecting the activity of SrtA and screening its inhibitors, the green fluorescence intensity of LPETG-motif displaying on the surface of *P. pastoris* was increased, and it was detected by flow cytometry (in Fig. 4.4) and fluorescence spectrophotometry (in Fig. 4.5). The results are a clear indication that fluorescence intensity change of the LPETG-motif becomes clearer with time. Moreover, these LPETG-motifs were correctly folded under the conditions studied and rarely disturbed by other cell-surface proteins. Both methods could reflect the relationship between the change of fluorescence and the catalysis of SrtA well. These results also indicate that *P. pastoris* displaying the LPETG-motif may constitute immobilized and purified substrates which are widely applicable to detecting SrtA activity. In earlier studies, when SrtA or the substrates were expressed on the cell-surface, this would lead to a barely efficient interaction with each other as the interaction might be broken by the interference of the other cell-surface proteins, etc. The yeast-display system method has the following advantages: easy manipulation of gene clone, protein expression, and cell concentration, while the maximal difficulty was finding a different protocol to achieve a robust assay that is sensitive enough for investigation of SrtA.

With the aim of reaching a more robust assay, the protocol was adjusted in various ways. The routine SrtA assay is based on the production of enough native or synthesized substrates, but the complicated purification process and the high expenses greatly limit the screening for inhibitors. In the present study, a novel SrtA method was developed by applying the yeast-displaying substrates directly into the SrtA detection system instead of soluble protein substrates. The displayed-substrates were successful in the detection of SrtA during the time of the project. The results were confirmed by fluorescence spectrophotometry. All the results indicated that the control and the sample with the inhibitors (different concentration

of berberine chloride) were increased with respect to growth, which was fitted for screening inhibitors, but the positive control was less variant. Even though the routine method was much more sensitive, the yeast-displaying substrates method was easier and cheaper, making it well suited to large-screening inhibitors.

Recently, cell surface engineering has made significant progress and has been exploited to represent many new functions. After improving the operation process and increasing the sensitivity, SrtA activity could be checked with the yeast-displaying substrates by flow cytometry and fluorescence spectrophotometry. The yeast-displaying substrates could totally replace recombinant proteins or synthesized substrates.

4.2. Antimicrobial and cytotoxic characteristics of novel antibiotic streptofungin from *Streptomyces albus*

The culture of the streptomycetes *Streptomyces albus* has been studied for more than one decade in a numbers of scientific laboratories and used in development, primarily as a producer of a complex of bacteriolytic enzymes [154, 156]. All this, as well as the return of interest in streptomycetes in general among researchers over the last ten years [107, 109, 110], caused us to turn to the analysis of the antibiotic activity of the culture. The result was the establishment of the culture's ability to synthesize a complex of antibiotics (called streptofungin), its nature and methods of isolation [153]. Interesting from a practical point of view is the possibility of simultaneous production of two different antimicrobial products of this producer – a complex of enzymes and a complex of antibiotics – in one producing cycle due to their different nature, localization, and therefore methods of isolation from the culture liquid.

Such approaches are modern and relevant, as shown, for example, using some antibiotic-producing streptomycetes, which simultaneously synthesize peptidase complexes. The main advantage of streptomycete peptidases is their thermal stability and a wide pH range of action. Peptidase preparations are obtained as a by-product

from the culture liquid of *S.fradiae*, *S.griseus* and *S.rimosus* during the biosynthesis of antibiotics [203].

Therefore, at the next stage of our work, the task was to determine the main characteristics of streptofungin, which is actually a complex of a number of individual antibiotics that can be isolated together (as the sample obtained for this study) or can be extracted in various organic solvent systems shown before [153] and obtained as separate substances. The latter can obviously be promising plans, especially if the main characteristics of streptofungin are established, which will show its potential as a pharmaceutical substance [156].

At the previous stages of work with the *Streptomyces albus* culture a collection of mutants with increased and/or modified biosynthetic ability of the bacteriolysins synthesis was obtained by various methods from the original strain (2435) [204]. The revealed ability of the culture to produce antibiotics led to the urgency of additional study of the spectrum of the culture antimicrobial metabolites action in general and the properties of the actual new streptofungin antibiotic complex.

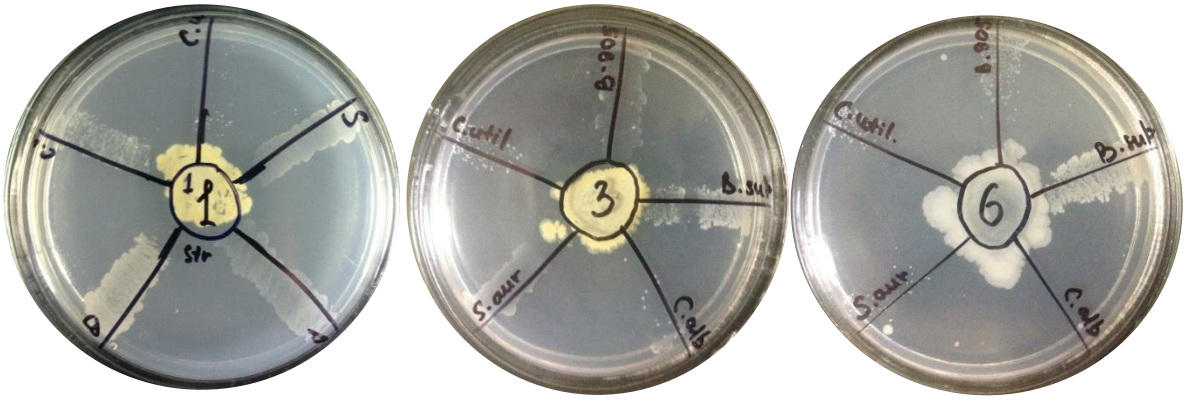
The results obtained at the first stage of the study of the antagonistic activity of *S. albus* against selected test-cultures (typical causative agents of inflammatory processes) indicate a similar spectrum of antimicrobial action, but differences in the productivity of different strains or the ratio of individual antimicrobial metabolites (Table 4.1, Fig. 4.6).

All strains are antagonistic to *S.aureus* and the represented *Candida* species. The highest antagonism towards *S.aureus* was found by *S.albus* strains UN44, AE6 and 105, forming zones of growth inhibition from 11 to 15 mm. Regarding representatives of *Candida*, there is a noticeable difference in antagonistic activity not only between strains, but also a difference in the ability to delay the growth of different species. In general, high antifungal activity was found in most of the studied strains of *S.albus* (zones of growth inhibition of *C.albicans* 20-30 mm and *C. utilis* from 11-20 mm), among which strain AE6 is the most active [156].

Table 4.1.

Antagonistic activity of *S. albus* strains

Strains of *S.albus*	Test-cultures				
	S.aureus ATCC 6538	*C.albicans* ATCC 10231	*C.utilis* LIA-01	*B.subtilis* ATCC 6633	*P.vulgaris* ATCC 6896
	Zone of growth inhibition, mm				
2435	6±0.06	9±0.1	16±0.2	14±0.2	0
2435/M	9±0.2	15±0.3	11±0.07	7±0.06	0
UN44	11±0.2	23±0.5	11±0.1	0	0
4S	7±0.1	14±0.4	2±0.03	3±0.02	0
US101	10±0.2	25±0.9	4±0.1	11±0.04	0
AE6	15±0.5	30±1.2	20±0.5	0	12±0.3
105	14±0.2	7±0.07	9±0.3	25±0.9	0
80/5	10±0.3	18±0.4	13±0.2	6±0.09	0



1 2 3

Fig. 4.6. Antagonistic activity of *Streptomyces albus* strains against conditionally pathogenic test cultures: 2435 (1), UN44 (2), AE6 (3)

The mutants with a high level of antagonism against *S.aureus* and the presented *Candida* species under these conditions did not show the ability to suppress *B.subtilis* (spore-forming bacteria), which was characteristic of the original strain

2435. And one of the mentioned mutants (strain AE6) turned out to be the only one that suppressed the growth of *P.vulgaris*.

The study of the antagonistic activity of different strains of the *S.albus* culture showed their close specificity, and the result of stepwise selection using various mutagens contributed to an increase in the synthesis of certain substances in antimicrobial complexes, but did not fundamentally affect their qualitative changes. From a practical point of view, this makes it possible to choose producers for different developments with increased target antimicrobial activity against different pathogens or with broad specificity. At the same time, the question of the activity of the final form of the same product of biosynthesis of different strains (for example, streptofungin), even in the case of a certain quantitative difference in their biosynthetic ability, is a question of the purification and concentration methods used for isolation [156].

We note that when analyzing the antagonistic activity of the producer of several antimicrobial compounds at the same time, especially of different nature (as in the case of the studied culture), it is difficult to separate the contribution of these components to the result. Also, of course, it will depend on the concentration of the component that is secreted into the agar nutrient medium.

A clear confirmation of such a "cautious" attitude to the conclusions regarding the biosynthetic capacity of cultures is the example of the strain *S. albus* UN 44, which in this study did not show antagonism against *B.subtilis* (see Table 4.1): in the work [153], fractions of antibiotics synthesized by it, active against another reference test strain of *B.subtilis*, were isolated, but at the same time, antagonism against it was not detected on the agar medium either. Obviously, the question is the amount of antibiotic produced and secreted by the culture when grown on an agar medium and the effective concentration in relation to different test strains.

The mutant strains of *S.albus* used in the study were obtained using various mutagens and their combinations, which determines the differences in the spectrum of their antagonistic activity. So, most remarkable is the biosynthetic activity of the *S. albus* AE6 (the only one among others, obtained by HNO_2 treatment), which lost

the ability to inhibit of *B. subtilis* (spore-forming bacteria), and synthesizes metabolites active against *P.vulgaris*. According to our previous analysis of the effect of various mutagens on the *S. albus* genome, which leads to the rearrangement of its nucleotide sequences, only in this type of mutants was the appearance of a fragment of 650 pairs of nucleotides, which can determine the above-mentioned changes in metabolism [204].

S. albus UN44 that obtained by treatment with a combination of HNO_2 and N-methyl-N-nitrosourea, like the AE6 strain, lost its ability to inhibit spore-forming bacteria, but does not synthesize metabolites active against proteus (see Table 4.1). However, it is obvious that both mutants have an increased synthesis of antibiotic, which determines the maximum growth inhibition zones of *C.albicans* (23-30 mm), and therefore this ability may also be related with the effect of the HNO_2 to the culture genome [156].

At the next stage of the work, deep cultivation of the *S.albus* 2435 strain was carried out, extraction of the antibiotic from the culture liquid with chloroform (1:1) and a sample of the streptofungin preparation by vacuum drying was obtained for further analysis [153].

The effect of different concentrations of streptofungin was determined in relation to the spectrum of microbial test-cultures strains recommended in the European Pharmacopoeia (https://pheur.edqm.eu/subhome/11-3), and the presented research results allow us to establish one of the defining characteristics of any antibiotic – the minimum inhibitory concentration (MIC) (Table 4.2, Fig. 4.7).

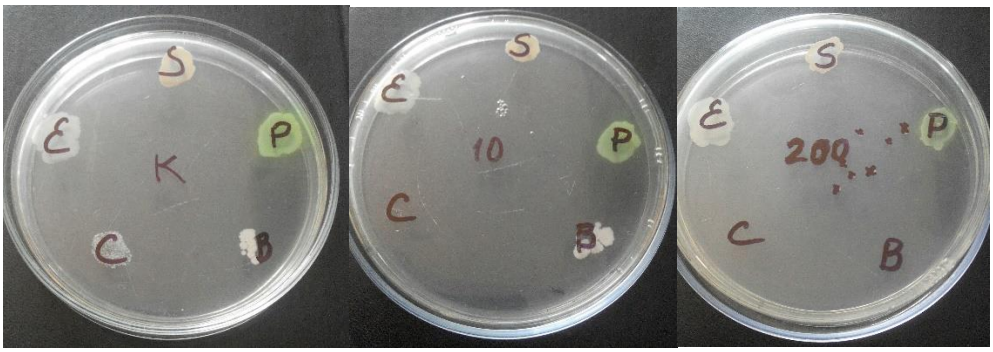
Streptofungin was the most active against *C.albicans*, and at the concentration of 10 μg/ml in the medium, there was no growth of the test-strain. As for the spore culture of *B.subtilis*, a similar picture was observed on the medium with a streptofungin concentration of 200 μg/ml, and the growth of *P.aeruginosa* was absent at an antibiotic concentration of 500 μg/ml [156].

Table 4.2

Activity of streptofungin against test-cultures strains

Test-cultures strains	Antibiotic concentration, μg/ml					
	1	10	50	100	200	500
B. subtilis ATCC 6633	+	+	+	+	–	–
C. albicans ATCC 10231	+	–	–	–	–	–
E. coli ATCC 8739	+	+	+	+	+	+
P. aeruginosa ATCC 9027	+	+	+	+	+	–
S. aureus ATCC 6538	+	+	+	+	+	+

Note: "+" – the presence of growth of the test strain;
"–" – no growth of the test strain



a) b) c)

Fig.4.7 Growth of test cultures on control medium (a) and media with 10 and 200 μg/ml of antibiotic (b, c):
B – *B. subtilis*, C – *C. albicans*, E – *E. coli*, P – *P. aeruginosa*, S – *S. aureus*.

It is obvious that streptofungin can be attributed to antifungal antibiotics by its specificity, and its MIC against a typical strain of *C.albicans* indicates its high activity. Such pharmaceutical substances as antimycotics are considered highly active and promising for development if their MIC is 4–16 μg/ml [205].

Along with such an important characteristic for an antibiotic as MIC, another indicator that determines its practical prospects is toxicity for the human. Analysis

of these two characteristics can provide an answer to the question of the potentiality of a certain substance as an API.

Therefore, at the next stage, the cytotoxicity of the antibiotic streptofungin was investigated on cell lines MDBK (Madin-Darby epithelial cells obtained from bull kidneys) and A549 (epithelial cells obtained from human lungs) using the resazurin test.

The interaction of cells with resazurin leads to a change in the color of the solution and its optical density (Table 4.3), which indicates the presence of metabolic activity and corresponds to the concentration of living cells [166].

Table 4.3

Color change of resazurin in the presence of MDBK and A549 cells pretreated with streptofungin and corresponding optical density (λ=538 nm)

Cell lines		**MDBK cells**			**A549 cells**		
Replicas		1	2	3	1	2	3
Without cells		0.529	0.901	0.899	0.616	0.611	0.718
Streptofungin, μg/ml	0	0.815	0.796	0.782	0.615	0.628	0.705
	2.5	0.736	0.797	0.808	0.634	0.668	0.774
	5.0	0.811	0.794	0.741	0.695	0.651	0.663
	7.5	0.899	0.942	0.803	0.652	0.683	0.647
	25.0	0.773	0.842	0.759	0.626	0.706	0.662
	50.0	0.768	0.802	0.766	0.665	0.839	0.724
	500.0	0.871	0.862	0.779	0.759	0.686	0.664

Taking into account the specific features of light filters installed on different devices, we analyzed the light absorption of all available light filters (405, 450, 492, 538, 620 nm) and determined that the highest optical absorption values were recorded at wavelengths of 538 nm and 620 nm, however, at the wavelength of 538 nm, the optical value increases linearly together with the metabolic activity of the cells, while at a wavelength of 620 nm these indicators are reversed [156].

According to statistical processing, the activity of MDBK cells (without antibiotic treatment) was 0.84±0.06; the activity of A549 cells was slightly lower and amounted to 0.65±0.05. (Fig. 4.8, Fig. 4.9) Under the action of the antibiotic, the activity of MDBK cells mostly decreased (except of concentrations of 7.5 µg/ml and 500 µg/ml) by 7-8% (Fig. 4.8), but this decrease was not significant (p>0.5).

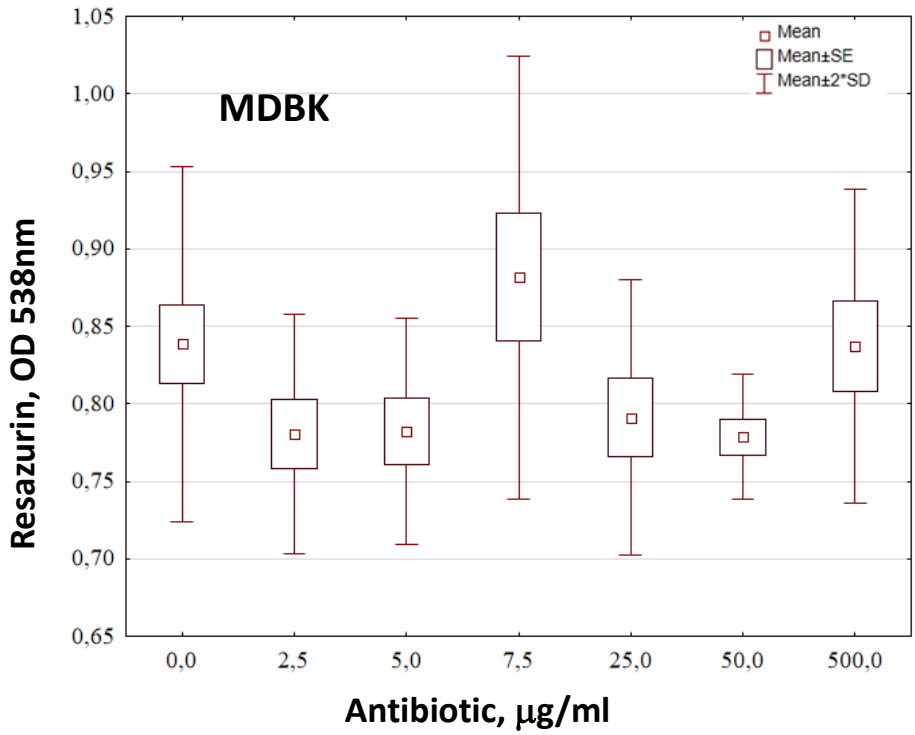


Fig. 4.8 The effect of the antibiotic streptofungin on the viability index of MDBK cells

The data represent the corresponding results of the statistical analysis of the data for the detection of a significant difference between the average activity indicators of the cells without treatment with the antibiotic (0 µg/ml) and treated with antibiotics at different concentrations (2.5–500 µg/ml).

Thus, according to the data of the resazurin test, the studied antibiotic in concentrations of 2.5 – 500 µg/ml did not show a cytotoxic effect, and therefore it can be considered a potentially permissible compound for humans and animals in the studied concentrations.

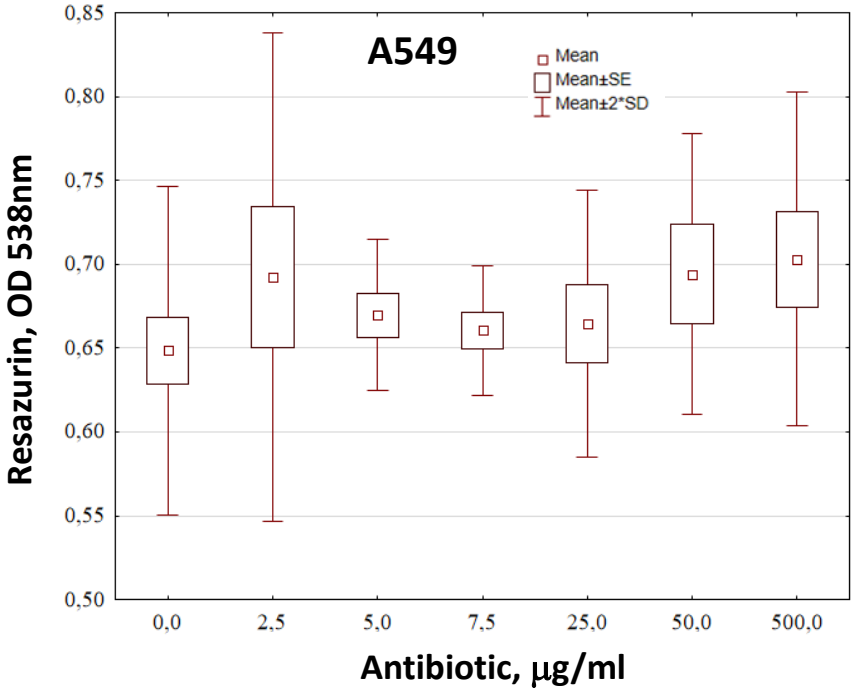


Fig. 4.9 The effect of the antibiotic streptofungin on the viability index of A549 cells

MIC is the important characteristic of any antibiotic, which will determine its practical value as an API. The activity of streptofungin at low concentrations (less than 10 µg/ml) against *C.albicans* makes it a promising antimicrobial agent, and the limited range of antifungal agents in general adds to this importance. Regarding higher MICs for pathogens such as *P.aeruginosa* and spore bacteria (*B.subtilis*), this issue can be further explored with a streptofungin substance of a higher degree of purification than the currently obtained test sample.

A comparison of the streptofungin MICs and other recently isolated antibiotics from streptomycetes shows their close values, and some studies show the possibility of combining different compounds and lowering their MICs. For example, echinomycin from *Streptomyces* sp. LS462 demonstrated antifungal activity (MIC of 6.25 µg/mL) and synergistic antifungal activity with a significantly reduced dose (up to 60-fold) in combination with posaconazole on *C.albicans* 5314 [206].

Therefore, the activity of streptofungin against *P.aeruginosa*, *B.subtilis* may be manifested differently in combination with other antimicrobial compounds, including bacteriolysins of the producer *S.albus* itself. Such a difference in the spectrum of activity of various antimicrobial metabolites of the culture is noticeable if you compare its antagonistic activity (see Table 4.1) and the activity of streptofungin (see Table 4.2) against *S.aureus*: significant antagonism (10-30 mm zone of growth inhibition) is shown by all strains, and streptofungin even at a concentration of 500 μg/ml does not affect the growth of the test strain. The answer is clear, because staphylolytic activity is the leading specificity of the enzyme complex of the culture, the strains of which were selected based on this feature [156, 204]. Therefore, the shown antagonism against *S. aureus* is due exclusively to the action of bacteriolysins produced by the culture, which are also active against *P.aeruginosa*.

The different spectrum of antimicrobial activity of *S.albus* products along with different mechanisms of its manifestation opens the possibility of creating antimicrobial compositions based on them. The presence of enzymes in such a composition will not only determine a certain spectrum of antimicrobial action, but also "help" the antibiotic to act, destroying individual defense mechanisms of pathogens. For example, we previously showed the ability of the *S.albus* enzyme complex to destroy the *P.aeruginosa* biofilm due to the content of proteinases, which can allow streptofungin in much lower concentrations (than shown in Table 4.2) to inhibit its reproduction [156].

But no one substances can be used as an antimicrobial agent if its effective doses against microbial pathogens are toxic to humans. For example, the antibiotic cycloserine isolated from streptomycetes *S.orchidaceus*, *S.garyphalus* and *S.lavendulae* inhibits the biosynthesis of peptidoglycan of the cell wall of bacteria and is active against G+ and G– microorganisms. However, its use is limited due to high toxicity, which causes serious negative side effects [207].

High toxicity (including against human cancer cell lines) was shown by three new complexes of angucycline-type antibiotics isolated from *Streptomyces* sp.

XZHG99T, which obviously have potential mainly as antitumor agents despite their significant activity against *Mycobacterium smegmatis* and *S.aureus* [208].

Therefore, we analyzed the cytotoxicity of streptofungin in doses that were determined as MIC for different test strains. The chosen method of studying the cytotoxicity of an antibiotic with resazurin allows to detect a wider range of its effect on cells than the known MTT test, since resazurin is restored (changing its color) by a greater number of living cell enzymes. The established effect of streptofungin on the used epithelial cell lines MDBK and A549 allows us to talk about its lack of cytotoxicity in concentrations effective against microbial pathogens, and therefore about its potential as an API in antimicrobials [156].

Antagonistic activity of investigated *S.albus* strains is due to the action of two different antimicrobial products – antibiotic complex streptofungin and complex of lytic enzymes, which have a different antimicrobial spectrum. The leading activity of streptofungin is determined against Candida fungi, and lytic enzymes actively destroy bacterial cells, primarily *S.aureus*.

The obtained results give reason to consider the new antibiotic streptofungin as a promising API with antifungal action, and its combination as an antimicrobial agent with the *S.albus* enzyme complex can create an antimicrobial composition with a wide spectrum of action and enhance the effectiveness of each of these components.

Conclusion for Chapter 4.

1. The novel SrtA detection could be applied further to form a new protein-free LPXTG-motif production method which would avoid the purification of recombinant proteins and refrain from relying on there being enough catalytically recombinant protein in the traditional LPXTG-motif production. The course of LPXTG-motif production would take much less time from gene cloning to SrtA assessment than the traditional way. Presumably, the yeast-displaying substrates could interact with SrtA directly, thereby enabling them to behave more efficiently.

It would accelerate the new, high-throughput, protein-free SrtA assay, which is promising and means that it could even replace the conventional method.

2. Minimal inhibitory concentrations of the antibiotic streptofungin were established for *C.albicans* ATCC 10231 (10 μg/ml), *B.subtilis* ATCC 6633 (200 μg/ml) and *P.aeruginosa* ATCC 9027 (500 μg/ml). According to the resazurin test, streptofungin in concentrations of 2.5–500 μg/ml does not show a cytotoxic effect in relation to MDBK and A549 epithelial cells, and therefore can be considered potentially permissible for humans and animals in the studied concentrations.

The results presented in Chapter 4 were published in these papers:

Lin Wu, Huijun Li, Tianle Tang. A Novel Yeast Surface Display Method for Large-Scale Screen Inhibitors of Sortase A. Bioengineering. 2017; 4,6. DOI:10.3390/bioengineering4010006 **(Scopus, Q2)**

Klochko V, Todosiichuk T, **Lin W**, Kobzysta O, Bobyr V. Antimicrobial and Cytotoxic Characteristics of Antibiotic Streptofungin. Innov Biosyst Bioeng [Internet]. 2023.Aug.22;7(2):13-21. DOI:10.20535/ibb.2023.7.2.286158 **(Scopus)**

CHAPTER 5. PROPOSALS AND RECOMMENDATIONS REGARDING THE DEVELOPMENT OF PROMISING ANTIMICROBIALS

5.1 Combined antimicrobials based on substances from *Streptomyces albus* with different mechanisms of action

The characteristics of the antibiotic streptofungin from *S.albus* 2435 established at the previous stage of the work gave reasons to consider it as a potential component of the drug, and the ability of the same culture to synthesize a complex of lytic enzymes also raised questions about the possibility of combining these substances in the composition of an antimicrobial agent. Complex of bacteriolytic enzymes cytal from this producer is active to varying degrees against such pathogens as *S.aureus, P.aeruginosa, E.coli, Proteus rettgeri*, etc. [154].

Additional interest in complex development using this producer is the possibility of simultaneously obtaining two products (enzymes and antibiotics) and combining them into a broad-spectrum antimicrobial agent. Such combination of APIs with different mechanisms of action is noted as an effective technique for increasing the effectiveness of antimicrobial drugs against resistant pathogens, and most importantly, for inhibiting the development of their antibiotic resistance in general [105].

Important data, on the basis of which the possibility of obtaining two products of S.albus 2435 (enzymes and antibiotics) is determined the dynamics and interrelationship of the biosynthesis of these two products.

In the process of cultivating the producer (Fig. 5.1), it was established that the maximum level of these two products of the culture coincides in time, which determines the technological advantages of such development and the possibility of isolating products from the culture liquid at the stage of maximum concentration (48-60 hours). At that moment, the activity of lytic enzymes was 1200 U/ml, and the antibiotic was 1.4 mg/ml.

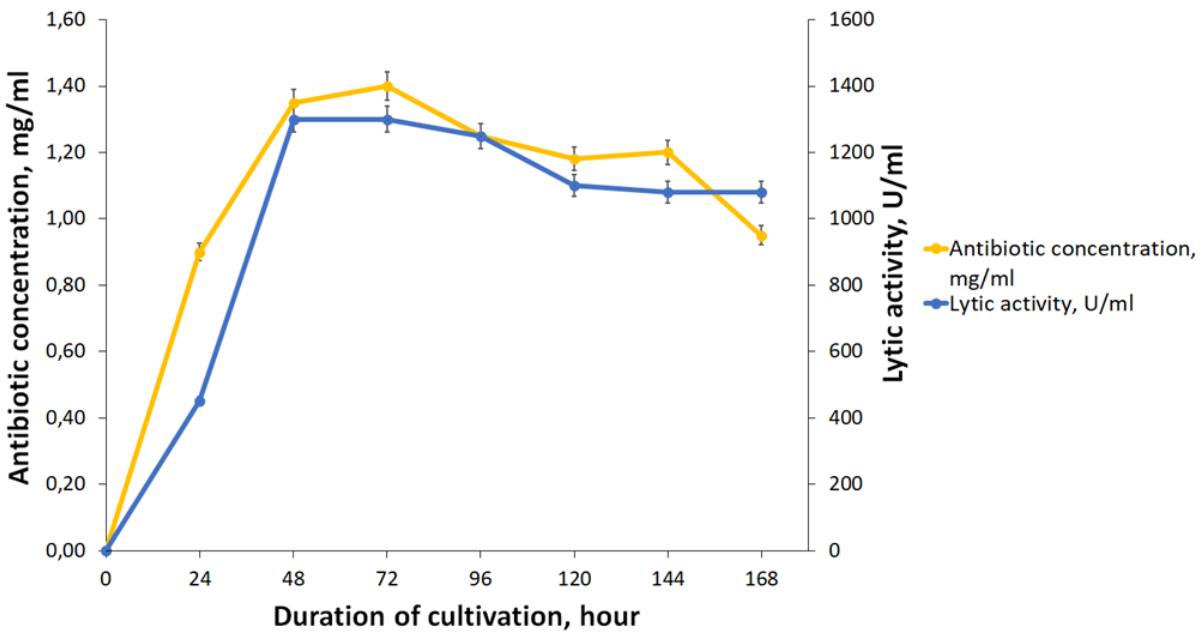
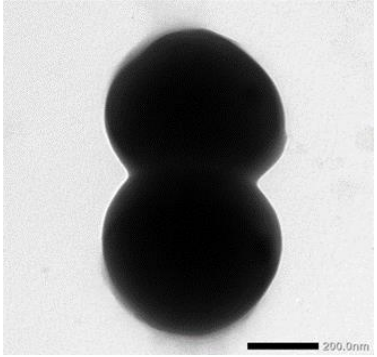


Fig. 5.1 Dynamics of biosynthesis of antibiotics and bacteriolysins by *S.albus* 2435

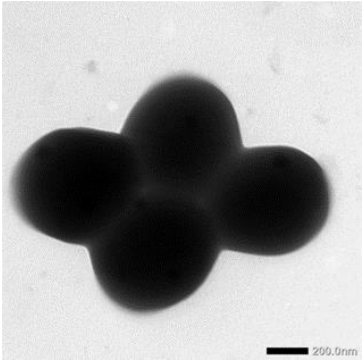
After the completion of biosynthesis, experimental samples of the antibiotic streptofungin and the complex of lytic enzymes cytal were obtained and used for analysis and creation of the composition of an antimicrobial combine pharmaceutical composition (as described in Chapter 2).

The antimicrobial spectrum of the lytic enzyme complex of *S.albus* 2435 has been established by many previous studies and has leading staphylolytic activity, both with to varying degrees cytal is active against *B. cereus, C. gravis, P. aeruginosa, E. coli, P. rettgeri, S. thermophiles , S. sonnei, S. typhi, K. pneumonia* [154].

However, there were no previous studies that allowed us to visualize the process of cell destruction under the action of the enzyme, so we analyzed the specified process by transmission electron microscopy (Fig. 5.2).

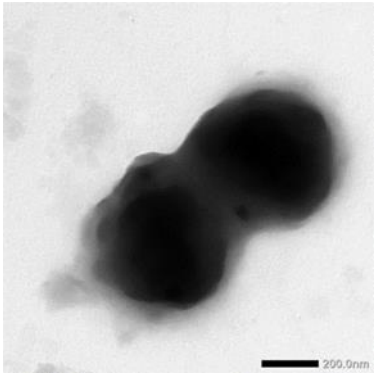


measurement segment
200.0 nm

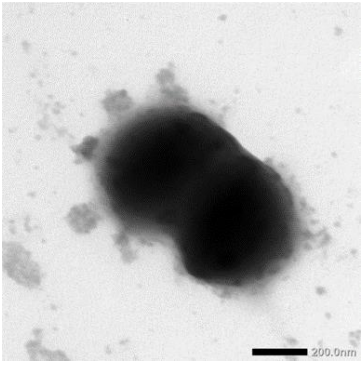


measurement segment
200.0 nm

S. aureus
(without adding the enzyme) – standard form of cocci cells

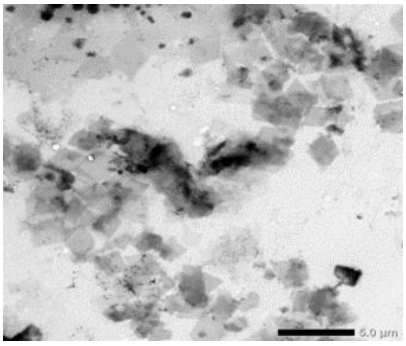


measurement segment
200.0 nm

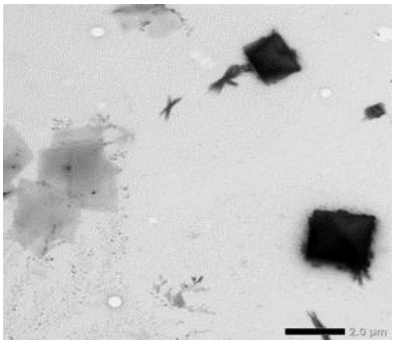


measurement segment
200.0 nm

After 20 minutes of cytal addition:
The beginning of cell lysis under the action of the enzyme
– cocci begin to lose their form



measurement segment
6.0 µm



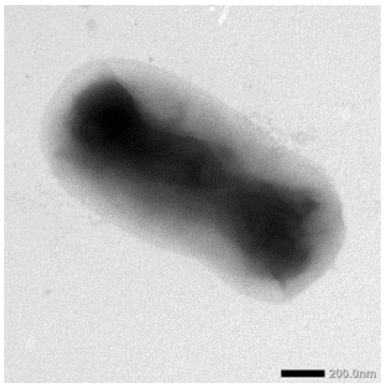
measurement segment
2.0 µm

After 24 hours of cytal addition:
– complete absence of staphylococcal cells, only opaque crystals in the shape of squares (more likely of a protein nature)

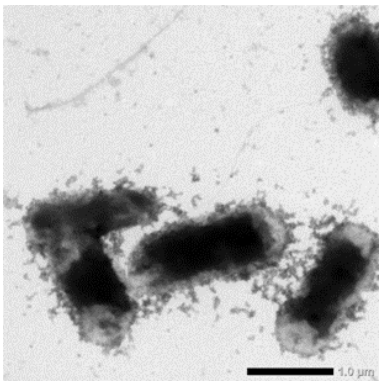
Fig. 5.2 Electron microphotography of lysis process of *S. aureus* cells by the enzyme cytal from *S. albus* 2435

Already after 20 minutes, you can see the beginning of the loss of the shape of cells, such as staphylococcus and *E. coli*, and after 24 hours, individual structures or cells with a destroyed cell wall or lost forms were observed in the field of vision. It is obvious that this mechanism of action of cytal, as well as lytic enzymes in

general, does not allow microbial cells to develop protective actions and acquire resistance to them.

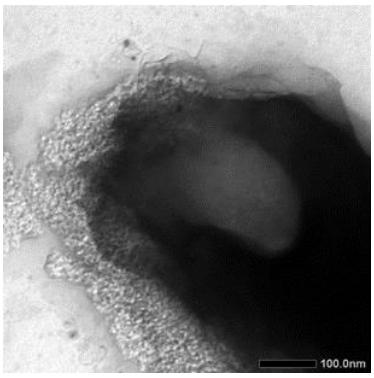


measurement segment 200.0 nm

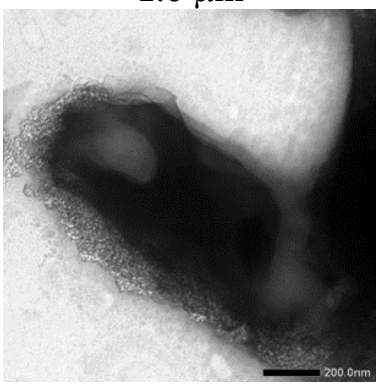


measurement segment 1.0 µm

E. coli - control (without adding the enzyme) - the standard form of rod cells surrounded by their own metabolites, rather polysaccharides

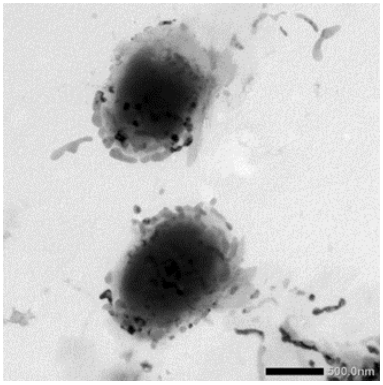


measurement segment 100.0 nm

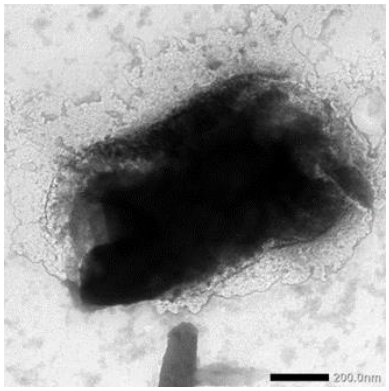


measurement segment 200.0 nm

After 20 minutes of cytal addition – the beginning of cell lysis under the action of an enzyme (cells begin to lose their form). The photo shows the enveloping of cells with enzyme



measurement segment 500.0 nm



measurement segment 200.0 nm

After 24 hours of cytal addition:
- there are single structures that faintly resemble rod cells

Fig. 5.3 Electron microphotography of lysis process of *E. coli* cells by the enzyme cytal from *S. albus* 2435

Substances of such type are defined as promising in the modern development of antiseptics and even not so long ago received another name - enzybiotics [153]. Namely, a number of experimental medicinal forms have already been developed on

the basis of cytal - liquid, ointment, powder, which have shown their effectivenes [210].

Among modern developments of ready-made forms of antiseptics for external use, soft forms (ointments, gels, liniments) predominate, created on the basis of a wide range of auxiliary substances that have a forming function, stabilizing and prolonging effect [209] In such compositions, enzymes or antibiotics are mainly used as active substances, but promising developments involve the combination of several antimicrobial substances with different spectrum and mechanism of action, which allows to increase their effectiveness.

The combination of two active substances with different antimicrobial specificity and mechanism of action in an antimicrobial pharmaceutical composition allows to expand the areas of its application and increase its effectiveness. The latter is due to the ability of cytal enzymes to directly destroy pathogen cells and their biofilms [210], and the antibiotic effect of streptofungin will additionally prevent the development of pathogen cells that can survive.

Since ointment compositions have already been developed on the basis of cital [210], one of them was used to create a dosage form with the introduction of two active substances - cytal (in previously selected concentrations) and streptofungin in variable concentrations taking into account the determined MIC.

Polyethylene oxide-400 (PEO-400), polyethylene glycol-600 (PEG-600), polyethylene glycol-1000 (PEG-1000) and proxanol 268 form the forming basis of the composition, which give the tool a gel-like state convenient for use. In addition, the polymers used in the composition of the base actively absorb wound exudate, and with it microbial toxins, tissue decay products and create favorable conditions for the manifestation of local cellular immunity. The composition of the ointment base, wt. %: PEO-400 – 10, PEG-600 – 25, PEG-1000 – 10, proxanol 268 – 20, glycerin – 10, lidocaine – 5, dimexide – 5, water – the rest.

In the experiment, variants of compositions were prepared that differed in the content and concentration of cytal and streptofungin, and the antimicrobial activity of the composition was determined in two ways (Table 5.1).

Table 5.1

Antimicrobial activity of combined pharmaceutical composition

Concentration of streptofungin, %	Concentration of cytal, %	**Lytic activity, % degradation of test culture (suspension)**				**Growth inhibition test cultures (surface): "+" available growth "-" there is no growth**			
		S. aureus ATCC 25923	*P. aeruginosa ATCC 27853*	*C. albicans ATCC 10231*	*E. coli ATCC* 25922	*S. aureus ATCC* 25923	*P. aeruginosa ATCC 27853*	*C. albicans ATCC 10231*	*E. coli ATCC* 25922
0	0	2	0	0	1	+	+	+	+
0	3.0	65	10	0	15	–	–	+	+
0.5	1.0	50	6	0	5	+	+	+	+
1.0	2.0	57	8	0	10	+	–	+	+
1.5	3.0	65	10	0	15	–	–	–	–
1.5	0	2	0	0	1	+	–	–	+

It is obvious that the pharmaceutical composition does not show lytic activity to *C. albicans*, because the enzyme does not have such a spectrum, and the antibiotic has a different mechanism. It is already capable of inhibiting the development of this pathogen, the result of which is the absence of growth on the surface of the medium treated with the agent. Growth inhibition of *C. albicans* occurs at the maximum concentration of the antibiotic (1.5%).

And when considering the results of inhibition of *P. aeruginosa*, the results of the combined action of the two components are visible, and at concentrations of 1.0% and 2.0% of the enzyme, there is already no growth of this representative of nosocomial infections. This indicates the synergistic effect of the studied antimicrobial substances and the obvious possibility of developing both broad-spectrum and targeted agents (by optimizing the concentrations accordingly).

Due to the wide spectrum of antimicrobial action, the composition can be used for the treatment of wounds of superficial localization of various etiologies

(traumatic, burn, postoperative, trophic ulcers), and the presence of proteinases among the cytal components will prevent the removal of necrotic tissues and wound healing.

The use of streptofungin, as an active substance in the composition, also allowed to exclude the additional introduction of other antibiotics, which are usually added as preservatives for the storage of the drug. However, a number of other components were introduced to give the composition the necessary properties. Thus, the composition includes the use of dimexide (dimethyl sulfoxide), which enhances the penetration of drugs through the skin, and also has an anti-inflammatory, analgesic, antiseptic, and fibrinolytic effect. Trimecaine or lidocaine can be used as an anesthetic in the composition of the composition to reduce pain sensations during treatment, which also have antiarrhythmic properties and retain their activity (unlike novocaine) in an acidic environment, which is characteristic of infected wounds.

In addition, the polymers used in the composition of the base actively absorb wound exudate, and with it microbial toxins, tissue decay products and create favorable conditions for the manifestation of local cellular immunity.

To date, there are no known ointments on the Ukrainian market that contain antibiotics and enzymes at the same time, therefore the relevance of such developments, as well as the adjustment of the production of the substances themselves, is very relevant. Until recently, Streptolaven ointment (FF "Darnytsia") containing the antibiotic miramistin and an enzyme was present on the market, but currently the drug is not on sale, perhaps because of the imported substance (enzyme ultralysin) contained in the drug.

An additional advantage of the possible introduction into production of a biotechnological method of obtaining the studied substances is the producer's ability to simultaneously accumulate them and the possibility of obtaining two products during the same production process.

5.2 Development of recommendations for the implementation of monitoring the resistance of nosocomial infections and the principles of modern antimicrobials work out

RECOMMENDATIONS

for the implementation of monitoring the resistance of nosocomial infections and the principles of development of modern antimicrobial drugs

1. The problems of the development of resistance of microbial pathogens to the used agents and the acquisition of resistance to new drugs are primarily associated with errors in the administration of antimicrobial therapy in hospitals, where a large number of pathogen strains circulate and mass use of antimicrobial drugs occurs.

2. In hospitals with infectious diseases departments, a strict system of monitoring and documenting the appointment of antimicrobial agents to each patient should be implemented in accordance with previously obtained results of the sensitivity of isolated pathogens. At the end of the treatment, an analysis of the effectiveness and compliance of the prescribed drugs should be carried out.

3. Special monitoring should be carried out in intensive care units when treating older patients who have weakened mechanisms of protection against infection with nosocomial pathogens, as a result, for example, intensive adhesion of pathogens on the mucous membrane of the upper respiratory tract, etc.

4. Problems during surgical intervention are one of the sources of infection with opportunistic microorganisms, including anaerobic ones, which are part of the normal microflora of a person, but when they get into the bloodstream, they can cause bacteremia with numerous variants of complex consequences. Therefore, it is important to monitor the postoperative conditions of patients and regarding possible infection both by representatives of one's own microflora and when using catheters, instruments, etc.

5. In the clinical practice of infectious disease departments of hospitals, the large-scale use of one class of drugs should be avoided in order to reduce their

selective pressure on pathogens, as well as periodically replace the spectrum of antibacterial drugs used.

6. Large hospitals with an infectious disease department should include scientific and analytical laboratories equipped with modern methods of pathogen identification and molecular genetic analysis. The functions of such laboratories should include the storage and analysis of strains of infectious agents isolated from patients (origin, characteristics of their sensitivity to antimicrobial agents, results of applied therapy), summarization of data to provide recommendations for the treatment of both an individual patient, and the use of the spectrum of antimicrobial agents in the hospital in general.

7. The development of modern antimicrobial drugs of various nature should be based on the selection of a target that makes it impossible or minimizes the induction of protective mechanisms in individual pathogens, and therefore the acquisition of resistance to an increasing number of agents. For this, it is important to study and establish the factors and mechanisms of resistance of the causative agents of the most common infectious diseases and, first of all, representatives of nosocomial infections.

8. Among the important targets for antimicrobial agents are surface structures of microbial cells and substances responsible for the adhesion of pathogens to tissue in the human body. Blocking the adhesion process determines the impossibility of infection, and therefore human disease. Thus, the ideal target that catalyzes the covalent attachment of tissue surface proteins to the cell wall of gram-positive bacteria is the enzyme Sortase A, so its inhibitors are promising agents, for example, against staphylococcus.

9. The development of drugs aimed at inhibiting the quorum sensing (QS) systems of pathogens as the main target, avoids the rapid development of resistance, as such substances do not have bactericidal or bacteriostatic action on pathogenic bacteria. Such drugs lead to the suppression of pathogenicity and are called "poisons of pathogenicity". Inhibition of QS systems can be achieved in several ways. One of the strategies is to inhibit the synthesis of precursor molecules of autoinducers or

autoinducers themselves (acylhomoserine lactones (AGL), peptides, amino acids and similar amine compounds). Second, drugs may be targeted by inhibiting the binding of autoinducers to the corresponding receptor proteins.

10. Successfully development focuses on the destruction of the biofilm of pathogens as an important factor in their stability. It is shown that the combination of antimicrobial enzyme and fluoroquinolone antibiotic causes a synergistic effect against *S. aureus*, which is based on the breakdown of the biofilm layer by the enzyme and the subsequent bactericidal action of the antibiotic

11. Among the new classes of antiseptics developed by pharmaceutical companies, new peptides are attracting special attention; drugs that block fatty acid synthesis or early stages of protein synthesis in the microbial cell, as well as β-lactamase inhibitors that do not have their own antibacterial activity.

12. Another promising direction in the search for *new antibiotic compounds* is the selection of microbial producers from exotic and non-studied ecotopes. One of them is new antibiotics hexalactin and hexamycin, related to ansamycins, which include the currently used rifampicin. In addition to finding new compounds among microbial producers*, screening sensitivity-based techniques* are proposed: when the intracellular level of the target affected by the desired antibiotic is reduced by the action of the corresponding antisense-RNA, test strains become more sensitive to this antibiotic. Thus, it is possible to detect compounds that under normal conditions do not inhibit the growth of test strains.

13.The experience of using bacteriophages as a basis for antimicrobial drugs already has a history but continues to be a promising way to combat resistant microbial pathogens. Preparations of bacteriophages as antimicrobial agents have advantages because they do not affect the normal human microflora, do not cause resistance to pathogens, but their activity depends on the effectiveness of their replication. The development of this direction is the use of bacteriophage enzymes as an antimicrobial substance.

14. Among other promising compounds that can solve the problem of fighting nosocomial infections - enzybiotics, which now include substances with a specific

mechanism of action (bacteriocins, cathelicins, lysines, bacteriophages, immunobiotics). The authors identify the benefits and broad prospects of such drugs, which significantly increase the effectiveness of antimicrobial action without causing the emergence of resistant forms of pathogens.

15. Genome sequencing and gene annotation of common microbial pathogens, as a modern tool, makes it possible to establish targets for the action of new antiseptics at the gene level. Blocking the genes encoding the resistance of individual pathogens can be a complex task for genetic engineers, bioinformaticians, microbiologists and other specialists on the way to the development of novel drugs against multiresistant strains.

16. Combined drugs based on substances with different mechanisms of action, mainly antibiotics and enzymes, allow to strengthen the effect of each of the substances, and different mechanisms of action on microbial pathogens lead to more effective destruction of cells at various stages of its development.

The results presented in Chapter 5 were published in these papers:

1.Tetiana S. Todosiichuk, Serhii O. Soloviov, **Lin Wu**, Iryna V. Dzyublyk, Olena P. Trokhimenko, Magdalena Dudek, Artem Symchuk, Volodymyr Vasylenko. Directions in the development of modern and promising antimicrobial agents. BIOLOGIJA. 2022; 68 (4): 218–229. DOI:10.6001/biologija.v68i4.4838

2. Korneva O.M., **Lin Wu**. Influence of mutagens of various nature on antagonistic activity of *Streptomyces albus*. "Biotechnology of the 21st century": materials of the 14th All-Ukrainian Scientific and Practical Conference (Kyiv, May 20, 2020). Kyiv:Igor Sikorsky KPI, "Polytechnic", 2020. P. 48.

3. Korneva O.M., Ryzhkova T.S., **Wu Lin**. Peculiarity of Streptomyces albus antimicrobial complex′s biosynthesis / Problems and achievements of modern biotechnology: materials of the 1st international science and practice. Internet Conf. (March 25, 2021, Kharkiv). - Electron. data. - Kh.: NPhU, 2021. – P.12.

CONCLUSIONS

The dissertation theoretically substantiates the approaches and provides new ways of solving the scientific and practical problem related to the creation of modern antimicrobial drugs against bacterial pathogens of nosocomial infections. The research results make it possible to draw the following conclusions.

1. On the basis of a systematic analysis of factors and mechanisms of nosocomial infections pathogens resistance, both with conducted investigation with bacterial strains of *Acinetobacter baumannii, Pseudomonas oryzihabitans, Bacteroides sp aff. thetaiotaomicron* shows their complex influence on the pathogens resistance formation and directions for solving the problem.

2. The results of monitoring the level of in-hospital infection with *A.baumannii* showed the impact of regular disinfection measures, and identified risk groups: the number of infections did not depend on gender, but almost doubled in patients of intensive care units and those over 60 years old.

3. A decrease in the sensitivity of clinical strains of *A.baumannii* to third-generation cephalosporins (up to 55-77%), which are widely used, was determined, both with the effectiveness of monitoring the sensitivity of nosocomial infection while selection the agents and their periodic change.

4. The analysis of the 16S rRNA sequence of the clinical strain *P. oryzihabitans* JN 873340 and its comparison with 29 other strains of the species from GenBank showed the possibility of identifying its geographical origin by two hypervariable regions V4 and V5, which can be used for rapid identification and taking into account the characteristics of strains of different origins when choosing treatment.

5. The genome of the isolated clinical strain *B. thetaiotaomicron* DSMZ 2079 was sequenced and annotated, showing that it has a one- and two-component system for recognizing environmental signals, four homologues of the self-transmitted conjugative transposon CTnDOT, which provides the extension of resistance to tetracycline and erythromycin. The genes *rpoB* and *tuf* (in loci JHR92_RS03155 and

JHR92_RS03195, respectively) were identified, which determine the resistance of the strain to antibiotics.

6. A new method of broad screening for Sortase A inhibitors, which catalyzes the processes of adhesion of gram-positive bacteria on body cells, and therefore is an ideal target for new antimicrobial agents, was developed. In the method implemented into practice the modified *Pichia pastoris* yeast cells instead of expensive synthetic substrates for the detection of enzyme activity were used.

7. The prospects for the search for new antibiotics among streptomycetes were determined and the characteristics of the antibiotic streptofungin isolated from *Streptomyces albus* 2435 were determined as minimal inhibitory concentrations against *C. albicans* ATCC 10231 (10 μg/ml), *B. subtilis* ATCC 6633 (200 μg/ml) and *P. aeruginosa* ATCC 9027 (500 μg/ml), combine with the absence of toxicity in a wide range of concentrations (from 2.5 to 500 μg/ml).

8. A pharmaceutical composition of an antiseptic ointment based on the products of *Streptomyces albus* 2435 with a different spectrum and mechanism of action – the enzyme cytal and the antibiotic streptofungin – was proposed. A synergistic antimicrobial effect was demonstrated by the combination of these substances, aimed to overcome such a mechanism of resistance of pathogens as biofilms.

9. Based on the results of the work, the recommendations regarding the implementation of nosocomial infections resistance monitoring and the principles of development of modern antimicrobial drugs were developed and approved.

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ANNEXE 1

海南醫學院
HAINAN MEDICAL UNIVERSITY

«APPROVED»
Assitantor-Head of the school of
Tropical Medicine

Dr/prof
_____ **Yu Chunwei**
«_01_» _09_ 2023

IMPLEMENTATION ACT
of the results of WU Lin dissertation work
«Resistance factors of bacterial nosocomial infections causative agents as background for the modern antimicrobials development»
at the education process of the school of Tropical Medicine

Object of implementation: scientific and scientific-methodological developments regarding the research and analysis of bacterial infection causative agent characteristics and properties.

The author of the development: WU Lin, PhD-student of National Technical University of Ukraine "Igor Sikorsky Kyiv Polytechnic Institute"
Sources:

- 1. **WU Lin,** LI Li-hua, WU Li-xian. Phylogenetic analysis of *Pseudomonas oryzihabitans* of different geographical populations based on partial sequences of 16S rRNA gene (in Chinese). China Tropical Medicine. 2012; 12(12):1453-1456.
- 2. WU Zhi-Cheng, **WU Lin.** Clinical distribution and drug resistance change of respiratory nosocomial infections of *Acinetobacter baumannii* (in Chinese). Journal of Hainan Medical University. 2013; 19(2):271-274.
- 3. ZC Wu, **L Wu,** M Zhang, W Zhou. Genome sequence and annotation of *Bacteroides sp aff. Thetaiotaomicron* strain isolated from blood. Infection, Genetics and Evolution. 2021; 91: 104816. PMID: 33771725.

Implementation results: scientific and scientific-methodological developments regarding the research and analysis of bacterial infection causative agent characteristics and properties were introduced into the educational process of students majoring in "Tropical Medicine" within the framework of lectures and laboratory (practical) classes in the

disciplines "Medical Immunology", "Pathobiology", "Medical Microbiology", "Environmental Microbiology", "Hygienic Microbiology", "Environmental and Health". The implementation of the above-mentioned developments made it possible to improve the teaching of the following sections (topics): "Resistance analysis", "Infection analysis", "Development, testing and organization of production of medical products of antimicrobials".

Responsible for implementation:

Prof. of Environmental ecology and Health teaching and research office	Signature	唐天乐 Tang Tianle
PhD-student of National Technical University of Ukraine "Igor Sikorsky Kyiv Polytechnic Institute"	Signature	吴琳 WU Lin

ANNEXE 2

海南醫學院
HAINAN MEDICAL UNIVERSITY

«APPROVED»
Assitantor-Head of the School of Tropical Medicine

Dr/prof
Yu Chunwei
«04» 09 2023

IMPLEMENTATION ACT
of the results of WU Lin dissertation work
«Resistance factors of bacterial nosocomial infections causative agents as background for the modern antimicrobials development»
at the Environmental Comprehensive laboratory of the School of Tropical Medicine

Object of implementation:
Method for Large-Scale Screen Inhibitors of Sortase A.

The authors of the development:
WU Lin, PhD-student of National Technical University of Ukraine "Igor Sikorsky Kyiv Polytechnic Institute",

Sources:
Lin Wu, Huijun Li, Tianle Tang. A Novel Yeast Surface Display Method for Large-Scale Screen Inhibitors of Sortase A. Bioengineering. 2017; 4,6.

Implementation results: developed during the dissertation work of WU Lin the Method for Large-Scale Screen Inhibitors of Sortase A using flow cytometry and fluorescence spectrophotometry. With the aim of reaching a more robust assay, the protocol was adjusted in various ways. The routine SrtA assay is based on the production of enough native or synthesized substrates, but the complicated purification process and the high expenses greatly limit the screening for inhibitors. The present method, a novel SrtA method was developed by applying the yeast-displaying substrates directly into the SrtA detection system instead of soluble protein substrates. The displayed- substrates were successful in the

detection of SrtA during the time of the project. The results were confirmed by fluorescence spectrophotometry. All the results indicated that the control and the sample with the inhibitors (different concentration of berberine chloride) were increased with respect to growth, which was fitted for screening inhibitors, but the positive control was less variant. Even though the routine method was much more sensitive, the yeast-displaying substrates method was easier and cheaper, making it well suited to large-screening inhibitors.

The yeast-display system method has the following advantages: easy anipulation of gene clone, protein expression, and cell concentration, while the maximal difficulty was finding a different protocol to achieve a robust assay that is sensitive enough for investigation of Sortase A. The method that was implemented is a simple and cheap method, which is very suitable for high throughput analysis. Despite the traditional method is much more sensitive, this approach is expected to lead to large-scale screening of SrtA inhibitors. It can be used to decrease the risk of drug resistance development.

Responsible for implementation:

Prof. of Environmental Comprehensive laboratory — Wen Shaobai

PhD-student of National Technical University of Ukraine "Igor Sikorsky Kyiv Polytechnic Institute" — WU Lin

ANNEXE 3

UKRAINE
MINISTRY OF EDUCATION AND SCIENCE OF UKRAINE
NATIONAL TECHNICAL UNIVERSITY OF UKRAINE
"IGOR SIKORSKY KYIV POLYTECHNIC INSTITUTE"

FACULTY OF BIOTECHNOLOGY AND BIOTECHNICS

03056, Kyiv-56, Prospect Beresteiskyi, 37 tel.: 204-94-52, tel.fax: 204-83-12
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№ 3300/1-2
"20" 09 2023

Acting Dean
Ass. Prof., PhD
Vita Linovytska

IMPLEMENTATION ACT
of the results of **WU Lin** dissertation work
«Resistance factors of bacterial nosocomial infections causative agents as background for the modern antimicrobials development»
at the education process of the
Department of Industrial Biotechnology and Biopharmacy

Object of implementation: scientific and scientific-methodological developments regarding the research and analysis of bacterial infection causative agent characteristics and properties.

The author of the development: **WU Lin**, PhD-student of National Technical University of Ukraine "Igor Sikorsky Kyiv Polytechnic Institute"

Sources:

- **L. Wu**, Z.C. Wu, T.S. Todosiichuk, O.M. Korneva. Nosocomial infections: pathogenicity, resistance and novel antimicrobials. Innov Biosyst Bioeng. 2021; 5(2):73–84. DOI: 10.20535/ibb.2021.5.2.228970
- Tetiana S. Todosiichuk, Serhii O. Soloviov, **Lin Wu**, Iryna V. Dzyublyk, Olena P. Trokhimenko, Magdalena Dudek, Artem Symchuk, Volodymyr Vasylenko. Directions in the development of modern and promising antimicrobial agents. BIOLOGIJA. 2022; 68 (4): 218–229. DOI:10.6001/biologija.v68i4.4838

• Klochko V, Todosiichuk T, **Lin W**, Kobzysta O, Bobyr V. Antimicrobial and Cytotoxic Characteristics of Antibiotic Streptofungin. Innov Biosyst Bioeng. 2023Aug.22;7(2):13-21. DOI:10.20535/ibb.2023.7.2.286158

Implementation results: scientific and scientific-methodological developments regarding the research and analysis of bacterial infection causative agent characteristics and properties were introduced into the educational process of students in framework of lectures in the disciplines "General Microbiology and Virology" and "Fundamentals of Pharmaceutical Production". The implementation of the above-mentioned developments made it possible to improve the teaching of the following sections (topics): "Effect of physical, chemical and biological factors on the microbial cells», «Morphological differentiation of bacteria", "Viruses of bacteria. Bacteriophages", "Development of the modern antimicrobials", «Promising active substances for antimicrobial agents».

Responsible for implementation:

Acting Head of the Department
of Industrial Biotechnology and Biopharmacy, Valentyna Polischuk
PhD

Senior Lecturer of the Department
of Industrial Biotechnology and Biopharmacy, Larisa Titova
PhD

ANNEXE 4

"APPROVED"
Director
LLC "PHARMA INTERNATIONAL GROUP 2"
_____ O.H. Sydorenko
"20" 07.2023

NOTICE OF USE
of the results of the dissertation of WU Lin (WU Lin)
"Resistance factors of bacterial nosocomial infections causative agents as background for the modern antimicrobials development"
in the production program of "PHARMA INTERNATIONAL GROUP 2" LLC

The compositions of combined antiseptics for external use based on a complex of enzymes and antibiotics of microbial origin proposed by the results of the dissertation research are promising for implementation, given their leading antistaphylococcal and antifungal effects. The presence of proteinases among the enzyme complex can provide additional functions to antiseptic means, such as purification from necrotic tissues, accelerating granulation and wound healing, and for cosmetic skin problems - anti-inflammatory effect, biopeeling, etc.

The results of the work were used to develop technological documentation to produce a liquid form of a combined antiseptic for cosmetic and pharmaceutical purposes. The principles and approaches to creating preparations with biologically active substances with different mechanisms of action proposed as a result of the dissertation research were used in the production program for the development of functional cosmetics.

Specialist Storozhilov A.S

ANNEXE 5

«APPROVED»
The first vice-rector
Shupyk National Healthcare
University of Ukraine
corresponding member of the National
Academy of Sciences of Ukraine
Professor Vdovychenko Yu.P.

_____ 2023 p.

ACT OF IMPLEMENTATION

1. **Title of the proposal for implementation:** Recommendations for the implementation of monitoring the resistance of nosocomial infections and the principles of developement of modern antimicrobial drugs.
2. **The institution, address, authors:** Kyiv, 03056, Prospekt Beresteyskyi, 37, building 4; Dean of the Faculty of Biotechnology and Biotechnic of Igor Sikorsky Kyiv Polytechnic Institute professor T.S. Todosiichuk, PhD-student of Igor Sikorsky Kyiv Polytechnic Institute Wu Lin
3. **Source of information:**

RECOMMENDATIONS

for the implementation of monitoring the resistance of nosocomial infections and the principles of development of modern antimicrobial drugs
as the results of **WU Lin** dissertation work
«Resistance factors of bacterial nosocomial infections causative agents as background for the modern antimicrobials developement»

1. The problems of the development of resistance of microbial pathogens to the used agents and the acquisition of resistance to new drugs are primarily associated with errors in the administration of antimicrobial therapy in hospitals, where a large number of pathogen strains circulate and mass use of antimicrobial drugs occurs.

2. In hospitals with infectious diseases departments, a strict system of monitoring and documenting the appointment of antimicrobial agents to each

patient should be implemented in accordance with previously obtained results of the sensitivity of isolated pathogens. At the end of the treatment, an analysis of the effectiveness and compliance of the prescribed drugs should be carried out.

3. Special monitoring should be carried out in intensive care units when treating older patients who have weakened mechanisms of protection against infection with nosocomial pathogens, as a result, for example, intensive adhesion of pathogens on the mucous membrane of the upper respiratory tract, etc.

4. Problems during surgical intervention are one of the sources of infection with opportunistic microorganisms, including anaerobic ones, which are part of the normal microflora of a person, but when they get into the bloodstream, they can cause bacteremia with numerous variants of complex consequences. Therefore, it is important to monitor the postoperative conditions of patients and regarding possible infection both by representatives of one's own microflora and when using catheters, instruments, etc.

5. In the clinical practice of infectious disease departments of hospitals, the large-scale use of one class of drugs should be avoided in order to reduce their selective pressure on pathogens, as well as periodically replace the spectrum of antibacterial drugs used.

6. Large hospitals with an infectious disease department should include scientific and analytical laboratories equipped with modern methods of pathogen identification and molecular genetic analysis. The functions of such laboratories should include the storage and analysis of strains of infectious agents isolated from patients (origin, characteristics of their sensitivity to antimicrobial agents, results of applied therapy), summarization of data to provide recommendations for the treatment of both an individual patient, and the use of the spectrum of antimicrobial agents in the hospital in general.

7. The developement of modern antimicrobial drugs of various nature should be based on the selection of a target that makes it impossible or minimizes the induction of protective mechanisms in individual pathogens, and therefore the acquisition of resistance to an increasing number of agents. For this, it is important to study and establish the factors and mechanisms of resistance of the causative

agents of the most common infectious diseases and, first of all, representatives of nosocomial infections.

8. Among the important targets for antimicrobial agents are surface structures of microbial cells and substances responsible for the adhesion of pathogens to tissue in the human body. Blocking the adhesion process determines the impossibility of infection, and therefore human disease. Thus, the ideal target that catalyzes the covalent attachment of tissue surface proteins to the cell wall of gram-positive bacteria is the enzyme Sortase A, so its inhibitors are promising agents, for example, against staphylococcus.

9. The development of drugs aimed at inhibiting the quorum sensing (QS) systems of pathogens as the main target, avoids the rapid development of resistance, as such substances do not have bactericidal or bacteriostatic action on pathogenic bacteria. Such drugs lead to the suppression of pathogenicity and are called "poisons of pathogenicity". Inhibition of QS systems can be achieved in several ways. One of the strategies is to inhibit the synthesis of precursor molecules of autoinducers or autoinducers themselves (acylhomoserine lactones (AGL), peptides, amino acids and similar amine compounds). Second, drugs may be targeted by inhibiting the binding of autoinducers to the corresponding receptor proteins.

10. Successfully development focuses on the destruction of the biofilm of pathogens as an important factor in their stability. It is shown that the combination of antimicrobial enzyme and fluoroquinolone antibiotic causes a synergistic effect against *S. aureus*, which is based on the breakdown of the biofilm layer by the enzyme and the subsequent bactericidal action of the antibiotic

11. Among the new classes of antiseptics developed by pharmaceutical companies, new peptides are attracting special attention; drugs that block fatty acid synthesis or early stages of protein synthesis in the microbial cell, as well as β-lactamase inhibitors that do not have their own antibacterial activity.

12. Another promising direction in the search for *new antibiotic compounds* is the selection of microbial producers from exotic and non-studied ecotopes. One of them is new antibiotics hexalactin and hexamycin, related to ansamycins, which

include the currently used rifampicin. In addition to finding new compounds among microbial producers, *screening sensitivity-based techniques* are proposed: when the intracellular level of the target affected by the desired antibiotic is reduced by the action of the corresponding antisense-RNA, test strains become more sensitive to this antibiotic. Thus, it is possible to detect compounds that under normal conditions do not inhibit the growth of test strains.

13.The experience of using bacteriophages as a basis for antimicrobial drugs already has a history but continues to be a promising way to combat resistant microbial pathogens. Preparations of bacteriophages as antimicrobial agents have advantages because they do not affect the normal human microflora, do not cause resistance to pathogens, but their activity depends on the effectiveness of their replication. The development of this direction is the use of bacteriophage enzymes as an antimicrobial substance.

14. Among other promising compounds that can solve the problem of fighting nosocomial infections - enzybiotics, which now include substances with a specific mechanism of action (bacteriocins, cathelicins, lysines, bacteriophages, immunobiotics). The authors identify the benefits and broad prospects of such drugs, which significantly increase the effectiveness of antimicrobial action without causing the emergence of resistant forms of pathogens.

15. Genome sequencing and gene annotation of common microbial pathogens, as a modern tool, makes it possible to establish targets for the action of new antiseptics at the gene level. Blocking the genes encoding the resistance of individual pathogens can be a complex task for genetic engineers, bioinformaticians, microbiologists and other specialists on the way to the development of novel drugs against multiresistant strains.

16. Combined drugs based on substances with different mechanisms of action, mainly antibiotics and enzymes, allow to strengthen the effect of each of the substances, and different mechanisms of action on microbial pathogens lead to more effective destruction of cells at various stages of its development.

4. **Implemented:** by the Department of Microbiology, Virology and Immunology of Shupyk National Healthcare University of Ukraine

5. **Effectiveness of implementation:** The results of scientific research are used in the formation of methodological support for educational components in the courses of specialization and thematic improvement. The use of the development showed that the effectiveness of the implementation meets the criteria that are given in the sources of information.

6. **Remarks, suggestions:** none.

Head of the Department of Microbiology,
virology and immunology
Doctor of Medicine, Professor I.V. Dzyublyk