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Review

# Addressing Antibiotic Resistance with Bacterial Cytological Profiling: A high-throughput method for antibiotic discovery

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**Abstract:** Developing new antibiotics poses a significant challenge in the fight against antimicrobial resistance (AMR), a critical global health threat responsible for approximately 5 million deaths annually. Finding new classes of antibiotics that are safe, have acceptable pharmacokinetic properties, and are appropriately active against pathogens is a lengthy and expensive process. Therefore, high-throughput platforms are needed to screen large libraries of synthetic and natural compounds. In this review, we present bacterial cytological profiling (BCP) as a rapid, scalable, and cost-effective method for identifying the mechanisms of action of antibiotics offering a promising tool for combating AMR and drug discovery. We present the application of BCP for different bacterial organisms and different classes of antibiotics and discuss BCP's advantages, limitations, and potential improvements. Furthermore, we highlight the studies that have utilized BCP to investigate pathogens listed in the Bacterial Priority Pathogens List 2024 and we identify the pathogens whose cytological profiles are missing. Lastly, we explore the most recent artificial intelligence and deep learning techniques that could enhance the analysis of data generated by BCP, potentially advancing our understanding of antibiotic resistance mechanisms and the discovery of novel druggable pathways.

**Keywords:** antibiotic resistance; bacterial cytological profiling; high-throughput screens; antibiotic mechanism of action; bacterial priority pathogen list; cell segmentation; machine learning; deep learning

## 1. Introduction

Antimicrobial resistance (AMR) has been declared by the World Health Organization (WHO) as one of the top ten global public health threats facing humanity. It impacts both public health and global development, resulting in severe morbidity and mortality worldwide. AMR is defined as a condition characterized by the ability of microorganisms (bacteria, fungi, viruses, and parasites) to live and grow in the presence of antimicrobial agents that were previously reported to be effective against them. Resistance occurs through pathogen evolution, either naturally over time or acquired by the use of antimicrobial drugs, which make these drugs ineffective [1].

It has been estimated that in 2019 alone, antimicrobial resistance killed at least 1.27 million people globally, more deaths than HIV/AIDS or malaria, with 4.95 million deaths associated with AMR [2]. According to the Centers for Disease Control and Prevention's Antibiotic Resistance Threats Report [3], in the United States, over 2.8 million antibiotic-resistant infections occur each year, leading to over 35,000 deaths. Furthermore, it has been estimated that, if the AMR trend continues, the cumulative loss to world economies might be as high as \$100 trillion by 2050 [4]. These alarming statistics underscore the urgent need to develop effective therapeutics to combat antimicrobial resistance.

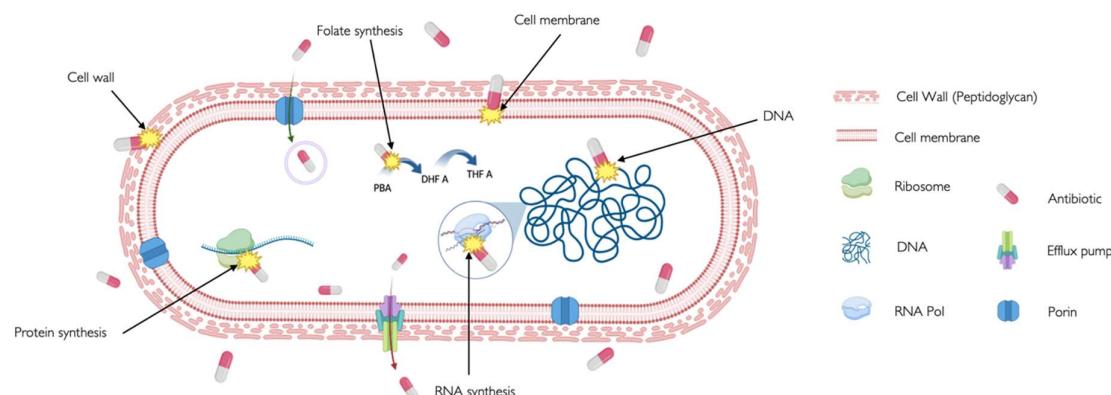
The efforts undertaken in the field of AMR until now have not been enough despite the research effort and inventive therapeutic approaches. Since 1940 antimicrobials have been used widely [5], [6], in addition to treating infectious diseases, antibiotics made many modern medical procedures possible, including cancer treatment, organ transplants and open-heart surgery [7]. Even before 1940 and for about 60 years after, most antibiotics were discovered by screening soil samples for such natural products that kill bacteria, including known pathogens, first on culture plates and then in

animal infection models [8]-[7]. However, in the past 20 years, only two new antibiotic classes have been developed that are only effective for Gram-positive bacteria [9]. To overcome the burden of antibiotic resistance, new high-throughput screens based on bacterial cytological profiling have been developed.

This review emphasizes the use of bacterial cytological profiling (BCP) as a highly effective method for discovering novel antibiotics and rapidly identifying antibiotic targets in a cost-effective manner. BCP initially creates a library of bacterial cell shapes and sizes induced by antibiotics with known mechanisms of action. This library is then used to discover new antibiotics and classify antibiotics based on the components of bacterial cells they target. In this review, we highlight how BCP is used to improve our quantitative understanding of antibiotic pharmacodynamics and bacterial stress responses, as well as how BCP enhances the development of non-traditional antibacterial strategies such as phage therapies[10-12].

## 2. Antibiotic Mechanism of Action and Antibiotic Targets

AMR is a natural process that happens over time through genetic alterations or phenotypic changes [13] in pathogens. Its emergence and spread are accelerated by human activity, mainly the misuse and overuse of antimicrobials to treat, prevent or control infections in humans, animals, and plants. To effectively tackle antibiotic-resistant bacteria, it is essential to understand how antibiotics work, which is known as their mechanism of action (MOA), see Table 1. Understanding MOA involves studying how antibiotics affect bacterial physiology and molecular interaction with bacterial targets (Figure 1). However, determining MOA remains a serious challenge that limits both basic research and antibiotic discovery programs.



**Figure 1. Antibiotic targets in bacteria.** Antibiotics typically kill bacteria by targeting at least one of the five cell components: cell wall, cell membrane, ribosome, DNA and RNA. Antibiotics interfere with the synthesis of or directly damage cell structures to inhibit bacterial growth or irreversible damage bacterial integrity. Antibiotics can inhibit the synthesis of some essential components, such as folate synthesis, a precursor for DNA synthesis. Antibiotics are transported into cells across the cell membranes or through membrane porins that facilitate antibiotic transport. Bacterial cells can also eliminate antibiotics by using efflux pumps, which are transport proteins that actively expel antibiotics out of the cell.

**Table 1.** General classification of antibiotics based on their target and chemical structure, including their mechanism of action (MOA), examples of each antibiotic type, and their typical clinical uses.

Target	Chemical Structure	Mechanism Of Action (MOA)		Generic Name Examples	Use
		Inhibit	penicillin-binding proteins (PBPs) that crosslink peptidoglycan chains in the bacterial cell wall [16],		
Cell Wall	$\beta$ -Lactams	Inhibit	penicillin-binding proteins (PBPs) that crosslink peptidoglycan chains in the bacterial cell wall [16],	Penicillins, cephalosporins, cephemycins, carbapenems,	To treat a variety of infections, including skin infections, chest infections, urinary and others.

		disrupting cell wall integrity and causing cell lysis [17].		tract infections sepsis and meningitis.
	Glycopeptides	Target gram-positive bacteria by binding to the acyl-D-Ala-D-Ala terminus to the growing peptidoglycan and then cross-linking peptides within and between peptidoglycan [18].	Vancomycin	Last resort medication for the treatment of sepsis and lower respiratory tract, skin, and bone infections caused by Gram-positive bacteria.
Membrane	Lipopeptides	Insert in the cell membrane and cause depolarization, reducing the ability to create ATP and cell death [19].	Daptomycin, Colistin	For treatment of complicated skin and skin-structure infections associated to Gram-positive bacteria.
Fatty Acid Synthesis	Chlorophenol	Block the reduction step of the fatty acid synthesis pathway by inhibiting an enoyl-ACP reductase ( <i>fabI</i> ) [20].	Triclosan	Added to many consumer products as soaps, body washes and toothpastes, intended to reduce or prevent bacterial contamination.
	Oxirane carboxylic acids	Irreversibly binds to fatty acid synthase, specifically b-ketoacyl-acyl carrier protein synthase. In sterol synthesis, inhibits HMG-CoA synthetase activity [21].	Cerulenin	Antifungal agent whose activity interferes with or otherwise acts to prevent the formation of fatty acids and sterols. With selective cytotoxicity to cancer cells
Protein Synthesis	Aminoglycosides	Interact with the 30s ribosomal subunit of 16S RNA causing misreading and/or truncated proteins and cell death [17] [22].	Gentamicin, tobramycin, kanamycin	To treat mainly very serious illnesses and infections such as sepsis, as they can cause very serious side effects.

	Tetracyclines	Inhibit translation by binding to 16S rRNA of the 30S ribosomal subunit, preventing tRNA binding to 30S [23].	Tetracycline, doxycycline and lymecycline	To treat a wide range of infections, acne and skin conditions as rosacea.
	Macrolides	It binds to the 23S rRNA of the 50S ribosomal subunit, leading to the production of incomplete peptide chains [24].	Azithromycin, erythromycin and clarithromycin	Particularly useful to treat lung and chest infections, as an alternative for people with a penicillin allergy or penicillin-resistant strains.
	Lincosamide	It binds to the 50S ribosome subunit to stimulate dissociation of the peptidyl-tRNA molecule from the ribosomes during elongation [25]	Clindamycin	Primarily used to treat gram-positive bacterial infections in which there is resistance or intolerance to penicillin.
	Oxazolidinones	Limit translation by binding to 23S rRNA of the 50S subunit and preventing the formation of a functional 70S subunit [26].	Linezolid	Active against multidrug-resistant staphylococci, streptococci, and enterococci.
<i>DNA Synthesis</i>	Fluoroquinolones	Inhibit DNA replication by targeting DNA gyrase and topoisomerase IV[27,28].	Ciprofloxacin and levofloxacin	Broad-spectrum antibiotics that are used to treat a wide range of infections, especially respiratory and urinary tract infections. Not commonly used due to their risk of serious side effects.
	Sulfonamides	Competitive inhibitor of Dihydropteroate synthase (DHPS) involved in folate synthesis [29]	Sulfamethazine, sulfapyridine	Utilized in the treatment of tonsillitis, septicemia, meningococcal meningitis, bacillary dysentery, and number of infections of urinary tract

RNA <i>Synthesis</i>	Rifamycins	It binds to the RNA polymerase and blocks the RNA synthesis [30]	Rifapentine, Rifampin	Effective against mycobacteria, and are therefore used to treat tuberculosis, leprosy, and mycobacterium avium complex (MAC) infections.
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\*Classification of frequently used antibiotics. For more detailed classifications based on the antibiotic targets see [20,29,30], or classifications based on chemical structure, see [31].

Traditionally, a variety of assays are performed to determine the MOA, by the evaluation of whether one of the five basic pathways is inhibited (Figure 1). These efforts typically begin with macromolecular synthesis (MMS) assays that use radioactively labeled precursors to determine whether a compound specifically inhibits peptidoglycan, lipid, protein, RNA or DNA synthesis or whether it blocks all simultaneously [14]. Despite being an important technique, MMS assays are time consuming and suffer from low resolution, low accuracy, and relatively low throughput [15].

To address the limitations associated with MMS assays, various alternative techniques for determining the mechanism of action (MOA) have been devised [32]. These include biochemical approaches, such as affinity chromatography that identify direct biophysical interactions between antimicrobials and their targets through an array where the antibiotic interacts with protein from whole-cell extracts[33–35]. Genetic approaches, such as selection for resistance and resistance screening [33], focus on the genetic comparison between non-resistant strains and strains that have evolved and mutated to become resistant to a specific antibiotic, this approach is often able to identify the molecular target of an antibiotic, the specific amino acid residues important for its interaction and the frequency with which resistance occurs.

While these methods offer various benefits, they also come with limitations that hinder their effectiveness. The main disadvantage of these methods is the required time to conduct the assay, moreover, these methods necessitate substantial quantities of purified compounds, which can be challenging as newly discovered lead compounds are frequently available in limited amounts.

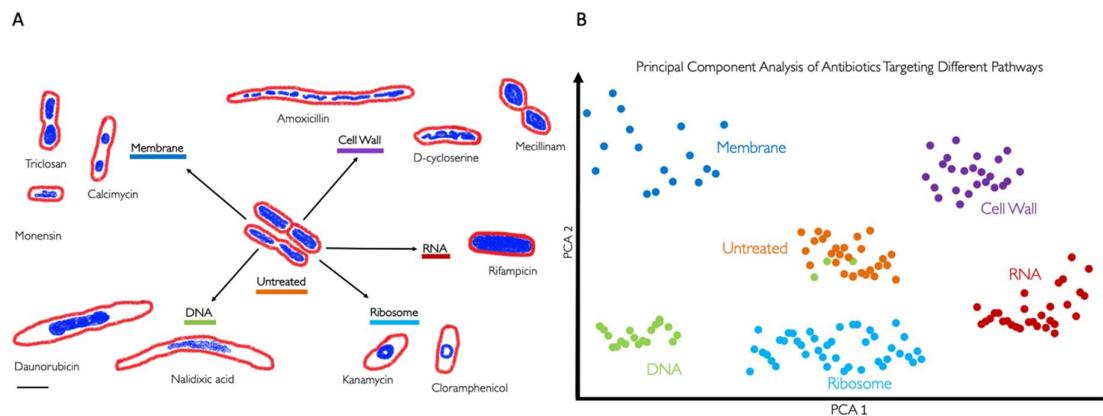
Apart from all limitations in determining MOA, discovering new compounds that are active against Gram-positive and Gram-negative bacteria remains challenging. In the past 20 years, only two new antibiotic classes (lipopeptides and oxazolidinones) have been developed and approved by international drug agencies (US Food and Drug Administration and European Medicines Agency)—both of which provide coverage against Gram-positive bacteria [9]. The quinolones, discovered in 1962, were the last novel drug class identified to be active against Gram-negative bacteria [36].

From a technical perspective, finding new classes of antibiotics that are safe, have acceptable pharmacokinetic properties, and are appropriately active is a challenging issue [36]. Additionally, the profit margins for producing antibiotics are relatively low given the high cost for production and the lengthy timeline for research, testing, and approval [36]. Therefore, new, high-throughput screening platforms are needed for the fast and inexpensive screening of large libraries of synthetic and natural compounds that are highly effective against human pathogens[37,38]. The following section reviews the quick and scalable bacterial cytological profiling methods (BCPs) and discusses their availability for some of the most important human pathogens as outlined in the latest WHO 2024 report.

### 3. BCP to Identify the Mechanism of Action

In 2013, Poochit *et al.* designed Bacterial Cytological Profiling (BCP) analysis for *E. coli* cells using different classes of antibiotics [15] (Figure 2). BCP data are obtained using fluorescent microscopy of *E. coli* cells stained with fluorescent membrane and DNA dyes as well as fluorescent reporter for membrane permeability. Using image analysis software, various bacterial cell parameters, such as cell length, width, solidity, and DNA content, are extracted [15]. Subsequently, complex multidimensional data are analyzed using the *Principal Component Analysis (PCA)* technique, to cluster cells based on their cytological profile to identify the mechanism of action of known and unknown antibacterial compounds (Figure 2B, Box 1). Since the cytological profiling method

produces data at a single-cell level [41], it identifies the metabolic pathways targeted by drugs or other toxins through their effects on bacterial chromosomal condensation, cell shape and overall cellular morphology [15] (Figure 2B). Furthermore, this approach can lead to the identification of antibiotics that are effective against multidrug-resistant bacteria [15].



**Figure 2. Bacterial Cytological Profiling.** (A) Different morphologies adopted by bacteria under the stress of antibiotics targeting different pathways. The cartoon drawings are based on the microscopy images from Poochit et al [15], where bacterial cells were treated with antibiotics targeting five major biosynthetic pathways (DNA, Ribosome, RNA, Cell Wall, Membrane), using fluorescent dyes FM4-64 (red) and DAPI (blue) to stain bacterial membranes and DNA respectively. Scale bar, 1  $\mu$ m. (B) Principal Component Analysis (PCA) is used to cluster different bacterial cell shapes based on the antibiotic mechanism of action. Each point on the graph represents a single cell. The graph also illustrates that when green dots, representing a characteristic morphology of a DNA-targeting antibiotic, cluster with orange dots, representing untreated bacteria, it indicates no morphological change and suggests possible antibiotic resistance or persistence[42,43].

#### Box 1. Principle Component Analysis (PCA)

PCA is a widely used statistical technique for analyzing data by identifying a set of principal components that capture the maximum variance in the data with the fewest components [44]. It involves transforming the original variables into a new set of uncorrelated variables, known as principal components, which are linear combinations of the original variables. These principal components are ordered so that the first component explains the maximum variance in the data, followed by the second component, and so on [44]. The process of PCA starts by calculating a matrix that shows how the original variables are related to each other. Then, it finds special vectors (eigenvectors) and values (eigenvalues) from this matrix. The eigenvectors show the new directions for the data, while the eigenvalues tell us how much of the data's variation is captured by each new direction. The principal components are then derived from these eigenvectors, and the data is projected onto these components to obtain the transformed dataset. PCA has diverse applications across almost all scientific fields, including biology, medicine, computer science, and geology. In the context of biomedical research, PCA has been utilized to analyze human cell atlas and prostate cancer risk prediction[45,46]. In computer science, PCA is employed for data processing, dimensionality reduction, and feature extraction[47–49]. In Bacterial Cytological Profiling, PCA is used to analyze microscopy data by grouping the phenotypic changes in bacteria after antibiotic exposure, thereby determining the target of the antibiotics (Error! Reference source not found.B). Together, Principal Component Analysis is a powerful statistical method that enables the transformation of complex data into a new simplified coordinate system to reveal underlying patterns and structures. Its wide-ranging applications underscore its significance in various scientific disciplines.

The cytological profiles triggered by antibiotics reveal their damaging effects. For example, compounds that block translation led to chromosome compaction due to the interference with coordinated translation and insertion of proteins into the membrane, whereas compounds that block transcription lead to chromosome decondensation due to the absence of active RNA polymerase (**Error! Reference source not found.**). BCP takes advantage of the limited presence of cell-cycle checkpoints in bacteria, combined with the precision of high-resolution imaging.

When stressed by antibiotics, bacteria show phenotypical changes that are characteristic of the antibiotic target. Treated bacteria transform into spheroplast, protoplast, ovoid or filamenting cells [50]. Spheroplast and protoplast are cells that have lost their peptidoglycan layer, spheroplasts are Gram-negative bacteria that keep their outer membrane, whereas protoplasts are formed from Gram-positive bacteria that lack both a peptidoglycan layer and an outer membrane [51]. Bacterial variants that lack a cell wall, encompassing both Gram-negative and Gram-positive bacteria, are known as *L-forms*[52–55].

Ovoid cells refer to bacterial rods (bacilli) that have decreased in length and become oval or round-shaped during antibacterial treatment[56,57]. Nomenclature in the literature varies, with some authors referring to them as 'round forms'[58,59], 'round cells'[60–62], 'spherical forms', 'spherical cells'[63–65], or 'coccoid forms'[66,67]. Filamentation, or cell elongation, occurs when rod-shaped bacteria (or sometimes cocci) produce peptidoglycan for their side walls but not for their division walls during growth, causing the cells to become unusually elongated [50]. Filamentation can occur following inhibition or disruption of peptidoglycan synthesis [56] but can also occur if DNA synthesis is inhibited[68,69] or DNA is damaged[70–72] by a process known as the SOS response that inhibits cell division [28] (**Error! Reference source not found.**).

Other phenotypic changes can be shown by bacteria under antibiotic exposure as altered cell size, localized swelling, bulge formation, blebbing, peptidoglycan thickening [50]. Phenotypical changes confer an increase in fitness to bacteria in the presence of antibiotics [73]. Resistance to antibiotics is commonly attained via reducing the intracellular concentration of the antibiotic or by reducing antibiotic binding affinities for their specific intracellular targets [74]. By using available BCP data, recent studies have shown that by reducing the surface-to-volume ratio (*S/V*), bacteria can effectively reduce the antibiotic concentration inside a cell, thereby promoting cell growth by decreasing antibiotic influx [74]. Similarly, an increase in *S/V* can also provide adaptive benefits by increasing the rate of nutrient uptake or by increasing the rate of antibiotic efflux[74–76]. These studies explain how cell shape transformations promote bacterial survival under antibiotic treatments – pointing towards potential new druggable targets that control cell shape and size under stress.

BCP has been successfully employed to study the MOA of various antibacterial agents, including azithromycin [77], diphenylureas [78] and thailandamide [79]. It has also been used to identify the cellular pathways targeted by anticancer metal complexes [80], to study the response of bacteria to antibiotics in different growth conditions [81]. Additionally, BCP has been used to identify the cellular pathways targeted by antibacterial molecules affecting different cellular pathways [82]· [83], making it a valuable tool not only for determining antibacterial targets but also to potentially identify novel MOA i.e., ones that target new proteins or new pathways (Figure 3).

BCP offers unprecedented insights not only into antibiotic therapies but also into phage therapies, revealing how phages exploit or disrupt bacterial cellular processes[10–12,84]. BCP enables the visualization of distinct cytological changes within bacterial cells during phage infection. These cytological signatures not only uncover the pathways and cellular targets that phages manipulate to propagate their lifecycle but also highlight the role of bacterial defense mechanisms in combating phage infection [11]. BCP has demonstrated how the overexpression of phage-related proteins can induce specific cytological changes that inhibit phage replication, showcasing the potential for targeting bacterial metabolic pathways to modulate phage activity [10]. Additionally, BCP has been instrumental in assessing the impact of various antibiotics on phage replication, revealing that certain antibiotics can synergize with phages to enhance bacterial cell lysis, while others inhibit phage propagation by disrupting essential bacterial processes [11]. This dual capability of BCP to monitor both the direct effects of phage infection and the influence of external agents such as antibiotics makes it an invaluable tool in studying phage-bacteria dynamics.

#### 4. BCP of Important Human Pathogens

Most importantly, BCP has been successfully used to study some of the most important human pathogens from the WHO Bacterial Priority Pathogens List (**Error! Reference source not found.**). In 2017, using a multicriteria decision analysis technique, WHO experts together with researchers from the Division of Infectious Diseases at the University of Tübingen, Germany, developed the first Bacterial Priority Pathogen List (BPPL) to guide investment into the R&D of new antibacterials for guiding AMR surveillance, prevention and control [85]. During the past 7 years, the antibiotic development pipeline brought to the market nine new antibiotics with in-vitro or in-vivo activity against the 2017 BPPL “critical” priority pathogens, although resistant strains have since been described for almost all of them[86,87]. In 2024, WHO published an updated list to address current challenges and provide essential guidance for policymakers, national health authorities and others involved in decisions about R&D and investment. The 2024 BPPL includes 15 families of antibiotic-resistant pathogens, grouped into critical, high and medium categories of priority for R&D and public health measures [88] (**Error! Reference source not found.**). Out of 15 families, bacterial cytological profiling is not available for 30 % of them: *Non-typhoidal Salmonella*, *Neisseria gonorrhoeae*, Group A and B *Streptococci*, *Haemophilus influenzae*. Therefore, urgent BCPs regarding these severe pathogenic organisms are needed.

**Table 2. BCP Analysis for the WHO Bacterial Priority Pathogens List (2024).** In third column, this table indicates whether bacterial have been studied using Bacterial Cytological Profiling (BCP) or not. We consider any BCP done in the wild-type strain rather than in the resistant strain.

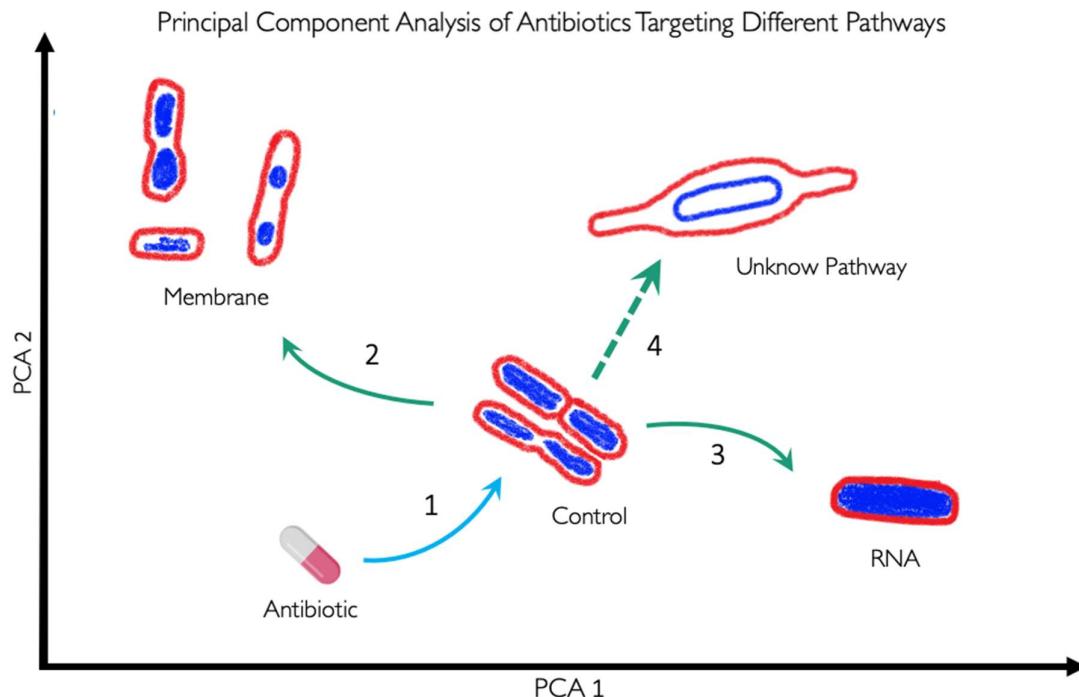
Bacteria	Resistant to	Bacterial Cytological Profiling (BCP)
<b>Priority 1. Critical group</b>		
<i>Acinetobacter baumannii</i>	Carbapenems	Yes[41,77,81,89]
<i>Enterobacteriaceae*</i>	Third generation cephalosporine	Yes[15,80,90–92]
<i>Enterobacteriaceae**</i>	Carbapenems, ESBL-producing	Yes[77,93,94]
<i>Rifampicin-Resistant Tuberculosis (RR-TB)***</i>	Rifampicin	Yes[95,96]
<b>Priority 2. High group</b>		
<i>Salmonella Thypi</i>	Fluoroquinolones	Yes [94]
<i>Shigella spp.</i>	Fluoroquinolones	Yes [97]
<i>Enterococcus faecium</i>	Vancomycin	Yes [98]
<i>Pseudomonas aeruginosa</i>	Carbapenems	Yes[11,77,93,94]
<i>Non-typhoidal Salmonella</i>	Fluoroquinolones	No
<i>Neisseria gonorrhoeae</i>	Cephalosporin, Fluoroquinolonas	No
<i>Staphylococcus aureus</i>	Methicillin and vancomycin	Yes[78,99–101]
<b>Priority 3. Medium group</b>		
<i>Group A Streptococci</i>	Macrolide	No

<i>Streptococcus pneumoniae</i>	Macrolide/No sensitivity to penicillin	Yes [102]
<i>Haemophilus influenzae</i>	Ampicillin	No
<i>Group B Streptococci</i>	Penicillin	No

\* The BCP column uses *Escherichia coli* as a reference for this group \*\* The BCP column uses *Klebsiella pneumoniae* as a reference for this group \*\*\*RR-TB was evaluated independently in a tailored approach so it was technically "not" included in the list but after the evaluation by specialists, it was determined as a critically dangerous bacteria therefore. RR-TB stands apart from the list due to the distinct nature of its evaluation process.

## 5. BCP to Identify New Druggable Cell Pathways

BCP is used to scan new antibacterial components to identify their specific targets (Figure 3). As demonstrated, BCP effectively differentiates between various morphological changes induced by different antibiotics, thereby providing insights into the antibiotic's mechanism of action. If a novel antibiotic places bacteria in a distinct region of the PCA plot compared to known antibiotic targets, it could indicate a new pathway target or MOA previously uncharacterized (Figure 3). For example, if the PCA analysis shows that the morphology of bacteria treated with a new antibiotic clusters in a region associated with membrane or RNA targets (Arrows 2 and 3 in **Error! Reference source not found.**), it directly indicates the antibiotic's mode of action. Conversely, if the antibiotic's effect causes a morphology change that places bacteria in a novel zone, as illustrated with Arrow 4, it may suggest the discovery of a new antibacterial pathway or target.



**Figure 3.** Representation of Principal Component Analysis (PCA) using bacterial morphologies to determine the mechanism of action (MOA) of a novel antibiotic. Arrow 1 indicates the antibiotic used against certain bacteria, which can change their shape depending on the antibiotic's MOA. If the bacteria exhibit a morphology as indicated by arrow 2, the antibiotic targets the membrane. Conversely, if the bacteria display a morphology as indicated by arrow 3, the antibiotic targets RNA. However, if the morphology is completely different from the known and clustered morphologies, as shown by arrow 4, it suggests that the antibiotic targets a novel pathway and if the bacteria do not show any change, it suggests that they are not susceptible to this antibiotic or, in the worst-case scenario, that they are resistant to the antibiotic.

Together, BCP significantly enhances drug development processes by offering a precise, fast and systematic method for characterizing the effects of new antibacterial agents. Its ability to identify

target-specific morphological changes provides a comprehensive tool for uncovering novel antibiotic targets and advancing our understanding of bacterial physiology.

## 6. BCP Limitations

Even with all the advantages we mentioned about BCP, it has certain limitations. BCP can identify the general target of an antibiotic, but it cannot provide precise information about the exact site within the target that is affected. For instance, while BCP can indicate that an antibiotic targets the ribosome, it cannot specify which part of the ribosome is involved.

BCP requires staining chemicals to evaluate DNA content and cell size and shape, with fluorescent dye intensity being a crucial variable (Table 3). These factors are essential for determining the mechanism of action of antibiotics. A plethora of dyes, protein fusions, and reporter strains are available and have been used in BCP, enabling both rapid mode of action categorization and in-depth analysis of antibiotic mechanisms. However, the plethora of possible dyes, strains, and assays can make it difficult to choose which specific phenotypic experiments are the most suited for a given purpose (Table 3). Furthermore, bacterial cell physiology is complex, and many cellular processes are intimately interconnected, e.g., by co-dependent regulation or metabolic flux [103]-[104].

**Table 3.** Organisms used in BCP and how images were analysed.

Organism	Dyes/Fluorophores	Processed Data available	Segmentation	Feature extraction	Source
<i>Acinetobacter baumannii</i> and <i>E. coli</i>	FM4-64 DAPI SYTOX-Green	Yes	CellProfiler	CellProfiler	[105]
<i>Acinetobacter baumannii</i>	FM4-64 DAPI SYTOX-Green	No	Ilastic	CellProfiler	[106]*
<i>Pseudomonas aeruginosa</i>	FM4-64 DAPI	Yes	Manually (FIJI/ImageJ)	Manually (FIJI/ImageJ)	[107]*
<i>S. aureus</i>	FM4-64 DAPI SYTOX-Green	Yes	Semi-Manual (FIJI/ImageJ)	Semi-Manual (FIJI/ImageJ)	[15]*
<i>S. aureus</i>	FM4-64 DAPI SYTOX-Green WGA-647	Yes	CellProfiler	CellProfiler	[99]*
<i>S. aureus</i> , <i>S. Typhimurium</i> , and <i>K. pneumoniae</i>	FM4-64 DAPI SYTOX-Green	Yes	Harmony	Harmony	[94]
<i>B. subtilis</i>	FM 4-64 DAPI SYTOX Green	Yes	CellProfiler	CellProfiler FIJI	[108]*
<i>B. subtilis</i>	FM4-64	No	CellProfiler	CellProfiler	[109]*

	DAPI SYTOX-Green				
<i>B. subtilis</i>	Nile red DAPI	No	MicrobeJ	MicrobeJ	[110]*
<i>Bacillus subtilis</i> , <i>E. coli</i>	FM4-64 DAPI GFP	No	Wasabi software (Hamamatsu)	Wasabi software (Hamamatsu)	[111]
<i>E. coli</i>	FM4-64 Hoechst-33342 Dendra2 protein	No	FIJI/ImageJ	FIJI/ImageJ	[80]
<i>E. coli</i> <i>Caulobacter crescentus</i>	FM4-64 DAPI	No	Oufti	Oufti	[112]*
<i>E. coli</i>	FM4-64 DAPI SYTOX-Green	No	Semi-Manual (FIJI/ImageJ)	Semi-Manual (FIJI/ImageJ)	[113]*
<i>Achromobacter xylosoxidans</i>	FM4-64 DAPI SYTOX-Green NBD Azithromycin	No	FIJI/ImageJ and CellProfiler	FIJI/ImageJ and CellProfiler	[114]
<i>M. smegmatis</i>	ParB-mCherry	Yes	MicrobeJ	MicrobeJ	[115]*
<i>M. tuberculosis</i> Erdman	FM4-64FX SYTO 24	Yes	MorphEUS	MorphEUS	[116]*
<i>Shewanella putrefaciens</i>	Ffh-mVenus FtsY-mVenus uL1-mVenus	Yes	FIJI/ImageJ	FIJI/ImageJ	[117]*
<i>V. parahaemolyticus</i>	FM4-64 DAPI	No	FIJI/ImageJ	FIJI/ImageJ	[84]
<i>Bacillus subtilis</i>	FM4-64 DAPI SYTOX-Green SYTO-9	No	-	Manual w/ FIJI	[118]*
<i>M. smegmatis</i> <i>M. tuberculosis</i>	FM4-64 GFP CellROX	Yes	Omnipose	Manual w/ FIJI or with Cell Counter installation in FIJI Custom python script	[119]
<i>E. coli</i> <i>B. subtilis</i>	FM4-64 DAPI GFP	No	FIJI/ImageJ and MicrobeJ	FIJI/ImageJ and MicrobeJ	[120]

	DiSC				
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\*Pipelines, scripts, or instructions are detailed and/or included in the paper. Programs are also widely accessible.

## 7. BCP Potential Improvements

Despite the cell walls being one of the primary antibiotic targets, direct visualization methods of cell wall synthesis and remodeling during antibiotic exposure have been lacking in BCP approaches developed so far (Table 3). In 2012, Kuru *et al.* discovered a groundbreaking method for bacterial cell wall staining using fluorescent amino acids [121]. Cell wall provides the shape and structural integrity to the cell. It is made of peptidoglycan (PG), which consists of glycan strands cross-linked by d-amino acid (DAA) [122]. The team introduced HADA and NADA, two fluorescent d-amino acids (FDAs) attached to a d-amino acid backbone (3-amino-d-alanine). This chemical biology approach aims to detect and visualize the exact location and amount of new peptidoglycan layer synthesis in bacteria. By using HADA or NADA as fluorescently labeled peptidoglycan components during cell wall synthesis, the technique allows researchers to observe morphological changes in bacteria over time. This provides a chronological account of shifts in peptidoglycan synthesis within individual cells across various bacterial species. This is relevant as HADA and NADA can be implemented in the methodology of BCP as dyes to unravel the growth modes of bacteria under antibiotic exposure as they exhibit a diverse array of growth patterns that confer selective advantages in their environments [123] [74].

Other fluorescent dyes are available to quantitatively probe bacterial physiological states: ThT and DiBAC<sub>4</sub> for bacterial membrane potential [124–127], carboxy-H<sub>2</sub>DCFDA for reactive oxygen species (ROS) [127], and DAF-FM for reactive nitrogen species (RNS) [127]. By integrating membrane potential, ROS, and RNS into cytological profiles could provide additional information regarding bacterial physiology and bacterial stress response during antibiotic treatment.

## 8. Image Analysis Tools for BCP and Data Availability

Although fluorescent microscopy is a valuable tool for characterizing cellular and subcellular structures and dynamics, quantitative analysis of microscopy images remains a persistent challenge [119,128]. Once the fluorescence microscopy is done, analyzing the images obtained is a critical step for accurately determining cell morphologies. This is especially evident in the study of bacterial shapes since their cell body is composed of a small number of pixels (for example, ~ 100–300 px [2] for *E. coli* in typical experiments) [129]. At this scale, accurate subcellular localization requires defining the cell boundary with single-pixel precision or more desirable sub-pixel resolution [130].

In addition to their small size, bacteria exhibit a diverse array of shapes. While many frequently studied bacteria can be adequately represented as rods or spheres, there is growing interest in those with more complex forms or the atypical shapes that emerge after exposure to antibiotics, such as seen in *Caulobacter crescentus* [73]. Cell segmentation allows us to analyze microscopy images more precisely to identify these different shapes. Segmentation is a method of partitioning an image into distinct regions with similar attributes, such as texture, intensity, or gray level, with the goal of distinguishing the cells from the background to enable quantitative analysis such as cell feature extraction [130].

Cell segmentation is a complex problem that extends far beyond microbiological research where advances can be lagging as expertise is less common; thus, many solutions are currently available within ImageJ [128,131,132] which broaden the number of potential users, but many more also use stand-alone image-analysis programs which can at times be less supported and less accessible [133,134]. Many traditional solutions use image-processing techniques such as the Otsu thresholding method to segment isolated cells [130]; however, this approach performs poorly on cells in close contact and it requires image-by-image tuning to optimize parameters. Furthermore, whilst implementing image-by-image thresholds instead of one static threshold can potentially improve segmentation, it also introduces bias [135].

Classical image segmentation techniques have been in use since the 1960s [136,137], laying the groundwork for the more advanced artificial intelligence methods used nowadays. In 2016 SuperSegger was developed to address thresholding issues in classical segmentation specifically in bacterial phase-contrast images and is an interesting example of classical segmentation combined

with Deep learning [138]. This program utilizes traditional image filtering techniques now with a shallow neural network to correct for errors that thresholding and watershed segmentation tend to produce [139]. This would soon be overshadowed by other Deep neural networks (DNNs) which have now become the backbone of most Deep learning segmentation methods and are widely recognized as superior tools for cell segmentation [140]. This has marked a successful trend in using classical techniques to supplement or in conjunction with DNN methods for cell segmentation. However, as showcased in Table 3 there is currently a significant gap in Deep learning being used in BCP. Unlike traditional image processing, machine-learning approaches such as DNNs require training on a ground-truth dataset of cells and corresponding labels. Trained DNNs are thus limited in applicability to images that are representative of those in the training dataset and require greater knowledge to set up than popular classical segmentation methods [141]. Early DNN approaches were based on the Mask R-CNN architecture, whereas more recent algorithms such as StarDist, Cellpose and MiSiC are based on the U-net architecture[131,132,142,143] but new Transformer-based object detection frameworks may begin to surpass them. We have already seen remarkable results from team Osi lab in the multimodality cell segmentation challenge, with their algorithm outcompeting all other entries and some established and popular algorithms[128,133].

Among easily available Deep learning segmentation algorithms, Pachitariu *et al.* showed that Cellpose outperforms the popular programs Mask R-CNN and StarDist on a variety of cell types and cell-like objects, distinguishing it as a relatively powerful general solution for cell segmentation [131]. Cutler *et al.* evaluated the performance of (at the time) state-of-the-art cell segmentation algorithms on a diverse collection of bacterial cells. Their findings motivated the design of a new algorithm, *Omnipose*, which substantially outperforms all segmentation algorithms tested across a wide range of bacterial cell sizes, morphologies, and optical characteristics and as such has been used extensively in literature [129]. *Omnipose*, whilst well supported and widely used in bioimage analysis as a generalist segmentation algorithm is now demonstrably worse than more recent generalist algorithms for many cellular morphologies. The winner of the Multimodality cell segmentation challenge published in 2024, a competition for the best generalist bioimage segmentation algorithms was a team led by Gihun Lee presenting their algorithm, MEDIAR[128,133]. The competition used 1000 images of multiple organisms from over 20 labs; 50 different experiments; using brightfield, fluorescent, PC and DIC microscopy techniques. Importantly, there was also a direct comparison between models submitted by the contestants, and models from the widely acclaimed Cellpose and *Omnipose* showcasing the higher accuracy of the entries.. Beyond segmentation, Deep learning has been used to predict morphologies of unlabeled cells, allowing researchers to simulate more tags than are currently possible to image at once and potentially extending the power of cytological profiling[144–146].

Segmented data availability (Table 3) is invaluable for scientific communities and accelerates new findings. By using published BCP data and mathematical modelling, the researchers uncovered the robustness of scaling behavior between cell surface area and volume in *E. coli* [75] and *B. subtilis* [76], inferred cell physiological alterations upon antibiotic treatments [147], and proposed a new antibiotic resistance pathway mediated by cell surface-to-volume ratio (S/V) transformations [74]. Therefore, the availability of BCP data is good practice and should be considered as a benchmark for all future BCP platforms, especially for pathogenic bacteria (Table 2).

## 9. Conclusions

Despite significant advances in research and the development of new tools, combating antimicrobial resistance (AMR) requires a multifaceted approach. Continued investment in research and development, global collaboration, and the effective implementation of surveillance and prevention strategies are crucial. Bacterial Cytological Profiling (BCP) stands out as a rapid and cost-effective technique that facilitates drug discovery by revealing the mechanism of action of novel antibacterial agents through detailed physiological and morphological analysis. Furthermore, BCP could be used to identify phenotypic changes when multiple antibiotics are used, revealing unique or overlapping cell morphologies induced by these combinations [41]. However, systematic explorations of cytological profiles for drug combinations are still missing.

Apart from bacteria, cytological profiling methods are also widely used for other organisms such as yeast[148,149], fungi [150], and human cells[151–153]. However, deep learning techniques

employed for yeast and human cells, unfortunately, have not yet been applied to bacteria. Therefore, wider availability, applications and integration of machine learning tools across different scientific fields are needed.

Besides BCP being used to discover new antibiotics, BCP has been used to investigate complex interactions between bacteria and their predators – bacteriophages[10–12]. By leveraging high-resolution imaging, BCP enables the identification of metabolic pathways and cellular processes targeted by phages and antibiotics, both individually and in combination, offering valuable insights into the molecular mechanisms governing the phage-bacteria interaction, ultimately paving the way for more effective phage-based antibacterial therapies.

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