# Witnessing the process of bacterial cell death: novel antimicrobials and their mechanisms of action

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# Strategies for Identification of the Mechanism of Action of Antimicrobial Agents

## CHAPTER 2

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## ABSTRACT

Identification of the mechanism of action (MoA) of an antimicrobial agent is one of the vital parts in antimicrobial discovery and development, because MoA provides information about the biochemical process through which an antimicrobial produces its pharmacological effect on bacteria. However, the MoA identification process is tedious and has become one of the bottlenecks in antimicrobial discovery. Here, we provide an overview of the strategies that were developed successfully over the years. This overview describes both classic approaches, which were established several decades ago but have been renovated using the advances of novel techniques, and modern approaches, which were developed recently taking advantage of high-tech solutions. This review will provide insight into the selection of MoA identification strategies, valuable for antimicrobial research.

## INTRODUCTION

Antimicrobial agents kill bacteria or inhibit bacterial growth and thus antagonize bacterial infections, which has a huge impact on human health and animal welfare. The mechanism of action (MoA) of antimicrobial agents describes the biochemical process through which an antimicrobial produces its pharmacological effect on bacteria [1]. An antimicrobial agent may achieve its effects on inhibition of any of the macromolecular synthesis pathways, or destruction of the sub-cellular structures. To elucidate the MoA of an antimicrobial agent is one of the vital parts in antimicrobial discovery and development [2,3]. Nowadays, MoA information of a drug has become more important than ever. As a consequence of increasing cases of antimicrobial resistance, development of alternative anti-infective therapies, such as the combination of different antimicrobials, is important for saving human lives. These therapies require more accurate elucidation of the interaction between antimicrobials and microbes. Therefore, understanding antimicrobial MoA is fundamental to optimize patient care, including antimicrobial selection, infection control and resistance prevention [4]. However, the target candidates in bacterial cells are very broad, including not only proteins, but also DNA and lipids. Whereas many new MoA identification strategies have been discovered by development of new techniques, the process of identifying antimicrobial targets remains complex and laborious [3], which makes it a bottleneck in the discovery of novel antimicrobial agents, including those from nature products.

Natural products have played important roles in yielding compounds with novel structures and novel bio-activities [5]. They are impacting the world by their unique chemical and biological properties that were gained by evolutionary processes to survive in harsh environments and would not be simply achieved using synthetic chemistry. Approximately two-thirds of the antimicrobials in clinical application are derived from natural products [6]. However, over the last few decades, the natural product antimicrobial research has attracted less attention at many pharmaceutical companies, due to the low yield of useful compounds [7]. Despite remarkable achievements in the discovery of active natural product antimicrobials, the procedure of turning these compounds into clinical application is relatively time-consuming. For a significant part, this can be attributed to the difficulty in identification of the MoA of natural products [8]. Unfortunately, there is no universal method that will accurately facilitate identification of the MoA of all antimicrobials. Thus, it is essential to select an approach or to combine multiple approaches for a faster and more accurate MoA identification.

The purpose of this review is to provide some insight into MoA identification with an overview of both traditional and modern strategies that were developed successfully over the past 20 years. We will first focus on some classic approaches that were established several decades ago but are still in use today. Some of the old methods are renovated using the advances of novel techniques. Next, we will review modern omics approaches, which are considered as the "high hopes" in the field of antimicrobial discovery [9]. Finally, we will discuss some novel imaging-based strategies, which were initially developed for eukaryotic cells, and then developed further for bacterial cells. This review will not include all MoA identification strategies available in the antimicrobial research area, but rather provides a first peek into the realm of MoA identification strategies.

## CLASSIC APPROACHES

In order to determine the targets of specific antimicrobial agents, several classic strategies are available, including target-based screenings, in which the direct interaction between the antimicrobial agent and its target is expected to be assessed, and phenotype-based screenings, where the pathway that an antimicrobial agent affects is explored by phenotypic read-out [3]. In the following section, we will discuss three kinds of widely used classic approaches: macromolecular synthesis (MMS) assays, genetic approaches and affinity approaches.

### *MMS assays*

An important way to determine the pathway of inhibition is to measure the effect of test compounds on MMS. These types of assays are indirect approaches that have been applied for almost 50 years [10]. MMS assays monitor the incorporation of radiolabeled tracers of the major biosynthetic pathways. Inhibition of DNA, RNA, protein, and cell wall synthesis will lead to a lower incorporation of radio-activity at a certain time of incubation (Fig. 1) [11–13]. Originally, MMS assays were laborious with large volumes of culture, trichloroacetic acid (TCA) precipitation, followed by washing and drying steps [13]. In addition, the incubation time with radiolabeled tracers sometimes had to be long (1-2 h) to be detected, and this could affect more than one pathway and thus confound the results [14]. If performed properly, the results of MMS still are informative. For example, Crumplin and Smith tried to identify the MoA of nalidixic acid using MMS approaches in 1975 [13]. Initially, all the tested pathways including DNA, RNA and protein synthesis were affected, although

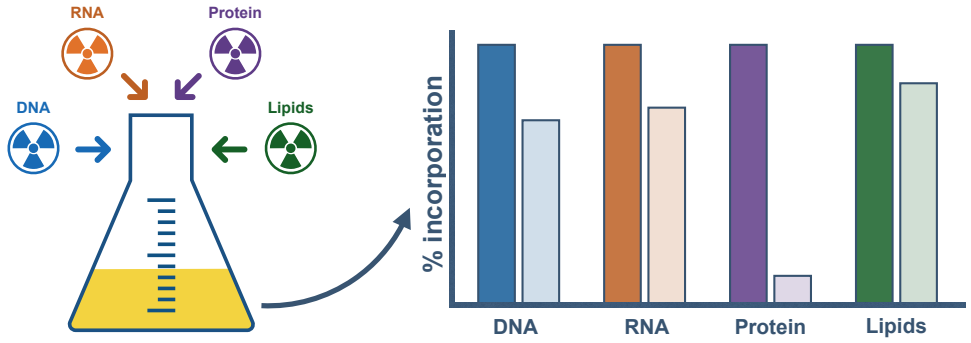


**Fig. 1. Schematic representation of MMS assay.** This assay monitors the incorporation of radiolabeled tracers of the major biosynthetic pathways. Inhibition of DNA, RNA, protein, or lipid synthesis will lead to a lower incorporation of radio-activity.

to a different extent. Afterwards, they adjusted the concentration of nalidixic acid to lower levels and performed these assays on different bacterial strains, and finally identified DNA synthesis to be the initial target. However, they also assumed RNA synthesis to be a secondary target, which was not proven in later studies [15,16]. Yet, their research still provides valuable information about the MoA of nalidixic acid.

Over the years, improvements have been achieved to shorten the period of incubation and increase the throughput. Cunningham et al. successfully developed a modified MMS assay in microplate format with shorter duration by optimizing the bacterial growth rates and precursor concentrations, and establishing simultaneous determination of all the pathways in adequate signal-to-noise ratios [10]. The modified assay is completed within half the bacterial generation time and therefore limits unexpected changes and facilitates the assessment of almost 100 compounds per week against four pathways.

Whereas MMS assays are informative and widely used in antimicrobial research, they have drawbacks. For antimicrobials that specifically target one of the MMS pathways, incorporation of radiolabeled tracers will provide direct information about the inhibition of that specific pathway [17,18]. However, if a nonspecific mechanism of inhibition occurs, like for instance in the case of nisin, all pathways are inhibited within a narrow concentration range, making it difficult to distinguish between them. In addition, for an antimicrobial with novel MoA, MMS assays may also fail to generate useful information [7]. Nevertheless, despite all these disadvantages, MMS assays are still considered to be a good prediction tool at an early stage of research. For example, at the start stage of the analysis of the MoA of daptomycin,

MMS assays successfully suggested a cell wall defect. A strong disruption of cell wall synthesis was observed, whereas less effects were evident on the synthesis of DNA, RNA, and protein [18]. Together with a series of subsequent assays including 2D SDS/PAGE, membrane property and protein delocalization measurements, Müller et al. have added new perspective onto the interaction between daptomycin and the cell envelope.

### *Genetic approaches*

Genetic approaches are direct approaches to identify antimicrobial targets. Normally, genetic approaches are being used to confirm or reject hypotheses of the MoA, which are based on prior information. One of the approaches is to regulate the expression level of the candidate target protein (Fig. 2). Overexpression of a target gene may lead to drug-resistance and may be achieved using an inducible promoter [19]. The overexpressed putative target protein may bind the antimicrobial agent, and therefore lower its working concentration, resulting in increased tolerance to that antimicrobial. However, whereas the overexpressed protein may interact with the antimicrobial, it is not definitive proof that this causes growth inhibition [8]. For instance, overproduction of efflux pumps may lead to resistance to a large variety of unrelated antimicrobials, but these are certainly not the targets of these antimicrobials [20]. Downregulation of the putative target may sensitize the bacteria to an inhibitor, providing evidence that the gene is involved in the antibacterial activity of the compound. To downregulate gene expression, one method is to upregulate the production of antisense RNA, which may reduce target protein expression [21]. However, interpretation of these experiments is often challenging. If multiple genes/proteins act in parallel, inactivation of each of these may sensitize the other proteins, which leads to the identification of multiple targets including potential false positive hits. For example, β-lactam antibiotics specifically target PBPs. It has been reported that if peptidoglycan biosynthesis proteins other than PBPs are under-expressed, bacteria may also become sensitive to β-lactam antibiotics [22]. This may provide evidence for the function of β-lactam antibiotics to affect cell wall synthesis. Interestingly, genetic downregulation of the target protein sometimes may reduce the sensitivity. In a study of quinolones, it was suggested that decreased expression of one of their targets, topoisomerase IV, generates low-level quinolone resistance [23]. This is due to the reduced amount of quinolone-topoisomerase complex, which is the real “poison” that inhibits bacterial growth. In those cases, further work is required to elucidate the MoA.

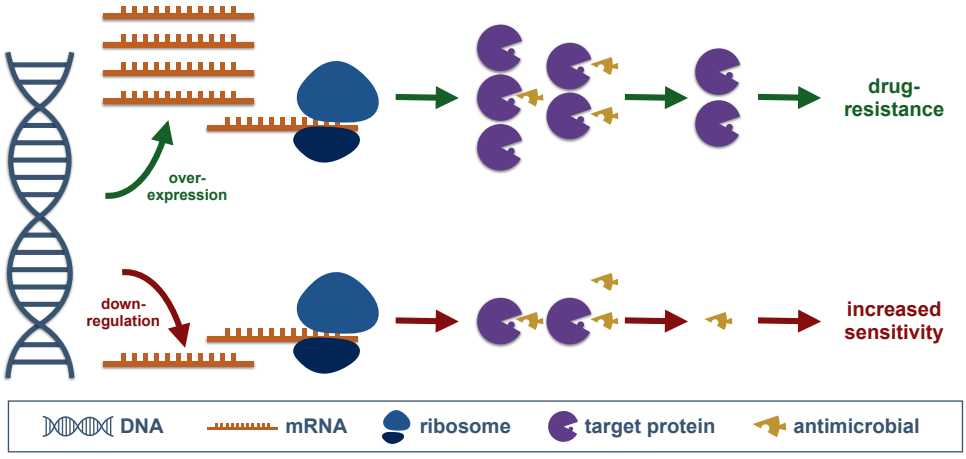


**Fig. 2. Schematic representation of genetic approaches.** Overexpression of putative target protein may bind the antimicrobial agent, and therefore lower its working concentration, resulting in increased resistance to that antimicrobial. In contrast, downregulation of the putative target may sensitize the bacteria to an inhibitor, providing evidence that the gene is involved in the antibacterial activity of the compound.

Whereas most genetic approaches are considered to be verifying prior hypotheses, there are also genetic approaches that are applied for initial screening. These make use of a mutant library of bacteria with a panel of inactivated genes. A drawback is that only non-essential genes are inactivated in these libraries and hence this approach is not feasible for antimicrobials targeting essential genes. By using higher or lower concentrations of an antimicrobial, more resistant or more sensitive strains are selected from the mutant library. Studying the gene functions of these hits will generate a first impression of the antimicrobial mechanism. An example of a *Staphylococcus aureus* mutant library is the Nebraska Transposon Mutant Library (NTML), which consists of 1,920 unique transposon mutants with inactivated non-essential genes [24]. In a recent study, low concentrations of oxacillin were tested against the NTML to search for mutants with increased susceptibility, which led to identification of *mecA* as one of the hits. The *mecA* gene encodes the alternative penicillin binding protein 2a (PBP2a), which is in line with oxacillin's MoA of binding to penicillin-binding proteins (PBPs) [25]. In the same study of oxacillin [25], daptomycin failed to provide any informative hits. The development of CRISPR or antisense RNA may complement the use of mutant libraries. Instead of complete gene inactivation by knock-out, the use of CRISPR technology or antisense RNA to

knockdown essential genes may facilitate investigation of essential gene functions [21,26], and thus benefits the antimicrobial research area.

Another genetic approach is to select drug resistant colonies by growing bacteria in antimicrobial agents for a long time. Identification of mutated genes that cause resistance in bacteria may provide insight into the underlying mechanism. Whereas evolving drug resistant colonies is not very complicated in the lab, traditionally, the identification of mutations in resistant colonies was time-consuming. However, due to the development of next-generation sequencing, the task of mapping resistant mutations became rapid and affordable [27]. Although there are several traits to develop bacterial resistance to a drug, and some of them may not be associated with the drug's MoA, this method has proven powerful for a variety of well-known antimicrobials [7].

Overall, genetic approaches are useful tools for identification of antimicrobial targets, but they also have limitations. If an antimicrobial binds to DNA or lipids, which are also proven targets for compounds [3,28], genetic approaches might be invalid. Whereas genetic approaches alone might not be definitive in demonstrating antimicrobial MoA, they definitely help to provide insight into the targets of antimicrobial agents.

### *Affinity approaches*

Affinity chromatography is another direct approach with a rather simple workflow (Fig. 3). Traditionally, the tested antimicrobial is immobilized onto a solid matrix and then exposed to bacterial cell lysates. After multiple washing steps to remove non-binding proteins, the matrix-bound proteins are eluted for identity analysis using N-terminal amino acid sequencing [29,30]. An alternative approach for affinity binding is to attach the tested antimicrobial covalently to a cell-permeable analytical tag, which is then allowed to bind the target protein in live bacterial cells [31].

In principle, affinity chromatography is applicable for the identification of any antimicrobial binding protein, but actually, to select the appropriate modification site on the antimicrobial without affecting its original target-binding property is not an easy task. Over the years, several improvements have been made to address this issue [32–36]. One interesting method is the non-selective universal labeling method, which is able to attach structurally different molecules using a photo-crosslinking reaction [32]. This reaction produces a mixture of differently modified ligands, some of which are assumed to still bind the target. Other promising methods are label-free strategies, such as "pulse proteolysis" [37], "stability of proteins from rates of

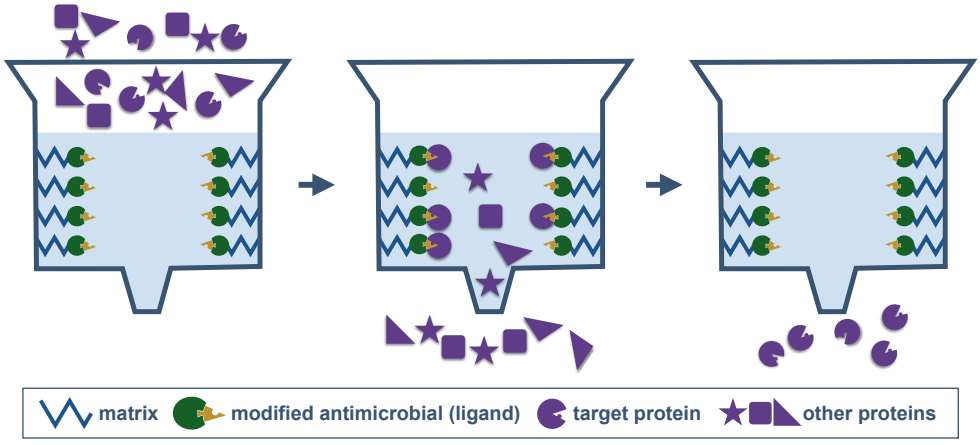


**Fig. 3. Schematic representation of affinity chromatography.** The tested antimicrobial is modified as a ligand, immobilized onto a solid matrix and then exposed to bacterial cell lysates. After multiple washing steps to remove non-binding proteins, the matrix-bound protein (target protein) is eluted for identification.

oxidation" (SPROX) [38] and "drug affinity responsive target stability" (DARTS) [39]. The underlying concept of these strategies is that the thermodynamic stability of the target protein will change when binding to ligand. These strategies have nice perspective because of their intrinsic advantage of being "label-free".

The development of mass spectrometry (MS) has greatly improved the identification of eluted proteins and straightforward workflows now include gel electrophoresis, protein band extraction and MS analysis [31]. The resolution, sensitivity, and throughput of MS-based methods has reached another level compared to the traditional N-terminal amino acid sequencing using Edman degradation. In addition, MS-based methods also benefit the affinity purification process. For example, Ong et al. labeled their cell lysates for negative control or positive probe with light or heavy isotopes using a quantitative proteomics method named SILAC, separately, and then MS was applied to identify the target proteins in combined protein samples without separation by gel electrophoresis [40]. But MS mediated approaches also have limitations. One of which is the requirement of specialized researchers and sophisticated instruments, which may not be available for all labs.

Lately, affinity approaches have contributed much to the MoA research of vancomycin [41–44]. However, they have not been broadly used in MoA identification of natural antimicrobials [7]. An intrinsic drawback is that these approaches require high-affinity interactions. There is a variety of interacting traits between

antimicrobials and their targets, and the interactions are not always high-affinity. Otherwise, affinity approaches are excellent tools for direct target identification of antimicrobials.

Above, we have compared the advantages and drawbacks of three classic approaches and have discussed their improvements over the years. Whereas these methods were established several decades ago, there is a reason why they are still in use today. These approaches do provide insight into the MoA of antimicrobials. The combination of classic approaches with continuously developing novel high-tech methodology may give the old methods a second and modern life.

## OMICS APPROACHES

Next to classic approaches, several modern approaches, including omics technologies, have been developed and used extensively in recent years. The 'age of omics' has shed light onto the discovery of new antimicrobial compounds by developing global analysis tools such as genomics, transcriptomics and proteomics [9]. Genomics has been applied not only to explore silent antimicrobial biosynthetic pathways, but also to search for novel antibiotic targets. Transcriptomic and proteomic analyses generate information about the expression of genes in response to antimicrobials at the RNA and protein level, respectively, and changes in response to antimicrobial treatment provide insight into the working mechanism of these antimicrobial agents [45–47]. Here, we will focus on the impact of the global analysis studies and provide insight into each of these promising approaches. A brief schematic of introductions and major applications of these approaches can be found in Fig. 4.

### *Genomic approaches*

Genomic methods are applied to explore the hidden property of bacterial genomes, such as searching for essential bacterial genes as the potential targets for novel antimicrobials, searching for genes that contribute to drug resistance or searching for potential antimicrobial synthetic gene clusters [48,49]. These approaches enable rapid antimicrobial target, resistance or synthesis prediction and therefore facilitate the search for antimicrobials with novel MoA. Unlike the other approaches reviewed here, genomic methods will not provide direct targets for antimicrobial compounds. Nevertheless, given the bright perspective of genomic methods to contribute to antimicrobial discovery, it is still interesting to discuss those here.

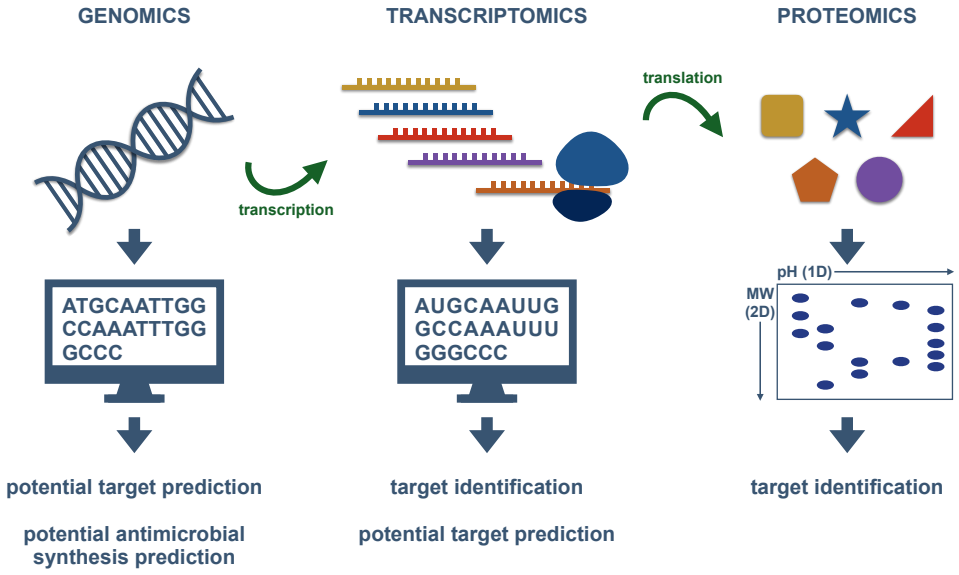


**Fig. 4. Concepts and applications of omics approaches.** The concept of genomics, transcriptomics and proteomics are schematically represented. Next-generation sequencing is performed to analyze genome and transcriptome, whereas 2D SDS-PAGE is the most common method for proteome analysis. Their application in antimicrobial discovery are listed below each approach.

Two commonly used genomic methods for antimicrobial discovery are genome mining and comparative genomics. The former one has achieved some goals by successfully characterizing several antimicrobial biosynthesis loci, including cyclic lipopeptide antimicrobials from *Pseudomonas aeruginosa*, polymyxin from *Paenibacillus polymyxa*, and polyketides from *Bacillus amyloliquefaciens* [9,50–52].

The focus of comparative genomics is on the identification of potential antimicrobial targets [53–55]. It is believed that conserved genes in different bacterial genomes often turn out to be essential, and therefore have the potential to become targets of broad-spectrum antimicrobials. Subtractive genome analysis is a method to search for conserved bacterial genes that are not present in eukaryotes. Admittedly, little of these efforts have arrived at clinical stages. One limitation to this approach may be the lack of distant gene relationships [48]. Essential bacterial functions are generally encoded by gene pairs [56], and these distant gene relationships are essential for target identification. Another obstacle is that the “essential gene” is relative rather than absolute, because this is dependent on the growth conditions. A study found pyrimidine–imidazole compounds to be novel anti-tuberculosis drugs

*in vitro*, but had no effects in an infected mouse model *in vivo* [57]. The reason for this discrepancy is that pyrimidine-imidazole inhibits glycerol metabolism, which is required for bacterial growth, but not for *Mycobacterium tuberculosis* during infection.

Lately, comparative genomics has also contributed to the identification of antimicrobial resistance gene clusters [58–60]. These studies illustrate the more complex mechanism of resistance than what was assumed previously. Comparative genomics suggests that antimicrobial resistance is not only due to mutations in the targets, but also to mutations in genes in charge of DNA repair, energy metabolism or transmembrane transporters. In addition, some studies reveal that many mutations encoding unannotated proteins may also contribute to antimicrobial resistance [61,62]. This provides leads for genetic studies in the future.

Different from genomic approaches, transcriptomic and proteomic approaches provide insight into the global response of bacterial cells to antimicrobials, which in turn provides insight into the MoA of certain compounds. In the following paragraphs, we will discuss their recent applications.

### *Transcriptomics approaches*

The transcriptome is the signature of bacterial gene expression, which may be established under different environmental conditions and/or at specific growth stages. Categorizing the transcriptome is fundamental to investigate the functional elements of the genome and can therefore lead to a better understanding of bacterial status. One of the key purposes of transcriptomics is to quantify the changes in each transcript under different conditions [63]. This may benefit antimicrobial research, because an antimicrobial may influence many cellular pathways [64] and analysis of differential gene expression may help to predict the MoA of antimicrobial agents.

Traditional quantification techniques of RNA expression levels, such as real-time PCR and northern blotting, were widely used to characterize cellular transcriptomes in the 1990s [45]. However, these techniques do not allow analysis of the expression levels of all genes in one go. Therefore, multiple replicates need to be performed, which hampers interpretation of the results. Recently, with the development of next-generation sequencing, whole-cell analysis approaches have attracted more interest. One approved approach is RNA-Seq, which is developed to profile the transcriptome using deep-sequencing technologies. Compared to the traditional approaches, RNA-Seq has several advantages: it provides a far more precise resolution with high throughput and low background noise; it offers a high dynamic range for quantifying

the level of gene expression; it requires low amount of input RNA, and the cost for mapping transcriptomes is relatively low [63].

RNA-Seq has successfully been used for antimicrobial MoA elucidation. In a MoA study of two natural products, transcriptomic analysis of methicillin-resistant *Staphylococcus aureus* was performed by RNA-seq following treatment with ursolic acid and resveratrol [65]. The results illustrate the reduction of amino acid metabolism and adhesin expression in response to ursolic acid. Resveratrol causes disruption of surface proteins and capsular polysaccharides synthesis. RNA-Seq is also used to show the mechanism how one drug improves the efficiency of another drug. In this study, *Acinetobacter baumannii* was treated with sub-MIC of tigecycline, a drug that inhibits the initial codon recognition step of tRNA accommodation [66]. Transcriptomic analysis clearly shows the downregulation of β-Lactamases, such as OXA-23 and AmpC, by tigecycline, which results in a decrease of the MIC of ceftazidime, an antimicrobial targeting the cell wall.

Another aspect of transcriptomics in the antimicrobial field is that RNA-seq may predict potential antimicrobial targets, similar to comparative genomics [45]. For instance, biofilm formation is an important virulence trait of infection [67]. Instead of growth inhibition, the inhibition of biofilm formation of pathogenic bacteria may be an alternative approach to treat infections. RNA-seq analysis of *A. baumannii* shows huge differences in gene expression between biofilm cells and planktonic cells, with overexpression of 1621 genes and new expression of 55 genes [68]. These differentially expressed genes are involved in a variety of biological processes including transcriptional regulation, motility, amino acid metabolism, quorum sensing as well as other important pathways. Disruption of some of the differentially expressed genes results in a considerable decrease in biofilm formation. This suggests that this large panel of genes contains potential targets of novel antimicrobials or biofilm inhibitors.

Whereas transcriptomics is technically relatively easy to perform, the analysis of the outcome may be challenging. The lack of a “golden standard” protocol makes it complicated to compare the results between individual studies [9]. The growth status of cells, the culture media, the antimicrobial concentration and even the antimicrobial incubation time may all greatly affect the outcome of gene expression patterns. However, as a first step in MoA identification, transcriptomics may still provide much valuable information.

### *Proteomics approaches*

The bacterial proteome responds quickly to changes in the surrounding environment. Antimicrobial treatment of bacteria results in direct and specific responses in their proteome. The goal of proteomics approaches is to globally analyze protein changes over time by identifying and quantifying proteins. This may help to formulate a hypothesis of the antibiotic target and MoA [69]. An additional advantage of proteomics is the quantification of post-translational modifications (PTM), which are not detectable by transcriptomic approaches.

In most cases, proteomic analysis is performed using two-dimensional electrophoresis (2-DE) and MS. With 2-DE, proteins are separated by gels on the basis of their isoelectric points in the first dimension and of their molecular weights in the second dimension. This results in a proteome map showing a variety of spots, each corresponding to a different protein. This method is routinely performed to study bacterial protein expression under given conditions. In a study using *Bacillus subtilis*, such proteome maps demonstrate the complex cellular responses to 30 distinct antimicrobial compounds [70]. Each of the antimicrobials induces a complex and unique protein expression profile. However, overlaps are evident in the expression of some proteins in response to distinct antimicrobials with similar MoAs. Analysis of the similarities results in identification of 122 marker proteins with at least two-fold overexpression compared to the untreated control. These markers are excellent references for the MoA study of novel antimicrobials. The marker protein concept was successfully applied to a novel compound, BAY 50-2369, and it is classified as a protein synthesis inhibitor. A recent MoA study of daptomycin, mentioned above in the "*MMS assay*" part [18], is also a successful example of 2-DE. The proteome map of daptomycin-treated bacteria was compared with maps from previous studies [70,71], resulting in identification of matching marker proteins of bacteria treated with cell membrane-active antimicrobials, which provides evidence for the membrane defects induced by daptomycin. However, the gel-based separation system has limitations. For example, it has low throughput and high inter-gel variability. More importantly, it can only be applied to proteins with certain properties, because it has poor recovery of very small and very large proteins as well as hydrophobic proteins.

Recently, with the improvements in liquid chromatography (LC) and tandem MS (MS/MS) technology, several gel-free proteomics methods have been established. These methods facilitate identification and quantification of complex peptide mixtures containing proteins digested by proteolytic enzymes. Subsequently, computational

approaches are being used to assemble these peptides back to proteins [72]. As a consequence of the LC separation process, gel-free methods are more sensitive and less laborious, compared to the gel-based methods. However, the popularity of gel-free approaches is less than gel-based methods in the antimicrobial fields. The greatest challenge in gel-free approaches is the computational approach, which is not as accurate as one would like for high-resolution data, although significant advances in analysis programs have been made over the years [72,73]. With the continuous improvement of bioinformatic analysis tools, gel-free approaches may become the superior application in the future. However, for now, gel-based methods are still dominant in proteomic analysis of antimicrobial MoA. Considering the limitations of the gels, transcriptomics data may be useful supplements. The combination of the two omics approaches may provide an interesting perspective for antimicrobial MoA identification.

Similar to the omics approaches above, other omics methods, like metabolomics and lipidomics, are also widely used to generate bacterial signatures in antimicrobial research [45]. Whereas all of these omics methods provide valuable information about antimicrobial targets, they are indirect approaches and therefore do not necessarily provide information about the direct target(s) of antimicrobials. These methods are developed for the initial stages of target identification. Despite that these approaches are modern and fascinating, they do not have significant advantages for MoA identification by themselves. Since the time spent and cost involved in the omics approaches is relatively high, it is wise for each application to choose the most appropriate approach.

## MORPHOLOGICAL PROFILING

Inhibition of a specific pathway in a cell may result in specific changes in the cell phenotype, including changes of molecular signatures (e.g. transcriptome or proteome) and cellular morphology [74]. Above, we have illustrated the application of omics approaches that use molecular signatures in identification of antimicrobial MoAs. Assessment of cellular morphology is also an important way to generate information about pathway defects and antimicrobials generating similar phenotypes may target the same pathways. With the advances in microscopy, cellular morphological profiling is another attractive way to hypothesize about the MoA of antimicrobials by reflecting the global cellular effects. For eukaryotic cells, many aspects of cellular morphological changes are easily observed under a microscope, followed by automated computational analysis [75,76].

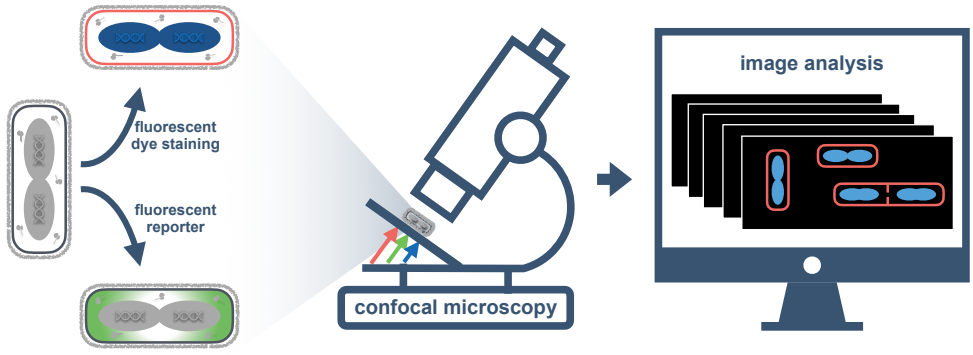


**Fig. 5. Concepts of BCP.** Fluorescent bacterial cells (by fluorescent dye staining or fluorescent protein expression) are imaged by confocal microscopy. Images are then analyzed using specific programs.

However, to apply this strategy onto antimicrobial MoA identification, i.e. to profile the cellular changes in prokaryotic cells, is a more challenging task. This is mainly due to the smaller size of bacterial cells, high phototoxicity and enhanced movement of bacterial cells. With the development of microscopy, high resolution images reflecting bacterial sub-cellular structures are gradually achievable. In this part, we will discuss the advances of imaging-based strategies in antimicrobial MoA identification.

### *Bacterial cytological profiling (BCP)*

The concept of BCP was initially validated in 2013 by Nonejuie and colleagues [77]. With micrographs of Gram-negative bacteria, *Escherichia coli*, treated with different antimicrobials, they successfully classified 41 antimicrobials into five groups, based on the five major pathways, including DNA synthesis, RNA synthesis, protein synthesis, lipid synthesis and peptidoglycan synthesis. In addition, the small differences within each group may also be target related, and may therefore allow further distinguishing. For this approach, *E. coli* cells are visualized by staining with DAPI (cell permeable nucleoid dye with blue fluorescence), FM4-64 (membrane dye with far red fluorescence) and SYTOX-Green (cell impermeable nucleoid dye with green fluorescence), immobilized by agarose pads and magnified by a 100× objective. Cell morphological differences are distinguished based on the features both from the shape parameters and the intensity of each of the dyes. As a proof-of-principle, the MoA of a natural antimicrobial, spirohexenolide A, was predicted to be active on the cell membrane.

Compared to the classic and omics approaches, BCP provides reliable MoA information, but with a less tedious workflow (Fig. 5) and lower amount of compound required. But it also has limitations. Whereas it provides information which pathway is affected, this phenotype-based indirect approach does not provide information about the molecular targets. Another limitation is that the hypothesis can only be derived from the reference set, which is based on profiling of control-treated antimicrobials with defined MoA. If an antimicrobial has a MoA that has not been described before, BCP cannot provide information. Last, but not least is the limitation that whereas BCP can be used on Gram-negative bacteria [77,78], the application of BCP on Gram-positive bacteria seems to be more complicated. Due to the strong cell wall, the shape of Gram-positive cells normally does not change significantly. Fortunately, some efforts have been made to improve these limitations.

To address these limitations, several improvements were made. To visualize potential new target defects, Lamsa et al. established a method, dubbed Rapid inhibition profiling (RIP) [79,80], which demonstrates that degradation of essential proteins in some of the major pathways, for example DNA synthesis, RNA synthesis, fatty acid synthesis, and peptidoglycan synthesis, matches the profiles of antimicrobials inhibiting the same pathways. This suggests that RIP has the potential to generate a library of profiles by inactivating each of the pathways, including the ones that are not known to be targeted by antimicrobials and thus reflect novel MoAs.

To apply BCP to Gram-positive bacteria, several avenues were developed. The first possible solution is to generate a panel of GFP-reporters covering many pathways. This panel has been successfully applied for several in-depth analyses of antimicrobial targets [18,81,82]. However, no single reporter was generated covering more than two main antimicrobial classes. This suggests that for classification of unknown antimicrobial candidates, at least five reporters will be needed each time. This approach is rather laborious, because for each reporter an accurate minimum inhibitory concentration (MIC) of the antimicrobial has to be established. Another possible solution is to add a new dimension to BCP, i.e. time, by taking advantage of time-lapse imaging and perform dynamic bacterial cytological profiling (DBCP). We have just developed this strategy and have acquired several benefits from it.

### *DBCP*

DBCP is a method to record bacterial changes before, during and after antimicrobial treatment using time-lapse imaging. The term dynamic is added ahead of BCP because the same cell is imaged over time, which is fundamentally different from

individual images of distinct cells or cultures at different time points. Bacterial long-term imaging was facilitated by an improved imaging protocol, which is based on a previously described technique [83]. The technique we developed contains several improvements, including the advantage to add antimicrobials at any time, which is less time consuming and less laborious (**Chapter 4**). In addition, several adjustments have been made to reduce the chance of being affected by environmental factors, such as staining toxicity and phototoxicity. Finally, DBCP has been successfully performed on a Gram-positive bacterium *B. subtilis* and allows to rapidly distinguish between anti-Gram-positive drugs from all of the five different pathways. As a proof-of-concept, a naturally occurring antimicrobial was identified to target the cell envelope. DBCP has several benefits. It allows to record the direct response from single cells to specific antimicrobial agents over time and to provide a first glance of timepoints at which a certain antimicrobial starts to have effects, cell growth starts to be inhibited and/or cells to be killed. These parameters are all important to establish antimicrobial MoA.

DBCP has its limitations. This imaging-based strategy results in the 'most similar' signatures, and thereby has an intrinsic danger of false positive matches. Therefore, subsequent validation experiments are fundamental to this approach. Nevertheless, considering the ease of accessibility and the affordability, DBCP is an excellent approach for the initial stage of MoA identification.

## CONCLUSION AND OUTLOOK

Antimicrobial resistance is one of the major threats to human health. Although many classes of antimicrobials are available on the market, none of them escapes emerging resistance. We have a strong need for new classes of antimicrobials with novel MoA to resolve the infections that are caused by the so-called "superbugs", which are pathogens that are resistant to multiple antimicrobials. MoA identification of newly screened antimicrobials has become more important than ever before. However, this process remains to be the bottleneck in drug discovery.

Here, we have reviewed various approaches that are developed for MoA identification. It will be smart to choose the most appropriate strategy depending on the expertise of the lab and the properties of the antimicrobial agent. Classic approaches were developed for slow processes decades ago, but two approaches from it, genetic approaches and affinity chromatography, are the only methods to directly identify the real targets at the molecular level. Even though they are relatively laborious and time-consuming, an appropriate and accurate hypothesis about the

pathway of the antimicrobial target might significantly shorten the process. All the modern approaches (omics, imaging-based methods etc.) as well as the improved MMS assay are actually designed for generating the hypothesis in a fast and accurate process. Although the "high hopes from omics" is not yet fulfilled, the global profiling methods of omics are definitely shedding light on future antibacterial discovery. They are high throughput and easily performed. The MMS assay and the imaging-based methods also contribute to some aspects of antimicrobial research and provide more options for researchers in the field. But they all have specific limitations.

It is important to note that no perfect method has been established for antimicrobial MoA identification yet, at least until now. However, due to the development of methodologies and technologies, it will be interesting to see how these approaches will influence the antimicrobial research in the future. There might be new ideas and new hopes coming in and old strategies dying out. But overall, until the emergence of the next "penicillin", we will never have the chance to take a short break from the battle with the kingdom of microorganisms.

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