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A Review

Phenotypic antibiotic resistance: Involvement of genes and additional factors

Santhi Sanil Nandini and Dipankar Nandi*

Department of Biochemistry, Indian Institute of Science, Bengaluru-560 012, Karnataka, India

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The rise of antibiotic resistance poses a critical threat to humanity. This review explores phenotypic antibiotic resistance, a mechanism by which bacteria transiently adapt to survival in the presence of antibiotics. We explore the underlying mechanisms, including alterations in the bacterial envelope, efflux pumps, and enzyme activity. The roles of the *mar* operon, a key regulatory system, are also discussed. Recent discoveries in compounds that induce phenotypic resistance and novel detection methods are also highlighted. We conclude by emphasizing the clinical significance of this phenomenon and exploring future research directions to combat this growing challenge.

Keywords: Antibiotic resistance, Efflux pumps, Lon protease, Mar operon, Sodium salicylate

Introduction

Antibiotics are chemical substances produced mainly by living organisms that are detrimental to bacteria. The first antibiotic, salvarsan, was discovered in 1910, followed by penicillin in 1928, marking the beginning of the golden age of antibiotics that peaked between the 1940s and 60s. Post the golden age, the decline in the antibiotic discovery pipeline and the evolution of antimicrobial resistance have led to the antimicrobial resistance crisis[1]. Antibiotic resistance emerges when bacteria develop the ability to bypass the mechanisms used by antibiotics to kill them. As a result, the medicines become ineffective, increasing morbidity and mortality. Microorganisms that develop resistance to drugs are often referred to as superbugs. Around 5 million deaths caused by AMR bacteria are reported yearly; poor research and the shrinking pipeline of new antibiotics coupled with this have led AMR to be highlighted as one of the top ten public health threats faced by humanity in 2019. The deaths contributed by AMR are predicted to reach 10 million by 2050, increasing the burden on the healthcare system[2].

The major reasons for the emergence of resistant strains can be either natural or anthropogenic. The natural/biological causes of the evolution of antibiotic resistance can be mutation, gene transfer and natural selection. The anthropogenic causes include the widespread misuse of antibiotics in humans, animal farming, and poultry, lack of proper diagnostic methods, poor sanitation, and vaccination reluctance[3]. However, bacteria may transiently become resistant to antibiotics without any genetic changes, referred to as phenotypic antibiotic resistance[4]. The most studied instances of phenotypic antibiotic resistance are drug indifference, growth in biofilm and persistence. Few studies have also focused on the link between antibiotic susceptibility and metabolic state or situations of growth arrest (Fig. 1) [5]. Phenotypic resistance can also be achieved by induction of a specific resistance mechanism such as the induction of β-lactamase by β-lactams, efflux pumps by different aromatic compounds, *etc*[6-8]. The review will discuss the history and other mechanisms by which phenotypic antibiotic resistance is induced and recent advances in the field of phenotypic antibiotic resistance research from the laboratory.

Antimicrobials and antimicrobial resistance: past and present

Penicillin, discovered in 1928, was the first antibiotic used successfully. However, the Penicillin-resistant Staphylococcus strain was described in 1940. To counteract the resistance mediated by penicillinase, methicillin was deployed in 1959 and 1960, and methicillin resistance to *staphylococcus* was also described[9]. Reports of several antibiotics from microorganisms encouraged Selman Waksman to systematically study microbes for their potential to

*Correspondence:
E-mail: nandi@iisc.ac.in

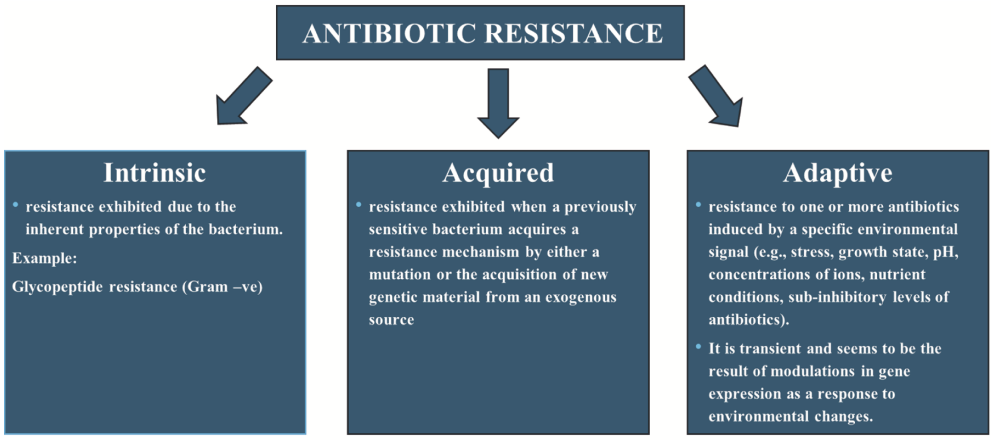

Fig. 1 — Different types of antibiotic resistance. Antibiotic resistance is broadly classified into intrinsic, acquired and adaptive

produce various antimicrobial compounds in the 1930s. His studies led to identifying actinomycetes and streptomyces as copious producers of antimicrobials. His discoveries were followed by the introduction of many other antibiotics, marking the Golden Age of Antibiotics from the 1940s to the 60s. Ample availability and ease of discovery of several new classes of drugs led to the misuse and overuse of these drugs. However, the emergence of resistant bacterial strains and the reduction of new antibiotic discoveries in the following decade disclosed the shortcomings of these compounds. They urged the policymakers to acknowledge the threats of a post-antibiotic era. Scientific challenges in antibacterial discovery research, regulatory and clinical challenges, and commercial challenges due to the low economic attractiveness of investing in antibacterial R&D lead to low industry success rates. Seventy High throughput screening (HTS) campaigns conducted by GlaxoSmithKline (GSK) over seven years using a collection of approximately 50,0000 compounds, but with very few leads and no candidates for development. Sixty-five HTS campaigns by AstraZeneca with a few leads but none that were active against multi-drug-resistant Gram-negative bacteria. These are two major examples of unsuccessful R&D initiatives in the near past. However, new tools for genome mining and the discovery of new strains capable of producing potent antibiotics from previously unexplored environments are giving the field and policymakers new hopes[1].

Mechanisms of phenotypic antibiotic resistance

Role of Bacterial Envelope

One of the significant factors contributing to the effectiveness of antibiotics is the bacterial envelope, which determines the penetration of the molecule inside the cell. Modifications in the cell envelope can significantly contribute to the penetration of the antibiotic molecule[10].

Lipopolysaccharide layer

In Gram-negative bacteria, the lipopolysaccharide (LPS) layer acts as the first defence barrier against antibiotics. The an ionically charged LPS layer allows the positively charged cationic antibiotics to bind, facilitating the uptake. Alterations in the negative charge of LPS can affect the uptake of these antibiotics[11]. In *Pseudomonas aeruginosa,* induction of the arnBCADTEF operon by any of the two-component systems PhoP-PhoQ, PmrA-PmrB, and ParS-ParR release products that can alter the charge and induce resistance. Low concentrations of cationic antimicrobial peptides or divalent cations such as Mg_2^+ and Ca_2^+ can induce this system[12]. Host defence peptides in humans can cause resistance to antibiotics of specific pathogens in a clinical setup. Instance of alteration of the growth temperature altering LPS pattern and induces resistance against aminoglycosides has been reported in *Stenotrophomonas maltophilia*[13].

Porins

Antibiotics use porins and inner membrane transporters to enter bacterial cells. Low expression of porins due to environmental signals reduces the uptake of antibiotics, rendering resistance to certain antibiotics. In *E. coli,* two major porins, *OmpC* and *OmpF*, are involved in the uptake of antibiotics such as β-lactams, tetracyclines and quinolones. The two-component system EnvZ-OmpR controls the transcription of *ompF* and *ompR* by sensing the osmolarity of the environment. The Mar-Sox-Rob regulon also controls the expression

of porins *via* antisense RNA *micF* and *micC.* This regulon responds to salicylate and has consequences for antibiotic resistance[14].

Efflux pumps

The multidrug efflux pump systems also play a crucial role in antibiotic resistance. The expression of these efflux pumps is regulated by effect or molecules, allowing upregulation of transient expression in specific environmental conditions. In *E. coli*, the efflux pump system AcrAB-TolC is tightly regulated by the Mar-Sox-Rob regulon. Previous studies have shown that sodium salicylate, non-steroidal anti-inflammatory drugs, and uncouplers can induce antibiotic resistance through this efflux pump through the Mar regulon[7,8,15]. Oxidative agents which produce superoxide anions can also upregulate the expression of the pump system *via* SoxS[16]. The SmeDEF efflux pump from *S. maltophilia* is induced by triclosan found in toothpaste. Efflux pumps induced by bile, cationic peptides or fatty acids have also been reported. Bacteria which overproduce efflux pumps can also act as a nucleus for high-level multidrug resistance[17].

Role of proteases

In prokaryotes, energy-dependent protein degradation is mainly controlled by two ATP-dependent proteases, Lon and Clp, which fall under the ATPases Associated with various cellular Activities (AAA^+) family of proteins[18]. ATP hydrolysis permits the unfolding of the protein followed by recognition and binding; the unfolded protein substrate is then translocated to the inner proteolytic chamber for cleaving peptide bonds. The high expression of Lon protease may lead to lethality. Thus, the appropriate level of protease is inevitable for the stable functioning of a cell (Fig. 2). In *E. coli, lon* belongs to the Heat shock (HS) regulon, whose transcription activation is mediated by the σ^{32} factor[19]. Proteomics studies have identified several additional substrates for Lon in *E. coli*, hinting at a far more significant influence of this protease on bacterial physiology than was previously understood. Deletion of *lon* also results in increased levels of *MarA* and drug efflux, making the Lon-deficient *E. Coli* tolerate higher concentrations of several antibiotics, such as tetracycline and ciprofloxacin[7,8]. Given its role in the pathogenesis of bacteria, Lon has been placed on the AMR map and inhibitors of Lon have been suggested as novel antimicrobials.

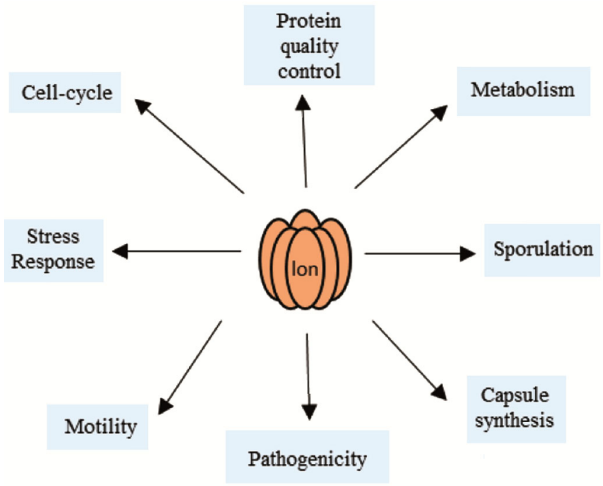

Fig. 2 — Multiple functions of Lon protease: Lon protease is a hexamer regulates several biological functions through cellular proteolysis

Roles of *mar* Operon

The multiple-antibiotic-resistance locus was first described in *E. coli* and is also present among other enteric bacteria[20]. The locus has been characterized by *E. coli*, *Salmonella enterica* serovar Typhimurium, *Shigella flexneri, etc*. The locus consists of two differently transcribed units, *marC* and *marRAB*, regulated by two different promoters in the *marC-marR* intergenic operator region. *marRAB* unit transcribes two regulatory proteins, MarR, the auto repressor of the operon and *MarA*, the transcriptional activator. On the other hand, MarB represses the *marRAB* promoter by an indirect mechanism and reduces the transcription rate of *MarA*. MarR is inactivated by binding to sodium salicylate, 2,4-DNP, and plumbagin[21,22]. Copper acts as a natural signal by directly binding to MarR and derepressing the *marRAB* operon[23]. Salicylate can also induce oxidative stress increasing amount of ROS which further alters membrane permeability and metabolism leading to the formation of persisters[24]. In *E. coli,* the multi-drug resistance pathway is mediated by the upregulation of the AcrAB-TolC multidrug efflux pump by *MarA*[33]. *MarA* activates transcription by binding to an asymmetric degenerate 20 bp DNA sequence in the promoter region of target genes, the *mar box* (with an AYnGCACnnWnnRYYAAAYn consensus where R = A or G; Y = C or T; W = A or T; n = A, T, C or G)[25]. The *MarA* protein of *E. Coli* consists of 164 residues and a Molecular weight of 30.18 kDA (Fig. 3). It belongs to the AraC/XylS family of transcriptional regulators. It has two helix-

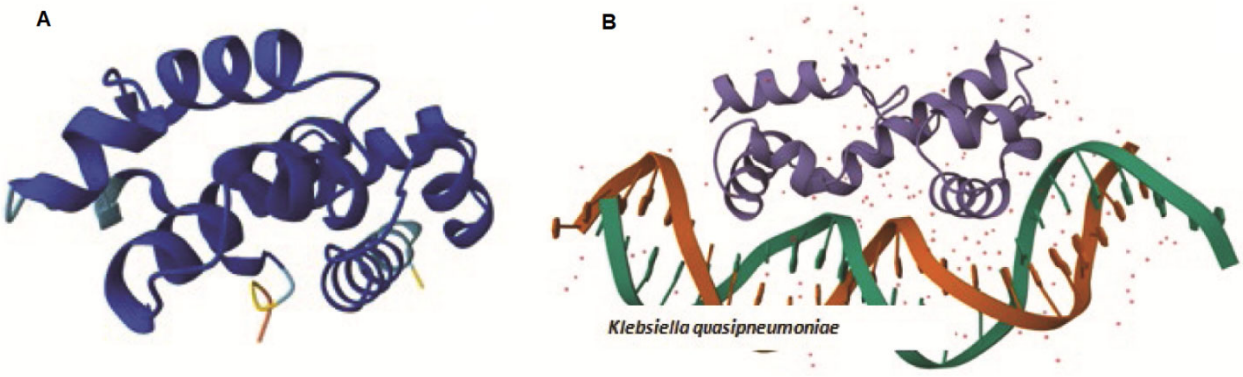

Fig. 3 — Structure of *MarA* transcription factor(A) 3D structure of *MarA* protein with two helix-turn-helix motifs. Protein (https://www.rcsb.org/structure/1bl0) ; and (B) 3D structure of *MarA*-DNA complex showing helix -turn -helix binding to major groove of DNA (https://alphafold.ebi.ac.uk/entry/P0ACH5)

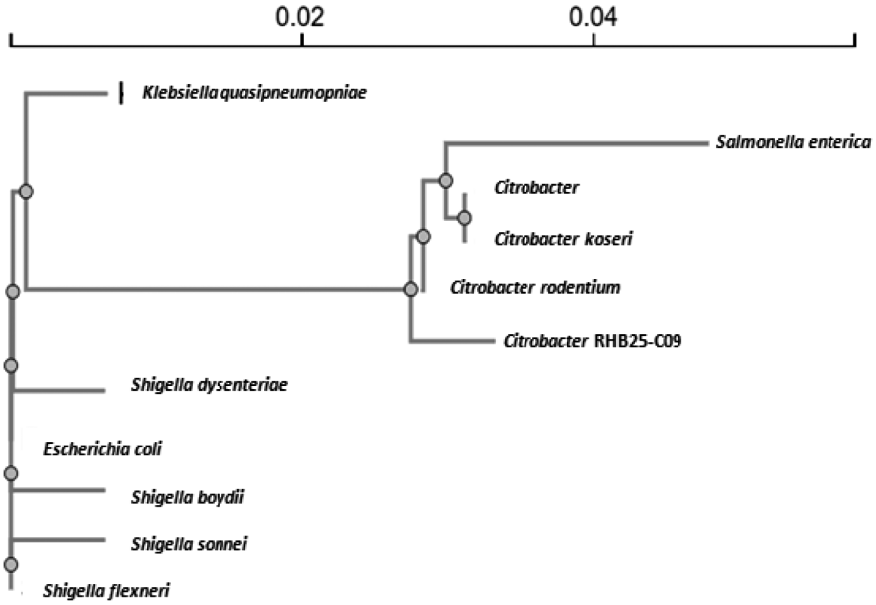

Fig. 4 — *MarA* orthologs are present in different bacteria. Phylogenetic tree generated from multiple sequence alignment of *MarA*-like proteins in different bacterial species

turn-helix (HTH) motifs for DNA binding, one located in the N terminal interacting with RE1 and the other in the C terminal interacting with RE2. Three residues, W42, R46 and R96, have been identified as involved in the DNA binding process (Fig. 3) [26].

Multiple sequence alignment of protein sequences which had similar sequences to *E. coli MarA* was performed to understand the phylogeny of the protein. Three different branches could be identified from the input sequences. The first branch was comprised of *Klebsiella* spp. alone, the second comprised of *Salmonella* spp. and *Citrobacter* spp. The third branch was comprised of *E. coli* and *Shigella* spp. This shows the close similarity between *MarA* of *E. coli* and *Shigella* spp (Fig. 4) [27].

Recent advances in the field of phenotypic antibiotic resistance research from our laboratory

A serendipitous observation was made in our lab that a *lon* mutant of *E. Coli* was more sensitive to NaSal due to higher *MarA*, leading to phenotypic antibiotic resistance, a new area of research in the laboratory[7].Since NaSal is an anti-inflammatory compound, non-steroidal anti-inflammatory drugs (NSAIDs) were screened for their potential to induce phenotypic antibiotic resistance, and we identified that acetaminophen and ibuprofen to induce resistance but are not as effective as NaSal. As NSAIDs are routinely administered to treat inflammatory diseases, continuous use of higher amounts of these drugs may induce phenotypic antibiotic resistance[8]. Following

this, we screened for environmentally relevance and has the potential to induce phenotypic antibiotic resistance; this screening led us to the identification of some uncouplers mostly used in sludge treatment and herbicides used in agriculture as potential inducers of resistance[28,29].

Importantly, the model system developed in our lab can be used to quickly identify resistant bacteria by differentiating them from the susceptible ones. Identification of resistant bacteria using conventional techniques like PCR and MIC requires species-specific methodologies and is time-consuming, leading to delayed detection and treatments. Raman spectroscopy was employed to differentiate between two genetic mutants of *E. coli,* Δ*lon* and Δ*acrB*, which display differential susceptibilities towards antibiotics[30]. We have also used our model system and established the use of solvatochromic dyes based on 4-amino phthalimide(4-AP) attached with different alkyl groups to determine antibiotic-induced membrane changes at a sub-nanometre scale through peak maxima difference ratio of the dyes 4-AP-C9 and 4-AP-C13[31].

Conclusion

The rise of antibiotic resistance is of significant public health concern. While genetic mutations contribute to resistance, phenotypic resistance mechanisms pose an additional challenge due to their transient nature and difficulty in detection. This review highlighted various phenotypic resistance mechanisms, including alterations in the bacterial envelope, efflux pumps, and proteases. The Mar operon's role in regulating these mechanisms was also discussed.

Recent research has shed light on the involvement of Lon protease and *MarA* in inducing phenotypic resistance. Additionally, advancements in detection methods using Raman spectroscopy and solvatochromic dyes offer promising avenues for tackling this challenge.

Further research on the complex interplay between these mechanisms and developing rapid and accurate diagnostic tools are crucial steps in combating phenotypic antibiotic resistance. This will ultimately aid in developing effective strategies to preserve the efficacy of existing antibiotics and ensure their continued usefulness in treating infections.

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Conflict of interest

Both the author declares no conflicts of interest.

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