



The Action of Efflux Pump Genes in Conferring Drug Resistance to *Klebsiella* Species and Their Inhibition

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Abstract

Nosocomial infections caused by *Klebsiella* species are characterized by high rates of morbidity and mortality. The emergence of the multidrug-resistant (MDR) and extensive drug-resistant (XDR) Gram-negative bacteria reduces the antibiotic efficacy in the treatment of infections caused by the microorganisms. Management of these infections is often difficult, due to the high frequency of strains resistant to multiple antimicrobial agents. Multidrug efflux pumps play a major role as a mechanism of antimicrobial resistance in Gram-negative pathogens. Efflux systems are significant in conferring intrinsic and acquired resistance to the bacteria. The emergence of increasing drug resistance among *Klebsiella pneumoniae* nosocomial isolates has limited the therapeutic options for treatment of these infections and hence there is a constant quest for an alternative. In this review, we discuss various resistance mechanisms, focusing on efflux pumps and related genes in conferring resistance to *Klebsiella*. The role of various efflux pump inhibitors (EPIs) in restoring the antibacterial activity has also been discussed. In specific, antisense oligonucleotides as alternative therapeutics in combatting efflux-mediated resistance in *Klebsiella* species have focused upon.

Keywords

- ▶ efflux pump systems
- ▶ *K. pneumoniae*
- ▶ efflux pump inhibitors
- ▶ antisense oligonucleotides

Introduction

Enterobacteriaceae, in particular, *Klebsiella*, is one of the outstanding etiological agents of significant importance in causing nosocomial and community-associated infections in humans.¹ Bacteria belonging to the genus *Klebsiella* cause a wide range of infections such as community-acquired infections including pneumonia, wound infections, urinary tract infections, septicemia, and gastrointestinal diseases.² Recognized species of the genus *Klebsiella* include *K. pneumoniae*, *K. oxytoca*, *K. terrigena*, and *K. planticola*.³ *K. pneumoniae* is the principal species of the genus, gaining importance as it is developing multidrug resistance in hospital settings similar to *Acinetobacter* and *Pseudomonas*.⁴ The source of isolation of *K. oxytoca* from clinical specimens includes blood and

respiratory secretions. It causes infections in the immunocompromised individuals admitted in intensive critical care units. In the history of therapeutics, one of the major milestones was the discovery of antibiotics, which no doubt was effective in controlling infectious agents and considered a wonder drug. Yet, extensive use of antibiotics in the medical, agricultural, and veterinary sectors has led to the emergence of drug-resistant strains. Listed as one of the ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species) pathogens, *Klebsiella* has gained resistance to almost all the drugs.⁵ The possible reasons for frequent infections caused by *Klebsiella* in comparison to other Gram-negatives could be (a) the ability to naturally resist the antibiotics, (b) the ability to outcompete

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other bacteria, (c) the potential to withstand starvation, (d) the capability to exchange genetic material readily with other members of the human microbiome, and (e) mobile genetic elements that encode broad-spectrum of virulence and antibiotic genes.^{6,7} Utmost strains of *K. pneumoniae* produce the capsular polysaccharide, conferring resistance to antimicrobial peptides and phagocytosis of the host immune response. Mobile genetic elements play a crucial role in the dissemination of several factors that play an essential role in the spread of drug-resistant *Klebsiella*.⁸ *K. pneumoniae* surpasses the host immune responses via several virulence genes harbored such as siderophores, fimbriae, adhesins, lipopolysaccharide (LPS), and outer membrane proteins for the survival and immune evasion during infection.⁵ The potential of the resistant bacteria and its genes to survive the hospital sewage water treatments contribute to the spread and act as a reservoir of antimicrobial resistance causing constant risks to humans and animals.⁹ The bacteria can be directly transmitted via direct contact, contaminated environments, and medical equipment are in patients affected with *Klebsiella* infections.

Drug Resistance

Under constant antibiotic pressure, the bacteria acquire antimicrobial resistance genes via mutations, plasmids, and transferable genetic elements resulting in the multidrug-resistant and extremely drug-resistant strains (XDR) possessing a “super resistome.”⁵ This evolution over the years has enabled the bacteria to gain resistance to all possible antibiotics with no treatment options left behind.¹⁰ The likelihood of dissemination of these drug-resistant pathogens is recognized as a global threat. In the era of drug resistance, *K. pneumoniae* is one of the most alarming nosocomial pathogens associated with antibiotic resistance, labeled as a significantly important MDR strain and listed under the ESKAPE groups of pathogens.¹¹ *Klebsiella* species harbor plasmids responsible for resistance to β -lactams, specifically extended-spectrum cephalosporins and also carbapenems.¹² All these make the treatment options limited and end up in the usage of last line of drugs like fluoroquinolones. Unfortunately, *K. pneumoniae* resistance to fluoroquinolones has drastically increased and has been reported in the recent past. Resistance toward fluoroquinolones occurs due to specific mutations in the DNA gyrase, topoisomerase IV, and overexpression of the multidrug efflux system.¹³ The European Antimicrobial Resistance Surveillance Network data for the years 2005 to 2015 demonstrate the *K. pneumoniae* nonsusceptibility toward four major classes of drugs: (a) carbapenems, (b) third-generation cephalosporins, and (c) fluoroquinolones and aminoglycosides (<http://atlas.eccdc.europa.eu/public/index.aspx?Instance>). Multifactorial dissemination of the resistance genes increases the occurrence of the MDR and XDR strains of *K. pneumoniae*. In this regard knowledge on various mechanisms of resistance conferred by the bacteria to various drugs has been described in this review. However, the main focus is on efflux-mediated resistance and alternatives to combat this particular mode of resistance in *Klebsiella*.

As stated earlier, development of resistance in bacteria is an evolutionary process. Resistance to antibiotics could be developed either by spontaneous mutations or through the acquisition of resistance genes. Drug resistance can evolve through several mechanisms. Two broadly categorized mechanisms are the intrinsic (innate) and the acquired resistance. The acquired resistance may be due to the (a) acquisition of exogenous genes by conjugation (transposons) or transformation (plasmids) and transduction (integrons and bacteriophages), (b) mutation of cellular genes, and (c) combination of these mechanisms.¹⁴

Plasmid-Mediated Resistance

In general, the plasmids are double-stranded deoxyribonucleic acid (DNA) encoding almost 10% of the host cell chromosome.¹⁵ In conferring resistance to toxic heavy metals, antimicrobial agents, and virulence determinants, transfer of genes is very efficient in helping the bacterial cell survive in the environment regardless of the lethal antibiotic dose.¹⁴

Transposons and Insertion Elements

The multidrug resistance genes located in a DNA sequence can be transferred from one plasmid to the other, or between genomes via transposon (jumping gene) systems.¹⁶ The insertion (IS) elements have terminal repeat sequences and can recognize a protein necessary to insert or remove a transposon from a specific region.¹⁷ The integrons (gene capture) systems are also responsible for conferring resistance and drive the expression of resistance genes by the use of a specific recombination mechanism. The three main components of the integrons encoded in the conserved 5' segment are (i) enzyme integrase (*int*) that serves as a specific recombination system to insert or remove a new gene cassette, (ii) a specific recombination site (*attI* site), and (iii) a promoter to initiate gene transcription. Nearly all the integrons of class I in the 3' conserved segment have a supplementary gene (*sull*) responsible for conferring resistance to sulphonamide.¹⁸

Mutations are the genetic changes occurring naturally, yet influence the bacteria to sustain and grow under extreme environmental pressure (antimicrobials).¹⁹ The selection of mutants involves factors such as the size of the bacterial population, immune status of the host, presence of other microbiota.²⁰ Frequently, random mutations of the genes that encode antibiotic lytic enzymes generate modified catalysts resulting in an increased spectrum of antibiotic resistance enzymes.

Efflux Pumps and Outer Membrane Permeability

Besides enzymatic modifications, overexpression of the MDR efflux systems substantially contribute to drug resistance. Efflux pumps, specifically the multidrug efflux, is of major concern to the drug therapy as they expel a wide range of substrates and clinically significant drugs.^{21,22} Efflux pumps are dominant in mediating resistance to diverse classes of

drugs and biofilm structures.²³ The efflux pumps not only extrude the given antibiotics out of the bacterial cell but also act as virulence factors and adaptive responses that bestow antimicrobial resistance during infections. In brief, these efflux systems allow the microorganisms in balancing the internal environment by extruding the toxic substances, such as metabolites, antimicrobial agents, and quorum-sensing-regulated expression of virulence determinant. So far, *acrAB*, *oqxAB*, and *kexD* efflux systems have been associated with the antibiotic resistance of *K. pneumoniae*. In addition to *acrAB*, *kexD*, *kdeA*, *kmrA*, *kpnEF*, and *oqxAB* few other efflux genes such as *EefAB*, *ketM*, and *CepA* are also reported in *K. pneumoniae*.^{24,25}

Several efflux systems have so far been functionally characterized—(a) resistance nodulation division (RND) family (*acrAB* and *kexD*), (b) multidrug and toxic compound extrusion family (MATE) (*kdeA*) efflux gene, (c) major facilitator superfamily (MFS) (*kmrA*) gene, and (d) small MDR family (*kpnEF*). Schematic representation of different efflux systems in Gram-negative bacteria is shown in ►Fig. 1.

RND-type Efflux Pump

Reports demonstrate overexpression of the RND-type efflux pump in the clinical isolates of *K. pneumoniae*. The RND type of efflux system consists of three components: inner membrane protein, periplasmic protein, and outer membrane protein. An inner component consists of a multisite binding pocket and recognizes several chemicals and the respective

substrates.²⁶ A trimeric complex formed by the protein component gains access, binds, and finally expels out the substrates. Besides its role in intrinsic resistance toward a wide range of drugs, the RND efflux system facilitates the bacteria in colonizing the gastrointestinal tract and release virulence factors.²⁷ Majority of them describe the overexpression of AcrB and OqxAB increases the opportunity of multidrug resistance in *K. pneumoniae*.²⁸ AcrAB efflux system in *K. pneumoniae* is encoded by *acrRAB* operon, wherein the *acrR* encodes the AcrAB repressor, while the *acrA* and *acrB* encode a periplasmic lipoprotein attached to the inner membrane connecting the outer, inner membranes, and an integral membrane protein situated in the cytoplasmic membrane.²⁹ Resistance to quinolones (ciprofloxacin and nalidixic acid) and other antibiotics (erythromycin, ceftioxin, tigecycline, and chloramphenicol) is associated with the multidrug efflux system AcrRAB. The efflux system is also associated with conferring resistance to antimicrobial peptides in the lungs.²⁷

OqxAB Efflux System

Resistance toward chloramphenicol, nalidixic acid, ceftioxin, and ciprofloxacin is related to the OqxAB efflux system. It is a part of the *rarA-oqxABR* locus, wherein RarA acts as a transcriptional regulator of *oqxAB* and OqxR acting as a transcriptional repressor of *oqxAB* and *rarA*.³⁰ KpnEF efflux pump in *K. pneumoniae* confers resistance to several dyes, detergents, and antimicrobial compounds such as benzalkonium chloride, ceftriaxone, chlorhexidine, acriflavine, cefepime,

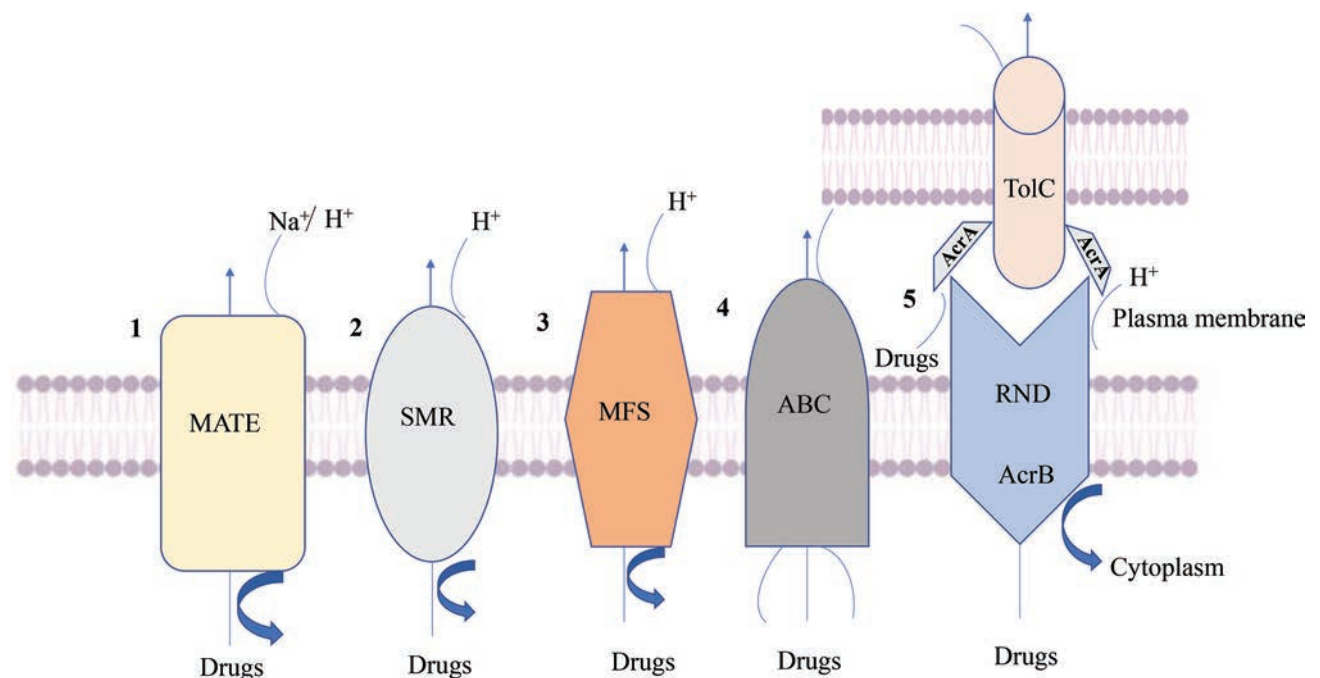


Fig. 1 Schematic representation of different efflux systems in Gram-negative bacteria. (1) MATE: multidrug and toxic compound extrusion superfamily, (2) SMR: small multidrug resistance protein, (3) MFS: major facilitator superfamily, (4) ABC: ATP-binding cassette transporter, and (5) RND: resistance nodulation division superfamily.

colistin, erythromycin, streptomycin, rifampin, triclosan, and tetracycline.³¹

Other Related Efflux Systems

A subset of inner membrane proteins concerned with the MFS function as efflux pumps, decrease the intracellular concentration of a broad spectrum of drugs and chemicals, thus conferring resistance to the bacteria.³² The efflux pump *EefAB*, though not associated with the antimicrobial drug resistance, was observed to colonize the murine digestive tract²⁵; *ketM* was not significantly found related to contributing toward resistance to antibiotics.

In *K. pneumoniae* loss of outer membrane protein in synchronization with the acquisition of ampC β -lactamase and new generation carbapenemase A confers resistance to carbapenems and aminoglycosides, and reduced sensitivity toward fluoroquinolones, tetracycline, chloramphenicol, erythromycin, trimethoprim, ethidium bromide, and meropenem.¹⁵ Mutations in the quinolone resistance-determining region in *K. pneumoniae* and *K. oxytoca* are associated with the resistance phenotypes.³³

Efflux Pump Inhibitors

Inhibiting these efflux pumps might seem like an effective strategy at times when the conventional antibiotics remain no longer effective. Establishment of natural substrates and efflux pump inhibitors (EPIs), that enable effective accumulation of drug inside the bacterial cell, enhances the antibacterial activity. Some of the plant-derived EPIs used are reserpine, pipeline, catechin gallates, flavonoids, and geraniol.³⁴ Frequently used synthetic efflux inhibitors to detect the efflux activity in *Klebsiella pneumoniae* are carbonyl cyanide-chlorophenylhydrazone (CCCP) and phenylalanine-arginine β -naphthylamide (PA β N).³⁵ General mechanism of EPIs is depicted in ►Fig. 2.³⁶ Many studies have

demonstrated the efficacy of the efflux inhibitors in inducing sensitivity to the bacteria, which still restores hope within us.

Fang et al³⁷ exposed the *K. pneumoniae* carbapenemase-producing tigecycline-resistant isolates to 1-(1-naphthylmethyl)-piperazine (NMP). On exposure to EPI *K. pneumoniae* isolates previously resistant to multiple drugs showed an obvious decrease in the minimum inhibitory concentration. Several other studies were conducted to evaluate the efficacy of both natural and synthetic EPIs on MDR strains of *K. pneumoniae* and other clinically significant nosocomial pathogens and proved it efficacious in inducing sensitivity toward various classes of drugs.^{28,38-60} Related results were obtained where PA β N and reserpine tested against commonly used antimicrobial agents in the case of clinical isolates of *Enterobacteriaceae*.^{61,62} Van Acker and Coenye⁶³ reported the role of PA β N, NMP, CCCP, and thioridazine in significantly reducing the biofilm formation in *E. coli*, *K. pneumoniae*, *Pseudomonas aeruginosa*, *Burkholderia cenocepaci*, and *Salmonella*. Aghayan et al⁶⁴ studied the potential effects of beberine and palmatine, naturally derived EPIs on the MexAB-OprM efflux system of *P. aeruginosa* isolated from burn infections. Rodrigues et al⁶⁵ suggested the use of EPI as therapeutic adjuvants to increase the efficacy of the existing antituberculosis drugs and to accelerate the treatment efficacy. Lv et al⁶⁶ identified a novel RND multidrug-efflux pump gene cluster (TMexCD1-TOprJ1) in *K. pneumoniae* isolated from animal source. Similar efflux pump sequence was also found in *P. aeruginosa*, *E. coli*, and *Salmonella*, which threatens the possibility of global dissemination. EPIs against the novel efflux system could restore the antibiotic sensitivity by decreasing the MIC of the drugs. Grimsey et al⁶⁷ demonstrated the efficacy of chlorpromazine and amitriptyline as EPIs to restore the antibacterial activity of the given antibiotics and inhibit the AcrB-mediated efflux. Abbas et al⁶⁸ investigated the potential inhibition activity of novel EPI (metformin) on AcrAB and MdtK efflux pumps of *K. pneumoniae*. Their reports suggest the use of metformin over the

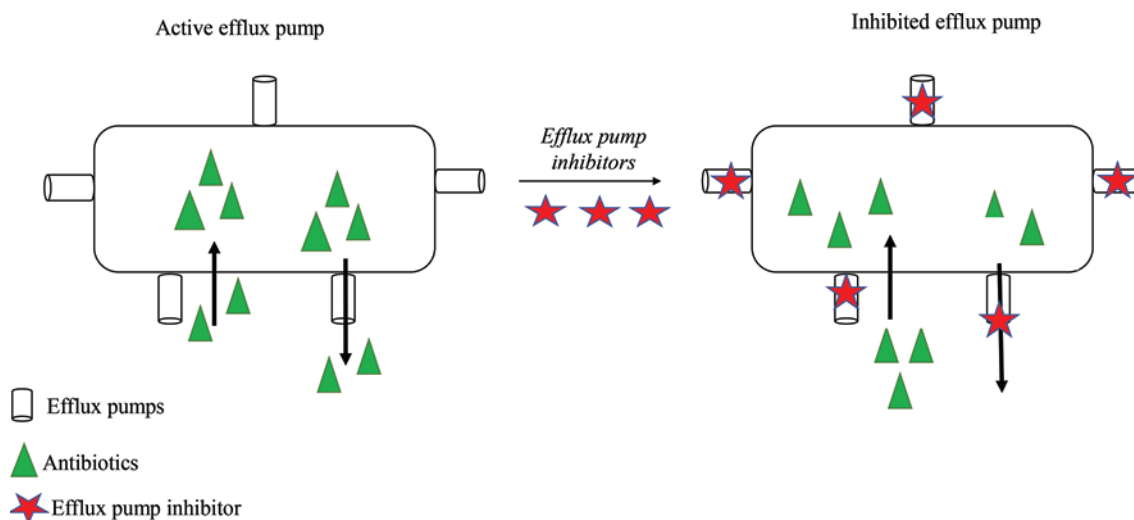


Fig. 2 Representation of mechanism of efflux pump inhibitor (EPI). (Modified from source Shriram et al. 2018).

existing EPIs ascorbic acid and verapamil in restoring the efficacy of the antibiotics. Reza et al⁶⁹ reported several EPIs as effective biofilm disruptors in the ESKAPE pathogens. Seukep et al⁷⁰ listed the use of numerous plant-derived secondary metabolites as nontoxic source of EPIs that would allow the use of antibiotics that were clinically ineffective due to resistance. Adam et al⁷¹ developed six new classes of EPIs effective against *E. coli* AcrAB-TolC, clinical strains of *A. baumannii*, and *K. pneumoniae*.

Nevertheless, efflux pumps are a major resistant determinant in the increasing issue of bacterial infections worldwide. Identification of small-molecule EPI in restoring the effectiveness of the available antibiotics has restored hope. The greatest challenge in the generation of active EPI against Gram-negative bacteria, is that the penetration is controlled by porins, favorable to only zwitterionic and smaller hydrophobic molecules. On the other hand, major RND efflux pumps prefer large hydrophobic molecules.⁷² In this instance, penetration into the bacteria could be improved by identification of suitable position on the EPI, by rational design by which the charge groups could be attached enhancing the EPI penetration. A group of pyranopyridine derivatives has been clearly shown to exhibit inhibitory effects against the AcrAB-TolC efflux system to date. Likewise, a series of pyridopyrimidinones were proved best against MexAB-OprM. D13-9001 was a well-optimized candidate which served effectively against *P. aeruginosa* efflux system (MexAB-OprM), but could not continue in clinical trials because it failed to inhibit MexXY-OprM.⁷² The PABN-derived EPIs are currently the most studied and developed families of inhibitors against *P. aeruginosa*.⁷² To date, pyranopyridines, peptidomimetics, and indole derivatives have been patented as promising compounds. No EPIs have been clinically approved to date, mainly because of poor pharmacokinetic properties and low in vivo efficacy.⁷²

Antisense Oligonucleotides

There is always a quest for an alternative therapeutic strategy in combating drug resistance conferred by efflux pumps in drug-resistant pathogens. In the quest, the antisense therapeutics contribute as an alternative antibiotic therapy with the aid of short single-stranded nucleic acid sequences modified to form stable oligomers. The molecules are termed antisense oligonucleotides (ASOs) due to their sequence complementarity to their messenger RNA (mRNA). ASOs modify the gene expression in a sequence-specific manner by binding to its complementary mRNA and thus inhibit its translation into protein.⁷² Zamenick and Stephenson, pioneers in the field of antisense therapy, reported in Rou's sarcoma virus in infected chicken embryo fibroblast cells the addition of a 13-mer oligonucleotide complementary to the repeated sequences located at the ends of the genome inhibited their replication.⁷³ These scientists set a benchmark for the utilization of the antisense technology. Ever since this pioneering experiment was put forth, various strategies were designed as therapeutics effects for the treatment of diverse diseases like bacterial, viral, genetic disorders, and cancer. Toxicity,

inability to penetrate the target, and nonspecific effects were the major challenges during the early years of antisense research which slowed down the progress. Following which, several antisense drugs of diverse chemical nature working through different mechanisms, including the utilization of siRNAs have been approved for trials.

ASOs could be exploited to suppress the bacterial antibiotic resistance determinants and increase its susceptibility. Increased antibiotic-resistant bacterial pathogens not only affect the ability to treat infectious diseases and complicate the medical procedure but also impose an economic burden on the health care system. ASOs technology permits the generation of antisense drugs that either have antibiotic activity or help to restore the ability of certain antimicrobials. Several studies have demonstrated the therapeutic efficacy and applications of the ASOs as mechanisms of inhibition to design therapies for bacterial infections.⁷⁴⁻⁸³ A similar strategy of ASOs could be exploited against the MDR efflux system of *K. pneumoniae*. Versatility and high specificity of complementarity of the antisense oligonucleotides allow the generation of antisense drugs that disable resistance to antimicrobials and act as adjuvants to join the armamentarium to fight multidrug resistance.

This technology holds hope in the treatment of a diverse group of diseases including bacterial, viral, genetic disorder, and cancer. In the years to come we could witness the antisense adjuvants become a reality.

Conclusion

Bacterial resistance to antibiotics is a critical issue and efflux systems are influential in this phenomenon. Efflux systems are authoritative for intrinsic resistance in several pathogens. As they are located on the plasmids, they could be acquired by other microbes. Diverse efflux systems have been characterized, but the MDR transporters of the RND and MFS superfamilies are the most implied in the clinical resistance of Gram-negative bacteria. EPIs for such systems have been identified. Although selectivity and stability are the concerned drawbacks the results are yet encouraging to prospect in this field. Future works could consider improvising the inhibitory activity of the molecules. The antisense oligonucleotide is a promising alternative strategy with potential therapeutic applications in the field of medicine and industrial microbiology.

Conflict of Interest

None declared.

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