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REVIEW

Proteomic Insights into Antimicrobial Resistance Mechanisms in Human-Associated Bacterial Pathogens

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Abstract
Antimicrobial resistance (AMR) has emerged as a critical global health challenge, supported in part by bacterial taxa that act as reservoirs of resistance determinants, facilitating their dissemination across microbial communities under antibiotic selective pressure. Among the diverse mechanisms underlying AMR, enzyme-mediated resistance plays a dominant role. These enzymes typically inactivate antibiotics or modify their targets by transferring specific functional groups or hydrolyzing the antibiotic molecules. Recent evolutionary pressures have fostered the emergence of bifunctional resistance enzymes, which provide simultaneous protection against multiple antibiotic classes and significantly enhance bacterial fitness. The remarkable diversity of these enzymes largely arises from divergent evolution, which has produced extensive enzyme superfamilies within bacterial systems. Collectively, they encompass a wide range of molecules that execute distinct resistance strategies, thereby fortifying microbial survival. To drive the discovery of novel therapeutics, it is essential to elucidate the expression patterns, structural dynamics, and functional mechanisms of these enzymes. Recent advances in omics technologies have transformed our capacity to investigate microbial defence systems, offering deep, high-throughput insights into the molecular underpinnings of resistance. This review provides an overview of critical resistance enzymes responsible for antibiotic modification and target protection in human-associated bacterial pathogens. Enhancing our understanding of these mechanisms is essential for developing next-generation therapeutics, including antibiotic potentiators, to effectively combat bacterial drug resistance.

Keywords HGT · Antibiotics · Drug resistance · Proteome · LC-MS

Introduction

Microbial evolution has had a profound impact on human health and disease. Over the course of more than two billion years, microbes have evolved remarkable adaptive mechanisms, including antimicrobial resistance [1]. However, the rate of resistance evolution has accelerated dramatically in recent times. The rising prevalence of antimicrobial-resistant microbes within and outside the human body is significantly influenced by global human mobility, compromised immune function, and the increasing burden of metabolic disorders. Consequently, the global reliance on antibiotics has surged over the past two decades. This extensive antibiotic use exerts strong selective pressure, promoting the survival and spread of resistant strains worldwide. AMR poses a critical global threat not only to humans but also to animals [2]. Despite significant advancements in medicine and healthcare, bacterial resistance continues to be one of the most pressing challenges to humanity. Bacteria possess an exceptional ability to adapt to environmental stresses and evolve rapidly in response to each new class of antibiotics.

A key concern is that bacteria can persist both in environmental niches and within the human body while disseminating resistance to a wide spectrum of antibiotics. Resistance can emerge through spontaneous mutations (e.g., base substitutions, SNPs, frameshifts) or horizontal gene transfer

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(HGT). The transfer of resistance genes via mobile genetic elements (MGEs), such as plasmids, transposons, and integrons, further accelerates the global spread of AMR. Genomic sequencing studies reveal that many resistance-associated enzymes belong to large superfamilies [3], some of which evolved from genes that originally encoded unrelated, non-resistance proteins. The localization of such enzymes on mobile DNA is a key factor driving their rapid dissemination across microbial populations. Therefore, studying MGEs and their encoded enzymes provides critical insight into the molecular mechanisms and epidemiology of antibiotic resistance.

Multiple mechanisms underlying antibiotic resistance have been identified and can be grouped into those that utilize enzymes or not for the bacterial defense strategies. Resistance due to the non-enzymatic actions primarily includes: reduced cell wall permeability, active efflux pumps, bypass of targeted metabolic pathways, and biofilm formation. Whereas resistance due to enzymatic actions can be broadly classified according to their biochemical functions, but two major mechanisms dominate: enzymatic degradation or transformation of antibiotics and enzymatic modification of antibiotic targets, notably a large proportion of these enzymes belong to the transferase class [4]. A comprehensive understanding of these underlying mechanisms is essential for developing more effective chemotherapeutic strategies.

Antibiotic Degradation or Transformation

Microbes employ a variety of mechanisms to develop drug resistance; however, enzymatic degradation or structural modification of antibiotics remains one of the most prevalent and effective strategies used by these pathogens. The following sections summarize key enzymes identified through functional studies that remodel antibiotic molecules, providing deeper insights into the molecular basis of AMR (Fig. 1).

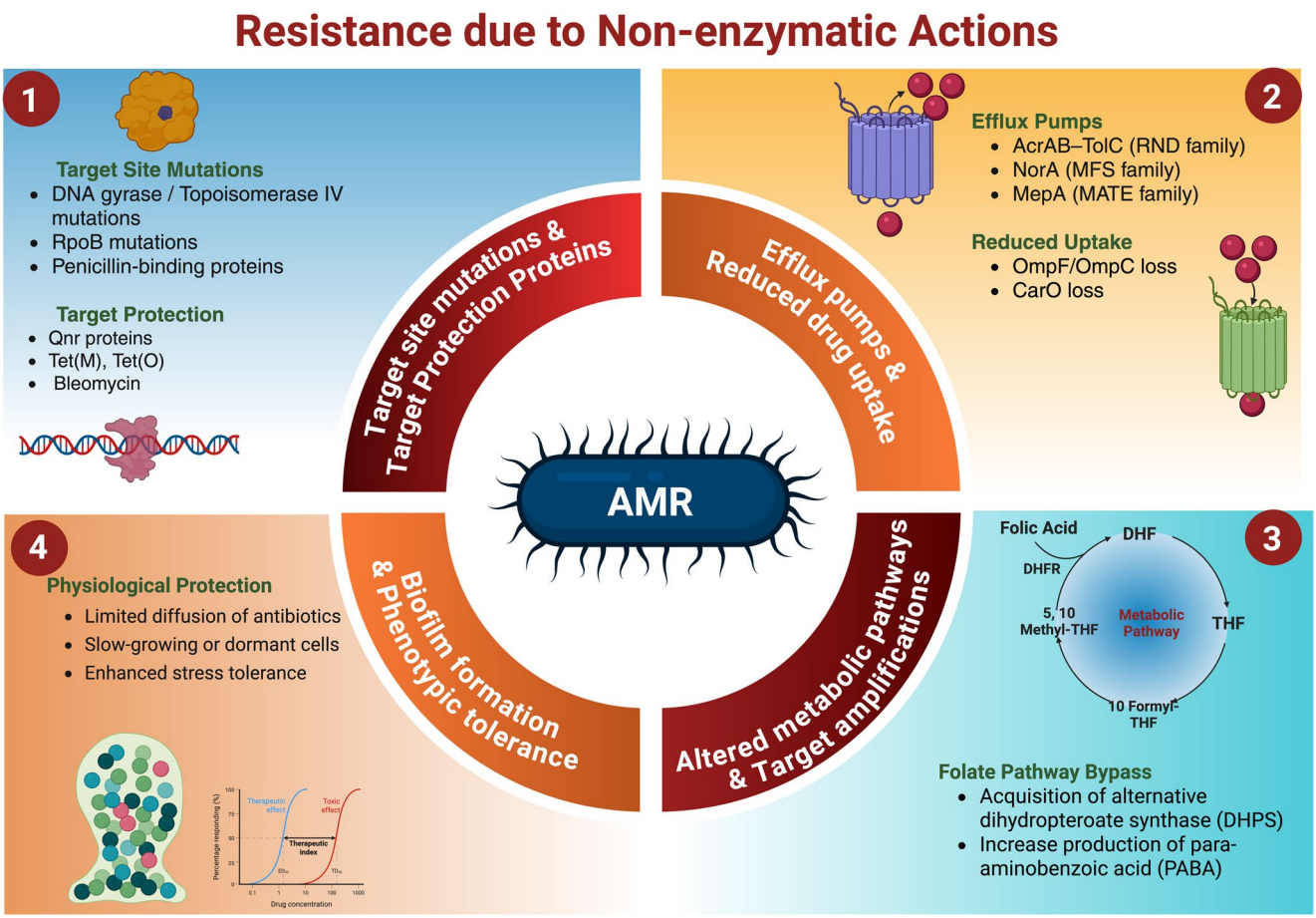

Fig. 1 Schematic illustration of non-enzymatic mechanisms contributing to antibiotic resistance in bacterial pathogens. Spontaneous mutations in antibiotic targets reduce drug binding and efficacy. Metabolic bypass pathways enable bacteria to circumvent inhibited biochemical steps. Efflux pumps actively expel antibiotics from the cell, lowering intracellular drug concentrations. Biofilm formation creates a protective multicellular matrix that restricts antibiotic penetration and promotes tolerant physiological states

Cell Envelope Targeting Antibiotics

β-lactams

β-lactam antibiotics represent one of the most vital and widely used classes of antibacterial agents globally. Since the groundbreaking discovery of penicillin, the first β-lactam antibiotic, numerous derivatives, including cephalosporins, carbapenems, thiopenems, and monobactams, have been developed and extensively employed to combat bacterial infections [5]. The bacterial cell wall, a critical structure for maintaining cell shape and integrity under hostile conditions, is composed of alternating units of N-acetylmuramic acid (NAM) and N-acetylglucosamine (NAG). To strengthen this structure, penicillin-binding proteins (PBPs), which function as transpeptidases, catalyze the cross-linking of pentapeptide chains attached to the D-alanine-D-alanine termini of NAM units. The β-lactam ring in β-lactam antibiotics closely mimics the D-alanine-D-alanine moiety of NAM, leading PBPs to mistakenly recognize the antibiotic as their natural substrate. This results in irreversible acylation and inactivation of the PBPs, disrupting cell wall synthesis and ultimately causing bacterial cell lysis [6, 7].

β-lactamases (Hydrolase) The effectiveness of β-lactam antibiotics has been greatly reduced due to the emergence of β-lactamase enzymes, which confer resistance by hydrolyzing the β-lactam ring essential for antibacterial activity. These enzymes form a large superfamily of over 2000 members and are classified under Ambler's scheme into four classes based on amino acid sequence: A (penicillinases), B (metallo-β-lactamases), C (cephalosporinases), and D (oxacillinases) [8]. Serine β-lactamases (classes A, C, and D) contain an active-site Ser residue that attacks the β-lactam carbonyl carbon, forming a covalent acyl-enzyme intermediate. Subsequent hydrolysis by water breaks this intermediate, opening the β-lactam ring and inactivating the antibiotic. In contrast, class B metallo-β-lactamases utilize one or two Zn^{2+} ions to activate a water molecule, which directly attacks the β-lactam ring without forming a covalent intermediate. These enzymes are clinically significant because they hydrolyze a broad spectrum of β-lactams, including carbapenems, and are not inhibited by conventional β-lactamase inhibitors [9]. Further, β-lactamases can also be categorized by substrate range into narrow-, moderate-, broad-, and extended-spectrum β-lactamases (ESBLs). The first plasmid-mediated β-lactamase, TEM-1, was identified in Gram-negative bacteria during the 1960s [10], while CTX-M-type β-lactamases have since become the most prevalent ESBLs, driving the global "CTX-M pandemic" [11]. The β-lactamase genes are either encoded within the bacterial chromosome or acquired on plasmids, transposons, or insertion sequences, facilitating HGT among bacterial species [12]. The major chromosomally encoded β-lactamase, AmpC have been found in human pathogens, such as *E. cloacae*, *K. aerogenes*, *S. marcescens*, *M. morganii*, and *C. freundii* [13]. Key plasmid-encoded acquired β-lactamases genes include bla_{TEM}, bla_{SHV}, and $bla_{CTX-M-15}$ (serine penicillinases) [14].

Several studies have shown the potential of mass spectrometry (MS) - based platforms to quantify and detect novel resistance enzymes in bacterial isolates. Proteomic investigations consistently demonstrate differential expression of β-lactamases across major human pathogens [15]. In carbapenem-resistant *K. pneumoniae*, MS-based studies have confirmed the contribution of NDM-5 and other β-lactamases to carbapenem resistance, providing mechanistic resolution beyond genomic prediction alone [16]. Similarly, in MDR *A. baumannii*, proteomic profiling has revealed upregulation of OXA-type carbapenemases (e.g., OXA-23, OXA-72), metallo-β-lactamases, and metal-dependent hydrolases of the aminoacylase-2/carboxypeptidase-Z family, underscoring the coordinated expression of multiple enzymatic defenses [17]. Quantitative analyses of membrane fractions further demonstrated > 2-fold increases in AmpC and OXA-51 in carbapenem-resistant isolates [18, 19], while adaptive responses to ceftriaxone exposure included altered expression of OXA-165, OXA-146, and OXA-23 [20]. Notably, proteogenomic studies identified OXA-23 packaged within outer membrane vesicles of MDR strains following imipenem treatment, suggesting a potential extracellular dissemination and protective mechanism [21]. The identification of the β-lactamase AXC in *A. xylosoxidans* as a marker of acquired carbapenem resistance and the demonstration that heterologous expression in *E. coli* confers increased carbapenem resistance, highlights the capacity of proteomics to reveal previously unannotated resistance determinants [22]. In clinical *E. coli* isolates, differential expression patterns associated with ESBL production, including TEM-52, have further clarified the molecular response to β-lactam stress [23]. Comparative label-free proteomics of methicillin-resistant versus methicillin-susceptible *S. aureus* (MRSA vs. MSSA) demonstrated marked upregulation of β-lactamase and PBP2a in oxacillin-treated MRSA, reinforcing their central roles in resistance phenotypes [24].

Importantly, MS-based analyses extend beyond β-lactamases. Phosphoproteomic profiling of *mcr-1* positive, colistin-resistant *E. coli* revealed induction of AmpC in response to colistin or ciprofloxacin exposure, alongside modulation of transcriptional regulators and efflux systems, illustrating the interconnected regulatory networks underpinning multidrug resistance [25]. However, not all

post-translational modifications (PTMs) enhance resistance. Quantitative acetylome analysis in ampicillin-, kanamycin-, and polymyxin B-resistant *E. coli* indicated that lysine acetylation can negatively impact central metabolism while promoting motility, suggesting complex trade-offs between fitness, stress adaptation, and resistance [26]. Therefore, direct measurement of resistance proteins and PTMs provides definitive, quantitative, and functional evidence of AMR, eliminating ambiguity inherent in the gene-based predictions.

Glycopeptides

Glycopeptides exert their antibacterial effects by binding to the terminal D-alanine residues of the peptidoglycan layer in the bacterial cell wall, thereby inhibiting cell wall synthesis. These glycosylated antibiotics' class includes first-generation natural compounds such as vancomycin and teicoplanin, as well as second-generation semi-synthetic derivatives like telavancin, oritavancin, and dalbavancin [27].

Glycopeptide Nucleotidyltransferase (Transferase) Glycopeptide nucleotidyltransferase is a resistance enzyme that inactivates glycopeptide antibiotics by catalyzing their nucleotidylation. The enzyme transfers a nucleotidyl group (commonly AMP) from ATP to hydroxyl group on the glycopeptide molecule. This modification alters the chemical structure of the antibiotic, reducing its ability to bind to its target in the bacterial cell wall. In studies contrasting vancomycin-susceptible and vancomycin-intermediate *S. aureus* strains (VSSA vs. VISA), upregulation of O-nucleotidyltransferase (Ant9) was observed in VISA isolates, together with increased expression of canonical resistance determinants such as PBP2a (MecA), MurE, MsrA2, and FmtA [28]. These findings suggest that nucleotidyltransferase activity may be embedded within a coordinated resistance network involving peptidoglycan biosynthesis, oxidative stress mitigation, and altered penicillin-binding protein function. Notably, vancomycin-induced adaptations were also associated with cross-resistance to structurally unrelated antibiotics, underscoring the pleiotropic consequences of cell wall-directed stress responses. Proteomic comparisons of isogenic VSSA and heterogeneous VISA (hVISA) strains further revealed upregulation of stress- and cell wall-associated proteins, including MsrA2, the transglycosylase precursor IsaA, and alkyl hydroperoxide reductase subunit C (AhpC), reinforcing the concept that intermediate glycopeptide resistance reflects global physiological reprogramming rather than a single enzymatic event [29]. Additional studies in clinical VISA-derived isogenic strains identified differential expression of bleomycin resistance protein and kanamycin nucleotidyltransferase, suggesting co-selection or co-regulation of multiple antibiotic-modifying enzymes under glycopeptide pressure [30]. Beyond staphylococci, integrative proteomic and transcriptomic analyses in vancomycin-resistant *E. faecalis* have delineated extensive alterations in stress response pathways, regulatory networks, and cell wall biosynthetic machinery, further emphasizing the multifactorial nature of glycopeptide resistance [31]. Collectively, these data indicate that while glycopeptide nucleotidyltransferases may contribute directly to antibiotic inactivation, their functional significance must be interpreted within the broader context of cell envelope remodeling, oxidative stress adaptation, and regulatory rewiring that collectively shape glycopeptide resistance phenotypes.

Fosfomycin

Fosfomycin is a broad-spectrum antibiotic that is primarily used for the treatment of urinary tract infections. It is a member of the phosphonic acid class of antibiotics that functions by inhibiting the enzyme MurA (UDP-N-acetylglucosamine enolpyruvyl transferase), a key catalyst in the biosynthesis of the bacterial cell wall. Acting as a structural analogue of UDP-N-acetylglucosamine (UDP-GlcNAc), one of MurA's natural substrates, fosfomycin disrupts peptidoglycan precursor formation, thereby impairing cell wall synthesis [32, 33].

Fosfomycin Epoxidase (Hydrolase) Fosfomycin-modifying enzymes inactivate the antibiotic and include metalloenzymes, such as FosA, FosB, and FosX/FosE/FosI. FosA is a glutathione-*S*-transferase that uses Mn^{2+} and K^{+}, FosB is a bacillithiol-*S*-transferase requiring Mg^{2+} as a cofactor, whereas FosX is a Mn^{2+}-dependent epoxide hydrolase that neutralizes fosfomycin through a hydration reaction [33]. Evolutionarily, FosX is related to a broader superfamily of metalloenzymes, which includes glyoxalase I, methylmalonyl-CoA epimerase, and estradiol dioxygenase. Enzymes of the FosX/FosE/FosI family mediate resistance to fosfomycin by catalyzing the hydrolytic cleavage of its epoxide ring, effectively inactivating the antibiotic. Proteomic profiling of resistant *L. monocytogenes* has revealed the presence of FosX/FosE/FosI thiol transferases associated with fosfomycin resistance [34].

Glutathione S-transferase and Bacillithiol S-transferase (Transferase) The thiol transferases are enzymes involved in the biosynthesis of glutathione and bacillithiol. These compounds act as scavengers of reactive oxygen species and are also involved in the regulation of the thioredoxin system and resistance to antibiotics and other toxic compounds. FosA transfers glutathione to open up the oxirane

ring of fosfomycin, leading to its inactivation. Similarly, Gram-positive bacteria lacking glutathione use bacillithiol or L-Cys (FosB) for fosfomycin neutralization. Although no proteomic report has been published to date on the expression levels of FosA and FosB enzymes, many genomic accounts have confirmed the existence of *fos* genes in drug resistance, such as *S. epidermidis* [35].

Lipopeptides

Lipopeptide antibiotics are a potent class of antibacterial agents primarily active against Gram-positive bacteria. Lipopeptides contains peptide core with a lipid tail and are widely employed in the treatment of skin and soft-tissue infections, respiratory tract infections, and sepsis. Representative members of this group include daptomycin, ramoplanin for Gram-positive, and polymyxin B for Gram-negative bacteria [36].

Daptomycin Acetyltransferase (Transferase) Daptomycin acetyltransferase confers antibiotic resistance by acetyl-CoA-dependent modification and inactivation of daptomycin. Transcriptomic analyses of clinically isolated daptomycin-resistant *S. aureus* strains have revealed altered expression of Gcn5-related N-acetyltransferases (GNATs) and metallo-β-lactamase genes, implicating enzymatic modification pathways in adaptive resistance [37]. Complementary quantitative proteomics subsequently validated the differential upregulation of acetyltransferases in resistant isolates, with nearly 30% of the induced proteins exhibiting catalytic activities linked to antibiotic resistance [38]. Together, these findings underscore a coordinated enzymatic reprogramming in daptomycin-resistant *S. aureus*, highlighting the contribution of acetyltransferase-mediated modification and broader metabolic adaptation to resistance phenotypes. Thus, proteomics distinctly predicts resistance spectrum of bacterial pathogens by identifying which enzymes are present.

Replication Targeting Antibiotics

Quinolones

Quinolone antibiotics are a class of drugs that inhibit bacterial DNA synthesis by targeting DNA gyrase and topoisomerase IV, enzymes responsible for supercoiling and relaxing bacterial DNA during replication and transcription.

Ciprofloxacin Phosphotransferase, CrpP (Transferase) CrpP has been discovered as a novel ciprofloxacin-modifying enzyme in *P. aeruginosa* pUM505 plasmid [39]. The enzyme catalyzes the transfer of a phosphate group from ATP to the hydroxyl group of ciprofloxacin. By decreasing the affinity of ciprofloxacin for these essential enzymes, CrpP lowers the intracellular activity of the drug and contributes to bacterial survival in the presence of fluoroquinolone antibiotics. Enzymatic assays and mass spectrometry have confirmed the detection of a phosphorylated form of ciprofloxacin in *E. coli* expressing the enzyme. The study revealed *crpP* gene encodes a small protein that confers decreased ciprofloxacin susceptibility with recombinant CrpP exhibits ATP-dependent activity consistent with phosphorylation of ciprofloxacin. However, subsequent reports have suggested that the presence of CrpP does not guarantee ciprofloxacin resistance [40].

Transcription Targeting Antibiotics

Rifamycins

Rifamycins antibiotics constitute a vital class of antibacterial agents that target bacterial RNA polymerase. Members of this group, including rifampicin, rifabutin, and rifapentine, act by binding to the β-subunit of RNA polymerase, specifically within its "fingers" domain, thereby inhibiting RNA synthesis. Rifamycins are a first-line therapy for tuberculosis and are typically administered in combination with other drugs to enhance treatment efficacy and prevent resistance development.

Rifamycin ADP-Ribosyltransferase, RifR (Transferase) RifR, a member of the ADP-ribosyltransferase family, confers resistance by transferring an ADP-ribose group from NAD^+ to the hydroxyl group at the C23 position of rifamycin molecules, effectively inactivating the antibiotic. Using *M. smegmatis* as a surrogate model, Giddey et al. applied quantitative MS-based proteomics to dissect phenotypic resistance mechanisms and characterize the temporal response to sub-lethal rifampicin exposure. Their analysis demonstrated a progressive induction of rifampin ADP-ribosyltransferase, implicating enzymatic drug modification as a key contributor to adaptive resistance under antibiotic stress [41].

Rifamycin Monooxygenase, Rox (Oxidoreductase) Rox is an enzyme that belongs to the family of flavin (FAD)-dependent monooxygenases, which are a group of enzymes that catalyze the oxidation of a variety of organic compounds by using molecular oxygen (O_2) as an oxidant. Rox monooxygenase is found in several bacteria, specifically in *Actinobacteria*, and is responsible for the inactivation of the rifamycin antibiotics. The enzyme causes monooxygenation of the 2-position of naphthyl group of the antibiotic, which subsequently results in ring opening and linearization. The most commonly acquired genes include *iri* and *rox*. A study

demonstrated that the *rox* gene from *N. farcinica* inactivates rifampicin by converting it into 2′-N-hydroxy-4-oxo-rifampicin, a non-functional derivative. Heterologous overexpression of *rox* in *E. coli* significantly increased rifampicin resistance, directly linking Rox-mediated drug modification to acquired resistance [42].

Rifamycin Phosphotransferase, RPH (Transferase) Unlike other common kinases, which generally add γ-phosphate of the ATP, RPHs are dikinases, transferring β-phosphate of ATP to the -OH group at C21 of rifamycin antibiotics. *rph* genes have been identified as the gene encoding for the resistant protein. Spanogiannopoulos et al. identified a conserved genetic element encoding a previously unrecognized resistance determinant that mediates rifamycin inactivation via phosphorylation. This discovery, validated in pathogens including *L. monocytogenes*, expands the known repertoire of enzymatic rifamycin resistance mechanisms and underscores the evolutionary conservation of drug-modifying strategies [43].

Translation Targeting antibiotics

Aminoglycosides

Aminoglycosides exert their antibacterial activity by binding to the 30 S subunit of the bacterial ribosome, thereby disrupting tRNA binding and impairing protein synthesis. Common examples of this antibiotic class include streptomycin, gentamicin, and tobramycin. Due to their frequent localization on MGEs, the genes encoding aminoglycoside-modifying enzymes (AMEs) are widespread among pathogenic bacteria like *P. aeruginosa*, *S. aureus*, etc., facilitating rapid dissemination of resistance traits [44, 45]. Resistance to aminoglycosides often arises through the coordinated action of three major transferase families: aminoglycoside N-acetyltransferases (AACs), aminoglycoside O-phosphotransferases (APHs), and aminoglycoside O-nucleotidyltransferases (ANTs).

Aminoglycoside N-Acetyltransferases, AACs (Transferase) Among the three major families, AACs represent the largest and most diverse group, with over 48 known variants, further classified into AAC(1), AAC(2'), AAC(3), AAC(6') main subfamilies based on site-specific activity. AACs confer resistance by catalyzing the acetylation of amino groups on aminoglycosides using acetyl-coenzyme A as the acetyl donor [46]. Proteomic investigations have delineated diverse enzymatic resistance determinants across bacterial species. In *Listeria* spp., enzymes such as aminoglycoside N(3)-acetyltransferases, GNAT family acetyltransferases, etc. have been identified [34]. Quantitative iTRAQ-based proteomics further revealed significant physiological remodeling and differential overexpression of AACs in MDR *A. baumannii* compared to susceptible strains [47], with AAC(1) and GNAT specifically implicated in gentamicin resistance [20]. Therefore, proteomic analysis reflects that higher expression of AAC leads to high-level resistance. Additionally, streptothricin acetyltransferase encoded on plasmid pIE636 in *E. coli* mediates antibiotic neutralization via C16 acetylation, reinforcing the central role of drug-modifying enzymes in resistance evolution [48].

Aminoglycoside Phosphotransferases, APHs (Transferase) The APHs constitute the second largest class of AMEs, with seven variants of the *aph* gene family identified. APHs mediate resistance by transferring a phosphate group from ATP or GTP to the hydroxyl moieties on aminoglycoside antibiotics, thereby impairing their ability to bind the aminoacyl-tRNA (A) site of the bacterial 30 S ribosomal subunit [49, 50]. Structural and functional analyses have revealed that APHs possess active-site architectures and tertiary folds resembling those of eukaryotic protein kinases (ePKs). Notably, several inhibitors originally developed against ePKs have also been shown to inhibit APHs activity, supporting the hypothesis that these enzymes share an evolutionary relationship and may have originated from a common ancestral kinase [50, 51]. Integrated proteomic and glycoproteomic analyses of MDR *A. baumannii* reveal coordinated overexpression of APH(3′)-Ia alongside elevated β-lactamases and increased tolerance to gentamicin, underscoring the convergence of aminoglycoside modification and β-lactam resistance pathways [17, 20, 47]. Similarly, clinical isolates of *S. typhimurium* phage type DT104B exhibited upregulation of StrA (APH(3″)-Ib) following ciprofloxacin exposure, suggesting antibiotic-induced modulation of resistance determinants [52]. Complementing discovery-based approaches, targeted PRM (parallel reaction monitoring) proteomics has enabled sensitive detection of APH(3′)-VI in aminoglycoside-resistant *E. coli* and *K. pneumoniae* isolates, highlighting its translational utility for resistance surveillance [53].

Aminoglycoside Nucleotidyltransferases, ANTs (Transferase): ANTs inactivate antibiotics through adenylation. These enzymes catalyze a metal-dependent reaction in which a deprotonated hydroxyl group of the aminoglycoside performs a nucleophilic attack on the α-phosphate of ATP, resulting in the formation of an AMP-antibiotic

phosphoester and the release of pyrophosphate. ANTs are grouped into five major classes based on the specific position of the aminoglycoside molecule that is modified. Within each class, enzymes are further subdivided according to amino acid sequence homology, giving rise to multiple variants, including ANT(2″)-Ia, ANT(3″), ANT(4′)-Ia, ANT(4′)-IIa, ANT(4′)-IIb, ANT(6)-Ia to -If, and ANT(9)-Ia and ANT(9)-Ib [54–56]. Zhang et al. identified a novel ANT(3″)-IIa subclass in *Acinetobacter* spp., where heterologous expression of *ant(3")-IIa* in *E. coli* conferred a 32-128-fold increase in aminoglycoside resistance, functionally validating its role as a potent resistance determinant [57]. Complementing these findings, Foudraine et al. employed targeted LC-MS/MS to detect ANT(2″)-I in clinical *E. coli* and *K. pneumoniae* isolates, demonstrating strong concordance between proteomic detection and whole-genome sequencing (2,416/2,460 isolate-resistance combinations) [53]. Collectively, these studies highlight both the expanding diversity of aminoglycoside-modifying enzymes and the reliability of MS-based approaches for resistance surveillance and validation.

Tetracyclines

Tetracycline antibiotics are a class of antibiotics that inhibit bacterial protein synthesis by binding to the A site of the bacterial ribosome, which is the site where amino acids are added to the growing peptide chain during protein synthesis. By binding to the A site, tetracyclines prevent the binding of the aminoacyl-tRNA, the molecule that carries the next amino acid to be added to the peptide chain, thus inhibiting protein synthesis. Tetracyclines have a broad spectrum of activity; they are active against Gram-positive and Gram-negative bacteria, as well as some types of mycoplasma and chlamydia. Some of the most well-known tetracyclines are tetracycline, doxycycline, and minocycline.

Tetracycline Monooxygenase (Oxidoreductase) Tetracycline monooxygenase belongs to the FAD-dependent monooxygenase family and catalyzes an oxidative inactivation reaction. Mechanistically, the enzyme uses molecular oxygen to generate a reactive flavin-hydroperoxide intermediate, which in turn introduces a hydroxyl group onto the tetracycline through an oxidative hydroxylation reaction. Enzymatic tetracycline inactivation mediated by *tet* genes represents a significant resistance mechanism. Biochemical characterization of TetX2 using UV-visible spectroscopy and reverse-phase HPLC confirmed its tetracycline-modifying activity [58]. Expanding this paradigm, TetX7 was identified in a clinical *P. aeruginosa* (Pa-3) isolate from a cystic fibrosis patient, with disk diffusion and broth microdilution assays verifying its resistant phenotype [59]. Together, these findings underscore the functional diversification and clinical emergence of TetX-mediated tetracycline monooxygenation.

Phenicols

Phenicols including, chloramphenicol, florfenicol, and thiamphenicol, all share a similar mechanism of action, which is to inhibit bacterial protein synthesis. They do this by binding to the 50 S ribosomal subunit, which is responsible for reading the mRNA and assembling the corresponding amino acids into proteins.

Chloramphenicol Acetyltransferases, CATs (Transferase) CATs transfer an acetyl group from acetyl-CoA to one or both hydroxyl groups of chloramphenicol. This acetylation prevents the antibiotic from binding to the 50 S ribosomal subunit. Two types of resistant genes (*cat* and *cfr*) are typically found on plasmids together with other AMDs. Alcalá et al. comprehensively characterized CATs from pathogenic *V. cholerae* and *V. vulnificus* through integrated sequence analysis, crystallographic structural determination, and kinetic evaluation. Their findings classified these enzymes within the Type B CAT family and demonstrated their efficient acetylation-mediated inactivation of chloramphenicol, providing structural and mechanistic insight into chloramphenicol resistance in pathogenic vibrios [60].

Macrolides, Ketolides, Lincosamides, and Streptogramins (MKLS)

MKLS antibiotics inhibit bacterial protein synthesis by targeting the ribosome. Macrolides (erythromycin, azithromycin, clarithromycin) bind to the large ribosomal subunit near the peptidyl transferase center, where they obstruct the ribosomal exit tunnel, leading to premature termination of translation [61]. Ketolides (telithromycin) are structurally related to macrolides but contain modifications at the C-11 and C-12 positions of the lactone ring. These changes allow ketolides to overcome common macrolide resistance mechanisms, including ribosomal target methylation and efflux-mediated resistance. Nafithromycin represents a new ketolide antibiotic designed for the treatment of drug-resistant community-acquired bacterial pneumonia [62]. Lincosamides (clindamycin) also target the 50 S subunit but bind at sites distinct from macrolides. Unlike macrolides, lincosamides lack a lactone ring and are composed of a 1-propyl hydroxy acid linked by an amide bond to an eight-carbon amino sugar. These antibiotics function as structural mimics of peptidyl-tRNA and deacylated tRNA during the early stages of ribosomal translocation, thereby inhibiting protein synthesis [61]. Streptogramins (quinupristin, alfopristin)

represent another class of antibiotics that reversibly bind to the 50 S subunit of the bacterial ribosome and inhibit protein synthesis.

Macrolide Esterase (Hydrolase) The macrolide esterase is considered as an important enzyme against macrolide resistance in bacteria. The enzyme acts by cleaving the ester bond within the large macrocyclic lactone ring of the antibiotic. A nucleophilic residue in the enzyme's active site attacks the carbonyl carbon of the lactone ring, leading to hydrolysis and opening of the ring structure. LC-MS analyses have validated macrolactone ring-opening as the principal inactivation mechanism mediated by EreB, EreC, and EreD across diverse clinically relevant macrolides [63]. Complementing these biochemical findings, Arthur et al. sequenced the *ereB* gene from plasmid pIP1527, demonstrating its role in conferring high-level erythromycin resistance in *E. coli* and highlighting the plasmid-borne, horizontally transferable nature of macrolide resistance determinants [64].

Macrolide Phosphotransferase, Mph (Transferase) Mph mediates transfer of γ-phosphate group from GTP to the antibiotic and grants resistance to a diverse group of bacteria such as *Staphylococcus*, *Klebsiella*, *Pseudomonas*, *Shigella*, *Serratia*, etc. Seven macrolide phosphotransferases have been described so far, coded by *mph* genes. O'Hara et al. purified and functionally characterized Mph(2′) from erythromycin-resistant *E. coli*, identifying it as an inducible intracellular macrolide phosphotransferase. The enzyme displayed marked substrate specificity, exhibiting higher catalytic activity against 14-membered macrolides such as erythromycin compared to 16-membered counterparts, thereby underscoring the structural determinants governing macrolide resistance [65].

Lincosamides Nucleotidyltransferase, Lnu (Transferase) Lincosamide resistance mediated by nucleotidyltransferases has been documented in clinical isolates. In *E. faecium* HM1025, LinB was shown to confer resistance by adenylating the C3 hydroxyl group of lincomycin and clindamycin, thereby impairing antibiotic activity [66]. Similarly, Lnu(E) expression in *S. aureus* RN4220 resulted in a 16-fold increase in lincomycin MIC, with mass spectrometry confirming its function as a nucleotidyltransferase catalyzing lincomycin modification [67]. Collectively, these findings highlight enzymatic nucleotidylation as a clinically relevant mechanism of lincosamide inactivation.

Streptogramin Lyase (Lyase) Streptogramins consist of a mixture of two components: group A, cyclic polyunsaturated macrolactones and group B, cyclic hexadepsipeptides. Hexadepsipeptides are cyclized through the esterification between hydroxyl group of an N-terminal Thr and C-terminal carboxyl. While most antibiotic resistance involves adding a molecule to "clog" the drug, this enzyme performs a "search and destroy" mission by physically breaking the antibiotic's structure. Group B streptogramin resistance in *S. aureus* is mediated by virginiamycin B lyase (VgbB), a divalent metal-dependent enzyme that inactivates cyclic hexadepsipeptides through lactone ring linearization, generating an N-terminal dehydrobutyrine residue [68]. This metal-assisted structural disruption underscores a distinct enzymatic strategy for streptogramin B neutralization.

Streptogramin Acetyltransferase, Vat (Transferase) A chloramphenicol acetyltransferase-related enzyme, virginiamycin acetyltransferase (VatA), sharing ~ 38% sequence similarity with CATs, was identified on *S. aureus* plasmids pIP680 and pIP1156. VatA confers resistance to streptogramin A-type compounds, including virginiamycin M and pristinamycin IIA, via antibiotic acetylation [69]. Expanding this family, VatH was subsequently characterized in quinupristin/dalfopristin-resistant *E. faecium* JS79, further illustrating the plasmid-mediated dissemination and diversification of streptogramin A acetyltransferases [70].

DNA targeting antibiotics

1Bleomycin

Bleomycin is a glycopeptide antibiotic that functions as an anti-neoplastic agent by binding to DNA and creating free radicals. These radicals in turn causes single- and double-stranded breaks in the DNA.

Glyoxalase/Bleomycin Resistance Protein/Dioxygenase, BLMR (Oxidoreductase) BLMR is a member of the glyoxalase enzyme family, participates in metal-dependent (Fe^{2+}, Ni^{2+}, or Zn^{2+}) detoxification of reactive aldehydes such as methylglyoxal. It contributes to bleomycin resistance by binding the antibiotic and preventing it from generating DNA-damaging radicals. Proteomic profiling of resistant *Listeria* spp. has revealed substantial proteome coverage, with the identification of 2,990 non-redundant peptides corresponding to 2,727 proteins. Notably, 395 of these peptides were assigned to proteins implicated in virulence and pathogenicity, including BLMR, suggesting that resistance-associated strains retain or potentially co-regulate key determinants of host adaptation [34]. Several homologous bleomycin-resistance proteins have also been characterized through genomic studies, including BLMS in

methicillin-resistant *S. aureus*, BLMA in soil bacterium *S. verticillus*, and BMLT in various Gram-negative bacteria [71].

Target Modifications

Modification of antibiotic targets through the action of versatile enzymes, rather than through genetic mutations, is becoming increasingly common among resistant bacteria, largely due to HGT (Fig. 2) [72]. Based on the reactions they mediate, these target-modifying enzymes can be categorized into protection proteins, transferases, oxidoreductases, ligases, hydrolases, and other functional classes. Gaining a detailed understanding of the mechanisms by which these enzymes alter molecular targets is clinically crucial for controlling the rise of AMR.

Cell envelope targeting antibiotics

β-lactams

Penicillin-Binding proteins, PBPs (Low-Affinity Target Protein) PBPs are classified based on molecular weight into high-molecular-weight (> 50 kDa) and low-molecular-weight (< 50 kDa) types. High-molecular-weight PBPs are further divided into Class A and Class B: Class A PBPs are bifunctional with transpeptidase and glycosyltransferase activities, whereas Class B PBPs are monofunctional with only transpeptidase activity. Low-molecular-weight PBPs, grouped as Class C, exhibit hydrolase activities such as carboxypeptidase and endopeptidase functions that aid in the regulation of peptidoglycan structure and recycling during cell growth and division [73]. The N-terminal domain exhibits transglycosylation activity, elongating glycan

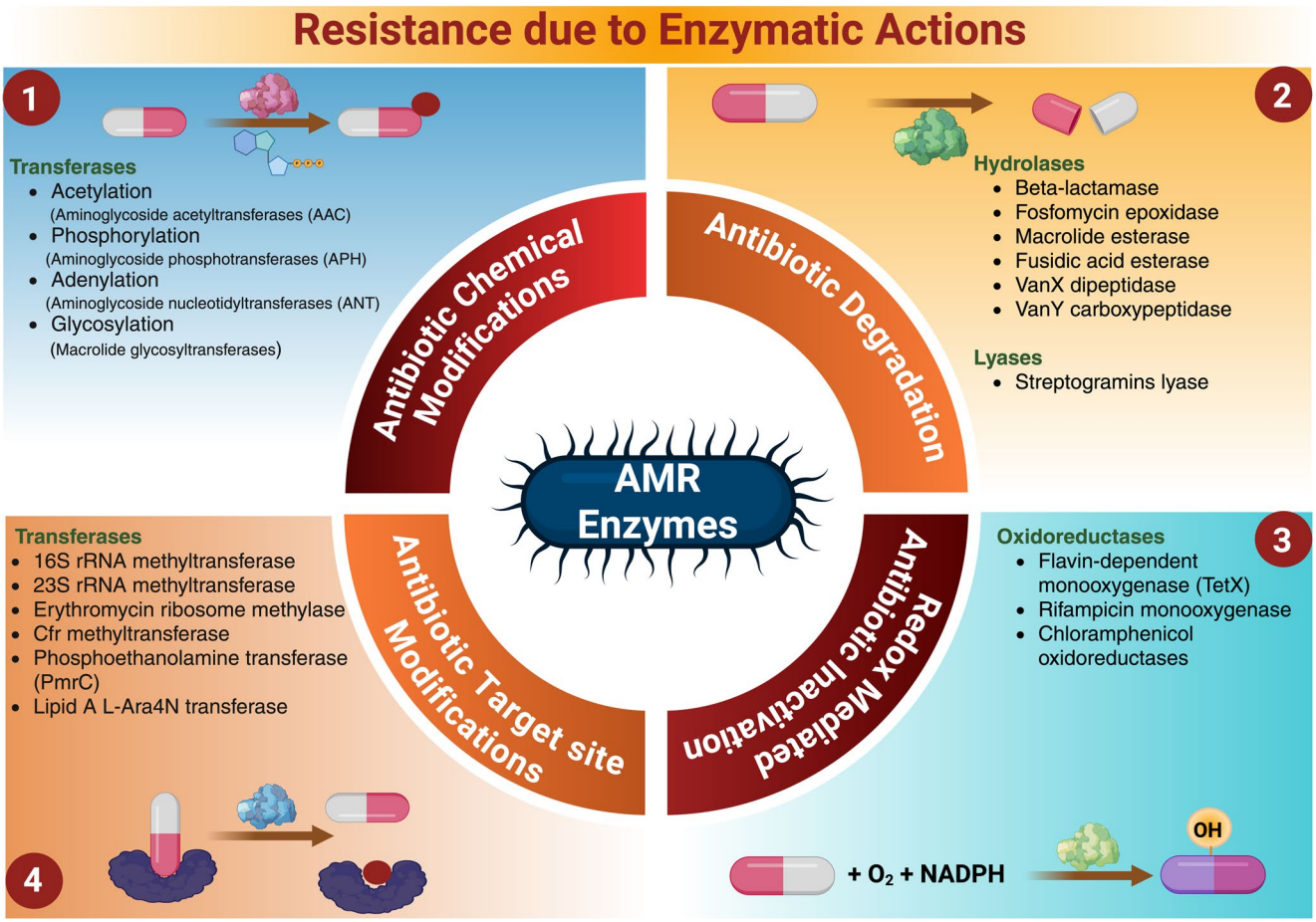

Fig. 2 Schematic illustration of enzymatic mechanisms of antibiotic resistance in bacterial pathogens. The figure illustrates enzymes that confer resistance by chemically modifying antibiotics (e.g., hydrolases, transferases, oxidoreductases) or by enzymatic alteration of antibiotic targets (e.g., rRNA and cell wall-modifying enzymes), thereby reducing drug binding and antimicrobial efficacy

chains by adding NAG and NAM units, while the C-terminal domain catalyzes cross-linking of adjacent glycan chains via pentapeptide linkers (transpeptidation), which is the primary target of β-lactam antibiotics, as described above. A major resistance mechanism involves the production of low-affinity PBPs that retain transpeptidase activity but are poorly inhibited by β-lactams. PBP2a, widely distributed among pathogenic bacteria through MGEs, exemplifies this mechanism.

In drug-resistant *S. aureus*, the *mec* gene family *(mecA*, *mecB*, *mecC*, *mecD)* underpins β-lactam resistance, with comparative proteomics consistently demonstrating differential overexpression of *mecA*-encoded PBP2a in VISA and MRSA strains [74, 75]. Integrated proteomic and in silico analyses further indicate that both elevated expression and structural alterations of PBP2a reduce β-lactam binding affinity. Beyond *S. aureus*, quantitative proteomics has implicated PBP6 and PBP1b in carbapenem resistance in *(A) baumannii* [19], underscoring the broader role of PBPs in β-lactam evasion. Notably, PBPs also contribute to adaptive responses beyond β-lactam pressure. In *(B) subtilis* W168, exposure to sub-lethal daptomycin and friulimicin induced a 3.8-fold upregulation of class B PBPs, as revealed by combined proteomic and transcriptomic analyses [76]. Although β-lactams and lipopeptides target distinct cellular components, both trigger cell envelope stress responses that activate compensatory pathways, including enhanced PBP expression, illustrating the interconnected nature of bacterial stress adaptation networks.

Monofunctional Biosynthetic Peptidoglycan Transglycosylase, MGT (Transferase) In a pioneering comparative proteomic study, Vashist et al. employed 2D-DIGE coupled with MALDI-TOF/TOF and ESI-MS/MS to profile the outer membrane proteome of *A. baumannii* ATCC 19,606 and a carbapenem-resistant clinical isolate. Several proteins with ≥ 2-fold upregulation (confidence > 90%) were identified, including membrane-bound MGT [77]. Unlike bifunctional PBPs, MGT specifically catalyzes the glycan chain polymerization of lipid II on the periplasmic side of the cytoplasmic membrane, suggesting a contributory role in peptidoglycan remodeling and carbapenem resistance-associated cell wall adaptation.

Glycopeptides

VanA Ligase (Ligase) VanA ligase is an ATP-dependent enzyme that mediates high-level resistance to glycopeptides by binding to the terminal D-Ala-D-Ala via hydrogen bonds and replacing the terminal amide with an ester in D-Ala-D-Lac. Proteomic analyses have provided functional insight into glycopeptide resistance in enterococci and staphylococci. Using 2-DE coupled with MS/MS, Wang et al. demonstrated vancomycin-induced upregulation of key resistance determinants (VanA, VanX, VanB, and VanXB) in *E. faecalis* strains V583 and V309 [78]. Similarly, comparative proteomics of vancomycin-resistant *E. faecium* SU18 revealed induction of multiple proteins, including VanA ligase, upon antibiotic exposure [79], underscoring the inducible and coordinated nature of the van gene cluster. Extending these observations to *S. aureus*, peptide mapping-based analyses implicated D-Ala-D-Ala ligase in vancomycin resistance through its role in peptidoglycan remodeling [30]. Collectively, these findings highlight cell wall biosynthetic enzymes as central mediators of glycopeptide resistance and potential therapeutic targets in nosocomial pathogens.

VanX Dipeptidase (Hydrolase) VanX dipeptidase is a metal-dependent cytoplasmic enzyme involved in vancomycin resistance, particularly in VanA- and VanB-type resistant enterococci. VanX contains a Zn^{2+} in its active site that activates a water molecule for a nucleophilic attack on amide bond of the D-Ala-D-Ala dipeptide. By eliminating D-Ala-D-Ala from the cytoplasmic pool, VanX ensures that only the altered precursor D-Ala-D-Lac (synthesized by other Van proteins) is incorporated into peptidoglycan. Proteomic expression of VanX dipeptidease has been found in *E. faecium* as described above [79] and hence the proteomic technology detects complete pathway activation, not just gene presence. As part of the Tn1546-encoded VanA-type operon, VanX functions synergistically with other cluster-encoded enzymes to remodel peptidoglycan precursors, thereby reducing vancomycin binding and conferring high-level glycopeptide resistance.

VanY Carboxypeptidase (Hydrolase) VanY also contributes to glycopeptide resistance by removing the terminal D-Ala from peptidoglycan precursors, trimming those that escaped VanX activity. In *A. baumannii*, Wang et al. integrated label-free quantitative proteomics, TMT labeling, and glycoproteomics to compare nine MDR and ten drug-susceptible clinical isolates, identifying VanY carboxypeptidase as significantly upregulated in MDR strains [17]. The differential expression of VanY highlights its potential contribution to cell wall remodeling and glycopeptide resistance, reinforcing the value of multi-platform proteomics in uncovering resistance-associated enzymes.

Fosfomycin

UDP-N-Acetylglucosamine Enolpyruvyl Transferase, MurA (Transferase) MurA catalyzes the first committed step in bacterial peptidoglycan biosynthesis. It transfers an

enolpyruvyl group from phosphoenolpyruvate (PEP) to UDP-NAG, forming UDP-NAG enolpyruvate. This reaction initiates the synthesis of UDP-NAM, a key precursor of the bacterial cell wall. Further, MurA is the molecular target of fosfomycin and has been reported to undergo mutations in a limited number of reports. A study from Japan identified two *murA* mutants in resistant clinical isolates of *E. coli*, having amino acid substitutions at Asp369Asn or Leu370Ile in the MurA protein. Expression of mutant MurA in *E. coli* strains showed at least eight-fold more resistance to fosfomycin than the strain overproducing wild-type MurA [80].

Antimicrobial Peptides

Antimicrobial peptides (AMPs) constitute a diverse group of antibiotics that primarily act on bacterial cell membranes, compromising their integrity and leading to cell death. By interacting with the phospholipid bilayer, AMPs destabilize and rupture membranes. Their modes of action are varied and include pore formation (e.g., melittin), electrostatic interactions (e.g., defensin), lipid binding (e.g., magainin), enzyme inhibition (e.g., LL-37), and RNA or protein binding (e.g., microcin J25, apidaecin). This multifunctionality positions the AMPs as promising alternatives to traditional antibiotics. Among these, cationic polymyxins, such as polymyxin B and colistin (polymyxin E), are notable.

Phosphoethanolamine (pEtN) Transferase (Transferase) Resistance to polymyxins is often mediated by modification of Lipid A, which prevents drug penetration into the cell. pEtN transferases, including the mobilized colistin resistance (MCR-1), catalyze the transfer of ethanolamine to the 1-phosphate of Lipid A, reducing the net negative charge of the lipid A therby reducing polymyxin binding. Plasmid-borne *mcr-1* has been reported in commensal *E. coli* from both animals (21%) and humans (16%) in China, highlighting its substantial dissemination potential [81]. MS-based interactomic analysis further showed that MCR-1 extends beyond lipid A modification-mediated colistin resistance, interacting with ribosomal proteins and the AcrA-TolC efflux system, suggesting broader impacts on protein synthesis and multidrug resistance physiology [82].

MltA-Interacting Protein A, MipA (Binding Protein) Peptidoglycan remodeling is essential for bacterial growth and division, and membrane-bound lytic transglycosylase A (MltA) catalyzes the cleavage of β-1,4-glycosidic bonds in peptidoglycan. MipA acts as a scaffold, forming a complex with MltA, which is important for cell wall expansion and septation. Quantitative LC-MS proteomics has increasingly implicated MipA as a multifaceted resistance-associated protein. Schmidth et al. demonstrated marked upregulation of MipA in *E. coli* exposed to escalating concentrations of the proline-rich antimicrobial peptide apidaecin 1b, suggesting its involvement in adaptive peptide resistance [83]. Consistently, SWATH-based label-free proteomics of *mcr-1* mediated colistin-resistant *E. coli* revealed elevated MipA levels under high-dose colistin and polymyxin B stress [84].

Beyond polymyxins, constitutive or inducible MipA overexpression has been observed across diverse antibiotic classes. Comparative proteomics linked MipA to quinolone resistance in *S. typhimurium* DT104B [85], while MALDI-TOF based outer membrane profiling of kanamycin-resistant *E. coli* K12 identified MipA as a novel resistance-associated protein under multiple antibiotic exposures [86]. Additionally, continuous-culture MS analyses of erythromycin-resistant *E. coli* populations showed increased MipA abundance [87]. Collectively, these findings position MipA as a stress-responsive envelope-associated factor potentially contributing to broad-spectrum antibiotic tolerance rather than drug-specific resistance alone.

PagL Deacylase (Hydrolase) PagL deacylase is an outer membrane enzyme found in Gram-negative bacteria that deacylates lipid A of LPS. It hydrolyzes the ester bond linking a secondary acyl chain (typically at the 3-position of lipid A) to the glucosamine backbone. The enzyme contains a catalytic triad (Ser-His-Asp), where Ser functions as a nucleophile. This deacylation reduces the endotoxic activity of lipid A and decreases recognition by the host receptor TLR4-MD2, resulting in increased resistance to AMPs. Using proximity ligation assay, small regulatory RNA profile was elucidated in *P. aeruginosa* to identify the antibiotic susceptibility and resistance. Sr006 positively regulates the expression of PagL deacylase, an enzyme that targets lipid A to reduce its proinflammatory property, resulting in polymyxin resistance [88].

AlmG Glycyltransferase (Transferase) Like PagL deacylase, AlmG glycyltransferase is also involved in lipid A modification in certain Gram-negative bacteria; however, it catalyzes the transfer of glycine to lipid A. This modification reduces the electrostatic attraction between the negatively charged lipid A and positively charged AMPs, resulting in polymyxin resistance. Deletion of *almG* gene resulted in a 100-fold decrease in resistance in *V. cholerae* compared with wild-type levels, whereas resistant phenotype could be restored when *almG* was expressed in trans [89].

Gcn5-related N-acetyltransferases, GNATs (Transferase) As reported earlier, GNATs are a large and diverse superfamily of enzymes transferring an acetyl group to an amino group of a wide range of substrates (e.g., on aminoglycosides

by AAC(6')-Ib). Mechanistically, GNAT action proceeds through a direct nucleophilic attack by -NH_2 of the substrate on the carbonyl carbon of acetyl-CoA. This leads to the formation of a tetrahedral intermediate, which then collapses to form the acetylated product. Unlike some acyltransferases, GNATs generally do not form a covalent acyl-enzyme intermediate; instead, the transfer occurs directly from acetyl-CoA to the substrate. In MDR *A. baumannii* MDR-ZJ06, multi-omics analyses demonstrated that GNAT plays a significant role in colistin resistance and multidrug resistance [90].

Replication Targeting Antibiotics

Quinolones

Qnr Pentapeptide Repeat Protein, PRP (Target Protection Protein) Quinolones normally act by binding to the DNA-enzyme complex formed by either DNA gyrase or topoisomerase IV during DNA replication, thus preventing their re-ligation. Qnr PRP interferes with this process by binding directly to the complex with their β-helix motif, reducing the quinolone binding. This confers low-level quinolone resistance, which can facilitate the selection of additional chromosomal mutations that lead to high-level resistance. The *qnr* gene was the first plasmid-mediated quinolone resistance determinant identified, encoding a protection protein that shields type II topoisomerases from quinolone inhibition. To date, five *qnr* families have been described, each containing multiple variants. In *S. typhimurium* DT104B, the presence of Qnr PRP was shown to be associated with enhanced quinolone resistance [91], providing a direct functional map of resistance mechanisms in the pathogen.

Nitroimidazoles

Nitroimidazole antibiotics, such as metronidazole and tinidazole, exert their antimicrobial activity by targeting anaerobic bacteria and protozoan parasites. Nitroimidazoles are reduced by intracellular enzymes to generate a reactive intermediate that binds to DNA. This binding causes the formation of DNA adducts, which leads to the inhibition of DNA synthesis and replication.

Nitroimidazole Reductase (Oxidoreductase) 5-Nitroimidazole (5-NI) resistance in *Bacteroides* species had already been reported early with the discovery of *nimA* and *nimB* genes on plasmid pIP417 and insertion sequence IS1168, encoding nitroimidazole reductases [92, 93]. 5-NI (metronidazole, tinidazole, dimteridazole, pretomanid, delamanid) are prodrugs activated anaerobically by the native reductase enzyme, by one-electron reduction of their nitro group. Whereas reductases, encoded by *nim* genes, cause complete (six-electron) reduction of nitro group to a non-bactericidal amine derivative, preventing the formation of DNA-damaging radicals [93]. In some cases, resistance also arises from decreased expression or mutation of activating nitroreductases, leading to reduced drug activation.

Translation Targeting Antibiotics

Aminoglycosides

16S rRNA Methyltransferase (Transferase) Aminoglycosides, including gentamicin, neomycin, amikacin, tobramycin, streptomycin, kanamycin, and spectinomycin, target bacterial ribosomes, which are composed of rRNA and proteins. Methylation of rRNA is a key resistance mechanism that protects ribosomes by reducing the binding affinity of aminoglycosides for their target nucleotides. Methyltransferases are versatile enzymes, encompassing classes such as N-methyltransferases, 2'-O-ribose methyltransferases, and radical SAM methyltransferases. Methylation at nucleotide G1405 of the 16 S rRNA confers resistance to most clinically important 4,6-disubstituted 2-deoxystreptamine (2-DOS) aminoglycosides, including gentamicin, kanamycin, amikacin, and the last-resort drug plazomicin. In contrast, methylation of A1408 results in resistance to a broader range of aminoglycosides, encompassing 4,6- and 4,5-disubstituted 2-DOS compounds (such as neomycin and paromomycin) as well as the monosubstituted aminoglycoside apramycin. These resistance mechanisms are naturally present in several aminoglycoside-producing *Streptomyces* and *Micromonospora* species and were later identified in pathogenic bacteria in the early 2000s.

To date, twelve acquired 16 S rRNA methyltransferases have been described, with ten targeting G1405 *(armA*, *rmtA-rmtI)* and only two targeting A1408 *(npmA and npmB)*. Although A1408 methyltransferases confer pan-aminoglycoside resistance, they are rarely reported, with sporadic detection of *npmA* in Enterobacteriaceae and *C. difficile*, and only four documented *E. coli* isolates carrying *npmB* [94–96]. These genes are often co-located with other resistance determinants on conjugative plasmids or transposons, facilitating horizontal transfer. Using an iTRAQ proteomic workflow, one study on *A. baumannii* isolates identified 16 S rRNA (guanine(1405)-N7)-methyltransferase (ArmA) as a prominent enzyme contributing to gentamicin resistance [20].

A previously unreported gene, *npmC*, was identified through analysis of globally available metagenomic datasets within a One Health framework. The gene shares 91.5% nucleotide similarity with *npmA* and up to 92.7% similarity at the amino acid level. The encoded protein contains

conserved motifs essential for A1408 methylation. Synthetic *npmC* was reported to be expressed in *E. coli*, resulting in high-level resistance to 4,5-disubstituted and 4-monosubstituted 2-DOS aminoglycosides, along with moderate resistance to 4,6-disubstituted 2-DOS aminoglycosides, including the last-resort drug plazomicin [97].

Tetracyclines

ABC-F Ribosomal Protection Protein, RPP (Target Protection Protein) The ABC family of proteins is an integral part of bacterial resistance mechanisms by functioning as the efflux transporters using energy derived by hydrolyzing ATP. However, ABC subfamily F (ABC-F) does not act as transporter rather behave as the target protection proteins. Tetracycline comes second in world consumption only after the penicillins due to their wide spectrum of activity, low cost, and relative safety. There are over ten *tet* (tetracycline resistance) and one *otr(A)* (oxytetracycline resistance) genes present coding for RPP, mostly on mobile elements. RPP, as the name suggest protects ribosome by dislodging the binding of antibiotics; they are basically translational GTPases with sequence similarity to the EF-G [98]. An iTRAQ-based quantitative proteomic study (2020) detected ≥ 8-fold overexpression of Tet(O) in clinical *C. jejuni* isolates, concordant with genomic data and elevated tetracycline MICs [99]. This integrative evidence underscores the reliability of proteomics for functional validation of resistance determinants in clinical settings.

Macrolides, Ketolides, Lincosamides, and Streptogramins (MKLS)

23 S rRNA Methyltransferase (Transferase) Methylation of A2058 of 23S rRNA drastically abrupts the therapeutic potential of MKLS antibiotics. A total of 39 genes have been identified for 23S rRNA methyltransferases. They differ from 16S rRNA methyltransferase in that these enzymes belong to either of N-methyltransferase *(erm*, *emt)*, 2'-O-ribose methyltransferase *(avi*, *tsr)*, or radical SAM methyltransferase *(cfr*, *clb)* classes. Resistance to different antibiotics by a single methylation event is possible due to the extensive overlap of target binding sites. Plasmid-mediated resistance to macrolide, lincosamide, streptogramin (MLS) antibiotics was first reported in 1983 in *S. sanguinis*, linked to the ErmAM *(ermB)* methylase class [100]. Subsequent sequencing of the erythromycin rRNA methylase gene on conjugative transposon Tn1545 from *S. pneumoniae* revealed 98% homology to *erm*-bearing plasmid pAM77; however, unlike the inducible expression of the pAM77-encoded gene, Tn1545 conferred constitutive methylase production [101]. Expanding beyond gene presence, acetyl-proteome profiling in *S. aureus* highlighted lysine acetylation, particularly of ribosomal proteins, as an additional regulatory layer influencing erythromycin resistance [102]. Collectively, these findings underscore both genetic mobility and PTMs as key drivers of MLS resistance evolution.

Fusidic Acid

Fusidic acid is a steroidal antibiotic primarily active against Gram-positive bacteria, especially *Staphylococcus* species, including MRSA. After GTP hydrolysis, it binds to EF-G in its GDP-bound state while it is still associated with the ribosome, preventing its release and blocking further rounds of translocation.

FusB Family Proteins (Target Protection Protein) FusB-family proteins (e.g., FusB, FusC) confer resistance to fusidic acid by binding to the antibiotic-target, EF-G, and facilitating its release from ribosomes, effectively rescuing stalled ribosome-EF-G-GDP complexes. Recently, a study concluded time-resolved single-particle cryo-EM structures explaining the mechanism of FusB-mediated rescue. They performed MS-based relative quantification of FusB and EF-G in a clinical *S. aureus* isolate where expression of FusB from resistance plasmid pUB101 was regulated by translational attenuation. Under exponential growth in the presence of fusidic acid, a 16-fold above the MIC for susceptible *S. aureus* strains, the molar ratio of EF-G to FusB was found to be 2.3X [103].

Metabolism Targeting Antibiotics

Sulfonamides

Sulfonamides (sulfamethoxazole, sulfisoxazole, sulfadiazine) inhibit bacterial growth by targeting dihydropteroate synthase, an enzyme critical for folic acid synthesis. Without folic acid, bacteria cannot produce thymine, which is essential for DNA synthesis, leading to impaired growth and cell death.

Dihydropteroate Synthase, DHPS (Transferase) DHPS, encoded by the *folP* gene, plays a central role in folate biosynthesis and bacterial cell proliferation. Clinical resistance arises from plasmid-borne genes that encode alternative DHPS variants. The *sul1* and *sul2* genes, first identified in the 1980s, are widely distributed among sulfonamide-resistant bacteria. Comprehensive quantitative proteomics (iTRAQ and LC-MS/MS) comparing MDR *A. baumannii* BAA-1605 with drug-susceptible ATCC 17,978 identified multiple resistance-associated proteins, including

sulfonamide resistance determinants, with nine differentially expressed proteins collectively correlating with resistance to 18 antibiotics [47]. Proteomics thus also helps to detects resistant enzyme variants against the native ones. Phylogenetic analyses further suggest that *sul13* genes originated from mobilized *folP*, underscoring HGT as a central driver of sulfonamide resistance dissemination [104].

Notably, proteomic profiling of quinolone-resistant *S. typhimurium* DT104B exposed to ciprofloxacin revealed differential expression of dihydropteroate synthase type-2 *(sul2)* among 186 regulated proteins [52]. This finding highlights the epidemiological and functional interplay between antibiotic classes, where exposure to one agent can co-select mobile elements harboring resistance genes to multiple drugs, reinforcing the complexity of multidrug resistance evolution.

Trimethoprim

Trimethoprim is a bacterial dihydrofolate reductase inhibitor, which is responsible for converting dihydrofolate (DHF) into tetrahydrofolate (THF), a necessary cofactor for the synthesis of nucleic acids, proteins, and other essential molecules in bacteria.

Dihydrofolate Reductase, DHFR (Oxidoreductase) Because trimethoprim is synthetic, resistance does not arise from drug-modifying enzymes but from acquisition of alternative DHFRs encoded by plasmid-borne *dfr* genes. These nonallelic DHFR variants retain catalytic conversion of DHF to THF while exhibiting reduced trimethoprim affinity, thereby sustaining folate metabolism under drug pressure. HGT has been implicated in the dissemination of *dfr*-mediated resistance among uropathogenic *E. coli* in Europe and Canada [105]. Complementing epidemiological data, native mass spectrometry of resistant DHFR mutants (P21L, W30R) demonstrated destabilized drug-enzyme ternary complexes relative to wild-type, directly linking structural perturbations to diminished trimethoprim binding and resistance [106].

Bifunctional Enzymes

Variation in a single gene typically produces a limited impact on the activity of a given enzyme. However, the recent emergence of bifunctional enzymes, which contain two or more linked genes, has been observed. These linked genes, often under the control of the same promoter, allow for the coordinated expression of distinct enzymatic activities, a strategy commonly seen in bacterial adaptation under antibiotic selection pressure. This arrangement provides bacteria with enhanced evolutionary flexibility and enables broad-spectrum resistance against multiple antimicrobial agents. Bifunctional enzymes offer several advantages, including simultaneous mobilization from a single gene, substrate channeling for efficient catalysis, and the ability to coordinate two enzymatic activities to counter a single antibiotic challenge.

Cell Envelope Targeting Antibiotics

Polymyxins

Bifunctional Polymyxin Resistance Protein, ArnA (Transferase-Oxidoreductase) ArnA is a bifunctional enzyme comprising an N-terminal formyltransferase and a C-terminal dehydrogenase domain, both required for the biosynthesis and incorporation of 4-amino-4-deoxy-L-arabinose (Ara4N) into lipid A, a key modification that reduces polymyxin binding and sustains resistance. Its clinical relevance is underscored in a carbapenem-resistant *K. pneumoniae* isolate, where ArnA contributed to a multidrug-resistant phenotype [16]. Additionally, acetyl-proteome profiling of ciprofloxacin-exposed resistant *S. typhimurium* implicated ArnA in adaptive resistance responses [107], suggesting its broader role in cell envelope remodeling and cross-class antibiotic tolerance.

Replication and Translation Targeting Antibiotics

Quinolones-Aminoglycosides

Fluoroquinolone-Acetylating Aminoglycoside 6′-N-Acetyltransferase, AAC(6′)-Ib-cr (Transferase) The *aac(6')-Ib-cr* gene, encoded on plasmids, produces an enzyme belonging to the AAC family. AACs have been identified in multiple bacterial species, including *E. coli* and *K. pneumoniae*. Robicsek et al. reported that clinical isolates carrying AAC(6')-Ib-cr, a variant of AAC(6')-Ib, exhibited reduced susceptibility to ciprofloxacin. Two amino acid substitutions, Tyr102Arg and Asp179Trp, in this variant enabled N-acetylation of ciprofloxacin at the amino nitrogen of its piperazinyl group [108]. Additionally, studies on *S. typhimurium* DT104B have also shown that AAC(6')-Ib-cr

contributes to resistance against both aminoglycosides and fluoroquinolones [52, 91].

Translation Targeting Antibiotics

Aminoglycosides

Aminoglycoside N-acetyltransferase-aminoglycoside O-phosphotransferase, AAC(6″)-APH(2″) (transferase) The bifunctional aminoglycoside-modifying enzyme AAC(6′)-Ie-APH(2″)-Ia confers broad-spectrum resistance by sequentially acetylating (N-terminal AAC domain) and phosphorylating (C-terminal APH domain) aminoglycosides, thereby inactivating most clinically relevant agents except streptomycin and spectinomycin. Epidemiological analysis of clinical *E. faecalis* and *E. faecium* isolates demonstrated that high-level gentamicin resistance is strongly associated with the *aac(6')-Ie-aph(2")-Ia* gene linked to the transposable element Tn5281, highlighting the role of mobile genetic elements in disseminating high-level aminoglycoside resistance [109].

Proteomic insights of antibiotic resistance

Proteomics offers a powerful approach to understand the emergence of new antibiotic resistance mechanisms in bacterial pathogens by enabling the large-scale identification and characterization of proteins and their functional states (Fig. 3). Since many established resistance mechanisms are mediated by proteins such as antibiotic-degrading enzymes and efflux pumps, proteomic analyses can directly detect changes in their expression, structure, and activity. In intracellular pathogens, resistance often arises not only from the acquisition of new proteins but also from subtle alterations in existing ones, particularly through amino acid substitutions in antibiotic target proteins that reduce drug binding [110]. Additionally, resistance can emerge through

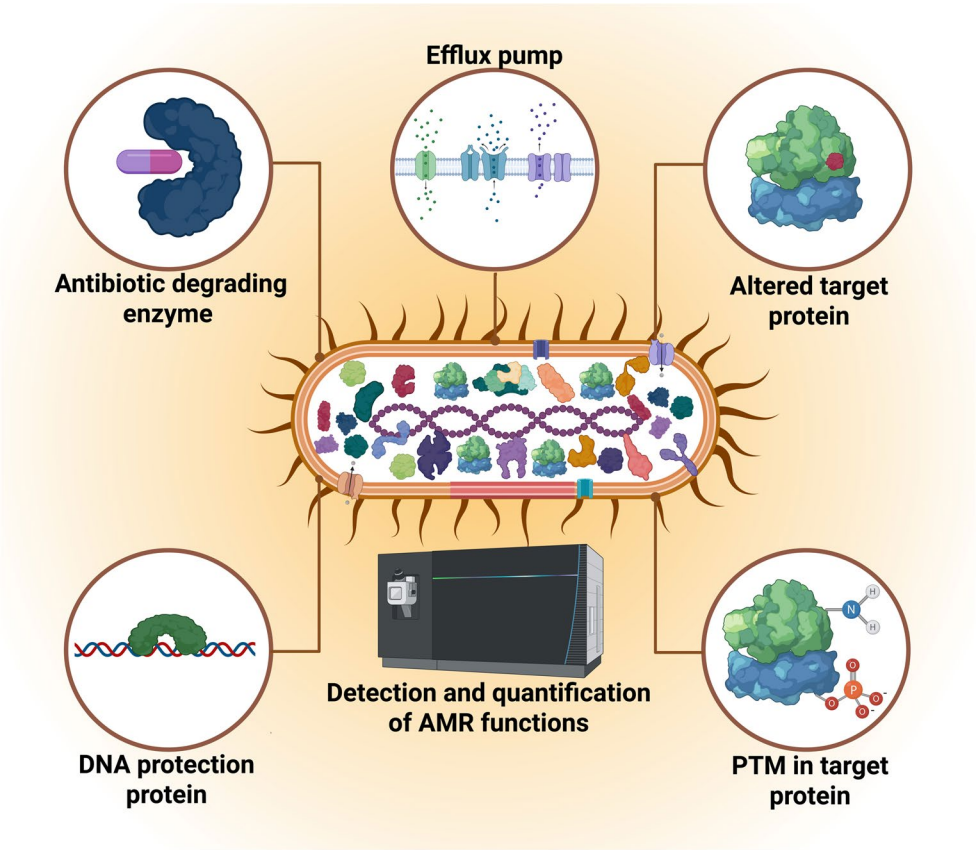

Fig. 3 Proteomic identification of proteins associated with antibiotic resistance. Representative antibiotic modification and target proteins and their structural or expression changes associated with resistance are shown. Comparative proteomics can identify altered targets, including ribosomal proteins (30S/50S), penicillin-binding proteins, DNA protection proteins, and RNA polymerase. Resistant strains exhibit amino acid substitutions, post-translational modifications, or differential abundance that reduce antibiotic binding. These changes are detected and quantified by mass spectrometry (MS/MS), with mutation-specific peptides and differential expression confirming resistance-associated variants

Table 1 List of enzymes involved in antibiotic resistance in human-associated bacterial pathogens. Summarizes a systematic classification of the major drug-modifying and resistance-associated enzyme families identified in clinically relevant human pathogens that underpin bacterial antimicrobial resistance. The term resistome refers to the complete set of antibiotic resistance functions within a bacterial species, while enzystome denotes the collection of antibiotic resistance-related enzymes and their variants. Unlike the resistome, which is generally gene-based description, the enzystome is function-based and expression-dependent analyses, often characterized using proteomics and enzymatic assays. Not all genes in the resistome are necessarily expressed; therefore, proteomic profiling of the enzystome provides insight into which resistance mechanisms are actively contributing to antimicrobial resistance at a given time. Advances in omics technologies have greatly enhanced our knowledge of resistance-related enzymes, providing a foundation for the design of next-generation drugs capable of overcoming bacterial adaptation. Large-scale omics-based studies offer a broader perspective on resistance dynamics and can accelerate the research and development of novel therapeutics. The proteomic insights into multidrug-resistant (MDR) enzymes discussed in this review aim to support further exploration of MDR human-associated pathogens and expedite the discovery of innovative treatments.

No.	Antibiotics	AMR enzymes	Pathogens	References
Antibiotic transformation				
1	β-lactams	β-lactamase	*K. pneumoniae*	16
2			*A. baumannii*	17–21
3			*A. xylosoxidans*	22
4			*E. coli*	23, 25–26
5			*S. aureus*	24
6	Glycopeptides	Glycopeptide nucleotidyltransferase	*S. aureus*	28–30
7			*E. faecalis*	31
8		Glyoxalase/bleomycin resistant protein/dioxygenase	*Listeria spp.*	32
9		Bleomycin binding protein	*Listeria spp.*	32
10			*S. aureus*	33
11	Fosfomycin	Fosfomycin epoxidase	*L. monocytogenes*	32
12		Glutathione S-transferase and Bacillithiol S-transferase	*S. epidermidis*	36
13	Lipopeptides	Daptomycin acetyltransferase	*S. aureus*	38–39
14	Quinolones	Ciprofloxacin phosphotransferase	*P. aeruginosa*	40
15	Rifamycins	Rifamycin ADP-ribosyltransferase	*M. smegmatis*	42
16		Rifamycin monooxygenase	*N. farcinica*	43
17		Rifamycin phosphotransferase	*L. monocytogenes*	44
18	Aminoglycosides	Aminoglycoside N-acetyltransferase	*Listeria spp.*	32
19			*A. baumannii*	20, 48
20			*E. coli*	49
21		Aminoglycoside phosphotransferase	*A. baumannii*	17, 20, 48
22			*S. typhimurium*	53
23			*E. coli*	54
24			*K. pneumoniae*	54
25		Aminoglycoside nucleotidyltransferase	*Acinetobacter spp.*	58
26			*E. coli*	54
27			*K. pneumoniae*	54
28	Tetracyclines	Tetracycline monooxygenase	*P. aeruginosa*	60
29	Phenicols	Chloramphenicol acetyltransferase	*V. cholerae*	61
30			*V. vulnificus*	61
31	MKLS	Macrolide esterase	*E. coli*	65
32		Macrolide phosphotransferase	*E. coli*	66
33		Lincosamide nucleotidyltransferase	*E. faecium*	67
34			*S. aureus*	68
35		Streptogramin lyase	*S. aureus*	69
36		Streptogramin acetyltransferase	*S. aureus*	70
37			*E. faecium*	71
Antibiotic target modifications				
1	β-lactams	Penicillim-binding proteins	*S. aureus*	74–75
2			*A. baumannii*	19
3			*B. subtilis*	76
4		Monofunctional biosynthetic peptidoglycan transglycosylase	*A. baumannii*	77

Table 1 (continued)

No.	Antibiotics	AMR enzymes	Pathogens	References
Antibiotic transformation				
5	Glycopeptides	VanA ligase	*E. faecalis*	78
6			*E. faecium*	79
7			*S. aureus*	30
8		VanX dipeptidase	*E. faecium*	79
9		VanY carboxypeptidase	*A. baumannii*	17
10	Fosfomycins	MurA protein	*E. coli*	80
11	Antimicrobial peptides	Phosphoethanolamine transferase	*E. coli*	81–82
12		MipA protein	*E. coli*	83–84, 86–87
13			*S. typhimurium*	85
14		PagL deacylase	*P. aeruginosa*	88
15		AlmG glycyltransferase	*V. cholerae*	89
16		Gcn5-related N-acetyltransferase	*A. baumannii*	90
17	Quinolones	Qnr pentapeptide repeat protein	*S. typhimurium*	91
18	Nitroimidazoles	Nitroimidazole reductase		93
19	Aminoglycosides	16 S rRNA methyltransferase	*A. baumannii*	20
20	Tetracyclines	ABC-F ribosomal protection protein	*C. jejuni*	99
21	MKLS	23 S rRNA methyltransferase	*S. pneumoniae*	101
22			*S. aureus*	102
23	Fusidic acid	FusB protein	*S. aureus*	103
24	Sulfonamides	Dihydropteroate synthase	*A. baumannii*	48
25			*S. typhimurium*	53
26	Trimethoprim	Dihydrofolate reductase	*E. coli*	105–106
Bifunctional proteins				
1	Polymyxins	ArnA protein	*K. pneumoniae*	16
2			*S. typhimurium*	107
3	Quinolones-Aminoglycosides	AAC(6')-Ib-cr	*E. coli*	108
4			*K. pneumoniae*	108
5			*S. typhimurium*	53
6	Aminoglycosides	AAC(6")-APH(2")	*E. faecalis*	109
7			*E. faecium*	109

enzymatic modification of target proteins via the transfer of specific functional groups, altering their susceptibility to antibiotics. Advanced proteomic techniques, especially when combined with post-translational modification (PTM) analysis, allow for the detection of such modifications, including phosphorylation, acetylation, or methylation, which may play critical roles in resistance phenotypes. By capturing both quantitative and qualitative changes in the proteome, these approaches can reveal early molecular signatures of emerging resistance before they become clinically apparent. Therefore, comprehensive proteomic profiling, supplemented with detailed PTM analysis, represents a promising strategy for the early detection and mechanistic understanding of novel antibiotic resistance functions, ultimately aiding in surveillance and the development of targeted therapeutic interventions. (Table 1)

Conclusion

The growing body of evidence presented in this review highlights enzymatic antibiotic degradation, transformation, and target modification as central drivers of AMR. Across diverse antibiotic classes, microorganisms have evolved highly specialized transferases, hydrolases, oxidoreductases, and protection proteins that either chemically inactivate antibiotics or remodel their molecular targets. These resistance determinants are frequently encoded on mobile genetic elements, enabling rapid horizontal dissemination across clinical and environmental bacterial populations. The emergence of bifunctional enzymes and multifunctional resistance determinants underscores the evolutionary plasticity of bacteria under antibiotic pressure. Such enzymes provide coordinated catalytic advantages, broaden

substrate specificity, and enhance the efficiency of resistance mechanisms. Their increasing prevalence further complicates therapeutic strategies and highlights the need for surveillance beyond single-gene detection. Proteomics-based investigations have substantially advanced our understanding of AMR by enabling the direct detection, quantification, and functional characterization of resistance-associated proteins. Proteomics adds a layer of functional, real-time biology, especially critical in AMR, where what is expressed and active matters more than just what genes are present. These findings demonstrate that resistance is not merely gene acquisition, but a coordinated, dynamic adaptive response involving metabolic rewiring, stress response pathways, and cell envelope remodelling. Ongoing progress in high-throughput proteomic technologies enables direct confirmation and quantitation of actual effectors, PTMs or variants driven resistance, global functional pathway reprogramming, detection of heteroresistance, membrane-associated resistance, and drug mechanism of action and target validation, Thus proteomics will be critical for the early and rapid identification of emerging resistance determinants, enhancement of diagnostic accuracy, and the establishment of robust molecular surveillance systems to monitor the evolving enzymatic landscape of bacterial resistance. Such approaches will also support the rational development of next-generation antimicrobial agents and enzyme inhibitors and hence bridging the gap between genetic potential and real world drug resistance. However, further research is needed to accurately detect resistance due low-abundant resistant proteins. Future progress depends not only on discovering new antibiotics but also on systematically targeting the enzymatic machinery that enables bacterial survival under antimicrobial pressure.

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Declarations

Competing interests The authors declare no competing interests.
The authors declare that the submitted work is original and has not been submitted / published elsewhere in any form or language (partially or in full).

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