Emerging Mechanisms of Antimicrobial Resistance in Bacteria and Fungi: Advances in the Era of Genomics

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Review

Emerging mechanisms of antimicrobial resistance in bacteria and fungi: advances in the era of genomics

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Abstract

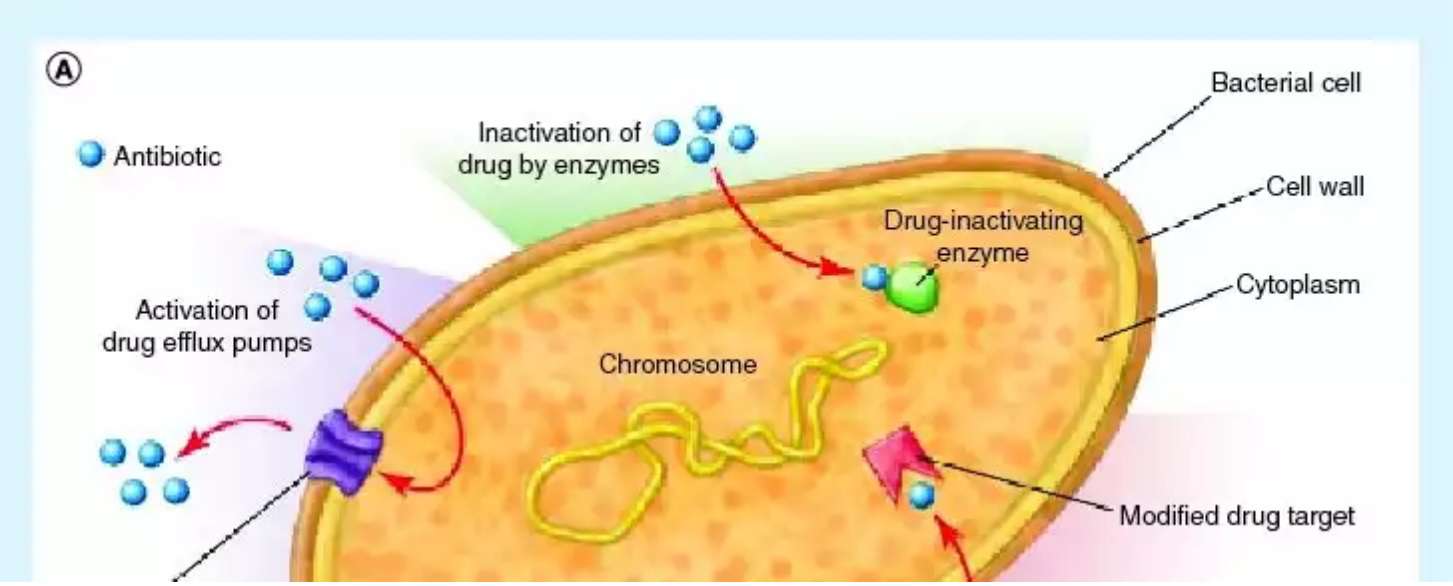
Bacteria and fungi continue to develop new ways to adapt and survive the lethal or biostatic effects of antimicrobials through myriad mechanisms. Novel antibiotic resistance genes such as *lsa*(C), *erm*(44), *VCC-1*, *mcr-1*, *mcr-2*, *mcr-3*, *mcr-4*, $bla_{\text{KLUC-3}}$ and $bla_{\text{KLUC-4}}$ were discovered through comparative genomics and further functional studies. As well, mutations in genes that hitherto were unknown to confer resistance to antimicrobials, such as *trm*, *PP2C*, *rpsJ*, *HSC82*, *FKS2* and *Rv2887*, were shown by genomics and transcomplementation assays to mediate antimicrobial resistance in *Acinetobacter baumannii*, *Staphylococcus aureus*, *Enterococcus faecium*, *Saccharomyces cerevisae*, *Candida glabrata* and *Mycobacterium tuberculosis*, respectively. Thus, genomics, transcriptomics and metagenomics, coupled with functional studies are the future of antimicrobial resistance research and novel drug discovery or design.

Keywords:
antimicrobial resistance mechanisms • fungi • Gram-negative bacteria • Gram-positive bacteria • *Mycobacterium tuberculosis* • next-generation sequencing • whole-genome sequencing

Background

Antimicrobial resistance (AMR) is becoming an issue of global concern, particularly with the development and spread of known as well as hitherto unknown/undescribed resistance mechanisms [1]. Antimicrobials have played a major role in the fight against infectious diseases, but their consistent use, misuse and abuse over decades have selected for resistant bacteria. Specifically, their introduction in human and veterinary medicine has played a key role [2-4]. AMR presents one of the biggest challenges to global public health, and it was estimated to have claimed 700,000 lives globally in 2014. Furthermore, it has been predicted that its attributable mortality will hit 10 millions by 2050 if measures are not taken to tackle it. Thus, it has become critical to safeguard the integrity of antimicrobials currently in use, considering that discovery of novel antimicrobials has stalled over recent decades [1]. Factors such as inappropriate use of antibiotics, inadequate infection prevention and control programs, limited laboratory capacity, poor surveillance, population growth and migration, as well as inadequate sanitation have compounded the problem of AMR [5].

Microorganisms, specifically bacteria, develop resistance through mutations in intrinsic chromosomal genes or by the acquisition of extraneous DNA such as plasmids, with multiple drug resistance being caused by the simultaneous expression of multiple resistance mechanisms (Figure 1) [6]. The effectiveness of antibacterial agents has been compromised by the clonal and polyclonal expansion of resistant bacteria as well as horizontal transmission of resistant plasmids [2,7-8]. [Query-Q4: Dinesh Kumar(CE) to All(AU)] Please check and confirm that any figures/tables that appear in the article have been redrawn correctly, and if not, please describe any corrections that need to be made.



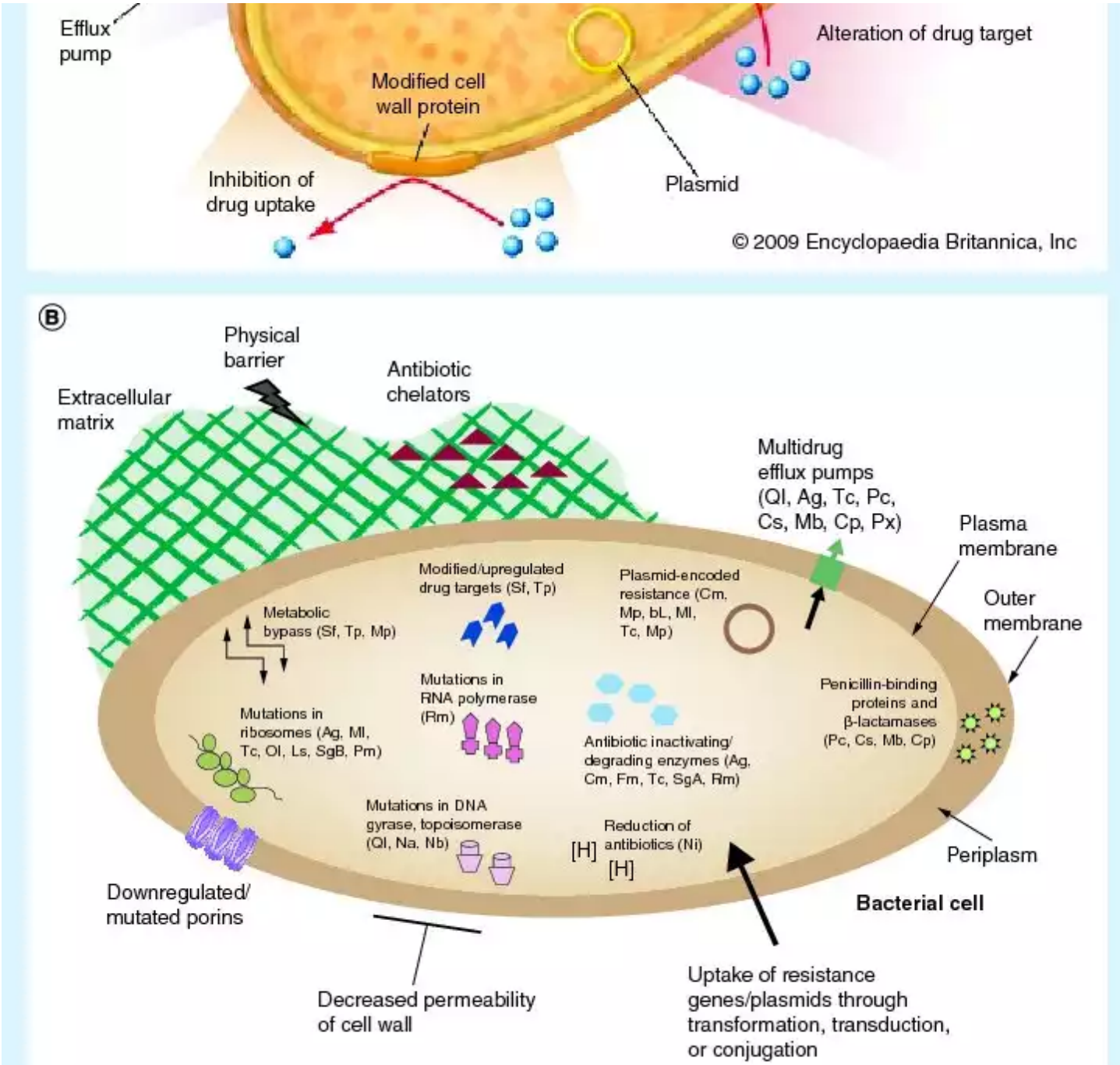


Figure 1. **XXXX.** [Query-Q5: Dinesh Kumar(CE) to All(AU)] Please provide the general/main title for Figure 1. Antibiotic resistance mechanisms of bacteria showing modifications in drug-target sites, impermeable cell walls, active efflux and drug-inactivating enzymes **(A)**. Porin downregulation in the cell wall to prevent drug entry, formation of physical barriers/matrixes, ribosomal mutations, etc. **(B)** are shown.
Adapted with permission from Morier (2016) and Sriramulu (2013) by kind courtesy of the © Encyclopaedia Britannica, Inc. (2009). [Query-Q6: Dinesh Kumar(CE) to All(AU)] Please provide the references for 'Morier, 2016' and 'Sriramulu, 2013' with complete publication details in the reference list.

Even more worrying is the emergence of resistance toward last-resort antimicrobial agents such as the echinocandins (caspofungin, anidulafungin and micafungin) in fungi as well as carbapenem and

colistin in Gram-negative bacteria; the effectiveness of these drugs have been weakened by the emergence of mutations in *FKS2* and *CDC6* genes [9], NDM-1 (first reported in 2008) [4] and MCR-1 (first reported in 2016) [10], respectively. Evidently, finding effective solutions to the menace of AMR requires cooperation among players from various scientific disciplines [2].

Underlying the gradual switch from susceptible to resistant bacterial populations is the presence of 'so-called' persister cells. Bacterial persister cells, which were first described in 1944 by Bigger [11], are extremely tolerant to traditional antibiotics due to their metabolic quiescence. They embody an important drug resistance mechanism and are difficult to control [12]. In contrast to antibiotic-resistant cells, which are able to multiply in the presence of antimicrobials due to changes at the genetic level [13], persister cells are metabolically inactive and can withstand antibiotics without undergoing genetic changes [14]. Persister cells comprise a maximum of approximately 1% of all cells in the stationary phase and in biofilms [15].

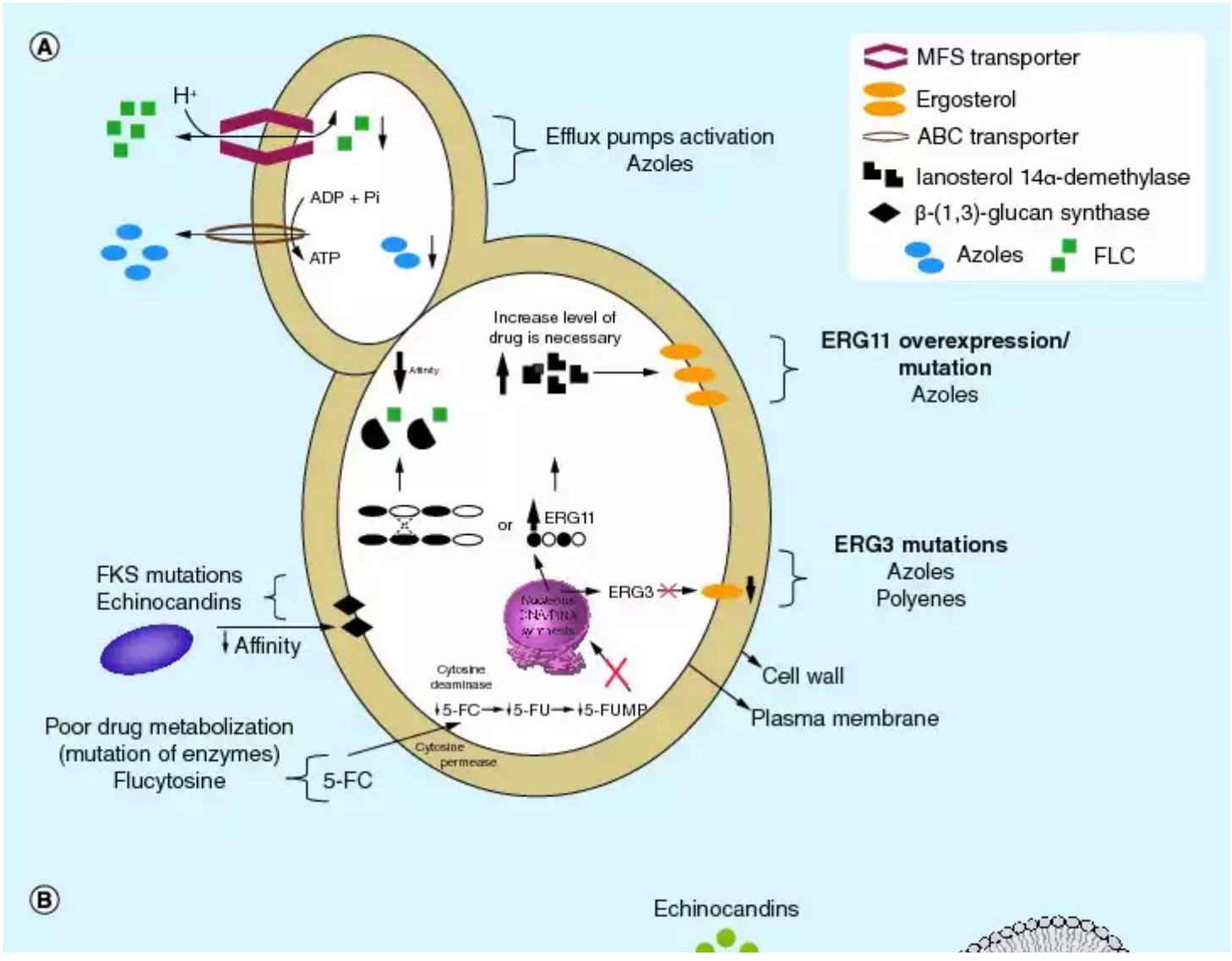
Persister cells' resistance to antimicrobials is primarily due to their nondividing nature, which makes antimicrobials binding to drug target sites unable to alter the cells' activities. Mechanisms underlying persister states remain unclear although toxin–antitoxin systems are thought to be involved in their formation, by inhibiting metabolic activity [14].

Bacteria have well-differentiated mechanisms by which they evade and develop resistance to antibiotics [4,7-8]. Generally, bacteria elude the effects of antibiotics by three primary mechanisms, which usually function synchronously with one another (Figure 1). These mechanisms include inactivation of the drug by enzymes such as β-lactamases and aminoglycoside/fluoroquinolone acetyl transferases [3,4], target modification such as DNA gyrase and topoisomerase for fluoroquinolone resistance [8] and decreased uptake of the drug through efflux upregulation and porin downregulation [3,8]. [Query-Q7: Dinesh Kumar(CE) to All(AU)] Please check and confirm that the preceding sentence has been edited correctly. These three major mechanisms could be intrinsically encoded in the bacterial chromosome or through spontaneous mutations in existing chromosomal genes [8]. In addition, transformation or conjugal acquisition of extraneous resistance plasmids, in other words, plasmids harboring resistance genes, can confer resistance to antimicrobials in hitherto susceptible bacteria [1].

Over the past decades, fungal infections have become a major problem in clinical practice, with immunocompromised patients being easily susceptible. Notably, systemic fungal infections are usually associated with high mortalities [16]. In addition, there are increasing reports of fungal infections in healthy populations due to, for example, the rising incidence of fungal pathogens such as *Aspergillus fumigatus*, making fungi as dangerous threat to human health as are bacteria, viruses and protozoa. Recently, *Candida auris* made news as an emerging pathogen with pandrug resistance to available

antifungal agents [16]. Although systemic fungal infections contribute to at least 10% of deaths in hospitals [9,16-17], there are limited drug targets for fungi due to their conserved metabolic pathways.

Antifungal drugs can be grouped into four main classes, namely, the polyenes, azoles, allylamines and echinocandins. Most antifungal drugs target ergosterol (polyenes), its biosynthetic pathway (azoles and allylamines) or β-glucan synthesis (echinocandins); however, those target proteins can induce the development of resistance [18]. Polyenes and azoles interfere with ergosterol biosynthesis and distribution; ergosterol is the sterol in fungal membranes (Figure 2). These drugs have the advantage of a broad-spectrum antifungal activity, but their use in humans is limited by their toxicity, which arises as a result of similarity in structure between fungal ergosterol and mammalian cholesterol [18]. The echinocandins, the newest class of antifungals introduced into clinical practice, target cell wall β-1,3-glucan synthase. The echinocandins have low toxicity in humans, but have a narrow spectrum of activity. Unfortunately, resistance has already been reported against them, with clinically resistant strains being isolated shortly after their introduction [19]. Studies investigating resistance mechanisms to current antifungals are important, considering that drug options are limited.



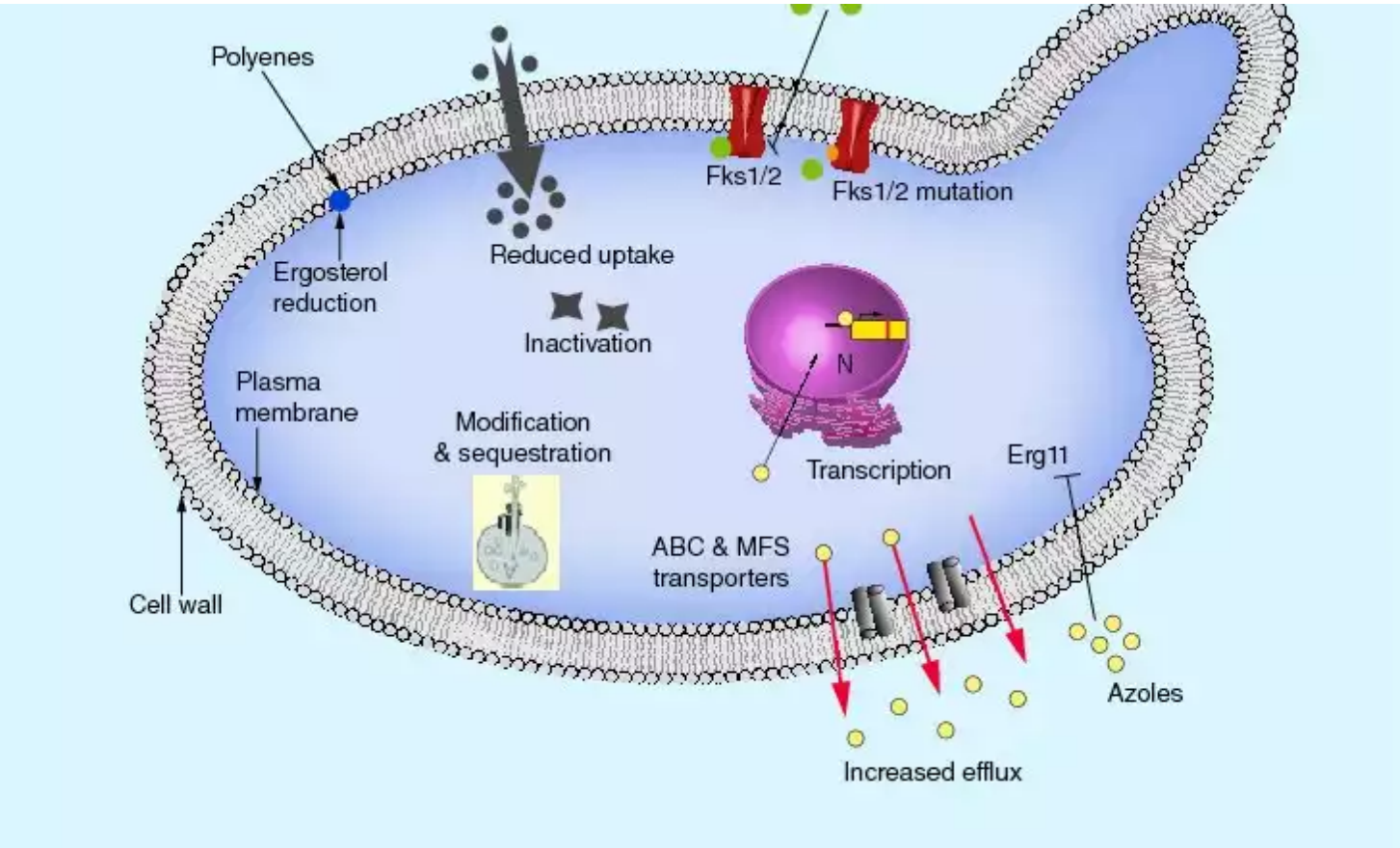


Figure 2. **xxx.** Antifungal resistance mechanisms to azoles (fluconazole, voriconazole, itraconazole, posaconazole and isavuconazole), echinocandins (anidulafungin, caspofungin and micafungin), flucytosine and polyenes (amphotericin B) as mediated by MFS and ABC transporters, ergosterol reduction in cell wall, ergosterol hyperexpression, ergosterol target site mutations or aneuploidy, *FKS* mutations and mutation(s) in flucytosine metabolizing enzymes **(A & B)**. [Query-Q8: Dinesh Kumar(CE) to All(AU)] Please provide the general/main title for Figure 2.
Adapted with permission from [20] and [21], respectively, by kind courtesies of the authors and publisher. [Query-Q9: Sharon Salt(PE) to All(AU)] Please could you let me know if you would like any of your figures printed in colour? The cost of colour printing is UK£155 per figure, with a discount of £95 per subsequent figure for articles with two or more figures. Thus, the price to print all your figures in colour would be £250.

Antifungal drug resistance mechanisms are categorized as either primary or secondary, and they can be either intrinsic or acquired mechanisms that interfere with drug action at drug target sites or that lower intracellular drug levels [22]. For example, in yeasts, resistance to echinocandins is mediated by target-site modifications resulting from point mutations. In molds, altered membrane ergosterol content, accumulation of other sterols, reduced intercalation and increased catalase activity decrease access to polyene drug targets resulting in polyene resistance [22]. In *Candida* species, the induction of efflux pumps that pump out azole antifungals from the fungal cell, leading to decreased drug concentrations within the cell are responsible among others for the decreased susceptibility or resistance of *Candida* to azole antifungals (Figure 2) [22].

Next-generation sequencing & AMR research

The role of technological advances in the detection and monitoring of antimicrobial resistance cannot be overemphasized. Technology provides the advantage of rapidity and cost–effectiveness in antimicrobial resistance surveillance and detection. It further translates such benefits into hospitals and communities, leading to improved stewardship of currently available antimicrobials and informed public health decisions/policies [4,23]. Subsequently, there has been a shift toward genome-wide investigations of AMR mechanisms following the decreasing costs of next-generation sequencing (NGS) within this decade. Thus, whole-genome sequencing (WGS) has played a key role in revolutionizing medical microbiology [24]. WGS provides vital information concerning emerging resistance genes at the national and global levels, which aids in tailoring approaches for stemming the tide of AMR. It has also resulted in a shift from phenotypic- to genome-based diagnoses of AMR [23,24]. Indeed, WGS of an isolate reveals all or almost all the information needed for diagnostic and typing purposes as well as inform decision making at the clinical- and public-health levels, although the analysis and interpretation of these data are sometimes challenging [23,24].

WGS also includes large gigabytes of extra data that are not available currently from routine diagnostic tests or even from other molecular methods such as PCR [23,24]. Rapid genome-sequencing methods technically can help forecast antibiotic resistance phenotypes via the identification of genetic resistance determinants, thereby aiding to arrive at swift clinical decisions [23,24]. Molecular methods of characterization, such as PCR, may be used to predict resistance phenotypes, for example, the detection of *mec*A (which encodes the alternative penicillin binding protein, PBP 2a) to predict methicillin resistance. However, WGS data technically improve upon previous molecular tests as it is easier to investigate more resistance determinants and also because computational querying of the sequence may be more sensitive than the use of PCR primers [23,24].

Although molecular techniques such as PCR have been used in the detection of novel molecular resistance mechanisms [25], WGS represents an upgrade on traditional methods to elucidate the epigenetic and genetic basis for observed antibiotic resistance. For instance, bacteria sometimes show resistance to certain antibiotics phenotypically, but lack the classical resistance genes commonly associated with such resistance. However, whole-genome comparison enables the easy detection of these resistance mechanisms and several genetic changes associated with antibiotic resistance throughout the genome [23,26-27].

WGS & functional characterization of resistance genes

Functional characterization of genes or the determination of gene function in cells has seen a major boost under NGS, which shifted the focus of research from individual genes and proteins to the study of entire genomes [28]. Through comparative genomics, using susceptible and resistant strains, the

putative or potential role of genes that were not known to confer resistance to antibiotics are becoming clearer. The antibiotic resistance effects of such hitherto uncharacterized genes are further confirmed through gene knockout/mutagenesis studies and/or gene cloning [3]. More interestingly, the interactions of several genes to confer resistance to antibiotics are also easily studied through comparative genomics, RNAseq and mutagenesis studies [26-27,29].

With the emergence of clustered regularly interspaced short palindromic repeats, CRISPR–Cas9, genome editing has become easier and faster [30,31]. With this technology, precise cuts can be affected on every site of the genome using the appropriate guide RNA, resulting in mutations and/or transformation/insertion with a customized DNA template. By using several guide RNAs that are complementary to particular sections of the genome, several precise mutations and transformations can be affected simultaneously to study the functions of various genes by observing phenotypic changes in the mutants [30,32].

RNAseq aids in the detection of differentially expressed genes (DEGs) in isogenic bacterial and fungal strains, particularly those exposed to different conditions of antibiotic stress. These DEGs are further studied through mutagenesis and cloning to ascertain their roles in antibiotic resistance. Once these DEGs are cloned into wild-type strains, their effect on antibiotic resistance is measured by determining the MICs of the new host to the antibiotic [3]. Advanced methods are being developed to enable *in vivo* transcriptional profiling of bacterial and fungal pathogens to determine genes that are differentially expressed during infection and antibiotic therapy. Such DEGs identified *in vivo* can aid in the design of novel antibiotics or therapies with different targets. The advantages of *in vivo* transcriptional profiling are evidently enormous as bacteria and fungi behave differently *in vivo* to antimicrobial chemotherapy compared with *in vitro* [33]. Through ChIP-Seq, transcription factors and their target genes/promoters which control or regulate drug resistance in bacteria and fungi can be easily studied to understand the transcriptional network or circuitry initiating and sustaining resistance. This can in turn aid in characterizing the roles of all genes and transcription factors involved in the resistance circuitry and gene regulation, respectively [31,34].

The benefits of WGS in helping characterize & identify hitherto unknown resistance mechanisms (genes) in Gram-positive bacteria since 2008

Novel resistance mechanisms have so far been detected in five Gram-positive bacterial species, namely, *Staphylococcus* spp., *Streptococcus* spp., *Enterococcus* spp., *Listeria monocytogenes* and *Bacillus subtilis*, using NGS and transformation/mutagenesis studies. *Staphylococcus aureus* (including methicillin-resistant *S. aureus* [MRSA]), *Staphylococcus xylosus*, *Streptococcus pneumoniae*, *Streptococcus agalactiae*,

Enterococcus faecalis, *Enterococcus faecium*, *L. monocytogenes* and *B. subtilis* were species in which novel resistance mechanisms were found between 2008 and 2017.

Staphylococcus aureus & Staphylococcus xylosus

One of the most-common nosocomial (healthcare-associated), livestock-associated and community-associated bacterial pathogens implicated in multiple drug resistance worldwide is MRSA, which usually cause skin infections such as boils in communities, as well as pneumonia, sepsis, bacteremia, etc. in hospitals with high attributable mortalities [35]. In a study involving MRSA isolates in human and bovine populations in the UK and Denmark [35], a novel *mec*A homolog, *mec*A_{LGA251}, was detected in isolates displaying phenotypic resistance to methicillin and yet lacking *mec*A, the gene implicated in methicillin resistance. This *mec*A homolog, detected by WGS of a *S. aureus* genome, was identified in a novel staphylococcal cassette chromosome *mec* element, labeled type-XI SCC*mec*. The isolate tested negative to *mec*A upon PCR analysis with corresponding primers, and reacted negatively to the latex agglutination test for detecting PBP2a. It was also negative to a PCR amplification of a region of the SCC*mec* (SCC*mec-orfX*). Although phylogenetic evaluation of *mec*A_{LGA251}, which has been renamed *mec*C [36], showed that it is part of the PBP2a family, it was different from other *mec*A homologs when compared with proteins from Genbank.

The inability of current confirmatory methods to identify the isolates as MRSA underscores the need for new diagnostic guidelines and next-generation diagnostics, without which this novel *mec*C would have been undetected (Table 1). Moreover, the virulence and higher mortality rates of MRSA make this finding very concerning as several MRSA strains can go undetected by available diagnostics, subtly spreading and infecting many patients and livestock. The public health benefits of thus adopting NGS into routine clinical microbiology laboratories to aid in diagnoses, identification and infection control cannot be gainsaid. [Query-Q10: Dinesh Kumar(CE) to All(AU)] Please provide the city/state code and country for the following manufacturers at their first occurrence in the main text: Applied Biosystems, Illumina and Roche.

Table 1. Novel resistance mechanisms discovered in Gram-positive bacteria after 2008.

Year of study	Microbial species	NGS platform used (e.g., Ion Torrent, Illumina, PacBio, etc.)	Resistance mechanism detected	Antibiotic/antibiotic class to which resistance mechanism was found	Ref.
2008	*Staphylococcus aureus*	NS[†]	Mutated response regulator *graR*	Vancomycin and daptomycin	[37]
2009	*Streptococcus pneumoniae*	NimbleGen (comparative genome sequencing)	Mutations in the protein spr0333	Linezolid	[38]
2011	*Streptococcus agalactiae*	454 Life Sciences (Roche) GS-FLX system	*lsa*(C) gene	Lincosamides, streptogramins A and pleuromutilins	[39]

2011	*S. pneumoniae*	454 Life Science (Roche) GS-FLX DNA sequencing platform	spr1178 (a putative iron permease)	β-lactams	[40]
2011	*Enterococcus faecalis*	Illumina Hiseq	Mutations in LiaF and a GdpD family‡	Daptomycin	[41]
2011	*S. aureus* (MRSA; LGA251)	Sanger capillary sequencing (on ABI 3730xl analyzers; Applied Biosystems) and pyrosequencing (on 454 instruments)	*mecA*LGA251 methicillin resistance	β-lactams	[35]
2012	*S. aureus*	454 Titanium and Junior platforms	A mutation in the *PP2C* phosphatase gene	Vancomycin and daptomycin resistance	[42]
2013	*S. aureus*	Illumina HiSeq 2000	*dacA*	Beta lactams	[43]
2013	*S. pneumoniae*	454 Life Sciences (Roche) GS-FLX Titanium System and HiSeq1000 next-generation sequencer (Illumina)	Mutations in spr1333, spr0981, spr1704 and spr1098§	Cefotaxime	[44]
2014	*Listeria monocytogenes*	Illumina MiSeq Benchtop sequencer (ProfileXpert-LCMT, Lyon, France)	FepA (efflux pump of the MATE family)	Fluoroquinolones	[45]
2014	*Bacillus subtilis*	Illumina HiSeq 2000	Mutations in sigA, *rho*	Beta-lactams and glycopeptides	[46]
2014	*Staphylococcus xylosus*	Ion Torrent semiconductor sequencing	*erm*(44)	lincosamide and streptogramin B (MLSB) [Query-Q11: Dinesh Kumar(CE) to All(AU)] Please check the term 'lincosamide and streptogramin B (MLSB)' in Table 1 for correctness.	[47]
2015	*S. pneumoniae*	Illumina HiSeq sequencing system	Large genomic duplication Genomic rearrangement	Fluoroquinolones	[48]
2015	*Enterococcus faecium*	Illumina MiSeq Sequencing System	Mutations in the *rpsJ* gene	Tigecycline	[49]
2015¶	*Mycobacterium tuberculosis*	Ion Torrent	Loss-of-function mutations in *Rv2887*	MPIII-71, a new antitubercular agent	[50]

† Particular sequencing platform used not specified.

‡ GdpD is glycerophosphoryl diester phosphodiesterase family protein.

§ These encode a peptidoglycan N-acetylglucosamine deacetylase, a glycosyltransferase, an ABC transporter and a sortase, respectively.

¶ *M. tuberculosis* can appear either as Gram-positive or Gram-negative because of its waxy coating, making it impervious to Gram-staining.

MATE: Multidrug and toxic compound extrusion; MRSA: Methicillin-resistant *S. aureus*; NGS: Next-generation sequencing; NS: xxx. [Query-Q12: Dinesh Kumar(CE) to All(AU)] Please define the term 'NS' in the footnote of Table 1.

Dairy production in cattle ranches/farms are drastically affected by mastitis, resulting in huge economic losses to farmers and affected countries. The importance of genomics in veterinary medicine diagnostics and AMR was delineated by Wipf *et al.* in 2014 [47], when they reported of a bovine mastitis mediated by an *S. xylosus* strain that was found by phenotypic screening to exhibit erythromycin and inducible clindamycin resistance. Whereas an MLS_B *erm* methylase gene was suspected, no methylase gene was found. Whole-genome analysis identified a novel gene, *erm*(44), which encodes a macrolide ribosomal methylase that conferred resistance to macrolides, lincosamides and streptogramin B (MLS_B) antibiotics (Table 1). It is worth mentioning that resistance to macrolides in bovine mastitis by *S. xylosus* has formerly been associated with the *msr* gene, which is responsible for drug efflux [51]. Early diagnosis and containment of antibiotic-resistant mastitis-causing pathogens such as *S. xylosus* from spreading in cattle farms will save millions of dollars [52].

Streptococcus agalactiae & Streptococcus pneumoniae

Although *S. agalactiae* is a commensal and a common intestinal denizen in a large population of humans, it is also the leading cause of invasive infections such as pneumonia, meningitis and septicemia among neonates, resulting in substantial fatalities per 1000 live births [53]. Furthermore, it causes serious morbidities and mortalities among nonpregnant adults such as the elderly and those with underlying disease, besides its association with bovine intramammary infections [53]. Malbruny *et al.* [39] identified a novel gene in *S. agalactiae*, named *lsa*(C), which mediates resistance toward lincosamides, streptogramins A and pleuromutilins (LS_AP phenotype) as observed from the increase in MICs of lincomycin, clindamycin, dalfopristin and tiamulin (a pleuromutilin) after cloning/transcomplementation (Table 1).[Query-Q13: Dinesh Kumar(CE) to All(AU)] In the preceding sentence, year '(2011)' of Malbruny et al. has been replaced with the reference citation [108] of reference Malbruny et al. Please confirm the change. The phenotype was renamed from LS_A (lincosamides and streptogramins A), which has already been reported in *S. agalactiae*, to LS_AP on account of cross-resistance to lincosamides, streptogramins A and pleuromutilins [54].

The ability of WGS to facilitate the identification and functional characterization of this gene is very welcoming as the streptogramins are among the most potent and reserved antibiotics for Gram-positive pathogens [55]. An early detection of a novel resistance gene or emerging resistance to clinically important and reserved antibiotics is crucial to the fight against AMR. This is because it enables faster detection of such resistance gene-expressing pathogens to prevent further spread. It also informs clinicians of the best antimicrobials to use to prevent further selection and dispersal of the novel resistance gene. Notably, many neonates and adults can be saved from this resistance gene, resistant *S.*

agalactiae strain and a certain death, should these resistant pathogens be easily and quickly detected and contained with well-chosen antibiotics.

NGS is an effective tool in the study of gene amplifications and its effects on gene expression and antibiotic resistance. Gene amplification is the transient generation of tandem repeats of large chromosomal regions, with data from a study indicating that about 10–30% of *Salmonella* cells in a nonselected culture, at any point in time, have a duplication somewhere in their genome [56]. In a study by Baylay *et al.* [48], a novel gene amplification was found to be the cause of *patAB* (an ABC transporter) hyperexpression, leading to fluoroquinolone resistance in *S. pneumoniae* (Table 1). In this study, duplication was detected in a region of the genome of an *S. pneumoniae* strain. In several bacterial species, gene amplifications have been found to cause antibiotic resistance and are the subject of a review elsewhere [57].

Listeria monocytogenes

In another vein, Guérin *et al.* analyzed a *L. monocytogenes* strain and showed that overexpression of a novel fluoroquinolone efflux pump of the multidrug and toxic compound extrusion family, designated *Fep*A, resulted in fluoroquinolone resistance [45]. Although fluoroquinolone resistance in Gram-positive bacteria is mainly due to the buildup of mutations in topoisomerases, which are the molecular targets of fluoroquinolones, resistance to this drug class is found to be mediated by active efflux in *L. monocytogenes* [45] (Table 1). Being an intracellular parasite and foodborne infectious agent implicated in foodborne infections that result in considerable morbidities and mortalities, *L. monocytogenes* is an important Gram-positive pathogen.

Resistance to antibiotics caused by novel mutations in both known & unknown nonantibiotic-resistance genes among Gram-positive bacteria

Mutations in certain bacterial genes sometimes correlate with AMR. These mutations may be single nucleotide polymorphisms (SNPs) or insertion/deletions (InDels) [58]. Resistance to antimicrobials may be mediated by nonantibiotic-resistance genes (NARGs), instead of known antibiotic-resistance genes (ARGs) such as *mcr-1*, $bla_{\text{NDM-1}}$ and $bla_{\text{CTX-M-15}}$. NARGs are defined herein as genes that have other cellular functions, but can mediate resistance due to SNPs and InDels. Such was the case with colistin resistance prior to the detection of plasmid-borne *mcr-1*/2/3/4 genes, and such is the case with tigecycline resistance mechanisms currently [49,59-60]. NARGs have become common due to comparative genomics. Resistance mediated by NARGs usually occurs in isolates in which the classical ARGs are absent and yet resistance is expressed by the bacteria. Interestingly, resistance-conferring mutations found in NARGs are always absent in wild-type strains of the same species [3]. In *M.*

tuberculosis, resistance-conferring mutations in genes such as *rpoB*, *katG*, *inhA*, etc. are commonly known to confer resistance to important antitubercular drugs [61,62].

Staphylococcus aureus

Renzoni *et al.* [63] investigated glycopeptide (teicoplanin) resistance in *S. aureus*. Glycopeptides, such as vancomycin, oritavancin and dalbavancin, are important reserve antibiotics that are effective against MRSA [55] and other multidrug-resistant pathogens. In this study, SNPs were found in the selected strain with nonsense mutations occurring in the *stp1* gene (encoding a serine/threonine phosphatase) and in *yjbH* (encoding a post-transcriptional negative regulator of the redox/thiol stress sensor and global transcriptional regulator, *Spx*). It was concluded that a combination of these mutations in the individual genes contributed to decreased teicoplanin susceptibility.

Dengler *et al.* showed that the diadenylate cyclase gene, *dacA*, increases methicillin resistance in *S. aureus* (Table 1). Diadenylate cyclases synthesize the new second messenger, cyclic diadenosine monophosphate, whose levels when increased, have been shown to influence methicillin resistance. A mutation detected in *dacA* (resulting in decreased cyclic diadenosine monophosphate levels), when introduced into methicillin-resistant strains, reduced the resistance or MICs [43]. Hence, molecules that can attack the *dacA* gene or its expressed protein could serve as lead compounds that can be further developed into potential drugs to combat methicillin resistance in *S. aureus*. The identification of novel drug targets to fuel the design of new drugs is one of the promising legacies of genomics and transcriptomics.

Using a 454 Titanium sequencer, Passalacqua *et al.* [42], identified InDel mutations in the *PP2C* gene, a protein phosphatase gene in an *S. aureus* isolate (USA 300) with reduced susceptibility to vancomycin and daptomycin. Further complementation studies confirmed their role in the observed phenomenon (Table 1). Neoh *et al.* [37] discovered that a mutation in the response regulator of the *graSR* two-component regulatory system was responsible for the conversion of heterogeneous vancomycin intermediate *S. aureus* to vancomycin intermediate *S. aureus* (Table 1). Increased expression of this regulator gene increases vancomycin resistance, which could be explained by the role of the gene in increasing cell wall thickness and in decreasing autolytic activity.

In this study, introduction of the mutated *graR* gene in a strain (Mu50) significantly increased the MICs of vancomycin and daptomycin whereas the introduction of the intact *graR* did not. This finding is very important for drug design or development in the future to treat vancomycin-resistant *S. aureus* strains. For instance, drug molecules that can inhibit the *graSR* two-component regulatory system could be co-administered with vancomycin to prevent the development of resistance against vancomycin.

Streptococcus pneumoniae

Feng *et al.* [38] showed that mutations in the spr0333 protein, a hypothetical protein with hitherto unknown functions, were implicated in linezolid resistance in *S. pneumoniae*. The protein contains an RNA methyltransferase domain, and WGS of the above-mentioned resistant strains revealed that it contained mutations within that domain. The study employed primer extension studies to establish that spr0333 was responsible for the methylation of G2445 of the 23S rRNA, which was eliminated by the mutations in spr0333. Reintroduction of an intact spr0333 in resistant bacteria restored G2445 methylation and re-established susceptibility of the bacteria to linezolid and other antibiotics. The clinical importance of linezolid in treating Gram-positive bacterial infections [55] make this novel finding worrying but important as it can inform the design of novel linezolid analogs that can overcome this resistance mechanism.

In another study [44], it was observed that mutations in PBPs were not sufficient to fully explain the levels of cefotaxime resistance observed in *S. pneumoniae*. Therefore, non-PBP mutations were investigated for their effect on cefotaxime resistance. Mutations detected in *spr1333*, *spr0981*, *spr1704* and *spr1098* genes, encoding a peptidoglycan N-acetylglucosamine deacetylase, glycosyltransferase, an ABC transporter and a sortase, respectively, were linked to cefotaxime resistance; these were confirmed by transformation experiments. The molecular epidemiology of antibiotic-resistant pathogens can easily be determined from genomic data while that cannot be said for other earlier molecular techniques, which makes the use of NGS more advantageous in infection control, drafting of antibiotic stewardship guidelines as well as in AMR research. Thus, the detection of these novel resistance mechanisms in *S. pneumoniae*, a leading cause of pneumonia, meningitis and sepsis in infants [64], will aid in future molecular epidemiology, surveillance and infection control programs that can contain the spread of this pathogen and save the lives of many infants.

As well, a mutation in an iron permease gene (named *spr1178*), was implicated in β-lactam resistance in *S. pneumoniae*. After the genome of two penicillin-resistant *S. pneumoniae* isolates were sequenced, a nonsense mutation was found in *spr1178*, designated as a putative iron permease. The introduction of this mutation by transformation confirmed that it reduced penicillin susceptibility by reducing the accumulation of reactive oxygen species following the introduction of penicillin and other antibiotics (Table 1). The *spr1178* gene is not known to mediate antibiotic resistance and its inactivation in this study did not affect penicillin susceptibility [40]. It is thus imperative for clinicians, infection control officials and clinical molecular epidemiologists to consider these mutations when surveilling for β-resistant *S. pneumoniae*, particularly in pediatric wards.

Enterococcus faecalis & Enterococcus faecium

Besides MRSA, multidrug resistance in *Enterococci*, specifically vancomycin-resistant *Enterococci*, is notorious for being a leading cause of nosocomial bacteraemia, surgical wound and urinary tract infections. They are also known to be resistant to many, and at times all, available antibiotics due to their ability to acquire or assemble a large repertoire of resistance genes [65]. In a study carried out in 2011, the genetic basis for daptomycin resistance in *E. faecalis* was investigated and mutations in two genes previously not linked with antibiotic resistance in *Enterococci* were detected after comparative genome alignments. The genes encode putative membrane proteins namely, LiaF and a glycerophosphoryl diester phosphodiesterase (GdpD) family protein. LiaF is thought to belong to the three-component regulatory system (LiaFSR), which is involved in the stress-sensing response of the cell envelope to antibiotics. The *lia*F allele alone and in combination with the *gdpD* allele contributed sufficiently to the development of daptomycin resistance [41].

Similarly, Niebel *et al.* [49], in a study investigating tigecycline resistance in *E. faecium*, detected that mutations (deletions) in the *rpsJ* gene, which encodes a structural protein forming part of the 30S ribosomal subunit, played a role in reducing tigecycline susceptibility in *E. faecium* (Table 1). Resistance to reserve antibiotics such as daptomycin and tigecycline suggests the easy acquisition or development of resistance among *Enterococcus* spp. and the need to carefully monitor them through advanced technology to pre-empt any outbreak or spread of multidrug-resistant *Enterococci* [55].

Bacillus subtilis

Lee and Helmann [46] discovered mutations in the primary sigma factor and termination factor, rho (ρ), that were responsible for reduced susceptibility of *B. subtilis* to the cell wall antibiotics vancomycin and cefuroxime. Mutations in the major sigma (σ) factor of RNA polymerase (sigA) were found to decrease vancomycin susceptibility. Also, in combination with mutations in the major sigma factor, mutations in the gene encoding the ρ termination factor (rho) also contributed to vancomycin nonsusceptibility. The danger with resistance to vancomycin and cefuroxime, which are reserved antibiotics used for fatal Gram-positive bacterial infections such as MRSA, in nonpathogenic bacteria such as *B. subtilis*, is the possible transmission of such resistance determinants horizontally via plasmids to pathogenic ones [26,29,66].

The benefits of WGS in helping characterize & identify hitherto unknown resistance mechanisms in Gram-negative bacteria

Novel resistance mechanisms, including the notorious *mcr-1* and *vcc-1* genes, have been described in various Gram-negative bacterial species, most of which are known pathogens: *Acinetobacter baumannii*, *Campylobacter coli*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pasteurella multocida*, *Photobacterium damselae*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Stenotrophomonas maltophilia* and *Vibrio*

cholerae. Reports of AMR determinants among Gram-negative pathogens, particularly to last-resort antibiotics such as carbapenems, colistin and tigecycline have been relatively numerous than for Gram-positive ones [2]. Furthermore, the transmissibility of resistance genes from commensals to pathogens have been greatly reported and established in Gram-negative bacteria, particularly in the GI tract [66]. Due to the exposure of intestinal bacteria to antibiotic stress, they are more prone to develop resistance and share it among themselves [26-27,29].

Pasteurella multocida

P. multocida is an opportunistic pathogen and commensal that is commonly associated with domestic, wild and farm animals and causes pasteurollosis, an acute or chronic infection that results in severe morbidities (such as cellulitis, meningitis, hemorrhagic septicemia, pneumonia, etc.) and mortalities in animals. Pasteurollosis is also a zoonotic disease that can be transferred to humans from pets or wild animals through bites, exposure/close contact, wounds or scratches [67]. The value of WGS in determining hitherto unknown resistance mechanisms in *P. multocida* was highlighted by Kadlec *et al.* Macrolide–triamilide resistance mechanisms in this organism had been unknown, but three novel resistance genes, namely, *erm*(42), *msr*(E) and *mph*(E) encoding an rRNA methylase, a macrolide transporter and a macrolide phosphotransferase, respectively, were identified by WGS of a multiresistant bovine *P. multocida* strain (Table 2) [68]. The roles of these genes in macrolide resistance were confirmed by PCR amplification and resequencing to corroborate the sequence acquired by WGS as well as by cloning studies.

Table 2. Novel resistance mechanisms discovered in Gram-negative bacteria after 2008.

Year of study	Microbial species	NGS platform used (e.g., Ion Torrent, Illumina, PacBio, etc.)	Resistance mechanism detected	Antibiotic/antibiotic class to which resistance mechanism was found	Ref.
2011	*Pseudomonas aeruginosa*	Illumina genome analyzer	Mutations in the ParR protein	Polymyxins, aminoglycosides, fluoroquinolones and β-lactams	[69]
2011	*Pasteurella multocida* strain 36950	454 Life Sciences (Roche) GS-FLX system (Eurofins MWG, Ebersberg, Germany)	*erm(42)*, *msr(E)* and *mph*(E)[†]	Macrolide–lincosamide Macrolide–triamilide	[68]
2012	*Escherichia coli*[2‡] *Campylobacter jejuni*	NS[§]	FRAMP1.1 CRAMP2.1 CRAMP2.2 CRAMP2.3 CRCHL2.2 FRSPE1.1	Ampicillin, spectinomycin and chloramphenicol	[70]
2012	*E. coli*	Illumina/Solexa technology	bla_{KLUC-3} and bla_{KLUC-4}	β-lactams	[71]
2012	*Acinetobacter baumannii*	Solexa single-reads sequencing technology	Mutation in the *adeN*	Tetracyclines, phenicols, fluoroquinolones,	[72]

				sulfonamides and macrolides	
2013	*Campylobacter coli*	454 GS FLX platform (Life Sciences, CT, USA)	*aph(2)-Ig* (phosphotransferase gene)	Aminoglycosides	[73]
2013	*E. coli*	Illumina	Mutations *in lpcA*, *rfaE*, *rfaD*, *rfaC* and *rfaF* genes	Tigecycline	[58]
2013	*A. baumannii*	Illumina Hiseq	Deletion mutation in *trm*	Tigecycline, minocycline and doxycycline	[74]
2013	*Salmonella typhimurium*	Illumina sequencing technology	Mutations in *waaY*, pmrB and phoP	Antimicrobial peptides	[75]
2014	*Klebsiella pneumoniae*	Illumina MiSeq	IS5 insertion element in *kpgABC* (a putative efflux pump operon)	Tigecycline	[76]
2014	*Stenotrophomonas maltophilia*	Illumina Hiseq	*floRv* (phenicol resistance gene)	Chloramphenicol and florfenicol	[77]
2014	*E. coli*	PacBio	a 12-nucleotide deletion in ribE and encoding lumazine synthase	nitrofurantoin	[78]
2015	*E. coli*	Illumina MiSeq	*mcr-1* gene (phosphoethanolamine transferase enzyme)	Polymyxin	[10]
2015	*Vibrio* and *Photobacterium damselae* subsp. *damselae*	PCR[¶]	*mef*(C) and *mph*(G)	Macrolides	[79]
2016	*Vibrio cholera* (N14–02106)	Illumina MiSeq and PacBio RSII single-molecule real-time sequencing	VCC-1 (Ambler Class A carbapenemase)	β-lactam antibiotics (penicillin, carbapenem and first-generation cephalosporins)	[80]
2016	*E. coli*	Illumina MiSeq	*mcr-2* (encodes phosphoethanolamine transferase)	Colistin	[81]
2017	*E. coli*	Illumina HiSeq	*mcr-3*	Colistin	[82]
		Illumina MiSeq	*Mcr-4*	Colistin	[83]
	S. typhimurium	Illumina MiSeq	*Mcr-4*	Colistin	

[†] rRNA methylase gene, a macrolide transporter gene and a macrolide phosphotransferase gene, respectively.

[‡] FRAMP1.1, CRAMP2.1, CRAMP2.2 and CRAMP2.3 encode β-lactamases whereas CRCHL2.2 and FRSPE1.1 encode a chloramphenicol acetyltransferase and adenyl transferase, respectively.

[§] Particular sequencing platform used not specified.

[¶] A follow-up study to determine the functions of genes discovered by WGS in a previous study.

IS5: Insertion sequence 5; NGS: Next-generation sequencing; NS: xxx; WGS: Whole-genome sequencing. **[Query-Q14: Dinesh Kumar(CE) to All(AU)] Please define the term 'NS' in the footnote of Table 2.**

Campylobacter coli

Chen *et al.* [73] discovered a novel plasmid-mediated aminoglycoside resistance gene, *aph (2'')-Ig*, in the foodborne pathogen *C. coli*, which together with *Campylobacter jejuni* is a major cause of infectious intestinal diseases or gastroenteritis [84]. The gene, which codes for a phosphotransferase enzyme, was

found by WGS of gentamicin-resistant *C. coli* strains obtained from retail meats (Table 2). This gene had not been previously described, although six other types/variants of the *aph (2'')* genes have been previously described and two of them have been reported to cause gentamicin resistance in Campylobacter [85]. Comparative genomics of two closely related species offered additional insight with respect to the factors that affect resistance as was the case in this study.

Due to the association of *C. coli* with poultry, pigs and food, it is necessary to continuously monitor the molecular epidemiology and resistance mechanisms of this pathogen to inform clinicians of the best antibiotic choices to use in *Campylobacter*-mediated gastroenteritis. Evidently, the detection of novel/emerging resistance mechanisms and associated clones or plasmids will necessitate a revision of antibiotic guidelines and enhance efficient infection control, respectively [86].

Klebsiella pneumoniae

Resistance to last-resort antibiotics such as carbapenems, colistin and tigecycline are commonly reported in *K. pneumoniae* worldwide, making this bacteria a notorious pathogen implicated in several resistant infections and subsequent mortalities [3,4]. In investigating the mechanism underlying tigecycline resistance in *K. pneumoniae*, Nielsen *et al.* [76] discovered a novel efflux pump operon, *kpgABC* as the cause; an increased expression of this gene was mediated by an insertion sequence 5 element (Table 2). The expression of *kpgABC* was independent of known efflux pumps like the functional AcrAB-TolC, whose activity is regulated by AraC-type regulators such as RamA, MarA and RarA [87]. The isolates in this study were multidrug- and extensively drug-resistant strains obtained from neonates, making it particularly concerning as tigecycline is reserved as an agent of last resort in this group [76].

Vibrio cholerae

Mangat *et al.* [80] reported the discovery of a novel Ambler class A carbapenemase, named VCC-1 from *V. cholerae* isolated from sea food (shrimp) in Canada (Table 2). The presence of a carbapenemase gene in a dangerous pathogen such as *V. cholerae* is especially worrying as infection from such carbapenemase-producing strains will be unresponsive to almost all β-lactam antibiotics. As *V. cholerae* is known to cause epidemics and pandemics in tropical regions [88], and carbapenemase genes are commonly associated with other resistance genes that confer resistance to fluoroquinolones, aminoglycosides, sulfamethoxazole–trimethoprim, etc. [4], an efficient genomics-based AMR surveillance and epidemiology is necessary to pre-empt the outbreak of pandrug-resistant *V. cholerae* strains, which can waste hundreds of lives.

The examined *V. cholerae* strain was observed to show resistance to β-lactam antibiotics, namely carbapenems, penicillins and monobactams, albeit no known β-lactamases were detected upon PCR screening. Sequencing of the genome of the strain, however, detected the novel carbapenemase, VCC-1, which was sufficiently inhibited by clavulanic acid and tazobactam. It is noteworthy that WGS, which directly facilitated the detection of this novel gene, was being used for the first time to discover an enzyme of this class. Again, this was the first report of a class A β-lactamase discovered in the Vibrionaceae, and the first report of a novel class A carbapenemase discovered in food.

Escherichia coli & Salmonella typhimurium

An equally important diarrheal-, bloodstream- and urinary-tract infection-causing pathogen is *E. coli*, which is one of the most well-studied model bacterial organisms of public health importance. It is also commonly associated with multidrug-resistance determinants and known to easily receive and/or transfer resistance plasmids to other species [89]. Liu *et al.* [10], made headlines with the discovery of *mcr-1*, which is the first plasmid-mediated colistin resistance mechanism described, by whole-plasmid sequencing. [Query-Q15: Dinesh Kumar(CE) to All(AU)] In the preceding sentence, year '(2016)' of Liu et al. has been replaced with the reference citation [10] of reference Liu et al. Please confirm the change. Before then, polymyxin resistance was due to chromosomal mutations [7,59-60]. In a study in Belgium, a novel plasmid-mediated colistin resistance gene, *mcr-2* (encoding phosphoethanolamine transferase), was found by WGS in *E. coli* isolates (from porcine and bovine sources), which had displayed resistance to colistin but lacked the known colistin-resistance gene *mcr-1*, with which it shares 76.7% nucleotide identity. Data from this study showed that the prevalence of *mcr-1* (11/53) was higher than that of *mcr-2* (7/53), highlighting the necessity of introducing screening methods for the novel gene during molecular surveillance (Table 2) [81].

In similar fashion, another novel plasmid-mediated colistin resistance gene, *mcr-3*, showing 45.0 and 47.0% nucleotide sequence identity to *mcr-1* and *mcr-2*, respectively, was detected in a colistin-resistant *E. coli* isolate (which was negative for *mcr-1* and *mcr-2*) using Illumina HiSeq sequencing technology. This comes on the heels of the discovery of the colistin-resistance genes *mcr-1* and *mcr-2*, which generated considerable worldwide attention. It was found in this study that the amino acid sequence of MCR-3 aligned closely with phosphoethanolamine transferases from Enterobacteriaceae and *Aeromonas* species originating from both clinical infections and environmental samples collected in 12 countries on 4 continents [82]. This is a significant event because of the potential transfer of *mcr-3* between Enterobacteriaceae and *Aeromonas* species, in light of the fact that aeromonads are abundant in the environment. The widespread use of colistin in veterinary medicine and the recent increase of its use in human medicine mean that there is the need to constantly monitor colistin-resistant

determinants in Gram-negative bacteria. This will provide insights into the dissemination of *mcr* genes to eventually tackle its attendant problems in the agricultural and healthcare sectors [82].

Recently, Carattoli *et al.* [83] identified a novel *mcr-4* colistin resistance gene from historical *S.* Typhimurium and *E. coli* isolates from pigs in 2013 in Italy, 2015 in Spain and 2016 in Belgium. This novel gene, which was found on non-selfconjugable ColE plasmids, was thus present in the pigs and pig farms prior to the detection of the first *mcr-1* gene in 2016 [10]. Obviously, this gene escaped detection until the whole genome of the isolates were analyzed. Subsequently, this novel gene should be added to all surveillance and screening protocols to increase its detection and prevent further spread into humans as consumption of these pigs will likely transfer these strains through the food chain. *E. coli* and *S.* Typhimurium are known zoonotic pathogens and the presence of these colistin resistance genes in these pathogens should be a cause for concern for food safety and public health.

Xu *et al.* [71] identified a novel bla_{KLUC} resistance gene, named $bla_{\text{KLUC-3}}$ that conferred resistance to some broad-spectrum β-lactams including cefotaxime, cefazolin and ceftriaxone (Table 2) in *E. coli* using whole plasmid sequencing. Notably, a substantial number of *E. coli* infections are community associated instead of nosocomial and their ability to transfer resistance plasmids to other species makes these novel discoveries especially troubling as they can be easily disseminated in both the community and hospitals to compromise the efficacy of important antibiotics [90,91].

Stenotrophomonas maltophilia

Using Illumina Hiseq to screen *S. maltophilia* from porcine sources, a novel variant of the known phenicol resistance gene, *floR*, named *floRv*, was detected in a genomic island (Table 2). The *floR* gene encodes an exporter protein and has been identified on diverse bacterial plasmids as well as on genomic islands. The emergence of *S. maltophilia* as a notable pathogen implicated in nosocomial infections means that the carriage of the *floR* gene or its variant can cause clinical treatment failure with the use of chloramphenicol [77]. As an emerging multidrug-resistant opportunistic pathogen increasingly associated with community-acquired and nosocomial infections including pneumonia, bacteremia, biliary sepsis, urinary tract infections, endocarditis, meningitis, etc., *S. maltophilia* is receiving worldwide attention. Thus, a comprehensive assessment of its resistance mechanism and molecular epidemiology is necessary as *S. maltophilia* is associated with substantial mortality rates and multidrug resistance [92].

Metagenomes of poultry microbiome

Most bacteria cannot be cultured under laboratory conditions, and for such species metagenomics comes in as a handy tool that enables scientists to characterize their genomes and resistance genes

without the need to culture them [93]. Six novel genes were described by Zhou *et al.* [70] in a microbiome study of chicken gut using metagenomics: FRAmp1.1 (ampicillin resistance), CRAmp2.1 (ampicillin resistance), CRAmp2.2 (ampicillin resistance), CRAmp2.3 (ampicillin resistance), CRChl2.2 (chloramphenicol resistance) and FRSpe1.1 (spectinomycin resistance). The first four encoded β-lactamases while the last two encoded a chloramphenicol acetyltransferase and adenyltransferase, respectively (Table 2). The ability of metagenomics in deciphering novel genes in various microbiomes is one of the fascinating endowments of NGS as it reveals resistance genes that can be transferred from commensals to pathogens in the future [93].

Photobacterium damselae

Nonaka *et al.* [79] determined the function of two novel genes, *mef*(C) and *mph*(G), which had been discovered by genome sequencing in *P. damselae* subsp. *damselae* in a previous study [94]. The zoonotic nature of *Photobacterium* infections and their association with marine animals (and as foodborne pathogens) and AMR make them of utmost public health concern. It was shown that the novel genes were macrolide resistance genes. In this study, the transformation of *E. coli* with *mph*(G) alone resulted in an increase in the MICs of erythromycin, clarithromycin and azithromycin, but no change was observed in macrolide susceptibility when *mef*(C) alone was introduced. However, introduction of both genes dramatically increased MICs of the same macrolides. The *mph*(G) gene encodes a macrolide phosphotransferase whereas *mef*(C) encodes an efflux pump. The results of this study suggested that the macrolide-inactivating effect of Mph(G) is essential for macrolide resistance and that Mef(C) is essential for the expression of high-level macrolide resistance.

Resistance to antibiotics caused by novel mutations in known and unknown NARGs in Gram-negative bacteria

Escherichia coli

In a study investigating reduced tigecycline susceptibility in *E. coli* mutants, various genes were identified to play a role. A group of mutations in genes designated as lipopolysaccharide (LPS) genes due to their effect on the LPS biosynthesis pathway and their involvement in heptose biosynthesis or transport was for the first time implicated in reduced tigecycline susceptibility (Table 2). The genes in the heptose biosynthetic pathway as well as the transport proteins transferring and incorporating heptose residues into the LPS inner-core structure were found to confer reduced tigecycline susceptibility in mutants [58].

Similarly, a 12-nucleotide deletion in *ribE*, which encodes 6,7-dimethyl-8-ribityllumazine synthase (lumazine synthase), a vital enzyme involved in the biosynthesis of flavin mononucleotide, which is an important cofactor for NfsA and NfsB, was found to contribute to nitrofurantoin resistance in *E. coli*. The importance of these mutations were confirmed with complementation studies. The 12-nucleotide

deletion in *ribE* resulted in a four-amino acid loss in the active site of lumazine synthase, at positions 131–134, compromising the ability of the strain to synthesize flavin mononucleotide. This resulted in decreased nitrofurantoin susceptibility [78].

Clinically, tigecycline and nitrofurantoin are important broad-spectrum antibiotics used for treating several bacterial and uncomplicated urinary-tract infections, respectively. These findings, particularly in *E. coli*, should be alarming enough to encourage the adoption of NGS in clinical microbiology laboratories if the fight against antibiotic resistance will be won. The development of antibiotic resistance seems dynamic, and the use of nongenome-wide-based approaches will make novel resistance determinants elude detection [26,27].

Acinetobacter baumannii

The dynamic nature of bacteria to evolve with novel resistance mechanisms that can evade available and last-resort antimicrobials was recently underscored by the discovery of Chen *et al*. They investigated the mechanisms underlying reduced tigecycline susceptibility in *A. baumannii*, using the whole genome of an isolate. A deletion in a gene therein named tigecycline-related methyltransferase gene, *trm*, was found to decrease susceptibility to tigecycline as well as to minocycline and doxycycline. The effect of the deletion in *trm*, which encodes S-adenosyl-L-methionine-dependent methyltransferase, was confirmed by complementation studies [74].

Loss-of-function mutations in the *adeN* gene in *A. baumannii* has been discovered to be responsible for the overexpression of the resistance-nodulation-division efflux system AdeIJK, leading to resistance to various antibiotics (Table 2) [72]. The results showed that AdeN is a repressor of AdeIJK expression and the mutation found in the gene abolished that function. The robust and multiple efflux pumps available in *A. baumannii* makes it resistant to many antibiotics [95].

Increasingly, *A. baumannii* is becoming a notorious extra-drug-resistant nosocomial pathogen, with resistance to almost all antimicrobials being reported. As part of the ESKAPE (*E. faecium*, *S. aureus*, *K. pneumoniae*, *A. baumannii*, *P. aeruginosa* and *Enterobacter* spp.) pathogens that present serious clinical challenges and multidrug resistance, the public health threat and economic losses posed by this pathogen cannot be overestimated, particularly when few antibiotics can be used to manage their infections, which includes pneumonia, bacteremia, wounds, meningitis, etc. [95].

Salmonella Typhimurium

Millions of people are affected by salmonellosis yearly throughout the world and *S. typhimurium*, which is commonly associated with animals such as pigs, is increasingly being implicated in foodborne salmonellosis as a foodborne pathogen and zoonosis. The ability of this pathogen to affect several hosts

makes it economically important with serious public health threats; hence, the development of AMR in *S. typhimurium* is of great concern [96-98]. Lofton *et al.* [75] subjected an antimicrobial peptide (AMP)-resistant *S. typhimurium* to WGS and incriminated mutations in both the LPS core biosynthesis pathway gene, *waaY*, and the two-component signal transduction systems, *pmrB* and *phoP*. It was found that mutations in *waaY* resulted in a reduction in the bacterial susceptibility to the AMPs tested, suggesting that resistance to AMPs can occur quickly in bacteria. The *waaY* gene attaches a phosphate group to the heptose II residue of the LPS molecule [99], thus creating enough negative charges on the membrane to allow for the interaction with positively charged AMPs. Mutations in *waaY* interrupt this process, thereby reducing the affinity and interaction of AMPs with the outer membrane.

AMPs and colistin have similar mechanisms of action, meaning that resistance to AMPs could select for colistin resistance. As a last-resort antibiotic for carbapenem-resistant bacterial infections, colistin is a very critical drug [3]. As such, the identification of AMP resistance is very concerning, particularly as *S. typhimurium* affects millions of people and animals yearly and can also spread such resistance determinants to other bacteria. Such ominous threats make the need for genome-based AMR studies and molecular epidemiology dire.

Pseudomonas aeruginosa

Similarly, Muller *et al.* [69] showed that peptide-adaptive resistance regulator and sensor (ParRS), a two-component regulatory system, mediates multidrug resistance to polymyxins (colistin), aminoglycosides, fluoroquinolones and β-lactams in the opportunistic multidrug resistant pathogen *P. aeruginosa*, which is one of the most resilient and resistant microbes on the planet. As the leading cause of death among Gram-negative bacterial infections and a major cause of infections in patients with cystic fibrosis, *P. aeruginosa* is a clinical conundrum [100]. WGS of a multidrug-resistant phenotype revealed a mutation in the ParR protein, the response regulator of the two-component system, with ParS being a sensor kinase. The role of the mutated ParRS in the resistance phenotype was determined by complementation studies. Only a few among the several two-component regulatory systems found in *P. aeruginosa* have been shown to confer substantial antibiotic resistance in clinical strains. In fact, this was the first reported study showing the role played by ParRS in the adaptation of bacteria to antibiotics.

Resistance to antibiotics caused by novel mutations in known & unknown NARGs in mycobacteria

Winglee *et al.* [50] discovered a loss-of-function mutation in the *Rv2887* gene in *M. tuberculosis* that conferred resistance to MPIII-71, an imidazole-based agent with a strong activity against *M. tuberculosis*. *Rv2887* encodes a MarR-like transcriptional regulator. It was found that mutations in *Rv2887* abrogated the activity of the efflux pump inhibitors verapamil and chlorpromazine. Resistance

to antitubercular agents is on the increase, which makes tuberculosis difficult to treat and makes antibiotic resistance studies in *M. tuberculosis* particularly of paramount importance [50]. Resistance to first-line antitubercular drugs such as isoniazid and rifampin, and to second-line drugs (including streptomycin, pyrazinamide, ethambutol, etc.) in tuberculosis patients claimed hundreds of lives in 2015 alone as second-line drugs, which are expensive, toxic and scarce, could respectively only treat 52 and 28% of multidrug-resistant and extra-drug-resistant infections [101]. [Query-Q16: Sharon Salt(PE) to All(AU)] I have moved the website URL mentioned in the preceding sentence to the reference list as per our house style. Please may you let me know if this is alright with you?

Apparently, WGS has made it possible to detect these resistance mechanisms, which would not be possible if, for instance, a method such as PCR or microarray was used to target known resistance genes. WGS is therefore a formidable procedure that can be used to identify resistance-conferring mutations that occur in clinical bacterial isolates and to delineate their molecular epidemiology and evolutionary biology concurrently.

The benefits of WGS in characterizing resistance mechanisms in fungi

Drug resistance in fungi is increasingly being reported, particularly in *C. albicans*, *C. glabrata* and *C. auris*, making the management of their infections in normal and immunocompromised patients especially challenging [21,31,102]. The high attributable mortalities, prolonged hospitalization and expensive treatment associated with fungi infections in the face of limited antifungal drugs make the problem of antifungal drug resistance a grave public health menace [20]. The relatively less studied mechanisms, epidemiology and effects/burden of antifungal resistance, when candidemia is becoming difficult to treat with fluconazole and the echinocandins, is disadvantageous to public health; increasing research into the burden, levels, effects and mechanisms of antifungal drug resistance is warranted [103]. [Query-Q17: Sharon Salt(PE) to All(AU)] I have moved the website URL mentioned in the preceding sentence to the reference list as per our house style. Please may you let me know if this is alright with you?

Candida glabrata

Singh-Babak *et al.* presented the first global analysis of mutations associated with antifungal drug resistance using a series of *C. glabrata* isolates and identified mutations in the drug target, *FKS2* gene (Figure 2). These mutations were associated with resistance to caspofungin, an echinocandin which targets cell wall biosynthesis in fungi. *FKS2* encodes 1,3-β-D-glucan synthases, which are required for the synthesis of 1,3-β-D-glucan, a key fungal cell wall component (Figure 2) [9]. As well, nonsynonymous mutations were observed in a gene (*CDC6*) not known previously to confer echinocandin resistance. The *CDC6* gene product, in other words, CDC6 protein, is involved in DNA replication initiation. The

effects of the *FKS2* (S663P) and *CDC6* mutations were confirmed by transformation [9] and could help in the design of new drugs to affect different drug targets or inform drug combinations to overcome the effect of these mutations in future.

Candida albicans & Saccharomyces cerevisiae

Investigating the evolution of resistance to antifungal drug combinations in *S. cerevisiae* and *C. albicans*, Hill *et al.* [104] discovered drug-target mutations that conferred resistance to geldanamycin or FK506. Geldanamycin and FK506 are inhibitors of Hsp90 and calcineurin, respectively. Hsp90 and calcineurin are two important cellular regulators of fungal stress responses, in this case drug-induced responses [104]. Seeking out stress response cellular regulators has appeared as an auspicious strategy to improve the effectiveness of antifungal drugs and to abolish drug resistance [20]. The stability and role of different client proteins are regulated by the molecular chaperone Hsp90, which also controls stress responses essential for drug resistance by stabilizing the protein phosphatase calcineurin [104].

The inhibition of the functions of Hsp90 and calcineurin change antifungals from fungistatic to fungicidal, improving the effectiveness of antifungals in mammalian models of systemic and biofilm-associated fungal infections. This suggests that combining azoles, which are fungistatic, with Hsp90 or calcineurin inhibitors may be a highly useful approach to combating life-threatening fungal infections. Considering that combination therapies have brought enormous success to the treatment of HIV, tuberculosis and malaria, combination therapy of fungal infection is promising. In this study, sequencing revealed a nonsynonymous point mutation (I117N) in the *HSC82* gene, which encodes Hsp90 in *S. cerevisiae*. The impact of this mutation on azole and geldanamycin resistance was confirmed by performing an allele swap, which showed that the mutation conferred resistance to azole and geldanamycin drug combinations (Table 3) [104].

Table 3. Novel resistance mechanisms discovered in fungi after 2008.

Year of study	Microbial species	NGS platform used (e.g., Ion Torrent, Illumina, PacBio, etc.)	Resistance mechanism detected	Antibiotic/antibiotic class to which resistance mechanism was found	Ref.
2012	*Candida glabrata*	Illumina	Mutations in the *FKS2* and CDC6 genes	Echinocandin	[9]
2013	*Candida albicans*	Illumina HiSeq	Mutations in *ERG2*, *ERG3* and *ERG11* encoding C-8 sterol isomerase, C-5 sterol desaturase and lanosterol 14a-demethylase, respectively	Amphotericin B	[17]
2013	*Saccharomyces cerevisiae*	Illumina HiSeq	Mutations in the HSC82 gene (which encodes Hsp90)	Azole, geldanamycin and FK506	[104]
2014	*Aspergillus nidulans*	Illumina HiSeq	Mutation in ANID_10647 (predicted cytochrome P450 protein) and ANID_03445	Calcofluor white	[18]

2015	*Fusarium graminearum*	Illumina Hiseq	Mutations in myosin-5	Phenamacril	[105]

NGS: Next-generation sequencing.

It has long been observed that *C. albicans*, a prominent fungal pathogen of humans, quickly develops resistance to two main antifungal classes, the triazoles and echinocandins. Conversely, with regards to amphotericin B, another major antifungal in clinical use, resistance has been found to be extremely rare even though it has been used for five decades as monotherapy [17]. Vincent *et al.* used WGS to analyze a rare amphotericin B-resistant clinical isolate as well as laboratory-evolved strains, to probe mutations conferring amphotericin B resistance *in vitro*. Mutations in *ERG2*, *ERG3* and *ERG11* encoding C-8 sterol isomerase, C-5 sterol desaturase and lanosterol 14a-demethylase, respectively, were detected in test strains and confirmed by complementation studies to be responsible for the resistance observed. Triazoles inhibit Erg11 (lanosterol 14a-demethylase), preventing ergosterol biosynthesis [17]. It was also discovered in this study that these mutations, which resulted in resistance, concurrently set up various stresses that required high levels of the molecular chaperone Hsp90 for survival, even in the absence of amphotericin B (Table 3).

Aspergillus nidulans

Antifungal drug resistance mutations in *A. nidulans* were characterized by He *et al.* [18] and they identified a C1198T mutation in ANID_10647, which had been previously characterized and annotated as a predicted cytochrome P450 protein [106]; however, the mutation created a premature stop codon in the predicted protein product. The study also detected a G1081T mutation in ANID_03445, a previously undescribed gene in *A. nidulans*, but its *CHS4* ortholog has been characterized previously in *S. cerevisiae* as encoding the activator of the major chitin synthase (CHS3) [107], which positively regulates chitin formation. This mutation in similar fashion gave rise to a stop codon in the middle of the predicted protein product, cutting out several amino acids from the C-terminal sequence. These two mutations in the isolates concerned, resulted in the development of resistance to calcofluor white, used therein as a model antifungal drug. The chitin synthase activity, which the ANID_03445 G1018T mutation possibly compromised, could have led to reduced chitin in the *A. nidulans* cell wall and resistance to calcofluor white. The effects of these two specific mutations were verified by further confirmatory tests.

Fusarium graminearum

Zheng *et al.* [105] discovered that point mutations in the gene encoding myosin-5 were responsible for conferring resistance to the fungicide phenamacril in *F. graminearum*, the causal agent of *Fusarium* head blight. Phenamacril is a fungicide of the cyanoacrylate class and is used to control *Fusarium* head

blight due to its specificity toward *Fusarium* [105]. Following the development of resistance to this fungicide, WGS was used to screen an *F. graminearum*-resistant strain. Myosins play critical roles in cytokinesis, transcriptional regulation, vesicle/organelle transport, actin filament bundle organization, cell polarization, signal transduction and intracellular transport. In *F. graminearum*, myosin-5 is encoded by the FGSG_01410.1 gene [105]. The results of this study indicated that the mode of action of phenamacril involves binding to the motor domain of myosin-5, and the mutation in this protein compromises the action of the fungicide; transformation experiments confirmed the effect of this mutation.

Experimental protocols & bioinformatic tools

The steps involved in the functional characterization of novel AMR genes begins with the identification of the selected isolate through molecular, proteomic and/or biochemical methods such as 16S PCR, MALDI–TOF MS and API micro-organisms' identification kits (BioMérieux), respectively. [Query-Q18: Dinesh Kumar(CE) to All(AU)] Please provide the city/state code and country for 'BioMérieux' in preceding sentence. Disc diffusion and/or broth microdilution, Etest, VITEK-2, BD Phoenix, MicroScan Walkaway antimicrobial sensitivity testing platforms and agar dilution methods are subsequently used to determine the isolates' susceptibility to and/or MICs of selected antimicrobials [23]. Genomic DNA of drug-resistant isolates are then extracted using extraction kits (with instruments such as NucleoBond buffer set III and NucleoBond AX-G 100) with manufacturer's instructions [105].

Library preparation and genome sequencing are then performed on test isolates using an appropriate NGS platform such as Illumina, PacBio, Ion Torrent and 454 Life Sciences GS-FLX system (Tables 1 & 2) to investigate the genetic basis of the observed resistance. The reads obtained from sequencing are normally quality controlled using software such as cutadapt [108], fastx [109], trimmomatic [110], etc. to remove sequencing artifacts/adaptors and poor quality reads prior to assembly with the appropriate software (e.g., Newbler 1.1.03.24, Velvet, Spades or Phrap) [10]. [Query-Q19: Sharon Salt(PE) to All(AU)] I have moved the website URLs mentioned in the preceding sentence to the reference list as per our house style. Please may you let me know if this is alright with you? SAMtools [111], GATK [112], Bowtie and BWA aligners are also commonly used by researchers to align/map the sequenced reads/genomes (resistant against reference genomes) and call SNPs to identify differences between the resistant and susceptible strain. [Query-Q20: Sharon Salt(PE) to All(AU)] I have moved the website URLs mentioned in the preceding sentence to the reference list as per our house style. Please may you let me know if this is alright with you? On the other hand, raw reads can be first assembled and annotated prior to comparing with the genomes of susceptible strains to call for SNPs [7-8,113]. ANNOVAR and SnpEff are common software that help scientists predict the effect of SNPs on the proteins products [114].

Identified genes as well as mutations in genes suspected to be responsible for the observed resistance phenotypes are then subjected to transformations/complementation/functional cloning studies to investigate their effects in the transformants or knock-out mutants, which confirms the function(s) or effect(s) of the gene(s) or mutation(s) [3].

Methods & results obtained

Literature search

PubMed and Science Direct were systematically searched for English research articles, using the following combined search term: '(whole-genome sequencing OR next-generation sequencing) AND (antibiotic resistance/persistence OR resistance/persistence mechanisms) AND (bacteria)'; '(whole-genome sequencing OR next-generation sequencing) AND (antifungal resistance mechanisms OR antifungal resistance)'.

Study selection

Any article published in 2008 or later (from 2008 to 2017), describing the discovery of antimicrobial resistance mechanisms previously not known, using NGS in bacteria and fungi were included. Articles not published in English were not included. Only studies reporting on unknown resistance mechanisms (novel genes or mutations in NARGs) that were verified by further confirmatory studies such as transformations/complementation/functional cloning were included. The focus of the review was primarily to assess how advances in genomics have contributed to the identification of emerging antimicrobial-resistance mechanisms in bacteria and fungi. All papers that only addressed classical (already known) resistance mechanisms such as *mec*A, ESBLS, *gyr*A, *par*CE, etc. were excluded. Last, all reviews, abstracts and conference proceedings or papers were not included.

The search yielded more than 200 articles. Of these, 38 articles were included based on the inclusion criteria discussed above. No article was found that addressed novel mechanisms of persistence with respect to advances in genomics.

Future perspective

The evolution and evolutionary drivers of antimicrobial resistance in bacterial and fungal populations are gaining greater attention in this age of genomics as more researchers try to understand the phenomena of antimicrobial resistance and attempt to design novel drugs with new target sites to tackle this menace [26-27,29]. History attests to the fact that the search for and design of novel antimicrobials are partly driven by the inexorable development of antimicrobial resistance among prokaryotes and fungi [4,23].

Recently published studies showing the relatively easy acquisition of surrounding genes and their subsequent mobilization through plasmids between bacteria [29], the ability of bacterial chromosomes and plasmids to mutate in the presence of antibiotic stress through intragenomic coevolution for their mutual benefit and greater antibiotic resistance [26] and the ability of bacteria to undergo compensatory mutations to acquire and maintain various plasmids should be a wake-up call [27]. These recent findings tell us of the advanced capacities of bacteria to generate and share resistance mechanisms, even to drugs they have not been exposed to, to permit, adapt to and maintain various forms of resistance-bearing plasmids for surviving antibiotic stress. Such alarming capacities obviously require advanced technologies and skill to overcome.

Increasing interest in *in vivo* transcriptional profiling is welcoming as it will open up novel target genes that enable pathogens to infect and persist in the host. As these technologies advance, it is our belief that the complexities and intricacies underlying the selection and emergence of ARGs and/or NARGs, in addition to antibiotic persistence shall be deciphered [2-3,8].

Clinicians and epidemiologists should strive to keep abreast with these technologies and with novel resistance determinants as bacteria and fungi are ever evolving newer resistance mechanisms. Protocols for diagnosis, either for clinical or epidemiological purposes should consider recently detected determinants, considering the implications of not screening for such novel resistance mechanisms. Given the merits of NGS technologies, which include speed and cost–effectiveness, they are potentially in line to replace or at least complement large-scale AMR gene detection methods such as microarray.

Executive summary

- Earlier molecular tools that have been in use before the introduction of next-generation sequencing (NGS) technologies, such as PCR and microarrays, are unable to detect novel or unknown resistance mechanisms in bacteria.
- With the introduction of NGS, both unknown resistance genes and resistance-conferring mutations are being detected and confirmed with functional studies.
- Genes such as *graR* (in *Staphylococcus aureus*), *adeN* (in *Acinetobacter baumannii*), *lpcA*, *rfaE*, *rfaD* and *rfaC* (in *Escherichia coli*) that do not confer resistance in wild-type strains, are now known to confer resistance when they undergo mutations under antibiotic stress.
- Compared with PCR and microarrays, NGS provides advanced tools to identify almost all resistance mechanisms in resistant bacteria and fungi.

Author's contributions

J Asante collated the data and wrote the paper. JO Sekyere conceived, designed and supervised the study, collated the data, verified included papers, wrote and formatted the paper. All authors agreed to the final formatting and state of the paper for publication.

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