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Transiently silent acquired antimicrobial resistance: an emerging challenge in susceptibility testing

Theresa Maria Wagner [1], Benjamin Peter Howden [2], Arnfinn Sundsfjord[1,3]† and Kristin Hegstad [1,3]*†

[1]Research Group for Host-Microbe Interactions, Department of Medical Biology, Faculty of Health Sciences, UiT the Arctic University of Norway, Tromsø, Norway; [2]Microbiological Diagnostic Unit Public Health Laboratory, The Department of Microbiology and Immunology, The University of Melbourne at The Peter Doherty Institute for Infection and Immunity, Melbourne, Australia; [3]Norwegian National Advisory Unit on Detection of Antimicrobial Resistance, Department of Microbiology and Infection Control, University Hospital of North Norway, Tromsø, Norway

*Corresponding author. E-mail: Kristin.Hegstad@uit.no
†Equal contributions.

Acquisition and expression of antimicrobial resistance (AMR) mechanisms in bacteria are often associated with a fitness cost. Thus, evolutionary adaptation and fitness cost compensation may support the advance of subpopulations with a silent resistance phenotype when the antibiotic selection pressure is absent. However, reports are emerging on the transient nature of silent acquired AMR, describing genetic alterations that can change the expression of these determinants to a clinically relevant level of resistance, and the association with breakthrough infections causing treatment failures. This phenomenon of transiently silent acquired AMR (tsaAMR) is likely to increase, considering the overall expansion of acquired AMR in bacterial pathogens. Moreover, the augmented use of genotypic methods in combination with conventional phenotypic antimicrobial susceptibility testing (AST) will increasingly enable the detection of genotype and phenotype discrepancy. This review defines tsaAMR as acquired antimicrobial resistance genes with a corresponding phenotype within the wild-type distribution or below the clinical breakpoint for susceptibility for which genetic alterations can mediate expression to a clinically relevant level of resistance.

References to *in vivo* resistance development and therapeutic failures caused by selected resistant subpopulations of tsaAMR in Gram-positive and Gram-negative pathogens are given. We also describe the underlying molecular mechanisms, including alterations in the expression, reading frame or copy number of AMR determinants, and discuss the clinical relevance concerning challenges for conventional AST.

Background

The increased prevalence of antimicrobial resistance (AMR) in bacterial pathogens emphasizes the need for rapid and accurate antimicrobial susceptibility testing (AST) to guide antibiotic therapy. Conventional AST is based on evaluating phenotypic inhibition of growth of a bacterium in pure culture by an antibiotic.[1] Complementary methods include the genetic detection of acquired AMR determinants, which may allow inference of resistance to the corresponding antibiotic.[2] However, the expression of acquired AMR determinants is often thought to be costly to the bacteria.[3] Thus, with reduced selection pressure from antibiotics, evolution may support the expansion of subpopulations with genetic modifications that tightly regulate or silence the expression of the acquired AMR determinant, suppressing the resistant phenotype allowing discrepancies between the genotype and phenotype. This poses a clinical concern as antibiotic exposure may select for genetically altered subpopulations with clinically significant expression of those quiescent acquired AMR determinants.

In the last decade, several observations of genetic modifications that reverse the expression of transiently silent acquired AMR (tsaAMR) to a clinically resistant phenotype have been described.[4,5] This phenomenon has been thoroughly characterized for VanA-type vancomycin-resistant *Enterococcus faecium* and MRSA, also associated with therapeutic failures.[6,7] Conversion of tsaAMR to clinical resistance has also been observed in Enterobacterales and *Pseudomonas aeruginosa*.[8–11]

In this review, we define tsaAMR, describe the underlying molecular mechanisms and discuss challenges for AST.

Defining tsaAMR

We define tsaAMR as acquired AMR genes with a corresponding phenotype within the wild-type distribution or below the clinical

breakpoint for susceptibility. When these determinants are present, genetic alterations can turn on or change their level of expression to a clinically relevant level of resistance. We define clinically relevant as a ≥8-fold increase in MIC or an increase in MIC to a level that needs antibiotic dosing adjustment (I - Susceptible, increased exposure) or is regarded as resistant according to internationally accepted guidelines.[12,13] Within the scope of tsaAMR we exclude genetic modifications in inherent chromosomal genes that cause a significant increase in resistance. These are well-described phenomena related to antibiotic target site mutations occurring at certain frequencies as well as mechanisms within the concept of monoclonal heteroresistance (see below).[14] Thus, we frame the concept of tsaAMR to acquired AMR genes and their ability to be transiently silent causing discrepancies and diagnostic challenges when comparing antibiotic resistance phenotype and genotype. Alterations in acquired determinants is an emerging field as their expansion in number due to clonal dispersion and dissemination by multicopy mobile genetic elements (MGEs) makes them more exposed to genetic changes.

The current literature describes several complex phenomena that are named differently but potentially associated to tsaAMR in different ways. These phenomena are depicted in Table 1, which also provides a short description of these phenomena and their potential relationship to tsaAMR. The concept of heteroresistance is a population-wide variation of antibiotic resistance, whereby different subpopulations of an isolate exhibit various susceptibilities to a particular antimicrobial agent.[14,28] This definition covers a broad range of phenomena at defined frequencies (greater than 1×10^{-7}) as well as a defined increase in MIC (≥8-fold) compared with the main population. The underlying molecular mechanisms include chromosomal mutations in regulatory genes or determinants for antibiotic permeability (porins) and efflux as well as increased gene dosage due to tandem gene amplification. Importantly, recent studies have shown that various Gram-negative and Gram-positive bacteria can express heteroresistance towards many classes of antibiotics, of which some species-antibiotic combinations have been associated with treatment failures.[14] It is outside the scope of this review to elaborate further on the other concepts or differences in nomenclature except for silencing of antibiotic resistance by mutation (SARM) and variable resistance that is mechanistically clearly associated with the evolution of tsaAMR.[4–6]

Underlying molecular mechanisms of tsaAMR

The genetic basis of tsaAMR will be discussed within the frame of mutational resistance and relevant characteristics of MGEs. Mutations and MGEs are major players in bacterial genome plasticity and the development of antimicrobial resistance. In particular MGEs play a crucial role in the capture, accumulation and spread of AMR genes, as recently reviewed.[29]

The extensive study of antibiotic resistance phenotype/genotype discrepancies (10.3%) in a collection of 1470 *Staphylococcus aureus* isolates clearly underlines the importance of mutations in transiently silent acquired resistance determinants.[4] The genetic basis for silencing by mutations, including IS insertions, positioned within or upstream of the antibiotic resistance determinants, could explain most of the discrepancies. Importantly, SARM was reversible in most strains at clinically relevant frequencies after exposure to the corresponding antibiotics.

The potential role of MGEs in the evolution of tsaAMR can be illustrated in at least two different contexts. The first one is related to MGEs as major vehicles for horizontal gene transfer (HGT) of AMR determinants. The multicopy and often replicative nature of MGEs make them exposed to genetic alterations including mutations that may affect expression of the inherent resistance gene and contribute to subpopulations that favour the development of tsaAMR. The second one is related to the mobile nature of major classes of MGEs. The MGE itself may mediate silencing or conversion to phenotypic resistance through insertion and excision events directly affecting the resistance gene integrity or its expression. Figure 1 illustrates molecular mechanisms in the conversion to a resistant phenotype: modifications in the promoter (Figure 1a), the coding region (Figure 1b) or increased copy number of the AMR gene (Figure 1c); these will be discussed in detail in sections below.

Intrinsic characteristics of MGEs related to the occurrence of tsaAMR

Small MGEs, like ISs and transposons (Tns), encode their own intracellular mobility (transposition). Through insertion and excision, they can disrupt or restore an AMR determinant and/or its promoter, enabling the switch between silence or expression in a single genetic event. ISs carry a transposase gene (*tnp*) encoding their transposition but can also capture accessory DNA, including AMR genes. Transposition can occur by non-replicative cut-and-paste or by replicative mechanisms. In replicative transposition, the element joins the donor and recipient DNA site in a co-integrate as for Tn*3* and IS*6* family elements, which is then resolved to the original donor plus the recipient with the element[30]. Alternatively, non-replicative transposition most commonly takes place by a copy-paste mechanism, which involves many IS families such as IS*3*, IS*21*, IS*30*, IS*256* and IS*L3*, where a double-stranded circular intermediate is formed and then integrates into the recipient[31] (reviewed in[32–34]). The replicative mechanisms support gene amplification events.

Several IS families carry a strong promoter facilitating gene expression.[35] Thus, the IS insertion can increase the expression of a downstream resistance gene mediating a resistance phenotype (Figure 1a). IS families known to carry complete outward-directed promoters include the IS*3*, IS*4*, IS*5*, IS*6* and IS*1380*. Besides providing a complete promoter, ISs may also activate genes through the formation of a hybrid promoter. The IS can provide a −35 box promoter sequence, which is functionally aligned with the −10 box promoter sequence of the adjacent gene. IS families related in hybrid promoter formation include the IS*256*, IS*1*, IS*3*, IS*6* and IS*30* families.[34] IS*256* has also been described to form hybrid promoters upstream of intrinsic AMR genes.[36–38] Moreover, ISs can also modify resistance phenotypes by excision from or disrupting AMR-encoding ORFs.[4]

Stress-induced elevated levels of IS transposition—transposition burst—support mechanisms associated with silencing or restoring resistance.[39,40] Antibiotic exposure, such as subinhibitory concentrations of chloramphenicol, linezolid, spectinomycin, ciprofloxacin and vancomycin, have been shown to induce transposition of IS*256*,[41,42] whereas cefotaxime, ceftazidime and piperacillin enhanced the transposition of IS*Ecp1B*.[43] Excision

Table 1. Terminology of phenomena potentially related to tsaAMR

Term	Description	Relation to tsaAMR	Ref.
Silencing of antibiotic resistance by mutation (SARM)	Defined as mutationally silenced acquired AMR genes Exemplified in *S. aureus*, which in most cases can revert to antibiotic resistance at frequencies $\geq 10^{-9}$	Covered within tsaAMR, but SARM makes no distinction whether the isolate can revert to resistance or not	4
Variable resistance	Defined as an initially antibiotic-susceptible isolate that can become resistant during exposure with the corresponding antibiotic Typically used for vancomycin variable enterococci (VVE)	Covered within tsaAMR. Mutational resistance and increased copy number affecting the functional expression of the acquired AMR gene	6,15–17
Cryptic resistance (1)	Defined as hidden resistance escaping diagnostic testing Description of various antibiotic resistance mechanisms in *S. aureus* that might escape phenotypic detection including oxacillin-susceptible MRSA (OS-MRSA), hVISA (heteroresistant vancomycin-intermediate *S. aureus*) and inducible clindamycin resistance	Partly related to tsaAMR as it includes the mutational conversion of OS-MRSA to a full MRSA phenotype	18
Cryptic resistance (2)	Defined as unclassified resistance genes that confer resistance upon amplification Experimental manipulation of plasmid cloned *E. coli* chromosomal DNA copy number revealed intrinsic genes that conferred resistance to several antibiotic classes when amplified	Not related to tsaAMR as it involves intrinsic chromosomal genes only and gene amplification that might be related to heteroresistance	19
Intermediate resistance	Defined as an isolate with a MIC between susceptible and resistant Exemplified in experimental evolution of reduced daptomycin susceptibility and vancomycin resistance in vancomycin-intermediate *S. aureus* (VISA) during vancomycin selection and experimental evolution of VISA and reversion to vancomycin susceptibility after serial passages without vancomycin selection	Not related to tsaAMR as it only involves changes in intrinsic chromosomal DNA	20–23
Adaptive resistance	Defined as a temporary increase in the ability of a bacterium to survive antibiotic exposure due to alterations in chromosomal DNA after environmental stress	Not related to tsaAMR as it only involves transient changes in intrinsic chromosomal DNA	24
Heteroresistance	Defined as a population-wide variation of antibiotic resistance towards a defined antibiotic, where subpopulations at a high frequency ($>1\times10^{-7}$) exhibit at least 8-fold higher MIC than the clinically susceptible main population	Not related to tsaAMR as it involves host tandem gene amplifications and mutations in intrinsic chromosomal genes including regulatory genes, permeability and efflux	14
Interniche heteroresistance	Defined as a phenomenon where susceptible and resistant isolates of the same strain are located in different sites Exemplified by susceptible and resistant *Helicobacter pylori* isolates of the same strain located in different anatomical sites—the antrum versus corpus ventriculi in humans	Not related to tsaAMR. The phenomenon is associated with monoclonal heteroresistance	25,26
Phenotypic heterogeneous resistance (PHR)	Defined as growth of colonies within the inhibition zone of antibiotics Exemplified by carbapenem heteroresistance in *Acinetobacter baumannii*	Not related to tsaAMR. The phenomenon is associated with monoclonal heteroresistance	27

(1) and (2) are used when there is more than one definition described by a single term.

and transfer rates of integrative and conjugative elements or cassette rearrangements are also influenced by environmental factors[44,45] including antibiotics.[46] Moreover, β-lactams and antibiotics targeting DNA replication and repair have been shown to trigger the excision of the staphylococcal cassette chromosome *mec* via the SOS response,[46] and erythromycin stimulated transposition of the macrolide resistance-encoding Tn*917*.[47]

Importantly, antibiotic exposure has also been shown to promote genetic rearrangements supporting clinical resistance development *in vivo*. Metronidazole treatment was associated with an activated SOS response, increased integrase expression, integron gene cassette rearrangements and subsequently increased β-lactamase (OXA-28) expression mediating high-level ceftazidime resistance in an epidemic *P. aeruginosa* strain.[10]

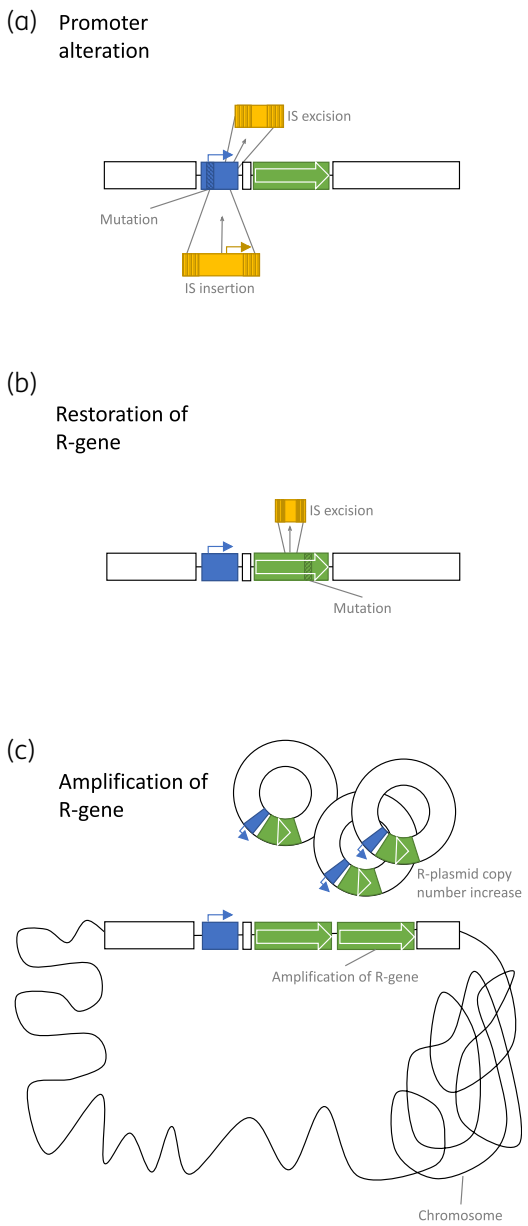

Figure 1. Molecular mechanisms in resurrection of transiently silent acquired AMR. Genetic alterations can occur in the promoter region upstream of the acquired resistance gene (R-gene), in the acquired R-gene itself, or change the R-gene copy number. (a) Promoter alterations may occur through mutations, insertions of ISs that provide functional or stronger promoters enhancing gene expression or excision of ISs reversing blockage of promoters. (b) Acquired R-genes may be restored through (point) mutations or excision of silencing ISs. (c) Amplification of acquired chromosomal or plasmid R-genes can restore resistance. Promoter regions are illustrated as boxes with bent arrows, R-genes as boxes with double lined arrows indicating reading frame direction, mutations as boxes with diagonal stripes, and ISs as boxes flanked with vertical stripes.

Furthermore, MGEs are enriched in extreme environments, such as antibiotic-exposed niches, where they contribute to niche adaptation.[48] In *E. faecium* MGEs, including plasmids, phages, genomic islands and especially ISs (IS*3*, IS*110*, IS*256* and IS*16*), are more prevalent in clinical than in commensal strains.[49–51] MGEs, like plasmids, prophages, ISs (IS*256*) and other transposons, are numerous in clinical *Staphylococcus haemolyticus* strains,[52] and specific MGEs are associated with epidemic *S. aureus*,[53,54] *Escherichia coli*[55,56] and *Klebsiella pneumoniae* clones.[57,58] Extended examples of the molecular mechanisms involved in tsaAMR are given in Table 2, and some are discussed below. The mechanisms encompass alterations in the regulation of AMR gene expression, reading frame or copy number, predominantly through mutations. Silencing typically comprises truncations of AMR genes or their promoters and corresponding mutations. Conversion to the resistant phenotype can either be a direct reversion or a compensatory genetic change in a different location. Silencing may also be irreversible.[4]

Alterations in the regulation of AMR expression

Rapid *de novo* evolution of functional promoters (Figure 1a) from random sequences has been demonstrated in an experimental *E. coli* system.[70] Correspondingly, single nucleotide mutations in silent *E. faecium vanB* clusters were shown to be associated with restored expression of clinical vancomycin resistance upon antibiotic exposure.[59] Similarly, selection of deletions predicted to remove a transcription inhibitory secondary structure and introduce a constitutive promoter for *vanHAX* expression was observed during vancomycin exposure of vancomycin variable enterococci (VVE).[61] VVE are *van*-positive isolates with a vancomycin-susceptible phenotype that can convert to resistance and be selected for during vancomycin selection. Likewise, a 44 bp deletion in the promoter region leading to constitutive *vanHAX* expression was observed in a VVE strain with a 5′-truncated *vanR* gene.[15] Moreover, constitutive *vanHBX* expression and teicoplanin resistance in *E. faecium* were caused by an 18 bp deletion in the vancomycin sensor gene $vanS_B$. In combination with an insertion in the housekeeping *ddl* gene inactivating the host D-alanine:D-alanine ligase, the constitutive synthesis of D-alanyl-D-lactate terminating precursors supported peptidoglycan synthesis.[60]

Corresponding findings have been observed in Gram-negative bacteria and MRSA. A 118 bp deletion upstream of the Tn*4401*-associated *K. pneumoniae* carbapenemase gene bla_{KPC}, created the novel isoform Tn*4401*h with a stronger promoter and enhanced bla_{KPC} expression in *E. coli*, increasing the meropenem MIC from 0.5 mg/L to ≥16 mg/L.[9] The expression of *mecA*, encoding methicillin resistance in staphylococci, can be regulated by the *bla* operon in the absence of the homologous *mecRI–mecI*.[71] In *S. aureus*, a frameshift mutation in the *blaRI* regulator gene silenced *mecA*.[4] However, the truncated *blaRI* failed to sense the antibiotic and derepress *mecA*. Upon cefoxitin exposure, the resistant revertant lost the *bla* operon, allowing constitutive *mecA* expression.

Several of the referred deletions appeared between tandem repeats.[8,15,60,72,73] Therefore, it has been hypothesized that the repeats in the AMR gene loci may act as emergency points allowing bacteria to express resistance genes when challenged. Apart from the mechanisms described here, it is likely that unknown *trans*-acting elements can provide resistance on and off switches. It has been suggested that chromosomally encoded transcriptional control could override standard R-plasmid gene

Table 2. Transiently silent acquired antimicrobial resistance: mechanisms and relevant characteristics

	Species	Antibiotics	AMR gene	Mechanism of silencing	Mechanism of resistance conversion	Conversion rate	Increase in MIC	Ref.
Alterations in the regulation of AMR expression: mutations and deletions								
Gram-positive	*E. faecium*	Glycopeptide, vancomycin	*vanA*	Truncation of *vanR*	44 bp deletion upstream of *vanHAX* moved alternative promoters for constitutive *vanHAX* expression	2×10^{-8}	1 mg/L to >256 mg/L (with both deletion and increased copy number)	15
	E. faecium	Glycopeptide, vancomycin	*vanA*	Truncation of *vanX*	17 bp deletion in *vanS* together with point mutation in *ddl* or IS*fae4* insertion in *vanS* together with mutation in *ddl*	ND	<4 mg/L to ≥256 mg/L	17
	E. faecium	Glycopeptide, vancomycin	*vanB*	Nucleotide substitutions in *vanB* gene cluster	Point mutations in $vanS_B$ led to vancomycin-dependent *vanB* expression	2×10^{-6} to 4×10^{-7}	3 mg/L to ≥16 mg/L	59
	E. faecium	Glycopeptide, teicoplanin	*vanB*	Mutation in promoter region of *vanB*	18 bp deletion between two tandem direct repeats in *vanS* created promoter for constitutive *vanB* expression	ND	2 mg/L_{TEC} to 4096 mg/L_{TEC}; 512 mg/L_{VAN} to 1024 mg/L_{VAN}	60
	S. aureus	β-Lactam, cefoxitin	*mecA*	Truncated *blaRI* regulator gene repressed *mecA* expression	Loss of *blaZ-blaRI-blaI* operon enabled *mecA* expression	4×10^{-6}	4 mg/L to 32 mg/L	4
Gram-negative	*E. coli*	β-Lactams, carbapenem, meropenem and cefepime	bla_{KPC}	Weak bla_{KPC} promoter	188 bp deletion upstream of bla_{KPC} created a stronger promoter and enhanced bla_{KPC} expression	ND	1 mg/L_{MEM} to 4 mg/L_{MEM}; 8 mg/L_{FEP} to 64 mg/L_{FEP}	9
	P. mirabilis	β-Lactams, meropenem, ertapenem, imipenem and temocillin	bla_{OXA-48}	Weak bla_{OXA-48} promoter	Single base pair deletion upstream of bla_{OXA-48} created a stronger promoter	ND	0.125 mg/L_{MEM} to 2 mg/L_{MEM}	11
Alterations in the regulation of AMR expression: ISs inserting alternative promoters								
Gram-positive	*E. faecium*	Glycopeptide, vancomycin	*vanA*	Absence of *vanRS* due to partial deletion	Insertion of IS*1251* in intergenic region between *vanS* and *vanH* led to expression of *vanHAX*	ND	1 mg/L to >256 mg/L	5,61
Alterations in the regulation of AMR expression: insertion of ISs forming hybrid promoters								
Gram-positive	*E. faecium*	Glycopeptide, vancomycin	*vanA*	Absence of *vanRS* due to partial deletion	Insertion of IS*1167* in intergenic region between *vanS* and *vanH* provides −35 box and led to high-level expression of *vanHAX*	ND	1 mg/L to >256 mg/L	61
Alterations in the regulation of AMR expression: excision of ISs from promoters restoring resistance								
Gram-positive	*E. faecium*	Glycopeptide, vancomycin	*vanA*	Insertion of IS*L3* between *vanRS* and *vanHAX*	Excision of IS*L3* between *vanRS* and *vanHAX* restored *vanRS–vanHAX* proximity and thus their expression	3×10^{-8}	1 mg/L to >256 mg/L	6
Alterations in the AMR gene								
	S. aureus	Macrolide, erythromycin	*ermA*	Nucleotide substitution in *erm*	Direct reversion of original mutation in *erm*	2×10^{-7} to 1×10^{-9}	0.5 mg/L to 256 mg/L	4

Continued

Table 2. *Continued*

	Species	Antibiotics	AMR gene	Mechanism of silencing	Mechanism of resistance conversion	Conversion rate	Increase in MIC	Ref.
	S. aureus	Macrolide, erythromycin	*ermA*	Insertion of IS*256* into *erm*	Excision of IS*256* from *erm*	4×10^{-7}	0.5 mg/L to 256 mg/L	4
	S. aureus	β-Lactam, cefoxitin	*mecA*	Frameshift due to insertion within *mecA*	Compensatory mutation, frameshift due to deletion restored *mecA* reading frame	3×10^{-9}	4 mg/L to 64 mg/L	4
	S. aureus	β-Lactam, cefoxitin and oxacillin	*mecA*	Single base pair insertion in *mecA* led to premature stop codon	Single nucleotide insertion restored reading frame of *mecA*	$1x10^{-6}$ to $1x10^{-7}$	<4 mg/L_{FOX} to >4 mg/L_{FOX}; ≤0.25 mg/L_{OXA} to >2 mg/L_{OXA}	62
	S. aureus	β-Lactam, oxacillin	*mecA*	Insertion of IS*1181* into *mecA*	Excision of IS*1181* from *mecA*	4×10^{-7}	0.25 mg/L to >64 mg/L	7
	S. aureus	β-Lactam, oxacillin	*mecA*	Single nucleotide deletion in *mecA* led to premature stop codon	Direct reversion of original mutation or insertion close to original mutation restored reading frame of *mecA*	9×10^{-8}	0.5 mg/L to 64 mg/L	7
	S. aureus	Mupirocin	*mupA*	Frameshift due to single nucleotide deletion in poly(A) tract	Direct reversion of original mutation in *mupA*	1×10^{-7}	0.5 mg/L to >256 mg/L	4
Gram-positive	*S. aureus*	Aminoglycosides, gentamicin and tobramycin	*aacA-aphD*	Insertion of IS*256* in *aacA-aphD*	Excision of IS*256* from *aacA-aphD*	$3\times10^{-8}_{GEN}$; $9\times10^{-8}_{TOB}$	0.25 mg/L_{GEN} to 32 mg/L_{GEN}; 0.5 mg/L_{TOB} to 16 mg/L_{TOB}	4
	S. aureus	Aminoglycosides, tobramycin	*ant4*	Frameshift due to single nucleotide insertion in *ant4*	Compensatory deletion, single nucleotide deletion in *ant4* restored reading frame	8×10^{-10}	0.25 mg/L to 64 mg/L	4
	S. aureus	Aminoglycosides, spectinomycin	*aad9*	Single nucleotide deletion in poly(A) tract led to frameshift	Direct reversion of original mutation in *aad9*	7×10^{-9}	64 mg/L to >512 mg/L	4
	S. aureus	Aminoglycosides, spectinomycin	*aad9*	Nucleotide substitution led to nonsense mutation and premature stop codon	Direct reversion of original mutation in *aad9*	2×10^{-6} to 9×10^{-7}	64 mg/L to >512 mg/L	4
	S. aureus	Tetracyclines, tetracycline	*tetM*	Single nucleotide deletion in poly(A) tract led to frameshift	Direct reversion of original mutation in *tetM*	8×10^{-8}	1 mg/L to 128 mg/L	4
Gram-negative	*Campylobacter coli*	Aminoglycoside, streptothricin	*sat4*	7 bp duplication disrupted *sat4* reading frame	7 bp deletion restored reading frame	5×10^{-8}	1 mg/L to >1024 mg/L	8

Alterations in the AMR gene copy number								
Gram-positive	*E. faecium*	Glycopeptide, vancomycin	*vanM*	ND	Amplification of *vanM* cluster	ND	1 mg/L to >256 mg/L	[63]
	E. faecium	Glycopeptide, vancomycin	*vanA*	Truncation of *vanX*	Increased *vanA* plasmid copy number and addition of a *vanA* plasmid containing a functional *vanX*	ND	<4 mg/L to ≥256 mg/L	[17]
	E. faecium	Glycopeptide, vancomycin	*vanA*	Truncation of *vanR*	Increased *vanA* plasmid copy number	2×10^{-8}	1 mg/L to >256 mg/L (with both deletion and increased copy number)	[15]
	S. aureus	Lincosamides, clindamycin	*vga(A)v*	ND	Amplification of *vga(A)v* resistance-bearing transposon Tn*5406*	2×10^{-9}	0.25 mg/L to 1 mg/L	[4]
Gram-negative	*E. coli*	β-Lactam and β-lactam inhibitor piperacillin-tazobactam	bla_{TEM}	ND	4-fold amplification of bla_{TEM} for higher β-lactamase activity	ND	≤16/4 mg/L to ≥128/4 mg/L	[64]
	E. coli	Piperacillin-tazobactam	bla_{CMY-2}	ND	Point mutation in *inc* antisense RNA gene led to increase in bla_{CMY-2} plasmid copy number and increase in bla_{CMY-2} expression	1×10^{-6} to 1×10^{-7}	4 mg/L to 64–512 mg/L	[65]
	E. coli	Piperacillin-tazobactam	bla_{TEM-1B}	ND	IS*26*-mediated increase in bla_{TEM-1B} copy number led to hyperproduction of β-lactamase	ND	2–4/4 mg/L to 64/4 mg/L	[66]
	K. pneumoniae	Carbapenem, imipenem	$bla_{OXA-232}$	ND	Increase in $bla_{OXA-232}$ plasmid copy number increased $bla_{OXA-232}$ expression	ND	0.5–1 mg/L to 16–32 mg/L	[67]
	K. pneumoniae	Carbapenem, meropenem and imipenem	bla_{KPC-2}	ND	Increase in bla_{KPC-2} plasmid copy number increased bla_{KPC-2} expression	ND	ND	[68]
	E. coli	Carbapenem and cephalosporin and β-lactamase inhibitor, meropenem, ceftazidime/avibactam	bla_{KPC-3}	ND	Increase in bla_{KPC-3}-Tn*4401a* plasmid copy number increased bla_{KPC-3} expression	ND	1 mg/L_{MEM} to 32 mg/L_{MEM}; 0.5 mg/L_{CZA} to 8 mg/L_{CZA}	[69]

CZA, ceftazidime/avibactam; FEP, cefepime; FOX, cefoxitin; GEN, gentamycin; MEM, meropenem; ND, not determined; OXA, oxacillin; TEC, teicoplanin; TOB, tobramycin; VAN, vancomycin.

expression in *E. coli*.[74] Resistance resurrection of intact silent plasmid-encoded AMR determinants (bla_{OXA-2}, *aadA1*, *sul1* and *tetA*) was observed only after introducing the plasmid into another host.

ISs may insert alternative or hybrid promoters restoring expression of AMR (Figure 1a). Examples include those related to the concept of VVE.[6,16] For example, the silent *E. faecium* VanA-type vancomycin-resistance gene cluster *vanHAX* was recovered by an IS*1251*-like element promoter implant[5]. The insertion of an IS*1542* hybrid promoter upstream of *vanHAX* in an *E. faecium* isolate, provided a −35 box sequence supporting constitutive expression of glycopeptide resistance.[75] The latter mechanism was also described in resistance reversion of another VVE, by insertion of an IS*1167*, providing the −35 box sequence adding to the *van* plasmid −10 box itself.[61]

Excision of ISs from promoter regions can also enable expression of resistance (Figure 1a). In *E. faecium* an IS*L3* element silenced vancomycin resistance by interrupting the binding site of the VanR activator and the *vanHAX* promoter. Resistance was restored upon excision.[6] In *E. coli*, IS*26* insertion in the promoter region of $bla_{CTX-M-15}$ separated the AMR determinant from its native promoter, which was associated with a significantly reduced MIC to third-generation cephalosporins, allowing a similar mechanism.[76]

A recent review summarizes observations supporting the importance of epigenetics in AMR, in particular related to adaptive resistance, heteroresistance and persistence.[77] Epigenetic regulation can affect mutation rates and gene expression thus modifying phenotypic expression of resistance. Genetic changes and epigenetics are tightly linked because mutations, also induced by antibiotic stress, affect methyltransferases and thus may alter the overall epigenetic landscape.[77] In the future epigenetic modifications will probably expand our understanding of AMR, where genetic changes alone fail to fully explain the dynamic nature of resistance phenotypes.

Alterations in the AMR gene

Mutations can silence or revive (Figure 1b) quiescent resistance genes and antibiotic exposure can increase mutagenesis.[78–80] A single missense mutation in the coding region of an AMR determinant can silence and revert resistance. In a large clinical collection of *S. aureus*, 10% of the strains harboured a resistance gene silenced by mutations.[4] The relevant mutations were mainly single nucleotide insertions or point deletions introducing a frameshift. Importantly, 90% of the silenced AMR gene-carrying strains converted to resistance upon exposure to the corresponding antibiotic *in vitro*, whereas silencing was irreversible in the other 10%. Likewise, a single base-pair deletion resulted in a frameshift silencing the *mupA* gene encoding high-level mupirocin resistance in *S. aureus*.[81] Reinsertion of the single nucleotide restored high-level resistance. In a similar manner, removal of a 7 bp duplication restored the reading frame of the streptothricin acetyltransferase gene, *sat4*, and the expression of streptothricin resistance in *Campylobacter coli*.[8]

ISs can silence AMR genes and subsequent excision may resurrect resistance (Figure 1b). Oxacillin exposure of a silenced MRSA was associated with reversion to resistance by excision of IS*1181* from the *mecA* reading frame.[7] Similarly, excision of IS*256* from *aacA-aphD* in *S. aureus* restored the gentamicin/tobramycin-resistant phenotype.[4]

Apart from direct reversion of the mutation causing the silenced resistance, the occurrence of compensatory mutations enabling functional resistance has been observed.[4] A nucleotide insertion into *mecA* caused inactivation through frameshift. After antibiotic exposure resistant mutants had a deletion of a different nucleotide restoring the reading frame and expression of a functional *mecA*.

Alterations in the AMR gene copy number

AMR gene dose can alter resistance phenotypes. The mechanisms include increased copy number of the antibiotic resistance gene itself or the MGE conferring AMR, as illustrated in Figure 1(c).

This notion has particularly been observed in Gram-negative bacteria.[82] A panel of almost 800 bacteria-drug combinations was experimentally examined for emerging resistance in subpopulations involving clinical isolates of *E. coli*, *K. pneumoniae*, *Salmonella typhimurium* and *Acinetobacter baumannii*. Resistance appeared at a median frequency of 4.9×10^{-5} in a quarter of the bacteria-antibiotic combinations, and more than half of these were associated with amplification of known resistance genes located at both plasmids and chromosomes. In *K. pneumoniae*, elevated carbapenem resistance correlated with higher β-lactamase OXA-232 expression, the copy number of the β-lactamase-encoding plasmid, and increased carbapenemase activity.[67] Likewise, carbapenem resistance levels correlated with bla_{KPC-2} plasmid copy number,[68] and ceftazidime-avibactam resistance of *K. pneumoniae* correlated with a higher bla_{KPC-3} gene dose as well as bla_{KPC-3} plasmid copy number.[69]

Similar findings have been observed in clinical isolates of Gram-positive bacteria. Vancomycin exposure selected for high-level vancomycin-resistant variants in a *vanM E. faecium* strain.[63] The high-level resistant phenotype was associated with multiple copies of the *vanM* operon, flanked by IS*1216* on plasmids and the chromosome. Resistance through increased gene dosage has also been described in *vanA E. faecium*[15,17] and *S. aureus*.[4] Clindamycin-susceptible *S. aureus* strains carrying a functional clindamycin resistance gene, *vga(A)v*, became resistant after clindamycin exposure, associated with an increase in the copy number of the resistance-conferring transposon.[4]

Clinical relevance

The observed resistance conversion rates in tsaAMR strains vary between 10^{-6} and 10^{-10} (Table 2). These numbers are comparable to the mutation rates for chromosomal *ampC* derepression for Enterobacterales, which have resulted in breakthrough infections and treatment failure during oxyimino-cephalosporin monotherapy.[83,84] In terms of clinical relevance, these conversion rates can also be compared to the bacterial load (in cfu) during infection. In human bacterial infections, $\geq10^4$ bacterial genome copies/mL in bloodstream and urinary tract infections (UTIs) as well as $\geq10^8$ cfu/mL in tissue infections have been observed.[85–87] During bacteraemia, 10^4 cfu *S. aureus* per mL were described, [88] and 10^5 cfu *S. aureus* per mL in UTI,[89] 10^5 cfu *E. coli* per mL in UTI,[85] 10^3 cfu *E. faecalis* per mL blood were detected during bacteraemia, and 10^5 cfu *E. faecium* per gram of

human faeces during colonization.[90] Overall, these numbers show that at least 5×10^7 cfu *E. coli* in the urinary tract (assuming a volume of 500 mL) or $5\times10^{7-9}$ cfu *S. aureus* in blood (assuming a volume of 5 L) or 2.5×10^7 cfu *E. faecium* in the intestinal faeces (assuming an amount of 250 g), can be obtained within patients (Figure 2a). These bacterial population sizes allow the spontaneous conversion of tsaAMR genotypes (Table 2), which could be enriched during antibiotic exposure.

Accordingly, *in vivo* resistance development and therapeutic failures due to breakthrough infections caused by selected resistant subpopulations of tsaAMR Gram-positive and Gram-negative pathogens have been reported. These observations include the emergence of high-level vancomycin-resistant *E. faecium* during treatment of systemic infections with VVE strains[6,15,61] converting at a frequency of 10^{-8}. The ability of silenced vancomycin-susceptible *vanA*-positive *E. faecium* to spread unnoticed causing hospital-associated outbreaks has been documented in several countries including Canada,[5] Denmark[92] and Norway.[6]

Similarly, an MRSA strain emerged during therapy of an initially methicillin-susceptible *mecA*-positive *S. aureus*, termed *mecA*-positive, oxacillin-susceptible *S. aureus* (OS-MRSA).[7,62] Importantly, some OS-MRSA isolates recently observed in Brazil appear to be related to epidemic clones, emphasizing their potential to spread unrecognized.[93] The referred *in vivo* development of ceftazidime resistance in an epidemic *P. aeruginosa* strain illustrates the inherent possibility of activation of integron-borne quiescent AMR determinants in Gram-negative pathogens.[10]

Diagnostic challenges and opportunities

The resistance conversion rates have implications for the interpretation of phenotypic AST. The bacterial inoculum size in conventional AST is of particular relevance, and the ability to detect resistant subpopulations needs to be compared with the observed conversion rates. The inoculum varies from 2.5×10^4 cfu in broth microdilution, to 1×10^4 cfu per spot in agar dilution,[94,95] and 2×10^5 cfu per plate in disc diffusion or gradient tests[96] (Figure 2b). Thus, compared with the observed resistance conversion rates[4,7,59,62,81] of between 10^{-6} and 10^{-10} standard bacterial inoculum sizes in routine AST are not able to detect resistant subpopulations in most tsaAMR strains (Figure 2c). Phenotypic AST methods are validated with a standard inoculum and do not allow a higher inoculum to overcome the problem with undetectable tsaAMR. The current population analysis profiling method,[14] considered the gold standard for determining heteroresistance, is far too laborious and time consuming in a routine laboratory. Thus, we must still rely on genetic methods in combination with phenotypic methods for the detection of tsaAMR. Knowledge of the circulating strains through genetic methods can help to adapt phenotypic methods in order to improve detection of challenging pathogens.[97] A composite approach of genetic and phenotypic methods is currently recommended in the Nordic countries for the detection of VVE in cases of ampicillin-resistant *E. faecium* bacteraemia.[92] The identification of tsaAMR is of particular importance in controlling nosocomial infections. For example, regional or national clonal spread of the originally tsaAMR-*vanA E. faecium* strains in Canada and Denmark, has been documented recently.[92,98]

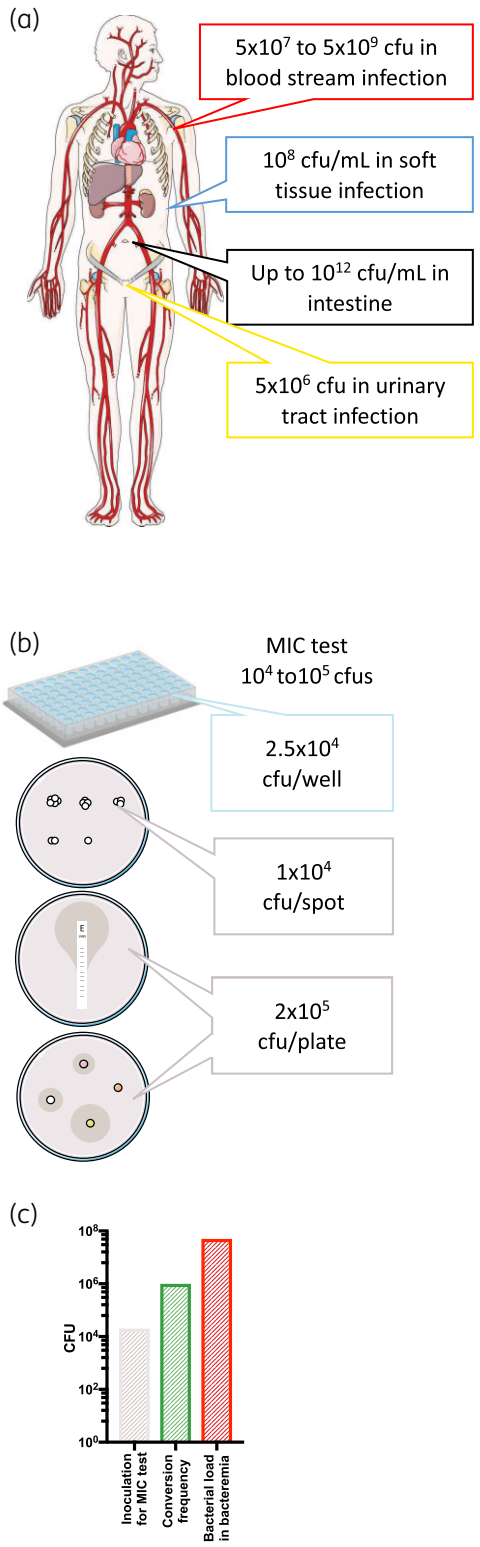

Figure 2. Challenges in detection of tsaAMR. Bacterial loads that can be reached during infection (a), the bacterial inoculum size for conventional AST (b), and their comparison with the resistance conversion rate (c). The numbers for the graph are based on Smith and Kirby,[91] Kime *et al.*[4] and Peters *et al.*[88]

In the future, increasing use of genomics in combination with phenotypic methods for AMR pathogens will likely reveal more cases of tsaAMR. This provides an opportunity to continue and expand the understanding of the prevalence and mechanisms of tsaAMR, optimizing detection methods of tsaAMR strains.

Conclusions and future perspectives

The acquisition of AMR determinants by HGT often impairs bacterial fitness.[3] Fitness cost compensation may support the advance of mutants with a silent resistance phenotype in the absence of antibiotic selection.[14] Thus, divergence in AST genotypes and phenotypes is likely to increase due to the expansion of acquired AMR and the increased use of genotypic AMR detection. This notion is supported by the recent study of the prevalence and nature of silent acquired AMR genes in a large collection of MDR clinical isolates of *S. aureus*. A total of 10% of the strains harboured silent AMR determinants. Most of those strains (90%) were able to recover their resistance phenotype at clinically relevant frequencies during antibiotic selection *in vitro*.[4]

The epidemic potential of clinically relevant tsaAMR strains and the ability to spread unnoticed was recently illustrated by the clonal shift for *vanA E. faecium* in Denmark during 2015 and 2019.[17] The VVE ST1421-CT1134 *vanA E. faecium*, first described in Denmark in 2016, soon became the most dominant *vanA E. faecium* clone occurring in all five regions in Denmark as well as the Faroe Islands.[92] Corresponding expansion of VVE has been reported from Canada and the Republic of Korea.[98,99]

The observed discrepancies between phenotypic and genotypic AST are a challenge for conventional AST, where the bacterial inoculum is too low to be able to detect the observed frequencies of resistance conversion. Extended use of genotyping, including pathogen WGS, will be a necessary supplement to phenotypic AST to overcome such challenges. This is most important in species where tsaAMR is a particular clinical problem such as *S. aureus* and *E. faecium*.[100–103]

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References

1 Kahlmeter G, Brown DFJ, Goldstein FW *et al*. European harmonization of MIC breakpoints for antimicrobial susceptibility testing of bacteria. *J Antimicrob Chemother* 2003; **52**: 145–8. https://doi.org/10.1093/jac/dkg312

2 Boolchandani M, D'Souza AW, Dantas G. Sequencing-based methods and resources to study antimicrobial resistance. *Nat Rev Genet* 2019; **20**: 356–70.

3 Andersson DI, Hughes D. Antibiotic resistance and its cost: is it possible to reverse resistance? *Nat Rev Microbiol* 2010; **8**: 260–71. https://doi.org/10.1038/nrmicro2319

4 Kime L, Randall CP, Banda FI *et al*. Transient silencing of antibiotic resistance by mutation represents a significant potential source of unanticipated therapeutic failure. *mBio* 2019; **10**: e01755-19. https://doi.org/10.1128/mBio.01755-19

5 Szakacs TA, Kalan L, McConnell MJ *et al*. Outbreak of vancomycin-susceptible *Enterococcus faecium* containing the wild-type *vanA* gene. *J Clin Microbiol* 2014; **52**: 1682–6. https://doi.org/10.1128/JCM.03563-13

6 Sivertsen A, Pedersen T, Larssen KW *et al*. A silenced *vanA* gene cluster on a transferable plasmid caused an outbreak of vancomycin-variable enterococci. *Antimicrob Agents Chemother* 2016; **60**: 4119–27. https://doi.org/10.1128/AAC.00286-16

7 Proulx MK, Palace SG, Gandra S *et al*. Reversion from methicillin susceptibility to methicillin resistance in *Staphylococcus aureus* during treatment of bacteremia. *J Infect Dis* 2016; **213**: 1041–8. https://doi.org/10.1093/infdis/jiv512

8 Jacob J, Evers S, Bischoff K *et al*. Characterization of the sat4 gene encoding a streptothricin acetyltransferase in *Campylobacter coli* BE/G4. *FEMS Microbiol Lett* 1994; **120**: 13–17.

9 Cheruvanky A, Stoesser N, Sheppard AE *et al*. Enhanced *Klebsiella pneumoniae* carbapenemase expression from a novel Tn*4401* deletion. *Antimicrob Agents Chemother* 2017; **61**: e00025-17. https://doi.org/10.1128/AAC.00025-17

10 Hocquet D, Llanes C, Thouverez M *et al*. Evidence for induction of integron-based antibiotic resistance by the SOS response in a clinical setting. *PLoS Pathog* 2012; **8**: e1002778. https://doi.org/10.1371/journal.ppat.1002778

11 Pedraza R, Kieffer N, Guzmán-Puche J *et al*. Hidden dissemination of carbapenem-susceptible OXA-48-producing *Proteus mirabilis*. *J Antimicrob Chemother* 2022: **77**: 3009–15. https://doi:10.1093/jac/dkac267

12 The European Committee on Antimicrobial Susceptibility Testing - EUCAST. Homepage. https://www.eucast.org/

13 The Clinical and Laboratory Standards Institute. Homepage. https://clsi.org/

14 Andersson DI, Nicoloff H, Hjort K. Mechanisms and clinical relevance of bacterial heteroresistance. *Nat Rev Microbiol* 2019; **17**: 479–96. https://doi.org/10.1038/s41579-019-0218-1

15 Wagner TM, Janice J, Sivertsen A *et al*. Alternative *vanHAX* promoters and increased *vanA*-plasmid copy number resurrect silenced glycopeptide resistance in *Enterococcus faecium*. *J Antimicrob Chemother* 2020; **76**: 876–82. https://doi.org/10.1093/jac/dkaa541

16 Coburn B, Low DE, Patel SN *et al*. Vancomycin-variable *Enterococcus faecium*: in vivo emergence of vancomycin resistance in a vancomycin-susceptible isolate. *J Clin Microbiol* 2014; **52**: 1766–7. https://doi.org/10.1128/JCM.03579-13

17 Hansen TA, Pedersen MS, Nielsen LG *et al*. Emergence of a vancomycin-variable *Enterococcus faecium* ST1421 strain containing a deletion in *vanX*. *J Antimicrob Chemother* 2018; **73**: 2936–40. https://doi.org/10.1093/jac/dky308

18 Saeed K, Marsh P, Ahmad N. Cryptic resistance in *Staphylococcus aureus*: a risk for the treatment of skin infection? *Curr Opin Infect Dis* 2014; **27**: 130–6. https://doi.org/10.1097/QCO.0000000000000046

19 Suarez SA, Martiny AC. Gene amplification uncovers large previously unrecognized cryptic antibiotic resistance potential in *E. coli*. *Microbiol Spectr* 2021; **9**: e00289-21. https://doi.org/10.1128/Spectrum.00289-21

20 Cui L, Tominaga E, Neoh H-M *et al*. Correlation between reduced daptomycin susceptibility and vancomycin resistance in vancomycin-intermediate *Staphylococcus aureus*. *Antimicrob Agents Chemother* 2006; **50**: 1079–82. https://doi.org/10.1128/AAC.50.3.1079-1082.2006

21 Boyle-Vavra S, Berke SK, Lee JC *et al*. Reversion of the glycopeptide resistance phenotype in *Staphylococcus aureus* clinical isolates. *Antimicrob Agents Chemother* 2000; **44**: 272–7. https://doi.org/10.1128/AAC.44.2.272-277.2000

22 Maki H, McCallum N, Bischoff M *et al*. *Tcaa* inactivation increases glycopeptide resistance in *Staphylococcus aureus*. *Antimicrob Agents Chemother* 2004; **48**: 1953–9. https://doi.org/10.1128/AAC.48.6.1953-1959.2004

23 Tenover FC, Lancaster MV, Hill BC *et al*. Characterization of staphylococci with reduced susceptibilities to vancomycin and other glycopeptides. *J Clin Microbiol* 1998; **36**: 1020–7. https://doi.org/10.1128/JCM.36.4.1020-1027.1998

24 Fernández L, Hancock REW. Adaptive and mutational resistance: role of porins and efflux pumps in drug resistance. *Clin Microbiol Rev* 2012; **25**: 661–81. https://doi.org/10.1128/CMR.00043-12

25 Matteo MJ, Granados G, Olmos M *et al*. *Helicobacter pylori* amoxicillin heteroresistance due to point mutations in PBP-1A in isogenic isolates. *J Antimicrob Chemother* 2008; **61**: 474–7. https://doi.org/10.1093/jac/dkm504

26 Kim JJ, Kim JG, Kwon DH. Mixed-infection of antibiotic susceptible and resistant *Helicobacter pylori* isolates in a single patient and underestimation of antimicrobial susceptibility testing. *Helicobacter* 2003; **8**: 202–6. https://doi.org/10.1046/j.1523-5378.2003.00145.x

27 Fernández-Cuenca F, Gómez-Sánchez M, Rodríguez-Baño J *et al*. Epidemiological and clinical features associated with colonisation/infection by *Acinetobacter baumannii* with phenotypic heterogeneous resistance to carbapenems. *Int J Antimicrob Agents* 2012; **40**: 235–8. https://doi.org/10.1016/j.ijantimicag.2012.05.005

28 El-Halfawy OM, Valvano MA. Antimicrobial heteroresistance: an emerging field in need of clarity. *Clin Microbiol Rev* 2015; **28**: 191–207. https://doi.org/10.1128/CMR.00058-14

29 Partridge SR, Kwong SM, Firth N *et al*. Mobile genetic elements associated with antimicrobial resistance. *Clin Microbiol Rev* 2018; **31**: e00088-17. https://doi.org/10.1128/CMR.00088-17

30 Hallet B, Sherratt DJ. Transposition and site-specific recombination: adapting DNA cut-and-paste mechanisms to a variety of genetic rearrangements. *FEMS Microbiol Rev* 1997; **21**: 157–78. https://doi.org/10.1111/j.1574-6976.1997.tb00349.x

31 Chandler M, Fayet O, Rousseau P *et al*. Copy-out–paste-in transposition of IS911: a major transposition pathway. *Microbiol Spectr* 2015; **3**: 3.4.01. https://doi.org/10.1128/microbiolspec.MDNA3-0031-2014

32 Siguier P, Gourbeyre E, Varani A *et al*. Everyman's guide to bacterial insertion sequences. *Microbiol Spectr* 2015; **3**: MDNA3-0030-2014. https://doi.org/10.1128/microbiolspec.MDNA3-0030-2014

33 Hickman AB, Dyda F. Mechanisms of DNA transposition. *Microbiol Spectr* 2015; **3**: 3.2.12. https://doi.org/10.1128/microbiolspec.MDNA3-0034-2014

34 Vandecraen J, Chandler M, Aertsen A *et al*. The impact of insertion sequences on bacterial genome plasticity and adaptability. *Crit Rev Microbiol* 2017; **43**: 709–30. https://doi.org/10.1080/1040841X.2017.1303661

35 Kamruzzaman M, Patterson JD, Shoma S *et al*. Relative strengths of promoters provided by common mobile genetic elements associated with resistance gene expression in Gram-negative bacteria. *Antimicrob Agents Chemother* 2015; **59**: 5088–91. https://doi.org/10.1128/AAC.00420-15

36 Couto I, Wu SW, Tomasz A *et al*. Development of methicillin resistance in clinical isolates of *Staphylococcus sciuri* by transcriptional activation of the *mecA* homologue native to s. *J Bacteriol* 2003; **185**: 645–53. https://doi.org/10.1128/JB.185.2.645-653.2003

37 Maki H, Murakami K. Formation of potent hybrid promoters of the mutant *llm* gene by IS*256* transposition in methicillin-resistant *Staphylococcus aureus*. *J Bacteriol* 1997; **179**: 6944–8. https://doi.org/10.1128/jb.179.22.6944-6948.1997

38 Jellen-Ritter AS, Kern WV. Enhanced expression of the multidrug efflux pumps AcrAB and AcrEF associated with insertion element transposition in *Escherichia coli* mutants selected with a fluoroquinolone. *Antimicrob Agents Chemother* 2001; **45**: 1467–72. https://doi.org/10.1128/AAC.45.5.1467-1472.2001

39 Schneider D, Lenski RE. Dynamics of insertion sequence elements during experimental evolution of bacteria. *Res Microbiol* 2004; **155**: 319–27. https://doi.org/10.1016/j.resmic.2003.12.008

40 Wu Y, Aandahl RZ, Tanaka MM. Dynamics of bacterial insertion sequences: can transposition bursts help the elements persist? *BMC Evol Biol* 2015; **15**: 288. https://doi.org/10.1186/s12862-015-0560-5

41 Nagel M, Reuter T, Jansen A *et al*. Influence of ciprofloxacin and vancomycin on mutation rate and transposition of IS*256* in *Staphylococcus aureus*. *Int J Med Microbiol IJMM* 2011; **301**: 229–36. https://doi.org/10.1016/j.ijmm.2010.08.021

42 Schreiber F, Szekat C, Josten M *et al*. Antibiotic-induced autoactivation of IS*256* in *Staphylococcus aureus*. *Antimicrob Agents Chemother* 2013; **57**: 6381–4. https://doi.org/10.1128/AAC.01585-13

43 Lartigue M-F, Poirel L, Aubert D *et al*. In vitro analysis of ISEcp1B-mediated mobilization of naturally occurring β-lactamase gene blaCTX-M of *Kluyvera ascorbata*. *Antimicrob Agents Chemother* 2006; **50**: 1282–6. https://doi.org/10.1128/AAC.50.4.1282-1286.2006

44 Delavat F, Miyazaki R, Carraro N *et al*. The hidden life of integrative and conjugative elements. *FEMS Microbiol Rev* 2017; **41**: 512–37. https://doi.org/10.1093/femsre/fux008

45 Baharoglu Z, Bikard D, Mazel D. Conjugative DNA transfer induces the bacterial SOS response and promotes antibiotic resistance development through integron activation. *PLoS Genet* 2010; **6**: e1001165. https://doi.org/10.1371/journal.pgen.1001165

46 Liu P, Wu Z, Xue H *et al*. Antibiotics trigger initiation of SCC*mec* transfer by inducing SOS responses. *Nucleic Acids Res* 2017; **45**: 3944–52. https://doi.org/10.1093/nar/gkx153

47 Grueter L, Koenig O, Laufs R. Transposon mutagenesis in *Staphylococcus epidermidis* using the *Enterococcus faecalis* transposon Tn*917*. *FEMS Microbiol Lett* 1991; **82**: 215–18. https://doi.org/10.1111/j.1574-6968.1991.tb04867.x

48 Sáenz JS, Airo A, Schulze-Makuch D *et al*. Functional traits co-occurring with mobile genetic elements in the microbiome of the Atacama desert. *Diversity (Basel)* 2019; **11**: 205. https://doi.org/10.3390/d11110205

49 Lebreton F, van Schaik W, McGuire AM *et al*. Emergence of epidemic multidrug-resistant *Enterococcus faecium* from animal and commensal strains. *mBio* 2013; **4**: e00534-13. https://doi.org/10.1128/mBio.00534-13

50 Leavis HL, Willems RJL, van Wamel WJB, *et al*. Insertion sequence-driven diversification creates a globally dispersed emerging

multiresistant subspecies of *E. faecium*. *PLoS Pathog* 2007; **3**: e7. https://doi.org/10.1371/journal.ppat.0030007

51 Arredondo-Alonso S, Top J, McNally A *et al*. Plasmids shaped the recent emergence of the major nosocomial pathogen *Enterococcus faecium*. *mBio* 2020; **11**: e03284-19. https://doi.org/10.1128/mBio.03284-19

52 Pain M, Hjerde E, Klingenberg C *et al*. Comparative genomic analysis of *Staphylococcus haemolyticus* reveals key to hospital adaptation and pathogenicity. *Front Microbiol* 2019; **10**: 2096. https://doi.org/10.3389/fmicb.2019.02096

53 Jamrozy D, Coll F, Mather AE *et al*. Evolution of mobile genetic element composition in an epidemic methicillin-resistant *Staphylococcus aureus*: temporal changes correlated with frequent loss and gain events. *BMC Genomics* 2017; **18**: 684. https://doi.org/10.1186/s12864-017-4065-z

54 Lindsay JA. Hospital-associated MRSA and antibiotic resistance—what have we learned from genomics? *Int J Med Microbiol* 2013; **303**: 318–23. https://doi.org/10.1016/j.ijmm.2013.02.005

55 Johnson TJ, Wannemuehler YM, Johnson SJ *et al*. Plasmid replicon typing of commensal and pathogenic *Escherichia coli* isolates. *Appl Environ Microbiol* 2007; **73**: 1976–83. https://doi.org/10.1128/AEM.02171-06

56 McNally A, Oren Y, Kelly D *et al*. Combined analysis of variation in core, accessory and regulatory genome regions provides a super-resolution view into the evolution of bacterial populations. *PLoS Genet* 2016; **12**: e1006280. https://doi.org/10.1371/journal.pgen.1006280

57 DeLeo FR, Chen L, Porcella SF *et al*. Molecular dissection of the evolution of carbapenem-resistant multilocus sequence type 258 *Klebsiella pneumoniae*. *Proc Natl Acad Sci U S A* 2014; **111**: 4988–93. https://doi.org/10.1073/pnas.1321364111

58 Chen L, Mathema B, Chavda KD *et al*. Carbapenemase-producing *Klebsiella pneumoniae*: molecular and genetic decoding. *Trends Microbiol* 2014; **22**: 686–96. https://doi.org/10.1016/j.tim.2014.09.003

59 Hashimoto Y, Kurushima J, Nomura T *et al*. Dissemination and genetic analysis of the stealthy *vanB* gene clusters of *Enterococcus faecium* clinical isolates in Japan. *BMC Microbiol* 2018; **18**: 213. https://doi.org/10.1186/s12866-018-1342-1

60 Depardieu F, Courvalin P, Msadek T. A six amino acid deletion, partially overlapping the VanSB G2 ATP-binding motif, leads to constitutive glycopeptide resistance in VanB-type *Enterococcus faecium*. *Mol Microbiol* 2003; **50**: 1069–83. https://doi.org/10.1046/j.1365-2958.2003.03771.x

61 Thaker MN, Kalan L, Waglechner N *et al*. Vancomycin-variable enterococci can give rise to constitutive resistance during antibiotic therapy. *Antimicrob Agents Chemother* 2015; **59**: 1405–10. https://doi.org/10.1128/AAC.04490-14

62 Goering RV, Swartzendruber EA, Obradovich AE *et al*. Emergence of oxacillin resistance in stealth methicillin-resistant *Staphylococcus aureus* due to *mecA* sequence instability. *Antimicrob Agents Chemother* 2019; **63**: e00558-19. https://doi.org/10.1128/AAC.00558-19

63 Zhou Y, Yang Y, Ding L *et al*. Vancomycin heteroresistance in *vanM*-type *Enterococcus faecium*. *Microb Drug Resist* 2020; **26**: 776–82. https://doi.org/10.1089/mdr.2019.0321

64 Schechter LM, Creely DP, Garner CD *et al*. Extensive gene amplification as a mechanism for piperacillin-tazobactam resistance in *Escherichia coli*. *mBio* 2018; **9**: e00583-18. https://doi.org/10.1128/mBio.00583-18

65 Kurpiel PM, Hanson ND. Point mutations in the inc antisense RNA gene are associated with increased plasmid copy number, expression of *bla*CMY-2 and resistance to piperacillin/tazobactam in *Escherichia coli*. *J Antimicrob Chemother* 2012; **67**: 339–45. https://doi.org/10.1093/jac/dkr479

66 Hubbard ATM, Mason J, Roberts P *et al*. Piperacillin/tazobactam resistance in a clinical isolate of *Escherichia coli* due to IS*26*-mediated amplification of *bla*TEM-1B. *Nat Commun* 2020; **11**: 4915. https://doi.org/10.1038/s41467-020-18668-2

67 Shen Z, Zhang H, Gao Q *et al*. Increased plasmid copy number contributes to the elevated carbapenem resistance in OXA-232-producing *Klebsiella pneumoniae*. *Microb Drug Resist* 2020; **26**: 561–8. https://doi.org/10.1089/mdr.2018.0407

68 van Dorp L, Wang Q, Shaw LP *et al*. Rapid phenotypic evolution in multidrug-resistant *Klebsiella pneumoniae* hospital outbreak strains. *Microb Genomics* 2019; **5**: e000263. https://doi.org/10.1099/mgen.0.000263

69 Coppi M, Di Pilato V, Monaco F *et al*. Ceftazidime-avibactam resistance associated with increased *bla*KPC-3 gene copy number mediated by pKpQIL plasmid derivatives in sequence type 258 *Klebsiella pneumoniae*. *Antimicrob Agents Chemother* 2020; **64**: e01816-19. https://doi.org/10.1128/AAC.01816-19

70 Yona AH, Alm EJ, Gore J. Random sequences rapidly evolve into de novo promoters. *Nat Commun* 2018; **9**: 1530. https://doi.org/10.1038/s41467-018-04026-w

71 Liu P, Xue H, Wu Z *et al*. Effect of *bla* regulators on the susceptible phenotype and phenotypic conversion for oxacillin-susceptible *mecA*-positive staphylococcal isolates. *J Antimicrob Chemother* 2016; **71**: 2105–12. https://doi.org/10.1093/jac/dkw123

72 Mazodier P, Genilloud O, Giraud E *et al*. Expression of Tn*5*-encoded streptomycin resistance in *E. coli*. *Mol Gen Genet MGG* 1986; **204**: 404–9. https://doi.org/10.1007/BF00331016

73 Trinh TQ, Sinden RR. The influence of primary and secondary DNA structure in deletion and duplication between direct repeats in *Escherichia coli*. *Genetics* 1993; **134**: 409–22. https://doi.org/10.1093/genetics/134.2.409

74 Enne VI, Delsol AA, Roe JM, Bennett PM. Evidence of antibiotic resistance gene silencing in *Escherichia coli*. *Antimicrob Agents Chemother* 2006; **50**: 3003–10. https://doi.org/10.1128/AAC.00137-06

75 Brown AR, Townsley AC, Amyes SG. Diversity of Tn*1546* elements in clinical isolates of glycopeptide-resistant enterococci from Scottish hospitals. *Antimicrob Agents Chemother* 2001; **45**: 1309–11. https://doi.org/10.1128/AAC.45.4.1309-1311.2001

76 Woodford N, Ward ME, Kaufmann ME *et al*. Community and hospital spread of *Escherichia coli* producing CTX-M extended-spectrum beta-lactamases in the UK. *J Antimicrob Chemother* 2004; **54**: 735–43. https://doi.org/10.1093/jac/dkh424

77 Ghosh D, Veeraraghavan B, Elangovan R *et al*. Antibiotic resistance and epigenetics: more to it than meets the eye. *Antimicrob Agents Chemother* 2020; **64**: e02225-19. https://doi.org/10.1128/AAC.02225-19

78 Long H, Miller SF, Strauss C *et al*. Antibiotic treatment enhances the genome-wide mutation rate of target cells. *Proc Natl Acad Sci U S A* 2016; **113**: E2498–505. https://doi.org/10.1073/pnas.1601208113

79 Blázquez J, Rodríguez-Beltrán J, Matic I. Antibiotic-induced genetic variation: how it arises and how it can be prevented. *Annu Rev Microbiol* 2018; **72**: 209–30. https://doi.org/10.1146/annurev-micro-090817-062139

80 Andersson DI, Hughes D. Microbiological effects of sublethal levels of antibiotics. *Nat Rev Microbiol* 2014; **12**: 465–78. https://doi.org/10.1038/nrmicro3270

81 Driscoll DG, Young CL, Ochsner UA. Transient loss of high-level mupirocin resistance in *Staphylococcus aureus* due to MupA polymorphism. *Antimicrob Agents Chemother* 2007; **51**: 2247–8. https://doi.org/10.1128/AAC.00241-07

82 Nicoloff H, Hjort K, Levin BR *et al*. The high prevalence of antibiotic heteroresistance in pathogenic bacteria is mainly caused by gene amplification. *Nat Microbiol* 2019; **4**: 504–14. https://doi.org/10.1038/s41564-018-0342-0

83 Kohlmann R, Bähr T, Gatermann SG. Species-specific mutation rates for *ampC* derepression in Enterobacterales with chromosomally encoded inducible AmpC β-lactamase. *J Antimicrob Chemother* 2018; **73**: 1530–6. https://doi.org/10.1093/jac/dky084

84 Choi S-H, Lee JE, Park SJ *et al*. Emergence of antibiotic resistance during therapy for infections caused by Enterobacteriaceae producing AmpC beta-lactamase: implications for antibiotic use. *Antimicrob Agents Chemother* 2008; **52**: 995–1000. https://doi.org/10.1128/AAC.01083-07

85 Stamm WE. Criteria for the diagnosis of urinary tract infection and for the assessment of therapeutic effectiveness. *Infection* 1992; **20**: S151–4. https://doi.org/10.1007/BF01704358

86 König C, Simmen HP, Blaser J. Bacterial concentrations in pus and infected peritoneal fluid—implications for bactericidal activity of antibiotics. *J Antimicrob Chemother* 1998; **42**: 227–32. https://doi.org/10.1093/jac/42.2.227

87 Bacconi A, Richmond GS, Baroldi MA *et al*. Improved sensitivity for molecular detection of bacterial and *Candida* infections in blood. *J Clin Microbiol* 2014; **52**: 3164–74. https://doi.org/10.1128/JCM.00801-14

88 Peters RPH, van Agtmael MA, Gierveld S *et al*. Quantitative detection of *Staphylococcus aureus* and *Enterococcus faecalis* DNA in blood to diagnose bacteremia in patients in the intensive care unit. *J Clin Microbiol* 2007; **45**: 3641–6. https://doi.org/10.1128/JCM.01056-07

89 Lafon T, Hernandez Padilla AC, Baisse A *et al*. Community-acquired *Staphylococcus aureus* bacteriuria: a warning microbiological marker for infective endocarditis? *BMC Infect Dis* 2019; **19**: 504. https://doi.org/10.1186/s12879-019-4106-0

90 Noble CJ. Carriage of group D streptococci in the human bowel. *J Clin Pathol* 1978; **31**: 1182–6. https://doi.org/10.1136/jcp.31.12.1182

91 Smith KP, Kirby JE. The inoculum effect in the era of multidrug resistance: minor differences in inoculum have dramatic effect on MIC determination. *Antimicrob Agents Chemother* 2018; **62**: e00433-18. https://doi.org/10.1128/AAC.00433-18

92 Hammerum AM, Justesen US, Pinholt M *et al*. Surveillance of vancomycin-resistant enterococci reveals shift in dominating clones and national spread of a vancomycin-variable *vanA Enterococcus faecium* ST1421-CT1134 clone, Denmark, 2015 to March 2019. *Euro Surveill* 2019; **24**: 1900503. https://doi.org/10.2807/1560-7917.ES.2019.24.34.1900503

93 Andrade-Figueiredo M, Leal-Balbino TC. Clonal diversity and epidemiological characteristics of *Staphylococcus aureus*: high prevalence of oxacillin-susceptible *mecA*-positive *Staphylococcus aureus* (OS-MRSA) associated with clinical isolates in Brazil. *BMC Microbiol* 2016; **16**: 115. https://doi.org/10.1186/s12866-016-0733-4

94 Wiegand I, Hilpert K, Hancock REW. Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. *Nat Protoc* 2008; **3**: 163–75. https://doi.org/10.1038/nprot.2007.521

95 The European Committee on Antimicrobial Susceptibility Testing. EUCAST reading guide for broth microdilution. Version 2.0, 2020. http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Disk_test_documents/2020_manuals/Reading_guide_BMD_v_2.0_2020.pdf

96 bioMérieux SA. Antimicrobial Susceptibility Testing. 2012. https://www.ilexmedical.com/files/E-test-Package-Insert/AST_WW.pdf

97 Grabsch EA, Chua K, Xie S *et al*. Improved detection of *vanB2*-containing *Enterococcus faecium* with vancomycin susceptibility by etest using oxgall supplementation. *J Clin Microbiol* 2008; **46**: 1961–4. https://doi.org/10.1128/JCM.01778-07

98 Kohler P, Eshaghi A, Kim HC *et al*. Prevalence of vancomycin-variable *Enterococcus faecium* (VVE) among *vanA*-positive sterile site isolates and patient factors associated with VVE bacteremia. *PLoS One* 2018; **13**: e0193926. https://doi.org/10.1371/journal.pone.0193926

99 Jung Y-H, Lee YS, Lee SY *et al*. Structure and transfer of the *vanA* cluster in *vanA*-positive, vancomycin-susceptible *Enterococcus faecium*, and its revertant mutant. *Diagn Microbiol Infect Dis* 2014; **80**: 148–50. https://doi.org/10.1016/j.diagmicrobio.2014.06.012

100 Aanensen DM, Feil EJ, Holden MTG *et al*. Whole-genome sequencing for routine pathogen surveillance in public health: a population snapshot of invasive *Staphylococcus aureus* in Europe. *mBio* 2016; **7**: e00444-16. https://doi.org/10.1128/mBio.00444-16

101 Gordon NC, Price JR, Cole K *et al*. Prediction of *Staphylococcus aureus* antimicrobial resistance by whole-genome sequencing. *J Clin Microbiol* 2014; **52**: 1182–91. https://doi.org/10.1128/JCM.03117-13

102 Lee T, Pang S, Stegger M *et al*. A three-year whole genome sequencing perspective of *Enterococcus faecium* sepsis in Australia. *PloS One* 2020; **15**: e0228781. https://doi.org/10.1371/journal.pone.0228781

103 Adamecz Z, Nielsen KL, Kirkby NS *et al*. Aminoglycoside resistance genes in *Enterococcus faecium*: mismatch with phenotype. *J Antimicrob Chemother* 2021; **76**: 2215–7. https://doi.org/10.1093/jac/dkab137