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

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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 Tn3 and IS6 family elements, which is then resolved to the original donor plus the recipient with the element³⁰. Alternatively, non-replicative transposition most commonly takes place by a copy-paste mechanism, which involves many IS families such as IS3, IS21, IS30, IS256 and ISL3, where a double-stranded circular intermediate is formed and then integrates into the recipient³¹ (reviewed in³²⁻³⁴). 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 IS3, IS4, IS5, IS6 and IS1380. 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 IS256, IS1, IS3, IS6 and IS30 families.³⁴ IS256 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 IS256,^{41,42} whereas cefotaxime, ceftazidime and piperacillin enhanced the transposition of ISEcp1B.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 <i>S. aureus</i> , 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 <i>S. aureus</i> that might escape phenotypic detection including oxacillin-susceptible MRSA (OS-MRSA), hVISA (heteroresistant vancomycin-intermediate <i>S. aureus</i>) 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 <i>E. coli</i> 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 <i>S. aureus</i> (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 ($>1 \times 10^{-7}$) 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 <i>Helicobacter pylori</i> 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 <i>Acinetobacter baumannii</i>	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 Tn917.⁴⁷

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

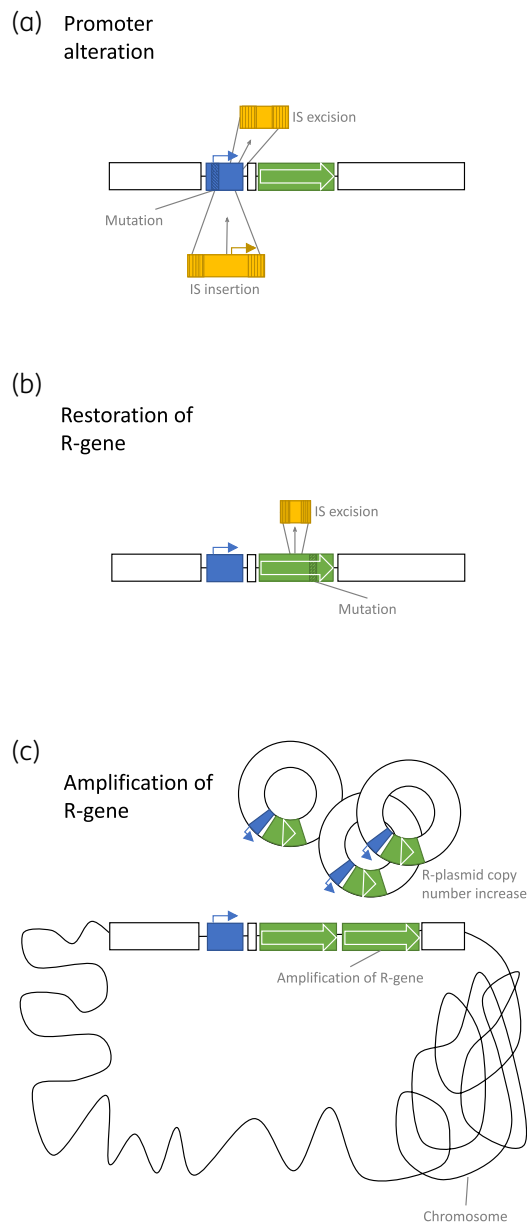


Figure 1. Molecular mechanisms in resurrection of transiently silent acquired AMR. Genetic alterations can occur in the promoter region upstream of the acquired resistance gene (R-gene), in the acquired R-gene itself, or change the R-gene copy number. (a) Promoter alterations may occur through mutations, 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. This figure appears in colour in the online version of JAC and in black and white in the print version of JAC.

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 (IS3, IS110, IS256 and IS16), are more prevalent in clinical than in commensal strains.^{49–51} MGEs, like plasmids, prophages, ISs (IS256) 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 Tn4401-associated *K. pneumoniae* carbapenemase gene *bla_{KPC}*, created the novel isoform Tn4401h 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 <i>E. faecium</i>	Glycopeptide, vancomycin	<i>vanA</i>	Truncation of <i>vanR</i>	44 bp deletion upstream of <i>vanHAX</i> moved alternative promoters for constitutive <i>vanHAX</i> expression	2×10^{-8}	1 mg/L to >256 mg/L (with both deletion and increased copy number)	15
<i>E. faecium</i>	Glycopeptide, vancomycin	<i>vanA</i>	Truncation of <i>vanX</i>	17 bp deletion in <i>vanS</i> together with point mutation in <i>ddl</i> or <i>ISfae4</i> insertion in <i>vanS</i> together with mutation in <i>ddl</i>	ND	<4 mg/L to ≥ 256 mg/L	17
<i>E. faecium</i>	Glycopeptide, vancomycin	<i>vanB</i>	Nucleotide substitutions in <i>vanB</i> gene cluster	Point mutations in <i>vanS_B</i> led to vancomycin-dependent <i>vanB</i> expression	2×10^{-6} to 4×10^{-7}	3 mg/L to ≥ 16 mg/L	59
<i>E. faecium</i>	Glycopeptide, teicoplanin	<i>vanB</i>	Mutation in promoter region of <i>vanB</i>	18 bp deletion between two tandem direct repeats in <i>vanS</i> created promoter for constitutive <i>vanB</i> expression	ND	2 mg/L _{TEC} to 4096 mg/L _{TEC} ; 512 mg/L _{VAN} to 1024 mg/L _{VAN}	60
<i>S. aureus</i>	β -Lactam, ceftioxin	<i>mecA</i>	Truncated <i>bla_{RI}</i> regulator gene repressed <i>mecA</i> expression	Loss of <i>bla_Z-bla_{RI}-bla_I</i> operon enabled <i>mecA</i> expression	4×10^{-6}	4 mg/L to 32 mg/L	4
Gram-negative							
<i>E. coli</i>	β -Lactams, carbapenem, meropenem and cefepime	<i>bla_{KPC}</i>	Weak <i>bla_{KPC}</i> promoter	188 bp deletion upstream of <i>bla_{KPC}</i> created a stronger promoter and enhanced <i>bla_{KPC}</i> expression	ND	1 mg/L _{MEM} to 4 mg/L _{MEM} ; 8 mg/L _{LFEF} to 64 mg/L _{LFEF}	9
<i>P. mirabilis</i>	β -Lactams, meropenem, ertapenem, imipenem and temocillin	<i>bla_{OXA-48}</i>	Weak <i>bla_{OXA-48}</i> promoter	Single base pair deletion upstream of <i>bla_{OXA-48}</i> 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 <i>E. faecium</i>	Glycopeptide, vancomycin	<i>vanA</i>	Absence of <i>vanRS</i> due to partial deletion	Insertion of <i>IS1251</i> in intergenic region between <i>vanS</i> and <i>vanH</i> led to expression of <i>vanHAX</i>	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 <i>E. faecium</i>	Glycopeptide, vancomycin	<i>vanA</i>	Absence of <i>vanRS</i> due to partial deletion	Insertion of <i>IS1167</i> in intergenic region between <i>vanS</i> and <i>vanH</i> provides -35 box and led to high-level expression of <i>vanHAX</i>	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 <i>E. faecium</i>	Glycopeptide, vancomycin	<i>vanA</i>	Insertion of <i>ISL3</i> between <i>vanRS</i> and <i>vanHAX</i>	Excision of <i>ISL3</i> between <i>vanRS</i> and <i>vanHAX</i> restored <i>vanRS-vanHAX</i> proximity and thus their expression	3×10^{-8}	1 mg/L to >256 mg/L	6
Alterations in the AMR gene							
<i>S. aureus</i>	Macrolide, erythromycin	<i>ermA</i>	Nucleotide substitution in <i>erm</i>	Direct reversion of original mutation in <i>erm</i>	2×10^{-7} to 1×10^{-9}	0.5 mg/L to 256 mg/L	4
<i>S. aureus</i>	Macrolide, erythromycin	<i>ermA</i>	Insertion of <i>IS256</i> into <i>erm</i>	Excision of <i>IS256</i> from <i>erm</i>	4×10^{-7}	0.5 mg/L to 256 mg/L	4

<i>S. aureus</i>	β -Lactam, cefoxitin	<i>mecA</i>	Frameshift due to insertion within <i>mecA</i>	Compensatory mutation, frameshift due to deletion restored <i>mecA</i> reading frame	3×10^{-9}	4 mg/L to 64 mg/L	4
<i>S. aureus</i>	β -Lactam, cefoxitin and oxacillin	<i>mecA</i>	Single base pair insertion in <i>mecA</i> led to premature stop codon	Single nucleotide insertion restored reading frame of <i>mecA</i>	1×10^{-6} to 1×10^{-7}	<4 mg/L _{Fox} to >4 mg/L _{Fox} ; ≤ 0.25 mg/L _{Oxa} to >2 mg/L _{Oxa}	62
<i>S. aureus</i>	β -Lactam, oxacillin	<i>mecA</i>	Insertion of IS1181 into <i>mecA</i>	Excision of IS1181 from <i>mecA</i>	4×10^{-7}	0.25 mg/L to >64 mg/L	7
<i>S. aureus</i>	β -Lactam, oxacillin	<i>mecA</i>	Single nucleotide deletion in <i>mecA</i> led to premature stop codon	Direct reversion of original mutation or insertion close to original mutation restored reading frame of <i>mecA</i>	9×10^{-8}	0.5 mg/L to 64 mg/L	7
<i>S. aureus</i>	Mupirocin	<i>mupA</i>	Frameshift due to single nucleotide deletion in poly(A) tract	Direct reversion of original mutation in <i>mupA</i>	1×10^{-7}	0.5 mg/L to >256 mg/L	4
Gram-positive	Aminoglycosides, gentamicin and tobramycin	<i>aacA-aphD</i>	Insertion of IS256 in <i>aacA-aphD</i>	Excision of IS256 from <i>aacA-aphD</i>	$3 \times 10^{6\text{GEN}}$; 9×10^{-8} _{TOB}	0.25 mg/L _{GEN} to 32 mg/L _{GEN} ; 0.5 mg/L _{TOB} to 16 mg/L _{TOB}	4
<i>S. aureus</i>	Aminoglycosides, tobramycin	<i>ant4</i>	Frameshift due to single nucleotide insertion in <i>ant4</i>	Compensatory deletion, single nucleotide deletion in <i>ant4</i> restored reading frame	8×10^{-10}	0.25 mg/L to 64 mg/L	4
<i>S. aureus</i>	Aminoglycosides, spectinomycin	<i>aad9</i>	Single nucleotide deletion in poly(A) tract led to frameshift	Direct reversion of original mutation in <i>aad9</i>	7×10^{-9}	64 mg/L to >512 mg/L	4
<i>S. aureus</i>	Aminoglycosides, spectinomycin	<i>aad9</i>	Nucleotide substitution led to nonsense mutation and premature stop codon	Direct reversion of original mutation in <i>aad9</i>	2×10^{-6} to 9×10^{-7}	64 mg/L to >512 mg/L	4
<i>S. aureus</i>	Tetracyclines, tetracycline	<i>tetM</i>	Single nucleotide deletion in poly(A) tract led to frameshift	Direct reversion of original mutation in <i>tetM</i>	8×10^{-8}	1 mg/L to 128 mg/L	4
Gram-negative	<i>Campylobacter coli</i>	<i>sat4</i>	7 bp duplication disrupted <i>sat4</i> 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	<i>E. faecium</i>	<i>vanM</i>	ND	Amplification of <i>vanM</i> cluster	ND	1 mg/L to >256 mg/L	63
<i>E. faecium</i>	Glycopeptide, vancomycin	<i>vanA</i>	Truncation of <i>vanX</i>	Increased <i>vanA</i> plasmid copy number	ND	<4 mg/L to ≥ 256 mg/L	17

Continued

Table 2. Continued

Species	Antibiotics	AMR gene	Mechanism of silencing	Mechanism of resistance conversion	Conversion rate	Increase in MIC	Ref.
<i>E. faecium</i>	Glycopeptide, vancomycin	<i>vanA</i>	Truncation of <i>vanR</i>	and addition of a <i>vanA</i> plasmid containing a functional <i>vanX</i> Increased <i>vanA</i> plasmid copy number	2×10^{-8}	1 mg/L to >256 mg/L (with both deletion and increased copy number)	15
<i>S. aureus</i>	Lincosamides, clindamycin	<i>vga(A)v</i>	ND	Amplification of <i>vga(A)v</i> resistance-bearing transposon Tn5406	2×10^{-9}	0.25 mg/L to 1 mg/L	4
Gram-negative <i>E. coli</i>	β -Lactam and β -lactam inhibitor piperacillin-tazobactam	<i>bla_{TEM}</i>	ND	4-fold amplification of <i>bla_{TEM}</i> for higher β -lactamase activity	ND	$\leq 16/4$ mg/L to $\geq 128/4$ mg/L	64
<i>E. coli</i>	Piperacillin-tazobactam	<i>bla_{CMY-2}</i>	ND	Point mutation in <i>inc</i> antisense RNA gene led to increase in <i>bla_{CMY-2}</i> plasmid copy number and increase in <i>bla_{CMY-2}</i> expression	1×10^{-6} to 1×10^{-7}	4 mg/L to 64–512 mg/L	65
<i>E. coli</i>	Piperacillin-tazobactam	<i>bla_{TEM-1B}</i>	ND	IS26-mediated increase in <i>bla_{TEM-1B}</i> copy number led to hyperproduction of β -lactamase	ND	2–4/4 mg/L to 64/4 mg/L	66
<i>K. pneumoniae</i>	Carbapenem, imipenem	<i>bla_{OXA-232}</i>	ND	Increase in <i>bla_{OXA-232}</i> plasmid copy number increased <i>bla_{OXA-232}</i> expression	ND	0.5–1 mg/L to 16–32 mg/L	67
<i>K. pneumoniae</i>	Carbapenem, meropenem and imipenem	<i>bla_{KPC-2}</i>	ND	Increase in <i>bla_{KPC-2}</i> plasmid copy number increased <i>bla_{KPC-2}</i> expression	ND	ND	68
<i>E. coli</i>	Carbapenem and cephalosporin and β -lactamase inhibitor, meropenem, ceftazidime/avibactam	<i>bla_{KPC-3}</i>	ND	Increase in <i>bla_{KPC-3}</i> -Tn4401a plasmid copy number increased <i>bla_{KPC-3}</i> 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, ceftoxitin; 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 IS1251-like element promoter implant⁵. The insertion of an IS1542 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 IS1167, 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 ISL3 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*, IS26 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 IS1181 from the *mecA* reading frame.⁷ Similarly, excision of

IS256 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 IS1216 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 Enterobacteriales, 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, $\geq 10^4$ bacterial genome copies/mL in bloodstream and urinary tract infections (UTIs) as well as $\geq 10^8$ 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 $5 \times 10^{7-9}$ 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 Denmark⁹² 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 tests⁹⁶ (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}

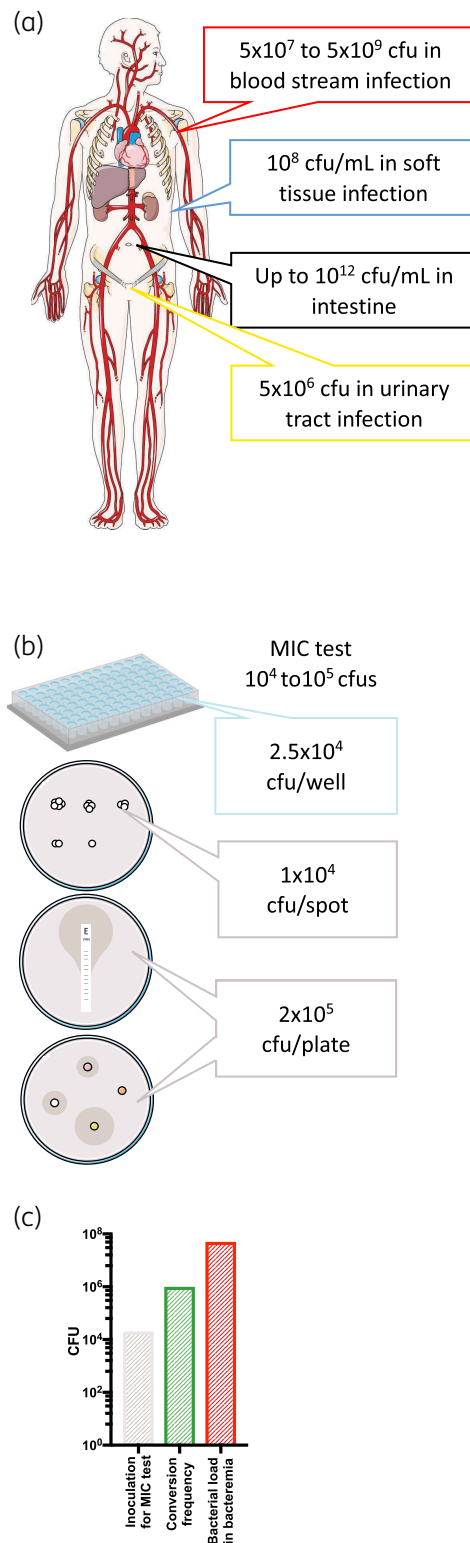


Figure 2. Challenges in detection of tsaAMR. Bacterial loads that can be reached during infection (a), the bacterial inoculum size for conventional AST (b), and their comparison with the resistance conversion rate (c). The numbers for the graph are based on Smith and Kirby,⁹¹ Kime *et al.*⁴ and Peters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

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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Transparency declarations

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