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Update on prevalence and mechanisms of resistance to linezolid, tigecycline and daptomycin in enterococci in Europe: Towards a common nomenclature

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ABSTRACT

Vancomycin-resistant enterococci (VRE) are important nosocomial pathogens. Invasive VRE infections are difficult to treat since common therapeutic options including ampicillin and glycopeptides often fail. *In vitro*, most VRE remain susceptible to last-resort antibiotics such as linezolid, tigecycline and daptomycin. However, neither tigecycline nor linezolid act in a bactericidal manner, and daptomycin has proven activity only at high dosages licensed for treating enterococcal endocarditis. Despite these pharmacological and therapeutic limitations, reports on resistance to these last-resort drugs in VRE, and enterococci in general, have increased in recent years. In this review, we briefly recapitulate the current knowledge on the mode of action as well as the known and novel mechanisms of resistance and describe surveillance data on resistance to linezolid, tigecycline and daptomycin in enterococci. In addition, we also suggest a common nomenclature for designating enterococci and VRE with resistances to these important last-resort antibiotics.

Introduction

Enterococci are important commensal bacteria residing in the intestines of humans and many other animals, including invertebrates. They are known as early colonizers of neonates and constitute a substantial part of the healthy intestinal microbiota of adults. Due to their beneficial impact on gut health, enterococcal mixtures have been licensed as probiotics. Various enterococci are used for food fermentation and preservation (Franz et al., 2011) due to their abilities to produce highly effective bacteriocins active against food-borne pathogens such as *Listeria monocytogenes*. The two species *Enterococcus faecium* and *Enterococcus faecalis* have been recognized for decades as major nosocomial pathogens in humans. Moreover, *E. faecalis* is occasionally implicated in community-associated infections such as urinary tract infections and endocarditis.

Members of the genus *Enterococcus* are known to be versatile

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microorganisms capable of surviving under harsh conditions and quickly adapting to a changing environment. Their biological constitution allows for a high rate of DNA recombination against a background level of mutation, which then facilitates survival under varying situations at the population level. Within the *E. faecium* species, frequent DNA exchange associated with niche separation and niche adaptation have shaped a subpopulation, called clade A1, that has clearly adapted to the hospital setting (Lebreton et al., 2013). Different models exist that describe the origin of these hospital-associated *E. faecium* lineages (Lebreton et al., 2013; Numminen et al., 2016); however, it is still under debate as to whether today's hospital-associated *E. faecium* strains originated from commensal strains in humans and animals or, in fact, evolved directly from livestock animals (Lebreton et al., 2013; Raven et al., 2016; Willems et al., 2012). Niche adaptation and host speciation is much less pronounced in *E. faecalis*, although a number of clonal lineages exhibit an association with hospitals, to a certain extent (CC2 and CC9; (Kuch et al., 2012; Numminen et al., 2016; Tedim et al., 2015).

Currently, *E. faecium* and *E. faecalis* rank as the second and third most important nosocomial pathogens worldwide (Kolonen et al., 2017; Mendes et al., 2016a; Weisser et al., 2017; Zhang et al., 2017). Even though the pathogenicity of these species is controversial, enterococcci can cause life-threatening infections in humans, mainly among elderly, multimorbid and/or immunocompromised patients (Arias and Murray, 2012; Murray, 1990). A wide armamentarium of natural resistance in this genus prevents a number of frequently used modern drugs such as cephalosporins and fluoroquinolones from being effective against enterococcal infections (Arias and Murray, 2012; Murray, 1990). Whereas ampicillin and glycopeptides are important first-line therapeutics, almost all hospital-associated strains of *E. faecium* have acquired ampicillin resistance (Lester et al., 2008; Top et al., 2007; Weisser et al., 2012). Moreover, since the mid-1980s, enterococci have garnered increased medical and public attention when vancomycin resistance first appeared in clinical isolates of *E. faecium* and *E. faecalis* (vancomycin-resistant enterococci; VRE) (Johnson et al., 1990; Uttley et al., 1989). Increased prevalence of ampicillin and vancomycin resistance, mostly among hospital-associated *E. faecium* isolates, has led to the therapeutic dilemma of multidrug-resistant (MDR) pathogens. Hence, these isolates, along with other superbugs such as *Staphylococcus aureus*, *Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa* and *Enterobacter* species, have been dubbed the "ESKAPE" pathogens (Boucher et al., 2009). Several reports and reviews have assessed rates of VRE among clinical enterococcal isolates and in specific clinical indications (such as bloodstream infections) (Adams et al., 2016; Akpaka et al., 2017; Simner et al., 2015; Weisser et al., 2017; Werner et al., 2008a). In general, the number of infections caused by enterococcal isolates, mainly *E. faecium*, and of those that are vancomycin resistant, has increased in many industrialized countries all over the world. Consequently, VRE are also considered a significant antibiotic resistance threat, leading to in excess of 20,000 drug-resistant *Enterococcus* infections and approximately 1300 deaths due to VRE in the US each year (CDC 2013, https://www.cdc.gov/drugresistance/pdf/ar-threats-2013-508.pdf). Through participation in the European Antimicrobial Resistance Surveillance Network (EARS-Net), coordinated by the European Center for Disease Prevention & Control (ECDC), many European countries have experienced an increased proportion of ampicillin resistant enterococci in invasive infections, mainly *E. faecium*, which are also resistant to vancomycin (Tedim et al., 2017). However, the European-wide trend of VRE causing systemic infections has remained inconsistent (https://ecdc.europa.eu/sites/portal/files/documents/antibiotics-EARS-Net-summary-2016_0.pdf). Ireland has been reporting the highest rate of vancomycin resistance among *E. faecium* bloodstream isolates in Europe in the last 10 years. Since 2012, more than 40% of *E. faecium* bloodstream isolates have been identified as vancomycin-resistant.

In the hospital setting, most VRE are also resistant to first line antibiotics (ampicillin, quinolones) with high MIC values. Furthermore, *E. faecium* and *E. faecalis* are often high-level resistant to aminoglycosides (HLAR). Hence, only a few therapeutic options, including oxazolidinones (linezolid, tedizolid), novel tetracyclines (tigecycline) and lipopeptides (daptomycin) remain to treat infections with multidrug-resistant VRE (Barber et al., 2015; Mutters et al., 2015; Whang et al., 2013; Zhao et al., 2016). The increasing prevalence of VRE in many countries and health-care settings, the expanded use of such last-resort antibiotics, and the ability of enterococci to easily acquire novel DNA fragments encoding resistance determinants currently enhance the selective pressure for developing resistance to these compounds. The genetic constitution of enterococci with a high recombination rate and the frequent occurrence of mobile genetic elements within the genome, as well as their natural habitat, predestine enterococci as a suitable indicator for novel emerging resistance phenotypes. We herein aim to summarize the general knowledge on non-susceptibility to last-resort antibiotics in *Enterococcus* spp. with a special focus on linezolid, tigecycline and daptomycin resistance. There are international surveillance systems that monitor the *in vitro* activities of these antimicrobial compounds, such as T.E.S.T. (Tigecycline Evaluation and Surveillance Trial) (Marco and Dowzicky, 2016; Vega and Dowzicky, 2017). They further serve as a basis for comprehensive databases from which resistance development can be analyzed retrospectively on a global scale (ATLAS, Antimicrobial Testing Leadership And Surveillance; supported by Pfizer). For the vast majority of the European countries, a generally low prevalence (< 1%) of isolates resistant to last-resort antibiotics has been noted (ATLAS database; https://atlas-surveillance.com). For this review article, we have comprehensively compared susceptibility data on enterococci with regard to linezolid, tigecycline and daptomycin and have summarized recent advances in studying the underlying resistance mechanisms in order to propose a common nomenclature for VRE with resistance to last-resort antibiotics.

Vancomycin resistance in Enterococcus spp.

Although this is not in the main focus of this report, we provide a short overview of the current state of knowledge of the different genotypes of vancomycin resistance in enterococci. At present, eight types (VanA to VanN) of vancomycin resistance are known, named according to its ligase gene (*vanA* to *vanN*). In addition to the ligase gene, two other genes encoding the sensor (VanS) and response regulator (VanR) are essential to confer the corresponding phenotype. Glycopeptide antibiotics bind to the N-terminal D-Ala-D-Ala residue present in disaccharide-pentapeptide cell wall precursors. The interplay between several expressed resistance genes allows for the downregulation of housekeeping cell wall precursor synthesis and the production of an alternative pentapeptide (the so-called pentadepsipeptide) side chain ending with D-lactate (e.g., VanA, VanB) or D-serine (e.g., VanG) (Courvalin, 2006). This alternative ending exhibits an up to 1,000-fold lower binding capacity to vancomycin (in case of D-Ala-D-Lac), which results in clinical resistance. The *vanA* and *vanB* resistance genotypes are by far the most frequently found variants in clinical *E. faecium* and *E. faecalis* isolates worldwide. The *vanA* gene cluster (carried by transposon Tn*1546*) is mainly plasmid-located, whereas the *vanB* gene cluster (carried by members of the integrative conjugative elements Tn*1549*-Tn*5382*) is usually present on the bacterial chromosome. For more details, the reader is referred to a number of review articles dedicated to this topic (Courvalin, 2006; Hegstad et al., 2010; Werner et al., 2013).

A specific variant of VRE is represented by vancomycin-variable enterococci (VVE); these strains were noticed only very recently. VVE are vancomycin-susceptible enterococci with a *vanA* genotype (VVE-S), which can become resistant to vancomycin (VVE-R) upon exposure to vancomycin or teicoplanin (Coburn et al., 2014; Sivertsen et al., 2016). Development of vancomycin resistance in VVE-S can arise from different mechanisms and result in the expression of *vanHAX* genes, in

either a restored vancomycin-inducible fashion (Sivertsen et al., 2016) or constitutively (Thaker et al., 2015). Outbreaks of VVE have been reported in Canada (Szakacs et al., 2014), Norway (Sivertsen et al., 2016) and Denmark (Hansen et al., 2018). Sporadic isolates of VVE have also been reported in other European countries (Jung et al., 2014; Loens et al., 2016; Sivertsen et al., 2017). Most of the reported VVE isolates have been *E. faecium*, but this phenomenon can also occur in *E. faecalis* (Sivertsen et al., 2016). The overall prevalence of VVE-S is unknown. Since the expression of vancomycin resistance genes is turned off in VVE-S, they are not easily detected with phenotypic methods. However, since vancomycin and teicoplanin exposure over days promote the development of resistance, VVE have caused treatment failure and could be considered an emerging threat (Downing et al., 2015; Sivertsen et al., 2016).

Last-resort antimicrobials to treat VRE infections

Oxazolidinones

Linezolid: mode of action

Linezolid (LZD) is the first representative of the oxazolidinones that received approval for clinical practice by the US Food and Drug Administration (FDA) in 2000 (and later by the European Medicines Agency, EMA). This bacteriostatic antibiotic exhibits activity against a broad range of Gram-positive bacteria, including Methicillin-resistant *Staphylococcus aureus* (MRSA), and has received licensing for the treatment of nosocomial pneumonia, complicated or uncomplicated skin and soft tissue infections (SSTIs) and for infections caused by VRE (Brickner et al., 2008; Plosker and Figgitt, 2005). Although it adds to the substances that inhibit bacterial protein synthesis, cross-resistance triggered by antibiotics with proximal target sites have not been described, which is most likely due to the distinct mechanism of LZD action (Fines and Leclercq, 2000; Kloss et al., 1999). By targeting bacterial protein translation, oxazolidinones prevent the formation of the initiation complex by binding to domain V of the 23S rRNA (Swaney et al., 1998; Wilson et al., 2008). Nevertheless, during a compassionate use program and following implementation in clinical daily routine, the detection of LZD-resistant bacteria has been reported, including those that emerged during LZD therapy (Bassetti et al., 2003; Birmingham et al., 2003; Krull et al., 2016; Seedat et al., 2006). LZD is the only antibiotic of last resort discussed in this overview that can be given also orally in addition to an intravenous application suggesting a wider selective pressure, for instance, also on bacteria with an intestinal reservoir.

Linezolid: mechanisms of resistance

Resistance to LZD (MIC > 4 mg/L; www.eucast.org) can be mediated by either mutational mechanisms or by gene acquisition (summarized in Table 1). The most common resistance mechanism in enterococci is based on a G-to-T conversion at position 2576 (according to *E. coli* K-12 numbering) within the genes encoding for 23S ribosomal RNAs (Klare et al., 2015; Mendes et al., 2014; Pfaller et al., 2017b). The LZD MIC increases with the copy number of mutated 23S rDNA alleles and LZD resistance in enterococcal isolates is rapidly selected under LZD therapy (Bassetti et al., 2003; Marshall et al., 2002; Seedat et al., 2006). The initial 23S rDNA mutation is a crucial step in resistance progression, as additional 23S rDNA mutation sites in the genome are most likely generated by recombination rather than by individual point mutations (Marshall et al., 2002). The classical G2576 T mutations also seem to determine resistance to tedizolid, the second clinically available oxazolidinone (Klupp et al., 2016). Acquired resistance-determining mutations appear to be stable, as 40 to 50 daily passages of a clinical LZD-resistant *E. faecium* isolates in non-selective media (ca. 300 bacterial generations) were not sufficient to revert resistance associated with heterozygous G2576 T mutations (Freitas et al., 2016; Klare et al., 2015). For one of these experiments, *E. faecium* strains (n = 3) were

Table 1
Overview of acquired mutations/genes possibly contributing to linezolid resistance in enterococci.

Species	Target (gene)	Function of target	Comment	References
E. faecalis/ *E. faecium*	23S rDNA	Ribosomal RNA	$G_{2576}T$ mutation Initial point mutation, most likely followed by further allele replacement due to homologous recombination MIC levels depend on the number of alleles affected	(Klare et al., 2015; Marshall et al., 2002; Mendes et al., 2014; Pfaller et al., 2017a)
	RplC/RplD	Ribosomal proteins		(Chen et al., 2013)
	Cfr, Cfr (B), Cfr (B) variants	23S rRNA methyltransferase	Expression of *cfr* does not necessarily result in LZD insusceptibility	(Bender et al., 2016; Cercenado et al., 2010; Deshpande et al., 2015; Liu et al., 2014a, 2012; Long et al., 2006)
	OptrA	ABC-F protein		(Brenciani et al., 2016; Cai et al., 2016; Cavaco et al., 2017; Cui et al., 2016; He et al., 2016; Huang et al., 2017; Li et al., 2016; Sharkey and O'Neill, 2018; Vorobieva et al., 2017; Wang et al., 2015)
E. faecalis	PoxtA	ABC-F protein		(Antonelli et al., 2018)
	DEGs	Upregulation of genes associated with efflux pumps and biofilm formation		(Hua et al., 2018)

isolated from a Portuguese patient taking linezolid for 61 days, who developed three variants of the same LZD-resistant clone presenting different antibiotic resistance and virulence profiles. Yet another mechanism based on nucleotide mutations has been described for genes encoding for ribosomal proteins. Here, mutations in *rplC* (L3) and *rplD* (L4) are predominantly linked to LZD resistance in enterococci that lack G2576 T mutations and that do not express the multidrug resistance gene *cfr* (Chen et al., 2013).

Cfr was first isolated from *Staphylococcus sciuri* of animal origin and soon received enormous attention, as it is capable of conferring resistance to at least five classes of antibiotics, including linezolid (but not tedizolid); it has been detected in various bacterial species and genera (Locke et al., 2014; Long et al., 2006; Schwarz et al., 2000; Shen et al., 2013). The resistance phenotype mediated by the Cfr methyltransferase is commonly referred to as $PhLOPS_A$, an acronym for resistance to the antibacterial agents phenicol, lincosamide, oxazolidinone, pleuromutilin and streptogramin A (Long et al., 2006). Most importantly, the transfer of plasmid-borne *cfr* has been demonstrated to occur even across bacterial species and genera (Bender et al., 2016; Cafini et al., 2016).

Regarding enterococci, *cfr* was first reported from *E. faecalis* of animal origin (Liu et al., 2012). Follow-up studies in enterococci identified several distinct conjugative plasmids that harbored the multidrug resistance gene (Liu et al., 2013, 2012). Transfer of the *cfr*-harboring plasmids was demonstrated and the wildtype *cfr* (staphylococci homolog) and derivatives thereof, named *cfr(B)* and *cfr(B)* variants, located on plasmids or within the bacterial chromosome emerged in *Enterococcus* spp. from animal and human samples alike (Bender et al., 2016; Deshpande et al., 2015; Diaz et al., 2012; Liu et al., 2014a, 2013; Patel et al., 2013). It is worth noting that *E. faecium* isolates containing the *cfr* gene are increasingly observed (Cercenado et al., 2010). Nevertheless, the contribution of *cfr* and *cfr(B)* variants to elevated LZD MICs in *Enterococcus* spp. is still under debate. It has been shown that, probably due to yet unknown strain-specific reasons, Cfr and Cfr(B) variants fail to demonstrate LZD resistance and the entire $PhLOPS_A$ phenotype in enterococci (Bender et al., 2016; Liu et al., 2014b).

Further studies on enterococci with a silent *cfr* gene, but exhibiting LZD resistance, have demonstrated a role for a second LZD (and tedizolid) resistance determinant, the oxazolidinone and phenicol transferable resistance A (OptrA) (Brenciani et al., 2016; Wang et al., 2015). First detected in *E. faecalis* of human origin, further studies have been published reporting *optrA* in isolates of *E. faecalis* and *E. faecium* and most recently also in *S. aureus, S. sciuri* and *Streptococcus suis* (Cai et al., 2016; Cui et al., 2016; Guo et al., 2018; He et al., 2016; Huang et al., 2017; Li et al., 2016). Surveillance studies from China have shown that *optrA* is detected more frequently in enterococci from food-producing animals than in humans (Wang et al., 2015). *OptrA* encodes for an ATP-binding cassette (ABC)-F protein and mediates resistance through target protection (Sharkey and O'Neill, 2018). Releasing ribosome-targeted antibiotics such as phenicols, compounds commonly used in the animal production, highlights the relevance of selected antibiotic usage in animal husbandry for the emergence of bacteria carrying this resistance determinant (Wang et al., 2015). Moreover, reports from animals raised in Korea, Tunisia and Colombia or residual wastewaters in Tunisia where *E. faecalis* isolates with *optrA* have been described pointed towards an important animal reservoir as well as the possibility of ongoing environmental dissemination of this resistance gene [(Cavaco et al., 2017; Tamang et al., 2017) A.R.F. and L.P., unpublished data]. Recently, the analysis of 26 *E. faecalis* isolates from humans with LZD MIC > 4 mg/L obtained from the SENTRY program (see above) revealed a high prevalence (53.5%) of *optrA*-positive *E. faecalis* from humans; however, not all of these isolates exhibited LZD resistance (Mendes et al., 2016b). Congruently, the German National Reference Center (NRC) for staphylococci and enterococci at the Robert Koch Institute (RKI) have observed an increase in *optrA*-positive and LZD-resistant clinical (human) *E. faecalis* from 2007 to 2016, with 90.9% of all LZD-resistant *E. faecalis* (n = 35) isolates being *optrA*-positive in 2016 (Bender et al., 2018). Moreover, all LZD-resistant *E. faecalis* isolates reported to the Norwegian National Advisory Unit on Detection of Antimicrobial Resistance since December 2015 harbored *optrA* (Hegstad et al., unpublished). In a study on Polish LZD-resistant enterococci (Gawryszewska et al., 2017), two *E. faecalis* strains were positive for *optrA* with the resistance determinant located on a transferable plasmid identical to pE394, initially reported in China (Wang et al., 2015). With respect to the One Health dimension, *optrA* has been reported from an ST22 *E. faecium* strain isolated from imported turkey meat and an ST22 *E. faecalis* strain isolated from Danish veal meat (Cavaco et al., 2017). Likewise, an ST16 *E. faecalis* isolate with *optrA* has been reported in a Danish patient (Vorobieva et al., 2017).

Exceeding what is known for *cfr*, a high diversity of *optrA* nucleotide sequences and a plethora of variable genetic environments embedding the resistance gene in either the chromosome or on diverse plasmids and/or within transposon structures (Tn*554*, Tn*558*, flanked by IS*1216* or similar IS elements) have been reported (Bender et al., 2018; He et al., 2016; Huang et al., 2017; Mendes et al., 2016b). Hence, rapid spread of the resistance locus is conceivable with the possibility of global distribution being underestimated to date.

Recently, another member of the ABC-F protein subfamily, PoxtA, has been discovered in MRSA of clinical origin, clostridia and enterococci, exerting its protective effect against phenicols, oxazolidinones and tetracyclines (Antonelli et al., 2018). The prevalence of *poxtA* amongst the enterococcal population is under current investigation.

Although insufficiently studied, cell wall thickness and biofilm formation were discussed as putative alternative resistance pathways to acquire LZD resistance (Tian et al., 2014); however, the authors had no knowledge of *optrA* or *poxtA* at the time when the article was published and thus did not screen for this particular resistance loci. Also, it remains to be determined as to how the combination of determinants and/or genetic background with respect to species impacts LZD MICs. A first report by Mendes and co-workers and as also observed by the NRC for Staphylococci and Enterococci in Germany and the Norwegian National Advisory Unit on Detection of Antimicrobial Resistance demonstrated that the frequency of particular LZD resistance mechanisms seems to differ between *E. faecalis* and *E. faecium*. A tendency of 23S rDNA mutation being mostly common in *E. faecium* and *optrA* dominating in *E. faecalis* was generally observed (Mendes et al., 2016b).

A plethora of LZD resistance mechanisms already exists in enterococci, and novel ones have emerged relatively rapidly. Hence, it is conceivable that additional factors might be discovered with the potential to equally contribute to infections of and outbreaks caused by linezolid-resistant enterococci (LRE). In fact, LRE without any of the currently known resistance mechanisms have been reported, both associated with infection and colonization (A.R.F. and L.P., pers. communication; J.K.B. and G.W.; pers. communication; K.H. pers. communication). Recently, Hua et al. demonstrated differential expression of genes related to efflux pumps and or biofilm formation in strains showing low-level resistance to LZD and lacking known resistance mechanisms using a whole transcriptomic approach (Hua et al., 2018). Alarmingly, gene acquisition or mutational changes do not seem to impact bacterial fitness, as Hegstad and colleagues noticed the persistence of an ST117 LZD-resistant *E. faecium* clone in a Scandinavian hospital for over one year (Hegstad et al., 2014). Likewise, acquisition of an *optrA*-plasmid by *E. faecalis* did not affect the growth rates of the transconjugant compared to the plasmid-free recipient (Gawryszewska et al., 2017).

Thus, prudent use of LZD is indispensable to prevent the emergence and dissemination of multidrug-resistant strains. Again, it must be stressed that the occurrence of enterococci susceptible to linezolid but carrying silent *cfr* and/or *optrA* might hamper their identification in a clinical setting (Brenciani et al., 2016). The potential to disseminate a highly promiscuous resistance locus to other bacteria imposes an

unpredictable risk to different sectors.

Linezolid: resistance surveillance data

Infections due to LRE and their nosocomial spread have been described in several case reports and outbreak investigations for both vancomycin-resistant and vancomycin-susceptible *E. faecium* and *E. faecalis* (Gomez-Gil et al., 2009; Gonzales et al., 2001; Hegstad et al., 2014; Herrero et al., 2002; Ntokou et al., 2012; O'Driscoll et al., 2015; Rahim et al., 2003). LZD exposure, prolonged antibiotic treatment, hospitalization in a hematological ward, immunodeficiency, prior surgery and patient-to-patient transmission have been identified as risk factors to acquire LRE infections (Hayakawa et al., 2012; Kainer et al., 2007; Scheetz et al., 2008; Souli et al., 2009). However, LZD resistance does not necessarily only emerge upon LZD therapy (Mutters et al., 2015).

Several national and international programs exist that started to monitor LZD resistance development in the early 2000s. The SENTRY Antimicrobial Surveillance Program, the Linezolid Experience and Accurate Determination of Resistance (LEADER) initiative, ZAAPS (Zyvox® Annual Appraisal of Potency and Spectrum) and T.E.S.T. concordantly report a sustained high potency of LZD against the entire spectrum of tested bacteria with very low rates of resistance development over the last two decades (Flamm et al., 2016; Mendes et al., 2016b, c; Mutnick et al., 2003; Pfaller et al., 2017a, b; Streit et al., 2015). As for other Gram-positive bacteria, the general prevalence of LRE still remains below 1%. It is also worth noting that no MIC creep was observed amongst the isolates tested (Mendes et al., 2016c; Streit et al., 2015). In a very recent review, enterococcal data from the US LEADER and the global ZAAPS studies were summarized, again confirming the overall low prevalence of < 1% LZD resistance among clinical *Enterococcus* spp. (Bi et al., 2017). The presence of common resistance mechanisms was also analyzed, demonstrating the presence of *cfr* in 4.7% of linezolid-resistant *E. faecalis* and in 4.8% of linezolid-resistant *E. faecium* isolates. Altogether, 32 cases of *optrA*-positive enterococci were found.

Specialized reference laboratories throughout Europe have reported a greater recognition of LRE. However, differences in resistance rates might also depend on the species analyzed; for instance, a recent report from China described that the occurrence of LZD non-susceptibility was greater in *E. faecalis* compared to *E. faecium* (Tian et al., 2014).

In Germany, the national resistance surveillance scheme ARS (https://ars.rki.de) has not observed any trend for linezolid resistance among clinical *E. faecalis* or *E. faecium* isolates in recent years (< 1%). However, the NRC recognized an increased frequency of LRE from < 1% in 2008 to > 9% in 2014 among all strains received annually for subsequent strain characterization on a voluntary basis (Klare et al., 2015).

Since the first finding of LZD-resistant *E. faecalis* in 2012 (Thilesen et al., 2014), only 20 LRE have been reported in Norway up to January 2, 2017 (https://unn.no/fag-og-forskning/k-res/betydelig-okt-forekomst-av-linezolidresistente-enterokokker-i-2016-i-norge). Half of these isolates were found in 2016, representing a significant increase in prevalence compared to previous years.

In Denmark, LRE have not been send to the antimicrobial reference laboratory on a routine basis. However, during 2016, 14 LZD-resistant *E. faecalis* (1.3%) and 27 LZD-resistant *E. faecium* (4.2%) were detected from Rigshospitalet, in the capital region in Denmark (DANMAP 2016). In the first nine months of 2017, the incidence of LZD-resistant *E. faecalis* was reduced at Rigshospitalet to 0.5%, and that of LZD-resistant *E. faecium* to 1.7%. This correlated with a reduction program for linezolid usage at Rigshospitalet (J. D. Knudsen, pers. communication). In 2017, two other Copenhagen hospitals had three isolates of LZD-resistant *E. faecium* (0.1%) (H.W., pers. communication).

In Spain, an *E. faecalis* clinical isolate was the first identified LRE in 2003 (Sanchez-Gomez et al., 2006). Since then, LRE, either *E. faecalis* or *E. faecium*, have been detected in different hospitals, mostly associated with the use of this antibiotic in neutropenic patients (Alonso et al., 2014; Sanchez-Diaz et al., 2014), eventually causing nosocomial outbreaks (Gomez-Gil et al., 2009). Data from T.E.S.T. for tigecycline and comparator compounds in Spain, which included isolates from 27 centers obtained from 2004 to 2014 (Marco and Dowzicky, 2016), reflected linezolid resistance of 0.1% for *E. faecalis* (MIC_{90} = 2; range 0.5–8 mg/L) and 0.6% for *E. faecium* (MIC_{90} = 2, range 0.5–16 mg/L,). Another collaborative study performed in Spain from 2015, including data from 23,907 patients in 200 intensive care units as part of a strategic program to reduce the risk of selection and spread of antibiotic resistance in this country (Plan Nacional de resistencias a los antibióticos, Programa PRAM, http://www.resistenciaantibioticos.es), documented the occurrence of resistance to linezolid in 1.5% of *E. faecalis* and 3.3% of *E. faecium* strains (Palomar et al., unpublished data). Finally, the first phenotypically LZD-resistant *E. faecalis* strain in wild animals was documented in Spain; however, LZD resistance was not confirmed by a second, independent method (Navarro-Gonzalez et al., 2013).

When analyzing T.E.S.T. data, no LRE were detected in Italy between 2012 and 2014 (Stefani and Dowzicky, 2016). The international ZAAPS program assessing LZD resistance rates in 32 countries reported in 2015 a single Italian *E. faecium* isolate with a confirmed resistance phenotype and a known G2756 T mutation (see below) (Pfaller et al., 2017a). The national Italian Surveillance system AR-ISS has reported, amongst others, linezolid resistance in isolates from bloodstream infections. Linezolid resistance was detected in 0.6% (n = 9/1,582) of *E. faecalis* strains in 2015 and 0.3% (n = 5/1,579) in 2016 and in 0.4% (n = 3/745) of *E. faecium* strains in 2015 and 1.1% (n = 10/930) in 2016.

Poland obtained the first LZD-resistant isolate of *E. faecium* as early as in 2003 (Krawczyk et al., 2004). Since 2012, the National Reference Center for Susceptibility Testing (NRCST) has witnessed both increasing numbers of strains that were received by the NRCST as well as centers that reported the occurrence of LRE. An analysis performed on 50 isolates in the period from 2008 to 2015 revealed the predominance of hospital-associated *E. faecium* (82%); LZD-resistant *E. faecalis* and a single isolate of *E. avium* were identified as well (Gawryszewska et al., 2017). In that study, the G2576 T mutation was the most prevalent mechanism of resistance, but isolates with *optrA* were also observed.

Out of 3,974 enterococcal clinical isolates sent to the French NRC from 2006 to 2016, only 9 (0.2%) were resistant to linezolid (MIC > 4 mg/L; V.C. unpublished data). The first isolate (found in 2010) possessed ribosomal mutations, whereas the more recent isolates (one in 2013, one in 2014, two in 2015 and four in 2016) harbored the plasmid-mediated *optrA* gene. Five strains isolated before 2016 were all *vanA*-positive *E. faecium*, whereas strains isolated in 2016 were all susceptible to vancomycin (one *E. faecium* and three *E. faecalis*).

In Ireland, LZD resistance in *E. faecium* blood culture isolates was reported for the first time in 2004, when four of 78 *E. faecium* isolates tested against LZD were reported as being resistant (5.2%). In subsequent years (2005–2016), LZD resistance continued to be reported from blood culture isolates. Overall, among 4073 isolates tested against LZD from 2002 to 2016, 75 were found to be resistant to LZD (1.8%). Of 3100 *E. faecalis* isolates tested against LZD between 2002 and 2016, 40 were found to be resistant (1.0%). LZD resistance in VRE-*E. faecium* isolates from sites other than the bloodstream, along with outbreaks of LZD-resistant VRE-*E. faecium* in tertiary hospitals, have also been reported in Ireland since 2012. Surveillance data in one institution showed an increase in LZD resistance in VRE-*E. faecium* from four isolates in 2015 to 16 isolates in 2016 from both clinical and screening specimens. The international ZAAPS program reported an Irish *E. faecalis* isolate from 2015 with LZD resistance and an *optrA* gene (Pfaller et al., 2017a).

In Portugal, available data from three major hospitals in the north and south of the country from 2009 to 2016 showed the occurrence of 35 *Enterococcus* spp. with LZD resistance. In each hospital, the incidence

Table 2
Overview of acquired mutations/genes possibly contributing to tigecycline resistance in enterococci.

Species	Target (gene)	Function of target protein	Comment	Reference
E. faecalis/ E. faecium	RpsJ	Ribosomal protein S10 (positions 54-61, *E. coli* numbering)	Some mutations are also present in TGC-susceptible isolates	(Beabout et al., 2015a, b; Cattoir et al., 2015; Fiedler et al., 2016; Niebel et al., 2015)
	Tet(M)	Ribosomal protection protein		(Beabout et al., 2015b; Fiedler et al., 2016)
	Tet(L)	MFS-type efflux pump		(Fiedler et al., 2016)

of linezolid resistance was below 1% (L.P., pers. communication). Data from T.E.S.T., including 400 isolates from four Portuguese centers collected from 2004 to 2016, confirmed the very low incidence of *Enterococcus* spp. resistant to LZD. Some of those isolates were also co-resistant to vancomycin or tigecycline. LRE (n = 7) and enterococci with intermediate resistance (n = 10; according to the Clinical & Laboratory Standards Institute criteria) were identified among 14 sampled hospital wastewaters (Santos Arronches, 2014).

Besides the occurrence of LZD-resistant enterococci in hospitals, their occurrence in other sources and habitats is of special concern. Recent reports from the European Food Safety Authority (EFSA), published together with the ECDC, indicated the presence of LZD resistance in enterococcal indicator isolates (i.e., non-selective screening) collected in 2012 and 2013 from food, especially from bovine and broiler samples, but also from pigs and cattle. The samples originated from various European countries including the Netherlands, France, Croatia, Belgium and Spain (http://ecdc.europa.eu/en/publications/Publications/antimicrobial-resistance-zoonotic-bacteria-humans-animals-food-EU-summary-report-2013.pdf; last access 30/10/2018).

Tigecycline

Tigecycline: mode of action

Tigecycline (TGC) is the first member of the class of glycylcyclines (Pankey, 2005). Structurally, it is a derivative of minocycline with a bulky ter-butyl-glycylamido side chain at the position 9 of the D ring (Thaker et al., 2010). It exerts bacteriostatic activity against a broad range of bacterial pathogens, including Gram-positive and Gram-negative bacteria, anaerobes and atypical organisms (Pankey, 2005; Stein and Babinchak, 2013). It is currently approved for the treatment of complicated SSTIs and complicated intra-abdominal infections (2005), as well as community-acquired bacterial pneumonia (2008) (Stein and Babinchak, 2013).

Like other tetracyclines, TGC inhibits bacterial protein synthesis at the elongation stage by preventing the binding of the ternary complex amino acid-tRNA·EF-Tu·GTP to the small 30S ribosomal subunit (Brodersen et al., 2000; Chopra and Roberts, 2001; Pioletti et al., 2001) due to an overlap in the primary binding site (named Tet-1) with the position of the anticodon stem loop of the A-site tRNA (Wilson, 2014). Both tetracycline (TET) and TGC reversibly bind through an Mg^{2+}-mediated interaction to the Tet-1 site, located in a pocket formed between helices h34 and h31 (16S rRNA) but in different orientations (Bauer et al., 2004; Grossman, 2016). As compared to classical tetracyclines, TGC has an increased binding affinity that results from a stacking interaction between the 9-ter-butyl-glycylamido moiety and nucleotide C1054 in h34 (Jenner et al., 2013; Olson et al., 2006).

Interestingly, glycylcyclines are not or only poorly affected by major classical TET resistance determinants, i.e. TET-specific MFS efflux pumps [e.g. Tet(A) and Tet(B) in Gram-negative bacteria; Tet(K) and Tet(L) in Gram-positives] and ribosomal protection proteins [e.g. Tet(M) and Tet(O)] (Grossman, 2016; Thaker et al., 2010). This stronger activity is related to the inhibition of bacterial protein synthesis with a potency three- and 20-fold greater than those of minocycline and TET as well as to a binding to 30S and 70S ribosomes with five- and > 100-fold greater affinity than minocycline and TET, respectively (Bergeron et al., 1996; Olson et al., 2006; Rasmussen et al., 1994).

Tigecycline: mechanisms of resistance

Clinical resistance to TGC is almost exclusively described in multi-drug-resistant Gram-negative bacteria, for which this antibiotic has been used as a last-resort option, usually in combination therapy (Sun et al., 2013). It commonly results from the overexpression of resistance-nodulation-cell division (RND) efflux pumps, such as AdeABC in *A. baumannii*, AcrAB-TolC in *K. pneumoniae* and *Enterobacter* spp. or SdeXY-HasF in *Serratia marcescens* (Sun et al., 2013). Of note, both *P. aeruginosa* and *Proteus mirabilis* show an intrinsic reduced susceptibility to TGC associated with the expression of MexXY-OprM and AcrAB-TolC RND-type systems, respectively (Dean et al., 2003; Visalli et al., 2003).

The role of active efflux has also been extensively studied in Gram-positive *S. aureus* mutants obtained *in vitro*, whereas no naturally-occurring clinical isolates with decreased TGC susceptibility have been reported to date (McAleese et al., 2005). In these *in vitro* mutants, TGC resistance was due to overexpression of the *mepA* gene (coding for an efflux pump belonging to the MATE family) caused by the inactivation of its transcriptional repressor MepR (Kaatz et al., 2005; Kumaraswami et al., 2009; McAleese et al., 2005).

In enterococci, the mechanism of TGC decreased susceptibility was first deciphered in *E. faecium* (Cattoir et al., 2015); resistance is defined by a MIC > 0.5 mg/L (www.eucast.org). TGC resistance determinants are summarized in Table 2. Mutants with reduced susceptibility to TGC were obtained *in vitro* by serial passages using two *E. faecium* reference strains, Aus0004 and HM1070 (Cattoir et al., 2015). The derivative mutants, AusTig and HMtig, exhibited MICs 8-fold higher than those of the parental strains (0.25 mg/L vs. 0.03 mg/L, respectively), with a concomitant 4-to-8-fold increase in MICs of TGC, minocycline and doxycycline. Importantly, decreased susceptibility was stable over two weeks without selective pressure, and no decrease in the MIC of TGC was evident in the presence of efflux pump inhibitors such as reserpine or pantoprazole. In addition, two *vanA*-positive *E. faecium* clinical isolates (named EF16 and EF22), collected in 2010 and 2013 in France, were found to have reduced susceptibility to TGC (Cattoir et al., 2015). EF16 was positive for *tet*(M) with a MIC of 0.5 mg/L while EF22 harbored both *tet*(M) and *tet*(L) genes with a MIC of 0.25 mg/L. Following a comparative genomic analysis between AusTig and Aus0004, four different non-synonymous nucleotide mutations were found, including one in the *rpsJ* gene, which encodes for the S10 ribosomal protein of the 30S ribosomal subunit. This mutation led to the D60Y substitution (*E. coli* numbering), which was also detected in HMtig mutants. A close mutation in the S10 ribosomal protein (K57E) was also detected in EF16, whereas no *rpsJ* mutation was found in EF22.

The role of RpsJ in TGC-reduced susceptibility in *E. faecium* was subsequently confirmed (Niebel et al., 2015). In this study, three different pairs of isogenic clinical isolates were characterized, each pair comprising a susceptible strain before TGC therapy and a non-susceptible strain following TGC exposure. The three pre-treatment strains (1S, 2S, and 3S) presented MICs of 0.12 mg/L, whereas among the post-exposure isolates, two (1R and 2R) were resistant (MICs of 8 mg/L) and one isolate (3I) showed reduced susceptibility (MIC of 0.5 mg/L). Comparative genomic analysis showed that all modifications occurred in a small portion of the S10 ribosomal protein comprised between positions 52 and 60, including A54E and H56R substitutions in 3I, a Δ52-56IRATH deletion in 2R and a Δ52I deletion plus Y58H in 1R.

The role of the ribosomal S10 protein as a general target of TGC

insusceptibility was confirmed across several bacterial species. Before *E. faecium*, it was first described in a KPC-producing *K. pneumoniae* clinical isolate, with the description of a substitution V57 L in RpsJ (Villa et al., 2014). Using an experimental evolution approach, several strains of *A. baumannii*, *E. faecium*, *E. coli* and *S. aureus* were adapted to growth in elevated TGC concentrations (Beabout et al., 2015a). At the end of the adaptation period, 35 out of 47 replicate populations presented clones with a mutation in the *rpsJ* gene. The residues where mutations in *rpsJ* were identified in Gram-positives were at nucleotide positions 53–56, 58 and 60 while a mutation observed in both Gram-positive and Gram-negative species was found only at position 57. All identified mutations were found on the vertex of an extended loop of the S10 ribosomal protein (positions 53–60), which is in close proximity to the 16S rRNA TGC binding pocket (Beabout et al., 2015a). The mechanism of reduced susceptibility could result from changes in the conformation or conformational dynamics of 16S rRNA, leading to reduced TGC binding affinity and/or a reduction in translational inhibition by TGC favoring tRNA entry and binding to the ribosome. Of note, the role of *rpsJ* mutations was also described in the early steps of TGC resistance in mutants of *Streptococcus pneumoniae* obtained *in vitro* (Lupien et al., 2015).

A recent study explored the acquisition of TGC resistance in *E. faecalis* using an experimental evolution strategy in a bioreactor setup (Beabout et al., 2015b). In two biological replicates, 24 mutations were found, but only two candidate loci seemed tightly linked to resistance: the 5′UTR of the *tet*(M) gene carried by Tn*916* (Δ87-bp and Δ125-bp 51-bp and 36-bp upstream of *tet*(M), respectively) and the *rpsJ* gene (R53Q-Δ54-57ATHK). In this study, the authors also showed that deletions of regulatory elements in the 5′UTR of *tet*(M) led to constitutive overexpression of *tet*(M) and a hyper-conjugative phenotype of Tn*916*. This study highlights the possibility of rapid spread of a resistance allele throughout the population.

An increase in TGC-resistant enterococci was reported in Germany from 2007 to 2015 (n = 73 *E. faecalis* and *E. faecium*) (Fiedler et al., 2016). In this study, the authors analyzed three isogenic *E. faecium* strains, including the resistant parental strain UW8175 (MIC = 8 mg/L), a susceptible revertant UW8175- (MIC = 0.03 mg/L) and a high-level resistant mutant UW8175+ (MIC = 64 mg/L). By comparative genomic analysis, they found some differences with the parental strain: six SNPs in both UW8175+ and UW8175- mutants, a loss of a 38-kb plasmid harboring *tet*(L) and *tet*(M) in UW8175- and a F62 V substitution in Tet(L) in UW8175 + . The authors also demonstrated increased expression of *tet*(M) and *tet*(L) (that were co-transcribed) and an increased plasmid copy number of the very same plasmid in UW8175+ as compared to the parental strain. The putative role of plasmid-associated *tet* genes was confirmed with other clinical isolates. Finally, it was also demonstrated that Tet(M) and Tet(L) were jointly responsible for TGC resistance in another bacterial host (namely *L. monocytogenes* EGD-e).

An uncommon mechanism of TGC resistance is provided by enzymatic inactivation mediated by the flavin-dependent monooxygenase Tet(X) (Forsberg et al., 2015). First described in *Bacteroides fragilis*, then in environmental and clinical isolates of Enterobacteriaceae and Pseudomonadaceae (Grossman, 2016; Moore et al., 2005), it has not been yet described in Gram-positive bacteria. However, *tet*(X) has been detected in the human gut microbiota and demonstrated to be harbored by mobile genetic elements (Aminov, 2013). The overexpression of *tet*(X) in *E. coli* is responsible for a 32-fold increase in the MIC of TGC, and it has also been predicted that its activity against TGC can be mutationally increased (Grossman, 2016; Linkevicius et al., 2016).

Tigecycline: resistance surveillance data

Recent surveillance and application studies have shown high levels of TGC susceptibility among Gram-positive clinical isolates worldwide, with no increase in resistance over time (Hoban et al., 2015; Sader et al., 2014b). Out of 20,782 enterococcal isolates (14,615 *E. faecalis* and 6167 *E. faecium*) collected globally between 2004 and 2013, 99.7% were categorized as susceptible according to the CLSI susceptibility breakpoint (≤0.25 mg/L) with a MIC_{50} of 0.25 mg/L (MIC range from ≤0.008 to 1 mg/L) (Hoban et al., 2015). Similar rates of < 1% were reported based on the most recent T.E.S.T. data from Latin America (Vega and Dowzicky, 2017). TGC susceptibility is not included in EARS-Net reporting, so available data on resistance to this agent derive from other sources, studies and surveillance schemes.

In enterococci, only a few TGC non-susceptible clinical isolates have been initially reported with no characterization regarding the mechanism of resistance. This includes one clinical isolate of *E. faecalis* (MIC = 2 mg/L) recovered in Germany in 2007 (Werner et al., 2008b), 10 clinical and non-clinical isolates (MICs = 0.5–1 mg/L) collected in Portugal between 1999 and 2007, of which seven were *E. faecalis*, one *E. gallinarum*, one *E. hirae* and one *Enterococcus* spp. (Freitas et al., 2011), and one clinical isolate of *E. faecalis* (MIC = 2 mg/L) reported in the UK in 2011 (Cordina et al., 2012).

In Spain, TGC resistance is still sporadic for *E. faecalis* (0.2%; range = 0.008–2; MIC_{50} = 0.12 mg/L and MIC_{90} ≤0.25 mg/L) and not reported for *E. faecium* (range = 0.015-0.5; MIC_{50} = 0.06 mg/L and MIC_{90} ≤0.12 mg/L), according to data from the T.E.S.T. survey that analyzed 1205 *E. faecalis* and 535 *E. faecium* isolates collected from 27 medical centers in Spain between 2004 and 2014 (Marco and Dowzicky, 2016).

In Portugal, data from four major hospitals in the north, center and south of the country showed the occurrence of two TGC non-susceptible *E. faecalis* of ST34 and ST319 from 2002, two from 2015 and two from 2016 [(Freitas et al., 2011); L.P., pers. communication]. In each hospital, the incidence of isolates non-susceptible to TGC was below 1% (L. P., pers. communication). Data available from T.E.S.T. for TGC and comparator compounds in Portugal, including 400 isolates from four centers assessed from 2004 to 2016, showed a single TGC-resistant *E. faecalis* isolate (MIC = 1 mg/L). Five *E. faecalis,* one *E. gallinarum,* one *E. hirae* and eight *Enterococcus* spp. were non-susceptible to TGC (MIC = 0.5–1.0 mg/L), originating from human colonization, chicken carcasses, piggeries and hospital wastewaters, respectively (Freitas et al., 2011); https://fenix.tecnico.ulisboa.pt/downloadFile/1970719973965985/Dissertacao_Ana%20Arronches.pdf).

In Germany, only limited data exist for TGC susceptibility among clinical enterococcal isolates. As part of the T.E.S.T. surveillance study (2004–2009) performed in France, Germany, Italy, Spain and the UK, overall very low resistance rates were reported for *E. faecium* (2/893; 0.2%) (Aznar et al., 2012). Earlier data for Germany (2004–2007) also revealed very low TGC resistance rates of < 1% (Seifert and Dowzicky, 2009). A German TGC resistance trial called G-TEST (parts I and II) flanking the introduction of this antibiotic into clinical practice in Germany revealed zero (part I) (Kresken et al., 2009) or very low TGC resistance rates (part II and subsequent years) (Kresken et al., 2011, 2009). However, the German NRC recognized an increasing number of confirmed TGC-resistant enterococci, mainly *E. faecium*, in recent years (unpublished data).

Out of 3974 enterococcal clinical isolates received by the French NRC from 2006 to 2016, none were categorized as resistant (MIC > 0.5 mg/L), but two isolates (0.05%) exhibited decreased susceptibility to TGC (MICs = 0.25-0.5 mg/L) (Cattoir et al., 2015) (V. C., unpublished data).

In Ireland, 560 *E. faecium* isolates from bloodstream infections (source: EARS-Net Ireland) were tested against TGC between 2009 and 2016; of these, four were resistant (0.7%). In one institution, TGC resistance was detected in 11 VRE isolates between 2012 and 2017, in one VR-*E. faecalis* and ten VR-*E. faecium* strains. The isolates were cultured from eight clinical specimens and three screening samples. One TGC-resistant VR-*E. faecium* isolate was resistant to LZD as well. Of note, TGC susceptibility was analyzed using automated testing (VITEK II) only and resistance was not confirmed by a second independent method.

TGC resistance was low among enterococcal isolates from

Table 3
Overview of acquired mutations possibly contributing to daptomycin resistance in enterococci.

Species	Target (gene)	Function of target	Comment	References
E. faecalis	LiaFSR (*liaFSR*)	Regulators involved in cell envelope stress response	Initial tolerance MIC 3-4 mg/L	(Arias et al., 2011; Miller et al., 2013)
	Cls (*cls*)	Cardiolipin synthetase involved in phospholipid metabolism	Results in non-susceptibility in combination with *liaFSR* mutations	(Miller et al., 2013; Palmer et al., 2011)
	GdpD (*gdpD*)	Glycerophosphoryl diester phosphodiesterase involved in phospholipid metabolism	Mutations in *gdpD* alone do not modify daptomycin susceptibility	(Arias et al., 2011)
	YvlB (*yvlB*)	Putative LiaFSR pathway target protein	* Mutation in *yvlB* alone results in non-susceptibility	(Miller et al., 2013; Palmer et al., 2011)
	GdpP (*yybT*)	Cyclic dinucleotide phosphodiesterase associated with the membrane stress response	*	(Miller et al., 2013; Palmer et al., 2011)
	GshF (*gshF*)	Glutathione synthase involved in the oxidative stress response	*	(Miller et al., 2013)
	MdpA (*mdpA*)	Putative multidrug resistance pump	*	(Miller et al., 2013)
	EF2470	Protein with unknown function Contains an HD domain, which may be involved in nucleic acid metabolism and signal transduction	*	(Palmer et al., 2011)
	EF1797 (*drmA*)	Membrane protein with a serine incorporator domain May aid in serine and phospholipid biosynthesis and promote transport of polar serine into the hydrophobic cell membrane	*	(Miller et al., 2013; Palmer et al., 2011)
	EF1753	Protein with auto-transporter adhesin domain that is likely involved in cell adhesion	*	(Palmer et al., 2011)
	EF0716	Membrane protein with EamA-like transporter family domain	*	(Palmer et al., 2011)
E. faecium	LiaFSR (*liaFSR*)	Regulators involved in the cell envelope stress response	Most common initial mutations in daptomycin non-susceptible isolates	(Diaz et al., 2014; Panesso et al., 2015) (Sinel et al., 2016a)
	YycFG (*yycFG*)	Regulators of cell wall synthesis and homeostasis	Detected in both daptomycin resistant and tolerant isolates	(Diaz et al., 2014; Tran et al., 2013a)
	Cls (cls)	Cardiolipin synthetase involved in phospholipid metabolism	Mutations in additional target needed to mediate non-susceptibility	(Diaz et al., 2014; Sinel et al., 2016a; Tran et al., 2013a)
	GdpD (*gdpD*)	Glycerophosphoryl diester phosphodiesterase involved in phospholipid metabolism		(Diaz et al., 2014)
	Cfa (*cfa*)	Cyclopropane synthase involved in phospholipid metabolism		(Diaz et al., 2014; Tran et al., 2013a; Werth et al., 2014)
		HD domain containing protein similar to that in *E. faecalis*	*	(Diaz et al., 2014; Lellek et al., 2015; Tran et al., 2013a)
	RrmA	23S rRNA methyltransferase	*	(Diaz et al., 2014; Tran et al., 2013a)
	TelA	Tellurite resistance protein	*	(Diaz et al., 2014)
		Alpha-mannosidase	*	(Humphries et al., 2012)
	aad	Alcohol dehydrogenase	*	(Humphries et al., 2012)
	EzrA	Transmembrane negative regulator of the septation ring formation protein FtsZ	*	(Humphries et al., 2012)
		Protein with unknown function	*	(Humphries et al., 2012)
	PTS IID	Mannose-specific IID component of PTS	*	(Humphries et al., 2012)
	relA	Involved in the stringent response	* Tolerance to daptomycin	(Honsa et al., 2017)

* Potentially contributing to non-susceptibility.

bloodstream infections reported by the national Italian surveillance system (AR-ISS). Among the *E. faecalis* isolates tested in 2015 and 2016, 0.8% (n = 9/1,105) and 0.16% (n = 2/1,244) were TGC-resistant, respectively. Corresponding data for *E. faecium* revealed TGC resistance in 0.4% (n = 2/490; 2015) and 0.3% (n = 2/611; 2016).

In Poland, Norway and Denmark TGC-resistant enterococcal isolates have not been reported to date.

Daptomycin

Daptomycin: mode of action

Daptomycin (DAP) is a cyclic anionic lipopeptide synthesized from the lipopeptide antibiotic A21978C complex produced by *Streptomyces roseosporus* (Debono et al., 1988; Eliopoulos et al., 1985). In 2003, the USA FDA approved the use of DAP for the treatment of complicated SSTIs at a dosage of 4 mg/kg/day, and later for the treatment of *S. aureus* bacteremia with or without right-sided infective endocarditis at a dosage of 6 mg/kg/day (https://www.fda.gov/Drugs/). The EMA approved DAP for the same indications in January 2006. Treatment dosage ranges from 4 to 10 mg/kg/day. Different studies have documented the safety and tolerability of high-dose daptomycin (8–12 mg/kg/day) (Casapao et al., 2013).

DAP shares structural and functional similarities with cationic antimicrobial peptides produced by the mammalian innate immune system (Jung et al., 2004). The bactericidal effect of DAP involves alterations to bacterial cell envelope homeostasis through interactions with membrane phospholipids; however, this process is not yet fully understood (Tran et al., 2015). Insertion of DAP into the bacterial cytoplasmic membrane is dependent on the divalent cation form of calcium (Ca^{2+}) (Jung et al., 2004; Taylor et al., 2016). In addition, calcium is required for the formation of DAP micellar structures proposed to facilitate delivery of the antibiotic to the bacterial cytoplasmic membrane (Ho et al., 2008; Scott et al., 2007; Taylor et al., 2016). Important steps in the DAP interaction with Gram-positive cell membranes after insertion include oligomerization of DAP-calcium complexes in the outer leaflet (Muraih et al., 2012, 2011) and subsequent translocation of DAP oligomers into the inner leaflet of the cell membrane to form functional ring-like pores of two opposing oligomers. Oligomerization is dependent on interactions with the phospholipid phosphatidylglycerol (Muraih et al., 2012, 2011), and translocation of the oligomers is negatively influenced by the presence of the negatively charged phospholipid cardiolipin (Zhang et al., 2014b). DAP oligomerization is required for its antibacterial activity (Zhang et al., 2013). DAP pores are cation- and size-selective with greater permeability to Na^+, K^+ and other metal cations (Zhang et al., 2014a). The presence of pores in the membrane presumably results in ion leakage, membrane depolarization and cell death without lysis (Silverman et al., 2003; Zhang et al., 2014a).

In addition, DAP induces abnormal septation events (Cotroneo et al., 2008; Hachmann et al., 2009) and cell wall stress responses (Muthaiyan et al., 2008), suggesting that this compound causes significant cell wall damage. A second explanation for cell death due to the insertion of DAP into the cell membrane could be that DAP oligomers induce curved membrane patches (Jung et al., 2008; Pogliano et al., 2012). These curved patches are then incorrectly identified by an essential cell division protein as a potential site of division, inducing local activation of peptidoglycan biosynthesis which causes striking cell wall and membrane deformities and finally a rupture in the cell membrane and cell death (Pogliano et al., 2012).

Chen et al. proposed the extraction of lipids from the cell membrane as yet another alternative mode of action for DAP (Chen et al., 2014). They discovered that the interaction between DAP and giant unilamellar vesicles resulted in a lipid extraction effect, i.e. removing lipids from vesicles. This effect was only observed in the presence of phosphatidylglycerol and calcium. However, the authors did not present any evidence that lipid extraction is required for the antibacterial action of DAP.

Daptomycin: mechanisms of resistance

Neither the EUCAST nor the CLSI have established (clinical) breakpoints for DAP resistance in enterococci, but they have set the epidemiological cut-off (ECOFF) value for *E. faecium* and *E. faecalis* to ≤ 4 μg/ml for DAP (http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/General_documents/EUCAST_daptomycin_guidance_note_20160924.pdf) (Humphries et al., 2013). Unfortunately, this value is considered as a clinical breakpoint by some experts (Shukla et al., 2016). For the purposes of this review, enterococci with DAP MIC ≥ 8 μg/mL will be referred to as "DAP-resistant".

DAP resistance in enterococci has been associated with distinct structural alterations to the cell envelope, including increased cell wall thickening and decreased ability of DAP to alter cell membrane integrity. Moreover, the development of DAP resistance is associated with a compelling reduction in cell membrane fluidity and phosphatidylglycerol content and an accompanying increase in glycerolphosphodiglycodiacylglycerol (Arias et al., 2011; Mishra et al., 2012; Steed et al., 2011). Acquired DAP resistance is associated with mutations in intrinsic genes (summarized in Table 3). Much of the knowledge regarding which mutations are involved in DAP resistance comes from studies comparing the genomes, membrane potential and cell envelope composition of susceptible -and resistant more-or less isogenic mutants developed either in the laboratory or in patients during DAP therapy. In enterococci, DAP resistance can be linked to mutations in two major groups of genes, including genes encoding regulatory pathways involved in the coordination of the cell envelope stress response, such as LiaFSR and YycFGHIJ, and genes encoding enzymes involved in the metabolism of phospholipids important for cell membrane homeostasis, such as glycerophosphoryl diester phosphodiesterase (*gdpD*) and cardiolipin synthetase (*cls*) (Arias et al., 2011; Miller et al., 2016; Tran et al., 2015).

In *E. faecalis*, initial DAP tolerance is due to the redistribution of cell membrane cardiolipin-rich microdomains, which diverts DAP from its preferred binding site at the septum. Tolerance with a MIC close to the susceptibility breakpoint is associated with mutations within LiaFSR. High levels of MICs can be reached when additional mutations occur in *gdpD* and *cls* that alter the phospholipid content of the cell membrane (Arias et al., 2011; Mishra et al., 2012; Munita et al., 2013; Reyes et al., 2015; Tran et al., 2015, 2013b). A mutation in *gdpD* does not modify DAP susceptibility on its own (Arias et al., 2011). In agreement with this, adaptation of *E. faecalis* to gradually increasing concentrations of DAP revealed that the critical first step to resistance involves changes in the LiaFSR pathway. Almost half of the endpoint strains also had a mutation in *cls* (Miller et al., 2013).

Mutations in several other genes have been described in DAP-resistant *E. faecalis*, but further experiments are needed to reveal the exact function of these genes in *E. faecalis* and confirm that the mutations are causatively linked to DAP resistance. Potential resistance mutations have been reported in genes encoding: 1) GdpP (encoded by *yybT*), a cyclic dinucleotide phosphodiesterase associated with the membrane stress response (Miller et al., 2013; Wang et al., 2017); 2) YvlB, a putative LiaFSR pathway target protein; 3) GshF, a glutathione synthase; 4) MdpA putative multidrug resistance pump (Miller et al., 2013); 5) a protein with unknown function containing an HD domain found in an enzyme superfamily with phosphohydrolase activity, which may be involved in nucleic acid metabolism and signal transduction; 6) a membrane protein with a serine incorporator domain that may aid in serine and phospholipid biosynthesis and promote transport of the polar serine into the hydrophobic cell membrane; 7) a protein with an auto-transporter adhesin domain that likely is involved in cell-adhesion; and 8) a membrane protein with an EamA-like transporter family domain (Palmer et al., 2011).

DAP resistance development seems to be more common in *E. faecium* than in *E. faecalis* (Miller et al., 2014). Initial DAP resistance in *E.*

faecium is probably due to electrostatic repulsion of the positively charged DAP-Ca^{2+} complex from the cell membrane, as seen in *S. aureus* and *Bacillus subtilis*. Resistance is not associated with the redistribution of anionic microdomains as in *E. faecalis*, although mutations in *liaFSR* are the most common changes seen in daptomycin-resistant *E. faecium* (Diaz et al., 2014; Tran et al., 2015). Deletion of *liaR* from clinical strains harboring mutations in both the LiaFSR and YycFG pathways reverses DAP resistance and restores the binding of DAP to the *E. faecium* cell surface (Panesso et al., 2015). The second most common modifications in *E. faecium* are mutations in YycFG and the accessory protein YycHIJ, detected in both DAP-resistant and tolerant isolates. Mutations in *cls* are further commonly related with DAP resistance in *E. faecium*, and mutations in *gdpD* can occasionally be found (Diaz et al., 2014; Tran et al., 2013a). Cls and YycFG changes only are probably not sufficient to mediate resistance (Diaz et al., 2014; Tran et al., 2015, 2013a). In *E. faecium*, mutations in LiaSFR appear before *cls* mutations, indicating a sequential progression towards resistance. Interestingly, when selective pressure is removed, *E. faecium* reverts to become hyper-susceptible through the insertion of IS elements in *liaSFR* (Sinel et al., 2016a). There are also several reports of mutations in the *cfa* gene encoding a cyclopropane synthase involved in phospholipid metabolism in *E. faecium* (Diaz et al., 2014; Tran et al., 2013a; Werth et al., 2014). The involvement of *cfa* mutations in resistance is supported by a significant increase in cyclopropane fatty acids in DAP-resistant *E. faecium*. These fatty acids aid in maintaining the cell membrane in the presence of different stressors (Mishra et al., 2012).

Comparing the genomes of DAP-susceptible and DAP-resistant *E. faecium* strains has suggested several other mutant genes potentially associated with DAP resistance, but further functional evidence is needed to support these genetic findings. These genes encode: 1) an HD domain-containing protein also linked to DAP resistance in *E. faecalis* (Diaz et al., 2014; Lellek et al., 2015; Tran et al., 2013a); 2) RrmA 23S rRNA methyltransferase (Diaz et al., 2014; Tran et al., 2013a); 3) the tellurite resistance protein TelA also linked to DAP resistance in *E. faecalis* (Diaz et al., 2014); 4) alpha-mannosidase; 5) alcohol dehydrogenase; 6) EzrA, a transmembrane negative regulator of the septation ring formation protein FtsZ; 7) a protein with unknown function; and 8) the mannose-specific IID component of PTS (mannose-specific PTS mutations in the IIC and IID domains have been linked to resistance to class IIa cationic antimicrobial peptide bacteriocins; (Humphries et al., 2012). Furthermore, whole genome sequencing of isogenic strains revealed the loss of a 43 kb chromosomal fragment with 42 genes, presumably leading to DAP resistance as no mutations were observed in genes previously described to confer DAP resistance (Gumpert et al., manuscript submitted). Altered cell surface charges or the modification of PTS as a receptor are likely to result in DAP resistance in *E. faecium* (Humphries et al., 2012). A mutation in *relA* developed during vancomycin-resistant *E. faecium* bacteremia in a leukemia patient caused a constitutively activated stringent response with elevated baseline levels of the alarmone guanosine tetraphosphate and tolerance to high doses of both DAP and LZD in a biofilm. This explained why this infection persisted despite appropriate antibacterial treatment (Honsa et al., 2017). Analyses of a DAP-resistant *E. faecium* that emerged during DAP therapy of endocarditis in a leukemia patient showed among other mutations alterations in the DNA repair proteins MutL and RecJ. These changes caused a significantly enhanced mutation frequency that possibly contributed to development of high-level DAP resistance (Matono et al., 2016).

So far, no mutations were identified in *mprF* (multiple peptide resistance factor) or the *dltABCD* operon that could be directly linked to DAP resistance in enterococci (Arias et al., 2011; Montero et al., 2008).

The examples above demonstrate that changes in many possible target genes can result in DAP tolerance and resistance and that these mutations are easily acquired, especially under selective pressure in *E. faecium*.

Daptomycin: resistance surveillance data

Most enterococcal isolates (> 99.8%) are susceptible to DAP worldwide (Sader et al., 2014a), and this trend did not change in European and US hospitals in the period from 2009 to 2013 (Sader et al., 2015). The occurrence of DAP-resistant enterococci is most often associated with prior DAP exposure (DiPippo et al., 2016; Lellek et al., 2015; Lewis et al., 2016) and may correspond to the use of too low dosages of DAP not providing high enough plasma concentrations to kill enterococci (Hall et al., 2012; Sinel et al., 2016b; Werth et al., 2014). A recent study provided evidence of the frequent *in vitro* increase of MIC values to DAP among *E. faecalis* strains highly resistant to gentamicin (Pericas et al., 2017). The results suggest the convenience of testing the MIC values of combination compounds in the presence of subinhibitory concentrations of DAP to predict the efficacy of combination therapy for multidrug resistant enterococci (e.g. DAP plus ampicillin). When DAP is used in combination with other antibiotics, the possibility of selecting MDR phenotypes might be increased (Cercenado and Pachon, 2012). DAP susceptibility data for enterococci cannot be included in EARS-Net data (due to a missing EUCAST breakpoint), so resistance data are derived from other sources, studies and surveillance schemes.

Although *in vitro* resistance arises spontaneously at low frequencies, the emergence of resistance is increasingly selected during DAP therapy. It could be related to the mutant selection window (MSW), which lies between the MIC and the mutant prevention concentration (MPC) and corresponds to the concentration range within which resistant mutants may be selected. Indeed, it has been shown that free C_{max} (taking into account the protein binding of DAP, approx. 90%) values for DAP usually fell into the MSW when using low dosages (4–6 mg/kg), thus suggesting a risk of *in vivo* selection of resistant mutants, especially at sites of infection where the concentration of DAP is low (Sinel et al., 2016b). Therefore, high daily doses of DAP (≥10 mg/kg) may be considered for the treatment of infections due to *E. faecium*.

The transmission of DAP- and vancomycin-resistant enterococci within the hospital environment has been documented (Lellek et al., 2015). Moreover, population genetics assessing DAP-resistant *E. faecium* from a medical center in New York showed that a novel clone, ST736, was associated with resistance in this region (Wang et al., 2014).

The European Cubicin Outcomes Registry and Experience (EU-CORE) had been introduced as a retrospective, European, post-marketing, non-interventional registry of DAP use in patients who received at least one DAP dose. The database provides information from 6075 patients representing 18 countries and 314 study sites. The primary objective was to evaluate the clinical outcomes of patients treated with this drug (Cogo et al., 2015; Gonzalez-Ruiz et al., 2015).

In Spain, DAP is mostly used as a rescue therapy, at 6 mg/kg/day, in both hospitalized and non-hospitalized patients according to data from the EU-CORE database (Spain, with 47 hospitals, is the best represented country in this database) (Cercenado and Pachon, 2012; Sandoval et al., 2015). The first DAP-resistant enterococcal isolate in Spain was an ST117 *E. faecium* strain (MIC = 12 mg/L) recovered from a 46-year-old woman following kidney transplantation in 2005. The isolate showed a novel substitution in the response regulator LiaR and also harbored a change in the active site of the cardiolipin synthase (Sorlozano et al., 2015). The proportions of DAP-resistant *E. faecalis* and *E. faecium* are 3.23% and 10.53%, respectively, according to a national collaborative study performed in Spain (M. Palomar, unpublished data). The mechanisms of resistance were not explored in this study.

In France, a recent increase in the MIC of DAP in *E. faecium* has been observed, particularly in the proportion of strains with MICs > 4 mg/L (6–12 mg/L): 0% for the period 2006–2013, 1.1% (3/267) in 2014, 7.0% (24/344) in 2015 and 8.2% (31/379) in 2016.

In Ireland, among 60 *E. faecium* isolates from bloodstream infections (source: EARS-Net Ireland) tested against DAP between 2009 and 2014, four were resistant (7%). A tertiary care referral center has detected 12

Table 4
Proposal for a common nomenclature of VRE demonstrating resistance to linezolid, daptomycin and/or tigecycline. *We propose abbreviating Vancomycin (V), Linezolid (L), Daptomycin (D) and Tigecycline (T) as single letters; however, combinations of several resistances should be written as given in the Table with abbreviated antibiotics in an alphabetical order.*

Resistance to	Vancomycin VRE	Linezolid LRE	Daptomycin DRE	Tigecycline TRE
Vancomycin		LVRE	DVRE	TVRE
Linezolid			DLRE	LTRE
Daptomycin				DTRE

DAP-resistant *E. faecium* isolates since 2016; six *E. faecium* isolates were sensitive to vancomycin and resistant to DAP, the other six isolates were VR-*E. faecium* resistant to DAP. All isolates were susceptible to LZD and TGC. One patient who had received a lengthy course of DAP while in the ICU was found to carry VR-*E. faecium* resistant to DAP both in the intra-abdominal drain fluid and blood culture. All DAP-resistant isolates were grown from clinical specimens (six blood cultures, one wound swab and five drain fluids).

Data on DAP susceptibility in clinical enterococci in Italy is limited, as only one laboratory tests and reports data from enterococcal bacteremia to the national Italian surveillance system (AR-ISS). In 2015 and 2016, a total of 181 isolates were tested, of which 125 were *E. faecalis*, with two that were DAP-resistant. Of 56 *E. faecium* isolates, none were DAP-resistant.

In Denmark, DAP-resistant enterococci are not sent to the antimicrobial reference laboratory on a routine basis. However, during 2017, resistance to DAP was detected in two *E. faecalis* strains and one *E. faecium* isolate at Rigshospitalet, Denmark (H.W. and J. D. Knudsen, pers. communication).

In Germany, DAP-resistant enterococci are sent to the reference centre on a voluntary basis. During recent years, resistance to DAP was confirmed for a few *E. faecalis* and *E. faecium* isolates per year only, performed and analysed by two independent methods (broth microdilution and Etest®; J.B. and G.W. unpublished data).

No DAP-resistant enterococcal isolates have been noted in Poland, Norway and Portugal so far.

Individual resistance development under therapy and subsequent transmission of successful DAP-resistant clones warrant careful surveillance of these isolates in order to prevent their wider spread and to keep DAP as a valuable therapeutic alternative.

Conclusions and proposal for a common nomenclature

The review summarizes the current knowledge on mechanisms of resistance to the last-resort antibiotics linezolid, tigecycline and daptomycin in *Enterococcus* spp., and where available provides relevant surveillance data, partly unpublished, for many European countries.

We advocate using a suitable method for confirmation of these resistances, such as the broth dilution assay, and additionally recommend submitting relevant strains to reference laboratories for external confirmation, to support their reference and alert functions and to elucidate the corresponding resistance mechanisms. Using a common and agreed-upon standard is essential and available for Europe using the EUCAST methodology, clinical breakpoints and guidelines (www.eucast.org).

What has been causally indicative for VRE prediction on the basis of the presence of *vanA* or *vanB* genes is far from easy for the resistance phenotypes discussed in this review, which in turn require more sophisticated confirmatory assays based on whole genome sequence (WGS) data, as well as sophisticated transcriptomic and genetic analyses. Loss of susceptibility to linezolid, tigecycline and daptomycin in enterococci may be associated with a variety of resistance mechanisms (target mutations, gene acquisitions, “gene dosage effects” and many others); a number of them also appear in a combined fashion. Resolving complex resistance mechanisms requires high throughput data entering and hypothesis-free approaches, thus allowing an “open view” (Chacko et al., 2018). Considering this potential complexity, extracting a clinical breakpoint (an MIC) for linezolid, tigecycline and daptomycin for *Enterococcus* isolates or predicting potential therapeutic success from WGS data seems challenging and, at present, requires additional and comprehensive isolate collections and more dedicated studies to support this concept. Genome-wide association studies will potentially pave the way towards the better prediction of resistance phenotypes out of multiple genome comparisons (Power et al., 2017).

Resistance to last-resort antibiotics in enterococci which, in general, serve as important indicator bacteria, requires contemporaneous and strict surveillance to detect the emergence and dissemination of these important resistance mechanisms. With this review, we also propose a common nomenclature for VRE additionally exhibiting these resistance phenotypes (Table 4).

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