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Mobile genetic elements causing plasticity in *E. faecium*

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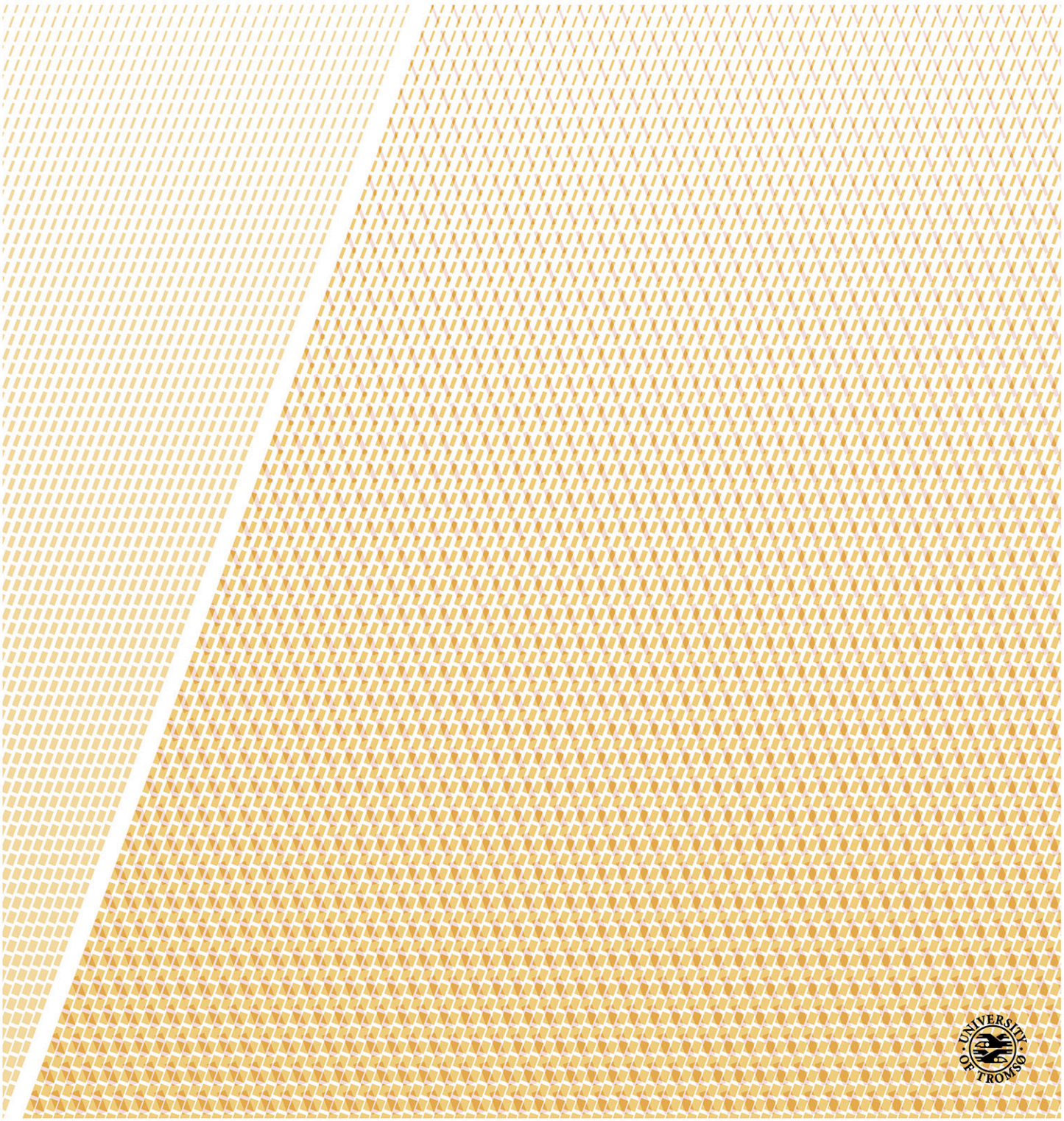
Audun Sivertsen

A dissertation for the degree of Philosophiae Doctor – December 2016



Mobile genetic elements causing plasticity in *Enterococcus faecium*

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Preface

Clinical microbiology is a vast topic with some overarching questions researchers aim to answer. We want to know how to identify and treat infections. We want to avoid the spread of infectious agents by stopping transmission through surveillance and appropriate containment measures. We want to understand the basal biological processes explaining how pathogens spread, produce disease, and evade treatment. Pathogen discovery, surveillance and disease prevention has evolved and matured greatly through the latest centuries (1), and the problems we encounter today and the methods we use to solve them are both in rapid change. Infections is a major cause of morbidity and mortality in humans, and during the last century, the revolution of the antimicrobial made us optimistic about eradicating this problem. Indeed, many easily treated diseases are no longer such a likely cause of death. As antimicrobials have been increasingly used and misused, the bugs have evolved themselves, and some are no longer responding to antimicrobial treatment. WHO estimates that resistant bugs are again to become a major cause of death relative to other diseases because of resistance development in important pathogens (2) even though these projections still need to be validated (3). Resistant organisms pose a threat to modern medicine as we know it, as many of the species which have developed the most resistance, are particularly adapted to persist in health care facilities and cause disease in patients with other serious conditions who otherwise enjoys the best and most advanced treatment modern health care is able to provide.

The research presented in this thesis focus on basal biological processes governing the spread, evolution and resistance to treatment in enterococci, and *Enterococcus faecium* in particular. This is in nature a ubiquitous bug, which recently has emerged as a multi-resistant pathogen within healthcare institutions.

The basic traits of enterococci

E. faecium was first described by distinct phenotypic traits in 1899 by Thiercelin, who isolated the bacterium from a case of infective endocarditis (4, 5). *E. faecium* was described as a sturdy Gram-positive diplococcus able to survive at 60°C, grow at 10-45°C, grow in salty conditions up to 6,5% NaCl, grow in basic media of up to pH 9,6, and to grow in media containing 40% bile. Although two different species today, *E. faecium* and *E. faecalis*, the two enterococci most prone to causing infections in humans, weren't considered two different species before the mid-sixties (6, 7). Enterococci reacted to serum of group D by the Lancefield's precipitin test, causing them to be classified as group D streptococci (8–10) until they were reclassified to enterococci in 1984 (11) due to advances in molecular classification schemes showing that enterococci and streptococci are too distantly related genetically to belong to the same genus. Although enterococci first were identified as parts of the human intestinal microbiota, we now know them as ubiquitous in nature (12). Found in and on other animals, insects and fish, on plants, in water and food, we now know of 54 species within the *Enterococcus* genus (12–19). Most of them have been characterized during the last 20-30 years. Thus, this genus is likely to expand further as todays screening methods yield substantially higher throughputs.

Clinical features of *E. faecium*

Historically, enterococci has been isolated predominantly as causes of bacteraemia, endocarditis and UTIs (20). Enterococci are also capable of invading other foci as an opportunistic pathogen, if a patient's ability to clear the infection has been compromised. *E. faecium* has been included as one of the five major multi-resistant pathogens predominantly causing the lion's share of nosocomial infections – the ESKAPE pathogens – the other ones being *Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumanni, Pseudomonas aeruginosa* and *Enterobacter* species (21). The complexities of modern medicine lead to a severely compromised immune defence in many patients for many treatments. Enterococci and *E. faecium* in particular have become increasingly prevalent as causes of nosocomial serious infections the last thirty years (22). This development is

paired with emergence of a hospital-associated clade demonstrating an increasing antimicrobial resistance pattern as well as presence of factors likely involved in virulence and adaptation to hospital environments (23, 24). These themes will be discussed in later sections.

Epidemiology

Enterococci have become more prevalent as cause of nosocomial infections the last 30 years. This development has been associated with use of third generation cephalosporins in U.S.A., of which enterococci are naturally resistant (25). In Europe, vancomycin resistant enterococcus (VRE) occurrence has been linked to the glycopeptide farm-animal growth-promoter avoparcin (26), but the overall prevalence is still lower than the U.S. The larger impact of enterococcal infections during the last two decades can be explained by a rise of *E. faecium* infections (22, 27), as the rate of *E. faecalis* infections has remained stable. Increased numbers of enterococcal infections has co-occurred with increased antimicrobial resistance, which mostly has occurred in *E. faecium* (27). The latest systemic surveys assessing hospital-acquired infections described enterococci as the second or third most common pathogen in the U.S.A. and Europe respectively (28, 29).

Virulence

Causal factors of pathogenicity and virulence in *E. faecium* has to be seen in connection to the extensive resistance levels this species demonstrates when encountered in hospital settings, permitting survival and transfer to patients susceptible to infection. No single defining virulence factor has been identified in *E. faecium*, but several genes are enriched in clinical lineages that may aid in invasiveness. As an opportunistic pathogen mostly causing severe disease in immunocompromised hosts, *E. faecium* has proven to be elusive to accurately characterise in terms of virulence.

It is difficult to assess the contribution of any particular putative virulence determinant in *E. faecium* as there are few reliable ways of knocking out or inserting a gene into experimental strains (30). The search for virulence factors in *E. faecium* has often consisted of finding secretion- and cell wall-associated genes

enriched in hospital-associated strains (31). This includes genes associated with biofilm formation [*esp* and *sgrA* (32, 33)], collagen adhesion [*acm*, (34)] and other genes associated with the enterococcal cell-wall [$efaA_{fm}$, *hyl, ecbA, scm, orf903, orf2010 and orf2514* (33, 35, 36)]. Hospital-acquired infection (HAI) isolates are also enriched with genes constructing pili [*pilA, pilB*, ebp_{fm} (37, 38)]. Some genes have been associated with virulence in *E. faecalis* and corresponding virulence in *E. faecium* is extrapolated, but of the few genes of which experimental data exist, *acm*, *esp*, and ebp_{fm} have been shown to contribute to virulence by exerting the assumed phenotypes in *in vivo* settings (38–40). In addition, two genes associated with two different phosphotransferase systems (PTS) are associated with improved intestinal colonization during antibiotic treatment [*ptsD* (41)] or implicated in biofilm formation and pathogenesis of endocarditis [*bepA* (42)].
Although these factors may contribute to virulence, it is important to consider that infection with *E. faecium* is most likely to occur in debilitated hosts. Obtaining a more comprehensive pathogenesis model would require examining the whole system in which infection occurs, which includes health state of the host, the intestinal environment where *E. faecium* resides, and obtaining a richer picture of how all parts interact with each other.

Host-microbe interactions

There is a deep and currently not fully defined interaction between humans and the microbiota residing in and on us. Many recent articles have emerged that refer to the imbalance of bacterial species in the gut where overgrowth of pathogenic bacteria (of which *E. faecium* is one) leads to increased risk of disease, and refer to this imbalance as 'dysbiosis' (43). Disentangling how changes in gut microbiota occur and what consequence they confer is a complex subject, and some controversy exists over whether dysbiosis/imbalance is a satisfactory explanation or whether other models need to be used in order to gauge how bacterial composition in the gut contributes to human diseases (44, 45). Outside the hospital and the selective effect of antimicrobial exposure, *E. faecium* from the commensal clade seem to outcompete nosocomial isolates in persistence over time (46). This suggests that modern advanced medical treatment, mostly done in medical institutions, may create an ignition for dysbiosis and acts as a "virulence

enabler" by itself. The papers presented in the next paragraphs aim to describe how VRE have been implied in dysbiosis, disease and protection from disease.

Brandl *et al.* (47) presents a model of how enterococci may end up dominating the gut flora as a result of broad-spectrum antibiotic use, thereby facilitating host invasion. They experimented on a feedback-loop where Paneth cells in the gut lining excretes an antimicrobial peptide with activity against Gram-positive bacteria called RegIIIγ in response to presence of lipopolysaccharides originating from Gram-negative bacteria (48). When Gram-negative bacteria are cleared from the gut, the intraluminal concentration of RegIIIγ decreases, permitting VRE to dominate and thereby creating a dysbiosis. VRE overgrowth is thus perceived to increase the risk of invasiveness.

As a continuation, Hendrickx *et al.* (49) created a similar VRE-dominant dysbiosis and found dramatic changes of the gut lining of mice as a response to VRE injection and concurrent antimicrobial treatment, with host factors segregating from the gut wall and creating an extracellular matrix around the VRE to protect the gut epithelium. The apical cell wall mucus layer thickness decreased during VRE dysbiosis. Epithelial architecture was also altered, which they were able to connect to intraluminal biochemical changes resulting in displacement of intercellular adherence junctions from the cell wall to the extracellular matrix surrounding the VRE. All these intra-luminal changes were clinically observed only as mild non-inflammatory diarrhoea.

Enterococci inhabit a crowded environment inside the human gut and compete with other species to survive. Their low virulence in immunocompetent hosts perturbs clear assertions of whether enterococci and their hosts co-exists in antagonistic, neutral or synergistic fashion (50). In fact, VRE introduced prior to induced cecal puncture giving a polymicrobial invasive infection actually protected mice by giving lower polymicrobial bacterial loads, milder inflammatory reactions and swifter recovery compared to VRE negative control mice (51). *E. faecium* excretes the biofilm-associated peptidoglycan hydrolase SagA (52), which according to recent studies is able to prevent *Salmonella* pathogenesis through degrading parts of the *Salmonella* cell wall (53). These cell wall fragments induce changes in the immunological pathways of the gut

epithelium which prevent *Salmonella* pathogenesis during infection (54) suggesting synergy between patient and *E. faecium* during *Salmonella* infection of immunocompetent hosts.

Antimicrobial resistance and treatment options

Already in 1929, Alexander Fleming noted that enterococci were resistant to penicillin (55), as a prelude to reports of the limited treatment options of today. Intrinsic resistance refers to an antimicrobial drug not working due to inherent features in a species, like restricting drug accessibility to target or not having the drug target at all. Acquired resistance occurs when the bacterium is baseline susceptible, but develops resistance either by somatic mutation or by acquisition of genes by horizontal transfer. To start with, *E. faecium* is on baseline intrinsically non-susceptible to penicillin, ampicillin, cephalosporins and other ß-lactams (25, 56) due to mutations in the penicillin-binding protein PBP5 (57–60) (gene is also horizontally transferrable (61)) as well as presence of other genetic determinants (62). Enterococci are *in vivo* resistant to clindamycin by efflux pumps, trimethoprim-sulfamethoxazole by missing target, and clinically achievable levels of most aminoglycosides due to enzymatic degradation (56, 63). *E. faecium* has also swiftly gained resistance through mutations or by acquiring resistance determinants towards antimicrobials such as quinolones, rifampicin and chloramphenicol, precluding their use (56, 64–68).

Even though enterococci are inherently low-level resistant towards ß-lactams and aminoglycosides such as gentamicin, *in vivo* these drugs in combination appears to have had synergistic effect as long as the bacterium does not harbour any additional gentamicin resistance determinants providing high-level resistance (60, 69–71), and has been considered standard treatment for decades. Mobile-element located enzymes modifying aminoglycosides are widespread in enterococci (60, 72), effectively obstructing the ampicillin plus gentamicin treatment alternative. In the case of high-level resistance and likely treatment failure using this regimen, vancomycin has been a reliable alternative.

Vancomycin

Vancomycin was isolated from *Amycolatopsis orientalis* in 1953 as a cell wall compound active against Gram-positives, but was considered secondary to other antimicrobials with better bactericidal effects, and as initially thought, better toxicity profiles (73). This changed in the 70s and 80s, as ß-lactam-resistant staphylococci (MRSA) began to emerge, prompting better characterizations of

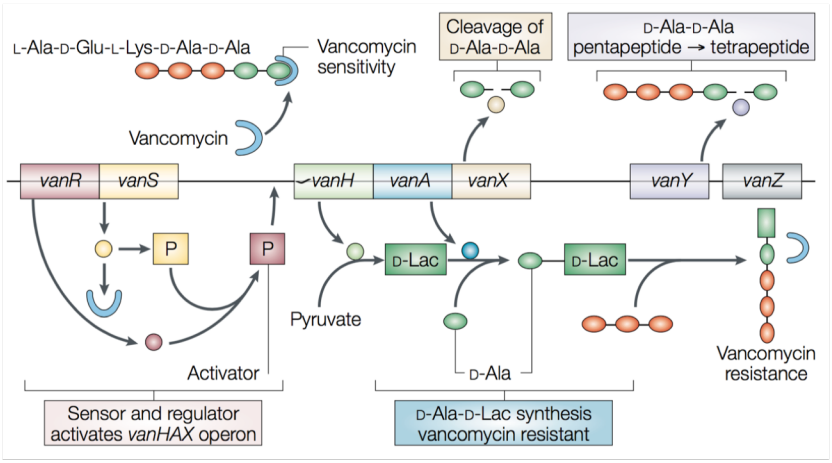
Figure 1. Global prevalence of antimicrobial resistance, and trends in Scandinavia Left: maps showing global burden of resistance towards vancomycin, gentamicin and aminopenicillins from resistancemap.cddep.org. Data from 2014. Regions for which data does not exist is marked in grey, resistance data as percentage of isolates in blue shades, the darker shade the higher percentage are resistant. Right: Resistance trends as percentage resistant isolates/year, relating to Norway, Sweden and Denmark for the corresponding antimicrobials. Note differences in Y-axis. Surveillance data and figures downloaded from EARS-Net http://atlas.ecdc.europa.eu/public/index.aspx

vancomycin use (74) and subsequent entry into treatment recommendations (75) as a last-resort antibiotic when other options were depleted. Vancomycin's glycopeptide cousin, teicoplanin, was also introduced in this period (76, 77), and several other less-used glycopeptides have appeared from the drug pipelines since (78–81).

Normally, the cell wall is constructed by interlinking peptidoglycans through the terminal D-alanyl-D-alanine (D-ala-D-ala) amino acids of the peptide moieties by transpeptidases (82). Vancomycin attaches to D-ala-D-ala through a hydrogen bond and denies transpeptidases to access peptidoglycans and thus polymerisation (82), see Figure 2. Vancomycin is a big molecule largely inefficient as an antimicrobial towards Gram-negative pathogens since it cannot cross the Gram-negative outer membrane, but recent research on vancomycin analogues show that increasing polarity through modification may circumvent this issue (83).

Vancomycin became increasingly used in the 80s, and the first reports of a vancomycin-resistant *E. faecium* (VREfm) outbreak (84) and first described occurrence of a plasmid-mediated vancomycin resistance determinant (85) (both in 1988) signalled a development to the troubling situation we have today. The dissemination of vancomycin-resistant *E. faecium* occurred swiftly in North American hospitals after that initial outbreak, while the later vancomycin resistance spread in Europe has been associated to the use of the glycopeptide avoparcin as a growth promoter in farms (86–88). North America still experiences higher prevalence of vancomycin resistance in clinical isolates compared to Europe, as seen in the point prevalence figure obtained from resistancemap.cddep.org and earssnet.org (Figure 1).

E. faecium, called a "drug resistance trafficker", has acquired no less than eight different vancomycin resistance gene clusters – *vanA*, *vanB*, *vanD*, *vanE*, *vanG*, *vanL*, *vanM* and *vanN* to defend itself (89–92). Additionally, a *vanC* gene cluster has been found in *E. casseliflavus* and *E. gallinarum* chromosomes (89). The function of these genes is to provide a pathway to alter the terminal D-alanine of the D-ala-D-ala vancomycin binding site to D-lactate (D-lac - *vanA, B, D, M*) or D-serine (D-ser - *vanC, E, G, L, N*). In modified peptidoglycan amino acid side-chain products terminating in D-Lac, VanH produces D-Lac from pyruvate, VanX cleaves



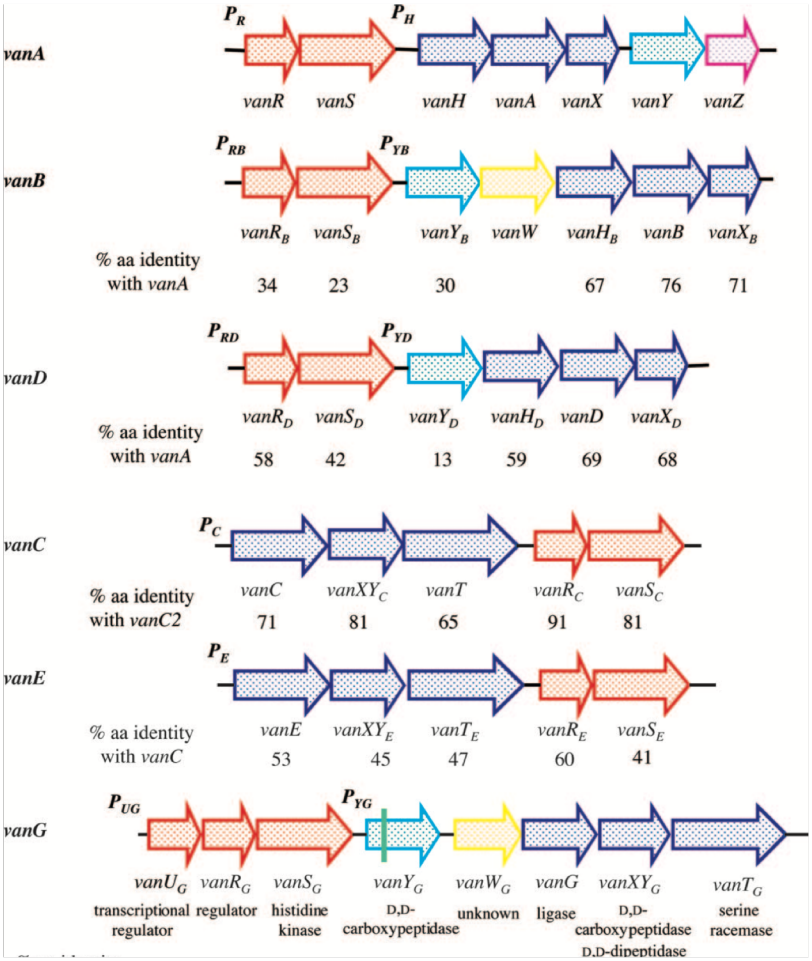


Figure 2. The vancomycin resistance gene clusters and resistance mechanism Functions of the proteins encoded by each gene in the *vanA* gene cluster (upper). Comparisons of several vancomycin resistance clusters found in enterococci, showing the variation in organization of the operons of *vanA*, *vanB*, *vanC*, *vanD*, *vanE*, and *vanG* gene clusters (lower). Genes with similar functions share colours. Upper figure reprinted with permission from Hughes (265), lower figure from Depardieu *et al.* (94).

off D-ala from the vancomycin-binding region of the amino acid side-chain, and VanA/B adds D-lac as the terminal peptide (93). See Figure 2 for graphical depiction of the VanA resistance mechanism and variations in operon structure of some of the vancomycin resistance clusters encountered in enterococci. The terminal peptide alteration provides significant reduction in glycopeptide binding affinity. D-ala-D-lac provides higher resistance level than D-ala-D-ser. Vancomycin resistance generally occurs via activation of resistance effectors by a feedback loop consisting of a sensor (VanS) that phosphorylates a regulator/activator (VanR) when vancomycin is present. Phosphorylated VanR subsequently binds to nucleotides in vicinity of the promoters of the resistance effector genes, and activates transcription. Vancomycin resistance itself is mediated by several genes (*vanH/T, vanA/B/C/D/E/G/L/M/N* and *vanX*) that when expressed forms a pathway replacing D-ala-D-ala to D-ala-D-lac (or D-ala-D-ser). In addition to these essential effector genes, accessory effectors that increases glycopeptide MIC (VanY) or have poorly characterized functions ($vanZ_A$ $VanW_B$) exist. See Departieu *et al.* for an extensive review of the topic (94). The origins of these gene clusters seem to be diverse, and it is likely that vancomycin resistance predates antibiotic use by millennia according to DNA extractions from glaciers (95).

Only *vanA* and *vanB* seem to be epidemiologically significant to provide resistance in clinical isolates (22). Globally, *vanA* has been and is still the most abundant resistance cluster in clinical isolates, whereas *vanB* has increasingly been found the last decade in Europe, and is the most abundant vancomycin resistance mechanism found in Australia (22, 23, 96). They are associated with mobile genetic elements. The *vanA* gene cluster is normally part of the Tn*3*-family transposon *Tn1546* (89) and the most prevalent *vanB* subtype is an integral part of the integrative conjugative element *Tn1549/5382* (61, 97). This may explain their high relative abundance compared to the other vancomycin resistance clusters.

Beyond vancomycin – available antibiotics to treat VRE*fm* and their resistance mechanisms

As vancomycin resistance, especially in the U.S.A. has become rampant, several recently approved drugs for which *E. faecium* often show susceptibility have

entered into treatment recommendations to clear VRE infections. Thus, several recent reviews have addressed options beyond the 'last resort treatment' vancomycin (98, 99). I will refer to these for further documentation.

Daptomycin, a lipopeptide bactericidal against enterococci through bacterial cell membrane disturbance, is used to treat VRE either by itself or in combination with ß-lactams. Resistance towards daptomycin has been shown to arise through mutations in genes associated with cell membrane construction pathways (*liaFSR*, *yycFG*) after prolonged daptomycin exposure (98), and *de* novo resistance development may spontaneously arise over time due to clinical daptomycin use (100).

Lipoglycopeptide drugs like telavancin, dalbavancin and oritavancin are modified versions of glycopeptides such as vancomycin. They bind to the same target as the glycopeptides but are thought to possess superior bactericidal action by closer association with the cell membrane by appendage of a lipophilic moiety. Both telavancin and dalbavancin have poor antimicrobial effects against VRE due to the altered biding site provided by vancomycin resistance gene clusters, and are thus not used clinically. Oritavancin on the other hand show activity against both *vanA*- and *vanB*-containing enterococci due to wider interactions to the peptidoglycan precursors. Since it has recently been introduced into the market, large studies describing oritavancin activity have not been published yet. Oritavancin is consequently not in wide therapeutic use.

Other antimicrobials, the oxazolidinones linezolid and tedizolid, act by binding to ribosomes and prohibiting mRNA-protein translation through abrogation of aminoacyl-tRNA docking. This mechanism ensures bacteriostasis in enterococci unless specific mutations occur in the 23S rRNA gene. Such mutations generally confer cross-resistance to linezolid and tedizolid (101). Enterococci possess several copies of 23S rDNA, and become increasingly resistant as more of the gene copies gain these mutations. The horizontally transmissible resistance determinants *cfr* and *optrA*, respectively encoding an rRNA methylase conferring resistance to linezolid and an ABC transporter pumping out both linezolid and tedizolid have also been found in enterococci (102, 103).

Streptogramins also attack the ribosome through binding to the 50S subunit, and the two drugs dalfopristin and quinopristin (Q/D) are delivered together since

they synergistically provide bactericide by irreversible inhibition of the ribosome. Resistance towards Q/D is mediated by multiple identified resistance determinants that alter the ribosome, provide hindrance to target, pump Q/D out of the cell or break either one or both Q and D thus hampering the synergistic effects and therefore bactericide.

Tigecycline also binds to the ribosomal subunit 16S and prohibits docking of aminoacyl-transfer RNA resulting in translation halt. Reservations against the use of this drug has arisen since it has a high volume of distribution, which causes low concentrations of free tigecycline at infection sites. This becomes a problem as observed mutations in relevant ribosomal genes, which slightly increases MIC for this antimicrobial rapidly create problems since obtainable antibiotic levels are so low.

Why does resistance accumulation occur?

In *E. faecium,* resistance development seems to occur in an additive manner in which certain strains amass one resistance determinant after another, while others stay susceptible and easier to manage during infection. As mentioned above, rise in *E. faecium* infection incidence is able to explain the rise of the *Enterococcus* (22).

A highly interesting theoretical paper by Chang *et al*. (104) attempts to identify the origins and proliferation of multi-resistance in bacteria and attempt to single out individual causes. Several of these multi-drug resistance development mechanisms shown in Figure 3 have already been shown effective in *E. faecium.* Resistance genes may be co-localised on mobile genetic elements [examples: (61, 103, 105–107)] and may as such be subject to linkage selection. The nosocomial clade of *E. faecium* is known to contain a wealth of MGEs and lack 'immune defences' against them such as restriction/modification systems and CRISPR-cas (24), which imply a potential for increased mutation rate in terms of

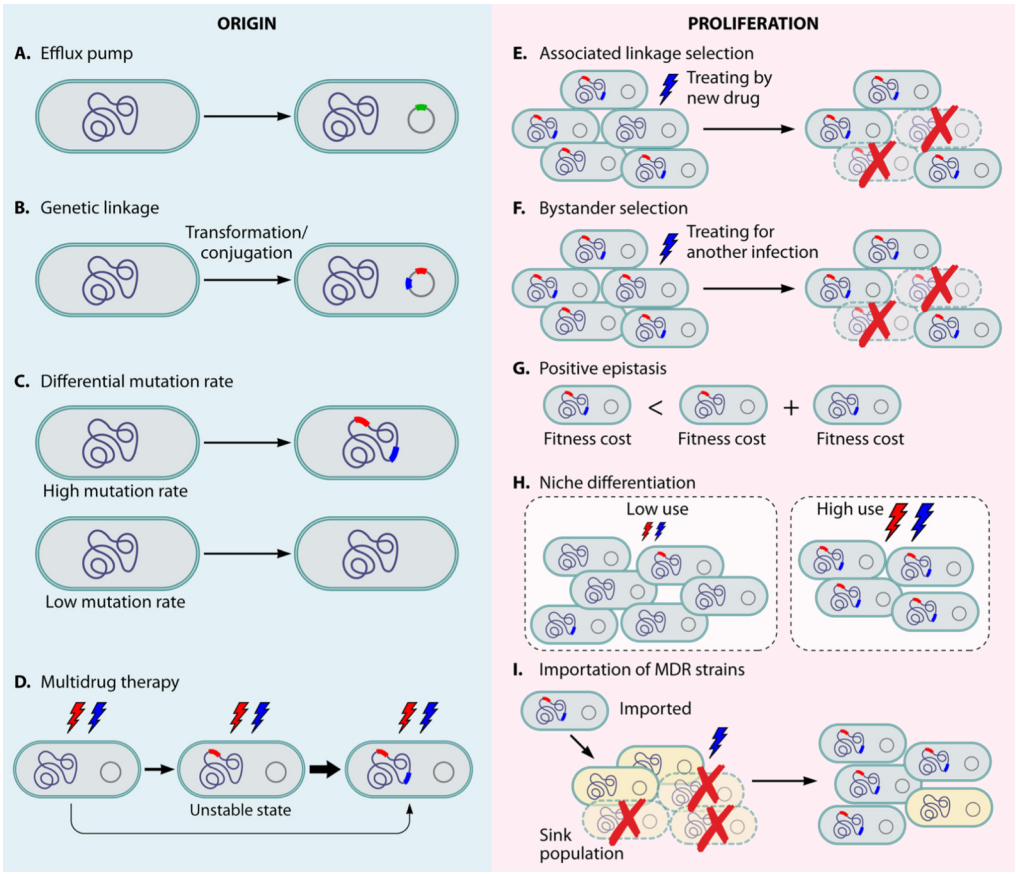


Figure 3. **Mechanisms by which multiple resistance (MDR) patterns arise in bacteria (A->D) as well as their dissemination and persistence within susceptible bacterial communities (E->I).** Resistance genes or gene clusters may confer resistance to multiple antibiotics or even multiple classes of antibiotics, here exemplified by a multi-drug efflux pump (A). Diverse resistance mechanisms often co-reside on MGEs and/or in proximity on the chromosome, and are co-inherited (B). High mutation/recombination rates permit swifter acquisition of resistance trough mutations or horizontally transferred DNA (C), but may also be an effect of antimicrobial exposure since bacterial stress increases mutation rates. The "slippery slope" thesis states that minorities of a bacterial population statistically may be resistant to one or some of the antimicrobials used during multidrug therapy (using several antibiotics at once to fight off infections) and has a higher propensity to acquire additional resistance (D). Resistance determinants may persist within bacteria even without antibiotic exposure due to genetic linkage to other genes encoding separate favorable traits to the bacterium (E). The bystander effect (F) happens as systemically administered antibiotics kill susceptible bacteria wherever they are present in the body (not only wherever the infection is at), leaving resistant bystanders to proliferate in their dead comrade's absence. Resistance phenotypes may confer a fitness cost to bacteria, meaning that they grow more slowly and lose their place in the population to susceptible ones. On the other hand, the combination of fitness effects (epistasis) may be positive (leading to better fitness) in MDR strains compared to single-resistant strains (G). Niche differentiation (H) refers to MDR bug proliferation in niche environments (such as hospitals) that contain frequent exposure to selecting stressors (like antibiotics). Finally, a MDR strain introduced into a bacterial population of susceptible strains (in for instance a patient) are well able to replace them if there is antibiotic selection of which the MDR strain is resistant. Printed with permission from Chang *et al*. (104).

MGE reorganisation. In addition, due to the intrinsic resistance towards a range of commonly used antimicrobials that *E. faecium* demonstrates, bystander selection during treatment for other infections is intuitively a present factor. Use of antimicrobials is more densely occurring in hospitals than in the community, which may explain how high-risk *E. faecium* clones are often found in health institutions. As demonstrated by Brandl *et al.* and Hendrickx *et al.* (47, 49), *E. faecium* has a potential of 'taking over' the gut during broad spectrum antimicrobial treatment as other bacteria succumb to the treatment while *E. faecium* survives. Finally, a host and a resistance gene containing element may co-evolve to alleviate any initial fitness cost introduction of the element may have burdened the host with (108, 109).

Horizontal gene transfer and mobile genetic elements

E. faecium is considered a master of HGT and understanding how genes move around in the bacterial population and which traits they confer is a complex but important task in order to reveal their basal biology. Mobile genetic elements blend in a Pandora's box where multiple movement mechanisms often co-exist in vicinity of each other, creating multiple recombination- and gene arrangement possibilities.

HGT as a phenomenon is a large field to embrace in some small sentences and paragraphs, and has to be seen in the larger scope than the spread of antimicrobial resistance and putative virulence genes (110). If we consider any stretch of DNA as under selection, following the thinking of Dawkins' selfish gene (111) or Baquero's term 'piece' – an ordered structure that forms part of a separate higher-ordered structure (112) – HGT enlarges the 'playground' in which any DNA is able to exist, and contribute to disseminate genetic structures throughout different lifeforms. Indeed, horizontal transfer is shown as the primary driver of expansion of protein families through prokaryotes as opposed to slower processes like gene duplication and subsequent specialisation (113). The transferred genetic structures themselves often seem to code for peripheral functions predominately under neutral selection (114) and tend in aggregate to be less expressed than core genes to mitigate the potential for reduced fitness in hosts that harbour them (115, 116) in order to persist in new hosts. Gene exchange between bacteria

seems dependent on a 'habitat-specific gene pool' where niche-adaptive genes are shared (114, 117). The majority of HGT events occur between genomes of high sequence similarity and similar GC content (genomes vary in amount of guanines and cytosines compared to adenines and thymines), and these two traits represent the largest barrier of HGT between different strains and species (118).

Taking this discussion down to the ground again, several mechanisms are responsible for shuttling genes from one bacterium to another, and those will be briefly explained. In addition, specific MGE types like plasmids, transposons, ICEs and so forth will conceptually be described. Furthermore, bacterial immune defences affecting the dynamics by which MGEs may or may not enter and survive in the cell are described, as the nosocomial clade of *E. faecium* seemingly lacks these systems.

Horizontal gene transfer mechanisms

Horizontal gene transfer is a process permitting the exchange of genetic material between organisms. Genetic exchange happens through different mechanisms.

- Conjugation is a process where type IV secretion systems create channels between bacterial cells through which DNA is transferrable, and is the dominant HGT mechanism of enterococci.
- Transformation occurs when bacteria are able to assemble an apparatus able to internalize naked DNA floating in the cells' immediate environment.
- Transduction happens when DNA is trapped within bacteriophages (bacterial viruses) that have infected a host, and is released and inserted into the genome of a new host after bacteriophage transmission.

In addition, several less characterized gene transfer mechanisms such as nanotubes (119), micro-vesicles (120) and gene-transfer agents (121) have been described, no documentation for the existence of these mechanisms in enterococci has been published to my knowledge.

Type 4 secretion systems

Type 4 secretion systems (T4SS) are the channels by which plasmids, ICEs and sometimes other DNA may pass through from cell to cell. In *E. faecium*, conjugation

is considered the most important HGT mechanism (122) and thus need additional functional explanation. There are several system variants as determined by comparison of T4SS from Gram-positive, Gram-negative and mobile element sources (123–126), but most systems share some basal common features. The process of conjugation starts with nicking the double-stranded (ds) DNA into single-stranded (ss) DNA by a relaxase, often referred to as a MOB protein. The relaxase recognises a specific site, the origin of transfer (*oriT*), which must be present for initiation of transfer to occur (127). See Figure 4. Using the nomenclature of the model T4SS found in *Agrobacterium tumefaciens*, the relaxase then interacts with a Type 4 Coupling Protein (T4CP), called VirD4. VirD4, an ATPase fuelling propagation of transfer-DNA, subsequently interact with other ATPases sometimes present (VirB4, VirB11) to pass the DNA through the channel (123, 128). The T4SS channel, also called Mating Pair Formation (MPF) complex, is a large cell membrane-spanning assembly of multi-copy proteins guiding the DNA from donor to recipient (126).

Plasmids

Plasmids are extrachromosomal structures able to autonomously replicate within its host. They are widespread in enterococci, and enterococcal strains often harbour several plasmids. Plasmids vary in complexity, and may range in size from a few kilobases to 0,2-0,3 megabases (127). This size range is also reflected in the number of genes they possess and thus what range of functions they encode. The simplest and smallest plasmids often just encode their own replication apparatus, are often present in many copies within a bacterial cell, and are referred to as "cryptic" meaning that there is no apparent functional benefit to harbour them. Plasmids that are more complex contain additional genes encoding functions such as antimicrobial resistance genes, stability modules (discussed below) and conjugation modules. They need other mechanisms to persist in the cell than smaller ones, and by looking at gene content, are more likely to contain genes encoding central functions to their hosts survival (127). Plasmids are conjugative when they encode the T4SS described above, and mobilisable if they contain *oriT* and parts of the conjugation apparatus, most commonly the relaxase and sometimes a T4CP. Recent compilations of plasmid sequences and attempts to

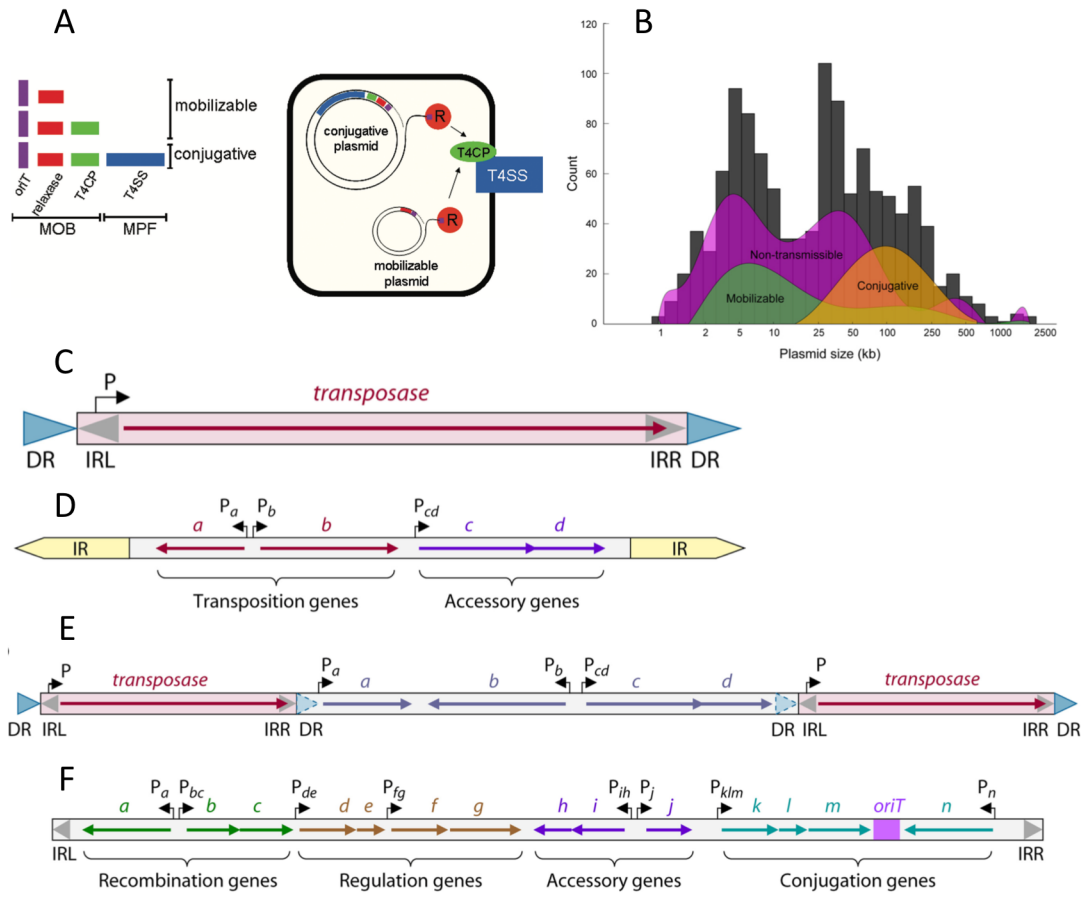


Figure 4. Mobility of plasmids (A, B), and organization of mobile elements (C-F). A: basal elements of a mobilisable or conjugative plasmid, and the size distribution of these plasmids (B). C: IS-element. D: typical transposon. E: composite transposon. F: Integrative Conjugative element. A and B printed with permission from Smillie *et al.* (127), C-F from Darmon & Leach (137).

classify them have shown that every scheme carries downsides due to the observed complexity. As reported by Shintani *et al.* (129) which analysed over 4600 plasmids deposited in NCBI, classification by replication genes is muddled by frequent mosaicism with presence of multiple replicons. Classifications using relaxases/MOB genes excludes non-mobilisable plasmids, which accounted for the majority of the dataset.

In enterococci, a large compilation and classification of enterococcal plasmids has recently been produced (122), to serve as a subset of that reported in Shintani *et al*. In short, plasmid replication proteins may be classified by mode of replication, sequence similarity and subdomains present within the translated gene.

Replication proteins may replicate plasmids by to ways – uni-directional leading strand Rolling Circle Replication (RCR) and bi-directional Theta (θ) replication (130, 131). RCR plasmids are often cryptic and small, as this replication method is prone to mistakes and becomes unstable when they are over 10-15 kb. θ-replicating plasmids are subdivided into replicon families in enterococci; Rep_3, Inc18 and RepA_N. Briefly, Rep_3 plasmids are narrow host range plasmids of similar size as RCR plasmids and likewise are often cryptic. Inc18 plasmids are often conjugative 25-50 kb broad host-range plasmids frequently harbouring resistance determinants. RepA_N plasmids are prevalent in low GC content Gram-positives, and are present in a wide size range (10-300 kb). Individual plasmids show a narrow host range. This classification scheme is often disturbed by recombination and merging of especially Inc18 and RepA_N plasmids, creating mosaic structures (127, 132–135).

Transposons, integrative conjugative elements and genomic islands

Ever since Barbara McClintock discovered genes which only apparent function was to encode their own transposition (movement) from one genomic location to another (136), multiple classes of transposable elements using a diverse array of transfer mechanisms located throughout all lifeforms including bacteria have been described (137). As extensively reviewed by Darmon & Leach (137) and Siguier *et al*. (138), transposases come in many shapes and forms in bacteria, and combine to create an enormous amount of elements able to jump around the genome through diverse mechanisms. The basal unit of mobile elements is the transposase (Tnp) itself. Tnps normally binds to specific inverted (IR) or direct (DR) repeats up- and downstream of the *tnp* gene, and excises and integrates the region between the repeats by two main mechanisms – cut-and-paste or copy-and-paste. Tnps are organized into different families based on their properties. First and foremost is the active protein site cleaving DNA during transposition, of which there are five main motifs: DDE, DEDD, HUH, and site-specific transposases/resolvases containing serine or tyrosine in the active site [also functionally reviewed (139, 140)]. General mode of transposition, length and sequence of IRs/DRs, functional domains in transposase proteins, and target

specificity are also among the properties considered to place each element within an IS/transposon family.

A unit transposon carries passenger genes in addition to the *tnp* within the IRs/DRs. Composite transposons are recognised by two transposons of the same IS family surrounding a stretch of DNA containing genes, which may be passengers if the transposase recognises the IR's on either side and thus moves both IS elements and the intermediate DNA stretch.

Mobile elements may grow even larger, engulfing multiple passenger genes or even complete pathways of gene clusters supporting complex functions. These larger elements, often called genomic islands (GIs), integrative conjugative elements (ICEs) if they are conjugative or integrative mobilisable elements (IMEs) if they are able to hitch-hike with other conjugative systems, are very diverse and likely more prevalent in nature than currently shown as we still struggle to identify them (141). Evidence suggests that ICEs may be more abundant than conjugative plasmids in prokaryotes (142). ICEs and IMEs most often demonstrate site-specific integration and excision mediated by tyrosine or serine recombinases, may recombine by stacking themselves in their insertion site, and in some cases demonstrate replicative properties once they are excised and in an extrachromosomal, circular form (141).

How do mobile genetic elements persist in hosts?

Bacteria and the MGEs that transfers between them are taking parts in an arms race where both parties harbour mechanisms designed to defend against MGE inclusion for the bacterial host, or ensuring persistence for the MGE. These systems may also demonstrate other functions in the cell, which introduces exiting dynamics we do not fully understand. The three most studied attack/defence mechanisms interacting with HGT are described below.

Toxin/Antitoxin systems

Toxin-Antitoxin (T/A) systems, also called post-segregational killing systems, consist of a T/A pair designed to kill the bacterium if the genes encoding them become segregated (that is: lost) from the genome. This works as the antitoxin inhibits toxin function, but is inherently less stable in the cell than the toxin. As the

genes encoding T/A are lost, the unstable antitoxin will not be transcribed anymore and since it is swiftly degraded, the stable toxin will kill the cell (122). There exist five types of T/A systems, of which only Type 2 seem to be prevalent in enterococci. Briefly, T/A types are divided by the toxin/antitoxin interactions; antitoxin binding to toxin for inhibition: $mRNA^{antitoxin}/mRNA^{toxin}$ (Type 1), $protein^{antitoxin}/protein^{toxin}$ (Type 2), and $mRNA^{antitoxin}/protein^{toxin}$ (Type 3), antitoxin binding to toxin substrates (type 4), and antitoxin degrading the toxin (143, 144). Even though T/A systems were originally associated with MGEs as a factor ensuring their persistence in cells, T/A systems are also found in chromosomes of bacteria without association to MGEs. This surprising finding has led to a nuanced view of T/A systems function, as they also seem to be a part of down-regulating cell growth and division under stressful conditions, apoptosis and/or other cellular processes (144). Type 2 T/A-systems found in *E. faecium* includes Axe/Txe and omega/epsilon/zeta (145, 146). These plasmid-located T/A systems are enriched in clinical MDR isolates (147, 148). Also, chromosomally located $HigBA_{Ef}$ and $MazEF_{Ef}$ T/A systems have been associated with expression patterns of several enterococcal virulence genes (149).

Restriction/modification (R/M) systems

R/M systems consists of a restriction enzyme cleaving unmethylated DNA in a site-specific manner and a methylation enzyme attaching a methyl group to the same site thereby prohibiting DNA cleavage (150). There are four main types of R/M systems, of which type II composed of a separate methylase and restriction enzyme and type I consisting of a complex of methylation/restriction/specificity subunits are the most common in prokaryotes (151). 3^{rd} generation sequencing by PacBio enables identification of methylated sites on genomes indicative of R/M activity, and a recent survey of 230 diverse bacteria and archaea found methylation in 93% of the genomes. These methylation motifs could in most cases be connected to a R/M system (151). Broader searches identifying R/M systems in genomic datasets also found dispersal of R/M systems in prokaryotes, implying that they serve important and currently unknown functions to prokaryote biology (150).

R/M systems has been proposed as a bacterial defence against foreign DNA as the host genome will be protected against cleavage due to methylation, whereas the unmethylated foreign DNA will be cleaved at arrival (152–154). This genomic defence is only active as long as the invading MGE does not succeed in inhabiting the cell for long enough to methylate its restriction-susceptible sites therefore becoming immune (155). R/M systems are also found on many MGE types, and are then thought to act as post-segregational killing systems by killing the new host if it doesn't allow persistence of the acquired MGE by methylation of its chromosome (150, 156). This "genetic addiction" also appear to alter dynamics of movement of MGEs through bacterial populations (157). Type II methylases are frequently found without their corresponding restriction enzyme, and these appear to alter expression of genes selectively leaving gene-regulatory regions unmethylated (151, 158).

CRISPR-Cas systems

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and Cas enzymes work by cleaving foreign DNA and RNA through sequence specific cleavage by Cas nucleases through hybridization of guiding sequences (spacers/crRNA) to the DNA or RNA, see review by Hille & Charpentier (159). The CRISPR region consists of short sequences bearing high identity to DNA/RNA of phages and conjugative elements previously encountered by the isolate and its ancestors. Spacers are separated by short repeat sequences that serve to separate each unique spacer through cleavage of the fully transcribed CRISPR array, and to provide interaction with the Cas nuclease apparatus. In addition to adaptive immune defence, CRISPR-Cas has also been implicated in DNA repair (160, 161), gene regulation (162) and structural genomic rearrangements (163) by containing self-recognising spacers. This would imply that bacteria are prone to auto-immune disease (164) and that presence of CRISPR-Cas may have both adaptive and maladaptive effects.

Interestingly, the nosocomial clade of *E. faecium* is largely deficient of the CRISPR-Cas systems (24, 165), which is associated with increased presence of MGEs. This pose a question of whether amount of HGT and activity of CRISPR-Cas systems is negatively correlated, of which the answer seems to be no (166). Conversely,

CRISPR-Cas presence and activity is positively associated with resource availability (food) (167) and inversely correlated to mesophilic (temperate habitat) lifestyle (167, 168). Authors suggests mutation rate is correlated with temperature, as bacteria and MGEs in mesophilic environments are more rapidly mutating. High mutation rate would imply that spacers need to be frequently exchanged to accommodate mutated targets, which is a strenuous task likely to cause negative fitness effects and so adaptive systems are likely less efficient than innate defences like R/M systems. This provides an alternative theory for absence of CRISPR-Cas in *E. faecium*.

How do we compare bacteria?

An important aspect to consider when dealing with bacteria is the notion of how they evolve. Unlike us, who seemingly only accumulate mutations through the vertical route – that is, from parent to child – many prokaryotes additionally possess the ability to transfer genes between themselves and thereby to both gain and lose them in a process called horizontal gene transfer (HGT). Treating genes as a commodity, a bacterial isolate may rapidly alter its gene content and thereby phenotype to accommodate a different environment, and as such, evolve.

This aspect thus initiate thoughts of a concept where we no longer merely are interested in whether there is an *E. faecium* infection or not, but also which isolate. Can this isolate be related to other isolates also causing infection? Does it contain specific traits able to at least give some indication of why exactly this isolate invaded a patient and caused serious problems?

Being able to answer some of these questions require the help of certain methods. Pathogen surveillance obviously require recording data of when and where a pathogen caused an infection to seed suspicions of an outbreak – that one particularly virulent bug was able to disseminate itself to several patients from a common source. Early methods for bacterial typing included phenotypic typing systems using serotypes, biotypes, phage-types and antibiograms (169). In addition to that, there exist several methods able to assess genomic relations between bacteria, which have followed the general scientific development. Widely used early adoptions in pathogen surveillance include wet-lab based molecular

methods which takes advantage of subtle genomic changes which can be visualized through patterns on a gel and thus create a "fingerprint" of each strain.

DNA fingerprints

Pulse-Field Gel Electrophoresis (PFGE) (170, 171) has been the most widely adopted method in enterococcal outbreaks since the nineties. It involves immersing isolate cultures in agarose plugs, chemical and enzymatic cell lysis and subsequent cutting of intact whole-genome DNA by a restriction enzyme that cleaves DNA sequences in a sequence-specific manner, ideally fragmenting the chromosome into 20-30 fragments. The resulting large genomic fragments are separated by agarose electrophoresis overnight by an electrophoresis chamber with electric pulse fields alternating in angle respective of the direction DNA runs through the gel to improve separation of these large fragments. The DNA is then dyed, and the fragments appear as fingerprint patterns, which can be directly compared to other isolates in the same gel. From there, comparison between bands (number of matches and relative size and presence/absence of mismatches) is used to determine relatedness by defined criteria (170).

Other methods in the same "fingerprint" category includes amplified fragment length polymorphism (AFLP) (172) and restriction fragment length polymorphism (RFLP) (173) which are variations of cutting DNA with restriction enzymes and amplification of DNA by PCR. Ribotyping is a third method, involving cutting whole-genome DNA with restriction enzymes, perform a gel electrophoresis and then transfer the fragments onto a membrane (Southern blotting) which is then hybridized with probes specific to labelled rRNAs, producing fingerprints (174, 175).

Reasons to perform comparisons of bacteria using DNA sequences

Given that the "fingerprint methods" mentioned above are just proxies for actually comparing the DNA sequences themselves and are costly and time-consuming to perform, further development has largely centred around comparing sequences directly. Other drawbacks to above-mentioned methods include poor portability between labs and maybe within labs, as well as difficulty assessing level of

difference between strains. Different methods needed to be used to address different questions: methods with high discrimination sensitivity are needed to group outbreak strains within a hospital, whereas lower discrimination thresholds are needed to analyse global evolution.

Allele-based clustering methods

The most important early adoption of genomic global-scale comparisons of bacteria on strain level came with multi-locus sequence typing (MLST) (176), first developed for *Neisseria meningitidis* and later adopted to many other bacteria. The method is based on choosing seven house-keeping genes ideally dispersed as much as possible throughout the bacterial chromosome, sequence them, and then adopt them into an allele-based scheme. 'House-keeping genes' refer to genes present in all strains of a species which are not super-conserved and never mutating, and not actively selected upon – neutral selection pressure. Each allele is assigned a number, and if the allele in your gene is different to those already present in the database by any means, it gets a new number. The allele combinations combine to create a Sequence Type (ST), which clusters by number of allele differences between the STs. As clinical isolates are sequenced in increasing numbers, additional genes are included in these schemes to gain resolution. Other approaches extending the allele comparison principle include MLST of ribosomal genes (rMLST), genes common to all or most of the isolates of a species (core-gene/cgMLST), or comparisons of all encountered genes in the entire bacterial population (pan/wgMLST) (177).

Whole genome-based comparisons

Many comparative studies in the microbiological field seek to explain what the population structure of a species looks like. Can strains be separated by ecology, geographical boundaries, pathogenicity, amount of antimicrobial resistance or by other traits? Can the phylogeny ('family tree') of a species tell anything about the evolution of that species, and possibly anything about adaptation to certain environments?

Mostly, such studies start with a phylogeny, which may be created by multiple methods (178). The basal process is to identify which stretches of DNA is present

in all isolates in the dataset (the core genome – interchangeable with common genes), and then identify individual bases that are different between the isolates – so-called single-nucleotide polymorphisms, or SNPs. As most of these studies use large amounts of isolates sequenced by short-read technologies like Illumina, a common method to identify SNPs is to find one or a few well-assembled reference(s), and map all the output from the sequencer to that reference to find these SNPs. The output from the sequencer is paired-end reads – normally 1000 bases long DNA molecules with ≈100 to 300 known bases in either end. There are several land-mark papers using this approach (179, 180). It is also possible to assemble the reads to obtain whole genome sequences, and identify common DNA through whole-genome alignment, see Treangen *et al.* and references therein (181).

Several additional analyses may be done on these SNPs to validate them. You may use statistics of each SNP to ensure that this particular SNP occur in all reads and is not produced by a fluke during read-mapping, for example with GATK (182). Phylogenetic software producing trees tend to assume that all SNPs are vertically inherited, which is often not the case as many/most of the species transfer genes horizontally, thus skewing inference of how they are related. Several methods exist to remove or account for SNPs that likely originate from horizontal transfer and recombination, to create a less 'polluted' collection of SNPs from which a more correct tree can be inferred – for example Gubbins (183), BratNextGen (184) and ClonalFrameML (185). The finished dataset of SNPs will then be fed into a phylogenetic software, of which there are several algorithms to choose from (178).

Identifying common DNA content between strains is also possible to do by clustering genes by similarity, subsequently gaining a matrix of which encountered genes are present in which genomes as output (186). Size of the core genome (genes present in all strains – interchangeable with common DNA segments) and pan-genome (genes present in some or one strain) is then computed (187). These questions are then often put in context with metadata pertaining to each isolate to possibly say something about the evolution and adaptation of the species based on presence and absence of genes. Many software packages are available to perform such analyses (188–191), but not all scale well

to large datasets due to differences in computational efficiency. As an extension of pan-genomics, bacterial Genome-Wide Association Studies (GWAS) pairing phenotypes with SNPs, presence and absence of genes and other genetic regions like regulatory sequences has begun to emerge (192–194).

The field is rapidly changing with introduction of new softwares which do the same type of analysis as the previous ones, but faster, slightly more accurately, and/or with additional available analysis steps and outputs.

What do comparative methods tell about *E. faecium*?

Comparative analyses of *E. faecium* from different origins using molecular fingerprinting methods like AFLP and ribotyping were able to show that *E. faecium* could be divided by which hosts they originate from, as well as identifying a subgroup associated with hospital-acquired infections (195–199). An MLST scheme for *E. faecium* was created (200), which permitted global comparisons of which STs and ST clusters (clonal complexes - CCs) dominated in different environments regionally and globally. Soon after, reports arrived which indicated that STs located in CC17 were overrepresented among clinical samples, and likely were more resistant to antimicrobials, and more virulent than strains in other CCs (201–203). The method normally used to assess relatedness of STs is eBURST (204, 205), which cluster STs to the likely ancestor and create relation networks based on number of shared alleles. In a comparison of eBURST-generated relation networks, Turner *et al.* showed that excessive recombination within a species will distort the network, with consequently straggled eBURST networks where likely unrelated CCs became incorrectly linked (206). The *E. faecium* eBURST network was one of these. As a result, Willems *et al.* addressed the issue by using a different analysis (Bayesian Analysis of Population Structure – BAPS) on the same MLST data. This analysis revealed that nosocomial isolates largely could be found in two subgroups, comprised of three CCs of founders ST17, 18 and 78 (207). They also performed an analysis aimed at assessing how much recombination occurred between the different defined groups, and found it to occur rarely. The overall conclusion was that eBURST-generated clusters were indeed artificially linked. They also found association of the nosocomial CCs to STs originating from strains found in livestock. At this point, not many strains had been whole-genome

sequenced, but the few that were got included in a whole genome sequence (WGS) phylogeny showing that the nosocomial CCs (and thus the two assumed separate BAPS groups) were distinct from livestock- and commensal isolates. Initial whole-genome studies assessing the population structure of enterococci (208, 209) also suggested a deep division between strains found in nosocomial settings and elsewhere to the point where there was suggested to create a new subspecies based on average nucleotide identity (ANI) scores dividing this clade from the others. In all these studies, the nosocomial strains contained more resistance- and virulence determinants, in addition to enrichment of certain mobile genetic elements.

With access to more isolates, Lebreton *et al.* could ascertain with better resolution the results from initial WGS analyses. They also further elaborated the assertions of how long ago the different *E. faecium* lineages diverged, and found indications that the livestock lineage departed from the commensal lineage roughly 3000 years ago, corresponding with humans domesticating animals, and that the nosocomial lineage departed from the livestock lineage within a decade ago, parallel with the use of antimicrobials. They also found that HAIs tended to lack CRISPR-cas9 and restriction/modification systems, commonly regarded as bacterial immune defence systems (24), an aspect also specifically investigated before (165, 210). Further characteristics dividing these lineages are genome size. Nosocomial isolates are on average larger with an additional gene content associated with MGEs, resistance and virulence determinants (24, 211).

There exists a cgMLST-scheme for *E. faecium* (212), but it is not open source, and has not been widely implemented yet since you have to pay to use it. However, cgMLST has shown that the nosocomial- and livestock lineage may be parted into several sub-clusters where nosocomial strains cluster by themselves, commensals likewise, and some strains from nosocomial- and livestock origins cluster in between (23).

Summary of papers

Paper 1: A multicentre hospital outbreak in Sweden caused by introduction of a *vanB2* transposon into a stably maintained pRUM-plasmid in an *Enterococcus faecium* ST192 clone

In **paper 1**, the aim was to determine the origin and molecular characteristic of a vancomycin-resistant *E. faecium* clone spreading through hospitals in three counties in Sweden.

- A nosocomially adapted ST192 *E. faecium* clone caused an outbreak in Sweden. The clone spread into different hospitals in three counties.
- The outbreak clone was resistant to vancomycin at variable levels, in addition to ciprofloxacin and ampicillin at high levels.
- Vancomycin resistance was caused by a *vanB2* resistance cluster located on a Tn*1549* ICE, which was inserted on a rep_{pRUM} plasmid containing an *axe/txe* toxin/antitoxin stabilization module.
- This plasmid was able to transfer between strains, and in the process merged with a rep_{pRE25} plasmid co-localised in the clinical donor. After the merge, the rep_{pRUM}+rep_{pRE25} plasmid was able to transfer at a higher frequency in subsequent filter mating experiments.
- The occurrence of clonal *E. faecium* both with and without *vanB* indicate that the clone initially was a successful vancomycin susceptible colonizer of hospitalized patients in which Tn*1549* containing *vanB2* was introduced, likely via anaerobe gut bacteria within the same environment.
- The phenotypic method to screen for VRE prior to the outbreak used a high concentration of vancomycin (32 mg/L), which produced an unnecessary risk of false negative results for low-MIC (4-32 mg/L) *vanB* VRE, thus underestimating factual VRE incidence.

Paper 2: Silenced *vanA* gene cluster on a transferable plasmid cause outbreak of Vancomycin-Variable Enterococci

In two patients, an *E. faecium* strain containing the *vanA* gene but was susceptible to vancomycin gained resistance towards vancomycin during the course of

treatment. A subsequent screening revealed dissemination of this strain in several hospitals. The aim in **paper 2** was to determine why this strain converted from vancomycin susceptible to resistant.

- A clonal outbreak of ST203 *E. faecium* occurred in multiple hospitals in Trøndelag, Norway
- All studied isolates carried the *vanA* gene cluster with additional IS elements inserted in the intergenic regions.
- One of these IS elements (an IS*L3* element) silenced the *vanHAX* operon resulting in susceptible phenotypes by hampering transcription activation by the *vanRS* regulator loop.
- Prolonged *in vitro* exposure to vancomycin at sub-lethal concentration lead to excision of the IS*L3* element upstream of the *vanHAX* operon. This restored the regulator loop and resulted in high-level glycopeptide resistance within two days of vancomycin exposure. Two patients from the start of the outbreak suffered treatment failure as a consequence of this IS element silencing the resistance expression.
- The *vanA* gene cluster was located on a rep_{pRE25} plasmid, which was transferrable to an *E. faecium* lab strain and *in vivo* into clinical *E. faecium* isolates not belonging to the outbreak clone. This plasmid was even found in a clinical *E. faecalis* isolate sampled late in the outbreak.

Paper 3: The *Enterococcus Cassette Chromosome*: an SCC*mec*-like mobilisable element in enterococci

There are possible orthologues of *ccrAB*, the serine recombinases responsible for transposition of SCC*mec*, in several species of the *Enterococcus* genus. The aim in **paper 3** was to determine if the $ccrAB_{Ent}$ genes mobilised a larger MGE in enterococci.

- We succeeded to horizontally transfer an SCC*mec*-like element from an *E. faecium* donor to two *E. faecium* recipients through letting the element hitchhike with a conjugative rep_{pLG1} megaplasmid.

- In accordance with the mechanism of SCC*mec*, the enterococcal element contained *att* sites permitting site-specific integration in the host genome downstream of the *rlmH* gene, and the *att* site motifs between *S. aureus* and *E. faecium* were similar.
- The donor region surrounding $ccrAB_{Ent}$ contained many transposons, and the presence of two IS*Efm1* elements contained within a 10 kb repeat on either end of $ccrAB_{Ent}$ also enabled this region to transfer onto the plasmid through homologous recombination.
- After downloading all enterococci assemblies from NCBI, we found *ccrAB* genes in 48 *E. faecium* genomes and one *E. durans*. Elements residing downstream of *rlmH* and containing *att* sites were extractable from 25 of these 49 genomes.
- Comparisons of identified elements show very high variation of gene content suggesting high plasticity of this genomic region, in agreement with similar studies done with *SCCmec*.

Discussion

Epidemiology of *E. faecium* in Scandinavia

As reported from several sources [(213–219), **paper 1, paper 2**)] clinical infections with VRE in Scandinavia first occurred in the nineties and has now been the cause of several outbreaks in hospital environments throughout this region. According to the national surveillance institutes (220–222) receiving reports of any encountered VRE, a surge of VRE has been seen from 2008 in Sweden and 2010 in Norway and Denmark, which seems to have flattened and started declining in Sweden and Norway since 2014. It should be noted that there have occurred several outbreaks in this period, and that these numbers also include identification of carriers during outbreak investigations, which outnumber clinical infections. VRE were rarely encountered before the late nineties, and the outbreaks of VRE in Scandinavia described in **paper 1**, **paper 2** and others indicate that VRE are a problem in hospital environments likely to persist. The sources of VRE seem to be a complex subject to disentangle. Several reports has linked VRE transmission to farm-animal related sources, and identified zoonotic transmission as a likely source (109, 223–225), in addition to import of VRE from foreign patients (220–222), international flight travel (226) and hospital-related reservoirs (227, 228). Another important pathway for multi-resistance development relates to horizontal gene transfer of resistance genes from environmental co-inhabitants into hospital-related lineages of enterococci, as reported in **paper 1** and by others (96, 229).

The isolates originating from the two outbreaks were from ST192 and ST203. These STs are single locus variants of ST78, one of the major lineages which has spread globally and have become more common than the lineage which emerged and dominated earlier, ST17 (207). ST78 and daughter STs were by Willems *et al.* connected to BAPS group 2-1, which consisted of a mesh of clinical and farm-animal related strains. Pinholt *et al.* (213, 214) find other single and double-locus variants of ST78 (ST117, ST80) making a lion's share of their data set. This suggests that this lineage is prevalent in Scandinavia, and causes most regionally encountered pathogenic VRE.

Whole genome sequencing enhances pathogen surveillance

Paper 1 and **paper 2** used a hybrid of PFGE of all isolates and WGS of subsampled isolates as the foundation for genetic comparison, due to the high cost of sequencing all strains at those time points. The WGS strains were thought to provide the "prototype" of the strain and PFGE the larger analysis assessing relationship between strains.

In **paper 1**, we concluded that all representative isolates from the prolonged outbreak were clonal in spite of multiple band differences between some strains and attributed the band differences to the long sampling period. Whole genome sequencing of all strains would have provided a richer dataset by which one could strengthen such conclusions.

In **paper 2**, the presence of IS elements in the *vanA* gene cluster was determined by PCRs linking contigs to each other. We initially found the *ISL3* element insertion by observing a contig break in the interoperonic region between *vanRS* and *vanHAX* in susceptible isolates but not in resistant ones after WGS and assembly. Maybe other variants could have been hidden in the isolates we did not sequence? Sequencing all the strains we analysed in this outbreak, including the *E. faecalis* VVE isolate, could have given more details than the PCRs, and possibly help determine the whole sequence of the *vanA*-containing plasmid. HGT of the plasmid containing the silenced *vanA* gene cluster from *E. faecium* to *E. faecalis* was confirmed by S1 nuclease PFGE and Southern hybridization of *vanA* and replicon probes. We knew the size of the plasmid (45-50 kb), but only 22 kb of the sequence. WGS analyses of the presumably identical plasmid in two different species backgrounds could possibly have identified the rest of the plasmid sequence by DNA identity scores between contigs. Presently, long-read technologies can close these plasmids, and would be the superior alternative.

Global population structures can be analysed by MLST (207, 230), but only sampling seven genes is unlikely to provide necessary resolution for local and regional outbreak investigations. Recently, the MLST scheme has also been shown to be unreliable in certain cases, as one gets superior and zoomable resolution using the whole sequence and may see untypeable *E. faecium* due to deletion of MLST loci (229, 231).

Importantly, the common unit for comparison of isolates has become the sequenced genome (232). Data sharing on a global scale has not been implemented yet due to challenges related to data storage, limitations in analytic methodology and speed and form of submission among other things, see Aarestrup *et al.* (233). This opinion article highlights the potential for global open access to sequence data and current initiatives trying to accomplish this. Several early papers using sequence data to compare global population structures in bacteria by MLST (for instance Maiden *et al*. (176)) mentioned portability as a key measure as assembled genomic data are contained in small files which are easily transferred online. Portability of WGS data is currently not possible with the envisioned ease, as the data preferred shared between scientists is sequence reads which for each strain amass to huge files that are not easily transferred. Aarestrup *et al.* suggest bringing analysis tools to the data through cloud-based storage and analysis resources, which may provide a plausible pathway for allowing data mining of complete data sets and global surveillance of important pathogens.

Data mining became an issue during searches for *ECC* elements in **paper 3**. We decided to use published assemblies from NCBI as search queries and omitted published raw data sets as downloading them, assembling them and thereafter probing for complete *ECC* elements would take a long time and create issues with data storage and data analysis and presentation. Second generation sequencing with Illumina is currently done at a massive scale. Semi-automated quality checks, assembly and annotation are already important initial steps for analysis of these datasets. Submission of these annotated assemblies with proper description of the employed software should be possible to do at a larger scale than what is presently done. Publication of assemblies may become more common as long-read technologies connecting the genomic fragments into full-size replicons gain traction by lower costs and increased availability. We could thus improve on both global, regional or even local pathogen surveillance as in **paper 1** and **paper 2**, as well as easing data mining to answer basal microbiological questions, as in **paper 3**.

Phylogenetic- and transmission analyses using PFGE and/or core genome SNPs only tell parts of the story

Connecting a clone causing an outbreak locally to a global phylogeny in order to identify likely origins is a challenging task, and present methods seem to have issues with this. An observation not mentioned in **paper 2** was an attempt to find internal phylogenetic relations between the six sequenced strains, which produced results too unlikely to be included in the paper. We wanted to map the six Vancomycin-Variable Enterococci (VVE) to Aus0085 (234) which was genetically closest according to the parsnp WGS phylogeny. After the read mapping, we got help from Marc Stegger at the Statens Serums Institut in Copenhagen to identify probabilistically sound SNPs by validation through the GATK pipeline (182). The result showed that the index isolate was identical (0 SNPs) to Aus0085, and that the others were separable to these two strains by 0-4 SNPs. The reads from our sequenced strains were able to cover 96% of the Aus0085 chromosome, implying that these isolates indeed shared much of the chromosome, and that the chromosomal DNA did not differ. The strong relation was highly surprising as Aus0085 was isolated on the other side of the world seven years before our isolates. We examined the accessory genome through Roary (190), which found significant differences in gene content between Aus0085 chromosome and plasmids on one side, and our fragmented VVE assemblies on the other. Subsequently, we attempted to use the cgMLST scheme of colleagues in Utrecht (212), where they were able to find that Aus0085 and the VVE were not particularly close in the network (163 alleles different from the index isolate), but acted with surprise when our isolates were the same cluster type (under 20 alleles difference) as an isolate (E7987) from Amsterdam, isolated in December 2013.

It has been a challenge to create a comprehensive population structure of nosocomial *E. faecium*. Constructing this with MLST data using the eBURST algorithm produced likely skewed results, and performing Bayesian analyses on the same data (207) yielded more accurate, but still contested results. The present consensus states that there is a clear distinction between a commensal/ environmental lineage and a nosocomial lineage (208). There has been debate on whether livestock- and nosocomial strains are actually two separate clades, as

phylogenetic distinctions vary by the datasets used (23, 24). In addition to the unpublished analyses done by us, others have remarked this seemingly lack of core-genome discrimination between isolates connected by phylogeny but not in period and location, and attribute such observations to silent global circulation of *E. faecium* clones (96, 235). A recent study of *E. faecium* gathered from Australia (235) suggest extensive intra-species recombination as a prominent force of species evolution, and also finds limited difference measured as single nucleotide variants between isolates gathered at different places within Australia. This suggests that analysis of patient transmission of *E. faecium* is a difficult task with present methods [exemplified by Brodrick *et al.* (236)], and that recruitment of currently un-used data may yield better resolution.

An excellent example comes from McNally *et al.* (237), who had a global dataset of ST131 *E. coli* found in different environments at different times, but appeared clonal by SNPs extracted from the common genetic content. By differentiating these very similar genomes through accessory genome content and variations in regulatory (inter-genic) regions, a better resolved population structure with sub-clusters could be achieved. Similar analyses are currently being pursued for a large *E. faecium* dataset too (Rob Willems, personal communication), and could provide additive knowledge here.

Lastly, phylogenetic analyses that also take into account the accessory genome could enhance how we view outbreaks and what we regard as clinically important transmission events. **Paper I and II** describe that resistance markers are horizontally transferrable. Plasmids harbouring resistance determinants may transfer to create multi-resistant strains which reduce treatment options, and may crucially disturb clinical decisions made on basis of whether an outbreak is occurring or not. As Sheppard *et al*. (238) found, rapid plasmid dissemination and frequent MGE rearrangements caused resistance genes to spread into several MGEs residing in several strains of several species of *Enterobacteriaceae*. The transferred resistance gene bla_{KPC} or the MGE on which it resided could as such be regarded the "functional unit" of the outbreak, and molecular or genetic/genomic comparative analyses of the harbouring strains would give misleading results. As *E. faecium* is also known to frequently perform intra- and interspecies HGT, mobile

genetic element analysis may also contribute to explain the relative success of this pathogen in nosocomial settings, as well as the origins of pathogenic strains.

Challenges with vancomycin susceptibility determination

As was already noted in the epidemiological paper by Söderblom *et al*. which coupled **paper 1** (215), the Swedish guidelines performed vancomycin phenotypic susceptibility tests with enrichment broth containing 32 mg/L vancomycin. This practice stopped in 2009 as *vanB*-containing isolates phenotypically may have MICs well under 32 mg/L, as was seen in some of the isolates included for molecular analysis in **paper 1** and in Söderblom *et al*. The high concentration of vancomycin in the enrichment broth was primarily designed to identify enterococci containing *vanA*, which phenotypically give consistently higher MICs.

Little is known of the observed variability of vancomycin MICs in *E. faecium* containing *vanB*, but some studies exist on *vanB* activation by glycopeptides. An important feature of *vanB* is the inability to be activated by teicoplanin, thereby only providing resistance to vancomycin (239). The failure of *vanB* protection against teicoplanin exposure relates to presence of hydrophobic moieties on this glycopeptide which leads to impaired interaction with the *vanB* activator loop (240, 241). Changes within $VanS_B$ has previously been shown to modulate the activation loop thereby causing constitutive, teicoplanin-induced or variable $vanHAX_B$ expression (242, 243). To my knowledge, no systematic study assessing the molecular reasons for variable MICs in *vanB* positive clinical strains has been done, although several studies indicate that low-MIC *vanB*-containing strains may escape identification in standardized tests (244–247), making this issue important.

The vancomycin resistance clusters contain a whole pathway consisting of different genes, and changes in this pathway is likely to alter resistance phenotype. As mentioned in **paper 2**, altered phenotypes associated with IS element insertions and other rearrangements of the *vanA* gene cluster had already been seen in isolates gathered for Tn*1546* typing purposes (248–253), and should hint towards existence of VVE.

As we currently report vancomycin resistance based on phenotypic traits, silent vancomycin resistance operons are not likely to be registered. Routine WGS of both VSE and VRE for international pathogen surveillance has not been implemented yet. We are incentivised to enable WGS as standard method of pathogen characterization as this better enables discovery of susceptible isolates containing silent vancomycin resistance operons. Such discoveries are already occurring. According to Raven *et al.* (229), *vanB*-containing VSE were also found in their data set, and in these isolates, the $vanR_B$ and $vanS_B$ regulatory genes were missing, likely explaining the susceptible phenotype. They found that out of 256 VSE, 10 contained *vanB* and one contained *vanA*. Why the *vanA*-containing VSE was susceptible was not discussed. Recently, Knight *et al*. discovered a Tn*1549*-located *vanB2* operon in *Clostridium difficile* from a ruminant carcass silenced by an insertion in the 5' end of $vanR_B$, likely abrupting transcription of the activator loop (254). These results, in conjunction with our results from **paper 2**, suggest that silenced vancomycin resistance clusters of both major types (*vanA* and *vanB*) may exist, located on unknown MGEs in unknown bacteria. Future third-generation sequencing technologies such as Oxford Nanopore may improve genotypic resistance determination by providing real-time genetic content in addition to structural composition by long reads. Even though a large report by Woodford *et al.* (255) recently cautioned against using WGS in general for antimicrobial susceptibility testing (or rather – resistance testing) of strains in clinical settings as the technology still appears slightly immature, testing for vancomycin resistance – especially with long-read technologies – seems like a good idea.

Several mechanisms may contribute to phenotypic vancomycin resistance variability in *vanA* and *vanB*-containing strains. Insertions of mobile elements into gene clusters represent one pathway, either causing silencing of an operon (**paper 2**) or providing an accessory promoter giving constitutive expression (256). Polymerase slippage in promoter regions of *vanHAX* may create a better promoter for un-induced expression of *vanHAX* genes (257). SNPs in the vancomycin gene clusters or promoter regions may give rise to variable expression and protein function. Vancomycin resistance gene operons coming from other bacteria may contain promoters, which due to variable adaption to the transcription apparatus

in diverse strains or species can cause variable expression of *vanRS* and/or *vanHAX*. Promoter adaptation is likely to play a role in *vanB* resistance operons, as they frequently seem to transfer between anaerobe species in the gut and into *E. faecium* (96). Also, vancomycin is shown to act as a zinc chelator (258). It is unclear if Zn(II)-depletion have deleterious effects on survival and/or replication of *E. faecium*, and how transport and retention of this ion is affected by vancomycin exposure.

A recent article by San Millan *et al.* (259) assessed the evolution of the ampicillin resistance gene bla_{TEM-1} on the chromosome or a small multi-copy-number plasmid given a challenge of ceftazidime against which bla_{TEM-1} may evolve to additionally provide resistance. Results showed that plasmid located bla_{TEM-1} was able to gain ceftazidime resistance faster than when chromosomally located, and that adaptations leading to increasing plasmid copy-number also contributed to the quicker ceftazidime resistance development. The vancomycin resistance clusters described here (*vanA* and *vanB*) are frequently harboured by plasmids, as seen in **paper 1** and **paper 2**. The role of plasmids in accelerating the evolution of plasmid-encoded genes such as resistance determinants could further be addressed in enterococci. For instance, in larger low-copy-number plasmids like those frequently harbouring *vanA* or *vanB*, other adaptations may be at play. These adaptations include structural rearrangements of genes caused by homologous recombination and movement of replicative transposons, as seen experimentally in Enterobacteriaceae (260) and *in vivo* in collections of clinical and surveillance isolates (261, 262).

MGE analyses: wet gunpowder in the starting gun

Discovery of horizontal gene transfer of particular MGEs through WGS is precluded by short read technologies, as MGEs often become fragmented due to presence of repeated regions such as IS elements within them. *E. faecium* contain a wealth of such repetitions, and circularization of chromosomes and large plasmids is thus impossible with short-read technologies. Thus, identification of horizontally transferred genetic elements is currently often done by extracting and connecting contigs if (as most often is the case) the element has remained fragmented after genome assembly.

Vancomycin resistance determinants are often located on conjugative plasmids as seen in **paper 1** and **paper 2**. Interestingly, in **paper 1** we encountered that the plasmid harbouring *vanB* had merged with another plasmid after conjugation. HGT and large structural rearrangements are not rare events (132, 133), but the molecular mechanisms behind are difficult to precisely disentangle with PFGE and Southern hybridisation, or with short-read sequence data. In **paper 2**, we saw HGT of the *vanA*-harbouring plasmid into both *E. faecium* and *E. faecalis*, which suggests that this plasmid poses a higher dissemination risk due to a broad host range. Even though we were able to link 22 kb of genomic sequence containing the *vanA* gene cluster, a *cat* chloramphenicol resistance gene and other plasmid-related genes in all six sequenced isolates, the plasmid was larger, about 25 kb according to Southern hybridization of S1 nuclease PFGE separated plasmids. Locating a possible source or species distribution and degree of conservation in genomic content of this particular plasmid would have added a strength to the analysis. Several scientific questions are difficult to assess due to hampered analysis of larger structural rearrangements.

One of the principal problems we encountered in **paper 3** related to circularization of the element. First, when we chose the $ccrAB_{Ent}$ containing strain UWECC^{cat} for mobilisation experiments, we did not know there was a 10 kb repeat on either side of the MGE. This repeat is visible in yellow in lower sequence depictions in Figure 2A and 2B in **paper 3**, and show that any product with inverse primers located adjacent to *attL* and *attR* in UWECC^{cat} would show chromosomal DNA surrounding *attR* if it was sequenced. This product would appear as a band after few cycles, and would likely be a false positive, or at least would drown out any PCR product originating from a circularized *ECC*. UWECC^{cat} was thus not a suitable isolate to test excision of ECC^{cat}. The second feature of the observed duplication in UWECC^{cat} which created several problems, was the duo of IS*Efm1* elements which inconveniently also was present in the rep_{pLG1} plasmid and permitted homologous recombination of the entire *ECC* into the plasmid. Remarkably, if IS*Efm1* would create a circular excised composite transposon by excising from UWECC^{cat} or the plasmid containing ECC^{cat}, the DNA circle would be identical to ECC^{cat}, except containing two adjacent IS*Efm1*. Unfortunately, how IS*Efm1* movement occurs is still an open question and finding out how would be

outside the scope of **paper 3**. We therefore had to design primers facing outwards from the 5' and 3' ends of *ECC*^{cat} and perform the PCR in BM*ECC*^{cat}. We observed bands corresponding to a circularized element, but they reached saturation in the PCR after <30 cycles, which was surprisingly early, but not indicative of any specific excision rate. A possible way of assessing rate of excision could be qPCR as has been done for SCC*mec* by Stojanov *et al.* (263), which we have yet to do.

Another way of demonstrating and quantifying *ECC* excision has presented itself through identifying additional *ECC* elements in other strains by searching published *E. faecium* genome sequences. These elements could be verified to not contain sequence duplications bioinformatically. Specifically, we have four strains (*E. faecium* DO, 9-F-6, C68 and K59-68) in our collection, whose chromosome have been closed and *ECC* boundaries have been identified. A positive circularization qPCR of cultured cells would further show that CcrAB serine recombinases recognise and recombine identified *att* sites in enterococci as well as quantifying excision in several isolates, and is thus something we intend to do before publication.

We are also planning to do experiments adding functional data to CcrAB$_{\text{Ent}}$ since we have observed transfer of an element but not that the *ccrAB*$_{Ent}$ genes are solely responsible for mobilizing the *ECC*^{cat} element. In theory, knock-out of *ccrAB*$_{Ent}$ in UW*ECC*^{cat} and repetition of a filter mating experiment should yield no transconjugants. Unfortunately, *ECC* mobilisation by homologous recombination into the mobilisation plasmid has already been demonstrated and results from *ccrAB*$_{Ent}$ knock-out and filter mating therefore cannot be trusted in this system. Misiura *et al*. (264) have designed a reporter system which shows promise. Expression of *ccrAB*$_{Ent}$ in *E. coli* and CcrAB$_{\text{Ent}}$ action against vectors containing *att* sites could show action of CcrAB$_{\text{Ent}}$ on *att* sites in a cleaner system. Properly designed vectors containing both *attL/R1* and *attL/R2* (see **paper 3,** figure 3) could determine specifically whether CcrAB$_{\text{Ent}}$ activity on *att* sites occur, and whether base substitutions in the presumed central recognition motif affect recombination frequency. As UW*ECC*^{cat} contains *attL1/R1* and C68 *ECC* contains *attL2/R2*, compatibility between *att* site variants may also be assessed.

Discovery and analysis of MGEs like *ECC*: What's next?

In addition to issues concerning experiments and the extent of conclusions to be drawn from them, we also leave a central question unanswered: what mechanism(s) create the observed gene variability in the *ECC* region? How does the gene synteny of this region compare to the overall gene synteny of *E. faecium* chromosomes, or even other mobile genetic elements encountered in this species? Prevalence of *ECC* elements is 9% in published genomes (**paper 3**). Does this mean that it is rarely occurring, or is it a common GI in *E. faecium*? It is possible to argue that it is common, as *ECC* may be more prevalent than for example elements harbouring vancomycin gene clusters, for which active selection occurs through use of glycopeptides. As seen by the global resistance map (Figure 1) and prevalence data from cddep.org, VRE are often occurring more rarely than *ECC* elements. VRE from the clinical setting (as is reported to the databases combined in cddep.org) are skewed towards nosocomial clades which results in a higher reported prevalence of vancomycin resistance since commensal isolates are rarely VRE. As we find *ECC* elements throughout several environments and dispersed through the whole *E. faecium* lineage, it is possible to state that it is a successful element able to exist in many different backgrounds.

Similarly, obtaining structural information of genomes can extend the scope of mobile genetic elements from 'prototypes' as public sequences now are, to a larger network of MGEs uniting the basal constituents they are now classified from – transposase or replication gene, mobilization modules, resistance genes and so forth – to a more comprehensive network showing genetic relation and variation using all the DNA.

It is possible to gain knowledge by shifting methods, and new 3rd generation sequencing technologies permitting structural information of genomes is likely to allow a better view of the accessory genome and the structure of mobile elements than has been possible with short-read technologies. Currently, the commercialized technologies (Pacific Biosciences and Oxford Nanopore) are still too expensive to allow for widespread sequencing and closure of genomes, but this may rapidly change as new apparatuses and SMRTcell/flow cell disposables with improved throughputs are released.

Another benefit of 3rd generation sequencers is the addition of methylation data to the mix (151), which allows for analysis of R/M systems. As described earlier, R/M systems influence the dynamics of both host defence against MGEs and MGE stabilisation in prokaryotes. MGEs harbouring R/M systems include *ECC*, *SCCmec* and large conjugative plasmids encoding resistance determinants, and it would be interesting to experimentally analyse whether these systems are active, and if they confer benefits to these MGEs.

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