Genome sequence of enterococcus faecalis

Genomic sequencing of E. faecalis V583 and the recent E. faecium Aus0004 showed presence of multiple mobile genetic elements in the genomes. Mobile genetic elements present in the strains are prophages, insertion sequence elements, transposons and plasmids. For this chapter, plasmids of the Incompatibility group 18 and pheromone-responsive plasmids are described alongside with transposons of the Tn3-family, composite and conjugative classes. Some of the plasmids and transposons described contain virulence and antibiotic resistance factors, which were very likely to be acquired from elsewhere. Plasmids and transposons also contribute to the dissemination of this factors and has been noted to contribute to the transfer of vancomycin resistance to methicillin-resistant S. aureus (MRSA).

Genomics of the Enterococcus

Sequencing of bacteria genomes has created a treasure trove of data and started off many research areas. Studying of virulence factors are now made easier with knowledge of this.

It is of no surprise that a bacteria with vancomycin resistance has already been sequenced. The strain, E. faecalis V583, a clinical isolate from the United States has already been fully sequenced at The Institute for Genome Research (TIGR).

V583

Based on data that is widely available to the public, V583 contains a 3218030 base pair chromosome and 3 circular plasmids. The GC content of the chromosome is 37. 53% and has 3257 open reading frames. The 3 plasmids, identified as pTEF1, pTEF2 and pTEF3 contains 66320, 17963 and

57660 bp respectively. Table 1 shows the distribution and the function of the genes in the genome.

pTEF1 and pTEF2 are similar to pAD1 and PCF10, making them pheromone conjugative plasmid, while pTEF3 is similar to pAMÎ²1, which is an Inc18 plasmid.

Vancomycin resistance in V583 is encoded within a mobile element, at EF2282 to EF2334. EF1955 to EF1963 also encodes a vancomycin resistance which has the same sequence to the vanB genes found on a conjugative transposon, Tn1549. A pathogen island in the gene, from EF0479 to EF0628 has been found in V583. It contains genes for aggregation, cell lysis and possibly many others and evidence on the islands show that it has been integrated into the genome from elsewhere by looking at the different percentages in GC content in different parts of the island. There are also presence of other remnants of integrated plasmids in the chromosome and adding on to the fact that they have 3 plasmids shows that V583 depend a lot on plasmids for genome evolution.

Aus0004

Aus0004 is a vanB-positive, vancomycin resistant E. faecium that was isolated from a patient in Austin Hospital, Melbourne, Australia. The strain contains a circular chromosome that is 2, 955, 294 bp in length and has three circular plasmids. The plasmids are named Aus0004_p1, Aus0004_p2 and Aus0004_p3 and are 56, 520 bp, 4, 119 bp and 3, 847 bp respectively.

In the genome itself, Tn1549-like conjugative transposon containing the vanB phenotype is found, along with several pathogenic islands. The

pathogenic island contains the esp gene which is needed for colonization in urinary tract infection and may play a role in biofilm formation.

Three prophages were present in the genome of Aus0004 and are similar to many other phages found in other species such as Clostridium and Staphylococcus.

21 different insertion sequence elements that are in 76 distinct copies were found in Aus0004 and distributed among the chromosome and plasmids. A certain insertion element, ISEf1 is not only commonly found in Aus0004 but is also one of the dominant ISEs found in V583.

Present in Aus0004 are genes that code for hemolysin, collagen-binding adhesin but unlike other strains of E. faecium and E. faecalis, gelatinase, aggregation substance, lipase and hemagglutinin were not found in this genome.

Just like V583, a large portion of Aus0004 is comprised of mobile DNA. This is shown by the presence of mobile genetic elements such as prophages, insertion sequence elements, pathogenic islands and plasmids.

Mobile Genetic Elements

The mobile genetic elements in the enterococcus are mainly the plasmids and transposons. This section is of particular significance to the clinical community due to the fact that it usually contains antibiotic resistance genes and directly participate in the dissemination of it.

Not only that, it also contains haemolysins, bacteriocins and even resistance to ultraviolet light.

As shown in the 2 example strain of E. faecalis and E. faecium, mobile genetic elements are heavily evolved in the evolution of the bacteria. The phenomenon is known as genomic plasticity, where the microbes evolve to counter the pressure exerted from different environments, such as going from the colon then to the urinary bladder to cause infection, or the presence of antibiotics.

In this section, some of the mobile genetic elements involved in the spread of virulence factors and resistance in the Enterococci would be explored.

Plasmids

There are three types of plasmids in the enterococcus bacteria: the rolling circle replicating (RCR) plasmids, the Inc18 (incompatibility group 18 plasmids and the pheromone-responsive plasmids.

The RCR plasmid is capable of replication in gram-positive and gram-negative species while Inc18 plasmids can only replicate in gram-positive bacteria.

The pheromone-responsive plasmids are only capable of replication in the enterococcus spp. only.

In this section, the Inc18 plasmids and the pheromone-responsive plasmids would be discussed but not the RCR. This is because RCR is not native to the enterococci but it is used a vector that is constructed for the use in this species.

Inc18 plasmids

There are two well-characterized Inc18 plasmids that are isolated. First, the pAMÎ²1 was first isolated from E. faecalis strain DS5 and the other one is pIP50 which was found in Streptococcus agalactiae. However, this particular type of plasmid is commonly found in E. faecium. There are other plasmids that fall under this category, such as pIP816 and pRUM, and each of it contain its own genes genes coding for antibiotic resistance. Some of the properties of this plasmid are that the sizes range from 25 to 30kb in size, copy numbers range from 10 to 15 per chromosome. Many are self-conjugative and can be transferred to and replicated in several species of the Streptococci. The pAMÎ²1 plasmid contains erythromycin resistance due to the presence of ermB, which also contains other resistance to antibiotics like macrolides and streptogramin B.

Numerous studies have shown that these plasmids can transfer antibiotic resistance to other gram-positive bacteria, such as Listeria. spp and Lactococcus. spp.. pIP501, in particular, is able to confer antibiotic resistance to gram-negative bacteria, such as E. coli.

The replication of the Inc18 plasmids have received considerable attention and it has been found that it replicates via a unique type of mechanism Initiation of replication of the pAMÎ²1 plasmid requires the RepE initiator protein, a short 44bp origin located downstream of the repE gene which is the initiating site for leading-strand synthesis and the host’s DNA polymerase I. Synthesis begins at the promoter of RepE with the making of a 10bp primer, then DNA polymerase I and replication would proceed for approximately 150bp until a site for lagging strand priming is exposed. At

this point, DNA polymerase I is replaced by DNA polymerase III which will direct replication for both lagging and leading strands.

RepE is rate limiting for replication, so therefore, synthesis should be tightly controlled. There are two mechanisms to its regulation, one being the CopF protein and the other being an antisense RNA which is produced from the 5′ end of the initiator gene. Antisense RNA binds to the 5′ end that is complementary to the non-coding region that is upstream of the repE mRNA and it results in termination of transcription. Cop proteins stops transcription of their respective rep genes by binding to a rep promoter sequence.

Pheromone-responsive plasmids

Plasmid transfer by pheromone is an important mechanism for distribution antibiotic-resistance and virulence in the enterococcus bacteria.

Map of pAD1 shows notation of ORFs in the sequence. Red contains genes related to replication and maintenance. Green regulates pheromone response. Dark blue contains structural genes related to conjugation and purple contains resistance to ultraviolet light. Picture taken from Clewell et all, 2002)The pheromone-responsive plasmids are a family of 20 different plasmids which has been isolated from 14 different enterococcal isolates. These plasmids range in size from 37 to 91kb maintained in low copy number of up to 2-4 per chromosome equivalent and are generally found in E. faecalis only except for one which was isolated in E. faecium. They are characterized by its ability to form aggregates in broth when mixed with plasmid-free cells which is formed due to mating.

They are so named because conjugative transfer starts off with at least 5 different “ sex pheromone” secreted by plasmid-free cells to other cells that contain plasmids. The donor cell, containing the plasmids that are specific to the pheromone, will respond by synthesizing plasmid-encoded aggregation substance (AS) which would bind to the surfaces of both cells, containing enterococcal binding substance (EBS). This binding allows for the formation of a channel which plasmid DNA can transfer from donor to recipient. As a result, it is not surprising to find that a strain would be carrying multiple pheromone-responsive plasmids.

Plasmids that have been described for it are pAD1, pCF10, pPD1 and pAM373. pAD1 encodes haemolysin or bacteriocin, pCF10 codes for tetracycline resistance and pPD1 encodes bacteriocin.

pAM373 plasmid’s function is still not known yet. Nomenclature of the plasmids is dependent on the pheromone it responds to. For example, pAD1 responses to the pheromone cAD1. A map of pAD1 can be seen in figure 4. 1. Pheromones are small peptide from 7 to 8 amino acid residues that are generally hydrophobic in nature. They are synthesized as a precursor, usually up to 275 amino acids depending on the plasmid strain, that requires processing to form the pheromone (see figure 4. 2).

Further studies has shown that pheromone signaling is not as simple as it was thought to be, where a mating response is conferred by donor cell after receiving of a pheromone. After some time, it became apparent that it does not explain enough, as pheromones are chromosomally encoded while response is plasmid determined. This would logically mean that the bacteria

itself could respond to its own sex pheromone. However, this has never seen to be happening. This was explained by donor cells demonstrating the ability to encode small peptides that act as competitive inhibitors to prevent its own plasmids from recognizing its endogenously secreted pheromone. The inhibitor peptide is secreted as a precursor up to 23 amino acids, where the last 7-8 amino acids in the C-terminal constitute mature inhibitors (see figure 4. 2). To explain how this inhibitor peptide does not inhibit exogenous pheromones, Mori et al showed that E. faecalis cells secrete a mixture of iCF10 and cCF10 in a molar ratio of 50-100/1, which allows the donor cells to remain sensitive to ‘ legit’ pheromones while neutralizing endogenous pheromones.

As mentioned earlier in this chapter, pheromone-responsive plasmids have not been identified in other species of bacteria. The cAM373 peptide, which induces a response from pAM373, has a similar activity produced by Staphylococcus aureus. However, the structure is different, with a difference of one amino acid at the C-terminus and there is currently no evidence that this is more than just a coincidence. Recent studies using a pAM373:: PAD2 conintegrate plasmid showed that the plasmid can be stably transferred from E. faecalis to S. aureus and was able to replicate. Add on to the fact that cAM373 is able to induce a response from a recently identified pAM368 plasmid that encodes a vancomycin-resistance gene shows that S. aureus attaining this virulence factor is coming closer.

Shows the virulence, plasmids, pheromone, inhibitors and their corresponding precursors. Taken from Clewell et al., 2000.

Transposons

Transposon in the Enterococcus spp. generally falls into 3 classes : the Tn3-family, composite and Transposon in the Enterococcus spp. generally falls into 3 classes : the Tn3-conjugative transposons. All 3 transposons are widespread in many bacteria and have been well-characterized in other gram-negative bacteria for the first 2 classes and gram-positive bacteria for the last class.

Transposons have a role to play in the dissemination of antibiotic-resistance genes, as it has a broad host range and some classes are able to undergo conjugative transposition even in the absence of plasmid DNA.

In this section, the role of transposons in the Enterococcus would be discussed.

Tn3 transposons

The Tn3 family of transposons commonly encode ampicillin resistance in gram-negative bacteria. The family are classed together by their similarity in their inverted repeat and transposition proteins and in their mechanisms of transposition. This group of transposons do not encode any conjugative functions, which means that transfer between bacteria cells require help by integrating into a plasmid. At least even without a conjugative function, they have one advantage over the conjugative and composition transposons, is that they are replicative and both donor and target molecules possess a copy.

Tn917 and Tn1546 are two transposon under this family that are well studied. Tn917 was the first transposon identified in enterococcus and contains a 5. 4kb erythromycin resistance gene on the plasmid pAD2. It also encodes resistance to lincosamides and streptogramin B due to the ermAM gene, which protects the ribosome from being binded by macrolides through methylation. Expression of it is can be induced by macrolides and erythromycin, but not clindamycin, even if ermAM is resistant against the latter drug. Tn551, a transposon found in S. aureus is essentially the same as Tn917 despite a few differences except that it can constitutively express the MLS resistance.

Tn1546, a 10. 9kb Tn3 -family transposon that encodes vancomycin resistance whose phenotype is the VanA-type glycopeptode, was isolated from E. faecalis. The phenotype can be seen expressed in one-quarter of the patients carrying the enterococcus bacteria in intensive care unit. Like Tn917, it does not have any conjugative functions and its spread is done by integrating itself into conjugative plasmids.

A review by Palmer et al has mentioned that Tn1546 resides in plasmids of the Inc18 class, such as pIP816 which is similar to the pAMÎ² class. It also mentioned that Inc18-type plasmid is associated with Tn1546 for most of the transfer between the enterococci and MRSA.

The replication of the Tn3 transposon is mediated by a transposase and resolvase. First, the plasmid containing Tn3 forms a cointegrate by fusing with the target plasmid with the help of the transposase enzyme. The target plasmid would receive the copy of the transposon through DNA replication

into double stranded DNA. After that, resolvase will separate the target and donor DNA and the end result is that each plasmid would have its own copy of the transposon.

Composite transposons

Structure of a composite transposon. Note the antibiotic resistance genes sandwiched in between two IS. (Taken from http://www. zo. utexas. edu/faculty/sjasper/images/18. 18. gif)

A composite transposons, in this context, consist of an antibiotic gene flanked by presence of terminal insertion sequences, or IS elements. Both IS elements, usually of the same type, provide mobility for the gene.

Tn5281, a 4. 7kb plasmid containing a resistance to aminoglycosides and flanked by inverted copies of IS256 and IS257 was first described in a gentamicin-resistant strain of enterococcus on pBEM10. The plasmid is similar to Tn4001 and Tn4031 found in the staphylococcus. Like Tn5281, it is also flanked with IS256. This group of transposons carry the aacA-aphD bifunctional aminoglycoside modifying enzyme gene that also shows resistance against gentamicin.

IS256 is common among enterococci, even those without any antibiotic resistance and this situation could contribute to the formation of plasmids with multiple antibiotic resistance. IS1216 is also another IS of importance in this transposon family, as it has been implicated in the spread of antibiotic resistance genes. A study by Heaton et al has shown that the transposon, with the help of IS1216, was able to transfer itself from a nonconjugative

plasmid to a pheromone-responsive-plasmid in E. faecium. They concluded that similar events such as this could happen elsewhere in nature, contributing to the spread of antibiotic resistance.

Tn5384 is a transposon of interest. It contains antibiotic resistance elements against erythromycin and gentamicin. The formation of this transposon is through the cointegration of a plasmid from enterococcus and staphylococcus, each having properties of being a broad host range and anti beta-lactam antibiotics respectively. This provide support for the role of staphyloccocus in the evolution of the enterococcus.

Conjugative transposons

Conjugative transposons are mobile genetic elements that could transfer itself from one genome of a bacteria to another genome without the use of a plasmid DNA, but just through intercellular contact. Not only that, its name was also given because of its ability to also transpose intracellularly.

Identification of this transposon was discovered through DS16, a E. faecalis strain in 1981. In the study where it was discovered, E. faecalis was used which contained 2 plasmids, pAD1 and pAD2 contain multiple antibiotic resistance genes. When DS16 was mated with plasmid-free recipients, it was reported that the tetracycline determinant is capable of conjugation even with the absence of plasmid DNA. It was found that this determinant is present on a transposon, called Tn916.

Tn916, a 18kb transposon, contain genes required for conjugation, antibiotic resistance and DNA cleavage. There is a similar transposon, also well-

characterized and has many appearances alongside Tn916 in literature, which is called Tn1545. A difference between the two plasmid is that Tn1545 carries a erythromycin and kanamycin resistance on top of tetracyclin. Tn916 is promiscuous, able to conjugate into an extremely broad host range, up to 50 species into numerous sites in the chromosome of the recipient bacterium, which has been shown in vitro. It can also transfer itself onto low-copy number plasmids and pAD1.

Conjugation of Tn916 involves an excision event and a non-replicative circular intermediate and it also must bear an oriT site, which is the origin of transfer. They are transposon like because they can excise from and then also integrate itself into DNA but their mechanism is different than other transposons, such as Tn5 and Tn10. The transposition is similar to plasmids, in that they have a circular intermediate, but the main difference is that it does not replicate. Figure 3 shows intercellular transposition.

A transfer intermediate is formed by first excision of the integrated transposon. A single strand is transferred into the recipient, where it the other strand gets synthesized. After that, integration back or into the chromosome occurs.

Conjugative transposons are common in enterococci and generally reside on the chromosome. This transposons plays an important role in the spread of antibiotic resistance. Being tetracycline resistance, the spread of this would result in more bacteria being antibiotic resistant.

Implications of the Mobile Genetic Elements

Mobile genetic elements are commonly associated with acquisition of antibiotic resistance in clinical settings and it is well known that this can cause a wide variety of problems in treating patients, especially MRSA.

Horizontal gene transfer has actually devastating consequence, especially when taking into consideration the antibiotic-producing gram-positive Actinomycetes spp. and the pathogenic Enterococcus spp. and Staphylococcus aureus. This allows bacteria that commonly causes disease in humans easily acquire antibiotic resistance. There is also another problem of potentially fatal MRSA acquiring vancomycin resistance with the use of conjugative transposons and researchers have investigated the possibility of acquisition through vancomycin-resistant Enterococci, which by nature also employs the use of mobile genetic elements for survival.

Back then, conjugative transfer of vancomycin-resistance between S. aureus and enterococci have only been achieved in the lab. Nobel et al have noted that conjugative transfer of vancomycin was achieved in the laboratory setting between enterococci and staphylococcus though there was no transfer between staphylococcus itself.

However, 4 years later after Noble et al’s paper, the first MRSA to acquire vancomycin resistance has been isolated in a Japanese patient and incidence of now what is called VRSA (vancomycin-resistant S. aureus) infections has been increasing after that.

There are possible explanations for this phenomenon. Showsh et al has noted that conjugative plasmid pAM368 containing vancomycin-resistance

found in E. faecalis responses to the pheromone peptide cAM373 produced by S. aureus. Another study done by Weigel et al has found that Tn1546 encoding vancomycin-resistance was able to transfer itself from E. faecalis to S. aureus onto its pLW1043 plasmid. This plasmid is then able to spread to other staphylococcus, which has the potential to make the growth of resistance against the only currently useful drug more widespread.

Conclusion

Mobile genetic elements are essential for the continued evolution of the enterococci. What was used to counteract environmental changes has been used against antibiotics.

In this chapter, each fully sequenced strain of E. faecalis and E. faecium has been analyzed from the current data available. Unlike V583, Aus0004 have only just been out for a few months when this chapter was written and therefore it is not very well characterized yet. However, current analysis of it showed that it is very similar to V583, in the fact that it contains many insertion sequences and other signs of mobile genetic elements being involved in its evolution.

Furthermore in this chapter, it has been shown that each of the plasmids and transposons found in the enterococcus has been noted to have at least one antibiotic resistance gene. The implications of this, especially composite transposons, is that it can be transferred to a broad host range, including the already multi-antibiotic resistant S. aureus. In fact, it has already been reported that strains of vancomycin resistant S. aureus has already appeared.

However, more research remains to be done, especially on the genomic differences between E. faecalis and E. faecium. Also, an approach to proteomic analysis could tell us more about the different proteins produced depending on the conditions.