

REVIEW OF VIRULENCE FACTORS OF ENTEROCOCCUS: AN EMERGING NOSOCOMIAL PATHOGEN

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Abstract

Enterococcus, considered a normal commensal of intestinal tract, is fast emerging as a pathogen causing serious and life threatening hospital borne infections. This is attributed to acquisition of multi drug resistance and virulence factors of the organisms. The sequencing of *Enterococcus faecalis* has given a lot of insight into its genetic makeup. The *E. faecalis* strain V583, which has been sequenced, contains a total of 3182 open reading frames (ORFs) with 1760 of these showing similarity to known proteins and 221 of unknown functions. Strikingly unique to this genome is the fact that over 25% of the genome is made up of mobile and exogenously acquired DNA which includes a number of conjugative and composite transposons, a pathogenicity island, integrated plasmid genes and phage regions, and a high number of insertion sequence (IS) elements. This review addresses the genomic arrangement and the study of virulence factors that have occurred since the sequencing of the genome.

Key words: *Enterococci, nosocomial infection, virulence factors*

Introduction

Enterococci have attracted much attention in recent times due to their increased recognition as a cause of nosocomial 'super infection' in patients receiving antimicrobial agents.^[1] They are intrinsically resistant / tolerant to many antibiotics and are able to acquire drug resistance either by chromosome, transfer of plasmid or transposon acquisition containing genetic sequences that confer resistance in other bacteria.^[2]

Until recently these ordinary bowel commensals languished as misclassified streptococci and were commonly perceived 'as organisms which did not cause major or serious infection'.^[3] The medical importance of Enterococci far outweighs the relatively insignificant proportion of the total adult human commensal they represent.^[4] It is ranked as one of the leading organisms causing hospital-borne infection. In USA alone, eight lakh cases of enterococcal infections occur each year.^[5]

Vancomycin has been used as the drug of choice in many resistant strains of gram positive bacterial infections, especially those caused by Enterococci. There has been an increase in number of Vancomycin Resistant Enterococci [VRE] in recent times. This has posed a serious problem

not only in the treatment of enterococcal infections but also because the organism can horizontally transfer this resistant determinant to other Vancomycin-susceptible species.^[6] Until recent times, the VRE strains were found sensitive to Linezolid. Resistance to Linezolid is slowly developing, posing several questions on the virulence factors and their survival mechanisms.^[7]

This review article focuses on the development of virulence factors of the organism and their significance in survival of the organism.

Genome of Enterococci

The two commonly isolated species of Enterococci associated with nosocomial infections are *E. faecalis* and *E. faecium*. The sequencing of the *E. faecalis* and *E. faecium* genomes^[8] has been done by the Institute for Genomic Research [TIGR] and Joint Genomic Institute of the Dept of Energy in USA respectively. The genome of *E. faecalis* V583 and *E. faecium* ATCC BAA-472 has been sequenced.

E. faecalis strain V583 is the first VRE exhibiting Van B phenotype. Some unique features of the genome are that over 25% of genome is made up of mobile and/or exogenously acquired DNA which includes a number of conjugative and composite transposons, a pathogenicity island, integrated plasmid genes phage regions, and high number of insertion sequence [IS]. A comprehensive genome-wide analysis identified 134 putative surface exposed proteins that might be associated with colonisation or virulence.

The genome of *E. faecium* is estimated to be around 2.9 Mb, slightly smaller than 3.2 Mb of *E. faecalis* strain V583. It consists of 2,928,706 base pairs in 300 contigs, 20 reads

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or greater, with an inferred 37.8% G+C content and 3309 potential protein-coding genes

Virulence Factors of Enterococcus

It is well known now that virulence of an organism is regulated with virulence coding genes present on the genome in special regions which are termed pathogenicity islands [PAI].^[9] The PAI of Enterococcus was first identified in the genome of multi-drug-resistant strain of *E. faecalis* [MMH594] that had caused an outbreak of nosocomial infection in the 1980's.^[10]

The size of the gene is around 150kb and encodes for 129 open reading frames (ORF). The G+C content was found to be 32.2%. There were genes which encoded for transposases, transcriptional regulators and proteins with known potential roles in virulence.^[9] Virulence traits encoded within the *E. faecalis* PAI were cell surface-associated protein namely enterococcal surface protein (ESP), the secreted toxin cytolysin and aggregation substance.

A striking feature identified by comparison of sequences comprising the PAI in strain MMH594 with VRE, isolates V583 and V586,^[11] was the ability to modulate virulence of the organism by selective high-frequency deletion of specific regions. The comparison also showed a high degree of sequence identity only with the exception being presence or absence of IS elements. It has been proved that at least one-third of this PAI appeared to have evolved from integration into chromosome of a conjugative plasmid sequence.^[12]

The PAI also encoded 18 ORFs without any functions. The commensal-derived Enterococci did not contain these ORFs which suggests their contribution to enterococcal survival in the hospital or in the process of disease transmission or pathogenesis.

Although Enterococcus inhabits the GIT as a commensal, certain predisposing conditions may allow this organism to invade extra intestinal regions and cause infections. The ability of the organism to acquire newer traits makes it more virulent enabling it to colonise in newer areas in the host and cause infection.^[12] It is not a single factor which is responsible for the virulence of the organism. A number of studies^[13-20] have identified different virulence factors, most important among them being haemolysin, gelatinase, enterococcal surface protein [Esp], aggregation substance [AS], MSCRAMM Ace (Microbial surface component recognizing adhesive matrix molecule adhesin of collagen from Enterococci), serine protease, capsule, cell wall polysaccharide and superoxide.

Haemolysin

It is a cytolytic protein capable of lysing human, horse

and rabbit erythrocytes. Haemolysin producing strains of Enterococci have been shown to be virulent in animal models and human infections^[5,21,22] and to be associated with increased severity of infection.^[23] Haemolysin production can be detected by inoculating Enterococci on freshly-prepared beef heart infusion agar supplemented with five per cent horse blood. Plates incubated overnight at 37°C in a carbon dioxide chamber and evaluated after 24 and 48 hours. A clear zone of β haemolysis around colonies on horse blood agar is taken as positive.^[24] Expression of haemolysin or cytolysin was found to be regulated by a novel, two-component regulatory system via quorum sensing mechanism.^[19]

Gelatinase

It is a protease produced by Enterococci that is capable of hydrolyzing gelatin, collagen, casein, haemoglobin and other peptides.^[25] Gelatinase producing strains of *E. faecalis* have been shown to contribute to virulence of endocarditis in an animal model.^[26] Gelatinase production in the laboratory can be detected by inoculating the Enterococci on freshly-prepared peptone-yeast extract agar containing gelatin plates incubated at 37°C overnight and cooled to ambient temperature for two hours show a turbid halo or zone around the colonies if it is positive for gelatinase production.^[24] A locus, *fsr* with sequence similarity to components of staphylococcal accessory gene regulator (AGR) locus,^[27] positively regulates the expression of gelatinase in *E. faecalis*. In a study, out of 219 *E. faecalis* blood isolates, 141 [64%] showed positive for gelatinase production.^[24] In another study, *fsr* was detected in 12 of 12 [100%] endocarditis isolates tested as compared with 10 of 19 [53%] stool isolates.

Surface Adhesions [Enterococcal Surface Protein (ESP)]

Enterococcal surface protein [ESP] is a cell wall-associated protein in *E. faecalis* isolates. Frequency of gene coding for Esp has been higher among clinical isolates than commensal isolates.^[18] PCR amplification of the Esp gene can be done by use of primers Esp 11 [5'-TTGCTAATGCTAGTCCACGACC-3'] and Esp 12 [5'-GCGTCAACACTTGCATTGCCGAA-3'], which correspond to nucleotide positions 1217-1238 and 2149-2171, respectively, within the N-terminal region of Esp.^[24] The PCR reaction mixture consists of 250 ng of DNA; 0.2 μ L each of dATP [2'-deoxyadenosine 5'-triphosphate], dCTP [2'-deoxycytosine 5'-triphosphate], dGTP [2'-deoxyguanosine 5'-triphosphate], and dTTP [2'-deoxythymidine 5'-triphosphate]; 2.5 mM MgCl₂; and 2.5 U of AmpliTaq DNA polymerase in 1 x reaction buffer.

The samples undergo initial denaturation at 95°C for two minutes and are subjected to 30 cycles of denaturation [94°C for 45s], annealing [63°C for 45s], and extension [72°C for one min]. Five micro litres of the amplification

mixture should be mixed with gel loading buffer and subjected to electrophoresis in a one per cent agarose gel. The reaction products can be visualised by ethidium bromide staining. DNA from *E. faecalis* isolates MMH594 and FA2-2 can be used as positive and negative controls respectively for the ESP gene.^[24]

ESP is shown to enhance the persistence of *E. faecalis* in urinary bladder during experimental urinary tract infections. ESP positive strain of *E. faecalis* and isogenic ESP-deficient mutant strain were taken for study. In a case study, groups of mice were challenged transurethrally with 100,000 CFU of either the parent or mutant strain and bacteria was enumerated in urine, bladder and kidneys after 5 days.^[28] Mice challenged by the ESP-bearing parent strain showed more bacteria. This shows that the ESP may help the organism adhere to bladder epithelium through specific components of the bladder wall such as mucin or uroplakin.

Aggregation Substance

It is a pheromone inducible surface protein of *E. faecalis* which promotes mating aggregate formation during bacterial conjugation.^[29] Aggregation Substance (AS) mediates efficient enterococcal donor- recipient contact to facilitate plasmid transfer. In vivo, aggregation substance may contribute to the pathogenesis of enterococcal infection through a number of mechanisms.^[30] The different functions ascribed to AS, in addition to promoting cell-cell contact, is adhesion to host cells, include adhesion to extracellular matrix [ECM] proteins and increased cell surface hydrophobicity. Enterococci expressing AS were also found to resist phagocytosis significantly better than isogenic AS-negative strain by inhibition of the respiratory burst [production of reactive oxygen species {ROS}] in the macrophages.^[31] *In vivo* studies, the role of aggregation substance in endocarditis has revolved around the rabbit endocarditis model. Using the purified N-terminal region of AS lacking the signal sequence, it was demonstrated that prior immunisation of rabbits with these purified AS fragments did not give protection against subsequently induced enterococcal endocarditis.^[32] This suggests that AS may not be involved in early establishment of the vegetation and that its primary role in virulence may be evasion of immune system as shown by macrophage and PMN binding studies.^[31,33]

MSCRAMM Ace

Ace is a collagen binding MSCRAMM (Microbial surface component recognizing adhesive matrix molecule adhesin of collagen from Enterococci) on Enterococci and is structurally and functionally related to staphylococcal Cna adhesion.^[34] Its presence among both commensal and pathogenic isolates of *E. faecalis*^[35] is apparently expressed during infection in humans. Human derived antibodies to Ace can block adherence to extracellular matrix proteins in

vitro.^[16,36] Employing anti-Ace antibodies, Ace was detected in 90% enterococcal endocarditis patient sera samples, suggesting that Ace is expressed in vivo. An Ace homolog, designated Acm, was identified in *E. faecium*.^[37]

Capsular Polysaccharide and Cell Wall Carbohydrate

An operon encoding synthesis of capsular polysaccharide of the type most commonly expressed by clinical isolates of *E. faecalis* has been identified.^[38] A second capsular polysaccharide present on the surface of both *E. faecalis* and *E. faecium* was also purified and chemically characterised.^[39] The protective efficacy of antibodies produced against this purified carbohydrate fraction was demonstrated in a subsequent study using a mouse infection model, suggesting the possibility that these antibodies may be useful for prevention of enterococcal infections.^[40] The purified cell wall carbohydrate fraction showed to consist of glycerol phosphate, glucose and galactose residues.^[40]

Extra-cellular Superoxide

E. faecalis isolates from blood stream are unique in their ability to produce superoxide.^[41] Superoxide production was observed to enhance in vivo survival of *E. faecalis* in mixed infection with *Bacteroides fragilis* in a subcutaneous infection.^[42]

Molecular Methods of Diagnosis

Molecular methods are available which can provide supportive evidence for epidemiological findings.^[43,44] These tools should be carefully employed after the hypothesis as their use is well formulated. Factors to consider for the use of Polymerase chain reaction (PCR), Multilocus sequence typing (MLST) and Pulse field gel electrophoresis (PFGE) tools include introduction of a new strain, dissemination of Vancomycin resistant genetic elements, out-break due to the spread of a single clone, and confirmation of initial clonal spread followed by establishment and maintenance of an endemic state. A coordinated investigation of clinical outcomes is linked with enterococcal virulence associated with enterococcal infections.^[45-47]

Conclusion

Enterococcus has clearly emerged as a medically important organism, causing outbreaks of many nosocomial infections. An organism, once considered a harmless commensal residing in the intestine, has emerged as a multiple-drug-resistant, virulent pathogen accounting for more hospital borne infections.

The sequencing of *E. faecalis* and *E. faecium* as well as identification of the PAI in these species have highlighted numerous genes encoding protein products of unknown function and provided compelling evidence for genetic

differences between commensal and clinical isolates.^[4]

More research is needed to characterise molecular and cellular interactions between the host and Enterococci which lead to intra-species genetic transfer and virulence factors in species beyond *E. faecalis*, current strategies to prevent and control the spread of VRE and Linezolid-resistant Enterococci.

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