# ROLE OF THE TRANSCRIPTION REGULATOR RPON (SIGMA 54) IN *ENTEROCOCCUS FAECALIS* BIOFILM DEVELOPMENT, METABOLISM AND VIRULENCE.

by

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B.Sc., University of Mumbai, India, 2003
M.Tech., Dr. D.Y. Patil Vidyapeeth, India, 2005

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Division of Biology
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# Abstract

Enterococci are the third leading cause of nosocomial infections including urinary tract infections (UTI), surgical site infections (SSI) and blood stream infections. Enterococci are also found in the gastrointestinal tracts of humans, and other mammals.

We elucidated the influence of the transcriptional regulator RpoN on enterococcal biofilm formation, virulence potential and cell wall architecture and proposed a potential involvement for carbohydrate metabolism in these processes. Biofilms are held together by matrix (BM) components such as extracellular DNA (eDNA) released by cell death from a sub-population of cells. The *rpoN* mutant (Δ*rpoN*) was resistant to autolysis as well as fratricide-mediated cell death and eDNA was not detected in planktonic as well as biofilm cultures. Unlike the parental strain V583, the Δ*rpoN* mutant formed proteinase K sensitive biofilms, suggesting that protein as well as eDNA serves as an important matrix component. The rabbit model of endocarditis was used to assess the effect of *rpoN* deletion on enterococcal virulence. Rabbits infected with Δ*rpoN* had reduced bacterial burden in heart, blood, liver, kidney and vegetation in comparison to the parental strain. The growth defect of Δ*rpoN* in physiologically relevant glucose levels partially explains the reduced bacterial burdens observed in the virulence study. Microarray analysis of Δ*rpoN* showed that 10% of the genome is differentially regulated by RpoN. Deletion of *rpoN* also protects *Enterococcus faecalis* from lysis in the absence of known modulators of cellular lytic events such as O-acetylation and D-alanylation. Of the four identified enhancer binding proteins in *E. faecalis*, MptR regulates the RpoN-dependent mannose/glucose uptake system (MptABCD) and the Δ*mptR* mutant phenocopied the Δ*rpoN* mutant in the eDNA release and growth assays. Because MptC and MptD have

been shown to be the cellular receptors for Class IIa and IIc bacteriocins, we are presently testing the hypothesis that these receptors may serve as a global receptor for bacteriocins.

In conclusion, our data demonstrates that alterations in the metabolic state of the bacterium, as observed in the Δ*rpoN* mutant could be responsible for the switch in biofilm matrix composition, and this switch in turn likely influences the virulence potential of the bacterium.

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Major Professor
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# Chapter 1 - Introduction

## 1.1 Enterococci

In 1899, Thiercelin identified enterococci as a Gram positive intestinal commensal capable of forming long chains. It was initially classified as *Streptococcus* but in 1984, it was recognized as the separate genus of *Enterococcus*on the basis of its distinct genetic differences from the streptococci (99). The progressive transition of enterococci from a benevolent intestinal commensal to a pathogen is due to the extended use of broad-spectrum antibiotics and horizontal gene transfer (81). As opportunistic pathogens, enterococci, rank third after coagulase negative staphylococci and *Staphylococcus aureus*. Out of the total hospital acquired or associated infections reported, enterococci are responsible for 11.2 % surgical site infection (SSI), 14.9% of urinary tract infection (UTI) and 16% of the reported blood stream infection (38). Of all the known enterococci, two main species *Enterococcus faecalis* and *Enterococcus faecium* predominantly colonize the intestine and have been causative agents for several infections (44). Despite the increasing incidence of *E. faecium* infections and its slow emergence as a nosocomial pathogen (3), most enterococcal infections reported continue to be caused by *E. faecalis* (39). The intrinsic and acquired resistance of *E. faecalis* to antimicrobial agents makes it important to understand the pathogenesis of this organism. The development of vancomycin resistance in *E. faecalis* strains, severely limited the therapeutic options as vancomycin had been the only therapeutic for the penicillin and aminoglycoside resistant *E. faecalis* (34, 43, 62). The recently acquired resistance to newer antibiotics such as linezolid, dalfopristin and daptomycin (12, 37, 49, 68) raises clinical concerns about the ongoing effectiveness of treatments for *E. faecalis* infections.

This alarming increase in multiple antibiotic resistances over a period of years and its spread through mutation and horizontal gene transfer (80, 81, 124, 128) has increased the need to deepen our understanding about these organisms and find alternative therapies to combat such infections. The virulence properties of *E. faecalis* thus need to be well understood in order to combat infections produced by this multiple antibiotic resistant organism. Several factors such as cytolysins, aggregation factors, *Enterococcus*surface proteins, biofilm formation and capsule production play an important role in its pathogenesis (47, 58, 98)**.**

## 1.2 Enterococcal cell wall physiology

The enterococcal cell wall is composed of several polymers including the capsular polysaccharide, the integral cell wall teichoic acid, group D antigen or lipoteichoic acid and rhamnopolymer that bears close resemblance the streptococcal group us antigens (29).

### Capsular polysaccharide:

In *E. faecalis*, the surface exposed carbohydrate (capsule) consists of a repeating polymer of glucose and galactofuranose with substitutions of O-acetyl and lactic acid at positions 5 and 3 on the Gal*f* residue, respectively (29, 116). Bacterial surface polysaccharides are known to play an important role in virulence by preventing the engulfment and digestion of the organism by host macrophages and neutrophils, allowing it to evade the immune system (3). Highly encapsulated stains of *E. faecalis* (9) and isolates that are resistant to phagocytosis in the absence of opsonic antibodies (40) have been reported. It has also been shown that mutants that lack the ability to produce capsule are readily engulfed by macrophages. The capsule in *E. faecalis*, has been shown to mask

other cell surface antigens such as lipoteichoic acid (LTA), which is a known stimulator of the human immune response during enterococcal infection (119).

The first report describing a locus involved in polysaccharide biosynthesis in *E. faecalis* was reported by Xu et al. (1997) in the strain OG1RF (130)**.** However, later investigations led to the conclusion that the polysaccharide synthesized by this gene was not extracellular and hence was disregarded as a locus responsible for capsule polysaccharide biosynthesis (131). In 2002, Hancock et al. reported an operon in *E. faecalis* strain FA2-2, consisting of 11 open reading frame (CpsA to CpsK) under the control of two promoters, responsible for capsule polysaccharide production (29). Later it was shown that CpsA and CpsB were not involved in capsular polysaccharide synthesis however, their presence was important for cell survival (31). The same group reported two regions of diad symmetry to the 5' end of the *cpsC* promoter. The deletion of the first region caused a two-fold increase in the promoter activity from the *cpsC* promoter indicating a potential regulatory role in capsule polysaccharide synthesis (31).

The expression of *cps* genes in *E. faecalis* has also been reported to be maximum in the mid logarithmic phase in comparison to the stationary phase (>10 fold) (31). Qualitative estimation of capsule production from *E. faecalis* strain FA2-2 at different stages of growth has also shown a mid-logarithmic increase in capsule production (Iyer VS, unpublished data). According to the proposed model for polysaccharide synthesis, in the first step, monosaccharide must be first activated by linkage to a nucleotide molecule. However, the trigger for this activation is unknown. Recently, Thurlow et al. characterized the nine gene operon responsible for sysnthesis of capsular polysaccharide in *E. faecalis* and showed that only seven out of nine genes are required for capsule

production (119). Using immunodetection methods, *E. faecalis* has been shown to produce four distinct cell wall polymers named A, B, C and D (41), however only serotypes C and D make a capsular polysaccharide (119). Out of the seven genes in the operon, it was postulated that the gene product of *cpsF* was responsible for the glucosylation of serotype C capsular polysaccharide and determing the serospecificity in serotypes C and D as CpsF is found only in *E. faecalis* serotype C (119).

**Lipoteichoic Acid, Wall Teichoic Acid and Rhamnopolymer:**

Noncovalently inserted into the cell membrane by a glycolipid anchor is the lipoteichoic acid (LTA) or the group D antigen which is an amphiphilic polymer made up of polyglycerol phosphate. The C-2 of glycerol residue in LTA is modified by the addition of D-alanine. LTA has been associated with several bacterial functions such as anchoring of cell wall proteins, membrane elasticity and porosity , determination of hydrophobicity and net charge as well as trafficking of molecules (76). Antibodies directed to non-encapsulated *E. faecalis* target the exposed enterococcal LTA and result in opsonophagocytosis. Thus LTA in enterococci is also a major antigenic determinant (115). LTA is also partially responsible for the inflammatory response elicited by *E. faecalis* because it stimulates the murine macrophages to secret tumor necrosis factor alpha (TNF-α) and nitric oxide and activates the Toll like receptor 2 (TLR-2) (4).

In addition to lipoteichoic acid, enterococci and other Gram positive bacteria contain a wall teichoic acid which is covalently attached to the cell wall and is composed of D-glucose, D-galactose, 2-acetamido-2-deoxy-D-galactose, 2-acetamido-2-deoxy-D-glucose, D-ribitol, and phosphate in a molar ratio 1:2:1:1:1:1 (114) . Similar to LTA, the wall teichoic acid also contains D-alanylated esters in their structure (76) .

Another cell wall associated polymer of *E. faecalis* is the rhamnopolysaccharide also called as Epa which is encoded by a highly conserved *epa* locus (113, 131). Because of the inability to detect the Epa antigen with the antiserum in several strains of *E. faecalis* (131), Hancock et al (2002) proposed that this rhamnopolymer is located in the deeper layers of the enterococcal cell wall (29). The *epa* mutants are more susceptible to phagocytic killing (112) and show gross changes in their cell shape (113) which may partly be responsible to the phenotypic effects observed in their virulence studies (131, 132).

In many Gram positive bacteria the D-alanylation of teichoic acid plays an important role in susceptibility to lysis, adherence, resistance to antimicrobial peptides and virulence. D-alanine incorporation is catalyzed by the gene products of the four gene *dlt* operon (*dltA-D*) (1, 50, 53, 82-84, 90). Similarly, in *E. faecalis* the D-alanylation partly contributes to lysis resistance (57) and a *dltA* mutant is defective in biofilm formation, adherence to eukaryotic cells and increasing susceptibility to antimicrobial peptides (25). Apart from D-alanylation, O-acetylation of peptidoglycan has also been reported as a cell wall modification that alters autolysis in some bacteria, including *Enterococcus* (7, 55, 85). The O-acetylation occurs at the C6 residue of N-acetylmuramic acid of the peptidoglycan (67) in a growth phase dependent manner (85). There has been conflicting reports about the contribution of this modification to enterococcal lysis. It is reported by Pfeffer et al. (2006) and Jeune et al. (2010) that O-acetylation of peptidoglycan protects the cells from action of lysozymes and autolysis (57, 85) while Emerian et al. (2009) showed very marginal effect of this modification to cell lysis and

also suggested an increase in the activity of autolysin B in the absence of this modification (24).

## 1.3 Carbon Metabolism in Enterococci

### Phosphoenolpyuruvate phosphotransferase systems (PTS) in Bacteria.

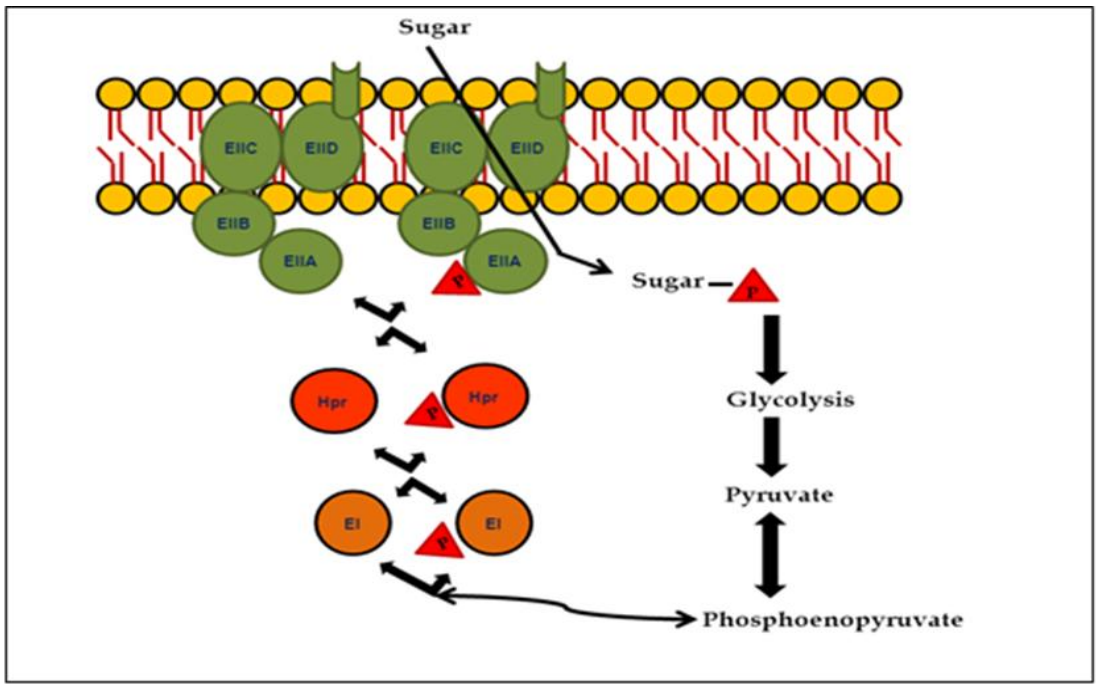


**Figure 1-1 Phosphotrasferase system in low GC Gram positive bacteria adapted from (27).**

Bacteria possess transport systems that facilitate the uptake of carbohydrates and their conversion to phosphosugars that feed into the catabolic pathway to generate energy. These transport systems are called phosphoenolpyruvate:carbohydrate phosphotransferase system or the PTS system. These are exclusive to the prokaryotes and have not been identified in eukaryotes. They were first discovered in *Escherichia coli* by Kundig, Ghosh and Roseman in 1964. They identified this novel system in their attempt to find an enzyme similar to mammalian kinases that are involved in metabolism of sialic

acids in bacteria (such as *E.coli*) that can metabolize N-acetyl-D-mannosamine. They showed that phosphoenolpyruvate (PEP) could phosphorylate different hexoses sugars such as glucose, mannose, glucosamine, mannosamine, N.acetylyglucosamine and N-acetylmannosamine by sequential transfer of phosphate group (54). It is the phosphorylation and dephosphorylation of the PTS components that mediates the regulation of this system (Figure 1-1).

**Composition of PTS:**

The PTS components of both Gram negative and Gram positive organisms are very similar and consists of three main components, Enzymes I, Enzyme II and heat-stable, histidine containing protein Hpr (87). Enzyme I and Hpr are cytoplasmic in nature. In general Enzyme II is multidomain protein with one/two (EIIC/EIID) hydrophobic integral membrane domain and two hydrophilic cytoplasmic domains (EIIB and EIIA). Enzyme I and Hpr are common to all the PTS carbohydrates however, enzyme II is specific and thus determines the specificity of a PTS system. Enzyme II is responsible to transport across the membrane and subsequently phosphorylates the sugar to feed into glycolysis. PTS systems are very diverse in nature due to the specificity bestowed upon them by the EII complex. On the basis of substrate specificity and phylogeny of EII's, the PTS systems have been grouped into four families with lucid evolutionary origin and termed by the authors as 'a snapshot of evolution in transition' (96). The four families are (i) the glucose-fructose-lactose (Glu-Fru-Lac) superfamily, (ii) the ascorbate-galactitol (Asc-Gat) superfamily, (iii) the mannose (Man) family, and (iv) the dihydroxyacetone (Dha) family.

Enzyme I (EI) encoded by the gene *pstI* (EF0710) is 140 kDa containing homodimer protein that is phosphorylated at the conserved histidyl residue by the glycolytic intermediate phosphoenolpyruvate (PEP) (2) .

## Carbon catabolite repression

The stringent regulation of carbon uptake by bacteria is termed as carbon catabolite repression (CCR). In the presence of preferred and easily metabolizable carbon source, bacteria prevent the expression of and function of catabolic systems that facilitate the use of secondary carbon source or secondary substrates thus exerting CCR. Being able to select the most energy rich carbon source that provides more energy and minimalizes the expenditure of the same is the basis of CCR. Being able to exert CCR provides bacteria an advantage in nature as well as in host environments because the ability to adapt and use the available nutrient source determines their survival in such environments.

Carbon catabolite repression is globally regulated in both Gram-positive and Gram-negative bacteria and PTS systems play a key role in both systems (27). The oldest and the most studied system of CCR is the diauxic growth curve exhibited by *E.coli* when grown in media containing glucose and lactose (61). Diauxic refers to the sequential use of carbon source with a short lag period before switching from the readily metabolizable carbon source such as glucose to the secondary substrate i.e. lactose. In Gram negative bacteria (model organism *E.coli*), the main players in CCR regulation are (i) enzyme II (EII) of the PTS system (ii) the secondary messenger molecule cyclic adenosine monophosphate (cAMP) (iii) cyclic AMP receptor protein (CRP) which is a transcriptional activator (iv) the enzyme adenylate cyclase. The phosphorylation state of

EII influences CCR. In the presence of preferred carbon source, EII is dephosphorylated because it transfers the phosphoryl group to the sugar that can then enter glycolysis. Phosphorylated EII activates the membrane bound adenylate cyclase. Activation of adenylate cyclase leads to increased cAMP levels and thus to the formation of cAMP-CRP complex. The cAMP-CRP complex binds to the promotors and activates the expression of catabolic genes (27).

In low GC Gram positive organisms, the phosphorylation state of Hpr of the PTS plays a pivotal role in the global regulation of CCR. Other than Hpr, the catabolite control protein A or CcpA, and the glycolytic intermediates Fructose-1,6-bisphosphate and glucose-6-phosphate are the key mediators of CCR (120). Hpr protein can be phosphorylated at two positions (Ser 46 and His 15) and depending on which site gets phosphorylated; it either activates or alleviates CCR. E1 of the PTS phosphorylates Hpr at position His-15 (87). In the presence of favorable growth conditions, a bifunctional enzyme HPrK/P phosphorylates Hpr at Ser-46. P-Ser-Hpr binds to CcpA and forms a complex that bind to operator sites leading to CCR (86).

## 1.4 Biofilm Formation

Complex infections caused by enterococci are generally nosocomial in nature associated with implanted medical devices leading to surgical site infection, urinary tract infections, endocarditis and bacteremia. Biofilms are the source of most of these infections. Biofilm, in simple terms, is a congregation of bacteria held together by substances secreted by the bacteria and contributed by the environment. Biofilm formation occurs in four distinct stages with various players involved in each of these stages: attachment, recruitment, maturation and dispersion.

A mature biofilm consists of loosely packed clusters of cells called microcolonies that are held together by extracellular matrix components. These loosely held microcolonies are also interspersed with water channels that serve to transport nutrients in the deeper part of the biofilm (78). In enterococci, dispersion of biofilms is an open area of research since very little is known regarding dispersal of enterococcal biofilms.

The initiation of biofilm formation occurs when bacteria adhere to a biotic (native heart valves, urinary tracts etc.) or abiotic (polystyrene plate, prosthetic valves, catheters etc) surface. These surfaces, similar to the bacterial surface, carry a net negative charge under normal physiological conditions (122). Hence the bacteria (including enterococci) develop modifications on their outer surface to lower their surface negative charge to prevent electrostatic repulsion that hinder adherence surfaces. The correlation between the natural charge occurring on a bacterial surface and the number of ionizable groups exposed on the surface is termed as a zeta potential of the bacteria and is influenced by the pH and ionic strength in the environment. It has been shown that the heterogeneity of the zeta potential in an enterococcal population enhances adhesion of enterococci and a homogenous culture is attenuated in adhesion (122). Recently, the endocarditis and biofilm associated pili locus of *E. faecalis* OG1RF (*ebp*) has been shown to play a role in heterogeneity of zeta potential. Deletion of EbpR, the regulator of *ebpABC* operon resulted in a homogenous culture with the least negative zeta potential. Similar results were observed when the genes of *ebp* operon were deleted. The differential expression of *ebp* operon contributed to the heterogeneity of zeta potential in a population. Thus, in addition to a role in promoting physical adhesion while establishing an infection, Ebp indirectly contributes by governing the zeta potential of the bacteria cell (108). Besides

adhesion to the surface, cell-cell adhesion also plays an important role in biofilm development. Several enterococccal surface proteins containing the LPxTG anchoring motif such as aggregation substance (15, 52, 107), enterococcal surface protein (Esp) (111, 123), collagen binding proteins (Ace (51, 71), Acm (72, 73), Scm (105)), *faecalis* surface protein (fss) (104) and pili (Ebp (74), bee (100) , pilA and pilB (35)) aid in adhesion, influence biofilm formation and promote pathogenesis. LPxTG type proteins are a class of surface proteins that are characterized by the presence of LPxTG motif at the C-terminal that are recognized and cleaved by a transpeptidase enzyme called sortase. Upon cleavage, these enzymes covalently mobilize the surface protein to the peptidoglycan (22).

Horizontal gene transfer is responsible for the acquisition of several virulence traits and antibiotic resistance by enterococci (81). One of the most prevalent forms of horizontal gene transfer is conjugation or transfer of genetic elements via physical contact. Bacterial surface bound glycoprotein termed the aggregation substance mediates contact between donor and recipient cells during conjugation and thus enhances plasmid transfer. Aggregation substances are encoded by the plasmids carried by the donor cells in response to sex-pheromones secreted by the recipient cells (23, 126). Asa1 is an aggregation substance encoded by the plasmid pAD1. In *E. faecalis* OG1X (pAD1) cells, Asa1 expression is induced by serum in addition to the pheromone secreted by the recipient cells suggesting a role beyond just plasmid transfer. Also, Asa1 contains an Arg-Gly-Asp-Ser motif similar to the fibronectin protein which binds eukaryotic cells. Giving credence to those observations, it was shown that Asa1 mediates bacterial adherence to renal tubular cells in addition to its known property to enhance bacterial

clumping for plasmid transfer (52). In addition to renal tubular cells, Ada1 was shown to augment adherence to and survival in macrophages (107). Another aggregation substance Asc10 encoded by the plasmid pCF10 has also been implicated in adherence to host tissue. Similar to *asa1* expression, mammalian bloodstream and growth on heart valves as a bacterial vegetation induced expression of the *prgB* gene encoding Asc10 suggesting its importance *in vivo* (15). *Enterococcus faecalis* was shown to bind porcine heart valves in both Asc10 dependent and independent fashion (15) although *E. faecalis* OG1Ssp (pCF10) strains expressing Asc10 were remarkably more virulent than the plasmid free counterpart in a rabbit model of endocarditis (16). The enterococcal surface protein (Esp) encoded by the *esp* gene on the pathogenicity island of *E. faecalis* enhances biofilm formation, although the presence of functional Esp is not absolutely required. Enhanced biofilm formation by Esp+ strains of *E. faecalis* could be attributed to increased adherence potential bestowed by the surface expression of Esp in those strains (65, 110, 121). Similarly, *E. faecium* also harbors a variant of the *esp* gene on its pathogenicity island (102) and its expression is temperature dependent with maximum expression observed at 37°C and under anaerobic conditions. Van Wamel et al. postulate this growth dependent expression of *esp* to be a niche dependent adaptation of *E. faecium*. Like *E. faecalis*, surface expression of Esp enhanced adherence to polystyrene plates and its ability to form a biofilms (123). The discrepancy about the contribution of Esp to adherence in *E. faecalis* also exists in *E. faecium*. Although Esp enhanced adherence of *E. faecium* to polystyrene plates, it was not essential for attachment to Caco-2 cells and intestinal colonization in mice (33).

In normal eukaryotic tissue the extracellular matrix such as collagen, fibronectin and laminin are usually not exposed and are covered by the epithelial or endothelial cells. They are exposed in the event of trauma and become vulnerable to microbial colonization. Several microrganisms have exploited these ECM for colonization and establishment of infection (60, 125). The enterococcal collagen binding MSCRAMM (Microbial Surface Component Recognizing Adhesive Matrix Molecules), Ace, promotes attachment of *E. faecalis* clinical isolates to collagen type I, type IV and laminin (71, 94). In *E. faecalis* OG1RF, the Ace dependent binding of bacterial cells to the ECM components is termed 'conditional' because the bacteria adhere to collagen and laminin only after incubation at 42°C and in ECM concentration dependent fashion (71). Although rare, *E. faecalis* has also been isolated from endodontic-treated root canals (133). Ace also mediates the binding of *E. faecalis* OG1RF to root canal dentin as the first step to establishing infection (51). 90% of root canal dentin is composed of Type I collagen (64). Similarly, a collagen binding adhesin in *E. faecium* (Acm) that shares homology with the A domain of *E. faecalis* Ace was detected in all the strains tested. However, only the clinical isolates of *E. faecium* harboring *acm* were found to bind type I collagen (75). It is also interesting to note that the presence of a functional *acm* gene was found to correlate with the hospital isolates of *E. faecium* CC17 suggesting that the adhesin Acm might have an important contribution in the emergence of *E. faecium* genogroup CC17 as a nosocomial pathogen (73). In addition to Acm, *E. faecium* also has second collagen binding adhesin Scm which binds efficiently to type V collagen in a concentration dependent manner (105). Recently two novel surface proteins have also been characterized in *E. faecium* that contributes to further enhance binding to host ECM.

A surface adhesion SgrA and another MSCRAMM called EcbA have been identified that enable binding to nidogen (I & II) and type V collagen respectively, apart from their contribution to fibrinogen binding (36). Three enterococcal surface proteins that are structurally and functionally similar to staphylococcal MSCRAMMs have been identified in *E. faecalis* V583. They are termed as *faecalis* surface proteins fss1, fss2 and fss3 also promote adherence to fibrinogen when enterococci are grown in serum supplemented media (104) suggesting that in-vivo, several host components trigger adaptation in eneterococci that enable them initiate colonization.

## 1.5 Quorum Sensing in Enterococci.

Cell density dependent signaling also termed as quorum sensing influences the behavioral changes that benefit the bacteria and also serve in the maturation of the biofilm on the basis of a cascade of genetic and hence phenotypic changes that occur as a result of quorum signaling (45). In *E. faecalis*, a gene cluster homologous to *Staphylococcusaureus agr* locus, called the *fsr* locus regulates the cell density dependent signaling cascade (69, 92). The *fsr* locus is comprised of four genes *fsrA, fsrB, fsr D* and *fsrC.* FsrA and FsrC constitute a two component signal transduction system with FsrC being the membrane localized sensor kinase while FsrA being the cytoplasmic response regulator. The C- terminus of FsrD encodes an 11 residue long quorum signaling molecule called the gelatinase biosynthesis activation pheromone (GBAP) which is processed and secreted by the membrane bound enzyme FsrB (70, 92). Once the concentration of GBAP in external milieu of the cell reaches a threshold value, GBAP binds to and activates FsrC which in turn activates the response regulator FsrA. Differential expression of over 300 genes is regulated by the activated FsrA. Most of

these genes are involved in other regulatory cascades, metabolism and virulence (11). Downstream the *fsr* locus and transcriptionally regulated by the same are two genes that encode the extracellular proteases, gelatinase (*gelE*) and serine proteinase (*sprE*) (93). The *fsr* system plays a pivotal role in enterococcal biofilm formation (30, 66). Hancock and Perego (2004) showed that mutation in *gelE* diminishes biofilm formation and thus *fsr* regulates biofilm formation via the downstream protease gelatinase (30). It has also been shown that the growth of fsr mutants in media supplemented with physiological concentrations of GelE restored biofilm formation by the *fsr* mutant (30). Death of a small population of cells is an important event for biofilm formation because cell death contributes extracellular DNA which is a key matrix component that binds the biofilm together (26). Thomas et al. (2008) showed that GelE contributed to biofilm formation by promoting cell death and providing eDNA. Using DNase I treatment of biofilms and confocal microscopy of *gelE* mutant biofilms, absence of eDNA was shown to correlate with the biofilm defect (118). They also proposed a fratricidal model of eDNA release where one population of cells that doesn't respond to the quorum is killed by the other population and supported the existence of heterogeneity in a single species population (117). Barnes et. al (2012) confirmed the presence of eDNA during early stages of biofilm formation however they suggest a cell death independent mechanism for eDNA release because eDNA was found in well-defined structures throughout the biofilm (5). Interestingly, *fsr* regulation of biofilm formation in *gelE* independent fashion has also been reported(66). It could be attributed to the role of environmental cues. Several studies have shown that nutrient factors such as glucose influence biofilm development in different organisms, including *Enterococcus*. (59, 106, 110). A study reported the

involvement of putative maltose specific PTS encoding genetic loci *bopABCD* (56) in enterococcal biofilm formation and bacteremia in mice (11, 42). They showed that BopD which is a sugar binding transcriptional regulator was essential for biofilm formation and was present in all biofilm-positive strains of *E. faecalis* (42).

## 1.6 RpoN

Controlled regulation of transcription initiation is one of the important stages of gene expression that plays a crucial role in helping an organism adapt to changing environmental conditions in addition to its general housekeeping functions. RNA polymerase core enzyme (cRNAP) that consists of various subunits (α2, β, β', ω) efficiently performs transcription in eubacteria inorder to accurately synthesize RNA and facilitate transcription termination. In prokaryotes, the evolutionarily conserved and cRNAP requires the binding of a dissociable sigma (σ) factor in order to identify the promoter specific DNA sequence and aid transcription initiation. The complex formed by the binding of cRNAP and σ is called the holoenzyme. Two distinct families of sigma factors have been identified on the basis of their protein homology. They have been named on the basis of their molecular weights as sigma 70 ($\sigma^{70}$) and sigma 54 ($\sigma^{54}$; RpoN) (129). The formation of holoenzyme strictly requires the dissociable σ subunit and hence the number of sigma factors possessed by organisms varies according to their environmental niche and their growth requirement. For example the Gram negative pathogen *Pseudomonas aeruginosa* has 24 sigma factors, some of which do not have a defined function (89). The $\sigma^{70}$ family of transcription regulators are abundant in most bacterial genomes. They direct RNA polymerase to specific promoter elements located between the -10 and -35 position upstream of the initiation start site of the gene. The $\sigma^{70}$

family also includes several related alternate sigma factors that are classified on the basis of their genetic structure and function. Sigma 70 controls the expression of most genes during the exponential growth phase and its related sigma factors have been associated with regulation of genes in response to specific environmental signals such as stress, sporulation, heat shock , presence of misfolded proteins etc. (79). Structurally divergent to sigma 70 are $\sigma^{54}$ family of transcription regulators. They recognize a distinct consensus binding sequence (TT**GG**CACNNNNNTT**GC**T) located at -24/-12 position upstream the initiation site (32, 63). One of the most striking difference between the two sigma factors is the way they initiate transcription. Once RNAP-sigma70 bind to the promoter consensus sequence at the -10/-35 position upstream the start site, it spontaneously triggers opening of the DNA and initiates transcription (79) . In contrast, once RNAP coupled to sigma54 binds to the promoter consensus sequence at -12/-24 position, it continuous to be in a closed inactive complex. For this inactive complex to open and for transcription to begin, the RNAP-sigma54 holoenzyme requires the interaction with activator proteins. Thus the initiation of transcription by sigma54 is termed to be activator dependent(63). The activator proteins that couple with RNAP-sigma54 holoenzyme are members of AAA (ATPase Associated with various cellular Activities) protein family that aid conformational changes for the active open complex formation using energy derived by ATP hydrolysis (101). In this dissertation, we restrict to $\sigma^{54}$ family of transcription regulators.

## Domain Organization of RpoN

In *E. faecalis* V583, sigma 54 or RpoN is a 447 amino acid long protein encoded by the gene *ef0782* on the chromosome. On the basis of data available regarding the

structure-function relationship, the protein is made up of three regions. Region I, Region II and Region III (Figure 1-2). The N-terminal region I is responsible for interaction with activator proteins to promote transcription initiation by forming an open complex. Region II strongly binds to RNA polymerase while region III is designated for DNA binding (8, 14, 127). The highly conserved 10 amino acid sequence (ARRTVAKYRE) at the C terminal end of RpoN in region III adopts an alpha helical structure. Mutational analysis attributed the function of promoter recognition by RpoN to this conserved sequence and it is termed as the'RpoN Box'(109). The C-terminal end of RpoN which contains the highly conserved RpoN box shares structural homology to the σ3 and σ4 domains of $\sigma^{70}$ This structural homology is interesting because there exists no sequence similarity between the two. Preceding the RpoN box is an unpredicted helix turn helix motif that enhances the binding of RpoN box to the -12/-24 consensus sequence (21).

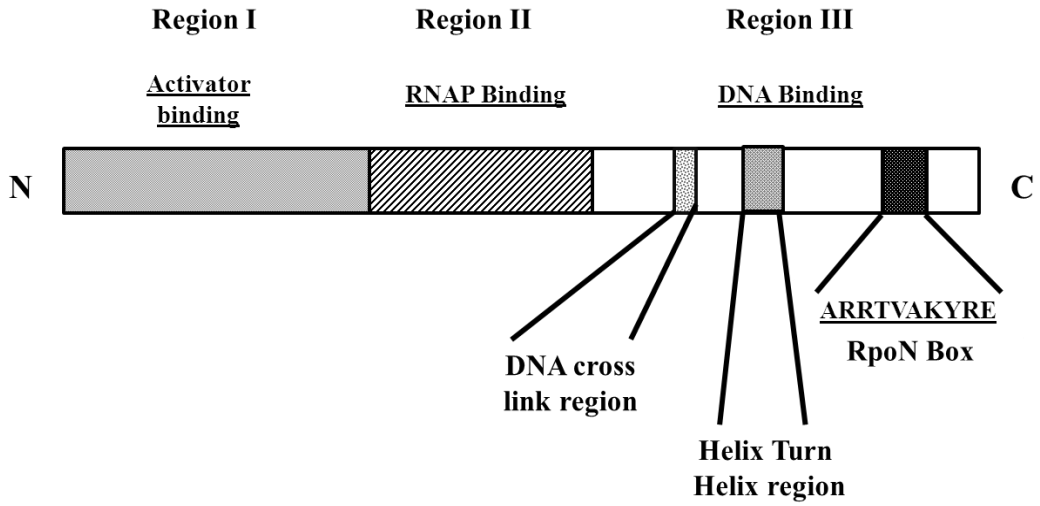


**Figure 1-2 Domain Organization in RpoN.**

## Role of RpoN in other bacteria

RpoN plays an important role in the virulence of several bacteria but does not share the same function in all pathogens (48). In the biology of *Vibrio* species, *rpoN* regulates several diverse functions which include motility, biofilm formation, and quorum sensing, luminescence and host colonization. In the fish pathogen *V. alginolyticus*, RpoN and the enhancer binding protein VasH along with the quorum regulator LuxR govern the expression of the genes involved in type 6 secretion system (T6SS). RpoN and VasH are also required for the expression of hemolysin regulated protein Hcp1 which is a hallmark for T6SS (103). Similarly in *V.cholerae*, expression of *hcp* which is regulated by quorum sensing requires functional RpoN. The quorum sensing regulator HapR positively regulates the expression of *hcp* and in an *rpoN* mutant expression of *hcp* was completely abolished despite the increased levels of HapR indicating that RpoN controls the expression of *hcp* in HapR-independent manner (46). In *V.fischeri*, RpoN regulates the activation of flagellar genes, influences biofilm formation and initial colonization of squids in a flagella independent fashion (91). The *rpoN* mutant in *V.fischeri* has no growth defect in rich medium and glucose supplemented minimal media (MM-G) that contain casamino acid, ammonium chloride and/or glutamine. However, in MM-G that contains serine as the sole source of nitrogen, the mutant is attenuated in growth suggesting the requirement of RpoN in nitrogen limiting conditions. In *Bacillus subtilis*, the sigma 54 equivalent SigL regulates the transcription of levanase operon and *sigL* mutants exhibit growth defect in media containing valine, ornithine, isoleucine or arginine as the sole nitrogen source (20). A seven gene operon, *bkd*, involved in the deamination of isoleucine and valine and degradation of branched

chain alpha keto acids in *B.subtilis* is also regulated by SigL along with the transcription activator BkdR (19). Sigma 54 is required for biofilm formation by *Burkholderia cenocepacia* as well as survival within macrophages (97). In the *Listeria monocytogenes*, RpoN regulates osmotolerance (77) and is responsible for mesentericin sensitivity (18, 77) whereas in *Pseudomonas aeruginosa*, it influences the activity of isocitrate lyase, alginate biosynthesis (10, 28) and in pilin and flagellin production in addition to several other virulence determinants (88). Sigma 54 also regulates biofilm formation, enterocyte effacement, acid tolerance, flagellar biosynthesis and several other processes in E. coli (6, 95, 134).

## RpoN in Enterococcus

In *E. faecalis*, RpoN is responsible for sensitivity to Class IIa bacteriocins such as mesentericin and divercin (13, 17). The basis for the Class IIa bacteriocin sensitivity is due to the role of $\sigma^{54}$ in regulating mannose sugar uptake system with the help of the enhancer binding protein MptR. In addition to mannose three distinct sugar phosphotransferase (PTS) systems with RpoN recognition sequence upstream the putative operons and their respective enhancer binding proteins LpoR, MphR and MpoR have been identified (32).

The focus of this dissertation is to understand the role of RpoN in the different aspects of enterococcal biology such as biofilm development, metabolism and its impact of virulence.

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# Chapter 2 - Deletion of σ(54) (rpoN) alters the rate of autolysis and biofilm formation in *Enterococcus faecalis*.

## 2.1 Abstract

Transcription initiation is a critical step in bacterial gene regulation and is often controlled by transcription regulators. The alternate sigma factor (RpoN) is one such regulator that facilitates activator dependent transcription initiation and thus modulates the expression of a variety of genes involved in metabolism and pathogenesis in bacteria. This study describes the role of RpoN in the nosocomial pathogen, *Enterococcus faecalis*. Biofilm formation is one of the important pathogenic mechanisms of *E. faecalis* that elevates its potential to cause surgical site and urinary tract infections. Lysis of bacterial cells within the population contributes to biofilm formation by providing eDNA as a key component of the biofilm matrix. Deletion of *rpoN* rendered *E. faecalis* resistant to autolysis which in turn impaired eDNA release. Despite the absence of eDNA, the *rpoN* mutant formed robust biofilms as observed using laser scanning confocal microscopy indicating and emphasizing the presence of other matrix components. Initial adherence to polystyrene plate was enhanced in the mutant. Proteinase K treatment of biofilm at different stages of development significantly reduced the accumulation of biofilm by the *rpoN* mutant. In conclusion, our data indicates the presence of proteins as an additional matrix component in *E. faecalis* V583 biofilms and suggests a regulatory role of RpoN in governing the nature and composition of the biofilm matrix.

## 2.2 Introduction

As opportunistic pathogens, enterococci, are the third leading cause of hospital acquired or associated infections responsible for 11.2 % surgical site infection (SSI), 14.9% of urinary tract infection (UTI) and 16% of the reported blood stream infections (25). The ability to form biofilm is an important aspect of the lifestyle of the organism as biofilm formation is thought to be a property associated with the establishment of SSI and UTI (34) both of which serve as foci to establish blood stream infections. Biofilms are aggregates of bacteria that are covered in exoploymer matrix and are more resistant to antibiotics than their planktonic counterparts (15, 26). In several bacterial species nucleic acids, polysaccharides, proteins and lipids constitute the exopolymer matrix (19). The components of the biofilm matrix form a physical barrier that enhance the inaccessibility of the biofilm cells to antibiotics and the immune system thereby making the infection difficult to eradicate (33). Extracellular DNA (eDNA) serves as an important biofilm matrix component in several microbial model systems including but not limited to *Neisseria meningitidis*, *Listeriamonocytogenes*, *Pseudomonas aeruginosa*, *E. faecalis*, *Staphylococcusaureus* and *S.epidermidis* (2, 23, 29, 32, 36, 47, 48, 54). The expression of the two secreted *E. faecalis* proteases, gelatinase and serine protease, is regulated in a quorum-dependent manner by the Fsr regulatory system (22, 45, 46), and these proteases direct biofilm development by modulating the eDNA matrix by regulating the extent of autolysis (54) in a fratricidal manner (51). In an attempt to identify other factors that govern eDNA release in *E. faecalis*, we identified *rpoN* which encodes $\sigma^{54}$ in a preliminary transposon mutagenesis screen.

Transcription initiation is one of the important stages of gene regulation and sigma factors play a crucial role in determining the controlled response of a subset of

genes tied to a given environmental stimulus. Sigma factors reversibly bind to RNA polymerases and drive promoter specific transcription initiation. In prokaryotes, two distinct families of sigma factors have been studied, sigma 70 ($\sigma^{70}$) and sigma 54 ($\sigma^{54}$). The $\sigma^{70}$ family also includes several related alternate sigma factors. Sigma 54 shares no structural homology with sigma 70, possesses a distinct consensus binding sequence (-24/-12;TTGGCACNNNNNTTGCT) and unlike sigma 70, facilitates activator dependent transcription initiation (24, 38).

Sigma 54 plays an important role in the virulence of several bacteria but does not share the same function in all pathogens (30). In *Vibrio fischeri*, $\sigma^{54}$ influences biofilm formation, motility and symbiotic colonization of squids and negatively regulates bioluminescence (58). Quorum sensing regulation in *V. cholerae* O1 strains is dependent on *rpoN* (28). Sigma 54 is required for biofilm formation by *Burkholderia cenocepacia* as well as survival within macrophages (51). In the major food-borne pathogen *Listeria monocytogenes* , $\sigma^{54}$ is essential for its osmotolerance potential (42) and is responsible for mesentericin sensitivity (14, 42) whereas in *Pseudomonas aeruginosa*, $\sigma^{54}$ influences the activity of isocitrate lyase (21) , alginate biosynthesis (6) and in pilin and flagellin production in addition to several other virulence determinants (44). Sigma 54 also regulates biofilm formation, enterocyte effacement, acid tolerance, flagellar biosynthesis and several other processes in *E. coli* (3, 49, 60).

In *E. faecalis*, $\sigma^{54}$ is responsible for sensitivity to class IIa bacteriocins such as mesentericin and divercin (9, 13). The basis for the class IIa bacteriocin sensitivity is due to the role of $\sigma^{54}$ in regulating four distinct sugar phosphotransferase (PTS) systems that are dependent on four known $\sigma^{54}$ enhancer binding proteins (LpoR, MphR, MpoR, and

MptR) (13) . MptD, a component of the mannose PTS system is thought to serve as the cellular receptor for the class IIa bacteriocins (24). However, additional roles for $\sigma^{54}$ in enterococcal biology remain to be elucidated.

In this study, we investigate the role of $\sigma^{54}$ in eDNA release, autolysis and biofilm formation and demonstrate a functional role for $\sigma^{54}$ in regulating initial adherence of cells to substrate as well as the overall composition of the biofilm matrix.

## 2.3 Materials and Method

### Bacterial strains and growth conditions:

The bacterial strains and plasmids used in this study are listed in Tables 1 and 2 respectively. *Escherichia coli* Electroten Blue (Strategene) was used for construction of plasmids and was cultured in Luria-Bertani (LB) broth supplemented with appropriate antibiotics. *Enterococcus faecalis* strains were cultured in Todd-Hewitt broth (THB; BD Biosciences) containing appropriate antibiotics whenever required. Chloramphenicol (Cm) and spectinomycin (Spec) were used for selection of *E. coli* at a concentration of 10µg/ml and 150µg/ml respectively. For *E. faecalis*, Cm, Spec and tetracycline (Tet) were used at 15µg/ml, 500µg/ml and 15µg/ml respectively.

**Table 2-1 *Enterococcus faecalis* strains used in this study**

| Strain | Genotype; Relevant Phenotype | Reference |
|---|---|---|
| V583 | Parental Strain | Clinical isolate (50) |
| VI01 | V583Δ*rpoN* | This Study |
| VI40 | VI01 full length *rpoN* markerless complement | This study |
| VT09 | V583 (pMV158GFP) Gfp, $Tet^R$ | (54) |
| VI29 | VI01 (pMV158GFP) Gfp, $Tet^R$ | This Study |
| VI41 | VI40 (pMV158GFP) Gfp, $Tet^R$ | This Study |

When required, X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; AMRESCO) was used at a concentration of 80µg/ml for both *E. coli* and *E. faecalis*.

### In-frame Markerless deletion of *rpoN*

An *E.coli*-enterococcal temperature sensitive cloning vector,pLT06 (56), was used to generate isogenic in-frame deletion of *rpoN* in *E. faecalis* V583. Upstream and downstream regions flanking *rpoN* (EF0782) were amplified by PCR from a V583

genomic template using the primer pair RpoNP1/RpoNP2 and RpoNP3/RpoNP4 respectively (Table 2-3). The primers RpoNP1/RpoNP2 and RpoNP3/RpoNP4 were designed with EcoRI/BamHI and BamHI/PstI restriction sites respectively. The resultant PCR products were digested with BamHI, ligated and re-amplified with primers RpoNP1 and RpoNP4. For the construction of the deletion vector, the amplified product was digested with EcoRI and PstI followed by ligation to similarly digested pLT06. The ligation was electroporated into competent E10-Blue cells for propagation and blue colonies were selected on LB agar containing chloramphenicol and X-Gal at 30°C. Clones were screened for the appropriate insert using the primers OriF and SeqR. A positive plasmid designated pKS70 was confirmed by restriction digest and electroporated into *E. faecalis* V583 cells (12) and VI01 was subsequently generated following the protocol previously described (56) and confirmed by PCR using primers RpoNUp and RpoNDown. Using pKS70, ~ 98% of the *rpoN* gene was deleted leaving seven codons at the 5' end and two codons at the 3' end. The next adjacent gene is *ef0783*, which encodes an O-acetyltransferase. This gene is located approximately 200 bps downstream of *rpoN* and the strategy used to delete *rpoN* does not alter the expression of *ef0783* (data not shown).

**Table 2-2 Plasmid Constructs used in this study.**

| Plasmid | Description | Reference |
|---|---|---|
| pLT06 | Deletion vector; chloramphenicol resistance. | (56) |
| pKS70 | pLT06 containing engineered *rpoN* deletion (2kb EcoRI/PstI fragment) | This study. |
| pVI12 | pLT06 containing full length *rpoN* | This study. |
| pMV158GFP | Gram-positive replicative vector: Gfp; $Tet^R$ | (1) |

## Markerless Complementation of VI01 [Δ*rpoN*]

The temperature sensitive cloning vector pLT06 (56), was used to generate markerless gene complementation of *rpoN* in VI01. The *rpoN* gene (EF0782) along with flanking regions was amplified by PCR from a V583 genomic template using primers RpoNP1 and RpoNP4 (Table 2-3). For the construction of *rpoN* markerless complementation vector pVI12, the amplified product was digested with EcoRI and PstI followed by ligation with similarly digested plasmid vector pLT06. The ligation was electroporated into competent E10-Blue cells for propagation and blue colonies were selected on LB agar containing chloramphenicol and X-Gal at room temperature. Clones were screened for the appropriate insert using the primers OriF and SeqR. A positive plasmid designated pVI12 was confirmed by restriction digest and electroporated into E. *faecalis* VI01 cells (12) and VI40 (markerless complement) was generated following the protocol previously described (56) and confirmed by PCR using primers RpoNUp and RpoNDown.

## 2-Deoxy-D-Glucose (2DG) resistance

*Enterococcus faecalis* V583, VI01 and VI40 were grown on LB agar containing 0.2% fructose and 10 mM 2-Deoxy-glucose (2DG) (24). 2DG is a toxic homologue of glucose and enters the cells via the mannose PTS permease (5). In *E. faecalis*, the mannose PTS expression is controlled by $\sigma^{54}$. Strains resistant to 2DG do not express a functional mannose PTS permease (24). Hence growth on media containing 2DG was used as a marker to confirm deletion of *rpoN*.

**Table 2-3 Oligonucleotides used in this study.**

| Primers | Sequence (5'-3') |
|---|---|
| RpoNP1 | GAGAGAATTCACAACGGTACAGTAAAATGG |
| RpoNP2 | CTCTGGAATCCCATTCGTTGCTCAAATTTCAT |
| RpoNP3 | GAGAGGATCCGAGTAAACAACCAAAGATTAT |
| RpoNP4 | CTCTCTGCAGGAACTAAGGCACTTAAACCA |
| RpoN UP | AGTCCAAGGAAGAGTCGTG |
| RpoN DOWN | AAGACAGTGGCTGCCAAAC |
| OriF | CAATAATCGCATCCGATTGCA |
| SeqR | CCTATTATACCATATTTTGGAC |

## Detection and precipitation of extracellular DNA

Overnight cultures were centrifuged for 10 minutes at 13,000 rpm and the resulting supernatant filtered (0.2 µm pore size; Nalgene) to obtain cell free supernatants. The supernatants were tested for the presence of e-DNA using 1µM SYTOX® Green (Invitrogen, Molecular Probes).

The eDNA was also precipitated from the culture filtrate using an equal volume of isopropanol. The precipitated eDNA was washed in 75% ethanol, air-dried and dissolved in TE buffer (10 mM Tris-Cl, 1 mM EDTA, pH 8.0) and visualized on 1% agarose gels after staining with ethidium bromide.

## Autolysis Assay

Autolysis assay was performed as previously described (15).

## Quantitative Detection of eDNA in Biofilm

eDNA in biofilm was quantified using a previously described protocol (36). Briefly, biofilms were grown in 96-well polystyrene plate in TSB for 24 hrs at 37°C.

After 24 hrs the supernatant was discarded and the biofilm was re-suspended in resuspension buffer (50 mM Tris-Cl pH 8, 10 mM EDTA, 500 mM NaCl). The resuspended biofilm was centrifuged and eDNA was quantified in the supernatant using 1μM SYTOX® Green (Invitrogen, Molecular Probes).

## Confocal Laser Scanning Microscopy (CLSM)

CLSM was performed on one-day old biofilms as described previously (54). *Enterococcus faecalis* strains VI01 and VI40 were transformed with pMV158GFP (39) to generate VI29 and VI41, respectively, and both express Gfp constitutively. VT09 [V583 (pMV158GFP)] (54) along with VI29 and VI41 were used for confocal imaging. Briefly, biofilms were grown on sterile glass coverslips placed in six-well tissue culture plates. The coverslip was submerged in 5ml of TSB broth containing tetracycline for plasmid maintenance. After 24 hrs of growth, the biofilm was gently washed with sterile phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM $Na_2HPO_4$, 2 mM $KH_2PO_4$ pH 7.4) and stained with 1μM SYTOX Orange® ( Invitrogen ) for 6 to 7 minutes. The coverslip were inverted on a clean glass slide and sealed using clear nail polish. The biofilm was visualized using Zeiss LSM 5 Pascal laser scanning confocal microscope.

## Macroscopic Biofilm

To visualize the biofilms formed by VT09, VI29 and VI41 macroscopically, biofilms were grown as per confocal analysis, with the exception that after 24 hours of growth, the biofilms were gently washed with sterile phosphate buffer and then fresh TSB was added, and the biofilms were grown for an additional 24 hours, at which time the biofilm was washed and imaged with a document camera.

## Adherence Assay

Adherence of *E. faecalis* strains to polystyrene plates was tested using a previously described protocol (27) with some modification. Overnight grown cultures were diluted 1:10 in fresh TSB and 200µl was transferred to a flat bottom 96 well polystyrene microtiter plates. After 2 hrs of incubation at 37°C, the supernatant was discarded and the wells were gently washed with sterile PBS. The adherent cells were resuspended in 200µl PBS by vigorous pipetting, diluted and plated on THB agar for colony counting. Also, the initial load was calculated by plating the diluted culture on THB agar for colony counting. The adherence potential of the strains was calculated as the percentage of initial load that adhered.

## Proteinase K treatment of Biofilm

Biofilm were grown on 96 well polystyrene plates as previously described (22). At 6-, 12-, and 24-hr time points, the biofilm were treated with 1µg/ml proteinase K (Amresco). An untreated control was included to determine the effect of treatment. After 24 hrs of growth, the biofilm was quantified with the crystal violet staining method (22). Each assay was performed in triplicate and repeated four times.

## Statistical Analysis

Statistical analysis of quantitative detection of eDNA, adherence assay and comstat analysis of biofilm was performed using GraphPad Prism 4 software (San Diego, CA). One way analysis of variance followed by Dunn's multiple comparison tests was performed to determine statistical significance.

## 2.4 Results

### Construction of the *E. faecalis* V583 isogenic *rpoN* mutant and its complement

The *rpoN* deletion mutant VI01 [Δ*rpoN*] was constructed using the markerless deletion vector pKS70. The 2DG resistant phenotype was confirmed by growth on media containing 2DG. VI01 grew to the final dilution of $10^{-8}$, whilst the parental strain V583 and the *rpoN* complement VI40 were significantly inhibited and grew only at a dilution of $10^{-3}$ and $10^{-4}$. Complementation confirmed that there were no polar effects of the gene deletion and attributed the 2DG resistant phenotype to the targeted deletion of *rpoN* (Figure 2-1).

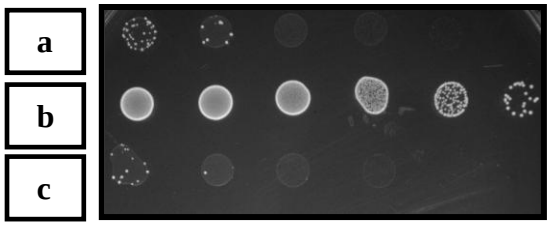



**Figure 2-1 2-Deoxy-D-Glucose (2DG) resistance analysis of VI01**

Wild type strain, V583, (a) and the complement strain VI40 (c) are sensitive to 2DG because of functional *mpt* operon under the control of intact *rpoN*. The *rpoN* mutant VI01 (b) is resistant to 2DG. This confirms the deletion and complementation of *rpoN* in *E. faecalis*.

### Sigma 54 alters eDNA release in the supernatant of planktonic and biofilm cultures.

On the basis of phenotype characterization of a preliminary transposon mutagenesis screen, we tested for eDNA in the supernatants of planktonic cultures using SYTOX green. A lower amount of eDNA was detected in VI01 culture supernatant in comparison to the wild type V583 strain whereas the markerless complementation of *rpoN* mutant restored the phenotype to wild type levels (Figure 2-2 A). eDNA in the

supernatants of the various strains was also confirmed by visualization on an EtBr-stained 1% agarose gel after precipitation of eDNA with isopropanol (Figure 2-2 B). Given the fact that planktonic growth and biofilm are two different lifestyles of the bacteria, we tested to see the effect of *rpoN* deletion on eDNA during biofilm development. As observed in planktonic cultures, a lesser amount of eDNA was detected in VI01 biofilm than the wild type which was attributed to the deletion of *rpoN* as the complementation restored the eDNA detected in the biofilm to wild type levels (Figure 2-2 C)

**Sigma 54 alters the rate of autolysis in *E. faecalis* V583.**

Because eDNA release in *E. faecalis* is dependant upon cell death by autolysis (54) and the *rpoN* mutant is defective in eDNA release, we hypothesized that $\sigma^{54}$ may differentially modify the rate of autolysis in *E. faecalis*. In the autolysis assay, we observed that VI01 showed a significant decrease in the rate of autolysis, a phenotype readily complemented by introducing the gene in single copy to its native locus (Figure 2-3).

**Sigma 54 alters the biofilm development of *E. faecalis***

eDNA has been shown to be an important matrix component in *E. faecalis* biofilm (54). The decreased levels of eDNA in VI01 led us to the hypothesis that VI01 may form less dense biofilm in comparison to the wild type V583. However, CLSM analysis of 24 hr old biofilm grown on glass coverslip showed that VI29 [Δ*rpoN*, $Gfp^{+}$] formed thicker biofilm (as measured by the Z stack thickness using LSM image examiner) than those formed by the wild type strain VT09 or the complement strain VI41 (Figure 2-4II and Table 2-4).

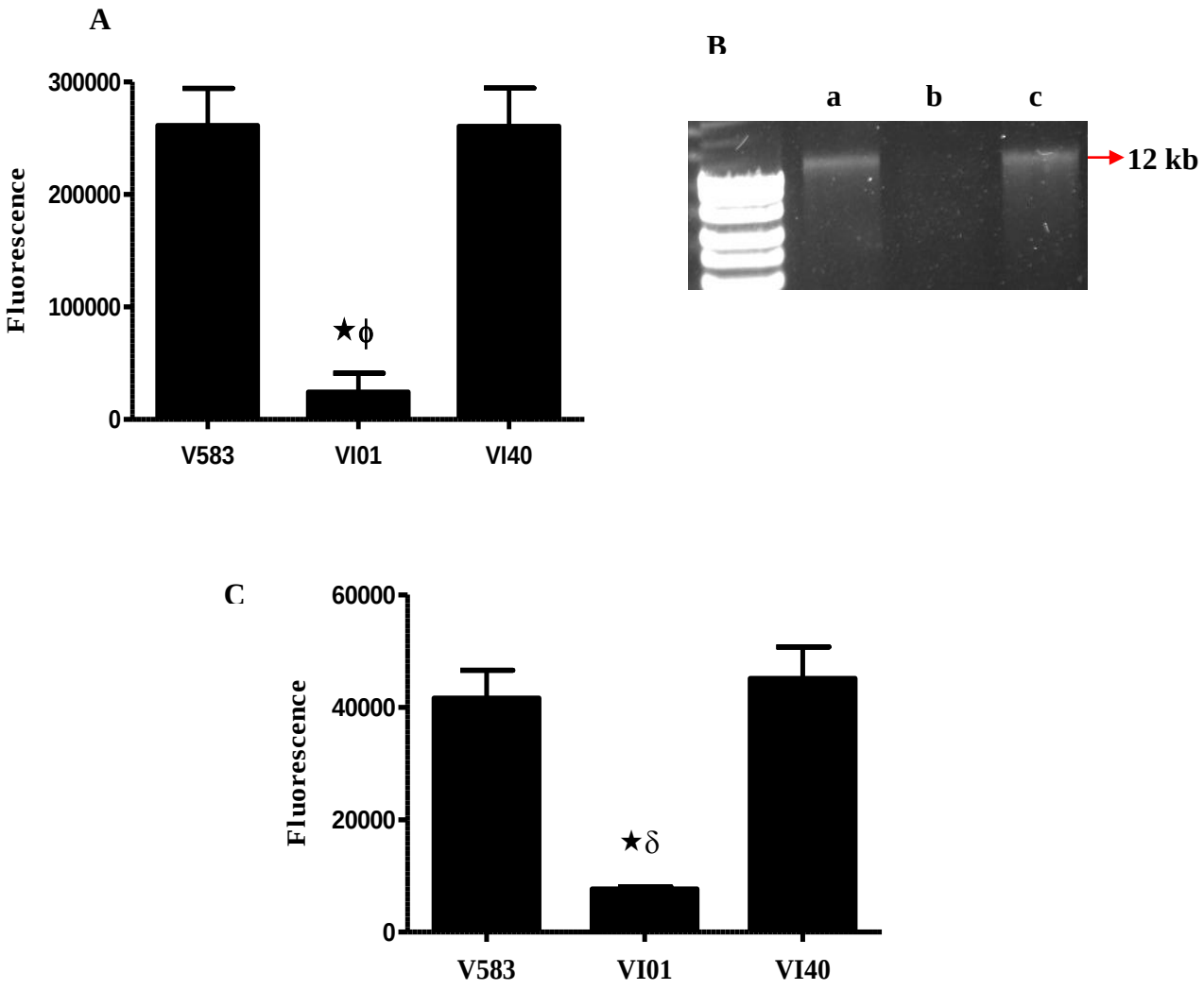



**Figure 2-2 Extracellular DNA detection**

A) Quantitative detection of eDNA in culture supernatants using SYTOX green. eDNA was quantified in the culture supernatants of overnight grown cultures using 1µM SYTOX green. Assays were performed in quadruplets and error bars indicate the standard error of mean. ★, significant P values of less than 0.001 relative to wild type V583; ϕ, significant P values of less than 0.001 relative to *rpoN* complement (VI40). B) Qualitative detection of eDNA in culture supernatant by isoporpanol precipitation. a, Wildtype (V583); b. *rpoN* mutant (VI01); c. *rpoN* complement (VI40). C) Detection of eDNA in biofilm using SYTOX green. Assays were performed in sextuplets and error bars indicate standard error or mean. ★, significant P values of less than 0.001 relative to wild type V583; δ, significant P values of less than 0.001 relative to *rpoN* complement (VI40).

The appearance of the VI29 biofilm suggested early initiation of microcolony development, which was confirmed by macroscopic examination of the biofilms after 2 days growth on coverslips (Figure 2-4I). Despite the increased thickness and overall biofilm biomass of the *rpoN* mutant [VI29] compared to the parental and complemented strains (Figure 2-4), very few random dead cells and DNA (as detected by SYTOX Orange staining) were observed within the biofilm. Consistent with earlier observations on the role of cell death and eDNA as a matrix component (54), regions within the wild type VT09 contained concentrated foci of DNA and dead cells, which was phenocopied by the complement strain VI41 (Figure 2-4II).

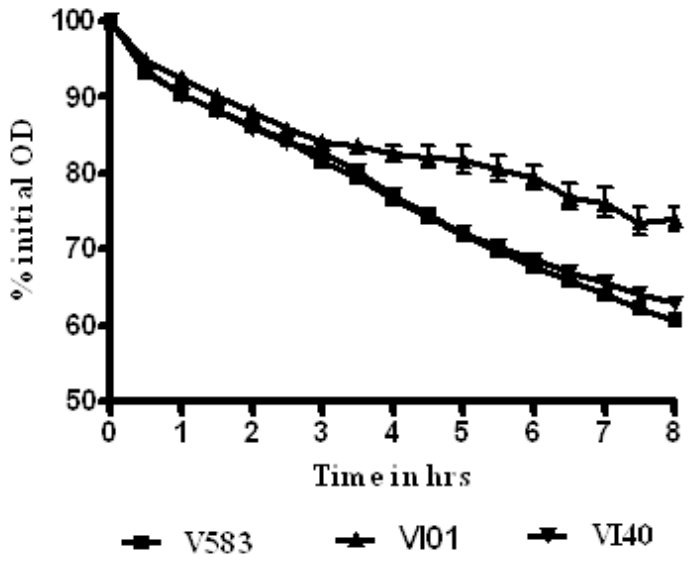
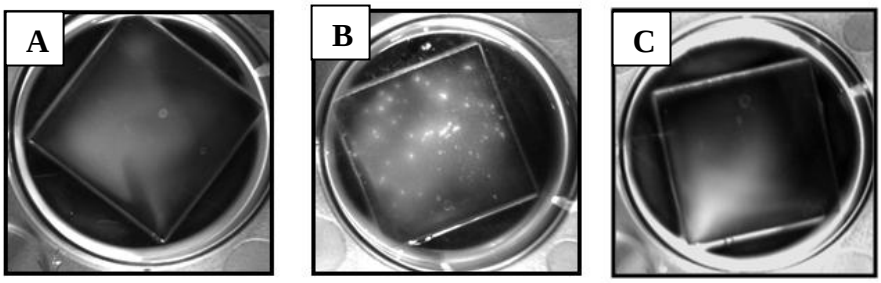



**Figure 2-3 RpoN alters the rate of autolysis in *E. faecalis***
RpoN alters rate of autolysis in *E. faecalis*. Difference in autolysis rates of wild type (V583), *rpoN* mutant (VI01) and complemented strain (VI40) are plotted as percent of initial optical density at 600 nm. Assays were performed in triplicates and repeated four times; error bars represent standard error of mean.

I




II

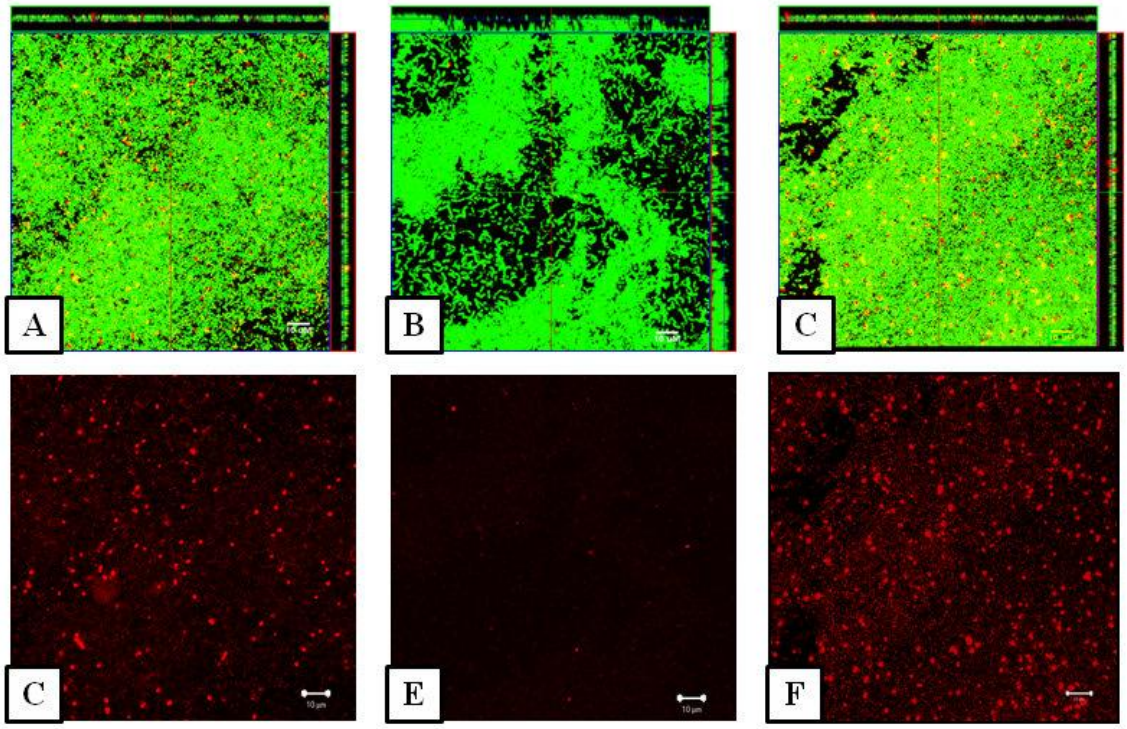



**Figure 2-4 *E. faecalis* Biofilm Analysis**

I) Macroscopic view of biofilm grown on glass coverslip. Biofilms were grown on glass coverslip in TSB media. A : VT09 ; B: VI29 ; C: VI41

II) Confocal analysis of 1-day old biofilms grown on glass coverslip. The wild type, mutant and complement constitutively express Gfp from pMV158gfp as mentioned in materials and methods. Biofilms were grown on glass coverslips in TSB media. Dead cells and eDNA were stained with SYTOX orange (1µM). Live bacteria appear green while dead cells and eDNA are red. Panel A, B and C represent biofilm orthogonal projections for VT09, VI29 and VI41 respectively, exhibiting merged green and red staining. Panel D, E and F correspond to dead cell and eDNA staining in VT09, VI29 and VI41 biofilm respectively (matched pair to biofilms in Panel A, B and C). The scale bar represents 10 µm.

.

**Table 2-4 Comstat Analysis of one-day old biofilms**

| Strain | Mean ± SD | |
|---|---|---|
| | Biomass ($\mu m^3 / \mu m^2$) | Average Thickness (µm) |
| **VT09** | 2.09 ± 0.522 § | 2.40 ± 0.589 § |
| **VI29** | 4.723 ± 1.28 | 5.97 ± 2.01 |
| **VI41** | 2.19 ± 0.638 § | 2.14 ± 0.624 § |

§, significant p-value of less than 0.05 in comparison to VI29

## Deletion of *rpoN* increases adherence to polystyrene plates

In order to determine whether increased biofilm formation by the *rpoN* mutant was due to its initial adherence ability, we calculated the percentage of initial inoculum that adhered to 96 well microtiter plates after two hours. Adherence of VI01 to a polystyrene plate was significantly enhanced in comparison to the wild type. In addition, complementation of VI01 reduced the adherence potential to wild type levels (Figure 2-5).

## Sigma 54 modulates the composition of *E. faecalis* V583 biofilm.

On the basis of the macroscopic observation and CLSM of the VI29 biofilm and the relative lack of eDNA detection in this mutant, we hypothesized a role for a different polymer matrix that promotes biofilm formation in the *rpoN* mutant. To test the role of proteins in VI01 biofilm, we examined the effect of Proteinase K treatment on biofilm development. The wild type and complemented strain exhibited decreased biofilm when treated with proteinase K only after 24 hr of biofilm growth. In contrast, reduction in VI01 biofilm was significant when treated with proteinase K after 6 hr biofilm growth and continued to respond to treatment after 12 hr and 24 hr of biofilm growth. (Figure 1-1Figure 2-6)

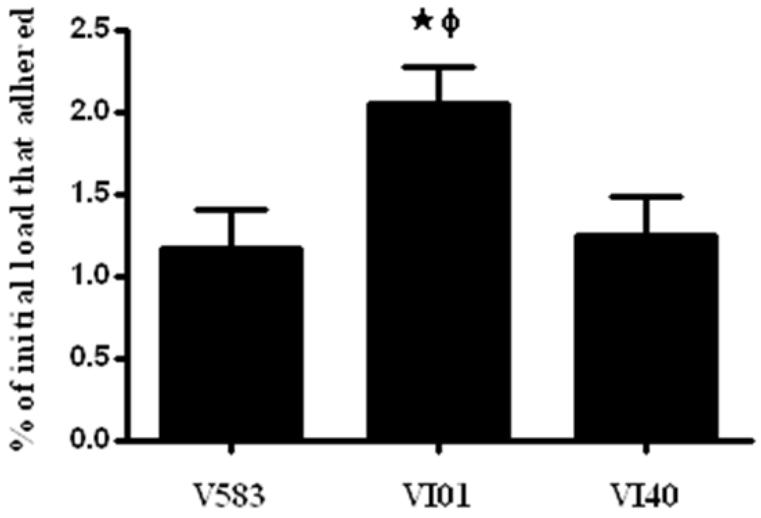



**Figure 2-5 Adherence of *E. faecalis* on Polystyrene plate.**
Deletion of *rpoN* increases adherence of *E. faecalis* to 96-well polystyrene plates. ★, significant *P* values of less than 0.05 relative to wild type V583; ϕ, significant *P* values of less than 0.05 relative to *rpoN* complement (VI40)

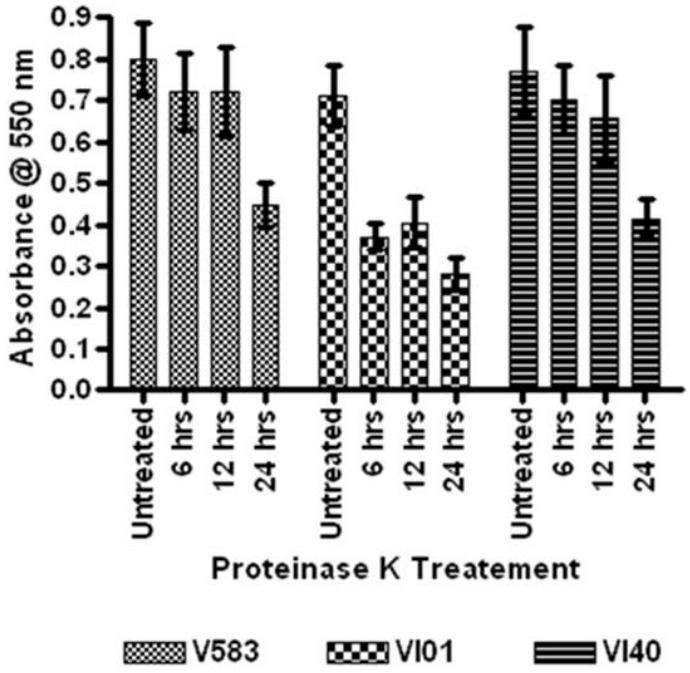



**Figure 2-6 Proteinase K inhibits biofilm development of *rpoN* mutant on polystyrene plates.**
Biofilms were seeded at time zero for V583, VI01, and VI40 and the untreated biofilms were stained 24 hours later. At the indicated times, proteinase K (1µg/ml) was added at either 6, 12, or 24 hours after seeding the biofilm, and the treatment was allowed to stand for the reminder of the assay. Each assay was performed in triplicate and repeated four times. Error bars indicate standard error of the mean.

## 2.5 Discussion

The role of $\sigma^{54}$ in regulating numerous biological properties, including those that relate to virulence has been well documented in a variety of bacterial species (3, 10, 21, 41, 49, 51, 52, 55, 58, 59). However its role in *E. faecalis* has been limited to observations made regarding its contribution to sensitivity to class IIa bacteriocins through the regulation of sugar PTS systems (9, 13, 22). Identification of $\sigma^{54}$ as a potential regulatory protein in the cascade of biofilm development was an interesting breakthrough and we focused our efforts on elucidating its affect on *E. faecalis* V583 biofilm. The role of autolysis (54) and fratricide (53) has been well documented in enterococcal biofilm formation and has been shown to be important in providing eDNA as a key biofilm matrix component. However, the observation that biofilm formation was enhanced in the *rpoN* mutant despite the increased resistance to autolysis and the absence of eDNA was an unexpected finding.

One possible explanation for increased resistance to autolysis observed in the *rpoN* mutant could be novel modifications of the cell wall or altering the modifications such as O-acetylation (43) or D-alanylation (17) on the cell wall that protect against lysis. Deletion of *rpoN* did not alter the autolysin profile of *E. faecalis* when using micrococcal cell wall as a zymoGram substrate (data not shown) ruling out the possibility of inactive autolysins. Also, the deletion of *rpoN* does not have a measurable affect on the secretion of the extracellular proteases, GelE and SprE, which have been previously shown to contribute to autolysis in *E. faecalis* (54, 57) (data not shown). A significant reduction in cell death due to impaired cell lysis occurred in *E. faecalis* V583 Δ*rpoN* planktonic and biofilm cultures suggesting the requirement of a functional $\sigma^{54}$ to regulate susceptibility

to cell lysis. In *P. aeruginosa*, deletion of *rpoN* abolishes cell death in the microcolonies during biofilm maturation and has been related to the expression of surface structures (Type 4 pili and flagella) whose expression is regulated by $\sigma^{54}$ (56). Additionally, $\sigma^{54}$ dependent gene regulation promotes phage induced lysis in *P. aeruginosa* (10). There are seven phages associated with *E. faecalis* V583 with one of them being a part of the core genome (37). It will be interesting to test the role of $\sigma^{54}$ dependent transcription of phage particle proteins and host lysis and its contribution to biofilm development.

Enhancement of biofilm formation in the absence of a well characterized matrix component in the *rpoN* mutant indicates that a substantial knowledge gap still exists to unravel factors associated with *E. faecalis* biofilm development. Cellular processes regulated by $\sigma^{54}$ will be attractive in this regard to begin revealing the interplay between metabolism and biofilm development, as one of the few characterized roles for $\sigma^{54}$ is the regulation of four sugar PTS pathways. It is noteworthy that deletion of the genes encoding the four known enhancer binding proteins (LpoR, MphR, MpoR, and MptR) did not reduce eDNA release, impair autolysis or alter biofilm development (data not shown), suggesting that $\sigma^{54}$ might act as a repressor of genes independent of enhancer protein function. The idea that $\sigma^{54}$ levels in the cell or within the population might be regulated raises an interesting experimental question. Our observation that the wild-type and *rpoN* complement strain could grow on 2DG at a much lower frequency ($10^{-4}$ and $10^{-5}$) relative to the *rpoN* mutant parallels a recent report by Flanagan et al. (18) in which resistance to the *E. faecalis* plasmid encoded bacteriocin MC4-1 (a class IIa bacteriocin) was dependent on point mutations within the *rpoN* gene that occurred at high frequency ($10^{-3}$ to $10^{-4}$). This resistance was shown to be reversible to a susceptible phenotype by

point mutations that also occurred within *rpoN* as second-site suppressors. These combined observations suggest that there are hot spots for mutation within *rpoN*, and could be a mechanism for phase variation within the *E. faecalis* population.

In *Vibrio vulnificus*, $\sigma^{54}$ positively regulates the gene encoding an ADP-glycero-manno-heptose-6-epimerase (*gmhD*) which is responsible for production of lipopolysaccharide and exopolysaccharide, both of which are required for biofilm formation (31) while in *Burkholderia cenocepacia*, $\sigma^{54}$ controls motility which in turn plays a role in biofilm formation (51). However, in *E. coli* K12, *rpoN* deletion enhances biofilm formation (3). Such different effects of *rpoN* on biofilm forming potential of bacteria provides a clear example of how a gene whose function was first reported to be restricted to nitrogen assimilation has evolved to govern virulence related functions in addition to bacterial metabolism. Our data provide additional support for the expanding role of $\sigma^{54}$ in the world of low-GC Gram-positive bacteria.

Biofilm formation is a multistep process that begins with the attachment of bacteria to the substrate followed by colonization via further recruitment of more bacteria or by cell division. Initial attachment of a bacterial cell to a surface is an important stage in biofilm development and determines the fate of this process. In *P. aeruginosa* (8, 35), *Staphylococcus aureus* (11) and Streptococci (40) it has been shown that defect in initial adherence of the bacteria affect biofilm formation and subsequently influences the virulence of these pathogens. Our data indicates a similar influence of attachment process in enterococcal biofilm development wherein deletion of *rpoN* increases the adherence potential of the pathogen which subsequently results in a more dense biofilm.

Other than DNA, other molecules such as proteins and polysaccharides have been suggested to be important constituents in the polymer matrix of several bacteria (16, 19). Robust biofilm in VI01 despite the significant reduction in eDNA led us to test for the presence of other matrix components using compounds capable of dissolving the aforementioned components. The reduced ability of VI01 (Δ*rpoN*) to form a biofilm when treated with proteinase K suggests a role for protein in either adhesion or matrix composition to promote *E. faecalis* biofilm and is consistent with recent observations by Guiton et al. (20). These authors observed that colonization of an implanted piece of urinary catheter as well as the bladder epithelium was dependent on a functioning sortase enzyme for the proper anchoring of proteins to the cell wall, which in turn promotes cellular adhesion. In *S. aureus*, a biofilm defect in mutants that over produce extracellular protease was rectified by the addition of α2 macroglobulin – a general protease inhibitor, indicating a vital role for proteins in either cellular adhesion or biofilm matrix (4) . Similarly in *B. subtilis*, TasA is required for the structural integrity and development of biofilms (7). In *E. faecalis* biofilms (54), eDNA is known to be a crucial matrix component in the early stages of biofilm development, but by 24 hours growth in the biofilm DNase has a minimal affect at disrupting the biofilm. Here we show that in *E. faecalis* V583, proteins are likely to serve as important matrix components during the later stages of biofilm development as a reduction in biomass was observed following only at 24 hours and not at earlier timepoints. This suggests the time dependent involvement of different polymers in the overall development of the biofilm.

Complementation studies of the *rpoN* mutant using a low copy plasmid did not result in complete reversal of the phenotype to wild type levels in experiments that

involved stressing of cells (osmotic shock and 2DG toxicity)(data not shown). This was primarily due to plasmid loss in the absence of selectioin, and suggested a survival advantage for *E. faecalis* in the absence of $\sigma^{54}$ under certain stress conditions. The inability to fully complement an *rpoN* mutant has also been reported in *L. monocytogenes* (42). Similarly, in a *V. fischeri* squid colonization model (58), the level of colonization varied with the complemented strain and only some animals exhibited wild type levels of colonization. For this reason, we utilized a complementation strategy that restored the function of the gene by placing it at its native locus in single copy.

A literature survey for $\sigma^{54}$ and its biological roles revealed a bias towards Gram-negative species with *P. aeruginosa*, *Vibrio* spps and *E. coli* being the most studied. In an attempt to identify the distribution of *rpoN* in low-GC Gram-positive organisms, we performed a BLAST search using $\sigma^{54}$ of *E. faecalis* V583 as the query. Amongst the organisms queried, only *L. monocytogenes*, *B. subtilis*, *C. difficle* and *C. perfringens* appeared to have homologues, whereas in *S. aureus, S. pneumoniae*, and *S. pyogenes* homologs to $\sigma^{54}$ were absent. The basis for this distribution among enteric adapted organisms as well as the potential genes regulated by $\sigma^{54}$ awaits further study.

In conclusion, the results from this study show that $\sigma^{54}$ in *E. faecalis* V583 contributes to cell death and eDNA release, and that in its absence, *E. faecalis* adapts an alternate matrix to establish biofilms. Understanding the mechanism underlying the phenotypes observed in this study is the main focus of ongoing studies in our laboratory.

## 2.6 Acknowledgments

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# Chapter 3 - Influence of the alternate sigma factor RpoN on global gene expression and carbon catabolite repression in *Enterococcus faecalis* V583.

## 3.1 Abstract

In this study, we show that the Enterococcus faecalis alternate sigma factor σ54 (RpoN) and its cognate enhancer binding protein MptR are essential for mannose utilization and contribute to glucose uptake through the Mpt phosphotransferase system. To gain further insight into the E. faecalis RpoN-dependent genes, we performed microarray transcriptional analysis of strain V583 and an isogenic rpoN mutant grown in a chemically defined medium with glucose as the sole carbon source. In comparison to V583, 340 genes were differentially regulated in the rpoN mutant, and the majority of genes encoding known functions related to nutrient acquisition. A comparative analysis of the transcription profiles of the rpoN mutant and a previously characterized mptD mutant (a gene known to be RpoN dependent) revealed significant overlap in the differentially expressed genes, including genes with predicted cre sites, consistent with loss of repression by the major carbon catabolite repressor, CcpA. To determine if the inability to efficiently metabolize glucose/mannose as a carbon source affected infection outcome we utilized two distinct infection models, namely the rabbit endocarditis model and the murine model of catheter associated urinary tract infection (CAUTI). Here we show that the rpoN mutant is significantly attenuated in both infection models. We also identified a link with carbon catabolite control by examining a ccpA mutant in the CAUTI model. This analysis showed that the absence of carbon catabolite repression significantly attenuates bacterial tissue burden. Our data emphasize that proper regulation of central carbon metabolism contributes to in vivo growth of E. faecalis..

## 3.2 Introduction

Enterococci have recently emerged as one of the leading causes of biofilm associated infections, including endocarditis and catheter associated urinary tract infections (CAUTI) (1). Infective endocarditis (IE) in simple terms is the infection of damaged heart valves. Three Gram-positive genera (Streptococcus, Staphylococcus and Enterococcus) represent 80% of the total reported cases of IE. Enterococci account for 5-18% of the cases or approximately 3000 cases per year in the United States (2). Among enterococcal species, E. faecalis causes 80% of the reported endocarditis cases (3). The increasing emergence of antibiotic resistance in Enterococcus is of heightened concern while treating patients with IE due to the potential of recurrent infections with resistant species. Healthy endothelium is generally considered resistant to bacterial infection. Endothelial damage is brought about by a variety of conditions such as rapid blood flow associated with congenital heart disease, inflammation due to rheumatic carditis, degenerative valve lesions in the elderly or due to catheterization (4). Fibrin and platelets are deposited on the damaged endocardium and serve as an ideal focal point for bacterial adherence (5) resulting in bacterial colonization on the heart valves. These vegetations have the potential to embolize, disseminate and infect other organs leading to the onset of a systemic infection.

In recent decades, a growing number of enterococcal virulence factors have been identified that contribute to endocarditis, including aggregation substance (6, 7), pili (8, 9) and two metalloproteases, the extracellular gelatinase and the membrane protease Eep (10-12).

Like endocarditis, CAUTI is also thought to be a biofilm-mediated disease and enterococci now rank as the second leading cause of CAUTI in U.S. hospitals (1). A growing number of virulence factors are associated with enterococcal CAUTI, including the membrane protease Eep and the transcriptional regulator AhrC (13) as well as the chromosomally encoded pili and associated sortases (14-16). Not surprisingly, these aforementioned virulence factors also contribute to in vitro biofilm formation by E. faecalis (12, 13, 16).

A large gap in our knowledge base pertains to how organisms, including E. faecalis, survive in nutrient poor environments, as would be expected to occur within a host. While several studies have examined the transcriptional profile of cells grown in urine and serum to approximate the in vivo condition (17, 18), our understanding of preferred nutrients and how E. faecalis acquires them in the host is in its infancy.

Because carbon acquisition and its regulation have received much attention in the last decade and are now being proven to be key drivers to in vivo fitness in a growing number of bacterial pathogens(19-25), we addressed the question of whether altered carbon utilization might affect the ability of E. faecalis to grow in vivo using two biofilm mediated infection models (lupine endocarditis and murine CAUTI). The choice of these two models was based on the differences in nutrient availability at these two sites. We recently showed that the alternate sigma factor σ54 (RpoN) contributes to in vitro biofilm formation in E. faecalis as an rpoN deletion mutant was shown to have an altered biofilm matrix composition (26). The rpoN mutant was less efficient at autolysis (less eDNA in the matrix) and the biofilm became more labile to protease K treatment compared with

the parental strain. In addition, σ54 (RpoN) is also known to regulate several phosphotransferase systems (PTS) including the mannose/glucose permease Mpt (27) making it a good candidate to explore its contribution to in vivo fitness.

In contrast to other sigma factors, σ54 is unable to initiate open complex formation upon association with target DNA and the core RNA polymerase and requires the assistance of an enhancer binding protein (EBP). EBPs are ATPases that act as mechano-transcriptional activators to facilitate the transition of the inactive RpoN-RNA polymerase closed complex to active open complex essential for transcription initiation (28). In E. faecalis, four EBPs are encoded on the V583 genome (MptR, MpoR, MphR, LpoR) with a 5th (XpoR) disrupted by an insertion element (27). The genes encoding each of these EBPs are positioned immediately upstream of their respective sugar PTS genes (27).

The best characterized PTS in E. faecalis is the aforementioned Mpt mannose/glucose permease (27, 29, 30), owing to the fact that components of this PTS complex are known cellular receptors for class IIa and IIc bacteriocins (31). The Mpt PTS complex is comprised of four proteins, Mpt A-D. Like other characterized PTS systems (32), MptC and MptD are positioned as integral membrane proteins where they are thought to bind and channel the sugar inside the cell. Upon entry, the sugar is phosphorylated by MptB (EIIB), which is in turn phosphorylated by MptA (EIIA) in the protein complex. As an EIIA enzyme, MptA is thought to be phosphorylated by Hpr, which gains the phosphate from enzyme EI after phosphorylation of EI by phosphoenol pyruvate (PEP).

Recent work by Opsata et al. (30) characterized the transcriptional profile of a mutant of mptD that conferred resistance to pediocin PA-1, a known class IIa bacteriocin. These authors identified a number of differentially expressed genes in the mptD mutant that contained putative catabolite responsive elements (cre) sites (WTGNAANCGNWNNCW) based on similarity to consensus cre sites from Bacillus subtilis (33). In low G-C Gram-positive bacteria, cre sites on DNA are bound by a protein complex consisting of the carbon catabolite repressor CcpA and the phosphorylated Ser-46 form of Hpr (34). Cre sites are generally positioned within or immediately downstream of the target gene promoter where the bound CcpA-Hpr complex blocks transcriptional procession of the target gene.

Because σ54 has been shown to regulate the expression of various genes involved in metabolism and virulence in other bacteria (35-39), we performed a microarray transcriptional analysis to identify genes whose expression was differentially regulated in the E. faecalis rpoN mutant. This dataset allowed a comparative analysis with the mptD mutant profile reported by Opsata et al. (30); as σ54 is known to directly regulate the expression of the mpt operon. We also show that the absence of σ54 results in derepression of CcpA controlled genes in E. faecalis. We similarly observed that genes with cre sites that overlapped the promoter or were positioned downstream of the promoter region were up-regulated in the rpoN mutant.

Furthermore, as the array data indicated an increased expression level of catabolite repressed genes, we also evaluated a ccpA mutant in a murine model of CAUTI. To our knowledge, this report represents the first examination of the contribution of either σ54 (RpoN) or CcpA to in vivo fitness in E. faecalis.

Overall, this study provides important evidence linking basic metabolism with in vivo growth, and provides the rationale for several distinct pathways that could be targeted as a potential therapeutic for treating enterococcal infections.

## 3.3 Materials and Methods

### Bacterial Strains and growth conditions:

Bacterial strains used in this study are listed in Table 3-1. For propagation of plasmids, *Escherichia coli* ElectroTen-Blue from Stratagene was cultivated in Luria-Bertani (LB) broth supplemented with appropriate antibiotics whenever necessary. Unless otherwise mentioned, *E. faecalis* was cultured in Todd-Hewitt broth (THB; BD Biosciences) containing appropriate antibiotics. For antibiotic selection, chloramphenicol (Cm) at a concentration of 10µg/ml and 15µg/ml was used for *E.coli* and *E. faecalis* respectively.

**Table 3-1 *Enterococcus faecalis* strains used in this study.**

| Strain | Genotype or Description | Reference |
|---|---|---|
| V583 | Parental strain | Clinical isolate (70) |
| VI01 | V583Δ*rpoN* | (26) |
| VI40 | VI01::*rpoN* | (26) |
| MG07 | V583Δ*mptR* | This study |
| MG08 | V583Δ*lpoR* | This study |
| MG09 | V583Δ*mpoR* | This study |
| MG10 | V583Δ*mphR* | This study |
| EH01 | V583Δ*ccpA* | This study |
| VI70 | EH01Δ*ef0114* | This study |
| AH05 | EH01Δ*ef0362-61* | This study |
| AH10 | EH01Δ*ef2863* | This study |
| IH12 | EH01Δ*ef2863*Δ*ef0362-61* | This study |

### In-frame markerless deletion of activator proteins:

Using the temperature sensitive cloning vector, pLT06 (65), isogenenic in-frame deletion mutants of the four activator proteins were generated in *E.faecalis* V583. Upstream and downstream flaking regions of the targeted activator proteins were amplified using primers listed in Table 3-2. The primer pairs MptRP1/MptRP2 and MptRP3/MptRP4 were used to amplify flanking regions upstream and downstream of

*mptR* respectively. To facilitate cloning, primers MptRP1/ MptRP2 were designed with EcoRI/BamHI restriction sites respectively whereas MptRP3/MptRP4 were designed with BamHI/PstI sites respectively. For the construction of the insert, the amplified regions were digested with BamHI, ligated and re-amplified with MptRP1 and MptRP4. To generate pMG07 (*mptR* deletion vector), the amplified insert fragment was digested and ligated with EcoRI/PstI cut pLT06 cloning vector. The ligated vector and insert was electroporated into *E. coli* ElectroTen - Blue and correct constructs were identified by colony PCR. The construct was screened by restriction digest analysis and then electroporated into competent *E. faecalis* V583 cells. MG07 [V583Δ*mptR*] was subsequently generated as previously described (66) and confirmed by PCR using the primers MptR-Up and MptR-Down. A similar approach was used to create all the mutants used in this study (Table 3-1).

**Growth Assessment in Nutrient limiting conditions:**

Using a single colony of each strain, liquid cultures were started in THB. Bacterial cultures were grown at 37°C overnight. For growth analysis, overnight cultures were diluted 1:100 in complete defined medium (CDM) (40, 41) supplemented with a range of either glucose, mannose or N-acetyl glucosamine concentrations (10mM, 50mM, 100mM and 200mM). Growth was monitored for 12 hours in an Infinite M200 Pro plate reader (Tecan Trading AG, Switzerland) at 37°C with orbital shaking at 250 rpm. The experiment was performed with 5 internal replicates and repeated twice.

**Microarray analysis:**

Colony biofilms (67) were grown similar to those reported previously (68). Briefly, *E. faecalis* strains were grown overnight in 2mL of CDM cultures with 100 mM

glucose and 20μM hematin added, shaking at 150 rpm at 37°C. Overnight cultures were sub-cultured 1:1000 in the same growth conditions and allowed to grow to an OD of approximately 0.2 then diluted in fresh medium to an A600 of 0.1 (approximately $10^8$ cells/mL). 10μL of this solution was added in three discrete spots to a 25 mm, 0.2 μm pore polycarbonate membrane affixed to the surface of CDM+1% agarose solid medium in a 100mm Petri dish. Plates were incubated ~16 hours at 37°C and polycarbonate membranes were moved to unoccupied areas of the dish and further incubated at 37°C for an additional 4 hours. Membranes were then transferred to 1.5mL microcentrifuge tubes containing 1mL of RNALater (Ambion) and vortexed until no visible cells remained attached to the membrane surface. Cells were pelleted by centrifugation (2 mins at 10,000xg), supernatants removed and cell pellets stored for RNA purification. RNA purification and Affymetrix microarray preparations were performed as described elsewhere (68, 69).

Array analyses were performed using RMA analysis through the University of Oklahoma Bioinformatics Core Facility (http://www.ou.edu/microarray/). 2Sigma analysis was used to determine the significant difference of fold change with a 95% confidence interval. Significant fold changes were considered for values greater than 3 fold for the *rpoN* mutant vs. wild-type and greater than 1.8 fold for the complement vs. wild-type comparisons.

**Table 3-2 Oligonucleotides used in this study,**

| Primer | Sequence (5'-3') |
|---|---|
| LpoRP1 | GAGAGAATTCGATTGACAAGTTAAAAAAACG |
| LpoRP2 | CTCTGGATCCCTTCCGAGATTTCATGGAATC |
| LpoRP3 | GAGAGGATCCGAAGCGGAAGTTGCGCAT |
| LpoRP4 | CTCTCTGCAGGATAGCCAATGTACCATTCC |
| LpoR-Up | ATGCAAATTGCTGAAGTTGCT |
| LpoR-Down | GCTAGCTCATTCACATTGTAT |
| MphRP1 | GAGAGGATCCGCTTCAATATCATCAATCGTTTA |
| MphRP2 | CTCTTCTAGATAACATAAAATCACCTCTCGC |
| MphRP3 | GAGATCTAGAACGGAGTTTAACAACGCAGA |
| MphRP4 | CTCTCCATGGATGCAACCTAAAAGAATCGCT |
| MphRUp | GTGATGAAGTGAAATGCTTTC |
| MphRDown | CTTGAAAGCAACGAGGTTCC |
| MpoRP1 | GAGAGGATCCGAGCACATTCGTCAACGAATG |
| MpoRP2 | CTCTCTGCAGGTGAAACTGTTTTAACGCTTGATG |
| MpoRP3 | GAGACTGCAGAAGGCTTAATTCAAACGGAAATG |
| MpoRP4 | GCTGAGACAGCATGCCTGAA |
| MpoRUp | GCAGCAGTAACGATGGAAGA |
| MpoRDown | GTGGAGGTGACTCCGAGATA |
| MptRP1 | GAGAGAATTCATTTTGTCGCTAGTTGGCTTG |
| MptRP2 | CTCTGGATCCAATCCGCTCATTTTTGATGG |
| MptRP3 | GAGAGGATCCACCGAAATTGATACACTAGAT |
| MptRP4 | CTCTCTGCAGAAAGATCATTGCACCAGATTG |
| MptRUp | CGAGAGGAAGGCTTGAATGTC |
| MptRDown | CGATGCAATGGCTTCTTGCA |
| CcpAP1 | GAGAGAATTCCAGAAGGTTCCAAGTAGCTG |
| CcpAP2 | CTCTGGATCCATTTGCCTCTCTAGCAACATC |
| CcpAP3 | GAGAGGATCCACAGTTGTTTTACCTTATGGAATTG |
| CcpAP4 | CTCTCTGCAGGACTTATGCTGATGGTCGTG |
| CcpA-Up | GCTGTAACACCAGGTTTCAC |
| CcpA-Down | GATCGTCAAGTTGGTTCTACG |
| EF0114P1 | GAGAGAATTCCGTGAAGTTACACTGAACAAG |
| EF0114P2 | GAGAGGATCCTTTCTGCACTCCGTTCAT |
| EF0114P3 | GAGAGGATCCCGCTAATGGATACGTTCTCG |
| EF0114P4 | CTCTCTGCAGCTTGATTGACAGCTTCCAGC |
| EF0114-Up | GATGGGAAGCCAGTAATAAGAG |

| | |
|---|---|
| EF0114-Down | CGTGCTGGCATATAATTGACC |
| EF0362-61P1 | GAGAGAATTCGATGGATAGTATCTTTCATGCC |
| EF0362-61P2 | CTCTGGATCCAACAAAACTAAAAGCCAGGACG |
| EF0362-61P3 | GAGAGGATCCGCGACACGTTATAGTAACCTTG |
| EF0362-61P4 | CTCTCTGCAGCGACTAAAGTGTTAATGAACAG |
| EF0362-61 Up | GCGATATTACGAGATACATG |
| EF0362-61 Down | TGAAGTCATGGCAGTGACGT |
| EF2863P1 | GAGAGAATTCTCGATACGTTGGCCATCTTC |
| EF2863P2 | CTCTGGATCCGGTCTTGCGAAGGACGCTC |
| EF2863P3 | GAGAGGATCCCGAACTATTCACCAACTGTTC |
| EF2863P4 | CTCTCTGCAGATCCTCTAAAGTTGAAGAGACG |
| EF2863-Up | CTGATCCGGAACTGTTACTG |
| EF2863-Down | TATCATTAACTGCTTGTCGG |
| EF0019f | ATTGGCGGAATGAGTTTCAG |
| EF0019r | GCTGGCGTTATTTTCTTTGC |
| EF0082f | TGTCAAAGGTGAACGCTACG |
| EF0082r | TGAGGACTTGGGCTTTCTTG |
| EF2223f | AACATCGGCGGTATCTTCAG |
| EF2223r | TGGTTCAATTCGACGAACAA |
| EF0891f | TGTCACAGCAAGCAGGAATC |
| EF0891r | TTCCACAAAAGGAACGGAAG |
| EF0013f | TTGGATGATGCAGAACGAAG |
| EF0013r | CTTGCAAGCCAGCAGTCAT |

**Table 3-3 Plasmids used in this study**

| Plasmid | Description | Reference |
|---|---|---|
| pLT06 | Parent Deletion vector; chloramphenicol resistance | (19) |
| pMG07 | pLT06 + engineered *mptR* deletion | This study |
| pMG08 | pLT06 + engineered *lpoR* deletion | This study |
| pMG09 | pLT06 + engineered *mpoR* deletion | This study |
| pMG10 | pLT06 + engineered *mphR* deletion | This study |

## Quantitative real time (q-RT) PCR:

Synthesis of cDNA was performed using Superscript II reverse transcriptase (Invitrogen) from RNA templates utilized in the above transcriptome studies following

the manufacturer's instructions. Random hexamer primers (Qiagen) were used in the initial synthesis reaction. The primers used in q-RT PCR analysis are listed in Table 3-2. The q-RT PCR reaction was performed with 100 ng of cDNA and 300 nM of each primer using Quanta SyBr Green fast mix (VWR) on a Biorad MJ MiniOption Real time PCR system. Following denaturation at 95°C for three minutes, the q-RT PCR reaction was set for 50 cycles with 95°C for 10 seconds, 60°C for 20 seconds and 72°C for 10 seconds. Differential gene expression was calculated using the ΔΔCt method using the threshold cycle values for the gene of interest (*ef0019, ef0082. ef2223, ef0891*) and the endogenous control [*ef0013*(*dnaB*)].

## RNAse B Assay

The glycosyl hydrolase activity was determined using the high mannose containing glycoprotein RNAse B (New England Biolabs) as the substrate. The bacteria were grown overnight at 37°C in CDM containing 20mM glucose and 20μM heme, shaking at 250rpm. To assess the glycosyl hydrolase activity , 5μg of RNAse B was incubated with the filtered supernatants in 1X G7 Reaction buffer (New England Biolabs) at 37°C overnight. EndoH (New England Biolabs) used as a positive control. The samples were separated on 15% SDS-PAGE followed by staining with Coomassie blue.

## Chitinase Assay

The fluorogenic glycanase substrate 4-methylumbelliferyl b-N, N', N''-triacetylchitotrioside (Sigma) was used to test for chitinase activity in the filtered supernatants of the bacterial cultures grown overnight in CDM containing 20mM glucose and 20μM heme. Briefly, 50μl of the filtered supernatant was incubated with 50μM substrate in 25mM citrate buffer (pH 6) for 15 minutes. The fluorescence intensity

(excitation 360 nm, emission 450 nm) was measured in an Infinite M200 Pro plate reader (Tecan Trading AG, Switzerland). Chitinase activity is expressed as relative fluorescence calculated by dividing the fluorescence emitted at 450 nm by the OD 600nm of the culture.

## Animal Models

All the procedures in the rabbit model to study experimental endocarditis and murine model for catheter associated urinary tract infection were performed in compliance with Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals.

### Experimental Endocarditis and determination of bacterial burden

Left sided endocarditis was induced in New Zealand white rabbits (Charles River Laboratories International, Inc.) by introducing a polyethylene catheter with an internal diameter of 0.86 mm (Becton Dickinson, MD) followed by injection of bacterial cultures ($10^7$ CFU ; *E.faecalis* V583(70) and VI01(26)) via marginal ear vein after 24 hours of catheterization as previously described (10). In preparation for injections, bacterial cultures grown to stationary phase were washed twice and diluted to achieve a concentration of $10^7$ CFU/ml in sterile saline. Group of eight rabbits were injected with each bacterial strain (V583 and VI01) and two negative controls were injected with sterile saline. The rabbits were monitored for 48 hrs after bacterial inoculation and euthanized by intraperitoneal administration of sodium pentobarbital. Immediately after euthanasia, a cardiac stick was performed to determine bacterial CFU in blood at the time of sacrifice. Bacterial burden in the heart, liver, spleen and kidney was assessed by plate

count following previously described protocol (10) and expressed as log10 CFU/gm of tissue

**Murine model for Catheter Associated Urinary Tract infection (CAUTI)**

Catheter associated urinary tract infections using six-seven week old female wild-type C57BL/6 mice. The mice were anesthetized by isoflurane inhalation and a 5-6 mm platinum cured silicone implant tubing (Renasil Sil025; Braintree Inc) was transurethrally placed in the urinary bladder of the mice as previously described (14). Post implantation, the mice were injected with 50 µl inocula of either sterile PBS or bacterial suspension (~ $2x10^7$ CFU) by transurethral catheterization. The mice were monitored for 48 hours post implantation and infection. They were euthanized by cervical dislocation after inhalation of isoflurane. To determine the degree of infection, kidneys and bladder were harvested aseptically and their bacterial burden was determined. Also, the silicone implant tubing was retrieved from the bladder and the bacterial burden enumerated using THB media.

## Bioinformatics and Statistical Analysis:

Cre (Catabolite responsive elements) sites were identified upstream of the differentially expressed genes using the pattern analysis option in Regulatory Sequence Analysis Tools (http://rsat.ulb.ac.be/rsat/).

The statistical analysis of the chitinase assay and bacterial burden determined in the various organs in the endocarditis study and CAUTI was performed using GraphPad Prism 5 software (San Diego, CA).

Statistical significance was measured using a non-parametric T-test (Mann-Whitney Test).

## 3.4 Results

### RpoN and MptR regulate glucose uptake and are required for mannose uptake:

We have previously demonstrated that deletion of *rpoN* has no growth defect in an enriched medium such as tryptic soy broth (26) or Todd-Hewitt broth. However, several sugar uptake systems in *E.faecalis* are known to be regulated by RpoN (27). Therefore we hypothesized that in a chemically defined media with specific carbohydrates as the major carbon nutrient source, RpoN might be indispensable for the bacteria. To this end, we assessed the fitness of the mutant in a chemically defined medium (CDM) (40, 41) with varying concentrations of sugars. Figure 3-1 shows the growth pattern in CDM with glucose as the sole carbon source. As evident from the growth curves, the mutants (*rpoN* and *mptR*) grew poorly in glucose concentrations at 10 mM and 50 mM glucose. At 100 mM glucose, the wild-type and mutants grew at similar rates into the early exponential phase and then the mutants grew more slowly than the wild-type, presumably as glucose levels were depleted below an optimal growth threshold for the mutants. In higher concentrations of glucose (200 mM) the mutants did not achieve maximal growth as compared to the parental strain, but were capable of sustaining growth equivalent to the parent through the mid-exponential phase, again likely attributed to a threshold glucose concentration being exhausted followed by growth delay in the mutant.

Strikingly, the growth defect of the mutants in CDM + mannose was not compensated by supplementation with increasing concentration of mannose as was

observed with glucose (Figure 3-2), suggesting that the Mpt PTS controlled by $\sigma^{54}$ and MptR represents the sole mannose transporter in the cell. To ascertain the sugar specificity of the PTS controlled by MptR and $\sigma^{54}$, we grew cells in CDM + N-acetylgucosamine (NAG) as a sugar source. It is clear from Figure 3-3 that the absence of either $\sigma^{54}$ or MptR does not impede the growth on N-acetylglucosamine as the major carbon source. In addition to NAG, galactose was also tested and no observed differences were noted between the growth pattern of the mutants and the wild type (data not shown). Together the growth curve analysis confirms that RpoN and MptR regulate the glucose/mannose specific permease.

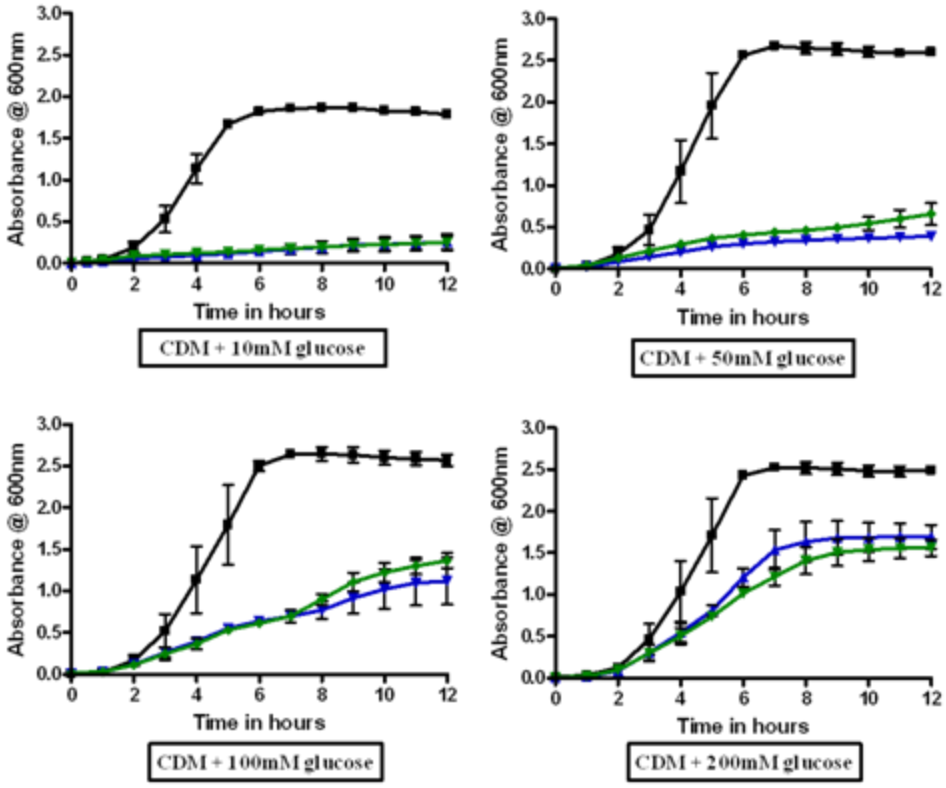


**Figure 3-1 Growth of *E.faecalis* in chemically defined medium with glucose as the carbohydrate source.**

The glucose concentrations are indicated below each panel. Each graph is the average of the replicates with the standard error of the mean show. The growth curves are shown in black (V583), blue (VI01 [Δ*rpoN*], and green [Δ*mptR*].

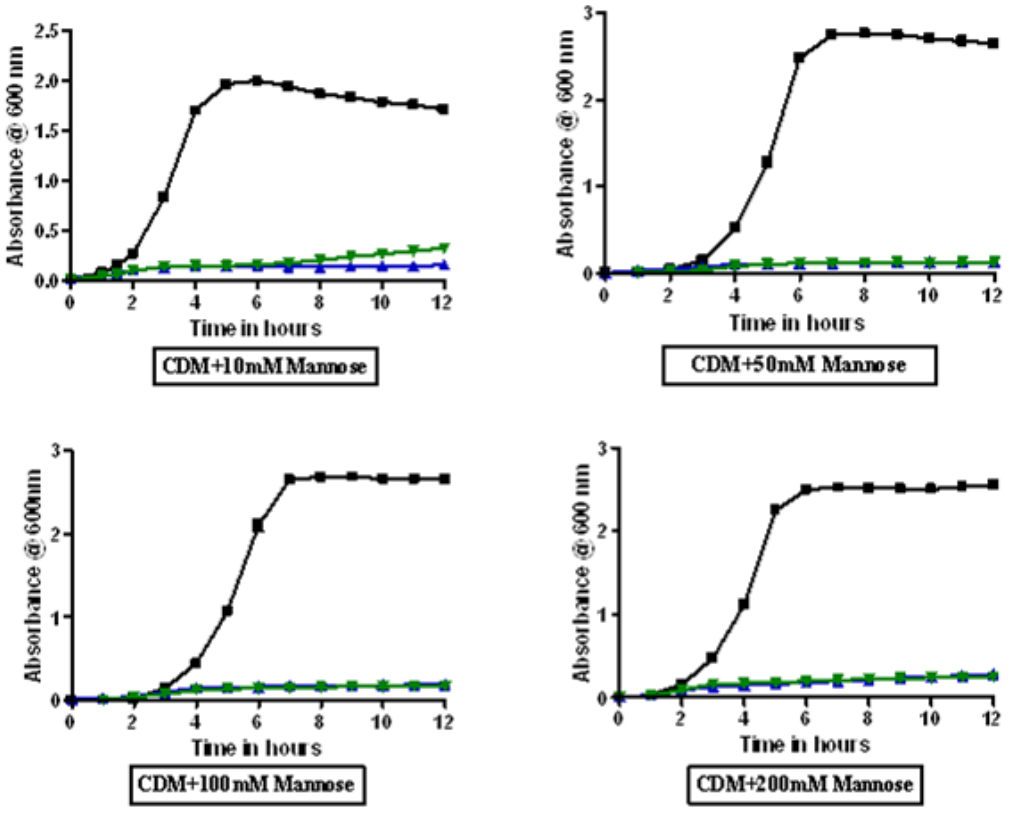


**Figure 3-2 Growth of *E.faecalis* in chemically defined medium with mannose as the principle carbon source.**

The concentration of mannose is indicated below each panel. Each graph is the average of ten replicates wirh the standard error of the mean shown. The growth curves are shown in black (V583), blue (VI01 [*ΔrpoN*]) and green (MG07 [Δ*mptR*]).

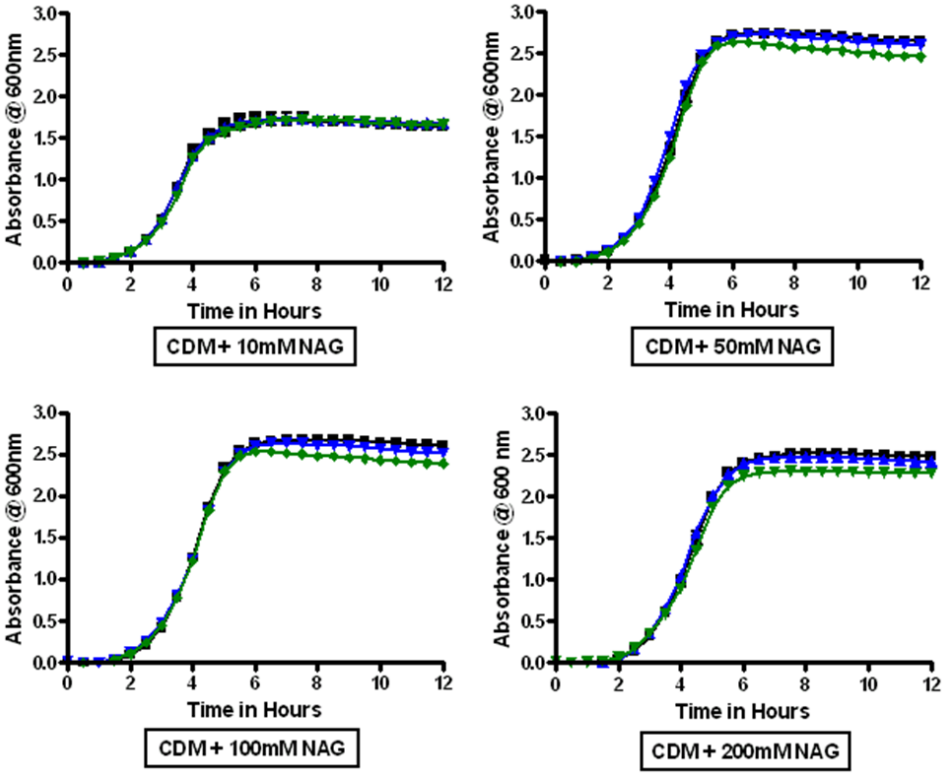


**Figure 3-3 Growth of *E.faecalis* in chemically defined medium with N-acetylglucosamine as the sole carbon source.**

The concentration of N-acetlyglucoamine is indicated below each panel. Each graph is the average of ten replicates with the standard error of mean shown. The growth curves are shown in black (V583), blue (VI01 [Δ *rpoN*] and green (MG07 [Δ*mptR*]).

**Transcriptional Analysis of *E. faecalis* V583 Δ*rpoN* (VI01):**

RpoN is known to impact the global gene expression profile in several bacteria and the genes it regulates are functionally divergent (38, 42, 43), indicative of functional divergence between the Gram-negative and Gram-positive bacteria under study. In *E.faecalis*, the only genes that have been described to be regulated by RpoN are the four putative PTS related operons (27). On the basis of the phenotypes we observed in our previously published work (26) and the present effect of RpoN on growth in glucose and mannose, we hypothesized that in *E.faecalis*, RpoN would have an impact on a larger regulatory gene network. Therefore, using DNA microarrays of *E. faecalis* strain V583, the transcriptional profile of VI01(Δ*rpoN*) was compared to that of the parental strain V583 and an *rpoN* complemented strain (VI40) grown on polycarbonate filter discs placed on 1% agar growth medium containing CDM + 100 mM glucose. The filters were passed twice over a 20 hour period to fresh sections of the agar medium to allow transcriptional analysis in the early exponential phase of growth, before glucose became exhausted below a level that would sustain adequate growth of the mutant. The overall cellular yield of all strains grown under these conditions was similar. The microarray data were taken from two biologic replicates and differentially expressed genes were identified by RMA analysis. The raw array data has been deposited in the NCBI Gene Expression Omnibus (http//:www.ncbi.nlm.nih.gov/projects/geo/) under accession number GSE40237.

Compared to the parental strain and the *rpoN* complemented strain, the *rpoN* mutant displayed 340 genes that were differentially regulated (Figure 3-4, SI Table 1) out of which 34% are hypothetical genes or proteins with no known functions, 17% are genes related to transport and binding proteins, 14% are energy metabolism related and 12% are

genes that encode PTS proteins. Of the 340 differentially expressed genes in VI01, 255 genes were upregulated and 85 genes were downregulated (≥ 3-fold) compared to the parental and complemented strain. Of the four PTS systems known to be regulated by RpoN, two were significantly downregulated (*mpt* and *lpo;* average 134 fold and 4 fold respectively) and no significant difference in expression was observed in the *mpo* and *mph* PTS systems.

## Quantitative real time PCR confirmation of the microarray data

To validate the microarray data, we performed qRT-PCR on a set of genes that represented both up (*ef0891* and *ef2223*) and down regulated (*ef0019* and *ef0083*) genes in an *rpoN* mutant. This list of validated genes included those whose expression profile was found be in common with the *mptR/mptD* transcriptome (*ef0019* and *ef2223*) (30) as well as those genes that were unique to *rpoN* (*ef0082* and *ef0891*). We confirmed the reduced expression of *ef0019* (*mptB)* and *ef0082* in an *rpoN* mutant and also showed that *ef0891* and *ef2223* were significantly upregulated in the mutant background relative to the parental strain V583 consistent with the levels of gene expression observed in the microarray (Table 3-4)

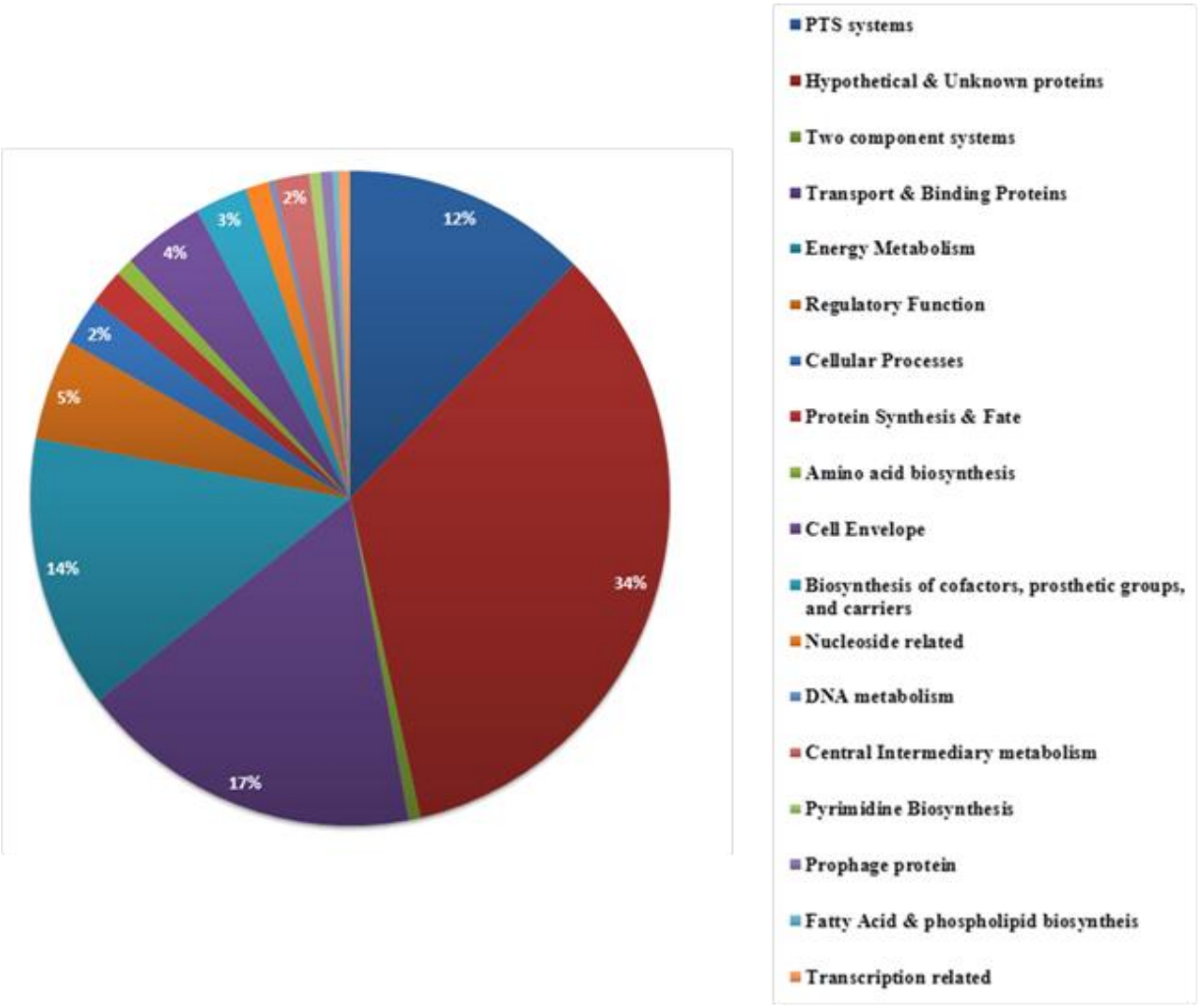


**Figure 3-4 Pie chart depicting the functional distribution of 340 differentially expressed genes in VI01 in comparison to the wildtype V583.**
The number in each functional category is the percentage of the differentially expressed genes.

**Table 3-4 Microarray expression profile validated with quantitative Real time PCR**

| Gene | Function | Fold change | |
|---|---|---|---|
| | | Microarray Analysis | Quantitative Real time PCR |
| **EF0082** | Major facilitator family transporter | -6.82 | -8.64 |
| **EF0019** | EIIB component of *mpt* operon | -152.11 | -190.90 |
| **EF0891** | Aspartate aminotransferase, putative | 47.93 | 140.86 |
| **EF2223** | ABC transporter, permease protein | 257.25 | 1175.42 |

### Catabolite repression elements (*cre*) in RpoN-dependent genes.

In order to gain additional insights into the genes regulated by RpoN which might be independent of its influence on Mpt, we compared the transcription profiles of the *rpoN* mutant with the published data for an *mptD* mutant (30). Both studies were performed with mutants in the V583 lineage, thus eliminating strain background biases. Other aspects of the experimental conditions were more variable (M17-Glucose vs. CDM-Glucose). Despite these varied growth conditions, we felt that this comparison would provide a rough estimate of those genes that might be unique to RpoN, and independent of the Mpt system. 58.9% of the genes that were differentially regulated in the *mptD* mutant were also found to be significantly altered in gene expression in the *rpoN* mutant but that represented only 31.2% of the differentially expressed genes in *rpoN* mutant. It was surprising to note that 42.1% of genes in the *mptD* mutant appeared to be independent of RpoN as the *mpt* operon is regulated by RpoN (Figure 3-5). This again could be attributed to the different experimental growth conditions used in the two studies.

The increased expression levels of genes encoding several sugar uptake systems as well as ABC transporters in the transcription profile of the *rpoN* mutant suggested a

loss of catabolite control. In order to determine the presence of such regulation, we searched the regions upstream of the differentially regulated genes in the *rpoN* mutant to identify *cre* sites. *Cre* sites have also been reported for genes whose expression was up regulated in an *mptD* mutant (30), most of these were also found to be upregulated in VI01 (Supplemental Table 3-2). We used the *cre* consensus sequence suggested for *E. faecalis* 5`-WTGWAARCGYWWWCW-3`(30) as our pattern query using the Regulatory Sequence Analysis Tool (http://rsat.ulb.ac.be/) to identify *cre* sites upstream of the genes that were unique to the *rpoN* mutant but that were not shown to be differentially regulated in the *mptD* mutant (30). Of the 154 *rpoN* mutant specific genes that were upregulated, *cre* sites were present in the promoter regions of 22 genes (14.28 %) (Supplemental Table 3-5). Of the 80 downregulated genes, one gene (*sdhB-2*) had a predicted *cre* site in the region downstream of the predicted promoter. The identified *cre* sites matched the exact consensus (5'- WTGWAARCGYWWWCW-3') or possessed a one base mismatch from the consensus sequence.

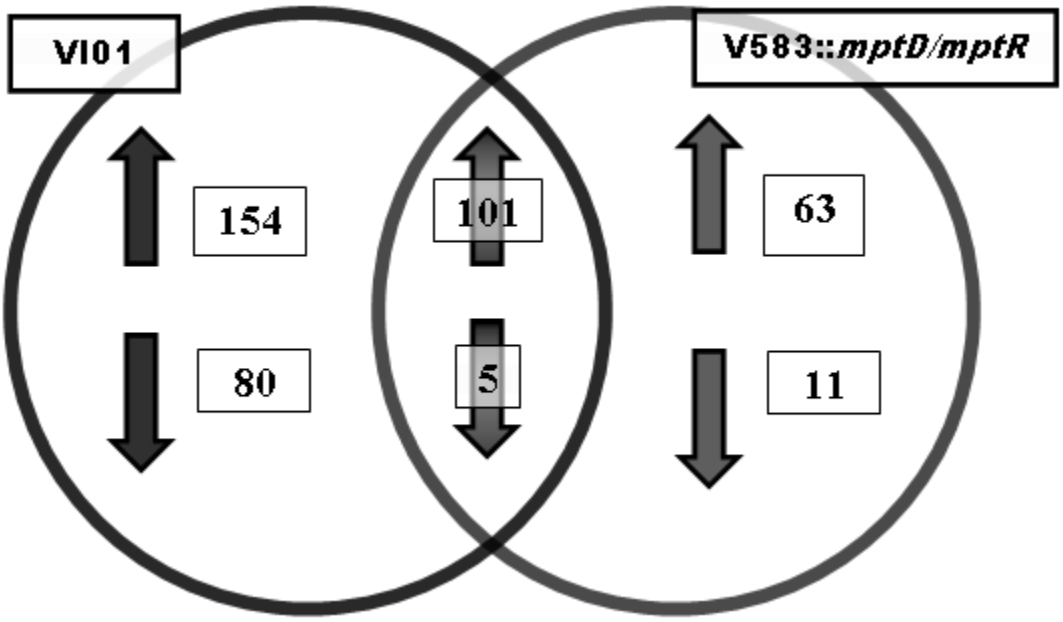


**Figure 3-5 Venn diagram illustrating the overlap between differentially expressed genes in VI01 (Δ*rpoN*) and in the *mptD* insertion mutant in *E. faecalis* V583.**

**Carbon catabolite repression regulates glycozyl hydrolase activity.**

Three gene clusters (*ef0114*[*ndoE*], *ef2863*, and *ef0362-61*) encoding putative endoglycosyl hydrolases were highly upregulated in the *rpoN* mutant (SI Table 1) and putative *cre* sites were identified downstream of their promoter regions (SI tables 4 and 5), indicating that these genes are under catabolite control. In order to test the activity of their gene products, the three gene clusters encoding the predicted glycosyl hydrolases were deleted in the *ccpA* mutant background to eliminate the influence of catabolite repression on the expression of these glycosyl hydrolase genes. The glycosyl hydrolase activity present in the filtered supernatant of the various strains was tested for the ability to breakdown the high mannose containing glycoprotein RNAseB, as well as the chitin fluorogenic substrate 4-methylumbelliferyl b-N, N', N''-triacetylchitotrioside (4-MU-chitotrioside). EF0362-61 has been examined previously for chitinolytic activity based on homology to chitin-binding proteins (EF0362=ChiB) and chitinases (EF0361=ChiA) (44, 45). Since the genes in the *ef0362-61* operon were highly upregulated [84 and 69-fold, respectively] in the Δ*rpoN* mutant and a *cre* site was found upstream of the *ef0362* gene, we hypothesized that deletion of the *ef0362*-61 operon would affect the chitinolytic activity in *E.faecalis* V583. As evidenced in Figure 3-6, culture supernatant from V583 possessed chitinolytic activity (~ 3900 rfu), but this activity was significantly increased ~ 2-fold (~ 7800 rfu) in the *ccpA* mutant (EH01). Surprisingly, AH05 [Δ*ccpA*Δ*ef0362-61*] was not significantly different from EH01 [Δ*ccpA*], suggesting that EF0362-61 does not contribute in a significant way to the breakdown of 4-MU-chitotrioside under these assay conditions. Although the purified proteins EF03612-61 have been previously shown to exhibit chitinolytic activity (44, 45), our results demonstrate that the gene products of *ef0362-61* contribute little activity to the overall breakdown of the fluorogenic chitin

substrate. The absence of correlation with chitinolytic activity for EF0362-61 may be due in part to the fact that multiple glycosyl hydrolases are known to target 4-MU-chitotrioside including lysozyme (46). Further analysis showed that both EF0114 and EF2863 contribute to the chitinolytic activity, as deletion of either gene (VI70 [Δ*ef0114*] and AH10 [Δ*ef2863*]) resulted in a significant reduction in chitinolytic activity compared to the *ccpA* control.

The gene products for EF0114 and EF2863 have previously been characterized as possessing activity that removes high mannose and hybrid-type N-linked glycans from glycoproteins, including RNAse B (47, 48) and human IgG (48). To confirm these previous observations, we used the glycoprotein RNase B to assess glycolytic activity. The removal of the N-linked glycoconjugate (deglycosalation) reduces the apparent molecular weight of RNase B and results in a downward size shift on SDS-PAGE and this was confirmed with the EndoH control. As the wild-type still produces some hydrolase activity, a mixture of glycosylated and deglycosylated forms of RNase B was observed (Figure 3-7). However, only the deglycosylated form of RNase B was observed after treating with supernatant from strain EH01 (Δ*ccpA*). As shown in Figure 3-7, deletion of *ef2863* in the *ccpA* mutant background (AH10) results in the elimination of nearly all of the lower deglycosylated form of RNase B, suggesting that EF2863 contributes substantial glycosyl hydrolase activity towards this substrate, and is consistent with published observations (47).

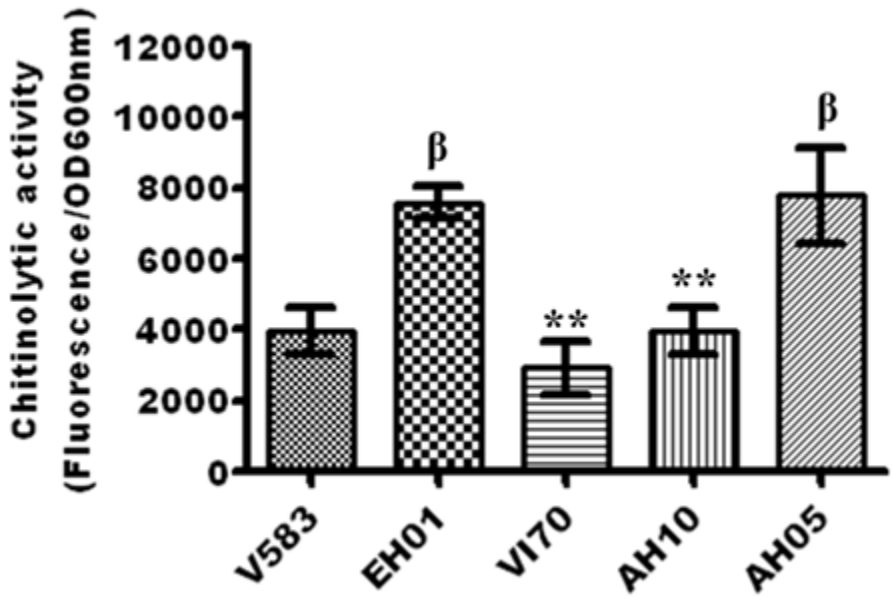


**Figure 3-6 Chitinolytic activity detected in the culture supernatant of wild-type V583, EH01 [Δ*ccpA*] and the endoglycosyl hydrolase mutants in the ΔccpA background: VI70 [Δ*ccpA*Δ*ef0114*], AH10 [Δ*ccpA*Δ*ef2863*] and AH05 [Δ*ccpA*Δ*ef0362-61].***

Chitinase activity was determined using the fluorogenic substrate 4-methylumbelliferyl b-N, N', N''-triacetylchitotrioside and is expressed as relative fluorescence calculated by dividing the fluorescence emitted at 450 nm by the absorbance of the culture at OD 600 nm. The statistical significance is calculated using Mann-Whitney test and is indicated as: β, p value less than 0.001 relative to V583; **, $p<0.05$ relative to Δ*ccpA*. The experiment was repeated 3-5 times and the error bars depict standard error of the mean.

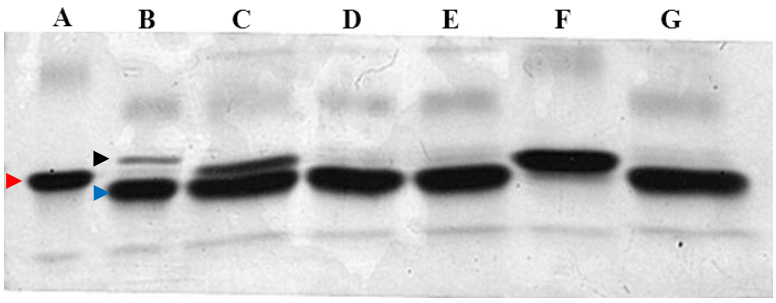


**Figure 3-7 Endoglycosidase activity against the RNase B substrate in the culture supernatants of V583, ΔccpA and the glycosyl hydrolase mutants in the ccpA mutant background.**
(A) RNAse B control; (B) RNase B treated with EndoH (New England Biolabs); (C-F) RNase B treated with supernatants from (C) V583; (D) EH01 [Δ*ccpA*]; (E) VI70 [Δ*ccpA*Δ*ef0114*], (F) AH10 [Δ*ccpA*Δ*ef2863*] and (G) AH05 [Δ*ccpA*Δ*ef0362-61*]. Deglycosylation of RNase B substrate results in increased mobility through an SDS-PAGE resulting in a size shift. The red arrow depicts the fully glycosylated form of RNase B, the blue arrow shows the fully deglycosylated form of RNase B, and the black arrow shows the location of the EndoH enzyme in the control treated lane.

## Role of RpoN in enterococcal virulence.

We have previously shown that *E.faecalis* V583 Δ*rpoN* (VI01) exhibited resistance to autolysis and formed altered biofilm structures in which the matrix was more protease K labile (26). On the basis of these results and the fact that RpoN plays a key role in regulating the uptake of mannose and glucose, we hypothesized an important contribution of RpoN to the *in vivo* fitness of *E. faecalis*. To determine the role of enterococcal RpoN in virulence we used two models of infection: rabbit endocarditis (10) and a murine model of catheter associated urinary tract infection (14). In the rabbit endocarditis model, the parental strain (V583) was compared to its isogenic *rpoN* mutant (VI01) for the ability to establish infective endocarditis, and mean bacterial burden on the valve, heart, liver, spleen and kidneys as well as in the blood was assessed. As observed in figure 6, a significant reduction ($p < 0.05$) in the mean bacterial burden was noted in the

vegetation, blood, spleen and kidneys of the rabbits infected with VI01 in comparison to the parental strain suggesting that *rpoN* contributes to infective endocarditis in rabbits (Figure 6). The histopathological staining of VI01 infected heart valves also revealed fewer bacteria and increased heterophil influx in contrast to the parental V583 strain (data not shown) emphasizing the importance of a functional RpoN in establishing infection. Due to the cost associated with the rabbit model of endocarditis, the *ccpA* mutant was not included in the study. We did, however, assess the contribution of both RpoN and CcpA in the murine model of CAUTI. Similar to the observation in the endocarditis model, the *rpoN* mutant was significantly attenuated in CAUTI as observed by the reduced number of bacteria ($p<0.05$) recovered from the bladder and catheter of the *rpoN* infected mice compared to the wild-type strain V583 (Fig. 7). In comparison to the wild-type and the *rpoN* mutant, the *ccpA* mutant was more highly attenuated for *in vivo* fitness as the mean bacterial numbers for the *ccpA* mutant isolated from the catheter were 15-fold lower than the *rpoN* mutant and 500-fold lower than the wild-type ($p<0.005$). The mean bacterial burden in the bladder for the *ccpA* mutant was 90-fold lower than the wild-type and 13-fold lower than the *rpoN* mutant ($p<0.005$).

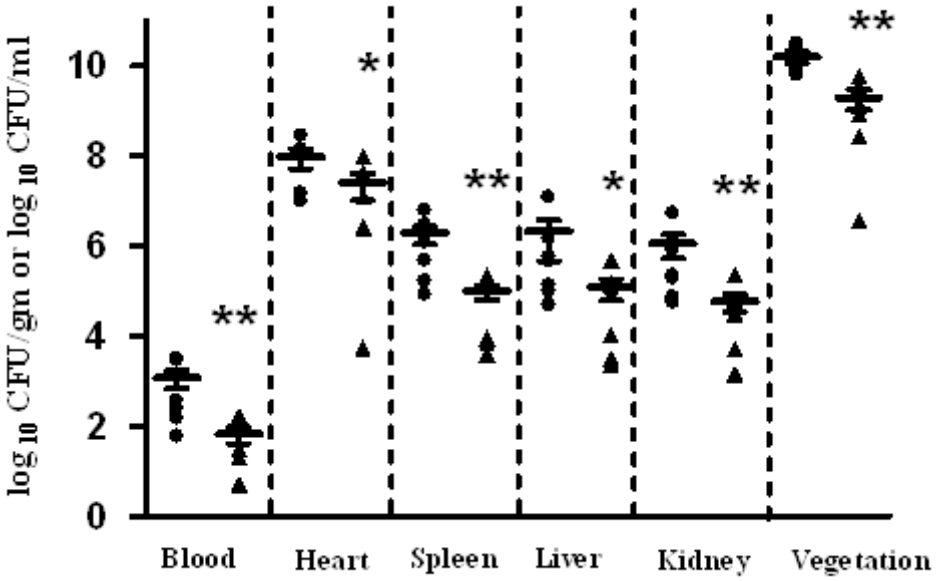


**Figure 3-8 Enterococcal burden in rabbits infected with *E. faecalis* strains.**
Rabbits were euthanized post-infection and organs were harvested to enumerate bacterial burden. Bacterial burden for wild-type V583 (●) and VI01 [Δ*rpoN*] (▲) are expressed as log10 CFU/gram of harvested tissue. The horizontal line represents the median value for each group. Mann-Whitney test was used to determine significance and is indicated as follows: **, significant p-value less than 0.05 relative to V583; *, significant p value less than 0.1 relative to V583.

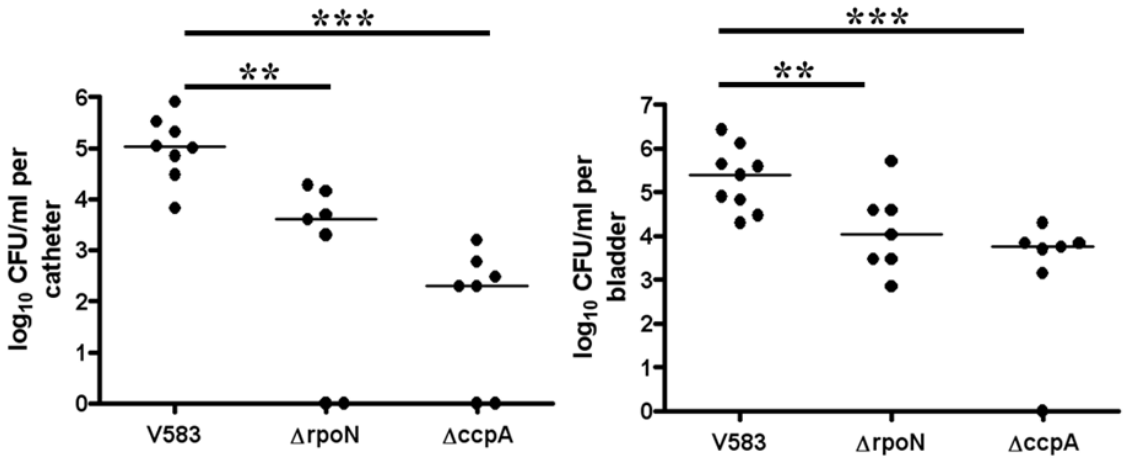


**Figure 3-9 RpoN and CcpA contribute to enterococcal virulence in the murine model of CAUTI.**

Female C57BL/6 mice were euthanized after 48 hours post infection. Bacterial burden is expressed in logarithmic scale for wild type V583, VI01 [Δ*rpoN*] and EH01 [Δ*ccpA*] in (A) implanted catheters retrieved from the mice and (B) homogenized bladders. The horizontal bar represents the median of each group of mice. Statistical significance as determined by Mann-Whitney test is represented as follows: **, significant p value less than 0.05 relative to V583; ***, significant p value less than 0.005 relative to V583.

## 3.5 Discussion

Binding sites for a consensus sequence (TTGGCACNNNNNTTGCT) recognized by sigma 54 (RpoN) have been identified upstream of genes predicted to encode sugar PTS systems (Mpt, Mpo, Lpo and Mph) in *E. faecalis*. The mesentericin Y105 (a class IIa bacteriocin) resistance phenotype of an *rpoN* mutant (29) has also been linked to the RpoN-dependent regulation of the *mpt* operon (27). In the present study, the *mptBADC* operon was approximately 100-fold downregulated in an *rpoN* mutant when grown in the presence of 100 mM glucose compared to the parental strain V583, and likely explains the growth defects observed in CDM media with glucose or mannose as the major carbon source. This is also in agreement with the observation that an *mptR* mutant is also impaired for growth on mannose and glucose, whereas the other genes encoding EBPs grow similar to the parental strain in either glucose or mannose (Supplemental Figure 3-1). It also suggests that glucose uptake and metabolism in *E. faecalis* is primarily dependent on the Mpt permease. We have previously shown that VI01 (Δ*rpoN*) is resistant to 2-deoxyglucose (a toxic homologue of glucose; 2DG) (26) and mutants resistant to 2DG have been shown to localize mutations within *mptR* or the *mptBACD* genes (27, 49) thus strengthening the notion that Mpt is the major glucose uptake system in *E. faecalis*.

Mpt has been the most extensively studied PTS system regulated by RpoN in *E. faecalis* (27, 30, 50). In a study designed to identify EBPs for $\sigma^{54}$ in *E. faecalis* (27), Héchard et al. described five activator proteins in the *E. faecalis* V583 genome. However, in the genome sequence immediately downstream of one of the activators, XpoR, the $\sigma^{54}$ consensus binding site was not detected and no putative PTS operon was found. Moreover, the gene *xpoR* was shown to be interrupted by an insertion element. Our

microarray analysis revealed the presence of a putative PTS operon (*ef3210-ef3213*) upstream of *xpoR* which was approximately six-fold downregulated in an *rpoN* mutant suggesting that this operon is induced in the presence of RpoN under the conditions tested here. Furthermore, a sigma 54 binding site with 100% match to the consensus sequence was identified upstream of *ef3210*, the first gene of the operon implying a potential role for RpoN in the expression of this operon. Comparison of the *xpoR* sequence in other *E. faecalis* genomes revealed that only V583 possesses the transposon insertion, and that this insertion likely dissociated the domains of XpoR into two separate gene products (EF3214 and EF 3216). EF3214 is a DEAH-box family ATP dependent helicase which is a characteristic of the *E. faecalis* RpoN activator proteins while EF3216 is a putative DNA binding protein. Most activator proteins are located upstream of the genes they regulate but in some instances activators are also found downstream of the genes they regulate (51). Also some organisms encode functional EBPs that lack the DNA binding domains (52-54) and experimentally truncated activator proteins that lack one of the two domains have also been shown to stably aid in open complex formation (51). Whether these two proteins EF3214 and EF3216 still function as an XpoR equivalent remains to be determined.

One of the common themes observed with the transcriptional profile of the *rpoN* and *mptD* mutants was the significant up-regulation of genes with predicted *cre* sites, suggestive of an involvement with the major catabolite control protein CcpA. Amongst the cohort of *cre*-regulated genes, we focused our attention on three gene clusters encoding endo glycosyl hydrolases (endoglycosidases). Endoglycosidases are enzymes that function to release oligosaccharides from glycoproteins or glycolipids, and do not

require the presence of a terminal sugar residue to affect cleavage, thus distinguishing them from known exoglycosidases.. The endoglycosidases also serve to release available nutrients from the host, and can therefore be thought of as nutrient acquisition systems. The genes for each of the endoglycosidases under study (*ef0114* [*ndoE*]; *ef0362-61* [*chiBA*] and *ef2863*) were ~ 50-fold upregulated in the *rpoN* deletion mutant compared to V583. Both EF0114 and EF2863 have previously been shown to possess endoglycosidase activity (47, 48, 55) and purified EF0362-61 has been shown to possess chitinolytic activity (45). Isogenic deletion mutants of the aforementioned endoglycosidases in a *ccpA* mutant background revealed that EF2863 is the major endoglycosidase for the high-mannose containing glycoprotein RNase B, whereas EF0114 appears to only play a minor role in this context. Work by Collin and Fischetti (48) demonstrated glycosyl hydrolase activity towards RNase B for EF0114, that they designated EndoE. The relative contribution of EndoE to RNase B deglycosylation in the context of other glycosyl hydrolases produced by *E. faecalis* was however not assessed in their study (48). While we did not observe a substantial contribution from EF0114 in the RNase B assay, we were able to detect a small accumulation of deglycosylated RNase B in strain IH12 (Δ*ccpA*Δ*ef0362-61*Δ*ef2863*), suggesting that this activity might be attributed to EF0114 (data not shown). Collin et al. also established a role for EndoE from *E. faecalis* in the deglycosylation of human IgG, suggesting that EndoE might play a role in immune evasion. Recent work by Flock et al. (56) demonstrated that an endoglycosidase targeting IgG served as a viable vaccine candidate against *Streptococcus equi* infection, and that antibodies against EndoSe were also cross-protective against *Streptococcus pyogenes* challenge. The *E. faecalis* EndoE (EF0114) shares 42% sequence similarity

with the endoglycosidases from both *S. equi* and *S. pyogenes*. Whether EndoE also contributes to immune evasion by *E. faecalis* awaits further study, and will be a component of ongoing studies in the lab.

In *Listeria monocytogenes* (57, 58) and *Legionella pneumophila* (59), the chitinase gene products (ChiA and ChiB) contribute to infection outcome in mouse models. EF0362 shares 47% sequence identity with the ChiB from *L. monocytogenes* and EF0361 shares 65% sequence identity with ChiA. On the basis of the sequence conservation between these resident Gram-positive enteric species, we anticipate that EF0362-61 will likely play a role in nutrient acquisition in the intestinal environment, but the nature of the substrates that it works on remain to be defined.

In order to assess the role of RpoN in *E. faecalis* virulence, we used a rabbit model of endocarditis and CAUTI in mice. On the basis of bacterial burden, the *rpoN* mutant was significantly attenuated in comparison to the wild type in both the models tested. The carbon catabolite protein A (CcpA) mutant in *E.faecalis* V583 was also tested for its ability to cause an infection in the catheter associated urinary tract infection model as several genes that were differentially regulated in the *rpoN* and *mptD* mutant appeared to be regulated by CcpA due to the presence of *cre* sites proximal to predicted promoter regions in the regulated genes. In the catheter associated UTI model, the *ccpA* mutant was also attenuated in comparison to the wild type. We previously showed that deleting *rpoN* in *E. faecalis* does not impact the growth of the organism in enriched medium such as TSB or THB (26). However, *in vivo* the organism will face more hostile growth conditions, as preferable nutrient sources are kept at growth limiting conditions (i.e. glucose is present in normal human serum at 4-8 mM (60) and similar blood glucose

levels are also observed in rabbits (61). The ability of microorganisms to adapt and grow in such limiting conditions will determine their infection potential. Our assessment of the *rpoN* mutant for its growth ability in a chemically defined medium with varying glucose concentration revealed that in lower glucose concentration (10 and 50 mM), the *rpoN* mutant grows poorly (as measured by absorbance at 600 nm) in comparison to the parental strain. This growth defect is less pronounced but still readily distinguishable from the parent strain at higher glucose concentrations (100-200 mM). The poor growth under limiting glucose concentrations likely explains the *rpoN* mutant's attenuated phenotype *in vivo*.

In contrast to the poor growth observed for the *rpoN* mutant in CDM + glucose, a *ccpA* mutant displays growth defects at higher glucose concentrations (50-200 mM), indicative of a metabolic overload (Supplemental Figure 3-2). A similar growth defect is also observed for the *ccpA* mutant under high concentrations of mannose (Supplemental Figure 3-3) or N-acetylglucosamine (Supplemental Figure 3-4) as the principal carbon source. The disregulation of normal metabolism that would occur in a *ccpA* mutant likely explains its attenuated phenotype and is consistent with a growing body of evidence for the role of CcpA in Gram-positive bacterial pathogenesis (24, 62, 63). . So despite the fact that nutrient acquisition systems are overexpressed in a *ccpA* mutant, including endoglycosidases that would likely contribute to immune evasion (EndoE), this mutant performs poorly *in vivo*. We hypothesize that a redox imbalance in the *ccpA* mutant would contribute to hypersensitivity to oxidative stress, as this has been recently observed in a *S. pyogenes* colonization model (63). We will be investigating this line of evidence in future studies. Collectively, the observations with the *rpoN* and *ccpA*

mutants both *in vitro* and *in vivo* suggest that regulated metabolism is key to successful colonization and infection. Too little nutrient acquisition of essential sugars in the case of *rpoN* and overactive metabolism in *ccpA,* results in poor fitness compared to the parental strain.

We present a model for how RpoN and CcpA interface in the cell to regulate central carbon metabolism. In the presence of high glucose concentrations (100-200), the growth defect in both the *mptR* and *rpoN* mutants appeared to be partially rescued suggesting that at higher concentrations, glucose is being transported via other uptake systems. The upregulation of several PTS systems and transport proteins in the *rpoN* mutant gives credence to this hypothesis. Of note is gene *ef1516* which encodes an EIIABC multi-domain PTS component, and is 14.22 fold upregulated in the *rpoN* mutant and was also found to be upregulated in the *mptD* mutant (30). EF1516 shares 57% sequence similarity and 39% identity with PtsG, a known glucose specific transporter in *Bacillus subtilis* (64) that possesses all the functional domains of the Enzyme II components of a typical PTS in a single protein, and perhaps represents the ancestral glucose transporter. We hypothesize that Mpt might confer a growth advantage to *E. faecalis* enabling the organism the ability to outcompete other microflora for limited supplies of glucose. This enhanced growth may also be the reason why the Mpt PTS is targeted by class IIa and IIc bacteriocins to prevent outgrowth of the organism by competitors. In the absence of a functional Mpt, the ancestral glucose transporter EF1516 could substitute a redundant role, but only when glucose is in excess. In the absence of RpoN there could be an alteration in the relative abundance of the glycolytic intermediates glucose-6-phosphate and fructose-1,6,-bisphosphate within the cell

indicating insufficient carbon flow which would trigger the phosphatase activity of the bifunctional enzyme HprK/P that dephosphorylates the PTS intermediate Hpr(Ser-P) (Figure 3-10). A dephosphorylated Hpr no longer binds to the catabolite control protein A (CcpA), and the dissociation of the Hpr(Ser-P) – CcpA complex would alleviate the repression of transcription of *cre*-regulated genes (39). In the Δ*rpoN* mutant, the carbon catabolite repression (CCR) may be perpetually derepressed, and the array data with respect to *cre* site containing genes is consistent with this interpretation. The CCR derepression can also be attributed in part to the RpoN-regulated *mpt* operon because *cre* sites have also been identified upstream of genes that are also differentially expressed in an *mptD*/*mptR* mutant transcriptional microarray (30) (Supplemental Table 3-4).

In addition to PTS systems and transport proteins, several proteins with regulatory functions were also differentially expressed (Supplemental Table 3-1). The differential expression of several genes in the *rpoN* mutant may be attributed to their dependence on these transcription regulators.

Thus the transcription profile of *rpoN* mutant clearly shows that this sigma factor contributes to several diverse functions in enterococcal biology in addition to controlling the four known PTS systems. Approximately 10% of the genome is differentially regulated by RpoN and understanding these complex metabolic circuits controlled by RpoN that feed into the virulence potential of *E. faecalis* is the focus of ongoing studies.

## 3.6 Acknowledgment

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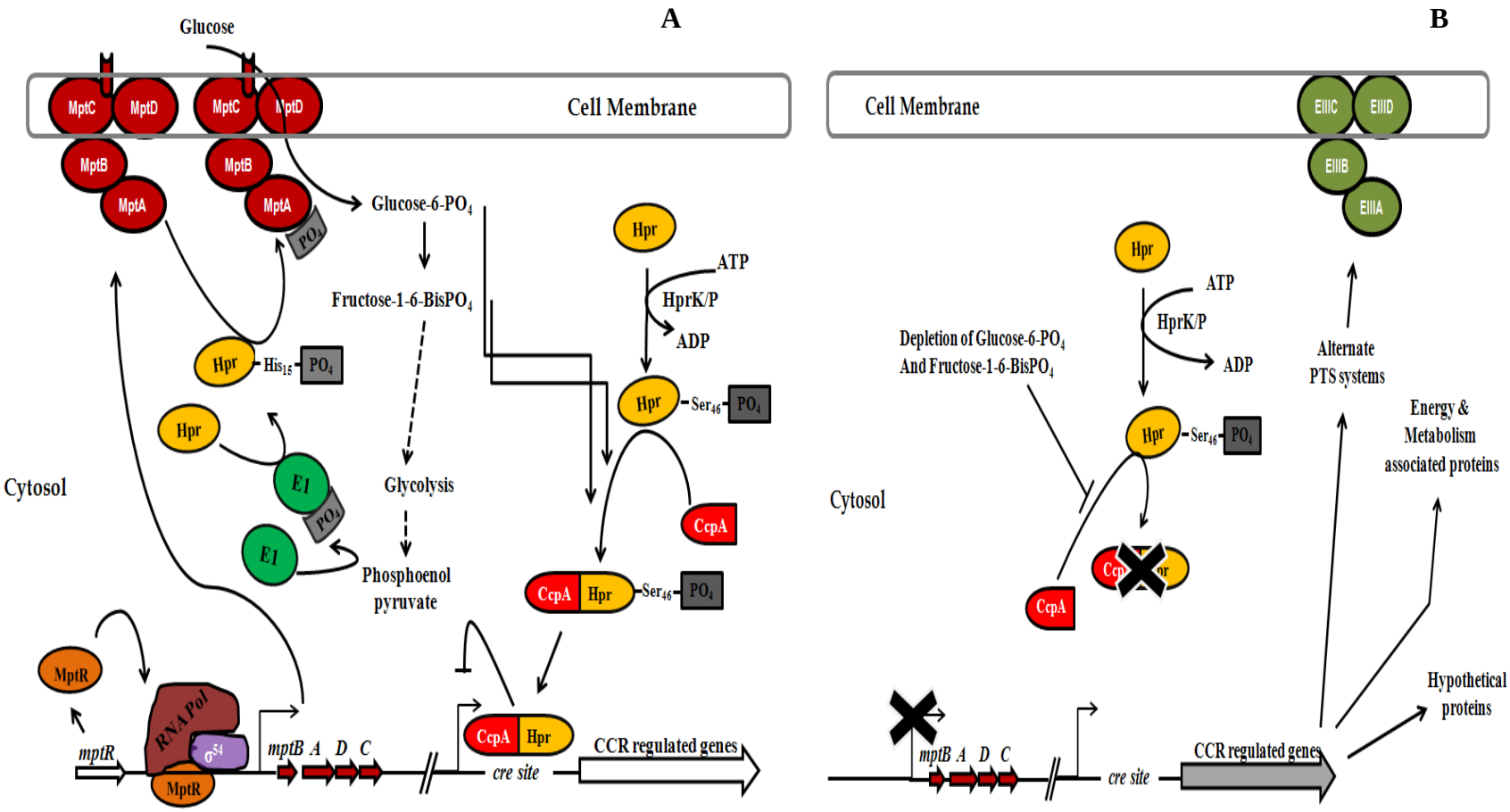


**Figure 3-10 Model for sigma 54 mediated carbon catabolite repression (CCR).**

Panel A: Regulation of CCR dependent genes in wild type *E. faecalis* V583; Panel B Alleviation of CCR in an *rpoN* mutant or *mptR/mptD* mutant.

CcpA, Catabolite Control Protein A; mpt, Mannose PTS; cre, catabolite responsive elements; Hpr, Histidine containing phosphocarrier protein; EI, Enzyme I; EII, Sugar specific multidomain enzyme II; HprK/P, Bifunctional ATP-dependent Hpr kinase/phosphatase; ATP, Adenosine triphosphate; ADP, Adenosine diphosphate.

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## 3.8 Supplemental Tables

**Supplemental Table 3-1 Differentially expressed genes in VI01[Δ*rpoN* mutant] compared to V583 expressed as fold change.**

| Gene | Function | Fold change |
|---|---|---|
| **HYPOTHETICAL PROTEINS AND PROTEINS WITH UNKNOWN FUNCTIONS** | | |
| EF0054 | hypothetical protein | **58.53** |
| EF0713 | conserved hypothetical protein | **58.00** |
| EF0384 | hypothetical protein | **34.97** |
| EF0711 | conserved hypothetical protein | **32.33** |
| EF1394 | conserved hypothetical protein | **29.49** |
| EF0383 | protein FdrAconserved hypothetical protein | **29.34** |
| EF0714 | hypothetical protein | **29.26** |
| EF0664 | hypothetical protein | **27.97** |
| EF1227 | conserved hypothetical protein | **21.74** |
| EF2569 | conserved hypothetical protein | **21.29** |
| EF2570 | aldehyde oxidoreductase, putative | **21.17** |
| EF1226 | oxidoreductase, putative | **21.02** |
| EF2566 | conserved hypothetical protein | **19.48** |
| EF2568 | aminotransferase, class V | **18.70** |
| EF2220 | conserved hypothetical protein | **18.53** |
| EF2236 | conserved hypothetical protein | **18.24** |
| EF0678 | acetyltransferase, GNAT family | **16.96** |
| EF0743 | hypothetical protein | **16.44** |
| EF0392 | hypothetical protein | **15.39** |
| EF2564 | conserved domain protein | **14.57** |
| EF0108 | C4-dicarboxylate transporter, putative | **13.04** |
| EF2565 | conserved hypothetical protein | **12.22** |
| EF2563 | conserved hypothetical protein | **11.38** |
| EF3142 | 6-phosphogluconate dehydrogenase family protein | **8.83** |
| EF2576 | hypothetical protein | **8.60** |
| EF1237 | conserved hypothetical protein | **8.26** |
| EF3105 | hypothetical protein | **7.74** |
| EF0390 | N-acyl-D-amino-acid deacylase family protein | **7.61** |
| EF1912 | ROK family protein | **7.19** |
| EF2237 | lipoprotein, putative | **7.17** |
| EF0405 | hydrolase, haloacid dehalogenase-like family | **7.16** |
| EF1229 | conserved hypothetical protein | **6.71** |
| EF1035 | lipoprotein, putative | **6.62** |
| EF1066 | hexapeptide-repeat containing-acetyltransferase | **6.47** |
| EF3087 | hypothetical protein | **6.39** |
| EF1228 | hypothetical protein | **6.29** |

| | | |
|---|---|---|
| EF0389 | membrane protein, putative | **6.19** |
| EF1919 | acetyltransferase, GNAT family | **5.87** |
| EF0377 | ankyrin repeat family protein | **5.56** |
| EF2441 | conserved hypothetical protein | **5.50** |
| EF0052 | hypothetical protein | **5.41** |
| EF3088 | hypothetical protein | **5.34** |
| EF1075 | acetyltransferase, GNAT family | **5.25** |
| EF0808 | hypothetical protein | **5.24** |
| EF1077 | acetyltransferase, GNAT family | **5.09** |
| EF3103 | membrane protein, putative | **5.04** |
| EF1503 | fructose-1,6-bisphosphatase, putative | **4.95** |
| EF3326 | conserved hypothetical protein | **4.85** |
| EF3102 | hypothetical protein | **4.82** |
| EF2268 | conserved hypothetical protein | **4.73** |
| EF2440 | celC-related protein | **4.71** |
| EF1359 | conserved hypothetical protein | **4.70** |
| EF1360 | dihydroxyacetone kinase family protein | **4.70** |
| EF1535 | conserved hypothetical protein | **4.69** |
| EF1061 | N-acyl-D-amino-acid deacylase family protein | **4.53** |
| EF2581 | oxidoreductase, pyridine nucleotide-disulfide family | **4.41** |
| EF0062 | 5-nucleotidase family protein | **4.40** |
| EF2577 | aspartateornithine carbamoyltransferase family protein | **4.30** |
| EF1230 | hypothetical protein | **4.28** |
| EF3092 | glyoxalase family protein | **4.16** |
| EF2996 | conserved hypothetical protein | **4.13** |
| EF1407 | hypothetical protein | **4.07** |
| EF1800 | conserved hypothetical protein | **4.01** |
| EF1361 | dihydroxyacetone kinase family protein | **3.84** |
| EF3032 | conserved hypothetical protein | **3.77** |
| EF0459 | glucokinase regulator-related protein | **3.77** |
| EF_B0056_x_at | hypothetical protein | **3.72** |
| EF3158 | hydrolase, haloacid dehalogenase-like family | **3.69** |
| EF1062 | N-acyl-D-amino-acid deacylase family protein | **3.68** |
| EF3008 | conserved hypothetical protein | **3.56** |
| EF0244 | acetyltransferase, GNAT family | **3.54** |
| EF0460 | conserved hypothetical protein | **3.48** |
| EF2994 | aminotransferase, class V | **3.41** |
| EF1362 | conserved domain protein | **3.41** |
| EF1933 | hypothetical protein | **3.39** |
| EF1512 | conserved hypothetical protein | **3.37** |
| EF0113 | hypothetical protein | **3.35** |
| EF0382 | conserved hypothetical protein | **3.32** |
| EF1322 | conserved hypothetical protein | **3.31** |

| | | |
|---|---|---|
| EF3101 | conserved domain protein | **3.26** |
| EF1959 | conserved domain protein | **3.23** |
| EF3009 | conserved hypothetical protein | **3.20** |
| EF3007 | conserved hypothetical protein | **3.15** |
| EF1239 | conserved hypothetical protein | **3.08** |
| EF1707 | glycosyl hydrolase, family 38 | **3.07** |
| EF3010 | conserved hypothetical protein | **3.05** |
| EF2933 | DNA-binding protein,putative | **3.03** |
| EF1528 | hypothetical protein | **3.01** |
| EF3245 | cell-envelope associated acid phosphatase | **-3.10** |
| EF2944 | hypothetical protein | **-3.10** |
| EF0708 | conserved hypothetical protein | **-3.16** |
| EF1198 | conserved hypothetical protein | **-3.26** |
| EF0672 | hypothetical protein | **-3.31** |
| EF0341 | hypothetical protein | **-3.33** |
| EF1168 | hypothetical protein | **-3.40** |
| EF3207 | dihydrouridine synthase family protein | **-3.64** |
| EF1968 | conserved hypothetical protein | **-3.66** |
| EF2500 | GcvH family protein | **-3.69** |
| EF1841 | HD domain protein | **-3.79** |
| EF1199 | conserved hypothetical protein | **-4.04** |
| EF0443 | LysM domain protein | **-4.25** |
| EF0747 | conserved hypothetical protein | **-4.26** |
| EF2899 | oxidoreductase, pyridine nucleotide-disulfide family | **-4.35** |
| EF0419 | conserved hypothetical protein | **-4.54** |
| EF0634 | decarboxylase, putative | **-4.90** |
| EF2750 | conserved hypothetical protein | **-4.95** |
| EF1258 | hypothetical protein | **-5.02** |
| EF1812 | hypothetical protein | **-5.03** |
| EF3244 | hypothetical protein | **-5.05** |
| EF2216 | conserved domain protein | **-5.66** |
| EF2214 | glyoxylase family protein | **-6.21** |
| EF2215 | conserved hypothetical protein | **-7.27** |
| EF2896 | hypothetical protein | **-7.39** |
| EF0083 | hypothetical protein | **-7.77** |
| EF1231 | conserved hypothetical protein | **-10.70** |
| EF0802 | hypothetical protein | **-29.61** |
| | | |
| **TRANSPORT AND BINDING PROTEINS** | | |
| EF2223 | ABC transporter, permease protein | **257.25** |
| EF2221 | ABC transporter, substrate-binding protein | **128.09** |
| EF2222 | ABC transporter, permease protein | **114.80** |
| EF0892 | amino acid ABC transporter, ATP-binding protein | **40.84** |

| | | |
|---|---|---|
| EF0893 | amino acid ABC transporter, amino acid-bindingpermease protein | **39.81** |
| EF1927 | glycerol uptake facilitator protein | **36.40** |
| EF0385 | major facilitator family transporter | **27.11** |
| EF1398 | molybdenum ABC transporter, permease protein | **26.30** |
| EF1397 | molybdenum ABC transporter, molybdenum-binding protein | **23.85** |
| EF2234 | sugar ABC transporter, sugar-binding protein, putative | **17.24** |
| EF0387 | sodiumdicarboxylate symporter family protein | **16.14** |
| EF1233 | ABC transporter, permease protein | **13.83** |
| EF1920 | C4-dicarboxylate anaerobic carrier | **13.23** |
| EF1399 | molybdenum ABC transporter, ATP-binding protein, putative | **13.10** |
| EF0938 | ABC transporter, ATP-bindingTOBE domain protein | **12.53** |
| EF1234 | ABC transporter, substrate-binding protein, putative | **12.52** |
| EF1345 | sugar ABC transporter, sugar-binding protein | **11.76** |
| EF1344 | sugar ABC transporter, permease protein | **10.11** |
| EF1232 | ABC transporter, permease protein | **8.63** |
| EF1207 | citrate carrier protein, CCS family | **8.54** |
| EF2233 | ABC transporter, permease protein | **8.46** |
| EF2992 | major facilitator family transporter | **7.38** |
| EF3327 | citrate transporter | **7.22** |
| EF0556 | xylose isomerase | **7.01** |
| EF1343 | sugar ABC transporter, permease protein | **6.65** |
| EF1400 | cadmium-translocating P-type ATPase | **6.43** |
| EF3104 | ABC transporter, ATP-binding protein | **5.46** |
| EF2442 | phosphate transporter family protein | **5.45** |
| EF3000 | cytosinepurines, uracil, thiamine, allantoin permease family protein | **4.56** |
| EF1513 | pheromone binding protein | **4.23** |
| EF2232 | ABC transporter, permease protein | **4.20** |
| EF3109 | peptide ABC transporter, ATP-binding protein | **4.07** |
| EF3108 | peptide ABC transporter, permease protein | **3.84** |
| EF3110 | peptide ABC transporter, ATP-binding protein | **3.53** |
| EF1060 | pheromone binding protein | **3.49** |
| EF0063 | pheromone binding protein, putative | **3.42** |
| EF3107 | peptide ABC transporter, permease protein | **3.40** |
| EF3106 | peptide ABC transporter, peptide-binding protein | **3.35** |
| EF0243 | branched-chain amino acid transport system II carrier protein | **3.22** |
| EF0429 | TRAP dicarboxylate transporter, DctP subunit | **3.16** |
| EF0807 | pheromone binding protein, putative | **3.15** |
| EF2593 | ABC transporter, ATP-bindingpermease protein | **-3.05** |

| | | |
|---|---|---|
| EF0569 | potassium-transporting ATPase, subunit C | **-3.16** |
| EF3069 | formatenitrite transporter family protein | **-3.16** |
| EF0804 | amino acid ABC transporter, amino acid-binding protein | **-3.19** |
| EF0568 | potassium-transporting ATPase, subunit B | **-3.37** |
| EF0805 | amino acid ABC transporter, ATP-binding protein | **-3.56** |
| EF3004 | sulfate transporter familySTAS domain protein | **-3.57** |
| EF0806 | amino acid ABC transporter, permease protein | **-3.72** |
| EF0420 | drug resistance transporter, EmrBQacA family protein | **-3.84** |
| EF0567 | potassium-transporting ATPase, subunit A | **-3.95** |
| EF1304 | magnesium-translocating P-type ATPase | **-4.12** |
| EF0635 | amino acid permease family protein | **-5.11** |
| EF1053 | ABC transporter, ATP-binding protein | **-5.23** |
| EF0636 | Na+H+ antiporter | **-6.49** |
| EF0082 | major facilitator family transporter | **-6.82** |
| EF1814 | drug resistance transporter, EmrBQacA family protein | **-8.11** |
| EF1192 | aquaporin Z | **-11.87** |
| EF1054 | ABC transporter, permease protein | **-17.50** |

| **CELL ENVELOPE** | | |
|---|---|---|
| EF0362 | chitin binding protein, putative | **83.75** |
| EF0361 | chitinase, family 2 | **69.31** |
| EF0114 | glycosyl hydrolase, family 20 | **48.20** |
| EF1657 | membrane protein, putative | **5.80** |
| EF0673 | membrane protein, putative | **4.05** |
| EF2662 | choline binding protein | **3.50** |
| EF2627 | teichoic acid glycosylation protein, putative | **-3.11** |
| EF1340 | pheromone cAM373 precursor lipoprotein | **-3.16** |
| EF2746 | dltD protein | **-3.45** |
| EF0468 | LemA family protein | **-3.71** |
| EF2749 | D-alanine-activating enzyme, putative | **-3.83** |
| EF2748 | basic membrane protein DtlB | **-4.01** |
| EF2747 | D-alanyl carrier protein | **-4.07** |
| EF0746 | penicillin-binding protein, putative | **-11.48** |

| **ENERGY METABOLISM** | | |
|---|---|---|
| EF2863 | endo-beta-N-acetylglucosaminidase | **62.34** |
| EF1929 | glycerol kinase | **53.22** |
| EF1928 | alpha-glycerophosphate oxidase | **43.44** |
| EF0253 | aldehyde dehydrogenase | **35.88** |
| EF2562 | Flavodoxin | **19.95** |
| EF0106 | carbamate kinase | **19.07** |
| EF1068 | aldose 1-epimerase | **18.97** |

| | | |
|---|---|---|
| EF0677 | phosphoglucomutasephosphomannomutase family protein | **18.13** |
| EF0386 | carbamate kinase | **16.90** |
| EF0104 | arginine deiminase | **15.83** |
| EF1661 | branched-chain alpha-keto acid dehydrogenase, E3 component, dihydrolipoamide dehydrogenase | **14.95** |
| EF1658 | branched-chain alpha-keto acid, E2 component, dihydrolipoamide acetyltransferase | **14.28** |
| EF3135 | mannonate dehydratase, putative | **13.37** |
| EF3134 | 2-dehydro-3-deoxyphosphogluconate aldolase4-hydroxy-2-oxoglutarate aldolase | **10.43** |
| EF2559 | pyruvate flavodoxinferredoxin oxidoreductase family protein | **9.99** |
| EF0388 | ureidoglycolate dehydrogenase | **9.27** |
| EF1659 | branched-chain alpha-keto acid dehydrogenase, E1 component, beta subunit | **8.46** |
| EF1349 | glycosyl hydrolase, family 13 | **7.96** |
| EF2235 | glucuronyl hydrolase, putative | **7.81** |
| EF1824 | glycosyl hydrolase, family 31fibronectin type III domain protein | **6.82** |
| EF0900 | aldehyde-alcohol dehydrogenase | **6.69** |
| EF1206 | malate dehydrogenase, decarboxylating | **6.26** |
| EF2579 | diaminopropionate ammonia-lyase, putative | **6.21** |
| EF1236 | acetyl xylan esterase, putative | **5.82** |
| EF3325 | sodium ion-translocating decarboxylase, biotin carboxyl carrier protein | **5.29** |
| EF1347 | glycosyl hydrolase, family 13 | **5.18** |
| EF1660 | branched-chain alpha-keto acid dehydrogenase, E1 component, alpha subunit | **5.14** |
| EF1662 | butyrate kinase | **5.13** |
| EF1805 | glycosyl hydrolase, family 35 | **4.81** |
| EF2575 | carbamate kinase | **4.52** |
| EF0271 | glycosyl hydrolase, family 1 | **4.33** |
| EF0291 | glycosyl hydrolase, family 1 | **4.06** |
| EF0551 | glycosyl hydrolase, family 31 | **3.89** |
| EF2265 | carbohydrate kinase, pfkB family | **3.88** |
| EF2264 | 4-deoxy-l-threo-5-hexosulose-uronate ketol-isomerase | **3.87** |
| EF1238 | glycosyl hydrolase, family 3 | **3.79** |
| EF3157 | glycosyl hydrolase, family 65 | **3.78** |
| EF2272 | glucuronyl hydrolase, putative | **3.74** |
| EF1348 | glucan 1,6-alpha-glucosidase, putative | **3.61** |
| EF2709 | glycosyl hydrolase, family 2 | **3.56** |
| EF1158 | N4-(beta-N-acetylglucosaminyl)-L-asparaginase, putative | **3.52** |
| EF1071 | galactose-1-phosphate uridylyltransferase | **3.33** |
| EF2646 | glycerate kinase, putative | **3.32** |

| | | |
|---|---|---|
| EF1069 | Galactokinase | **3.17** |
| EF2722 | L-serine dehydratase, iron-sulfur-dependent, alpha subunit | **-3.19** |
| EF2721 | L-serine dehydratase, iron-sulfur-dependent, beta subunit | **-3.77** |

| **PTS SYSTEMS** | | |
|---|---|---|
| EF2213 | PTS system, IIBC components | **23.30** |
| EF3138 | PTS system, IID component | **17.41** |
| EF3137 | PTS system, IIB component | **16.00** |
| EF1516 | PTS system, IIABC components | **14.22** |
| EF3136 | PTS system, IIA component | **12.08** |
| EF0553 | PTS system, IID component | **11.35** |
| EF0554 | PTS system, IIB component | **9.60** |
| EF1529 | PTS system, IIC component, putative | **8.56** |
| EF1803 | PTS system, IIC component | **8.28** |
| EF_A0067_at | PTS system, IIABC components | **7.93** |
| EF3139 | PTS system, IIC component | **7.55** |
| EF1802 | PTS system, IID component | **7.35** |
| EF3031 | PTS system, IIB component | **7.03** |
| EF0270 | PTS system, beta-glucoside-specific IIABC component | **6.91** |
| EF0292 | PTS system, IIC component | **6.62** |
| EF1804 | PTS system, IIB component | **6.36** |
| EF0552 | PTS system, IIC component | **6.21** |
| EF1836 | PTS system, IIA component, putative | **5.84** |
| EF1837 | PTS system, IIB component, putative | **5.74** |
| EF0816 | PTS system, IIC component | **5.32** |
| EF3030 | PTS system, IIC component | **4.58** |
| EF1801 | PTS system, IIA component | **4.42** |
| EF3029 | PTS system, IID component | **4.32** |
| EF2269 | PTS system, IID component | **4.14** |
| EF0958 | PTS system, IIABC components | **3.91** |
| EF0815 | PTS system, IIAB components | **3.81** |
| EF0817 | PTS system, IID component | **3.77** |
| EF0555 | PTS system, IIA component | **3.75** |
| EF0456 | PTS system, IID component | **3.75** |
| EF2270 | PTS system, IIC component | **3.59** |
| EF2267 | PTS system, IIA component | **3.42** |
| EF1018 | PTS system, IIA component | **-3.05** |
| EF1019 | PTS system, IIC component | **-4.12** |
| EF3213 | PTS system, IID component | **-4.30** |
| EF1017 | PTS system, IIB component | **-4.49** |
| EF3212 | PTS system, IIC component | **-5.34** |
| EF3210 | PTS system, IIA component, putative | **-6.27** |
| EF3211 | PTS system, IIB component | **-6.57** |

| | | |
|---|---|---|
| EF0020 | PTS system, mannose-specific IIAB components | **-90.38** |
| EF0021 | PTS system, mannose-specific IIC component | **-100.36** |
| EF0022 | PTS system, mannose-specific IID component | **-107.49** |
| EF0019 | PTS system, IIB component | **-152.11** |

| **REGULATORY FUNCTIONS** | | |
|---|---|---|
| EF0107 | transcriptional regulator, CrpFnr family | **18.06** |
| EF1515 | transcription antiterminator, bglG family | **14.09** |
| EF0432 | transcriptional regulator, AraC family | **12.37** |
| EF1656 | transcriptional regulator, LysR family | **8.75** |
| EF1591 | transcriptional regulator, AraC family | **8.39** |
| EF3144 | phosphosugar-binding transcriptional regulator, RpiR family | **8.25** |
| EF2711 | transcriptional regulator, AraC family | **7.88** |
| EF0103 | transcriptional regulator, ArgR family | **5.68** |
| EF0102 | transcriptional regulator, ArgR family | **3.33** |
| EF3328 | transcriptional regulator, GntR family | **3.07** |
| EF0676 | arginine repressor | **3.05** |
| EF2594 | transcriptional regulator, TetR family | **-3.45** |
| EF2703 | transcriptional regulator | **-4.07** |
| EF1302 | transcriptional regulator, putative | **-4.41** |
| EF1224 | transcriptional regulator, CroCI family | **-4.56** |
| EF1303 | transcriptional regulator, LysR family | **-4.92** |
| EF0782 | RNA polymerase sigma-54 factor | **-132.88** |

| **CELLULAR PROCESSES** | | |
|---|---|---|
| EF0439 | immunity protein PlnM, putative | **13.54** |
| EF3023 | polysaccharide lyase, family 8 | **8.85** |
| EF1076 | streptomycin 3-adenylyltransferase, putative | **5.84** |
| EF0818 | polysaccharide lyase, family 8 | **3.61** |
| EF1502 | beta-lactamase, putative | **3.25** |
| EF2739 | alkyl hydroperoxide reductase, C subunit | **-3.04** |
| EF1300 | cell division protein, FtsWRodASpovE family | **-3.69** |
| EF1301 | cell division protein, FtsWRodASpovE family | **-3.89** |

| **AMINOACID BIOSYNTHESIS** | | |
|---|---|---|
| EF0891 | aspartate aminotransferase, putative | **47.93** |
| EF0105 | ornithine carbamoyltransferase | **19.35** |
| EF2560 | glutamate synthase (NADPH), homotetrameric | **10.50** |

| **TWO COMPONENT SYSTEMS** | | |
|---|---|---|
| EF2218 | DNA-binding response regulator, AraC family | **12.18** |

| | | |
|---|---|---|
| EF2219 | sensor histidine kinase | **11.03** |
| | | |
| **BIOSYNTHESIS OF COFACTORS,PROSTHETIC GROUPS AND CARRIERS** | | |
| EF1395 | molybdenum cofactor biosynthesis family protein | **38.35** |
| EF1396 | molybdenum cofactor biosynthesis family protein, putative | **35.07** |
| EF1393 | molybdopterin cofactor biosynthesis protein A, putative | **30.09** |
| EF1225 | thiamin biosynthesis ApbE, putative | **23.88** |
| EF1392 | molybdenum cofactor biosynthesis protein MoaC | **4.44** |
| EF1655 | 2-dehydropantoate 2-reductase, putative | **4.30** |
| EF1391 | molybdenum cofactor biosynthesis family protein | **3.06** |
| EF1969 | phosphomethylpyrimidine kinase, putative | **-3.38** |
| EF2445 | 2-dehydropantoate 2-reductase, putative | **-4.14** |
| | | |
| **DNA METABOLISM** | | |
| EF0053 | DNA polymerase III, epsilon subunit | **7.74** |
| | | |
| **TRANSCRIPTION** | | |
| EF0115 | endoribonuclease L-PSP, putative | **30.17** |
| EF3214 | ATP-dependent helicase, DEAH-box family, putative | **-3.63** |
| | | |
| **PRYRIMIDINE RIBONUCLEOTIDE BIOSYNTHESIS** | | |
| EF2561 | dihydroorotate dehydrogenase electron transfer subunit, putative | **13.65** |
| EF2999 | allantoinase, putative | **5.82** |
| | | |
| **NUCLEOTIDE RELATED** | | |
| EF1921 | inosine-uridine preferring nucleoside hydrolase | **15.75** |
| EF1036 | nucleoside diphosphate kinase | **11.54** |
| EF2580 | D-hydantoinase | **6.39** |
| EF1958 | deoxyguanosinetriphosphate triphosphohydrolase, putative | **3.61** |
| | | |
| **CENTRAL INTERMEDIARY METABOLISM** | | |
| EF3141 | D-isomer specific 2-hydroxyacid dehydrogenase family protein | **16.20** |
| EF0895 | glycerol dehydrogenase, putative | **15.54** |
| EF3140 | alcohol dehydrogenase, iron-containing | **9.99** |
| EF1358 | glycerol dehydrogenase, putative | **4.88** |
| EF1364 | acetyl-CoA acetyltransferasehydroxymethylglutaryl-CoA reductase, degradative | **-3.41** |
| EF1813 | sulfatase domain protein | **-11.88** |

| | | |
|---|---|---|
| **PHAGE PROTEINS** | | |
| EF0354 | holin, putative | **-3.02** |
| EF0339 | major capsid protein, putative | **-3.15** |
| | | |
| **FATTY ACID AND PHOSPHOLIPID METABOLISM** | | |
| EF1663 | branched-chain phosphotransacylase | **10.28** |
| | | |
| **PROTEIN SYNTHESIS AND FATE** | | |
| EF2567 | selenide, water dikinase | **16.82** |
| EF2471 | arginyl-tRNA synthetase | **7.80** |
| EF2997 | peptidase, M20M25M40 family | **3.64** |
| EF2578 | peptidase, M20M25M40 family | **3.02** |
| EF2858 | threonyl-tRNA synthetase | **-3.14** |
| EF0633 | tyrosyl-tRNA synthetase | **-4.39** |

**Supplemental Table 3-2 Genes common to the transcriptional profile of VI01 & V583::*mptD***

| Gene | Function | Fold change |
|---|---|---|
| EF2223 | ABC transporter, permease protein | 257.25 |
| EF2221 | ABC transporter, substrate-binding protein | 128.09 |
| EF2222 | ABC transporter, permease protein | 114.80 |
| EF0362 | chitin binding protein, putative | 83.75 |
| EF0361 | chitinase, family 2 | 69.31 |
| EF2863 | endo-beta-N-acetylglucosaminidase | 62.34 |
| EF0054 | hypothetical protein | 58.53 |
| EF1929 | glycerol kinase | 53.22 |
| EF0114 | glycosyl hydrolase, family 20 | 48.20 |
| EF1928 | alpha-glycerophosphate oxidase | 43.44 |
| EF1395 | molybdenum cofactor biosynthesis family protein | 38.35 |
| EF1927 | glycerol uptake facilitator protein | 36.40 |
| EF1396 | molybdenum cofactor biosynthesis family protein, putative | 35.07 |
| EF0115 | endoribonuclease L-PSP, putative | 30.17 |
| EF1393 | molybdopterin cofactor biosynthesis protein A, putative | 30.09 |
| EF0664 | hypothetical protein | 27.97 |
| EF1398 | molybdenum ABC transporter, permease protein | 26.30 |
| EF1225 | thiamin biosynthesis ApbE, putative | 23.88 |
| EF1397 | molybdenum ABC transporter, molybdenum-binding protein | 23.85 |
| EF2569 | conserved hypothetical protein | 21.29 |
| EF2570 | aldehyde oxidoreductase, putative | 21.17 |
| EF2562 | Flavodoxin | 19.95 |
| EF0105 | ornithine carbamoyltransferase | 19.35 |
| EF0106 | carbamate kinase | 19.07 |
| EF1068 | aldose 1-epimerase | 18.97 |
| EF2568 | aminotransferase, class V | 18.70 |
| EF2220 | conserved hypothetical protein | 18.53 |
| EF0677 | phosphoglucomutasephosphomannomutase family protein | 18.13 |
| EF3138 | PTS system, IID component | 17.41 |
| EF0678 | acetyltransferase, GNAT family | 16.96 |
| EF0743 | hypothetical protein | 16.44 |
| EF3141 | D-isomer specific 2-hydroxyacid dehydrogenase family protein | 16.20 |
| EF3137 | PTS system, IIB component | 16.00 |
| EF0104 | arginine deiminase | 15.83 |
| EF1921 | inosine-uridine preferring nucleoside hydrolase | 15.75 |

| | | |
|---|---|---|
| EF1516 | PTS system, IIABC components | 14.22 |
| EF1515 | transcription antiterminator, bglG family | 14.09 |
| EF2561 | dihydroorotate dehydrogenase electron transfer subunit, putative | 13.65 |
| EF1920 | C4-dicarboxylate anaerobic carrier | 13.23 |
| EF0108 | C4-dicarboxylate transporter, putative | 13.04 |
| EF0938 | ABC transporter, ATP-bindingTOBE domain protein | 12.53 |
| EF3136 | PTS system, IIA component | 12.08 |
| EF1345 | sugar ABC transporter, sugar-binding protein | 11.76 |
| EF2563 | conserved hypothetical protein | 11.38 |
| EF0553 | PTS system, IID component | 11.35 |
| EF2560 | glutamate synthase (NADPH), homotetrameric | 10.50 |
| EF3134 | 2-dehydro-3-deoxyphosphogluconate aldolase4-hydroxy-2-oxoglutarate aldolase | 10.43 |
| EF1344 | sugar ABC transporter, permease protein | 10.11 |
| EF3140 | alcohol dehydrogenase, iron-containing | 9.99 |
| EF2559 | pyruvate flavodoxinferredoxin oxidoreductase family protein | 9.99 |
| EF3142 | 6-phosphogluconate dehydrogenase family protein | 8.83 |
| EF1529 | PTS system, IIC component, putative | 8.56 |
| EF1659 | branched-chain alpha-keto acid dehydrogenase, E1 component, beta subunit | 8.46 |
| EF1237 | conserved hypothetical protein | 8.26 |
| EF3144 | phosphosugar-binding transcriptional regulator, RpiR family | 8.25 |
| EF1349 | glycosyl hydrolase, family 13 | 7.96 |
| EF_A0067_at | PTS system, IIABC components | 7.93 |
| EF2711 | transcriptional regulator, AraC family | 7.88 |
| EF0053 | DNA polymerase III, epsilon subunit | 7.74 |
| EF3139 | PTS system, IIC component | 7.55 |
| EF1802 | PTS system, IID component | 7.35 |
| EF1912 | ROK family protein | 7.19 |
| EF0405 | hydrolase, haloacid dehalogenase-like family | 7.16 |
| EF3031 | PTS system, IIB component | 7.03 |
| EF0270 | PTS system, beta-glucoside-specific IIABC component | 6.91 |
| EF1343 | sugar ABC transporter, permease protein | 6.65 |
| EF1066 | hexapeptide-repeat containing-acetyltransferase | 6.47 |
| EF1206 | malate dehydrogenase, decarboxylating | 6.26 |
| EF1919 | acetyltransferase, GNAT family | 5.87 |
| EF0377 | ankyrin repeat family protein | 5.56 |
| EF2441 | conserved hypothetical protein | 5.50 |
| EF2442 | phosphate transporter family protein | 5.45 |

| | | |
|---|---|---|
| EF3325 | sodium ion-translocating decarboxylase, biotin carboxyl carrier protein | 5.29 |
| EF1347 | glycosyl hydrolase, family 13 | 5.18 |
| EF1358 | glycerol dehydrogenase, putative | 4.88 |
| EF2440 | celC-related protein | 4.71 |
| EF1359 | conserved hypothetical protein | 4.70 |
| EF1535 | conserved hypothetical protein | 4.69 |
| EF1801 | PTS system, IIA component | 4.42 |
| EF0271 | glycosyl hydrolase, family 1 | 4.33 |
| EF3109 | peptide ABC transporter, ATP-binding protein | 4.07 |
| EF1800 | conserved hypothetical protein | 4.01 |
| EF0958 | PTS system, IIABC components | 3.91 |
| EF1361 | dihydroxyacetone kinase family protein | 3.84 |
| EF3108 | peptide ABC transporter, permease protein | 3.84 |
| EF1238 | glycosyl hydrolase, family 3 | 3.79 |
| EF3157 | glycosyl hydrolase, family 65 | 3.78 |
| EF0459 | glucokinase regulator-related protein | 3.77 |
| EF0456 | PTS system, IID component | 3.75 |
| EF3158 | hydrolase, haloacid dehalogenase-like family | 3.69 |
| EF1348 | glucan 1,6-alpha-glucosidase, putative | 3.61 |
| EF0818 | polysaccharide lyase, family 8 | 3.61 |
| EF3110 | peptide ABC transporter, ATP-binding protein | 3.53 |
| EF0460 | conserved hypothetical protein | 3.48 |
| EF3107 | peptide ABC transporter, permease protein | 3.40 |
| EF3106 | peptide ABC transporter, peptide-binding protein | 3.35 |
| EF1069 | Galactokinase | 3.17 |
| EF3328 | transcriptional regulator, GntR family | 3.07 |
| EF1707 | glycosyl hydrolase, family 38 | 3.07 |
| EF1528 | hypothetical protein | 3.01 |
| EF0082 | major facilitator family transporter | -6.82 |
| EF0020 | PTS system, mannose-specific IIAB components | -90.38 |
| EF0021 | PTS system, mannose-specific IIC component | -100.36 |
| EF0022 | PTS system, mannose-specific IID component | -107.49 |
| EF0019 | PTS system, IIB component | -152.11 |

**Supplemental Table 3-3 List of genes differentially regulated in VI01[Δ*rpoN*] but not in the V583::*mptD* mutant.**

| Gene | Function | Fold Change |
|---|---|---|
| EF0713 | conserved hypothetical protein | 58.00 |
| EF0891 | aspartate aminotransferase, putative | 47.93 |
| EF0892 | amino acid ABC transporter, ATP-binding protein | 40.84 |
| EF0893 | amino acid ABC transporter, amino acid-bindingpermease protein | 39.81 |
| EF0253 | aldehyde dehydrogenase | 35.88 |
| EF0384 | hypothetical protein | 34.97 |
| EF0711 | conserved hypothetical protein | 32.33 |
| EF1394 | conserved hypothetical protein | 29.49 |
| EF0383 | protein FdrAconserved hypothetical protein | 29.34 |
| EF0714 | hypothetical protein | 29.26 |
| EF0385 | major facilitator family transporter | 27.11 |
| EF2213 | PTS system, IIBC components | 23.30 |
| EF1227 | conserved hypothetical protein | 21.74 |
| EF1226 | oxidoreductase, putative | 21.02 |
| EF2566 | conserved hypothetical protein | 19.48 |
| EF2236 | conserved hypothetical protein | 18.24 |
| EF0107 | transcriptional regulator, CrpFnr family | 18.06 |
| EF2234 | sugar ABC transporter, sugar-binding protein, putative | 17.24 |
| EF0386 | carbamate kinase | 16.90 |
| EF2567 | selenide, water dikinase | 16.82 |
| EF0387 | sodiumdicarboxylate symporter family protein | 16.14 |
| EF0895 | glycerol dehydrogenase, putative | 15.54 |
| EF0392 | hypothetical protein | 15.39 |
| EF1661 | branched-chain alpha-keto acid dehydrogenase, E3 component, dihydrolipoamide dehydrogenase | 14.95 |
| EF2564 | conserved domain protein | 14.57 |
| EF1658 | branched-chain alpha-keto acid, E2 component, dihydrolipoamide acetyltransferase | 14.28 |
| EF1233 | ABC transporter, permease protein | 13.83 |
| EF0439 | immunity protein PlnM, putative | 13.54 |
| EF3135 | mannonate dehydratase, putative | 13.37 |
| EF1399 | molybdenum ABC transporter, ATP-binding protein, putative | 13.10 |
| EF1234 | ABC transporter, substrate-binding protein, putative | 12.52 |
| EF0432 | transcriptional regulator, AraC family | 12.37 |
| EF2565 | conserved hypothetical protein | 12.22 |

| | | |
|---|---|---|
| EF2218 | DNA-binding response regulator, AraC family | 12.18 |
| EF1036 | nucleoside diphosphate kinase | 11.54 |
| EF2219 | sensor histidine kinase | 11.03 |
| EF1663 | branched-chain phosphotransacylase | 10.28 |
| EF0554 | PTS system, IIB component | 9.60 |
| EF0388 | ureidoglycolate dehydrogenase | 9.27 |
| EF3023 | polysaccharide lyase, family 8 | 8.85 |
| EF1656 | transcriptional regulator, LysR family | 8.75 |
| EF1232 | ABC transporter, permease protein | 8.63 |
| EF2576 | hypothetical protein | 8.60 |
| EF1207 | citrate carrier protein, CCS family | 8.54 |
| EF2233 | ABC transporter, permease protein | 8.46 |
| EF1591 | transcriptional regulator, AraC family | 8.39 |
| EF1803 | PTS system, IIC component | 8.28 |
| EF2235 | glucuronyl hydrolase, putative | 7.81 |
| EF2471 | arginyl-tRNA synthetase | 7.80 |
| EF3105 | hypothetical protein | 7.74 |
| EF0390 | N-acyl-D-amino-acid deacylase family protein | 7.61 |
| EF2992 | major facilitator family transporter | 7.38 |
| EF3327 | citrate transporter | 7.22 |
| EF2237 | lipoprotein, putative | 7.17 |
| EF0556 | xylose isomerase | 7.01 |
| EF1824 | glycosyl hydrolase, family 31fibronectin type III domain protein | 6.82 |
| EF1229 | conserved hypothetical protein | 6.71 |
| EF0900 | aldehyde-alcohol dehydrogenase | 6.69 |
| EF0292 | PTS system, IIC component | 6.62 |
| EF1035 | lipoprotein, putative | 6.62 |
| EF1400 | cadmium-translocating P-type ATPase | 6.43 |
| EF2580 | D-hydantoinase | 6.39 |
| EF3087 | hypothetical protein | 6.39 |
| EF1804 | PTS system, IIB component | 6.36 |
| EF1228 | hypothetical protein | 6.29 |
| EF0552 | PTS system, IIC component | 6.21 |
| EF2579 | diaminopropionate ammonia-lyase, putative | 6.21 |
| EF0389 | membrane protein, putative | 6.19 |
| EF1076 | streptomycin 3-adenylyltransferase, putative | 5.84 |
| EF1836 | PTS system, IIA component, putative | 5.84 |
| EF1236 | acetyl xylan esterase, putative | 5.82 |
| EF2999 | allantoinase, putative | 5.82 |

| | | |
|---|---|---|
| EF1657 | membrane protein, putative | 5.80 |
| EF1837 | PTS system, IIB component, putative | 5.74 |
| EF0103 | transcriptional regulator, ArgR family | 5.68 |
| EF3104 | ABC transporter, ATP-binding protein | 5.46 |
| EF0052 | hypothetical protein | 5.41 |
| EF3088 | hypothetical protein | 5.34 |
| EF0816 | PTS system, IIC component | 5.32 |
| EF1075 | acetyltransferase, GNAT family | 5.25 |
| EF0808 | hypothetical protein | 5.24 |
| EF1660 | branched-chain alpha-keto acid dehydrogenase, E1 component, alpha subunit | 5.14 |
| EF1662 | butyrate kinase | 5.13 |
| EF1077 | acetyltransferase, GNAT family | 5.09 |
| EF3103 | membrane protein, putative | 5.04 |
| EF1503 | fructose-1,6-bisphosphatase, putative | 4.95 |
| EF3326 | conserved hypothetical protein | 4.85 |
| EF3102 | hypothetical protein | 4.82 |
| EF1805 | glycosyl hydrolase, family 35 | 4.81 |
| EF2268 | conserved hypothetical protein | 4.73 |
| EF1360 | dihydroxyacetone kinase family protein | 4.70 |
| EF3030 | PTS system, IIC component | 4.58 |
| EF3000 | cytosinepurines, uracil, thiamine, allantoin permease family protein | 4.56 |
| EF1061 | N-acyl-D-amino-acid deacylase family protein | 4.53 |
| EF2575 | carbamate kinase | 4.52 |
| EF1392 | molybdenum cofactor biosynthesis protein MoaC | 4.44 |
| EF2581 | oxidoreductase, pyridine nucleotide-disulfide family | 4.41 |
| EF0062 | 5-nucleotidase family protein | 4.40 |
| EF3029 | PTS system, IID component | 4.32 |
| EF1655 | 2-dehydropantoate 2-reductase, putative | 4.30 |
| EF2577 | aspartateornithine carbamoyltransferase family protein | 4.30 |
| EF1230 | hypothetical protein | 4.28 |
| EF1513 | pheromone binding protein | 4.23 |
| EF2232 | ABC transporter, permease protein | 4.20 |
| EF3092 | glyoxalase family protein | 4.16 |
| EF2269 | PTS system, IID component | 4.14 |
| EF2996 | conserved hypothetical protein | 4.13 |
| EF1407 | hypothetical protein | 4.07 |
| EF0291 | glycosyl hydrolase, family 1 | 4.06 |
| EF0673 | membrane protein, putative | 4.05 |

| | | |
|---|---|---|
| EF0551 | glycosyl hydrolase, family 31 | 3.89 |
| EF2265 | carbohydrate kinase, pfkB family | 3.88 |
| EF2264 | 4-deoxy-l-threo-5-hexosulose-uronate ketol-isomerase | 3.87 |
| EF0815 | PTS system, IIAB components | 3.81 |
| EF0817 | PTS system, IID component | 3.77 |
| EF3032 | conserved hypothetical protein | 3.77 |
| EF0555 | PTS system, IIA component | 3.75 |
| EF2272 | glucuronyl hydrolase, putative | 3.74 |
| EF_B0056 | hypothetical protein | 3.72 |
| EF1062 | N-acyl-D-amino-acid deacylase family protein | 3.68 |
| EF2997 | peptidase, M20M25M40 family | 3.64 |
| EF1958 | deoxyguanosinetriphosphate triphosphohydrolase, putative | 3.61 |
| EF2270 | PTS system, IIC component | 3.59 |
| EF2709 | glycosyl hydrolase, family 2 | 3.56 |
| EF3008 | conserved hypothetical protein | 3.56 |
| EF0244 | acetyltransferase, GNAT family | 3.54 |
| EF1158 | N4-(beta-N-acetylglucosaminyl)-L-asparaginase, putative | 3.52 |
| EF2662 | choline binding protein | 3.50 |
| EF1060 | pheromone binding protein | 3.49 |
| EF0063 | pheromone binding protein, putative | 3.42 |
| EF2267 | PTS system, IIA component | 3.42 |
| EF2994 | aminotransferase, class V | 3.41 |
| EF1362 | conserved domain protein | 3.41 |
| EF1933 | hypothetical protein | 3.39 |
| EF1512 | conserved hypothetical protein | 3.37 |
| EF0113 | hypothetical protein | 3.35 |
| EF0102 | transcriptional regulator, ArgR family | 3.33 |
| EF1071 | galactose-1-phosphate uridylyltransferase | 3.33 |
| EF2646 | glycerate kinase, putative | 3.32 |
| EF0382 | conserved hypothetical protein | 3.32 |
| EF1322 | conserved hypothetical protein | 3.31 |
| EF3101 | conserved domain protein | 3.26 |
| EF1502 | beta-lactamase, putative | 3.25 |
| EF1959 | conserved domain protein | 3.23 |
| EF0243 | branched-chain amino acid transport system II carrier protein | 3.22 |
| EF3009 | conserved hypothetical protein | 3.20 |
| EF0429 | TRAP dicarboxylate transporter, DctP subunit | 3.16 |
| EF0807 | pheromone binding protein, putative | 3.15 |
| EF3007 | conserved hypothetical protein | 3.15 |

| | | |
|---|---|---|
| EF1239 | conserved hypothetical protein | 3.08 |
| EF1391 | molybdenum cofactor biosynthesis family protein | 3.06 |
| EF0676 | arginine repressor | 3.05 |
| EF3010 | conserved hypothetical protein | 3.05 |
| EF2933 | DNA-binding protein,putative | 3.03 |
| EF2578 | peptidase, M20M25M40 family | 3.02 |
| EF0354 | holin, putative | -3.02 |
| EF2739 | alkyl hydroperoxide reductase, C subunit | -3.04 |
| EF2593 | ABC transporter, ATP-bindingpermease protein | -3.05 |
| EF1018 | PTS system, IIA component | -3.05 |
| EF3245 | cell-envelope associated acid phosphatase | -3.10 |
| EF2944 | hypothetical protein | -3.10 |
| EF2627 | teichoic acid glycosylation protein, putative | -3.11 |
| EF2858 | threonyl-tRNA synthetase | -3.14 |
| EF0339 | major capsid protein, putative | -3.15 |
| EF0708 | conserved hypothetical protein | -3.16 |
| EF0569 | potassium-transporting ATPase, subunit C | -3.16 |
| EF3069 | formatenitrite transporter family protein | -3.16 |
| EF1340 | pheromone cAM373 precursor lipoprotein | -3.16 |
| EF0804 | amino acid ABC transporter, amino acid-binding protein | -3.19 |
| EF2722 | L-serine dehydratase, iron-sulfur-dependent, alpha subunit | -3.19 |
| EF1198 | conserved hypothetical protein | -3.26 |
| EF0672 | hypothetical protein | -3.31 |
| EF0341 | hypothetical protein | -3.33 |
| EF0568 | potassium-transporting ATPase, subunit B | -3.37 |
| EF1969 | phosphomethylpyrimidine kinase, putative | -3.38 |
| EF1364 | acetyl-CoA acetyltransferasehydroxymethylglutaryl-CoA reductase, degradative | -3.41 |
| EF2746 | dltD protein | -3.45 |
| EF2594 | transcriptional regulator, TetR family | -3.45 |
| EF1168 | hypothetical protein | -3.46 |
| EF0805 | amino acid ABC transporter, ATP-binding protein | -3.56 |
| EF3004 | sulfate transporter familySTAS domain protein | -3.57 |
| EF3214 | ATP-dependent helicase, DEAH-box family, putative | -3.63 |
| EF3207 | dihydrouridine synthase family protein | -3.64 |
| EF1968 | conserved hypothetical protein | -3.66 |
| EF2500 | GcvH family protein | -3.69 |
| EF1300 | cell division protein, FtsWRodASpovE family | -3.69 |
| EF0468 | LemA family protein | -3.71 |
| EF0806 | amino acid ABC transporter, permease protein | -3.72 |

| | | |
|---|---|---|
| EF2721 | L-serine dehydratase, iron-sulfur-dependent, beta subunit | -3.77 |
| EF1841 | HD domain protein | -3.79 |
| EF2749 | D-alanine-activating enzyme, putative | -3.83 |
| EF0420 | drug resistance transporter, EmrBQacA family protein | -3.84 |
| EF1301 | cell division protein, FtsWRodASpovE family | -3.89 |
| EF0567 | potassium-transporting ATPase, subunit A | -3.95 |
| EF2748 | basic membrane protein DtlB | -4.01 |
| EF1199 | conserved hypothetical protein | -4.04 |
| EF2703 | transcriptional regulator | -4.07 |
| EF2747 | D-alanyl carrier protein | -4.07 |
| EF1019 | PTS system, IIC component | -4.12 |
| EF1304 | magnesium-translocating P-type ATPase | -4.12 |
| EF2445 | 2-dehydropantoate 2-reductase, putative | -4.14 |
| EF0443 | LysM domain protein | -4.25 |
| EF0747 | conserved hypothetical protein | -4.26 |
| EF3213 | PTS system, IID component | -4.30 |
| EF2899 | oxidoreductase, pyridine nucleotide-disulfide family | -4.35 |
| EF0633 | tyrosyl-tRNA synthetase | -4.39 |
| EF1302 | transcriptional regulator, putative | -4.41 |
| EF1017 | PTS system, IIB component | -4.49 |
| EF0419 | conserved hypothetical protein | -4.54 |
| EF1224 | transcriptional regulator, CroCI family | -4.56 |
| EF0634 | decarboxylase, putative | -4.90 |
| EF1303 | transcriptional regulator, LysR family | -4.92 |
| EF2750 | conserved hypothetical protein | -4.95 |
| EF1258 | hypothetical protein | -5.02 |
| EF1812 | hypothetical protein | -5.03 |
| EF3244 | hypothetical protein | -5.05 |
| EF0635 | amino acid permease family protein | -5.11 |
| EF1053 | ABC transporter, ATP-binding protein | -5.23 |
| EF3212 | PTS system, IIC component | -5.34 |
| EF2216 | conserved domain protein | -5.66 |
| EF2214 | glyoxylase family protein | -6.21 |
| EF3210 | PTS system, IIA component, putative | -6.27 |
| EF0636 | Na+H+ antiporter | -6.49 |
| EF3211 | PTS system, IIB component | -6.57 |
| EF2215 | conserved hypothetical protein | -7.27 |
| EF2896 | hypothetical protein | -7.39 |
| EF0083 | hypothetical protein | -7.77 |

| | | |
|---|---|---|
| EF1814 | drug resistance transporter, EmrBQacA family protein | -8.11 |
| EF1231 | conserved hypothetical protein | -10.70 |
| EF0746 | penicillin-binding protein, putative | -11.48 |
| EF1192 | aquaporin Z | -11.87 |
| EF1813 | sulfatase domain protein | -11.88 |
| EF1054 | ABC transporter, permease protein | -17.50 |
| EF0802 | hypothetical protein | -36.40 |
| EF0782 | RNA polymerase sigma-54 factor | -132.88 |

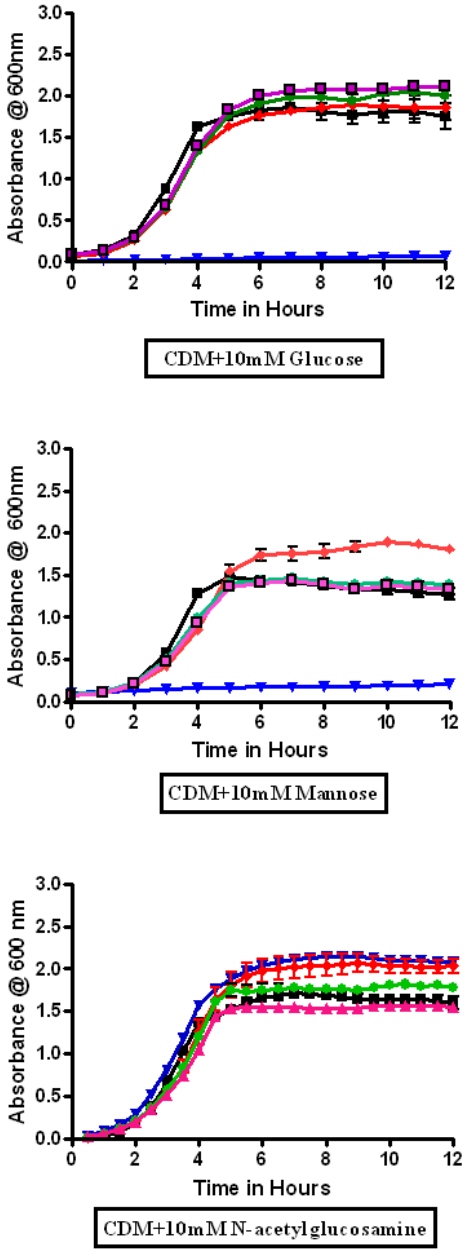
**Supplemental Table 3-4 Putative cre sites in differentially expressed genes common to VI01[Δ*rpoN*] and V583::*mptD* transcriptome.**

| Gene | Function | Fold change | Start | End | Cre sequence (WTGWAARCGYWWWCW) |
|---|---|---|---|---|---|
| EF0104 | arginine deiminase | 15.83 | -146 | -132 | ATGAAAGCGCATTCT |
| EFA0067 | PTS system, IIABC components | 7.93 | -154 | -140 | TAGTAAACGTTTTCT |
| EF0054 | hypothetical protein | 58.53 | -92 | -78 | TCGAAAGCGCTTTCT |
| EF0115 | endoribonuclease L-PSP, putative | 30.17 | -49 | -35 | ATGTAAGCGGAATCA |
| EF0362 | chitin binding protein, putative | 83.75 | -41 | -27 | CTGTAAGCGCATACA |
| EF0377 | ankyrin repeat family protein | 5.56 | -295 | -287 | ATTAAAACGCTTTCT |
| EF0405 | hydrolase, haloacid dehalogenase-like family | 7.16 | -71 | -57 | ATGTAAACGGATTCT |
| EF0664 | hypothetical protein | 27.97 | -17 | -3 | ATGAAAGCGGATACA |
| EF0938 | ABC transporter, ATP-bindingTOBE domain protein | 12.53 | -60 | -46 | ATGAAAACGCTATCT |
| EF1800 | conserved hypothetical protein | 4.01 | -41 | -27 | ATGAAAGCGTGTTCA |
| EF2223 | ABC transporter, permease protein | 257.25 | -38 | -24 | ATGAAAACGCTATTA |
| EF2711 | transcriptional regulator, AraC family | 7.88 | -37 | -23 | ATGTAAAGGCTTTCT |
| EF2863 | endo-beta-N-acetylglucosaminidase | 62.34 | -45 | -31 | TTGTAAGCGCTAACA |
| EF3142 | 6-phosphogluconate dehydrogenase family protein | 8.83 | -374 | -360 | ATGAAAAGGCATTCA |
| | | | -68 | -34 | ATGTAAACGATTACA |
| EF3144 | phosphosugar-binding transcriptional regulator, RpiR family | 8.25 | -359 | -345 | ATGTAAACGATTACA |
| | | | -53 | -39 | ATGAAAAGGCATTCA |
| EF1069 | galactokinase | 3.17 | -109 | -95 | TTGTACACGTTTTCA |
| EF1068 | aldose 1-epimerase | 18.97 | -126 | -112 | TTGAAAACGTGTACA |
| EF1929 | glycerol kinase | 53.22 | -146 | -132 | TTGAAAGCGTTGTCT |
| | | | -36 | -22 | TTGAAATCGTTTTCT |

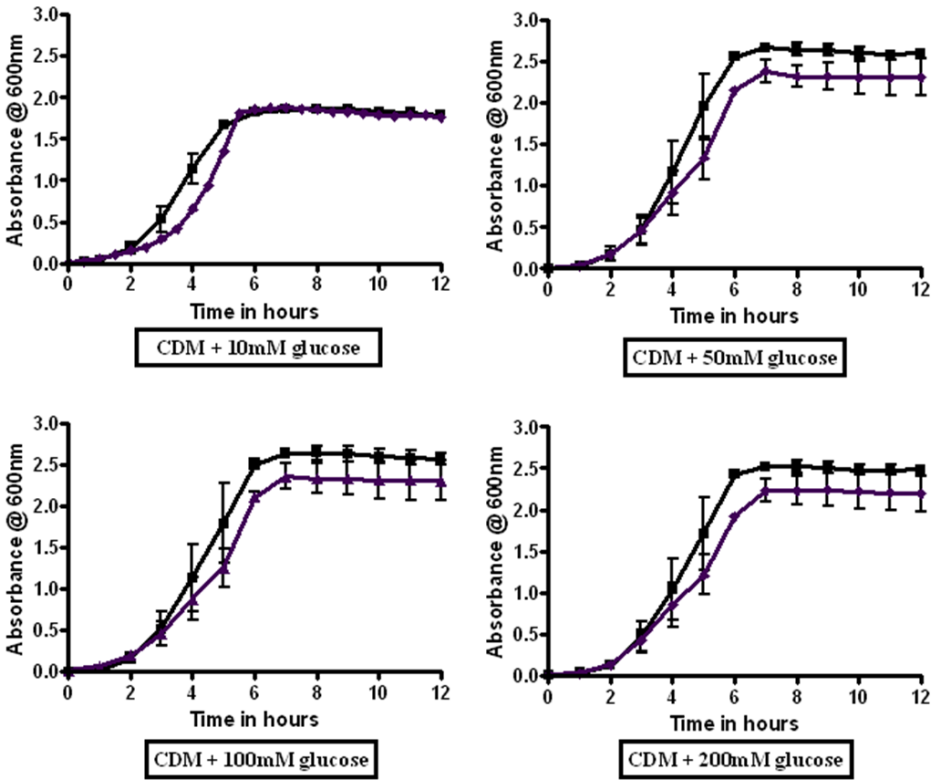
**Supplemental Table 3-5 Putative cre sites in genes exclusive to VI01 [Δ*rpoN* mutant]**

| Gene | Function | Fold Change | Start | End | Cre sequence WTGWAARCGYWWWC |
|---|---|---|---|---|---|
| EF0253 | aldehyde dehydrogenase | 35.88 | -38 | -24 | TTGTAAGCGGATACA |
| EF0292 | PTS system, IIC component | 6.62 | -51 | -37 | ATGTAAACGGATACA |
| EF0382 | conserved hypothetical protein | 3.32 | -76 | -62 | ATGAAAACACTTTCT |
| EF0383 | protein FdrA conserved hypothetical protein | 29.34 | -61 | -47 | ATGAAAACACTTTCT |
| EF0439 | immunity protein PlnM, putative | 13.54 | -49 | -35 | ATGAAAACGTTATCA |
| EF1036 | nucleoside diphosphate kinase | 11.54 | -75 | -61 | ATGAAAGCGGATACT |
| EF1207 | citrate carrier protein, CCS family | 8.54 | -53 | -39 | ATGTAAACGTTTTCT |
| EF1232 | ABC transporter, permease protein | 8.63 | -81 | -67 | ATGTAAGGGTTTACA |
| EF1392 | molybdenum cofactor biosynthesis protein MoaC | 4.44 | -38 | -24 | GTGTAAACGTTAACA |
| EF1655 | 2-dehydropantoate 2-reductase, putative | 4.3 | -158 | -144 | TTAAAAGCGCTTACA |
| EF1656 | transcriptional regulator, LysR family | 8.75 | -33 | -19 | TTAAAAGCGCTTACA |
| EF1663 | branched-chain phosphotransacylase | 10.28 | -61 | -47 | ATGTAAACGCATACA |
| EF1805 | glycosyl hydrolase, family 35 | 4.81 | -30 | -16 | TTGAAAGCGTTTACT |
| EF1824 | glycosyl hydrolase, family 31fibronectin type III domain protein | 6.82 | -255 | -241 | ATGAAAACGCATTCA |
| EF1836 | PTS system, IIA component, putative | 5.84 | -63 | -49 | TTGAAAGCGTTTTAT |
| EF2237 | lipoprotein, putative | 7.17 | -35 | -21 | ATTAAAGCGCTTTCT |
| EF2721 (sdhB2) | L-serine dehydratase, iron-sulfur-dependent, beta subunit | -3.77 | -33 | -19 | ATGAAAACATATTCT |
| EF3000 | cytosinepurines, uracil, thiamine, allantoin permease family protein | 4.56 | -118 | -104 | TTGTAAGCGCTTTTT |
| EF3023 | polysaccharide lyase, family 8 | 8.85 | -209 | -195 | GTGAAAGCGTAAACA |
| EF3087 | hypothetical protein | 3.62 | -102 | -88 | ATAAAAACGTTTTCT |
| EF3088 | hypothetical protein | 5.34 | -58 | -44 | ATAAAAACGTTTTCT |
| EF3326 | conserved hypothetical protein | 4.85 | -373 | -359 | TTGTAAGCGTTAACA |
| EF3327 | citrate transporter | 7.22 | -45 | -31 | TTGTAAGCGTTAACA |

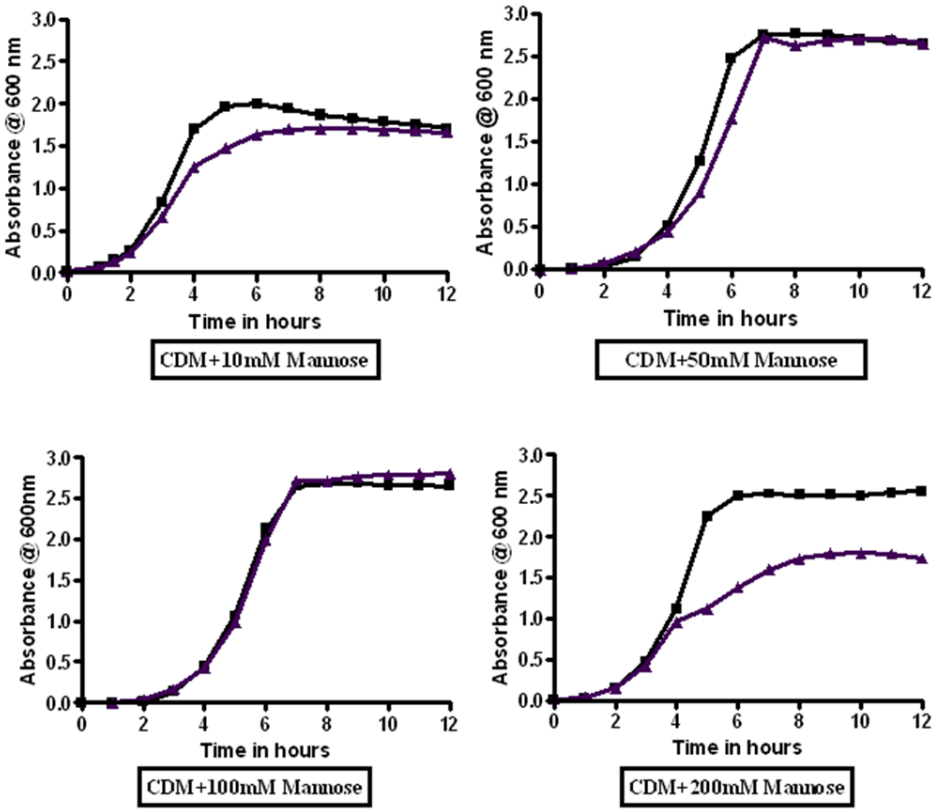
## 3.9 Supplemental Figures




**Supplemental Figure 3-1 Growth pattern of activator mutants in CDM containing 10mM glucose; 10mM Mannose or 10mM N-acetlglucosamine.**
The growth curves are represented as Black: V583, Blue: MG07 [ΔmptR], Red: MG09 [Δ*mpoR*]. Green: MG10 [Δ*mphR*] and Purple, MG08 [Δ*lpoR*]

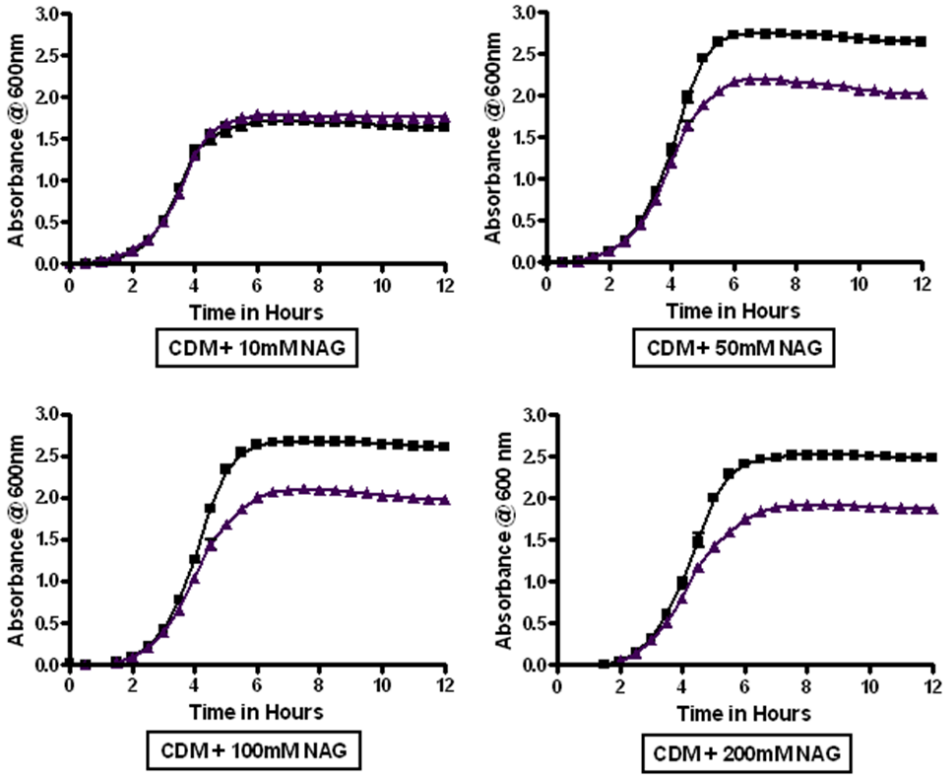



**Supplemental Figure 3-2 Growth of *E.faecalis* in chemically defined medium with the glucose as the sole carbon source.**
Each graph is the average of ten replicates with standard error of the mean shown. The growth curves are shown in black (V583) and purple (EH01[Δ*ccpA*]).




**Supplemental Figure 3-3 Growth of *E.faecalis* in chemically defined medium with the mannose as the sole carbon source.**

Each graph is the average of ten replicates with standard error of the mean shown. The growth curves are shown in black (V583) and purple (EH01[Δ*ccpA*]).




**Supplemental Figure 3-4 Growth of *E.faecalis* in chemically defined medium with the N-acetylglucosamine (NAG) as the sole carbon source.**

Each graph is the average of ten replicates with standard error of the mean shown. The growth curves are shown in black (V583) and purple (EH01[Δ*ccpA*

# Chapter 4 - Novel modification to enterococal cell wall architecture regulated by sigma 54.

## 4.1 Introduction

The bacterial exoskeleton or cell wall protects the bacteria from environmental disturbances. It acts as a physical barrier and also relays the changes in the surrounding as signals to the interior that then prompts the bacterial response system to take appropriate measures to stabilize the cell to the alterations. In-vivo it protects the cell from the host immune system. The enterococcal cell wall is made up of a thick peptidoglycan layer, proteins (6), lipoproteins (32), polysaccharides (40), glycolipids (35) and glycopolymer such as wall teichoic acid and lipoteichoic acid (34, 39). In several strains of enterococci, covering the cell wall and covalently attached to it is a layer of capsular polysaccharide which protects the bacteria from opsonophagocytic killing by the host (16, 38). These structures function to regulate cells from autolytic breakdown as well as antimicrobial peptides encountered by the enterococci in-vivo.

Specific modification in these components have helped enterococci to resist the damage that might be caused by several cell wall targeting agents such as antimicrobial peptides and also protect them from the host immune system. For instance, the wall teichoic acid (WTA) and lipoteichoic acid (LTA) is D-alaninated at the C2 position of glycerol residue by the protein products of the *dlt* operon. This modification has been shown to affect bacterial autolysis and antimicrobial peptide sensitivity (14). In addition, the O-acteylation of the peptidoglycan at the C-6 hydroxyl group of the muramoyl residue inhibits the lytic action of muramidases such as lysozyme of the immune system and autolysins of the bacterial cell wall metabolic machinery (30) in a concentration dependent manner. The main lipid constituents of bacterial cell wall, phosphatidylglycerol and diphosphatidylglycerol impart a net negative charge to the surface. Bacteria reduce the negative charge by incorporating positively charged

substitutions such as lysine on these phospholipids which enables the repulsion of antimicrobial cationic peptides (27). In *Staphylococcus aureus*, an intergral membrane protein MprF generates the lysyl-phophatidylglycerol which renders *S.aureus* resistant to antibiotics such as gentamycin and vancomycin and antimicrobial peptides (25). It also protects *S.aureus* from neutrophil mediated killing and the *mprF* mutant was attenuated in a mouse model of sepsis and septic arthritis (28). Two paralogs of *mprF* (*mprF1* & *mprF2*) were recently characterized in *E. faecalis* 12030 and it was shown that only mprF2 contributed to amino-phospholipid formation, antimicrobial peptide resistance and unlike in *S.aureus mprF* in *E. faecalis* did not play a role in virulence (2). The peptidoglycan hydrolyzing enzymes (autolysins) also play an important role in cell wall turnover and lysis. Three autolysins have been described in *E. faecalis* AtlA, AtlB and AtlC. Septum digestion for cell division and cell wall separation are major functions of AtlA while AtlB can substitute for AtlA, although at a very low efficiency. No prominent function has been attributed to AtlC (24). The two extracellular proteases gelatinase and protease mediate cell lysis via proper cleaving and activation of AtlA. Hence, both gelatinase and AtlA mutants are completely resistant to autolysis (36).

We have previously shown that a sigma54 (*rpoN*) mutant in *E. faecalis* V583 is resistant to lysis. Mutation in the genes (*oatA ,dlt, tag, pgdA, atlA ,gelE* and *sprE*) whose products play an important role in cell wall modifications, render the cell susceptible to lysis. Here we show that deletion of *rpoN* in the lysis susceptible mutant backgrounds protect the otherwise susceptible strains suggesting that RpoN regulates cell lysis in a manner that is independent of the known modifications. Thus, on the basis of data

presented in this study, we postulate the presence of a certain cell wall modification in *E. faecalis* V583 has not previously been already described.

## 4.2 Materials and Methods

### Bacterial Strains and Growth Conditions

*Enterococcus faecalis* strains were cultured in Todd-Hewitt broth (THB) or Trypticase soy broth (TSB; BD Biosciences) unless otherwise stated. The media was supplemented with appropriate antibiotics whenever required. The bacterial strains used in this study are listed in Table 4-1. *Escherichia coli* electron blue from Strategene was used for propagation of plasmids and cultivated in Luria-Bertani (LB) broth supplemented with appropriate antibiotics whenever necessary. For *E. faecalis* chloramphenicol (Cm) and spectinomycin were used at a concentration of 15 µg/ml and 500 µg/ml respectively whereas for *E.coli* chloramphenicol was used at 10 µg/ml while spectinomycin was used at 150 µg/ml.

### Construction of Isogeneic Deletion Mutants.

Isogenic deletion of genes encoding proteins that play a role in enterococcal cell wall modification was achieved using a previously published protocol with the *E.coli*-enterococcal shuttle vector pLT06 (38). The primers and plasmids used in this study are listed in Table 4-2 and Table 4-3 respectively. For cloning purposes, the P1/P2 and P3/P4 primer pairs were designed with EcorI/BamHI and BamHI/PstI restriction sites respectively. Using primer pair EF0783P1/P2 and EF0783P3/P4, the upstream and downstream regions of o-acetlytransferase gene (oatA) were PCR amplified. Post amplification, the products were digested using BamHI enzyme, religated to one another and amplified using EF0783P1/P4 to generate the insert fragment to delete *oatA*. The

amplified insert was digested with enzymes EcoRI/PstI and ligated with a similarly cut pLT06 deletion vector to generate pVI08. E10B was used to propagate the vector and it

**Table 4-1 *E. faecalis* strains used in this study.**

| Name | Description | Reference |
|---|---|---|
| **V583** | Parental strain | Clinical isolate |
| **VI01** | V583 Δ*rpoN* | (20) |
| **VI02** | V583 Δ*oatA* | This study |
| **MG07** | V583 Δ*mptR* | Chapter 3 |
| **MG08** | V583 Δ*lpoR* | Chapter 3 |
| **MG09** | V583 Δ*mpoR* | Chapter 3 |
| **MG10** | V583 Δ*mphR* | Chapter 3 |
| **VI09** | V583 Δ*rpoN*Δ*oact* | This study |
| **VI10** | V583 Δ*rpoN*Δ*dlt* | This study |
| **VI11** | V583 Δ*oatA*Δ*dlt* | This study |
| **VI12** | V583 Δ*rpoN*Δ*oatA*Δ*dlt* | This study |
| **VT03** | V583 Δ*gelE* Δ*sprE* | (37) |
| **VI14** | VT03 Δ*rpoN* | This study |
| **VI21** | VT03 pKS82; spec$^r$ | This study |
| **VI22** | VI14 pKS82; spec$^r$ | This study |
| **VI29** | VI01 pMV158GFP; tet$^r$ | This study |
| **VI30** | VI14 pMV158GFP; tet$^r$ | This study |
| **VI37** | VI01 pKS12(A); spec$^r$ | This study |
| **VI44** | V583 Δ*atlA* | This study |
| **VI45** | VI01 Δ*atlA* | This study |
| **VI48** | V583 Δ*tag* | This study |
| **VI49** | VI01 Δ*tag* | This study |
| **V150** | V583 Δ*pgdA* | This study |
| **VI51** | VI01 Δ*pgdA* | This study |
| **KS20** | V583 Δ*dlt* | This study |

**Table 4-2 Oligonucleotides used in this study.**

| Primers | Sequence (5'-3') |
|---|---|
| **EF0783P1** | GAGAGAATTCTTGATGCCTTAGTGAGTCGT |
| **EF0783P2** | CTCTGGATCCCTTGCTATTTGCTGTCTGATT |
| **EF0783P3** | GAGAGGATCCCAGTAGCAAGAAACTTCCAG |
| **EF0783P4** | CTCTCTGCAGGCCAAACCAGCAGCAACAAT |
| **EF0783-Up** | GTTACAACAGCTAGATCCAGA |
| **EF0783-Down** | AGATCACTCGTACCAAACGTA |
| **DltP1** | GAGAGAATTCTAAGTGGTATGTCTCGTTATG |
| **DltP2** | CTCTGGATCCCATTATCATTCACCTCCTAAG |
| **DltP3** | GAGAGGATCCTAGTTTCAGAAAGGATGGAATG |
| **DltP4** | CTCTCTGCAGAGTCAATTTCATGTGTGGACA |
| **Dlt-Up** | CCTTCTCCAACTACCGCAAC |
| **Dlt-Down** | AATGTCGTACTGCCTGCATC |
| **TagP1** | TGAAGAATTCAAAGGTACAGG |
| **TagP2** | CTCTGGATCCCGGTCTCCATCTTAATACTGT |
| **TagP3** | GAGAGGATCCCAGAAGTATCAACCACACAG |
| **TagP4** | CTCTCTGCAGGCAGCATATGGATTTTGAATG |
| **Tag-Up** | GGAATAGATCCAGCTGCG |
| **Tag-Down** | CCTTCTAAACGAACTTCTGG |
| **PgdAP1** | GAGAGAATTCGCTTGATTTGCTTGCAGTGC |
| **PgdAP2** | CTCTGGATCCATGTCGCATACTTTCACTCCT |
| **PgdAP3** | GAGAGGATCCTAGAGCAACTCGGAGCAC |
| **PgdAP4** | CTCTCTGCAGGCCACCTTATGATCCAAGAG |
| **PgdA-Up** | GCGCTTGGCTACTGTTGTGC |
| **PgdA-Down** | TCGCTTGGCTACTGTTGTGC |
| **AtlAP1** | GAGAGAATTCATCACCTCGTAAAACAGCA |
| **AtlAP2** | CTCTGGATCCTGCTTTCCTTCTTTCGATACG |
| **AtlAP3** | GAGAGGATCCAATGCAATACGGAATCAGCAT |
| **AtlAP4-SphI** | CTCTGCATGCATAACCGACACGTTCACTTT |
| **AtlA-Up** | TGGTATAAGGTCCTTTGCCT |
| **AtlA-Down** | GAACGTCCAATCCAATTTCG |

**Table 4-3 Plasmids used in this study.**

| Plasmid | Description | Reference |
|---|---|---|
| **pLT06** | Parent Deletion vector; chloramphenicol resistance | (23) |
| **pVI01** | *rpoN* deletion vector | (20) |
| **pVI08** | pLT06 containing engineered *oatA* deletion | This study. |
| **pVI10** | pLT06 containing engineered *rpoN* + *oatA* deletion | This study. |
| **pVI14** | pLT06 containing engineered *tagB* deletion | This study. |
| **pVI15** | pLT06 containing engineered *pgdA* deletion | This study. |
| **pVI17** | pLT06 containing engineered *atlA* deletion | This study. |
| **pKS101** | pLT06 containing engineered *dlt* deletion | This study |
| **pMV158GFP** | Gram-positive replicative vector: $GFP^+$ $Tet^r$ | (1) |
| **pKS12 (A)** | Derivative of pTCV - LacSpec (Del Papa MF & Perego M (9)) devoid of erythromycin resistance cassette; Promoter less lacZ $Spec^r$ | L.E. Hancock (This study) |
| **pKS82** | pKS12 (A) derivative carrying the constitutive *malM* promoter from pMV158GFP; $LacZ^+$ $Spec^r$ | This study |

was then transformed into *E. faecalis* V583 cells. The temperature shift and generation of the mutant was achieved as previously described (38). The deletion was confirmed using EF0783-Up and EF0783-Down primers. Similarly, Δ*dlt,* Δ*tagA and* Δ*pgdA* were generated in *E. faecalis* V583 as well as *E. faecalis* V583 Δ*rpoN* (VI01) (20) backgrounds.

To generate the *rpoN* mutant in a gelatinase and serine protease free background, the deletion vector construct pKS70 was transformed into a previous described protease mutant *E. faecalis* VT03 strain (37).

## Extracellular DNA Release Assay

eDNA release was used an indicator of lysis and measured using a double stranded DNA specific dye SYTOX® green as previously described (20).

## Fratricide Assay

Co-culture assay was performed as described earlier using VT02 (37) as the attacker VT03 (37) or VT03Δ*rpoN* as the target.

For quantitative detection of lysis, the target strain harboring pKS12 (A) (low copy *lacZ* vector) and hence expressing LacZ was used. After 24 hrs of co-culture, lysis was determined by β-galactosidase assay.

## Statistical Analysis

Statistical analysis of quantitative detection of eDNA release was performed using GraphPad Prism 4 software (San Diego, CA). Non-parametric t-test was used to assess the statistical significance between the mutants, the wild type parental strain *E.faecalis* V583 and the *rpoN* mutant VI01.

## 4.3 Results & Discussion

The bacterial cell wall functions as a physical barrier and protects the cell from the lytic action of several bacterial enzymes as well as host defense molecules such a lysozymes. Lysozymes are muramidases produced by the innate immune system cells and cleave at the beta 1-4 linkages between the N-acetylmuramic acid (NAM) and N-actyl glucosamine (NAG) in the bacterial peptidoglycan causing the lysis of bacterial cells (19). Similarly the bacterial autolysins such as AtlA in enterococci (11) and staphylococci (5), LytA in pneumococci (33) and MurA in *Listeria*(7) are responsible for proper septation of these bacteria. Like lysozyme, these autolysins also target the beta 1-4 linkages of NAM and NAG, giving credence to their role in bacterial lysis. The *atlA* mutant is resistant to autolysis. Sensing the susceptibility to such lytic agents, bacteria have evolved to be better prepared against their action and several modifications to the bacterial cell wall architecture are great examples of that adaption. The cell wall polymers associated with enterococcal cell wall are the type specific antigen on the outer surface responsible for the serospecificity of enterococci and include the lipoteichoic acid which is the Group D antigen, the wall teichoic acid, NAM and NAG (16). Enterococci have developed modifications in some of these polymers to protect the cells from lysis. The resistance of *E. faecalis rpoN* mutant to lysis led us to hypothesize that deletion of *rpoN* might have resulted in the increase in the expression of one of the several protective modifications to the enterococcal cell wall. O-acetylation of the peptidoglycan at the C6-hydroxyl group of the muramyl residue on NAM (12) and D-alanylation at the C2-position of the glycerol residue in LTA and WTA (14) are known to protect against the lytic action of muramidases (3, 26, 29). Our data confirms their protective role in *E. faecalis* because deletion of the *oatA* gene and *dlt* operon resulted in an increase in eDNA

release (Figure 4-1). Also, O-acetylation and D-alanylation provide protection in a balanced manner because deletions of both *oatA* and *dlt* together had a cumulative effect on lysis as observed by greater eDNA release suggesting that one modification compensated for the absence of another and when both are absent the organism becomes more susceptible to lysis. Surprisingly, in the double mutants, Δ*oatA*Δ*rpoN* and Δ*dlt*Δ*rpoN* and the triple mutant Δ*oatA*Δ*dlt*Δ*rpoN,* the absence of RpoN provided protection from lysis and reverted the phenotype to mimic the Δ*rpoN* (Figure 4-1)

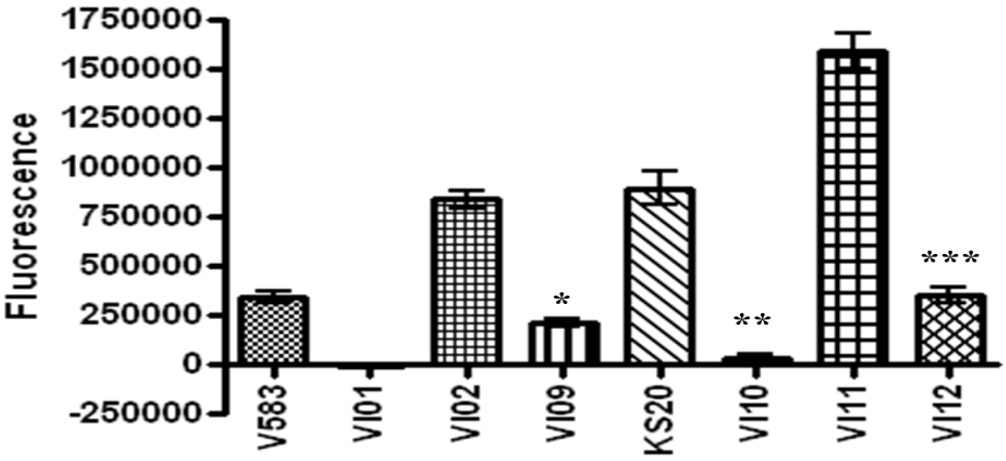



**Figure 4-1 Extracelleular DNA release Assay using mutants defective in cell wall modification**
Quantification of eDNA released in the culture supernatants of overnight grown cultures using SYTOX green DNA dye. V583 (parent strain); VI01 (V583 Δ*rpoN*); VI02 (V583 Δ*oatA*); VI09 (V583 Δ*rpoN* Δ*oatA*); KS20 (V583 Δ*dlt*); VI10 (V583 Δ*rpoN*Δ*dlt*); VI11 (V583 Δ*oatA*Δ*dlt*); VI12 (V583 Δ*rpoN* Δ*oatA* Δ*dlt*). *, significant p value less than 0.001 in comparison to VI02; **, significant p value less than 0.001 in comparison to KS20; ***, significant p values less than 0.001 in comparison to VI11.

It has been previously shown that a fratricidal (sibling killing sibling) mechanism is responsible for eDNA release in *E. faecalis* (36). In a homogenous population of cells, certain heterogeneity arises when a small group of cells fail respond to quorum and this population is subject to killing by the larger population of cells that respond to the

quorum. This population becomes the source of eDNA to the rest of the population and aid in biofilm formation. The fratricidal mechanism is mediated by the two extracellular proteases gelatinase and serine protease and is a result of their activity on the autolysin AtlA. In the absence of these proteases the cells become increasingly susceptible to lysis when co-cultured with cells that produce both these enzymes (36). In order to assess the role of RpoN in fratricide and to determine whether the protection observed in the absence of RpoN is protease independent, we deleted *rpoN* in a previously described mutant lacking both the proteases and tested it as a target strain in the fratricide assay. Similar to eDNA release assay, deletion of *rpoN* in VT03 confered protection to the target strain and reduced death as measured by beta galactosidase activity (Figure 4-2). Thus, independent of the mode of lysis (autolysis or fratricicde) absence of *rpoN* conferred novel mechanism of protection to the susceptible cells.

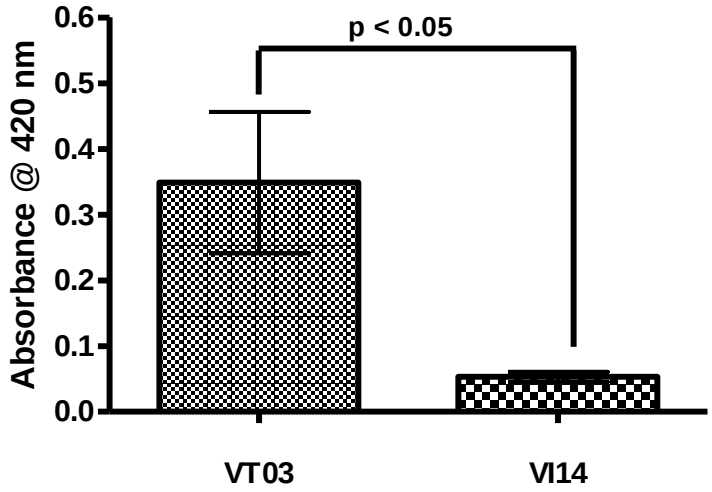



**Figure 4-2 Beta Galactosidase Assay for quantitative detection of fratricide using VT02 (Δ*sprE*) as the attacker strains.**

In addition, the charge on bacterial cell surface also plays an important role in maintaining the integrity of cells when faced by unfavorable osmotic conditions. The polymers on bacterial cell wall make them sturdy and help withstand harsh environmental conditions and provide protection from the action of antimicrobial cationic peptides.

By mutational analysis and NMR spectroscopy, the *tag* operon has been proposed to be responsible for the cell wall rhamnopolysaccharide synthesis in *E. faecalis*. It also mediates evasion from the complement system (15). In *S.aureus*, the reduction in proton gradient across the bacterial membrane in a *tag* negative mutant was shown to regulate the action of autolysin by modulating the pH because AtlA exhibits lower activity in acidic pH (4). In this study, we deleted the entire tag operon in *E. faecalis* and assessed its role in lysis and eDNA release. In contrast to the *Staphylococcus* study, *E. faecalis tag* mutant was not susceptible to lysis and had no effect on eDNA release (Figure 4-3).

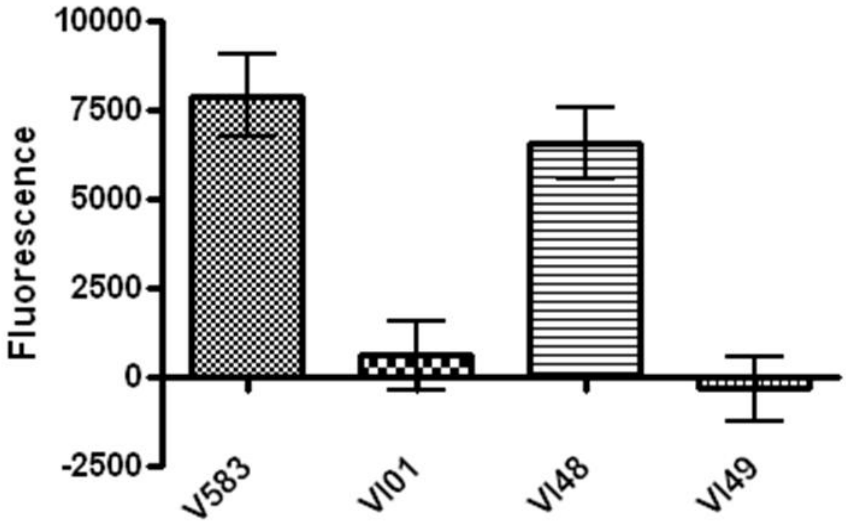



**Figure 4-3 Quantitative detection of eDNA release using SYTOX Green DNA staining dye. V583, parent strain; VI01, Δ*rpoN*; VI48, Δ*tag*; VI49 Δ*rpoN* Δ*tag*.**
No statistical significance observed between V583 and VI48 as well as VI01 and VI49.

Interestingly, in the conditions tested by us, the *tag* mutant was defective is capsule biosynthesis. The higher molecular weight band in the capsule gels (130 kDa) was absent in the *tag* mutant and *rpoN* deletion did not revert that phenotype suggesting that RpoN has no role in capsule synthesis (Figure 4-4). To the best of our knowledge, this is the first report where we describe the presence of a second capsule biosynthesis operon in addition to the *cps* operon in *E. faecalis* V583. Blastp analysis of tag operon from *E. faecalis* V583 with the other sequenced strains of enterococci revealed that all the strains that harbor the capsule operon also contain the tag operon suggesting the requirement of both operons for capsule synthesis. These two operons do not appear to be transcriptional linked because they are located far apart on the chromosome. Our future studies will entail the understanding of *tag* operon regulation and its functional characterization in *E. faecalis* V583.

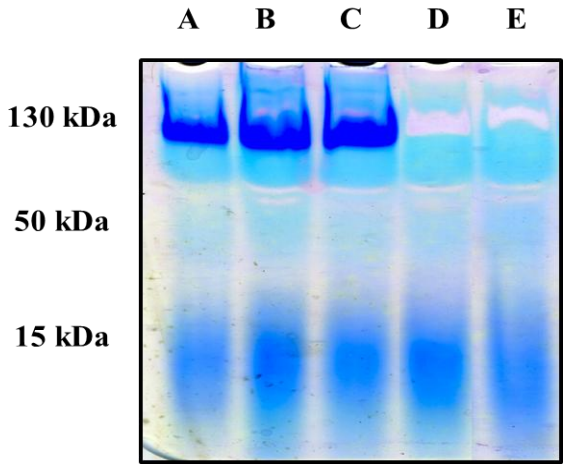



**Figure 4-4 Detection of cell wall polymers on 10% polyacrylamide gels stained using cationic dye, Stains All.**
Lane A, V583; Lane B, VI01 (Δ*rpoN*); Lane C, VI40 (*rpoN* complement); Lane D, VI48 (Δ*tag*); Lane E, VI49 (Δ*rpoN*Δ*tag*)

In addition to O-acetylation and D-alanylation, deacetylation of NAG residues by peptidoglycan N-acetyl glucosamine deacetylase (*pgdA*) has also been proposed.

However, in *E. faecalis* JH2-2, no obvious changes to cell wall composition were observed in a *pgdA* mutant (17). Similarly, in our study, the *pgdA* mutant phenocopied the parental strain in eDNA release and lysis and deleting *pgdA* in VI01 background resulted in phenotype similar to VI01 (data not shown) ruling out the possibility of this modification being involved in RpoN mediated resistance. In *E. faecalis* V583, RpoN regulates the expression of four sugar uptake systems namely Mpt, Mpo, Mph and Lpo with the help of specific activator proteins that are located upstream of the respective sugar uptake systems (18). Of the four sugar uptake systems, *mpt* operon is the major glucose/mannose uptake system in most firmicutes including enterococci (41). We have previously shown in Chapter 3 that the mutant lacking the mannose PTS system activator protein MptR, phenocopies the growth pattern of *rpoN* mutant in CDM medium with 10 mM glucose. The absence of all the known cell wall modifiers in an *rpoN* mutant did not alter its lysis resistance (Figure 4-1). This led us to hypothesize that RpoN could regulate lysis via the PTS systems whose expression is controlled by RpoN. Inorder to test this hypothesis we assessed the role of the aforementioned activator mutants of the PTS system in eDNA release. Similar to the phenotype observed in growth pattern, of the four PTS system specific activator protein mutants, only *mptR* mutant phenocopied the *rpoN* mutant. Extracellular DNA release was significantly decreased in the Δ*mptR,* as observed by Sytox Green staining (Figure 4-5).

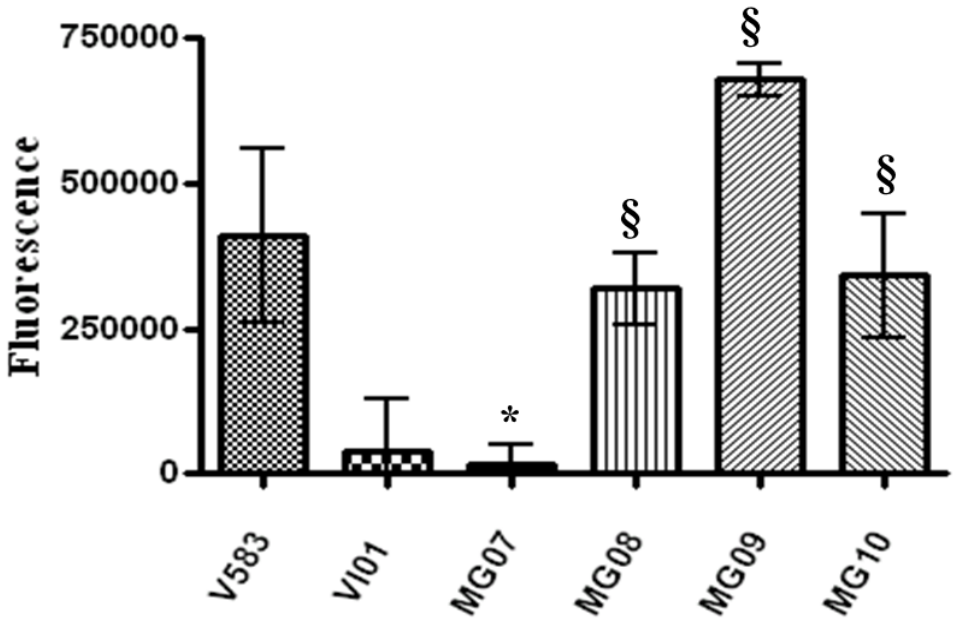



**Figure 4-5 Quantitative detection of extracellular DNA in activators mutants using SYTOX Green.**
VI01, Δ*rpoN*; MG07, Δ*mptR*; MG08,Δ*lpoR*; MG09,Δ*mpoR*; MG10,Δ*mphR*.
*, significant p value less than 0.001 in comparison to V583; §, significant p value leass than 0.001 in comparision to VI01 and MG07.

The *mpt* operon consists of four genes namely *mptB*, *mptA* , *mptC* and *mptD* that encode of cytosolic proteins EIIB & EIIA and transmembrane proteins EIIC and EIID respectively. Certain bacteria produce small antimicrobial peptides called the bacteriocins that target other closely related bacteria and/or a broader spectrum of bacteria including pathogens. Pediocins like bacteriocins or Class IIa bacteriocins are of 38-47 amino acid long peptides that display a broad spectrum of activity against several organisms including *Listeria* and *Enterococcus* (13). It has been well established that the Class IIa bacteriocins use the mannose PTS system (*mpt*) as a cellular receptor to lyse susceptible organisms (8) (10, 18). It has been previously shown that the cytosolic EIIAB complex is

not involved in conferring the sensitivity to these bacteriocins (10). There have been conflicting reports regarding the involvement of MptC and/or MptD as receptors to the Class IIa bacteriocins (8, 10, 31). However, it is clear that MptC and MptD, the two transmembrane proteins of the *mpt* pathway have a role in determining the sensitivity of the bacteria to Class IIa bacteriocin and the lactococcal bacteriocin lactococcin A (a Class IIc bacteriocin). The extracellular loop in MptC has been shown to interact with class IIa bacteriocins and a complex interaction occurs between the lactococcinA and regions in IIC and IID (22). Interestingly, development of spontaneous resistance to bacteriocins has been described and one mechanism of resistance is the down regulation of the mannose PTS system (21). These observations and our results that an *mptR* mutant mimics the resistant phenotype of the *rpoN* mutant led us to the hypothesis that the MptC/MptD receptor complex may play an important role in susceptibility of enterococci to autolysis during normal growth conditions as a part of a global regulatory mechanism of controlled lysis. The ongoing studies in our laboratory are focused on understanding the regulation of lysis resistance mediated by RpoN via MptC/MptD.

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# Chapter 5 - Summary Conclusion and Future Directions.

Enterococci have emerged as third leading causative agents of hospital acquired infections (19). Antibiotic resistance and acquisition of several virulence traits have contributed to its emergence as a pathogen. Development of resistance to the drug of last resort, vancomycin (8, 16, 20) and to the newer antibiotics such as linezolid (3, 50), daptomycin (23, 28, 34) and dalfopristin (17) have made enterococcal infections difficult to treat. In addition to acquiring antibiotic resistance, a number of virulence factors associated with enterococci such as capsule, aggregation substance, cytolysins, extracellular proteases, pili, biofilm enhancing factors etc. play an important role in the progression of a benevolent microorganism to a pathogen (4, 5, 13, 22, 25, 29, 35, 41, 43, 47-49). Enterococci are sensitive to class IIa bacteriocins or pediocin like bacteriocins produced by lactic acid bacteria (10). The sensitivity to class IIa bacteriocins is due to the mannose/glucose sugar uptake system or Mpt system in enterococci because Mpt system functions as a receptor for these bacteriocins (7). In enterococci and other bacteria such as *Listeria*, the *mpt* operon is regulated by the alternate sigma factor RpoN (6, 15).

In this study we tried to understand the role of RpoN in enterococcal biology beyond its known role in sensitivity to class IIa bacteriocins. RpoN is an alternate sigma factor that recognizes the signature -12/-24 sequence upstream the genes it regulates (27). It requires the binding of a transcriptional activator located upstream from the RpoN signature consensus for open complex formation and transcription initiation. These transcription activators possess both ATPase activity that provides the required energy and helicase activity that cause the bending of DNA (42). Four such activators have been identified upstream of the four sugar uptake systems (*mpt, mpo, lpo, mph*) regulated by

RpoN (15). Biofilm is a characteristic of infective endocarditis, urinary tract infections and surgical site infections caused by enterococci. Planktonic or sessile bacteria adhere to an available surface such as damaged heart valves, urinary tracts, catheters etc. and form microcolonies that are held together by matrix components secreted by the bacteria and contributed by the host immune systems such as fibrins and platelets (33). In enterococci, two extracellular proteases, gelatinase and serine proteases play an important role in regulating cell lysis (49). Gelatinase is pro-lytic in nature and causes lysis of cells while serine protease is anti-lytic in nature and protects the cells from lysis (45). Both these proteases are co-regulated and under the control of the *fsr*, the quorum sensing operon (39). The killing of a by-stander population that do not respond to quorum is called the 'bystander effect' and contributes one of the main matrix component i.e. extracellualar DNA that binds the biofilms (44). Autolysis and fratricide contribute to eDNA release. Apart from GelE and SprE mediated release of eDNA, no other regulation of eDNA release has been described. Our results described in Chapter 2 provide supporting information about the involvement to the alternate sigma factor RpoN in eDNA release. The *rpoN* mutant was resistant to lysis and no eDNA was detected in the culture supernatant of the *rpoN* mutant. The findings reported in Chapter 4 describe the dominance of the lysis resistant phenotype of Δ*rpoN* even in the presence of mutations that make enterococci susceptible to lysis. In Chapter 3 and Chapter 4, we further explore the potential involvement of sugar uptake systems in modulating lysis. In other bacteria, proteins and polysaccharides have been shown to be important matrix components (2, 12, 26) but little is known about other matrix components in enterococcal biofilms. In Chapter 2 we provide evidence for the presence of proteins in enterococcal biofilms at

later stages in biofilm accumulation. Proteinase K treatment of Δ*rpoN* biofilms affected accumulation of biofilms at early time points whereas in the parental strain, the treatment perturbed biofilms only at later stages. This led us to the conclusion that proteins play a role in later stages of enterococcal biofilm development. However, it does not rule out the possibility that in the parental strain, proteins in biofilm are masked by other unknown components that may be absent in Δ*rpoN* biofilms. Thus in Chapter 2, we substantiate the presence of protein in enterococcal biofilms and describe the role of RpoN in regulating eDNA release by affecting autolysis in a protease independent manner. The finding in Chapter 2 has been published in Journal of Bacteriology (21).

RpoN regulates diverse functions in several bacteria. For example in *E.coli, V.cholerae P.aeruginosa* and *V.fischeri*, RpoN controls flagellar biosynthesis (37, 38, 46, 51, 53). In *L.monocytogenes*, RpoN is responsible for mesentericin sensitivity osmotolerance and regulates several carbohydrate metabolism related genes (1, 6, 30). RpoN is required for the survival of *B.cenocepacia* within macrophages (40) and in *Borrelia burgdorferi* RpoN is required to maintain the tick-mammalian enzootic life cycle (32)

However, the knowledge about its role in *E. faecalis* V583 is restricted to bacteriocin sensitivity and regulation of sugar uptake PTS systems. In order to gain insight into the nature of genes regulated by RpoN in *E. faecalis*, we generated a transcriptional profile of Δ*rpoN* in comparison to the parental strain and the complemented strain using microarray analysis. In Chapter 3, the finding of this study has been described. Almost 10% of the genome is differentially regulated by RpoN and that includes a number of genes encoding transport and binding proteins, proteins related

to metabolic function and several other transcription regulators. Since, RpoN regulates the function of Mpt system; there was an extensive overlap of differentially expressed genes between the transcriptional profile of Δ*rpoN* and that of the previously described *mpt* mutants (31). Our data indicate a role for RpoN in carbon catabolite repression or CCR in enterococci because several genes with catabolite responsive elements (*cre*) in their promoter regions were differentially regulated in Δ*rpoN*.

In Chapter 3, using a rabbit model of infective endocarditis and mouse model for catheter associated urinary tract infections (CAUTI), we assessed the role of RpoN in enterococcal virulence. The bacterial burden in the organs of rabbits and mice infected with Δ*rpoN* was significantly lower in comparison to the wild type. The *rpoN* mutant grew poorly in chemically defined medium with glucose at 10mM and 50mM concentration. At physiologically relevant glucose concentrations i.e. 5 mM (18, 52) Δ*rpoN* was not able to reach a cell density similar to the wild type. This defect justifies the inability of Δ*rpoN* to establish an infection in the rabbit endocarditis and CAUTI studies. Our data in chapter 3 describes the indispensability of RpoN for growth of enterococci in nutrient limiting conditions and to establish infection. It also highlights the regulatory effect of RpoN on genes in addition to the sugar PTS systems.

Presence of capsular polysaccharide (47) and modification on the cell wall polymers of enterococci such as O-acetylation (9, 36), D-alanylation (11) and deactylation (14) that have a protective function have been described. The findings in Chapter 4 show that the lysis resistant phenotype of RpoN is independent of any of the known modifications. In Chapter 4, a new mechanism of resistance to lysis involving the mannose PTS system components, MptC and MptD has been proposed. It is based on the

knowledge that these two transmembrane proteins of *mpt* operon play an important role in bacteriocin mediated sensitivity to lysis (7). Using a series of permutations and combinations of MptC and D protein fusions, it has been shown that these proteins acts as receptors for docking bacteriocins and thus regulate sensitivity (24). There exists as possibility that these transmembrane proteins could bind to unidentified bacteriocins in *E. faecalis* genome that modulate lysis. In order to test this hypothesis, experiments involving expression of *mptCD* under the control of a constitutive or inducible promoter independent of *mptBA* in Δ*rpoN* is underway. The rationale underlying this approach is that expression of *mptCD* in Δ*rpoN* may revert the lysis resistant phenotype of Δ*rpoN*.

Another project of interest stems from the identification of a second pathway involved in capsule biosynthesis. During the course of our research we found that presence of teichoic acid biosynthesis genes (EF1172-EF1175) was essential for capsule synthesis. Both capsule operon (*cps*) and teichoic acid biosynthesis genes (*tag)* are required for capsule biosynthesis because no capsule is observed when cell wall preparations were analyzed on polyacrylamide gels stained using the Stains-All dye in the absence of either one of the operon. Interestingly, the *cps* and *tag* operon are located at a distance on the chromosome and do not appear to be transcriptionally linked. Given these exciting preliminary finding, the role of *tag* operon should be further tested to understand its contribution to enterococcal capsule biosynthesis.

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