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*Enterococcus faecalis* capsular polysaccharide and mechanisms of host innate immune evasion

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Running Title: *E. faecalis* capsule and innate immunity

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1 **Abstract**

2 It has become increasingly difficult to treat infections caused by *Enterococcus faecalis* due to  
3 the high levels of intrinsic and acquired antibiotic resistances. However, few studies have  
4 explored the mechanisms that *E. faecalis* employs to circumvent the host innate immune  
5 response and establish infection. Capsule polysaccharides are important virulence factors that  
6 are associated with innate immune evasion. We demonstrate that capsule producing *E. faecalis*  
7 strains of either serotype C or D are more resistant to complement-mediated opsonophagocytosis  
8 compared to un-encapsulated strains using cultured macrophages (RAW 264.7). We show that  
9 differences in opsonophagocytosis are not due to variation in C3 deposition, but due to the ability  
10 of capsule to mask bound C3 from detection on the surface of *E. faecalis*. Similarly, *E. faecalis*  
11 capsule masks detection of lipoteichoic acid which correlates with decreased TNF- $\alpha$  production  
12 by cultured macrophages in the presence of encapsulated strains compared to unencapsulated  
13 strains. Our studies confirm the important role of the capsule as a virulence factor of *E. faecalis*,  
14 and provide several mechanisms by which the presence of the capsule influences evasion of the  
15 innate immune response, and suggest that the capsule could be a potential target for developing  
16 alternative therapies to treat *E. faecalis* infections.

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**Introduction**

*Enterocoocus faecalis* is an important nosocomial pathogen associated with many types of infections including surgical site infections, bacteremia, urinary tract infections, and endocarditis (32). Many infections caused by *E. faecalis* are difficult to treat due to increasing resistance to conventional antibiotic therapies including vancomycin (11-13). Apart from studies on the roles of gelatinase and cytolysin (6, 23, 28), relatively little is known about the mechanisms employed by *E. faecalis* to circumvent host innate immune responses.

In other bacterial pathogens, the production of capsular polysaccharide is a known virulence factor as it aids in avoidance of the host innate immune response (26, 31, 37). *E. faecalis* is known to produce two capsular polysaccharide serotypes (C and D)(11, 13, 15, 41) that contribute to pathogenesis and evasion of the host innate immune response (11). Hufnagel *et al.* reported decreased neutrophilic killing of encapsulated serotype C and D strains compared to the un-encapsulated A and B strains (16). In addition, a recent comprehensive analysis of clinical *E. faecalis* isolates indicated that most pathogenic strains of *E. faecalis* belonged to serotypes C (20). Despite a link between capsule and virulence, little is known about the specific mechanism(s) of how capsule enhances pathogenesis.

The complement system plays a central role in the activation of the immune system and in the clearance of pathogens. Cleavage of C3 to C3b provides a highly effective opsonin in the absence of antibodies. Several reports have shown that capsule producing species of bacteria are more resistant to opsonophagocytosis by inhibiting the deposition and/or detection of C3b on the

1 surface of the organism (29, 33, 43). Encapsulated bacteria employ numerous mechanisms to  
2 resist C3 opsonization and subsequent phagocytosis, including overall reduction in C3 deposition  
3 (7). The abundance of C3 deposition is known to differ between capsule producing serotypes of  
4 *Streptococcus pneumoniae* (21). In *Staphylococcus aureus*, C3 is buried beneath the surface of  
5 the capsule rendering C3 less accessible to complement receptors on the surfaces of  
6 macrophages and neutrophils (42).

7

8 Bacterial capsular polysaccharides are also known to aid in the avoidance of innate immune  
9 responses including immune surveillance. Immune surveillance relies on pathogen recognition  
10 receptors (PRRs), including Toll-like receptors, to sense pathogen associated molecular patterns  
11 (PAMPs). Two common PAMPs associated with Gram-positive microorganisms are  
12 lipoteichoic acid (LTA) and peptidoglycan (PGN). Detection of these PAMPs by Toll-like  
13 receptor 2 in conjunction with Toll-like receptors 1 and 6 induces the production of cytokines.  
14 In other instances, capsule prevents the detection of PAMPs by PRRs which leads to decreased  
15 or altered cytokine production (10). The altered cytokine response to encapsulated pathogens  
16 appears to contribute to pathogenicity and virulence.

17

18 Our data indicate that the *E. faecalis* capsular polysaccharides from serotypes C and D attenuate  
19 C3 opsonized phagocytosis, and that this attenuated response is likely due to decreased  
20 recognition of bound C3 on the bacterial surface. Similarly, capsule inhibits detection of *E.*  
21 *faecalis* LTA on the surface and the absence of recognition of this molecule and/or other surface  
22 PAMPs in the presence of capsule results in decreased TNF- $\alpha$  production by macrophages.

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**Material and Methods**

**Bacterial Strains, plasmids, and growth conditions.** All relevant bacterial strains are listed in table 1. *E. faecalis* strains were cultivated in Todd-Hewitt broth supplied with the appropriate antibiotics when needed (THB; Becton, Dickinson and Company, Sparks, Maryland).

**Culture of Macrophages.**

The macrophage like RAW 264.7 (ATCC TIB-71) cells were cultured in DMEM (Invitrogen, Grand Island, N.Y.) supplemented with 100 U penicillin per mL, 100 µg streptomycin per mL, 2 µg L-glutamine per mL, and 5% heat inactivated fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA).

**Complement C3 Deposition**

Overnight cultures of *E. faecalis* were diluted 1:100 in fresh media. The cultures were allowed to reach mid-log phase (O.D. 600 of 0.6), and were washed 3X in sterile phosphate buffered saline (PBS) pH 7.4. Approximately  $2 \times 10^7$  cells of each strain were re-suspended in 10% normal CD1 mouse serum containing complement (Innovative Research, Southfield, MI) diluted in PBS. Serum for negative controls was heat inactivated prior to the addition of bacteria by incubating at 56°C for 30 minutes. Bacteria were incubated in 10% serum for 30 minutes at 37°C with agitation. Complement deposition was stopped by addition of EDTA to a final concentration of 10mM followed by incubation on ice for 5 minutes. The bacteria were pelleted at 4°C, washed 3 times with sterile PBS to remove unbound complement, and finally re-suspended in 30 µL of 1X SDS-PAGE loading buffer. Whole bacteria were boiled vigorously for five minutes, and the cell debris was removed by centrifugation. The remaining supernatants were loaded on an SDS-PAGE gel and electrophoresed. Proteins in the gel were transferred to nylon membranes, and detection of C3 was carried out by western blot analysis using goat anti-

1 mouse C3 polyclonal antibodies (Bethyl Laboratories, Montgomery, TX) and rabbit anti-goat  
2 conjugated with horse radish peroxidase (HRP) as secondary antibody (Bethyl Laboratories,  
3 Montgomery, TX) followed by development with SuperSignal<sup>®</sup> West Pico Chemiluminescent  
4 Substrate (Thermo Scientific, Rockford, IL).

## 5 **ELISA**

6 The concentration of naturally occurring anti-enterococcal antibodies present in the CD1  
7 (Innovative Research, Southfield, MI) mouse serum (used for subsequent phagocytosis assays)  
8 was analyzed by ELISA. In addition, ELISA was performed to investigate the serotype  
9 specificity conferred by the presence of CpsF among *E. faecalis* isolates using serotype C-  
10 specific antibodies. Briefly, log phase *E. faecalis* strains were washed 3 times in PBS and  
11 aliquoted (50  $\mu$ L) into high binding 96 well Costar plates (Corning). The washed cells were  
12 allowed to adhere overnight at 4°C. Bound cells were then incubated with either CD1 mouse  
13 serum or rabbit anti-serotype C serum (19) followed by incubation with either goat anti-mouse  
14 IgG HRP conjugate (Sigma, Saint Louis, MO) or goat anti-rabbit IgG HRP conjugate (Jackson  
15 ImmunoResearch, West Grove, PA). ELISAs were developed using o-phenylenediamine  
16 dihydrochloride (OPD, Sigma) as the HRP substrate, and the results were read at O.D. 490 on a  
17 Bio-Tek PowerWave XS 96 well plate reader.

18

## 19 **Opsonophagocytosis assay**

20 *E. faecalis* strains V583, LT02 (V583  $\Delta$ *cpsF*), and LT06 (V583  $\Delta$ *cpsC*) were transformed by  
21 electroporation with the plasmid pMV158GFP (25) giving rise to LT12, LT13, and LT14  
22 respectively (Table 1). Strains LT12, LT13, and LT14 constitutively express GFP allowing  
23 fluorescent detection during the opsonophagocytosis assay.

1 Log phase bacteria were washed three times in PBS prior to re-suspending in HBSS (Invitrogen)  
2 media. Harvested RAW 264.7 cells were also re-suspended HBSS media. A concentration of  
3  $2 \times 10^6$  CFU/mL bacteria were added to  $2 \times 10^5$  RAW 264.7 cells/mL followed by the addition of  
4 complement containing CD1 mouse serum to a concentration of 10% to give a final volume of  
5 500  $\mu$ L and a bacteria to macrophage ratio of 10:1. The samples were incubated at 37°C for 20  
6 minutes to allow uptake of bacteria by macrophages. Trypsin was then added at 0.25% final  
7 concentration and incubated for 10 minutes to remove any bacteria bound to the external surfaces  
8 of the RAW 264.7 cells. The free bacteria were removed by three PBS washes with low speed  
9 centrifugation (750Xg) (8, 9). The washed cells were fixed to glass slides by cyto-  
10 centrifugation. The samples were viewed under 100X oil immersion using a Ziess Axioplan 2  
11 fluorescent microscope to visualize the GFP expressing bacteria inside the RAW 264.7 cells.  
12 The intracellular bacteria of at least 100 RAW 264.7 cells were counted for each experimental  
13 replicate. The phagocytic index was calculated by dividing the number of phagocytic cells (cells  
14 that had consumed bacteria) by the total number of macrophages counted and multiplying that  
15 number by the number of bacteria per phagocytic macrophage ( $\frac{\# \text{phagocytic cells}}{\text{total cells counted}} \times$   
16  $\frac{\text{bacteria}}{\# \text{phagocytic cells}}$ ) as previously described (22, 30). Data are presented as percent phagocytic  
17 index with the phagocytic index of LT14 (V583 $\Delta$ *cpsC*, capsule -) set to 100%. Data were  
18 compiled from three separate experiments and the standard error of the mean and statistical  
19 significance were calculated with Graphpad Prism software.

20

## 21 Slide Agglutination

22 Un-encapsulated and encapsulated strains were tested for their reactivity to serotype A  
23 antiserum, previously reported to be specific for enterococcal lipoteichoic acid (LTA) (40).



1 Slide agglutination assays were performed as previously described (19, 41). Briefly, log phase  
2 bacteria were washed three times with PBS. Following the PBS washes, 5.0  $\mu$ L of LTA  
3 antiserum or pre-immune serum was added to 15.0  $\mu$ L of test cells on a glass slide, and gently  
4 rotated for one minute. Agglutination was determined by visual clumping of the cells. Sterile  
5 PBS and pre-immune serum were used as negative controls.

6

### 7 **Flow Cytometry**

8 Flow cytometry was used to determine if C3 or LTA accessibility to antibodies was altered by  
9 the presence of capsule. Log phased bacteria were washed three times in PBS, diluted 1:2, and  
10 blocked in 5% donkey serum (Jackson ImmunoResearch). Bacteria used for analyzing C3  
11 accessibility were incubated in 50  $\mu$ L of CD1 mouse serum for 20 minutes at 37°C to allow for  
12 C3 deposition and washed three times in PBS prior to blocking with donkey serum. Blocked  
13 cells were incubated for 15 minutes on ice with 2.0% goat anti-C3 antibodies followed by three  
14 washes in PBS. Similarly diluted goat serum was used as an isotype control. The bacteria were  
15 then incubated with FITC conjugated donkey anti-goat antibody (1:1000) (Jackson  
16 ImmunoResearch) for 15 minutes on ice in the dark. The bacteria were again washed three times  
17 with PBS and analyzed by flow cytometry. For detection of LTA accessibility, washed and  
18 blocked bacterial cells were incubated on ice for 15 minutes with 2.0% anti-LTA rabbit serum  
19 (40). Similarly diluted pre-immune rabbit serum was used as an isotype control. Cells were then  
20 washed three times in PBS and incubated for 15 minutes on ice in the dark with FITC conjugated  
21 donkey anti-rabbit antibody (1:100) (Jackson ImmunoResearch). Bacteria were washed three  
22 times in PBS and analyzed by flow cytometry. For both the C3 and LTA experiments, flow  
23 cytometry analysis of 50,000 bacteria was performed using a FACSCalibur flow cytometer

1 (Becton and Dickinson, San Jose, CA) at a flow rate of ~2000 cells per second. Data were  
2 analyzed using the WinList software program (VerityHouse, Topsham, ME).

3

#### 4 **TNF- $\alpha$ production**

5 Log phase bacteria were washed three times in PBS and heat killed by incubation at 80°C for 30  
6 minutes. RAW 264.7 cells were harvested and re-suspended in fresh DMEM culture media to a  
7 concentration of  $1 \times 10^6$  cells per mL. RAW cells at a concentration of  $1 \times 10^6$  cells/mL in a total  
8 volume of 2.0 mL were seeded in 24 well plates. The cells were allowed to adhere to the plate  
9 surface for two hours prior to induction. Bacteria were added to each well at a concentration of  
10  $1 \times 10^7$  Cfu. Lipopolysaccharide (LPS) from *Salmonell enterica* serotype typhimurium (Sigma)  
11 was used as a positive control for TNF- $\alpha$  production at a concentration of 10 ng per mL.

12 Clarified supernatants were collected from each well at four hours after the bacterial inoculation.  
13 The amount of TNF- $\alpha$  present in the supernatants was determined by ELISA (eBioscience, San  
14 Diego, CA) following the manufacturer instructions. One way ANOVA in correlation with a  
15 Newman-Kuels post hoc test were used to evaluate statistical significance (GraphPad Prism).

16

## 17 **Results**

18

### 19 **Protective effects of capsule on opsonophagocytosis**

20

21 The capsular polysaccharides of many bacterial species confer resistance to complement  
22 mediated opsonophagocytosis. We examined whether *E. faecalis* capsule conferred resistance to  
23 C3 opsonophagocytosis mediated by macrophages. We used ELISA to confirm that our  
24 complement source (CD-1 mouse serum) was free of detectable *E. faecalis* antibodies (Data not  
25 shown).

26

1 Previous studies have shown that *E. faecalis* opsonizing antibodies exist in normal human serum;  
2 however, these antibodies are only directed towards the un-encapsulated serotype A and B  
3 strains of *E. faecalis* (2, 15). In view of these studies, we determined if *E. faecalis* capsule  
4 serotypes C or D conferred resistance to complement mediated opsonophagocytosis compared to  
5 an isogenic acapsular mutant. The encapsulated *E. faecalis* strains LT12 (serotype C), LT13, an  
6 isogenic *cpsF* deletion mutant which results in the production of a serotype D capsular  
7 polysaccharide (40) and LT14, an isogenic *cpsC* deletion mutant which is un-encapsulated (40)  
8 were compared. For this assay, we followed the method of Drevets et al. (8, 9) which calls for  
9 trypsin treatment and subsequent washes to remove externally bound bacteria as opposed to  
10 antibiotic treatment with gentamicin which has been shown to be internalized by macrophages  
11 leading to antibiotic killing affects independent of macrophage activity (9). Our data shows a  
12 50% reduction in the opsonophagocytosis of capsule producing strains by macrophages in the  
13 presence of complement compared to unencapsulated strains (Fig. 1). These data also show that  
14 there is no statistical difference in opsonophagocytosis between isogenic serotype C (LT12) and  
15 serotype D (LT13) strains (Fig. 1), suggesting that the mere presence of capsule regardless of  
16 serotype provides protection against bacterial uptake by macrophages .

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### 18 **Complement C3 deposition and surface accessibility**

19 Bacterial resistance to complement mediated opsonophagocytosis has been attributed to  
20 decreased amounts of C3 deposition on the surface of encapsulated strains (7). We used western  
21 blot analysis to assess the abundance of complement C3 deposited on both encapsulated and un-  
22 encapsulated *E. faecalis* strains. Two encapsulated strains FA2-2 and LT01(FA2-2 $\Delta$ *cpsF*) and  
23 two un-encapsulated LT05 (FA2-2 $\Delta$ *cpsC*) and OG1RF strains were used in this experiment.

1 Complement C3 is composed of an  $\alpha$  and a  $\beta$  chain (35). The 75 kDa C3  $\beta$  chain is left intact  
2 through the processing events of C3, and was used to determine differences in overall C3  
3 deposition. Figure 2 shows the deposition of the 75 kDa  $\beta$  chain of C3 on different strains of *E.*  
4 *faecalis*. There is no difference in the amount of C3 deposited on the surfaces of the un-  
5 encapsulated strains OG1RF and LT05 when compared to the encapsulated V583 and LT01  
6 strains (Fig. 2). The other detected fragments in this blot are known breakdown products of C3  
7 and C3b.

8  
9 The amount of complement deposition does not vary between strains, but the presence of  
10 complement on the encapsulated strains could be masked from detection by complement  
11 receptors leading to decreased phagocytosis. We used complement opsonized strains of V583  
12 (serotype C), LT02(V583 $\Delta cpsF$ , serotype D) and LT06 (V583 $\Delta cpsC$ , capsule -) in conjunction  
13 with flow cytometry to determine C3 surface accessibility to antibodies. Our data show that C3  
14 deposited on the surface of LT06 is more detectable than C3 deposited on the surface of  
15 encapsulated strains V583 and LT02 (Fig. 3). Statistical analysis using one-way ANOVA in  
16 conjunction with a Newman-Keuls post hoc test show a significant statistical difference (p-  
17 values  $< 0.05$ ) between V583 and LT06, and also between LT02 and LT06 (Fig. 3). There was  
18 also a statistically significant difference between V583 and LT02 even though they appear to be  
19 equally resistant to complement mediated opsonophagocytosis (Fig. 1). The basis for this  
20 difference is not known at the present time, but may relate to structural differences in the  
21 capsular polysaccharides between these two serotypes.

22

23

1 **Lipoteichoic acid and capsule**

2 Lipoteichoic acid (LTA) and peptidoglycan are PAMPs present on *E. faecalis* that are known to  
3 stimulate the immune system through pathogen recognition receptors including TLR-2 (36). The  
4 capsules produced by other bacteria shield PAMPs resulting in altered cytokine production (31).  
5 We examined differences in LTA accessibility between encapsulated and un-encapsulated strains  
6 by slide agglutination assays. *E. faecalis* serotype A anti-serum is directed against enterococcal  
7 LTA (40). We tested the ability of these antibodies to agglutinate either encapsulated or un-  
8 encapsulated *E. faecalis* strains. The encapsulated strains V583 (serotype C) and LT02( $\Delta cpsF$ ,  
9 serotype D) were not agglutinated by the anti-serum, whereas the un-encapsulated strains  
10 LT06( $\Delta cpsC$ ) and 12030 (serotype A reference strain) were both agglutinated (data not shown).

11

12 As agglutinating antibodies are generally of the IgM class, we also used flow cytometry to  
13 quantify the differences of LTA availability to the IgG class. Strains V583, LT02, and LT06  
14 were incubated with Serotype A antiserum followed by a FITC conjugated secondary antibody.  
15 Figure 4A and B show the percentage of the cells that were positive for FITC labeling. One-way  
16 ANOVA followed by a Newman-Keuls post hoc test showed significant statistical differences  
17 ( $p$ -values  $< 0.05$ ) in the amount LTA detected between V583 (serotype C) and LT06 (capsule -),  
18 and also between LT02 (serotype D) and LT06. However, there was no significant statistical  
19 difference when the encapsulated strains V583 and LT02 were compared. These data indicate  
20 that capsule produced by either serotype C or D strains masks LTA from antibody detection.

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1 **TNF- $\alpha$  production in response to capsule**

2 The presence of a capsule is known to alter the macrophage cytokine response in other  
3 microorganisms (10). To examine this possibility in *E. faecalis*, we used ELISA to assess the  
4 ability of capsule producing and non-producing strains to induce TNF- $\alpha$  production by RAW  
5 264.7 cells. We predicted that the ability of capsule to inhibit detection of LTA (Fig. 4) would  
6 translate to less TNF- $\alpha$  production by RAW 264.7 cells. The capsule producing strains T-5  
7 (serotype D), V583 (serotype C), LT02 (serotype D) along with the un-encapsulated strains  
8 (LT06, 12030, OG1RF) were heat-killed and incubated with RAW 264.7 cells. Clarified  
9 supernatants were collected at 4 hours post inoculation, and were analyzed for TNF- $\alpha$   
10 production. The TNF- $\alpha$  produced in response to the un-encapsulated strains is significantly  
11 higher than that produced in response to encapsulated strains with p-values < 0.05 using one-way  
12 ANOVA and a Newman-Keuls post hoc test analysis (Fig. 5). However, there is no statistically  
13 significant difference when comparing the encapsulated strains with each other or when  
14 comparing the un-encapsulated strains with each other. Strikingly, there is no statistically  
15 significant difference in the amount of TNF- $\alpha$  produced by RAW cells when comparing the  
16 strains T-5, V583, and LT02 to the un-induced RAW control cells.

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1 **Discussion**

2 Capsular polysaccharides contribute to the virulence of microorganisms through multiple  
3 mechanisms including resistance to opsonophagocytosis, and by masking bacterial surface  
4 antigens from detection by the host immune system (1, 10). Several Gram-positive cocci  
5 including *S. aureus* (26), *S. pneumoniae* (1), and group-B streptococci (4) produce capsular  
6 polysaccharides that are known to contribute to virulence. Previous reports have indicated that  
7 *E. faecalis* strains can be classified by the presence or absence of capsular polysaccharide (11,  
8 15, 16, 40). Hancock and Gilmore (11) showed that the presence of capsule enhances  
9 persistence at infectious sites using a murine infection model, and subsequently showed that  
10 encapsulation protects the bacteria from killing by neutrophils, whereas an unencapsulated  
11 isogenic mutant was readily killed by neutrophils. The killing of the unencapsulated mutant by  
12 neutrophils was dependent on the opsonic activity of complement.

13

14 Here, we demonstrate that *E. faecalis* capsular polysaccharide serotypes C and D provide  
15 resistance to complement opsonized phagocytosis by macrophages. In good agreement with  
16 previously reported work on the role of the *E. faecalis* capsule in affecting resistance to opsonic  
17 killing by neutrophils (11, 16), we observed a 50% reduction in phagocytic killing in  
18 encapsulated strains compared to the isogenic acapsular mutant. An additional cell wall  
19 polysaccharide in *E. faecalis* termed Epa has also been shown to contribute to resistance to  
20 phagocytic killing (38), and may account for why the protective affect of the capsule is not more  
21 substantial in *E. faecalis*. Unlike the capsule, the Epa polymer and its genetic locus appear to be  
22 highly conserved in *E. faecalis* (11, 38). A direct comparison on the relative contribution of Cps  
23 and Epa in the same strain background has not possible to date, because the OG1RF strain in

1 which Epa mutants were created lacks the capsule locus (15, 41), and in our hands we have been  
2 unable to generate Epa mutants in encapsulated strain backgrounds (L.T., unpublished data). A  
3 recent report by Teng et al. (39) demonstrated gross changes in the bacterial cell shape of Epa  
4 mutants in the OG1RF background and this may account for our inability to generate such  
5 mutants in our encapsulated strains and may partially explain the pleiotropic affects ascribed to  
6 the Epa locus in virulence studies (39, 44).

7

8 An additional benefit of the macrophage system is the use of cultured cells that are less likely to  
9 vary from experiment to experiment compared to the neutrophil assay, which requires fresh  
10 isolation of neutrophils from human blood donors. Furthermore, because the strains used in this  
11 comparative study were isogenic derivatives we can make a direct assessment on the role of  
12 capsule and serotype differences in host immune evasion as has been observed in other microbial  
13 pathogens (29, 33, 43) 43). Our findings show that *E. faecalis* capsular polysaccharides alter the  
14 detection of C3 and LTA by antibodies (Figs. 3-4). Paralleling these findings, we also  
15 demonstrate that the presence of capsule also abrogates TNF- $\alpha$  production by macrophages (Fig.  
16 5). Together these data provide a mechanism by which the presence of capsule alters  
17 complement-mediated opsonophagocytosis by altering accessibility of the bound C3b opsonin,  
18 as well as limiting the recruitment of phagocytes to sites of infection by altering the production  
19 of TNF- $\alpha$  in response to encapsulated *E. faecalis*. It is noteworthy that capsule serotype  
20 differences in an isogenic background did not result in significant changes in resistance to  
21 opsonin-mediated phagocytosis, or in altered TNF- $\alpha$  response. McBride et al. (20) recently  
22 showed that clinical isolates of *E. faecalis* possessing multiple virulence factors, as well as multi-  
23 drug resistance were more likely to be identified as capsule serotype C. Our findings suggest



1 that either of the encapsulated serotypes (C or D) benefit the bacterium in evasion of the host  
2 innate response. We did however observe a significant difference in the amount of bound C3  
3 detectable on the surface of isogenic serotype C compared with serotype D capsule, but this  
4 difference did not correlate with changes in the phagocytic index of these strains, leaving open  
5 the question as to why the more pathogenic and drug-resistant clinical isolates are more  
6 frequently identified as serotype C as opposed to D. In *S. aureus*, comparison of the contribution  
7 of type 5 and type 8 capsule in the same strain background revealed that the presence of N-  
8 acetylation on the type 5 capsule structure conferred a fitness advantage *in vivo* (42). Whether a  
9 similar affect will also be observed in the comparison of *E. faecalis* serotype C and D strains *in*  
10 *vivo* will be the focus of future studies.

11  
12 The mechanism by which capsule alters the phagocytic response in the presence of complement  
13 opsonization differs depending on the microorganism. The capsule of group-B streptococci  
14 contains a sialic acid side chain that inactivates C3b, whereas the capsule serotypes 5 and 8 of *S.*  
15 *aureus* shield C3 from detection by the cognate receptors (4, 42). In contrast, the presence of  
16 capsule in *S. pneumoniae* decreases the amount of complement deposited on the cell surface  
17 (21). Our results suggest that the mechanism of evading opsonophagocytosis by encapsulated *E.*  
18 *faecalis* is dependent on the ability of the capsule to mask the bound C3b to prevent it from being  
19 recognized by host effector cell receptors targeted to bound complement.

20  
21 Aside from anti-phagocytic properties, bacterial capsules also act as barriers that limit detection  
22 of PAMPs by PRRs (1, 10). A common PAMP shared by all strains of enterococci is LTA. The  
23 LTA of *E. faecalis* is known to stimulate TNF- $\alpha$  production via TLR-2 and TNF- $\alpha$  is thought to

1 play a key role in *E. faecalis*-mediated inflammatory responses (3), though the full role of TNF- $\alpha$   
2 in *E. faecalis* infections is not fully understood (27). A study involving *Enterococcus faecium*,  
3 which produces serologically identical LTA to *E. faecalis*, showed that TLR-2 mediated  
4 signaling was critical for early immune response and clearance of *E. faecium* (18). Based on  
5 these studies, recognition of enterococcal LTA and/or peptidoglycan by TLR-2 would appear  
6 critical for an efficient host immune response, and the masking of these integral wall components  
7 by capsule could result in increased pathogenesis by limiting the host response to the organism..  
8 Interestingly, a study by Kau et al. (17) demonstrated that the response to *E. faecalis* in a urinary  
9 tract infection model is not TLR-2 dependent. The capsule phenotype of the clinical isolate used  
10 in this study is not known, and based on our finding that the presence of the capsule alters  
11 recognition of an important PAMP (LTA) known to be recognized by TLR-2, it would suggest  
12 that TLR-2 signaling might only be of benefit against *E. faecalis* strains that lack capsule.

13  
14 Our goal was to understand the mechanism of how encapsulation enhances the resistance of *E.*  
15 *faecalis* to innate immunity. Taken together, our results show that the two capsule serotypes  
16 produced by *E. faecalis* can subvert host innate immune responses by conferring resistance to  
17 complement-mediated phagocytosis, as well as altering the innate response to the pathogen. This  
18 study provides mechanistic evidence demonstrating that the *E. faecalis* capsule alters the  
19 accessibility of bound C3 supporting the observation that the most pathogenic lineages of *E.*  
20 *faecalis* are encapsulated (20, 40). By masking PAMPs on the surface of *E. faecalis*, the capsule  
21 also alters the host response to infection by encapsulated strains. It is our contention that the  
22 capsule produced by *E. faecalis* serotypes C and D is an important virulence determinant that

1 plays multi-faceted roles in evasion of host innate immune responses. Because of this, we  
2 believe that the *E. faecalis* capsule could serve as a target for developing future therapeutics.

3

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10 Terry C. Johnson Cancer Center at Kansas State University (V.C.T).

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## List of Figures

1

2 **Figure 1.** Capsule serotypes C and D are resistant to opsonophagocytosis in the presence of  
3 complement. **A.** Representative micrographs depicting from left to right LT12 (V583 expressing  
4 Gfp), LT13 ( $\Delta cpsF$  expressing Gfp), and LT14 ( $\Delta cpsC$  expressing Gfp) incubated with RAW  
5 264.7 macrophage like cells. **B.** Quantification of phagocytic index expressed as the percentage  
6 of the un-encapsulated LT14 strain (see Materials and Methods for calculating Phagocytic  
7 index). The light gray bar (LT12: serotype C) and the dark gray bar (LT13: serotype D) both  
8 show a significant reduction in phagocytic index when compared to LT14 (black bar). Error bars  
9 represent SE of three replicates.

10 **Figure 2.** The amount of C3 deposition does not differ between strains. Western blot analysis  
11 was employed to examine the amount of the C3 deposited on the cell surface of serotype C  
12 (FA2-2), serotype D (LT01), and un-encapsulated (LT05 and OG1RF) strains. The blot shows  
13 the 75 kDa  $\beta$  chain of C3 for FA2-2 (A), LT02 (B), LT05 (C), OG1RF (D) ,and the negative  
14 control, FA2-2 incubated with heat inactivated serum (E).

15 **Figure 3.** Complement C3 is masked from detection by capsule. Flow cytometry was used in  
16 conjunction with anti-C3 antibodies and FITC conjugated secondary antibodies to evaluate the  
17 availability of C3 to detection. **A.** Representative histograms depicting (from left to right) flow  
18 cytometry results for serotype C (V583), seroypte D (LT02) and un-encapsulated (LT06) *E.*  
19 *faecalis* strains. The isotype controls are light gray and the C3 antibody treated cells are dark  
20 gray. **B.** Quantification of the C3 positive cells. Using one-way ANOVA in conjunction with a  
21 Newman-Keuls post test, statistical analysis for three replicates showed statistically significant  
22 differences (p-value < 0.05) in the amount of positively labeled bacteria when V583 (light gray

1 bar) and LT06 (black bar) were compared, and when LT02 (dark gray bar) and LT06 were  
2 compared. Statistical analysis also revealed a significant difference in C3 detection between  
3 V583 and LT02 ( $P < 0.05$ ). Error bars represent SE for three replicate. Approximately 50,000  
4 bacteria were analyzed for each replicate.

5 **Figure 4.** The presence of capsule masks LTA from detection by antibodies. Flow cytometry  
6 was used in conjunction with LTA antiserum and FITC conjugated secondary antibodies to  
7 evaluate the levels of LTA accessibility. **A.** Representative histograms depicting (from left to  
8 right) flow cytometry results for serotype C (V583), seroypte D (LT02) and un-encapsulated  
9 (LT06) *E. faecalis* strains. The isotype controls are in light gray and the C3 antibody treated  
10 cells are dark gray. **B.** Quantification of LTA detection by flow cytometry. Statistical analysis  
11 for three replicates using a one-way ANOVA in conjunction with a Newman-Keuls post test  
12 showed significant differences ( $P < 0.05$ ) in the amount of LTA detected between V583 (light  
13 gray bar) and LT06 (black bar), and between LT02 (dark gray bar) and LT06 with p-values less  
14 than 0.05. However, there is no statistical difference in LTA detection when LT02 is compared  
15 V583. Error bars represent SE for three replicate. Approximately 50,000 bacteria were analyzed  
16 for each replicate.

17 **Figure 5.** *E. faecalis* capsule reduces TNF- $\alpha$  production by RAW 264.7 cells. Macrophage like  
18 RAW 264.7 cells were incubated with serotype C (V583), serotype D (T-5 and LT02), and un-  
19 encapsulated (LT06, 12030, and OG1RF) *E. faecalis* strains. Supernatants were collected and  
20 analyzed by ELISA for TNF- $\alpha$  content. Results show pg/mL of TNF- $\alpha$  production by RAW  
21 264.7 cells in the presence of each strain. Statistical analysis of three replicates using one way  
22 ANOVA and a Newman-Kuels post hoc test shows significant differences between the amount  
23 of TNF- $\alpha$  produced in response to T-5, V583 and LT02 when compared to LT06, 12030, and



1 OG1RF. Interestingly, there is no statistically significant difference between the amount of  
2 TNF- $\alpha$  produced by un-induced RAW cells when compared to the three encapsulated strains.  
3 Error bars represent SE for three replicate experiments.

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**Table 1**Table 1: *E. faecalis* Strains used in this study

<b>Strain</b>	<b>Description</b>	<b>Reference</b>
FA2-2	Capsule + (Serotype C)	(5)
V583	Capsule + (Serotype C)	(34)
OG1RF	Capsule -	(24)
12030	Capsule -	(14)
LT01	FA2-2 $\Delta cpsF$ Capsule + (Serotype D)	(41)
LT02	V583 $\Delta cpsF$ Capsule + (Serotype D)	(41)
LT05	FA2-2 $\Delta cpsC$ Capsule -	(41)
LT06	V583 $\Delta cpsC$ Capsule -	(41)
LT12	V583 + pMV158gfp	This Study
LT13	LT02 + pMV158gfp	This Study
LT14	LT06 + pMV158gfp	This Study

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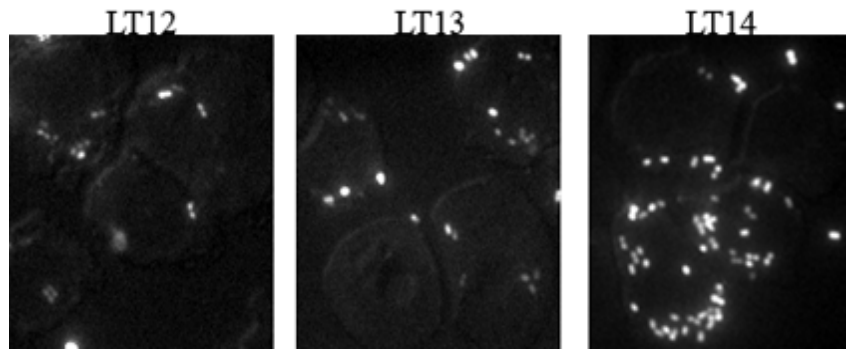
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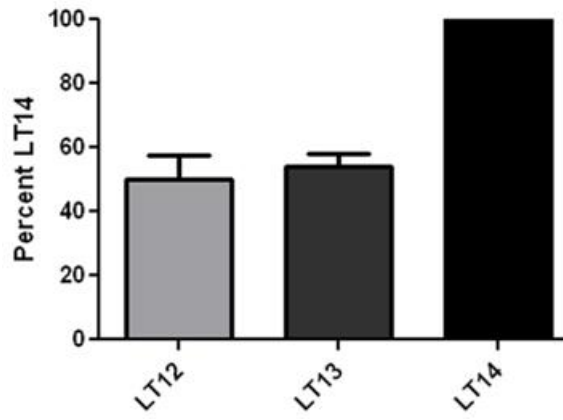
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1 **Figure 1**

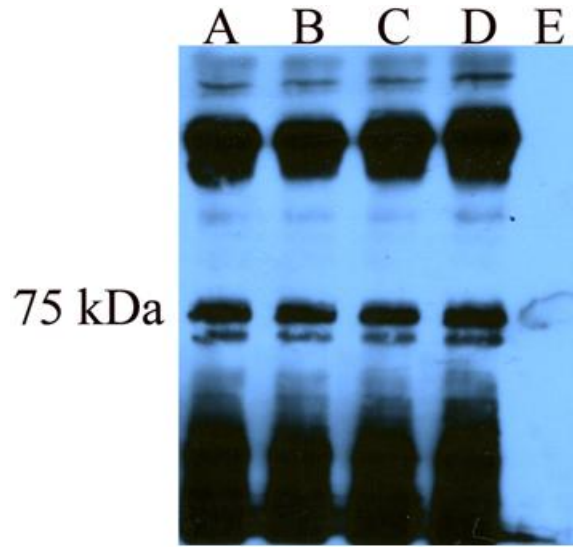
2 **A.**



6 **B.**



1 **Figure 2**



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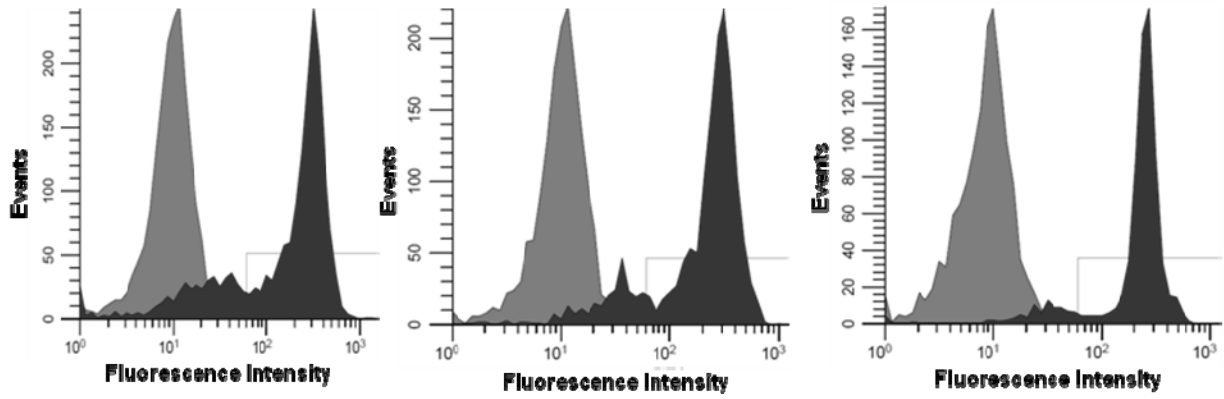
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1 **Figure 3:**

2 **A.**

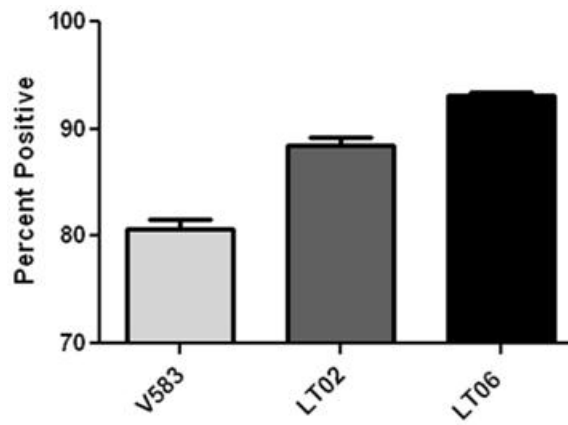


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5 **B.**

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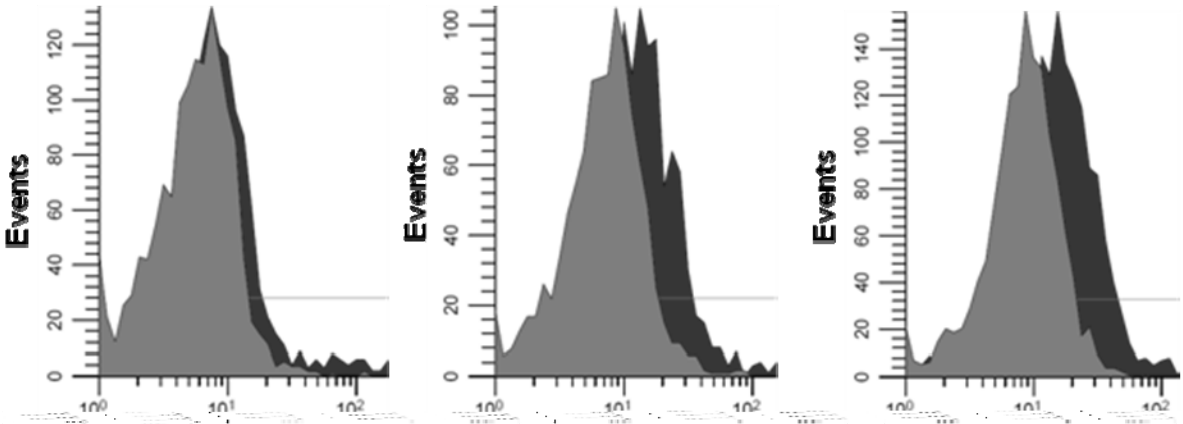
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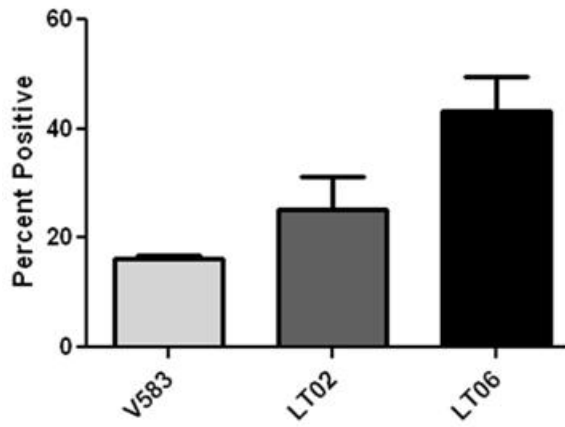
2 **A.**



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5 **B.**



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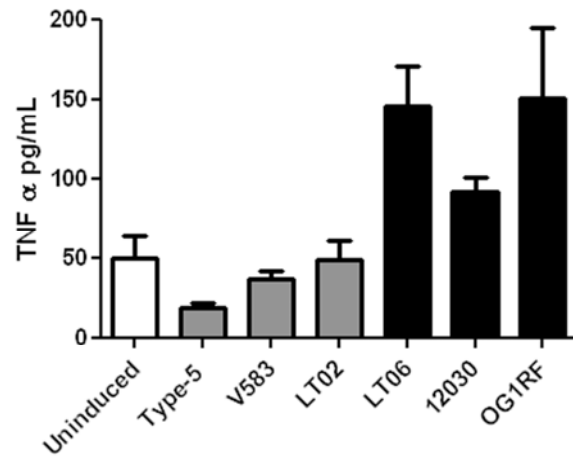
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1 **Figure 5:**

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