Importance of the Collagen Adhesin Ace in Pathogenesis and Protection against *Enterococcus faecalis* Experimental Endocarditis

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Abstract

Ace is an adhesin to collagen from *Enterococcus faecalis* expressed conditionally after growth in serum or in the presence of collagen. Here, we generated an ace deletion mutant and showed that it was significantly attenuated versus wild-type OG1RF in a mixed infection rat endocarditis model (P < 0.0001), while no differences were observed in a peritonitis model. Complemented OG1RFΔace (pAT392:ace) enhanced early (4 h) heart valve colonization versus OG1RFΔace (pAT392) (P = 0.0418), suggesting that Ace expression is important for early attachment. By flow cytometry using specific anti-ace recombinant Ace (rAce) immunoglobulins (Igs), we showed in vivo expression of Ace by OG1RF cells obtained directly from infected valves, consistent with our previous finding of anti-Ace antibodies in *E. faecalis* endocarditis patient sera. Finally, rats actively immunized against rAce were less susceptible to infection by OG1RF than non-immunized (P = 0.0004) or sham-immunized (P = 0.0475) by CFU counts. Similarly, animals given specific anti-rAce Igs were less likely to develop *E. faecalis* endocarditis (P = 0.0001) and showed fewer CFU in vegetations (P = 0.0146). In conclusion, we have shown for the first time that Ace is involved in pathogenesis of, and is useful for protection against, *E. faecalis* experimental endocarditis.

Citation: Singh KV, Nallapareddy SR, Sillanpää J, Murray BE (2010) Importance of the Collagen Adhesin Ace in Pathogenesis and Protection against *Enterococcus faecalis* Experimental Endocarditis. PLoS Pathog 6(1): e1000716. doi:10.1371/journal.ppat.1000716

Introduction

Enterococci are gram-positive cocci of intestinal origin first reported as a cause of infective endocarditis (IE) in 1899 [1]. They were recognized as the 3rd most common cause of IE as early as the 1920’s, and have remained the 3rd most common cause of community onset IE since then with *Enterococcus faecalis* accounting for >90% of isolates from enterococcal IE when identified to the species level [1,2,3,4,5]. Over the past 20 years, enterococci have also become the 2nd–3rd most common organisms isolated from nosocomial (healthcare-associated) infections including UTIs, bacteremia, intraabdominal and wound infections, endocarditis, sepsis in neonates, among others [1,3]. Indeed, among causes of endocarditis, enterococci (predominantly *E. faecalis*) have been variably reported as the #1 and #2 cause [6,7]. Since healthcare-associated infections, particularly those caused by antibiotic resistant bacteria, result in enormous increases in hospital stays and costs, enterococci clearly represent an important drain on healthcare dollars. In one study, the attributable mortality of enterococcal bacteremia was 31% [8], emphasizing the clinical, not just the financial, seriousness of these infections.

The first step in infective endocarditis is vascular tissue colonization, which can be mediated by cell-wall anchored adhesins such as MSCRAMMs (for microbial surface components recognizing adhesive matrix molecules) [9] of gram-positive bacteria. Our previous *in silico* analyses of the *E. faecalis* genome identified a family of genes encoding MSCRAMM-like proteins containing one or more regions of ca. 150 aa segments with deviant Ig-like fold(s), characteristic of the *Staphylococcus aureus* MSCRAMMs [10]. One of these, called Ace (for Adhesin to collagen of *E. faecalis*), has been studied in detail. Genetic and biochemical analyses showed that Ace mediates adherence of *E. faecalis* cells to bovine and rat collagen type I (CI), human collagen type IV (CIV), and mouse laminin [11,12,13], as well as human dentin [14].

Crystal structure analysis of the ligand-binding segment of Ace showed that the Ace A domain is composed of two sub-domains, N1 and N2, each adopting an Ig-like fold [15]. Subsequent point and truncation mutation analyses suggested that Ace binds to collagen by a mechanism called the “Collagen Hug” [15], a variant of the “Dock, Lock and Latch” ligand-binding mechanism shown for *Staphylococcus epidermidis* fibrinogen (Fg) adhesin SdrG [16,17]. The ace gene is ubiquitous [18] in *E. faecalis* and conserved among diverse isolates albeit with at least four variants due to variation in the number of repeats of the B domain [19]. *Conditional in vitro* production of Ace (i.e., markedly enhanced production after growth at 46°C, growth in brain-heart infusion plus 40% serum (BHIS) or growth in the presence of collagen...
versus growth in BHI broth at 37°C) by different strains correlates with conditional adherence of these \textit{E. faecalis} strains to collagens and laminin [19,20]. Most sera from patients with \textit{E. faecalis} IE show reactivity with rAce, indicating that different strains express Ace during human infection and that it is antigenic in \textit{vitro} [19].

Furthermore, anti-Ace antibodies (affinity purified from human serum or animals immunized with rAce) were shown to inhibit \textit{in vitro} adherence of \textit{E. faecalis} strains to collagen and laminin [11,19]. In a recent study, anti-Ace40 (ligand-binding A-domain of Ace) monoclonal antibodies were shown to completely inhibit binding of Ace40 to human CI and collagen type VI and inhibited binding of Ace-coated fluorescent beads to epithelial cell lines, thus suggesting Ace as a potential therapeutic target antigen against \textit{E. faecalis} infections [21].

In the present study, we have studied the role of Ace in the pathogenesis of \textit{E. faecalis} endocarditis by generating an \textit{ace} deletion mutant in \textit{E. faecalis} strain OG1RF, by complementing this mutant (OG1RF\textit{ace}::cat), by comparing these isogenic strains with OG1RF for adherence to various extracellular matrix (ECM) proteins and for their ability to infect aortic valves in a rat endocarditis model. Finally, we also determined the importance of Ace as a protective antigen against experimental endocarditis in a rat model by using active and passive immunization.

### Results

**Characterization of the \textit{Dace} mutant and complementation construct**

Our previous disruption mutant of \textit{ace} was found to be unstable \textit{in vitro} (see below). We therefore constructed an allelic replacement \textit{ace} deletion mutant of OG1RF (TX5467, OG1RF\textit{ace::cat}; resistant to chloramphenicol 10 μg/ml). Deletion of \textit{ace} from OG1RF was verified by sequencing confirming the correct deletion of \textit{ace} from −23 to +2200 (including the RBS, complete \textit{ace} gene, and 34 bp downstream of \textit{ace}), and by pulsed field gel electrophoresis (PFGE) and hybridizations (Table 1). Growth (OD_{600}) of the \textit{Dace} mutant was similar to wild-type (WT) OG1RF

### Table 1. Bacterial strains and plasmids used in this study.

| Strains/Plasmids | Relevant characteristics | Reference or source |
|------------------|--------------------------|---------------------|
| **Strains**      |                          |                     |
| \textit{E. faecalis} |                          |                     |
| OG1RF            | Laboratory strain; Rif', Fus', Chl', Gen', Kan' | [61] |
| TX5256           | \textit{ace} insertion disruption mutant of OG1RF; Rif', Fus', Kan', Chl', Gen' | [11] |
| TX5467           | OG1RF\textit{ace::cat}, \textit{ace} deletion mutant of OG1RF; Rif', Fus', Kan', Chl', Gen' | This study |
| TX5647           | TX5467 harboring pTEX5646 (for complementation with the \textit{ace} gene); Rif', Fus', Chl', Gen' | This study |
| TX5648           | TX5467 harboring pAT392 (for complementation); Rif', Fus', Chl', Gen' | This study |
| \textit{E. coli} |                          |                     |
| DH5s             | \textit{E. coli} host strain for routine cloning | Stratagene |
| XL1-Blue         | \textit{E. coli} host strain for routine cloning | Stratagene |
| LMG194           | \textit{E. coli} strain for expression of recombinant proteins | Invitrogen |
| TX5254           | LMG194 (pBAD::\textit{ace}); 1008 bp \textit{OG1RF} \textit{ace} (coding for complete A domain) cloned into pBAD/HisA expression vector; Amp' | [11] |
| TX5428           | DH5s (pTEX5428); Chl', Gen' | This study |
| TX5646           | XL1-Blue (pTEX5646); Gen' | This study |
| **Plasmids**     |                          |                     |
| pAT392           | Shuttle expression vector (Gen' Spc' ori\textit{R}_{bac} ori\textit{R}_{PAM1} ori\textit{R}_{P2} P_{3}) | [51] |
| pTEX5500ts       | Shuttle plasmid, ts in Gram' hosts; Chl', Gen' | [50] |
| pTEX5646         | Construct for complementation; a 2,186-bp fragment containing \textit{ace} cloned into pAT392 downstream of the P2 promoter | This study |
| pTEX5428         | Plasmid for \textit{ace} deletion with flanking regions of the \textit{ace} gene cloned on either side of the car gene into pTEX5500ts; Chl', Gen' | This study |

*Chl, chloramphenicol; Fus, fusidic acid; Gen, gentamicin; Kan, kanamycin; Rif, rifampicin; and ts, temperature-sensitive. Superscript “s” designates sensitivity and “r” designates resistance; “’” is defined for enterococci as MIC >500 for Gen and >2000 for Kan.

doi:10.1371/journal.ppat.1000716.t001
in BHI (data not shown). We have previously shown, using western blotting and RT-PCR, that ace is expressed at higher levels when grown in BHIS at 37°C or in BHI at 46°C [11] than in BHI at 37°C. Here, we assessed surface localization of Ace in OG1RF at 10 h using flow cytometry analyses with affinity-purified anti-rAce Igs. The mean fluorescence intensity levels for different culture conditions increased progressively with cells grown in BHI at 37°C, BHIS at 37°C and BHI at 46°C (Figure 1A), consistent with our previous western and immunofluorescence microscopy data [11,20]. The percentages (%) of Ace-expressing cells in BHIS cultures of OG1RF, OG1RFΔace, OG1RFΔace (pAT392) (empty vector control), and OG1RFΔace (pAT392::ace) (complementation) were >70%, <5%, <3%, and >90%, respectively, demonstrating the inability of OG1RFΔace to produce Ace and the efficient complementation of OG1RFΔace by pAT392::ace. In these experiments, pAT392-containing strains were grown without added gentamicin, the same conditions that we used for preparing inocula for the rat endocarditis experiments. When BHIS was supplemented with gentamicin, expression of Ace increased to >95% of cells in OG1RFΔace (pAT392::ace) (Figure 1B), likely due to improved plasmid stability (see below).

OG1RF and its isogenic Δace mutant as well as the complementation constructs were tested for their ability to adhere to immobilized ECM proteins and BSA. Consistent with our previous demonstration of adherence of OG1RF to CI, CIV and Fg after growth in BHIS at 37°C and to CI and CIV after growth in BHI at 46°C (but not in BHIS [11] at 37°C), we observed here that OG1RF adhered to CI and CIV when grown in BHIS at 37°C, unlike OG1RFΔace which showed markedly reduced adherence to CI (from ~36 to 15%) (Figure 2A), and CIV (43 to 3%) (Figure 2B), but no change in adherence to Fg (Figure 2C). This corroborates our earlier data with a mutant in an inserional disruption of ace [11,20]. Introduction of the ace gene in trans into OG1RFΔace resulted in even greater adherence to collagens than WT (>1.5-fold higher), whereas OG1RFΔace electroporated with pAT392 retained its reduced adherence phenotype (Figure 2); these results are consistent with Ace expression data from flow cytometry analysis.

**In vivo surface expression of Ace**

To determine if Ace is produced during infection, we performed flow cytometry analyses on extracts directly processed from IE vegetations infected with OG1RF grown in BHI at 37°C. Forward and side scatter pattern analyses of particles from processed vegetations and comparisons with those from in vitro grown OG1RF cells indicated that most of the gated particles detected by flow cytometry are likely OG1RF bacterial cells, thus confirming the removal of the majority of host tissue particles from the vegetations during the processing steps described in methods. Sterile processed vegetations from non-infected rats probed with anti-rAce-specific Igs (negative control) showed labeling of a minor fraction (<3%) of bacterium-sized particles (Figure 3A), while processed vegetations from OG1RF infected rats probed with Igs from an antiserum raised against formalin-killed *E. faecalis* strain HI22-whole-cells (positive control) bound 85% of bacterium-sized particles, further indicating that the majority of these particles were *E. faecalis* cells (Figure 3A). Affinity-purified anti-rAce-specific Igs bound to ~40 to 45% of bacterium-sized particles from different rat endocarditis vegetations infected with OG1RF (Figure 3), demonstrating that Ace is actively expressed in host vegetations during IE.

**In vivo testing of WT OG1RF and ace mutants in a mixed infection competition assay**

Although our initial mixed-infection competition experiments showed a clear advantage for the WT over an ace disruption mutant TX3256 [11,20] to develop IE in rat model (data not shown), subsequent experiments identified instability of this single cross-over ace disruption mutant during in vivo growth. Hence, we generated an OG1RFΔace mutant for further in vivo testing.

In an initial mono-infection experiment (n = 2) with our ace deletion mutant (OG1RFΔace), TX3467, we first determined the expression of cat (encoding chloramphenicol acetyl transferase) in OG1RFΔace, which carries this chloramphenicol resistance marker gene in place of ace, by analyzing individual colonies for chloramphenicol resistance and by high stringency hybridization using intragenic cat and ace DNA probes. We found that ~10% of the colonies recovered from vegetations were chloramphenicol (10 μg/ml) susceptible even though they were cat probe positive and ace probe negative, indicating that, although the cat gene was present, silencing of its expression had occurred in these colonies. Previously, it has been shown that some antibiotic resistance genes of *Escherichia coli* are silenced in vivo; specifically, expression of an intact antibiotic resistance gene was switched off during the course of gut colonization in pigs, a phenomenon suggested to be helpful for bacterial fitness [22]. Therefore, for our mixed infection animal experiments, all the results reported here are based on high}

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**Figure 1. Flow cytometry analysis of cell surface expression of Ace by *E. faecalis* OG1RF, its isogenic ace deletion mutant and its in trans complemented ace deletion mutant.** (A) Comparison of the effects of growth for 10 h under different conditions on expression levels of Ace in OG1RF using both pre-immune Igs (PI) and anti-Ace Igs. (B) Analysis of Ace expression by the OG1RF ace deletion mutant and the effect of its in trans complementation. OG1RFΔace, ace deletion mutant; OG1RFΔace (pAT392::ace), complemented ace deletion mutant; OG1RFΔace (pAT392), ace deletion mutant with the empty vector. Reactivity to affinity-purified specific anti-Ace Igs is shown for each isogenic strain. Bacteria were analyzed using side scatter as the threshold for detection. Binding by specific anti-Ace Igs is indicated as log fluorescence intensity on the X-axis. For each histogram, 50,000 events of bacterium-sized particles were counted.

doi:10.1371/journal.ppat.1000716.g001
The mean virulence index of the calculated using the equation shown in Materials and Methods.

a particular mutant in a mixed infection with the WT strain, was sensitive measure of the relative degree of virulence attenuation of mean virulence index or competitive index [23,24], which is a vegetations was 0.077; this indicates that in this endovascular infection.

OG1RF thus demonstrating a clear advantage of OG1RF versus recovered was 81.5% versus 18.5% for OG1RF (Figure 4). Bacterial CFUs from vegetations on aortic valves were recovered at 72 h from all 12 rats and are shown in Figure 4. The mean percentage (%) of OG1RF in the total CFU of bacteria, recovered 3.8 D 6

In the mixed infection competition assay, all 12 rats were infected with an approximately 1:1 mixture (as predicted by OD<sub>600</sub> of BHI-grown OG1RF (determined geometric mean (GM) CFU 3.8×10<sup>7</sup>/rat, representing 47% of the inoculum): OG1RF-<i>Δace</i> (GM CFU 4.4×10<sup>7</sup>/rat, representing 53% of the inoculum) (Figure 4). Bacterial CFUs from vegetations on aortic valves were recovered at ~72 h from all 12 rats and are shown in Figure 4. The mean percentage (%) of OG1RF in the total CFU of bacteria recovered was 81.5% versus 18.5% for OG1RF<i>Δace</i> (<i>P</i>&lt;0.0001), thus demonstrating a clear advantage of OG1RF versus OG1RF<i>Δace</i> at 72 h for heart valve colonization in rats. The mean virulence index or competitive index [23,24], which is a sensitive measure of the relative degree of virulence attenuation of a particular mutant in a mixed infection with the WT strain, was calculated using the equation shown in Materials and Methods. The mean virulence index of the <i>ace</i> mutant relative to WT in vegetations was 0.077, this indicates that <i>ace</i> has an important role in this endovascular infection.

In vivo testing of the complemented OG1RF<i>Δace</i> (pAT392<i>Δace</i>) construct and OG1RF<i>Δace</i> (pAT392) in the mono-infection model

In initial mono-infection experiments with complementation constructs and testing 24 h after inoculation, we observed loss of the plasmid from cells recovered from vegetations, with 94%–98% loss from OG1RF<i>Δace</i> (pAT392<i>Δace</i>) (7 rats) and 14%–100% loss from OG1RF<i>Δace</i> (pAT392) (8 rats) (data not shown). We also tried growing both constructs in BHIS supplemented with gentamycin for the preparation of inocula for infection, but in vivo loss of the plasmid still occurred 24 h after inoculation. To minimize in vivo growth time and to determine the role of Ace in the early stage of valve colonization in rats, we tested both OG1RF<i>Δace</i> (pAT392<i>Δace</i>) and OG1RF<i>Δace</i> (pAT392) in the rat model 4 h after inoculation. Two independent mono-infection experiments were done and the combined results are shown in Figure 5. Rats inoculated with OG1RF<i>Δace</i> (pAT392<i>Δace</i>) (n = 12) showed 1.4±0.6 log<sub>10</sub> more CFU/gm than OG1RF<i>Δace</i> (pAT392) (n = 11) (<i>P</i>= 0.0417) (Figure 5), thus demonstrating that Ace has a significant role in early colonization of heart valves in <i>E. faecalis</i> rat IE. Reduced time in vivo also resulted in much less loss of the plasmid from each construct. In the case of OG1RF<i>Δace</i> (pAT392<i>Δace</i>), gentamicin susceptible colonies were recovered in only 2/12 rats (2/2 colonies from one and 3/3 colonies from the other were gentamicin susceptible), while with OG1RF<i>Δace</i> (pAT392), 3–100% of colonies (among 8–48 tested) recovered from 3/11 rats were gentamicin susceptible. These results corroborated the above described complementation of <i>ace</i> surface expression (Figure 1B) and restoration of in vitro adherence of OG1RF<i>Δace</i> (pAT392<i>Δace</i>) to CI and CIV to similar levels as observed for OG1RF.

Active immunization with rAce and in vivo protection against <i>E. faecalis</i> experimental IE in rats

Since Ace was found to be an important virulence factor in rat experimental IE, an Ace-specific immune response might hinder the development of IE. To study this, rats were vaccinated s.c. thrice with 99% pure 100 μg rAce or PBS or Freund's complete adjuvant – Freund's incomplete adjuvant (FCA – FICA) and were challenged with 10<sup>7</sup> to 10<sup>9</sup> CFU of <i>E. faecalis</i> OG1RF per rat (see methods). Comparison of anti-Ace antibody levels of 10 immunized and three non-immunized animals by ELISA showed that all 10 immunized rats tested had high levels of anti-Ace titers (1: &gt;50,000), whereas no anti-Ace antibodies were detected in any of the three control rats (Figure 6). Sixteen of 16 no-treatment control rats (100%) developed <i>E. faecalis</i> endocarditis after challenge with 10 times the ID<sub>50</sub> of BHI-grown OG1RF compared with 5 of 14 rats (35%) in the rAce active-immunization group (<i>P</i>=0.0001) (Figure 7A). The no-treatment control rats

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Stringency hybridization with <i>ace</i> and <i>cat</i> probes using ~200 CFUs/rat vegetation for all the rats used. Of interest, we also tested for chloramphenicol resistance but did not observe further <i>cat</i> silencing.

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**Figure 2. Adherence of <i>E. faecalis</i> OG1RF and its derivatives to immobilized collagens.** (A) Adherence to collagen type I (CI). (B) Adherence to collagen type IV (CIV). (C) Adherence to fibrinogen (Fg). Mean % of cells adhering ± SD from two independent experiments representing 12 wells/sample are shown.

doi:10.1371/journal.ppat.1000716.g002
showed a mean of $4.2 \pm 1.0 \log_{10}$ more CFU/gm than the rAce active-immunized ($P = 0.0004$) in vegetations recovered from heart valves. In an independent experiment, in order to mimic in vivo growth conditions more closely and because we had found that Ace is expressed at higher levels by OG1RF when grown in BHIS [11], we used BHIS-grown OG1RF for the preparation of inocula. rAce (n = 10) rats were inoculated with a higher inoculum of $1.4 \times 10^9$ CFU/rat ($\sim 100$ times the ID$_{50}$), while non-immunized controls (n = 18) were inoculated with $3.1 \times 10^8 - 1.1 \times 10^9$ CFU/rat. Fifteen of 18 non-immunized control animal (83%) developed Ace expression) as well as in BHIS at 37°C, stained with pre-immune Igs and anti-Ace and the same batch of R-phycoerythrin-conjugated secondary antibody, are shown in panel B. doi:10.1371/journal.ppat.1000716.g003

Figure 4. E. faecalis OG1RF and OG1RF\textDelta ace (TX5467) in a competition (mixed infection) assay in the rat endocarditis model. Percentages of OG1RF and OG1RF\textDelta ace present in inocula and recovered from vegetations 72 h post infection of 12 rats are shown. Horizontal bars represent the means ($P < 0.0001$ by paired $t$ test) for percentages of total bacteria in the vegetations of OG1RF versus OG1RF\textDelta ace. Empty circles and empty triangles represent percentages of OG1RF and OG1RF\textDelta ace in inocula, respectively, while solid circles and solid triangles represent percentages of OG1RF and OG1RF\textDelta ace in vegetations, respectively. doi:10.1371/journal.ppat.1000716.g004

reproducibility of in vivo protection by active immunization with rAce against E. faecalis experimental endocarditis in rats using two different growth conditions to prepare the inocula.

**Mouse peritonitis model**

In this *in vivo* model, both OG1RF and OG1RF\textDelta ace caused animal mortality at similar rates with all the inocula tested (data with two inocula were shown in Figure 9) showing that OG1RF\textDelta ace was not attenuated versus OG1RF.

**Discussion**

Infective endocarditis, which affects the endothelial lining of the heart, is among the most severe of the wide range of enterococcal...
infections encountered in humans, presenting a major therapeutic challenge and resulting in considerable mortality even when treated with antibiotics [5,7,8,25]. Development of endocarditis can be initiated by injury to the valvular endothelium, which disrupts the normal valve structure and exposes underlying tissues, including ECM material. Deposition of host proteins, such as fibrin, as well as platelets at the site of injury then leads to the formation of a sterile thrombotic vegetation. This endovascular lesion may become colonized by circulating bacteria, leading to the growth of an infected vegetation. Valvular and aortic tissues are rich in collagen [26], and collagen is also found in sterile vegetations [26]. Previous studies have demonstrated that Ace plays a major role in the in vitro adherence of E. faecalis isolates to immobilized collagen [11,12,13]. Therefore, we reasoned that collagen could serve as a potential adhesion target for enterococci during bacteremia and that Ace could mediate bacterial attachment to these collagen-containing sites. To date, no studies have demonstrated a role for Ace in endocarditis and only very recently has a report appeared showing that Ace is important in a murine urinary tract infection model [27]. However, our previous demonstration of the role of host-derived cues (i.e., using moieties typically encountered in the host, such as serum or collagen [19,20]), for induction of both Ace expression and adherence of E. faecalis cells to collagen, suggested that both of these phenotypes are elicited by close association of this organism with a mammalian host/tissue. Moreover, we found that 90% of patients with prior E. faecalis endocarditis have Ace-specific antibodies in their sera, implying that Ace is expressed in vivo during the infection and that it is immunogenic [19]. For these reasons, we chose an experimental IE model in this study to explore the role of Ace in E. faecalis pathogenesis.

We first looked for direct evidence showing that Ace is expressed during E. faecalis infection. E. faecalis OG1RF cells recovered directly from infected vegetations showed surface expression of Ace with a much higher (P = 0.0417) by unpaired t test for mean log10 CFU/gm is shown. doi:10.1371/journal.ppat.1000716.g005

Figure 5. Complemented OG1RFΔace (pAT392::ace) [TX5647] and OG1RFΔace (pAT392) [TX5648] in early (mono-infection) colonization of aortic valves in the rat model. Data are expressed as log10 CFU/gm recovered from the vegetations and solid circles and solid triangles represent OG1RFΔace (pAT392::ace) and OG1RFΔace (pAT392) in vegetations, respectively. In the panel on the right, solid circles and solid triangles represent OG1RFΔace (pAT392::ace) and OG1RFΔace (pAT392) inocula, respectively. Data are expressed as log10 CFU/gm recovered from the vegetations 4 h post infection of 12 rats (OG1RFΔace (pAT392::ace)) and 11 rats (OG1RFΔace (pAT392)), respectively. Horizontal bars represent the geometric mean titers. Significantly enhanced (by a mean ± SD increase of 1.4±0.6 log10 CFU/gm) vegetation titer by OG1RFΔace (pAT392::ace) versus OG1RFΔace (pAT392) (P = 0.0417) by unpaired t test for mean log10CFU/gm is shown.

doi:10.1371/journal.ppat.1000716.g005

Figure 6. Comparison of serum anti-Ace titers in immunized and non-immunized rats. Rats were immunized and boosted twice with 100 μg rAce. Antibody levels were measured by ELISA. Mean serum anti-Ace titers were plotted for each antibody dilution tested. doi:10.1371/journal.ppat.1000716.g006

As anticipated, in vitro ECM protein adherence results with OG1RFΔace corroborated our previous results with an insertionally inactivated ace [11,20]. Collagen adherence of the ace deletion mutant was restored by complementation in trans and the adherence of the complemented strain was 1.5-fold above the level of the WT parent strain, likely due to the higher number of Ace molecules displayed on the surface of the complemented strain, as determined by flow cytometry.
Deletion of ace resulted in significant attenuation in the ability of the mutated *E. faecalis* OG1RF strain to compete successfully with its isogenic WT parent in infection of vegetations in a mixed-inoculum rat IE model. To the best of our knowledge, this is the first demonstration that Ace contributes to *E. faecalis* virulence in endocarditis. When we complemented the ace mutant in trans, significantly more colonization of heart valves was observed at 4 h after infection by this strain than by an isogenic strain containing the mutated ace deletion mutant and, furthermore, provide evidence that Ace plays an important role during the initial attachment and colonization stage of IE development, possibly by mediating adherence of *E. faecalis* cells to exposed collagen at the site of endovascular injury. This is consistent with the high surface expression levels of Ace in the complemented strain shown by our flow cytometry analysis. While stably maintained by the majority of *E. faecalis* cells during early colonization (4 h), the high instability of the complementation vector after extended growth in vegetations (94–98% of cells had lost the plasmid by 24 h) reduced its effect at later stages of endocarditis. The residual ability of OG1RF<sup>Δace</sup> to cause endocarditis in some rats indicates that Ace is not absolutely required for *E. faecalis* to cause endocarditis; this is in agreement with published studies that showed a role for additional factors in causing *E. faecalis* IE [28,29,30]. While the precise mechanism of action of Ace for initiating, maintaining and/or propagating IE has yet to be elucidated, we infer that the difference in virulence of OG1RF<sup>Δace</sup> may be due to its reduced ability to adhere to collagen. However, we cannot exclude the possibility of another ligand or another function of this protein.

Interestingly, deletion of ace did not result in observable effects in the mouse peritonitis model in terms of either time to death or total mortality, suggesting that ace is not important for this infection or that the direct administration of a large inoculum of bacteria into the peritoneal cavity may bypass an early infection stage where Ace might be involved. These results also indicate that deletion of ace did not affect growth or survival of OG1RF<sup>Δace</sup> in rat<sub>in vivo</sub> in general, consistent with the similar growth rate and viability of the ace deletion mutant and WT when grown in trans, significantly more colonization of heart valves was observed at 4 h after infection by this strain than by an isogenic strain containing the mutated ace deletion mutant and, furthermore, provide evidence that Ace plays an important role during the initial attachment and colonization stage of IE development, possibly by mediating adherence of *E. faecalis* cells to exposed collagen at the site of endovascular injury. This is consistent with the high surface expression levels of Ace in the complemented strain shown by our flow cytometry analysis. While stably maintained by the majority of *E. faecalis* cells during early colonization (4 h), the high instability of the complementation vector after extended growth in vegetations (94–98% of cells had lost the plasmid by 24 h) reduced its effect at later stages of endocarditis. The residual ability of OG1RF<sup>Δace</sup> to cause endocarditis in some rats indicates that Ace is not absolutely required for *E. faecalis* to cause endocarditis; this is in agreement with published studies that showed a role for additional factors in causing *E. faecalis* IE [28,29,30]. While the precise mechanism of action of Ace for initiating, maintaining and/or propagating IE has yet to be elucidated, we infer that the difference in virulence of OG1RF<sup>Δace</sup> may be due to its reduced ability to adhere to collagen. However, we cannot exclude the possibility of another ligand or another function of this protein.

We have recently shown that Acm, a collagen adhesin from *E. faecium*, is an important factor for endocarditis caused by that species [23]; this is similar to a previous observation with Cna of *S. aureus* [31], which is also involved in other infections, such as septic arthritis [32]. These MSCRAMMs share a large degree of sequence conservation in their collagen-binding domains; similar proteins are present in several other species of gram-positive pathogens, such as *Streptococcus equi* [33], *Arcanobacterium pyogenes* [34], *Bacillus anthracis* [35] and *Streptococcus gilvolitidis* [36], and they possibly share a similar collagen-binding mechanism, called the “Collagen Hug” that has been characterized for Cna and Ace [15,37]. Therefore, it seems plausible that this family of proteins.

![Figure 7](image-url)
Figure 8. Passive immunization (anti-rAce Ig versus PI Ig) in rat endocarditis model. In the panel on the left, empty circles and empty triangles represent OG1RF inocula for pre-immune (PI) Ig treated and affinity purified specific anti-rAce Ig treated rats, respectively. In the panel on the right, solid circles and solid triangles represent OG1RF recovered from rat vegetations 24 h post infection. Horizontal bars represent the geometric means. Significantly fewer rats were infected by OG1RF in rAce Ig (2 mg/kg) (2/10) versus PI Ig (2 mg/kg) (5/6) treated rats (P=0.0001 by Fisher’s exact test). Rats (PI Ig treated) showed a mean ± SD increase of 3.8±1.4 log10 OG1RF CFU/gm from vegetations versus anti-rAce Ig treated rats (P=0.0146 by unpaired t test).

doi:10.1371/journal.ppat.1000716.g008

has been preserved or acquired across different gram-positive species/genera as a generalized mechanism to provide a binding function, although the ligand in the ecological niches where enterococci are found in nature and the purpose for these adhesins is not known. Recently, we described Elp pili as another bacterium of the same species/genera as a generalized mechanism to provide a binding function, although the ligand in the ecological niches where enterococci are found in nature and the purpose for these adhesins is not known. Recently, we described Elp pili as another

important factor for E. faecalis endocarditis [29], as well as urinary tract infections and biofilm formation [29,30], a further indication of the significance of surface proteins of the MSCRAMM family for E. faecalis pathogenesis.

Our results with active immunization of rats using the collagen-binding domain of Ace showed that only 25% of immunized rats developed endocarditis, while the infection rate in the untreated group was 100%. Protection was also evident when bacterial counts were evaluated. Consistent with these results, prophylactic treatment of rats with affinity-purified anti-Ace antibodies raised against the collagen-binding domain of Ace significantly reduced bacterial numbers in vegetations, demonstrating that passive transfer of Ace-specific antibodies confers significant protection against E. faecalis IE in rat. The differences in pre-infection procedures between the active- and passive-immunized groups preclude direct comparison of results from these two methods. Based on the results presented in this study, it seems likely that these preventive strategies specifically target the initial attachment and colonization stage of endocarditis by blocking collagen adherence of E. faecalis cells. Consistent with this hypothesis, we have previously shown that Ace-specific polyclonal antibodies purified from immunized rabbits or from humans with a prior E. faecalis endocarditis infection were effective in inhibiting adherence of Ace-expressing E. faecalis isolates to collagen [11,19]. Furthermore, a recent study that generated monoclonal antibodies against rAce showed that some of the mAbs completely inhibited binding of rAce to collagen and Ace-coated fluorescent beads to epithelial cell lines [21]. The ace gene is ubiquitously present among isolates of E. faecalis and its encoded amino acid sequence, especially within the collagen-binding domain, is highly conserved [19]. Therefore, targeting ace could potentially offer protective immunization against a large spectrum of genetically diverse E. faecalis isolates, an advantage over other virulence-associated factors, such as aggregation substance, hemolysin and gelatinase, which were found to be produced by <45% of endocarditis isolates [39] and for which protective efficacy has not been shown [40]. So far, only one E. faecalis antigen, the capsular polysaccharide, has shown promise as a potential vaccine candidate, as passive and active immunization against it lowered bacterial counts in kidneys, spleens and livers in a mouse i.v. infection model [41]. To our knowledge, our study is the first report of an immunization strategy that reduces E. faecalis colonization of aortic valves and shows protection against the development of E. faecalis endocarditis, thus, suggesting Ace as a promising alternative target for prophylaxis of E. faecalis endocarditis in high risk patients. However, the ability of OG1RF to cause IE in some of the rAce-immunized rats and also in some anti-Ace antibody-treated rats indicates that targeting multiple MSCRAMMs may be necessary for a robust protection of E. faecalis IE. Consistent with this, a recent study [42] showed full vaccine protection against abscess formation or lethal challenge with S. aureus strains when a combination of four MSCRAMM antigens were used versus a moderate reduction in bacterial load when used as individual vaccine antigens.

In summary, we have demonstrated here that i) deletion of the ace gene resulted in significant attenuation of the ability of E. faecalis to colonize aortic valves and cause endocarditis in an experimental rat IE model, coinciding (ii) with reduced in vitro adherence by the ace deletion mutant to collagen types I and IV; we have also shown that (iii) Ace is actively expressed within host vegetations during endocarditis and that (iv) both active and passive immunization against the collagen-binding domain of Ace conferred significant protection against endocarditis and reduced the numbers of bacteria found in vegetations. Taken together,
these results demonstrate that Ace is an important virulence-associated factor and a likely target for prophylactic and therapeutic strategies against *E. faecalis* endocarditis. Since Ace-like proteins are widespread among streptococci and staphylococci, future cross-protection studies may reveal novel opportunities for the development of vaccines or immunotherapeutics that may be useful for the prevention and treatment of gram-positive infective endocarditis.

**Materials and Methods**

**Ethics statement**

The rat endocarditis model and surgical procedures were performed in accordance with the institutional policies and the guidelines stipulated by the animal welfare committee, University of Texas Health Science Center at Houston (AWC, UTHSC). This study was reviewed and approved by the University Institutional Review Board (AWC approval # HSC-AWC-08-067).

**Bacterial strains, plasmids, materials, standard molecular techniques, and growth conditions**

*E. coli* and *E. faecalis* strains and all plasmids used in this study are listed in Table 1. All constructs were given TX numbers and plasmids from these constructs were assigned respective pTEX numbers (Table 1). *E. coli* strains were grown in Luria-Bertani media (Difco Laboratories, Detroit, Mich.). Enterococci were grown either in BHII, BHIS, Todd-Hewitt (TH) broth/agar (Difco Laboratories) or Enterococcosel Agar (EA) (Becton Dickinson) at 37°C, unless a different growth temperature is specified. The following antibiotic concentrations were used with *E. faecalis*: chloramphenicol 10 μg/ml, kanamycin 2000 μg/ml, rifampicin 100 μg/ml and gentamicin 125 μg/ml. With *E. coli*, the concentrations used were chloramphenicol 10 μg/ml, kanamycin 50 μg/ml, and gentamicin 25 μg/ml. Resistance of enterococci to gentamicin and kanamycin was defined as MIC >500 and >2000 μg/ml, respectively [18].

**Materials**

All antibiotics were obtained from Sigma (St. Louis, Mo.), Tran 35S-label and bovine serum albumin (BSA) were purchased from MP Biomedicals Inc. (Irvine, Calif.), C I and C IV were from Sigma and Fg was from Enzyme Research Laboratories (South Bend, Ind.). Oligonucleotide primers were purchased from Invitrogen (Carlsbad, Calif.) or IDT (Coralville, Iowa) or Sigma and Fg was from Enzyme Research Laboratories (South Bend, Ind.). Oligonucleotide primers were purchased fromInvitrogen (Carlsbad, Calif.) or IDT (Coralville, Iowa) or Sigma and their sequences are provided in Table 2. Restriction enzymes and DNA modification enzymes were mostly from Invitrogen and New England BioLabs, Inc. (Beverly, Mass.). All other chemicals used in the investigation were of molecular biology grade.

**Standard molecular techniques**

Chromosomal DNA from *E. faecalis* isolates was prepared following thehexadecyltrimethyl ammonium bromide method described earlier [43]. Plasmid DNA was isolated from *E. coli* using the Wizard Plus SV miniprep DNA purification system (Promega Corporation, Madison, Wis.) and, from *E. faecalis*, by a previously described method [44]. General recombinant DNA techniques such as ligation and agarose gel electrophoresis were performed using standard methods [45]. When necessary, DNA fragments were purified with low melting temperature agarose gel followed by purification using QIAquick-gel extraction kit (Qiagen Inc., Valencia, Calif.). PCR reactions were performed with a Perkin-Elmer GeneAmp PCR system 9700 using the optimized buffer B (1 x buffer: 60 mM Tris-Cl [pH 8.5], 15 mM ammonium sulfate and 2 mM MgCl2) obtained from Invitrogen. PCR-generated fragments were purified using the Wizard PCR DNA Cleanup System (Promega Corporation). Recombinant plasmids were generated in *E. coli* DH5α or XL1-blue. Electroporation of *E. coli* and *E. faecalis* was carried out using a Gene Pulser (Bio-RAD Laboratories, Richmond, Calif.) as described previously [46,47]. Agarose plugs containing genomic DNA were digested with SmaI and PFGE was performed using a previously described method [1].Southern blotting was performed using Hybond-N + nylon membrane and 0.4 N sodium hydroxide solution. Preparation of colony lysate blots was described elsewhere [48]. The RadPrime DNA Labeling System (Invitrogen) was used for labeling DNA probes with [α-32P]dCTP (GE Healthcare, Piscataway, N.J.) and hybridizations were carried out using high stringency conditions [48,49]. DNA sequencing reactions were performed by the Taq dye-deoxy terminator method and an automated ABI Prism sequencer (Applied Biosystems, Foster city, Calif.).

**Construction of an ace deletion mutant in OG1RF and its complementation**

An *E. faecalis* ace mutant (OG1RFΔace:cat) was constructed by allelic replacement using pTEX5300ts as described earlier for *E. faecium* [50]. We used a replacement strategy in this study to facilitate distinguishing between WT and OG1RFΔace during in vivo animal experiments with mixed cultures; bioinformatics and mRNA analyses of the ace locus predicts the absence of a polar effect of ace deletion by the cat replacement (unpublished data). *E. faecalis* OG1RFΔace was constructed by allelic replacement using

| Table 2. Primers used in this study. |
|---|
| **Primer Name** | **Sequence (5’→3’)** | **Function** | **Amplicon** |
| AceDelF1 | CGCGGATCCGAGGGTTGTAATTGTTG | Deletion mutant generation | Upstream fragment of ace |
| AceDelR1 | CCCAACGCTCTTTTATTACCCGTTT | Deletion mutant generation | Upstream fragment of ace |
| AceDelF2 | AAAACCGCAATTGTTTCTATTGGAATGATGCTT | Deletion mutant generation | Downstream fragment of ace |
| AceDelR2 | CCGAAATCTTCCAAGGCGTATAGGCTACTTTAC | Deletion mutant generation | Downstream fragment of ace |
| AceUpF1 | CCAAACATTTGCTCATACCTCTAAA | Mutant confirmation | |
| AceOxR1 | CACCATCTCTTATGAGAATTGTTG | Mutant confirmation | |
| AceComF1 | GCGGGATCCTAGAAGGGTGAATATTATTTATGAC | Complementation | Complete ace |
| AceComR1 | GCGGGATCCTTAAATATCTGTATGATAAAC | Complementation | Complete ace |

*Introduced restriction sites are underlined. doi:10.1371/journal.ppat.1000716.t002*
pTEX5500ts as described earlier for *E. faecium* [50]. A 1027-bp DNA fragment (AceDelUp) encompassing the region upstream of ace was amplified from OG1RF genomic DNA template using primers AceDelF1 and AceDelR1 (Table 2), digested with BamHI and HindIII, and ligated with similarly digested pTEX5500ts. Similarly, a 909-bp DNA fragment (AceDelDn) encompassing the region downstream of ace was amplified from the same genomic DNA template using primers AceDelF2 and AceDelR2 (Table 2). The AceDelDn PCR product digested with PstI and EcoRI was ligated to similarly digested pTEX5500ts:AceDelUp and was then transformed into *E. coli* DH5α to obtain TX3428. The plasmid from this construct, pTEX5428 (pTEX5500ts:AceDelUp+AceDelDn), was introduced into electrocompetent cells of OG1RF and cells were then plated on gentamicin plates at the permissive temperature (28°C). A single gentamicin and chloramphenicol resistant colony from these plates was grown overnight at 42°C, then plated on chloramphenicol plates and incubated at 37°C. After confirming the specific single crossover integration (OG1R::FauUp::pTEX5428) by PCR (with primer sets AceUpF1 and CmRk as well as AceDnR1 and CmFk), one of the integrants was picked, grown for eight overnight serial passages at 42°C, and then plated on BHI to select for plasmid excision by double crossover recombination. The colonies from these BHI plates were then replica plated to chloramphenicol and gentamicin plates to identify colonies that retained the *cat* gene but not the vector.

To complement OG1RFΔace in trans, an ~2 kb fragment containing the *ace* open reading frame plus its ribosome-binding site (amplified using primers aceComF1 and aceComR1; Table 2) was cloned under the control of the P2 promoter of the shuttle vector, pAT392 [51]. This *in vitro*-ligated construct for complementation (designated as pTEX5646) was transformed into *E. coli* XL1-Blue to obtain TX5646 and was then introduced into electrocompetent cells of TX5467 to obtain TX5647 (OG1RF::pAT392::ace). Surface expression of Ace in OG1RF::ace (pAT392::ace::ace) was determined by flow cytometry (see below).

**Growth curves**

Overnight cultures were inoculated into BHI broth at a dilution of 1:100. Overnight cultures were grown at 37°C with shaking in an orbital shaker and aliquots were removed hourly from 0 to 12 h and at 24 h, for determining the absorbance at 600 nm (OD$_{600}$) with a spectrophotometer.

**In vitro adherence assay**

Adherence of *E. faecalis* to CI, CIV, Fg and BSA was determined in four independent experiments using Tran 35S-labeled bacteria by a previously described assay [11].

**Expression and purification of (His)$_6$ tagged Ace A domain**

Construction of the recombinant plasmid pTEX5254 (complete ace A domain of OG1RF cloned into pBAD/HisA expression vector) was described previously [11]. Expression cultures of TX5254 were induced with arabinose and the N-terminally His$_6$ tagged proteins were purified using nickel affinity chromatography and anion exchange chromatography, as described previously [11,52]. Protein concentrations were determined by absorption spectroscopy at 280 nm using calculated molar absorption coefficient values [53].

**Ace specific polyclonal antibodies**

Expression and purification of (His)$_6$-tagged recombinant Ace A domain was done using a previously described construct and methods [11]. Goat polyclonal serum against recombinant rAce A domain (rAce) was generated by Bethyl Laboratories (Montgomery, TX). Ace A-domain specific antibodies were eluted from rAceA coupled to cyanogen bromide-activated Sepharose 4B, according to the manufacturer’s protocol (Amersham Biosciences, Piscataway, N.J.). The antibodies were concentrated by ultrafiltration with a 10,000-Da molecular-weight-cutoff filter (Millipore, Bedford, Mass.), dialyzed against PBS and concentrations were determined by absorption spectroscopy.

**Protein extraction and Western blotting**

Surface protein extracts from *E. faecalis* isolates were prepared using mutanolysin (Sigma) as described earlier [11]. Protein extracts were electrophoresed in 4–12% NuPAGE Bis-Tris gels (Invitrogen) under reducing conditions in MOPS buffer, and transferred to a polyvinylidene difluoride (PVDF) membrane. Membranes were then incubated with either affinity-purified anti-Ace A-domain specific immunoglobulins (Igs) [11] or pre-immune rabbit serum Igs followed by horseradish peroxidase-conjugated anti-goat antibodies. The blots were then developed with Supersignal West Pico Chemiluminescent substrate (PIERCE, Rockford, Ill.). Purified recombinant Ace A-domain was used as a positive control.

**Flow cytometry**

**Bacteria.** Surface expression of Ace on OG1RF or OG1RFace (pAT392::ace) cells was detected by flow cytometry using affinity purified Ace A-domain (ligand-binding domain)-specific antibodies, as described earlier [54]. Bacteria grown for 10 h in appropriate conditions were probed with pre-immune or affinity purified anti-rAceA specific-antibodies followed by donkey anti-goat IgG F(ab’)$_2$ fragment conjugated with phycoerythrin (Jackson ImmunoResearch Laboratory, West Grove, Pa.). The cells were then fixed in paraformaldehyde and analyzed with a Coulter EPICS XL AB6064 flow cytometer (Beckman Coulter, Fullerton, Calif.) and System II software.

**Endocarditis vegetations.** Sterile vegetations were produced in 11 rats, as described below, and nine rats were infected (*i.e.* with OG1RF. Vegetations harvested after 48 h from the nine infected and two non-infected rat heart valves were processed to remove host tissue debris, using a previously described method [23]. Processed samples in groups of three infected vegetations were mixed and then divided into aliquots for labeling with a) Igs purified from antiserum raised against formalin-killed *E. faecalis* HH22-whole-cells (positive control) and b) affinity-purified anti-rAceA-specific Igs. To assess possible cross-reactivity of anti-rAce Igs with host tissue, the non-infected vegetation sample was probed with affinity-purified anti-rAceA-specific Igs. Forward scatter (for analysis of particle sizes in the sample) and side scatter (for analysis of cell granularity or internal complexity) of vegetation processed cells were analyzed and compared with the *in vitro* grown *E. faecalis* OG1RF cells.

**Antibody titers**

Anti-Ace antibody titers in rat sera were determined by ELISA as described by [19] with some modifications. Briefly, 96-well plates (Immundi 4HBX, Thermo Fisher Scientific, Waltham, Mass.) were coated with 1 µg of rAce in 0.05 M carbonate-bicarbonate buffer, pH 9.6. Rat sera were tested in duplicate with serial dilutions from 1:100 to 1:240,800, followed by detection with peroxidase-conjugated anti-rat secondary antibody (Jackson ImmunoResearch Laboratory, West Grove, Pa.) and TMB peroxidase substrate (Bethyl Laboratories, Montgomery, Tex.). The reaction was stopped with 2 M H$_2$SO$_4$. Antibody titers were
expressed as the highest serum dilution with an A_405nm ≥0.10 at 3 min after addition of the substrate [55].

Testing the effect of *E. faecalis* OG1RF, ace mutants, complemented mutants and immunizations in experimental endocarditis

Aortic valve endocarditis was produced in rats by following previously published methods [23,30,55,56,57,58]. In brief, for induction of endocarditis, white Sprague-Dawley rats (~200 gm) were used. The animals were anesthetized with isoflurane for placement of intravascular catheters. The right carotid artery was exposed and a sterile polyethylene catheter was inserted through a small incision and advanced to ~4 cm into the left ventricle. Proper positioning was assured by sensing resistance and vigorous pulsation of the line.

**Mixed infection competition assay using OG1RF and ace mutants.** For testing OG1RF and OG1RFΔace in a mixed infection competition assay, bacteria (~1:1 by OD) were inoculated via the catheter 20 min after catheter placement; the catheter was heat-scaled and left in place during the course of the experiment and the skin was closed with sutures. Bacterial geometric mean (GM) CFUs determined for OG1RF and OG1RFΔace from the inocula were 3.8×10^7/rat and 4.4×10^7/rat, respectively. Animals were sacrificed 72 h after infection. Hearts were aseptically removed and aortic valves were examined. The platelet-fibrin vegetations formed on aortic valves were excised, weighed, homogenized in 1 ml of saline and dilutions were plated onto BHI and EA media. Ninety-six colonies/rat were plated onto BHI and EA media. Ninety-six colonies/rat were excised, weighed, homogenized in 1 ml of saline and dilutions were plated onto BHI supplemented with chloramphenicol 10 µg/ml or BHI supplemented with kanamycin 2000 µg/ml in the case of the ace disruption mutant, to verify the phenotypic markers of OG1RF and OG1RFΔace. DNA lysates from colonies obtained from the OG1RFΔace plus OG1RF mixed infection were hybridized under high stringency conditions [48], using intragenic DNA probes of ace and cat in order to determine the percentage (%) of OG1RF and OG1RFΔace colonies of the recovered bacteria from vegetations. Rats with sterile cultures of undiluted vegetation homogenates (~1 ml) were considered to have had no induction of endocarditis. Data were expressed as percentages (%) of WT and mutant per vegetation. For vegetations showing only OG1RF colonies, for example, when 96/96 colonies were cat negative and ace positive and 0/96 colonies were cat positive and ace negative, the proportion of OG1RFΔace in the vegetation was assigned the value of 1/97 (~1%) (i.e., assuming the next colony picked would have been a mutant).

**Mono-infection testing using complemented OG1RFΔace (pAT392::ace) and OG1RFΔace (pAT392).** For testing OG1RFΔace (pAT392::ace) versus OG1RFΔace (pAT392) in the mono-infection model, rats were inoculated (i.e.) 24 h post-catheterization and were sacrificed at 4 h or 24 h post infection. Due to the instability of pAT392 seen in *in vivo* experiments, inocula were then grown in the presence of gentamicin (25 µg/ml) and animals were sacrificed at 24 h post infection. To minimize the loss of pAT392 over time and to determine the role of Ace in early colonization of aortic valves by OG1RFΔace (pAT392::ace) versus OG1RFΔace (pAT392), we also sacrificed a group of 11 to 12 animals administered with bacteria grown in BHIS at 4 h post inoculation. Rats with sterile cultures of undiluted vegetation homogenates were considered to have had no induction of endocarditis. In order to determine the *in vivo* stability of plasmid pAT392 in complemented OG1RFΔace (pAT392::ace) and OG1RFΔace (pAT392), ~95 random colonies recovered from vegetations were picked into microtiter wells and were replica plated onto BHI supplemented with gentamicin 125 µg/ml versus BHI to differentiate between gentamicin resistant (the marker of pAT392) [51] and gentamicin susceptible colonies.

**Estimation of WT OG1RF ID₅₀ in rat IE model.** ID₅₀ of OG1RF was determined for BHI and BHIS grown cultures. Twenty one rats were inoculated (i.e.) with either BHI or BHIS grown OG1RF in a range of 1.6×10⁶–2.6×10⁶ and 7.2×10⁵–1.1×10⁶ CFU/rat, respectively. The ID₅₀ was determined by the method of Reed and Muench [59].

**Immunizations.** For active immunization, animals were divided into three groups: (a) rAce active-immunized, (b) non-immunized and (c) FCA – FICA sham-immunized. Immunizations were done following previously published methods [55,56]. In brief, animals in group (a) received an initial dose of 100 µg of rAce in FCA (week 1) followed by a second and third dose of 100 µg of rAce in FICA at a two weeks interval. Animals in group (c) received FCA (week 1) followed by a second and third dose of FICA at a two weeks interval. Surgeries and catheter placement were done as described above for testing the ace mutant. Twenty-four hours post catheterization, animals were inoculated (i.e.) using BHI- or BHIS-grown OG1RF and were sacrificed at 48 h post infection. Three independent experiments were done and results were combined.

For the passive immunization group, animals were injected (i.e.) with 2 mg/kg of affinity purified Ace Igs purified from immunized goat serum 24 h post-catheterization and 1 h prior to bacterial inoculation; controls received 2 mg/kg of purified Igs from pre-immune goat serum. Animals were sacrificed at 24 h post infection. Two independent experiments were done and results were combined.

**Post euthanasia procedures.** Hearts were aseptically removed from all euthanized animals. The vegetations on aortic valves were excised, weighed, homogenized in 1 ml of saline and dilutions were plated onto BHI and EA media. Ninety-six colonies/rat were combined. For testing OG1RF and OG1RFΔace, DNA lysates from colonies obtained from the OG1RFΔace plus OG1RF mixed infection were hybridized under high stringency conditions [48], using intragenic DNA probes of ace and cat in order to generate the percentage (%) of OG1RF and OG1RFΔace colonies of the recovered bacteria from vegetations. Rats with sterile cultures of undiluted vegetation homogenates (~1 ml) were considered to have had no induction of endocarditis. Data were expressed as percentages (%) of WT and mutant per vegetation. For vegetations showing only OG1RF colonies, for example, when 96/96 colonies were cat negative and ace positive and 0/96 colonies were cat positive and ace negative, the proportion of OG1RFΔace in the vegetation was assigned the value of 1/97 (~1%) (i.e., assuming the next colony picked would have been a mutant).

Mouse peritonitis model

OG1RF and OG1RFΔace were tested following our previously published method [60]. In brief, mice were injected intraperitoneally with appropriate dilutions of bacteria (BHI or BHIS), premixed with sterile rat fecal extract (SRFE) and were observed for 5 days for survival. Two-fold inocula (range of ~1×10⁵–1×10⁹ CFU/ml) of both test bacteria were used to compare animal survival/mortality. LD₅₀ was determined using six mice per group and by the method of Reed and Muench [59].

**Statistics** To compare the mean ± SD values of the adherence results, an unpaired *t* test was used. Percentages (%) of OG1RF versus OG1RFΔace present in mixed infection vegetations were analyzed by the paired *t* test. Similar to the method previously described for *E. faecalis* and *E. faecium* endocarditis using a mixed infection [23,29], the mean virulence index of the mutant relative to WT
was calculated using the following equation:

\[
\text{Mean virulence index} = \frac{\sum \left(\% \text{ wild-type in inoculum}/\% \text{ mutant in inoculum} \right)}{\sum \left(\% \text{ wild-type in vegetation}/\% \text{ mutant in vegetation} \right)}
\]

Mean virulence index for the mutant should be 1.0, if the WT and the mutant have the same level of virulence, and lower values would indicate increasing levels of attenuation. Differences in bacterial log_{10} CFU (geometric mean) from vegetations of rAce-immunized versus non-immunized and FCA-FICA-immunized controls were analyzed by the unpaired \(t\) test. Fisher’s exact test was used for comparing the total number of infected/non-infected rats in the rAce-immunized group versus control groups. Graph Pad Prism version 4.00 for Windows (GraphPad Software, San Diego, Calif.) was used for statistical analysis.

**Acknowledgments**

We thank Karen Jacques-Palaz and L. Charlene Thomson for their technical assistance.

**Author Contributions**

Conceived and designed the experiments: KVS SRN BEM. Performed the experiments: KVS SRN JS. Analyzed the data: KVS SRN JS BEM. Contributed reagents/materials/analysis tools: KVS SRN JS. Wrote the paper: KVS SRN JS BEM.

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