

Adhesion of Vancomycin-Resistant *Enterococcus* to Human Intestinal Mucus

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Abstract. The intestinal mucus layer provides a potential niche for colonization by vancomycin-resistant *Enterococcus faecium* (VREF). We therefore examined the ability of six VREF strains to adhere to human intestinal mucus and determined binding kinetics. Four of six (67%) VREF strains demonstrated significant adhesion to immobilized intestinal mucus compared with a *Salmonella typhimurium*-negative control strain, but the level of adherence was low compared with *Lactobacillus rhamnosus* GG. Binding kinetics studies demonstrated that the maximum number of these four VREF strains that could adhere to a unit surface area of immobilized mucus was similar to or higher than the maximum number of *L. rhamnosus* GG that could adhere; however, *L. rhamnosus* GG demonstrated 20- to 130-times higher affinity than the VREF strains. These results demonstrate that VREF strains may adhere to human intestinal mucus and suggest that *L. rhamnosus* GG might be able to displace VREF strains.

Adherence of pathogenic microorganisms to mucosal surfaces may facilitate colonization of the intestinal tract [4]. The mucosal surface of the intestines includes both the layer of epithelial cells and the overlying mucus gel [2]. The mucus layer of the colon includes a firmly adherent layer (approximately 100 μm) adjacent to the epithelium and a thicker loosely adherent layer (approximately 550 μm) that can be removed by gentle suctioning [1, 7]. In addition to mucin glycoproteins, the mucus layer contains many smaller glycoproteins, proteins, glycolipids, lipids, and sugars [2, 7]. The intestinal mucus layer provides an important niche for colonization of the mouse intestinal tract with *Escherichia coli* and *Salmonella* species [9, 15, 16]. These organisms grow in mucus and adhere to specific receptors in the mucus layer [9, 15, 16]. We previously demonstrated that vancomycin-resistant *Enterococcus faecium* (VREF), an important nosocomial pathogen, is able to grow in vitro in cecal mucus and becomes associated in vivo with the cecal mucus layer of clindamycin-

treated mice [12]. In this study, we tested the hypothesis that VREF strains can adhere to human intestinal mucus in vitro.

Materials and Methods

Intestinal mucus. The Joint Ethics Committee of the University of Turku and Turku University Hospital approved the use of resected human intestinal material. The mucus samples were obtained from the ascending colon of a patient who had undergone surgery for reasons other than inflammation or malignancy. After resection, the tissues were washed gently in phosphate-buffered saline (PBS; pH 7.2; 10 mM phosphate) containing 0.01% gelatin until all contents were removed. Mucus was prepared as reported earlier [10, 11]. In short, mucus was collected by gently scraping the mucosa with a rubber spatula. The mucus was added to a small amount of HEPES (*N*-(2-hydroxyethyl)piperazine *N*-2(ethane sulfonic acid)-buffered Hanks' balanced salt solution (HH; 10 mM HEPES; pH 7.4) buffer and centrifuged twice for 10 minutes at 12,000 \times g and once for 15 minutes at 27,000 \times g to remove cell debris and bacteria. The mucus was stored at -70°C until use.

Bacterial strains and media. Six clinical VREF isolates with differing pulsed-field gel electrophoresis (PFGE) patterns were studied [3]. Five of the isolates were vanB-type, and one was vanA-type [3]. *L. rhamnosus* GG (ATCC 53103) was used as a positive

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control for adhesion, and *S. enterica* serovar Typhimurium (ATCC 14028) was used as negative control.

VREF strains were grown in brain–heart infusion broth, *L. rhamnosus* GG in de Man, Rogosa, and Sharpe broth, and *S. typhimurium* in Luria-Bertani (LB) broth for 20 hours at 37°C. To metabolically radiolabel the bacteria, 10 $\mu\text{l ml}^{-1}$ tritiated thymidine (methyl-1,2- ^3H -thymidine 120 Ci mmol^{-1}) was added to the medium. After growth, the bacteria were harvested by centrifugation (1300 \times g), washed twice with PBS, and resuspended in PBS. Absorbance at 600 nm was adjusted to 0.5 ± 0.02 to standardize the number of bacteria (1 to 5×10^7 colony-forming units [CFU] ml^{-1}) used in the adhesion assay.

In vitro adhesion assay. The adhesion of radioactively labeled bacteria to immobilized mucus was examined as reported earlier [10]. In short, intestinal mucus was passively immobilized on polystyrene Maxisorp microtiter plate wells (Nunc, Roskilde, Denmark) by overnight incubation at 4°C, 100 $\mu\text{l/well}$. The wells were washed three times with HH buffer, and 100 μl radioactively labeled bacteria was added. Also, 100 μl of the bacterial suspension was added to scintillation vials to be used as a measure of the bacteria added. After 1.5 hours of incubation at 37°C, the wells were washed three times to remove unbound bacteria, and the adhered bacteria were lysed with 1% sodium dodecyl sulfate (SDS) in 0.1 M NaOH at 60°C for 1 hour. The radioactivity of the lysed bacteria was assessed by liquid scintillation. Adhesion was expressed as the percentage of radioactivity recovered from the wells compared with the radioactivity added to the wells.

Binding kinetics. For the four VREF strains that demonstrated significant levels of adherence (i.e., C38, C37, C22, and C68), the binding kinetics were determined as previously described [8, 10]. In short, adhesion assays were performed as noted previously with dilution series of bacterial suspensions. The concentrations of bacteria that were incubated with the immobilized mucus were assessed by preparing serial dilutions in sterile saline and plating onto selective media. From the results, double reciprocal plots were prepared, with 1/bacteria added (expressed as CFU/ml) on the x-axis and 1/bacteria bound (expressed as bacteria per microtiterplate well) on the y-axis. After curve fitting with the least squares method, the intercepts with the ordinate and abscissa were calculated, which gave the value of 1/maximum number of binding sites) and $-1/(\text{dissociation constant})$.

Statistical analysis. The results for the adhesion assay were expressed as the average of three or four independent experiments. Each experiment was performed with four parallels to correct for intra-assay variation. Student *t*-test was used to evaluate the statistical significance ($P < 0.05$) of the differences in the ability of the different strains to adhere to intestinal mucus.

Results

Of the six VREF strains tested, four (67%) demonstrated significant adhesion to immobilized intestinal mucus compared with the *S. typhimurium*–negative control strain ($P < 0.05$) (Fig. 1). However, the level of adherence was low compared with the *L. rhamnosus* GG–positive control strain. Binding kinetics studies were performed on the four VREF strains that demonstrated significant adherence (C38, C37, C22, and C68) as well as the positive and negative control strains. The plots of the reciprocal of adhered cell concentration versus the

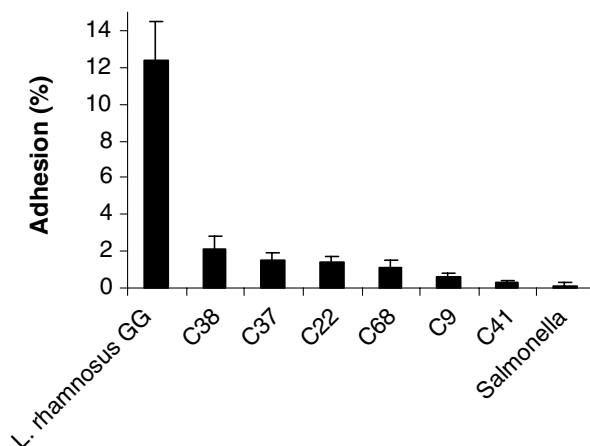


Fig. 1. Adhesion of six vancomycin-resistant *E. faecium* isolates to immobilized intestinal mucus compared with *L. rhamnosus* GG (positive control) and *S. enterica* serovar Typhimurium (negative control). Adhesion is expressed as the percentage of bacteria binding relative to the amount of bacteria added to the immobilized mucus. Error bars = SD.

reciprocal of the concentration of cells added are shown in Fig. 2. A linear relationship was observed for each strain. The intercept on the ordinate gives the value of the reciprocal of the maximum number of bacterial cells bound per well (e_m) [8, 10]. The intercept on the abscissa is $-1/k_x$, where k_x is the dissociation constant for the adhesion process [8, 10]. Thus, the values of maximum number of binding sites (cells per well) and dissociation constants (cells per well) were calculated and are shown in Table 1. The results of these studies suggest that the maximum number of the VREF strains that could adhere to a unit surface area of immobilized mucus was similar to or higher than the maximum number of *L. rhamnosus* GG that could adhere. However, the *L. rhamnosus* GG demonstrated 20 to 130 times higher affinity (Table 1) than the VREF strains. This suggests the possibility that *L. rhamnosus* GG might be able to displace the VREF strains.

Discussion

Previous studies have demonstrated that *E. faecium* strains may adhere to intestinal mucus [5, 6, 13]; however, these studies included only one or two *E. faecium* strains [5, 13] or primarily examined isolates from animal sources [6], and studies of binding kinetics were not performed. Rinkinen et al. [13] found that two *E. faecium* strains used in probiotic preparations (i.e., M74 and SF 68) could adhere to human intestinal mucus with percent adhesions of 3% and 18%, respectively. Jin et al. [5] found that approximately 9% of *E. faecium* strain

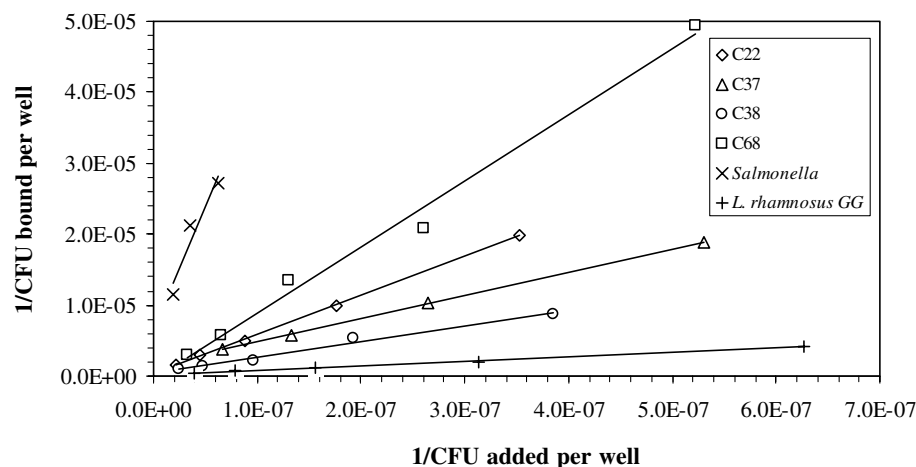


Fig. 2. Double-reciprocal representation of the adhesion of bacterial strains to immobilized human intestinal mucus. The lines indicate the linear fit according to least-squares method.

Salmonella = *S. enterica* serovar Typhimurium–negative control strain; C22, C37, C38, and C68 = vancomycin-resistant *E. faecium* test strains.

Table 1. Maximum number of adhered bacterial cells to human intestinal mucus and dissociation constant of the adhesion process for various strains

Bacterial strain	Maximum number of binding sites (cells/well)	Dissociation constant (cells/well)
<i>Lactobacillus</i> GG	3.74E + 05	1.2E + 06
C38	2.66E + 06 ^a	6.18E + 07 ^b
C37	6.57E + 05	2.23E + 07 ^b
C22	2.93E + 06 ^a	1.58E + 08 ^b
C68	6.21E + 05	4.48E + 07 ^b
<i>Salmonella typhimurium</i>	2.43E + 05	7.42E + 07 ^b

^a Statistically higher than negative and positive control ($P < 0.05$).

^b Statistically lower than positive control ($P < 0.05$).

18C23 adhered to porcine small intestine mucus and showed that binding of the enterococcal strain efficiently inhibited the adhesion of enterotoxigenic *E. coli*. Laukova et al. [6] found that *E. faecium* and *E. faecalis* strains from horse feces, dog feces, and dog feed demonstrated significant adherence to human or canine mucus. Our findings demonstrate that VREF strains may also adhere to human intestinal mucus. Although the percent adherence of the VREF strains to mucus was lower than has been described for the *E. faecium* strains that have been studied previously, it is notable that the percent adherence of *L. rhamnosus* strain GG in the current study was also three- to four-fold lower than the approximately 40% adherence reported previously, which may have been caused by differences in mucus source [13]. The relatively low percent adherence of the VREF strains compared with previous *E. faecium* strains could also be related to differences in the human mucus samples that were used in the assay. A previous study demonstrated that the use of radioactive labels in bacterial adhesion assays offers the best reproducibility and

sensitivity when poorly adherent (< 1%) bacterial strains are studied [14].

As noted previously, we showed that VREF becomes associated *in vivo* with the cecal mucus layer of clindamycin-treated mice [12]. After discontinuation of clindamycin treatment, the anaerobic microbiota recovered within 10 days, resulting in conditions that were inhibitory to replication of VREF within the cecal lumen [12] (and investigators' unpublished data). Because VREF colonization persists well beyond the period of recovery of the anaerobic microbiota within the lumen of the colon, we proposed that the mucus layer may provide a protected niche that facilitates persistence of colonization [12]. The findings of the present study suggest that adherence to mucus could facilitate the association of VREF with the mucus layer. Additional work is needed to determine if adherence to mucus plays a role in establishment or persistence of VREF colonization *in vivo*. Further studies are also needed to determine whether VREF strains bind to specific receptors in mucus.

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