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Influence of isolate origin and presence of various genes on biofilm formation by *Enterococcus faecium*

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Abstract

Enterococcus faecium, a major cause of nosocomial infections, is often isolated from conditions where biofilm is considered to be important in the establishment of infections. We investigated biofilm formation among *E. faecium* isolates from diverse sources and found that the occurrence and amount of biofilm formation was significantly greater in clinical isolates than fecal isolates from community volunteers. We also found that the presence of the *emp_{fm}* (*E. faecium* pilus) operon was associated with the amount of biofilm formation. Furthermore, we analyzed the possible association between the distribution of 16 putative virulence genes and the occurrence of biofilm production. Even though the prevalence of these virulence genes was significantly higher in clinical isolates, we did not observe any correlation with the occurrence of biofilm formation.

Keywords

Enterococcus faecium; biofilm; virulence

Introduction

Enterococci, found naturally as part of the digestive tract flora in humans and animals, have evolved into major opportunistic pathogens causing nosocomial infections including bacteremia in neutropenic patients, endocarditis and device infections (Arias & Murray, 2012). In the USA, there has been a rapid increase in *Enterococcus faecium* infections associated with hospital outbreaks since the 1980s, often attributed to the emergence of

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resistance to ampicillin, the increased use of advanced generation cephalosporins and vancomycin and the subsequent development of resistance to vancomycin (Murray, 2000, Somarajan & Murray, 2013). Also, a recent CDC survey reported that about 38% of hospital-associated enterococcal isolates identified to the species level were *E. faecium* (Hidron, *et al.*, 2008). Over the last two decades, it has been recognized that the vast majority of hospital infection-associated *E. faecium*, including clonal complex CC17, are part of the hospital-associated clade (Galloway-Pena, *et al.*, 2012, Willems, *et al.*, 2012, Gilmore, *et al.*, 2013); this clade is characterized by, in addition to high-level ampicillin and vancomycin resistance, increased frequency of genes encoding putative virulence determinants such as the enterococcal surface protein (Esp) (Willems, *et al.*, 2001) and MSCRAMMs (microbial surface component recognizing adhesive matrix molecules) (Sillanpaa, *et al.*, 2008, Sillanpaa, *et al.*, 2009), a plasmid encoding a hyaluronidase-like protein (Hyl_{fm}, now annotated as family 84 glycosyltransferase) (Rice, *et al.*, 2003), expression of collagen adhesin mediated by *acm* (Nallapareddy, *et al.*, 2006), and EmpABC (forming the *E. faecium* pili, previously known as EbpABC_{fm}) (Sillanpaa, *et al.*, 2009).

The clinical impact of *E. faecium* may be enhanced by biofilm formation and these organisms are frequently found in conditions where biofilm is thought to be important, such as endocarditis, catheter-associated urinary tract infections, periodontitis, and a variety of device-related infections, thereby making it more difficult to treat with antibiotics (Donlan & Costerton, 2002, Donelli & Guaglianone, 2004, Mohamed, *et al.*, 2004, Di Rosa, *et al.*, 2006, Paganelli, *et al.*, 2012). Biofilm formation has been reported to occur less commonly among *E. faecium* isolates compared to *E. faecalis* (Baldassarri, *et al.*, 2001, Di Rosa, *et al.*, 2006, Tsikrikonis, *et al.*, 2012) and several putative virulence determinants have been implicated in biofilm formation by *E. faecium* including Esp_{fm} (Heikens, *et al.*, 2007), SgrA (a surface adhesin binding to nidogen 1 and 2 of the basal lamina) (Hendrickx, *et al.*, 2009), EmpABC (*fms* 1-5-9) (Sillanpaa, *et al.*, 2010) and AtlAEfm (autolysin) (Paganelli, *et al.*, 2013). Although Esp_{fm} was the first determinant to be connected with biofilm formation in *E. faecium* (Eaton & Gasson, 2002, Heikens, *et al.*, 2007), there are conflicting reports about its contribution to biofilm formation and it appears that the *esp_{fm}* gene is neither essential nor sufficient for biofilm production in infectious clinical isolates (Ramadhan & Hegedus, 2005, Di Rosa, *et al.*, 2006). While data on the prevalence of various other putative virulence genes in *E. faecium* isolates are available (Rice, *et al.*, 2003, Hendrickx, *et al.*, 2007, Sillanpaa, *et al.*, 2008, Sillanpaa, *et al.*, 2009), there is only limited information about their association with or contribution to biofilm formation capacity (Di Rosa, *et al.*, 2006, Tsikrikonis, *et al.*, 2012). Also, there are no data available regarding the differences in the frequency and level of biofilm formation between *E. faecium* isolates of clinical and community origin.

Materials and Methods

The present study assessed the ability of *E. faecium* isolates from diverse sources to form biofilm and also examined whether the distribution of 16 genes including *esp_{fm}*, *hyl_{fm}*, *emp_{fm}* ABC, other *fms* genes (encoding MSCRAMM-like *E. faecium* surface protein) like *scm* (*fms*10), *fms*15, *fms*18, *fms*11-19-16 cluster, *fms*14-17-13 cluster and *fms*21-20 cluster

among *E. faecium* strains is associated with biofilm formation. For this, a total of 65 *E. faecium* strains obtained from different geographical regions (Argentina, Belgium, China, and various cities across the United States) isolated over a fourteen year period were analyzed. These 65 *E. faecium* strains were divided into groups based on their source of isolation, 21 from *E. faecium* endocarditis, 19 from other *E. faecium* clinical infections and 25 from healthy volunteer stools (Supplementary file 1). High-stringency colony hybridizations using intragenic *efa_{fm}* and *aac (6') - II* probes were performed to confirm the species identity of *E. faecium* isolates, after initial biochemical characterization (Singh, *et al.*, 1998, Baldassarri, *et al.*, 2001). *E. faecium* isolates were routinely grown in brain heart infusion (BHI) broth or agar (Difco Laboratories, Detroit, MI).

Biofilm Assay

Biofilm formation by these isolates was quantitated by measuring OD₅₇₀ of crystal violet stained wells as described previously (Mohamed, *et al.*, 2004). Briefly, bacteria that had been grown overnight were diluted 1:100 in tryptic soy broth (TSB) with 0.25% glucose and 200 µl of cells was added onto the wells of polystyrene microtiter plates (Falcon, Franklin Lakes, N.J.). After 24 h of incubation at 37°C, plates were gently washed three-times with phosphate buffered saline (PBS, pH 7.3) and bacteria were fixed with 200 µl of Bouin's fixative for 30 min, washed once with PBS, and stained with 200 µl of crystal violet (1%) for 30 min. After washing and air drying the plates, the biofilm-associated dye was solubilized in 200 µl of ethanol-acetone (80:20, vol/vol) and optical density at 570 nm (OD₅₇₀) was determined. Each strain was assessed on at least two occasions with 8 replicate wells each time.

Colony Hybridization

The prevalence of various genes was evaluated using DNA colony hybridizations, as described previously (Singh, *et al.*, 1998). Genomic DNA probes, prepared by PCR amplification of intragenic regions of various genes using specific primers (Sillanpaa, *et al.*, 2008) and radio labeled using a RadPrime DNA Labeling Kit (Invitrogen, Carlsbad, CA) were used. The gene *acm*, which has been shown to be present in >99% of isolates of various origins (Nallapareddy, *et al.*, 2008), was used as a positive control throughout this study.

Median OD₅₇₀ readings of strains from different sources were compared using Mann-Whitney tests. Association between biofilm formation and the distribution of the 16 genes in *E. faecium* strains belonging to different clinical or non-clinical subgroups was analyzed using two tailed Fisher's exact test and binary logistic regression (Pezullo, 2013). Most of the statistical analyses were performed using GraphPad prism 4 software and *P* values less than 0.05 were considered statistically significant.

Results and Discussion

Biofilm formation among *E. faecium* isolates from diverse sources

We observed that the maximum OD₅₇₀ absorbance value for crystal violet stained biofilms of *E. faecium* isolates was 1.2 (OD₅₇₀ 0.01 to 1.2) (Figure 1), which suggests that the

biofilm formation capacity by *E. faecium* isolates is less than that of *E. faecalis* isolates, which in our hands using the same technique, demonstrate a higher level of biofilm formation with a maximum OD value of 3.5 (OD₅₇₀ 0.2 to 3.5) (Mohamed, *et al.*, 2004, Mohamed & Murray, 2005, Mohamed & Murray, 2006). Of note, *E. faecium* isolates of endocarditis origin did not show any statistically significant difference in biofilm forming capacity compared to isolates from other clinical infections (median OD₅₇₀ of 0.16 and 0.21, respectively) whereas in *E. faecalis*, endocarditis isolates exhibited significantly more biofilm formation compared to isolates from other clinical infections (Mohamed, *et al.*, 2004). We did however find that isolates of clinical origin demonstrated a high level of biofilm production (median OD₅₇₀ of 0.19, interquartile range (IQR) 0.1 to 0.45) compared to isolates from healthy community volunteers (median OD₅₇₀ of 0.06, IQR 0.03 to 0.16, $P = 0.0008$).

The presence of putative virulence genes and the amount of biofilm formation

We next analyzed whether the presence of putative virulence genes was associated with the amount of biofilm formation and found that the presence of the *emp_{fm}* operon showed a significant association with the level of biofilm formation, as shown by the higher median OD₅₇₀ values for *E. faecium* isolates with *emp_{fm}* (0.04 and 0.14 for *emp_{fm}*-negative and *emp_{fm}*-positive respectively, $P = 0.03$); this also supports the previous findings by our group that deletion of the *emp_{fm}* operon resulted in significantly reduced biofilm formation (Sillanpaa, *et al.*, 2010). On the other hand, the presence of other putative virulence determinants did not show any significant association with the amount of biofilm formation (Table 1).

This suggests that the presence of virulence traits is not always associated with increased biofilm formation capacity; for example, we could not find any reduction in biofilm formation with our *acm* deletion mutant (unpublished data), even though it showed attenuation in a rat endocarditis model (Nallapareddy, *et al.*, 2008).

The presence of putative virulence genes and the frequency of biofilm formation

To further delineate the association between the presence of putative virulence determinants and the occurrence of biofilm formation among isolates of different origins, we followed the approach of others (Di Rosa, *et al.*, 2006, Tsikrikonis, *et al.*, 2012) and classified strains of *E. faecium* with OD₅₇₀ readings of 0.12 as biofilm formers (35 of 65, 53.8%). Using this criterion, 67.5% of *E. faecium* clinical isolates were found to produce biofilm including 13 of 21 clinical endocarditis strains (61.9%) and 14 of 19 isolates from other clinical infections (73.7%), compared to 8 of 25 community stool (32%). Thus, biofilm formation occurred at a higher frequency in clinical isolates compared to commensal isolates (odds ratio (OR) of 4.413, 95% CI 1.514 – 12.86, $P = 0.0099$). Our results also suggest that biofilm formation among *E. faecium* isolates is less common (53.75%) compared to *E. faecalis* isolates, which exhibit a higher frequency of biofilm formation (95%–100%) based on previous reports (Mohamed, *et al.*, 2004, Di Rosa, *et al.*, 2006).

We previously found that the 16 putative virulence genes, which were tested for association with biofilm in this study, were present in higher frequencies in clinical subgroups than in

non-clinical subgroups and their prevalence in clinical isolates compared to community stool isolates and animal isolates were significantly different (Sillanpaa, *et al.*, 2009). Based on these data, we analyzed whether there was an association between gene distribution and the occurrence of biofilm formation (Table 2). The genes *esp_{fm}* and *hyl_{fm}* were present only in clinical isolates and among the clinical isolates, *esp_{fm}* gene was present in 33% (9/27) of biofilm producing clinical isolates and 53.8% (7 /13) of non biofilm producing clinical isolates ($P=0.7475$). The observation that seven of the *esp_{fm}* positive isolates did not produce biofilm, support the reports of those who found that *esp* gene is not essential for biofilm formation (Ramadhan & Hegedus, 2005, Di Rosa, *et al.*, 2006, Tsikrikonis, *et al.*, 2012) and contrasts with the observations of others (Heikens, *et al.*, 2007). Similarly, 22% (6/27) of the biofilm producing clinical isolates and 38.5% (5/13) of the non biofilm producing clinical isolates carried the gene *hyl_{fm}* ($P=1$). The *fms* genes were variably present among all isolates and the *emp_{fm}* operon was present in 88.6% of total biofilm forming isolates and 76.7% (23/30) of the total non biofilm forming isolates ($P=0.32$). Thus, although *E. faecium* isolates with *emp_{fm}* operon showed significantly higher amount of biofilm formation compared to *emp_{fm}*-negative strains, the mere presence of *emp_{fm}* operon does not predict the occurrence of biofilm formation in *E. faecium* isolates (whether clinical or commensal). Likewise, other *fms* genes did not present any significant association with the occurrence of biofilm formation. Even though our study does not find a correlation between the presence of genes and the occurrence of biofilm formation, we recognize the possibility that recent isolates with substantial genomic diversity might yield different results.

In conclusion, our study finds that biofilm formation as measured in a microtiter plate assay occurs at a higher frequency and amount in clinical *E. faecium* isolates compared to isolates from healthy volunteers, thus suggesting a potential connection between biofilm formation and virulence potential. Our study also suggests that enrichment of *emp* (pilus) genes may account for the higher amount of biofilm formation associated with clinical isolates, which in turn could enhance their ability to cause infection through increased resistance to antibiotics and innate host defenses (Donelli & Guaglianone, 2004, Fedtke, *et al.*, 2004).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Analysis of biofilm formation among *E. faecium* isolates from diverse sources
 Circles and squares represent clinical isolates and community stool isolates respectively. The horizontal line indicates the median OD₅₇₀ of biofilm formation of each group. Each point represents the average OD₅₇₀ values from two independent experiments with 8 replicates each.

Table 1Median OD₅₇₀ readings of *E. faecium* isolates based on the presence of putative virulence determinants

<i>Virulence determinants</i>	Median	IQR	P
<i>esp</i> -positive	0.18	0.08–0.45	0.15
<i>esp</i> -negative	0.11	0.04–0.31	
<i>hyl</i> -positive	0.11	0.07–0.30	0.58
<i>hyl</i> -negative	0.15	0.05–0.35	
<i>emp</i> -positive	0.14	0.06–0.32	0.03
<i>emp</i> -negative	0.04	0.01–0.24	
<i>fms11-19-16</i> -positive	0.13	0.07–0.35	0.20
<i>fms11-19-16</i> -negative	0.06	0.03–0.29	
<i>scm</i> -positive	0.12	0.05–0.29	0.89
<i>scm</i> - negative	0.13	0.03–0.36	
<i>fms15</i> -positive	0.13	0.05–0.39	0.22
<i>fms15</i> -negative	0.11	0.04–0.23	
<i>fms18</i> -positive	0.15	0.06–0.39	0.47
<i>fms18</i> -negative	0.11	0.04–0.30	
<i>fms21</i> -positive	0.13	0.05–0.33	0.22
<i>fms21</i> -negative	0.05	0.03–0.33	
<i>fms20</i> -positive	0.13	0.06–0.32	0.52
<i>fms20</i> -negative	0.08	0.04–0.38	
<i>fms14</i> -positive	0.13	0.06–0.31	0.35
<i>fms14</i> -negative	0.06	0.03–0.51	
<i>fms17</i> -positive	0.12	0.05–0.31	0.86
<i>fms17</i> -negative	0.09	0.04–0.93	
<i>fms13</i> -positive	0.12	0.05–0.29	0.66
<i>fms13</i> -negative	0.09	0.06–0.11	

Table 2

The presence of virulence associated genes and occurrence of biofilm formation in *E. faecium* of diverse origin^a

Gene	Total no. (%) of isolates with gene /no. (%) of biofilm producing isolates with gene		
	Clinical (n=40/27) ^b	Community(n=25/8) ^b	Total (n=65/35) ^b
<i>esp</i>	16 (40)/9 (33)	0 (0)/0 (0)	16 (25)/9 (26)
<i>hyl</i>	11 (28)/6 (22)	0 (0)/0 (0)	11 (17)/6 (17)
<i>scm</i>	31 (78)/20 (74)	13 (52)/2(25)	44(68)/22(78)
<i>fms15</i>	29 (73)/18 (67)	6 (24)/1 (13)	35 (54)/19(54)
<i>fms18</i>	20 (50)/12(44)	1 (4)/0 (0)	21 (32)/12(34)
<i>emp</i> operon genes (<i>fms 1-5-9</i>)	37 (93)/25 (93)	17 (68)/6 (75)	54 (83)/31(89)
<i>fms11-fms19-fms16</i> cluster	31(78)/19 (70)	11 (44)/4 (50)	42 (65)/23 (66)
<i>fms14-fms17-fms13</i> cluster			
<i>fms14</i>	30 (75)/19 (70)	19 (76)/7 (87.5)	49(75)/26 (74)
<i>fms17</i>	30 (75)/19 (70)	22 (88)/8 (100)	52 (80)/27 (77)
<i>fms13</i>	31 (78)/20 (74)	23 (92)/8 (100)	64 (98)/28 (80)
<i>fms21-fms20</i> cluster			
<i>fms21 (pilA)</i>	36 (90)/24 (89)	20 (80)/8 (100)	56 (86)/32 (91)
<i>fms20</i>	30 (75)/19 (70)	18 (72)/8 (100)	48 (74)/27 (77)

Association between the presence of genes and the occurrence of biofilm formation among isolates was compared and none were found to be significant ($P > 0.05$).

^a An isolate was considered as a biofilm producer if the OD₅₇₀ was >0.12 (as in (3)).

^b Total no. of isolates in the indicated group/ No. of biofilm positive isolates in the group