Enterococcal surface protein contributes to persistence in the host but is not a target of opsonic and protective antibodies in *Enterococcus faecium* infection

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Enterococci are important nosocomial pathogens with multiple intrinsic and acquired resistances to antibiotics. In the past, the majority of infections were caused by *Enterococcus faecalis*; however, an increase in *Enterococcus faecium* clinical isolates has been observed in recent years. The enterococcal surface protein (Esp) is expressed on the surface of most *E. faecium* clinical isolates and has been shown to be involved in biofilm formation. Here, *E. faecium* E1162 and its previously created insertion-deletion mutant of the *esp* gene, *E. faecium* E1162 Δ *esp*, were compared in a mouse bacteraemia model. Anti-Esp serum was tested for its capacity to mediate opsonophagocytic killing of E1162 *in vitro* and to protect against *E. faecium* bacteraemia. The inactivation of *esp* attenuated *E. faecium* virulence with reduced numbers of bacteria recovered from the kidneys in animals infected with the mutant compared to the wild-type strain ($P=0.035$). Passive immunization with rabbit polyclonal serum raised against the recombinant N-terminal Esp protein did not protect mice against *E. faecium* bacteraemia ($P>0.05$). In contrast, mice passively immunized with polyclonal antiserum raised against lipoteichoic acid (LTA) from *E. faecalis* had lower numbers of *E. faecium* E1162 in the blood compared to mice immunized with normal rabbit serum. These results suggest that Esp contributes to *E. faecium* persistence in the host. However, in contrast to LTA, Esp does not seem to be a target for protective antibodies in *E. faecium* strain E1162 in mouse bacteraemia.

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INTRODUCTION

Enterococcus faecalis and *Enterococcus faecium* are currently the third to fourth most commonly isolated nosocomial pathogens worldwide (Werner *et al.*, 2008). The increased contribution of *E. faecium* relative to *E. faecalis* as a causative agent of nosocomial enterococcal infections, first observed in the USA and recently also found in Europe, is of major concern (Arias & Murray, 2009; Top *et al.*, 2008) because most *E. faecium* strains are increasingly resistant to both vancomycin and ampicillin. In the USA, resistance percentages against vancomycin and ampicillin for *E. faecium* isolated in critical care units increased to 80 % and almost 100 %, respectively (Arias & Murray, 2009). The rapid emergence of resistance to novel antimicrobial compounds in these bacteria significantly reduces options for antibiotic therapies. Population genetics of *E. faecium* disclosed that epidemic *E. faecium* strains are a genetically

distinct population compared to isolates from healthy persons (Willems *et al.*, 2005). Enterococcal surface protein (Esp) is an important marker in epidemic strains since its presence seems to be limited to hospital-acquired *E. faecium* clones (Willems *et al.*, 2001). In the present study, we investigated the role of Esp in the pathogenicity of bacteraemia and its potential role as a target for immunotherapy.

METHODS

Bacterial strains and growth conditions. *E. faecium* strain E1162 is a well-characterized clinical blood isolate harbouring the enterococcal surface protein gene (*esp*) (van den Bogaard *et al.*, 1997). An insertion-deletion mutant in the *esp* gene of *E. faecium* strain E1162 was constructed previously by deleting a 287 bp internal fragment and introducing a chloramphenicol resistance cassette (Heikens *et al.*, 2007). Enterococci were grown at 37 °C in tryptic soy broth (or CASO-broth; Merck) without agitation or on tryptic soy agar [TSA (or CASO agar); Merck].

Abbreviations: LTA, lipoteichoic acid; NRS, normal rabbit serum.

Mouse bacteraemia model. Virulence of the *E. faecium* E1162 and *E. faecium* E1162 Δ esp strains was evaluated in a mouse bacteraemia model as described previously (Hufnagel *et al.*, 2004), with minor modifications. Aliquots of bacterial overnight cultures were washed, resuspended in saline, shock-frozen and stored at -80°C . For each experiment, the concentration of bacterial stocks was confirmed by viable counts. Two groups of seven female BALB/c mice (6–8 weeks old) were infected intravenously via the tail vein with 1.02×10^9 c.f.u. per animal for the E1162 wild-type strain and 1.76×10^9 c.f.u. per animal for E1162 Δ esp. The animals were sacrificed after 24 h, blood was collected by cardiac puncture and the kidneys were harvested and homogenized. The number of bacteria in blood and kidney homogenates was determined by plating serial dilutions onto TSA plates and c.f.u. were counted the next day. The number of bacteria was expressed as c.f.u. (ml blood) $^{-1}$ or c.f.u. (g homogenized kidney) $^{-1}$.

Opsonophagocytic activity of the anti-Esp serum. An opsonophagocytic assay was used as previously described to test rabbit anti-Esp serum for the presence of opsonic antibodies directed against strain E1162 (Theilacker *et al.*, 2006). Fresh polymorphonuclear neutrophils (PMNs) were isolated from healthy volunteers and immune rabbit serum raised against lipoteichoic acid (LTA) purified from *E. faecalis* strain 12030 (Theilacker *et al.*, 2006) or against the recombinant N-terminal domain of Esp (anti-Esp serum) (Van Wamel *et al.*, 2007) was used in various concentrations ranging from 1:1000 to 1:25. Newborn rabbit serum (Cedarlane Laboratories) diluted 1:15 and absorbed with the target bacterial strain (*E. faecium* strain E1162) for 1 h at 4°C served as a source of complement. Bacteria were prepared by growing them to mid-exponential phase, with subsequent washing and diluting to obtain a final bacterium:PMN ratio of 1:1. Tubes lacking complement, PMNs or serum were used as negative controls. The per cent killing was calculated by comparing the mean of the negative controls with the colony counts obtained after incubation for 90 min at 37°C (T_{90}) on a rotor rack by use of the following formula: $[(\text{mean c.f.u. controls} - \text{mean c.f.u. at } T_{90}) / (\text{mean c.f.u. controls})] \times 100$.

Protection studies. Rabbit anti-Esp serum raised against the N-terminal portion of the protein (Van Wamel *et al.*, 2007) was tested for its ability to confer protection against *E. faecium* bacteraemia in mice. The N-terminal portion is probably the surface-exposed part of the Esp protein (Heikens *et al.*, 2007) and was shown to be sufficient for mediating biofilm formation in *E. faecalis* and for inhibiting initial adherence of *E. faecium* to polystyrene (Van Wamel *et al.*, 2007; Tendolkar *et al.*, 2005). Groups of eight BALB/c mice were injected in the tail vein with either 200 μl rabbit anti-Esp serum, 200 μl anti-LTA serum or 200 μl normal rabbit serum (NRS), respectively. After 24 h, all mice were challenged i.v. with 2×10^9 c.f.u. *E. faecium* strain E1162 and after an additional 24 h all mice were sacrificed. The blood was drawn and the kidneys were harvested and cultivated quantitatively as described above.

RESULTS AND DISCUSSION

Experimental bacteraemia model

To study the persistence of strain E1162 and its isogenic Esp-deficient mutant in a bacteraemia model, mice were injected via the tail vein with a high bacterial inoculum of both wild-type and mutant. In these experiments, the animals were sacrificed after 24 h because all the mice had completely cleared the bacteria from blood and kidneys after 72 h. At 24 h, quantitative counts of bacteria in the

blood did not differ between E1162 wild-type and E1162 Δ esp infection. The mean bacterial numbers recovered from blood were 70 c.f.u. ml $^{-1}$ for the wild-type bacteria and 50 c.f.u. ml $^{-1}$ for the Esp-deficient mutant. However, the bacterial load in homogenized kidneys from mice infected with the Esp-deficient mutant was significantly lower compared to infection with wild-type bacteria ($P=0.035$, Fig. 1), suggesting a specific role of Esp in invasion in the renal parenchyma and/or persistence in the kidney.

Opsonic and protective activity of anti-Esp serum

Since Esp seemed to be associated with infectivity in a mouse bacteraemia model, we evaluated the efficacy of immune serum raised against the N-terminal part of Esp using this serum as a source of antibodies in an opsonic killing assay. However, even at high concentrations of the serum (1:25), no significant bacterial killing (i.e. more than 50 %) was observed. In comparison, using serum raised against LTA from *E. faecalis*, more than 75 % killing of E1162 even at relatively low serum concentrations (i.e. 1:500) was observed (Fig. 2).

Mice were also passively immunized with anti-Esp serum or with NRS and subsequently infected with strain E1162. All mice showed similar bacterial counts in blood (data not shown) and in kidneys 24 h after infection (Fig. 3). This indicates that anti-Esp antibodies do not protect against *E. faecium* infection. In additional experiments, NRS and anti-LTA serum were compared for their ability to protect mice against bacterial infection with strain E1162 under the same experimental conditions. Passive immunization with anti-LTA serum significantly reduced bacterial counts in blood (2.71 log $_{10}$ reduction) and kidneys (3.45 log $_{10}$ reduction) ($P<0.05$).

Fig. 1. Mouse bacteraemia model. Mice were challenged i.v. with the E1162 strain (1.02×10^9 c.f.u. per animal) or with the E1162 Δ esp strain (1.76×10^9 c.f.u. per animal). After 24 h, the animals were sacrificed and the kidneys were harvested. The mean number of bacteria in kidney homogenates was expressed as c.f.u. (g homogenized kidney) $^{-1}$ ($P=0.035$, using two-tailed *t*-test).

Fig. 2. Opsonic activity of the anti-Esp serum (□) compared with anti-LTA serum (■). The target of the opsonic antibodies in strain E1162 was investigated using rabbit anti-Esp and anti-LTA sera in an opsonophagocytic assay. Bars represent the mean of four determinations and error bars represent SEM.

Several factors have been associated with virulence in enterococci (Jett *et al.*, 1994; Koch *et al.*, 2004). Shankar and colleagues described Esp in *E. faecalis*, which is contained on a 153 kb pathogenicity island (PAI) and has homology to alpha-C and Rib proteins in group B streptococci (Shankar *et al.*, 1999, 2002). A few years later, Willems *et al.* (2001) and Baldassarri *et al.* (2001) found an *esp* homologue in *E. faecium* and in addition discovered that epidemic *E. faecium* isolates causing hospital infections carry this *esp* gene more frequently than faecal isolates. Furthermore, it was determined that *E. faecium esp* is also part of a putative PAI that seems to be associated with nosocomial outbreaks (Leavis *et al.*, 2004). The *E. faecalis* Esp protein has been shown to be associated with biofilm

formation on polystyrene (Tendolkar *et al.*, 2004). However, since Kristich *et al.* (2004) and Toledo-Arana *et al.* (2001) were able to demonstrate that biofilm formation also takes place in the absence of Esp, it is likely that Esp represents only one of a number of different mechanisms by which *E. faecalis* produces biofilm. Insertion-deletion mutation of the *esp* gene in a hospital-acquired *E. faecium* isolate demonstrated the role of *E. faecium* Esp in initial adherence and biofilm formation on polystyrene surfaces (Heikens *et al.*, 2007). By using this Esp-deficient mutant, it was demonstrated that Esp is involved in adhesion of *E. faecium* to uroepithelial cells and that the presence of this protein prolonged infection in the kidneys and bladder of mice (Leendertse *et al.*, 2009). The role of Esp in a mouse urinary tract infection model was also demonstrated for *E. faecalis* (Shankar *et al.*, 2001), which emphasizes that Esp is an important virulence determinant in urinary tract infections caused by either *E. faecium* or *E. faecalis*. Esp disruption did not affect the course of peritonitis in mice (Leendertse *et al.*, 2009), and did not affect bacterial adhesion to colonic carcinoma cells *in vitro* or intestinal colonization and translocation to lymph nodes in mice (Heikens *et al.*, 2009). In *E. faecalis*, Esp is also not essential for intestinal cell adhesion and translocation (Pultz *et al.*, 2005), reaffirming the *E. faecium* results. The importance of *E. faecium* Esp in experimental rat endocarditis and mouse urinary tract infection probably can be explained by the fact that these models represent classical biofilm infections.

Our results suggest that passive immunization with antibodies against Esp does not confer protection against *E. faecium* in a mouse bacteraemia model. However, in other Gram-positive bacteria, antibodies to homologues of Esp, such as Rib and α -like protein of *Streptococcus agalactiae*, have been shown to confer immune protection in mice (Lachenauer & Madoff, 1996; Stalhammar-Carlén *et al.*, 1993). So far, only an ABC transporter in *E. faecium* (Burnie *et al.*, 2002), the Gls24 protein (Teng *et al.*, 2005) and the collagen adhesin Ace of *E. faecalis* (Singh *et al.*, 2010) have been experimentally shown to elicit a protective immune response in animal models. The susceptibility of E1162 to opsonic killing mediated by anti-LTA antibodies confirms previous studies by our group (Huebner *et al.*, 1999, 2000) in which LTA was shown to be the predominant target for protective antibodies in about 25% of *E. faecalis* and 33% of *E. faecium* strains. In summary, the data presented here confirm the role of Esp in the pathogenicity of *E. faecium*, but indicate that this antigen is probably not a suitable vaccine candidate.

Fig. 3. Protection against enterococcal bacteraemia using anti-Esp serum and NRS. Mice were challenged with 2×10^9 c.f.u. 24 h after vaccination and sacrificed 24 h later. The mean number of bacteria in kidney homogenates was expressed as c.f.u. (g homogenized kidney)⁻¹ ($P > 0.05$, using two-tailed *t*-test).

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