Conjugative transfer of the virulence gene, esp, among isolates of Enterococcus faecium and Enterococcus faecalis

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Received 20 November 2003; returned 5 January 2004; revised 5 February 2004; accepted 25 March 2004

Objectives: The enterococcal surface protein gene, esp, is a major putative pathogenicity marker in clinical isolates of Enterococcus faecium and Enterococcus faecalis. This study demonstrates in vitro conjugative transfer of the esp gene among E. faecium and E. faecalis.

Materials and methods: Enterococcal isolates from clinical samples, positive for esp, were mated on filters with enterococcal recipients. Transconjugants were checked for transfer of antibiotic resistance determinants and co-mobilization of the esp gene. They were also characterized by PCR and plasmid profiling/ PFGE typing including Southern hybridizations with labelled esp probes. Transfer as triggered by excision was tested using Taqman PCR.

Results: Two of five E. faecalis and five of nine E. faecium transferred antibiotic resistance determinants into a recipient. Of the transconjugants analysed by PCR for acquisition of esp, only isolates from two E. faecalis and a single E. faecium mating were positive. In the donor strains, the esp gene was located on the chromosome. Molecular analysis revealed a plasmid localization of esp in the E. faecium transconjugant and chromosome-to-chromosome transfer in E. faecalis.

Conclusion: The esp gene is transferable by conjugation among enterococcal isolates.

Keywords: enterococci, pathogenicity island, mating

Introduction

Enterococci are facultative pathogens. Isolates from infections are enriched in (putative) virulence factors, such as cytolysin, aggregation substance, specific surface proteins, and hyaluronidase, some of which are encoded by genes within a recently identified pathogenicity island (PAI) in Enterococcus faecalis. A similar genetic arrangement seems probable in Enterococcus faecium. An enterococcal surface protein gene, esp, also part of this PAI was found to be enriched among human E. faecalis and clinical E. faecium strains from outbreaks, suggesting esp is a marker for epidemic strains at least among the latter species. Acquired antibiotic resistance genes spread horizontally among enterococci mainly by conjugative transfer. The questions remain, if, how, and to what extent chromosomally encoded virulence factors, such as esp, are transferred between enterococcal strains. Attempts to date to show a transfer of esp or the entire PAI have failed. We describe here transfer of esp triggered by a conjugative event and co-selected by transfer of antibiotic resistance determinants.

Materials and methods

Bacterial strains

All esp-positive donor strains were from clinical samples (infection and colonization) and isolated between October 1998 and August 2001. Antibiotic susceptibilities were determined by micro-broth dilution according to Werner et al. All E. faecium were vancomycin-resistant, whereas all E. faecalis were vancomycin-susceptible. All strains were resistant to antibiotics of at least two independent classes, positive for esp by PCR and clonally diverse (Table 1).

Mating experiments

Mating experiments, plasmid preparations and PFGE were carried out as described elsewhere. E. faecium 64/3 and E. faecalis JH2-2, both high-level rifampicin- and fusidic acid-resistant (MICs > 256 mg/L) were chosen as recipients for matings. Selection was done on agar plates supplemented with antibiotics according to the resistance pattern of the donors (erythromycin, tetracycline or vancomycin) and the recipient (rifampicin or fusidic acid). Mating rates were calculated as transconjugants per recipient cell. Concentrations of antibiotics in the agar plates...
were: erythromycin 20 mg/L, tetracycline 15 mg/L, vancomycin 5 mg/L, rifampicin 30 mg/L and fusidic acid 20 mg/L.

**Molecular studies**

PCR for esp was carried out using 100 pM of primer 1 (5′-ACCTGGATGTAGATTGCC) and primer 2 (5′-GAATTCAGTCCTAAACCGGTAAC), 10 ng DNA, and 100 pM dNTPs. Parameters were as follows: initial step of 94°C for 2 min followed by 35 cycles of 94°C for 30 s, 50°C for 30 s and 72°C for 1 min; final step of 72°C for 4 min. RAPD typing PCR was carried out with primer RAPD 10 (5′-TGCTCTGGCC) and 50 ng DNA.

Labelling was done by incorporating digoxigenin-labelled dUTP (Roche Biochemicals, Mannheim, Germany) directly during PCR in a ratio dUTP/DIG dUTP = 4:1. Hybridization was carried out using buffers, kits and conditions as recommended by the manufacturer (DIG Easy Hybridization Kit, Roche Biochemicals). Real-time PCR was carried out according to the manufacturer’s recommendations (Applied Biosystems, Darmstadt, Germany). Two primers binding outside the integration site of the PAI in the reference isolate MMH594 were chosen to amplify the intact integration site in PAI (described in MMH594) was sought in the donor strain. 

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Fourteen esp-positive donors (nine *E. faecium*, five *E. faecalis*) were tested for a conjugative transfer of *esp* in filter-matings (Table 1). Five of nine *E. faecium* and two of five *E. faecalis* pairs yielded antibiotic-resistant transconjugants. Transconjugants were subsequently tested for growth on agar plates with the donor-selective antibiotic, the second selective marker of the recipient and were assessed as mating-negative (Table 1). Transconjugants from three mating pairs (one *E. faecium*, two *E. faecalis*) were positive for *esp* in PCR (Table 1). In each case, only a subpopulation of transconjugants acquired the *esp* gene indicating that the transferred antibiotic resistance determinants were not directly linked with the *esp* gene. This is not surprising since antibiotic resistance determinants have not been identified in the PAI structures reported to date in enterococci.4,9 The three donors (*E. faecalis* 3410 and 3114, *E. faecium* 3308) generating *esp*-positive transconjugants were used for mating experiments with recipients of the other species. Only matings with the *E. faecalis* donors yielded transconjugants (*E. faecalis* 3114 × *E. faecium* 64/3—mating rate 5.81 × 10⁻³; *E. faecalis* 3410 × *E. faecium* 64/3—mating rate 3.87 × 10⁻³). The identification and antibiotic susceptibility profiles of transconjugants were checked (not shown in detail). PCR for *esp* with transconjugant DNA of *E. faecalis* 3410 transconjugants and *E. faecalis* 3114 transconjugants revealed positive signals in 0/1 (only one transconjugant available) and 1/14 cases, suggesting that only a single *E. faecium* transconjugant acquired the *esp* determinant from *E. faecalis* 3114.

Each single pair of *E. faecium* and *E. faecalis* (UW3308, UW3114) and their corresponding *esp*-positive and negative transconjugants derived from matings between donors and recipients of the same species were chosen for a further detailed molecular analysis.

**Results and discussion**

**Mating experiments**

Fourteen *esp*-positive donors (nine *E. faecium*, five *E. faecalis*) were tested for a conjugative transfer of *esp* in filter-matings (Table 1). Five of nine *E. faecium* and two of five *E. faecalis* pairs yielded antibiotic-resistant transconjugants. Transconjugants were subsequently tested for growth on agar plates with the donor-selective antibiotic, the second selective marker of the recipient and were checked by RAPD PCR for clonal relatedness with the recipient. Transconjugants from the mating with strains UW3183 and UW2840 revealed a pattern identical to the corresponding donor strains and were assessed as mating-negative (Table 1). Transconjugants from three mating pairs (one *E. faecium*, two *E. faecalis*) were positive for *esp* in PCR (Table 1). In each case, only a subpopulation of transconjugants acquired the *esp* gene indicating that the transferred antibiotic resistance determinants were not directly linked with the *esp* gene. This is not surprising since antibiotic resistance determinants have not been identified in the PAI structures reported to date in enterococci.4,9 The three donors (*E. faecalis* 3410 and 3114, *E. faecium* 3308) generating *esp*-positive transconjugants were used for mating experiments with recipients of the other species. Only matings with the *E. faecalis* donors yielded transconjugants (*E. faecalis* 3114 × *E. faecium* 64/3—mating rate 5.81 × 10⁻³; *E. faecalis* 3410 × *E. faecium* 64/3—mating rate 3.87 × 10⁻³). The identification and antibiotic susceptibility profiles of transconjugants were checked (not shown in detail). PCR for *esp* with transconjugant DNA of *E. faecalis* 3410 transconjugants and *E. faecalis* 3114 transconjugants revealed positive signals in 0/1 (only one transconjugant available) and 1/14 cases, suggesting that only a single *E. faecium* transconjugant acquired the *esp* determinant from *E. faecalis* 3114.

Each single pair of *E. faecium* and *E. faecalis* (UW3308, UW3114) and their corresponding *esp*-positive and negative transconjugants derived from matings between donors and recipients of the same species were chosen for a further detailed molecular analysis.

**Molecular characterization**

Genomic and plasmid DNA were isolated from the donors. Non-digested plasmid patterns and *Hin* III-digested genomic DNA were resolved in a single agarose gel, blotted onto a nylon membrane and hybridized with a labelled *esp* probe. None of the plasmids showed a signal, whereas in all the genomic preparations definite bands could be visualized (not shown). This was as expected, as all the *esp* clusters identified so far in both enterococcal species have been exclusively chromosomally located.4,9 Association of *esp* in structures similar to the PAI in *E. faecalis* MMH594 was identified for all five *E. faecalis* donors using primers amplifying *cylM* and both ends of the PAI. All but one possessed identically sized PCR products when compared with MMH594 (not shown). Isolate 3410 showed no product for *cylM* and the left end structure of the PAI, suggesting deletion of fragment(s) upstream of the *esp* gene of the putative PAI in this strain. When *Hin* III-digested plasmids from both donor/transconjugant (esp+)-pairs were compared, several band differences were identified (Figure 1a). Whereas in the *E. faecalis* mating pair, the transconjugant showed more fragments than the donor, in *E. faecalis*, the picture was reversed. After Southern hybridization, a single band was labelled corresponding to a novel DNA fragment in the *esp*-positive *E. faecium* transconjugant. The most likely explanation is that the *esp* gene was mobilized from the donor chromosome, integrated into a conjugative plasmid which was then transferred into recipient 64/3, giving an *esp*-positive transconjugant (Figure 1b). When plasmids in the *E. faecalis* pair were digested, none of the fragments showed a signal (Figure 1b). Since *esp* was not on a plasmid either in the *E. faecalis* donor or in the *esp*-positive transconjugant, we postulated that chromosome-to-chromosome transmission had occurred. To test this hypothesis, we carried out *Smal* macrorestriction analysis (PFGE) with DNA from the donor, the recipient and both an *esp*-positive and an *esp*-negative transconjugant (Figure 1c). The patterns of the recipient and the *esp*-negative transconjugant were identical, while there was a single band loss in the pattern of the *esp*-positive transconjugant compared to the former two strains and a completely different donor pattern. The picture became clearer after Southern hybridization with a labelled *esp* probe. The largest band in the donor and the *esp*-positive transconjugant’s pattern showed a signal (Figure 1d), but no signal appeared in the other two lanes. The
Transfer of esp among enterococci

Table 1. Strains used, mating parameters and features of resulting transconjugants

<table>
<thead>
<tr>
<th>Donor</th>
<th>Origin (outbreak)</th>
<th>Resistance pattern</th>
<th>Recipient</th>
<th>Selection on</th>
<th>Transconjugant phenotype</th>
<th>Mating rate</th>
<th>Transconj. tested for esp</th>
<th>Number esp (+)</th>
</tr>
</thead>
<tbody>
<tr>
<td>UW 3114 (FC)</td>
<td>urine</td>
<td>PEN, GEN, STR, ERY, TET, CIP, RIF (1), MFL</td>
<td>JH2-2</td>
<td>TET/RIF</td>
<td>ERY/TET</td>
<td>3.28 x 10^{-5}</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>UW 2981 (FC)</td>
<td>wound</td>
<td>PEN, GEN, STR, ERY, TET, CIP, MFL</td>
<td>JH2-2</td>
<td>TET/FUS</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>UW 2982 (FC)</td>
<td>wound</td>
<td>PEN, GEN, ERY, TET, CIP, RIF, MFL</td>
<td>JH2-2</td>
<td>TET/FUS</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>UW 1978 (FM)</td>
<td>human (outbreak)</td>
<td>PEN, AMP, TET, VAN, TEC, CIP</td>
<td>64/3</td>
<td>VAN/RIF</td>
<td>TET, VAN</td>
<td>0.063 x 10^{-5}</td>
<td>6</td>
<td>–</td>
</tr>
<tr>
<td>UW 2384 (FM)</td>
<td>blood (outbreak)</td>
<td>PEN, GEN, ERY, TET, VAN, TEC, CIP, RIF (2), FUS (4), MFL</td>
<td>64/3</td>
<td>TET/RIF</td>
<td>VAN/RIF</td>
<td>0.031 x 10^{-5}</td>
<td>6</td>
<td>–</td>
</tr>
<tr>
<td>UW 2834 (FM)</td>
<td>urine (outbreak)</td>
<td>PEN, AMP, ERY, TET, VAN, TEC, CIP</td>
<td>64/3</td>
<td>TET/RIF</td>
<td>VAN/RIF</td>
<td>0.179 x 10^{-5}</td>
<td>6</td>
<td>–</td>
</tr>
<tr>
<td>UW 3308 (FM)</td>
<td>vagina</td>
<td>PEN, AMP, GEN, STR, ERY, VAN, TEC, CIP, RIF (2), MFL</td>
<td>64/3</td>
<td>TET/RIF</td>
<td>VAN/RIF</td>
<td>0.349 x 10^{-5}</td>
<td>15</td>
<td>13</td>
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<tr>
<td>UW 3185 (FM)</td>
<td>stool</td>
<td>PEN, AMP, STR, ERY, TET, VAN, TEC, CIP, RIF, MFL</td>
<td>64/3</td>
<td>VAN/FUS</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>UW 3410 (FC)</td>
<td>stool</td>
<td>ERY, TET, CHL, RIF (1)</td>
<td>JH2-2</td>
<td>ERY/FUS</td>
<td>ERY/TET</td>
<td>0.0325 x 10^{-5}</td>
<td>10</td>
<td>3</td>
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<tr>
<td>UW 2840 (FC)</td>
<td>wound</td>
<td>PEN, GEN, STR, ERY, TET, CHL, CIP</td>
<td>JH2-2</td>
<td>ERY/FUS</td>
<td>TET/RIF</td>
<td>0.0355 x 10^{-5}</td>
<td>–</td>
<td>–</td>
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<tr>
<td>UW 3181 (FM)</td>
<td>blood</td>
<td>PEN, AMP, GEN, ERY, TET, VAN, CIP, RIF, MFL</td>
<td>64/3</td>
<td>ERY/RIF</td>
<td>VAN/FUS</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>UW 3183 (FM)</td>
<td>blood</td>
<td>PEN, AMP, STR, ERY, TET, VAN, CIP, RIF, MFL</td>
<td>64/3</td>
<td>TET/FUS</td>
<td>VAN/FUS</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>UW 3311 (FM)</td>
<td>blood</td>
<td>PEN, AMP, GEN, STR, ERY, TET, VAN, CIP, RIF, MFL</td>
<td>64/3</td>
<td>TET/FUS</td>
<td>VAN/FUS</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>UW 3440 (FM)</td>
<td>sputum</td>
<td>PEN, AMP, ERY, CHL, VAN, TEC, CIP, RIF, MFL</td>
<td>64/3</td>
<td>TET/FUS</td>
<td>VAN</td>
<td>0.904 x 10^{-5}</td>
<td>6</td>
<td>–</td>
</tr>
</tbody>
</table>

Abbreviations: AMP, ampicillin; CIP, ciprofloxacin; CHL, chloramphenicol; ERY, erythromycin; FUS, fusidic acid; GEN, gentamicin; MFL, moxifloxacin; PEN, penicillin G; RIF, rifampicin; STR, streptomycin; TET, tetracycline; TEC, teicoplanin; VAN, vancomycin; numbers in parentheses indicate MICs.

a FC, E. faecalis; FM, E. faecium.

b Mating rate per recipient.

c Only one transconjugant gentamicin-resistant. Donors 1978, 2384 and 2834 are related isolates belonging to different subclusters of a German-wide disseminated outbreak strain type.

The largest band giving a signal with the esp probe in the esp-positive transconjugant’s pattern also appeared in the patterns of the esp-negative transconjugant and the recipient. This indicated that, in the former case, the band comprised two fragments of similar size, which were not resolved in PFGE, with one of them possessing esp. The size difference between the lost and the new fragment is about 150–180 kb, which is approximately the size of the entire PAI in E. faecalis. However, we were unable to investigate the possibility that the entire PAI had been transferred for a number of reasons. When we carried out PCR screening for acquisition of additional markers of this PAI (cytolysin operon genes, PCR for the integration site of the PAI using primers PAI164/167 as suggested by Shankar et al.), we have shown that parts of the PAI were already present in E. faecalis JH2-2 used as the recipient. JH2-2 is widely used as a recipient in mating experiments, mainly with pheromone-related plasmid transfer models. The finding that this strain already possesses parts of the PAI (although not esp) was not previously known. PCR with primers binding outside and inside the PAI at both ends of the PAI in JH2-2 (primers EF1/ER1 and EF2/ER2) generated a PCR product in both cases similar to MMH594 (not shown).

There is considerable evidence that PAIs in bacteria are exchanged. However, the mechanisms by which these structures are transferred are not understood. To investigate putative excision of the PAI in E. faecalis from the chromosome as a triggering factor...
Acknowledgements

Excellent technical assistance by B. Hildebrandt is kindly acknowledged.

References


