The hylEfm gene in pHylEfm of Enterococcus faecium is not required in pathogenesis of murine peritonitis

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Abstract

Background: Plasmids containing hylEfm (pHylEfm) were previously shown to increase gastrointestinal colonization and lethality of Enterococcus faecium in experimental peritonitis. The hylEfm gene, predicting a glycosyl hydrolase, has been considered as a virulence determinant of hospital-associated E. faecium, although its direct contribution to virulence has not been investigated. Here, we constructed mutants of the hylEfm-region and we evaluated their effect on virulence using a murine peritonitis model.

Results: Five mutants of the hylEfm-region of pHylEfmTX16 from the sequenced endocarditis strain (TX16 [DO]) were obtained using an adaptation of the PheS* system and were evaluated in a commensal strain TX1330RF to which pHylEfmTX16 was transferred by mating; these include i) deletion of hylEfm only; ii) deletion of the gene downstream of hylEfm (down) of unknown function; iii) deletion of hylEfm plus down; iv) deletion of hylEfm-down and two adjacent genes; and v) a 7,534 bp deletion including these four genes plus partial deletion of two others, with replacement by cat. The 7,534 bp deletion did not affect virulence of TX16 in peritonitis but, when pHylEfmTX16Δ7,534 was transferred to the TX1330RF background, the transconjugant was affected in in vitro growth versus TX1330RF (pHylEfmTX16) and was attenuated in virulence; however, neither hylEfm nor hylEfm-down restored wild type function. We did not observe any in vivo effect on virulence of the other deletions of the hylEfm-region.

Conclusions: The four genes of the hylEfm region (including hylEfm) do not mediate the increased virulence conferred by pHylEfmTX16 in murine peritonitis. The use of the markerless counterselection system PheS* should facilitate the genetic manipulation of E. faecium in the future.

Background

Enterococcus faecium is a common enterococcal species increasingly isolated from hospital-associated infections in the USA [1]. Compelling evidence suggests that this substantial increase in E. faecium nosocomial infections is due to the worldwide occurrence of a genetic subcluster (designated clonal cluster 17, CC17) which encompasses clones that appear to have evolved independently [2-4]. Several genes have been associated with CC17 E. faecium including i) espEfm, encoding a surface protein which has been associated with increased biofilm formation and urinary tract infection (UTI) [4-6]; II) some fms genes (two of which are also designated pilA and pilB), encoding putative microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) or components of enterococcal pili (including the pilus operon ebpABCfm, which appear to play a role in biofilm formation and experimental UTI) [2,7-10]; iii) an intact acm gene encoding a collagen adhesin which was shown to be important in the pathogenesis of endocarditis [8] and, iv) plasmids carrying the hylEfm gene [11-14].

It has been previously shown that hylEfm is carried by large transferable megaplasmids of different sizes (145 to 375 kb) in hospital-associated E. faecium which are widely distributed worldwide [11-13,15]. These plasmids also can harbour antibiotic resistance determinants and some pilus-encoding genes of E. faecium which are present with hylEfm in the same plasmid [15,16]. The
acquisition of the \textit{hyl}$_{Efm}$-plasmid by an \textit{E. faecium} laboratory strain (D344SRF) from a US clinical isolate (C68) increased the colonization of the gastrointestinal tract of mice, an effect that was independent of the presence of antibiotic resistance determinants [17]. Moreover, the acquisition of the \textit{hyl}$_{Efm}$-plasmid from another US clinical strain (TX16) increased the virulence of a commensal strain \textit{E. faecium} TX1330RF in experimental peritonitis [11].

The \textit{Hyl}$_{Efm}$ protein was initially predicted to have homology with hyaluronidases which have been associated with virulence in other gram-positive pathogens [18,19], although hyaluronidase activity has not been detected in \textit{E. faecium} isolates carrying this gene [15]. The most recent annotation and sequence comparisons indicate that this protein is likely to encode a family 84 glycosyl hydrolase with 61% similarity, was recently shown not to have any detectable hyaluronidase activity. Spy1600 was characterized as a family 84 glycosyl hydrolase with \(\beta\)-N-acetyl-glucosaminidase specificity after purification and substrate analysis [20] and expression of \textit{spy1600} in \textit{S. pyogenes} was found to be up-regulated during phagocytosis [21]. For this reason, and because of the almost exclusive occurrence of \textit{hyl}$_{Efm}$ in isolates from clinical origin in different surveillance studies [14,22–24], this gene has been postulated as an important pathogenic determinant of hospital-associated \textit{E. faecium}. However, its exact role in virulence has not been established. In this work, we assess the role of the \textit{hyl}$_{Efm}$-region in \textit{E. faecium} pathogenesis of experimental peritonitis.

**Methods**

**Bacterial strains and plasmids**

Table 1 and Figure 1 show the strains and plasmids used in this work and depict the genetic organization of the \textit{hyl}$_{Efm}$-region in \textit{E. faecium} strains and mutants.

**Table 1. \textit{E. faecium} strains and plasmids used in this work**

| Strains/Plasmids | Relevant Characteristics | Reference |
|------------------|--------------------------|-----------|
| **Strains** | | |
| \textit{E. faecium} | | |
| TX16 (DO) | Sequenced endocarditis clinical isolate, Em', Sm'. ST-16* | [35] |
| TX1330RF | F' and R' derivative of TX130, a faecal colonizing strain from a healthy human volunteer | [11] |
| TX1330RF (pHylEfmTX16) | Derivative of TX1330RF to which the \textit{hyl}$_{Efm}$-containing plasmid (pHylEfmTX16) was transferred by conjugation from TX16 (DO) (~250 kb) | [11] |
| TX1330RF (pHylEfmTX16Δhyl) | Mutant with deletion of part or all of 6 genes of the \textit{hyl}$_{Efm}$ region of TX1330RF(pHylEfmTX16) | This work |
| TX1330RF (pHylEfmTX16ΔhylΔ4genes) | Non-polar deletion of 4 genes of the \textit{hyl}$_{Efm}$ region of TX1330RF(pHylEfmTX16) | This work |
| TX1330RF (pHylEfmTX16ΔhylΔ4genesΔhyl) | Non-polar deletion mutant of \textit{hyl}$_{Efm}$ of TX1330RF(pHylEfmTX16) | This work |
| TX1330RF (pHylEfmTX16ΔhylΔdown) | Non-polar deletion of \textit{hyl}$_{Efm}$ plus its downstream gene of TX1330RF(pHylEfmTX16) | This work |
| TX1330RF (pHylEfmTX16Δdown) | Non-polar deletion of the gene downstream of \textit{hyl}$_{Efm}$ of TX1330RF(pHylEfmTX16) | This work |
| **\textit{E. faecalis}** | | |
| CK111 | OG15p upp4::P\(_2\)-lacP44 | [25] |
| **Plasmids** | | |
| pHylEfmTX16 | Conjugative and transferable megaplasmid (ca. 250 kb) of TX16 (DO) containing \textit{hyl}$_{Efm}$ | [11] |
| pCIK47 | Conjugative donor plasmid for markerless mutagenesis; ori\(_T\), pheS*, pORI280 derivative; confers Em' | [25] |
| pHOU1 | Derivative of pCIK47 in which the \textit{erm}(C) gene was replaced by \textit{aph}-2'-ID; confers Gm' | This work |
| pHOU2 | Derivative of pCIK47 in which the \textit{erm}(C) gene was replaced by \textit{aph}-2'-ID and \textit{cat} was incorporated in the cloning site for allelic replacements; confers Gm' | This work |
| pTEX5501ts | E. coli-enterococcal shuttle plasmid for mutagenesis using a temperature-sensitive replicon | [27] |
| pAT392 | ori\(_F\), ori\(_p\), ori\(_T\), ori\(_R\), ori\(_T\); cat, aac(6)-ID, aac(6)-aph(2') | [30] |
| pAT392::\textit{hyl}$_{Efm}$ | Derivative of pAT392 containing \textit{hyl}$_{Efm}$ (cloned with Sall and Smal) under the control of the P2 promoter | This work |
| pAT392::\textit{hyl}$_{Efm}$down | Derivative of pAT392 containing both the \textit{hyl}$_{Efm}$ plus downstream genes (cloned with Sall and Smal) under the control of the P2 promoter | This work |

\(Em'\), erythromycin resistance; F's, fusidic acid resistance; Gm', gentamicin resistance, R', rifampin resistance; Sm', high-level resistance to streptomycin. *ST refers to sequence type after multi-locus sequence typing. ST16 is part of CC17.
Construction of a deletion mutant of the hylEfm-region using the pheS* counter-selection system in TX16 (pHylEfmTX16) and its transfer to TX1330RF

The pheS* system (previously used in Enterococcus faecalis) [25] is based on the acquired sensitivity of bacteria to p-chloro-phenylalanine (p-Cl-Phe) if they carry a pheS* allele encoding a phenylalanine tRNA synthetase with altered substrate specificity [25,26]. In order to apply this approach to E. faecium strains, which are commonly macrolide resistant, we constructed a derivative of the pheS* vector pCJK47 by replacing its erm(C) gene with aph2"-ID, which confers resistance to gentamicin. The full aph2"-ID gene (including promoter and terminator regions) was amplified by PCR using plasmid pTEX5501ts [27] as the template with primers A and B (Table 2). The amplified fragment (1,089 bp) was digested with NsiI and BglII and ligated with pCJK47 digested with the same enzymes resulting in pHOU1 (Figure 2A). Subsequently, pHOU1 was digested with BamHI and PstI and ligated with a 992 bp fragment released from pTEX5501ts after digestion with the same enzymes and containing the chloramphenicol acetyltransferase gene (cat), obtaining a 7,906 bp vector designated pHOU2 (Figure 2B).

In order to create a deletion mutant of the hylEfm" region (which contains genes predicted to be involved in carbohydrate metabolism and transport; Figure 1), fragments upstream (977 bp) and downstream (999 bp) of this region were amplified by PCR (with primers C-D and E-F, respectively; Table 2) and cloned upstream and downstream of the cat gene in pHOU2, respectively, using BamHI and Xhol for the upstream fragment and ApaI and EcoRI for the downstream fragment; the correct insert was confirmed by sequencing in both
Table 2 Primers used in this work

| Primer Sequence (5’-3’) | Relevant Characteristics |
|------------------------|--------------------------|
| A gcagacgggttgataggttgctagggagt | Forward, BglII site (underlined), used amplification of aph-2'-ID |
| B ccaagaagttttttggtcatgcttactaaacagag | Reverse, NsiI site (underlined), used amplification of aph-2'-ID |
| C cgctctctctaagcttcttcttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttctttc |
with p-Cl-Phe were excisants, the corresponding colonies were grown simultaneously on BHI agar in the presence and absence of gentamicin. Colonies that were susceptible to gentamicin were further screened by PCR, pulsed field gel electrophoresis (PFGE) and hybridizations with hylEfnn and cat probes as described before [11]. The mutated region was also sequenced in order to confirm deletion of the corresponding genes. Subsequently, the mutated hylEfnn-containing plasmid (pHy-lEfnnTX16Δ7,534) was transferred from E. faecium TX16 to TX1330RF (a fusidic and rifampin resistant derivative of the commensal strain TX1330, Table 1) by filter mating as described previously [11] to obtain the strain TX1330RF(pHylEfnnTX16Δ7,534). Acquisition of the mutated plasmid by TX1330RF was also confirmed by PFGE, PCR, hybridizations and sequencing. S1 nuclease digestion and PFGE was performed with the mutant to confirm that no other plasmid had transferred during the conjugation event as previously described [11].

Complementation of the hylEfnn-region mutant TX1330RF (pHy-lEfnnTX16Δ7,534)
The hylEfnn gene was PCR amplified with primers G and H (including the ribosomal binding site and the stop codon of hylEfnn) (Table 2) using total DNA from TX16 as template, and the DNA fragment (1,685 bp) cloned into the shuttle plasmid pAT392 [30] under the control of the P2 promoter (which allows constitutive expression of the cloned genes) and upstream of the aac(6')-aph(2') gene (which is co-transcribed from the same promoter) using SacI and SmaI sites (plasmid pAT392::hylEfnn). In order to evaluate if the deletion of hylEfnn had an effect in the downstream gene (encoding a hypothetical protein of 331 amino acids of unknown function), the hylEfnn and down genes (Figure 1) were also cloned together into pAT392 following a similar strategy and using primers G and I (pAT392::hylEfnn-down). Recombinant pAT392-derivatives were purified from E. coli grown on Luria-Bertani agar containing gentamicin (25 μg/ml) and all their DNA inserts sequenced. Subsequently, they were introduced into E. faecium TX1330RF, and the TX1330RF(pHy-lEfnnTX16Δ7,534) mutant by electroporation. Stability of the plasmid constructs was tested by isolating ca. 100 colonies from overnight cultures (using BHI broth) and from the spleens of dead animals (in different experiments) after intraperitoneal inoculation of the corresponding strain (see below) and plating them simultaneously on BHI and BHI-gentamicin (125 μg/ml).

Construction of additional mutants of the hylEfnn-region in E. faecium TX1330RF(pHy-lEfnnTX16)
To investigate the specific role of the hylEfnn locus in E. faecium pathogenesis, complete in-frame deletions of four genes of the hylEfnn-region, hylEfnn alone, hylEfnn plus its downstream gene and the gene downstream of hylEfnn were generated using TX1330RF(pHy-lEfnnTX16). Fragments upstream and downstream of each region were amplified by PCR with the corresponding primers.

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**Figure 2** Physical map of the plasmids pHOU1 and pHOU2 for targeted mutagenesis of E. faecium. A, plasmid used for construction of TX1330RF (pHy-lEfnnTX16Δ4genes), TX1330RF(pHy-lEfnnTX16Δ2genes), TX1330RF(pHy-lEfnnTX16Δ3genes) and TX1330RF (pHy-lEfnnTX16Δdown) deletion mutants (Figure 1); B, plasmid used for construction of the TX1330RF(pHy-lEfnnTX16Δ534) deletion mutant (Figure 1).
(Figure 1 and Table 2). These fragments, with overlapping ends, were subsequently amplified by crossover PCR and cloned into pHOU1 using EcoRI and NotI (for hylEFm).

PFGE, hybridizations and DNA sequencing. Confirmation of the deletion was performed by PCR, containing medium, respectively, as previously described obtained by plating in BHI with gentamicin and p-Cl-Phe containing medium, respectively, as previously described [25]. Confirmation of the deletion was performed by PCR, PFGE, hybridizations and DNA sequencing.

RT-PCR RNA was extracted from bacterial cells (TX16, TX1330RF(pHylEFmTX16), TX1330RF and strains containing pAT392 derivatives) grown in BHI broth at 37°C with mild agitation (logarithmic phase of growth, A600 0.8) as described before [31], and using the RNA isolation kit RNAwiz (Ambion, Austin, TX). RNA was treated twice with DNase (DNase-Free solution, Ambion) and synthesis of cDNA was performed using the commercial kit SuperScript One-Step reverse transcription-PCR (RT-PCR) with Platinum Taq (Invitrogen), according to the manufacturer’s instructions. The mixture contained 0.2 μM of each primer, designed to detect overlapping transcripts of the four putative metabolic genes (Figure 3) and an internal transcript of hylEFm (Table 2). A primer pair directed to detect a 550-bp transcript of the housekeeping gene dAllE. faecium was used as an internal control for RT-PCR experiments [32,33].

Mouse peritonitis model Female (4 to 6 week old), outbred ICR mice (Harlan Sprague Dawley, Houston) were used as previously described [34]. Groups of 10 mice per inoculum (ranging from 2.3 \times 10^8 to 3.1 \times 10^9 CFU/ml) were included in each experiment. Inocula for each peritonitis experiment were prepared by growing bacteria initially on BHI agar plates. Subsequently, one colony was grown in BHI broth for 24 h at 37°C and the cells were concentrated in saline (0.9%) to an A600 of ca. 1.2. Strains containing pAT392 and derivatives were handled similarly before the intraperitoneal inoculation, except that the BHI agar and broth contained gentamicin (125 μg/ml). Comparison of the survival curves at similar inocula was performed using a log-rank test with Prism for Windows®. A P < 0.05 was considered significant. All experiments were approved by the Animal Welfare committee, University of Texas Health Science Center at Houston.

Results and Discussion Deletion of 6 genes in the E. faecium hylEFm-region altered in vitro growth and attenuated virulence of TX1330RF (pHylEFmTX16) but not TX16(pHylEFmTX16) in murine peritonitis

Since acquisition of the transferable pHylEFmTX16 by TX1330RF conferred increased virulence in experimental peritonitis [11], we explored the possibility that the hylEFm region was an important mediator of this effect. Using RT-PCR assays, we were able to detect in vitro expression of hylEFm during the exponential phase of growth in both TX16 and TX1330RF (pHylEFmTX16) (Figure 3). RT-PCR with primers located at the 3’ and 5’ ends of contiguous genes yielded products of the expected size in each case, suggesting that these genes are likely to be co-transcribed (Figure 3). Then, we adapted the pheS* counter-selection system [25] developed for E. faecalis to obtain several deletions of the hylEFm-region. The hylEFm gene in E. faecium TX16 (http://www.ncbi.nlm.nih.gov/genomeprj/30627, Genbank accession number ACIY00000000) is located in a cluster of genes whose putative function appears to involve the transport and breakdown of carbohydrates (Figure 1) [13]. As an initial step to test the mutagenesis system, a relatively large deletion (7,534 bp) from pHylEFmTX16 was obtained. The deletion involved three genes predicted to encode glycosyl hydrolases (including hylEFm) and a gene downstream of hylEFm whose function is unknown (Figure 1). Part (226 nucleotides) of a gene encoding a hypothetical transmembrane protein and located upstream of the putative family 20 glycosyl hydrolase gene and part (202 nucleotides) of a gene located 1,332 nt downstream of hylEFm encoding a putative GMP-synthase and likely transcribed in the opposite direction from the hylEFm cluster (Figure 1) were also deleted. As it is shown in Figure 4A, the deletion of 7,534 bp in the hylEFm-region did not affect the virulence of TX16 (DO) in murine peritonitis.

Next, we considered the possibility that an in vivo effect might be more clearly dissected if studies were performed in the background of a non-clinical strain. We hypothesized that an in vivo effect of a virulence determinant might more likely be seen in strains which are less successful clinically; that is, that a commensal strain such as TX1330RF [11] is likely to have decreased fitness or ability to produce disease compared to TX16 [35] and, thus, acquisition plus subsequent loss of a virulence determinant that alters such fitness would be easier to identify [11]. Thus, the mutated plasmid from strain TX16(pHylEFmTX16Δ7,534) was transferred to TX1330RF by conjugation and the in vivo effect of acquiring the intact plasmid [11] vs the plasmid carrying the deletion was evaluated. The two strains [TX1330RF
(pHylEfmTX16) and TX1330RF(pHylEfmTX16Δ7,534) appeared to differ only in the size of the hylEfm plasmid by PFGE and S1 nuclease assays [11] (not shown). Figure 4B shows that deletion of 7,534 bp in the hylEfm region of TX1330RF(pHylEfmTX16) caused an in vitro growth defect. The alteration of growth was also seen in a second transconjugant from the same mating experiment between TX16(pHylEfmTX16Δ7,534) and TX1330RF (TC-II in Figure 4B). The mutant strain TX1330RF (pHylEfmTX16Δ7,534) was attenuated in the mouse model of peritonitis (even when an increased intraperitoneal inoculum for the mutant were used) (Figure 4C and 4D) (P < 0.05). Due to the alterations produced in the growth of TX1330RF(pHylEfmTX16Δ7,534), these results suggest that the attenuation in virulence may have also been due to factors other than those specifically related to virulence.

Complementation of the hylEfm-region mutant with hylEfm and a combination of hylEfm and the downstream gene did not restore the virulence of TX1330RF (pHylEfmTX16Δ7,534) In order to further evaluate if the attenuation observed in TX1330RF(pHylEfmTX16Δ7,534) (as described above)
was mediated by a direct effect of hylEfm in the peritonitis model, we explored complementation of this mutant in trans with the full hylEfm gene and a combination of hylEfm and the downstream gene using the shuttle vector pAT392 [30]. The cloning strategy placed these genes upstream of the aac(6’)-aph(2’)-gene (which confers resistance to gentamicin) resulting in all open reading frames under the control of the constitutive P2 promoter. Up to 80% loss was observed with all strains in the absence of gentamicin; however, in the presence of the antibiotic during inoculum preparation, the TX1330RF (pHyfEfmTX16Δ7,534)-derivatives containing the pAT392 constructs were stable both in vitro and in vivo (5% maximum percentage of plasmid loss). Introduction of hylEfm or a combination of hylEfm plus its downstream gene (cloned into pAT392) did not restore the virulence of the mutant strain TX1330RF(pHyfEfmTX16Δ7,534), compared to pAT392 alone in the presence of gentamicin (Figure 5A and 5B). The results indicate that constitutive expression of hylEfm alone or in combination with its downstream gene (which was confirmed by RT-PCR, not shown) was not able to restore the phenotypic differences observed in the mutant strain TX1330RF(pHyfEfmTX16Δ7,534), supporting the fact that hylEfm may not be directly responsible of the attenuation observed in the mutant.

Under our experimental conditions, we cannot completely rule out that the in vivo attenuation observed with pHyfEfmTX16Δ7,534 in the TX1330RF background may have been caused by the partial deletion of the hypothetical transmembrane protein or the putative GMP-synthase located upstream and downstream of the hylEfm-cluster, respectively. Indeed, a deletion of 76 amino acids in the C-terminus of the hypothetical membrane protein occurred in this plasmid, resulting in the deletion of three predicted transmembrane helices.
Similarly, 68 amino acids in the C-terminus of the putative GMP-synthase were deleted; the removal of these amino acids is likely to disturb the dimerization domain of this protein [36] affecting its function in nucleotide metabolism. Moreover, a second TX1330RF(pHy1EfmTX16Δ7,534) mutant also exhibited an almost identical growth defect (Figure 4B). Thus, it is tempting to speculate that changes in these two genes may have affected the “metabolic” fitness of the TX1330RF(pHy1EfmTX16Δ7,534) strain. However, since no evident change in fitness or virulence was observed with the mutated plasmid in the TX16 background, another possibility is that an extraneous change elsewhere in the plasmid (or chromosome) occurred during the conjugation process that influenced the in vitro growth of the TX1330RF(pHy1EfmTX16Δ7,534) mutant(s) and its virulence.

Additional deletions of genes in the hylEfm-region did not alter the virulence of TX1330RF(pHy1EfmTX16) in the mouse peritonitis model

In order to dissect further the in vivo role of hylEfm and the adjacent genes, we produced several in-frame deletions of these genes (Figure 1) including: i) a four gene mutant of the hylEfm-region (including hylEfm) [TX1330RF(pHy1EfmTX16Δgenes)], ii) a deletion of hylEfm alone [TX1330RF (pHy1EfmTX16Δhyl)], iii) a deletion of hylEfm plus its downstream gene mutant [TX1330RF (pHy1EfmTX16Δhyl-down)] and, iv) a single deletion of the gene located downstream from hylEfm [TX1330RF (pHy1EfmTX16Δdown)]. The mutagenesis strategy removed the open reading frame from the start codon of the first gene to the stop codon of the last gene (in case of multiple genes). In case of single gene deletion, the complete ORF (start to stop codon) was removed, leaving the surrounding DNA intact as in the wild type plasmid. None of the four mutants of the hylEfm-region showed a deleterious effect in the growth kinetics compared to TX1330RF (pHy1EfmTX16) (harbouring an intact plasmid, Additional file 1). Moreover, we were unable to observe any attenuation of virulence in the mouse peritonitis model compared to the parental strain with the intact plasmid (Figure 6A-D), which further supports the fact that the four genes of the hylEfm region do not appear to be directly involved in increasing the pathogenic potential of pHylEfmTX16 in strain TX1330RF (pHy1EfmTX16).

Megaplasmids (>145 kb, with or without hylEfm) have been recently found to be widespread among clinical isolates of E. faecium worldwide [12,13,15]. The proportion of these plasmids carrying hylEfm appears to vary according to geographical location (ca. 11 to 36%) [12,13]. Our findings indicate that the four genes of the hylEfm-cluster studied here, including hylEfm, are not the main mediators of the virulence effect conferred by the plasmid carrying them in experimental peritonitis. Since the pHylEfm plasmids are large, it is presumed that other genes (i.e., upstream or downstream of the glycoside hydrolase-encoding genes) are more relevant in mediating this
effect. Additionally, we cannot exclude that the hylEfm cluster studied in this work may play a role in other infections such as endocarditis or urinary tract infections (a subject of our ongoing studies). As a final remark, the adaptation of the pheS* counter-selection system for targeted mutagenesis in plasmid and chromosomal genes of E. faecium will facilitate the understanding of the role of other specific plasmid genes in the pathogenesis of E. faecium infections in the near future.

Conclusions
We provided evidence that four genes of the hylEfm-region (including hylEfm) do not mediate the virulence effect of the E. faecium plasmid pHylEfm in experimental peritonitis. The adaptation of the PheS* counter-selection system for targeted mutagenesis in plasmid and chromosomal genes of E. faecium will facilitate the understanding of the role of other specific plasmid genes in the pathogenesis of E. faecium infections in the near future.

Additional material

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Authors’ contributions
DP carried out molecular genetics studies, animal experiments and participated in editing the manuscript. MCM, SR and MFM performed...
molecular genetics experiments. KVS carried out part of the animal work. BEM and LBR participated in editing the manuscript and data analysis. CAA is the principal investigator, conceived the study, designed the experiments, performed data analysis and wrote the manuscript. All authors read and approved the final version of the manuscript.

Competing interests
The authors declare that they have no competing interests.

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