

RESEARCH ARTICLE

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The *hyl_{Efm}* gene in pHyl_{Efm} of *Enterococcus faecium* is not required in pathogenesis of murine peritonitis

Diana Panesso^{1,2,5}, Maria C Montealegre^{1,2,8}, Sandra Rincón^{1,2,5}, Maria F Mojica^{1,2,8}, Louis B Rice^{6,7}, Kavindra V Singh^{1,3}, Barbara E Murray^{1,3,4}, Cesar A Arias^{1,2,5*}

Abstract

Background: Plasmids containing *hyl_{Efm}* (pHyl_{Efm}) were previously shown to increase gastrointestinal colonization and lethality of *Enterococcus faecium* in experimental peritonitis. The *hyl_{Efm}* 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 *hyl_{Efm}*-region and we evaluated their effect on virulence using a murine peritonitis model.

Results: Five mutants of the *hyl_{Efm}*-region of pHyl_{EfmTX16} 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 pHyl_{EfmTX16} was transferred by mating; these include *i*) deletion of *hyl_{Efm}* only; *ii*) deletion of the gene downstream of *hyl_{Efm}* (*down*) of unknown function; *iii*) deletion of *hyl_{Efm}* plus *down*; *iv*) deletion of *hyl_{Efm}*-*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 pHyl_{EfmTX16Δ7,534} was transferred to the TX1330RF background, the transconjugant was affected in *in vitro* growth versus TX1330RF (pHyl_{EfmTX16}) and was attenuated in virulence; however, neither *hyl_{Efm}* nor *hyl_{Efm}*-*down* restored wild type function. We did not observe any *in vivo* effect on virulence of the other deletions of the *hyl_{Efm}*-region

Conclusions: The four genes of the *hyl_{Efm}* region (including *hyl_{Efm}*) do not mediate the increased virulence conferred by pHyl_{EfmTX16} 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*) *esp_{Efm}*, 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 *ebpABC_{fms}* 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 *hyl_{Efm}* gene [11-14].

It has been previously shown that *hyl_{Efm}* 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 *hyl_{Efm}* in the same plasmid [15,16]. The

* Correspondence: cesar.arias@uth.tmc.edu

¹Department of Internal Medicine, Division of Infectious Diseases, Center for the Study of Emerging and Reemerging Pathogens, Houston, TX, USA
Full list of author information is available at the end of the article

acquisition of the *hyl_{Efm}*-plasmid by an *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 *hyl_{Efm}*-plasmid from another US clinical strain (TX16) increased the virulence of a commensal strain *E. faecium* TX1330RF in experimental peritonitis [11].

The 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 *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 [12,13]. In fact, the homolog of *hyl_{Efm}* in *Streptococcus pyogenes* (*spy1600*) encoded in a genetic locus with a similar organization to that of the *hyl_{Efm}*-region and sharing 42% identity at the amino acid level

(61% similarity), was recently shown not to have any detectable hyaluronidase activity. Spy1600 was characterized as a family 84 glycosyl hydrolase with β -N-acetyl-glucosaminidase specificity after purification and substrate analysis [20] and expression of *spy1600* in *S. pyogenes* was found to be up-regulated during phagocytosis [21]. For this reason, and because of the almost exclusive occurrence of *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 *E. faecium*. However, its exact role in virulence has not been established. In this work, we assess the role of the *hyl_{Efm}*-region in *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 *hyl_{Efm}*-region in *E. faecium* strains and mutants.

Table 1 *E. faecium* strains and plasmids used in this work

Strains/Plasmids	Relevant Characteristics	Reference
Strains		
<i>E. faecium</i>		
TX16 (DO)	Sequenced endocarditis clinical isolate, Em ^r , Sm ^r . ST-16 ^a http://www.hgsc.bcm.tmc.edu	[35]
TX1330RF	Fs ^r and Rf ^r derivative of TX1330, a faecal colonizing strain from a healthy human volunteer	[11]
TX1330RF (pHyl _{EfmTX16})	Derivative of TX1330RF to which the <i>hyl_{Efm}</i> -containing plasmid (pHyl _{EfmTX16}) was transferred by conjugation from TX16 (DO) (~250 kb)	[11]
TX1330RF (pHyl _{EfmTX16Δ7,534})	Mutant with deletion of part or all of 6 genes of the <i>hyl_{Efm}</i> region of TX1330RF(pHyl _{EfmTX16})	This work
TX1330RF (pHyl _{EfmTX16Δ4genes})	Non-polar deletion of 4 genes of the <i>hyl_{Efm}</i> region of TX1330RF(pHyl _{EfmTX16})	This work
TX1330RF (pHyl _{EfmTX16Δhyl})	Non-polar deletion mutant of <i>hyl_{Efm}</i> of TX1330RF(pHyl _{EfmTX16})	This work
TX1330RF (pHyl _{EfmTX16Δhyl-down})	Non-polar deletion of <i>hyl_{Efm}</i> plus its downstream gene of TX1330RF(pHyl _{EfmTX16})	This work
TX1330RF (pHyl _{EfmTX16Δdown})	Non-polar deletion of the gene downstream of <i>hyl_{Efm}</i> of TX1330RF(pHyl _{EfmTX16})	This work
<i>E. faecalis</i>		
CK111	OG1Sp <i>upp4::P₂₃ repA4</i>	[25]
Plasmids		
pHyl _{EfmTX16}	Conjugative and transferable megaplasmid (ca. 250 kb) of TX16 (DO) containing <i>hyl_{Efm}</i>	[11]
pCJ47	Conjugative donor plasmid for markerless mutagenesis; <i>oriT_{pCF10}</i> and <i>pheS*</i> pORI280 derivative; confers Em ^r	[25]
pHOU1	Derivative of pCJ47 in which the <i>erm(C)</i> gene was replaced by <i>aph-2'-ID</i> ; confers Gm ^r	This work
pHOU2	Derivative of pCJ47 in which the <i>erm(C)</i> gene was replaced by <i>aph-2'-ID</i> and <i>cat</i> was incorporated in the cloning site for allelic replacements; confers Gm ^r .	This work
pTEX5501ts	<i>E. coli</i> -enterococcal shuttle plasmid for mutagenesis using a temperature-sensitive replicon	[27]
pAT392	<i>oriR_{pAMB1}</i> , <i>oriR_{pUC}</i> <i>oriT_{RK2}</i> <i>spc lacZα</i> P2 <i>aac(6)-aph(2'')</i>	[30]
pAT392:: <i>hyl_{Efm}</i>	Derivative of pAT392 containing <i>hyl_{Efm}</i> (cloned with SacI and SmaI) under the control of the P2 promoter	This work
pAT392:: <i>hyl_{Efm}down</i>	Derivative of pAT392 containing both the <i>hyl_{Efm}</i> plus downstream genes (cloned with SacI and SmaI) under the control of the P2 promoter	This work

Em^r, erythromycin resistance; Fs^r, fusidic acid resistance; Gm^r, gentamicin resistance, Rf^r, rifampin resistance; Sm^r, high-level resistance to streptomycin. ^aST refers to sequence type after multi-locus sequence typing. ST16 is part of CC17.

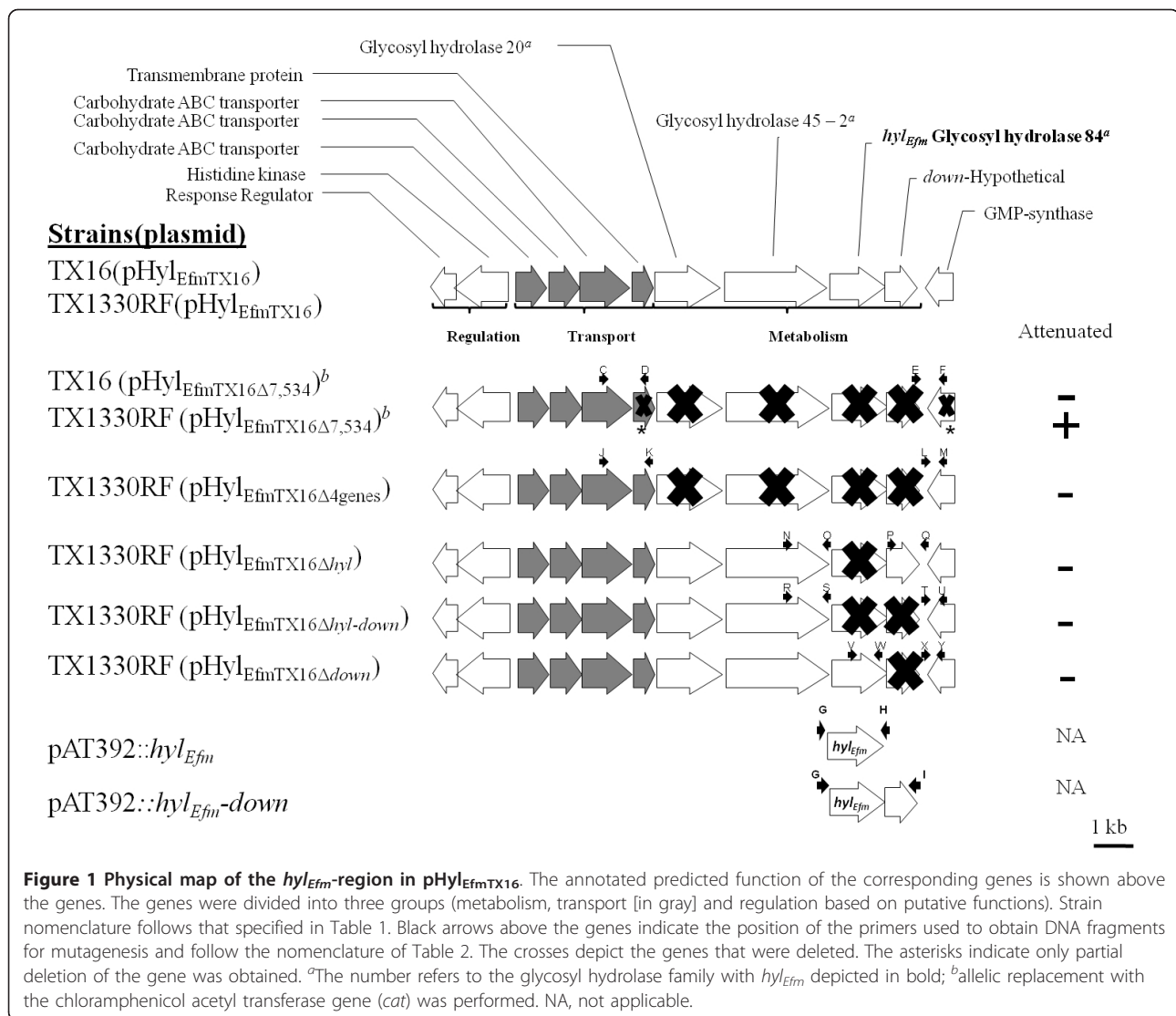


Figure 1 Physical map of the *hyl*_{Efm}-region in pHyl_{Efm}TX16. The annotated predicted function of the corresponding genes is shown above the genes. The genes were divided into three groups (metabolism, transport [in gray] and regulation based on putative functions). Strain nomenclature follows that specified in Table 1. Black arrows above the genes indicate the position of the primers used to obtain DNA fragments for mutagenesis and follow the nomenclature of Table 2. The crosses depict the genes that were deleted. The asterisks indicate only partial deletion of the gene was obtained. ^aThe number refers to the glycosyl hydrolase family with *hyl*_{Efm} depicted in bold; ^ballelic replacement with the chloramphenicol acetyl transferase gene (*cat*) was performed. NA, not applicable.

Construction of a deletion mutant of the *hyl*_{Efm}-region using the *pheS*^{*} counter-selection system in TX16 (pHyl_{Efm}TX16) 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 *aph*²-ID, which confers resistance to gentamicin. The full *aph*²-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).

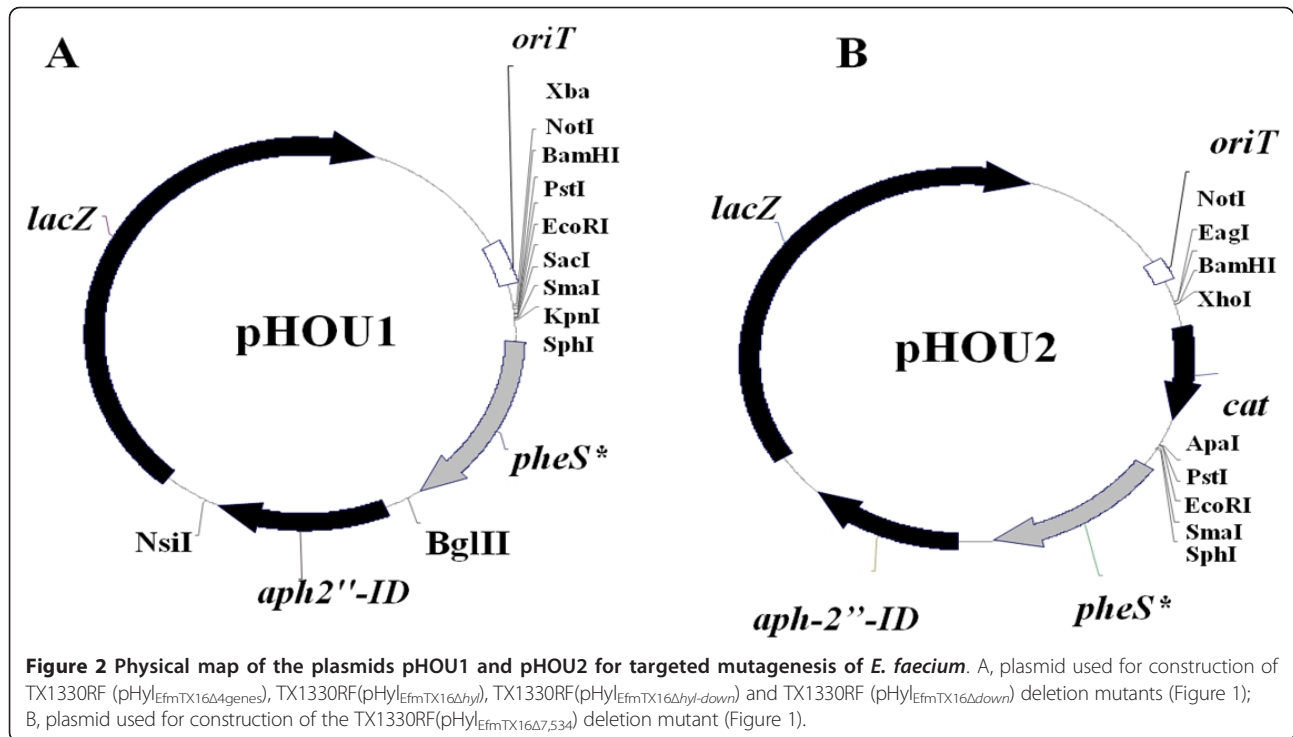
In order to create a deletion mutant of the *hyl*_{Efm}-region (which contains genes predicted to be involved in carbohydrate metabolism and transport; Figure 1), fragments upstream (977 bp) and downstream (999 bp) of this region were amplified by PCR (with primers C-D and E-F, respectively; Table 2) and cloned upstream and downstream of the *cat* gene in pHOU2, respectively, using BamHI and XhoI for the upstream fragment and Apal 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	gaag <u>atctg</u> agataggttatgcaagat	Forward, BglII site (underlined), used amplification of <i>aph-2''-ID</i>
B	ccaatgcatgattccggtacttaaaaaagg	Reverse, NsiI site (underlined), used amplification of <i>aph-2''-ID</i>
C	cggatccggttaaaaccagctggaaaag	Forward, BamHI site (underlined), located 1,251 nucleotides upstream of the start codon of the gene encoding a putative glycosyl hydrolase family 20 (Figure 1.)
D	ccgctcagagcaattcaacattgcaaaagac	Reverse, XhoI site (underlined), located 294 nucleotides upstream of the start codon of the gene encoding a putative glycosyl hydrolase family 20 (Figure 1.)
E	cgagggcccgtgaagtattgccagatgt	Forward, Apal site (underlined); located 592 nucleotides downstream of the <i>down</i> gene (hypothetical, Figure 1.)
F	ccggaattcaaaagcagaattggaatca	Reverse, EcoRI site, 1,571 nucleotides downstream of the <i>down</i> gene (hypothetical, Figure 1.)
G	gcgagctcgattacttcaaggaga	Forward, SacI site (underlined), ribosomal binding site of <i>hyl_{Efm}</i> (italics) (Figure 1.)
H	tccccgggctaacttttgataattgtct	Reverse, SmaI site, (underlined) and stop codon of <i>hyl_{Efm}</i> (Figure 1.)
I	tccccggggttagcattgatcgagc	Reverse, SmaI site (underlined), stop codon of <i>down</i> (Figure 1.)
J	cggatcccaatcaagaagtagcggatt	Forward, BamH site (underlined) 438 nucleotides upstream of the stop codon of carbohydrate ABC transporter gene (Figure 1.)
K	gcgccgctcgaggcccttagtgcgattgtatctgac	Reverse, stop codon of the gene that encodes to transmembrane protein (Figure 1.)
L	gggccctcagggcggccgcaaaataaaaaatgg	Forward, Apal, XhoI, NotI site, stop codon <i>down</i> (Figure 1.)
M	catgcatgaatcaggaactgaaactgc	Reverse, NsiI site, 1,091 nucleotides upstream of stop codon of GMP synthase (opposite orientation) (Figure 1.)
N	ccggaattccagtaaaaggcacagagc	Forward, EcoRI site (underlined), located 2,138 nucleotides down-stream of glycosyl hydrolase family 45-2 start codon (Figure 1.)
O	tcatctatcttctcttgaagaatcactatattcc	Reverse, stop codon of glycosyl hydrolase family 45-2 (Figure 1.)
P	tcaaaggagaaaatagatgaatattcttaaaaaataaaaagc	Forward, located 40 nucleotides upstream of <i>down</i> gene start codon (Figure 1.)
Q	ataagaatcgccgcttagcattgatcgagc	Reverse, NotI site (underlined), stop codon of <i>down</i> (Figure 1.)
R	ataagaatcgccgcttagcattgaaaggcacagagc	Forward, NotI site (underlined), located 2,138 nucleotides down-stream of glycosyl hydrolase family 45-2 start codon (Figure 1.)
S	tcatctatcttctcttgaagaatcactatattcc	Reverse, stop codon of glycosyl hydrolase family 45-2 (Figure 1.)
T	tcaaaggagaaaatagatgacaaaataaaaaatgg	Forward, 1,973 nucleotides upstream of stop codon of GMP synthase (Figure 1.)
U	cggaaattcgaattgtatgtctctcg	Reverse, EcoRI site (underlined), 994 nucleotides upstream of start codon of GMP synthase (opposite direction) (Figure 1.)
V	aaggaaaaaacgcccgcagaaatgataatcgatcg	Forward, NotI site (underlined), 902 nucleotides downstream of <i>hyl_{Efm}</i> start codon (Figure 1.)
W	ttgttctcttcttctgctttttatttttaag	Reverse, stop codon of <i>hyl_{Efm}</i> (Figure 1.)
X	gcaagaaaaaggagaacaaataaaaaataaaaatgg	Forward, 1,973 nucleotides upstream of stop codon of GMP synthase (opposite direction) (Figure 1.)
Y	ccggaattcgaatcaggaactgaaactgccc	Reverse, EcoRI site (underlined), 1,094 nucleotides upstream of stop codon of GMP synthase (opposite direction) (Figure 1.)
A1	cgctgctattaaaaatcat	Forward, 143 nucleotides upstream of stop codon of GH20 (Figure 3.)
A2	gatcgataaaactgctgctg	Reverse, 139 nucleotides upstream of start codon of GH42 (Figure 3.)
B1	acgctgcagacagcagctgatgctga	Forward, Sall site (underlined), 2,316 nucleotides downstream of start codon of GH42 (Figure 3.)
B2	ggaagatctccggttccagacttct	Reverse, BglII site (underlined), 159 nucleotides downstream of start codon of <i>hyl_{Efm}</i> (Figure 3.)
C1	gtagaagaagtctggaaccg	Forward, 138 nucleotides downstream of start codon of <i>hyl_{Efm}</i> (Figure 3.)
C2	tgtaagatattcttactctg	Reverse, 798 nucleotides upstream of stop codon of <i>hyl_{Efm}</i> (Figure 3.)
D1	acatgcatgcagaattggagccttggt	Forward, SphI site (underlined), 169 nucleotides upstream of stop codon of <i>hyl_{Efm}</i> (Figure 3.)
D2	cggaaattcgtctccgataaga	Reverse, EcoRI site (underlined), 319 nucleotides upstream of stop codon of <i>down</i> gene (Figure 3.)
E1	gcaaggcttcttagaga	Forward, <i>ddl</i> <i>E. faecium</i> [32,33]
E2	catcgtgtaagtaacttc	Reverse, <i>ddl</i> <i>E. faecium</i> [32,33]

directions. This recombinant plasmid was introduced into *E. faecalis* CK111 by electroporation as described previously [25,28] and blue colonies were recovered on brain heart infusion (BHI) agar plates containing gentamicin (125 µg/ml) and X-Gal (200 µg/ml). Subsequently, the pHO2 derivatives were introduced into strain TX16 by filter mating [29] with *E. faecalis* CK111 as the

donor. Single cross-over integrants were selected on gentamicin (170 µg/ml) and erythromycin (200 mg/ml) and purified colonies were then resuspended in 50 µl of normal saline and plated on MM9YEG media (salts and yeast extract) supplemented with 7 mM of *p*-Cl-Phe [25] and incubated for 48 h at 37°C. To confirm that colonies which grew on MM9YEG media supplemented



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 *hyl_{Efm}* 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 *hyl_{Efm}*-containing plasmid (pHyl_{Efm}TX16Δ7,534) 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(pHyl_{Efm}TX16Δ7,534). 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 *hyl_{Efm}*-region mutant TX1330RF (pHyl_{Efm}TX16Δ7,534)

The *hyl_{Efm}* gene was PCR amplified with primers G and H (including the ribosomal binding site and the stop codon of *hyl_{Efm}*) (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::*hyl_{Efm}*). In order to evaluate if the deletion of *hyl_{Efm}* had an effect in the downstream gene (encoding a hypothetical protein of 331 amino acids of unknown function), the *hyl_{Efm}* and *down* genes (Figure 1) were also cloned together into pAT392 following a similar strategy and using primers G and I (pAT392::*hyl_{Efm}*-*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* TX1330RE, and the TX1330RF(pHyl_{Efm}TX16Δ7,534) 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 *hyl_{Efm}*-region in *E. faecium* TX1330RF(pHyl_{Efm}TX16)

To investigate the specific role of the *hyl_{Efm}* locus in *E. faecium* pathogenesis, complete in-frame deletions of four genes of the *hyl_{Efm}*-region, *hyl_{Efm}* alone, *hyl_{Efm}* plus its downstream gene and the gene downstream of *hyl_{Efm}* were generated using TX1330RF(pHyl_{Efm}TX16). Fragments upstream and downstream of each region were amplified by PCR with the corresponding primers

(Figure 1 and Table 2). These fragments, with overlapping ends, were subsequently amplified by crossover PCR and cloned into pHO1 using EcoRI and NotI (for *hyl_{Efm}*, *hyl_{Efm}* plus its downstream gene and the downstream gene of *hyl_{Efm}* mutants); and BamHI and PstI (for the four gene mutant). The inserts were sequenced in both directions to confirm that no mutations had been introduced during the cloning process. The recombinant plasmids were electroporated or transferred by conjugation (using *E. faecalis* CK111) into TX1330RF(pHyl_{EfmTX16}). Single crossover events and deletions of targeted regions (Figure 1) were 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(pHyl_{EfmTX16}), TX1330RF and strains containing pAT392 derivatives) grown in BHI broth at 37°C with mild agitation (logarithmic phase of growth, *A*₆₀₀ 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 *hyl_{Efm}* (Table 2). A primer pair directed to detect a 550-bp transcript of the house-keeping gene *ddl_{E. faecium}* was used as an internal control for RT-PCR experiments [32,33].

Mouse peritonitis model

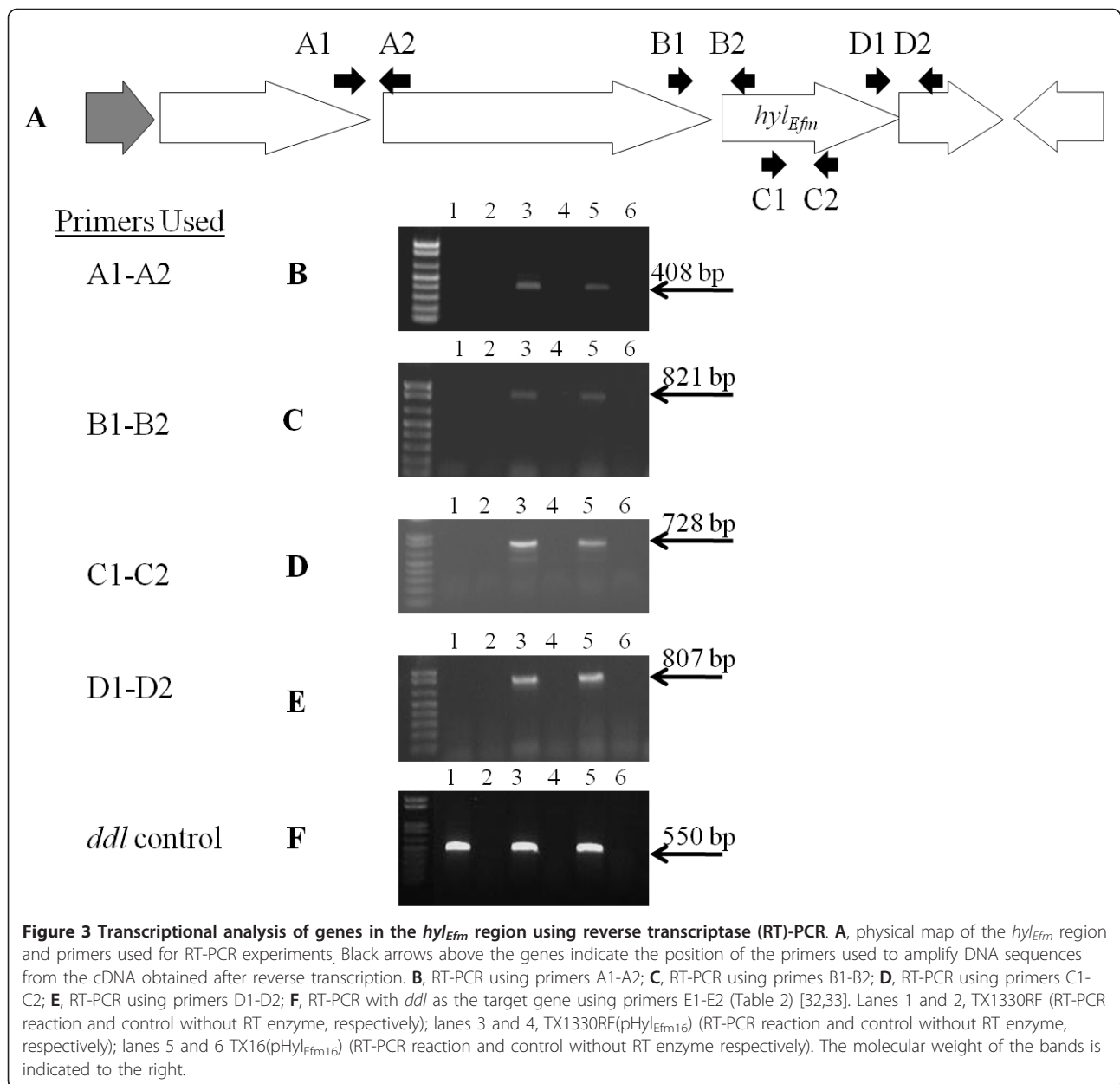
Female (4 to 6 week old), outbred ICR mice (Harlan Sprague Dawley, Houston) were used as previously described [34]. Groups of 10 mice per inoculum (ranging from 2.3×10^8 to 3.1×10^9 CFU/ml) were included in each experiment. Inocula for each peritonitis experiment were prepared by growing bacteria initially 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 *A*₆₀₀ 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 hyl_{Efm}*-region altered *in vitro* growth and attenuated virulence of TX1330RF (pHyl_{EfmTX16}) but not TX16(pHyl_{EfmTX16}) in murine peritonitis

Since acquisition of the transferable pHyl_{EfmTX16} by TX1330RF conferred increased virulence in experimental peritonitis [11], we explored the possibility that the *hyl_{Efm}* region was an important mediator of this effect. Using RT-PCR assays, we were able to detect *in vitro* expression of *hyl_{Efm}* during the exponential phase of growth in both TX16 and TX1330RF (pHyl_{EfmTX16}) (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 *hyl_{Efm}*-region. The *hyl_{Efm}* 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 pHyl_{EfmTX16} was obtained. The deletion involved three genes predicted to encode glycosyl hydrolases (including *hyl_{Efm}*) and a gene downstream of *hyl_{Efm}* 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 *hyl_{Efm}* encoding a putative GMP-synthase and likely transcribed in the opposite direction from the *hyl_{Efm}* cluster (Figure 1) were also deleted. As it is shown in Figure 4A, the deletion of 7,534 bp in the *hyl_{Efm}*-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(pHyl_{EfmTX16Δ7,534}) 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

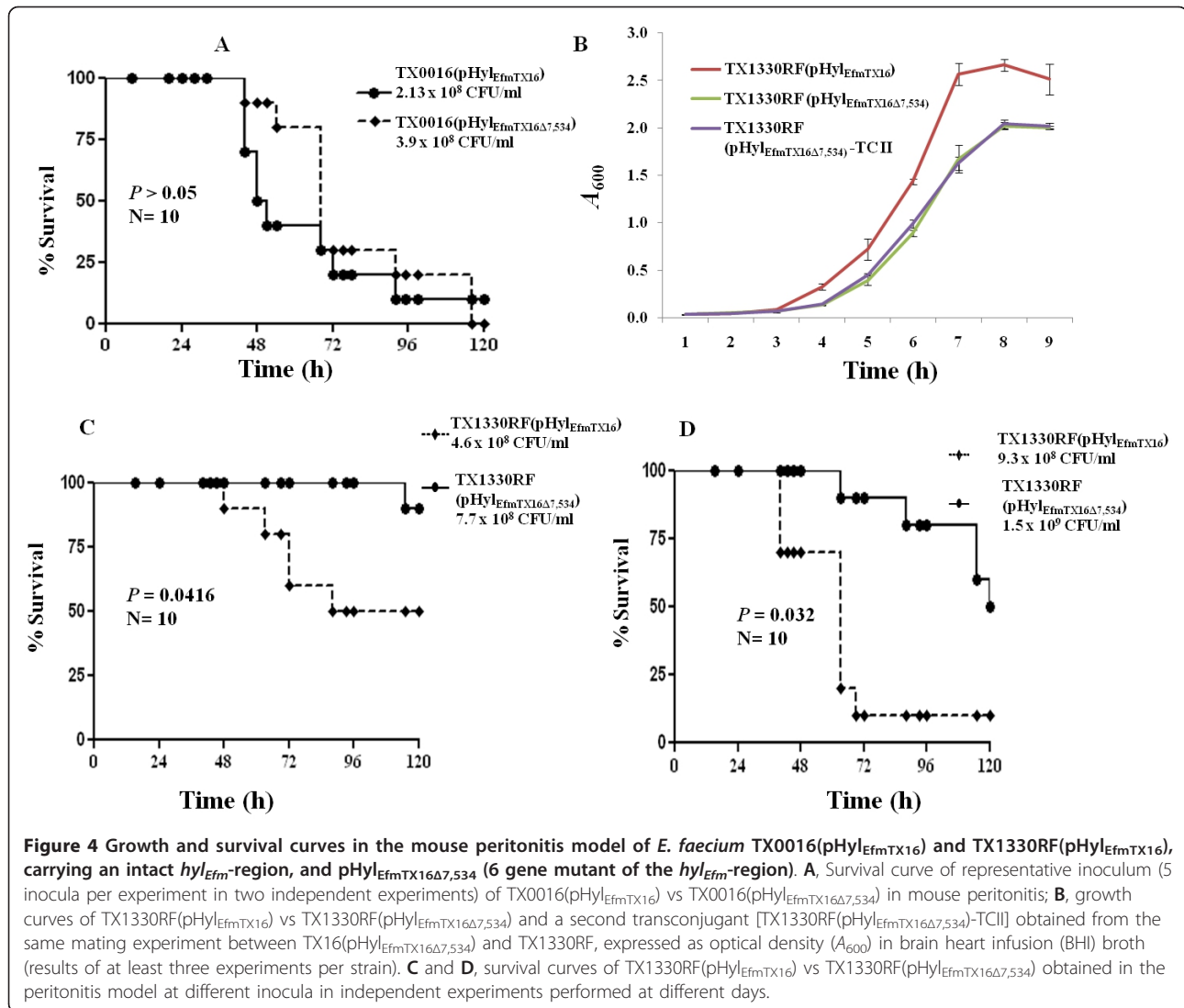


(pHyl_{EfmTX16}) and TX1330RF(pHyl_{EfmTX16Δ7,534}) appeared to differ only in the size of the *hyl_{Efm}* plasmid by PFGE and S1 nuclease assays [11] (not shown). Figure 4B shows that deletion of 7,534 bp in the *hyl_{Efm}* region of TX1330RF(pHyl_{EfmTX16}) caused an *in vitro* growth defect. The alteration of growth was also seen in a second transconjugant from the same mating experiment between TX16(pHyl_{EfmTX16Δ7,534}) and TX1330RF (TC-II in Figure 4B). The mutant strain TX1330RF(pHyl_{EfmTX16Δ7,534}) 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(pHyl_{EfmTX16Δ7,534}), 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 *hyl_{Efm}*-region mutant with *hyl_{Efm}* and a combination of *hyl_{Efm}* and the downstream gene did not restore the virulence of TX1330RF (pHyl_{EfmTX16Δ7,534})

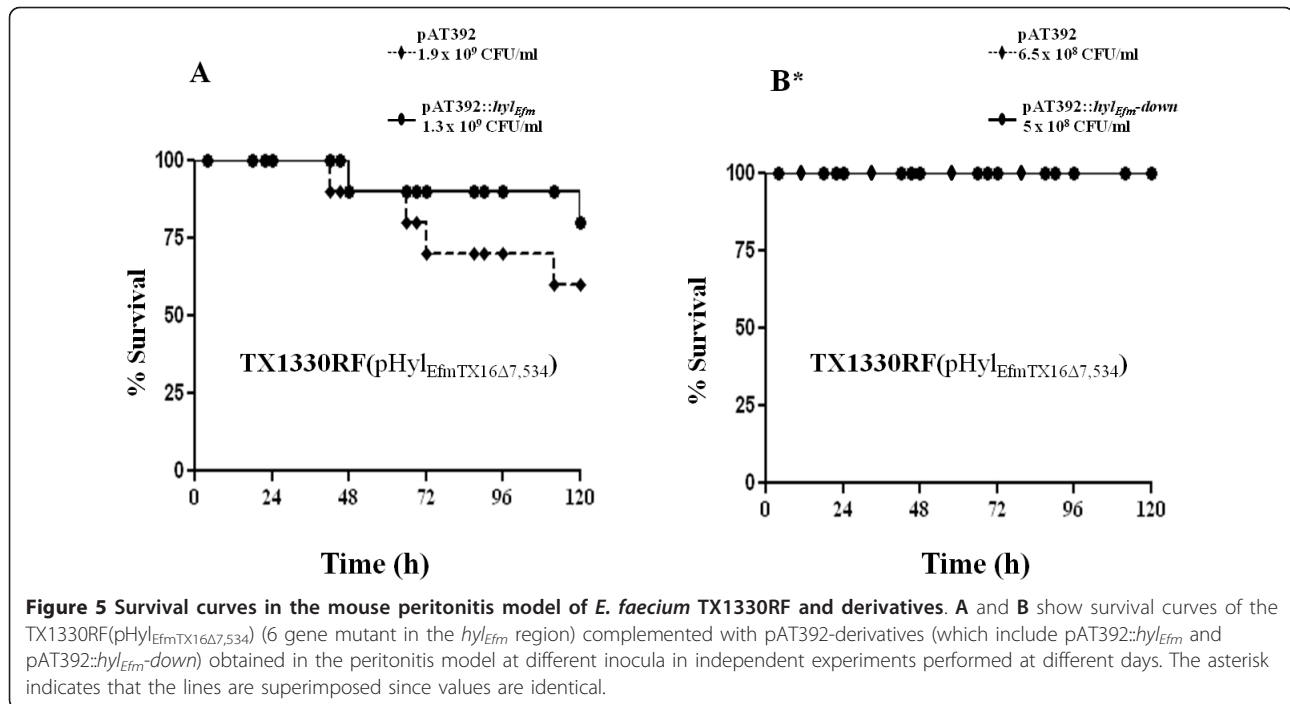
In order to further evaluate if the attenuation observed in TX1330RF(pHyl_{EfmTX16Δ7,534}) (as described above)



was mediated by a direct effect of *hyl*_{Efm} in the peritonitis model, we explored complementation of this mutant *in trans* with the full *hyl*_{Efm} gene and a combination of *hyl*_{Efm} 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(pHyl_{Efm}TX16Δ7,534)-derivatives containing the pAT392 constructs were stable both *in vitro* and *in vivo* (5% maximum percentage of plasmid loss). Introduction of *hyl*_{Efm} or a combination of *hyl*_{Efm} plus its downstream gene (cloned into pAT392) did not restore the virulence of the mutant strain TX1330RF(pHyl_{Efm}TX16Δ7,534), compared to pAT392 alone in the presence of gentamicin

(Figure 5A and 5B). The results indicate that constitutive expression of *hyl*_{Efm} 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(pHyl_{Efm}TX16Δ7,534), supporting the fact that *hyl*_{Efm} 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 pHyl_{Efm}TX16Δ7,534 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 *hyl*_{Efm}-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(pHyl_{Efm}TX16Δ7,534) 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(pHyl_{Efm}TX16Δ7,534) 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 (pHyl_{Efm}TX16Δ7,534) mutant(s) and its virulence.

Additional deletions of genes in the *hyl_{Efm}*-region did not alter the virulence of TX1330RF(pHyl_{Efm}TX16) in the mouse peritonitis model

In order to dissect further the *in vivo* role of *hyl_{Efm}* and the adjacent genes, we produced several in-frame deletions of these genes (Figure 1) including: *i*) a four gene mutant of the *hyl_{Efm}*-region (including *hyl_{Efm}*) [TX1330RF(pHyl_{Efm}TX16Δ4genes)], *ii*) a deletion of *hyl_{Efm}* alone [TX1330RF (pHyl_{Efm}TX16Δ*hyl*)], *iii*) a deletion of *hyl_{Efm}* plus its downstream gene mutant [TX1330RF (pHyl_{Efm}TX16Δ*hyl-down*)] and, *iv*) a single deletion of the gene located downstream from *hyl_{Efm}* [TX1330RF

(pHyl_{Efm}TX16Δ*down*)]. 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 *hyl_{Efm}*-region showed a deleterious effect in the growth kinetics compared to TX1330RF (pHyl_{Efm}TX16) (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 *hyl_{Efm}* region do not appear to be directly involved in increasing the pathogenic potential of pHyl_{Efm}TX16 in strain TX1330RF (pHyl_{Efm}TX16).

Megaplasmids (>145 kb, with or without *hyl_{Efm}*) have been recently found to be widespread among clinical isolates of *E. faecium* worldwide [12,13,15]. The proportion of these plasmids carrying *hyl_{Efm}* appears to vary according to geographical location (ca. 11 to 36%) [12,13]. Our findings indicate that the four genes of the *hyl_{Efm}*-cluster studied here, including *hyl_{Efm}* are not the main mediators of the virulence effect conferred by the plasmid carrying them in experimental peritonitis. Since the pHyl_{Efm} 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

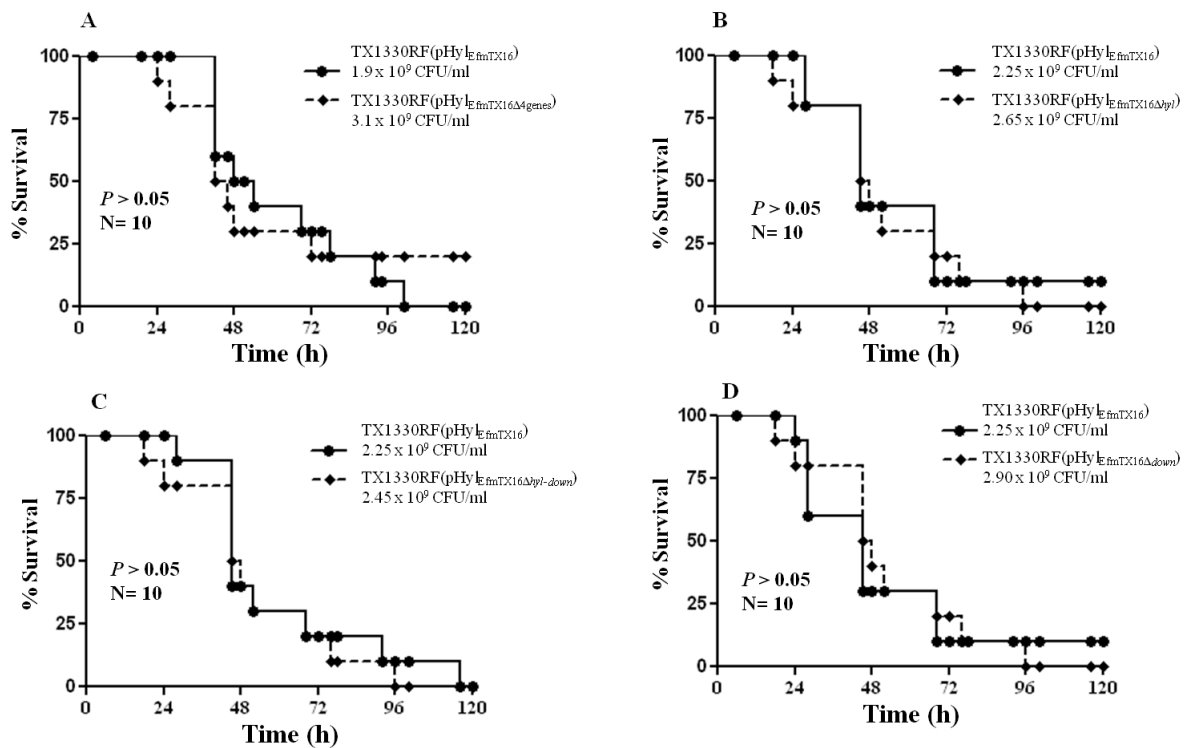


Figure 6 Survival curves in the mouse peritonitis model of *E. faecium* TX1330RF(pHyl_{Efm}TX16) and deletion mutants (Figure 1 and Table 1) showing representative inocula (5 inocula per each experiment). **A**, TX1330RF(pHyl_{Efm}TX16) vs TX1330RF(pHyl_{Efm}TX16Δ4genes); **B**, TX1330RF(pHyl_{Efm}TX16) vs TX1330RF(pHyl_{Efm}TX16Δhyl); **C**, TX1330RF(pHyl_{Efm}TX16) vs TX1330RF(pHyl_{Efm}TX16Δhyl-down); **D**, TX1330RF(pHyl_{Efm}TX16) vs TX1330RF(pHyl_{Efm}TX16Δdown).

effect. Additionally, we cannot exclude that the *hyl*_{Efm} 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 *hyl*_{Efm} region (including *hyl*_{Efm}) do not mediate the virulence effect of the *E. faecium* plasmid pHyl_{Efm} in experimental peritonitis. The adaptation of the *PheS** counter-selection system for targeted mutagenesis of *E. faecium* should facilitate the study of the role of other pHyl_{Efm} genes in the pathogenesis of murine peritonitis.

Additional material

Additional file 1: Growth curves of *E. faecium* and mutants. The strains were incubated in BHI broth and the *A*₆₀₀ were measured every hour.

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Author details

¹Department of Internal Medicine, Division of Infectious Diseases, Center for the Study of Emerging and Reemerging Pathogens, Houston, TX, USA. ²Laboratory for Antimicrobial Research, University of Texas Medical School at Houston, Houston, TX, USA. ³Laboratory of Enterococcal Research, University of Texas Medical School at Houston, Houston, TX, USA. ⁴Department of Microbiology and Molecular Genetics, University of Texas Medical School at Houston, Houston, TX, USA. ⁵Molecular Genetics and Antimicrobial Resistance Unit, Universidad El Bosque, Bogotá, Colombia. ⁶Medical and Research Services, Louis Stokes Cleveland Department of Veterans Medical Center, Cleveland, OH, USA. ⁷Department of Medicine, Case Western Reserve University, School of Medicine, Cleveland, OH, USA. ⁸Centro Internacional de Entrenamiento e Investigaciones Médicas (CIDEIM), Cali, Colombia.

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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