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The Recombinase IntA Is Required for Excision of *esp*-Containing ICE*Efm1* in *Enterococcus faecium*▽

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Comparative genome analysis of *Enterococcus faecium* recently revealed that a genomic island containing the *esp* gene, referred to as the *esp*-containing pathogenicity island (*esp* PAI), can be transferred by conjugation and contains a partial Tn*916*-like element and an integrase gene, *intA*. Here, we characterize the role of *intA* in the excision of the *esp* PAI. An *intA* insertion-deletion mutant in *E. faecium* E1162 (E1162Δ*intA*) was constructed and in *trans* complemented with wild-type *intA* (E1162Δ*intA*::pEF30). Circular intermediates (CI) of excised *esp* PAI were determined using inverse PCR analysis on purified chromosomal DNA from strains E1162, E1162Δ*esp*, E1162Δ*intA*, and E1162Δ*intA*::pEF30. In E1162 and E1162Δ*esp*, CI of the *esp* PAI were detected. No CI were detected in E1162Δ*intA*, while in the complemented strain E1162Δ*intA*::pEF30 CI formation was restored, indicating that *intA* is essential for excision and subsequent mobilization of the *esp*-containing genomic island in *E. faecium*. Based on the fact that this island can be mobilized and is self-transmissible, we propose to change the name of the *esp* PAI to ICE*Efm1*.

For a long time, the Gram-positive species *Enterococcus faecium* was considered a harmless commensal of the mammalian gastrointestinal (GI) tract. However, in the last 2 decades *E. faecium* has emerged as one of the leading nosocomial pathogens (16, 18, 20). Molecular epidemiological studies using multilocus sequence typing (MLST) identified host-specific genogroups, including a hospital-associated *E. faecium* subpopulation (18–20). Recently, whole-genome sequencing revealed the total sequence of a previously partially sequenced genomic island containing the enterococcal surface protein gene, *esp*, designated the *esp* pathogenicity island (*esp* PAI) in strain E1162 (GenBank accession number ABQJ00000000) (6, 17). In this strain, the *esp* PAI (contig 156; GenBank accession number ABQJ01000139) (Fig. 1A) is flanked by direct repeats (DR) of 54 bp, is integrated at the 3′ end of the *rpsI* gene, and contains a partial, conjugation module-containing, Tn*916*-like element. Conjugation experiments using E1162Δ*esp* as a donor revealed that the E1162 *esp* PAI is self-transmissible and integrates in a site-specific manner (17). This indicates that the *esp* PAI in E1162 is an integrative conjugative element (ICE) rather than a genome or pathogenicity island, which is a chromosomal region acquired through horizontal gene transfer that is no longer or never was self-transmissible (2).

The term ICE has been introduced for all elements that excise by site-specific recombination into a circular form, self-transfer by conjugation, and subsequently integrate into the host genome, very often at the 3′ end of tRNAs, whatever the specificity and mechanism of integration and conjugation (1, 2). ICEs are characterized by the presence of an integrase/excisionase gene belonging to the tyrosine or serine family of recombinases, a conjugation module, and a modular structure (9). The *intA* gene (locus tag EfmE1162_2326) is the only integrase encoded on the *esp* PAI. The IntA protein is homologous to a family of phage and phage-related integrases. BLAST searches with the amino acid sequence of the IntA protein revealed that this protein belongs to the tyrosine family of recombinases that is commonly located on the ICE, like Tn*916* (3, 4, 10, 13, 14). Remarkably, the closest homolog of IntA was found with an integrase of *Enterococcus faecalis* E1Sol (83% amino acid identity). This integrase was also found directly downstream of the *rpsI* gene of *E. faecalis* and is probably also part of a mobile element because of the presence of a partial Tn*916* element, which includes the tetracycline resistance gene *tetR*, downstream of the integrase. We therefore hypothesized that *intA* is involved in the excision and formation of circular intermediates (CI) of the *esp* PAI.

An *intA* insertion-deletion mutant was constructed in a clinical *E. faecium* strain (E1162), using the temperature-sensitive vector (pTEX5500ts) as previously described (5, 8). The *intA* mutant was named E1162Δ*intA*. The other bacterial strains and plasmids used in this study are listed in Table 1. Culture conditions and genetics methods were described previously (5).

IntA is involved in recipient-independent formation of circular intermediates. To determine whether conjugative transfer of the *esp* PAI occurs via CI, PCRs were performed on purified DNA from the conjugation mixture of E1162Δ*esp* and BM4105RF using outward-facing primers designed on the borders of the PAI (Fig. 1A and B). This PCR yielded a single amplification product with the expected size of 2.6 kb that can occur only when CI forms are present (Fig. 1C). Subsequent sequencing confirmed the specificity of the PCR product. To investigate whether CI formation is dependent on the presence of a recipient strain and on the presence of a functional *intA* gene, in total three different donor strains, comprising E1162,

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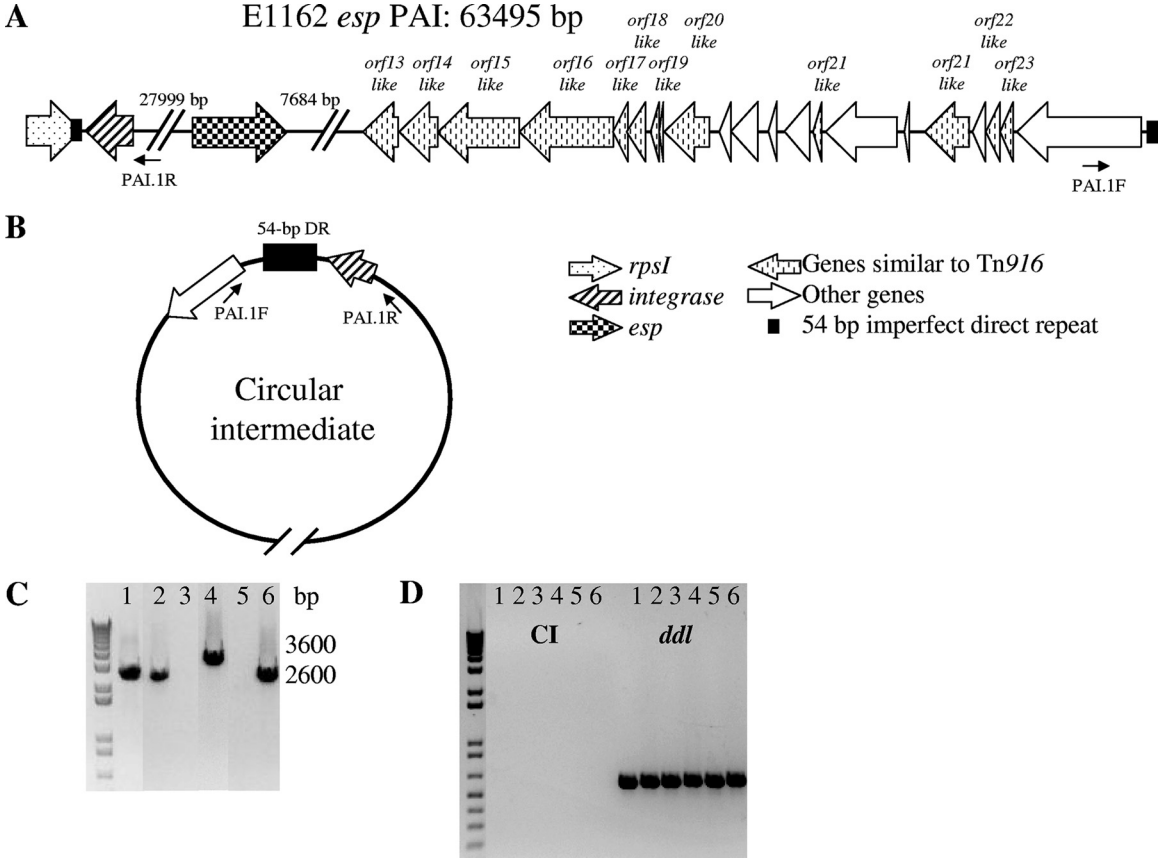

FIG. 1. (A) Structure of the *E. faecium* E1162 *esp* PAI. Arrows indicate coding sequences (CDS) and direction of transcription; only CDS that are relevant for this study are depicted. Indicated are binding sites for the outward-facing primers (PAI.1R and PAI.1F) used to detect circular intermediates. (B) A schematic representation of circular intermediate (CI) formation. Primers (PAI.1R and PAI.1F) for the detection of circular intermediates are indicated. (C) Detection of circular intermediates using inversed PCR analysis on purified chromosomal DNA with outward-facing primers at the borders of the *esp* PAI. (D) Detection of circular intermediates using inversed PCR analysis on purified chromosomal DNA after S1 nuclease treatment; *ddl* PCR analysis was performed as a positive control. Lanes: 1, conjugation mixture of *E. faecium* strain E1162Δ*esp* with BM4105RF; 2, E1162; 3, E1162Δ*intA*; 4, E1162Δ*intA*::pEF30; 5, E1162Δ*intA*::pEF25; and 6, the transconjugant (TC).

the *intA* insertion-deletion mutant E1162Δ*intA* (Table 1), and the previously described transconjugant (TC) obtained from the mating of E1162Δ*esp* with BM4105RF (17), were grown on filters in a manner similar to filter-mating experiments but in the absence of a recipient strain. CI PCR analysis revealed single bands for all donor strains except for E1162Δ*intA* (Fig. 1C). This indicates that CI formation is not dependent on the presence of the recipient strain in the conjugation mixture and that *intA* is involved in excision of the *esp* PAI (Fig. 1C). Sequencing of the CI PCR products of E1162

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics[a]	Reference or source
E. faecium strains		
E1162	Clinical blood isolate; Amp^r Van^s Chl^s Gen^s Spc^s; *esp* PAI^+; CI^+	5, 19
E1162Δ*intA*	*int* insertion-deletion mutant of E1162; Chl^r Gen^s; *esp* PAI^+; CI^-	This study
E1162Δ*intA*::pEF25	*int* insertion-deletion mutant of E1162, complemented with "empty" vector; Spc^r Chl^r Gen^s; PAI^+; CI^-	This study
E1162Δ*intA*::pEF30	*int* complementation strain of E1162Δ*intA*; Spc^r Chl^r Gen^s; *esp* PAI^+; CI^+	This study
BM4105RF::PAI	Transconjugant; Efm PAI^+	17
Plasmids		
pTEX5500ts	Shuttle plasmid; temperature sensitive in Gram-positive hosts; Chl^r Gen^r	8
pEF25	Shuttle plasmid pAT18 with spectinomycin resistance cassette cloned in the EcoRI site; Spc^r Ery^r	This study
pEF30	pEF25::*int*; Spc^r Ery^r	This study

[a] Amp, ampicillin; Van, vancomycin; Chl, chloramphenicol; Gen, gentamicin; Spc, spectinomycin; *esp* PAI, *E. faecium esp*-containing pathogenicity island; CI, circular intermediates; +, presence; −, absence.

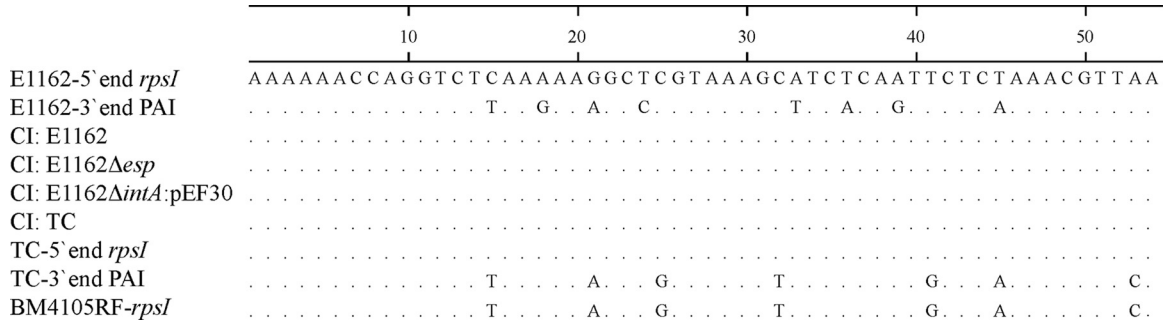

FIG. 2. Sequence alignment of the 54-bp direct repeat from the 5′-end *rpsI* and 3′-end PAI of strain E1162 and the transconjugant (TC), the *rpsI* in recipient strain BM4105RF, and the 54-bp direct repeat at the junction sites of the circular intermediates (CI) of E1162, E1162Δ*esp*, E1162Δ*intA*::pEF30, and the transconjugant.

and TC confirmed that the PCR products were specific for the junction site.

In *trans* complementation of *intA*. Complementation studies were performed to determine whether expression of *intA* in *trans*, from a plasmid (pEF30) (Table 1), was able to restore CI formation in the *intA* mutant strain. As a control the *intA* mutant strain was also complemented with plasmid pEF25 (Table 1), which does not contain wild-type *intA*. Both plasmids were transferred to E1162Δ*intA* by electroporation, resulting in E1162Δ*intA*::pEF25 and E1162Δ*intA*::pEF30 (Table 1). Restored CI formation was observed in E1162Δ*intA*::pEF30, but not in the *intA* mutant strain complemented with pEF25 (Fig. 1C), corroborating the finding that *intA* is involved in *esp* PAI excision. The size of the CI PCR product in E1162Δ*intA*::pEF30 is larger than that in the other donor strains due to the insertion of the *cat* resistance marker in the inactivated copy of *intA* in the *esp* PAI of E1162Δ*intA*.

Sequence analysis of CI PCR products. The whole-genome sequence project of E1162 enabled us to identify the borders of the *esp*-containing PAI (6, 17). In order to investigate whether CI formation occurs via recombination of the two imperfect 54-bp direct repeats leading to the presence of a single direct repeat at the junction site, as described for Tn*916* elements (13), all CI PCR fragments were subjected to sequencing. Furthermore, the 5′- and 3′-end direct repeats of the *esp* PAI in the transconjugant and the integration site (the *rpsI* gene) of the recipient BM4105RF strain were sequenced. Sequence analysis revealed that all CI contained a single 54-bp direct repeat at the junction site. Interestingly, in all cases the sequences were identical to the 54-bp sequence at the 5′ end of the *esp* PAI of E1162 and different from the 54-bp sequence at the 3′ end of the *esp* PAI of E1162 and the *rpsI* integration site of BM4105RF (Fig. 2). The 3′-end sequence of the TC was identical to the BM4105RF *rpsI* integration site. This suggests that recombination always occurs via the 5′ end of the *esp* PAI.

CI exist as ssDNA. The identification of the 54-bp sequence of the 5′ end of the *esp* PAI suggests that transfer occurs via single-stranded DNA (ssDNA), as has been hypothesized previously (9, 12, 13). In order to investigate the presence of single-stranded CI, the purified chromosomal DNA obtained to determine CI was treated with S1 nuclease, which specifically degrades ssDNA and not double-stranded DNA (dsDNA). In the following CI PCR analysis, no PCR products were obtained, indicating degradation of CI, while the PCR result on the chromosomally encoded *ddl* gene remained positive (Fig. 1D). This indicates that the *esp* PAI forms a single-stranded CI.

Mechanistic differences in excision of the *esp* PAIs in *E. faecium* and *E. faecalis*. Comparable to other ICEs, the *esp* PAI is flanked by two large direct (imperfect) repeats of 54 bp. Only the 54-bp DR at the 5′ end of the *esp* PAI is present at the junction site, suggesting that the *esp* PAI is similarly transferred as described for the ICE-like element Tn*916*. In contrast to the *esp* PAI, Tn*916* does not integrate in a site-specific manner, but prefers A · T-rich regions by using nonspecific coupling sequences (2, 9, 11, 13, 17). Site-specific integration using longer direct repeats has been described for several ICEs (2, 9).

Also in *E. faecalis*, the *esp* gene is harbored on a large PAI of 150 kb in size (15). Except for the *esp* gene itself and a 10-kb completely conserved gene cluster, both *esp* PAIs are different (17). Recently, Manson et al. investigated the mechanism of transfer of the *E. faecalis* PAI (7), and a striking difference can be observed between the transfer of the *E. faecium* and *E. faecalis esp* PAIs. In contrast to that of *E. faecium*, transfer of the *E. faecalis* PAI is independent of the phage-related integrase and conjugation functions contained on the PAI. Instead, transfer occurred only from donor strains possessing a pheromone-responsive type of conjugative plasmid (7).

In summary, the *E. faecium esp* PAI is a self-transmissible element that requires the IntA integrase contained on this element for excision. Based on the fact that the *esp* PAI contains an integrase and a partial copy of Tn*916*, including the conjugation module, and the fact that this element is self-transmissible in a fashion comparable to that of ICEs, we propose to change the name of this *esp* PAI to ICE*Efm1*.

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