Presence of Pathogenicity Island Genes in *Enterococcus faecalis* Isolates from Pigs in Denmark[▽]

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Enterococcus faecalis **isolates of porcine origin were screened for the presence of a previously identified pathogenicity island (PAI). By using the** *esp* **gene as a genetic marker for the presence of this PAI, 9** *esp***-positive and 10** *esp***-negative isolates of porcine origin were investigated by use of a designed oligonucleotide array. The results indicated the clustering of** *esp***-positive strains by multilocus sequence typing (MLST), but surprisingly, all strains investigated contained parts of the PAI. None of the strains of animal origin investigated belonged to previously identified MLST complex 2, where most isolates from patients cluster. Five of the nine** *esp***-positive** *E. faecalis* **isolates of animal origin belonged to the same PAI complex as human isolate MMH594 but differed in their sequence types, which strongly indicates the horizontal transfer of the PAI between enterococci of porcine and human origin.**

The *esp* gene is significantly associated with infection-derived isolates of *Enterococcus faecalis* (10) and *E. faecium* (13), contributes to the persistence of urinary tract infections in the bladder (9), and enhances biofilm formation by *E. faecalis* (11, 12). Recently, the *esp* gene has been shown to be part of a large pathogenicity island (PAI) harboring multiple virulence factors in *E. faecalis* (8) and *E. faecium* (4). These data suggest that *esp* may serve as a marker for the presence of the PAI and as a means of identifying virulent lineages of enterococci. The *esp* gene not only is detected in isolates of human origin but also can be detected in enterococcal isolates from both production and companion animals (2, 3; A. M. Hammerum and L. B. Jensen, Letter, Antimicrob. Agents Chemother. **40**:4396, 2002).

In a previous study, 75 selected *Enterococcus faecalis* isolates of porcine origin from 2001 were screened for the presence of the *esp* gene by PCR. Six isolates (isolates D1 to D6) were found to be positive (Hammerum and Jensen, letter). These isolates, together with newly identified *esp*-positive isolates (isolates D27, D34, and D38) and an additional 10 *esp*-negative isolates (D26, D28 to D33, and D35 to D37) representing randomly selected isolates with different resistance profiles, were included in this study. All *E. faecalis* isolates were obtained from pig feces in 2001 as part of the Danish Integrated Antimicrobial Resistance and Research Programme. Only one *E. faecalis* isolate was collected from a pig on each of the sampled farms (1). The *E. faecalis* isolates should therefore be epidemiologically unrelated. To test this hypothesis, pulsed-field gel electrophoresis was performed with all *esp*-positive strains. Only two of these isolates (isolates D4 and D5) showed highly similar profiles (data not shown).

All isolates were analyzed by the newly described multilocus sequence typing (MLST) scheme for *E. faecalis* (7; http://www.mlst.net). The allelic profiles of the *E. faecalis* strains were obtained by sequencing internal fragments of seven housekeeping genes, *gdh*, *gyd*, *pstS*, *gki*, *aroE*, *xpt*, and *yqiL* (http://www.mlst.net); and the sequence types (STs) of all isolates were determined.

Among the nine *esp*-positive isolates of porcine origin, five belonged to ST16, two belonged to ST40, and one isolate each belonged to ST26 and ST47 (Table 1). Isolates belonging to ST16 have previously been isolated from nonhospitalized humans and animals. Isolates belonging to ST40 have previously been isolated from a hospitalized patient, a food handler from Spain, and a seal from The Netherlands (7). *esp*-positive human isolate MMH594 has ST6, which belongs to MLST clonal complex 2. This complex comprises hospital-derived isolates exclusively (7). None of the animal isolates tested belonged to this complex. The *esp*-negative isolates were genetically more diverse than the *esp*-positive isolates. Two *esp*-negative isolates each were ST40 (isolates D32 and D37) and ST99 (isolates D33 and D35), while the other *esp*-negative isolates were of genetically unrelated STs (Table 1). Two STs, ST40 and ST16, were represented by both *esp*-positive and *esp*-negative isolates.

All isolates were then screened for the presence of genes colocalized with the *esp* gene in the previously identified PAI by using a custom array. The custom oligonucleotide array was designed to accommodate all of the 129 open reading frames (ORFs) represented on the *E. faecalis* PAI as duplicate spots of 50-mer oligonucleotides. Each computer-generated oligomer represented a unique region of each of the PAI genes, was amino modified, and was arrayed on pan epoxy glass slides by using an Affymetrix 417 arrayer (MWG Biotech, Ebersberg, Germany). Total genomic DNA was isolated as described earlier (10) and labeled with fluorescent dyes, and hybridization experiments were performed with a mixture of two target preparations applied to each array. The processed microarray slides

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TABLE 1. Allelic profiles and STs of the *E. faecalis* isolates

Strain no.	Origin	*esp* status[a]	ST	Allelic profile						
				gdh	*gyd*	*pstS*	*gki*	*aroE*	*xpt*	*yqiL*
OG1	Human	Neg	1	3	1	16	1	1	1	1
MMH594	Human	Pos	6	12	7	3	7	6	1	5
D2	Porcine	Pos	16	5	1	1	3	7	7	6
D4	Porcine	Pos	16	5	1	1	3	7	7	6
D5	Porcine	Pos	16	5	1	1	3	7	7	6
D6	Porcine	Pos	16	5	1	1	3	7	7	6
D29	Porcine	Neg	16	5	1	1	3	7	7	6
D34	Porcine	Pos	16	5	1	1	3	7	7	6
D38	Porcine	Pos	26	3	1	11	12	9	14	7
D1	Porcine	Pos	40	3	6	23	12	9	10	7
D27	Porcine	Pos	40	3	6	23	12	9	10	7
D37	Porcine	Neg	40	3	6	23	12	9	10	7
D32	Porcine	Neg	40	3	6	23	12	9	10	7
D3	Porcine	Pos	47	19	1	24	22	19	17	14
D26	Porcine	Neg	63	21	2	1	3	7	1	6
D28	Porcine	Neg	96	27	12	35	32	6	2	23
D30	Porcine	Neg	97	2	7	11	21	3	4	2
D31	Porcine	Neg	98	25	2	15	12	23	18	26
D33	Porcine	Neg	99	21	9	1	3	7	1	29
D35	Porcine	Neg	99	21	9	1	3	7	1	29
D36	Porcine	Neg	100	34	2	17	37	29	23	6

[a] Neg, negative; Pos, positive.

were read in the green (Cy3) and red (Cy5) channels of a ScanArray Express scanner (PE Life Sciences, Boston, MA). DNA from prototype PAI-positive human isolate MMH594 (8) and DNA from PAI-negative strain OG1RF (5), a well-characterized laboratory strain, were included as controls for the microarray hybridization. To confirm that the PAI genes were in fact organized in a contiguous manner and that the positive hybridization on the microarray was not indicative of the random distribution of genes throughout the genome, we performed overlapping long-range PCR throughout the length of the PAI, as described previously (8). Using isolate D34 as a prototype for the *esp*-positive isolates, we verified that the PAI genes were in fact contiguous both in *esp*-positive and in *esp*-negative strains. Furthermore, amplification across the left end of the PAI insertion site revealed that the PAI in the isolates tested was inserted at the same site where it is inserted in prototype human isolate MMH594, revealing the 10-bp duplication at the target site (8).

The pathochip hybridization results revealed a high degree of plasticity within the PAI region of the genomes of the enterococci isolated from pigs (Fig. 1). Surprisingly, all isolates, including the *esp*-negative isolates, contained fragments of the PAI. Interestingly, if single-linkage clustering (nearest-neighbor clustering), based on presence and absence of PAI genes, was used, the isolates tested could be grouped into two major complexes (PAI complexes 1 and 3) of 6 and 10 isolates, respectively, and a minor complex (PAI complex 2) of two isolates when an arbitrarily set cutoff of 85% similarity for grouping of the complexes was used (Fig. 2). Of the nine *esp*-positive *E. faecalis* isolates tested, five (55%) isolates, all of which were ST16, clustered in PAI complex 1, indicating that the PAI of porcine origin highly resembles the previously published PAI of strain MMH594 (8). The remaining 4 *esp*-positive isolates as well as the 11 *esp*-negative isolates contained a PAI that differed considerably from the published MMH594
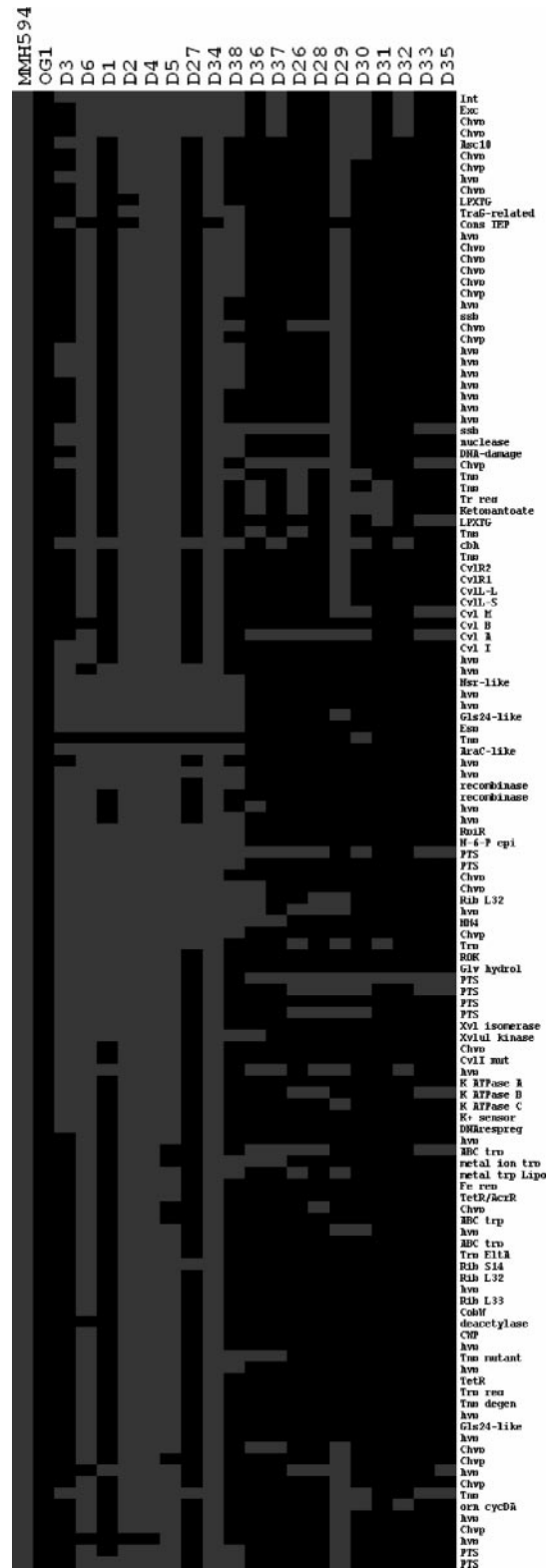

FIG. 1. Comparison of *esp*-positive *E. faecalis* isolates (isolates D3 through D38) and *esp*-negative *E. faecalis* isolates (isolates D36 through D35) (*x* axis) from pigs for the presence (gray) or the absence (black) of PAI genes (*y* axis) (4). MMH594 and OG1RF were included as controls for PAI-positive and PAI-negative strains, respectively.
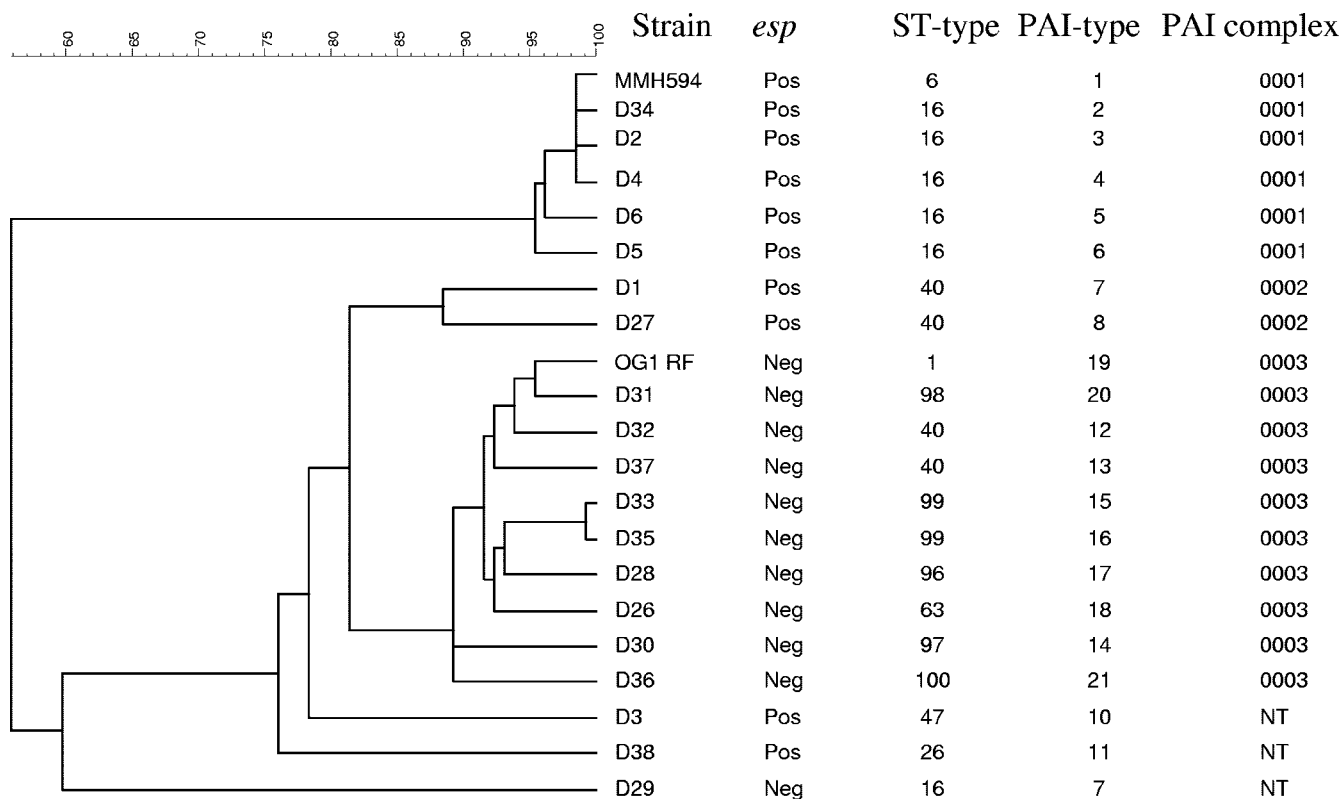
Strain	*esp*	ST-type	PAI-type	PAI complex
MMH594	Pos	6	1	0001
D34	Pos	16	2	0001
D2	Pos	16	3	0001
D4	Pos	16	4	0001
D6	Pos	16	5	0001
D5	Pos	16	6	0001
D1	Pos	40	7	0002
D27	Pos	40	8	0002
OG1 RF	Neg	1	19	0003
D31	Neg	98	20	0003
D32	Neg	40	12	0003
D37	Neg	40	13	0003
D33	Neg	99	15	0003
D35	Neg	99	16	0003
D28	Neg	96	17	0003
D26	Neg	63	18	0003
D30	Neg	97	14	0003
D36	Neg	100	21	0003
D3	Pos	47	10	NT
D38	Pos	26	11	NT
D29	Neg	16	7	NT

FIG. 2. Clustering of the tested *E. faecalis* strains by using single-linkage clustering (nearest-neighbor clustering), based on the presence and the absence of the individual genes composing the PAI. An arbitrarily set cutoff of 85% similarity for the grouping of complexes was used. NT, nontypeable.

PAI (8). Furthermore, these isolates, with the exception of strain D29, were of different STs and were not evolutionarily linked to the ST16 isolates. Previous studies by MLST based on seven housekeeping genes have defined distinct clonal complexes (CCs) of *E. faecalis* adapted to the hospital environment (CC2 and CC9). Of the isolates included in this study, only human isolate MMH594, which is ST6, grouped in one of these complexes, CC2. Porcine isolates of ST16 belonged to a previously reported prominent singleton clone represented by 11 isolates from hospitalized patients; 3 isolates from nonhospitalized individuals; and 3 isolates from animals, not including pigs, recovered from Spain and The Netherlands (7). Of the other STs reported in this study, four (ST1, ST26, ST40, and ST47) were found previously, although they were found infrequently and were not associated with pigs, whereas six STs (ST63, ST96, ST97, ST98, ST99, and ST100) represented novel STs (7).

The results presented here indicate the clonal expansion of most of the *esp*-positive *E. faecalis* isolates of porcine origin, with five of the nine isolates belonging to MLST group ST16. These five isolates all contained PAIs belonging to PAI complex 1. In addition, the finding of highly similar PAIs among genetically diverse *E. faecalis* isolates (like PAI complex 1 in isolates with ST6 and ST16, which do not belong to the same clonal complex, and PAI complex 3 in isolates with eight different STs that group in two different clonal complexes and five singleton clonal complexes) indicates the horizontal transfer of PAI gene sequences. Horizontal transfer of the *esp* gene has

already been demonstrated in *E. faecalis* (6), and recent unpublished work has revealed that this is accompanied by the transfer of large regions of the *E. faecalis* PAI. Our finding that different PAI variants could be grouped on the basis of the presence and the absence of ORFs suggests that the insertions and the deletions of PAI ORFs driving the evolution of the PAI do not occur randomly but occur between defined clusters. Ongoing work suggests that large, contiguous regions of the PAI may be lost or gained as an evolutionary process, but the mechanisms defining these remain to be characterized (N. Shankar, unpublished data). Whether the plasticity of the *E. faecalis* PAI may favor adaptation to different ecological niches, like the guts of humans and animals, is unknown and remains to be further investigated.

The identification of highly homologous PAIs among enterococci isolated from production animals could indicate the presence of a nonhuman reservoir of the *E. faecalis* PAI implicated in virulence. The existence of a nonhuman reservoir of enterococcal virulence genes is undesirable and calls for careful monitoring and further research to gain more insight into the impact of the horizontal transfer of these genes from animals to humans, directly or via the food chain, and its influence on the establishment of pathogenic *E. faecalis* strains capable of causing infections in humans.

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