Occurrence of Putative Pathogenicity Islands in Enterococci from Distinct Species and of Differing Origins

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Enterococci isolated from ewe’s milk and cheese, clinical isolates of human and veterinary origins, and reference strains obtained from culture collections were screened for the occurrence of putative pathogenicity island (PAIs). Results obtained after PCR amplification and hybridization point toward PAI dissemination among enterococci of diverse origins (food, clinical) and species (Enterococcus faecalis, Enterococcus faecium).

First, all enterococci were screened by PCR for PAI-related genes (see Table S1 in the supplemental material). The analyzed loci represent diverse regions of the E. faecalis PAI (15) and include the esp and araC-like genes also present in the putative PAI identified in E. faecium (4). All isolates showed between 11 and 22 genes (Table 1 and data not shown), with food strain QSE123 presenting the lowest incidence and four strains harboring the majority of the regions under analysis (22 genes). Eight of the analyzed loci were detected in more than 90% of the enterococci, pointing to a high conservation of

### TABLE 1. Incidence of PAI-related loci among the food, clinical, and reference enterococci tested in this study

| Locus (gene) | Reference strains | Food strains | Clinical strains | Total | Locus presence |
|-------------|-----------------|-------------|-----------------|-------|---------------|
|             |                  |             |                 |       |               |
| ef0481      | 20 (100)        | 13 (93)     | 6 (100)         | 39 (98) | +             |
| ef0482      | 20 (100)        | 13 (93)     | 6 (100)         | 39 (98) | +             |
| agg         | 20 (100)        | 14 (100)    | 6 (100)         | 40 (100) | +             |
| ef0495      | 20 (100)        | 13 (93)     | 6 (100)         | 39 (98) | +             |
| cbb         | 10 (50)         | 3 (21)      | 1 (17)          | 14 (70) | d             |
| cylL1       | 1 (5)           | 4 (29)      | 1 (17)          | 6 (15)  | d             |
| cylL4       | 2 (10)          | 6 (43)      | 1 (17)          | 9 (23)  | d             |
| cylM        | 9 (45)          | 8 (57)      | 3 (50)          | 20 (50) | d             |
| cylB        | 9 (45)          | 10 (71)     | 5 (83)          | 24 (60) | d             |
| cylA        | 9 (45)          | 9 (64)      | 5 (83)          | 23 (58) | d             |
| cylH        | 15 (75)         | 9 (64)      | 4 (67)          | 28 (70) | d             |
| esp         | 15 (75)         | 6 (43)      | 3 (50)          | 24 (60) | d             |
| araC        | ef0530          | 17 (85)     | 10 (71)         | 4 (67)  | 31 (78)       |
| ef0534      | 20 (100)        | 14 (100)    | 6 (100)         | 40 (100) | +             |
| ef0539      | 19 (95)         | 12 (86)     | 6 (100)         | 37 (93) | +             |
| ef0543      | 19 (95)         | 11 (79)     | 6 (100)         | 36 (90) | +             |
| ef0551      | 11 (55)         | 9 (64)      | 5 (83)          | 25 (63) | d             |
| xylA        | ef0556          | 10 (50)     | 9 (64)          | 4 (67)  | 23 (58)       |
| ef0571      | 1 (5)           | 0 (0)       | 1 (17)          | 2 (5)   | –             |
| ef0590      | 20 (100)        | 14 (100)    | 6 (100)         | 40 (100) | +             |
| gis24-like  | ef0604          | 16 (80)     | 10 (71)         | 1 (17)  | 32 (80)       |
| ef0609      | 16 (80)         | 8 (57)      | 4 (67)          | 28 (70) | d             |
| ef0617      | 13 (65)         | 7 (50)      | 6 (100)         | 26 (65) | d             |
| ef0628      | 7 (35)          | 2 (14)      | 3 (50)          | 12 (30) | d             |

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In 2005, Nallapareddy and coworkers (9) analyzed 454 *E. faecalis* isolates and identified isolate-specific deletions in four PAI regions: one included *cylM*, *araC*, and ef0534 and was also previously described for *E. faecalis* V583 (15); the second included ef0530 and ef0534; the third corresponded to region ef0571; and the fourth included loci ef0604 to ef0609. PAI variability was also reported by other authors (7, 8) and corroborates the findings observed in the present study, where deletions were found throughout the PAI, with major incidence among loci *cbh* to *esp*, ef0551 to ef0571, and ef0628. After chi-square analysis of contingency tables, a statistically significant association was found between the presence of the gene *cbh* and clinical origin (*P* = 0.00525). Distinct results were observed in previous studies (9) where the *esp*, *xylA*, and *gls24*-like genes were enriched in infection-derived isolates. The differences observed are probably due to sample size effects (454 isolates of *E. faecalis* versus 40 of enterococci).

In conclusion, after PCR screening for PAI-related genes, 13 putative PAI-harboring enterococci were selected, including reference, clinical human/veterinarian, and food isolates.

Subsequently, a genome-walking strategy was applied to establish the genetic organization of the putative PAIs by comparison with the positive controls included in this study (MMH594, *E. faecalis* complete PAI; V583, *E. faecalis* PAI with a deletion [15]; E300, *E. faecium* putative PAI [4]). The results obtained with 33 primer combinations (see Table S2 in the supplemental material) are summarized in Fig. 1 and show that the majority of the strains possess more than 20 of the analyzed loci, with the least-conserved regions being located between the genes *cbh* and *esp*, pointing to this region as a “deletion hot spot” and suggesting that some deletions are more favored than others, as can be inferred from the results reported by other authors (7, 8, 9, 15). Excluding the PAI-harboring control strains, the isolate harboring more PAI-related genes was LN11, an *E. casseliflavus* ewe’s milk isolate with an amplification profile almost 100% identical to that of MMH594. The diversity of genetic organizations observed in the putative PAIs is probably a reflection of the enterococcal ability to acquire and share material with other bacteria inhabiting the same ecological niche.
Subsequently, to test for physical linkage of the virulence-related genes in the same region of the bacterial chromosome, total cell DNA was cleaved with restriction enzymes (I-Ceu-I, SfiI, and SmaI), the macrorestriction fragments were separated by pulsed-field gel electrophoresis (PFGE; contour-clamped homogeneous electric field with pulse times increasing linearly from 5 to 35 s for 22 h at 220 V), transferred to nylon membranes by Southern blotting (11), and hybridized under high-stringency conditions (probes and signals detected with Dig-High Prime DNA labeling and detection starter kit II as recommended by the manufacturer (Roche Diagnostics).

The restriction enzyme I-Ceu-I recognizes a unique sequence within the 23S rRNA (5), so cleavage of total DNA, followed by hybridization with a 16S rRNA gene probe (6), highlights the fragments corresponding to genomic DNA, allowing the distinction between chromosomal DNA and extrachromosomal elements. When rehybridization of the same membrane with a PAI gene as the probe reveals a positive hybridization signal in the same fragment, this demonstrates its chromosomal nature. Subsequent hybridizations with other PAI-related probes confirm the physical linkage of those loci in the same region of the bacterial chromosome, pointing to the presence of putative PAIs.

Figure 2 shows the results obtained after DNA cleavage with I-Ceu-I (Fig. 2, left panel), followed by hybridization with the 16S rRNA gene (Fig. 2, center panel) and cylMB4 (Fig. 2, right panel) probes, which demonstrate the chromosomal location of the cyl operon in veterinary clinical strain V434 and food isolates LN11, LA78, and QCB54. Concerning the other cyl-positive enterococci included in this investigation, very weak or no hybridization signals were obtained (data not shown), suggesting that such traits must be carried by plasmids.

Subsequently, Southern blot assays of SfiI and SmaI macrorestriction profiles were hybridized with exp and cylMB4 (Fig. 3, left and right panels, respectively). Hybridization of the two probes within the same-size macrorestriction fragment was observed for strains MMH594 and LN11, pointing to the physical linkage of these PAI-related genes in ewe’s milk E. casseliflavus isolate LN11. For V583, with a deletion including this region, as expected, no hybridization signal was observed.

Positive amplification with primer combination EF1/ER2-2, directed to specific regions of E. faecalis V583 located before and after its PAI (10), demonstrated that in strains V95 and QSE123, either the PAI is absent or its insertion occurred at a distinct location. For the other 11 putative PAI-harboring enterococci, primer combinations EF1/ER1 (before the PAI) and EF2/ER2 (at the end of the PAI/outside of the PAI) confirmed a PAI insertion site similar to that of MMH594 and V583, as also reported for other enterococci (7, 8, 9, 14).

Although there are many differences within this genomic region of the isolates under analysis, the overall results point toward PAI dissemination among enterococci from diverse origins (ewe’s milk/cheese) and species (E. faecalis, E. faecium, E. casseliflavus, E. raffinosus, E. durans, and E. hirae), suggesting a wide pathogenicity potential of the genus Enterococcus.

REFERENCES

1. Abes, P. L., M. P. Martins, T. Semedo, J. J. Figueiredo-Marques, R. Tenreiro, and M. T. Barreto-Crespo. 2004. Comparison of phenotypic and genotypic taxonomic methods for the identification of dairy enterococci. Antonie van Leeuwenhoek 85:237–252.
2. Eaton, T. J., and M. J. Gasson. 2001. Molecular screening of Enterococcus virulence determinants and potential for genetic exchange between food and medical isolates. Appl. Environ. Microbiol. 67:1628–1635.
3. Hacker, J., and J. B. Kaper. 2000. Pathogenicity islands and the evolution of microorganisms. Annu. Rev. Microbiol. 54:661–679.
4. Leavis, H., J. Top, N. Shankar, K. Borgen, M. Bonten, J. van Embden, and R. J. Willems. 2004. A novel putative enterococcal pathogenicity island linked to the esp virulence gene of Enterococcus faecium and associated with epidemicity. J. Bacteriol. 186:672–682.
5. Liu, S., A. Hessel, and K. E. Sanders. 1993. Genomic mapping with I-Ceu I, an intron-encoded endonuclease specific for genes for ribosomal RNA, in Salmonella spp., Escherichia coli, and other bacteria. Proc. Natl. Acad. Sci. USA 90:6874–6878.
6. Massol-Deya, A., D. A. Odelson, R. F. Hickey, and J. M. Tiedje. 1995. Bacterial community fingerprinting of amplified 16S and 16-23S ribosomal DNA gene sequences and restriction endonuclease analysis (ARDRA), p. 1–8, In A. D. L. Akkermans, J. D. van Elsas, and F. J. de Bruijn (ed.), Molecular microbial ecology manual, 3.3.2. Kluwer Academic Publishers, Dordrecht, The Netherlands.
7. McBride, S. M., P. S. Coburn, A. S. Baghdayan, R. J. L. Willems, M. J.
8. McBride, S. M., V. A. Fischetti, D. LeBlanc, R. C. Moellering, Jr., and M. S. Gilmore. 2007. Genetic diversity among Enterococcus faecalis. PLoS ONE 2:e582. doi:10.1371/journal.pone.0000582.

9. Nallapareddy, S. R., H. Wenxiang, G. M. Weinstock, and B. E. Murray. 2005. Molecular characterization of a widespread, pathogenic, and antibiotic resistance-receptive Enterococcus faecalis lineage and dissemination of its putative pathogenicity island. J. Bacteriol. 187:5709–5718.

10. Oancea, C., I. Klare, W. Witte, and G. Werner. 2004. Conjugative transfer of the virulence gene, esp, among isolates of Enterococcus faecium and Enterococcus faecalis. J. Antimicrob. Chemother. 54:232–235.

11. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

12. Semedo, T., M. A. Santos, M. F. Lopes, J. J. F. Marques, M. T. Crespo, and R. Tenreiro. 2003. Virulence factors in food, clinical and reference enterococci: a common trait in the genus? Syst. Appl. Microbiol. 26:13–22.

13. Semedo, T., M. A. Santos, P. Martins, M. F. Lopes, J. J. F. Marques, R. Tenreiro, and M. T. Crespo. 2003. Comparative study using type strains and clinical and food isolates to examine hemolytic activity and occurrence of cyt operon in enterococci. J. Clin. Microbiol. 41:2569–2576.

14. Shankar, N., A. S. Baghdayan, R. Willems, A. M. Hammerum, and L. B. Jensen. 2006. Presence of pathogenicity island genes in Enterococcus faecalis isolates from pigs in Denmark. J. Clin. Microbiol. 44:4200–4203.

15. Shankar, N., A. S. Baghdayan, and M. S. Gilmore. 2002. Modulation of virulence within a pathogenicity island in vancomycin-resistant Enterococcus faecalis. Nature 417:746–750.