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Genetic Variation and Evolution of the Pathogenicity Island of *Enterococcus faecalis*▽†

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Enterococcus faecalis **is a leading cause of nosocomial infections and is known for its ability to acquire and transfer virulence and antibiotic resistance determinants from other organisms. A 150-kb pathogenicity island (PAI) encoding several genes that contribute to pathogenesis was identified among antibiotic-resistant clinical isolates. In the current study, we examined the structure of the PAI in a collection of isolates from diverse sources in order to gain insight into its genesis and dynamics. Using multilocus sequence typing to assess relatedness at the level of strain background and microarray analysis to identify variations in the PAI, we determined the extent to which structural variations occur within the PAI and also the extent to which these variations occur independently of the chromosome. Our findings provide evidence for a modular gain of defined gene clusters by the PAI. These results support horizontal transfer as the mechanism for accretion of genes into the PAI and highlight a likely role for mobile elements in the evolution of the *E. faecalis* PAI.**

Enterococcus faecalis is a core constituent of the intestinal flora of humans and a leading cause of nosocomial infections worldwide (35). Enterococci are associated with a variety of pathologies, including pelvic infections, intra-abdominal abscesses, postsurgical infection, bacteremia, endocarditis, and urinary tract infections (12, 20, 30). The ability of *E. faecalis* to cause serious infection is connected to the inherent hardiness of the bacterium, which enables it to tolerate desiccation, persist in the hospital environment, and then endure host defenses (20, 22). In addition, enterococci are particularly adept at acquiring resistance to antibiotics and disseminating these elements within and beyond the genus (30, 48).

Pathogenicity islands (PAIs) are large, horizontally transmitted elements found in many gram-positive and gram-negative pathogens (14). They are believed to contribute to the rapid evolution of nonpathogenic organisms into pathogenic forms (2, 26). The PAI of *E. faecalis* is approximately 150 kb and encodes multiple factors that contribute to its virulence, including the cytolysin toxin (3, 18), the enterococcal surface protein Esp (40), and Gls-24-like proteins (44), as well as traits suspected of contributing to pathogenicity or altering its relationship with the host, including a bile acid hydrolase, carbohydrate utilization pathways, and many additional genes of unknown function (38). The PAI, or parts thereof, has been identified in hundreds of *E. faecalis* isolates, and variation in genetic content has been noted (25, 30, 32, 39). It is enriched among infection-derived isolates (38) and highly clonal lineages containing multiple antibiotic resistance elements (30). Variation has been found in the occurrence of genes within the PAI, even within a genetic (clonal) lineage, suggesting that segments of the island can vary independently of the whole (38). Indeed, movement of genes derived from an internal portion of the PAI has been detected (6).

Little is known about the genesis of the enterococcal PAI, how it varies, or how it entered the species. The prototype PAI of strain MMH594 includes known and putative insertion sequence (IS) elements, transposases, conjugal transfer components, and other plasmid derived sequences and at the sequence level appears to have been assembled by a process of accretion. In the current work we investigated the variability of the PAI among a diverse set of clinical isolates and compared this variability to variation in the host chromosome.

MATERIALS AND METHODS

Bacterial strains and culture methods. The *E. faecalis* strains used in this study are listed in Table 1. PCR was used to confirm the species of each isolate using the species-specific primers $ddl_{E.\ faecalis}$ E1 and $ddl_{E.\ faecalis}$ E2 (11). To identify *E. faecalis* strains possessing the PAI for further study, isolates were screened by PCR for the presence of the *esp* gene, which so far has only been localized to the PAI (38) (in contrast to cytolysin or aggregation substance genes, which also can be plasmid mediated [4, 7]). The *E. faecalis* strains examined were obtained from a variety of geographical sources and were routinely grown on brain heart infusion (BHI) agar (1.5%, wt/vol; Difco) or BHI broth without aeration at 37°C.

Preliminary screening of strains for the presence of the PAI. Genomic DNA was purified from strains determined by PCR as being *E. faecalis* and positive for *esp*. Briefly, bacterial cultures were grown overnight in 5 ml of BHI broth containing appropriate antibiotics and 3% glycine. Cells were pelleted, resuspended in 200 μl of a solution containing 50 mM glucose, 25 mM Tris-HCl (pH 8.0), 10 mM EDTA, and 10 mg/ml lysozyme (Sigma-Aldrich), and incubated for

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TABLE 1. Bacterial strains used in this study

Strain	Isolation date (mo/day/yr)	Source	Description	MLST	Reference(s) or source[a]
126	8/24/1993	Blood	Buffalo, NY; from T. Russo	62	This study
EV98	3/1/1999	Blood	Hospital isolate	155	46, 47
EV609	4/4/1999	Blood	Hospital isolate	151	46, 47
28	3/8/1993	Blood	Buffalo, NY; from T. Russo	21	This study
W32944	Unknown	Urine	Unknown	21	9
EV68	3/1/1999	Blood	Hospital isolate	21	46, 47
E1198	Unknown	Blood	22A108; clinical isolate, Turkey	21	ENARE
E0115	1997	Feces	9700334; community survey, The Netherlands	145	NIVEL
EV479	4/4/1999	Blood	Hospital isolate	157	46, 47
EV172	3/1/1999	Blood	Hospital isolate	41	46, 47
EV108	3/1/1999	Blood	Hospital isolate	146	46, 47
613	1994	Endocarditis	MC0098; from M. Huycke	19	This study
T16	≤1951	Infant/fecal	NCTC 8729, s161 type 3, United Kingdom	19	28–30, 41, 49, 50
E19	10/4/1999	Urine	Little Rock, AK	4	This study
E99	1/14/2000	Urine	Little Rock, AK	4	This study
NZ4	2001–2002	Blood	Christchurch, New Zealand; from D. Murdoch	152	This study
545	1989	Urine	U17248; Oklahoma City, OK; from M. Huycke	6	This study
MMH594	1985	Blood	Wisconsin	6	16, 30, 38
V583	2/12/1987	Blood	ATCC 700802; St. Louis, MO	6	30, 37
V586	2/23/1987	Blood	St. Louis, MO	6	37
EV408	3/24/1999	Blood	Hospital isolate	155	46, 47
T4	≤1992	Urine	Otaru-104; Otaru, Japan	62	28–30, 49, 50
E103	1/18/2000	Urine	Little Rock, AK	64	This study
EV596	4/4/1999	Blood	Hospital isolate	64	46, 47
79-3	10/4/1999	Blood	Chicago, IL	64	30
EV586	4/4/1999	Blood	Hospital isolate	8	46, 47
EV147	3/1/1999	Blood	Hospital isolate	154	46, 47
EV217-2	4/4/1999	Blood	Hospital isolate	153	46, 47
E71	12/8/1999	Urine	Little Rock, AK	9	This study
EV638	4/4/1999	Blood	Hospital isolate	9	46, 47
Ned10	1961	Horse	D5278/61; The Netherlands	9	30
YI6-1	≤1992	Clinical	Japan	28	17
D6	Unknown	Pig	73-30318-4; Denmark	16	30, 39
A25	10/13/1989	Urine	3A1025; from Pfizer	30	This study
Ned2	1956	Throat	D939/56; The Netherlands; from R. Willems	156	This study
D3	Unknown	Pig	73-30245-2; Denmark	47	30, 39
EV650	4/4/1999	Blood	Hospital isolate	40	46, 47
EV419	3/24/1999	Blood	Hospital isolate	40	46, 47
EV447	4/4/1999	Blood	Hospital isolate	40	46, 47
EV632	4/4/1999	Blood	Hospital isolate	40	46, 47
326	7/14/1986	Blood	2081; from M. Huycke	40	This study
E73	12/10/1999	Urine	Little Rock, AK	40	This study
V105	Unknown	Animal	From Pfizer	40	This study
D1	Unknown	Pig	73-30082-2; Denmark	40	30, 39
A-3-1	Early 1980s	Infant/sepsis	Denver, CO	40	27, 30
NZ10	2001–2002	Blood	Christchurch, New Zealand; from D. Murdoch	150	This study
A27	10/13/1989	Urine	3A1027; from Pfizer	148	This study
NZ7	2001–2002	Blood	Christchurch, New Zealand; from D. Murdoch	144	This study
620	1994	Endocarditis	MC0105; from M. Huycke	144	This study
EV321	3/1/1999	Blood	Hospital isolate	144	46, 47
EV564	4/4/1999	Blood	Hospital isolate	55	46, 47
296	9/7/1986	Blood	2343: from M. Huycke	149	This study
D18	Unknown	Human	14821-V-2; Denmark; from L. B. Jensen	147	This study
OG1	≤1975	Oral	ATCC 47077	1	10, 15, 30
FA2-2	≤1973	Clinical	United Kingdom; Rif/Fus-resistant mutant derived from plasmid-free strain JH2	8	5, 19, 30

[a] ENARE, European Network for Antibiotic Resistance and Epidemiology; NIVEL, The Netherlands Institute for Health Services Research.

30 min at 37°C. Cells were lysed in 200 μl lysis buffer AL (Qiagen) and treated with a final concentration of 0.25 mg/ml RNase A for 10 min at 37°C (Sigma-Aldrich). To this mixture, 25 μl of a 20-mg/ml solution of proteinase K (Sigma-Aldrich) was added, and the solution was incubated for 15 min at 50°C. Proteinase K was inactivated by incubation at 70°C for 30 min. DNA was precipitated with 565 μl ethanol and resuspended in 200 μl Tris-EDTA buffer, pH 8.0.

Dot blot hybridization was used initially to verify the presence of additional PAI-associated genes. Hybridizations were conducted essentially as described by the manufacturer (Zeta Probe; Bio-Rad) using 200-ng spots of DNA from each isolate. The membranes were probed with PCR products generated from a strain MMH594 template, which harbors the prototype PAI (38). Probes were designed to query the length of the PAI as it exists in strain MMH594, one quarter at a time, and labeled using the RadPrime DNA labeling system (Invitrogen). The first quarter was assessed by generating probes using primers PAI110/PAI113, PAI112/PAI115, and CylB4224/CylB5535, which span bases 4999 to 47260 in the MMH594 PAI (38). The second quarter was detected using a mixture of labeled

amplicons generated with primers 5641/583R, PAI120/PAI123, PAI122a/PAI125a, and PAI124/PAI127, which span bases 47366 to 87880 in the MMH594 PAI. The third quarter of the PAI was probed with a mixture of amplicons generated by primers PAI126/PAI129, PAI128a/PAI131a, PAI130/PAI133, and PAI132/PAI135, which span bases 86853 to 117295 in the MMH594 PAI. Finally, the last quarter of the element was probed using amplicons made with primers PAI134/PAI137, PAI140/PAI143, and PAI142/PAI145, covering bases 116216 to 150328. Hybridization intensity was assessed and compared to those of MMH594 (full island) and OG1 (no island) controls.

Design of a custom pathoarray. To detect the occurrence of individual genes within putative PAIs, a custom oligonucleotide array that represented each of the 129 open reading frames of the prototype PAI of strain MMH594 was designed and termed a pathoarray. Additionally, selected *E. faecalis* genes potentially related to virulence or antibiotic resistance, such as those encoding surface proteins or encoding polysaccharide and lipid biosynthesis/transport and metabolic functions, were also represented on the array. A specific 50-mer oligonucleotide with theoretically optimum hybridization characteristics was generated for each open reading frame (MWG Biotech software). Each oligomer was synthesized, purified, amino modified, and arrayed on pan-epoxy glass slides using an Affymetrix 417 Arrayer (MWG Biotech) in duplicate 100-μm-diameter spots. All arrays were controlled for quality by scanning for physical probe deposition and by hybridization with labeled random oligonucleotides for covalent binding of probes to the slide surface. A full listing of the probe sets, genes represented, and array design is described in the ArrayExpress public repository (http://www.ebi.ac.uk/arrayexpress; accession number A-MEXP-876).

Oligonucleotide array hybridization and analysis. Microarrays were hybridized simultaneously with a mixture of DNAs from two strains being queried, each differentially labeled with Cy3 or Cy5. The microarray hybridization protocol was initially optimized using DNAs from the positive control strain MMH594 and a negative control strain, OG1, which shares the core genome but lacks the PAI. Briefly, RsaI-restricted genomic DNA (2 μg per strain) was denatured and annealed to random hexamers. Fluorescent probes were synthesized using Klenow fragment of DNA polymerase I (Invitrogen) in the presence of 1.2 mM each of dATP, dGTP, and dTTP; 0.6 mM of dCTP; and 1 mM of Cy5-dCTP or Cy3-dCTP (GE LifeSciences). The reaction was terminated by the addition of 0.5 M EDTA. Labeled probes were purified from unincorporated dye using Microcon-30 filtration units (Amicon). Cy3- and Cy5-labeled probes containing 200 pmol of each dye were concentrated to a volume of 13.5 μl in hybridization buffer (50% formamide, 5× Denhardt's solution, 6× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 0.5% sodium dodecyl sulfate, and 50 mM phosphate buffer), denatured at 95°C, chilled on ice, and then applied to a microarray slide and hybridized overnight at 42°C. Slides were washed at room temperature once in 2× SSC–0.1% SDS and four times in 0.2× SSC and finally rinsed in 0.01× SSC before air drying. Microarray slides were read in the green (Cy3) and red (Cy5) channels of a ScanArray Express scanner (PE Biosciences) at 10-μm resolution to generate separate red and green TIFF images. Fluorescence intensities and spot morphologies were analyzed using the quantitation functions of ScanArray Express version 2.1.8, and spots were excluded based on slide or morphology anomalies. Total fluorescence intensity was measured from a fixed circular area within each oligonucleotide spot, and signals with a statistically significant difference ($P < 0.01$) from the background level were considered positive. Reproducibility was verified by two replicate hybridizations (dye swap), using independent DNA preparations from the positive and negative control strains. Reproducibility of results for queried strains was confirmed with replicate hybridizations (dye swap) for six randomly chosen strain pair combinations (EV650-EV172, EV98-EV609, EV68-T16, T4-D1, 326-E0115, and E99-V105). The data obtained were converted to binary present (1) and absent (0) calls for analysis.

Long-range PCR was used to confirm the presence or absence of gaps within the PAI and to demonstrate contiguity between genes detected. Long-range PCR was performed using TaKaRa LA *Taq* polymerase (TaKaRa Biomedicals) according to the manufacturer's instructions with primers listed in Table S1 in the supplemental material. When PCR and sequencing data indicated a false-negative hybridization by pathoarray, the corrected data were used for constructing a gene presence profile (see Table S3 in the supplemental material). Where needed, nucleotide sequence information for PCR fragments was obtained using an ABI 3730 capillary sequencer at the Oklahoma Medical Research Foundation (Oklahoma City, OK) and an ABI 3100 sequencer at the DNA Sequencing Center for Vision Research (DSCVR) at the Massachusetts Ear and Eye Infirmary (Boston, MA).

Phylogenetic analysis. Multilocus sequence typing (MLST) was used to determine the relatedness of strain background and was performed by amplifying and sequencing regions of the housekeeping genes *gdh*, *gyd*, *pstS*, *gki*, *aroE*, *xpt*, and *yqiL*, as described elsewhere (http://www.mlst.net; 36), using primers listed in Table S1 in the supplemental material. eBURST (13, 42) was used to display the allele-based population snapshot of *E. faecalis*. In two steps, BioNumerics (version 5.1; Applied Maths, Saint-Martens-Latem) was used to construct the neighbor-joining tree for 52 *E. faecalis* isolates, based on the concatenated sequences of all seven MLST genes, In the first step, strain *Enterococcus faecium* DO was used as an outgroup by including the concatenated sequences of the *E. faecium* DO homologs of the *E. faecalis* MLST housekeeping genes. The *E. faecalis* isolate (D3) that branched off first from the *E. faecium*-rooted neighbor-joining tree was forced as a root in a second neighbor-joining tree composed only of *E. faecalis* isolates. Nonconcatenated (single-gene) MLST analysis yielded the same relatedness pattern as concatenated MLST (data not shown).

BioNumerics 5.1 was also used to construct a maximum-parsimony tree based on pathoarray hybridization data of 53 *E. faecalis* isolates. For this tree, isolate D3 was forced as root, because this isolate was closest to the *E. faecium-E. faecalis* last common ancestor according to the rooted neighbor-joining tree. Finally transversal clustering was performed based on pathoarray hybridization data for the 53 *E. faecalis* isolates, to visually associate groups of PAI genes with groups of isolates. In this clustering, the isolates are grouped by means of their PAI profile (presence and absence of genes), while the PAI genes are sorted by means of their value (0 = absent and 1 = present).

Gel electrophoretic evidence of chromosomal localization. Because the prototype PAI identified in strain MMH594 included a pAM373-like plasmid integrated within its structure, contour-clamped homogenous electric field (CHEF) gel analysis, performed as previously described (43), was used to assess integration of known plasmid-associated sequences into the PAIs of the strains studied here. Strains that contained more than half of the pAM373 element and did not give a PCR product indicating insertion of the plasmid into the PAI were analyzed by CHEF analysis and probed with portions of PAI gene EF0025, a gene highly related in sequence to that encoding open reading frame 8 of pAM373 (8). Using a strategy developed to discern plasmid-encoded from chromosomally encoded genes in *Clostridium perfringens*, (21), agarose plugs containing total DNA from isolates were digested with the homing endonuclease I-CeuI (New England Biolabs), which cleaves the genome within rRNA operons, at 37°C for 16 h. Southern hybridization employed probes specific for the *E. faecalis* 23S rRNA gene, to identify chromosomal fragments and for EF0025 to localize to the pAM373-like sequence to either a chromosomal fragment or the extrachromosomal pool. The 23S rRNA gene probe was generated using primers EF23sFor and EF23sRev, and the EF0025 gene probe was generated with primers PAI114 and PAI115. Strain MMH594 and plasmid-free strain OG1 were used as positive and negative controls, respectively.

Functional evidence for chromosomal localization of the cytolysin operon. In addition to pAM373, the cytolysin operon also has been shown to occur on highly transmissible plasmids, such as pAD1 (24), as well as on uncharacterized elements within the chromosome (17), and it was subsequently identified as occurring within the PAI (38). Though the cytolysin, when plasmid encoded, transfers at high rates (5), portions of the PAI, including the cytolysin, transfer at rates near the limit of detection (6, 38). To detect strains harboring the cytolysin operon on highly transmissible, pAD1-like plasmids, all isolates found to be hemolytic on 5% human blood were used as candidate donor strains in broth matings with rifampin (rifiampicin)- and fusidic acid-resistant recipient *E. faecalis* strain FA2-2 (19). Briefly, 50 μl of donor culture and 500 μl of recipient were combined with 4.5 ml BHI broth and incubated at 37°C for 4 hours with gentle agitation. Mating cultures were then vortexed and plated onto BHI agar containing 5% human blood, 50 μg/ml rifampin, and 25 μg/ml fusidic acid to select for hemolytic transconjugants. Since strain YI6-1 is inherently rifampin resistant, JH2SS was used as the recipient strain, and in this case streptomycin and spectinomycin (both at 500 μg/ml) were used to select for possible transconjugants. Hemolytic colonies were checked for additional unselected donor and recipient markers to verify their status as transconjugants. Conjugation tests were performed in duplicate.

RESULTS

Analysis of PAI variation. Isolates were initially selected for PAI analysis based on presence of the *esp* gene, which is known to be located on the prototype PAI, has not been described as occurring on any other element, and is associated with increased disease risk (40). To obtain preliminary evidence for variability in PAI content among *E. faecalis* strains, isolates positive for *esp* were further screened by dot blot hybridization

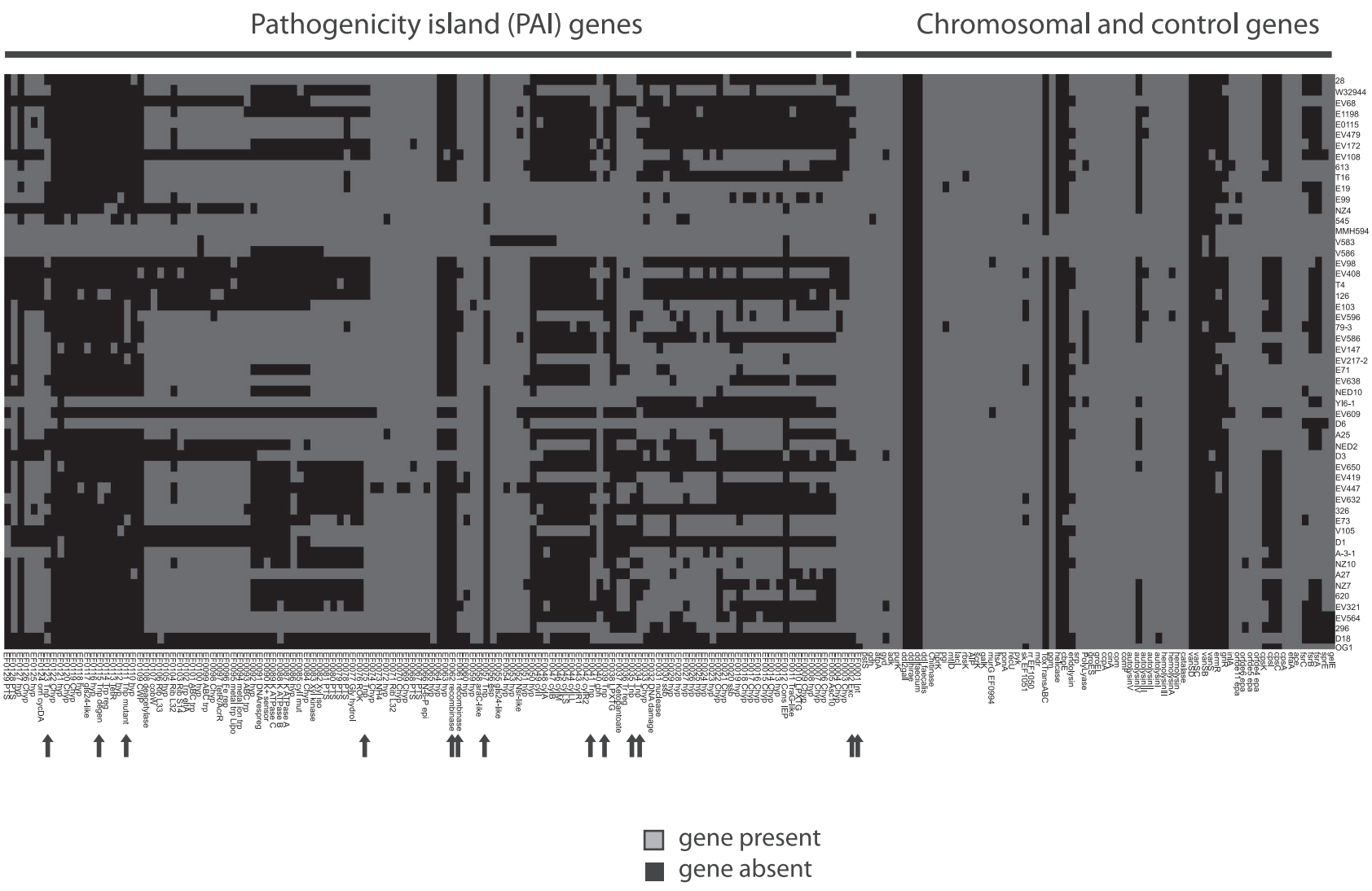

FIG. 1. PAI gene contents of *E. faecalis* isolates. Black squares represent absent calls for a specific probe; present calls are shown in gray. Arrows indicate sequences found in the PAI of strain MMH594 with sequence similarity to mobile genetic elements. Strains MMH594 and OG1 were included as representative PAI-positive and PAI-negative control strains, respectively.

TABLE 2. Analysis of pAM373 and cytolysin regions

Region and strain	Cytolysin activity	Integration (PCR)	CHEF gel electrophoresis[a]	Conjugation	Probable location
pAM373-like region					
MMH594		+[b]	C		Chromosome
V583		+	NT		Chromosome
V586		+	NT		Chromosome
545		+	NT		Chromosome
E99		+	NT		Chromosome
NZ4		+	NT		Chromosome
E19		+	NT		Chromosome
E103		+	NT		Chromosome
EV596		+	NT		Chromosome
D6		+	NT		Chromosome
EV638		+	NT		Chromosome
79-3		+	NT		Chromosome
EV147		+	NT		Chromosome
EV217-2		+	NT		Chromosome
W32944		−	E		Plasmid
E73		−	E		Plasmid
EV108		−	E		Plasmid
613		−	E		Plasmid
EV408		−	E		Plasmid
E71		−	E		Plasmid
Ned10		−	E		Plasmid
Ned2		−	E		Plasmid
EV419		−	E		Plasmid
V105		−	E		Plasmid
A-3-1		−	E		Plasmid
NZ10		−	E		Plasmid
A27		−	E		Plasmid
620		−	E		Plasmid
296		−	E		Plasmid
A25		−	E		Plasmid
EV447		−	E		Plasmid
EV632		−	E		Plasmid
NZ7		−	E		Plasmid
EV321		−	E		Plasmid
EV564		−	E		Plasmid
OG1		−	−		
Cytolysin region					
MMH594	+	+[b]		No	Chromosome
V583	−	+[b]		No	Chromosome
V586	−	+[b]		No	Chromosome
545	−	+		No	Chromosome
V105	+	−		Yes	Plasmid
Ned10	+	−		Yes	Plasmid
NZ4	+	−		No	Chromosome
E19	+	+		Yes	Chromosome
Ned2	+	−		Yes	Plasmid
D6	+	+		No	Chromosome
EV217-2	+	−		Yes	Plasmid
EV419	+	−		Yes	Plasmid
EV596	+	−		Yes	Plasmid
296	+	−		Yes	Plasmid
W32944	+	−		Yes	Plasmid
E99	+	+		No	Chromosome
YI6-1	+	−[b]		No	Chromosome

[a] See Materials and Methods. C, chromosomal; E, extrachromosomal; NT, not tested.
[b] Previously characterized.

using as probes sequences amplified from across the PAI from prototype isolate MMH594 (38). This analysis revealed a variety of hybridization patterns, indicating substantial heterogeneity within the PAIs of the strains queried. To determine the genetic basis for this heterogeneity, these strains were further investigated by microarray hybridization, using a custom oligonucleotide pathoarray designed for this study and also used in another study (39). The pathoarray hybridization results (Fig. 1), show the genetic basis for variability that occurs within the PAIs of the isolates tested.

Since the PAI was known to include sequences that also occur on plasmids in *E. faecalis* (38), it was important to verify

that these sequences were contiguous and incorporated into the PAI. These regions include sequences derived from a pAM373-like plasmid integrated into the prototype PAI (EF0005 to EF0033) and the cytolysin toxin operon (EF0042 to EF0049), both of which can occur extrachromosomally in some strains (4, 7). Verification of contiguity within the PAI was achieved by performing overlapping long-range PCR between putative PAI regions detected in strains being queried, using primers complementary to the PAI occurring in MMH594 (see Tables S1 and S2 in the supplemental material). Amplification primers were selected to amplify outward from the integrated pAM373 element in the prototype PAI (primers PAI28928/PAI35147) into flanking chromosomal sequences, such that an amplification product was generated only when the pAM373-related sequences were integrated within the PAI. This analysis revealed that some of the putative PAI sequences detected were not, in fact, located within the PAI in some strains. As further proof of either integration or PAI independence of detected pAM373-derived sequences, DNAs from strains that contained more than half of the pAM373 element by microarray analysis (36/53 strains) but did not give a PCR product indicating insertion of the plasmid into the PAI (22/36 strains) were digested with the homing restriction enzyme I-CeuI, analyzed by CHEF analysis, and probed with portions of pAM373 gene EF0025 to identify possible integration at another site (Table 2). This analysis confirmed that all isolates that did not yield PCR products indicative of integration of the pAM373 region were positive for the pAM373 probe in the extrachromosomal pool. These results indicate that the majority of isolates harboring pAM373-like genes by pathoarray hybridization possess this region as part of an extrachromosomal element.

For the cytolysin operon (EF0042 to EF0049), 17 of the 53 strains (including the MMH594 control) were positive for genes of this region by pathoarray analysis. Of the 17 *cyl*$^+$ strains, 3 (V583, V586, and 545) expressed no detectable cytolysin activity. Strains V583 and V586 had previously been shown to have deletions or disruptions in the cytolysin operon (38); however, no obvious lesion in the strain 545 cytolysin operon was detected by PCR analysis. Two additional isolates, D6 and W32944, expressed cytolytic activity but did not generate a positive signal for one or more cytolysin genes by pathoarray analysis. These were determined to be due to false-negative hybridizations, possibly stemming from observed sequence polymorphism (data not shown) and corrected in the analysis (see Table S3 in the supplemental material). Isolates positive for cytolysin genes by pathoarray analysis were tested for integration of the cytolysin operon within the chromosome by PCR using primers G5 (outside the *cyl* operon in the PAI) and cylR1F (within *cylR1*) (see Table S1 in the supplemental material). Cytolytic isolates were further evaluated for the ability to transfer cytolysin-expressing activity at high frequency to the recipient strain FA2-2 (or, in the case of YI-6, JH2SS) by mating in broth (Table 2), which is characteristic for plasmid mediation of this trait. Of the 14 cytolytic isolates, 9 readily transferred this ability by conjugation. One isolate, E19, yielded a PCR product indicating integration but nevertheless was able to transfer cytolysin activity through mating. To characterize the location of the cytolysin element in this strain, we digested E19 DNA with the restriction enzyme I-CeuI, separated the DNA by CHEF gel electrophoresis, and hybridized a blot to a labeled probe corresponding to the *cylB* gene. The *cylB* probe hybridized to a single chromosomal band in strain E19 and not to the extrachromosomal pool, demonstrating that the cytolysin operon is located only on the chromosome in this strain but, for reasons yet to be determined, also retains high-frequency transmissibility. Two additional isolates, YI6-1 and NZ4, were negative for integration of the cytolysin operon into the PAI using the PCR test performed but also did not transfer cytolysin by conjugation. YI6-1 was previously characterized as having the cytolysin determinant located on the chromosome (17). Whether it is integrated into another PAI-like element or into a defective, integrated plasmid remains unknown, as does the type of element harboring the cytolysin in strain NZ4.

Relationship between chromosomal divergence and PAI variability among isolates. To determine whether the PAI is evolving independently of the chromosomes of the strains tested, we characterized the host background by MLST (36) (Fig. 2A). MLST analysis showed that our collection consists of an assortment of both distantly related strains and several sequence types (STs) and clonal clusters (CCs) that are represented multiple times. In all, 29 distinct STs were identified, representing 24 different CCs. The relationship between isolates of our collection and the overall population of *E. faecalis* (the universe of currently known STs and CCs of the species [http://efaecalis.mlst.net/]) was determined by eBURST analysis (see Fig. S1 in the supplemental material). eBURST is an allele-based clustering method that identifies nonoverlapping CCs (groups of closely related genotypes sharing a recent common ancestor) and predicts patterns of evolutionary descent (13, 42). eBURST-based clustering of the MLST data supports the deduction that our collection consists of distantly related strains that do not share a recent common ancestor and belong to different CCs, as well as STs and CCs that are represented multiple times. For frequently recombining populations like *E. faecalis* (36), an allele-based phylogeny, such as eBURST, is usually more reliable than a sequenced-based phylogeny, although the sequenced-based tree in Fig. 2A is remarkably congruent with the eBURST-based clustering.

We next examined the arrangement of isolates based on the likeness of PAI gene content. To do so, we constructed a rooted maximum-parsimony tree based on the PAI content by comparative analysis of hybridization to the pathoarray, again with *E. faecalis* isolate D3 forced as the root (Fig. 2B). Comparison of the PAI contents among isolates of the same ST or CC reveals limited congruence between trees based on housekeeping gene sequence relatedness and PAI profile (presence and absence of PAI genes), indicating that strain backgrounds evolve differently and independently of the PAI. Exceptions where there is congruence are limited to closely related strains in CC 6 and CC 4, which all appear to have the majority of the island (note that the prototype island occurs in strain MMH594, which belongs to CC 6). The clear dissimilarity between the grouping of isolates by PAI content and MLST points to apparent differences in the evolutionary history of the PAI versus housekeeping alleles. Interestingly, the phylogenetic arrangement of isolates by PAI content partitioned the isolates into two major clusters, with a progression of gene content within these lineages (Fig. 2B). Cluster A contains the PAI prototype strain MMH594 and includes more known PAI

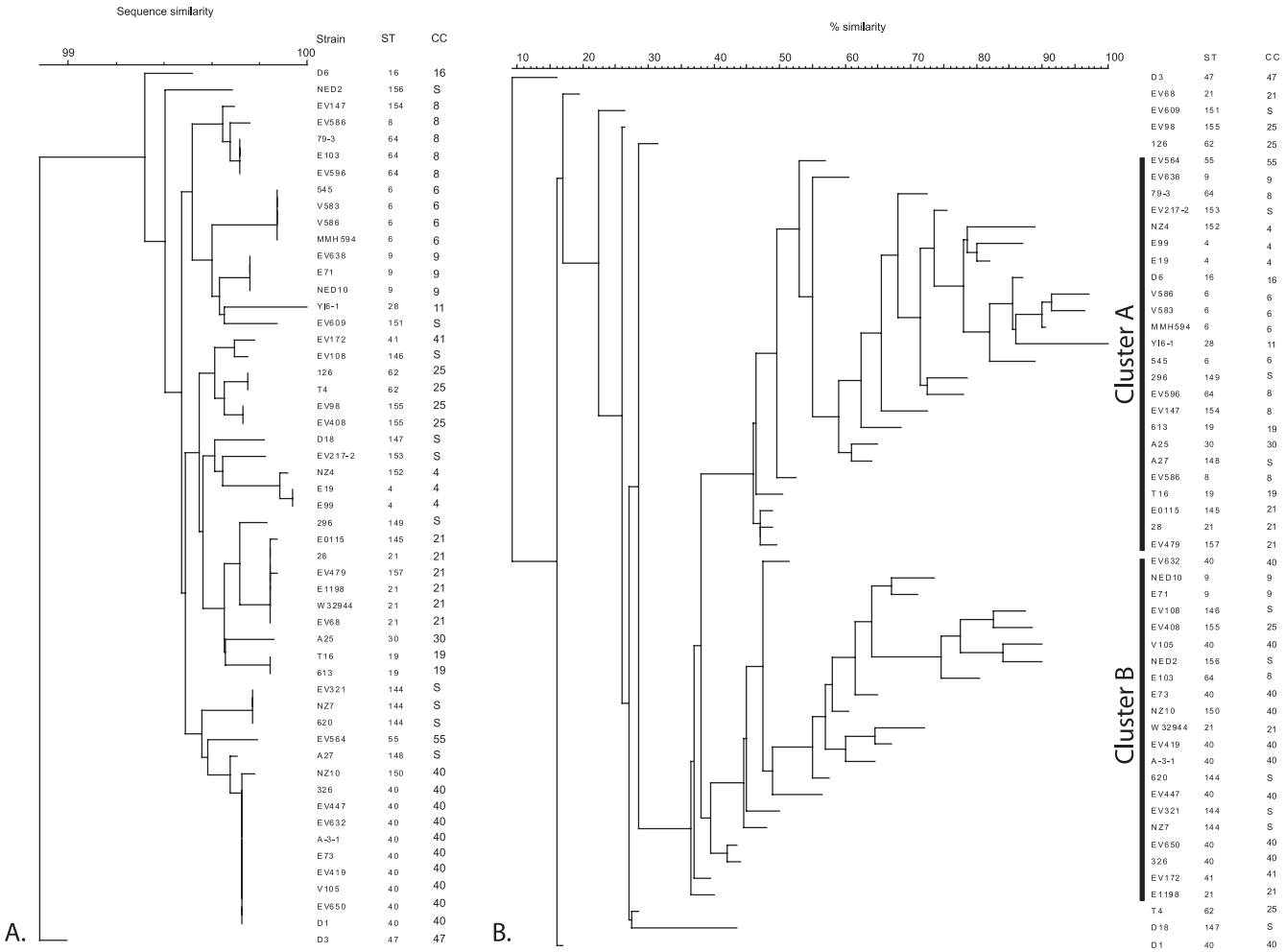

FIG. 2. (A) Rooted neighbor-joining tree of the concatenated sequences of all seven MLST genes of 53 *E. faecalis* isolates. The tree was constructed with BioNumerics 5.1 using the neighbor-joining algorithm with strain D3 forced as the root. Strain names, STs, and CCs based on clustering of allelic profiles using eBURST are indicated (S, single isolate [no CC]). (B) Phylogeny of 53 *E. faecalis* isolates based on PAI pathoarray hybridization data. The tree was constructed using BioNumerics 5.1 with the maximum-parsimony algorithm, and for comparison to panel A strain D3 was forced as the root. Strain names, STs, and CCs are indicated.

genes than isolates of cluster B. Cluster A also contains all isolates known to carry the end of the island that includes the *gls24*-like gene EF0117 (approximately EF0108 to EF0123). This region was previously implicated as a less common part of the island that, thus far, has been found only in strains isolated since 1980 (30). Since maximum-parsimony analysis of the PAI content creates a phylogenetic tree based on the least number of evolutionary changes, the two divergent clusters identified by maximum-parsimony analysis, combined with the progression of gene content within those clusters, provides evidence for a stepwise gain of PAI genes in the species.

Modular gain of elements into the PAI. Based on the incongruent topologies of PAI and MLST trees (Fig. 2), the variability within the PAI (Fig. 1), and the occurrence of several genes derived from known mobile elements, we propose a model where the PAI evolved within the species by the accretion of horizontally acquired segments rather than being acquired as one large element followed by divestiture of segments along with radiation into the species. To test this model, we performed a two-dimensional transversal clustering based on the pathoarray hybridization analysis of our isolates, to visually associate groups of PAI genes with groups of isolates (Fig. 3). In this analysis, the isolates are clustered based on their PAI profile, while the PAI genes are clustered by means of their value (absent or present) per isolate. This results in a matrix in which both the isolates and PAI genes are ordered according to their relatedness. For example, the first group of isolates, E1198, EV172, 326, and EV650, are separated from the others by the absence of PAI genes of regions A, D, B, and F but the presence of PAI genes of regions C and E. This analysis shows not only which strains group together by PAI profile but which genes cluster together as well. As a result, we can predict which genes were acquired simultaneously and how these regions may transfer between isolates or species. For example, PAI genes representing the pAM373-like element group together almost exclusively (region A), as do the cytolysin genes (region B). In all, we were able to identify six distinct clusters representing consecutive stretches of PAI genes (with

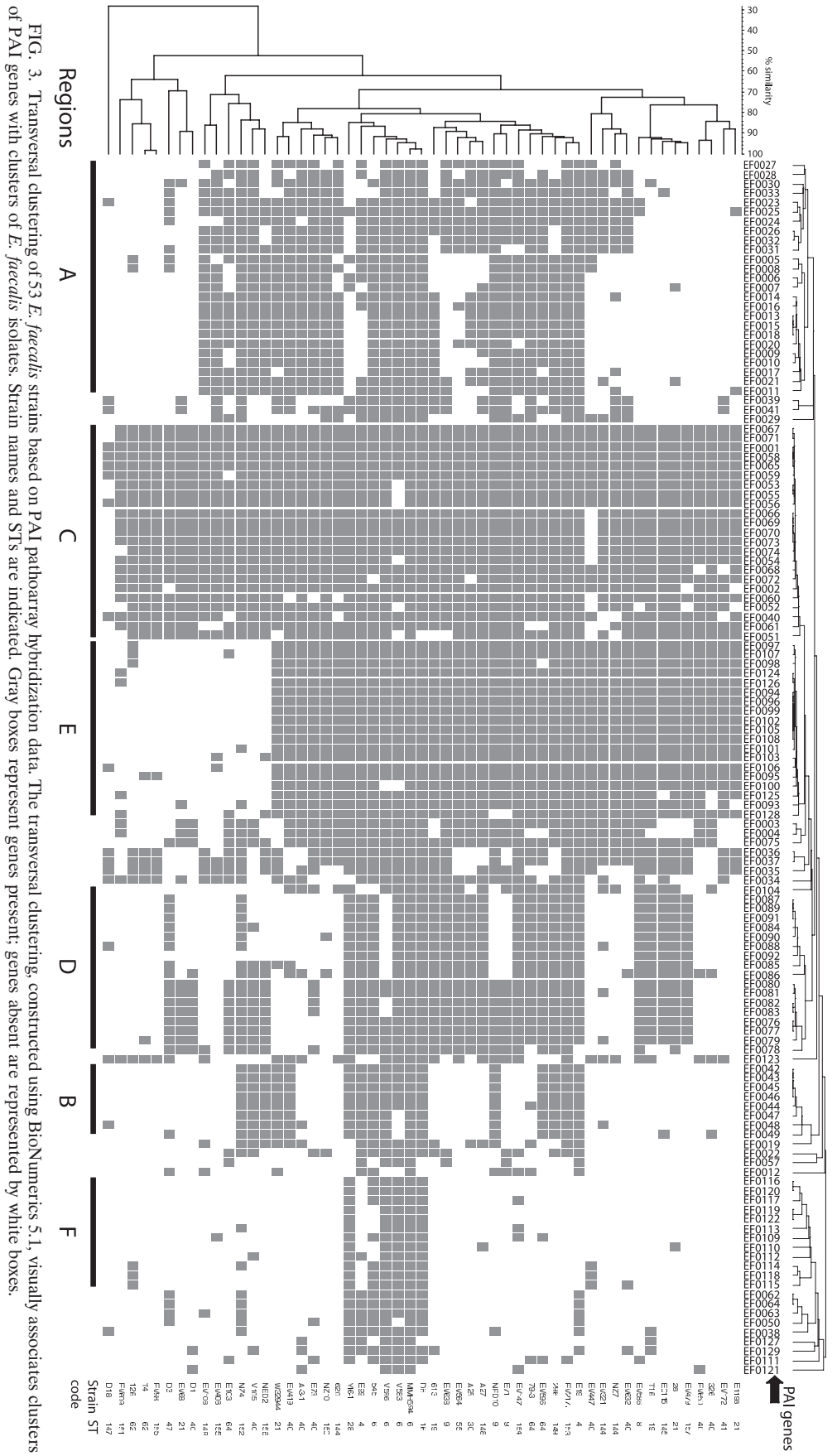

FIG. 3. Transversal clustering of 53 *E. faecalis* strains based on PAI pathoarray hybridization data. The transversal clustering, constructed using BioNumerics 5.1, visually associates clusters of PAI genes with clusters of *E. faecalis* isolates. Strain names and STs are indicated. Gray boxes represent genes present; genes absent are represented by white boxes.

the exception of region E) that appear to be inherited as units (regions A to F) (Fig. 3). The pAM373 and cytolysin elements could be anticipated to group together, but the other four regions do not exhibit sequence or organizational similarity to known genetic elements or gene clusters within enterococci or other species. However, their clustering indicates convergence into functional elements, which potentially were selected to enhance fitness in a specific, as-yet-unknown environment or niche.

In addition to identifying genes that may have entered the PAI together, the transversal clustering of PAI genes also gives clues as to how these segments entered. As previously mentioned, the PAI is littered with sequences that are related to mobile genetic elements (Fig. 1), and at least 13 potential mobile elements occur within the MMH594 PAI (38). We therefore examined each region to determine if it was bounded by putative IS or other mobile elements. Region A, the pAM373-like element, spans from EF0005 to EF0033 of the PAI and is flanked on either side by multiple genes with possible roles in mobility (Fig. 1). Close to the 5′ end of this region, as illustrated, are the integrase (EF0001) and excisionase (EF0002) genes, both of which are conserved variants of phage-encoded proteins involved in site-specific recombination. Immediately 3′ of the pAM373 element are two putative transposase genes: EF0034, which carries a point mutation, and EF0035, which has sequence similarity to IS*256* family transposases. All isolates tested positive for the integrase (EF0001) by pathoarray analysis, and all but four were positive for the excisionase (EF0002), while many isolates carried the transposase genes EF0034 and EF0035 (35 isolates were positive for each). Of the 14 strains that have pAM373 genes linked to the PAI (Table 2), 13 also have either EF0034 or EF0035.

Region B, which contains the cytolysin operon, extends from EF0042 to EF0049 of the PAI and is preceded by two transposase genes (EF0039 and EF0041). These transposons are situated on either side of the *cbh* gene (EF0040), which encodes a bile salt hydrolase (Fig. 1). All eight isolates that have cytolysin linked to their PAI are positive for both the EF0039 and EF0041 transposases, as well as the *cbh* gene. Though we did not separately test to determine if *cbh* is located in the PAI of every isolate, EF0039 and EF0041 transposase genes were found in all but two cytolysin-containing strains, including strains that possessed plasmid-encoded cytolysin operons. Thus, the EF0039 and EF0041 transposases may be involved in the cotransfer of *cbh* and the cytolysin operon, and the *cbh* and cytolysin genes may be related functionally.

Four additional regions (Fig. 3C to F) that had not been characterized previously as known functional elements were demarcated by transversal clustering, and each is represented by data from multiple isolates. Region C, which spans from EF0051 to EF0074, has several putative and uncharacterized genes, including transcriptional regulators, a lantibiotic resistance gene, a *gls24*-like stress response gene, phosphotransferase components, and the virulence-associated Esp surface protein (40). The *esp* gene has been reported to mobilize from the chromosome by conjugative transfer between *E. faecalis* and *E. faecium* isolates (33). That portions of region C are present in all isolates tested reflects the inherent selection bias in using *esp* positivity as the first criterion in detecting strains possessing the PAI for study. Region C also contains an internal transposase (EF0057) and two recombinases (EF0061 and EF0062) and is bordered on one side by a degenerate transposase, EF0075. Few isolates were positive for EF0057 and EF0062; however, almost all isolates tested positive for the recombinase EF0061 and the flanking transposase EF0075.

Immediately downstream of region C, region D extends from EF0076 to EF0092. Region D contains a cluster of sequences that have extensive homology to sugar uptake and metabolism genes. These genes appear to encode phosphotransferase modules, xylose utilization pathway components, and regulatory factors. This region is also bordered by the degenerate transposase EF0075.

Region E, which spans from EF0093 to EF108, includes several putative metal transporters, metal-dependent regulators, and genes encoding ribosomal proteins. The presence of a *cobW*-related gene within this group (EF0107) suggests a role in the synthesis of cobalamin (vitamin B_{12}). This suggests a potentially important role for this element in cofactor control of metabolism. Also grouping within this region are genes from the far 3′ end of the PAI, EF0124 through EF0128. Region E is present in 39 of the 53 isolates tested by pathoarray hybridization, yet (assuming region F, described below, is autonomous) there are no other mobile genes located within or immediately abutting this cluster that would indicate a mechanism for the transfer or integration of this region.

Region F, which is internal to region E, extending from EF0108 through EF0122, is the least common region found within our test group. This region is present in only seven isolates tested, four of which belong to CC 6. Region F includes three genes related to mobility: EF0111, a transposase gene with a frameshift mutation; EF0115, encoding a degenerate transposase; and EF0123, encoding an IS*200* family transposase. The EF0111 and EF0115 transposases cluster with genes of this region, as does the recombinase EF0062. The fact that this region and others are associated with specific mobile genes strongly suggests that these elements are involved in the transfer of these regions.

DISCUSSION

In the current work, we examined patterns of variation in the *E. faecalis* PAI to better understand how this element evolved and is transmitted among strains. Clustering PAI genes by their presence or absence yielded a comprehensive view of its organization (Fig. 3). This analysis led to the discovery of distinct genetic regions within the PAI that have entered into this element, which in most cases are associated with mobile genetic elements.

The PAI was found to be highly variable in gene content, although PCR evidence demonstrated that this element is organized in the same general pattern throughout the species as the PAI of the prototype strain MMH594 (see Table S2 in the supplemental material). The genetic content and organization of the PAI were found to correlate with genetic lineage in only a few, closely related isolates (e.g., those of CC 4 and CC 6). In general, however, the presence of specific PAI regions in different strains did not correlate closely with the predicted relatedness of strains discerned by MLST analysis of housekeeping genes. This indicates that although recombination is a key

factor in the diversification of the *E. faecalis* core genome (36), suggesting a relatively quickly evolving core genome, the *E. faecalis* PAI is evolving even faster, most likely by the accretion of mobile elements as evidenced by the occurrence of multiple PAI variants in a single ST. These results are in agreement with previous reports of variation in PAI content between isolates (1, 30). Maximum-parsimony analysis does not agree with a model that the entire PAI entered the species in an ancestral strain, with segments subsequently deleted in certain lineages as the species diverged (32). In fact, several strains tested by PCR with primers specific for the MMH594 PAI gave larger-than-expected products, suggesting that there may be a number of genes in the PAIs of other isolates that are not in the prototype island and are not widely distributed through the species (data not shown). Altogether, the distribution of PAI genes in different lineages and the composite nature of the island point to a model in which core elements of the PAI radiated outward throughout the species and, in the process, the PAI accrued new traits and continues to evolve through the deletion and addition of genes and through recombination.

Enterococci are well known for their ability to acquire genes, such as antibiotic resistance determinants, and disseminate them through horizontal transfer (31, 45, 48). Transposase genes and IS elements are commonly found on plasmids isolated from *E. faecalis*, and mobile DNA has long been thought to play a key role in the evolution of enterococci (23, 34). Previous studies demonstrated that transfer of some genes of the PAI can occur between *E. faecalis* isolates, as well as between *E. faecalis* and *E. faecium* through conjugative mechanisms (6, 33). The present study defined groups of genes that have been incorporated into the PAI and provides evidence for the acquisition of these segments as mobile functional elements. Though this investigation did not explore the transfer of the PAI genes between isolates, the delineation of these regions may allow more targeted study of transfer focusing on movement of specific regions of the PAI. Closer examination of these regions as functional units may provide clues to their contributions to fitness and their evolutionary origins. Applying this approach to the analysis of PAIs in other species may reveal new insights into their evolution and a greater understanding of how these elements transfer between organisms as well.

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