VanA-Type Enterococci from Humans, Animals, and Food: Species Distribution, Population Structure, Tn*1546* Typing and Location, and Virulence Determinants[∇]

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VanA-type human ($n = 69$), animal ($n = 49$), and food ($n = 36$) glycopeptide-resistant enterococci (GRE) from different geographic areas were investigated to study their possible reservoirs and transmission routes. Pulsed-field gel electrophoresis (PFGE) revealed two small genetically related clusters, M39 ($n = 4$) and M49 ($n = 13$), representing *Enterococcus faecium* isolates from animal and human feces and from clinical and fecal human samples. Multilocus sequence typing showed that both belonged to the epidemic lineage of CC17. *purK* allele analysis of 28 selected isolates revealed that type 1 was prevalent in human strains (8/11) and types 6 and 3 (14/15) were prevalent in poultry (animals and meat). One hundred and five of the 154 VanA GRE isolates, encompassing different species, origins, and PFGE types, were examined for Tn*1546* type and location (plasmid or chromosome) and the incidence of virulence determinants. Hybridization of S1- and I-CeuI-digested total DNA revealed a plasmid location in 98% of the isolates. Human intestinal and animal *E. faecium* isolates bore large (>150 kb) *vanA* plasmids. Results of PCR-restriction fragment length polymorphism and sequencing showed the presence of prototype Tn*1546* in 80% of strains and the G-to-T mutation at position 8234 in three human intestinal and two pork *E. faecium* isolates. There were no significant associations ($P > 0.5$) between Tn*1546* type and GRE source or enterococcal species. Virulence determinants were detected in all reservoirs but were significantly more frequent ($P < 0.02$) among clinical strains. Multiple determinants were found in clinical and meat *Enterococcus faecalis* isolates. The presence of indistinguishable *vanA* elements (mostly plasmid borne) and virulence determinants in different species and PFGE-diverse populations in the presence of host-specific *purK* housekeeping genes suggested that all GRE might be potential reservoirs of resistance determinants and virulence traits transferable to human-adapted clusters.

Enterococci are gram-positive, opportunistic bacteria that inhabit the gastrointestinal tracts of humans and many animals. They are also present in food, as starter cultures for the production of cheese and fermented sausages or as fecal contaminants of raw meat, milk, and milk products. Some specific strains are available as probiotics in animal feeds (20, 22). However, enterococci have gained notoriety as a major cause of nosocomial infection and are increasingly isolated from the bloodstream, urinary tract, and surgical sites. *Enterococcus faecalis* causes 80 to 90% of human enterococcal infections, and *Enterococcus faecium* causes most of the remaining cases (other enterococcal species being infrequently involved) (27). The emergence of multidrug resistance (i.e., resistance to multiple antibacterial agents), including high-level resistance to glycopeptides, among enterococci, particularly *E. faecium*, has resulted in clinical isolates resistant to all antibiotics of proven efficacy (7). Glycopeptide-resistant enterococci (GRE), which have emerged as nosocomial pathogens in the past 10 to 15 years (7), are a global problem despite major epidemiological differences between Europe and the United States. A high frequency of GRE has been reported in hospitals in the United States, whereas extremely low frequencies have been reported in the community, in animals, and in food of animal origin (7, 12, 29, 39, 51).

Results of studies conducted in northern European countries have revealed a low overall prevalence of GRE infection in Europe, with GRE being detected mostly in the nonhospitalized healthy population and among animals (7, 51). However, their incidence in clinical infections has been rising in southern Europe (Portugal, Greece, and Italy) and, though remaining generally lower that those described in the United States (European Antimicrobial Resistance Surveillance System, http://www.earss.rivm .nl, last accessed 10 October 2005), rates of 20% for clinical infections and of 7.5% for intestinal colonization in at-risk hospital wards have been reported in Italy (25).

The spread of GRE was linked to the use of the glycopeptide avoparcin as a growth promoter in animal husbandry (37, 51), until its ban in the European Union in 1997. Despite indirect evidence for dissemination to humans of glycopeptide resistance selected in animals by clonal spread or horizontal resistance gene transfer (51), in Italy and other southern European countries GRE isolation has so far been reported mostly in hospital settings (7, 25; http://www.earss.rivm.nl), whereas relatively few data are available with regard to isolation in the community and nonhuman sources (5, 7, 15).

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TABLE 1. Strains collected in this study

Strain and origin (n)	Source (n)	Area	Yr	Isolate(s)
Italian strains				
Human intestinal (43)	Inpatients[a] (37)	North	1997	VI1, VI46
			2002	MI24, MI25, MI26, MI28, MI29, MI30, MI31, MI32, MI33, MI34, MI54, MI55, MI56, MI57, MI58, MI59, MI60
		Center	1997	AN8
			2002	AN7, AN9, AN12, AN13, AN14, AN15, AN16, AN17, AN19, AN20, AN23, AN47, AN48, AN49, AN50, AN51, AN52
	Outpatients (6)	North	2002	MI27
		Center	2002	AN10, AN11, AN18
			2003	AN53, AN61
Animal (43)	Poultry (38)	North	1997	PD1, VI2, PD3, VI4, VR5, PD6, PD7, VR8, VI9, VI10, VI11, VR12, PD13, PD33, PD34, PD35, PD36, PD37, PD38, PD39, PD40, PD41, PD42
			2001	VE31, VE44, VE45, VE46, VE47, VE50
		Center	2003	AN14, AN25, AN26, AN27, AN28, AN29, AN30, AN43, AN49
	Pig (5)	Center	2003	AN21, AN22, AN23, AN24, PG15
Food (36)	Poultry meat (27)	North	1998	PM3, PM12, PM13
			2000	PM14, PM15, PM16, PM17, PM18, PM19, PM20, PM21, PM22, PM23, PM24, PM25, PM31, PM32
			2001	PM26, PM27, PM28, PM33
		Center	1998	PM10, PM11
			2003	PM2, PM30, PM35, PM36
	Pork meat (7)	Center	2003	KM1
		South	1998	KM4, KM5, KM6, KM7, KM8, KM9
	Cheese (2)	North	2003	CH29, CH34
Human clinical (21)	Blood (10)	North	1997	VI4, MI66
			1998	MI65
		Center	1998	R35, AN64
			2002	R36, R39, R40
			2003	AN113, AN338
	Urine (9)	North	1997	VI2, VI3, UD5
			2001	GE69
		Center	1997	AN21, AN67
			1998	AN22
			2002	R38, AN68
	Wound (1)	Center	2002	R37
	Bile (1)	North	1997	UD6
Non-Italian strains				
Human intestinal (5)	Community	Norway	1998	HI-N41 to HI-N45 (31)
Animal (6)	Poultry	Norway	1998	A-N16 to A-N20 (31)
		Belgium	1997	FAIR-E-16210 (EU-FAIR project CT97-3078)

[a] No enterococcal infection.

The VanA phenotype, expressing inducible, high-level vancomycin and teicoplanin resistance, is the most common in Europe (3, 5, 25, 29, 37). The *vanA* cluster—detected primarily in *E. faecium* and *E. faecalis* and less frequently in other enterococcal species—is carried by Tn*1546* and is transferable by conjugation (2, 9). Considerable heterogeneity may exist among Tn*1546* elements, largely resulting from the presence of insertion sequences or from deletions in nonessential genes and intergenic regions (32, 51, 57).

The pathogenesis of enterococcal infections is still poorly understood, although several virulence factors, such as aggregation substance(s) (AS), gelatinase (Gel), cytolysin (Cyl), and enterococcal surface protein (Esp), have been described (24, 27). AS are pheromone-inducible surface proteins of *E. faecalis* that facilitate the conjugative exchange of plasmids (carrying virulence and/or antibiotic resistance genes) and also contribute to pathogenicity by enhancing adhesion to and internalization by cultured human cells, as well as favoring intracellular survival within macrophages (10, 52, 55). Although sex pheromone plasmids are highly specific for *E. faecalis*, they have also been detected in vancomycin-resistant *E. faecium* strains (28, 40). Gel, a secreted Zn metalloprotease, and Cyl, a lytic toxin, have been implicated in the pathogenicity of *E. faecalis* on the basis of both epidemiological data and studies of animal models (24, 27, 48). Esp is a surface protein involved in the ability to colonize and in immune evasion in *E. faecalis* and *E. faecium* (21, 24). Enterococci are also known to produce slime (17, 18) and to form biofilms, which have been regarded as virulence features of clinical isolates (16, 18).

TABLE 2. Control strains used in the study

Strain	Purpose	Reference or source
E. faecium BM4147	Glycopeptide resistance genotype, Tn*1546* typing, probe synthesis and *ddl* PCR; *vanA* control strain	38
E. faecalis V583	Glycopeptide resistance genotype, *ddl* PCR; *vanB* control strain	45
E. gallinarum ATCC 49573	Glycopeptide resistance genotype, *ddl* PCR; *vanC-1* control strain	ATCC
E. casseliflavus ATCC 14432	Glycopeptide resistance genotype, *ddl* PCR; *vanC-2* control strain	ATCC
E. durans ATCC 19432	*ddl* PCR	ATCC
E. faecalis OG1RF(pCF10)	Clumping, aggregation substance identification; sex pheromone plasmid-harboring strain	10
E. faecalis OG1RF(pAD1)	Clumping, aggregation substance identification; sex pheromone plasmid-harboring strain	10
E. faecalis JH2-2	Sex pheromone production	40
E. faecalis OG1RF	Sex pheromone production	40

Several reviews have addressed the genetic basis, reservoirs, and spread of glycopeptide resistance in enterococci (7, 9, 51) and enterococcal virulence (24, 27, 47). A combination of glycopeptide resistance and virulence in enterococci could pose a serious threat to human health. However, data on the presence of virulence traits in GRE are scarce (42, 44).

The present study was undertaken to explore the relatedness of GRE of different origins (human, animal, and food) and from different geographic areas to gain a better understanding of the involvement of the different reservoirs in the emergence and spread of virulent clones, i.e., those that in addition to antibiotic resistance have also acquired a number of genes conferring infectivity and virulence. To do this, human, animal, and food GRE were analyzed for population structure (using pulsed-field gel electrophoresis [PFGE], *purK* allele sequence analysis, and multilocus sequence typing [MLST]) and Tn*1546* type and location (chromosome or plasmid) as well as for the presence and expression of the main virulence determinants.

MATERIALS AND METHODS

Bacterial strains and media. We studied a total of 154 VanA-type GRE from humans ($n = 69$), animals ($n = 49$), and food ($n = 36$). See Table 1 for details. Most of the strains ($n = 142$) were collected throughout Italy between 1997 and 2003 (64 human, 42 animal, and 36 food isolates). The 11 non-Italian GRE isolates were from Belgium ($n = 1$) and Norway ($n = 10$); of these, five were human intestinal and six were animal isolates. Reference and control strains are listed in Table 2.

GRE were isolated on Slanetz-Bartley agar (Becton Dickinson, Milan, Italy) containing 6 µg/ml vancomycin. Fecal and meat homogenates were previously enriched in selective tryptone soy broth (Oxoid, Basingstoke, United Kingdom) containing 0.4 mg/ml sodium azide and 6 µg/ml vancomycin. Brain heart infusion broth and agar (Oxoid) were used for routine culture. Tryptone soy broth supplemented with 1% glucose was used for slime production assays. Gelatin infusion broth containing 40 mg/ml gelatin (Bio-Rad Laboratories, Richmond, CA) was used to determine gelatinase production. Blood agar base (Oxoid) supplemented with fresh horse blood (5%) was used to investigate hemolysin production. Isolates were maintained in glycerol at −70°C and subcultured twice on Slanetz-Bartley agar before testing.

Species identification and PFGE typing. GRE were identified at the species level with API ID32-STREP kits (bioMérieux Italia, Rome, Italy) and additional biochemical tests (22) and species-specific enterococcal *ddl* PCR performed with the primers listed in Table 3. PFGE of SmaI (New England Biolabs, Beverly, MA)-digested total DNA was performed essentially as described previously (3) using a CHEF Mapper apparatus (Bio-Rad) with pulse time increasing from 1 to 20 s over 20 h at 200 V (6 V/cm). Genetic relatedness was interpreted according to the method of Tenover et al. (53). Strains differing by six or fewer bands were grouped into the same PFGE type (1, 2, 3, etc.) and subdivided into PFGE subtypes (1a, 1b, 1c, etc.) based on single-band differences. PFGE data were analyzed separately for each species, considering each band as a separate putative locus and scoring it as present (1) or absent (0) in each accession. Dendro-

grams were constructed by the use of the Dice coefficient and the unweighted-pair group method using arithmetic averages.

MLST. MLST was performed as described by Homan et al. (30) and included the genes *purK*, *adk*, *atpA*, *ddl*, *gdh*, *gyd*, and *pstS*. Sequence types (ST) were obtained from the MLST database at http://www.mlst.net. PCR was performed in a Gene Amp PCR system 2400 thermal cycler (Applied Biosystems, Foster City, CA). Amplification reactions were carried out in a 50-µl final volume containing 2.5 U AmpliTaq Gold (Applied Biosystems). Amplified fragments were purified from the reaction mix using a Montage PCR purification kit (Millipore Corporation, Bedford, MA). Sequencing was performed using ABI Prism (Applied Biosystems) with dye-labeled terminators; sequences were analyzed by the ClustalW method available at http://align.genome.jp.

Detection of glycopeptide resistance and virulence genes. Total DNA extraction was done as described previously (3). Vancomycin resistance and virulence genes were detected by PCR using a Hybaid PCR Express thermal cycler (Hybaid Ltd, Ashford, United Kingdom). Primers and target genes are listed in Table 3. Virulence genes were detected using primers internal to (i) highly conserved regions in the AS genes of pAD1, pPD1, and pCF10 of *E. faecalis*, (ii) *asa373* of pAM373, (iii) *gelE*, (iv) *cylB*, and (v) *esp*. EcoRI digestion of AGG amplicons was performed according to the manufacturer's instructions (Roche Molecular Biochemicals, Mannheim, Germany), and restriction fragments were separated by 2.0% agarose gel electrophoresis.

***vanA* gene location.** The plasmid or chromosomal location of *vanA* was assessed using three different methods: (i) *vanA* hybridization of plasmid content extracted by an alkaline lysis method; (ii) *vanA* hybridization of S1-digested total DNA; (iii) and *vanA* hybridization of I-CeuI-digested total DNA. The first two methods were performed as described previously (23) using a biotin-labeled *vanA* probe and a BrightStar BioDetect kit (Ambion, Huntingdon, United Kingdom). *vanA* hybridization of I-CeuI-digested total DNA was performed essentially as described previously (33). DNA was digested with 5 U of I-CeuI (New England Biolabs), separated by PFGE, transferred onto a nylon membrane, and hybridized sequentially with 16S rRNA gene and *vanA* biotin-labeled DNA probes.

Molecular analysis of Tn*1546*-like elements. The structure of Tn*1546*-like elements was analyzed by PCR and amplicon restriction analysis (PCR-restriction fragment length polymorphism) essentially as described by Palepou et al. (43). Long PCR was performed using TaKaRa Ex *Taq* (Cambrex Bio Science, Milan, Italy) and a Hybaid PCR Express thermal cycler. Primers (sequence and position) and target genes are listed in Table 3. Long PCR amplicons of the whole Tn*1546* were analyzed by digestion with ClaI, whereas amplicons of the *orf2-vanX* region were digested with DdeI to detect the point mutation at position 8234 (31). Strains giving different results from the prototype were amplified using primer pairs targeting the left (*orf1-orf2*) and right (*vanX-vanZ*) region of Tn*1546*.

Phenotypic assays. Clumping assays were performed as described previously (40). Production of Gel was determined as described previously (11). For β-hemolysis detection, strains were grown on horse blood agar plates for 1 to 2 days at 37°C. Biofilm formation was tested using the slime production assay described previously (17).

Statistical analysis. The prevalence of the different species, different Tn*1546* types, and virulence traits in the various reservoirs were compared using Fisher's test. Statistical analysis was performed with the S-PLUS 6 statistical program (S-PLUS 6.1 for Windows, Professional Edition, release 1). A P of <0.05 was regarded as statistically significant.

TABLE 3. PCR primers and products

Purpose and primers	Gene target(s)	Primer sequence (5′–3′)	Position[a]	Product length (bp)	Reference
Identification and 16S probe					
HiF	$ddl_{E. hirae}$	TTATGTCCCTGTTTTGAAAAA	485–506[b]	378	36
HiR		TTTTGATAGACCTCTTCCGGT	868–845[b]		
DuF	$ddl_{E. durans}$	TTATGTCCCAGTATTGAAAAA	485–506[b]	189	36
DuR		TGAATCATATTGGTATGCAGT	649–672[b]		
DDLM1	$ddl_{E. faecium}$	TAGAGACATTGAATATGCC	359–377	528	19
DDLM2		CTAACATCGTGTAAGCT	887–870		
DDLS1	$ddl_{E. faecalis}$	ATCAAGTACAGTTAGTCT	98–116	941	19
DDLS2		ACGATTCAAAGCTAACTG	1038–1021		
P1	rRNA gene 16S	GCGGCGTGCCTAATACATGC	39–58[b]	957	35
16S1		TGCATTAGCTAGTTGGTGAGG	240–260[b]	726	14
16S2[c]		TCGAATTAAACCACATGCTCC	964–944[b]		14
Glycopeptide resistance genotype					
VANA1	$vanA$	GGGAAAACGACAATTGC	175–191	732	19
VANA2		GTACAATGCGGCCGTTA	907–891		
VANB	$vanB$	ATGGGAAGCCGATAGTC	173–189	635	19
VANB2		GATTTGCTTCCTCGACC	807–791		
VANC1-1	$vanC1$	GGTATCAAGGAAACCTC	246–272	822	19
VANC1-2		CTTCCGCCATCATAGCT	1067–1051		
VANC2/3-1	$vanC2/3$	CTCCTACGATTCTCTTG	455–486	439	19
VANC2/3-2		CGAGCAAGACCTTTAAG	885–869		
Tn1546					
IR	Tn1546	GGAAAATGCGGATTTACAACGCTAAG	13–38, 10814–10839	10,826	43
INV2	$orf2$-$vanX$	ATGAGGTGATATTTTGCGGAAA	3174–3195*	5,405	23
VANX2		CTATTGGGGTATGGTTCGTCT	8579–8599*		4
ORF1A	$orf1$	AGGGCGACATATGGTGTAACA	170–190*		41
ORF1B	$orf1$	TGGTGGCTCCTTTTCCCAGTTC	907–928*		41
ORF1C	$orf1$	ACCGTTTTTGCAGTAAGTCTAAAT	1871–1894'*		41
ORF2R	$orf2$	TTTCCGCAAAATATCACCTCAT	3195–3174*		This study
INV3	$vanX$-$vanZ$	AGACGAACCATACCCCAATAG	8578–8596*	1,999	23
VANZ1		GGTACGGTAAACGAGCAATAATA	10577–10555*		4
Virulence factors					
AGG1	$prgB$, $asaI$, $aspI$	AAGAAAAAGAAGTAGACCAAC	601–622	1,555	20
AGG2		AAACGGCAAGACAAGTAAATA	2156–2133		
ASA373F	$asa373$	GGACGCACGTACACAAAGCTAC	3094–3115	619	13
ASA373R		CTGGGTGTGATTCCGCTGTTA	3693–3713		
GELE1	$gelE$	ACGCATTGCTTTTCCATC	729–746	419	20
GELE2		ACCCCGTATCATTGGTTT	1148–1129		
CYLB1	$cylB$	ATTCCTACCTATGTTCTGTTA	1199–1219	843	20
CYLB2		AATAAACTCTTCTTTTCCAAC	2041–2021		
ESP1	esp	TTGCTAATGCTAGTCCACGACC	1217–1238	932	20
ESP2		GCGTCAACACTTGCATTGCCGA	2149–2128		

[a] Positions are from the first base of the coding sequence, except for positions marked with asterisks, which are from the first base of IR_L of Tn1546.
[b] *Escherichia coli* numbering.
[c] The P1-16S2 pair was used for species identification; the 16S1-16S2 pair was used for probe synthesis.

RESULTS

Species and glycopeptide resistance genotype identification. Of the 154 GRE, there were 120 *E. faecium*, 18 *E. durans*, 12 *E. faecalis*, and 4 *E. gallinarum*. Species prevalence in the different reservoirs is reported in Table 4. As expected, *E. faecium* was always the prevalent species ($P < 0.02$), followed by *E. faecalis* (among human specimens) and by *E. durans* (among nonhuman samples). *E. faecalis* was not found among animal nor *E. durans* among human samples. *E. faecalis* was more frequently recovered from the clinical than the other reservoirs ($P < 0.05$). Among the other reservoirs (i.e., animal, food, and human intestinal), the sole significant difference in its frequency was between food and animal samples ($P = 0.03$).

The results of multiplex PCR targeting *vanA*, *vanB*, and *vanC* showed that all isolates carried the *vanA* gene, including the four *vanC-1 E. gallinarum*.

PFGE typing. All isolates were PFGE typed after SmaI digestion of total DNA, yielding 69 different PFGE types (*E.*

TABLE 4. Species distribution in the different reservoirs

Origin	No. of isolates			
	E. faecium	*E. faecalis*	*E. durans*	*E. gallinarum*
Human intestinal	47	1	0	0
Animal	35	0	12	2
Food	24	4	6	2
Human clinical	14	7	0	0

A

E. faecium (120)
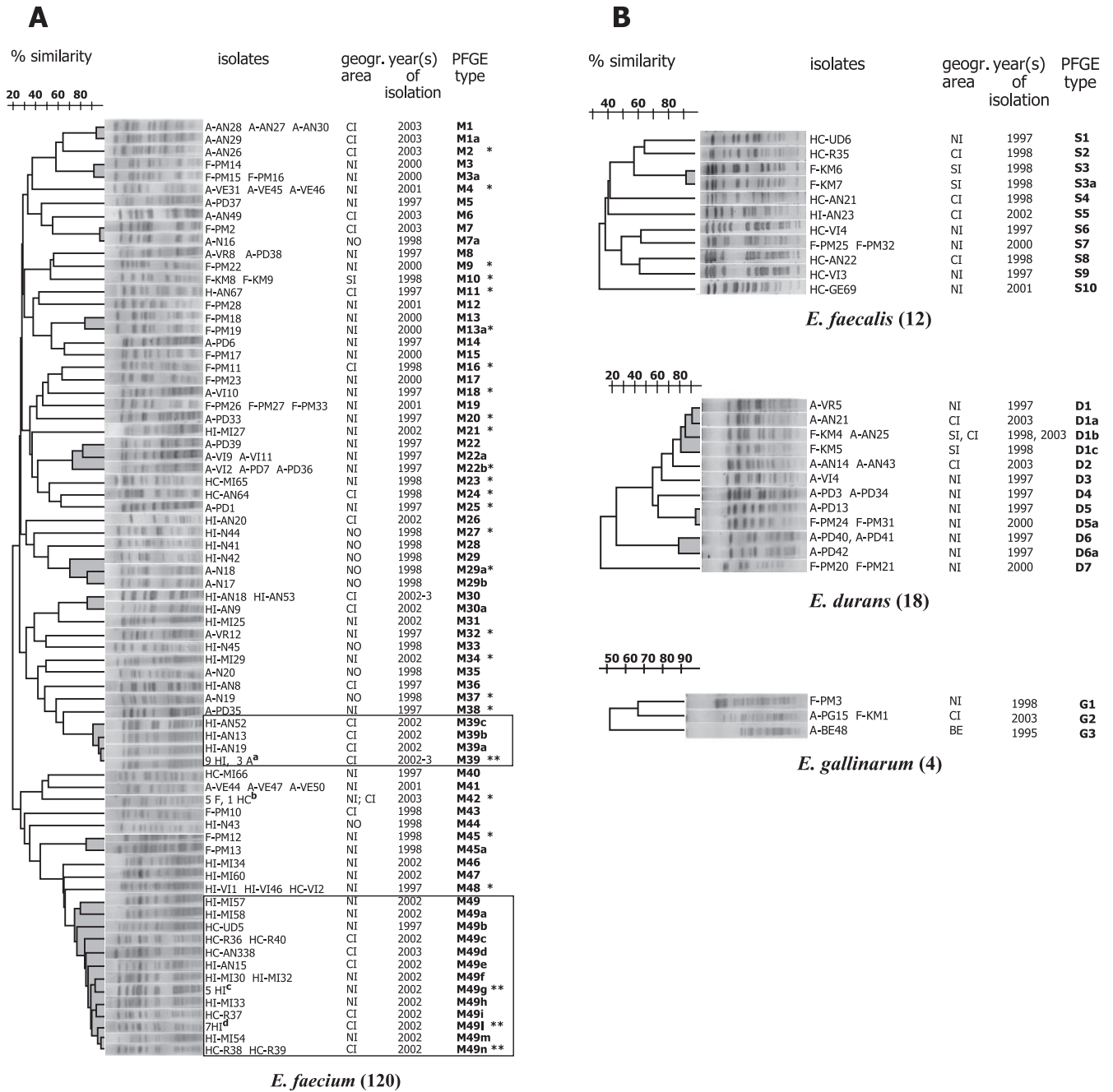
[a] HI-AN10, HI-AN11, HI-AN12, HI-AN17, HI-MI26, HI-AN49, HI-AN50, HI-AN51, HI-AN61, A-AN22, A-AN23, A-AN24

[b] F-CH29, F-PM30, F-CH34, F-PM35, F-PM36, HC-AN113

[c] HI-MI28, HI-MI31, HI-MI55, HI-MI56, HI-MI59

[d] HI-AN7, HI-AN14, HI-AN16, HI-MI24, HI-AN47, HI-AN48, HC-AN68

* *purK* allele determined; ** Sequence Type determined

B

E. faecalis (12)

E. durans (18)

E. gallinarum (4)

FIG. 1. Dendrograms showing the similarity index among the 154 isolates of *E. faecium* (A) and *E. faecalis*, *E. durans*, and *E. gallinarum* (B). Clusters sharing ≥70% similarity are shown in gray. A, animal isolate; F, food isolate; HC, human clinical isolate; HI, human intestinal isolate. CI, central Italy; NI, northern Italy; SI, southern Italy; NO, Norway; BE, Belgium. PFGE types showing a clonal spread are boxed.

faecium M1 to M49, *E. faecalis* S1 to S10, *E. durans* D1 to D7, and *E. gallinarum* G1 to G3) and 30 PFGE subtypes. Results are represented in four dendrograms, one per species (Fig. 1).

Ten clusters of *E. faecium*, one of *E. faecalis*, and three of *E.*

durans were evidenced. Two *E. faecium* PFGE types (M39 and M49) provided evidence of clonal spread; type M39 ($n = 15$) was isolated from both human ($n = 12$, in- and outpatients) and animal ($n = 3$, pig) samples, whereas type M49 ($n = 26$)

TABLE 5. *purK* allele in 28 strains of different origins and PFGE types

Origin and strain	Source	PFGE type	*purK* allele
Human intestinal			
HI-VI1	Inpatient	M48	1
HI-MI28	Inpatient	M49g	1
HI-MI29	Inpatient	M34	1
HI-AN47	Inpatient	M49l	1
HI-AN10	Community	M39	1
HI-MI27	Community	M21	6
HI-N44	Community	M27	6
Animal			
A-PD1	Poultry	M25	2
A-VI2	Poultry	M22b	3
A-VI10	Poultry	M18	3
A-VR12	Poultry	M32	3
A-N18	Poultry	M29a	6
A-N19	Poultry	M37	6
A-AN26	Poultry	M2	6
A-VE31	Poultry	M4	6
A-PD33	Poultry	M20	6
A-PD35	Poultry	M38	6
A-AN23	Pig	M39	1
Food			
F-PM11	Poultry	M16	3
F-PM12	Poultry	M45	6
F-PM19	Poultry	M13a	6
F-PM22	Poultry	M9	6
F-PM30	Poultry	M42	6
F-KM9	Pork	M10	9
Human clinical			
HC-AN64	Blood	M24	1
HC-BG65	Blood	M23	1
HC-R38	Urine	M49n	1
HC-AN67	Urine	M11	3

was isolated from inpatients (both fecal [*n* = 19] and clinical [*n* = 7] samples). Six additional PFGE types were recovered from different sources: M7, D1, D5, and G2 from both animals and food, M29 from both human intestine and animals, and M42 from both human clinical and food (meat and cheese) samples. Type M30 was detected in fecal samples from both in- and outpatients.

With regard to geographic spread, type M7 was recovered from two isolates, one from Italy (poultry meat) and one from Norway (chicken feces), type D1 was collected throughout Italy, type D5 in northern Italy, types M39, G2, and M30 in central Italy, type M29 in Norway, and types M49 and M42 in central and northern Italy.

***purK* allele analysis.** *purK* allele polymorphisms were determined in 28 isolates of different origins (5 clinical, 6 human intestinal, 11 animal, and 6 food) and PFGE types. Five *purK* alleles were found, with types 1, 6, and 3 being detected in multiple strains (Table 5). Type 1 was found mostly in human strains (3/5 clinical and 5/6 intestinal), type 6 in poultry (6/10 animal and 4/5 meat), and type 3 in poultry (3/10 animal).

MLST. To gain a better understanding of the clonal lineage of the two major PFGE clusters, M39 and M49, MLST was performed on five isolates representing different reservoirs, two from cluster M39 (HI-AN10 and A-AN23) and three from

M49 (HI-MI28, HI-AN47, and HC-R38, subtype M49g, M49l, and M49n, respectively). ST 18 was found in the two M39 isolates (one from outpatient feces and one from a pig), and ST 78 was found in the three M49 isolates (two from inpatient feces and one clinical).

One hundred and five isolates (9 *E. faecalis*, 78 *E. faecium*, 14 *E. durans*, and 4 *E. gallinarum* strains), encompassing different origins and PFGE types, were selected for further studies, i.e., *vanA* gene location, molecular analysis of Tn*1546* elements, and virulence traits.

***vanA* gene location.** Hybridization of plasmid content following alkaline lysis extraction demonstrated a plasmid location of *vanA* in 77 of the 105 strains (Fig. 2). Fifty-four isolates, including the 28 that did not hybridize and 26 isolates showing a positive reaction, were subjected to *vanA* hybridization of S1-digested total DNA, which allows better identification of high-molecular-weight plasmids. A plasmid location of *vanA* was demonstrated with this method in 24 of the previously negative 28 isolates and was confirmed in all of the 26 positive ones. The size of the *vanA*-carrying plasmids ranged from 150 kb to 250 kb in the former and from 25 kb to 150 kb in the latter isolates. The same 54 isolates were then analyzed by sequential hybridization of I-CeuI-digested total DNA with 16S rRNA gene and *vanA* probes. All tested strains hybridized with the 16S rRNA gene probe, and seven strains (*E. faecium* HI-MI30, HI-MI25, HI-MI31, HI-MI32, and HI-MI60 and *E. gallinarum* A-BE48 and F-PM3) also hybridized with the *vanA* probe, demonstrating a chromosomal location of the *vanA* gene. In S1 digestion experiments, none of the *E. gallinarum* isolates hybridized with the *vanA* probe, whereas in all of the five *E. faecium* isolates, *vanA* was detected on a 240-kb plasmid, thus demonstrating the presence of two copies of *vanA* (Fig. 3). In *E. faecium* HI-AN20 and HI-AN15, the *vanA* location could not be assessed with any experimental approach.

Molecular analysis of Tn*1546*-like elements. The 105 *vanA* isolates were analyzed for the structure of the Tn*1546* element and assigned to 12 different groups (Fig. 4). Overall, PCR experiments with primer IR gave a positive result in 93 isolates. In 83 isolates, they yielded a single amplicon identical in size to the prototype Tn*1546* element, as also confirmed by ClaI restriction analysis; in six isolates (HI-VI1, HI-MI29, HC-AN64, HC-VI2, F-KM5, and A-PD3), they gave rise to an amplicon larger than the prototype; and in the remaining four isolates (HI-MI25, HI-MI31, HI-MI32, and HI-MI60), they yielded two amplicons, one of the same size as the prototype and one larger.

The 10 isolates differing from the prototype and the 12 isolates not yielding amplicons were further studied by PCR. In the six isolates harboring a single Tn*1546*-like element larger than the prototype, the ORF1A-ORF2R primer pair yielded an amplicon corresponding to the prototype in four cases (*E. durans* F-KM5, *E. faecium* HI-VI1, *E. faecium* HC-VI2, and *E. faecium* HI-MI29), an amplicon larger by 900 bp in one (*E. durans* A-PD3), and no amplicon in the remaining isolate (*E. faecium* HC-AN64); the INV3-VANZ1 pair yielded an amplicon of the expected size in *E. durans* A-PD3 and *E. durans* F-KM5 (suggesting an insertion downstream of VANZ1 in this strain) and an amplicon larger by 800 bp to 1,900 bp in the remaining isolates (Fig. 4). The four isolates with two IR amplicons, one corresponding to and one larger
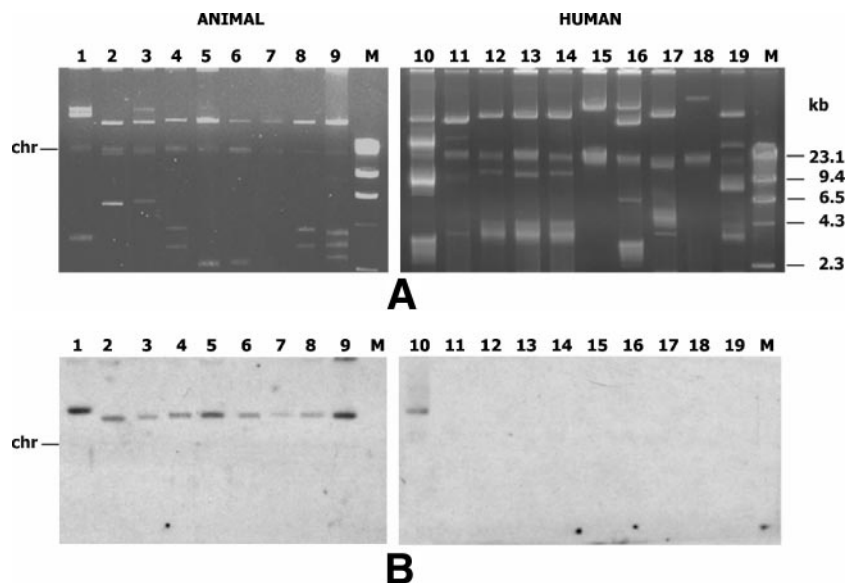
FIG. 2. Plasmid profile (A) and *vanA* hybridization (B) of animal (lines 1 to 9) and human (lines 10 to 19) isolates. Line 1, *E. durans* A-VI4; line 2, *E. faecium* A-PD33; line 3, *E. durans* A-VR5; line 4, *E. faecium* A-PD6; line 5, *E. faecium* A-VR8; line 6, *E. faecium* A-PD37; line 7, *E. faecium* A-VI9; line 8, *E. faecium* A-VI10; line 9, *E. faecium* A-VR12; line 10, *E. faecium* HI-MI29; line 11, *E. faecium* HI-MI30; line 12, *E. faecium* HI-MI57; line 13, *E. faecium* HI-MI58; line 14, *E. faecium* HI-MI31; line 15, *E. faecium* HI-MI32; line 16, *E. faecium* HI-MI27; line 17, *E. faecium* HI-MI60; line 18, *E. faecium* HI-MI34; line 19, *E. faecium* HI-MI28. M, molecular size marker (Marker II; Roche).

than the prototype, all gave the same results, i.e., ClaI digestion yielded three extra fragments, suggesting the presence of a Tn*1546* element 3 kb larger than the prototype. Tn*1546* amplification using ORF1A-ORF2R and INV3-VANZ1, targeting the ends of the transposon, and further PCR experiments using INV2-VANX1, targeting the core region, yielded amplicons comparable in size to those of the prototype, indicating the presence of insertions downstream of VANZ1 or upstream of ORF1A (data not shown).

Primer pair INV3-VANZ1 yielded an amplicon corresponding to the prototype in all 12 isolates giving no IR amplicons, indicating a prototype *vanA* cluster on the right end, whereas ORF1A-ORF2R yielded an amplicon of the expected size in only two isolates (*E. faecium* F-KM8 and HI-AN18), arguing for the presence in these strains of deletions upstream of nucleotide 170 (Fig. 4). Additional PCR experiments were performed to establish the size of the left-end deletion in the remaining 10 isolates. Primer pair ORF1B-ORF2R yielded a product corresponding to the prototype in one strain (*E. faecium* A-AN26), suggesting a left-side deletion until a nucleotide between 170 and 907 bp, while ORF1C-ORF2R yielded a product corresponding to the prototype in one of the remaining strains (*E. faecium* F-KM6), suggesting a left-end Tn*1546* deletion as far as a nucleotide between 907 and 1871. No amplicons were obtained from the remaining eight isolates, arguing for an *orf1* deletion until a nucleotide between 1871 and 3174.

Tn*1546* elements were also analyzed for the presence of the G-to-T mutation at position 8234 in the *vanX* gene using INV2-VANX1 and DdeI digestion of amplicons. Restriction analysis revealed the *vanX* mutation in five *E. faecium* isolates, three human intestinal (HI-AN9, HI-AN18, and HI-MI34) and two pork (F-KM8 and F-KM9) isolates (Fig. 4).

Fisher's test failed to evidence any association between Tn*1546* type and a particular source of GRE or a particular enterococcal species ($P > 0.5$).

Genetic detection and expression of virulence determinants. The 105 *vanA* enterococcal isolates were screened for the presence of AS genes, *gelE*, *cylB*, and *esp* and tested for clumping after growth in the presence of pheromone-containing supernatants of *E. faecalis* JH2-2 and *E. faecalis* OG1RF and for gelatinase and hemolysin production.

The presence of AS genes was determined by PCR using primers AGG and ASA373. To identify the specific AS gene, AGG amplicons were subjected to EcoRI restriction analysis, together with those obtained with *E. faecalis* OG1RF(pCF10, *prgB*) and *E. faecalis* OG1RF(pAD1, *asa1*) (Fig. 5). All tested *E. faecalis* isolates carried at least one AS gene. In particular, HI-AN23, HC-N22, and F-PM25 carried *prgB*, whereas HC-VI4 was shown to contain both *prgB* and *asa1* genes, as well as *asa373*. Four strains showed a restriction profile with an additional fragment, and the corresponding AS genes were indicated as *prgB*∗ (HC-UD6, F-KM6, and F-KM7) and *asa1*∗ (HC-R35). HC-AN21 contained both *prgB*∗ and *asa1*.

A total of 21 *vanA* isolates exhibited a positive clumping reaction. Growth in the presence of pheromones gave rise to different levels of aggregation: some strains generated moderate to large aggregates, whereas others elicited a barely detectable effect (Table 6). Clumps were particularly evident in six (five human and one food) *E. faecalis* isolates, all containing one or more of the AS genes tested (HC-VI4 [*prgB*, *asa1*, and *asa373*], HC-AN21 [*prgB*∗ and *asa1*], HC-AN22 [*prgB*], HC-UD6 [*prgB*∗], HI-AN23 [*prgB*], and F-PM25 [*prgB*]). Clumps were less pronounced in the remaining 15 isolates (8 human, 5 animal, and 2 food; 12 *E. faecium*, 2 *E. durans* and 1 *E. gallinarum*), none of which contained any of the AS genes tested.
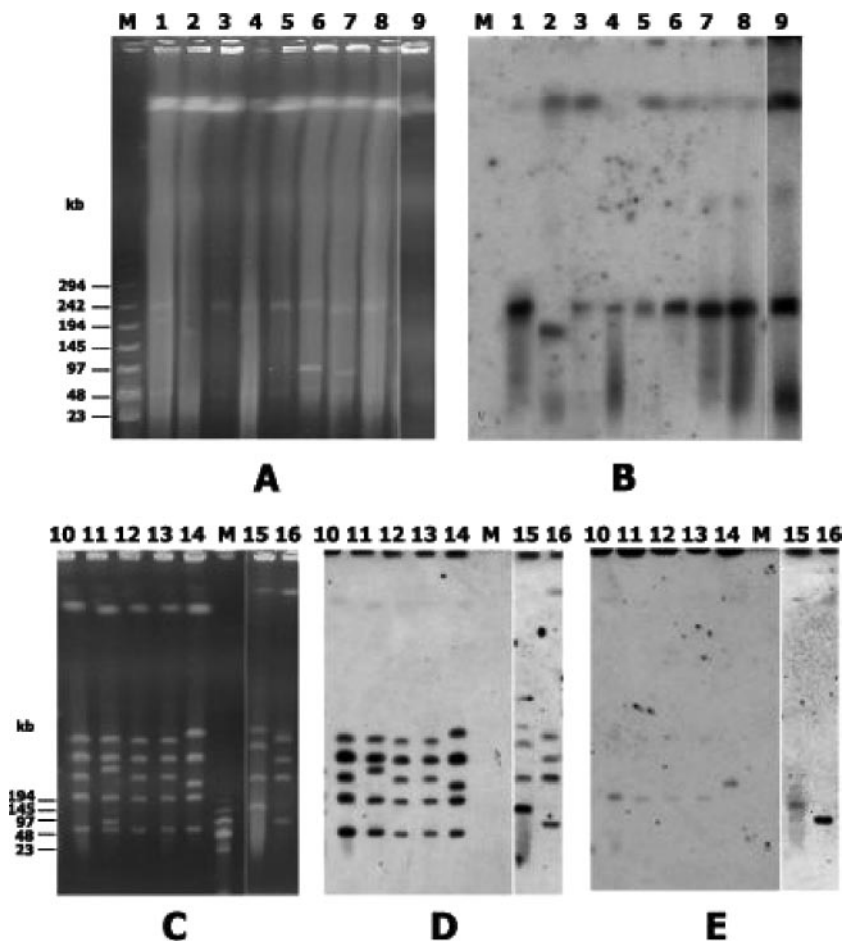
FIG. 3. PFGE of S1-digested (A) and I-CeuI-digested (C) total DNA and corresponding *vanA* (B and E) and 16S rRNA gene (D) hybridization. Lane 1, *E. faecium* HI-MI28; lane 2, *E. faecium* HI-MI34; lane 3, *E. faecium* HI-MI60; lane 4, *E. faecium* HI-MI32; lane 5, *E. faecium* HI-MI31; lane 6, *E. faecalis* HI-MI58; lane 7, *E. faecium* HI-MI57; lane 8, *E. faecium* HI-MI30; lane 9, *E. faecium* HI-MI25; lane 10, *E. faecium* HI-MI30; lane 11, *E. faecium* HI-MI25; lane 12, *E. faecium* HI-MI31; lane 13, *E. faecium* HI-MI32; lane 14, *E. faecium* HI-MI60; lane 15, *E. gallinarum* A-BE48; lane 16, *E. gallinarum* F-PM3. M, low range marker (BioLabs).

The clumping-negative phenotype correlated with the presence of an additional EcoRI site in *E. faecalis* HC-R35 (*asa1*∗), F-KM6 (*prgB*∗), and F-KM7 (*prgB*∗).

gelE was detected in 28 isolates, of which eight (five human and three food) were also Gel producers (Table 6). All the *gelE*-positive Gel producers were *E. faecalis* isolates and carried at least one AS gene (F-KM6, F-KM7, F-PM25, HC-VI4, HC-AN21, HC-AN22, HC-UD6, and HC-R35). No Gel production was detected in the remaining *gelE*-positive strains (17 *E. faecium* [A-PD1, A-VC2, A-PD35, A-PD6, A-VI9, A-VI10, A-VR12, F-KM9, F-PM12, F-PM15, HC-R38, HI-VI1, HI-AN19, HI-MI54, HI-MI30, HI-MI57, and HI-MI32], 2 *E. durans* [F-KM5 and F-PM20], and 1 *E. gallinarum* [A-PG15]). The five *esp*-positive strains (Table 6) included four *E. faecium* isolates (HC-AN64, HC-VI2, HC-UD5, and HI-VI1) and an *E. gallinarum* isolate (F-KM1). *cylB* was not detected in any of the 105 enterococcal isolates, all of which were negative for β-hemolysis.

Biofilm formation. When the 105 *vanA* isolates were tested in vitro for biofilm formation on abiotic surfaces, 10 strains were seen to have a strong or weak ability to produce biofilm (Table 6). Seven of these strains were *E. faecalis* (four human and three

food), two *E. faecium* (one human and one animal), and one *E. durans* (animal). None of the four *E. gallinarum* strains were able to form biofilm. Interestingly, the seven positive *E. faecalis* strains (five strong and two weak producers) were also positive for *prgB* and/or *asa1* and *gelE* and were negative for *esp*. These features were independent of the source of isolation. In contrast, the two *E. faecium* weak biofilm producers and the only *E. durans* isolate (a strong biofilm producer) were negative for all tested virulence traits (Table 6).

Overall, strains possessing suspected virulence genes were more frequent among clinical isolates than in the other reservoirs ($P < 0.05$), whereas there was no significant difference in their occurrence between human intestinal, animal, and food isolates ($P > 0.6$). However, the occurrence of strains carrying multiple virulence factors was peculiar to clinical and food reservoirs only.

DISCUSSION

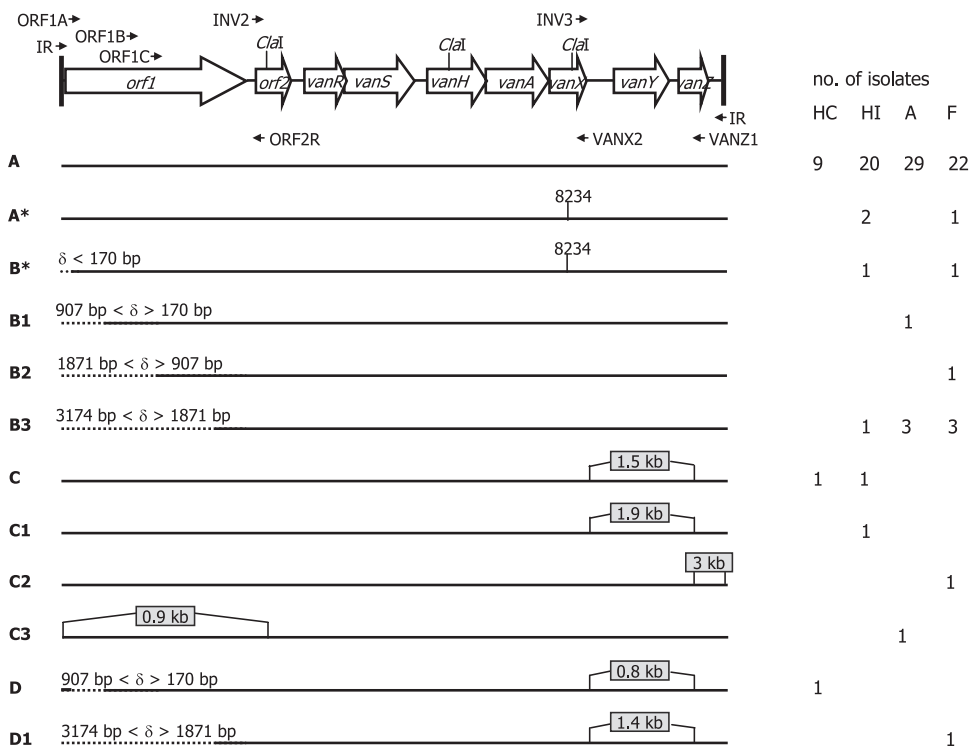Since the 1997 European Union ban forbidding the use of avoparcin in animal feeds, the prevalence of GRE has de-

FIG. 4. Schematic representation of the Tn*1546* prototype (A) and 11 different Tn*1546*-like elements (A* to D1) detected in the 101 *vanA* isolates carrying a single *vanA* element. Locations of primers, ClaI target sites, and the mutation at position 8234 are indicated. Left-side deletions (δ, deletion size) are indicated by dotted lines, insertions by gray boxes. The origin (HC, HI, A, and F) and number of isolates carrying the Tn*1546* type are reported on the right. The labels of the Tn*1546*-like elements of Palepou et al. (43) that may correspond to those characterized in the present study are reported in parentheses: A, A* (A); B* (D); B₁ (D/M); B₂ (M); B₃ (P); C, C1 (B/C); C2 (no correspondence); C3 (H-L); D (E); and D1 (Q-S).

creased among farm animals and in the community (15, 34, 54), even though a readily detectable persistence of GRE in avoparcin-exposed farm environments has been reported (1, 33). By contrast, the incidence of GRE in hospitals has remained substantially unchanged in northern Europe and has actually increased in southern European countries (http://www.earss.rivm.nl). The role of different reservoirs in the spread of glycopeptide resistance is thus still unclear.

In this study, we compared GRE isolates of different origins and geographic locations. GRE were initially identified at the species level, PFGE typed, and analyzed for their *van* genotypes. Results showed different species prevailing in the different reservoirs. As expected, *E. faecium* was the most prevalent species irrespective of the source, whereas *E. faecalis* was recovered only from human (mostly clinical) and food samples. *E. durans* and *E. gallinarum* were only isolated from animal and food samples. All isolates were confirmed to be *vanA* positive and *vanB* negative.

PFGE results showed a polyclonal distribution of *vanA* isolates in the different reservoirs; however, the presence of some clones in different reservoirs was observed. In particular, 26 *E. faecium* isolates belonged to type M49; they were first isolated in northern Italy in 1997 and since 2002 in northern and central Italy, thus showing both a temporal and a geographic spread. A different subtype characterized the different hospitals or towns of isolation. The same clone was isolated from both clinical and intestinal human samples, suggesting an ability of intestinal

isolates to act as pathogens. However, no difference in virulence determinants was detected between clinical and intestinal clonally related *E. faecium* isolates. Clinical isolates could act as opportunistic pathogens or could have acquired virulence traits still to be characterized. Notably, the M49 type and subtypes belong to ST 78, which has already been described as epidemic in Italy (6). Type M39 was recovered over a limited period of time (2002 to 2003) and geographic area (central Italy) from human and pig intestines, suggesting an ability to colonize both species. It was shown to belong to ST 18, which has never been found in either human epidemic or animal GRE according to a previous Italian study (6). Interestingly, ST 18 and ST 78 both belong to the clonal complex 17 (CC17), the first globally dispersed nosocomial-adapted clonal lineage of *E. faecium* (56). However, while type M49 (ST 78) was isolated from intestinal samples of inpatients only, type M39 (ST 18) was isolated from intestinal samples of inpatients as well as in the community and in pigs. Isolates belonging to types M7, M29, M42, D1, D5, and G2 were also recovered from samples of different origins, demonstrating an occasional clonal spread between different reservoirs also for *E. durans* and *E. gallinarum*. M29 and M42, as well as other PFGE types isolated from poultry in different geographic areas, carried the *purK* type 6 allele, whereas all human hospital isolates (except type M11) carried *purK* type 1, according to data reported by others (6, 33, 50). Overall, these data strongly suggest that human colonization by food and animal GRE is possible but
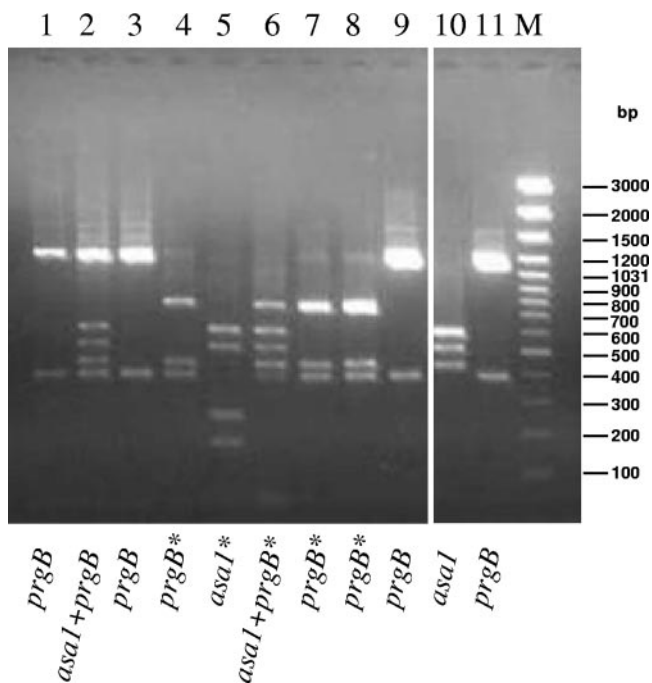
FIG. 5. EcoRI restriction analysis of AGG amplicons of nine *E. faecalis* isolates and of *E. faecalis* OG1RF(pAD1, *asa1*) and *E. faecalis* OG1RF(pCF10, *prgB*) reference strains. Lane 1, HI-AN23; line 2, HC-VI4 (also *asa373* positive); line 3, HC-AN22; line 4, HC-UD6; line 5, HC-R35; line 6, HC-AN21; line 7, F-KM6; line 8, F-KM7; line 9, F-PM25; line 10, OG1RF(pAD1; *asa1*); and line 11, OG1RF(pCF10; *prgB*). *, additional EcoRI site. M, GeneRuler 100-bp DNA Ladder Plus marker (M-Medical Genenco).

that vertical transmission between different reservoirs is infrequent. Although the colonization might be transient (49, 57), the possible transfer of resistance genes during this period could be crucial.

Results of Tn*1546* location analyses suggested an association among the *vanA* location, species, and origin of isolates. In the vast majority of our strains (98%), *vanA* was located on plasmids of either <150 kb (all the *E. faecalis* and *E. durans* strains) or >150 kb (intestinal *E. faecium*, both human—including M49 and M39—and animal). Notably, plasmids of >150 kb have already been described in an *E. faecium* clone widely disseminated among pigs (1). These results suggest that high-molecular-weight *E. faecium* plasmids might be involved in intestinal colonization of both humans and animals, thus contributing to the persistence of resistant strains. Moreover, large plasmids are likely to be conjugative, thus contributing to the horizontal transfer processes. A chromosomal *vanA* location was demonstrated in two of the four *E. gallinarum* strains, suggesting that in this species the chromosome is a more common location than in other enterococcal species, as also reported previously (23).

Tn*1546* typing showed a Tn*1546* element, indistinguishable from the prototype, in about 80% of the strains tested. The remaining Tn*1546*-like elements displayed insertions or left-end deletions. This finding agrees with other data from Italian *vanA* strains from different sources (5). In contrast, Tn*1546*-like elements different from the prototype seem to be more common in other countries (8, 26, 32, 46, 57). The same

Tn*1546* type was found in clonally unrelated poultry, swine, and human strains (Fig. 4), while different Tn*1546* types were found in isolates belonging to the same clone (Table 6), suggesting that horizontal gene transfer may have played a significant role in the spread of glycopeptide-resistant strains. The finding of the G-to-T mutation at position 8234 of Tn*1546* in pork and human isolates, suggesting a relationship between human and food *vanA* elements, supports this hypothesis.

When evaluated for virulence determinants and their expression, about half of the GRE showed at least one virulence trait, *gelE* and the pheromone response being the most frequent. Gelatinase production was found in all clinical and food *E. faecalis* isolates. Since gelatinase production has been more frequently described in clinical isolates than in those from other sources (11, 13), these results point to a link between clinical and food reservoirs, as suggested by previous reports (9, 47). Silent *gelE* was detected in isolates from other species (*E. faecium* [particularly human and animal feces], *E. durans* [food], and also *E. gallinarum* [animal feces]). *gelE* has been documented frequently in *E. faecalis*, rarely in *E. faecium* and *E. durans* (20, 24), but never in *E. gallinarum*. Thus, the spread of *gelE* from *E. faecalis* by horizontal gene transfer might be involved in the evolution of different pathogenic enterococcal species. Lack of expression in species other than *E. faecalis* might be explained by low levels or downregulation of gene expression, an inactive gene product, or experimental conditions. Growth in the presence of *E. faecalis* sex pheromones gave rise to clumps in all species, although the level of aggregation was higher in *E. faecalis*, suggesting a species-specific response. On the other hand, AS genes (prevalently *prgB*) were detected only in *E. faecalis*, with some strains harboring more than one gene (*prgB*, *asa1*, and *asa373*). AS genes were also detected in a few clumping-negative isolates. The presence in some strains of *prgB*∗ or *asa1*∗ correlated with the clumping-negative phenotype, suggesting an inactive gene product.

Although it is frequently carried by pheromone-response plasmids (24, 27), *cylB* was never detected; *esp* was only detected in a few *E. faecium* (human) and *E. gallinarum* (food) isolates, arguing against the association of these virulence traits with glycopeptide resistance. Although the ability to form biofilm was uncommon, it is worth noting that it was mostly present in *vanA* *E. faecalis* isolates harboring other virulence genes.

Overall, virulence studies of our *vanA* enterococci revealed different trends in the occurrence of virulence determinants among human, food, and animal isolates. Their higher incidence detected among human and food compared with animal isolates was associated with the presence of *E. faecalis* isolates carrying multiple virulence factors in the former reservoirs.

A similar virulence profile observed among clinical and food *E. faecalis* isolates and the absence of multivirulent animal enterococci suggest that food could be more closely involved than animals in the spread of virulent GRE in humans. Moreover, the different features observed in enterococcal strains isolated from breeding animals and animal food raise questions about the source of food contamination by *vanA* enterococci. On the other hand, GRE polyclonality suggests that horizontal transfer of the *vanA* cluster, rather than clonal spread, is responsible for their emergence and dissemination. Subsequently, the presence of *vanA*, combined with one or

TABLE 6. PFGE and Tn*1546* type, *vanA* location, genotype or phenotype of virulence, and slime production of 48 *vanA* enterococcal isolates of different origins harboring one or more virulence traits[a]

Origin (*n*) and strain	Species	PFGE type	*vanA* location	Tn*1546* type	gelE	Gel	esp	AS genes	Clumping activity	Slime production
Human intestinal (13)										
HI-AN19	*E. faecium*	M39a	P*	A	+	−	−	−	−	−
HI-MI32	*E. faecium*	M49f	P* + Chr	A/C2	+	−	−	−	−	−
HI-MI57	*E. faecium*	M49	P*	A	+	−	−	−	−	−
HI-VI1	*E. faecium*	M48	P	C	+	−	+	−	−	−
HI-AN8	*E. faecium*	M36	P	B3	−	−	−	−	+	−
HI-AN15	*E. faecium*	M49e	ND	A	−	−	−	−	+	−
HI-MI30	*E. faecium*	M49f	P* + Chr	A	+	−	−	−	+	−
HI-MI54	*E. faecium*	M49m	P*	A	+	−	−	−	+	−
HI-MI58	*E. faecium*	M49a	P*	A	−	−	−	−	−	WP
HI-N42	*E. faecium*	M29	P	A	−	−	−	−	+	−
HI-N43	*E. faecium*	M44	P	A	−	−	−	−	+	−
HI-N44	*E. faecium*	M27	P	A	−	−	−	−	+	−
HI-AN23	*E. faecalis*	S5	P	A	−	−	−	*prgB*	++	SP
Animal (15)										
A-VE44	*E. faecium*	M41	P*	B3	−	−	−	−	−	WP
A-VI2	*E. faecium*	M22b	P	A	+	−	−	−	−	−
A-PD1	*E. faecium*	M25	P	A	+	−	−	−	−	−
A-PD6	*E. faecium*	M14	P	A	+	−	−	−	−	−
A-PD35	*E. faecium*	M38	P	A	+	−	−	−	−	−
A-VI19	*E. faecium*	M22a	P	A	+	−	−	−	−	−
A-VI10	*E. faecium*	M18	P	A	+	−	−	−	−	−
A-VR12	*E. faecium*	M32	P	A	+	−	−	−	−	−
A-N19	*E. faecium*	M37	P	A	−	−	−	−	+	−
A-N20	*E. faecium*	M35	P	B3	−	−	−	−	+	−
A-VR8	*E. faecium*	M8	P	A	−	−	−	−	+	−
A-AN21	*E. durans*	D1a	P	A	−	−	−	−	−	SP
A-AN14	*E. durans*	D2	P	A	−	−	−	−	+	−
A-PD41	*E. durans*	D6	P	A	−	−	−	−	+	−
A-PG15	*E. gallinarum*	G1	P	A	+	−	−	−	−	−
Food (11)										
F-KM9	*E. faecium*	M10	P	A*	+	−	−	−	−	−
F-PM12	*E. faecium*	M45	P	A	+	−	−	−	−	−
F-PM15	*E. faecium*	M3a	P	A	+	−	−	−	−	−
F-PM13	*E. faecium*	M45a	P	A	−	−	−	−	+	−
F-KM6	*E. faecalis*	S3	P	B2	+	+	−	*prgB**	−	SP
F-KM7	*E. faecalis*	S3a	P	A	+	+	−	*prgB**	−	WP
F-PM25	*E. faecalis*	S7	P	A	+	+	−	*prgB*	+++	SP
F-KM5	*E. durans*	D1c	P	C2	+	−	−	−	−	−
F-PM20	*E. durans*	D7	P	B3	+	−	−	−	−	−
F-PM3	*E. gallinarum*	G1	Chr	D1	−	−	−	−	+	−
F-KM1	*E. gallinarum*	G2	P	A	−	−	+	−	−	−
Human clinical (9)										
HC-AN64	*E. faecium*	M24	P	D	−	−	+	−	−	−
HC-UD5	*E. faecium*	M49b	P	A	−	−	+	−	−	−
HC-VI2	*E. faecium*	M48	P	C	−	−	+	−	+	−
HC-R38	*E. faecium*	M49n	P*	A	+	−	−	−	−	−
HC-VI4	*E. faecalis*	S6	P	A	+	+	−	*prgB asa1 asa373*	+++	−
HC-AN21	*E. faecalis*	S4	P	A	+	+	−	*prgB** *asa1*	++	SP
HC-AN22	*E. faecalis*	S8	P	A	+	+	−	*prgB*	++	WP
HC-UD6	*E. faecalis*	S1	P	A	+	+	−	*prgB**	++	−
HC-R35	*E. faecalis*	S2	P	A	+	+	−	*asa1**	−	SP

[a] Abbreviations: P, <150-kb plasmid; P*, >150-kb plasmid; Chr, chromosome; Gel, gelatinase production; WP, weak producer (0.120 < optical density < 0.240); SP, strong producer (optical density > 0.240); ND, not detected.

more virulence genes in the same genome, could have favored particular clusters of *E. faecium*, e.g., type M49 in the hospital environment.

The *E. faecium* isolates were generally devoid of virulence determinants, albeit with notable exceptions. Although all *E. faecium* strains lacked the AGG genes, several strains formed clumps after pheromone induction. The same profile was occasionally observed in *E. durans* and *E. gallinarum* isolates. This phenomenon might depend on the presence of AS/pheromone systems in these species, different from those of *E.*

faecalis. By contrast, using *E. faecalis* primers, *gelE* and *esp* determinants were detected in several *E. faecium* and in some *E. durans* and *E. gallinarum* isolates, in line with data on *E. faecium* reported by Eaton et al. (20, 21). The presence in the different reservoirs of *vanA E. faecium*, *E. durans*, and *E. gallinarum* isolates carrying virulence factors might be linked with the increasing isolation rate of enterococcal species other than *E. faecalis* in hospital settings (4).

In conclusion, (i) indistinguishable plasmid-located *vanA* determinants in PFGE-diverse populations strongly suggest a common Tn*1546* reservoir readily accessible by horizontal gene transfer; (ii) large *vanA* plasmids (>150 kb) carried by intestinal *E. faecium* might be involved in GRE colonization/infection in humans (with colinked unknown virulence determinants possibly involved in adhesion) and represent an important target for further studies; and (iii) detection of the same *vanA* and virulence determinants in enterococci of different species and origins, in the presence of host-specific *purK* housekeeping genes, indicates a lack of host-specific markers and suggests that all GRE, irrespective of their origins and species, might be regarded as potential reservoirs of resistance determinants and virulence traits transferable to human-adapted clusters. Moreover, the finding of virulent *vanA E. faecalis* in meat suggests a food involvement (farm animal independent) in the spread of virulent GRE in humans.

ACKNOWLEDGMENTS

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