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# Population Structure of *Enterococcus faecium* Causing Bacteremia in a Spanish University Hospital: Setting the Scene for a Future Increase in Vancomycin Resistance?

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**Over an 8-year period (1995 to 2002), 86 *Enterococcus faecium* blood isolates from 84 patients, of which 54 were ampicillin resistant (AREF) and 32 were ampicillin susceptible (ASEF), were studied in a university hospital (1,200 beds; serving a population of 600,000) in Spain, a country characterized by a near-absence of resistance to vancomycin and very high rates of ampicillin resistance among enterococci. Clonal relatedness by pulsed-field gel electrophoresis (PFGE), antibiotic susceptibility, presence of the virulence/epidemicity genes $esp_{Efm}$ and $hyl_{Efm}$, and identification of *purK* alleles were studied. A group of isolates was also analyzed by amplified fragment length polymorphism (AFLP) and multilocus sequence typing. Medical charts (30 variables collected) were reviewed for 60/84 patients. ASEF showed high clonal diversity (32 PFGE types, 11 *purK* alleles, 4 AFLP genogroups), did not harbor putative virulence genes, and had no specific association with hospital acquisition. AREF isolates belonged to a clonal complex (CC) of genetically related strains (*purK-1*, AFLP genogroup C), occasionally harboring putative virulence traits, and were from patients with particular risk factors. Within this CC, previously associated with vancomycin-resistant *E. faecium* isolates causing outbreaks worldwide (W. L. Homan et al., J. Clin. Microbiol. 40:1963–1971, 2002), a great genetic diversity of antibiotic resistance and virulence/epidemicity profiles was found. Associations between *esp* and a >7-day hospital stay and between *purK-1*, hospital location, and nosocomial acquisition were noted ($P < 0.001$). These findings reflect the importance of local environmental differences in the evolution of this CC, suggesting that the emergence of vancomycin resistance among AREF strains in Spain may be a question of time.**

The emergence and spread of antibiotic-resistant enterococci are not fully understood, since striking differences in different geographical areas have been observed. In the United States, the emergence of *Enterococcus faecium* as a nosocomial pathogen started in the 1980s with an increase in resistance to ampicillin (7, 33). Acquisition of vancomycin resistance by a few of these ampicillin-resistant *E. faecium* (AREF) clones, and further dissemination of specific vancomycin resistance transposons to multiple genetic backgrounds, led to an increase in vancomycin-resistant enterococci (VRE) in the following decade (12, 16, 23, 24, 31, 39). The occurrence of VRE in European hospitals remained low throughout the '90s, with a polyclonal enterococcal population structure containing a great variety of Tn*1546* types in the community setting (7, 45, 48). However, the high prevalence of AREF in all European Union (EU) countries, with rates of VRE above 10% in six countries in recent years, suggests a change in the epidemiology of enterococci in Europe (Annual Report of the European Antibiotic Resistance Surveillance System [EARSS], 2002 [http//:www.earss.rivm.nl]). It has been demonstrated that endemic vancomycin-susceptible *E. faecium* (VSEF) clones have provided a key biological substrate for vancomycin resistance in European and American hospitals (20, 24, 31, 36). Widely disseminated VSEF clones have been described recently in Norway, Sweden, and Spain (11, 19, 40), and they might constitute the base for the emergence and successful spread of multiresistant enterococcal organisms in the EU.

*E. faecium* is a highly host adapted organism that has been divided into different ecovars or host-specific groups on the basis of phenotypic and/or genotypic characteristics (1, 47). Moreover, genetic differences between epidemic and nonepidemic strains among isolates colonizing humans have been reported (8, 17, 22, 47). Epidemic *E. faecium* strains belong to a human-specific ecovar classified as genogroup C on the basis of amplified fragment length polymorphism (AFLP) profiles (47), frequently harbor a specific allele of the housekeeping gene *purK* (coding for the phosphoribosylaminoimidazole carboxylase ATPase subunit), and eventually harbor a pathogenicity island (PAI) encoding an enterococcal surface protein (Esp) (5, 17, 21, 22, 46). Other traits potentially involved in the virulence processes, including the gene coding for $Hyl_{Ef}$, a possible hyaluronidase, have been found in a number of epidemic VRE strains (27, 32, 37).

In this work, the population structure of all invasive *E. faecium* isolates recovered over an 8-year period (1995 to 2002) in a single hospital in Spain was studied. Differences between ampicillin-susceptible *E. faecium* (ASEF) and AREF popula-

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tions were found; most of the AREF isolates constituted a highly clonal population with features of the previous identified clonal lineage 1 (C1), renamed clonal complex 17 (CC-17) (*purK-1*, AFLP genogroup C) (4, 17, 44). The reasons for the different evolutionary outcomes of this CC in different countries remain to be clarified, but local availability of resistance determinants and gene capture elements might contribute to explaining these differences.

## MATERIALS AND METHODS

**Bacterial strains.** All *E. faecium* blood isolates recovered from June 1995 to December 2002 at the Hospital Ramón y Cajal, a 1,200-bed teaching institution, were included in our study. One isolate per patient (or more than one when different susceptibility patterns were observed) was selected for further studies. Preliminary bacterial identification and antibiotic susceptibility testing were performed by the semiautomatic PASCO (Difco, Detroit, MI) or WIDER (Francisco Soria Melguizo, Spain) system. Identification was confirmed by testing for the presence of *aac(6′)-Ii*, which is specific for *E. faecium*, by PCR (10). Antimicrobial susceptibility to 13 antibiotics was determined by the standard agar dilution method (28).

**Virulence/epidemicity (VIEP) markers.** The presence of genes presumptively involved in virulence or epidemicity, *esp*, *hyl*, *cyl*, *gel*, and *agg*, coding for an enterococcal surface protein (Esp), hyaluronidase (Hyl), cytolysin/hemolysin (Cyl), gelatinase (Gel), and aggregation substance (Agg), respectively, was tested by multiplex PCR (14, 21, 32, 42). Tests with negative results were repeated in experiments using primers specific for individual genes. Three different oligonucleotide sets covering different regions of the pathogenicity island in which $esp_{Efm}$ is located were used to test isolates with a initial negative result for this gene (21): esp4F (5′-GGA ACG CCT TGG TAT G-3′) and esp6R (5′-CCG CTT TTG GTG ATT C-3′), esp14F (5′-AGATTT CAT CTT TGA TTC TTG G-3′), and esp–12R (5′-AAT TGA TTC TTT AGC ATC TGG-3′). Production of hemolysin and gelatinase was searched for as previously reported (9). The following strains were used as controls: *E. faecium* E-774 ($esp_{Efm}^{+}$ $hyl_{Efm}^{+}$ $gel^{-}$ $cyl^{-}$ $agg^{-}$), *Enterococcus faecalis* OG1X containing pAD1 ($esp_{Efm}^{-}$ $hyl_{Efm}^{-}$ $gel^{-}$ $cyl^{+}$ $agg^{+}$), and *E. faecalis* OG1RF ($esp_{Efm}^{-}$ $hyl_{Efm}^{-}$ $gel^{+}$ $cyl^{-}$ $agg^{-}$). The identities of the amplified $esp_{Efm}$ and $hyl_{Efm}$ DNA fragments were verified by sequencing different PCR products. *E. faecalis* control strains were kindly provided by Barbara Murray (University of Texas at Houston).

**Pulsed-field gel electrophoresis (PFGE).** Genomic DNA was prepared as previously described (10) and digested with SmaI or ApaI (Amersham Life Science, Uppsala, Sweden). Migration of DNA fragments was carried out in a 1.4% agarose (SeaKemGold; Iberlabo, Spain) gel with 0.5% Tris-borate-EDTA at 13°C in a CHEF DR-II device (Bio-Rad, La Jolla, CA). Pulse times were ramped from 1 to 20 s for 37 h. Strains with more than six distinct restriction fragments were considered different as previously described (26, 38).

**Computer-monitored fingerprinting analysis.** Acquisition of the gel images was performed by using a charge-coupled device EDAS 2000 camera (Kodak, PACISA, Spain) with a 587- by 768-pixel resolution. Computer analysis of the banding patterns obtained by PFGE was performed with the Phoretix software package (Nonlinear Dynamics, Newcastle, United Kingdom). The PFGE patterns were normalized by using SmaI-digested genomic DNA from *E. faecalis* strain OG1RF as an external size marker. Images analyzed included three reference lanes of DNA size markers, and all images were compatible after normalization. Bands were assigned by computer and corrected manually after visual checking when necessary. The similarity of the banding patterns was analyzed by the Dice coefficient, and cluster analysis was performed by the unweighted-pair group method using average linkages (UPGMA). We used a slightly different position tolerance to allow 100% matching of the banding patterns obtained by duplicate samples on different images. A cutoff equivalent to 80% fragment matching was used, in additional statistical analysis, to group possibly genetically related isolates classified as different PFGE types by standard criteria (see above) (35).

**MLST.** A subset of *E. faecium* isolates was selected for characterization by multilocus sequence typing (MLST) using seven housekeeping loci as previously described (17). Different sequences were assigned allele numbers, and different allelic profiles were assigned sequence types (STs) by interrogating the *E. faecium* MLST database (http://www.mlst.net).

**AFLP.** A subset of *E. faecium* isolates was selected for analysis by AFLP. The technique was performed as previously described with the addition of a final ethanol precipitation step to further purify the DNA (47). The amplification products were run on a capillary DNA sequencer (ABI PRISM 3700 DNA analyzer; Applied Biosystems, Foster City, CA). GeneScan collection software (Applied Biosystems) was used to collect data and to export the data to BioNumerics (version 3.5; Applied Maths, Sint-Martens-Lattem, Belgium) for further analysis. The Pearson product-moment correlation coefficient was used to calculate similarities of the AFLP-based densitometric curves, and UPGMA was used for cluster analysis (BioNumerics, version 3.5). AFLP banding patterns were matched with a library of 404 vancomycin-resistant *E. faecium* (VREF) strains representing the four different AFLP genogroups (A to D) discerned previously (47).

**Sequence analysis.** The *purK* gene was amplified using primers *purK*-1n and *purK*-2n (http://www.mlst.net), purified (Qiaquick PCR purification kit; QIAGEN, Hilden, Germany), and sequenced by using an ABI Prism 377 automated sequencer (Perkin-ElmerApplied Biosystems, Foster City, CA). *purK* alleles were assigned by interrogating the *E. faecium* MLST database (see above).

**Patients and chart review.** Patients' medical charts were reviewed, and the following demographic and clinical data were included: age, sex, nosocomial acquisition, hospital location, days of hospitalization, APACHE II score, baseline illness, recent surgery, assisted ventilation, central venous catheterization, hyperalimentation, urinary catheterization, renal insufficiency (serum creatinine level of >2 mg/dl), crude mortality, and related mortality. Exposure to antibiotics in the month prior to the date of the initial enterococcal blood culture was evaluated and included vancomycin (parenteral and oral), teicoplanin, metronidazole, clindamycin, beta-lactams, sulfonamides, quinolones, aminoglycosides, and tetracyclines. Patient outcomes were analyzed until hospital discharge or death while in hospital.

**Statistical analysis.** Characteristics of patients with bacteremia caused by AREF versus ASEF were compared using Student's *t* test for continuous data and chi-square analysis for categorical data. Yates' correction and a two-tailed Fisher exact test were performed when necessary. Epidemiological and clinical variables were related to molecular typing data provided by PFGE and AFLP and to the presence of the *esp*, *hyl*, and *purK-1* genes. For PFGE, isolates showing more than 80% similarity were included in the same group and compared with the rest of the isolates; for AFLP, isolates of genogroup C were analyzed as one class and isolates of genogroups A, B, and R as another. Putative virulence factors were categorized as follows: for *esp* or *hyl*, isolates containing these traits were compared with those lacking them; for *purK*, comparison was made between isolates harboring the *purK-1* allele and isolates containing other *purK* alleles. All variables with a *P* value of <0.1 were included in logistic regression modeling. Multivariate analysis was performed by using logistic regression, with significant variables selected by a backward stepwise procedure. Statistical significance for comparison proportions was calculated by the chi-square test (a *P* value of <0.05 was considered to be statistically significant). Epi-Info, version 6 (Centers for Disease Control and Prevention, Atlanta, GA), and SPSS 7.5 for Windows were used for analysis of the data.

## RESULTS

**Epidemiological background and bacterial strains.** We collected 626 enterococcal blood isolates from 374 patients. *E. faecalis* was the most common species isolated ($n = 481$; 77%), followed by *E. faecium* ($n = 96$; 15%) and *Enterococcus* spp. ($n = 49$; 8%). Enterococci represented the fourth most common pathogen isolated from bloodstream infections after *Escherichia coli*, *Staphylococcus aureus*, and coagulase-negative staphylococci. The yearly incidence of bloodstream infections caused by *E. faecium* ranged from 0.1% to 1.7% during the study period. The rate of ampicillin resistance among clinical isolates of this species increased steeply, from 17% in 1991 to 53% in 1995 and 75% in 2002 (data obtained from the microbiology laboratory) (13).

Ninety-six *E. faecium* isolates corresponding to 90 patients were initially collected. Eighty-six *E. faecium* blood isolates corresponding to 84 patients were selected for further studies after the identification was confirmed by molecular methods (2 patients each had 2 isolates with different susceptibility profiles). The distribution of the *E. faecium* clinical isolates over hospital units is shown in Table 1. Of the 84 patients, 39% were

TABLE 1. Features of *Enterococcus faecium* blood isolates recovered from 1995 to 2002 in the Ramón y Cajal Hospital: clinical data, PFGE, antibiotic resistance, and virulence profiles

| PFGE type | Date (mo/yr) | No. of isolates | AFLP[a] | Ward[b] | Antibiotic resistance profile[c] | | | | | | | | | | VIEP marker[d] | | *pur*K allele |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | A | S | E | C | QD | K | G | Sx | T | V | *esp* | *hyl* | |
| ASEF-9 | 7/96 | 1 | C | Pediatrics | | R | R | | | | | | R | | − | − | 1 |
| ASEF-12 | 6/97 | 1 | C | General surgery | | R | | | | | | | R | | − | − | 1 |
| ASEF-26 | 4/00 | 1 | R | Gastroenterology | | R | | R | | R | | R | R | | − | − | 1 |
| ASEF-3 | 12/95 | 1 | A | General surgery | | | | | | | | | | | − | − | 2 |
| ASEF-4 | 8/95 | 1 | B | Hematology | | | R | | | | | | | | − | − | 2 |
| ASEF-2 | 6/95 | 1 | B | Outpatient | | | R | | | | | | | | − | − | 2 |
| ASEF-5 | 9/95 | 1 | NT | Outpatient | | | | | | | | | R | | − | − | 2 |
| ASEF-13 | 7/97 | 1 | B | Outpatient | | | R | | | | | | | | − | − | 2 |
| ASEF-14 | 11/97 | 1 | B | Nephrology | | | | | | | | | | | − | − | 2 |
| ASEF-21 | 9/99 | 1 | B | Outpatient | | | | | | | | | | | − | − | 2 |
| ASEF-22 | 10/99 | 1 | B | Outpatient | | | R | | | | | | | | − | − | 2 |
| ASEF-10 | 11/96 | 1 | A | Outpatient | | | | | | | | | R | | − | − | 3 |
| ASEF-31 | 1/01 | 1 | NT | Outpatient | | R | R | R | | | | | | | − | − | 3 |
| ASEF-33 | 10/02 | 1 | R | Hematology | | | | | | | | | | | − | − | 4 |
| ASEF-6 | 11/95 | 1 | C | Cardiology | | | | | | R | | | R | | − | − | 6 |
| ASEF-8 | 1/96 | 1 | C | Rehabilitation | | | R | R | | | | | R | | − | − | 6 |
| ASEF-24 | 1/00 | 1 | C | ICU—general surgery | | R | | | | | | | R | | − | − | 6 |
| ASEF-1 | 6/95 | 1 | R | Neurosurgery | | | | | | | | | | | − | − | 8 |
| ASEF-16 | 11/97 | 1 | NT | Gastroenterology | | | | | | | | | | | − | − | 8 |
| ASEF-17 | 12/97 | 1 | B | ICU—medicine | | | R | | | | | | | | − | − | 8 |
| ASEF-19 | 8/99 | 1 | R | Maxillofacial surgery | | | | | | | | | | | − | − | 8 |
| ASEF-11 | 12/96 | 1 | A | General surgery | | | | R | | | | | | | − | − | 9 |
| ASEF-18 | 12/97 | 1 | NT | Gastroenterology | | | | R | | | | | | | − | − | 9 |
| ASEF-20 | 8/99 | 1 | C | Nephrology | | | | | | | | | | | − | − | 9 |
| ASEF-25 | 1/00 | 1 | NT | Outpatient | | | | | | | | | | | − | − | 9 |
| ASEF-29 | 11/01 | 1 | NT | Outpatient | | | R | R | | | | | | | − | − | 9 |
| ASEF-30 | 11/01 | 1 | A | Outpatient | | | R | R | | | | R | | | − | − | 11 |
| ASEF-32 | 5/02 | 1 | R | Outpatient | | | R | R | | | | R | | | − | − | 16 |
| ASEF-7 | 2/96 | 1 | NT | Liver transplant | | | | R | | | | | | | − | − | 17 |
| ASEF-15 | 11/97 | 1 | R | ICU—pediatrics | | | R | | | | | | | | − | − | 22 |
| ASEF-28 | 9/01 | 1 | R | Outpatient | | | R | | | | | | | | − | − | 22 |
| ASEF-23 | 11/99 | 1 | NT | ICU—neurosurgery | | | | | | | | | | | − | − | NT |
| AREF-A | 8/95–11/97 | 6 | C ($n$ = 2), NT ($n$ = 4) | Gastroenterology (5), ICU—cardiology (1) | R | (R) | R | (R) | | R | | | | | − | − | 1 |
| AREF-P | 7/95–10/95 | 2 | C ($n$ = 2) | Liver transplant (1), outpatient (1) | R | R | R | R | | R | | | | | + | − | 1 |
| AREF-B | 4/96 | 1 | NT | Traumatology | R | R | R | R | | R | | | R | | + | − | 1 |
| AREF-C | 5/96 | 1 | C | Infectious diseases | R | R | R | R | R | R | R | R | | | − | − | 1 |
| AREF-V | 10/96 | 1 | NT | Ophthalmology | R | | R | | | | | R | R | | − | − | NT |
| AREF-E | 1/97 | 1 | C | Vascular surgery | R | R | R | R | | R | | R | | | − | + | 1 |
| AREF-F | 2/97 | 1 | C | Outpatient | R | R | R | | | R | | | R | R | − | − | 1 |
| AREF-D | 4/97–12/02 | 16 | C ($n$ = 9), NT ($n$ = 7) | Abdominal surgery (12), gastroenterology (2), traumatology (1), outpatient (1) | R | (R) | R | R | (R) | R | (R) | R | | | −/+ | −/+ | 1 |
| AREF-W | 2/98–9/01 | 3 | C ($n$ = 3) | Internal medicine (1), ICU—abdominal surgery (1), ICU—vascular surgery (1) | R | (R) | (R) | R | R | R | | R | | (R) | + | −/+ | 1 |
| | 1/00–5/01 | 2 | C ($n$ = 2) | ICU—abdominal surgery (1), ICU—vascular surgery (1) | R | (R) | (R) | R | R | R | | R | | (R) | + | − | 21 |
| AREF-G | 2/98 | 1 | C | Cardiology | R | R | R | | | R | | R | | | − | − | 1 |
| AREF-H | 3/98 | 1 | C | Gastroenterology | R | R | R | R | | R | | | | | − | − | 1 |
| AREF-Z | 5/98 | 2 | C | Abdominal surgery | R | R | R | R | R | R | (R) | R | | | − | − | 1 |
| AREF-K | 5/99 | 1 | NT | Emergency room | R | R | R | | | | | | | | − | − | 1 |
| AREF-J | 12/99 | 1 | C | ICU—Pediatrics | R | R | R | | | R | | | R | | − | − | 1 |
| AREF-Q | 1/00 | 1 | C | ICU—pediatrics | R | | R | | | | | | R | | − | − | 1 |
| AREF-M | 7/00 | 1 | C | Oncology | R | R | R | | | R | R | R | R | | − | − | 1 |
| AREF-N | 8/00 | 1 | C | Infectious diseases | R | R | R | R | R | R | | R | | | − | − | 1 |
| AREF-L | 10/00 | 1 | A | Nephrology | R | | R | | | | | | | | − | − | 6 |
| AREF-R | 5/01 | 1 | NT | Oncology | R | | R | R | | | | R | | | − | − | 1 |
| AREF-Y | 7/01 | 1 | C | Infectious diseases | R | R | R | R | | R | | R | | | − | − | 1 |
| AREF-S | 11/01 | 1 | A | Gastroenterology | R | R | R | R | | R | | | | | − | − | 9 |
| AREF-T | 11/01–8/02 | 4 | C ($n$ = 1), NT ($n$ = 3) | Gastroenterology (3), ICU—medicine (1) | R | R | (R) | R | | R | | | | | + | + | 1 |
| AREF-I | 1/02 | 1 | C | Vascular surgery | R | R | R | | | R | | | R | | − | − | 1 |
| AREF-U | 7/02 | 1 | C | ICU—pediatrics | R | R | R | R | | R | | | R | | − | − | 1 |
| AREF-X | 12/02 | 1 | NT | Oncology | R | R | R | | | R | R | R | R | | − | − | 1 |

[a] NT, not tested.
[b] ICU, intensive-care unit.
[c] R, resistant, (R), variable resistance to a given antibiotic among isolates belonging to the same PFGE type. A, ampicillin; S, streptomycin (high level of resistance); E, erythromycin; C, ciprofloxacin; QD, quinupristin-dalfopristin; K, kanamycin (high level of resistance); G, gentamicin (high level of resistance); Sx, cotrimoxazole; T, tetracycline; V, vancomycin. All isolates resistant to vancomycin were also resistant to teicoplanin, corresponding to the *vanA* genotype.
[d] Presence (+) or absence (−). Some isolates belonging to the same PFGE type showed variable presence of a given VIEP marker (−/+).

TABLE 2. Antimicrobial susceptibilities of *Enterococcus faecium* blood isolates collected from the Ramón y Cajal Hospital (1995 to 2002)

| Antibiotic | Isolate type | MIC (μg/ml)[a] | | | Nonsusceptibility (%)[b] |
|---|---|---|---|---|---|
| | | Range | 50% | 90% | |
| Ampicillin | AREF | 16–128 | 64 | 128 | 100 |
| | ASEF | <0.12–8 | 1 | 4 | 0 |
| Ciprofloxacin | AREF | <0.5–16 | 4 | 32 | 71 |
| | ASEF | <0.5–32 | 4 | 8 | 22 |
| Erythromycin | AREF | <0.25–>128 | >128 | >128 | 86 |
| | ASEF | <0.25–>128 | 4 | >128 | 40 |
| Streptomycin (HLR) | AREF | ≤125–≥2,000 | ≥2,000 | ≥2,000 | 65 |
| | ASEF | ≤125–≥2,000 | ≤125 | 1,000 | 12 |
| Kanamycin (HLR) | AREF | ≤125–≥2,000 | 125 | 125 | 85 |
| | ASEF | ≤125–≥2,000 | 125 | 125 | 12 |
| Gentamicin (HLR) | AREF | ≤125–≥500 | 125 | 125 | 11 |
| | ASEF | ≤125–≥500 | 125 | 125 | 0 |
| Vancomycin | AREF | <0.5–>256 | <0.5 | 1 | 3 |
| | ASEF | <0.5–>2 | <0.5 | 1 | 0 |
| Teicoplanin | AREF | <0.5–>256 | <0.5 | <0.5 | 3 |
| | ASEF | <0.5–0.5 | <0.5 | <0.5 | 0 |
| Quinupristin-dalfopristin | AREF | <0.5–16 | 4 | 16 | 50 |
| | ASEF | <0.5–4 | 2 | 4 | 25 |
| Linezolid | AREF | 0.5–4 | 2 | 2 | 0 |
| | ASEF | 1–4 | 2 | 2 | 0 |
| Chloramphenicol | AREF | 4–32 | 4 | 8 | 6 |
| | ASEF | <0.5–32 | 8 | 8 | 3 |
| Tetracycline | AREF | <0.25–>128 | 0.25 | >128 | 24 |
| | ASEF | <0.5–>32 | 0.25 | 64 | 24 |

[a] 50% and 90%, MICs at which 50 and 90% of isolates are inhibited, respectively.
[b] Differences in susceptibility to ciprofloxacin, HLR to streptomycin, and HLR to kanamycin among AREF and ASEF isolates were statistically significant ($P < 0.001$). Differences in susceptibility to erythromycin among AREF and ASEF isolates were statistically significant ($P < 0.005$).

in medical wards, 26% were in surgical wards, 15% were in intensive-care facilities, and 19% were outpatients. This distribution differs for patients with bacteremia caused by ASEF versus AREF: 31% versus 44% in medical wards, 19% versus 31% in surgical wards, 12% versus 17% in intensive-care facilities, and 37% versus 8% outpatients.

**Antimicrobial susceptibility.** MICs of 13 antibiotics are shown in Table 2. AREF isolates were more resistant than ASEF isolates to the following antibiotics: ciprofloxacin (71% versus 22%), erythromycin (86% versus 40%), quinupristin-dalfopristin (50% versus 25%), streptomycin (high-level resistance [HLR]) (65% versus 12%), and kanamycin (HLR) (85% versus 12%). HLR to gentamicin was not found among ASEF isolates (11% versus 0%). All isolates were susceptible to linezolid. Isolates within a particular PFGE type (A, W, T, D, or Z) showed different susceptibility patterns (Table 1).

**PFGE.** The 86 isolates studied were classified into 57 PFGE types, 25 AREF and 32 ASEF. Four PFGE types were found in more than one patient (A, T, D, W). Identical banding patterns were found among all the isolates within type A and within type T. However, a diversity of banding profiles (0 to 6 bands) was observed for isolates within PFGE types D (16 isolates, 15 subtypes) and W (5 isolates, 5 subtypes) The genetic diversity observed in PFGE types D and W was also reflected by differences in antimicrobial resistance and epidemicity/virulence profiles among isolates of these types. Interestingly, PFGE types representing isolates that persisted in the hospital for more that one year (types A, D, T, W) clustered together (80% similarity) and were obtained from patients located on the same hospital floor.

**AFLP.** Fifty-eight *E. faecium* isolates (34 of 54 AREF and 24 of 32 ASEF isolates) were selected for typing by AFLP; they included isolates belonging to different PFGE types and isolates representing different subtypes within a PFGE cluster showing different VIEP profiles. All AREF isolates studied belonged to AFLP group C, except for two that belonged to group A. ASEF isolates were distributed among AFLP genogroups A ($n = 4$), B ($n = 7$), C ($n = 6$), and R ($n = 7$).

**Diversity of *purK* alleles.** Most of the AREF isolates, 49 strains from hospitalized patients, contained *purK-1*, while 2 harbored *purK-21*, 1 harbored *purK-6*, and 1 contained *purK-9*. The fact that *purK-21* differs from *purK-1* by only 1 nucleotide suggests that isolates carrying *purK-21* are evolutionarily closely related to *purK-1*-carrying isolates and thus probably belong to the same clonal complex. This was supported by a maximum-parsimony tree based on the *purK* gene sequences of the 12 alleles described in this study (Fig. 1).

Conversely, ASEF isolates had a diversity of *purK* alleles: *purK-1* ($n = 3$), *purK-2* ($n = 8$), *purK-3* ($n = 2$), *purK-4* ($n = 1$), *purK-6* ($n = 3$), *purK-8* ($n = 4$), *purK-9* ($n = 5$), *purK-11* ($n = 1$), *purK-16* ($n = 1$), *purK-17* ($n = 1$), and *purK-22* ($n = 2$). Certain *purK* alleles, such as *purK-2*, frequently encountered in isolates belonging to AFLP group B, *purK-3*, *purK-16*, and *pur K-11*, were found only in ASEF isolates from nonhospitalized patients. ASEF isolates containing *purK-6*, *purK-8*, or *purK-9* were collected from inpatients located in different hospital wards (Table 1).

**Virulence/epidemicity markers.** The distribution of different VIEP markers among the *E. faecium* isolates studied is shown in Table 1. We found $esp_{Efm}$ in 24/86 isolates (28%) corre-

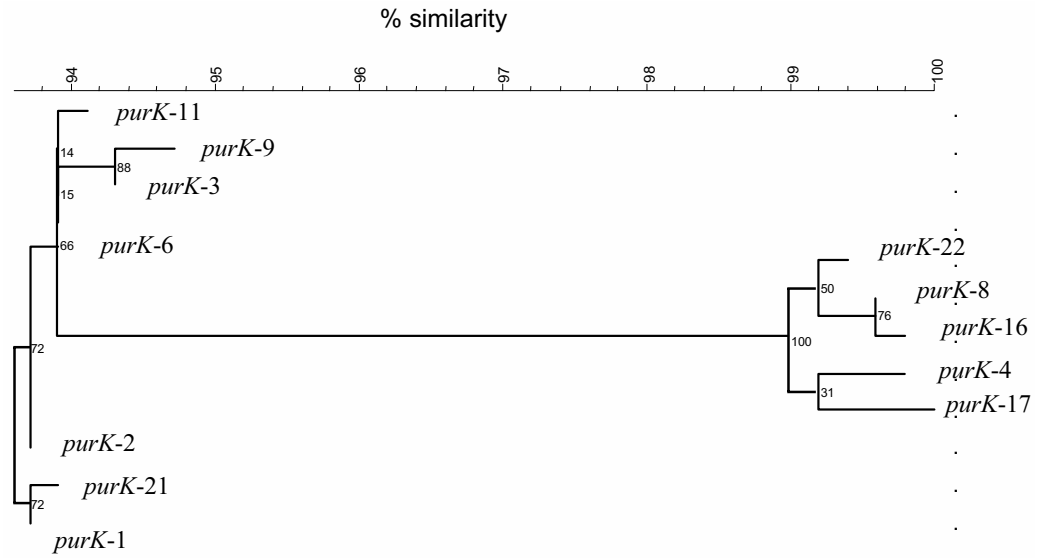


FIG. 1. Rooted maximum-parsimony tree using BioNumerics (version 4.0) based on the DNA sequences of 12 different *purK* alleles. Numbers on the branches refer to bootstrap percentages of 1,000 resamplings.

sponding to five PFGE types. All *E. faecium* isolates harboring the *esp* determinant were AREF and were assigned to AFLP group C. The $hyl_{Efm}$ determinant was detected in 8/86 isolates (9%) corresponding to four PFGE types of AREF isolates that belong to AFLP genogroup C. The first isolates harboring $esp_{Efm}$ or $hyl_{Efm}$ were recovered in 1995 and 1997, respectively. Within specific PFGE types (types D and W), Esp or Hyl positive as well as negative isolates were found. In these cases Esp- and Hyl-negative isolates in time gave way to positive ones. Only 6/86 isolates (7%) corresponding to three PFGE-types (types D, W, and T) contained both $esp_{Efm}$ and $hyl_{Efm}$. These isolates were recovered in 2001 and 2002 on the same floor of our hospital. The presence of gelatinase or hemolysin was not demonstrated for any isolate.

**MLST.** Fourteen AREF isolates representing PFGE types with different VIEP marker profiles and 6 ASEF isolates containing different *purK* alleles were studied by MLST (Table 3). STs obtained for AREF isolates with *purK-1* were ST-16, ST-17, and ST-18, and those obtained for AREF isolates with *purK-21* were ST-63 and ST-103; all clustered in a single clonal complex (CC-17). The only *purK-1* ASEF isolate studied had ST-18 and also belonged to this CC. This clonal complex encompasses most of the outbreak isolates collected from hospitalized patients on different continents (17, 44).

**Clinical data.** Data for analysis were available for 60 of the 84 patients included in the study. Data included in the clinical records of the other 24 patients were too scarce to be analyzed in detail, but they did not differ from those of the patients

TABLE 3. Diversity of STs among *E. faecium* blood isolates with different antibiotic and virulence profiles

| MLST | Clonal complex | PFGE type | AMP profile[a] | No. of isolates | Allelic profile[b] | | | | | | | AFLP type | VIEP marker[c] | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | *purK* | *adk* | *atpA* | *ddl* | *gyd* | *gdh* | *pstS* | | *esp* | *hyl* |
| ST-18 | CC-17 | AREF-A | R | 1 | 1 | 1 | 7 | 1 | 5 | 1 | 1 | C | − | − |
| ST-18 | CC-17 | AREF-D | R | 4 | 1 | 1 | 7 | 1 | 5 | 1 | 1 | C | −/+ | − |
| ST-18 | CC-17 | AREF-J | R | 1 | 1 | 1 | 7 | 1 | 5 | 1 | 1 | C | − | − |
| ST-18 | CC-17 | AREF-M | R | 1 | 1 | 1 | 7 | 1 | 5 | 1 | 1 | C | − | − |
| ST-18 | CC-17 | AREF-C | R | 1 | 1 | 1 | 7 | 1 | 5 | 1 | 1 | C | − | + |
| ST-18 | CC-17 | ASEF-9 | S | 1 | 1 | 1 | 7 | 1 | 5 | 1 | 1 | C | − | − |
| ST-17 | CC-17 | AREF-P | R | 2 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | C | + | − |
| ST-16 | CC-17 | AREF-T | R | 1 | 1 | 1 | 1 | 2 | 1 | 1 | 1 | C | + | + |
| ST-63 | CC-17 | AREF-W | R | 2 | 21 | 1 | 1 | 1 | 1 | 1 | 1 | C | + | − |
| ST-103 | CC-17 | AREF-W | R | 1 | 21 | 1 | 1 | 14 | 1 | 1 | 1 | C | + | − |
| ST-4 | | ASEF-31 | S | 1 | 3 | 5 | 8 | 4 | 1 | 1 | 1 | NT | − | − |
| ST-22 | | ASEF-2 | S | 1 | 2 | 1 | 2 | 3 | 1 | 1 | 1 | B | − | − |
| ST-71 | | ASEF-3 | S | 1 | 2 | 1 | 15 | 3 | 1 | 1 | 1 | A | − | − |
| ST-40 | | ASEF-32 | S | 1 | 16 | 6 | 10 | 8 | 6 | 8 | 10 | R | − | − |
| ST-74 | | ASEF-15 | S | 1 | 22 | 6 | 22 | 13 | 6 | 14 | 27 | R | − | − |

[a] AMP, ampicillin; R, resistant; S, susceptible.
[b] *adk*, gene coding for adenylate kinase; *atpA*, gene coding for ATP synthase, alpha subunit; *ddl*, gene coding for D-alanine:D-alanine ligase; *gyd*, gene coding for glyceraldehyde-3-phosphate dehydrogenase; *gdh*, gene coding for glucose 6-phosphate dehydrogenase; *pstS*, gene coding for phosphate ATP binding cassette transporter.
[c] Presence (+) or absence (−). Some isolates belonging to the same PFGE type showed variable presence of a given VIEP marker (−/+).

TABLE 4. Association between epidemiological variables of patients with bacteremia caused by *Enterococcus faecium* and the virulence/epidemicity traits *esp*, *purK-1*, and *hyl*

| Type of analysis and variable | Significance of association (OR; 95% CI; *P*) | | |
|---|---|---|---|
| | *esp* | *purK-1* | *hyl* |
| Univariate analysis | | | |
| Hospital location | NS | 0.06; 0–0.47; **0.002** | NS |
| Urinary catheterization | 0.18; 0.03–0.87; **0.013** | NS | NS |
| Therapy with β-lactams | NS | 0.18; 0.04–0.79; **0.019** | NS |
| Therapy with quinolones | NS | NS | NS |
| Hospital stay >7 days | 0.14; 0.02–0.79; **0.009** | NS | NS |
| Nosocomial acquisition | 0.13; 0.01–1; **0.04** | 0.14; 0.03–0.57; **0.003** | NS |
| Multivariate analysis | | | |
| Hospital location | | 0.06; 0.007–0.57; **0.002** | |
| Urinary catheterization | | | |
| Hospital stay >7 days | 0.14; 0.02–0.70; **0.005** | | |
| Nosocomial acquisition | | 0.16; 0.04–0.67; **0.008** | |

[a] OR, odds ratio; CI, confidence interval; NS, not significant. *P* values are boldfaced.

analyzed with respect to clinical data and outcome (data not shown). Of the 60 patients analyzed, bacteremia was caused by AREF in 39 (65%) and by ASEF in 21 (35%).

The following variables were significantly associated in univariate analysis with AREF: place of hospitalization (abdominal or digestive surgery departments versus other), urinary catheterization, previous exposure to beta-lactams or quinolones, length of hospitalization, and nosocomial acquisition. No significant association was found between the presence of central venous catheters, parenteral nutrition, abdominal surgery, or underlying diseases (diabetes, neoplasia, immunodeficencies) and bacteremia caused by AREF or ASEF. No association was found between previous therapy with vancomycin, metronidazole, clindamycin, or sulfonamides and isolation of AREF or ASEF.

Univariate analysis demonstrated a strong correlation between *purK-1* determinants and AFLP type C ($P < 0.001$), so both factors were considered together for subsequent analysis. Strains with *purK-1* also have a significantly higher proportion of $esp_{Efm}$ ($P = 0.001$) but not of $hyl_{Efm}$. The presence of *esp* was significantly associated with nosocomial acquisition, prolonged hospitalization, and urinary catheterization. *purK-1* was also significantly associated with nosocomial acquisition, particularly with a stay in the general surgery or gastroenterology ward, and with previous therapy with beta-lactam agents. In multivariate analysis, Esp was again significantly associated with prolonged hospitalization and *purK-1* with nosocomial acquisition, particularly in the above-mentioned wards (Table 4).

## DISCUSSION

AREF strains have been suggested to serve as substrates for the emergence of VREF outbreaks in European and American institutions (20, 24, 31, 36). MLST studies including epidemiologically unrelated isolates from different sources, hosts, and geographical areas demonstrated that recent *E. faecium* population biology has at least two main components: (i) a great number of genotypes at linkage equilibrium and (ii) a recently emerged clonal complex encompassing most of the VRE outbreak isolates collected from hospitalized patients on different continents (originally designated C1 and renamed CC-17) which are predominantly resistant to ampicillin (90%) and contain the *esp* trait (70%) (5, 17, 44). In our 8-year-follow-up study, we have also detected similar clear-cut differences between ASEF and AREF populations causing bacteremia in our institution. AREF isolates constitute a highly clonal population with features of CC-17 (*purK-1* and *-21*; AFLP genogroup C). The low diversity of AREF clinical isolates has also been demonstrated in Norwegian and Swedish hospitals, suggesting the emergence and spread of an *E. faecium* subpopulation in the clinical setting both in geographical areas where VRE are epidemic and in those where they are not (17, 19, 40; this study).

Our results showed clear differences in the host-ecovar specificity of the VSEF population causing bacteremia in our institution. Most AREF isolates from our study clustered in genogroup C (isolates from hospitalized humans), while ASEF isolates were equally distributed among genogroups A (isolates from pigs and nonhospitalized humans), B (isolates from poultry), C, and R (human strains that do not fit in any of the other groups mentioned) (47), indicating different acquisition routes for different invasive *E. faecium* strains. Previous studies in different countries have shown that most VREF and VSEF clinical isolates clustered in genogroup C, while VREF and VSEF from community sources or colonized inpatients clustered in different genogroups (8, 17, 22, 47). Differences between our study and previous work might be due to the bias created by the selection of isolates, since other series included smaller numbers of ASEF or blood isolates (8, 17, 19, 22, 47), thereby underestimating/overlooking infections caused by endogenous ASEF isolates (19).

It has been demonstrated previously that specific clones can acquire different antibiotic resistance and virulence traits, highlighting the relevance of the local gene pool for the dissemination and persistence of antibiotic-resistant enterococci (4, 29, 34, 36, 48). In this study, epidemic and nonepidemic AREF isolates with different antibiotic and virulence profiles belonging to AFLP genogroup C and containing *purK-1* or *purK-21* correspond to STs within CC-17 (ST-16, -17, -18, -63, and -103). This was also true for one ASEF isolate. Since isolates of this clonal complex were able to persist for long periods in our hospital, the lack of epidemic VREF in our country and other European locations might be due to differences in the availability, ability of transfer, or stability of specific genetic elements coding for vancomycin resistance (7, 12, 16, 39–41, 45, 48).

The *esp* gene has been defined as an epidemicity marker of VREF isolates (17, 21, 46); however, it has been also detected in nonepidemic VSEF isolates, and it is absent in some epidemic AREF strains (3, 11, 19, 21, 49). That suggests that other factors might play a role in the dissemination of the main AREF clonal complex. The variable presence of *esp* in isolates of PFGE type D could be explained by horizontal transfer of either DNA fragments containing *esp* or the whole *E. faecium*

PAI to only some isolates within the clone or by loss of this DNA region by some of the isolates. Transfer and deletion of PAIs is frequent (15), and interenterococcal transfer of large chromosomal fragments has been reported on different occasions (24). Furthermore, conjugative transfer of *esp* among isolates of *E. faecium* has been described recently (30). In addition to Esp, Hyl has also been associated with epidemic isolates (32). We detected only eight $hyl^+$ isolates corresponding to five PFGE types (9%), a frequency lower than that previously reported for American isolates (34%) (32). Interestingly, for the clones representing both *esp*- and/or *hyl*-positive and -negative isolates, the negative variant gave way in time to a positive variant, possibly suggesting horizontal transfer of these traits in the nosocomial setting. The fact that the presence of *esp* was associated with prolonged hospitalization and urinary catheterization suggest that these traits may have been acquired to enhance fitness at least in some of the clones. Differences in the prevalence of *esp* and *hyl* between this and other studies could be due to the bias imposed by the selection of the strains or, again, by differences in the local availability of *esp*- or *hyl*-containing elements.

PFGE-types D and W, repeatedly recovered in bacteremic cases from inpatients in high-risk areas over 5 years and 1 year, respectively, were highly variable (for type D, 16 isolates and 15 subtypes; for type W, 5 isolates and 5 subtypes). In contrast, two PFGE types recovered from inpatients in medical wards were highly stable over time (types A and T, persisting 1 and 3 years, respectively). A remarkable polymorphism among isolates corresponding to the same PFGE type (0 to 7 bands) has been shown previously in different long-term studies (2, 6, 26, 36, 43). Differences in clone plasticity might be explained by a variable presence of "hot spots" for rearrangements of genetic fragments in the genomes, the local availability (ward, hospital, host) of adaptive genetic elements and the further evolution imposed by the environment (interplay with a patient's own flora or antibiotic selective pressure).

The analysis of patients' clinical records confirms our findings, as it strongly supports the nosocomial acquisition, from specific wards in our institution, of strains harboring the *purK-1* allele and belonging to genogroup C, as well as the association of strains harboring *esp* with prolonged hospitalization. In agreement with previous studies (13, 25), the place of hospitalization (general surgery or gastroenterology ward versus other), urinary catheterization, previous exposure to β-lactams or quinolones, and lengthy hospitalization were associated with the isolation of AREF.

This paper documents the population structure of isolates belonging to the "epidemic" clonal complex CC-17 of *E. faecium* in a setting with a near-absence of VREF. The reasons for the different evolutionary outcomes of this CC in different countries remain to be clarified, although local availability of antibiotic resistance determinants and/or specific gene capture elements, previously demonstrated to be important for successful dissemination and persistence of VRE (18, 29, 39), might contribute to explaining these differences.

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