A Functional Collagen Adhesin Gene, acm, in Clinical Isolates of Enterococcus faecium Correlates with the Recent Success of This Emerging Nosocomial Pathogen

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Enterococcus faecium recently evolved from a generally avirulent commensal into a multidrug-resistant health care-associated pathogen causing difficult-to-treat infections, but little is known about the factors responsible for this change. We previously showed that some E. faecium strains express a cell wall-anchored collagen adhesin, Acm. Here we analyzed 90 E. faecium isolates (99% acm+) and found that the Acm protein was detected predominantly in clinically derived isolates, while the acm gene was present as a transposon-interrupted pseudogene in 12 of 47 isolates of nonclinical origin. A highly significant association between clinical (versus fecal or food) origin and collagen adherence (P ≤ 0.0003) was also demonstrated, and levels of adherence were highly correlated (r = 0.879) with the amount of cell surface Acm detected by whole-cell enzyme-linked immunosorbent assay and flow cytometry. Thirty-seven of 41 sera from patients with E. faecium infections showed reactivity with recombinant Acm, while only 4 of 30 community and hospitalized patient control group sera reacted (P ≤ 0.0003); importantly, antibodies to Acm were present in all 14 E. faecium endocarditis patient sera. Although pulsed-field gel electrophoresis indicated that multiple strains expressed collagen adherence, multilocus sequence typing demonstrated that the majority of collagen-adhering isolates, as well as 16 of 17 endocarditis isolates, are part of the hospital-associated E. faecium genogroup referred to as clonal complex 17 (CC17), which has emerged globally. Taken together, our findings support the hypothesis that Acm has contributed to the emergence of E. faecium and CC17 in nosocomial infections.

Enterococcus faecium, generally considered a normal mammalian gut commensal, has emerged in the last 2 decades as a significant nosocomial pathogen (11, 14, 22). In addition to an increased incidence of E. faecium infections, increased morbidity and mortality due to vancomycin-resistant E. faecium (VRE) bacteremia have also been reported, particularly among patients neutropenic from cancer chemotherapy, those receiving dialysis, and those who have undergone liver or bone marrow transplantation (6). For example, a recent study (30) reported VRE intestinal colonization in 40% of 92 neutropenic patients, of which 34% developed bacteremia with this organism, with a subsequent mortality rate of 36%, despite treatment with recently available drugs such as linezolid and daptomycin; this is in marked contrast to the decades before VRE emerged, when E. faecium infections were rarely seen.

Acquisition of resistance to vancomycin preceded by emergence of resistance to ampicillin, impacting the primary therapies of choice for nonresistant strains, has been assumed to be the major factor responsible for transforming this organism from its docile, commensal nature into a significant nosocomial pathogen (14). It has also been postulated that the recent clinical success of E. faecium exemplified by the hospital-associated E. faecium genogroup, also called clonal complex 17 (CC17), which accounts for the majority of clinical isolates (11), is due at least in part to the acquisition or evolution of putative virulence-associated traits, such as enterococcal surface protein (Esp) and a hyaluronidase-like gene (hyl) (23). However, a contribution of these traits to E. faecium pathogenesis has not been demonstrated, and we found them in only 49% and 28%, respectively, of clinical E. faecium isolates (23).

The recent emergence of resistance to newer antibiotics, including quinupristin-dalfopristin, linezolid, and daptomycin, by VRE strains emphasizes the need for alternative therapeutic strategies, which might include immunoprophylaxis or immunotherapy by targeting in vivo-expressed virulence-associated surface proteins. Evidence from other gram-positive pathogens suggests that the adhesin family of proteins may serve as potential candidates for the development of novel immunotherapies (24). It was recently shown that combination therapy with vancomycin plus anti-clumping factor A antibodies was more effective than vancomycin alone in sterilizing rabbit valvular vegetations in infective endocarditis caused by methicillin-resistant Staphylococcus aureus (29).

Recently, we identified the collagen-binding adhesin Acm as a primary and, to date, only documented adhesin of E. faecium (20). Acm has an N-terminal signal peptide, followed by a collagen binding A domain, a variable number of B repeats, and a C-terminal region for sorting and anchoring to peptidoglycan. Although we demonstrated that the gene encoding this adhesin (acm) was present in all 32 E. faecium isolates tested (20), unlike its staphylococcal homologue Cna, which is present in 38 to 56% of isolates (26), only 55% of 20 infection-derived clinical isolates and none of the 10 community-derived

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fecal isolates showed collagen adherence. Sequencing of the \textit{acm} locus for six non-collagen-adherent \textit{E. faecium} isolates identified a pseudogene in five of them (20), and genetic analysis confirmed that Acm is sufficient to mediate the attachment of \textit{E. faecium} strains to collagen (18, 20). In our most recent study, we identified the minimal and high-affinity binding subsegments of Acm and showed that antibodies against these Acm subsegments inhibited collagen adherence of \textit{E. faecium} cells (16).

In the current investigation, we first studied the collagen-adhering capability of an additional 90 \textit{E. faecium} isolates, derived from human infections, community feces, and animals, and found a highly statistically significant association between a clinical origin as the source of the isolate and collagen type I adherence. We next tested for the presence of an uninterrupted \textit{acm} gene and surface expression of Acm to confirm the involvement of Acm in this collagen adherence by the diverse collection of isolates. We have also taken advantage of our collection of sera from patients with \textit{E. faecium} endocarditis to look for evidence that Acm is produced during infections, even if the infecting strain does not produce Acm in vitro under standard laboratory growth conditions. The molecular epidemiological analyses of this study, supported by experimental endocarditis data from the companion paper (19), suggest that expression of Acm, the mediator of collagen adherence of \textit{E. faecium}, is important for successful competition of this species in the clinical setting.

\section*{MATERIALS AND METHODS}

\textbf{Bacterial strains and growth media.} Ninety \textit{E. faecium} strains isolated over 18 years from diverse locations (Argentina, Belgium, China, Spain, Germany, Norway, and several cities in the United States) were included in this study. These isolates included some from endocarditis specimens (n = 11), other clinical specimens (Other-Clin group) (n = 31), community-derived human feces (n = 20), and animals (either meat products or feces of cattle, chickens, turkeys, or swine) (n = 23). The sources of the Other-Clin isolates included blood, bile, catheters, sputum, urine, and several types of wounds, among others, and several isolates in this group are representative of epidemic and outbreak strains (1, 3, 8, 10, 13, 28). Six additional endocarditis isolates for which we assessed the adherence phenotype in our previous study (20) were included for further characterization. An allelic replacement \textit{acm} deletion mutant, \textit{TX0651 (TX0082 dacr::cat)} (18), was included as a control strain for flow cytometry analysis.

Brain heart infusion (BHI) broth and agar (Difco Laboratories, Detroit, MI) were used for routine \textit{E. faecium} growth.

\textbf{Colony hybridization for species identification and determination of gene presence.} All of the \textit{E. faecium} isolates, initially identified to the species level by standard identification methods and sequencing of the \textit{VOL. 76, 2008 CONTRIBUTION OF Acm TO E. FAECIUM CLINICAL SUCCESS} 4111

\textbf{biochemical tests, were confirmed by high-stringency colony hybridization, using \textit{E. faecium} strains to collagen (18, 20). In our most recent study, we identified the minimal and high-affinity binding subsegments of Acm and showed that antibodies against these Acm subsegments inhibited collagen adherence of \textit{E. faecium} cells (16).}

\textbf{Adherence assay.} All isolates were tested for adherence to collagen type I (Sigma Chemical Co., St. Louis, MO) and bovine serum albumin (BSA), using a previously described assay (20).

\textbf{Acn-specific polyclonal antibodies.} Production of rabbit polyclonal antibodies against the recombinant Acm A domain (\textit{rAcm A}) was described elsewhere (20). \textit{rAcm A} domain-specific antibodies were eluted from \textit{rAcm A} coupled to cyanogen bromide-activated Sepharose 4B according to the manufacturer’s protocol (Amersham Biosciences, Piscataway, NJ). The antibodies were concentrated by ultrafiltration with a 10,000-Da-molecular-mass-cutoff filter (Millipore, Bedford, MA) and diazylated against phosphate-buffered saline (PBS), and concentrations were determined by absorption spectroscopy.

\textbf{Whole-cell ELISA.} Surface expression of Acm on \textit{E. faecium} cells was detected by a whole-cell enzyme-linked immunosorbent assay (ELISA) using affinity-purified \textit{Acm A} domain (binding domain)-specific antibodies, as described earlier (20). Antiserum raised against formalin-killed \textit{TX0016} whole cells (21) was used as a control to confirm that whole cells of all strains were actually bound to the microtiter plates.

\textbf{Flow cytometry.} For flow cytometry analysis, bacteria were grown in BHI broth until entry into stationary phase and washed twice with PBS, 100 \mu l of bacteria adjusted to an optical density at 600 nm (OD\textsubscript{600}) of 0.2 in PBS was added to an Eppendorf tube containing 40 \mu l of newborn calf serum (Siga) and incubated for 30 min at room temperature. After centrifugation at 10,000 \times g for 6 min, 100 \mu l of 20-\mu g/ml preimmune or affinity-purified anti-Acm A specific antibodies in dilution buffer (PBS containing 20\% newborn calf serum and 0.1\% BSA) was added. Incubation was performed at 4\°C for 2 h. The bacteria were washed twice with 400 \mu l of 0.1\% BSA in PBS, and 100 \mu l of 1:100 dilution antisera with immunoglobulin G (IgG) conjugated with F(ab’\textsubscript{2}) fragment-specific R-phycocerythrin (Jackson ImmunoResearch, West Grove, PA) in dilution buffer was added and incubated at 4\°C for 2 h. After being washed twice with 400 \mu l of 0.1\% BSA in PBS, cells were resuspended in 500 \mu l of 1\% paraformaldehyde in PBS and were analyzed with a Coulter Epics XL AB6064 flow cytometer (Beckman Coulter) and System II software.

\textbf{PCR method for screening IS element interruptions and sequencing.} Chromosomal DNA from \textit{E. faecium} isolates were prepared as described earlier (33). PCR and sequencing of the \textit{acm} locus were performed using previously described primers (20). Screening for pseudogenes due to interruption by insertion elements (IS elements) was performed by PCR with 3 sets of overlapping primers, namely, \textit{AcmF1-AcmR4}, \textit{AcmF2-AcmR1}, and \textit{AcmF3-AcmR3} (20), that cover the entire \textit{acm} locus. Randomly chosen PCR products were sequenced by the Taq BigDye Terminator method (Applied Biosystems, Foster City, CA) to confirm the predictions and to identify the IS element.

\textbf{Human sera.} From our serum collection, four study groups were selected for analysis. Sera from 14 patients with \textit{E. faecium} endocarditis constituted one group. A second group consisted of 27 serum samples collected from patients with \textit{E. faecium} nonendocarditis infections in hospitals in Nebraska, New York, Delaware, Michigan, New Jersey, Illinois, and Pennsylvania. The third group consisted of 15 sera that had been obtained for routine chemistry testing from hospitalized patients with no knowledge of their diagnosis; these sera were included as a control group (nonhealthy control sera). The final group consisted of 15 human sera from healthy volunteers (normal human sera [NHS]); these sera were included as a healthy control group. Twenty additional NHS, previously pooled in groups of three to five, were used for the determination of serum titers, as described below.

\textbf{Western blotting and Acm antibody titer determination.} The cloning of the \textit{E. faecium} \textit{TX2555 acm} gene, coding for all 501 amino acids of the \textit{Acm} A domain, and purification and phosphorylation of Acm have been described elsewhere (20). \textit{rAcm A} protein was electrophoresed in 4 to 12\% NuPAGE bis-Tris gels (Invitrogen, San Diego, CA) under reducing conditions in 3\%-[\text{N-morpholino}] propanesulfonic acid buffer and transferred to a polyvinyldiene difluoride membrane. The presence of anti-Acm antibodies was detected by incubation with either \textit{E. faecium} endocarditis patient serum (antibody I) or NHS obtained from healthy volunteers, followed by horseradish peroxidase-conjugated goat anti-human IgG antibodies (antibody II) and development with 4-chloronaphthol in the presence of \textit{H}_2\text{O}_2.

\textbf{ELISAs using human sera and \textit{rAcm A} protein were performed as previously described (17).} For ELISA, each serum was assayed in duplicate in serial dilutions of 1:16 to 1:1,024 in 1\% NHS containing 200 \mu g/ml of 1:100 dilution antibodies to \textit{rAcm A} protein (Sigma Chemical Co., St. Louis, MO) and bovine serum albumin (BSA), using a previously described assay (20).

\textbf{PFGE.} Agarose plugs containing genomic DNA were digested with \textit{Smal} or \textit{BglII}, and pulsed-field gel electrophoresis (PFGE) was performed using a previously described method (15), but with ramped pulse times of 2 s and 30 s. Since \textit{Smal} profiles were more discriminatory for some isolates and \textit{ApaI} profiles were more discriminatory for others, we used both for analysis. PFGE was performed for all 17 endocarditis-derived \textit{E. faecium} isolates (including 6 previously described isolates) (20) and collagen-adhering isolates from the Other-Clin group with broad geographical and temporal diversity.

\textbf{MLST.} Multilocus sequence typing (MLST) and analysis of allelic profiles of \textit{E. faecium} isolates were performed as described previously (9), with the use of primer sequences available at http://ecaecium.mlst.net/misc/info.asp. Different sequences of a given locus were assigned allele numbers, and different allelic profiles were assigned sequence types (STs). MLST was carried out for one isolate of the PF1 pulotype, one of the PF2 pulotype, and seven distinct
pulotype strains. Eight of these were derived from endocarditis samples, and one was from the Other-Clin group (see Results for details on PF numbers).

Drug susceptibility testing. Vancomycin and ampicillin susceptibilities were determined by agar dilution, with concentrations ranging from 4 to 64 g/ml; MICs of $\geq 16$ g/ml of ampicillin and $\geq 32$ g/ml of vancomycin were considered resistant, according to CLSI guidelines (4, 5).

Statistical analyses. Fisher's exact test was used to determine the statistical significance of the differences observed in collagen adherence phenotypes of different strain groups. To determine if a correlation existed between collagen adherence and Acm surface detection, Pearson's correlation coefficient ($r$) was calculated. One-tailed Student’s $t$ test was used to compare Acm antibody levels (at a 1:16 dilution) among the three groups of subjects.

RESULTS AND DISCUSSION

Association of clinical origin and collagen adherence in *E. faecium* isolates from four continents. Since our previous pilot survey (20) with 32 isolates suggested a relationship between in vitro collagen adherence and clinical origin, we extended this analysis to a diverse group of 90 additional *E. faecium* isolates (including isolates of animal origin) from our collection. Eight of 11 (73%) endocarditis-derived isolates and 18 of 31 (58%) Other-Clin isolates showed in vitro adherence (defined as $\geq 5\%$ of cells adhering [20]) to collagen type I (see Fig. 1, which also shows data for the 30 nonlaboratory isolates previously reported [20]). All four catheter-derived infection isolates (Other-Clin group) showed collagen adherence, and the percentages of cells showing adherence among these strains ranged from 10.2 to 54.5%. In contrast, only 1 of 25 community-derived isolates from feces of healthy volunteers and 2 of 23 animal-derived isolates adhered to collagen, and this adherence was at a low level, as shown in Fig. 1. A highly statistically significant difference was observed between the percentages of adherent strains in the endocarditis group and the community-derived fecal isolate group or the animal isolate group ($P < 0.0001$ and $P = 0.0002$, respectively). Although the percentage of in vitro collagen-adhering isolates was found to be higher in the endocarditis group (73%) than in the nonendocarditis Other-Clin group (58%), this difference was not significant. The consistent association of clinical origin and adherence, observed in two independent studies (here and in our previous pilot survey [20]) with different isolates from four continents, suggests that collagen adhesion may be a risk factor that enhances the ability of *E. faecium* to survive, colonize, and/or cause infection in the clinical setting.

Our further investigations in this study were designed to test the hypotheses that (i) Acm is the primary collagen adhesin for...
isolates of diverse origins under the growth conditions tested; (ii) the acm gene may be expressed during severe E. faecium infections, such as endocarditis, even in isolates that do not produce Acm in vitro; and (iii) a functional acm gene may correlate with the increased occurrence of the hospital-associated E. faecium cluster of the distinct genogroup CC17.

The amount of surface Acm correlates with collagen adherence. To investigate the correlation between Acm and collagen type I adherence by diverse isolates, we assessed surface expression of Acm, using anti-Acm A domain affinity-purified antibodies in a whole-cell ELISA. Thirty-one E. faecium isolates, including 11 endocarditis isolates representing nine pulsortypes (see below), 10 Other-Clin isolates, and 5 isolates from each of the nonclinical groups, were tested (Fig. 2). All of the 19 collagen-adhering strains were positive in this assay, and the OD450 values of these strains ranged from 0.252 to 0.672 (Fig. 2), while the OD450 values for all 31 strains with preimmune Igs ranged from 0.002 to 0.112 (data not shown). When the collagen adherence percentages of the respective strains were plotted as a function of the level (OD450) of surface-exposed Acm, regression analysis showed that the amount of surface Acm correlated strongly (r = 0.879; P < 0.0001) with the degree of collagen adherence (Fig. 2).

To further test whether the observed variation in collagen adherence of different isolates is due to variability in Acm surface expression, we next quantified surface expression of all five nonadhering and four adhering endocarditis isolates by fluorescence-activated cell sorting analysis using Acm A-domain-specific antibodies and preimmune Igs. The binding of anti-Acm to the surfaces of all four collagen-adhering strains (TX0074, TX2535, TX0082, and TX2658) was readily detected (Fig. 3A to D) and correlated well with the various levels of collagen adherence observed in these strains. Furthermore, anti-Acm did not exhibit any measurable binding to the surfaces of three nonadhering strains (TX0016, TX0068, and TX0111) and bound to only a minor fraction (4.1 to 6.6%) (Fig. 3E to J) of three other nonadhering strains (TX0080, TX0110, and TX0111). Thus, our results indicate

FIG. 3. Quantitation of Acm surface expression by fluorescence-activated cell sorting analysis. (A to D) Analysis of four endocarditis-derived collagen-adhering E. faecium isolates exhibiting different levels of collagen adherence. Percentages of radiolabeled cells adhering to collagen were 43%, 36%, 11%, and 6% for TX0074, TX2535, TX0082, and TX2658, respectively. (E to J) Analysis of five non-collagen-adhering E. faecium endocarditis isolates and an acm deletion mutant of TX0082. Percentages of radiolabeled cells adhering to collagen were 2.3%, 2.7%, 3.9%, 2.3%, and 2.2% for TX0016, TX0068, TX0080, TX0110, and TX0111, respectively. Bacteria were analyzed by flow cytometry, using side scatter as the threshold for detection. Specific binding by anti-Acm antibodies is indicated as log fluorescence intensity on the x axis. Each histogram represents 50,000 events (bacterium-sized particles). PI, preimmune Igs; anti-Acm, anti-Acm A-domain-specific Igs.
that the level of collagen adherence is related to the amount of Acm on cell surfaces as well as to the percentage of cells expressing Acm.

Taken together, these surface Acm results corroborate our studies of acm constructs (18, 20), demonstrating that surface localization of Acm is necessary and sufficient for collagen type I adherence in clinical strains belonging to multiple pulsotypes (see below) under the growth conditions used.

**IS element-mediated disruptions of the acm gene are frequent in isolates of nonclinical origin.** Since a previous survey of 56 *E. faecium* strains from Brazil showed the absence of acm in one *E. faecium* isolate from a healthy human (2), we first screened the isolates of this study for the presence of the acm gene. Hybridization results showed that 89 of 90 (99%) *E. faecium* isolates carried the acm gene, thus confirming its very widespread nature; the exception was a purK allele 6 (non-CC17) poultry isolate susceptible to both ampicillin and vancomycin. Since this isolate served as a negative control for PCR with overlapping primers covering the entire acm locus, followed by sequencing of selected strains. The results showed interruption of acm (leading to a pseudogene) in 0% of endocarditis isolates, 6% of Other-Clin isolates, 24% of community fecal isolates, and 27% of animal isolates and accounted for the nonadherence phenotype of approximately one quarter of the nonclinical group isolates; disruptions mapped to three regions and were caused by four types of IS elements (Fig. 4). Although our earlier pilot study (20) found that two of six nonadherent isolates had a mutation leading to a premature stop codon, additional sequencing was not performed, except for that of endocarditis isolates included in our companion study (19).

**Precise excision of IS256 from the acm pseudogene of clinical strain TX2466 in the presence of collagen as a mechanism for increased expression of Acm.** Among two pseudogene-containing Other-Clin isolates, one (TX2466), in which acm was interrupted by IS256, was noted to show a faint band by PCR that corresponded to the size of an uninterrupted acm sequence, suggesting spontaneous excision of IS256 from acm. After growth in collagen-supplemented (0.1 mg/ml) BHI broth, this strain showed a slight increase in collagen adherence (Fig. 5A). In addition, comparison of this strain by fluorescence-activated cell sorting after passage of cells in collagen-coated wells versus passage in BHI showed an increase in the percentage of cells (from 2.8% to >16%) expressing Acm on the surface (Fig. 5B). Subsequent testing of ~700 colonies of TX2466 by PCR after repeated passages in wells coated with immobilized collagen showed no completely revertant colonies, although a higher-intensity, intact-size band was detectable in the mixture of cells after enrichment (Fig. 5C); sequencing of the eluted intact-size band identified precise excision of the 1,332-bp IS256, including the initially duplicated 8-bp target site (Fig. 5D). Whether “permanent” revertant colonies could be obtained under different conditions remains to be seen. Similar IS256-mediated modulation of expression of a polysaccharide intercellular adhesin was previously demonstrated for staphylococci (35).

Anti-Acm antibodies are present in all patients with *E. faecium* endocarditis, including those whose infecting isolates do not express Acm in vitro. An indirect assessment of in vivo

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**FIG. 4.** PCR detected acm disruptions in 14 different *E. faecium* isolates. (A) Schematic representation showing the positions of disruptions by different IS elements analyzed in isolates of this study and a previous study (20). RBS, predicted ribosomal binding site; S, signal peptide; A domain, nonrepetitive collagen binding domain; B domain, domain with variable number of repeats in different strains; W, cell wall domain with motif required for sortase-mediated surface anchoring; M, membrane-spanning domain; and C, charged C-terminal domain. (B) Distribution of disrupted acm genes among 89 acm-positive *E. faecium* isolates from this study. †, the four collagen-adherent and two nonadherent endocarditis isolates of our previous study included in this study for molecular epidemiological analyses and surface expression level studies also had an uninterrupted acm gene (20); *, a single isolate of animal origin lacked acm.
expression is the demonstration of a serological response. We initially screened five *E. faecium* endocarditis patient sera and eight NHS from a healthy control group by Western blotting. Among the five endocarditis sera, two reacted strongly and three reacted moderately, suggesting that in vivo surface expression of Acm by different strains had occurred in these patients (Fig. 6A); none of the sera from healthy controls reacted with Acm. We then quantitatively assayed the presence of Acm A-domain-specific antibodies from four different serum groups, described in Materials and Methods. Strikingly, all 14 *E. faecium* endocarditis sera in our collection (including the 5 used in Western blot analyses) showed reactivity (Fig. 6B) with rAcm A. Titers of the reactive *E. faecium* endocarditis sera against rAcm A varied from 1:16 to 1:1,024. The endocarditis patient serum that showed the lowest titer (1:16) also showed low titers against total enterococcal surface proteins (data not shown). Of particular interest, among the five endocarditis isolates that did not show collagen adherence (including two from a previous study [20]) (Fig. 1) or reaction with anti-rAcm A antibodies in vitro (Fig. 3), sera were available from four of the patients infected with these organisms. All four patient sera had substantial antibodies to Acm (Fig. 6B, black circles), despite our inability to demonstrate the presence of Acm or collagen adherence in vitro, supporting the notion that Acm was expressed by these strains in vivo, even though it was not expressed in vitro.

A total of 23 of 27 (85%) sera from *E. faecium* nonendocarditis Other-Clin infections showed Acm antibody levels that were greater than the control serum levels, and the remaining 4 sera from this group showed reactivity equal to that of controls. Of the 15 nonhealthy control sera from hospitalized patients, 12 reacted at levels that were the same or lower than those of NHS and three had elevated anti-Acm antibody levels. Clinical information was not obtained for these randomly chosen nonhealthy control sera. Thus, we predicted that the three positive patients may have had a prior *E. faecium* infection or had cross-reacting antibodies. Likewise, 1 of 15 healthy control group sera also had anti-Acm antibodies. A statistically significant difference was observed between the endocarditis group and the nonendocarditis Other-Clin group versus each of the control group sera (*P* ≤ 0.0003). Taken together, our results suggest that Acm is produced during most *E. faecium* infections and promotes a host immune response.

The presence of a functional *acm* gene correlates with increased occurrence of the hospital-associated *E. faecium* genogroup CC17. To examine the possibility that the collagen
adherence seen in isolates of clinical origin might represent dissemination of closely related isolates. PFGE typing was performed with a subset of isolates (see Materials and Methods). Among the 23 isolates analyzed, 8 isolates (6 endocarditis isolates, 1 catheter isolate, and 1 wound isolate, isolated between 1988 and 2002 in several hospitals in Texas) were categorized as “closely related” or “possibly related” (<3- or 3- to 6-band difference) (27) and were designated pulsotype PF1 (Fig. 7A). We also found another group (PF2) with two “possibly related” endocarditis isolates from Arizona and Texas (Fig. 7A). In Fig. 7B, 12 individual strains showing considerable heterogeneity (≥7- to 11-fragment difference) in PFGE fingerprints are shown, along with single representatives of PF1 and PF2. It is interesting that the adherence percentages of PF1 isolates also varied to a great extent, ranging from 6% to 36%. Hybridization of PFGE Southern blots of chromosomal digestion fragments with an acm probe showed hybridization to the same-sized bands in all but one of the related isolates and to 11 differently sized fragments in those that were different by PFGE (data not shown). Taken together, our PFGE analysis showed that collagen adherence was found in at least 15 clearly different pulsotypes, thus demonstrating that collagen adherence is not limited to closely related isolates.

To evaluate more long-term evolutionary relationships, we next used MLST (9) with a subset of distinct pulsotype isolates (see Materials and Methods) and compared the results with an international E. faecium MLST data set (available at www.mlst

FIG. 6. Reactivity of human serum with rAcm A protein. (A) Immunoblots of rAcm A protein probed with human sera. Lanes 1, 2, and 4 to 6, sera from different patients with E. faecium endocarditis; lane 3, molecular size standards; lanes 7 to 14, NHS obtained from healthy volunteers. (B) Distribution of anti-Acm A domain Ig titers in human sera. The four endocarditis patient sera that had antibodies to Acm but whose infecting strain lacked in vitro collagen adherence and surface Acm are shaded in black. Sera from hospitalized patients as well as healthy volunteers were used as two groups of controls.

FIG. 7. Analysis of 23 E. faecium isolates by PFGE. (A) SmaI PFGE profiles of PF1, PF2, and other strains. Six endocarditis, one catheter, and one wound isolate, isolated between 1992 and 2002 from several hospitals in Texas, were categorized as PF1. Two endocarditis isolates from Arizona and Texas were categorized as possibly related (PF2). (B) SmaI PFGE profiles of strains with distinct PFGE patterns. Nonendocarditis isolates belonging to the Other-Clin group are marked with the symbol “§”.
were ST17 (ancestrally related. As shown in Table 1, the STs obtained on the PFGE results) and are part of the distinct nosocomial group. Twenty-three of 25 clinical isolates contained collagen-adhering and 8 nonadhering isolates) isolates from the characteristic of the hospital-associated CC17 lineage, but the overall combined results of PFGE, MLST, and data support the previously proposed hypothesis of acquisition of ampicillin resistance and then vancomycin resistance, as well as activation (e.g., acm) or acquisition (e.g., esp or hyl) of other genetic traits to facilitate infection, colonization, and/or transmission, although the order of these events cannot currently be determined (11, 23, 31).

Since the purK allele used in MLST has been defined as an epidemic marker of CC17 lineage (31), we also characterized allele analyses implicated 39 of 42 clinical isolates and 0 of 16 nonclinical isolates as being CC17 related. Another striking observation was that among the 17 endocarditis isolates obtained from various geographical locations in the United States from 1992 to 2002, all but 1 belongs to the CC17 genomgroup. The overall combined results of PFGE, MLST, and purK allele analyses implicated 39 of 42 clinical isolates and 0 of 16 nonclinical isolates as being CC17 related. Another striking observation was that among the 17 endocarditis isolates obtained from various geographical locations in the United States from 1992 to 2002, all but 1 belongs to the CC17 genomgroup. Testing for vancomycin and ampicillin resistance showed a very strong association between ampicillin resistance, clinical origin, and collagen adherence (Table 2); however, vancomycin resistance (predominantly vanA) was found in both clinical (74%) and animal (48%) isolate groups. All vancomycin-resistant animal isolates were from Europe. Although the presence of esp and of hyl was independently associated with clinical origin, as anticipated from previous reports (11, 23), these genes were each present in only 41% of endocarditis isolates and 36 to 71% of Other-Clin group isolates (Table 2). These data support the previously proposed hypothesis of acquisition of ampicillin resistance and then vancomycin resistance, as well as activation (e.g., acm) or acquisition (e.g., esp or hyl) of other genetic traits to facilitate infection, colonization, and/or transmission, although the order of these events cannot currently be determined (11, 23, 31).

In summary, analysis of E. faecium strains of multiple pulsortypes showed a collagen adherence phenotype in 62% of clinical versus 6% of nonclinical isolates. In vitro production of Acm was found to be very highly correlated with a collagen adherence phenotype, supporting the concept that Acm is the primary collagen adhesin of E. faecium in vitro. Our results also demonstrate that the amount of Acm detected on bacterial cell surfaces is the primary determinant of the observed variation in collagen adherence of individual strains. Impor
dant cell surfaces is the primary determinant of the observed variation in collagen adherence of individual strains. Important

### TABLE 1. Ancestral relatedness of a subset of E. faecium isolates exhibiting distinct pulsortypes

<table>
<thead>
<tr>
<th>Strain</th>
<th>Clinical source; origin; yr of isolation/collection</th>
<th>Ampicillin/vancomycin MICs (µg/ml)a</th>
<th>Presence of esp/hylb</th>
<th>PFGE typec</th>
<th>MLST type (allelic profiles for afa, dda, gdh, purK, gnd, putS, and add)</th>
</tr>
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<tbody>
<tr>
<td>TX2400</td>
<td>Blood; Galveston, TX; 1994</td>
<td>&gt;64/&gt;64</td>
<td>+/–</td>
<td>PF9</td>
<td>ST16 (1, 2, 1, 1, 1, 1, 1)</td>
</tr>
<tr>
<td>TX0067</td>
<td>Endocarditis/blood; Houston, TX; 1994</td>
<td>&gt;64/&lt;4</td>
<td>–/–</td>
<td>PF4</td>
<td>ST17 (1, 1, 1, 1, 1, 1, 1)</td>
</tr>
<tr>
<td>TX0082</td>
<td>Endocarditis/blood; Houston, TX; 1999</td>
<td>&gt;64/&lt;4</td>
<td>–/–</td>
<td>PF1</td>
<td>ST17 (1, 1, 1, 1, 1, 1, 1)</td>
</tr>
<tr>
<td>TX0110</td>
<td>Endocarditis/blood; Houston, TX; 2002</td>
<td>&gt;64/&lt;4</td>
<td>+/–</td>
<td>PF2</td>
<td>ST17 (1, 1, 1, 1, 1, 1, 1)</td>
</tr>
<tr>
<td>TX0080</td>
<td>Endocarditis/blood; Worcester, MA; 1996</td>
<td>&gt;64/&lt;64</td>
<td>+/+</td>
<td>PF7</td>
<td>ST17 (1, 1, 1, 1, 1, 1, 1)</td>
</tr>
<tr>
<td>TX0016</td>
<td>Endocarditis/blood; Houston, TX; 1992</td>
<td>16/&lt;4</td>
<td>–/–</td>
<td>PF3</td>
<td>ST18 (7, 1, 1, 1, 1, 1, 1)</td>
</tr>
<tr>
<td>TX0068</td>
<td>Endocarditis/blood; Worcester, MA; 1994</td>
<td>64/&lt;4</td>
<td>+/–</td>
<td>PF5</td>
<td>ST18 (7, 1, 1, 1, 1, 1, 1)</td>
</tr>
<tr>
<td>TX0081</td>
<td>Endocarditis/blood; Baltimore, MD; 1996</td>
<td>&gt;64/&lt;64</td>
<td>+/+</td>
<td>PF8</td>
<td>ST154 (9, 2, 1, 1, 1, 1, 1)</td>
</tr>
<tr>
<td>TX0074</td>
<td>Endocarditis/blood; Valhalla, NY; 1995</td>
<td>64/&lt;64</td>
<td>+/+</td>
<td>PF6</td>
<td>ST333? (5, 3, 1, 38, 8, 6, 1)</td>
</tr>
</tbody>
</table>

a MICs of ≥16 µg/ml for ampicillin and ≥32 µg/ml for vancomycin were considered to indicate resistance.
b +, gene present; –, gene absent.
c A distinct PFGE type was defined as differing by more than six fragments from other types.

### TABLE 2. Frequencies of ampicillin and glycopeptide resistance as well as esp and hyl genes among E. faecium strains of diverse origins

<table>
<thead>
<tr>
<th>Epidemiologic source (n)</th>
<th>No. of isolates resistant to Ampicillin</th>
<th>Vancomycin (vanA, vanB)</th>
<th>esp</th>
<th>hyl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endocarditis patients (17)a</td>
<td>17</td>
<td>12 (10, 2)</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>Collagen adherent (12)</td>
<td>12</td>
<td>9 (7, 2)</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Nonadherent (5)</td>
<td>5</td>
<td>3 (3, 0)</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>Other-Clin isolates (31)</td>
<td>28</td>
<td>24 (17, 7)</td>
<td>20</td>
<td>10</td>
</tr>
<tr>
<td>Collagen adherent (18)</td>
<td>18</td>
<td>16 (11, 5)</td>
<td>12</td>
<td>4</td>
</tr>
<tr>
<td>Nonadherent (13)</td>
<td>10</td>
<td>8 (6, 2)</td>
<td>8</td>
<td>6</td>
</tr>
<tr>
<td>Community fecal isolates (25)</td>
<td>0</td>
<td>3 (3, 0)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Collagen adherent (1)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Nonadherent (24)</td>
<td>0</td>
<td>3 (3, 0)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Animal isolates (23)</td>
<td>2</td>
<td>11 (11, 0)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Collagen adherent (2)</td>
<td>0</td>
<td>2 (2, 0)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Nonadherent (21)</td>
<td>2</td>
<td>9 (9, 0)</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

a Includes six isolates from our previous study (20), including four collagen-adherent and two nonadherent strains.
tantly, the presence of antibodies to Acm in all 14 endocarditis patient sera and 85% of sera from other *E. faecium* infections tested provides evidence of Acm production in vivo during serious infections. Among the 17 endocarditis isolates, we found Acm production in vitro with 12 isolates and indirect evidence of Acm production in vivo during endocarditis with an additional 4 isolates. The extent of surface display of Acm and its highly conserved nature (19), along with our recent data showing that antibodies against Acm subsegments inhibit collagen adherence of *E. faecium* (16), underscores the possibility of using Acm as a target for immunoprophylaxis or in combination with antibiotics (29). Finally, while antibiotic resistance certainly contributes to the difficulty in managing *E. faecium* infections, its contribution to the increase in frequency of this organism in nosocomial infections remains less certain. Indeed, vancomycin resistance is not uncommon in strains of organism in nosocomial infections remains less certain. In fact, vancomycin resistance is not uncommon in strains of *Enterococcus faecium*, *Enterococcus faecalis*, and *Enterococcus hirae* (30, 32). Indeed, vancomycin resistance is not uncommon in strains of non-CC17 origin in animals and in commensal strains from healthy humans in Europe, which very rarely, if ever, cause infection. In conclusion, the results from our molecular epidemiological analyses, together with the data from our companion paper demonstrating Acm’s role in *E. faecium* endocarditis (19), suggest that a functional acm gene, a predictor of collagen adherence, helps to explain the increased virulence potential of the CC17 endemic group.

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