

## Survival of *Enterococcus faecalis* in Mouse Peritoneal Macrophages

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*Enterococcus faecalis* was tested for the ability to persist in mouse peritoneal macrophages in two separate studies. In the first study, the intracellular survival of serum-passaged *E. faecalis* 418 and two isogenic mutants [cytolytic strain FA2-2(pAM714) and non-cytolytic strain FA2-2(pAM771)] was compared with that of *Escherichia coli* DH5 $\alpha$  by infecting BALB/c mice intraperitoneally and then monitoring the survival of the bacteria within lavaged peritoneal macrophages over a 72-h period. All *E. faecalis* isolates were serum passaged to enhance the production of cytolysin. *E. faecalis* 418, FA2-2(pAM714), and FA2-2(pAM771) survived at a significantly higher level ( $P = 0.0001$ ) than did *E. coli* DH5 $\alpha$  at 24, 48, and 72 h. Internalized *E. faecalis* 418, FA2-2(pAM714), and FA2-2(pAM771) decreased 10-, 55-, and 31-fold, respectively, over the 72-h infection period, while internalized *E. coli* DH5 $\alpha$  decreased 20,542-fold. The difference in the rate of survival of *E. faecalis* strains and *E. coli* DH5 $\alpha$  was most prominent between 6 and 48 h postinfection ( $P = 0.0001$ ); however, no significant difference in killing was observed between 48 and 72 h postinfection. In the second study, additional *E. faecalis* strains from clinical sources, including DS16C2, MGH-2, OG1X, and the cytolytic strain FA2-2(pAM714), were compared with the nonpathogenic gram-positive bacterium, *Lactococcus lactis* K1, for the ability to survive in mouse peritoneal macrophages. In these experiments, the *E. faecalis* strains and *L. lactis* K1 were grown in brain heart infusion (BHI) broth to ensure that there were equal quantities of injected bacteria. *E. faecalis* FA2-2(pAM714), DS16C2, MGH-2, and OG1X survived significantly better ( $P < 0.0001$ ) than did *L. lactis* K1 at each time point. *L. lactis* K1 was rapidly destroyed by the macrophages, and by 24 h postinfection, viable *L. lactis* could not be recovered. *E. faecalis* FA2-2(pAM714), DS16C2, MGH-2, and OG1X declined at an equivalent rate over the 72-h infection period, and there was no significant difference in survival or rate of decline among the strains. *E. faecalis* FA2-2(pAM714), MGH-2, DS16C2, and OG1X exhibited an overall decrease of 25-, 55-, 186-, and 129-fold respectively, between 6 and 72 h postinfection. The overall reduction by 1.3 to 2.27 log units is slightly higher than that seen for serum-passaged *E. faecalis* strains and may be attributable to the higher level of uptake of serum-passaged *E. faecalis* than of *E. faecalis* grown in BHI broth. Electron microscopy of infected macrophages revealed that *E. faecalis* 418 was present within an intact phagocytic vacuole at 6 h postinfection but that by 24 h the infected macrophages were disorganized, the vacuolar membrane was degraded, and the bacterial cells had entered the cytoplasm. Macrophage destruction occurred by 48 h, and the bacteria were released. In conclusion, the results of these experiments indicate that *E. faecalis* can persist for an extended period in mouse peritoneal macrophages.

*Enterococcus faecalis* is a gram-positive, facultatively anaerobic, coccal bacterium that causes a variety of community- and hospital-acquired infections in humans (for reviews, see references 9, 28, 33, and 39), including infections of the blood, endocardium, genitourinary tract, abdomen, wounds, and skin and soft tissue (e.g., burns, decubitus ulcers, and diabetic foot ulcers). The two most life-threatening infections caused by *E. faecalis* are bacteremia and endocarditis. *E. faecalis* causes 5 to 8% of all cases of bacteremia and 5 to 20% of all cases of endocarditis (~55% in intravenous drug users [9, 17, 18, 33, 39, 40, 47]). Bacteremia due to *E. faecalis* can lead to septicemia, septic shock, and death or, alternatively, to the formation of acute or subacute endocarditis (17). Enterococcal endocarditis is a serious consequence of *E. faecalis* infection, with a mortality rate of 17 to 46% (37).

*E. faecalis* is part of the normal flora of the oral cavity and may also be found in the gastrointestinal tract, male urethra,

and female vaginal tract of humans (9, 17, 28, 39). Under certain circumstances (in patients with indwelling catheters, intravenous lines, previous antibiotic use, or abscesses), *E. faecalis* breaches the host defenses by contamination of instrumentation or by direct extension to the bloodstream to cause bacteremia and/or endocarditis (9, 37, 38, 40). In some 42% of nosocomial bloodstream infections, there is no obvious explanation of how *E. faecalis* gained entry to the blood. To successfully cause infection, the bacterium must overcome the clearance functions of the host immune system. *E. faecalis* produces several virulence factors, including cytolysin (hemolysin/bacteriocin) (16, 25, 27), aggregation substance (10, 22, 24, 31, 41), gelatinase (protease) (4), and superoxide dismutase (6, 44), which could potentially modify the effectiveness of host defenses. There have been a limited number of studies which have addressed the interaction of *E. faecalis* or its products with cellular host defenses.

The interaction of *E. faecalis* with primary macrophages or macrophage-like cell lines has received limited attention (3, 19). Human monocytes respond to *E. faecalis* lipoteichoic acid by simultaneously synthesizing the inflammatory cytokines tumor necrosis factor alpha, interleukin-6, and interleukin-1 $\beta$  (3). Despite this inflammatory response, the ability of macro-

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phages to eliminate *E. faecalis* may be inhibited or delayed in vivo. Wells et al. first hypothesized that intestinal bacteria such as *E. faecalis* can utilize macrophages as a vehicle for translocation across the intestinal epithelial cells to the mesenteric lymph nodes, where the bacteria could be released to proliferate and spread hematogenously to other sites (53). Animal studies support this hypothesis, since antibiotic-induced *E. faecalis* overgrowth in the intestines of mice led to the subsequent recovery of the bacterium from the mesenteric lymph nodes and livers of infected animals (52).

Wells et al. examined the survival of *E. faecalis* in mouse peritoneal macrophages infected in vivo following intraperitoneal injection of *E. faecalis* into mice (51). Their studies revealed that *E. faecalis* survived within mouse peritoneal macrophages for 2 h and that intracellular survival of *E. faecalis* over the infection period was comparable to that of the facultative intracellular pathogen *Listeria monocytogenes*. However, their studies were focused on determining the oral infectivity of various enteric bacteria rather than on the actual ability of *E. faecalis* to survive within macrophages for an extended period. Therefore, it is difficult to form a conclusion about the susceptibility of *E. faecalis* to killing by macrophages.

Since survival and sequestration within macrophages may contribute to the pathogenesis of *E. faecalis* infections and, furthermore, may hinder the efficacy of antimicrobial therapy, this study focused on determining whether *E. faecalis* isolates survive within mouse peritoneal macrophages for an extended period. Studies in this laboratory indicated that at least six *E. faecalis* isolates survived for 72 h in mouse peritoneal macrophages and that cytolysin or gelatinase had no effect on intracellular survival in an in vivo-in vitro macrophage infection model.

#### MATERIALS AND METHODS

**Bacterial strains.** *E. faecalis* 418 was isolated from a culture of *Fusobacterium necrophorum* ATCC 27852 after infection of a mixed *F. necrophorum*-*E. faecalis* culture into mouse peritoneal macrophages resulted in rapid destruction of the *F. necrophorum* and recovery of the *E. faecalis* isolate 6 h postinfection. *F. necrophorum* ATCC 27852 was originally recovered from a sheep with foot rot, and *E. faecalis* 418 presumably was a fecal contaminant present in the foot rot. *E. faecalis* 418 was repeatedly recovered from separate vials of *F. necrophorum* ATCC 27852. *E. faecalis* FA2-2(pAM714) (24), a cytolysin-positive strain, *E. faecalis* FA2-2(pAM771) (24), a noncytolytic isogenic mutant, and *E. faecalis* OG1X (26), a gelatinase mutant, were kindly supplied by Mike Gilmore (University of Oklahoma Health Sciences Center, Oklahoma City, Okla.). Strains FA2-2(pAM714) and FA2-2(pAM771) were assayed for the production of cytolysin by previously published methods (25). Don Clewell (University of Michigan School of Dentistry, Ann Arbor, Mich.) kindly provided *E. faecalis* DS16C2, a derivative of clinical strain DS16 that contains cytolysin but lacks plasmid pAD2 (15). *E. faecalis* MGH-2 (clinical isolate, mouse virulent) was kindly supplied by Michael Cohen (Parke-Davis Pharmaceutical Research, Division of Warner-Lambert Co., Ann Arbor, Mich.) (11). These *E. faecalis* strains were chosen for study since they were human clinical isolates (or derivatives of clinical isolates) and were sensitive to vancomycin (1 to 2 µg/ml) and gentamicin (16 µg/ml) when tested by the broth dilution method as specified by National Committee for Clinical Laboratory Standards guidelines. *Lactococcus lactis* K1, a nonpathogenic, gram-positive bacterium, served as a negative control and was kindly provided by John Thompson (Oral Infection and Immunity Branch, National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, Md.) (49). *Escherichia coli* DH5α [F<sup>-</sup> *endA1 hsdR17* (r<sub>k</sub><sup>-</sup> m<sub>k</sub><sup>+</sup>) *supE44* λ<sup>-</sup> *recA1 gyrA96 relA1 Δ*(*argF-lacZYA*)U169 φ80d*lacZ*ΔM15] was purchased from GIBCO/BRL, Life Technologies, Gaithersburg, Md.), and served as the negative control in these experiments.

**Reagents and mice.** Dulbecco's modified Eagle's medium (DMEM), penicillin G, vancomycin, Sarkosyl, and DNase I were obtained from Sigma Chemical Co., St. Louis, Mo. Gentamicin, HEPES buffer solution, MEM nonessential amino acids solution, and glutamine were obtained from Life Technologies. Fetal bovine serum was obtained from Summit Biotechnology, Fort Collins, Colo. Non-hemolyzed rabbit serum was supplied by Pel-Freez Biologicals, Rogers, Ark. The rabbit serum was heat inactivated at 56°C for 1 h prior to use. Brain heart infusion (BHI) broth and agar were purchased from Difco, Detroit, Mich. BALB/c mice (10-week-old males) were purchased from Harlan Sprague-Dawley, Indianapolis, Ind.

**Assay for survival in mouse peritoneal macrophages.** Experimental methods were based on those established for studying the survival and multiplication of *Salmonella typhimurium* in macrophages (5, 21, 34). *E. faecalis* 418, FA2-2(pAM771), and FA2-2(pAM714) were passaged twice by being grown in sterile, nonhemolyzed rabbit serum (heat inactivated at 56°C for 1 h) and *E. coli* DH5α was grown aerobically at 37°C in Luria-Bertani (LB) broth to an identical density prior to injection into mice. In separate experiments, *E. faecalis* FA2-2 (pAM714), DS16C2, OG1X, and MGH-2 were grown aerobically at 37°C in BHI broth for 16 h. After overnight growth, the bacteria were pelleted in a microcentrifuge at 13,800 × g for 2 min and the cell pellet was resuspended in an equivalent volume of phosphate-buffered saline (PBS) for injection. *E. faecalis* strains and *E. coli* DH5α (10<sup>7</sup> to 10<sup>8</sup> CFU) were injected intraperitoneally into 10-week-old BALB/c mice, and following an infection period of 4 h, peritoneal macrophages were harvested by peritoneal lavage (two applications of 5 ml of PBS [pH 7.2] [lacking Ca<sup>2+</sup> and Mg<sup>2+</sup>]). The infected peritoneal macrophages were centrifuged for 10 min at 900 × g (at room temperature) and suspended in DMEM containing 10 mM HEPES, 2 mM glutamine, 10% fetal bovine serum, and 1× nonessential amino acids (this combination is designated "DMEM complete medium" throughout this paper), supplemented with vancomycin (10 µg/ml) and gentamicin (150 µg/ml). The cell suspension was dispensed into 24-well tissue culture plates and incubated at 37°C under 8% CO<sub>2</sub>. After exposure to antibiotics for 2 h (i.e., 6 h postinfection) at 37°C under 8% CO<sub>2</sub> to kill extracellular bacteria, the infected macrophages were washed three times with DMEM containing 10 mM HEPES buffer, and duplicate wells of infected macrophages were lysed with 0.5% Sarkosyl containing 2 µg of DNase per ml. Dilutions of lysates were made in BHI broth and plated on BHI agar to quantitate viable intracellular bacteria. The remaining wells of infected macrophages were maintained in DMEM complete medium containing vancomycin (2 µg/ml) and gentamicin (10 µg/ml) for the duration of the experiment. At 6, 24, 48, and 72 h postinfection, supernatant fluids from each well were removed and extracellular bacteria were quantitated by plating the fluids on BHI agar. Duplicate wells of infected macrophages were lysed with detergent at 24, 48, and 72 h postinfection, and lysates were plated as described above to recover viable bacteria.

**Transmission electron microscopy.** Mice were injected intraperitoneally with *E. faecalis* 418 and *E. coli* DH5α, and peritoneal macrophages were harvested, plated into 24-well tissue culture plates, and exposed to antibiotics as described above. At 6, 24, 48, and 72 h postinfection, tissue culture medium was removed and cells were overlaid with 1 ml of a fixative solution consisting of 4% formaldehyde, 1% glutaraldehyde, and 0.1% sodium cacodylate (supplied by Paragon Biotech Inc., Baltimore, Md.). The fixed cells contained in the 24-well tissue culture plates were immediately transported to Paragon Biotech for processing and for transmission electron microscopy.

**Assessment of macrophage viability.** Infected and noninfected mouse peritoneal macrophages were quantitated at 6, 24, 48, and 72 h to determine whether infection resulted in death of the infected macrophages. The macrophages were detached from tissue culture wells (2 wells) with cell scrapers and mixed with trypan blue dye, and viable macrophages were visualized under an inverted microscope and counted with a hemacytometer.

**Statistical analysis of infection data.** All infection experiments described were performed three times and subjected to statistical analysis. The results of these experiments were analyzed by Sean Mahabir, Phillip Chapman, and Jill Smith, Colorado State University Statistics Department, with SAS computer software. Regression lines were fit for each strain for each time segment, and Bonferroni intervals were used to perform pairwise differences between slopes for strains at each time interval. A mixed-model analysis of variance was also performed to test for significance of strain-time interaction. A SAS-PROC MIXED program was used to perform a mixed-model analysis of variance.

#### RESULTS

**Comparative survival of *E. faecalis* strains, *E. coli* DH5α, and *L. lactis* K1 in mouse peritoneal macrophages.** In initial experiments, the intracellular survival of *E. faecalis* 418, FA2-2 (pAM714), and FA2-2(pAM771) was compared with that of *E. coli* DH5α by infecting mice intraperitoneally, recovering infected macrophages 4 h later, and then monitoring the survival of intracellular bacteria over a period of 72 h within peritoneal macrophages maintained in vitro. In these initial studies, all the *E. faecalis* isolates were passaged in nonhemolyzed rabbit serum prior to injection, since it was previously reported that growth of *E. faecalis* in rabbit serum is required for optimum expression of cytolysin. *E. coli* DH5α was used as a negative control, since this laboratory strain was found to be susceptible to killing by mouse peritoneal macrophages. By necessity, *E. coli* DH5α was grown in LB broth since it grew poorly in rabbit serum. In preliminary experiments, both *E. coli* DH5α and *E.*

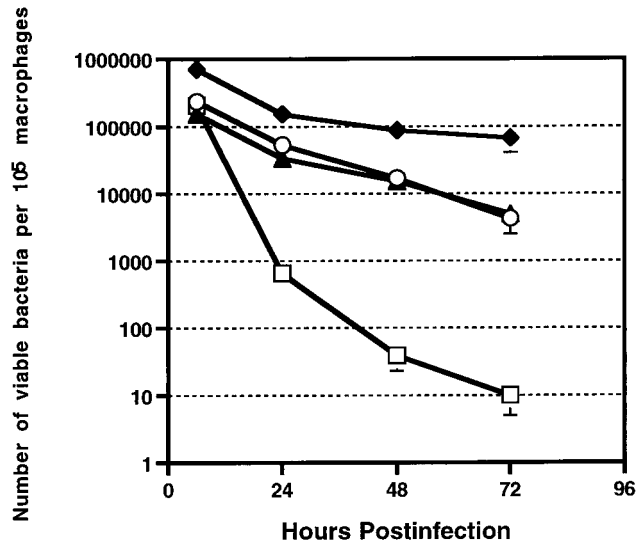


FIG. 1. Recovery of viable *E. faecalis* and *E. coli* DH5 $\alpha$  from infected murine peritoneal macrophages at 6, 24, 48, and 72 h postinfection. *E. faecalis* strains were passed through serum, while *E. coli* DH5 $\alpha$  was grown in LB broth prior to infection. The results represent the mean and standard error of three experiments. In some cases, the standard error is not indicated, because it is too small to be visible on the graph. Symbols:  $\circ$ , *E. faecalis* FA2-2(pAM714);  $\blacktriangle$ , *E. faecalis* FA2-2(pAM771);  $\square$ , *E. coli* DH5 $\alpha$ ;  $\blacklozenge$ , *E. faecalis* 418.

*coli* LE392 served as negative controls; however, there was no difference between the survival of the two *E. coli* strains and therefore only *E. coli* DH5 $\alpha$  was included as the negative control in subsequent studies.

All serum-passaged *E. faecalis* strains were equal in their ability to survive inside macrophages and exhibited a similar rate of decline over the 72-h period (Fig. 1). There was no significant difference in the levels of the *E. faecalis* strains and *E. coli* DH5 $\alpha$  at the 6-h time point. However, all *E. faecalis* strains were recovered at significantly higher levels ( $P < 0.0001$ ) than *E. coli* DH5 $\alpha$  at the 24-, 48-, and 72-h time points. By the 72-h time point, the *E. faecalis* strains exhibited a definite superiority in their ability to survive intracellularly, since numbers of internalized *E. faecalis* 418, FA2-2(pAM714), and FA2-2(pAM771) organisms decreased only 10-, 55-, and 31-fold, respectively, between 6 and 72 h postinfection while the number of *E. coli* DH5 $\alpha$  organisms decreased 20,542-fold. The number of intracellular *E. faecalis* organisms slowly declined at a rate of 1 to 1.5 log units over the 72-h period. In contrast, the number of viable *E. coli* DH5 $\alpha$  organisms declined more rapidly, with an initial reduction of 2 log units between 6 and 24 h postinfection and a similar reduction between 24 and 48 h postinfection, followed by a rate of decline of approximately 0.5 log unit between 48 and 72 h postinfection. Statistical analysis of the rate of reduction of *E. coli* DH5 $\alpha$  and *E. faecalis* over the infection period revealed that *E. coli* DH5 $\alpha$  was reduced at a significantly greater ( $P = 0.0001$ ) rate than were the *E. faecalis* strains between 6 and 48 h postinfection but that the differences in the rates diminished between 48 and 72 h postinfection. Another common laboratory strains of *E. coli*, LE392, declined at a rate similar to that of *E. coli* DH5 $\alpha$  (data not shown). The viability of both infected and uninfected mouse peritoneal macrophages decreased approximately 10-fold over the course of the experiment.

These initial experiments were extended to include *E. fae-*

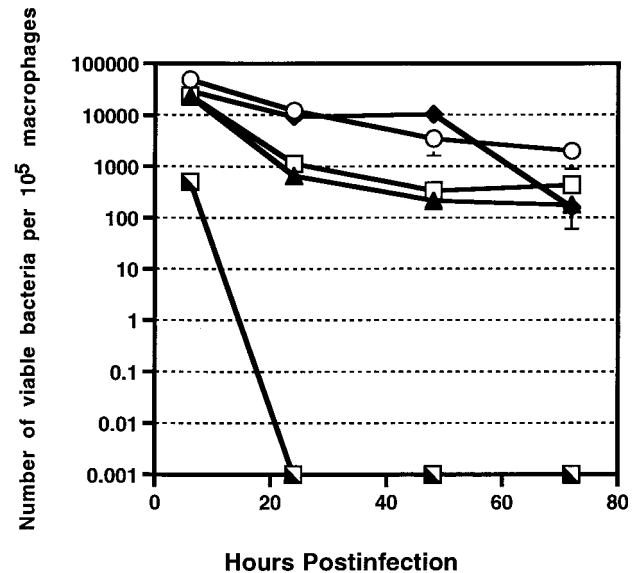


FIG. 2. Recovery of viable *E. faecalis* and *L. lactis* K1 from infected murine peritoneal macrophages at 6, 24, 48, and 72 h postinfection. All *E. faecalis* strains and *L. lactis* K1 were grown in BHI broth prior to infection. The results represent the mean and standard error of two experiments. In some cases, the standard error is not indicated, because it is too small to be visible on the graph. Symbols:  $\circ$ , *E. faecalis* FA2-2(pAM714);  $\blacktriangle$ , *E. faecalis* OG1X;  $\square$ , *E. faecalis* MGH-2;  $\blacklozenge$ , *E. faecalis* DS16C2;  $\blacksquare$ , *L. lactis* K1.

*calis* strains from different clinical sources and to include a more relevant negative control—the closely related, innocuous, gram-positive bacterium, *L. lactis* K1. These experiments were performed by growing *E. faecalis* FA2-2(pAM714), MGH-2, DS16C2, and OG1X and *L. lactis* K1 overnight in BHI broth, since *L. lactis* K1 did not grow at a rate similar to the *E. faecalis* strains in serum. The bacteria were collected by centrifugation, and a bacterial suspension was prepared in PBS for injection into mice. Macrophage survival was monitored as described above by determining the number of viable intracellular bacteria over the 72-h time course.

The *E. faecalis* strains grown in BHI broth [FA2-2(pAM714), MGH-2, DS16C2, and OG1X] showed no significant difference in their ability to survive in mouse peritoneal macrophages and exhibited similar rates of decline over the 72-h infection period (Fig. 2). However, it is interesting that the number of BHI-grown *E. faecalis* organisms recovered at 6 h postinfection was consistently 10-fold smaller than the number of serum-passaged *E. faecalis* organisms. *E. faecalis* FA2-2(pAM714), MGH-2, DS16C2, and OG1X (grown in BHI broth) showed an overall decrease of 25-, 55-, 186-, and 129-fold, respectively, between 6 and 72 h postinfection. The overall reduction of 1.3 to 2.27 log units is slightly higher than that seen for serum-passaged *E. faecalis* strains. This difference may be attributable to the fact that serum-passaged *E. faecalis* strains were phagocytosed at a higher rate than were BHI broth-grown strains and that the initial burden to the macrophage was higher with the serum-passaged *E. faecalis* strains. However, the overall reduction of the BHI broth-grown *E. faecalis* strains is still significantly smaller than the 4- to 4.5-log-unit reduction in *E. coli* DH5 $\alpha$  over the 72-h infection period described above.

All *E. faecalis* strains were markedly superior to the negative control, *L. lactis* K1, in their ability to survive in the macrophages. By 6 h postinfection, the level of *L. lactis* K1 was



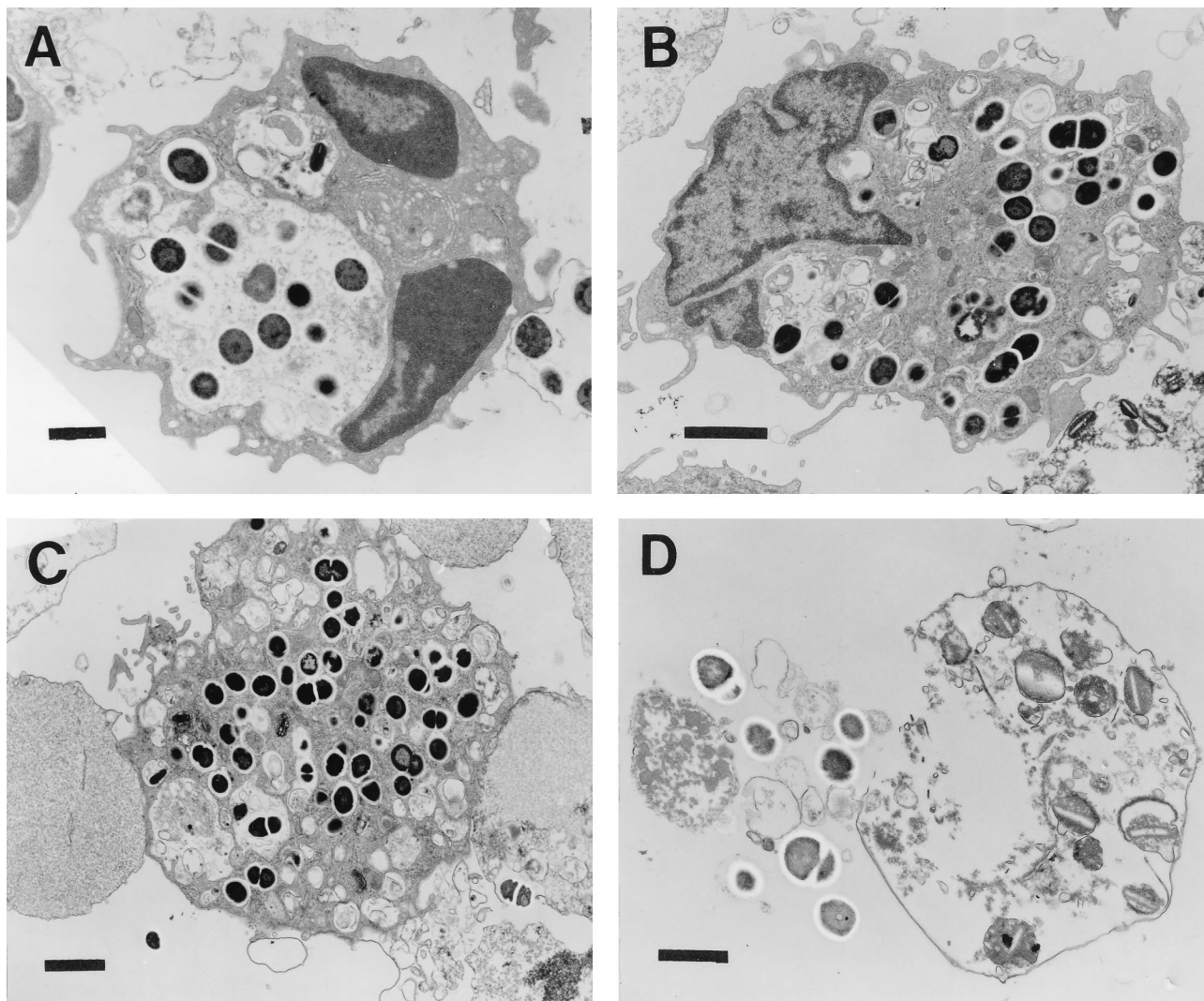


FIG. 3. Electron micrographs of mouse peritoneal macrophages infected with *E. faecalis* 418. Infected macrophages were processed for transmission electron microscopy (see Materials and Methods) at 6 h (A), 24 h (B), 48 h (C), and 72 h (D) postinfection. Bars, 1  $\mu\text{m}$  (A and D) or 2  $\mu\text{m}$  (B and C).

drastically reduced in the mouse peritoneal macrophages compared with those of the *E. faecalis* strains ( $P = 0.0001$ ); it recovered at a 63-fold lower level than the *E. faecalis* strains. *L. lactis* K1 succumbed rapidly to the killing effects of the macrophage, and by 24 h postinfection no viable *L. lactis* K1 was recovered from the infected macrophages.

**Electron micrographs of *E. faecalis*-infected mouse peritoneal macrophages.** *E. faecalis* 418-infected macrophages were subjected to transmission electron microscopy at 6, 24, 48, and 72 h postinfection to confirm that intact bacterial cells were present within the macrophage and to determine whether the bacteria were present in a membrane-bound vacuole or in the cytoplasm. Representative electron micrographs of *E. faecalis* 418-infected macrophages at the different time points are shown in Fig. 3. By 6 h postinfection, *E. faecalis* bacteria were phagocytosed by the macrophage and were found intact within a phagocytic vacuole. At all time points postinfection, it appeared that dividing, intact enterococci were present within the macrophage, suggesting that some of the phagocytosed bacteria were capable of replication. At 24 h postinfection, the infected macrophages were disorganized, the vacuolar mem-

brane appeared to be degraded in areas, and the bacterial cells were present in the cytoplasm. By 48 h postinfection, macrophages that had ingested numerous *E. faecalis* bacteria appeared to be disintegrating, whereas the majority of the intracellular *E. faecalis* isolates appeared to be intact and were surrounded by an electron-transparent halo. At the 72-h time point, infected macrophages were destroyed and both intact and degraded bacteria were present inside the remnants of the macrophages.

## DISCUSSION

*E. faecalis* is a significant cause of nosocomial infection, and therefore it must possess the ability to compete at some level with host cellular defense mechanisms in order to survive in tissue and blood. The experiments described above demonstrate that *E. faecalis* is superior to a closely related bacterium, *L. lactis*, and a nonpathogenic strain of *E. coli* in its ability to survive in mouse peritoneal macrophages for 72 h. This finding allows speculation that survival in macrophages contributes to the pathogenicity of this bacterium. It is of interest that viru-

lent strains of *Streptococcus bovis*, a bacterium that causes septicemia in pigeons, multiply within pigeon peritoneal macrophages whereas avirulent strains are killed by macrophages (13).

Preliminary experiments in this laboratory revealed that *E. faecalis* 418, a sheep foot rot isolate, possessed the ability to survive in mouse peritoneal macrophages. These experiments were repeated in this study, and the role of cytolysin in macrophage survival was examined since animal studies by other investigators showed that noncytolytic mutants are 10-fold less virulent than wild-type, cytolytic *E. faecalis* isolates when tested in a mouse peritoneal infection model (27). Mouse peritoneal macrophages were infected in vivo with the cytolytic strain *E. faecalis* FA2-2(pAM714) and the noncytolytic, isogenic mutant, *E. faecalis* FA2-2(pAM771). Quantitation of viable, intracellular bacteria in infected macrophages maintained in vitro over 72 h revealed that there was no significant difference between the cytolytic and noncytolytic strains, suggesting that cytolysin does not play a role in intracellular survival. It was initially surprising that *E. faecalis* cytolysin is not necessary for macrophage survival, since hemolysins of other intracellular pathogens, including *Listeria monocytogenes* (43, 48) and *Shigella flexneri* (46), are essential for release of the bacteria into the cytoplasm and intracellular survival. However, it is possible that the *E. faecalis* cytolysin is different from other latter hemolysins since it is a member of the lantibiotic family, a group of bacteriocins which are active against other gram-positive bacteria (16). Therefore, the *E. faecalis* cytolysin may not be important in macrophage survival but, as proposed by previous investigators (16), may contribute to the local ecology of infections by eliminating competing bacteria and allowing overgrowth of cytolysin-producing *E. faecalis* strains. Alternatively, the cytolysin may contribute to pathogenicity by causing localized tissue damage. Another explanation for the lack of an effect of cytolysin in macrophage survival is that cytolysin may not be produced under the conditions of the assay. Studies in this laboratory indicated that cytolysin is not produced by *E. faecalis* grown in tissue culture media under a carbon dioxide atmosphere. Further experiments involving reverse transcriptase PCR for detection of cytolysin mRNA are necessary to determine whether cytolysin is produced by bacteria residing in the macrophage.

The initial infection studies in mouse peritoneal macrophages were extended to include additional *E. faecalis* strains from clinical sources [i.e., DS16C2, MGH-2, OG1X, and FA2-2(pAM714)] and a more relevant negative control strain, *L. lactis* K1. *L. lactis* K1 was included as a control strain since it is closely related to *E. faecalis* and has not been associated with human disease. *L. lactis* K1 failed to grow at the same rate as *E. faecalis* in rabbit serum; therefore, in subsequent experiments all *E. faecalis* strains and the *L. lactis* control was grown in BHI broth prior to infection. The results of macrophage infection with the additional *E. faecalis* strains essentially mimicked the results of the initial experiments—all the *E. faecalis* strains survived within the macrophages for the duration of the experiment, and *E. faecalis* greatly exceeded the ability of the negative control, *L. lactis* K1, to survive intracellularly. These experiments indicated that gelatinase and cytolysin play no role in macrophage survival under these test conditions. *E. faecalis* strains grown in BHI broth exhibited a slightly increased rate of decline during intracellular growth compared to *E. faecalis* strains grown in serum. This difference is presumably due to an increased uptake of serum-passaged *E. faecalis* by macrophages, resulting in increased macrophage burden and reduced killing.

Examination of *E. faecalis* 418-infected mouse peritoneal

macrophages by transmission electron microscopy revealed that intact diplococci were present intracellularly at all times postinfection, suggesting either that the bacteria replicated intracellularly or that they remained in a viable, resting state. Furthermore, electron micrographs of infected macrophages revealed that *E. faecalis* could be found lying in the cytoplasm of the macrophage, an observation that was previously reported for *S. bovis* within splenic macrophages (13).

Quantitation of viable intracellular *E. faecalis* 418 over the course of the infection of the mouse peritoneal macrophages indicated that the number of bacteria did not increase, as has been reported for intracellular pathogens such as *Brucella abortus* (2, 30), *Listeria monocytogenes* (32, 35, 43), and *Salmonella typhimurium* (8, 34), but decreased slightly (approximately 10- to 55-fold) over the 72-h period in primary mouse macrophages. The slight reduction in the number of viable intracellular bacteria may be explained in three ways. First, there may be two populations of bacteria inside the macrophage, one which survives and multiplies and another which is killed intracellularly, an observation that has been made for macrophages infected with *Salmonella typhimurium* (1) and *Listeria monocytogenes* (12). Alternatively, *E. faecalis* may not replicate in the macrophage but may remain quiescent and undergo delayed death due to intrinsic resistance of the bacterium to the internal environment. Since *E. faecalis* is an opportunistic pathogen and has not previously been reported to survive intracellularly for extended periods, one might speculate that this bacterium lacks the mechanisms used by overt pathogens to multiply within the macrophage but may resist macrophage killing due to the production of enzymes that inactivate reactive oxygen intermediates generated by the oxidative burst. *E. faecalis* produces superoxide dismutase and NADH peroxidase, enzymes which may serve to reduce the detrimental effects of superoxide anion and hydrogen peroxide, respectively, in the macrophage (6, 42, 44). *E. faecalis* may simply persist in the macrophage, a property which might allow the bacterium to resist killing by antibiotics or to remain viable while translocating across the intestinal wall and which might also facilitate entry into the mesenteric lymph nodes and into the blood. The third possibility is that the decrease in the number of viable intracellular bacteria may reflect the slow uptake of gentamicin and vancomycin (or penicillin), antibiotics which are effective for killing *E. faecalis*. It is difficult experimentally to eliminate this last possibility, since for long-term survival assays it is necessary to include these antibiotics in the tissue culture media to prevent overgrowth of extracellular *E. faecalis*. The results of studies on whether various antibiotics enter the macrophage at a level sufficient to kill intracellular bacteria have been conflicting (7, 14, 23, 29, 36, 50). However, vancomycin and aminoglycosides are taken up slowly, and aminoglycosides localize primarily in lysosomes, where they are partially inactivated by the acidic pH (for a review, see reference 36).

The results reported in this study indicate that *E. faecalis* is capable of surviving for a prolonged period in mouse peritoneal macrophages. Knowledge of the detailed mechanisms used by *E. faecalis* to evade the bactericidal effects of the macrophage will require further studies of the bacterial products produced within the macrophage.

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