Antibiotic-induced lysis of enterococci.

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Research Article

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Antibiotic-induced Lysis of Enterococci

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ABSTRACT Enterococci are resistant to penicillin killing in vivo and in vitro. Because some bacteria resistant to penicillin killing have reduced autolytic activity, we examined the lysis of clinical enterococcal isolates suspended in buffer (spontaneous lysis), and compared it with their susceptibility to antibiotic-induced lysis and killing.

We found significant correlations between spontaneous and antibiotic-induced lysis, using five antibiotics that inhibit cell wall synthesis (penicillin, cephalothin, bacitracin, cycloserine, and vancomycin). Among isolates, strains more rapidly lysed by one antibiotic were more rapidly lysed by the other antibiotics, and more susceptible to spontaneous lysis. In studies involving a single strain grown in different media, spontaneous lysis also correlated closely with antibiotic-induced lysis. These results are consistent with a common mechanism for spontaneous and antibiotic-induced lysis, such as the autolytic enzyme system.

Human serum was one of the least permissive media tested for enterococcal growth and antibiotic-induced lysis and killing. We suggest that the inhibitory effect of human serum on growth and the activation of the enterococcal autolytic enzyme system may be a critical factor in the resistance of enterococcal endocarditis to treatment with penicillin alone.

INTRODUCTION

Although the mechanism by which penicillin inhibits bacterial cell wall synthesis has been described in detail (1), the mechanism by which this antibiotic kills bacteria remains poorly understood (2). The idea that inhibition of cell wall synthesis and killing induced by penicillin may have different mechanisms is supported by the occurrence of enterococci and other bacteria that are much more resistant to the lethal effect of penicillin than they are to its growth inhibitory effect. The resistance of enterococci to penicillin killing in vitro has been demonstrated by time-kill studies (3–5) and by the high ratio of the minimal bactericidal concentration (MBC)[1] to the minimal inhibitory concentration (MIC) of penicillin for these strains (4, 6–11). The clinical relevance of these in vitro results is supported by the observation that enterococcal endocarditis is resistant to treatment with penicillin alone (12, 13), in contrast to endocarditis caused by most other streptococci (14).

Recent work has implicated the autolytic enzyme system in the resistance of bacteria to penicillin-induced killing. In several studies, laboratory mutants known to be deficient in autolytic enzyme activity were found to be resistant to killing by penicillin and other inhibitors of cell wall synthesis (15–18). In a complementary set of studies, clinical isolates of *Staphylococcus aureus* selected for their tolerance to β-lactams (MBC:MIC ratio ≥ 32 for nafcillin or oxacillin) were subsequently shown to be deficient in autolytic enzyme activity (19, 20).

In the present study, we examined the antibiotic-induced lysis and killing of multiple strains of *Streptococcus faecalis*, the most common clinically encountered species of the enterococcus. We also examined the spontaneous lysis of these strains in sodium phosphate buffer as a measure of their endogenous autolytic activity. The close relationship we observed between spontaneous and antibiotic-induced lysis in these strains, supports the hypothesis that the lysis and killing of *S. faecalis* by antibiotics that inhibit cell wall synthesis are mediated by the autolytic enzyme system, and provides a framework for understanding the resistance of enterococci to penicillin killing.

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[1] *Abbreviations used in this paper*: BHI, brain heart infusion; HI, heart infusion broth; MBC, minimal bactericidal concentration; MHB, Mueller-Hinton broth; MIC, minimal inhibitory concentration; PAB, Pen-Assay broth; THB, Todd-Hewitt broth.

METHODS

Bacterial strains and media. All 30 bacterial strains used in these studies were originally isolated from bacteremic patients at Barnes Hospital and identified as *S. faecalis* by standard criteria (21). The MIC and MBC of penicillin and other cell wall-active antibiotics for these organisms have been reported previously (11). The eight strains subjected to more extensive study were selected before their lytic responses were known. With the exception of vancomycin, there was little variation (less than or equal to two tubes or a fourfold dilution) in the MIC of the five antibiotics for the eight strains tested. The median MIC were 4 μg/ml for penicillin (1–4), 31 μg/ml for cephalothin (31–62), 62 μg/ml for bacitracin (62–125), 250 μg/ml for cycloserine (all strains were identical), and 0.06 μg/ml for vancomycin (0.06–1.0).

Media used included Mueller-Hinton (MHB), Todd-Hewitt (THB), Pen-Assay (PAB), brain heart infusion (BHI), and heart infusion broth (HI) (Difco Laboratories, Detroit, Mich.); dextrose phosphate broth (Gibco Diagnostics, Gibco Invenox Div., Chagrin Falls, Ohio); and MS-2 broth (Oxoid Ltd., Hampshire, England). These media were dissolved in glass-distilled water and sterilized by steam autoclaving at 121°C and 15 psi for 15 min before use. The serum used for lysis and killing experiments was pooled from samples collected from out-patients who had routine tests for syphilis, and was kept at −20°C if stored for more than several days. Serum was sterilized by filtration through a 0.45 μm Nalgene filter (Sybron Corp., Rochester, N. Y.) and tested with an agar well-diffusion bioassay (22) to exclude the presence of antimicrobials. All cultures were grown at 37°C.

Antibiotics. Potassium penicillin G was kindly provided by the Wyeth Laboratories, Philadelphia, Pa.; sodium cephalothin and vancomycin hydrochloride were provided by the Eli Lilly & Co., Indianapolis, Ind. Bacitracin and D-cycloserine were obtained from the Sigma Chemical Co., St. Louis, Mo.

Antibiotic solutions were prepared fresh in sterile, glass-distilled water on the day of the experiment. The final concentrations used were in the range that produced maximal lysis of strain 953 in THB: 20 μg/ml for penicillin, 312 μg/ml for cephalothin, 625 μg/ml for cycloserine, 625 μg/ml for bacitracin, and 15 μg/ml for vancomycin. These concentrations were generally 2.5–15-fold greater than the median MIC of those drugs for the strains tested (11).

Antibiotic-induced lysis. After overnight growth in the desired medium, an aliquot of the culture was diluted 1:10 in fresh medium and reincubated for 1 h to permit the resumption of exponential growth. Using a Coleman 54 B spectrophotometer (Perkin-Elmer Corp., Instrument Div., Norwalk, Conn.), the absorbance of the culture at 675 nm was adjusted to 0.050–0.100 by addition of fresh medium. This corresponded to ~2×10^8 colony-forming units per milliliter. Stock antibiotic solutions were prepared at 20 times the desired final concentrations and added as 5% of the volume of these cultures. In each experiment, an identical volume of sterile, glass-distilled water was added to a control culture derived from the same inoculum. After the addition of antibiotics, absorbance measurements at 675 nm were used to detect lysis. Lysis was measured as the percent reduction in absorbance between 1 and 4 h after the addition of antibiotics, using absorbance measurements adjusted to agree with Beer's Law (23). Antibiotic killing was measured by viable colony counts using serial 10-fold dilutions in sterile normal saline. The number of colony-forming units per milliliter was determined by counting visible colonies on sheep blood agar plates that had been incubated overnight after inoculation with 0.1-ml aliquots of the bacterial suspensions in saline.

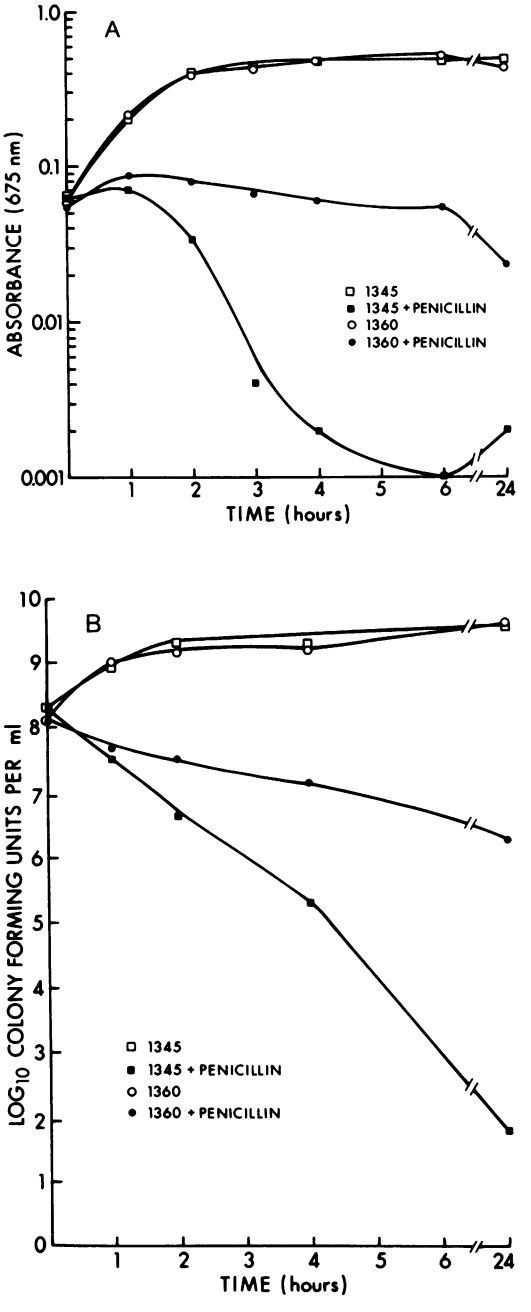

FIGURE 1 Strain 1345 was more rapidly lysed (A) and killed (B) by penicillin G (20 μg/ml) than strain 1360 in THB. This concentration was five times the penicillin MIC of both strains (4 μg/ml).

Spontaneous lysis. These studies were performed using a modification of the protocol of Shockman et al. (18, 24). After overnight growth, cells were harvested by centrifugation at 12,000 *g* and 4°C for 10 min, and washed once with phosphate buffer (0.3 M, pH 7.0). The cells were resuspended in the same buffer and adjusted to an absorbance of 0.300–0.400 at 450 nm. Spontaneous lysis was determined by serial absorbance measurements at 450 nm. The percent decline

TABLE I
*Increasing Penicillin Concentrations and the Lysis of S. faecalis, Strain 953**

Penicillin concentration	Lysis
μg/ml	%‡
2	41
4	81
10	87
20	88
50	66
100	41
200	29
1,000	32
2,000	32
10,000	31

* The penicillin MIC of strain 953 is 4 μg/ml.
‡ Lysis is the percent reduction in absorbance at 675 nm between 1 and 4 h after exposure to penicillin in THB. Each point is the average of three determinations.

in absorbance between 0 and 4 h was calculated using adjusted absorbance measurements (23).

Statistical methods. Correlation coefficients (Pearson's coefficient of correlation) were calculated according to standard methods (25) and evaluated for significance using a two-tailed t test (25).

RESULTS

Antibiotic-induced lysis. Enterococci began to lyse within 30–60 min after exposure to penicillin in THB. Viable colony counts fell approximately in parallel with absorbance (Fig. 1). Lysis was greatest with penicillin concentrations of 10–20 μg/ml, and was less with higher concentrations (Table I). This pattern was also observed with the other drugs tested (data not shown), although the antibiotic concentrations producing maximal lysis were substantially greater for cephalothin (312 μg/ml), bacitracin (625 μg/ml), and cycloserine (625 μg/ml). Vancomycin produced less lysis than the other antibiotics tested.

Variability in antibiotic-induced lysis among clinical isolates. In testing 30 bacterial strains we found considerable variability in penicillin-induced lysis. Among the eight strains tested against five antibiotics, those strains most rapidly lysed by one antibiotic were also most rapidly lysed by the others. Conversely, strains lysed slowly by one antibiotic were also lysed slowly by the others. The amount of penicillin-induced lysis correlated strongly with that produced by cephalothin ($r = 0.97$, $P < 0.001$), bacitracin ($r = 0.87$, $P < 0.01$), cycloserine ($r = 0.89$, $P < 0.01$), and less strongly with that produced by vancomycin ($r = 0.75$, $P = 0.04$) (Table II).

Spontaneous lysis. All 30 clinical isolates of *S. faecalis* underwent spontaneous lysis in sodium phosphate buffer although there was considerable strain variation. In general, strains that underwent rapid spontaneous lysis were rapidly lysed by penicillin, and strains that underwent slow spontaneous lysis were slowly lysed by penicillin ($r = 0.73$, $P < 0.001$). However, there was considerable variation in penicillin lysis among strains with similar amounts of spontaneous lysis, and in spontaneous lysis among strains with similar amounts of penicillin lysis (Fig. 2). For the eight strains tested against five antibiotics, spontaneous lysis also correlated with lysis produced by cephalothin ($r = 0.82$, $P = 0.01$), bacitracin ($r = 0.94$, $P < 0.001$), and cycloserine ($r = 0.90$, $P < 0.01$) (Table II). The correlation between spon-

TABLE II
*Spontaneous and Antibiotic-induced Lysis of S. faecalis**

Strain‡	Penicillin (20 μg/ml)	Cephalothin (312 μg/ml)	Bacitracin (625 μg/ml)	Cycloserine (625 μg/ml)	Vancomycin (15 μg/ml)	Spontaneous lysis
953	88	84	59	56	17	44
1345	90	87	66	66	14	58
1360	29	26	17	9	6	32
1398	86	76	48	47	9	45
1406	87	68	69	47	7	49
1411	66	48	11	13	8	17
1694	53	43	18	24	3	20
1905	28	23	7	12	2	20

* Lysis is expressed as the percent reduction in absorbance at 675 nm between one and four h after the addition of antibiotics, and between 0 and 4 h in the spontaneous lysis experiments. Each value represents the mean of three separate experiments.
‡ Median MIC for the strains tested are 4 μg/ml for penicillin (1–4), 31 μg/ml for cephalothin (31–62), 62 μg/ml for bacitracin (62–125), 250 μg/ml for cycloserine (all strains identical), and 0.06 μg/ml for vancomycin (0.06–1.0).

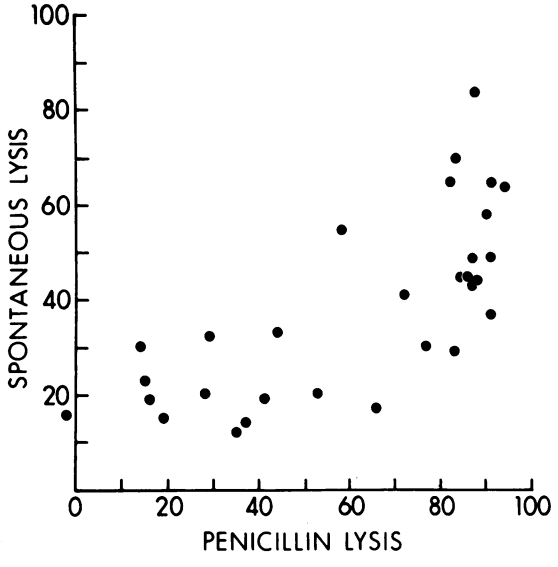

FIGURE 2 Testing of 30 clinical enterococcal isolates demonstrated a correlation between spontaneous and antibiotic-induced lysis ($r = 0.73$, $P < 0.001$).

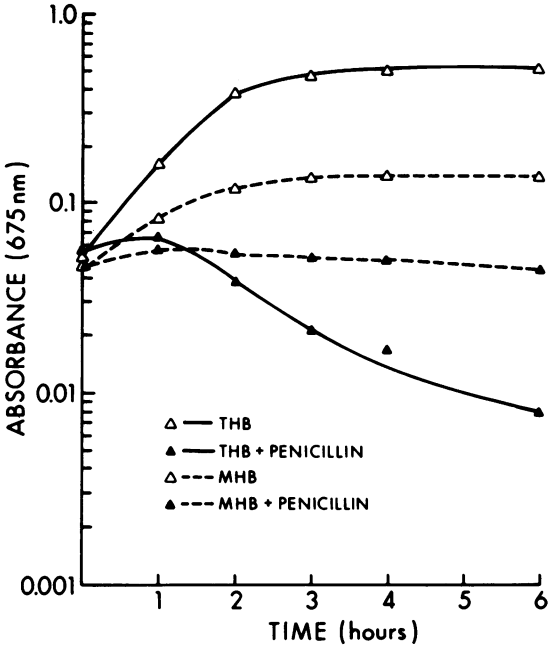

FIGURE 3 THB permitted more rapid growth and penicillin lysis (20 μg/ml) than MHB with strain 953 (penicillin MIC of 4 μg/ml).

taneous lysis and vancomycin-induced lysis was weaker, and of borderline significance ($r = 0.69$, $P = 0.06$).

Medium related differences. Growth medium was an important variable in antibiotic-induced lysis and killing. The eight strains tested against five antibiotics all showed more rapid lysis and killing in THB than in MHB for all antibiotics tested (e.g., Fig. 3). Human serum permitted only minimal growth, and was one of the least permissive media tested for antibiotic-induced lysis and killing. For example, the most lytic strain (1345) demonstrated only 15% lysis in serum vs. 90% in THB. The close relationship between spontaneous and antibiotic-induced lysis was also observed in experiments comparing the lysis of a single isolate (strain 953) after growth in seven different media. Spontaneous lysis was more rapid after growth in media that permitted rapid penicillin-induced lysis than it was after growth in media that permitted slower rates of penicillin-induced lysis ($r = 0.85$, $P = 0.02$). Likewise, both spontaneous and penicillin-induced lysis were enhanced by media that permitted more rapid growth (Table III). Because growth rate is exponentially related to the growth constant by the equation $N = N_0 e^{\mu t}$, where μ is the growth constant and N/N_0 is the growth for a given time period (t) (26), correlations were calculated between the antilog of the growth constant and both spontaneous and penicillin-induced lysis. Only the correlation with spontaneous lysis was significant ($r = 0.82$, $P = 0.03$; and $r = 0.62$, $P = 0.13$, respectively).

DISCUSSION

The mechanism of the bactericidal action of penicillin is incompletely understood. Early hypotheses suggested that penicillin killing was an osmotic catastrophe resulting from penicillin inhibition of cell wall synthesis (2). More recent hypotheses have focused on penicillin activation of endogenous autolytic enzymes (27). In addition, some investigators have sought to

TABLE III
*Spontaneous and Penicillin-induced Lysis of S. faecalis Strain 953 after Growth in Different Media**

	Growth constant (h^{-1})	Spontaneous lysis	Penicillin-induced lysis (20 μg/ml)
Dextrose phosphate broth	1.24	44	58
THB	1.16	46	87
BHI	1.13	30	48
PAB	0.99	22	7
MS-2 (Oxoid)	0.96	26	5
HI	0.66	21	39
MHB	0.51	21	7

* The penicillin MIC of strain 953 is 4 μg/ml. Spontaneous and penicillin-induced lysis are defined as in Table II. Growth constants are calculated using the equation $N = N_0 e^{\mu t}$ (26) (see text).

identify a specific penicillin-binding protein responsible for penicillin killing (28). The studies presented here suggest that the lysis and killing of enterococci (*S. faecalis*) by antibiotics that inhibit cell wall synthesis result from activation of the endogenous autolytic enzyme system.

Previous studies implicating the autolytic enzyme system in penicillin killing have employed mutants deficient in autolytic enzyme activity (15–18). In this study, we present a different line of evidence that derives from naturally occurring strain variation. In testing 30 clinical isolates of *S. faecalis*, we found substantial strain variation in penicillin-induced lysis, which correlated with spontaneous lysis in sodium phosphate buffer (Fig. 2). These data suggest that spontaneous and penicillin-induced lysis occur via the same mechanism, although the degree of scatter suggests that other factors also influence these processes. Spontaneous lysis in phosphate buffer is an index of endogenous autolytic activity and has been used extensively by Shockman et al. (18, 24, 29–33) in studies of *Streptococcus faecium* ATCC 9790. Thus, our findings suggest that the common mechanism of spontaneous and penicillin-induced lysis is the autolytic enzyme system.

Several studies have demonstrated that autolytic-defective mutants are resistant to killing by both β-lactam and non-β-lactam inhibitors of cell wall synthesis (15–18). Likewise, we found that individual strains of enterococci had similar lytic response to both β-lactam and non-β-lactam inhibitors of cell wall synthesis. Although the correlations between lysis induced by different antibiotics were not perfect ($r < 1.0$), the strength of the correlations observed and their statistical significance (Table II) suggest that both classes of antibiotics were producing lysis via the autolytic enzyme system. The similarities in the lytic response of enterococci to β-lactams and non-β-lactams included their dose-response relationships, which resembled the classical Eagle effect initially described for penicillin killing of gram-positive cocci (34). That is, beyond an optimal concentration, increased concentrations of both groups of antibiotics actually produced less lysis. The similar dose-response relationships of β-lactam and non-β-lactam inhibitors of cell wall synthesis support the idea that both groups of antibiotics share a common mechanism for inducing lysis.

If spontaneous and antibiotic-induced lysis involve the same mechanism, then conditions which affect one process should affect the other in a similar manner. To test this hypothesis, we measured spontaneous and penicillin-induced lysis using a single strain grown in seven different media. The hypothesis was supported by the observation that cells grown in media that permitted more rapid penicillin lysis were

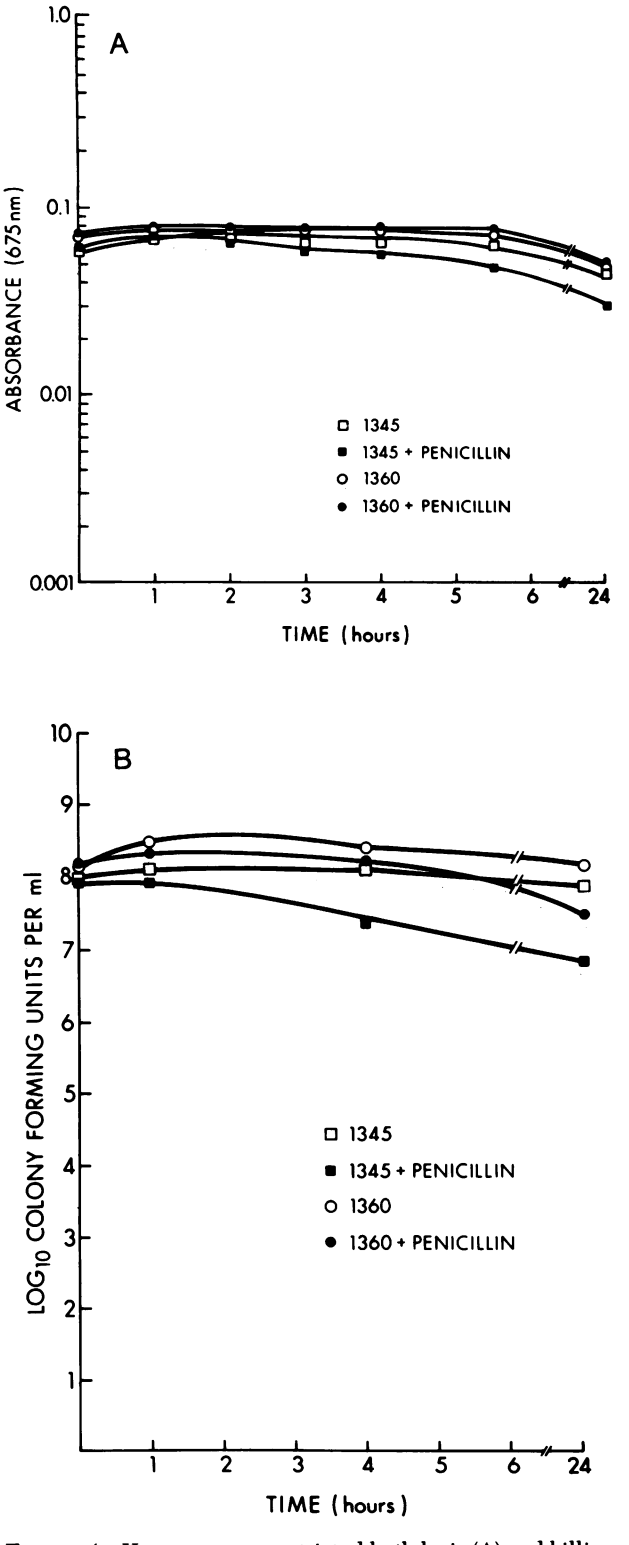

FIGURE 4 Human serum restricted both lysis (A) and killing (B) induced by penicillin G (20 μg/ml) with strains 1345 and 1360. This concentration was five times the penicillin MIC of these strains (4 μg/ml).

also more susceptible to spontaneous lysis. Conversely, cells grown in media that permitted less rapid penicillin lysis were less susceptible to spontaneous lysis (Table III).

Our studies have demonstrated more rapid and extensive penicillin lysis and killing of *S. faecalis* than those of most previous investigators (3–5, 35–36). We believe that the reasons for these differences include (*a*) strain variation and (*b*) differences in the specific testing conditions used. We observed substantial strain variation similar to that originally reported by Eagle (34) that raises the possibility that endocarditis caused by strains very susceptible to penicillin lysis and killing might not require synergistic treatment with penicillin plus an aminoglycoside. Additional in vivo studies will obviously be necessary to resolve this question. We also found that the rate of antibiotic-induced lysis and killing varied widely with different media (Figs. 3 and 4). We suggest that the resistance of enterococci to penicillin killing in certain media may result from medium-dependent suppression of autolytic enzyme activity. Tomasz and his colleagues (37–39) have previously demonstrated suppression of antibiotic-induced lysis in pneumococci, *Escherichia coli*, and other organisms by similar changes in growth conditions.

In an attempt to approximate more closely the in vivo environment, we used human serum as a growth medium. The experiments demonstrated that human serum permitted only minimal growth and was one of the least permissive media tested for antibiotic-induced lysis and killing (Fig. 4). We suggest that the restrictive nature of human serum as a growth medium for enterococci and as a medium for penicillin lysis and killing may be an important factor in the resistance of enterococcal endocarditis to treatment with penicillin alone.

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