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In Vitro Activities of Quinolones against Enterococci Resistant to Penicillin-Aminoglycoside Synergy

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The MICs and MBCs of CI-934, ciprofloxacin, difloxacin (A-56619), A-56620, norfloxacin, enoxacin, amifloxacin, and coumermycin were determined for 43 clinical isolates of *Enterococcus faecalis* known to be resistant to penicillin-aminoglycoside synergy. Results were compared with those obtained for 37 synergy-susceptible *E. faecalis* and 22 *Enterococcus faecium* strains. Although no substantial differences in quinolone activities were observed between synergy-resistant and -susceptible *E. faecalis* strains, CI-934 and ciprofloxacin were the drugs that demonstrated the greatest bactericidal activity against both types of *E. faecalis*. The MBCs of the other quinolones were generally within a single twofold dilution of the MICs, but their antienterococcal activity did not approach that of CI-934 or ciprofloxacin. The MBCs for 90% of the isolates of CI-934 for synergy-resistant and -susceptible *E. faecalis* strains were 1 and ≤0.5 μg/ml, respectively. The ciprofloxacin MBC for 90% of the *E. faecalis* strains tested was 1 μg/ml. For *E. faecium* isolates the CI-934 and ciprofloxacin MBCs for 90% of the isolates were 8 and 4 μg/ml, respectively. Time-kill assays performed with synergy-susceptible enterococcal strains showed that the bactericidal activities of both CI-934 and ciprofloxacin were less than those of the penicillin-aminoglycoside combinations tested. However, against synergy-resistant isolates the activities of these two quinolones were comparable with and sometimes greater than those of penicillin-aminoglycoside combinations.

Aminoglycoside and penicillin (or ampicillin) combinations have been the most effective treatment for serious enterococcal infections (13, 17, 22). The synergistic bactericidal activity that these drugs demonstrate is particularly important for their use in the treatment of enterococcal endocarditis. However, by acquiring plasmids that encode for various aminoglycoside-modifying enzymes, *Enterococcus faecalis* isolates can exhibit high-level aminoglycoside resistance (MIC, ≥2,000 μg/ml) and be refractory to antibiotic synergy (6, 12, 19). Infections caused by these resistant strains present serious therapeutic dilemmas (10), and their incidence at one medical center has been reported as high as 55% of all clinical enterococcal isolates (37). In addition, the therapeutic problems posed by the emergence of high-level aminoglycoside resistance have been complicated by the discovery of β-lactamase-producing strains of *E. faecalis* (25). The resistance emerging among *E. faecalis* isolates as well as *Enterococcus faecium* resistance to antibiotics, which has been known for some time (20, 23), indicates that the choice of antibiotics that may be selected for use against enterococci is extremely limited.

Several of the recently developed fluoroquinolone antibiotics have demonstrated a broad spectrum and a high level of antibacterial activity (3, 18, 30, 34), and certain of these drugs have shown good activity against some *E. faecalis* isolates (15, 18). However, thorough evaluations of fluoroquinolone activity against enterococci that exhibit resistance to penicillin-aminoglycoside synergy have not been reported. This study investigated the in vitro bacteriostatic and bactericidal activity of several quinolones against synergy-resistant enterococci and compared the results obtained with those of synergy-susceptible strains. Additionally, quinolones that exhibited the greatest antienterococcal activity were selected for comparative time-kill studies with penicillin and aminoglycoside combinations. The potential for quinolones to select for resistant *E. faecalis* and *E. faecium* mutants was also investigated.

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MATERIALS AND METHODS

Bacterial isolates. Eighty *E. faecalis* strains isolated from patient specimens submitted to the University of Chicago Clinical Microbiology Laboratories were used for this study. Duplicate strains isolated from the same patient were excluded. Organism identification was based on colony morphology, Gram stain, and recommended biochemical characteristics (8). The classification of each strain as being resistant or susceptible to penicillin-aminoglycoside synergy was based on results of high-level aminoglycoside resistance screens that had been performed with streptomycin, kanamycin, and gentamicin. A previously described agar dilution method was used for these screens (21, 29), and strains that exhibited an aminoglycoside MIC of >2,000 μg/ml were considered high-level resistant (HLR) and synergy resistant. Synergy was defined as a ≥100-fold increase in killing by the penicillin-aminoglycoside drug combination over the killing accomplished by the most active of the two drugs tested separately (22). Forty-three of the *E. faecalis* strains chosen were HLR, and 37 isolates were non-HLR. Among the 43 resistant strains, 3 were resistant only to streptomycin, 11 were resistant to streptomycin and kanamycin, 14 were resistant to gentamicin and kanamycin, and 15 were resistant to all three aminoglycosides tested.

Twenty-two strains of *E. faecium* also were studied. Twenty of these isolates were provided by the Antimicrobic Investigations Branch of the Centers for Disease Control, and two were obtained from patient specimens submitted to the University of Chicago Clinical Microbiology Laboratories. Because of the uncertain accuracy of the screening tests for detecting synergy resistance in *E. faecium* (29), isolates

were not screened for high-level aminoglycoside resistance but were subjected to time-kill studies (22).

Antimicrobial agents. The drugs used for this investigation were supplied by their respective manufacturers: ciprofloxacin (Miles Pharmaceuticals, West Haven, Conn.); difloxacin (A-56619) and A-56620 (Abbott Laboratories, North Chicago, Ill.); norfloxacin (Merck Sharp & Dohme, Rahway, N.J.); amifloxacin (Sterling-Winthrop Research Institute, Rensselaer, N.Y.); enoxacin and CI-934 (Warner-Lambert, Ann Arbor, Mich.); coumermycin (Hoffmann-La Roche Inc., Nutley, N.J.); penicillin and streptomycin (Sigma Chemical Co., St. Louis, Mo.); gentamicin (Schering Corp., Bloomfield, N.J.); kanamycin (Bristol Laboratories, Syracuse, N.Y.).

Antimicrobial susceptibility test. An MIC 2000 Dynatech microdilution system (Dynatech Laboratories, Alexandria, Va.) was used to prepare broth microdilution panels containing douoling dilutions of the various antibiotics in 0.1 ml of cation-supplemented Mueller-Hinton broth. Panels were inoculated with each bacterial isolate to give a final inoculum of 10^5 CFU/ml (10^4 CFU per microdilution well) and were incubated aerobically at 35°C for 20 h. The MIC was defined as the lowest concentration of antibiotic that inhibited visible growth in the microdilution wells. To minimize drug carryover in the determination of MBC, procedures recommended by the National Committee for Clinical Laboratory Standards were followed (26). The entire volume (0.1 ml) of each microdilution well that had not shown growth after 20 h of incubation was spotted onto the surface of a sheep blood agar plate (BBL Microbiology Systems, Cockeysville, Md.) and spread over the entire surface. After 24 h of incubation plates were examined for bacterial growth and quantitation of colonies. The MBC was defined as the lowest antibiotic concentration at which a 99.9% reduction (i.e., ≤10 CFU per plate) in the original inoculum was noted (26).

Time-kill assays. Based on the MIC data, certain *E. faecalis* and *E. faecium* isolates were selected for 24-h time-kill assays. Strains resistant to penicillin-aminoglycoside synergy were included among the isolates tested. The antibiotics tested, either alone or in combination with penicillin (10 U/ml), included streptomycin (25 μg/ml), gentamicin (5 μg/ml), ciprofloxacin (1, 2, and 4 μg/ml), and CI-934 (0.25, 1, and 5 μg/ml). All assays were performed with a final inoculum of 10^7 CFU/ml in dextrose phosphate broth (GIBCO Diagnostics, Madison, Wis.) as described previously (22, 26, 29). At 0, 4, and 24 h after inoculation 0.1-ml samples were taken from the broth and passed through serial 10-fold dilutions. From each dilution a 0.1-ml sample was taken, spotted onto a sheep blood agar plate, and spread over the entire surface. After 24 h of incubation plates were examined for bacterial growth and colonies were counted.

Mutational studies. The method of Tenney et al. (32) was used to determine whether exposing enterococcal inocula to twofold stepwise increases in ciprofloxacin and CI-934 concentrations would result in the emergence of isolates highly resistant to these quinolones. Overnight Mueller-Hinton broth cultures of synergy-resistant and synergy-susceptible *E. faecalis* and *E. faecium* isolates were swabbed onto Mueller-Hinton agar (Difco Laboratories, Detroit, Mich.) containing either ciprofloxacin or CI-934 at concentrations equal to one-half the MIC of each drug. By this method, any colonies that grow are subcultured to Mueller-Hinton broth not containing drugs, grown overnight, and again swabbed to agar containing twofold increments in drug concentration. Additionally, to study the emergence of spontaneous single-step mutants highly resistant to ciprofloxacin and CI-934, large inocula (10^8 to 10^9 CFU) were plated onto Mueller-Hinton agar containing drug concentrations that were 4, 8, and 16 times the MICs (18).

RESULTS

Antienterococcal activities of the various quinolones and coumermycin, a nonquinolone drug that acts on bacterial DNA gyrase (11), are given in Table 1. As shown by the MIC and MBC data, the drug activities against the 43 HLR *E. faecalis* isolates were comparable to the activities against 37 non-HLR *E. faecalis* strains. Among HLR isolates that had different aminoglycoside resistance profiles the activities of the different drugs were also comparable (data not shown). In general, CI-934 was the most active quinolone tested, followed by ciprofloxacin. However, because CI-934 activity endpoints less than 0.5 μg/ml were not established, exact comparisons of CI-934 activity between HLR and non-HLR isolates cannot be made. All other drugs tested had MICs for 90% of the isolates (MIC_{90}s) and MBCs for 90% of the isolates (MBC_{90}s) that were at least fourfold or more higher than those of either CI-934 or ciprofloxacin. Enoxacin, amifloxacin, and coumermycin demonstrated the lowest activities. Regardless of the drug being tested the MBC rarely exceeded the MIC by more than a single twofold dilution.

The 22 *E. faecium* strains were more resistant than were either the HLR or the non-HLR *E. faecalis* isolates (Table 1). CI-934 and ciprofloxacin demonstrated the greatest activity against *E. faecium*, but the MICs and MBCs were at least fourfold higher than for the *E. faecalis* isolates. Whereas CI-934 generally was more active than ciprofloxacin against *E. faecalis*, the activities of the two drugs against *E. faecium* were nearly the same. The poor activity of the other quinolones was evident by MIC_{90}s and MBC_{90}s that were never less than 16 μg/ml. As occurred with the *E. faecalis* strains, the MBCs of most drugs were within one twofold dilution of the MICs.

The key susceptibility characteristics of the *E. faecalis* and *E. faecium* strains chosen for time-kill assays are given in Table 2. Four strains of each species were selected to include synergy-resistant and -susceptible isolates with relatively high and relatively low ciprofloxacin and CI-934 MICs. For all *E. faecalis* strains tested, CI-934 MICs were ≤0.5 μg/ml (Table 1); therefore an isolate relatively resistant to CI-934 could not be included in the time-kill assays.

Results of time-kill assays with *E. faecalis* strains are demonstrated in Fig. 1. For both synergy-susceptible strains (A and B) the penicillin-aminoglycoside combinations demonstrated synergistic killing that was greater than the killing obtained with either CI-934 or ciprofloxacin tested alone at any of the three concentrations. For strain A the bactericidal activities of both quinolones were comparable to the activity exhibited by penicillin alone. For strain B, CI-934 at 0.25 μg/ml and ciprofloxacin at 1 μg/ml failed to show any appreciable bactericidal activity and were notably less active than penicillin alone.

Against the synergy-resistant *E. faecalis* strains (C and D) the bactericidal activities of penicillin-aminoglycoside combinations were comparable to the activity of penicillin alone, as were the activities of ciprofloxacin and CI-934 at most concentrations tested (Fig. 1). However, for strain C, which was relatively susceptible to the quinolones (Table 2), ciprofloxacin at each concentration tested and CI-934 at 1 and 5 μg/ml showed tendencies for slightly greater bactericidal activity than penicillin tested alone or with an aminoglyco-

TABLE 1. Comparative activity of quinolones and coumermycin against enterococci

Organism (no. of isolates)	Test agent	MIC (μg/ml)			MBC (μg/ml)		
		Range	50%	90%	Range	50%	90%
HLR[a] *E. faecalis* (43)	CI-934	≤0.5	≤0.5	≤0.5	≤0.5–1	≤0.5	1
	Ciprofloxacin	≤0.25–2	0.5	1	≤0.25–4	0.5	1
	Difloxacin	≤0.5–4	2	4	≤0.5–8	4	4
	Norfloxacin	1–8	4	4	1–8	4	8
	A-56620	2–16	4	8	2–16	4	8
	Enoxacin	2–16	8	8	2–32	8	16
	Amifloxacin	4–16	8	16	4–16	8	16
	Coumermycin	2–16	4	16	4–>32	16	32
Non-HLR[b] *E. faecalis* (37)	CI-934	≤0.5	≤0.5	≤0.5	≤0.5–1	≤0.5	≤0.5
	Ciprofloxacin	≤0.25–1	0.5	1	≤0.25–2	0.5	1
	Difloxacin	1–4	2	4	1–8	4	8
	Norfloxacin	1–8	4	8	1–8	4	8
	A-56620	2–16	4	8	2–16	8	16
	Enoxacin	4–16	8	8	4–16	8	16
	Amifloxacin	4–32	8	16	4–32	16	16
	Coumermycin	≤0.5–16	8	16	2–32	32	>32
E. faecium (22)	CI-934	≤0.5–8	2	4	≤0.5–16	4	8
	Ciprofloxacin	≤0.25–8	2	4	0.5–8	2	4
	Difloxacin	2–16	8	16	2–>32	8	16
	Norfloxacin	4–32	8	16	4–32	8	32
	A-56620	4–>32	16	32	4–>32	32	>32
	Enoxacin	16–>32	16	32	16–>32	32	>32
	Amifloxacin	16–>32	32	>32	32–>32	32	>32
	Coumermycin	2–>32	16	32	4–>32	>32	>32

[a] HLR (MIC, >2,000 μg/ml) indicates synergy resistance; included are 3 strains resistant to only streptomycin, 11 strains resistant to both streptomycin and kanamycin, 14 strains resistant to both gentamicin and kanamycin, and 15 strains resistant to all three aminoglycosides.
[b] Non-HLR indicates synergy susceptibility.

side. For the more quinolone-resistant strain D, only CI-934 at 5 μg/ml demonstrated bactericidal activity notably greater than that of any other drug or drug combination tested.

Figure 2 shows time-kill results obtained *E. faecium* strains. Against synergy-susceptible strains the penicillin-aminoglycoside combinations showed synergistic bactericidal activity that was 3 to 4 $\log_{10}$ units greater than that obtained with penicillin alone. For strain A ciprofloxacin at 2 and 4 μg/ml had bactericidal activities comparable to those of the penicillin-aminoglycoside combinations. Additionally, the activity of CI-934 at 5 μg/ml was similar to that of the penicillin-aminoglycoside combinations for both strains A and B. For *E. faecium* strains resistant to penicillin-aminoglycoside synergy (C and D), ciprofloxacin and CI-934 at the highest concentrations tested were the only drugs that showed any appreciable bactericidal activity. After 24 h ciprofloxacin at 4 μg/ml and CI-934 at 5 μg/ml substantially reduced the original inoculum of strain C. After 4 h these same drug concentrations also reduced the CFU per milliliter of strain D, which had fourfold higher ciprofloxacin and CI-934 MICs than did strain C (Table 2), but by 24 h active growth in the presence of these quinolones had resumed.

TABLE 2. Susceptibility characteristics of enterococci tested by time-kill studies

Strain	Synergy susceptibility[a]	MIC (μg/ml)	
		Ciprofloxacin	CI-934
E. faecalis A	S	≤0.25	≤0.5
E. faecalis B	S	1	≤0.5
E. faecalis C	R	≤0.25	≤0.5
E. faecalis C	R	2	≤0.5
E. faecium A	S	≤0.25	≤0.5
E. faecium B	S	4	8
E. faecium C	R	2	2
E. faecium D	R	8	8

[a] S, Susceptible to penicillin-gentamicin and penicillin-streptomycin synergy; R, not susceptible to the synergistic activity of either penicillin-aminoglycoside combination.

To determine whether synergy between ciprofloxacin (or CI-934) and penicillin could be demonstrated against the *E. faecalis* and *E. faecium* strains, time-kill studies were performed with these drug combinations. No synergy was observed; the penicillin-quinolone combinations failed to be any more bactericidal than were either ciprofloxacin or CI-934 tested alone, regardless of the concentration (data not shown).

E. faecalis and *E. faecium* strains subjected to both gradual step-wise and single-step spontaneous mutational studies failed to yield mutants highly resistant to either ciprofloxacin or CI-934. For the incremental resistance investigations, overnight broth cultures swabbed onto agar plates containing the MIC of either CI-934 or ciprofloxacin failed to yield growth. By the spontaneous mutation method the largest inoculum used was 10^9 CFU, but no resistant colonies were detected. Therefore, for both species studied the frequency of spontaneous mutations leading to either ciprofloxacin or CI-934 resistance was $<10^{-9}$.

DISCUSSION

For all HLR and non-HLR *E. faecalis* isolates studied, CI-934 and ciprofloxacin were more active than any of the other quinolone-type drugs tested. The activity of CI-934 against both HLR and non-HLR *E. faecalis* isolates was similar to that reported by other investigators, who found

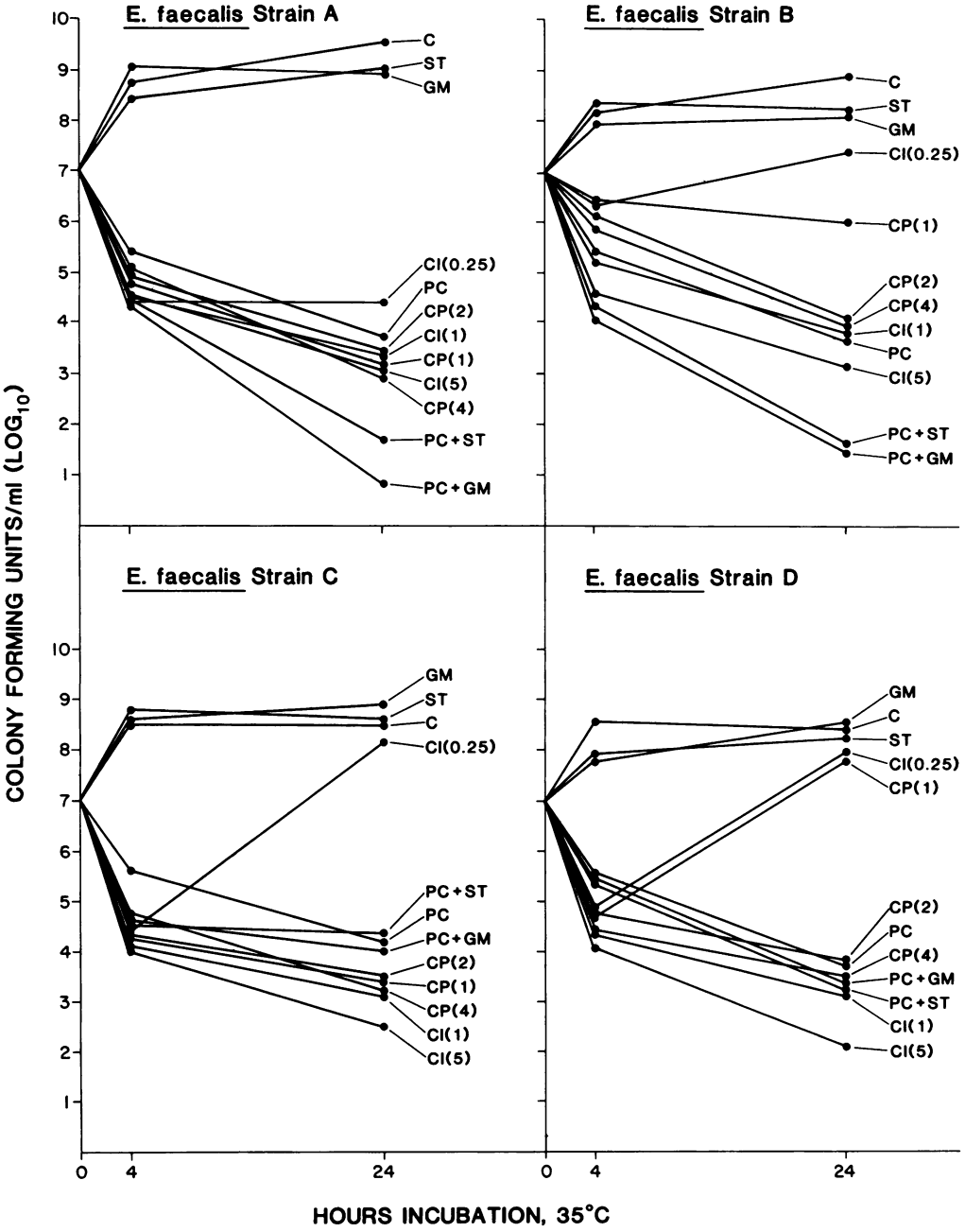

FIG. 1. Effect of penicillin and aminoglycosides (tested alone and in combination), ciprofloxacin, and CI-934 on the growth of *E. faecalis* strains A, B, C, and D (Table 2). Antibiotics were tested at the following concentrations: penicillin (PC), 10 U/ml; streptomycin (ST), 25 μg/ml; gentamicin (GM), 5 μg/ml; ciprofloxacin (CP), 1, 2, and 4 μg/ml; CI-934 (CI), 0.25, 1, and 5 μg/ml; control (C), no antibiotic.

CI-934 to be a potent antienterococcal quinolone (15, 16, 18). However, because the penicillin-aminoglycoside synergy susceptibility of *E. faecalis* isolates tested in these previous studies was not indicated, we do not know how our CI-934 results for HLR strains would compare with those of other investigators. Against most HLR and non-HLR *E. faecalis* isolates, ciprofloxacin was the second most active quinolone. Although our ciprofloxacin MIC_{90} of 1 μg/ml for 80 *E. faecalis* strains is slightly lower than that reported by other investigators, whose values ranged from 2 to 6.3 μg/ml, both our study and those of others indicate that this drug does have notable in vitro activity against some *E. faecalis* isolates (2, 3, 4, 7, 15, 18). Previous reports of ciprofloxacin studies did not indicate whether synergy-resistant strains of *E. faecalis* were included. Therefore, as with CI-934, comparison of ciprofloxacin susceptibilities with those of HLR *E. faecalis* isolates from other studies is not possible.

CI-934 and ciprofloxacin also were the most active quinolones against *E. faecium*. The CI-934 MIC_{90} of 4 μg/ml against *E. faecium* was higher than the MIC_{90} of 1 μg/ml reported by Kim et al. (15), 3.1 μg/ml reported by Cohen et al. (5), and 2 μg/ml reported by King et al. (16). On the other hand, the ciprofloxacin MIC_{90} of 4 μg/ml we obtained against *E. faecium* was the same as that reported by Kim et al. (15)

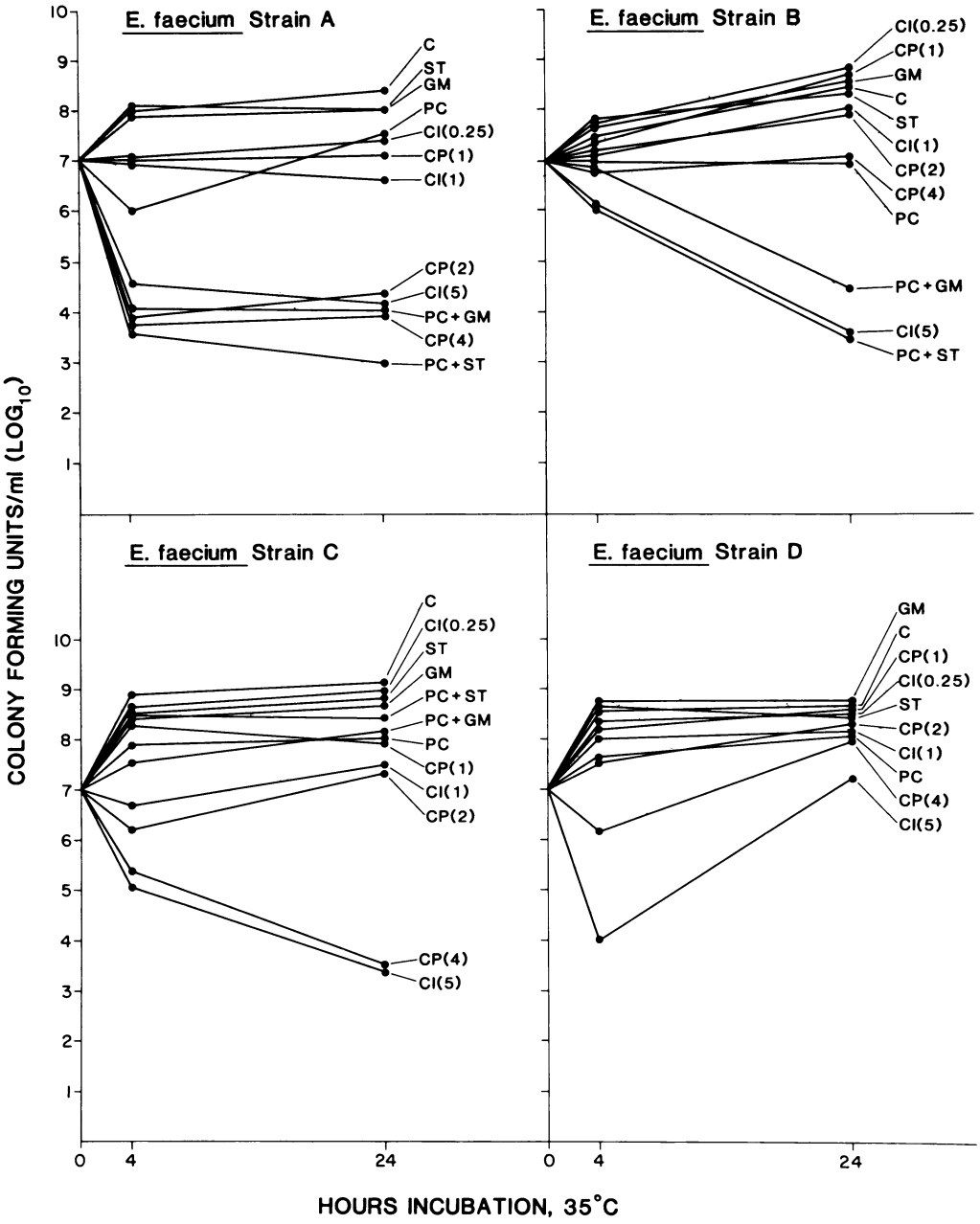

FIG. 2. Effect of penicillin and aminoglycosides (tested alone and in combination), ciprofloxacin, and CI-934 on the growth of *E. faecium* strains A, B, C, and D (Table 2). Antibiotics were tested at the following concentrations: penicillin (PC), 10 U/ml; streptomycin (ST), 25 μg/ml; gentamicin (GM), 5 μg/ml; ciprofloxacin (CP), 1, 2, and 4 μg/ml; CI-934 (CI), 0.25, 1, and 5 μg/ml; control (C), no antibiotic.

but twofold less than the MIC_{90} of 8 μg/ml reported by others (3, 7). The reasons for these moderate differences in CI-934 and ciprofloxacin MIC results among various investigators are not known but may be due to subtle interlaboratory variations in susceptibility testing techniques. The anti-enterococcal activities of all other drugs tested were comparable to those reported in previous studies (3, 15, 27, 30).

For all quinolones tested the MBCs were generally either equal to or only one twofold dilution higher than the MICs, which is consistent with the findings of others (4, 7, 15, 30). Because of their bactericidal activity and because they exhibited the greatest antienterococcal activity, CI-934 and ciprofloxacin were the two quinolones selected for comparative time-kill studies.

The bactericidal activities of ciprofloxacin and CI-934 demonstrated by the time-kill studies (Fig. 1) were consistent with those reported from previous studies (9, 31, 35). Although their activities against synergy-susceptible *E. faecalis* strains generally were comparable to those of penicillin alone, they were substantially less than those of the penicillin-aminoglycoside combinations. In support of these in vitro findings, in vivo studies with rabbit models have demonstrated that ciprofloxacin alone fails to reduce enterococcal bacterial counts as substantially as do penicillin-aminoglycoside combinations (9, 28). Against synergy-resistant

strains the higher concentrations of ciprofloxacin and CI-934 demonstrated bactericidal activities comparable to those of both penicillin alone and penicillin combined with an aminoglycoside. However, in one study that used a rat model for endocarditis, Ingerman et al. (14) showed that ciprofloxacin alone may not be efficaceous for endocarditis caused by synergy-resistant enterococci. Because the in vitro activity of these particular quinolones was comparable to that of penicillin alone, their potential use as alternatives to combination therapy for infections caused by either synergy-resistant or -susceptible *E. faecalis* is doubtful. However, the activity of these antimicrobial agents indicates that potential exists for development of quinolone-type drugs that could provide effective therapy for serious *E. faecalis* infections.

For synergy-susceptible *E. faecium* strains the two quinolones did not demonstrate any activity advantage over penicillin-aminoglycoside combinations (Fig. 2). However, against synergy-resistant *E. faecium* strain C the highest concentrations of CI-934 and ciprofloxacin were notably more bactericidal than the penicillin-aminoglycoside combinations. In contrast, synergy-resistant strain D with higher quinolone MICs was not effectively killed by any of the drugs tested. Therefore, against quinolone-susceptible isolates such drugs may expand our currently limited therapeutic choices for synergy-resistant *E. faecium*.

Combining either CI-934 or ciprofloxacin with penicillin did not synergistically enhance the activity of either drug against any *E. faecalis* or *E. faecium* strain. Except for Peterson et al. (28), who reported superior bactericidal activity with azlocillin-ciprofloxacin combinations tested by time-kill assays, our findings agree with those of other investigators who have studied the effects of combining quinolones with other drugs (1, 9, 18, 24). Currently there does not seem to be definitive evidence to suggest that combining quinolones with other antimicrobial agents synergistically enhances antienterococcal activity.

The low mutational frequency of $<10^{-9}$ found in our study agrees with that reported from other studies (4, 18) and would seem to indicate that *E. faecalis* and *E. faecium* isolates are not likely to spontaneously develop high-level resistance to ciprofloxacin or CI-934 during therapy. However, Zervos et al. (36) have recently reported two cases in which patients receiving ciprofloxacin developed superinfections caused by *E. faecalis* strains whose susceptibility to ciprofloxacin decreased substantially when the inoculum size was increased from 10^5 to 10^7 CFU/ml. In contrast, the enterococcal strains we tested appeared susceptible to quinolones even when a inoculum of 10^7 CFU/ml was used (Fig. 1). Whether the resistance reported by Zervos et al. (36) is due to an enterococcal propensity for mutationally acquired resistance or to an inoculum effect associated only with certain strains (9, 36) is not clear and requires further investigations. Although incremental development of quinolone resistance has been described for a variety of non-enterococcal organisms (7, 18, 32), we were unable to demonstrate such resistance development in the enterococcal isolates we tested. The reasons for these findings are currently unknown, and further studies to determine whether enterococci are relatively refractory to developing resistance by such methods are warranted.

In summary, the emergence of synergy-resistant enterococci signals a strong need for the research and development of alternative antimicrobial agents for the treatment of serious enterococcal infections (10). An example of one potential alternative is the newly developed lipopeptide daptomycin (LY146032), which has shown bactericidal activity against enterococci (31, 33). Similarly, our study has demonstrated that synergy-resistant enterococci with low CI-934 or ciprofloxacin MICs and MBCs may be effectively killed by these quinolones. Although these two particular drugs may not be therapeutic choices for serious enterococcal infections, their in vitro activity is indicative of the potential that quinolone-type antimicrobial agents have as alternatives to penicillin-aminoglycoside combinations for the treatment of enterococcal infections.

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