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Lytic Effect of Two Fluoroquinolones, Ofloxacin and Pefloxacin, on *Escherichia coli* W7 and Its Consequences on Peptidoglycan Composition

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Examination of biochemical changes in *Escherichia coli* W7 after exposure to ofloxacin or pefloxacin revealed distinct concentration-dependent responses. At levels close to the MIC, extensive filamentation was followed by a lytic event, which involved an active protein synthesis. This lysis was correlated with changes in the peptidoglycan composition, particularly a decrease in the average glycan chain length, involving the action of the autolysines. At higher concentrations, no lysis occurred and the growth was totally inhibited as well as the protein synthesis. The peptidoglycan composition exhibited an increase in the average glycan chain length, suggesting an apparent reduced activity of the lytic transglycosylase. These results show that exposure to low concentrations of quinolones leads to the induction of lysis and peptidoglycan modifications which might contribute to the bactericidal effects of quinolones.

Quinolones are broad-spectrum antibacterial agents for which the primary mechanism of action is the inhibition of DNA gyrase activity (23). The mechanism of quinolone-induced cell death has not been fully explained (3). It has been proposed that nicked DNA formed by the action of quinolone-inhibited DNA gyrase acts as a substrate for exonucleases, which degrade the DNA (23). It has also been shown that nalidixic acid induces the SOS response in *Escherichia coli*, leading to a cascade of events, including filamentation (18, 25). We have examined the effects of two fluoroquinolones, ofloxacin and pefloxacin, on *E. coli* W7 in an attempt to elucidate possible damages to the cell wall occurring during such treatment. Indeed, nalidixic acid and related compounds, such as ciprofloxacin and norfloxacin, have been shown to promote cell filamentation, vacuolization, and finally bacterial lysis (6, 8, 14, 16). In the present study, we present evidence that ofloxacin and pefloxacin induce peptidoglycan degradation and important structural modifications of the remaining sacculus, which may be, in part, responsible for lysis and cell death.

MATERIALS AND METHODS

Materials. Ofloxacin and pefloxacin were obtained, respectively, from Roussel-Uclaf (Paris, France) and Roger Bellon (Paris, France). *meso*-[3,4,5-^{3}H]diaminopimelic acid (DAP) (0.85 TBq/mmol) and L-[1-^{14}C]leucine (2.2 GBq/mmol) were purchased from the Commissariat à l'Energie Atomique, Service des Molécules Marquées (Gif-sur-Yvette, France).

Strain and culture conditions. *E. coli* W7 (*dapA lysA*) was routinely grown in Penassay medium 3 (M3) (Difco Laboratories, Detroit, Mich.) supplemented with DAP (20 μg/ml) at 37°C in a shaking water bath (generation time, 40 min). Bacterial growth was determined by monitoring the optical density at 650 nm (OD_{650}) in a Coleman Junior spectrophotometer.

Susceptibility testing. MICs were determined by plating 10^4 bacteria per spot on Mueller-Hinton agar (Diagnostic Pasteur, Marnes-la-Coquette, France) supplemented with DAP (20 μg/ml) and containing serially diluted antibiotics. Plates were incubated overnight at 37°C. The MIC of the antibiotic was defined as the first concentration for which no visible growth occurred.

Bactericidal activity. Bactericidal activity was monitored after the addition of quinolones to a mid-logarithmic-phase culture (OD_{650} = 0.18). Samples were removed at timed intervals. Viable counts were determined after the removal of unbound drug by washing and centrifuging the cells three times in distilled water. Suspensions were appropriately diluted in distilled water, and 0.1 ml of the dilution was spread on Mueller-Hinton agar plates containing DAP (20 μg/ml). Plates were incubated at 37°C overnight, and colonies were counted.

Measurement of peptidoglycan synthesis. At mid-logarithmic phase (OD_{650} = 0.18), quinolones and [^{3}H]DAP (1 μCi/ml) were added to cultures. Samples (0.5 ml) were withdrawn at timed intervals, added to 0.5 ml of boiling 8% sodium dodecyl sulfate (SDS), and kept at 100°C for 30 min. The radioactivity was determined by filtering with glass fiber filters (Whatman GF/F); filters were washed with distilled water, followed by 95% (vol/vol) ethanol, dried, and counted in a liquid scintillation counter (Tri-Carb model 460 CD; Packard Instrument Co.).

Measurement of peptidoglycan degradation. A culture (10 ml) was grown in M3 broth containing [^{3}H]DAP (1 μCi/ml) and nonlabeled DAP (4 μg/ml). When the OD_{650} reached 0.24, the cells were harvested by centrifugation (3,000 × *g*, 10 min, 4°C), washed twice in M3 broth supplemented with nonlabeled DAP (20 μg/ml), suspended in 50 ml of M3 broth containing DAP (20 μg/ml), and divided into 8-ml aliquots. These cultures were incubated at 37°C until the OD_{650} equaled 0.18 (more than three generations), and then quinolones were added and 0.5-ml samples were removed at timed intervals and treated as described above.

Measurement of protein synthesis. Quinolones and [^{14}C]leucine (0.02 μCi/ml) were added to mid-logarithmic-phase cultures. Samples (0.5 ml) were removed at timed intervals,

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added to 0.5 ml of ice-cold 10% (wt/vol) trichloroacetic acid, and kept at 4°C for 1 h. The samples were filtered through glass fiber filters (Whatman GF/F), washed with ice-cold 5% (wt/vol) trichloroacetic acid, followed by 95% (vol/vol) ethanol, dried, and counted as described above.

Purification of peptidoglycan. Peptidoglycan was isolated and processed essentially as previously described (10, 12, 13). Cultures (400 to 2,000 ml) were grown in M3 broth supplemented with DAP (20 μg/ml) and treated with antibiotics for 3 h (h). Cells were harvested by rapid cooling with ice-cold distilled water, followed by centrifugation (12,000 × *g*, 15 min, 4°C). Control cells were harvested after 3 h and treated in parallel to the cultures exposed to antibiotics. The pellets were resuspended in 3 ml of distilled water. Sacculi were prepared by boiling the cells in 4% (wt/vol) SDS (final concentration), centrifuged (100,000 × *g*, 1 h, 25°C), washed six times with distilled water, and treated successively in 10 mM Tris hydrochloride buffer (pH 7)–10 mM NaCl with α-amylase (100 μg/ml, 2 h, 37°C) and pronase E (200 μg/ml, 1 h, 60°C), which was preincubated at 60°C for 2 h. Samples were treated again with 4% (wt/vol) SDS (final concentration) and kept for 15 min at 100°C. The sacculi were washed three times and resuspended in 0.8 ml of 25 mM sodium phosphate buffer (pH 6.5) with 0.1 mM $MgCl_2$. M1-muramidase (Mutanolysin; Sigma Chemical Co., St. Louis, Mo.; 200 μg/ml) and sodium azide (0.05%, wt/vol) were added, and the material was incubated overnight at 37°C. The enzyme reaction was stopped by boiling the sample for 3 min. After centrifugation, the supernatants were lyophilized, redissolved in distilled water, and stored frozen (−20°C).

Reverse-phase high-pressure liquid chromatography analysis. The muropeptide mixtures obtained as described above were diluted 1:1 with 0.5 M borate buffer (pH 9) and reduced with $NaBH_4$ (10 mg/ml; 30 min at 20°C); after the pH was adjusted to 4 with *ortho*-phosphoric acid, the material was applied to a reverse-phase HPLC column (Hypersil ODS; 250 by 4.6 mm, 3-μm pore size; Bischoff, France). The composition of purified peptidoglycan was determined by using a linear gradient from 50 mM sodium phosphate buffer (pH 4.31) containing sodium azide at a concentration of about 1 μg/ml to 75 mM sodium phosphate buffer (pH 4.95) in 15% methanol over 135 min at a flow rate of 0.5 ml/min and at a temperature of 55°C.

RESULTS

Turbidimetric studies. The MICs of ofloxacin and pefloxacin for *E. coli* W7 were both 0.12 μg/ml. By using multiples of the MIC, liquid cultures were exposed to the antibiotics and the OD was determined every 30 min. Figure 1 shows the growth in the presence of ofloxacin (Fig. 1A) or pefloxacin (Fig. 1B). As described recently for ciprofloxacin or norfloxacin (8), these quinolones had two distinct effects depending on the concentration used. At concentrations higher than 4× to 8× MIC, the growth was totally inhibited. At lower concentrations, a phase of filamentation, explaining the increase in OD, was followed by an abrupt decrease in OD. This lytic effect began after 90 min of exposure.

Bactericidal effects. Two concentrations (3× and 128× MIC) were chosen to study the bactericidal activity of ofloxacin (Fig. 2). The rate of killing was greatest at the lower concentration, which caused lysis. This is consistent with the observations of others for ciprofloxacin (5), norfloxacin (3), and nalidixic acid (4).

Synthesis and degradation of peptidoglycan. Peptidoglycan metabolism was followed by assaying the incorporation of

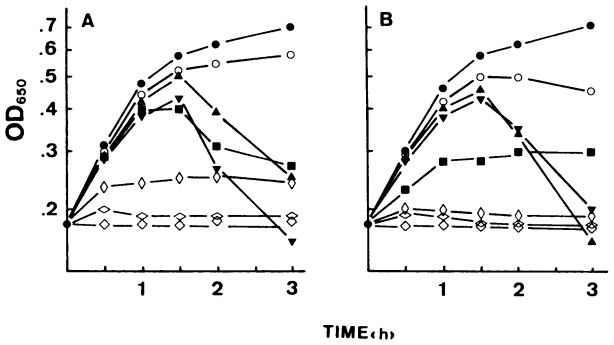

FIG. 1. Growth of *E. coli* W7 in presence of ofloxacin (A) or pefloxacin (B) at different multiples of the MICs (0.12 μg/ml for both antibiotics). Symbols: ●, control; ○, 1× MIC; ▲, 2× MIC; ▼, 4× MIC; ■, 8× MIC; ◊, 16× MIC; ◇, 32× MIC; ◇, 128× MIC.

[^{3}H]DAP in the SDS-insoluble fraction (Fig. 3). Treatment of *E. coli* W7 with 128× MIC of ofloxacin or pefloxacin resulted in a continuous incorporation of the label, slower than that in the control, for 2 h. In the presence of 3× MIC of antibiotics, the rate of synthesis was close to that of the control for the first 90 min, followed by an important and constant decrease which was parallel to the lytic effect (Fig. 1). Since this decrease seemed to result from the degradation of the cell wall, the degradation of peptidoglycan was studied by measuring the loss of label from prelabeled cultures during antibiotic exposure. In the presence of the high concentration (128× MIC) of ofloxacin or pefloxacin, as in the control, no degradation was observed (Fig. 4). On the other hand, with the low concentration (3× MIC) of antibiotics, 90% of the peptidoglycan was degraded between 90 and 180 min after addition of antibiotics.

Protein synthesis. In the presence of the high concentration of ofloxacin or pefloxacin (128× MIC), protein synthesis was immediately and totally inhibited (Fig. 5). With the low

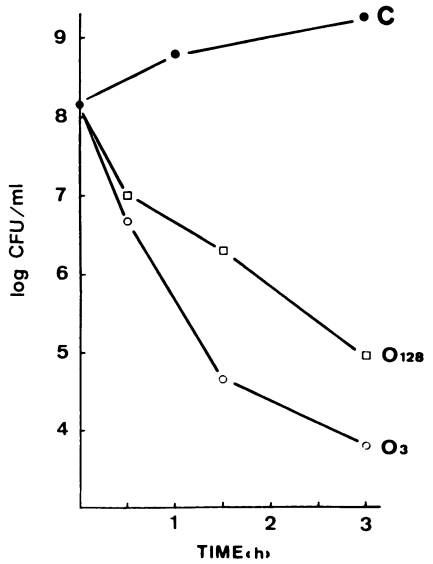

FIG. 2. Killing kinetics of *E. coli* W7 after addition of ofloxacin at time zero. Symbols: ●, control (no ofloxacin); ○, 3× MIC; □, 128× MIC.

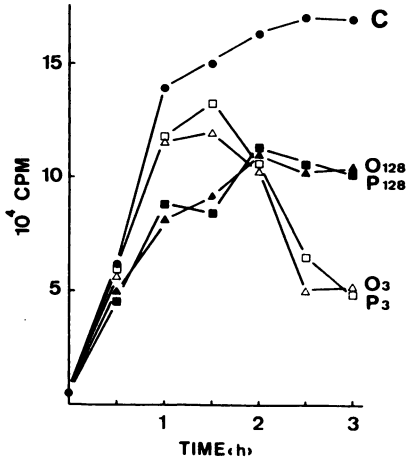

FIG. 3. Peptidoglycan synthesis of *E. coli* W7 after addition of ofloxacin or pefloxacin at 3× MIC (△ and □, respectively) and 128× MIC (▲ and ■, respectively) or with no antibiotic (control) (●).

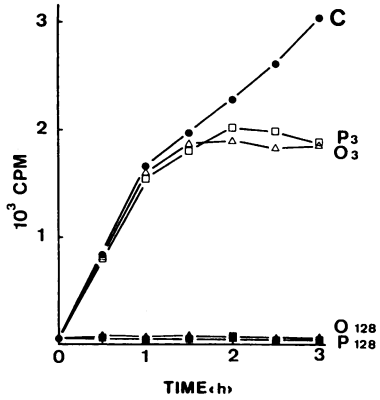

FIG. 5. Protein synthesis of *E. coli* W7 measured by the incorporation of [^{14}C]leucine into trichloroacetic acid-insoluble fraction, after addition of ofloxacin or pefloxacin at 3× MIC (△ and □, respectively) and 128× MIC (▲ and ■, respectively) or with no antibiotic (control) (●).

concentration of antibiotics (3× MIC), the protein synthesis was close to that of the control for the first 90 min.

Inhibition by chloramphenicol or magnesium of the lysis induced by the low concentration of ofloxacin. To check if active protein synthesis was necessary for the induction of lysis by low concentrations of quinolones, the effect of chloramphenicol on cultures exposed to 3× MIC of ofloxacin was monitored. Chloramphenicol (128 μg/ml) was added to the cultures at different times (0, 15, 30, and 90 min after addition of ofloxacin). Figure 6 shows that chloramphenicol totally inhibited the lysis that normally follows treatment with this concentration of ofloxacin, even when the chloramphenicol was added just before the usual onset of lysis (90 min).

Magnesium, which has been shown to be an inhibitor of lysis (17), totally inhibited the lytic effect of 3× MIC of ofloxacin at 10 mM (Fig. 7).

Peptidoglycan HPLC analysis. Reverse-phase HPLC was used to determine the composition of peptidoglycan remaining in the sacculus after exposure to quinolones. The relative amount of each muropeptide was calculated from the integration results, and related compounds were grouped. In this way, values for four basic parameters of peptidoglycan composition were obtained: (i) the average length of the glycan chains as calculated from the fraction of disaccharide peptides with a (1,6)-anhydromuramyl residue; (ii) the quantification of the different peptide side chains in monomers; (iii) the degree of the two types of cross-linkage (Ala-DAP, DAP-DAP) as calculated from the fractions of monomers, dimers, trimers, and tetramers; and (iv) the amount of covalently bound lipoprotein as calculated from the fraction of disaccharide peptides carrying a lysyl-arginine residue. From these parameters, one can speculate as to the presence of different autolytic activities.

Table 1 presents the results obtained for samples exposed to 128× and 3× MICs of ofloxacin or pefloxacin for 3 h. The peptidoglycan compositions of *E. coli* W7 exposed to 128× MIC of ofloxacin or to 128× MIC of pefloxacin were quite similar: the anhydro compounds accounted for 3.5 and 4.1%, respectively, of the total muropeptides. These values correspond to average glycan chain lengths of 28 and 24 disaccharide peptides, respectively (control, 17 disaccharide peptides), suggesting reduced lytic transglycosylase activities

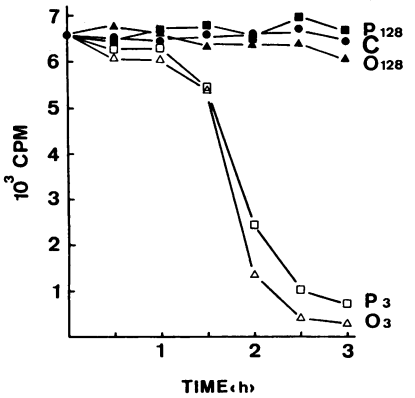

FIG. 4. Degradation of [^{3}H]DAP-prelabeled peptidoglycan of *E. coli* W7 after addition of ofloxacin or pefloxacin at 3× MIC (△ and □, respectively) and 128× MIC (▲ and ■, respectively) or with no antibiotic (control) (●).

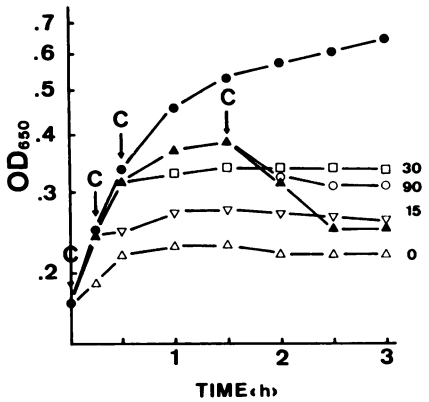

FIG. 6. Inhibition of ofloxacin (3× MIC)-induced lysis by timed addition of chloramphenicol (128 μg/ml) to *E. coli* W7. Symbols: ●, control; ▲, ofloxacin; C, chloramphenicol. Timed addition was at 0 (△), 15 (▽), 30 (□), and 90 (○) min.

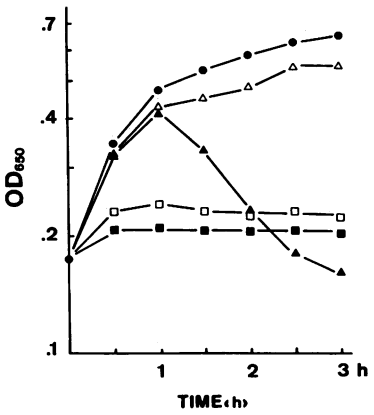

FIG. 7. Effect of magnesium (10 mM) on *E. coli* W7 exposed to ofloxacin. Symbols: ●, control; ▲, ofloxacin at 3× MIC; ■, ofloxacin at 128× MIC; △, ofloxacin at 3× MIC plus magnesium; □, ofloxacin at 128× MIC plus magnesium.

(13, 20). After ofloxacin and pefloxacin exposure, the disaccharide tetrapeptides (38 and 38.1%, respectively) and the disaccharide pentapeptides (0.26 and 0.30%, respectively) were increased, compared to those values in the control (tetrapeptides, 28.1%; pentapeptides, 0.18%), suggesting that the carboxypeptidase activities may also be reduced.

The degrees of total cross-linkage for samples exposed to ofloxacin or pefloxacin (26.1 and 26.4%, respectively) were similar to that of the control (26.05%). However, the DAP-DAP cross-linkage was twice that of the control, which is typical of cells growing under stress situations (11). This may explain the apparent decrease of the monomer tripeptide which would be engaged in the DAP-DAP cross-linkage. The amounts of lipoprotein-attached muropeptides were decreased (3.35 and 2.96%) compared to that of the control (4.1%). These amounts correspond to 1 residue of lipoprotein per 30 or 34 disaccharide peptides as compared to 1 residue per 24 disaccharide peptides for the control.

Table 1 also presents the peptidoglycan composition after a 3-h exposure to the low concentration (3× MIC) of ofloxacin or pefloxacin. In contrast to the results with the high concentration, the values for anhydro compounds, compared to that of the control (5.8%), were increased (10.3 and 7.8% for ofloxacin and pefloxacin, respectively), corresponding to an average glycan chain length of 17.3 (control), 9.7 (ofloxacin), and 12.7 (pefloxacin) disaccharide peptides. The increase of anhydro compounds could be explained by the increased activity of the hydrolytic transglycosylases. The amounts of the disaccharide dipeptides and tripeptides were reduced (for ofloxacin, 1.3 and 3.4%, respectively; for pefloxacin, 1.3 and 4%, respectively) compared to those of the control (2 and 10.7%, respectively), suggesting that the overall carboxypeptidase activity was less, similar to what was observed at high concentrations of the quinolones. No change in the degree of cross-linkage was observed, but again the DAP-DAP cross-linkage was significantly increased compared to that of the control. As described after penicillin exposure (15), lipoprotein-attached muropeptides were increased, suggesting that they were spared from the autolytic degradation.

DISCUSSION

The quinolones are bactericidal antibiotics for which the mechanism of action is supposed to be related to the inhibition of DNA supercoiling (7, 9, 23, 24, 27) and the activation of the SOS response (5, 18, 22, 25). However, the target site(s) and the mechanism of the bactericidal effect are still unclear. Protein synthesis may be required for killing by quinolones (3). As discussed by Wolfson et al. (26), neither DNA degradation nor inhibition of DNA synthesis alone are sufficient to explain killing. They suggest other associated mechanisms, which could involve cell division genes or autolysis genes via the SOS response after DNA damage. Data presented here represent an examination of different effects induced by two extreme concentrations of ofloxacin or pefloxacin on *E. coli* W7 and more specifically on its cell wall. As described for ciprofloxacin and norfloxacin (8), low concentrations of these antibiotics induce filamentation, followed by a lytic event. At high concentrations, quinolones totally inhibit bacterial growth and no lysis occurs. Exposure to 128× MIC of ofloxacin or pefloxacin involves complete inhibition of protein synthesis, while peptidoglycan synthesis is continuous and no degradation of this structure occurs. This situation is reminiscent of what was shown with inhibitors of protein synthesis (17, 19) and could be associated with a thickening of the peptidoglycan. The composition of residual peptidoglycan after exposure to 128× MIC of the two drugs was profoundly different from that of untreated cells, reflecting an apparent reduced activity of murein hydrolases. This analysis showed an increase of the average glycan chain length, which could be due to reduced lytic transglycosylase activity (13, 20). In comparison, after exposure to 3× MIC of quinolones, a phase of filamentation,

TABLE 1. Composition of the peptidoglycan of *E. coli* W7 after exposure to ofloxacin or perfloxacin

Drug group[a]	Molar fraction (%) in muropeptides										Glycan chain length (disaccharide peptides)	Cross-linkage (%)		
	Monomers[b]					Dimers	Trimers	Tetramers	Anhydromuropeptides	Lipoprotein muropeptides		Total	Ala-DAP	DAP-DAP
	Total	Dipeptides	Tripeptides	Tetrapeptides	Pentapeptides									
Control	49.80	2.05	10.70	28.10	0.18	44.80	5.00	0.4	5.80	4.10	17.20	26.05	22.51	3.54
OFLO 128	49.30	1.18	2.50	38.00	0.26	45.96	4.37	0.37	3.53	3.35	28.33	26.15	19.48	6.67
PEFLO 128	48.25	1.13	2.65	38.18	0.30	48.73	2.70	0.32	4.12	2.96	24.27	26.40	19.01	7.39
OFLO 3	50.21	1.27	3.38	35.00	0.20	45.52	4.27	0	10.32	5.75	9.70	25.60	20.31	5.29
PEFLO 3	46.31	1.36	3.96	31.73	ND[c]	46.50	6.79	0.4	7.85	6.50	12.74	28.08	21.72	6.36

[a] OFLO 128 and PEFLO 128, exposure to 128× MIC of ofloxacin and pefloxacin, respectively. OFLO 3 and PEFLO 3, exposure to 3× MIC of ofloxacin and pefloxacin, respectively.
[b] All peptides refer to peptide side chains.
[c] ND, not determined.

followed by a lytic effect, occurred which was completely abolished after the addition of chloramphenicol. The peptidoglycan and the protein synthesis were active for 90 min, after which the peptidoglycan was degraded. The HPLC analysis showed a decrease in the average glycan chain length, which may reflect increased transglycosylase activity. However, this effect may have been exaggerated by the late time point at which samples were taken. Given the dynamic state of the sacculus, i.e., it being the product of an equilibrium of synthetic and degrading activities, one cannot exclude that what we have assessed as an increased degrading activity may be due to a reduced activity of the synthetic machinery and vice versa.

In the present study, there was some evidence that exposure to low concentrations of quinolones leads to alterations of the peptidoglycan composition which are similar to those observed after exposure to β-lactam antibiotics (1, 2, 15). This suggests the possibility of a common pathway for killing by β-lactams and quinolones that involves cell division. This is also suggested by the difference between the bactericidal effect observed at a high concentration (no lysis) and that at a low concentration (lysis) of quinolones. Moreover, the isolation of *E. coli hipA* mutants, which are partially tolerant to ampicillin and which also exhibit a partial tolerance to nalidixic acid (21), norfloxacin, and ofloxacin (26), would reinforce this hypothesis. Finally, if the mechanism of bacterial killing by quinolones is still unclear, our results would suggest that lysis induced by low drug concentrations and peptidoglycan alterations may contribute to their bactericidal effect.

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