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Concentration of CCCP Should Be Optimized to Detect the Efflux System in Quinolone-Susceptible *Escherichia coli*

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Unlike eukaryotic efflux pumps energized by ATPase, bacterial efflux pumps are energized by the proton motive force. That is the reason why CCCP, an inhibitor of proton motive force, is widely used to study the bacterial efflux pump. In many cases, efflux systems have been observed only in quinolone-resistant bacteria. Most of the quinolone-susceptible strains have been found to maintain little efflux pump. However, some susceptible bacteria showed the increased intracellular quinolone concentration only at a low concentration (0.01 or 0.1 mM) but not at a high concentration (1 mM) of CCCP. If bacterial cells were killed at high concentrations of CCCP and lost the integrity of their membranes, the intracellular quinolone would leak out from cells with no efflux system. The efflux pump system in the quinolone-susceptible strains could not be detected at the same concentration used for resistant bacteria. To test this hypothesis, the intracellular quinolone concentration in the quinolone-susceptible and -resistant strains of *Escherichia coli, Pseudomonas aeruginosa,* and *Staphylococcus aureus* was assayed at various concentrations of CCCP. Since the effect of CCCP is very rapid, the survival of bacteria was observed by assaying the DNA synthesis in 5 min. In the case of *E. coli*, but not *P. aeruginosa* or *S. aureus*, the quinolone susceptible strain was more susceptible to CCCP than the quinolone resistant ones, especially when the incubation with CCCP was extended. Decrease of the intracellular quinolone concentration resulted in a false result-no or weak efflux system in the quinolone susceptible strains. Results suggested that the concentration of CCCP should be optimized in order to detect the efflux system in the quinolone susceptible strains of *E. coli*.

Key words: antibiotic resistance, CCCP, efflux, *E. coli*, quinolone

Antibiotic resistant bacteria have become a worldwide problem and their number is increasing as more antibiotics are used. Among various antibiotics, quinolone has been discovered and widely used more recently compared to other antibiotics (2, 8). Up until now, genes responsible for the quinolone resistance have been found on the main chromosome and this made people assume that quinolone resistant bacteria will not be easily found (3, 7, 9). In contrast, the number of quinolone resistant bacteria is increasing rapidly in recent years. Quinolone resistance is ascribed to three mechanisms: target site mutation in DNA gyrase and topoisomerase IV (5, 10, 23, 25, 26, 28); decreased permeability (1, 14, 15, 16, 22, 24, 28); and the existence of the efflux pump system (4, 6, 11, 12, 17, 18, 19, 21). Since the bacterial efflux pump is energized by the proton motive force (13, 18, 27), CCCP which dissipates the proton gradient has been widely used to detect the efflux pump. In most cases, 0.1 mM or 1 mM CCCP is added to bacterial cells to inhibit the efflux pump and the difference in the intracellular quinolone concentration before and after the addition of CCCP is considered as evidence for the existence of the efflux pump system. Many people found the active efflux system in the quinolone resistant bacteria and very weakly active efflux pump in the quinolone-susceptible bacteria.

When we added various concentrations of CCCP to bacterial cells, we found active efflux pumps in the quinolone susceptible bacteria at lower concentrations of CCCP (less than 0.1 mM). Interestingly, the activity of the efflux pump was higher at the low concentration than that at the high concentration. We hypothesized that CCCP must have a harmful effect on cells because it dissipates the proton gradient (20) and the quinolone susceptible bacterial cells are more easily damaged by CCCP than the quinolone-resistant bacteria and that results in leakage the of intracellular materials; there fore thereby intracellular quinolone concentration is detected less.

To test this hypothesis, we assayed the inhibitory activity of CCCP on bacterial cells by measuring the growth curve. Since the uptake assay is performed in a very short time period-less than 5~10 min, its inhibitory effect on the cells was assayed by measuring the DNA synthesis using

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radioactive thymidine. We determined the dose effect of CCCP on the intracellular quinolone concentration using both quinlone-resistant and susceptible *Escherichia coli, Pesudomonas aeruginosa*, and *Staphylococcus aureus*.

Materials and Methods

Bacterial cells

The quinolone-susceptible and resistant isolates (MICs to ofloxacin >128 μg/ml) of *E. coli, P. aeruginosa, and S. aureus* were from the Culture Collection of Antibiotic Resistant Microbes (CCARM). Bacterial cells at the log phase in Brain Heart Infusion (BHI) were collected by centrifugation, dispersed in new BHI making A_{600} and used for the experiments.

Assay of the DNA synthesis

[^{3}H] Thymidine (0.01 μCi) was added to 400 μl cells in log phase and cells were incubated at 37°C with shaking. After 5 min, 250 μl of the reaction mixture was mixed with the same volume of cold 40% TCA and out in ice to stop the reaction. The acid insoluble precipitate was collected on a filter (GF/C, Whatman, U.S.A.) using a vacuum manifold (Bio-Rad, U.S.A.) and washed with 5 ml each of 5% TCA, 0.1 N HCl, and 95% ethanol. Filters were soaked in 0.05% thymidine to prevent unspecific binding before use. The radioactivity in the acid insoluble precipitate on the filter was counted in a liquid scintillation counter (PACKARD TRI-BARB 4530, U.S.A.) after adding the scintillation cocktail (PACKARD, U.S.A.) to the air-dried filters.

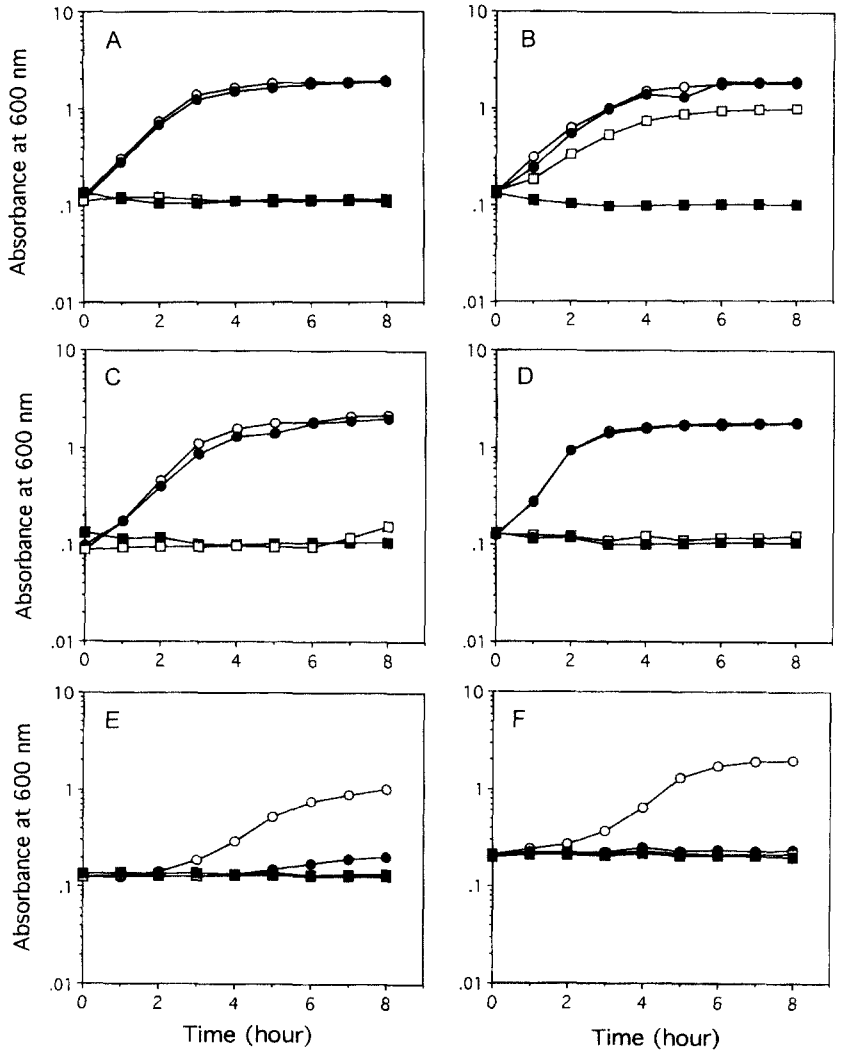

Fig. 1. Growth curves in the presence of CCCP. The quinolone-resistant and -susceptible *E. coli, P. aeruginosa*, and *S. aureus* were grown in the presence of CCCP. A, the quinolone-susceptible *E. coli*; B, the quinolone-resistant *E. coli*; C, the quinolone-susceptible *P. aeruginosa*; D, the quinolone-resistant *P. aeruginosa*; E, the quinolone-susceptible *S. aureus*; F, the quinolone-resistant *S. aureus*; ○, 0 mM CCCP; ●, 0.01 mM CCCP; □, 0.1 mM CCCP; ■, 1 mM CCCP.

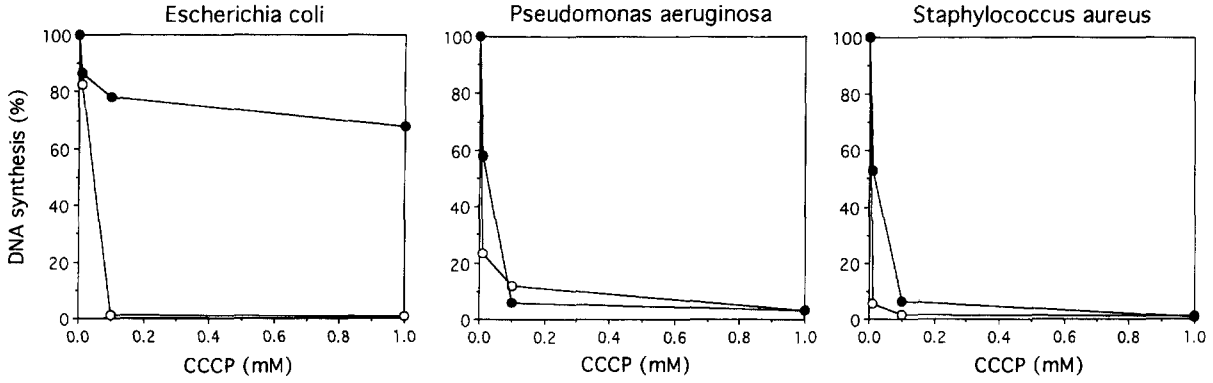

Fig. 2. DNA synthesis in the presence of CCCP. The quinolone-resistant and -susceptible *E. coli, P. aeruginosa, S. aureus* were grown in the presence of 1 mM CCCP and radioactive [^{3}H] thymidine was added to the cells. After 5 min incubation, the radioactivity in the acid insoluble precipitate was measured in a scintillation counter. (○ , the quinolone-susceptible strain; ● , the quinolone-resistant strain)

Assay of the intracellular ofloxacin concentration

The intracellular ofloxacin concentration was measured following a procedure described in a previous paper (14). Bacterial cells at the log phase were prepared as described above. Ofloxacin (final concentration, 50 μg/ml) was added to the cells and cells were incubated at 37°C. At various incubation times, cells were layered on the top of 1 ml of cold silicon oil and collected with centrifugation to remove the extracellularly bound ofloxacin. The lower part of the tube containing the bacterial cell pellet was cut with a tube cutter and transferred to a new tube containing 1 ml of millipore-filtered water. The intracellular ofloxacin was extracted from cells by boiling in water for 10 min and cells were removed by centrifugation. The concentration in the supernatant was assayed in a fluorospectrophotometer (Hitachi, Japan).

Results and Discussion

Inhibitory effect of CCCP on the bacterial growth

When CCCP was added to the quinolone-resistant and susceptible strains of *E. coli, P. aeruginosa, and S. aureus*, the growth rate of every strain increased as the concentration CCCP decreased. However, bacterial cells belonging to each genus have different susceptibilities to CCCP (Fig. 1). The difference in the inhibitory activity of CCCP on the quinolone-susceptible and resistant cells were the largest in the case of *E. coli*. The growth of the quinolon-susceptible cells was completely inhibited by 0.01 mM CCCP but the growth of the quinolone-resistant cells was not. In the case of *P. aeruginosa*, the growth of both quinolone-resistant and susceptible cells was completely inhibited by 0.1 mM CCCP. In the case of *S. aureus*, 0.01 mM CCCP could inhibit the growth of both quinolone-resistant and -susceptible strains. Conventionally, either 0.1 mM or 1 mM CCCP with incubation time less than 10 min has been used to detect the bacterial efflux system. It is possible that this is the reason why people observed the higher efflux activity in the resistant strain but not in the susceptible strain since the bacterial cells died at this CCCP concentration (4, 14, 19).

Assay of the DNA synthesis

To detect the DNA synthesis in a short time period, radioactive thymidine was added to bacterial cells and the resulting radioactive DNA synthesized in 5 min was assayed (Fig. 2). DNA synthesis was almost completely inhibited by the addition of 0.01 mM CCCP in all of the quinolone-susceptible bacterial cells- whether it was *E. coli, P. aeruginosa,* or *S. aureus* except the quinolone-resistant *E. coli*. In the case of quinolone-resistant *E. coli*, it synthesized 70% of the DNA even in the presence of 1 mM CCCP. These results coincided with the results in shown Fig. 1., which show that the quinolone-resistant *E. coli* could grow in the presence of 1 mM CCCP. The results showed that CCCP has an inhibitory effect on the bacterial growth in a very short time period. Thus, we concluded that high concentration of CCCP could give false results since the bacterial cells would die at this concentration resulting in the leakage of the intracellular materials.

Intracelluar ofloxacin concentration in the presence of CCCP

All of the quinolone-resistant strains showed higher efflux activity compared to the quinolone-susceptible strains (Fig. 3). The intracellular ofloxacin concentration in the quinolone-susceptible *E. coli* increased the most in the presence of 0.01 mM CCCP. The intracellular ofloxacin concentration decreased as the incubation time increased as we expected from Fig. 1 and Fig. 2. These results showed that the dead bacterial cells lose the integrity of cell membrane and the resulting leakage of the intracellular ofloxacin. Especially in the case of quinolone-susceptible *E. coli*, the intracellular concentration decreased

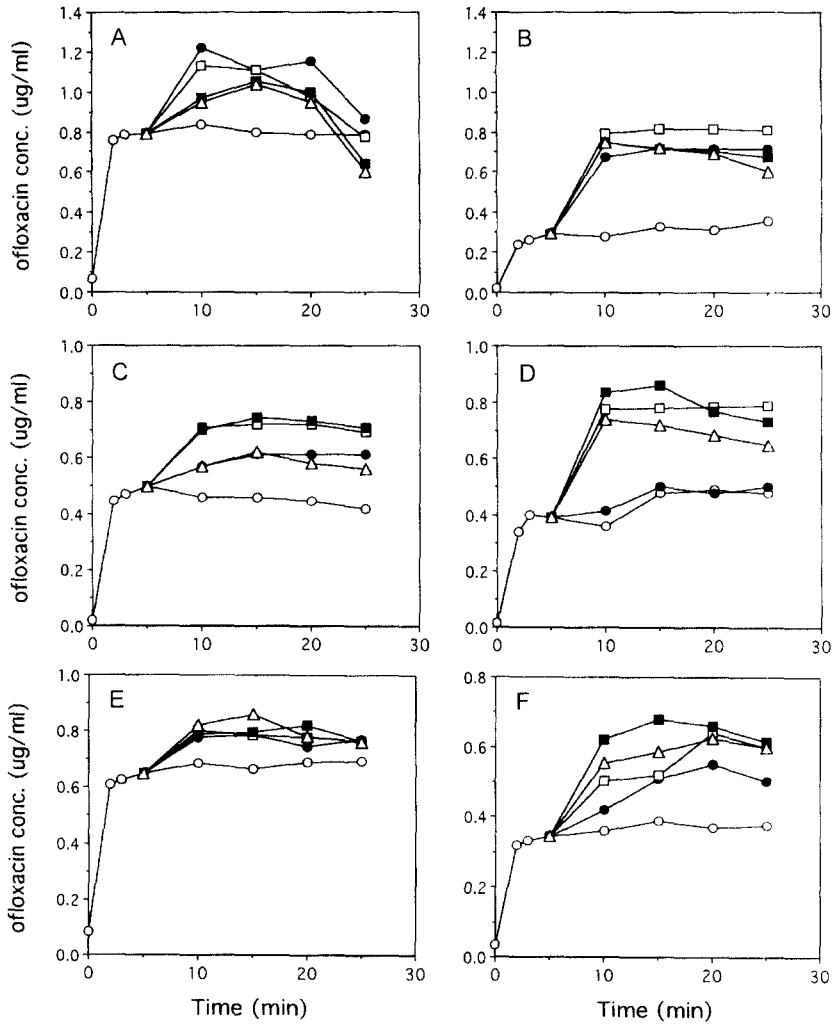

Fig. 3. Intracellular ofloxacin concentration in the presence of CCCP. Bacterial cells of the quinolone-resistant and -susceptible strains were incubated with 50 μg/ml ofloxacin in the presence of CCCP. At various time intervals, the intracellular ofloxacin concentration was assayed in a fluorescence spectrophotometer. A, the quinolone-susceptible *E. coli*; B, the quinolone-resistant *E. coli*; C, the quinolone-susceptible *P. aeruginosa*; D, the quinolone-resistant *P. aeruginosa*; E: the quinolone-susceptible *S. aureus*; F, the quinolone-resistant *S. aureus*; ○, 0 mM CCCP; ●, 0.01 mM CCCP; □, 0.1 mM CCCP; ■, 1 mM CCCP; △, 2 mM CCCP.

as the incubation time increased. The intracellular ofloxacin concentration in the quinolone-susceptible *E. coli* was the highest in the presence of 0.01 mM CCCP while that in the quinolone-resistant *E. coli* was at the 0.1 mM. Those in the quinolone-resistant and susceptible *P. aeruginosa* and *S. aureus* were the highest in the presence of 1 mM CCCP.

All the results presented above suggest clearly that CCCP must be optimized to detect and assay the efflux system in the quinolone-susceptible strain, especially with *E. coli*.

Acknowledgments

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