INHIBITION OF R-FACTOR TRANSFER AND PHAGE INFECTION BY REQUINOMYCIN

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To be effective in inhibiting R-factor transfer (between *Escherichia coli* strains) and infection of *E. coli* with phage f 2, requinomycin must be present at the time of initiation of both events. Delayed addition of requinomycin showed no or slight inhibition. No direct effect of requinomycin on phage f2 or *E. coli* strains employed was observed. The intact cell surface of *E. coli* must be impermeable to requinomycin as shown by the fact that DNA synthesis by toluene-treated *E. coli* cells (permeability modified) was markedly inhibited by requinomycin, in contrast to its ineffectiveness on intact *E. coli* cells. Therefore, requinomycin probably acts without penetrating the cells. Probable mechanism of action is discussed.

R-Factors which determine resistance to a wide range of antibiotics are transmitted between enteric bacteria by conjugation[1,2]. In a preceding paper we reported that a new anthracycline antibiotic requinomycin blocked R-factor transfer at concentrations which did not inhibit the growth of *E. coli*[3]. Since requinomycin had originally been screened for anti-phage activity, we became interested in learning if there is some biological process (es) common to both R-factor transfer and phage infection. As we shall report in the present paper, it seems that requinomycin exerts these effects by binding reversibly to some component (s) of the cell surface.

Materials and Methods

Anti-phage activity: The time sensitivity to requinomycin was determined as follows: *E. coli* S-26 was grown in Polypeptone broth at 37°C under shaking until an O.D. of 0.18 at 600 mμ was reached. From the cell suspension, 1.7 ml portions were distributed into small test tubes which were placed in a 37°C bath at minute "zero" and incubated under shaking. At minute 5, 10, 15, 20 or 30, one tube for each run received 0.2 ml of 200 μg/ml of requinomycin solution (or 0.85 % NaCl solution for a control run), while at minute 10, each tube received 0.1 ml suspension of phage f 2 (10^8 pfu*). Incubation was terminated at minute 110 by chilling and the mixtures were serially diluted with 0.85 % NaCl - 2 mM $CaCl_2$. One-tenth ml portions of the diluted suspensions were titrated for pfu using *E.coli* S-26 as an indicator strain by the pour plate method.

Possible inactivation of phage f 2 by requinomycin was examined as follows: Phage

* plaque forming unit

f 2 (2×10^9 pfu) were suspended in 2 ml of 0.85 % NaCl - 2 mM $CaCl_2$ and incubated at 37°C for 60 minutes under shaking in the presence or absence of 100 μg/ml of requinomycin. After chilling, each mixture was serially diluted and 0.1 ml portions were titrated for pfu.

Inhibition of R-factor trasfer: *E.coli* K-12 (T^-) R 1629[4] as a donor strain and *E.coli* K-12 as a recipient strain were grown separately in nutrient broth at 37°C under shaking. When the cultures reached an O.D. of 0.2 at 600 mμ, 0.9 ml portions withdrawn from each culture were mixed in Erlenmeyer flasks of 30 ml volume (the time of mixing will be referred to as "minute 0") and incubation was initiated under gentle shaking at 37°C. At minute 0, 30 or 60, an appropriate flask received 0.2 ml of 500 μg/ml of requinomycin solution (or 0.85 % NaCl solution for a control run) and incubation was continued through minute 90. After chilling, the mixtures were serially diluted with 0.85 % NaCl solution and 0.1 ml portions were submitted to counting total viable cells and R^+ recipient cells (recipient cells which acquired drug resistance by conjugation) on plates of nutrient agar and on plates of minimal agar (see below) supplemented with 70 μg/ml of kanamycin sulfate, respectively. Minimal agar consisted of K_2HPO_4 1.05 %, KH_2PO_4 0.45 %, $MgSO_4\cdot7H_2O$ 0.005 %, $(NH_4)_2SO_4$ 0.1 %, Na-citrate 0.045 %, glucose 0.2 %, agar 1.5 % (bottom layer) and 0.6 % (overlayer), and water.

Elimination of R-factor: An assay mixture contained, in 5 ml of nutrient broth, 4×10^8 cells of *E. coli* K-12 ML 1630[4] and 500 μg of requinomycin (no requinomycin in a control run). After incubation at 37°C for 15 hours, each mixture was 10-fold serially diluted and 0.1 ml portions were submitted to counting total viable cells and R^+ cells as described above.

DNA synthesis by toluenized *E. coli* cells: Experiments were conducted according to Moses and Richardson[5]. *E. coli* S-26 were grown in CG-medium** at 37°C under shaking until a density of 7×10^8 cells/ml was reached. Cells were collected by centrifugation, suspended in $^1/_{10}$ of the culture volume of cold 0.05 M potassium phosphate buffer at pH 7.4, containing toluene at 1 % (v/v), and incubated at 37°C for 10 minutes under shaking. The cells were collected by centrifugation in a refrigerated centrifuge, washed with 0.05 M potassium phosphate buffer by suspension and centrifugation, suspended in $^1/_{10}$ of the culture volume of 0.05 M potassium phosphate buffer and stored at −60°C until used. A reaction mixture (0.3 ml) consisted of 70 mM potassium phosphate (for a final pH 7.4), 13 mM $MgCl_2$, 1.3 mM ATP, 33 μM each 3 dXTP's, 33 μM ^{3}H-TTP (0.1 μCi), 1.5×10^8 of toluene-treated cells, and a desired amount of requinomycin. The mixtures were incubated at 37°C for 30 minutes and the reaction was terminated by adding 2 ml of ice-cold 10 % trichloroacetic acid - 0.1 M pyrophosphoric acid solution (TCA-PPi). Insoluble materials of each mixture were then filtered on a Whatman GF/C disk (2.4 cm diameter) and washed on the filter 3 times with ice-cold TCA-PPi. The paper disks were dried, placed in counting vials with scintillation solution (toluene-PPO system), and counted in a Beckman liquid scintillation counter.

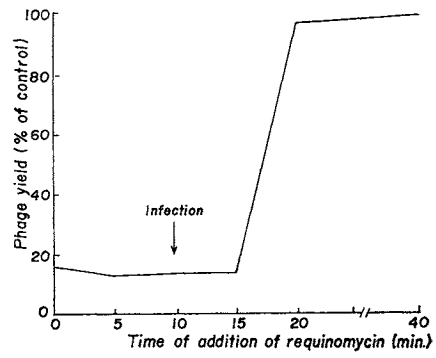
Results

Anti-phage Activity

Time Sensitivity to Requinomycin

Requinomycin was added to the host-cell suspension before, at or after the time of infection of phage f 2. As shown in Fig. 1, the latest effective time of addition of requinomycin was found to be 5 minutes after infection. Later additions showed no protection. Thus requino-

Fig. 1. Time sensitivity to requinomycin in the course of phage f2 infection



** Casein hydrolysate and glucose as constituents.

mycin possibly inactivates phage particles or inhibits some early process of phage infection such as adsorption to pili or introduction of phage RNA into host cells through the pili. Requinomycin does not inhibit later processes involved in phage production. As we have reported in a preceding paper, if requinomycin was added 10 minutes before infection at concentrations of 100 μg/ml, 10 μg/ml and 1 μg/ml, it inhibited phage production by 95 %, 85 % and 38 %, respectively, under similar conditions. It has been confirmed by a separate experiment that requinomycin does not inhibit the growth of the host cells at concentrations as high as 100 μg/ml.

Table 1. Direct effect of requinomycin on free phage f2

Concentration of requinomycin (μg/ml)	Number of plaques
0 (control)	1.10×10^9
100	1.16×10^9

Does Requinomycin Inactivate Phage Particles?

Even after a prolonged incubation of a phage suspension in the presence of 100 μg/ml of requinomycin, the phage titer of the solution, assayed after dilutions, was found unchanged as shown in Table 1. This result ruled out the possibility that requinomycin inactivates phage particles. Therefore, if requinomycin has any direct effect on phage particles, the effect should be of reversible nature since it can be abolished upon dilution.

Inhibition of R-Factor Transfer

Time Sensitivity to Requinomycin

In the preceding paper[3] we reported that requinomycin inhibited R-factor transfer by 92 %, 58 % and 19 % at concentrations of 50 μg/ml, 25 μg/ml and 5 μg/ml, respectively, if the antibiotic was present at the time of mixing of donor and recipient cells. Under these conditions, requinomycin did not affect the viability of either donor or recipient cells. In the present experiment, the time of addition of requinomycin was varied during the course of R-factor transfer. As shown in Fig. 2, requinomycin was very effective only when it was present throughout the incubation period. Later additions of requinomycin showed lesser inhibition. These results suggested that requinomycin inhibits the transfer process *per se* but has no effect on

Fig. 2. Time sensitivity to requinomycin in the course of R-factor transfer

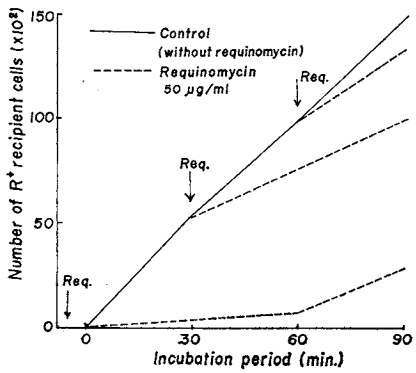


Table 2. Examination for curing of R-factors by requinomycin

	Total cells	Cells resistant to 50 μg/ml of kanamycin sulfate
Control	8.7×10^8 (cells/ml)	8.9×10^8
+Requinomycin (100 μg/ml)	4.1×10^8	3.6×10^8

Runs were triplicated and averaged.

The experiment was conducted as given under Materials and Methods. Under these conditions, 100 μg/ml of requinomycin appeared somewhat growth-inhibitory to this strain of *E. coli*; total cells in +requinomycin run were fewer than those in control run (4.1×10^8 *vs.* 8.7×10^8). This is in contrast to other observations that requinomycin showed no effect on the doubling time of any *E. coli* strain (based on turbidity measurement or cell counting) at a concentration as high as 100 μg/ml. This inconsistency may be due to difference either in growth conditions or the period of drug treatment. However, the conclusion drawn from this experiment is not affected by this inconsistency because most cells (3.6×10^8 in 4.1×10^8) retained resistance to kanamycin after the treatment with requinomycin.

cells which acquired drug-resistance. This point was made clearer by another experiment which examined possible elimination of R-factors (curing) by requinomycin. As shown in Table 2, requinomycin slightly converted the resistant cells to antibiotic sensitivity even after a prolonged treatment, indicating that requinomycin has no curing effect. In this respect, the mode of action of requinomycin is different from that of acriflavin[2], an intercalating dye. IYOBE *et al.* reported[6] that macarbomycin, an inhibitor of cell wall synthesis, preferentially inhibited the growth of drug-resistant cells which were derepressed for sex-pili formation. Requinomycin does not have this effect, either.

Table 3. Effect of requinomycin on DNA synthesis in toluene-treated *E. coli* cells

System	^{3}H-TMP Incorporated into the acid-insoluble fraction
Complete	100 % (995.4 cpm)
−ATP	23.5
−3dXTP	6.3
+Requinomycin, 100 μg/ml	7.9

Impermeability of *E. coli* Cell Surface to Requinomycin

Inhibition by Requinomycin of DNA Synthesis in Toluenized *E. coli* Cells

Considering the ineffectiveness of requinomycin to any *E. coli* strain used for the present study, the simplest explanation for the requinomycin action would be that requinomycin binds to the cell surface in a specific manner to modify the function of sex-pilus or its receptor but not to interfere with membrane transport of essential metabolites. At present, exact nature of the target molecule to which requinomycin binds is not known. However, it must localize on the cell surface because of the following observation. As shown in Table 3, requinomycin markedly inhibits DNA synthesis in toluenized *E. coli* cells in contrast to its ineffectiveness to intact cells. Interaction with DNA template could be characteristic to anthracycline antibiotics[7~9]. This result strongly suggests that the intact cell surface of *E. coli* is impermeable to requinomycin.

Discussion

Sex-pili are essential structures for both adsorption of phage f2 and conjugation[10], and therefore, sex-pili or their receptors are suspected to be the binding sites of requinomycin. However, it has been reported recently that levallorphan, a structural analogue of morphine, also inhibited both phage infection and R-factor transfer[11,12] and the latter action was thought to be due to inhibition of mating pair formation. Requinomycin may work in an analogous way. Considering dissimilarity in structure, it is unlikely that requinomycin and levallorphan can share the same target molecule of the cell surface. Accordingly, these effects could be a common result of various changes in the conformation of the cell surface.

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