ON THE MODE OF ACTION OF ASK-753, A NEW IRON-CONTAINING ANTIBIOTIC

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The effect of antibiotic ASK-753 on cell morphology DNA, RNA and protein syntheses in *E. coli*, *B. subtilis* and *B. mycoides* was studied. In the presence of the antibiotic, gram-positive bacteria formed long filaments, while gram-negative bacteria formed short filaments. It was found that this antibiotic causes inhibition of DNA, RNA and protein syntheses. This inhibition was reversible in *E. coli*, while it is irreversible in *B. subtilis* and *B. mycoides*. By programming the addition and removal of the antibiotic, DNA and RNA syntheses can be made to proceed in stepwise increments corresponding to doublings of the DNA. ASK-753 causes the release of 260 mμ absorbing materials from the ASK-753 sensitive strains of *B. subtilis*. It also lyses protoplasts of *B. subtilis* but not spheroplasts of *E. coli*. On the basis of this study, it was concluded that this antibiotic acts primarily on the cellular membrane and consequently leakage of intracellular material occurs.

ASK-753[1] is a new iron-containing antibiotic, active against gram-positive and some gram-negative bacteria. The antibiotic was found to induce various morphological changes in microorganisms, such as elongation in *Bacillus subtilis*, *Bacillus mycoides*, and a slight elongation in *Escherichia coli*. These morphological changes have prompted us to study not only the effects of this antibiotic on DNA, RNA and protein syntheses in *E. coli* (gram-negative) and *B. subtilis* and *B. mycoides* (gram-positive), but also its action on protoplasts of *B. subtilis* and spheroplasts of *E. coli* B.

Material and Methods

Bacteria. The bacteria employed were: *Escherichia coli* NRRL B-210, *Bacillus subtilis* (ice strain) and *Bacillus mycoides* (USSR).

Culture media. Media contained 2.0 g beef extract, 2.0 g yeast extract, 5.0 g peptone, 5.0 g NaCl per 1 liter distilled water. This medium was used for the cultivation of bacteria. The medium for agar plates was prepared from the liquid medium by the addition of 15.0 g of agar to 1 liter of the medium. All incubations were carried out on a rotary shaker at optimal temperature for bacterial growth.

Staining of bacteria. The smears were stained by Giemsa stain.

Viable-cell count *vs.* concentration of ASK-753. An over-night culture of *E. coli* was re-inoculated into fresh medium in a series of culture flasks, prepared by the addition of graded amounts of ASK-753, and incubated for a period of 6 hours. Samples for viable cell counts were removed from all flasks after every 2 hours incubation. The viable cell counts were determined by dilution of the cultures in sterile media at room temperature and by spreading 0.1 ml samples on the surface of nutrient agar plates. The colonies which developed were counted after 24 hours of incubation at 37°C. Results are presented in Fig. 1A.

Effects of ASK-753 on the synthesis of macromolecules in *E. coli* B, *B. subtilis* and *B. mycoides*. Samples (15 ml) of culture of *E. coli* B or *B. subtilis* or *B. mycoides* treated with

different concentrations of the antibiotic were chilled and washed with cold distilled water, and the washed cells were extracted with 5 ml of 0.25 N perchloric acid at 0°C for 30 minutes. After centrifugation, the pellet was extracted with 3 ml of 0.5 N perchloric acid at 70°C for 15 minutes. The extracts were used to assay for DNA by the diphenylamine method (BURTON's procedure)[2], calf thymus DNA being used as a standard, and for RNA by the orcinol reaction (MEJBAUM)[3], yeast RNA being used as a standard. The pellet in each centrifuge tube after extraction of DNA and RNA with perchloric acid, was dissolved in 2 ml 1 N sodium hydroxide at 90°C for 30 minutes, and protein was measured by the BIURET method[4]. The results are recorded in Figs. 1, 2 and 3.

Reversal of the inhibitory action of ASK-753. The reversibility of inhibition of DNA, RNA and protein syntheses caused by minimum inhibitory concentration of ASK-753 was determined for *E. coli*, *B. subtilis* and *B. mycoides*. Two flasks were prepared for each exercise by the same procedure as that used above for a determination of viable cell count *vs.* drug concentration. One flask contained no antibiotic, and the other contained the MIC of ASK-753 for the bacteria tested. Incubation was as before but at the end of the third hour of incubation, the antibiotic was removed from the drug-treated culture by centrifugation and washing with liquid medium. The cells from this flask were resuspended in the original volume of medium and incubation was resumed, samples were taken for DNA, RNA and protein assay at the end of 1 hour and 2 hours of incubation. Table 1 summarizes the results.

Control of DNA replication by ASK-753. The finding that DNA synthesis could be completely inhibited by non-toxic concentrations of ASK-753 suggested its use as an agent to control DNA synthesis. By programming the addition and removal of ASK-753, it was found that DNA replication proceeded in stepwise increments (Table 2). *E. coli* cells were exposed to 40 μg/ml of the antibiotic for 2 hours. The inhibitor was removed and after 20 minutes, 40 μg per ml was added, and incubation continued for 2 hours. The inhibition was again relaxed for 40 minutes, and the antibiotic was added again. A third cycle was repeated in a similar manner. Table 2 summarizes the results, and shows the effect of programming the addition and removal of ASK-753 on DNA and RNA syntheses.

Assay for the release of intracellular ultraviolet absorbing materials, protoplasts or spheroplast-lysing activities. The methods described by ITO and KOYAMA were used[5].

Results

Microscopic examination of bacterial cells treated with different concentrations of the antibiotic ASK-753 revealed filament formation in *E. coli* B, *B. subtilis* and *B. mycoides*. The filaments did not appear to be chains of bacteria as indicated in Plates 1, 2 and 3 respectively.

In nutrient media at concentrations of 10 and 20 μg per ml, the antibiotic had a slight effect on *E. coli* growth and viable cell count; however concentrations of 40 and 50 μg per ml completely inhibit multiplication of this bacterium (Fig. 1A).

In *B. subtilis* and *B. mycoides* turbidity of the cultures was used as a criterion of growth, we also found that the antibiotic was progressively more inhibitory as its concentration was increased. This was reflected by the decreased rate of growth (Figs. 2A and 3A).

Because disturbances in the rates of synthesis of macromolecules resulted in the development of filamentous bacterial cells[6,7], the effect of ASK-753 on DNA, RNA and protein synthesis was investigated.

Upon addition of different concentrations of the antibiotic to exponentially growing cultures of *E. coli*, there was no net synthesis of DNA, RNA and protein at higher concentrations, and after 6 hours, the amount of DNA had not doubled. On the other hand, at lower con-

Plate 1. *E. coli* cells treated with 40 μg/ml of ASK-753 antibiotic for 4 hours

Control Treated

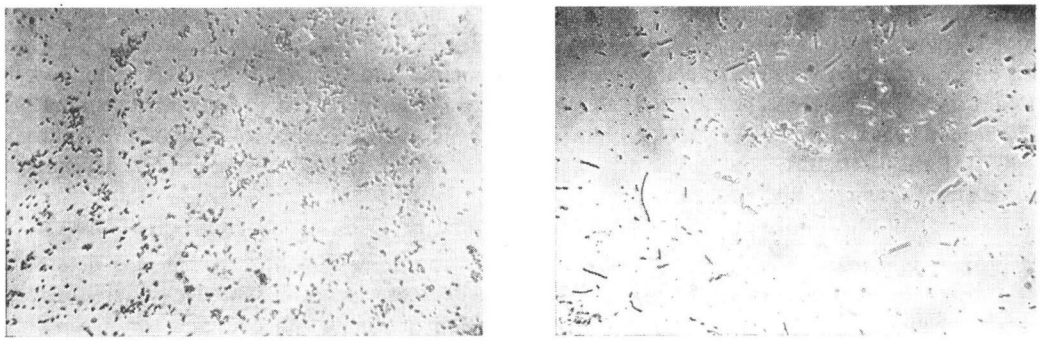


Plate 2. *B. subtilis* cells treated with 1.5 μg/ml of ASK-753 antibiotic for 4 hours

Control Treated

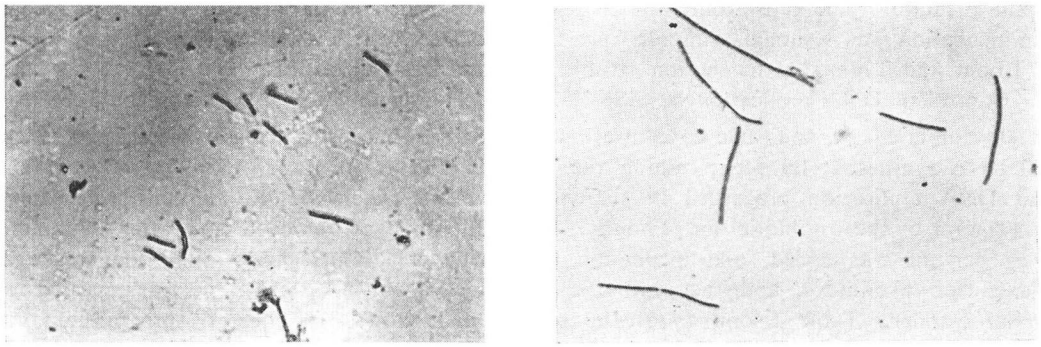
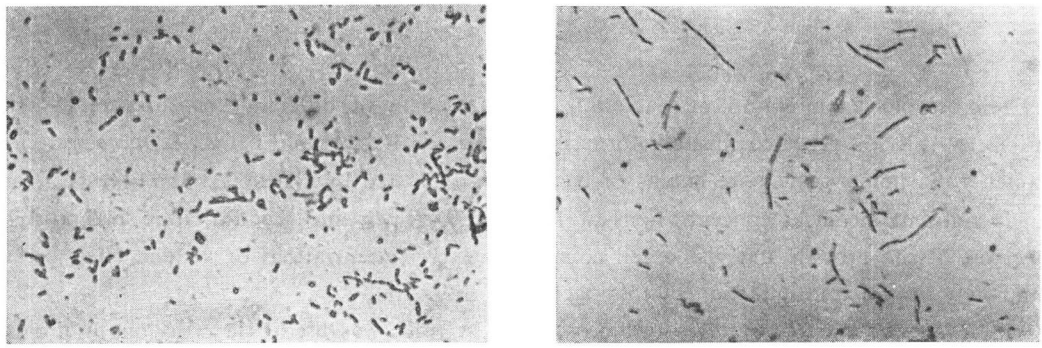


Plate 3. *B. mycoides* cells treated with 3.12 μg/ml of ASK-753 antibiotic for 4 hours

Control Treated



centrations of the antibiotic net syntheses of DNA, RNA and protein increased slightly but not at a rate comparable to the control as indicated in Figs. 1B, C and D respectively. Similar results were obtained with *B. mycoides* as indicated in Figs. 3B, C and D respectively. With *B. subtilis* a concentration of 0.75 μg per ml had a slight effect on growth, DNA, RNA and protein syntheses; late during incubation (between the 4th and 6th hour of incubation) there was complete inhibition of DNA, RNA but no effect on protein synthesis. The synthesis of these macromolecular components was progressively inhibited as the concentration of ASK-753

Fig. 1. Effect of ASK-753 on the growth of *E. coli* and the syntheses of DNA, RNA and protein

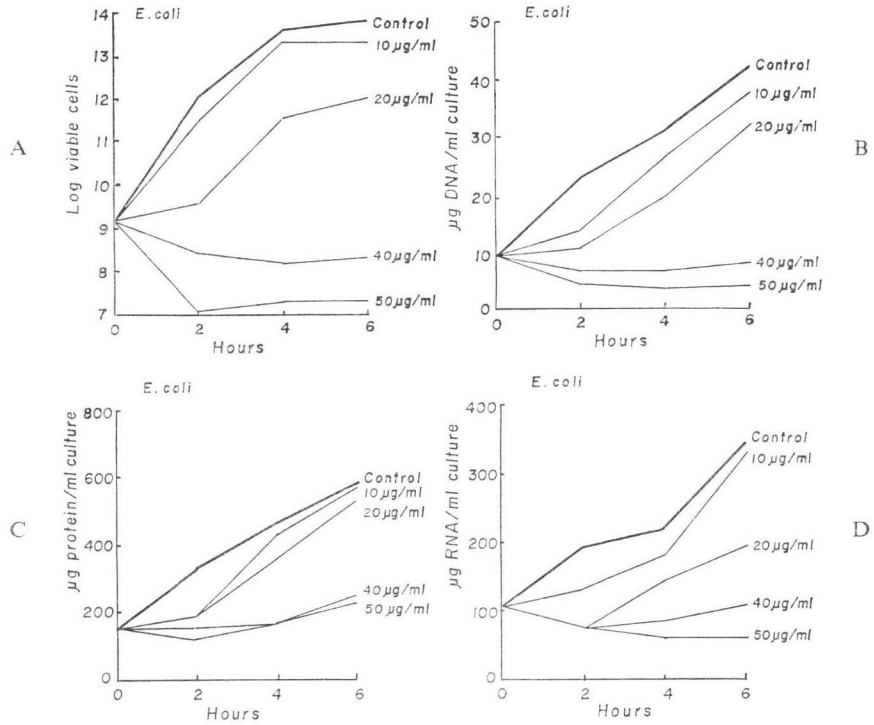


Fig. 2. Effect of ASK-753 on the growth of *B. subtilis* and the syntheses of DNA, RNA and protein

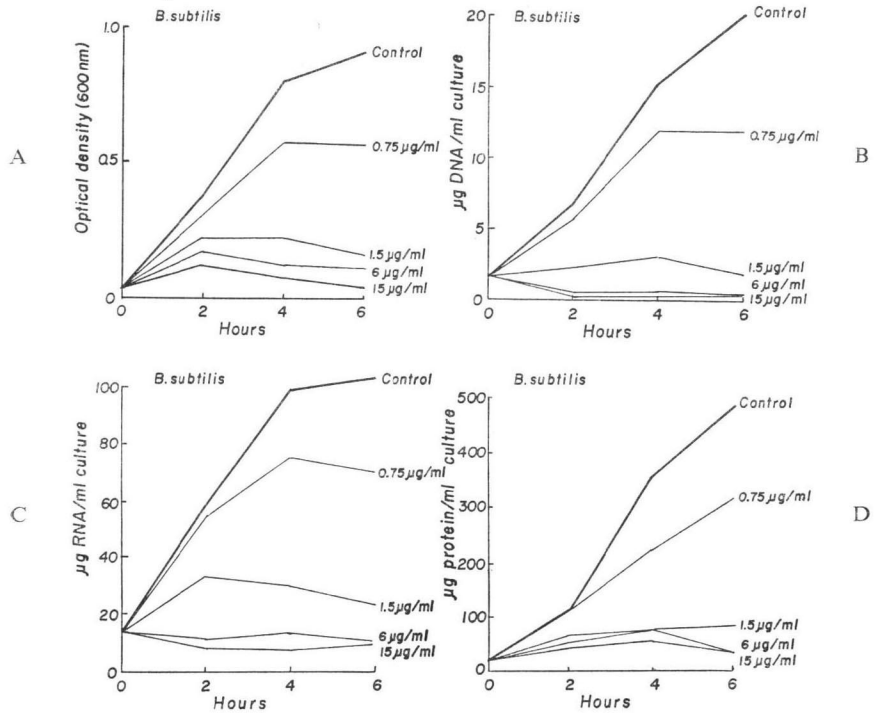


Fig. 3. Effect of ASK-753 on the growth of *B. mycoides* and the syntheses of DNA, RNA and protein

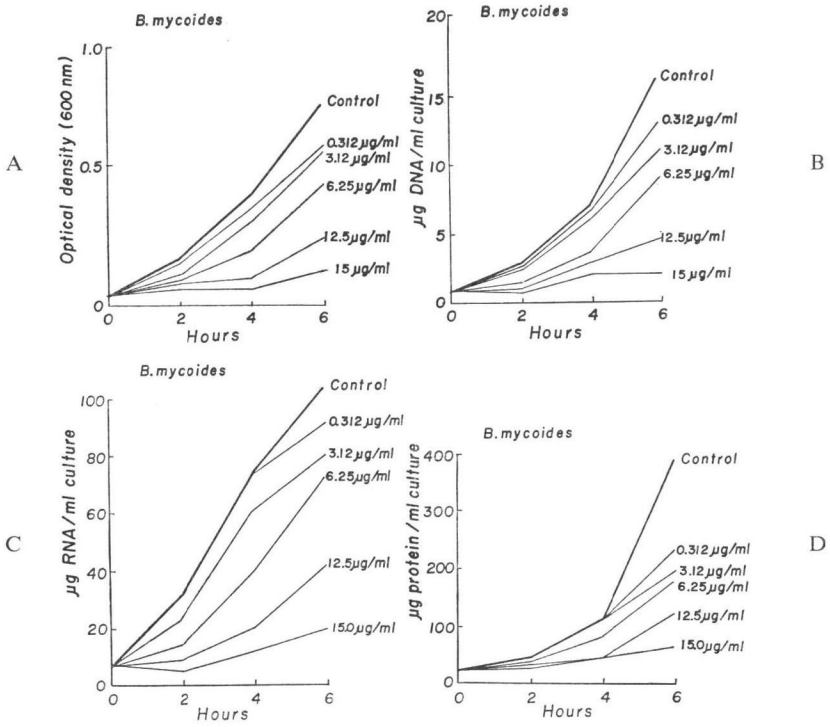


Table 1. Reversal of the inhibiting action of ASK-753 on DNA, RNA and protein syntheses

Test organism	μg/ml culture	Time in min.	μg Macromolecules/ml culture		
			DNA synthesis	RNA synthesis	Protein synthesis
E. coli (B-210)	40.00				
Inhibited		180	6.75	80.0	160.00
Inhibited (W)*		240	13.33	261.5	333.00
		300	50.00	312.8	717.00
B. subtilis (ICCB-543)	1.50				
Inhibited		180	6.90	77.25	295.20
Inhibited (W)*		240	7.20	89.9	316.45
		300	9.30	94.9	316.45
B. mycoides (U.S.S.R.)	3.12				
Inhibited		180	2.90	32.6	83.30
Inhibited (W)*		240	3.70	29.7	77.80
		300	3.80	30.9	77.80

* At the end of 180 minutes of incubation, 50 ml was removed from this culture, 15 ml was used to assay for DNA, RNA and protein; from the remaining culture the inhibitor was removed. The cells were then resuspended in the same volume of new medium. This culture was then designated Inhibited (W) for "Inhibited, washed". The 240- and 300-minute time indications are only 60 and 120 minutes after the completion of the removal of the inhibitor and the resumption of incubation at the end of 180 minutes.

Table 2. Control of DNA replication by ASK-753

	Time in (min.)	μg Macromolecules/ml culture	
		DNA synthesis	RNA synthesis
Control	0	5.242	27.775
	120	16.532	74.074
	240	36.935	101.852
	360	39.113	96.295
Inhibited*	0	5.242	27.775
	120	6.855	27.775
Inhibited (W)+	140	15.725	53.704
Inhibited++	260	15.725	66.665
Inhibited (W)§	300	18.952	96.295
Inhibited''	420	29.033	109.259
Inhibited (W)°	460	39.113	161.110

* Cells of *E. coli* was exposed to 40 μg/ml culture of ASK-753 for 120 minutes.
\+ The antibiotic was removed and the culture was incubated for 20 minutes. Inhibited (W) stand for "inhibited, washed".
++ At the end of 20-minutes, 40 μg/ml culture of the antibiotic was added and incubation was resumed for 120 minutes.
§ The antibiotic was again removed and the culture was incubated for 40 minutes.
'' The antibiotic was added again 40 μg/ml and the culture was incubated for 120 minutes.
° The antibiotic was again removed from the above culture and incubation resumed for 40 minutes.

increased, and at 1.5 μg per ml DNA synthesis stopped completely, while RNA and protein syntheses continued to a slight degree. Concentrations higher than 1.5 μg per ml completely block macromolecular synthesis.

The reversibility of the inhibition of cell division as well as DNA, RNA and protein syntheses in *E. coli* was shown by removal of the antibiotic from a culture in which it had been present for 3 hours. Once the inhibitor was removed from culture, these biosynthetic processes resumed and maintained rates parallel to those of control uninhibited cultures. However, in *B. subtilis* and *B. mycoides* removal of the antibiotic and reincubation did not result in restoration of the syntheses of DNA, RNA and protein as indicated in Table 1. From Table 2 it is evident that by programming the addition and removal of the antibiotic, DNA and RNA syntheses proceeded in a stepwise fashion.

Fig. 4. Effect of ASK-753 on the release of intracellular materials and on the turbidity of *B. subtilis*

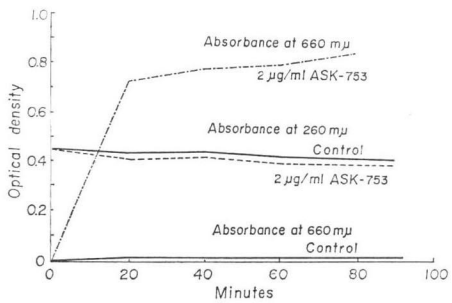


Fig. 5. Effect of ASK-753 on the release of intracellular materials and on the turbidity of *E. coli* B

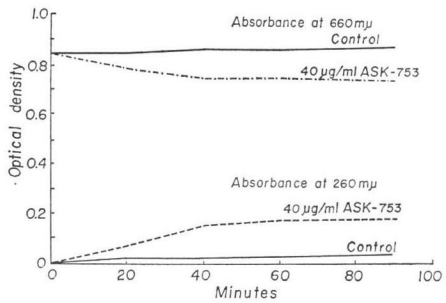


Fig. 6. Lysis of protoplasts of *B. subtilis* after treatment with ASK-753

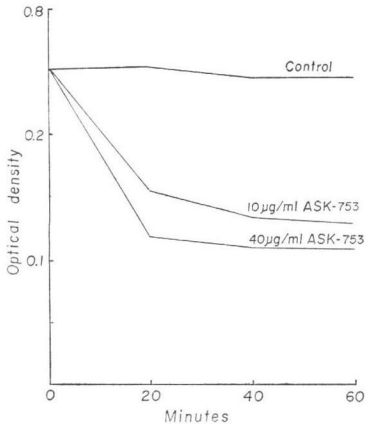
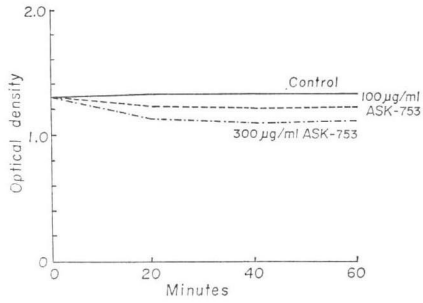


Fig. 7. Lysis of spheroplasts of *E. coli* B after treatment with ASK-753



Figs. 4 and 5 show the release of 260 mμ-absorbing materials from the cells in the presence or absence of ASK-753. Following the exposure of *B. subtilis* to this antibiotic the release of 260 mμ-absorbing materials from cells occurred immediately, while only a gradual and a slight release of 260 mμ-absorbing meterials was observed in *E. coli* B. ASK-753 also lyses protoplasts of *B. subtilis* but not spheroplasts of *E. coli* B (Figs. 6 and 7).

Discussion

From the results it is evident that concentrations of the antibiotic equal to or greater than that which causes complete inhibition of the synthesis of all three macromolecules in *B. subtilis* induce leakage of intracellular components and lysis of its protoplast. This might explain the irreversible effect of this antibiotic on DNA, RNA and protein syntheses in *B. subtilis*. In *E. coli* B, there was only a slight and gradual release of intracellular components in presence of the antibiotic; also it was found that the effect of the antibiotic on the syntheses of DNA, RNA and protein to be reversible.

These observations suggest that this antibiotic may not have a direct effect on the DNA replication cycle *per se*, RNA and protein syntheses, but, instead, it may cause alteration of the bacterial membranes (especially ASK-753 sensitive strain, *B. subtilis*) and thereby affect the attachment of the chromosome to the bacterial membrane.

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