THE MODE OF ACTION OF ASK-753 ON *BACILLUS SUBTILIS*

IBRAHIM R. SHIMI and SAFWAT SHOUKRY

Department of Biochemistry, Faculty of Science, Ain Shams University, Cairo-Arab Republic of Egypt

(Received for publication September 1, 1975)

The mode of action of ASK-753 on *Bacillus subtilis* was examined. Unlike proper sideromycin antibiotics ferrioxamine B failed to antagonize the antimicrobial effects of ASK-753. The antibiotic could inhibit the biosynthesis of nucleic acids; effect on the RNA was more pronounced. ASK-753 affected the stability of prelabelled DNA of *B. subtilis* in growing or resting cultures; the effect on the latter was more pronounced. Lysis of *B. subtilis* protoplasts could be attained at 30°C but not at 4°C which excludes a possible detergent effect of the drug. The drug exerted a potent inhibiting influence on protein synthesis by arresting the activity of lysyl-tRNA synthetase and thus could prevent the incorporation of ^{14}C-lysine.

The antibiotic ASK-753 is an iron-containing polypeptide firstly isolated by SHIMI *et al.*[1] The antimicrobial actions of ASK-753, ferramidochloromycin[2] and NRCS[3] could not be antagonized by ferrioxamine B and hence the term pseudosideromycin was given to these products.

The present work was conducted to elucidate the primary site of action of ASK-753 on *Bacillus subtilis*.

Materials and Methods

Organism and culture conditions.

A wild strain of *B. subtilis* prepared as described by FARMER[4] was grown in nutrient broth having the following composition (g/100 ml): Peptone, 0.5; NaCl, 0.5; meat extract, 0.15; yeast extract 0.15 and 10 μg thymine. Cultures were shaken at 220 rpm at 35°C and the cells were harvested during the early logarithmic phase of growth of the culture.

Synthesis of macromolecules

Synthesis of the cellular macromolecules was assessed by the incorporation of appropriate labelled precursors for the macromolecular fraction. The following amounts of tagged chemicals were added to 10 ml portions of culture medium: 5.0 μCi of ^{14}C-uridine, 5.0 μCi of ^{14}C-thymine and 1 μCi of ^{14}C-lysine to trace their incorporation into RNA, DNA and proteins respectively.

Radioactivity measurments

Samples (0.5 ml) from treated and untreated cultures were withdrawn at different time intervals and mixed with 5 ml of 5% ice-cold trichloroacetic acid. Insoluble materials were collected on Millipore membrane filter disks (pore size 0.45μ) and washed twice with 5 ml of cold 5% TCA, rinsed with diethyl ether, and finally counted in 10 ml scintillation liquid containing 6 g PPO and 0.49 dimethyl-POPOP/L toluene.

Stability of DNA in presence of ASK-753

Cells were prelabelled for three successive generations by the addition of 0.5 μCi of ^{14}C-thymine per 10 ml of culture medium then harvested by centrifugation at $15,000\times g$ and resuspended in either fresh prewarmed nutritive media or phosphate saline of pH 7.3 (*ca* 2×10^8 cells/ml). Three concentrations of the antibiotic ASK-753 were tried (1.0, 4.0 and 8.0 μg/ml) alongside with controls. The cultures were reincubated and 0.5 ml sample portions were with-

drawn at different time intervals and treated as described before. Results are given in Fig. 4a and b.

Effect of ASK-753 on stability of *B. subtilis* protoplasts

Protoplasts were prepared as described by WEIBULL[5] and suspended in hypertonic medium containing 20 % sucrose and phosphate buffer of pH 7.3. ASK-753 was added at 1.0, 4.0 and 8.0 μg/ml and its lysing property for *B. subtilis* protoplasts was assessed by measuring the turbidity of the culture at 660 nm.

Preparation of lysyl-tRNA synthetase

The method used was as that described for *E. coli*[6]. The S-150 fraction (free of ribosomes and mRNA) was precipitated with 46 % ammonium sulfate and then resuspended in 7.5 ml of 50 mM tris-HCl (pH 7.8) and 10 mM 2-mercaptoethanol and then dialysed against 200 volumes of 20 mM tris-HCl (pH 7.8), 0.01 mM mercaptoethanol and 10 % propylene glycol. The dialysed fraction (10 ml) was then added to DEAE-cellulose column equilibrated with 20 mM of tris-HCl (pH 7.8), 0.01 M 2-mercaptoethanol and 10 % propylene glycol. The enzyme was eluted with a linear KCl gradient, from 0～0.4 M and the active fraction was precipitated by 40 % saturation with ammonium sulfate. The precipitate was stored at 4°C, and was used within one week after being prepared. This preparation was found to contain 2,200 μg total protein with a specific binding activity of 1,000 unit. The membrane filter technique for the assay of amino-acylation of tRNA was that described by SCOTT.[7]

Total proteins either in the enzyme preparation or in the microbial cell treated with ASK-753 was assayed by the methed of LOWRY *et al.*[8]

Amino acid analysis of *B. subtilis* growth medium

The test organism was allowed to grow in a simple synthetic medium given by KNIGHT and PROOM.[9] The amino acid contents in the growth media of treated cultures was estimated by the zone strip technique.[10,11]

Results

Concentrations of ferrioxamine B as high as 1.0 μg/ml of growth medium did not antagonize the action of ASK-753 on *B. subtilis* cells, whereas it exerted a strong suppressing influence on the activity of albomycin (Table 1). When *B. subtilis* protoplasts were treated with the antibiotic at 30°C a lysing effect was recorded at a concentration of 4 μg/ml which is four times higher than the MIC (Fig. 1a). In contrast, such a treatment failed to exert any lytic effect on *B. subtilis* protoplast when carried out at 4°C (Fig. 1b). Thus such an effect on cellular membrane could not be considered as a primary one.

Table 1. The effect of ferrioxamine B on the antibiotic action of ASK-753 and albomycin against *B. subtilis*.

Antibiotics	Concentration of ferrioxamine B (mg/ml of media) and MIC (μg/ml)				
	0.0	0.1	0.2	0.5	1.0
ASK-753	1.0	1.0	1.0	1.0	1.0
Albomycin	1.5	30.0	100	100	100

The serial agar dilution technique was adopted

ASK-753 affects the biosynthesis of *B. subtilis* RNA and DNA as indicated by arresting the incorporation of ^{14}C-uridine and ^{14}C-thymine respectively (Figs. 2b and 3 respect.). The inhibition of RNA was more pronounced than that of DNA.

It was of interest to examine the effect of ASK-753 on the breakdown or *B. subtilis* DNA. The antibiotic at higher levels and longer incubation periods stimulated the breakdown of prelabelled DNA either in growing or a resting cells of *B. subtilis* (Fig. 4a and b respectively). The effect in the latter condition was more pronounced than the former.

Fig. 1. Effect of ASK-753 on *B. subtilis* protoplasts (a) at 30°C and (b) at 4°C.

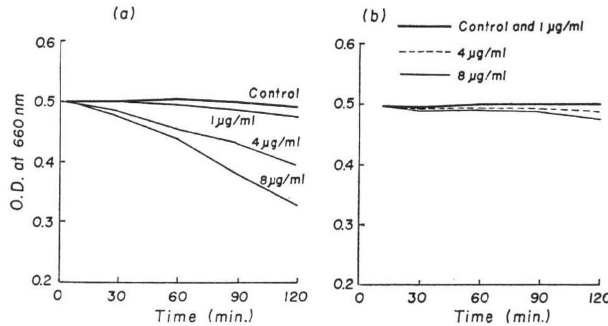
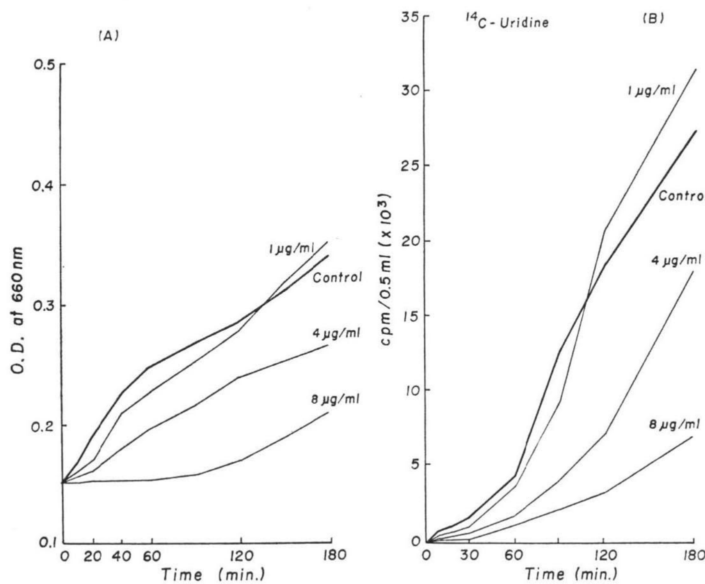


Fig. 2. Effect of ASK-753 on (A) the growth of *B. subtilis* and (B) synthesis of RNA.

A culture of *B. subtilis* in the log phase of growth was diluted with fresh medium. ^{14}C-Uridine and the different concentrations of the antibiotic were added simultaneously, portions of 0.5 ml were withdrawn from the culture at the appropriate time intervals. The rest of the experimental steps were as that described in the text.

Bacterial growth was followed by measuring the O.D. at 660 nm of cultures. Which could be converted into cell counts (No. of cells/ml medium=$O.D._{660nm} \times 8 \times 10^8$).



ASK-753 at its MIC level markedly prevented the biosynthesis of proteins (Fig. 5). The higher the concentration of the drug the more drastic was the effect. The antibiotic caused the accumulation of extracellular lysine which increased by progressing periods of the incubation (Table 2). Moreover the antibiotic curtailed the incroporation of ^{14}C-lysine into the cellular proteins as indicated by the decrease in radioactivity in the TCA insoluble macromolecules (Fig. 6). The effect of ASK-753 on the aminoacylation of the other amino acids was tested; its effect on acylation of lysine was the most pronounced (Table 3). The antibiotic in very dilute concentrations could inhibit the activity of lysyl-tRNA synthetase as demonstrated by the decrease in counts of TCA-insoluble fraction (Fig. 7).

Fig. 3. Effect of ASK-753 on the synthesis of *B. subtilis* DNA.

The experimental steps were as those of legend to Fig. 2 except that ^{14}C-thymine replaced ^{14}C-uridine.

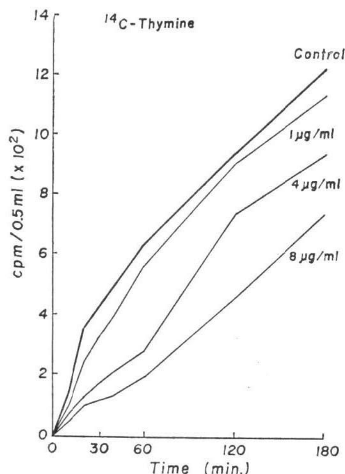


Fig. 4. Effect of ASK-753 on the stability of *B. subtilis* DNA.

(a) Prelabelled cells were resuspended in fresh nutrient medium. Stability of cellular DNA was assessed by measuring radioactivity in the TCA insoluble fraction.

(b) Prelabelled cells were resuspended in phosphate saline solution of pH 7.3. The rest of the experimental steps were as described before.

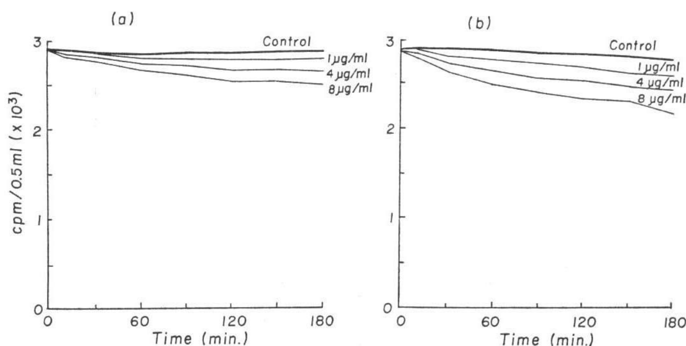


Fig. 5. Effect of ASK-753 on protein synthesis by *B. subtilis*.

Cultures of *B. subtilis* grown overnight were harvested by centrifugation at 15,000×*g* and their cells were suspended in fresh medium (*ca* 2×10^8 cells/ml) supplemented with various concentrations of the antibiotic. Bacterial cells were collected by centrifugation at the appropriate incubation period, washed and its protein content was quantitatively estimated.

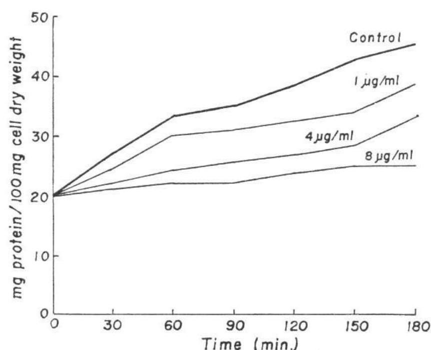


Table 2. Effect of ASK-753 on the release of lysine in the culture broth of *B. subtilis*.

Concentration of ASK-753 (μg/ml)	Concentration of lysine (mg/100 ml)				
	10 min.	30 min.	60 min.	90 min.	120 min.
0.0	1.21	1.63	1.70	1.89	2.12
1.0	2.83	3.62	4.16	4.83	4.90
4.0	4.01	4.56	4.91	5.12	5.32
8.0	5.08	5.32	5.68	6.14	6.28

The harvested cells from overnight cultures were suspended in 30 ml of simple synthetic medium (*ca* 2×10^8 cells/ml) KNIGHT & PROOM[9]) containing 1μg/ml thymine. ASK-753 1.0, 4.0 and 8.0μg/ml were supplemented and the flasks were shaken at 35°C. Samples were withdrawn at different time intervals, centrifuged and the supernatants were analyzed for the accumulated amino acids.

Discussion

ZAHNER, HUTTER and BECHMAN[12]) reported that iron-containing antibiotics (sideromycins) produced by actinomycetes are subject to the antagonistic action of ferrioxamine B whereas the action of the iron-containing polypeptide antibiotics ferramidochloromycin (FACM)[2]), ASK-753[1]) and NRCS-15[3]) could not be antagonised by the action of this growth factor. Thus the term pseudosideromycin was given. The primary site of action of FACM on bacterial cells has recently been elucidated by SHIMI, SHOUKRY and ALY[2]) who found that the antibiotic

Fig. 6. Effect of ASK-753 on the incorporation of ^{14}C-lysine into *B. subtilis* proteins

The experimental steps were as those of legend to Fig. 2 except that ^{14}C-lysine was added instead of ^{14}C-uridine.

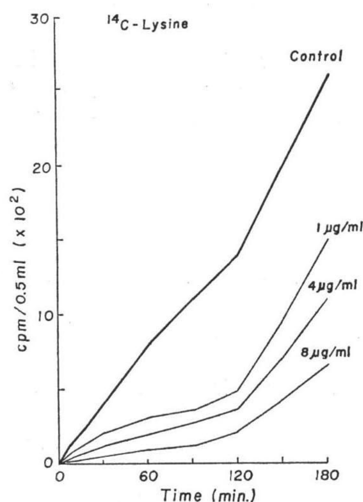


Table 3. Effect of ASK-753 on the activity of aminoacyl-tRNA synthetase.

^{14}C Amino acids	cpm/15µl of reaction mixture	
	Untreated (control)	Treated with 1µg/ml of ASK-753
Alanine	2,910	2,207
Arginine	3,150	3,010
Aspartic acid	3,901	3,095
Cysteine	2,980	2,560
Glutamic acid	3,503	3,483
Glycine	2,301	1,978
Histidine	3,007	2,702
Leucine	3,370	2,650
Lysine	3,550	1,032
Methionine	2,610	2,230
Phenylalanine	1,450	1,225
Serine	3,780	3,394
Threonine	2,101	2,011
Tyrosine	1,060	1,036
Valine	3,210	2,873

Aminoacylation of tRNA proceed for 30 minutes.

The reaction mixture was as that of legend to Fig. 7 with except ^{14}C-lysine was substituted with the tested amino acid. Partially purified aminoacyl-tRNA synthetase was used.

Fig. 7. Effect of ASK-753 on the activity of lysyl-tRNA synthetase

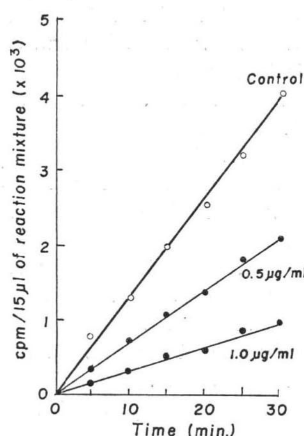
The reaction mixture contains the following:

a ATP, 0.1M (100µl); CTP, (10µl); phosphoenolpyruvate (PEP) 0.33M (30µl) tris-HCl pH 7.6, 2M (50µl); mercaptoethanol, 1.0M (50µl); $MgCl_2$, 1.0M (20µl); ^{14}C-lysine (5µCi) and water (130µl).

50µl of mixture (a) were set in ice-cold bath, 1µg in 10µl of PEP kinase was added followed by 30µl of *E. coli* tRNA (2 mg/ml) and then 30µl of the enzyme solution was added. The tubes were incubated at 28°C for 35 minutes. 15µl samples were withdrawn at different time intervals and the counts for the ice-cold 5% TCA insoluble fraction were measured.

Different levels of ASK-753 were added (5µl each) just before the addition of the enzyme.

The reaction mixtures in both cases were completed to 120µl by the addition of distilled water.



inhibits the activity of lysyl-tRNA synthetase.

KHAFAGY and HARON[18] reported that ASK-753 cause complete inhibition of the synthesis of *B. subtilis* DNA, RNA and protein. They attributed this inhibition to the alteration of the bacterial cell membrane. However they used 10 and 40 µg/ml ASK-753 which were appreciably higher than the reported MIC of ASK-753 on *B. subtilis* (1.0 µg/ml). Our results indicate that ASK-753 failed to lyse cellular membrane of the bacterial protoplasts when the treatment was carried out at 4°C where as surface-active agents could induce their effects at relatively low temperature. Therefore the lytic action of the antibiotic on *B. subtilis* protoplasts at 30°C can be considered as a delayed secondary effect.

The action of the antibiotic on the breakdown of the prelabelled DNA was higher in resting cultures than in growing ones. Such results might indicate that the repair enzymes could actively polymerise the damaged DNA in growing cells, while in resting cells the damaged DNA tem-

plate could not be repaired efficiently as long as the action of the drug was still retained.[14]

The results indicate that the most pronounced inhibiting effect of the drug was exerted on the biosynthesis of cellular proteins by arresting aminoacylation of lysine *via* inhibiting lysyl-tRNA synthetase. The antibiotic could also inhibit, though to a lesser extent the synthesis of bacterial RNA as well as of DNA, probably as a secondary consequence to the damage induced by the drug on the cellular membrane.

Comparing ferramidochloromycin and ASK-753, the two antibiotics possess strong inhibitory effect on lysyl-tRNA synthetase. Furthermore, ASK-753 could damage the cellular membrane whereas such an effect was not recorded with FACM. ASK-753 inhibited the synthesis of RNA and DNA while ferramidochloromycin failed.

References

1) SHIMI, I. R.; G. M. IMAM & B. M. HAROUN: ASK-753, a new iron-containing antibiotic. J. Antibiotics 22: 106~111, 1969
2) SHIMI, I. R.; S. SHOUKRY & F. T. ALY: On the mode of action of ferramidochloromycin on *Bacillus subtilis*. Int. J. Biochem. 6: 205~209, 1975
3) HAROUN, B. M.: Isolation and characterization of NRCS-15, a new iron-containing antibiotic. J. Antibiotics 27: 14~19, 1974
4) FARMER, J. L. & F. ROTHMAN: Transformable thymine-requiring mutant of *Bucillus subtilis*. J. Bact. 89: 262~263, 1965
5) WEIBULL, C.: The isolation of protoplasts from *Bacillus megaterium* by controlled treatment with lysozyme. J. Bact. 66: 688~695, 1953
6) KAJI, K.: Technique for measuring specific sRNA binding to *Escherichia coli* ribosomes. cited in Methods in Enzymology Vol. 12, p. 692. New York: Academic press. 1968
7) SCOTT, J. F.: Membrane filter technique for the assay of charged tRNA. cited in Methods in Enzymology, Vol. 12 p. 173, New York Academic Press. 1968
8) LOWRY, O. H.; M. J. ROSEBROUGH, A. L. FARR & R. J. RANDULL: Protein measurement with the FOLIN phenol reagent. J. Biol. Chem. 193: 265~275, 1951
9) KNIGHT, B. C. & H. PROOM: A comparative survey of the nutrition and physiology of mesophilic species in the genus *Bacillus*. J. Gen. Microbiol. 4: 508~512, 1950
10) SHIMI, I. R.; G. R. IMAM & A. DEWIDAR: Further studies on the zone-strip technique. Analyst 86: 744~747, 1961
11) SHIMI, I. R. & G. M. IMAM: Analytical results for the paper chromatographic zone-strip technique. Analyst 90: 564~567, 1965
12) ZAHNER, H.; R. HUTTER & E. BECHMAN: Stoffwechselprodukte von Actinomyceten. 23 Mitteil. Zur Kenntnis der Sideromycin Wirkung. Arch. Mikrobiol. 36: 325~328, 1960
13) KHAFAGY, E. Z. & B. M. HAROUN: On the mode of action of ASK-735, a new iron-containing antibiotic. J. Antibiotics 27: 874~880, 1974
14) BARKER, G. R. & N. HARDMAN: Elimination of F'Lac episome from deoxyribonucleic acid polymerase I-defecient *Escharichia coli* by acridine orange. Biochem. Soc. Trans. 2: 477~480, 1974