*MUTANTS OF ESCHERICHIA COLI PERMEABLE TO ACTINOMYCIN**

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Escherichia coli is generally impermeable to charged molecules unless specific permeation systems exist. The resistance of *E. coli* to some antibiotics could be attributed to impermeability of the cell to the compounds. For instance, actinomycin inhibits RNA synthesis by *E. coli* extract but has no effect on RNA synthesis or on growth of intact cells.[1]

In order to study the nature of cell permeability, it might be useful to isolate bacterial mutants possessing different permeability properties. The present paper describes the isolation and characterization of *E. coli* mutants which are permeable to actinomycin. Thus, RNA synthesis can be inhibited by actinomycin in the intact cell. It is also shown that these mutants are sensitive to lysozyme and can be readily infected by DNA of bacteriophage ϕX174.

Materials and Methods.—The parental strains were *E. coli* strain B, obtained from Dr. S. Benzer, and strain AB1157, a derivative of K12,[2] obtained from Dr. N. Otsuji of Osaka University. Crystalline actinomycin S_3[3] was supplied by the Daiichi Seiyaku Pharmaceutical Co., Ltd., Osaka, Japan. H^3-labeled actinomycin S_3 was furnished by Dr. J. Kawamata of Osaka University, and was purified free of contaminating radioactive materials by thin-layer chromatography on silica gel (benzene–ethyl acetate–methanol, 6 : 4 : 1).[4] Egg-white lysozyme was purchased from Sigma Chemical Co.

Actinomycin-sensitive mutants were isolated as follows. Bacteria (5×10^8 cells/ml) were treated with 100 μg/ml of N-methyl-N'-nitroso-N-nitroguanidine for 15 minutes at 37°C in 0.005 *M* Tris-maleic buffer, pH 6.0.[5] The treated cells were washed, resuspended in the original volume of nutrient broth, and shaken for three hours. The bacteria were plated on nutrient agar and incubated overnight. Colonies were transferred by replica plating to plates containing actinomycin (1–10 μg/ml). After incubation overnight the plates were examined, and colonies which did not grow on the actinomycin-containing plates were selected as mutants.

The incorporation of P^{32}-orthophosphate into nucleic acids was determined according to Sekiguchi.[6] RNA polymerase activity was determined by the method of Furth *et al.*[7] The procedures used in experiments of ϕX174 DNA were as previously described.[8]

Results.—General properties of actinomycin-sensitive mutants: Strains AS1 and AS12 (isolated from AB1157), and AS19 and AS27 (isolated from B) were used to study the general properties of actinomycin-sensitive mutants. Figure 1 shows the colony-forming abilities of the mutants on plates containing various concentrations of actinomycin. All mutant strains are more sensitive to actinomycin than are the parental strains (B and AB1157), both of which grow normally in the presence of more than 10 μg/ml of the antibiotic.

The actinomycin-sensitive mutants exhibit the following characteristics of *E. coli:* indole production (+), Voges-Proskauer test (−), methyl red test (+), Gram-stain (−). In addition, the mutant strains retain their original nutritional require-

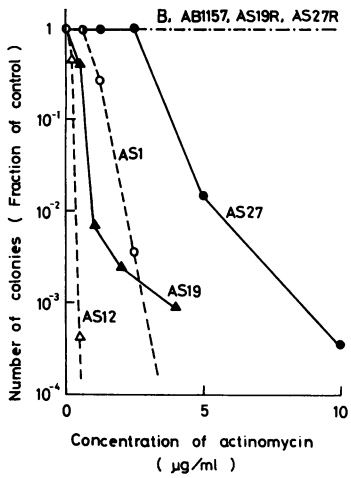

FIG. 1.—Plaque-forming abilities of various strains of *E. coli* in the presence of actinomycin. Appropriate concentrations of bacterial suspensions were plated on nutrient broth plates containing various levels of actinomycin S_3. The plates were incubated at 37°C overnight, and numbers of colonies formed counted.

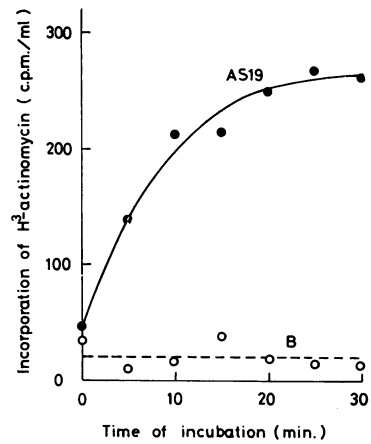

FIG. 2.—Uptake of H^3-labeled actinomycin by *E. coli* B and AS19. Bacteria (5×10^8 cells/ml) were incubated at 37°C in broth containing H^3-actinomycin S_3 (1 μg/ml, 1.9×10^4 cpm/ml). Aliquots were removed at times indicated, centrifuged at 2°C, and washed twice with ice-cold broth containing nonradioactive actinomycin S_3 (1 μg/ml). The washed cells were collected on a membrane filter, washed with 3 ml of cold actinomycin-containing broth, and the radioactivity retained on the filter was measured in a liquid-scintillation counter.

ments. AS1 and AS12 require threonine, leucine, proline, histidine, and arginine for growth, as does their parent, AB1157. AS19 is a prototroph, as is B. AS27 has acquired a new growth requirement for isoleucine-valine; however, this requirement does not seem to be related to actinomycin sensitivity because AS27R, an actinomycin-resistant revertant from AS27, still has the requirement. The generation times in broth show that the mutants grow somewhat more slowly than do the parental strains. As judged by cross-streak tests, the mutants also retain their original sensitivity to phage. B and AS19 plate all T phages with almost equal efficiency.

The character of actinomycin sensitivity in AS19 and AS27 is relatively unstable: they produce actinomycin-resistant revertants at frequencies of 10^{-3} to 10^{-5}. Therefore, it is advisable to start each culture from a single colony and, before use, to check its sensitivity to lysozyme, as described below. AS1 and AS12, on the other hand, are very stable; so far, no revertants have been isolated.

Uptake of actinomycin: To compare the abilities of actinomycin-resistant and sensitive cells to take up actinomycin, bacteria were incubated with H^3-labeled actinomycin, washed in the cold, and the incorporated isotope measured. Figure 2 shows the time-course of incorporation by *E. coli* B and by AS19. The actinomycin-sensitive cells took up actinomycin to a final level of 40 mμg/10^9 cells, while little uptake occurred with the parent strain B. Most radioactivity retained in AS19 was recovered as a compound identified with actinomycin S_3 on a radiochromatogram, suggesting that the actinomycin is taken up in AS19 as such, and not metabolized significantly. In addition, it was found that AS19R, an actinomycin-resistant revertant from AS19, also has diminished ability to incorporate

TABLE 1

COMPARISON OF ABILITIES OF VARIOUS *E. coli* STRAINS TO TAKE UP ACTINOMYCIN

Strains	Radioactivity Retained by Cells (cpm/ml) (0 min)	(20 min)	Actinomycin incorporated (mμg/5 × 10^8 cells/20 min)
B	9	38	1.6
AS19	53	353	16.7
AS19R	17	48	2.1
AS27	82	425	19.0
AS27R	13	22	0.5
AB1157	34	21	0
AS1	31	36	0.3
AS12	12	10	0

Procedures and conditions were as described in Fig. 2.

actinomycin (Table 1). Essentially similar results were obtained with the actinomycin-sensitive mutant AS27 and its revertant AS27R. Thus, in these mutants sensitivity to actinomycin is correlated with ability to take up the antibiotic.

However, there is a class of actinomycin-sensitive mutants which do not incorporate much actinomycin. These include AS1 and AS12 derived from strain AB1157, for which data are also shown in Table 1. The sensitivity of these mutants does not seem to be associated with a change in permeability.

Inhibition of RNA synthesis by actinomycin: The effect of actinomycin on the incorporation of P^{32}-orthophosphate into RNA and DNA in *E. coli* AS19 is shown in Table 2. It is found that both RNA and DNA synthesis by the actinomycin-sensitive mutant are strongly inhibited by relatively low concentrations of actinomycin. No such inhibition was observed with *E. coli* B. Similar results were obtained in other experiments in which the incorporation of P^{32} into nucleic acids in AB1157 and in AS12 were compared.

The effect of actinomycin on *in vitro* RNA synthesis was studied by using crude extracts of *E. coli* B and AS19. Since the removal of DNA by deoxyribonuclease from the system reduced the rate to 5 per cent or less of that of the complete system, most of the activity measured appears to represent DNA-dependent RNA polymerase activity. The results are shown in Table 3. In the cell-free system, *E. coli* RNA polymerase was effectively inhibited by actinomycin, as first found by Hurwitz *et al.*,[1] and the extent of inhibition with the two types of extracts were essentially the same. These results are compatible with the idea that ordinary *E. coli* has a permeability barrier which prevents access of actinomycin to its sites of action, and that the mutant is defective in that barrier.

The experiments described in the preceding section demonstrated that some actinomycin-sensitive mutants, such as AS1 and AS12, do not exhibit increased ability to take up actinomycin. The sensitivity of these mutants might be due

TABLE 2

EFFECT OF ACTINOMYCIN ON NUCLEIC ACID SYNTHESIS OF *E. coli* AS19

Concentration of actinomycin (μg/ml)	RNA		DNA	
	Incorporation (cpm/ml)	Per cent inhibition	Incorporation (cpm/ml)	Per cent inhibition
0	512	—	267	—
1	241	53	237	11
3	49	90	102	42
10	0	100	53	80

E. coli AS19 (5 × 10^8 cells/ml), washed once by 0.01 *M* tris-chloride (pH 7.5) in cold, was aerated for 30 min at 37°C in tris-glucose-yeast extract (0.2%) medium containing P^{32} (3 × 10^6 cpm/ml) and actinomycin S_3.

TABLE 3

INHIBITION OF *in vitro* RNA POLYMERASE ACTIVITIES OF *E. coli* B AND AS19 BY ACTINOMYCIN

Concentration of actinomycin (μg/ml)	B		AS19	
	C^{14}-UTP incorporated	Per cent inhibition	C^{14}-UTP incorporated	Per cent inhibition
0	0.51	—	0.78	—
1	0.39	23	0.47	40
2	0.21	59	0.33	58
4	0.13	75	0.21	73

The reaction mixture (0.5 ml) contained 40 mμmoles each of ATP, GTP, CTP, and C^{14}-UTP (1×10^6 cpm/μmole), 1 μmole of $MnCl_2$, 4 μmoles of $MgCl_2$, 1 μmole of 2-mercaptoethanol, 25 μmoles of tris-chloride buffer, pH 7.5, 40 mμmoles (as nucleotide) of *E. coli* DNA, extract (0.4 mg of protein), and varying amounts of actinomycin S_3. The mixture was incubated for 20 min at 37°C, and C^{14}-UTP incorporated into RNA (mμmoles per mg protein in 20 min) was determined.

to the existence of extraordinarily sensitive sites of action of the antibiotic. Therefore we investigated the effect of actinomycin on RNA polymerase, one of the possible sites. The results obtained for AB1157 and AS12 are the same as those of B and AS19, and thus eliminate the possibility that the mutant contains a RNA polymerase system which is unusually sensitive to actinomycin.

Sensitivity to lysozyme: Intact *E. coli* cells are resistant to the lytic action of lysozyme, but when treated with chloroform or EDTA they become susceptible. Presumably, these treatments cause an alteration in outer surface structures of the cell so that a glycopeptide layer can be attacked. If the increased sensitivity to actinomycin of the mutants is due to changes in their surface structure, the mutants might have become sensitive to lysozyme. To test this possibility, the sensitivity to lysozyme of actinomycin-sensitive cells was compared to that of the wild-type strain. Figure 3 demonstrates that AS19 and AS27 are, indeed, sensitive to lysozyme. An extensive decrease in turbidity occurred, due to cell lysis, when intact cells of the mutants were incubated with egg-white lysozyme. No decrease in turbidity was observed with the parental strain B.

The correlation of sensitivities to actinomycin and lysozyme was further demonstrated by the following experiments. (1) The actinomycin-resistant revertants, AS19R and AS27R, were resistant to lysozyme. When cells of AS19 were incu-

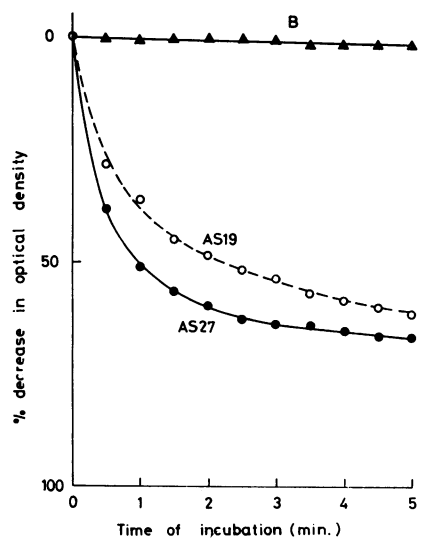

FIG. 3.—Lysis of actinomycin-sensitive cells by lysozyme. Bacteria (5×10^8 cells/ml) were incubated with 2 μg/ml of egg-white lysozyme in 0.05 *M* tris-chloride buffer, pH 8.0, at 22°C, and the decrease in turbidity was measured at 450 mμ.

TABLE 4

INFECTION OF *E. coli* AS27 WITH φX174 DNA

Host	No. phages produced (per ml)
AS27 cells	1.0×10^6
AS27 spheroplasts prepared by lysozyme	3.7×10^6
AS27 spheroplasts prepared by lysozyme and EDTA	1.8×10^6
B cells	$<1 \times 10^2$
C cells	$<1 \times 10^2$

Bacteria were incubated at 20°C for 10 min in 0.05 *M* tris-chloride, pH 8, 20% sucrose. To prepare spheroplasts, 5 μg/ml of lysozyme with or without 0.25 mM EDTA was added prior to the incubation. φX174 DNA (4×10^{10} phage equivalent DNA per ml) was added, and the mixture was incubated at 37°C in a growth medium containing 20% sucrose. After 2 hr of incubation, the mixture was diluted with saline and plated on agar layer with a culture of *E. coli* C.

bated with 100 μg/ml of lysozyme and the surviving cells tested, 40 out of 42 survivors were resistant to actinomycin. (2) The treatment of AS19 and AS27 with lysozyme in a hypertonic medium produced spheroplasts. Ethylenediaminetetraacetate (EDTA), which is essential in making spheroplasts from ordinary *E. coli* cells, was not required. (3) AS19 and AS27 were lysed by phage-induced lysozyme. Phage T4, plated on these mutants, produced plaques with large halos formed by lysis of surrounding cells by phage lysozyme. No such halo was formed by T4 plated on B, or when a lysozyme-deficient mutant of T4[9] was plated on AS19 or AS27.

In spite of their high susceptibility to actinomycin, AS1 and AS12 were resistant to lysozyme. Thus, also in this respect, these mutants derived from AB1157 are different from AS19 and AS27, derived from B.

Infection with φX174 DNA: It has been shown that DNA isolated from φX174 can infect *E. coli* spheroplasts but not normal cells.[8, 10, 11] This might be due to impermeability of the normal cell to DNA. Thus, it is of interest to see whether φX174 DNA can infect intact cells of actinomycin-sensitive mutants, which have enhanced permeability to actinomycin and possibly other compounds.

It was found that growing cultures of the actinomycin-sensitive mutants are not infectable with φX174 DNA, but when the cells are incubated in a hypertonic medium they become susceptible. The results shown in Table 4 indicate that AS27 cells incubated in a tris-sucrose medium are as active as spheroplasts in producing infective centers from DNA. No significant increase in infective centers was observed with the parental strain B, or with C, the usual host of φX174, incubated in the same medium. Thus, the actinomycin-sensitive mutants appear to be converted to a form susceptible to φX174 DNA more easily than do the wild-type strains.

Discussion.—The actinomycin-sensitive mutants isolated thus far fall into two major categories. The first type, including AS19 and AS27 (isolated from strain B), takes up greater amounts of actinomycin than does the parent type; thus the increased sensitivity of these mutants to actinomycin may be due to loss of a permeability barrier. The second type, such as AS1 and AS12 (isolated from strain AB1157), does not exhibit an increased ability to take up the antibiotic, and the mechanism of this type of mutation is not evident.

Greater sensitivity to lysozyme is another notable feature of the first type of actinomycin-sensitive mutant. Lysozyme attacks glycopeptide in a rigid layer of the cell wall of both gram-positive and -negative bacteria. However, the intact

cells of gram-negative bacteria are resistant to the action of lysozyme. The glycopeptide layer of gram-negative organisms such as *E. coli* is covered by layers of lipoprotein and lipopolysaccharide, so that removal of the outer layers by chloroform or phenol is necessary to digest the glycopeptide layer of these organisms by lysozyme.[12, 13] The fact that sensitivities to actinomycin and to lysozyme are closely related in this type of mutant suggests that the mutants are defective in some part of the cell-wall structure, possibly in the lipoprotein or lipopolysaccharide layers, which serve as permeability barriers. It is to be recalled that the treatment of *E. coli* with EDTA results in the rapid release of components of cell-wall lipopolysaccharide,[14] and that cells thus treated become sensitive to lysozyme as well as to actinomycin.[15, 16] Preliminary observations with the electronmicroscope have revealed no major difference in surface structure of the mutant and the wild-type strains,[17] and more extensive studies are necessary to elucidate how the mutation alters permeability.

There are obvious advantages in the use of these mutants in various biological studies. Nucleic acid and protein syntheses *in vivo* can be readily controlled by actinomycin. As indicated by the experiment with ϕX174 DNA, the mutant cells might take up various biologically active macromolecules more efficiently. It would be interesting to pursue the possibility of transformation of these cells.

Summary.—Two nitrosoguanidine-induced mutants of *Escherichia coli*, selected for their sensitivity to actinomycin, have been isolated and shown to be permeable to actinomycin and sensitive to lysozyme. Their RNA synthesis *in vivo* as well as *in vitro* are effectively inhibited by the antibiotic. Another type of actinomycin-sensitive mutant was also isolated, which has low permeability to actinomycin and resistance to lysozyme.

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