MECHANISM OF TETRACYCLINE RESISTANCE IN *STAPHYLOCOCCUS AUREUS*

I. INDUCIBLE RESISTANCE TO TETRACYCLINE

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This paper deals with kinetic studies on the induction of resistance to tetracycline (TC) in *Staphylococcus aureus*. It was found that TC-resistance is inducible and TC is an active inducer. Cell populations acquired high resistance to TC after prior exposure to subinhibitory concentrations of the drug, but the resistance of induced populations was lost when the cells were grown in the absence of inducer. Induction of TC-resistance did not take place when protein synthesis of bacteria was inhibited by addition of chloramphenicol or actinomycin D, and by histidine starvation in a histidine auxotroph. The acquisition of resistance to tetracycline was paralleled by a decrease in the accumulation of the drug in bacterial cells, resulting from a decrease in their permeability for tetracycline.

Many investigators have reported on the biochemical basis of resistance to tetracycline (TC) and have emphasized that a decrease in permeability for the drug is the main factor responsible for TC-resistance. Nevertheless, details still remain to be answered. IZAKI *et al.*[3,4] reported that growth of a TC-resistant strain of *E. coli* K12 in the presence of TC caused a decrease in the accumulation of the drug in the cells with a concomitant increase in the level of TC-resistance. Their data suggest an induction of TC-resistance by prior treatment with the drug, but detailed analysis was not described. FRANKLIN[1] also found that the level of TC-resistance in *E. coli* K12 strains carrying R(TC) factors increased after prior treatment with the drug, and observed a simultaneous decrease of TC-accumulation in the cells. This induction of TC-resistance was found to be inhibited by the presence of chloramphenicol or proflavine in the induction mixture, suggesting an inducible synthesis of enzyme (or enzymes) responsible for the formation of a permeability barrier for tetracycline. The present paper deals with the inducible resistance to tetracycline and kinetic studies of the induction in *Staphylococcus aureus*.

Materials and Methods

Bacterial Strains: *Staphylococcus aureus* MS353, S1419 and S1549 are stock cultures of this laboratory which were isolated from clinical sources. *S. aureus* PS81 is a propagating strain of typing phage 81 of the International Typing Series. Strain S1419 is a histidine (*his*$^-$) auxotroph and strain S1419-T is a *his*$^+$ transductant which was obtained by transduction with a phage lysate from strain S1549 *his*$^+$. *S. aureus* MS353 is resistant

to sulfanilamide (SA). Strains PS81 and S1419-T are resistant to both tetracycline (TC) and SA.

Media: Heart Infusion (HI) agar (Eiken, Tokyo) was used for the determination of drug resistance. Medium B was used for the kinetic studies of TC resistance. It consisted of 7.0 g of Na_2HPO_4, 2.0 g KH_2PO_4, 1.2 g $(NH_4)_2SO_4$, 0.4 g $MgSO_4 \cdot 7H_2O$, 2.0 g Bactopeptone (Difco), 1.0 g yeast extract (Difco), and 1,000 ml of deionized water. The pH was adjusted to 7.2. The synthetic medium was used for the test of inducibility for TC-resistance in a histidine auxotroph. Its composition was described previously[9].

Drugs and Drug Resistance: Tetracycline (TC), chloramphenicol and actinomycin D were supplied by the Taito Pfizer Co., Ltd., the Sankyo Seiyaku Co., Ltd., and Merck Sharp & Dohme, U.S.A., respectively. Drug resistance was determined by an agar dilution method as reported previously[8].

Determination of Bacterial Growth: Growth in liquid cultures was assayed turbidimetrically at 630 mμ with a "Simazu Spectronic 20" colorimeter. A standard curve was constructed by plotting the dry weight of bacteria *versus* optical density at 630 mμ.

Induction and Assay of TC Resistance: The method of WEAVER and PATTEE[9] was employed with a slight modification as follows: One ml of an overnight broth culture of strains to be tested was inoculated in 9 ml of medium B and shaken in a water bath at 37°C. After 2 hours of incubation, the culture had reached the middle of the exponential growth; 0.9 ml of this culture was inoculated in 1.0 ml of medium B containing different concentrations of TC and shaken in a water bath at 37°C. After appropriate time intervals of incubation, 0.2 ml samples were withdrawn, inoculated in 9.8 ml of medium B containing TC (50 mcg/ml), and shaken in a water bath at 37°C. At appropriate time intervals, the ability of the bacteria to grow in this medium was checked photometrically. Without pretreatment with TC, the tested strains could not grow in medium B containing 50 mcg of TC/ml. In control experiments 0.2 ml samples were inoculated in 9.8 ml of medium B without the addition of TC and treated as described above.

Growth Inhibition by Various Inhibitors: An overnight broth culture of strain PS81 was diluted 100-fold by fresh medium B and 10 ml of this diluted culture was shaken in a water bath at 37°C. After 2 hours of incubation, various inhibitors of bacterial growth were added to the cultures at the middle of exponential growth and further incubation was carried out to follow the growth of bacteria under these conditions. At appropriate time intervals, the extent of bacterial growth was determined photometrically and compared with the growth in broth without inhibitors.

Results

Inhibitory Effect of TC on Bacterial Growth

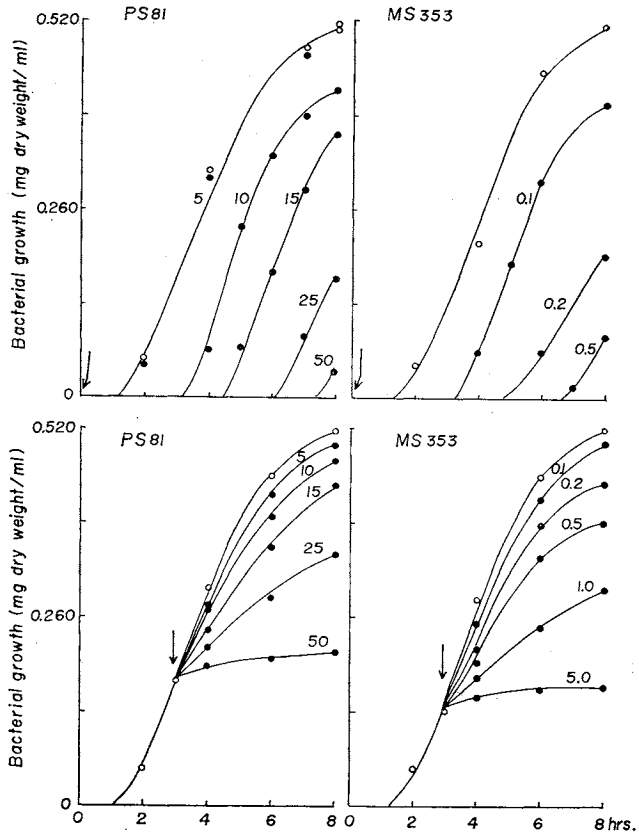
As shown in Fig. 1 (upper graphs), a prolongation of the lag period was observed with increased concentrations of TC in the culture medium, but the growth rates following the lag period were found to be similar to those of the control without TC. Within a period of 8 hours complete inhibition of strain PS81 occurred at a concentration of 50 mcg of TC/ml, and 0.5 mcg of TC/ml in strain MS353. When antibiotic was added to the cultures at the middle of logarithmic growth, it was found that 50 mcg of TC/ml caused an immediate cessation of growth of strain PS81, whereas an addition of only 5.0 mcg/ml had scarcely any effect (Fig. 1, bottom graphs). In contrast thereto, the growth of the TC-sensitive strain MS353 was completely inhibited in the medium containing 5.0 mcg of TC/ml. About 10% reduction of optical density (O. D.) of the cultures PS81 and MS353 was achieved by addition of 10 and 0.2 mcg of TC/ml, respectively.

Rise in Level of TC-Resistance by Prior Treatment with the Drug

It was found that prior treatment of strain PS81 with subinhibitory concentrations of TC enhanced the level of TC-resistance, and induced populations were capable of growing on plates containing 400 mcg of TC/ml. In order to determine the time required for complete induction of TC-resistance, strain PS81 was inoculated with shaking in medium B containing 0.5 mcg of TC/ml. At appropriate time intervals, bacterial cells were withdrawn and inoculated in medium B containing 50 mcg of TC/ml to see whether they are capable of growing in this medium. As shown in Fig. 2, prior treatment for 30 minutes caused a rise in level of TC-resistance, and the induction for TC-resistance was found to be complete within 2 hours of incubation in the induction mixture.

Fig. 1. Effect of various concentrations of TC on the growth of staphylococci.

Numbers indicate mcg of TC/ml (•——•). In the case of experiments represented in the bottom curves, TC was added at the time indicated by arrow. Bacterial strains used are indicated in the figure. Control (○——○), bacterial growth without TC.



The results of experiments determining the optical concentration of TC for induc-

Fig. 2. Effect of incubation time on the rise in level of TC resistance

Samples were withdrawn from the induction culture in the broth containing 0.5 mcg of TC/ml at various time intervals and inoculated in medium B containing 50 mcg of TC/ml to determine the rise in level of TC resistance. Details are described under Materials and Mdthods. ○——○, medium B without TC; •——•, medium B with TC (50 mcg/ml). Numbers indicate time (hours) of incubation in broth containing 0.5 mcg of TC/ml.

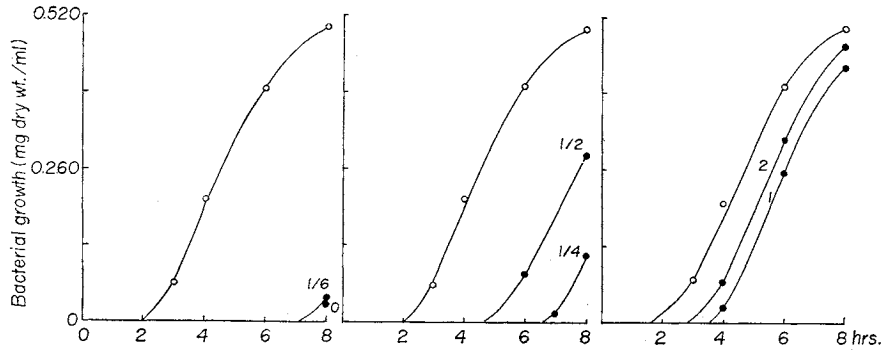


Fig. 3. Effect of TC concentrations in induction mixtures on the rise in level of TC resistance.

Induction of TC resistance was carried out by the method described in Materials and Methods in medium B containing various concentrations of TC. After 2 hours of incubation, the induction culture was inoculated in medium B containing 50 mcg of TC/ml to examine the rise in level of TC resistance. Numbers indicate mcg of TC/ml in induction culture. For circle symbols, see Fig. 2.

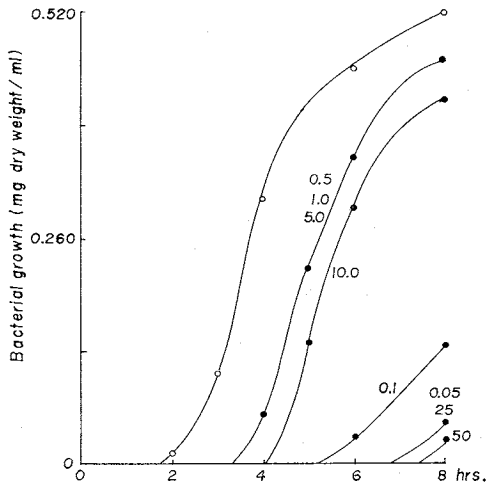
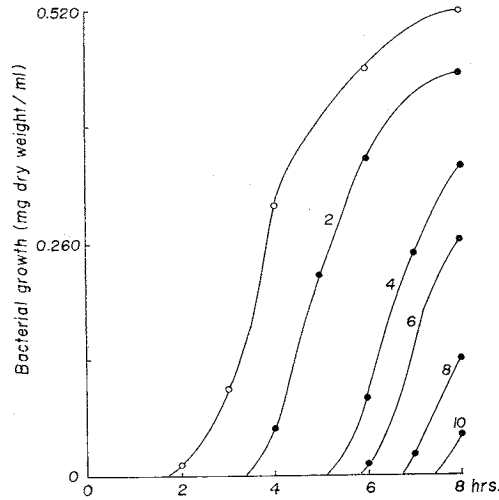


Fig. 4. Loss of TC resistance of the induced population of strain PS81 after incubation in TC-free medium.

Induction of TC resistance was carried out by incubation of strain PS 81 in medium B containing 0.5 mcg of TC/ml. After incubation for 2 hours at 37°C, bacterial cells were harvested by centrifugation, washed once with medium B and resuspended in the original volume B. After various time intervals of incubation, loss of TC resistance was examined by inoculating them in medium B containing 50 mcg of TC/ml. Numbers indicate time of incubation in TC-free medium. For circle symbols, see Fig. 2.



tion of TC-resistance are shown in Fig. 3. Concentrations of 0.5~10.0 mcg of TC/ml in induction cultures were found to be sufficient to complete the induction of TC-resistance within 2 hours of incubation. The induced populations could now grow in the medium containing 50 mcg of TC/ml. However, prior treatment of PS81 in the medium containing less than 0.5 mcg or more than 10.0 mcg of TC/ml did not enhance the level of TC-resistance. Accordingly, bacterial growth in the medium containing 50 mcg of TC/ml was delayed.

Loss of TC-Resistance after Incubation in Drug-free Medium

The loss of TC-resistance of induced populations was examined by inoculating the cells in TC-free medium. As shown in Fig. 4, the growth of induced cells in the medium containing 50 mcg of TC/ml was more and more delayed with increasing time of incubation in TC-free medium. Finally, the TC-resistance of previously induced populations was lost after incubating them for 10 hours in TC-free medium.

Inhibitors of Induction of TC-Resistance

Strain PS81 was inoculated into medium B containing either 5.0 mcg of chloramphenicol/ml or 2.0 mcg of actinomycin D/ml, and was shaken at 37°C. After 2 hours of incubation, the cells were spun down by centrifugation, washed once, and were inoculated into medium B containing 50 mcg of TC/ml to observe the rise in level of TC-resistance. Preliminary experiments had made it sure, that 5.0 mcg of chloramphenicol/ml or 2.0 mcg of actinomycin D/ml of medium B did not affect the growth of strain PS81.

As shown in Table 1, both chloramphenicol and actinomycin D inhibited the induction of TC-resistance by their presence in the induction mixtures.

In contrast thereto, they could not inhibit the induction of TC-resistance if they were added simultaneously with TC to the induction mixtures, *i.e.*, without prior pretreatment of the bacterial cells with these agents. These results suggest that induction of TC-resistance takes place rapidly after contact of PS81 with TC, and that chloramphenicol is only able to inhibit the inductive process for TC-resistance if it is in contact with the bacterial cells prior to the addition of TC. Moreover, it may be concluded from these results that protein synthesis is required for induction of resistance to TC.

Table 1. Effect of inhibitors on the induction of TC resistance

Induction mixture [a]	Turbidity at [c]			
	0 min.	120 min.	240 min.	480 min.
Tetracycline (C) [b]	0	0.04	0.690	0.990
TC+Chloramphenicol	0	0	0	0.03
TC+Actinomycin D	0	0	0	0.03
None	0	0	0	0.05

a) PS 81 was inoculated in medium B containing various drugs as shown in the table.
b) 0.5 mcg of TC/ml was used for induction.
c) After incubation for 30 minutes, bacterial cells were harvested by centrifugation, washed once with medium B and inoculated in medium B containing 50 mcg of TC/ml to follow the rise in level of TC resistance.

Table 2. Effect of histidine on the induction of TC resistance in S1419-T

First incubation [a]	Second incubation [b]	Turbidity at	
		0 min.	480 min.
With histidine	Tetracycline	0	0.750
	None	0	1.020
Without histidine	Tetracycline	0	0.020
	None	0	0.980

a) S1419-T was inoculated in synthetic medium containing 0.5 mcg of TC/ml with or without 0.05 mcg of histidine/ml.
b) After incubation for 2 hours, bacterial cells were harvested by centrifugation, washed once with synthetic medium and resuspended in medium B to make a 100-fold dilution of the original culture. The diluted culture was incubated with or without TC (50 mcg/ml) to examine the rise in level of TC resistance.

Effect of Histidine Auxotrophy on Induction of Resistance to TC

The effect of histidine deprivation on induction of TC-resistance was studied with strain *his*$^-$ S1419-T. Cells of this strain were harvested by centrifugation after 18 hours of incubation in medium B, washed twice with 0.9 % saline, and resuspended in 10 ml of synthetic medium devoid of histidine. The bacterial suspension was divided into two portions, each being filled into L-shaped tubes and shaken at 37°C to exhaust of histidine. After 2 hours of incubation, histidine was added to one of the tubes, and 0.5 mcg of TC/ml was added to both cultures to initiate the induction of TC-resistance. After further 2 hours of incubation with shaking, cells from each tube were examined for their ability to grow in the medium containing 50 mcg of TC/ml.

As shown in Table 2, strain S1419-T could not acquire TC-resistance by prior treatment with TC in the absence of histidine, but induction of TC-resistance took place if histidine was present in induction mixture.

Discussion

It is a known fact that there are two types of macrolide resistance in microorganisms: constitutive and inducible resistances[6]. Microorganisms carrying the genetic information for inducible resistance acquire resistance to a macrolide antibiotic by pretreatment with subinhibitory concentrations of the drug, but the resistance of induced populations is lost when they are grown in a medium without inducer[2,5].

According to the results described in this article, the rise in level of TC-resistance in *S. aureus* by prior treatment with subinhibitory concentrations of the drug is the result of the induced type of resistance. The characteristic features of induction for TC-resistance are summarized as follows:

(1) A short time of prior treatment of microorganisms with low concentrations of TC is enough to complete the induction of TC-resistance.

(2) Rapid loss of TC-resistance in previously induced populations took place by inoculating them in the medium without TC.

(3) Acquisition of TC-resistance after induction was to be paralleled by a decrease in the accumulation of TC in microorganisms (INOUE, unpublished data).

The observation that the presence of chloramphenicol in induction mixtures or histidine deprivation in culture of the *his*$^-$ auxotroph strain prevents the initiation of induction of TC-resistance indicates that protein synthesis may be necessary for this process. According to previous papers erythromycin[2,5,6,9], chloramphenicol[7] and tetracycline are active inducers in staphylococci in spite of their inhibion of protein synthesis. The mechanism of induction for resistance to such drugs including TC has not been fully investigated.

Since the initiation of induction of TC-resistance could be inhibited only after pretreatment of the staphylococci with chloramphenicol the process of induction seem to take place very rapidly when TC is accumulated in the cells and attached to the site responsible for the initiation of induction.

The TC-resistance of induced populations still persisted even after 8 hours of incubation in drug-free medium and was finally lost after 10 hours of incubation. These results may suggest that concentrations of TC sufficient for induction of drug resistance still exist in the cells even after 8 hours of incubation in drug-free medium.

According to a survey, 137 TC-resistant strains of *S. aureus* which were selected at random from our culture collection of more than 5,000 strains, were found to be all inducible for TC-resistance. We could not detect any strain constitutively resistant to TC. The genetic determinants for TC-resistance and those responsible for induction are not fully investigated. The mechanism of induction and accumulation of TC into microorganisms will be described elsewhere.

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