

TRANSDUCTION OF DRUG RESISTANCE TO TETRACYCLINE,  
CHLORAMPHENICOL, MACROLIDES, LINCOMYCIN AND  
CLINDAMYCIN WITH PHAGES INDUCED FROM  
*STREPTOCOCCUS PYOGENES*

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Strains of *Streptococcus pyogenes* isolated from pediatric patients with acute infections which were resistant to one or more of the antibiotics, tetracycline (TC), chloramphenicol (CP), macrolide antibiotics (erythromycin, kitasamycin, oleandomycin, josamycin), lincomycin (LCM) and clindamycin (CLM), were used for transduction of drug resistance. These drug-resistant strains were treated with mitomycin C to induce phages and transduction of drug resistance was attempted by means of phages so induced. It was found that transduction of resistance to the above antibiotics was possible. The transductants obtained on TC-containing selective agar plate were resistant to TC alone while those produced on CP- or erythromycin (EM)-containing selective agar plate were resistant to CP, macrolide antibiotics (Mac), LCM and CLM. From this finding, it was inferred that transduction of resistance to TC, CP, Mac, LCM and CLM *via* phages occurred in two different patterns, *i. e.*, transfer of resistance to TC alone and that of resistance to CP, Mac, LCM and CLM. All of the transductants obtained were found to belong to group A. In T-typing, they were of the same T-12 type as the donor and recipient strains in a majority of cases though some were not typable.

In recent years, the isolation of *Streptococcus pyogenes* resistant to various antibiotics has been reported abroad<sup>1-6)</sup> as well as in Japan.<sup>7,8,15)</sup> Some reports also indicate that these drug-resistant *S. pyogenes* are predominantly of the T-12 type.<sup>4,15)</sup> Since 1972, we have investigated and reported<sup>9)</sup> the drug resistance and its mechanism of *S. pyogenes* isolated from children with acute respiratory infections or scarlet fever. The present report deals with the transduction of drug resistance with phages induced from these drug-resistant *S. pyogenes*.

### Materials and Methods

#### Bacterial strains

*S. pyogenes* were isolated from clinical sources and 114 strains used. To select the recipient strains, phage lysates were obtained with the procedure described below from the 114 strains, and they were spotted onto blood agar plates previously spread with these strains. Observations were then made for bacteriolysis and those strains which showed a large number of phage lysates were selected as recipient strains. There were only TK-4958 and TK-4983 found among the 114.

The 8 donor strains were selected at random from 114 strains which held phages capable of lysing the selected recipient strains and which had different patterns of drug resistance. There were TK-4803, TK-4923, TK-4924, TK-4943, TK-4960, TK-4905, TK-4917 and TK-4919.

#### Media

Heart Infusion agar (HI agar, Eiken, Tokyo) supplemented with 5% defibrinated sheep blood was used for the determination of drug resistance. For the growth in liquid medium was used Todd Hewitt broth (TH broth, Difco) usually. Blood agar plates (TH broth +1.5%

agar +5% defibrinated sheep blood) were used for counting the number of phage plaques for phage titer and selection of transductants. The addition of blood to the agar plate makes the identification of plaques easier. As selective plates for transductants, blood agar plates containing TC 2  $\mu\text{g/ml}$ , CP 5  $\mu\text{g/ml}$  or EM 0.25  $\mu\text{g/ml}$  were used.

#### T-typing and grouping

This was done with the agglutination method described by TAKIZAWA *et al.*<sup>9)</sup> The cells used in the transduction study were all group A *S. pyogenes* of the T-12 type.

#### Preparation of phage lysates from donor strains

Bacteria (0.5 ml) cultured overnight in 10 ml of TH broth were inoculated into 10 ml of TH broth and incubated with shaking for 4 hours at 37°C. Mitomycin C (Kyowa Hakko Co., Ltd. Japan) was then added to the culture to a final concentration of 0.15  $\mu\text{g/ml}$  and the mixture was further incubated with shaking for 1 hour. After centrifugation, the supernatant was removed and the bacterial sediment was suspended in 10 ml of fresh TH broth and incubated with shaking for 5 hours. Then, the culture was centrifuged for 20 minutes at 3,000 r.p.m. and the supernatant was filtered through a 0.40  $\mu$  Millipore filter (MF-40, Kowa Kizai Co., Ltd. Japan) to obtain a phage lysate. The phage titer was determined by counting the number of plaques in recipient cells on a blood agar plates.

#### Transduction

The overnight culture of recipient cells was incubated in TH broth with shaking for 4 hours at 37°C. Then, 1 ml of the culture cells was mixed with 1 ml of phage lysate which was adjusted to yield a multiplicity of phage infection (MOI) about 0.1, and the mixture was shaken for 1 hour at 37°C. After shaking, it was immediately cooled and centrifuged. The sedimented cells were washed three times with cooled TH broth to remove free phage particles. They were then spread on selective agar plates containing antibiotics and incubated for 48 or 72 hours at 37°C. The colonies that grew on the selective agar plates were picked up, passed through three serial cultures on blood agar plate, subjected to grouping and T-typing, and finally tested for sensitivity to various antibiotics. The antibiotics used in sensitivity test were: penicillin G(PC-G), TC, CP, EM, kitasamycin (LM), oleandomycin (OM), josamycin (JM), LCM and CLM.

#### Electron microscopic observation of phages

The bacterial culture treated with mitomycin C in the procedure as described above was collected in a quantity of 10 ml every 1 hour. To each sample thus collected, 2% glutaraldehyde (0.15 M phosphate buffer at pH 6.1) was added, and it was centrifuged. To the sediment obtained, 2% glutaraldehyde was further added, and it was fixed for 2 hours at 4°C. After being washed three times with phosphate buffer and addition of 1%  $\text{OsO}_4$  solution, the sample was fixed overnight at 4°C. The following day, after dehydration with ethanol and acetone, it was embedded in Epon 812. The ultrathin section was prepared with a Porter-Blum MT-1 microtome using a glass knife. After double-staining with uranyl acetate and lead nitrate, the section was observed under Hitachi HU-11E electron microscope. For the observation of phage particles, the specimen was subjected to negative staining with 2% uranyl acetate in aqueous solution.

## Results

### Induction of Phages with Mitomycin C

The optimum concentrations of mitomycin C to induce phages from *S. pyogenes* ranged from approximately 0.1  $\mu\text{g/ml}$  to 0.2  $\mu\text{g/ml}$ ; a high titer of phages was obtained with these concentrations.

Shown in Fig. 1 is the ultrathin section of cells about 3 hours after treatment with mitomycin C. Within the cells, phages are seen as particles with a high electron density. Fig. 2

Fig. 1. Ultrathin section about 3 hours after treatment with mitomycin C  $\times 101,500$ .

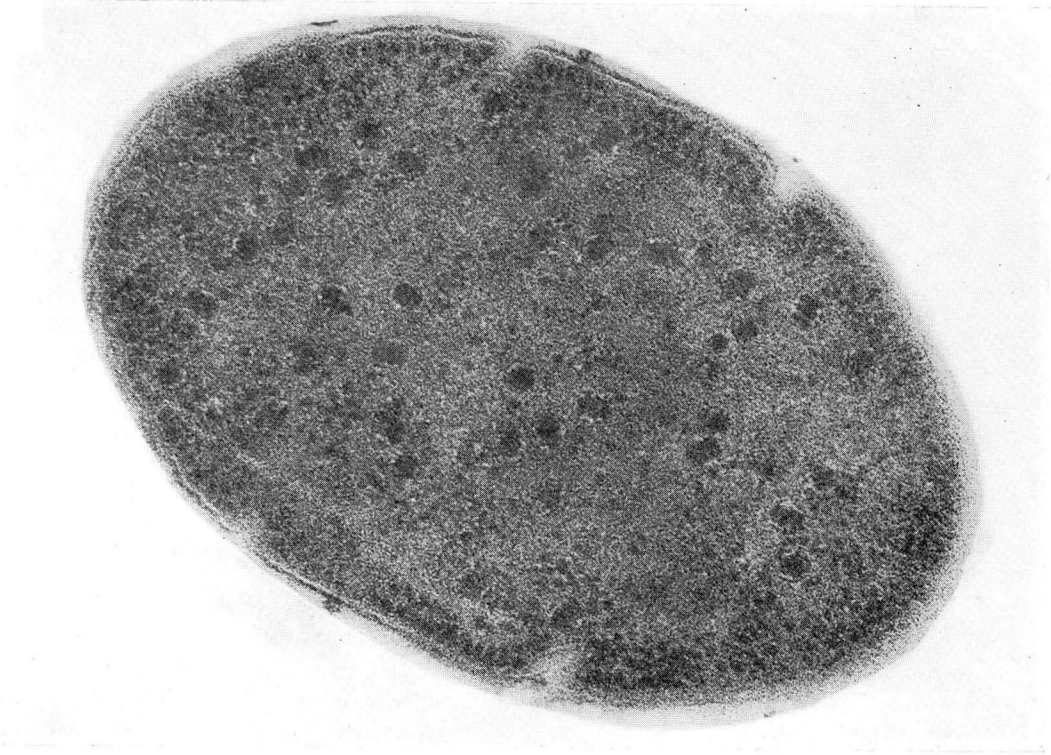


Fig. 2. The phage particles with negative staining  $\times 150,000$ .

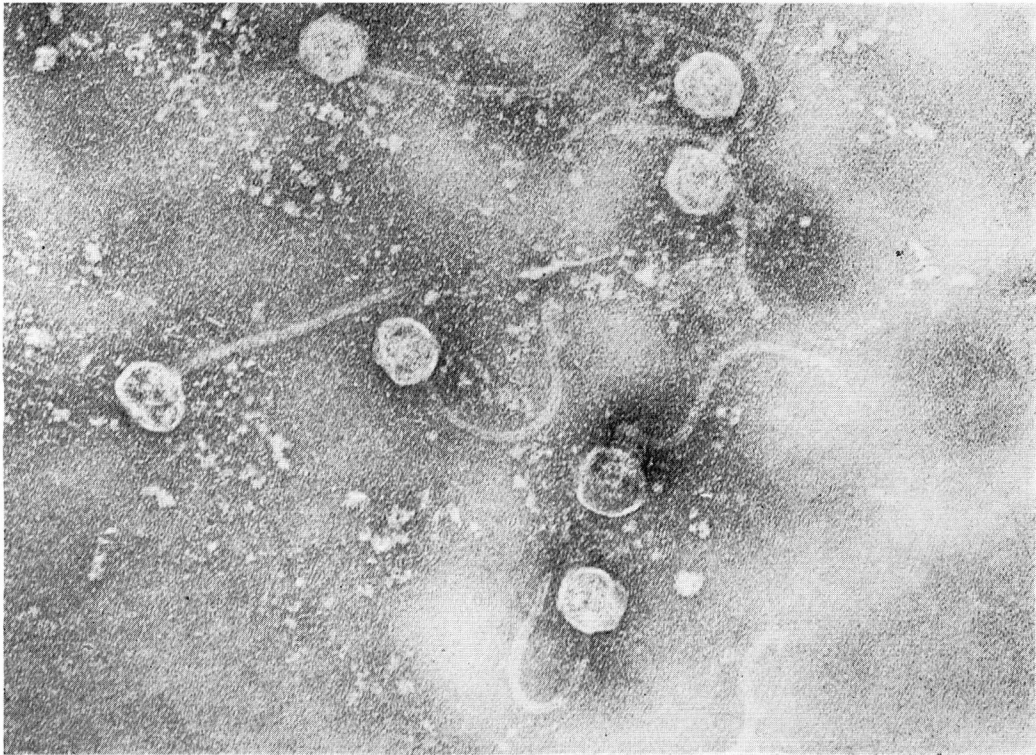


Table 1. Drug resistance and T-type of the strain used.

	Strain	T-type	Drug resistance (MIC: mcg/ml)							
			TC	CP	EM	LM	OM	JM	LCM	CLM
Donor strain	T K-4803	T 12	50	50	>100	>100	>100	>100	>100	>100
	T K-4923	T 12	50	50	>100	>100	>100	>100	>100	>100
	T K-4924	T 12	50	50	>100	>100	>100	>100	>100	>100
	T K-4943	T 12	50	50	>100	>100	>100	>100	>100	>100
	T K-4960	T 12	50	50	>100	>100	>100	>100	>100	>100
	T K-4905	T 12	100	25	0.05	0.39	0.78	0.39	0.1	0.05
	T K-4917	T 12	50	3.13	0.05	0.2	0.39	0.2	0.1	0.05
	T K-4919	T 12	0.2	25	0.1	0.78	0.78	0.78	0.1	0.03
Recipient strain	T K-4958	T 12	0.2	1.56	0.05	0.2	0.39	0.2	0.1	0.05
	T K-4983	T 12	50	3.13	0.05	0.39	0.78	0.39	0.1	0.05

Drug resistance was determined by the agar dilution method.  
 Medium: HI agar (Eiken Chem. Co., Tokyo)+5% sheep blood.  
 Inoculum size:  $10^7$ /ml.

shows the phage particles of the phage lysate observed with negative staining. The phages appear to have regular-hexagonal head and non-contractile tail. The head size is about 500 Å and the tail is about 2,400 Å.

#### Transduction of Drug Resistance

Shown in Table 1 are the donor and recipient strains used in the present study which were selected from our stock of 114 strains. The drug sensitivity of these strains expressed as MIC.

The results of transduction of drug resistance experiment are shown in Tables 2 and 3. Transduction was possible with phages induced from any donor strains. Table 2 summarizes the results obtained with the recipient strain TK-4958 which was sensitive to TC, CP, Mac, LCM and CLM. The transduction of drug resistance was attempted in this recipient strain using the phage lysates induced from the 5 donor strains (TK-4803, TK-4923, TK-4924, TK-4943 and TK-4960) which were resistant to TC, CP, Mac, LCM and CLM. It was found that the transductants obtained were classifiable into two different groups in terms of resistance pattern. Namely, the transductants grown on TC-containing selective agar plate were resistant only to TC while those grown on CP- or EM-containing agar plate were resistant to CP, Mac, LCM and CLM.

Table 2. Transduction of TC, CP, Mac, LCM and CLM resistance with phage lysates from donor strains to recipient TK-4958.

Strain	Donor		MOI	Selective drug	Transductants	
	Pattern of drug resistance	PFU of phage lysates			No. of transductants	Pattern of drug resistance
TK-4803	TC,CP, Mac, LCM, CLM	$2.0 \times 10^7$	0.1	TC	7	TC CP,Mac,LCM,CLM
				CP	5	
				EM	0	
TK-3923	TC,CP, Mac, LCM, CLM	$1.6 \times 10^7$	0.08	TC	1	TC CP,Mac,LCM,CLM CP,Mac,LCM,CLM
				CP	9	
				EM	1	
TK-4924	TC,CP, Mac, LCM, CLM	$1.2 \times 10^7$	0.06	TC	13	TC CP,Mac,LCM,CLM CP,Mac,LCM,CLM
				CP	13	
				EM	1	
TK-4943	TC,CP, Mac, LCM, CLM	$1.0 \times 10^7$	0.08	TC	99	TC CP,Mac,LCM,CLM
				CP	0	
				EM	2	
TK-4960	TC,CP, Mac, LCM, CLM	$2.0 \times 10^7$	0.15	TC	17	TC
				CP	0	
				EM	0	
TK-4905	TC,CP	$1.0 \times 10^7$	0.08	TC	62	TC CP
				CP	6	
				EM	0	
TK-4917	TC	$1.2 \times 10^7$	0.09	TC	8	TC
				CP	0	
				EM	0	
TK-4919	CP	$0.9 \times 10^7$	0.05	TC	0	CP
				CP	5	
				EM	0	

MOI: Multiplicity of phage infection.

Table 3. Transduction of TC, CP, Mac, LCM and CLM resistance with phage lysates from donor strains to recipient TK-4983 (TC).

Donor			MOI	Selective drug	Transductants	
Strain	Pattern of drug resistance	PFU of phage lysate			No. of transductants	Pattern of drug resistance*
TK-4803	TC.CP. Mac. LCM. CLM	$2.0 \times 10^7$	0.1	CP EM	4 0	CP.Mac.LCM.CLM
TK-4923	TC.CP. Mac. LCM. CLM	$1.6 \times 10^7$	0.08	CP EM	15 2	CP.Mac.LCM.CLM CP.Mac.LCM.CLM
TK-4924	TC.CP. Mac. LCM. CLM	$1.2 \times 10^7$	0.06	CP EM	4 0	CP.Mac.LCM.CLM
TK-4943	TC.CP. Mac. LCM. CLM	$1.0 \times 10^7$	0.08	CP EM	7 1	CP.Mac.LCM.CLM CP.Mac.LCM.CLM
TK-4960	TC.CP. Mac. LCM. CLM	$2.0 \times 10^7$	0.15	CP EM	1 0	CP.Mac.LCM.CLM

\* Pattern of drug resistance was expressed excluding TC as TK-4983 was resistant.

Transduction with phage lysates induced from TK-4905 resistant to both TC and CP, TK-4917 resistant to TC alone and TK-4919 resistant to CP alone yielded TC and CP resistance separately.

Table 3 shows the results of the transduction of drug resistance using the recipient strain TK-4983 which was resistant only to TC. In the transduction, two kinds of selective agar plate—CP-containing and EM-containing—were used with the phage lysates obtained from the 5 donor strains listed in Table 1. Transduction with these phage lysates on either of the agar plates yielded simultaneously resistance to CP, Mac, LCM and CLM. That is, all transductants produced were resistant to TC, CP, Mac, LCM and CLM.

The transductants obtained from the greatest number of donor strains were resistant to TC alone and the transduction frequency ranged from  $10^{-6}$  to  $10^{-7}$ .

The drug resistance of the transductants to each antibiotics tested was almost of the same order as that of the donor strain. The grouping and T-typing of the transductants obtained in the recipient strain TK-4983 revealed that all of these transductants were of group A and of T-12 type. However, some of the transductants produced in the recipient TK-4958 were found not T-typable.

### Discussion

It has been thought that *S. pyogenes* is frequently resistant to TC but at the same time not prone to acquire resistance to other antibiotics. Recently, however, it has been reported abroad<sup>1-6)</sup> as well as in Japan<sup>7,8)</sup> that this organism has begun to acquire resistance to CP, Mac, LCM and CLM, in addition to TC, thus posing a major problem in the clinical field. The *S. pyogenes* being reported throughout the world is predominantly of T-12 type,<sup>10)</sup> and drug resistance is found by far the most frequently in this T type and in such types as T-4. The strains of *S. pyogenes* which have become resistant to Mac, LCM and CLM also develop resistance to TC and CP in most cases.<sup>7,8)</sup> Some study has already been made by us<sup>8)</sup> as to a possible mechanism of drug resistance in the organism, and it has been assumed that the resistance to CP may be due to the presence of an inactivating enzyme while resistance to TC and Mac may be due not to some inducible factor. In our subsequent study, it was demonstrated



that CP resistance was induced by an inactivating enzyme and that transductants to CP resistance by transduction also came to produce an inactivating enzyme. The details of this study are now in the process of publication as a separate paper.

It has also been a matter of concern for us to look into how such drug resistance is transferred. Though we guessed that resistance was probably transferred via phages as in the case of *Staphylococcus aureus*,<sup>11,12)</sup> no experimental work was possible because there seemed to be no appropriate indicator strains available in Japan. Later, however, we found by chance some strains among our stock that could serve as recipient strains. As in *Staphylococci*, *S. pyogenes* is reported to be lysogenic in nearly all cases,<sup>13,14)</sup> prior to the present study, we tested the 114 strains of *S. pyogenes* for their lysogeny and found that 86 (75.4 %) of these strains were lysogenic. Among the widespread T-12 type of strains, as many as 80 (92.0 %) of the 87 strains tested were found lysogenic.<sup>15)</sup> From high incidence of lysogenic strains, we assumed that in the development of multiple drug resistance the lysogenic phages in the bacterial cells might play an important role.

Studies on transduction of drug resistance in *S. pyogenes* by means of phages began with transduction of SM resistance by LEONARD *et al*.<sup>16,17)</sup> and are still in an early stage. Later, MALKE,<sup>18,19)</sup> using phages, reported a success in the transduction of resistance to macrolide antibiotics produced with nitrosoguanidine treatment. However, this resistance was produced artificially and thus seems to be intrinsically different from that of the clinically isolated strains that are multiply resistant to drugs, particularly all macrolides.

The present study was an attempt at transduction of drug resistance with the use of phages induced from multiple drug-resistant strains of *S. pyogenes* which were isolated from pediatric patients with scarlet fever and other acute respiratory infections. The strains used as donors included 5 resistant to TC, CP, Mac, LCM and CLM; 1 resistant to both TC and CP; 1 resistant to TC alone; and 1 resistant to CP alone, totalling 8 strains. Though the transduction of drug resistance was possible with phages induced from any of these strains, the transductants obtained could be classified into two different groups in terms of resistance pattern: those which showed resistance to TC alone and those resistant to CP, Mac, LCM and CLM. From this, it may be inferred that so far as the phages induced from *S. pyogenes* resistant to multiple drugs are concerned there are two types, *i.e.*, those which carry a factor responsible for resistance to TC alone and those bearing a factor responsible for resistance to CP, Mac, LCM and CLM jointly.

Furthermore, on the basis of the finding that the strains which could be used as recipients were of T-12 type and that transduction of TC resistance occurred most frequently, it may be guessed that the multiple drug-resistant T-12 type strains of *S. pyogenes* now posing a problem in clinical situations may have acquired resistance to TC first and then to CP, Mac, LCM and CLM.

At present, it is impossible for us to determine genetically whether such drug resistance is present on chromosome or as plasmid in the cell since we are not yet successful in elimination of resistance. In performing these studies, it is necessary to separate cells one by one in a gentle state. A search is now being made for a proper technique to carry out this. However, the finding that CP and Mac are 100 % cotransducible, strongly suggests that a cluster of genes concerned with drug resistance exists. Analyses of extrachromosomal DNA as well as of incompatibilities also remain to be done.

Finally, when observed under the electron microscope, the phages induced from the donor strains in the present study were found to be morphologically identical with A25 phages,<sup>19)</sup> with their end-plate structure being not distinctive.

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