Association of a Penicillin Resistance Gene with a Tetracycline Resistance Plasmid (P_{TP-2}) in *Staphylococcus aureus*

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On transduction with a lysogenic strain of *Staphylococcus aureus* isolated from a clinical specimen and having tetracycline (TC)-penicillin (PC)-chloramphenicol (CP)-resistant plasmids, the three-drug-resistant strain was frequently obtained. By repeatedly transducing from this strain, a strain (TP-2) having stable resistance to TC and PC could be obtained. In transformation with the deoxyribonucleic acid (DNA) of TP-2 as donor, all of the transformants obtained by selecting with either TC or PC were both TC and PC resistant. According to electron microscopy study of the covalently closed circular DNA of TP-2, the plasmid DNA size was $1.37 \pm 0.03 \mu\text{m}$ (2.84×10^6 daltons). The plasmid (P_{TP-2}) is presumed to be a new plasmid in which the PC resistance gene was integrated into the TC-resistant plasmid.

It has been reported that many of the drug resistance genes of *Staphylococcus aureus* are present on plasmids (13, 16, 20). These resistance genes are frequently transferred into recipient bacteria by co-transduction of two or three markers. For example, co-transduced markers for penicillin (PC)-macrolide (Mac) resistance (17), tetracycline (TC)-streptomycin (SM) resistance (5, 6), TC-sulfonamide (SA)-SM resistance (9), and TC-chloramphenicol (CP) resistance have been reported (7, 8, 23). Among the co-transduced resistances, only PC-Mac resistance is stable after being transferred. TC-SM resistance appears to be eliminated separately after being transferred by transduction (6). Inoue et al. (7) also reported TC-CP resistance to be similar: it is eliminated separately.

We have been studying the co-transduction of drug resistance genes of *S. aureus* and have found the co-transduction of resistance to TC, PC, and CP: we also obtained from the three-drug-resistant co-transductants the strain TP-2, which demonstrated complete linkage of the TC and PC resistance genes after subsequent transductions with TC selection. The TC-PC resistance of the TP-2 strain was on a single plasmid, and after deoxyribonucleic acid (DNA) transformation both resistances were completely transferred together by selecting with either TC or PC. Considering the transforma-

tion and the electron microscopy findings, the plasmid (P_{TP-2}) is presumed to be a combination of the PC resistance gene and TC plasmid.

MATERIALS AND METHODS

Strains. *S. aureus* MS1477 and MS353 were used: the former was a strain possessing plasmids resistant to three drugs, PC, TC, and CP (12), and the latter, which is susceptible to these antibiotics, was used as a recipient. Each strain was isolated from clinical specimens and given to us by S. Mitsuhashi.

Media. Penassay broth (PB; Difco) and nutrient agar (NA) medium were used for the usual bacterial multiplication; CY medium [composition: K_2HPO_4 , 7.0 g; KH_2PO_4 , 3.0 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.25 g; $(\text{NH}_4)_2\text{SO}_4$, 1.0 g; trisodium citrate, 0.5 g; glucose, 5.0 g; Casamino Acids, 2.0 g; yeast extract, 1.0 g; and distilled water to 1.0 liter; with the pH adjusted to 7.0] was used for labeling of bacteria, and brain heart infusion (Difco) and brain heart agar medium were used for the experiments on transformation.

Antibiotics. Mitomycin C (MMC), TC, CP, and PC-G were used.

Transduction. For transduction experiments, temperate phages of resistant bacteria were exclusively used. The phage lysate was prepared according to the method of Inoue et al. (7) utilizing MMC induction. The titer of the phage lysate was determined using MS353 as an indicator strain. For transduction, the PB overnight culture of a recipient was diluted 10-fold with PB and incubated with shaking at 37°C. One milliliter of the recipient culture (5.5×10^8 colony-forming units/ml) in exponential growth was mixed with an equal amount of the phage lysate, and, after allowing the mixture to stand for 30 min at 37°C, the bacteria were collected by centrifugation and suspended in 1.0 ml of PB.

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Then several 0.1-ml aliquots were spread on a selection plate. The selection plate was NA containing CP (12.5 $\mu\text{g/ml}$) or TC (25 $\mu\text{g/ml}$).

Elimination of drug resistance. For eliminating the resistance with ethidium bromide (EB; Boots Pure Drug Co.), a platinum loop of an overnight culture of resistant bacteria was inoculated to 2 ml of PB medium containing 2 μg of EB per ml, and after incubation for 18 h at 37 C the culture was diluted adequately and spread on an NA plate. After overnight incubation, the resistance of the formed colony was examined by the replica method. When resistance was eliminated with sodium dodecyl sulfate (SDS; Wako Junyaku), 0.1 ml of an overnight culture of resistant bacteria was inoculated to 9.9 ml of PB containing 0.002% SDS and then treated like the EB preparation. When resistance was eliminated by thermal treatment, the PB overnight culture of resistant bacteria was diluted 100-fold with fresh PB, and after overnight incubation at 43 C with shaking the resistance of the culture was examined by the method described above. In the experiment where the loss of resistance with time was determined, a 100-fold dilution was done, and then a part of the culture was sampled every hour during incubation at 43 C, and the resistance was examined as described above.

Separation of plasmid DNA. ^3H labeling of bacteria, bacteriolysis, and the separation of plasmid DNA by ultracentrifugation were performed according to the methods of Cohen and Miller (3) and Clewell and Helinski (2).

Test bacteria were spread on an NA plate to produce colonies, which were then incubated overnight in CY medium at 37 C. One milliliter of the overnight culture was diluted 10-fold with CY medium and incubated at 37 C with shaking. After incubating for 3 h (absorbance at 600 nm, 0.35), deoxyadenosine (250 $\mu\text{g/ml}$) (Sigma) was added. After 3 min, 10 μCi of [6- ^3H]thymidine per ml (12 Ci/mmol) (Daiichi Pure Chemicals Co. Ltd., Tokyo) was added, followed by shaking-incubation for a further 2 h. Immediately after incubation, the culture was cooled with ice, and the bacteria were collected by centrifugation. The bacteria were twice washed with TES buffer [0.05 M tris(hydroxymethyl)aminomethane(Tris)-hydrochloride, 0.05 M NaCl, and 0.05 M ethylenediaminetetraacetic acid (EDTA); pH 8.0].

The lysostaphin-Brij 58-Sarkosyl method was employed for bacteriolysis. The bacteria labeled with [^3H]thymidine were suspended in 25% sucrose (in TES buffer), and after adding EDTA (to a final concentration of 0.1 M) and lysostaphin (final concentration, 50 $\mu\text{g/ml}$) (Schwarz/Mann) the mixture was incubated for 10 min at 37 C. Brij 58 was added (final concentration, 0.5%) (Kao-Atlas Chemicals, Tokyo), and the mixture was incubated for a further 10 min. Sarkosyl (final concentration, 2.5%) was added, and the mixture was shaken slightly at 50 C to yield a clear and viscous solution, which was rendered less viscous with a pipette. A Hitachi ultracentrifuge for separation (type 65P) was employed for the EB-CsCl density gradient equilibrium centrifugation. A mixture of 6.55 g of CsCl, 2 ml of

EB solution (700 $\mu\text{g/ml}$), and 4 ml of redistilled water was put in a centrifuging tube, and after solution was accomplished 1 ml of the sheared lysate was layered on top, and the tube was gently turned over to blend the contents. A layer of paraffin oil was placed on top, and the mixture was centrifuged at 44,000 rpm at 20 C for 40 h. The bottom of the tube was pierced, and fractions of 3 drops were collected. Ten microliters of each fraction was spotted on a Whatman glass-fiber filter (GF/A, 2.4 cm), and after drying ^3H radioactivity was determined in a liquid scintillation counter (Packard model 3330) with a toluene scintillator. EB was extracted with an *n*-octanol solution saturated with CsCl from the fractions containing satellite DNA, which were then dialyzed against $1\times$ SSC (0.15 M NaCl plus 0.015 M trisodium citrate) to remove the CsCl. The resulting satellite DNA solution was used for the electron microscopy examination.

Electron microscopy examination of plasmid DNA. Plasmid DNA was observed with an electron microscope after a DNA-cytochrome *c* solution was denatured and spread on water (11) according to the methods of Follett and Crawford (4). A mixture consisting of 20 μl of the DNA solution and 10 μl of formaldehyde (37%) was put in a capillary, treated at 42 C for 10 min, cooled with ice, mixed with 0.9 ml of 1 M ammonium acetate (containing 0.5% formaldehyde) and 0.1 ml of 0.1% cytochrome *c*, and then spread on the surface of a 0.3 M ammonium acetate (containing 0.5% formaldehyde) solution. Carbon-coated specimen grids were touched face down to the surface of the solution and, after dehydration with ethanol, were dried and made into specimens by rotary shadowing with platinum-palladium. A Nihon Denshi type JEM 100C instrument was employed for electron microscopy.

Transformation experiment. The transformation experiment was performed according to the method of Lindberg et al. (14) and Sjöström et al. (22), wherein transforming DNA was extracted from a clear lysate according to the method of Clewell and Helinski (2).

(i) **Extraction of DNA.** The bacteria, in exponential growth in 500 ml of PB, were washed twice with 0.02 M Tris-hydrochloride buffer (containing 0.8% NaCl, pH 8.0) and then suspended in 6 ml of the buffer. To the suspension, 0.6 ml of 0.2 M EDTA and 0.1 ml of lysostaphin solution (4 mg/ml) were added, and after incubation at 37 C for 10 min 11 ml of distilled water (0 C) and 2 ml of SDS solution (5%) were added, followed by gentle shaking to obtain lysis. The lysate thus obtained was centrifuged at 40,000 rpm for 20 min in the Hitachi RP 65T rotor, and the supernatant fluid was collected. After removal of protein from the supernatant fluid by extraction with an equal amount of 0.05 M Tris-hydrochloride buffer (pH 7.5), saturated phenol (redistilled phenol-buffer, 4:1), DNA was obtained by precipitation with a twofold quantity of isopropanol. The isopropanol precipitation was performed twice to remove residual phenol, and separated DNA was collected by low-speed centrifugation and dissolved in 1 ml of 0.02 M Tris-hydrochloride buffer (containing 0.8% NaCl).

(ii) **Transformation.** The recipient bacteria, in exponential growth in 100 ml of PB, were washed twice in 100 mM CaCl₂ and suspended in 1 ml of 100 mM CaCl₂. One milliliter of the bacterial suspension (containing 10⁸ colony-forming units/ml) was mixed with 0.1 ml of the DNA solution and 0.1 ml of 200 mM CaCl₂. As a control, 0.02 M Tris-hydrochloride buffer (containing 0.8% NaCl) was used in place of the DNA solution. The mixture was allowed to stand at 37 C for 20 min, and the bacteria were collected, washed twice with sterilized physiological saline, and suspended in PB medium. After overnight incubation at 37 C, the bacteria were inoculated onto the selection medium. For the selection medium, brain heart infusion agar plates were used with a drug concentration of 25 µg of TC per ml or 0.2 µg of PC per ml.

RESULTS

Isolation of transductant (TP-2) having TC-PC resistance. On transduction with a phage obtained from MS1477 by MMC induction, transductants that acquired TC-PC-CP resistance were obtained in the recipient strain MS353 (multiplicity of infection, 0.36; selection drug, CP; transduction frequency, 1.0×10^{-7} ; number of transductants, 20). From these 20 transductants we selected one strain (designated as Td-16) which had rather stable resistance to the three drugs and in which it was easy to induce the lysogenic phage. Retransduction of the three drug resistances to strain MS353 was attempted. The results are shown in Table 1: when TC was used as the selective drug, many transductants were obtained with the resistance patterns TC-CP, TC-PC-CP, and TC. TC-PC-resistant strains were also found, but they represented only 5% of all transductants. When CP was used as the selection drug, the resistant strains obtained were almost invariably of types TC-CP or TC-PC-CP. Transductant Td-16 was treated with EB to study the loss of resistance. Among 240 colonies, CP resistance alone and PC-CP resistance were eliminated in

each strain (0.4%), respectively, though the data are not shown here. The above results indicate the contradiction between co-transduction of the drug resistance marker and the elimination of the resistance, as has often been reported (5-7, 23) previously. However, there has been no report about the co-transduction of TC-PC-CP resistance or TC-PC resistance in staphylococci. Among the transductants produced by transduction from Td-16 to MS353, a single three-drug-resistant strain (designated TPC) obtained by TC selection was used as a donor for retransduction into MS353. Contrary to expectations, no three-drug-resistant transductants were obtained, but TC-PC-resistant co-transductants and TC-resistant ones were found (Table 2). The rate of co-transduction of TC-PC resistance from TPC was 62%, remarkably higher than that from Td-16, 5%. One of the co-transductants obtained from TPC (designated TP-1) was used in the further transduction experiment as described above, and TC-PC-resistant co-transductants were obtained at a rate of 97 to 98%. In this experiment, the multiplicity of infection was varied, but the rate of co-transduction of TC-PC resistance in the transductants was constant; consequently, transduction of TC resistance and PC resistance did not appear to result from multiple infection. It was also found that TC-PC resistance could be completely co-transduced by repeating transduction from this one strain (designated TP-2).

From the above results, there is the sugges-

TABLE 1. Transduction from strain Td-16 to strain MS353^a

Selective drug	MOI ^b	Transduction frequency	No. of transductants	Transductants resistant to (%)				
				TC-PC-CP	TC-PC	TC-CP	TC	CP
TC	0.1	1.0×10^{-6}	37	24	5	43	27	0
CP	0.1	6.0×10^{-6}	240	38	0	61	0	1

^a Strain Td-16 was treated with 1 µg of MMC per ml in PB medium for 50 min and then incubated for a further 2 h in a fresh medium. After sterilizing with a membrane filter, it was used as a phage lysate. The concentration of the selective drug was 25 µg of TC per ml or 12.5 µg of CP per ml.

^b MOI, Multiplicity of infection.

TABLE 2. Transduction from strains TPC, TP-1, TP-2, and TP-3 to strain MS353^a

Donor	MOI ^b	Transduction frequency	No. of transductants	Transductants resistant to (%)	
				TC-PC	TC
TPC	0.01	5.0×10^{-6}	47	62	38
TP-1	0.07	9.0×10^{-5}	3500	97	3
	0.035	1.0×10^{-5}	191	97	3
	0.007	2.0×10^{-5}	87	98	2
TP-2	0.01	9.0×10^{-5}	283	100	0
	0.01	3.0×10^{-4}	723	100	0
TP-3	0.01	3.0×10^{-5}	231	100	0

^a The phage lysate was similarly prepared from each strain as described in Table 1. Strain TPC was a three-drug-resistant transductant from Td-16; strain TP-1 was a TC-PC-resistant transductant from strain TPC; strains TP-2 and TP-3 were TC-PC-resistant transductants from strains TP-1 and TP-2, respectively. The selective drug was TC in all cases.

^b MOI, Multiplicity of infection.

tion that in strain TP-2 the genes for PC resistance and TC resistance are stably combined with one another.

Elimination of resistance genes from TP-2. Elimination of resistance to TC and PC was attempted in strain TP-2 by treatment with EB and SDS, and by thermal treatment. The results are shown in Table 3. EB treatment caused elimination of PC and TC-PC resistances in 5.3 and 0.17%, respectively, whereas thermal treatment caused elimination of PC and TC-PC resistances in 0.25 and 3.6%, respectively, of the resulting colonies. With SDS treatment, only PC resistance was eliminated in 0.25% of the colonies. In any treatment, the elimination of TC resistance alone did not occur; elimination of TC resistance was always accompanied by elimination of PC resistance. The results were similar to those on TC-CP-resistant strains reported by Stiffler et al. (23).

For examining the elimination phenomenon more precisely, the change with time in the frequency of elimination of the resistance by thermal treatment was studied. TC resistance was eliminated more frequently. As seen in Fig. 1, elimination of PC resistance alone always occurred to less than 0.1%, compared with untreated controls. Elimination of TC resistance began to occur when the growth of bacteria entered stationary phase and reached 3.6% after 18 h. Those strains in which TC resistance was lost also showed all elimination of PC resistance. Consequently, it was suggested that the PC resistance gene appeared to be combined with the TC resistance gene but could be separated from it by EB treatment, and that only the PC resistance gene would spontaneously separate at low frequency.

Separation of plasmid DNA by ultracentrifugation. A lysate was prepared from bacteria labeled with [^3H]thymidine according to the lysostaphin-Brij 58-Sarkosyl method, and covalently closed circular DNA was separated by EB-CsCl density gradient equilibrium centrifugation. Strains MS353 and TP-2 were used in

FIG. 1. Growth of TC^r-PC^s, TC^s-PC^s strains resulting from strain TP-2 on incubation at 43 C. Symbols: (○) growth of bacteria, (●) TC^r-PC^s colonies, (Δ) TC^s-PC^s colonies.

FIG. 2. EB-CsCl ultracentrifugation of the DNA from strains TP-2 and MS353 labeled with [^3H]thymidine. The solid line is the ultracentrifugal pattern of DNA from strain TP-2 and the dotted line is that of MS353.

TABLE 3. Loss of resistance to TC and PC in strain TP-2 after 43 C growth and in presence of EB or SDS

Eliminated marker	No. (%) of colonies susceptible after:			
	No treatment	EB	SDS	43 C
TC-PC	0 (0)	2 (0.17)	0 (0)	43 (3.6)
PC	1 ^a (0.08)	64 (5.3)	3 (0.25)	3 (0.25)

^a Number of colonies susceptible to drug of 1,200 tested.

the experiment. As seen in Fig. 2, no peak of DNA, aside from that of chromosomal DNA, was recognized in strain MS353, whereas in strain TP-2 an obvious peak of satellite DNA was found. Consequently, TC-PC resistance genes appear to be in the cytoplasm as covalently closed circular DNA.

Transformation of TC resistance and PC resistance by DNA of TP-2. Transforming DNA was extracted from strain TP-2, and the transfer of TC resistance and PC resistance by transformation was studied using strain MS353 as the recipient. Resistant bacteria were se-

lected on a plate containing either TC or PC. Bacteria selected as resistant to either TC or PC also possessed resistance to both antibiotics (Table 4). Consequently, it appears that the resistance genes for both TC and PC are completely linked in strain TP-2 and are on the same plasmid.

Electron microscopy of TC-PC plasmid (P_{TP-2}). The fractions at the peak of satellite DNA separated by ultracentrifugation were collected, and after extraction of EB with *n*-octanol CsCl was removed by dialysis. As described in Materials and Methods, a specimen was prepared by a spreading procedure, rotary shadowed with platinum-palladium, and then subjected to electron microscopy (Fig. 3).

The length of 44 circular DNA molecules was measured, from which the molecular weight was calculated. The mean length for 41 DNA molecules was $1.37 \pm 0.03 \mu\text{m}$, which corresponds to a molecular weight of 2.84×10^6 . The remaining three molecules appeared to be trimers or tetramers (Table 5). These values are identical with those reported for the TC plasmid by Stiffler et al. (23). As the plasmid obtained here had both TC and PC resistance genes, it was designated P_{TP-2} .

DISCUSSION

It has been shown that in *S. aureus*, PC resistance and erythromycin resistance genes are on the same plasmid (PC plasmid), but in general each gene responsible for drug resistance is considered to be on a separate plasmid in a cell. However, resistance genes that are thought to be independent, for example, TC and SM resistance genes and TC and PC resistance genes, are reported to be co-transduced. In retransducing from a co-transduced strain, individual resistances are sometimes seen to segregate, whereas simultaneous elimination of resistances is not always seen. The state of the resistance plasmid in a cell of *S. aureus* has not been sufficiently revealed.

Recently, Stiffler et al. succeeded in separating a strain that could completely co-transduce TC resistance on selection with CP (23). However, in this strain, CP resistance alone was eliminated by thermal treatment, and when

transformants were separated by TC selection the frequency of co-transformation of TC-CP resistance was greatly reduced. This result has suggested that both TC and CP resistance genes would not be stably combined on the same plasmid, as seen in a PC plasmid combined with a Mac resistance gene.

Plasmid P_{TP-2} , isolated in our experiments, was obtained from Td-16, which is a three-drug-resistant strain, and the gene structure for the drug resistance of Td-16 appears to be different from that of its parental strain, MS1477; that is, from MS1477, co-transduction of two- or three-drug resistance accompanied with PC resistance was obtained by chance, but from strain Td-16 it was, rather, the rule. It was similarly seen with another strain, Td-2, which was isolated simultaneously with Td-16 (unpublished data). It is thus suggested that in strain Td-16 PC resistance or CP resistance genes can be readily recombined to the TC resistance plasmid, though the state of each resistance gene in strain Td-16 has not been fully revealed as yet.

In strain TP-2, obtained by repeated transduction, the plasmid carries complete TC-PC resistance. This was proven by the fact that co-transfer of TC and PC resistance was shown by both transduction and transformation, and that TC and PC resistances were eliminated together on thermal treatment.

At present, PC, TC, and CP plasmid DNA has been separated by ultracentrifugation, and the shape and length has been observed by electron microscopy by many workers. Rush et al. reported that PC plasmid DNA was $9.4 \mu\text{m}$ long (21), TC plasmid DNA was $1.41 \pm 0.05 \mu\text{m}$ long (18), and CP plasmid DNA was $1.6 \pm 0.1 \mu\text{m}$ long (18). Stiffler et al. reported that TC plasmid DNA was $1.37 \pm 0.02 \mu\text{m}$ (23) and that the CP DNA plasmid was $1.47 \pm 0.01 \mu\text{m}$ (23) long. In each report, the TC and CP plasmids were miniplasmids of about 1.3 to 1.6 μm , whereas the PC plasmid appeared to be about 6 to 7 times larger. Odakura et al. (19) have reported that the β -lactamase gene in an R factor was about 2.5×10^4 daltons. If the PC resistance gene responsible for penicillinase production in the PC plasmid in *S. aureus* is the same size as the β -lactamase gene, it may be generally neglected in influencing the size of the TC plasmid. The plasmid obtained in our experiment, P_{TP-2} , was 2.4×10^6 daltons. Consequently, the plasmid size will be reasonable only if it is presumed that the PC resistance gene attaches to, or recombines with, the TC plasmid. The PC resistance gene, which is an exogenous genetic determinant for the TC plasmid, may segregate spontaneously with a low

TABLE 4. Co-transformation of TC resistance and PC resistance with DNA of strain TP-2

Selective drug	Transformation frequency	No. of transformants	Co-transformation (%)
TC	1.4×10^{-7}	43	100
PC	1.7×10^{-7}	53	100

FIG. 3. Electron micrographs of open circular DNA molecules from strain TP-2. Bar represents 0.25 μm .

frequency, and the genetic determinant may be diluted out during growth, since it lacks the genetic part determining autonomous replication. It may be more frequently segregated from the TC plasmid by chemicals such as EB and SDS. The most reasonable explanation for coelimination of TC and PC resistance genes by thermal treatment would be inhibition of plasmid replication itself, according to the suggestion of Stocker (24). May et al. (15) have also suggested this as the most attractive hypothesis for the loss of TC or PC resistance from

strain E169 by thermal treatment. The fact that the TC plasmid was generally more stable when compared with any other plasmid conferring drug resistance may be explained if the gene determining TC resistance or the gene(s) governing autonomous replication of the TC plasmid is homologous with chromosomal DNA. This speculation is based on the following evidence. Some strains having unstable TC resistance, such as strain E169, could be stabilized in a different host strain (10). It may be that the host chromosome of strain E169 has

TABLE 5. Contour length and estimated molecular weight of plasmid P_{TP-2} with DNA of strain TP-2

No. measured	Mean length $\pm$ SD ^a	Estimated molecular wt ^b
41	1.37 $\pm$ 0.03	2.84 $\times 10^6$
2	4.17 $\pm$ 0.24	8.64 $\times 10^6$
1	5.00	10.35 $\times 10^6$

^a SD, Standard deviation.^b Molecular weights were calculated by assuming a mass of 2.07×10^6 per μm .

lost some of the homologous region of the TC plasmid. Also, there is a report which indicates that in a variant of strain PS80 there is a duplication of the penicillin resistance region to give one copy on the chromosome and one copy linked to cadmium-mercury resistance genes on a plasmid (1). Grubb et al. (6) presumed that co-transduction would occur when resistant plasmids are associated reversibly with each other in a cell, or when different resistant plasmids are carried together into a phage particle. Considering the process of formation of P_{TP-2} , the plasmid obtained in our experiment, association among plasmids seems more certain.

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