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ISOLATION OF RECOMBINANTS DOUBLY AND TRIPLY DRUG-RESISTANT TO STREPTOMYCIN, TETRACYCLINE AND CHLORAMPHENICOL BY PEG-INDUCED CELL FUSION OF SINGLY RESISTANT *STAPHYLOCOCCUS AUREUS* L-FORMS

YOSHIYUKI HIRACHI, YOICHI KATO, TAKASHI MATSUMOTO, YOSHIYA UEYAMA, SUMIKO FURUYAMA, MASUMI KURONO, YOICHIRO TODA and SHOZO KOTANI

Department of Oral Microbiology and Microbiology, Osaka University Dental School, 4–3–48 Nakanoshima, Kita-ku, Osaka 530, Japan

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SUMMARY Various combinations of four substrains of *Staphylococcus aureus* L-form (strain STA-EMT-1), each of which was resistant to one of the following four drugs, streptomycin (SM), tetracycline (TC), chloramphenicol (CP) and erythromycin (EM), were submitted to polyethylene glycol (PEG)-induced cell fusion. PEG-induced cell fusion followed by enrichment culture in the liquid basal medium supplemented with penicillin G resulted in development of recombinants that were doubly drug-resistant to SM and TC, SM and CP, and TC and CP, but no recombinant doubly resistant to EM and TC, was obtained by treatment of a EM-resistant and TC-resistant substrains with PEG.

No recombinants resistant to SM, CP and TC could be obtained by treatment of substrains resistant to SM, CP and TC, respectively, with PEG. But recombinants triply resistant to these three drugs were produced by two-step cell fusion; that is by fusion of a recombinant doubly resistant to two of the three drugs with a substrain resistant to the third drug.

INTRODUCTION

In a previous paper (Hirachi, Kurono and Kotani, 1979), we reported that treatment of a mixture of streptomycin (SM)-resistant and erythromycin (EM)-resistant substrains (SM-R and EM-R, respectively) of *S. aureus* L-form with polyethylene glycol (PEG) and subsequent enrichment culture of the mixture in the presence of penicillin G (PC-G) resulted in development of a product that grew in assay medium supplemented with SM and EM. We showed (Hirachi, Kurono and Kotani, 1980) that the development of this doubly drug-resistant recombinant (SmEm-R) was due to cell fusion induced by PEG, not to other genetic transfer mechanisms, such as conjugation, transformation or transduction.

In the present study, using substrains singly resistant to chloramphenicol (CP) and

tetracycline (TC) (CP-R and TC-R, respectively) in addition to SM-R and EM-R strains, we have examined whether recombinants resistant to two or three of these drugs could be produced by PEG-induced cell fusion.

MATERIALS AND METHODS

1. *Organisms and growth media*

SM-R (MIC>1,000 μg/ml) and EM-R (MIC>10 μg/ml) substrains were obtained as described previously (Hirachi et al., 1979). The CP-R (MIC >60 μg/ml) and TC-R (MIC>5 μg/ml) substrains were isolated by serial subculture of the parent L-form strain (STA-EMT-1, generously given by Dr. Ichiro Tadokoro, Department of Bacteriology, Yokohama City University Medical School) in liquid or solid basal medium (Brain-Heart-Infusion Broth, Difco), supplemented with 4.5% NaCl and increasing concentrations of the respective drugs. The substrain (a recombinant) SmEm-R (MIC> 1,000 μg/ml of SM and MIC>10 μg/ml of EM) used in this study was isolated by PEG-induced cell fusion of SM-R and EM-R as described in a previous paper (Hirachi et al., 1980). Isolation of other doubly resistant recombinants is described under Results.

These substrains, singly resistant to one of the drugs or doubly resistant to SM and EM, were maintained by serial subculture in solid basal medium supplemented with 500 U/ml of PC-G (Meiji Seika Co., Tokyo) and the maximum tolerable concentration of the respective drug, SM (Meiji), 1,000 μg/ml; EM (Difco), 10 μg/ml; CP (Difco), 60 μg/ml; TC (Difco), 5 μg/ml, until they were used in PEG-induced cell fusion experiments.

2. *Polyethylene glycol (PEG) treatment*

PEG treatment was performed as described previously (Hirachi et al., 1979) with the following modification: Immediately after incubation of mixtures of L-form substrains with 50% PEG at 37 C for 5 min, 1 ml portions of the incubation mixture were mixed with 0.5 ml aliquots of fresh liquid basal medium to decrease the viscosity and specific gravity of the mixture. The diluted mixture was then carefully stirred in a Thermo-Mixer (Thermonics Co., Ltd. Tokyo). By this modification, PEG was more effectively removed from sedimented cells by centrifugation (at 8,000×g, for 20 min). The pellet of cells, from which PEG had been drained off as completely as possible, was transferred to 10–14 ml of enrichment medium (liquid basal medium supplemented with 500 U/ml of PC-G).

3. *Assay for formation of recombinants*

Assays were made in assay medium consisting of basal medium supplemented with various combinations of SM (500 μg/ml), EM (3 μg/ml), CP (10 μg/ml) and TC (1 μg/ml), solidified with 0.8% agar (Wako Pure Chemical Ind., Osaka) as follows. PEG-treated mixtures of the indicated combinations of L-form substrains were incubated in the enrichment medium overnight at 37 C (unless otherwise stated, the incubation or cultivation was carried out at 37 C) and then were serially diluted 10-fold with the liquid basal medium. Portions of 1 ml of appropriate dilutions were cultured in triplicate in assay media by the pour-plate method. The number of colonies developed in the assay medium was counted after incubation for 1 week (or 2 weeks in some experiments).

4. *Single colony analysis*

Test recombinants (SmTc-R) showing resistance to SM and TC were cultured in liquid basal medium supplemented with PC-G for 1–2 days. The cells were appropriately diluted with liquid basal medium and smeared on solid basal medium. About 25 well-isolated single colonies of recombinants developed on solid basal medium were picked up at random with a sterile toothpick and inoculated in a grid arrangement (5×5) on solid basal medium. The colonies developed after 3–5 days cultivation were replicated onto assay media containing either SM and TC or TC and grown for 7 days.

RESULTS

1. *Check of stability of the original drug resistance and the frequency of spontaneous development of drug-resistance of singly resistant substrains*

Four substrains (SM-R, EM-R, CP-R and TC-R), all derived from the same parent strain (STA-EMT-1), were each grown in liquid basal medium with or without the respective drug for 1–2 days. They were then

TABLE 1. *Frequency of spontaneous development of mutants of test L-form substrains resistant to drugs other than the drug to which they were originally resistant and stability of the original drug-resistance of test substrains*

Substrain	Drug[a] added to the enrichment culture	No. of colonies (ratio[b]) developed in medium supplemented with[a]				
		SM (500)	EM (3)	CP (10)	TC (1)	None
SM-R	SM (1,000)	1.2×10^{8} (0.86)	0	0	0	1.4×10^{8}
	0	5.0×10^{7} (1.0)	ND	ND	ND	5.0×10^{7}
EM-R	EM (10)	0	8.5×10^{7} (1.4)	Not estimated[c]	0	6.2×10^{7}
	0	ND	8.4×10^{6} (1.8)	ND	ND	4.8×10^{6}
CP-R	CP (60)	0	0	2.7×10^{8} (1.0)	0	2.7×10^{8}
	0	ND	ND	2.3×10^{7} (1.6)	ND	1.4×10^{7}
TC-R	TC (1)	0	0	0	3.9×10^{7} (0.98)	4.0×10^{7}
	0	ND	ND	ND	7.8×10^{6} (0.52)	1.5×10^{7}

[a] Figures in parentheses represent concentrations (μg/ml) of drugs added to the medium.
[b] Ratio of the number of colonies developed in the respective assay medium to that in the control medium containing no drugs.
[c] When undiluted or 1 : 10 diluted inoculum was cultured, numerous colonies developed, but when an inoculum diluted more than 1 : 100 was used, no colonies grew.
[d] ND : Not determined.

submitted to colony counting by the pour-plate method, using a series of solid assay media each supplemented with one of the drugs, to check the stability of the original drug resistance and spontaneous development of resistance to other drugs. As summarized in Table 1, the rate of spontaneous development of new resistance by mutation was calculated to be less than 7.1×10^{-9}, 1.6×10^{-8}, 3.7×10^{-9}, and 2.5×10^{-8} with SM-R, EM-R, CP-R and TC-R, respectively. EM-R cells, on the other hand, developed a large number of colonies on medium supplemented with a high concentration (up to 30 μg/ml) of CP, though the exact number of colonies developed could not be determined for the reason described in the legends to the table. This fact made it impracticable to pursue studies on recombinants between EM-R and CP-R.

The stability of the drug resistance of each singly drug-resistant substrain was examined by serial subculture in liquid basal medium containing no drugs. Each of the substrains obtained on the third subculture was pour-plated into assay medium supplemented with the respective drug and control medium. All test substrains gave similar numbers of colonies in the respective assay media to those in the control, indicating the stability of the drug resistance of the test substrains.

2. *Development of doubly drug-resistant recombinants by PEG-treatment of two or three substrains singly resistant to SM, CP and TC*

Table 2 a-c shows the development of various recombinants, namely the products doubly drug-resistant to two of the three drugs, SM, CP and TC, by PEG-induced fusion of

TABLE 2a. *Development of doubly drug-resistant recombinants between SM-R and CP-R substrains*

Expt. No.	PEG	Medium[a] with SM (500) and CP (10)		No. of colonies developed in medium[a] with		
		No. of colonies	Frequency	SM (500)	CP (10)	None
1	+	1.7×10^{2}	2.1×10^{-3}	ND	ND	8.0×10^{4}
	−	0	$<1.9\times10^{-6}$	ND	ND	5.4×10^{5}
2	+	2.1×10^{2}	1.1×10^{-5}	ND	ND	1.9×10^{7}
	−	0.7	5.0×10^{-8}	ND	ND	1.4×10^{7}
3	+	1.0×10	1.1×10^{-6}	6.2×10^{6}	7.3×10^{7}	8.8×10^{6}
	−	0	$<2.3\times10^{-8}$	1.6×10^{7}	6.2×10^{7}	4.4×10^{7}

Legends are the same as for Table 1.

TABLE 2b. *Development of doubly drug-resistant recombinants between SM-R and TC-R substrains*

Expt. No.	PEG	Medium[a] with SM (500) and TC (1)		No. of colonies developed in medium[a] with		
		No. of colonies	Frequency	SM (500)	TC (1)	None
1	+	3.0×10^{2}	2.5×10^{-4}	ND	ND	1.2×10^{6}
	−	0	$<3.0\times10^{-6}$	ND	ND	3.3×10^{5}
2	+	3.3×10^{3}	6.7×10^{-6}	ND	ND	4.9×10^{8}
	−	0	$<1.7\times10^{-8}$	ND	ND	5.8×10^{7}
3	+	1.7×10	3.3×10^{-7}	4.2×10^{7}	1.9×10^{5}	5.1×10^{7}
	−	0	$<3.6\times10^{-8}$	2.2×10^{7}	5.6×10^{5}	2.8×10^{7}

Legends are the same as for Table 1.

TABLE 2c. *Development of doubly drug-resistant recombinants between CP-R and TC-R substrains*

Expt. No.	PEG	Medium[a] with CP (10) and TC (1)		No. of colonies developed in medium[a] with		
		No. of colonies	Frequency	CP (10)	TC (1)	None
1	+	9.7×10	7.5×10^{-4}	ND	ND	1.3×10^{5}
	−	0	$<1.9\times10^{-6}$	ND	ND	5.4×10^{5}
2	+	2.6×10^{2}	6.3×10^{-7}	ND	ND	4.1×10^{8}
	−	1	4.3×10^{-8}	ND	ND	2.3×10^{7}
3	+	7.5×10^{2}	2.1×10^{-5}	1.0×10^{8}	9.0×10^{6}	3.6×10^{7}
	−	0	$<8.3\times10^{-8}$	8.5×10^{7}	2.0×10^{6}	1.2×10^{7}

Legends are the same as for Table 1.

TABLE 3. *Attempt to obtain triply drug-resistant recombinants by simultaneous PEG-induced cell fusion of SM-R, CP-R and TC-R substrains*

PEG	No. of colonies developed in medium[a] with					
	SM CP TC		SM CP —	SM — TC	— CP TC	None
	1 w	2 w[b]				
+	0	0	7.5×10^{2}	1.9×10^{2}	2.0×10^{2}	2.2×10^{8}
−	0	0	0	0	0	7.0×10^{7}

[a] SM 500 μ/ml, CP 10 μg/ml and TC 1 μg/ml.
[b] Period of cultivation.

TABLE 4. *Stability of double drug-resistance of recombinants (SmTc-R, SmCp-R and CpTc-R) obtained by PEG-induced cell fusion*

Substrain[a]	Number of colonies (ratio[b]) developed in medium[c] with					
	Appropriate combination of drugs		SM (500)	CP (10)	TC (1)	None
SmTc-R	SM (500) +TC (1)	Not estimated[e]	1.6×10^{8} (0.89)	ND[d]	Not estimated	1.8×10^{8}
SmCp-R	SM (500) +CP (10)	5.5×10^{6} (1.1)	6.4×10^{6} (1.3)	8.3×10^{6} (1.7)	ND	4.9×10^{6}
CpTc-R	CP (10) +TC (1)	5.3×10^{6} (0.41)	ND	1.2×10^{7} (0.92)	7.8×10^{6} (0.60)	1.3×10^{7}

[a] SmTc-R: Resistant to SM (1,000 μg/ml) and TC (5 μg/ml).
SmCp-R: Resistant to SM (1,000 μgml) and CP (60 μg/ml).
CpTc-R: Resistant to CP (60 μg/ml) and TC (5 μg/ml).
[b] Ratio of the number of colonies developed in the respective assay medium to that in the control medium containing no drugs.
[c] Figures in parentheses are concentrations (μg/ml) of drugs added to the medium.
[d] ND: Not determined.
[e] When undiluted or 1 : 10 diluted inoculum was cultured, many colonies developed, but when an inoculum diluted more than 1 : 100 was used, no colonies grew.

substrains singly resistant to each drug. On combinations of SM-R×CP-R (Table 2a), SM-R×TC-R (Table 2b) and CP-R×TC-R (Table 2c), recombinants doubly resistant to SM and CP, SM and TC, and CP and TC developed at frequencies of 10^{-3}–10^{-6}, 10^{-4}–10^{-7}, and 10^{-4}–10^{-7}, respectively. The frequency of induction of doubly resistant recombinants by PEG treatment varied considerably from one experiment to another, but was always significantly higher than that observed in the control experiment without PEG treatment.

Table 3 shows that simultaneous treatment of SM-R, CP-R and TC-R with PEG did not produce recombinants triply drug-resistant to the three drugs, even on prolonged incubation for two weeks under experimental conditions where recombinants doubly resistant to SM and CP, SM and TC, or CP and TC, developed at a fairly high rate.

A randomly selected doubly drug-resistant colony developed by PEG-induced cell fusion was cultivated in liquid basal medium sup-

TABLE 5. *Attempts to obtain recombinants showing triple drug-resistance to SM, CP and TC by PEG-induced cell fusion between a doubly drug-resistant recombinant and a substrain resistant to a third drug*

Cross by fusion	PEG	No. of colonies developed in medium[a] with								
		SM CP TC		SM CP —	SM — TC	CP TC	SM — —	— CP —	— — TC	None
		1 w	2 w[b]							
SmTc-R ×CP-R	+	1.3	2.3×10	1.2×10^{3}	Not estimated[c]	4.0×10^{2}	5.3×10^{7}	3.0×10^{7}	Not estimated	4.5×10^{7}
	−	0	0	0	Not estimated	0	6.0×10^{7}	1.5×10^{7}	Not estimated	4.7×10^{7}
CpTc-R ×SM-R	+	4.5×10	2.2×10^{2}	4.5×10^{2}	5.2×10^{2}	3.2×10^{6}	5.4×10^{7}	2.6×10^{7}	4.0×10^{6}	5.3×10^{7}
	−	0	0	0	0	3.4×10^{6}	4.4×10^{7}	1.1×10^{7}	3.5×10^{6}	1.9×10^{7}
SmCp-R ×TC-R	+	2.7	4.7	1.4×10^{7}	4.0×10^{2}	7.7×10	3.0×10^{7}	1.7×10^{7}	2.4×10^{6}	2.7×10^{7}
	−	0	0	4.1×10^{6}	0.3	0	1.7×10^{7}	1.9×10^{7}	1.9×10^{6}	2.3×10^{7}

[a] SM 500 μg/ml, CP 10 μg/ml and TC 1 μg/ml.

[b] Period of cultivation.

[c] When undiluted or 1 : 10 diluted inoculum was cultured, numerous colonies developed, but when an inoculum diluted more than 1 : 100 was used, no colonies grew.

plemented with PC-G but not with other drugs. The liquid culture was then transferred to solid assay medium supplemented with the two respective drugs (SM 1,000 μg/ml, CP 60 μg/ml and TC 5 μg/ml). A pure culture of each doubly resistant recombinant was then obtained by isolating a colony and was serially subcultured. These pure culture will hereafter be referred to as SmCp-R, SmTc-R and CpTc-R.

Of these three recombinants SmCp-R and CpTc-R remained doubly resistance on repeated (at least three) subcultures in liquid basal medium containing no drugs (Table 4). SmCp-R developed similar numbers of colonies in assay media supplemented with SM and CP, SM alone or CP alone, and CpTc-R produced similar numbers of colonies in media supplemented with CP and TC, CP alone or TC alone. Assay on the stability of the double drug-resistance of SmTc-R, however, was technically difficult due to the dilution effect of the inoculum, similar to that encountered with EM-R substrains (described above). It was thus difficult to estimate the number of colonies produced by SmTc-R on assay medium supplemented with SM and TC, or TC alone. Therefore, the homogeneity of SmTc-R recombinants was checked by the replica method: 25 colonies developed on the solid basal medium were stamped in quadriplicate on control basal medium with and without supplementation with both SM and TC, or TC only. The results indicated that all the test colonies were doubly resistance to SM and TC, suggesting that SmTc-R was homogeneous (data not shown).

3. *Development of PEG-induced recombinants showing triple drug-resistance by cross of a doubly drug-resistant recombinant with a substrain singly resistance to the third drug*

Table 5 shows that all three combinations (SmTc-R×CP-R, CpTc-R×SM-R and SmCp-R×TC-R) of PEG-induced cell fusion produced recombinants triply resistant to SM, CP and TC. In addition to the triply drug resistant recombinant, the above combinations gave recombinants that showed the newly developed doubly drug-resistance, and

TABLE 6. *Failure to obtain recombinants doubly resistant to EM and TC by PEG-induced cell fusion between EM-R and TC-R*

PEG	Medium[a] with EM (3) and TC (1)			No. of colonies developed in medium[a] with		
	No. of colonies		Frequency	EM (3)	TC (1)	None
	1 w	2 w[b]				
+	0	0	$<2.8\times10^{-7}$	1.3×10^{7}	5.5×10^{6}	3.6×10^{6}
+	0	0	$<3.0\times10^{-7}$	3.8×10^{6}	4.7×10^{5}	3.3×10^{6}
+	0	0	$<1.9\times10^{-7}$	1.7×10^{7}	7.2×10^{5}	5.3×10^{6}
−	0	0	$<5.9\times10^{-8}$	8.1×10^{6}	1.2×10^{6}	1.7×10^{7}
−	0	0	$<6.3\times10^{-8}$	2.1×10^{7}	4.7×10^{5}	1.6×10^{7}
−	0	0	$<1.3\times10^{-7}$	2.0×10^{7}	1.5×10^{5}	7.5×10^{6}

[a] Figures in parentheses represent concentrations (μg/ml) of drugs added to the medium.
[b] Period of cultivation.

TABLE 7. *Failure to obtain recombinants doubly resistant to EM and TC by PEG-induced cell fusion between SmEm-R and TC-R or SmTc-R and EM-R*

Cross by fusion	PEG	No. of colonies developed in medium[a] with							
		EM TC		EM SM	TC SM	EM	TC	SM	None
		1 w	2 w[b]						
EM-R ×TC-R	+	0.3	0.3	0	0	2.5×10^{7}	4.2×10^{6}	0	3.4×10^{7}
	−	0	0	0	0	9.8×10^{6}	4.3×10^{6}	0	2.5×10^{7}
SmEm-R ×TC-R	+	0	0	1.4×10^{5}	3.7×10^{2}	5.3×10^{5}	6.6×10^{7}	4.7×10^{5}	2.7×10^{7}
	−	0	0	1.4×10^{5}	0	1.2×10^{6}	1.1×10^{7}	6.0×10^{5}	4.4×10^{7}
SmTc-R ×EM-R	+	0	0	1.2×10^{3}	Not estimated[c]	1.2×10^{7}	Not estimated	4.0×10^{6}	1.4×10^{7}
	−	0	0	0	Not estimated	1.2×10^{6}	Not estimated	1.5×10^{6}	5.4×10^{6}

The legends are the same as those for Table 5 expect that the concentrations of drugs added to the medium were as follows: SM 500 μg/ml, EM 3 μg/ml, and TC 1 μg/ml.

grew on the assay media supplemented with SM+CP and CP+TC, SM+CP and SM+TC, and SM+TC and CP+TC, respectively.

The frequency of occurrence of newly developed doubly resistant recombinants can consistently be explained by assuming that the chromosomal genes of SM, CP and TC are lined up in this order and that the relative distances between SM- and CP-genes, CP- and TC-genes, and SM- and TC-genes are 3–6 : 1 : 4–7, respectively. Thus, the observed frequency of development of triply drug-resistant recombinants was low, but this low frequency seemed to be consistent with the relative distances of the SM, CP and TC genes described above.

4. *Difficulty in obtaining recombinants doubly resistant to TC and EM*

As shown in Table 6, repeated attempts have failed to produce a recombinant doubly resistant to EM and TC by cell fusion of EM-R and TC-R, unlike other combinations of two singly resistant substrains. So, we at-

tempted to produce a strain doubly resistant to EM and TC by PEG-induced cell fusion between either SmEm-R and TC-R or SmTc-R and EM-R (Table 7). No colonies developed in assay medium supplemented with TC and EM, even during prolonged incubation for two weeks, although new doubly drug-resistant recombinants, namely the products which grew well on assay medium supplemented with either SM and TC or SM and EM were obtained by cell fusion at a fairly high frequency.

DISCUSSION

It has been demonstrated in the present and previous studies that among six possible combinations of two singly drug-resistant substrains (SM-R, EM-R, CP-R and TC-R) derived from one strain of *S. aureus* L-form, four combinations (SM-R×CP-R, SM-R×TC-R, and CP-R×TC-R in this study, and SM-R×EM-R in the previous study) produced recombinants showing the expected double drug-resistance at a high frequency on PEG-induced cell fusion. Of the remaining two combinations, the cell fusion of EM-R×TC-R did not produce a recombinant doubly resistant to EM and TC at a significant frequency, and an attempt to study the combination of EM-R×CP-R was impracticable since the EM-R substrain showed high resistance to CP.

The observation that the combination of either SmEm-R×TC-R or SmTc-R×EM-R resulted in recombinants exhibiting at considerable frequency the double drug-resistance which was not shown by the parents suggests that failure to obtain recombinants (TcEm-R) resistant to TC and EM was not due to unsuccessful cell fusion.

The reason why recombinants showing double drug-resistance to EM and TC cannot be obtained by cross of EM-R with TC-R is not known, because the mechanism whereby the drug-resistance to TC emerges is unclear. There have so far been no reports on successful isolation of bacteria showing TC resistance of chromosomal nature.

An attempt to study PEG-induced cell fusion between EM-R and CP-R in terms of development of double drug-resistance to these drugs was not successful, since the EM-R substrain showed resistance to CP. A plausible explanation for this is that EM and CP show similar modes of antibacterial action, and share a common or similar attack point on ribosomes (Muñoz et al, 1971; Vazquez, 1974). Further work is required to explain fully the observed cross of resistance between EM-R and CP-R.

Electron microscopic studies by Frehel et al. (1979) showed multiple protoplast fusion of *Bacillus subtillis*. Thus, similar multiple fusion of *S. aureus* L-forms may have occurred in our cell fusion experiments.

However, we did not succeed in isolating a recombinant triply resistant to SM, CP and TC by simultaneous PEG-treatment of any combinations of three substrains, each of which was resistant to a single drug. However, a recombinant having triple drug-resistance was obtained by PEG-induced fusion of recombinants doubly drug-resistant to two of the three drugs with a substrain singly resistant to a third drug.

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