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SHORT COMMUNICATION

PROPHAGE TYPE OF RECOMBINANTS PRODUCED BY CELL FUSION BETWEEN VARIOUS COMBINATIONS OF LYSOGENIC OR NON-LYSOGENIC SUBSTRAINS FROM TWO *STAPHYLOCOCCUS AUREUS* L-FORMS

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SUMMARY Streptomycin (SM)- or erythromycin (EM)-resistant lysogenic and non-lysogenic substrains were produced from two *Staphylococcus aureus* L-form strains lysogneic for different prophages, namely, EMT-L (prophage α) and 209P (prophage β). Cells of these L-form substrains were fused in various combinations using polyethylene glycol (PEG), and the frequency of recombinants selected as double resistance to both SM and EM and the prophage types of these recombinants were examined. In all the combinations, the frequency of recombinants was greater when the cells were treated with PEG than when they were not, and the difference was statistically significant ($p<0.01$) in 13 combinations. Combination between the lysogenic SM-resistant EMT-L substrain [EMT(Sm^r-α)] and lysogenic EM-resistant 209P-L substrain [209P(Em^r-β)] and the reverse combination, between 209P(Sm^r-β) and EMT(Em^r-α), resulted in a majority of recombinants harboring prophage β. The former combination yielded recombinants that all held both prophage α and β.

In the first report of our studies on cell fusion of L-form bacteria, we showed that cell fusion induced with polyethylene glycol (PEG) between streptomycin (SM)-resistant and erythromycin (EM)-resistant L-form substrains, EMT-L of *Staphylococcus aureus* resulted in recombinants resistant to both drugs (Hirachi, Kurono and Kotani, 1979, 1980). Subsequent studies demonstrated that treatment with PEG of drug-resistant substrains of *S. aureus* L-forms, including those resistant to antibiotics other than SM and EM, in various combinations yielded recombinants resistant to two or three drugs (Hirachi et al., 1982).

Of the strains used in these studies, EMT-L and 209P-L were found to be lysogenic strains possessing prophages, tentatively designated as α and β (Takemasa et al., 1984).

Non-lysogenic strains could readily be isolated by ultraviolet irradiation of these bacteria or their treatment with Mitomycin C.

There have been few studies on bacterial cell fusion in relation to phage, the only reports being that of Sanches-Rivas and Garro (1979), who showed the fusion of protoplasts of *Bacillus subtilis* by the prophage complementation test, and that of Schaeffer, Cami and Hotchkiss (1976), who demonstrated the absence of zygotic induction in cell fusion of *B. subtilis* protoplasts. In this study, we treated various combinations of lysogenic and non-lysogenic substrains derived from the same or different *S. aureus* L-form strains, with PEG, and examined the prophage types of the recombinants obtained.

SM- and EM-resistant substrains (harboring α or β prophage) were isolated by the method described previously from two L-form *S. aureus* strains, namely EMT-L and 209P-L (Hirachi, Kurono and Kotani, 1979). Non-lysogenic substrains were obtained by ultraviolet (UV)-irradiation as described previously (Takemasa et al., 1984). Those that induced no phage capable of lysing any of the 24 indicator strains were considered to be non-lysogenic. Non-lysogenic EMT (Sm^r), EMT(Em^r) and 209P(Sm^r) substrains were thus obtain from substrains EMT(Sm^r-α), EMT(Em^r-α) and 209P(Sm^r-β), respectively. No non-lysogenic substrain could be obtained from substrain 209P(Em^r-β) in this way. The non-lysogenic substrain 209P (Em^r) was isolated by inoculation of the parent cells in Brain Heart Infusion (BHI, Difco laboratories, Detroit, Mich., USA) broth supplemented with 4.5% NaCl and Mitomycin C (1 μg/ml).

Cell fusion was performed as described previously (Hirachi et al., 1982). The frequencies of development of recombinants resistant to both SM and EM by PEG-induced cell fusion between SM-resistant and EM-resistant L-forms paired in various combinations were determined as follows. PEG-treated or untreated bacteria were cultured in triplicate in assay medium containing both SM and EM, or in drug-free control medium. The logarithm ($\log_{10}$) of the mean number of doubly drug-resistant colonies per 10^{10} of the total mean number of colonies developed on the drug-free control medium was calculated. The average value obtained for 4 or 8 sets of determinations on each combination was calculated to determine the frequency (mean$\pm$ standard error, SE) of doubly drug resistant fusion products in the combination. For cultures of L-forms not treated with PEG that showed no doubly drug-resistant colonies even on inoculation of 1 ml of the undiluted culture fluid, the calculation was made assuming that the number of doubly drug-resistant colonies was one for each plate. Phages induced by UV-irradiation of fusants (recombinants) were examined as described previously (Takemasa et al., 1984).

The phage derived from the EMT-L strain showed definite plaque formation against indicator *S. aureus* strains 6, 53 and 54, and the phage from the 209P-L strain showed plaque formation against strains 6, 77, 83A and St. 1125 (Takemasa et al., 1984). Thus, recombinants that released phage capable of lysing the former three indicator strains were regarded as prophage type α, and those that released phage that lysed the latter 4 indicators were regarded as prophage type β. Recombinants whose culture supernatant obtained by UV-irradiation lysed all 6 of the above indicators were identified as prophage type $\alpha+\beta$. Recombinants that did not produce phage that lysed any of above indicators or *S. aureus* strain, 52A/79, 3A, 3C or 42D were considered to be non-lysogenic.

Table 1 shows the emergence of doubly drug-resistant colonies of recombinants resulting from PEG-induced cell fusion between various combinations of substrains of EMT-L and 209P-L differing in drug resistance and lysogenicity. Although the frequency of doubly drug-resistant colonies varied in different combinations, all combinations showed higher values with PEG treat-

TABLE 1. *Frequency and prophage type of recombinants obtained by PEG treatment of various combinations of lysogenic or non-lysogenic substrains derived from S. aureus L-forms, strain EMT-L or 209P-L*

EM-resistant substrain	Test strain	PEG treatment	Test strain, SM-resistant substrain: EMT (Sm^r)	EMT (Sm^r-α)	209P (Sm^r)	209P (Sm^r-β)
	EMT (Em^r)	+	4.475±0.166	5.187±0.240	4.454±0.074	3.983±0.519[d]
		−	0.805±0.089	<2.540±0.189	<1.621±0.075	<2.595±0.336
			N 27 (100)	N 71 (99)	N 8 (100)	N 33 (92)
				α 1 (1)		β 3 (8)
			T 27 (100)	T 72 (100)	T 8 (100)	T 36 (100)
	EMT (Em^r-α)	+	3.540±0.131	3.431±0.298[e]	1.859±0.216[b]	2.285±0.079[e]
		−	<0.925±0.075	<2.752±0.261	<0 819±0.178	<1.465±0.238
			N 46 (96)	α 30 (100)	N 13 (87)	α 2 (2)
			α 2 (4)		α 2 (13)	β 103 (98)
			T 48 (100)	T 30 (100)	T 15 (100)	T 105 (100)
	209P (Em^r)	+	4.012±0.181	2.749±0.178[a]	3.644±0.077	3.880±0.123
		−	<1.775±0.054	<1.702±0.142	0.846±0.135	<1.461±0.222
			N 18 (100)	N 19 (56)	N 15 (100)	N 29 (97)
				α 15 (44)		β 1 (3)
			T 18 (100)	T 34 (100)	T 15 (100)	T 30 (100)
	209P (Em^r-β)	+	4.496±0.083	4.460±0.244[b]	3.565±0.042	3.064±0.071
		−	<0.681±0.148	<2.790±0.303	<1.256±0.176	<0.906±0.070
			N 8 (17)	N 4 (5)	N 2 (4)	β 32 (100)
			β 40 (83)	α 1 (1)	β 47 (96)	
				β 71 (89)		
				$\alpha+\beta$ 4 (5)		
			T 48 (100)	T 80 (100)	T 49 (100)	T 32 (100)

Data are expressed as logarithms, $\log_{10}$ (mean±SE) of the number of doubly drug-resistant recombinants per 10^{10} of the total number of colonies developed on the drug-free control medium.

[ ] Prophage type of recombinants N, Non-lysogenic type; α, α prophage type: β, β prophage type; $\alpha+\beta$, mixed type; T, numbers of recombinants tested. Figures in parentheses are numbers of recombinants harboring the respective prophage as percentages of the total number of recombinants tested.

The statistical significance of the difference between the values with and without PEG treatment for each combination was examined by the Student's *t* test. Significant difference from control value (without PEG treatment): no symbol, $P<0.001$; *a*, $P<0.005$; *b*, $P<0.01$ · *c*, $P<0.02$; *d*, $P<0.1$; *e*, $P<0.2$.

ment than without. Statistical analysis by Student's *t* test revealed that differences between the values with and without PEG were significant ($p<0.01$) in 13 of the 16 combinations examined. The frequency of doubly drug-resistant recombinants was not related to the substrains paired, namely their lysogenicity or the type of prophage.

Schaeffer, Cami and Hotchkiss (1976) found in cell fusion experiments with protoplasts of *B. subtilis* that the frequency of fusants resulting from fusion between lysogenic and non-

lysogenic bacteria was similar to that in fusion either between lysogenic bacteria or between non-lysogenic bacteria. This finding, which was confirmed in the present study, suggests that zygotic induction does not occur on fusion of *S. aureus* L-form cells.

In the present study we found that none of the recombinants between non-lysogenic substrains released phage, even when they were treated with UV. Recombinants between homologous substrains derived from the same parent strains and with the same prophage type [EMT(Sm^r-α)×EMT(Em^r-α) and 209P (Sm^r-β)×209P(Em^r-β)] all retained their respective prophages.

In most combinations between lysogenic and non-lysogenic substrains and those between substrains from different parent strains with different prophage types, the majority of the recombinants showed the prophage type of the EM-resistant substrain. However, in combinations involving EMT(Em^r-α), the majority of the recombinants showed the prophage type of the SM-resistant substrain. The finding that many of the recombinants resulting from fusion 209P(Em^r-β) with SM-resistant substrains and from fusion of EMT (Em^r-α) with 209P(Sm^r-β) possessed β prophage suggests that the β prophage is located proximally to the SM or EM resistance gene. On the contrary, the finding that on fusion of the lysogenic EMT-L substrain with other strains most of the recombinants did not harbor α prophage suggests that the α prophage is located distal to the SM or EM resistance gene.

Of the combinations between lysogenic heterologous substrains derived from different parent strains, that between EMT(Sm^r-α) and 209P(Em^r-β) resulted in all four possible prophage types (α, β, $\alpha+\beta$ and non-lysogenic type). The frequency of the four prophage types in 80 colonies obtained by this combination was 1:71:4:4 (1:89:5:5 as percentages). However, the reverse combination, between 209P(Sm^r-β) and EMT (Em^r-α), resulted in recombinants of only the α or β prophage type and none of the $\alpha+\beta$ or non-lysogenic type.

Then, we examined whether the colonies of recombinants showing the prophage $\alpha+\beta$ type resulting from fusion between EMT(Sm^r-α) and 209P(Em^r-β) were a mixture of two substrains of L-forms possessing prophage α and β, respectively, or whether they consisted of a single L-form possessing both prophages α and β. L-form cells from 2 of 4 colonies showing the prophage $\alpha+\beta$ type were subcultured for several generations in 4.5% NaCl-BHI broth, diluted with the same medium to a suitable cell density, and then cultured on 4.5% NaCl-BHI agar plates containing no drugs. L-Form cells harvested from 30 clearly separated colonies were cultured overnight in the above broth. All these colonies showed the prophage $\alpha+\beta$ type, indicating that each of the recombinants had both prophage α and prophage β.

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