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Title	Further Evidence of Polyethylene Glycol-Induced Cell Fusion of Staphylococcus aureus L-Forms
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Citation	Biken journal : journal of Research Institute for Microbial Diseases. 1980, 23(1), p. 43-48
Version Type	VoR
URL	https://doi.org/10.18910/82537
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BIKEN JOURNAL Vol. 23 43-48, 1980

SHORT COMMUNICATION

FURTHER EVIDENCE OF POLYETHYLENE GLYCOL-INDUCED CELL FUSION OF *STAPHYLOCOCCUS AUREUS* L-FORMS

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(Received September 26, 1979)

Further evidence is presented that on treatment with polyethylene glycol the emergence of doubly durg-resistant recombinants of *Staphylococcus aureus* L-forms from substrains resistant to streptomycin or erythromycin only is really due to cell fusion, and not to other genetic transfer mechanisms. The reason for the necessity of enrichment culture is also discussed.

In the preceding paper (Hirachi, Kurono and Kotani, 1979), we reported that incubation of a mixture of streptomycin (SM)-resistant and erythromycin (EM)-resistant substrains of the L-forms of *Staphylococcus aureus* with fusogenic polyethylene glycol (PEG) and subsequent enrichment culture in the presence of peinicillin G resulted in the emergence of recombinants, resistant to both SM and EM. The emergence of doubly-resistant recombinants was taken as an indication of cell fusion of SM- and EM-resistant substrains of the L-forms. However, because a number of mechanisms are known to cause genetic transfer in bacterial cells, the present study was undertaken to obtain further evidence that the emergence of the doubly resistant recombinants was really due to cell fusion induced by PEG, and not to other genetic transfer mechanisms.

The two substrains of *S. aureus* L-forms used in this study, an SM-resistant SM1000R-L mutant (MIC>1,000 μg/ml) and an EM-resistant EM10R-L mutant (MIC>10 μg/ml), were obtained as described previously (Hirachi et al., 1979). The composition of the basal medium and assay media (solid and liquid) were also as described previously (Hirachi et al., 1979). For cell fusion, SM1000R-L and EM10R-L were grown in the basal medium supplemented with penicillin G (PC-G, 500 U/ml), and with SM (1,000 μg/ml) and EM (10 μg/ml), respectively. Recombinants that were resistant to both SM and EM were assayed using basal medium with SM (500 μg/ml) or EM (3 μg/ml) or both. Enrichment culture was carried out in basal medium with PC-G (500 U/ml), but not deoxyribonuclease (DNase), unless otherwise noted. In some experiments, SM or EM or both were added to the enrichment medium at the same concentration as that in the assay medium.

A mixture of overnight cultures of SM 1000R-L and EM10R-L (one ml each) was distributed in small centrifuged tubes and

centrifuged at 5,000×*g* for 10 min. The precipitated L-forms were washed once with 1 ml of basal medium, and resuspended in 1 ml-portions of phosphate-buffered 4.5% NaCl solution (pH 7.5) supplemented with PEG (6,000) at 50% (w/w) concentration. The mixed suspension of the L-forms of the two substrains in PEG was incubated for 10 min at 37°C and then centrifuged at 12,000×*g* for 20 min. The supernatant fluid was removed and the residual PEG was drained off as completely as possible by inverting the centrifuge tube over sterilized paper. The pellet of cells was then transferred as quantatively as possible to 10 ml of enrichment culture medium. After overnight culture, appropriate 10-fold dilutions (1 ml each) of the enrichment culture with 4.5% NaCl solution were cultured in triplicate, by the pour plate method, in solid assay medium for 6 days at 37°C. Then the number of colonies was counted.

Some L-form cells may be ruptured by PEG treatment, even in the presence of 4.5% NaCl. Therefore, we first examined whether one ml of intact L-forms of one substrain could receive genetically active DNA molecules released by spontaneous rupture from one ml of the L-form culture of the other substrain. Lysates of the L-forms were prepared as follows: 10 ml portions of overnight cultures of the SM- and EM-resistant substrains, respectively, were centrifuged at 5,000×*g* for 10 min. The precipitated cells were washed with 5 ml of basal medium by centrifugation and then mixed with 0.8 ml of sterilized distilled water. The suspensions were stood at room temperature for 3 hr with occasional vigorous shaking in a Thermo-mixer (Thermonics Co., Tokyo), to burst the cells by osmotic shock. The burst cell suspensions were supplemented with 0.2 ml of NaCl solution (22.5 g/dl) to give a final concentration of 4.5% NaCl. Volumes of 0.1 ml of the lyzates derived from 1.0 ml of the L-form cultures, were used instead of intact L-form cells in the standard procedure. As shown in Table 1 (Experiment A), recombinants, resistant to

TABLE 1. *Frequency of emergence of recombinants doubly resistant to SM and EM*

Expt. No.	Composition of reaction mixture			Solid assay medium with			
	SM1000R-L	EM10R-L	PEG(6000) 50% (w/w)	SM+EM	SM	EM	None
A	Intact cells	Intact cells	+	6.4×10^3	4.3×10^6	3.5×10^5	2.9×10^7
B	Intact cells	Intact cells	−	0	8.9×10^6	6.0×10^4	2.0×10^7
C	Intact cells	Lysate[a]	+	2	1.1×10^7	7.6	3.4×10^7
D	Lysate[a]	Intact cells	+	0	0	8.5×10^6	6.9×10^6
A	Intact cells	Intact cells	+	6.7×10^3	6.3×10^7	5.7×10^7	3.9×10^7
B	Intact cells	Intact cells	−	0	1.6×10^8	2.5×10^8	3.4×10^8
E	Intact cells	Filtrate[b]	+	0	3.0×10^8	0	2.6×10^8
F	Filtrate[b]	Intact cells	+	0	2.3×10	7.8×10^7	1.3×10^7
A	Intact cells	Intact cells	+	8.3×10^3	3.7×10^5	4.0×10^5	8.0×10^5
B	Intact cells	Intact cells	−	0	3.0×10^5	6.3×10^5	9.0×10^5
G	PEG-treated cells	PEG-treated cells	(+)	0	3.3×10^6	1.0×10^6	4.2×10^6

Colony number/ml of enriched culture

[a] From 1 ml of culture.

[b] From 2 ml of culture.

both SM and EM were obtained when a mixture of intact SM1000R-L and intact EM10R-L substrains were exposed to PEG and then submitted to enrichment culture. But when either of the intact L-forms of the two substrains was replaced by a lysate of osmotically bursted L-forms, few if any doubly drug-resistant recombinant colonies were recovered (Experiment C and D in Table 1). The recovery of a very few doubly resistant colonies in Experiment C was probably due to survival of a few intact EM10R-L cells in the lysate. This finding is consistent with the previous observation that the emergence of doubly resistant recombinants was not effected by the presence of DNase during incubation of a mixture of the SM- and EM-resistant substrains with PEG and enrichment culture of the mixture (Hirachi et al., 1979). The results indicate that the L-form of one substrain does not incorporate DNA molecules that control EM or SM resistance from a lysate of the other L-form.

Next we examined whether the recombinants resistant to both SM and EM were formed by transduction, that is, with the aid of bacteriophages induced by PEG. To examine this possibility, L-form cells obtained by centrifugation ($5{,}000\times g$, for 20 min) of 8 ml of cultures of the respective substrains were suspended in 4 ml of PEG, and the suspensions were vigorously stirred with a Thermomixer for 10 min at 37°C. Then they were centrifuged at $12{,}000\times g$ for 20 min, and the supernatant fluids were passed through a Millipore filter of 0.45 μm pore size (Sartorius, West Germany). One ml of filtrate from 2 ml of each L-form culture was incubated with L-forms precipitated from one ml of the other substrain, under the standard experimental conditions. No recombinants developed in either combination, as shown in Experiments E and F in Table 1. In the combination of the filtrate of SM1000R-L cells and intact EM10R-L cells (Experiment F), some SM-resistant colonies were recovered. This seemed to be because some intact SM-resistant cells passed through the filter. A similar experiment was made to see if enrichment culture of a mixture of SM1000R-L and EM10R-L, which had been separately treated with PEG and then separated from PEG, produced doubly resistant recombinants. As shown in Table 1, Experiment G, no recombinants were obtained.

Previously we found that no doubly resistant recombinants could be produced without enrichment culture after PEG treatment. We examined the reason for the necessity of enrichment culture in the following experiment. Either SM (500 μg/ml) or EM (3 μg/ml) or both were added to the enriched culture. As summarized in Table 2, on enrichment culture with both SM and EM no recombinant colonies appeared that could grow on SM+ EM assay medium and only a few colonies were obtained that could grow even in assay medium without either drug. This finding suggests that almost all the L-forms, including fused cells, were killed by the combined actions of SM and EM during enrichment culture. However, the results presented in Table 2 (Experiment B) show that on enrichment culture in the presence of either SM or EM, unlike on enrichment culture with both SM and EM, a certain number of doubly resistant recombinants developed. This finding can be explained by the fact that diploid cells just after fusion contain SM-resistant ribosomes and EM-resistant ones (Ozaki, Mizushima and Nomura, 1969; Wittmann and Wittmann-Liebold, 1974; Pardo and Rosset, 1977) derived from the respective L-forms, and these cells can survive for a few division in enrichment culture medium with one of the two drugs, although their multiplication may be retarded. The reason for this is as follows: During early divisions, haploid cells that segregate from fused diploid cells before recombination revert to the respective parent cells, and thus about half of them will be able to grow in medium containing one drug, while haploid cells segregating after recombination can form ribosomes resistant to both SM and

TABLE 2. *Emergence of doubly resistant recombinants by enrichment culture with or without SM and EM*

Expt. No.	Enrichment culture with	None		SM+EM[a]		SM		EM	
	Solid assay medium with	SM+EM	None	SM+EM	None[b]	SM+EM	None	SM+EM	None
A		2.2×10^4	8.3×10^6	0	1.3×10				
		6.3×10^3	4.9×10^6	0	1.0×10				
		1.4×10^4	1.1×10^7	0	0.7×10				
		1.6×10^3	3.2×10^6	0	0.3×10				
		2.7×10^2	1.6×10^6	0	0.3×10				
B		7.8×10^2	4.5×10^6	0	2.7×10	9.0	9.3×10^6	2.7×10^2	2.6×10^7
		2.0×10^3	1.1×10^7	0	0.3×10	8.0×10	2.3×10^7	3.1×10^2	3.1×10^7
		3.2×10^3	1.2×10^7	0	0.3×10	6.1×10	5.1×10^7	4.2×10^2	1.1×10^7

Colony number/ ml of enriched culture

[a] No growth (no increase in turbidity) was recognized even after incubation for one week, unlike in other enriched cultures.

[b] In this experiment, 0.1 ml not 1.0 ml portions of the enriched cultures were inoculated into the solid assay medium, to reduce the amount of transfer of SM and EM from the enriched culture.

TABLE 3. *Time course of emergence of doubly resistant recombinants by enrichment culture without drugs*

Assay media	Time (hr)	Experiment				
		A	B	C	D	E
with SM+EM						
	1	0	0	0	0	0
	2	0	0	0	0	0
	4	0.3	2.4	0.3	0	0.7
	6	4.7×10	2.4×10	2.3×10	3	6.4×10
	8	4.8×10^2	2.4×10^2	1.0×10^2	2.4×10	4.9×10^2
	12	6.4×10^3	7.1×10^3	2.4×10^3	1.9×10^3	1.7×10^3
without drugs						
	0	1.6×10^5	2.4×10^5	4.5×10^5	1.2×10^5	1.9×10^5
	2	2.6×10^5	1.9×10^5	1.2×10^6	1.6×15^5	9.6×10^5
	4	1.7×10^6	1.6×10^6	1.0×10^7	1.3×10^6	6.0×10^6
	6	7.3×10^6	3.0×10^6	2.5×10^7	4.5×10^6	1.2×10^7
	8	1.0×10^7	7.6×10^6	6.0×10^7	1.2×10^7	2.8×10^7
	12	2.5×10^8	5.2×10^8	1.8×10^8	4.3×10^8	8.5×10^8

Colony number/ml of enriched culture

EM and thus will be able to survive in medium with both drugs. On the other hand, cells that remain as diploid cells can synthesize both SM-resistant ribosome proteins and EM-resistant ones, but since the probability that component proteins of two kinds of ribosomes are assembled in a single ribosome is very small considering the topography of the two ribosomes, very few diploid cells survived in medium with SM or EM.

Table 3 shows the time course of emergence of doubly resistant recombinants during enrichment culture without drugs. In this experiment (made in quintuplicate), the volumes of the L-form cultures, PEG and enrichment medium were 10, 2, and 10 times, respectively, those used in the standard experimental conditions, so that successive samples could be taken. It can be seen that recombinants resistant to both SM and EM emerged after 4 to 6 hr of enrichment culture.

Finally we examined the stability of the doubly-resistant recombinants that emerged by PEG-induced cell fusion of the two substrains. Fifteen primary colonies that developed on culture for 5 days in solid assay medium with SM and EM after enrichment culture for 6 hr were cut out as a block from the agar medium, and cultivated for 18 hr in basal medium supplemented with PC-G but not SM or EM. The overnight cultures were replated on basal solid medium without drugs to obtain secondary colonies. After 5 days cultivation, 10 secondary colonies per one primary colony were selected at random, and small agar blocks each containing an individual secondary colony were cut out, and suspended as homogeneously as possible in 2 ml of basal medium containg no drugs. Then 0.2 ml portions of the suspensions were inoculated into 2 ml volumes of liquid assay medium containing SM or EM or both and growth was examined daily for 4 days. All secondary colonies examined grew well in the assay media. Thus the double resistance to SM and EM of the ricombinants induced by cell fusion was stable. This stability of recombinants suggests that the doubly resistant haploid cells segregated from either diploid or multinucleated cells formed by cell fusion.

Foder and Alföldi (1976) and Schaeffer, Schaeffer, Gami and Hotchkiss, (1976) reported that fusion by PEG of bacterial protoplasts derived from two auxotrophic strains of *Bacillus subtilis* and *Bacillus megaterium*, respectively, resulted in recombinants of the wild type without the necessity for enrichment culture, and that some recombinants were unstable. There findings seem to be consistent with Lederberg's finding (Lederberg, 1947) that abilities of bacteria to synthesize essential metabolites were dominant to inabilities to synthesize the compounds in heterozygotic cells: the recombinants isolated in their experiments using the requirements for nutrients as markers, were not always haploid cells, and therefore, prototrophic colonies emerging on the minimal medium contained auxotrophic cells (Foder and Alföldi, 1976).

ACKNOWLEDGMENTS

The authors express their gratitude to Prof. T. Amano and A. Matsushiro for their valuable criticisms and advices.

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