BIKEN JOURNAL Vol. 22 25-29, 1979

SHORT COMMUNICATION

POLYETHYLENE GLYCOL-INDUCED FUSION OF L-FORMS OF *STAPHYLOCOCCUS AUREUS*

YOSHIYUKI HIRACHI, MASUMI KURONO and SHOZO KOTANI

Department of Microbiology, Osaka University Dental School, Nakanoshima, Kita-ku, Osaka 530, Japan

(Received October 13, 1978)

The pioneer work on the fusion of Ehrlich's tumor cells by HVJ virus in vitro by Okada and his colleagues (Okada, Suzuki and Hosaka, 1957; Okada, 1958) has been followed by a number of studies on the fusion of animal cells by various fusing agents, because this phenomenon is very useful in various fields of basic and applied genetics and in studies on the expression of differentiated functions (Harris, 1970; Steplewski and Koprowski, 1970; Ephrussi, 1972; Sell and Krooth, 1973; Okada, 1975). Studies have also been extended to plant cells (Carlson, 1973; Power, Evans and Cocking, 1978) and fungi (Ferenczy, Kevei and Zsolt, 1974; Binding and Weber, 1974; Ferenczy, Kevei and Szegedi, 1975a, 1975b; Ahkong et al., 1975; Ferenczy et al., 1976; Anné and Peberdy, 1976; Sipiczki and Ferenczy, 1977; van Solingen and van der Plaat, 1977; Genthner and Borgia, 1978), using either protoplasts or spheroplasts prepared by appropriate methods. Fusions of bacterial protoplasts or spheroplasts derived from *Bacillus megaterium*, *Bacillus subtilis* and several species of streptomyces have also been achieved with polyethylene glycol, a widely effective, fusing agent, or nascent calcium phosphate (Fodor and Alföldi, 1976; Schaeffer, Gami and Hotchkiss, 1976; Levi, Rivas and Schaeffer, 1977; Hopwood, Wright and Bibb, 1977; Fodor, Demiri and Alföldi, 1978). However, there seem to be no reports on hybridization by fusion of bacterial L-forms and mycoplasmas. This paper reports the fusion of two drug-resistant substrains of the L-form of *Staphylococcus aureus*.

The strain of *S. aureus* L-form used in this study was obtained by serial subculture of the STA-EMT-1 strain, generously given by Dr. Ichiro Tadokoro, Department of Bacteriology, Yokohama University Medical School, on 4.5% NaCl-brain heart infusion (BHI) broth (Difco) supplemented with decreasing amounts of horse serum, which was included in the medium recommended by Okuda et al. (1977). The strain thus obtained could grow well in 4.5% NaCl-BHI medium containing no horse serum (named basal medium, hereafter), with or without 1% agar (Grade I, Wako Pure Chemical Industries, Osaka), and was found to be susceptible to streptomycin (SM, Streptomycin sulfate, Meiji Seika Co., Tokyo) and erythromycin (EM, Erythrocin, Dainippon Pharmaceutical Co., Osaka). The minimum inhibitory concentrations (MIC) of these drugs for this parent L-form strain were 20 μg/ml and 0.16 μg/ml, respectively. An SM-resistant substrain (SM1000R-L, MIC>1,000

μg/ml) and an EM-resistant substrain EM10R-L, MIC>10 μg/ml) were isolated by serial subculture of the parent L-form strain in basal medium, either liquid or solid, with increasing concentrations of each of the drugs by the conventional method. Isolation of the EM-resistant substrain was accelerated by use of ultraviolet irradiation and a gradient plate method (Szybalski and Bryson, 1952).

Substrains, SM1000R-L and EM10R-L, were grown at 37 C in the basal medium supplemented with SM (1,000 μg/ml) and EM (10 μg/ml), respectively, and with penicillin G (Meiji, 500 U/ml). Overnight cultures were homogenized gently by agitation in a Thermo-Mixer (Thermonics Co., Tokyo). Samples of 2 ml of culture of each substrain or a mixture of the two (one ml each) were distributed in small centrifuge tubes (see Table 2) and centrifuged at 8,000×*g* for 15 min. The precipitated L-forms were washed with basal medium, and resuspended in 1 ml-portions of phosphate-buffered 4.5% saline solution (Na_2HPO_4 1.2 g, KH_2PO_4 0.7 g and NaCl 45 g/ liter, adjusted to pH 7.5 with 1 N NaOH) containing polyethylene glycols, 1,000, 4,000 and 6,000 (PEGs, Grade I, Wako) at various concentrations (10 to 50%, w/w) and deoxyribonuclease (DNase-I from bovine pancreas, Sigma; 10 μg/ml). Either PEG and DNase or DNase was omitted from the buffered 4.5% saline in control experiments. The suspensions were homogenized, taking care not to damage protoplasts appreciably. Then the suspensions were incubated for 15 min at 37 C and centrifuged at 12,000×*g* for 15 min. The residual PEGs were drained off by inverting the centrifuge tubes on sterilized filter paper. The pelleted cells were transferred quantitatively to 10 ml of basal medium containing penicillin G (500 U/ml) and DNase-I (10 μg/ml), and incubated at 37 C. DNase-I was omitted in some control experiments. After overnight culture for enrichment of drug-doubly resistant L-form cells, five replicate 0.2 ml samples of each culture were inoculated into 2 ml of basal medium supplemented with SM (500 μg/ml) or EM (3 μg/ml), or both drugs (liquid assay media). Growth was examined daily for 4 days. Further 1 ml samples of the above overnight culture or appropriate 10-fold dilutions were cultured in triplicate by the pour plate method, in assay medium containing 0.8% agar and after incubation for 6 days at 37 C the number of colonies was counted.

TABLE 2. *Frequency of emergence of recombinants doubly resistant to SM and EM by PEG-sence of DNase-I*

Tube No.	Composition of reaction mixture				SM+EM medium	
	SM1000R-L	EM10R-L	PEG (6000) 50% (w/w)	DNase-I 10 μg/ml	Liquid[a]	Solid[b]
A	1 ml	1 ml	+	−	5/5	2.0×10^3
B	2 ml	—	+	−	0/5	0
C	—	2 ml	+	−	0/5	0
D	1 ml	1 ml	−	−	0/5	0
E	2 ml	—	−	−	0/5	0
F	—	2 ml	−	−	0/5	0
G	1 ml	1 ml	+	+	5/5	4.6×10^3
H	2 ml	—	+	+	0/5	0
I	—	2 ml	+	+	0/5	0

[a] No. of liquid media showing growth/No. of liquid media inoculated.
[b] Colony number/ml of enrichment culture.

TABLE 1. *Emergence of the recombinant L-form resistant to both SM and EM by cell fusion of SM-resistant and EM-resistant substrains of S. aureus L-forms in the presence of PEGs of various molecular weights at various concentrations*

PEG as fusing agent	Concentration per cent (w/w)	Doubly resistant recombinant	
		Liquid assay	Solid assay
		medium[a]	medium[b]
PEG (1000)	10	0/5	0
	20	0/5	0.7
	30	1/5	3.7
	40	3/5	33.7
	50	4/5	215
PEG (4000)	10	0/5	3
	20	2/5	1
	30	3/5	9
	40	2/5	0.7
	50	5/5	385
PEG (6000)	10	0/5	0
	20	0/5	0.3
	30	1/5	3
	40	5/5	510
	50	5/5	4,200
None	0	0/5	0

[a] No. of liquid media showing growth/No. of liquid media inoculated.
[b] Colony counts/ml of enrichment culture.

Table 1 shows that incubation of a mixture of the SM-resistant and EM-resistant substrains of *S. aureus* L-forms with PEGs, as a fusing agent, and subsequent enrichment culture resulted in the emergence of recombinats doubly resistant to SM and EM. Of the PEGs tested at various concentrations, PEG (6,000) was found to be most effective at 50% concentration for inducing the recombinant. The recombination frequency could not be estimated under the present assay conditions, because enrichment culture was performed to raise the efficiency of detection of recombinants after interaction of singly resistant substrains in the presence of PEGs. Enrichment culture seemed to be necessary, because dominance of drug sensitivity delayed the initiation of expression of drug-resistant recombinations for several generations in heterozygous recombinants (Lederberg, 1951). Table 2 shows that the recombinants developed at a fairly high rate: while no recombinants doubly resistant to SM and EM were recovered by either liquid or solid culture of a mixture of

induced fusion of S. aureus L-form singly resistant to either SM or EM in the presence or ab-

SM medium		EM medium		Medium without drug
Liquid	Solid	Liquid	Solid	Solid
5/5	1.4×10^6	5/5	7.5×10^5	7.3×10^6
5/5	5.3×10^5	0/5	0	3.7×10^6
0/5	0	5/5	4.2×10^5	4.0×10^6
5/5	2.7×10^6	5/5	1.0×10^6	1.2×10^7
5/5	1.1×10^6	0/5	0	1.5×10^7
0/5	0	5/5	1.4×10^6	2.0×10^7
5/5	4.5×10^5	5/5	6.8×10^5	3.3×10^6
5/5	6.7×10^5	0/5	0	9.7×10^6
1[c]/5	0	5/5	2.5×10^6	1.6×10^6

[c] Presumably due to spontaneous mutation.

singly resistant substrains without PEGs, but doubly drug-resistant recombinants induced by 50% PEG (6,000) grew in all 5 liquid cultures and formed colonies in the order of 10^3/ml in the solid assay medium.

Although further studies are needed to exclude the possibility that other recombination mechanisms were involved, the findings that no doubly resistant recombinants appeared on mixing the SM- and EM-resistant substrains in the absence of PEGs, and that the emergence of recombinants was not affected by addition of DNase-I to the incubation mixture and enrichment culture strongly suggest that the recombinants developed as the result of PEG-induced cell fusion of SM- and EM-resistant substrains.

It is noteworthy that all recombinants so far induced by fusion of bacterial protoplasts (Fodor and Alföldi, 1976; Schaeffer, et al., 1976; Levi, et al., 1977; Hopwood, et al., 1977; Fodor, et al., 1978) were rod forms with regenerating cell walls, but the recombinants obtained in this study remained in the L-forms. It should be mentioned that omission of penicillin from the enrichment culture medium resulted in the emergence of the coccal recombinant, resistant to both SM and EM. This finding suggests that the reversion from L-forms to parent coccal forms may also be accelerated by PEG-induced cell fusion of L-forms, because reversion of the L-form strain used in this study to coccal forms is known to be rare (Eda, Matsuoka and Tadokoro, 1972).

ACKNOWLEDGMENTS

This research was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Science and Culture (Nos. 337018 and 348146).

REFERENCES

Ahkong, Q. F., J. I. Howell, J. A. Lucy, F. Safwat, M. R. Devey, and E. C. Cocking. 1975. Fusion of hen erythrocytes with yeast protoplasts induced by polyethylene glycol. Nature 255: 66–67.

Anné, J., and J. F. Peberdy. 1976. Induced fusion of fungal protoplasts following treatment with polyethylene glycol. J. Gen. Microbiol. 92: 413–417.

Binding, H., and H. J. Weber. 1974. The isolation, regeneration and fusion of phycomyces protoplasts. Mol. Gen. Genet. 135: 273–276.

Carlson, P. S. 1973. The use of protoplasts for genetic research. Proc. Natl. Acad. Sci. USA 70: 598–602.

Eda, T., S. Matsuoka, and I. Tadokoro. 1972. Studies on staphylococcal L-forms. II. Growth and morphological characteristics in liquid medium. Jpn. J. Bacteriol. 27: 795–800. [In Japanese]

Ephrussi, B. 1972. Hybridization of somatic cells. Princeton University Press, Princeton, New Jersey.

Ferenczy, L., F. Kevei, and J. Zsolt. 1974. Fusion of fungal protoplasts. Nature 248: 793–794.

Ferenczy, L., F. Kevei, and M. Szegedi. 1975a. Increased fusion frequency of *Aspergillus nidulans* protoplasts. Experientia 31: 50–52.

Ferenczy, L., F. Kevei, and M. Szegedi. 1975b. High-frequency fusion of fungal protoplasts. Experientia 31: 1028–1030.

Ferenczy, L., F. Kevei, M. Szegedi, A. Frankō, and I. Rojik. 1976. Factors affecting high-frequency fungal protoplast fusion. Experientia 32: 1156–1158.

Fodor, K., and L. Alföldi. 1976. Fusion of protoplasts of *Bacillus megaterium*. Proc. Natl. Acad. Sci. USA 73: 2147–2150.

Fodor, K., E. Demiri, and L. Alföldi. 1978. Polyethylene glycol-induced fusion of heat-inactivated and living protoplasts of *Bacillus megaterium*. J. Bacteriol. 135: 68–70.

Genthner, F. J., and P. T. Borgia. 1978. Spheroplast fusion and heterokaryon formation in *Mucor racemosus*. J. Bacteriol. 134: 349–352.

Harris, H. 1970. Cell fusion. Harvard University Press, Cambridge, Massachusetts. 108 p.

Hopwood, D. A., H. M. Wright, and M. J. Bibb. 1977. Genetic recombination through protoplast fusion in streptomyces. Nature 268: 171–174.

Lederberg, J. 1951. Streptomycin resistance: a genetically recessive mutation. J. Bacteriol. 61: 549–550.

Levi, C., C. S. Rivas, and P. Schaeffer. 1977. Further genetic studies on the fusion of bacterial protoplasts. FEMS Microbiol. Lett. 2: 323–326.

Okada, Y. 1958. The fusion of Ehrlich's tumor cells caused by HVJ virus in vitro. Biken J. 1: 103–110.

Okada, Y. 1975. Cell fusion and cell engineering. Kodansha Scientific, Tokyo. [In Japanese]

Okada, Y., T. Suzuki, and Y. Hosaka. 1957. Interaction between influenza virus and Ehrlich's tumor cells. III. Fusion phenomenon of Ehrlich's tumor cells by the action of HVJ Z strain. Med. J. Osaka Univ. 7: 709–717.

Okuda, K., T. Takahashi, I. Tadokoro, and T. Eda. 1977. Studies on the minimum reproducible unit of staphylococcal L-forms. Microbiol. Immunol. 21: 1–10.

Power, J. B., P. K. Evans, and E. C. Cocking. 1978. Fusion of plant protoplasts, p. 369–387. *In* G. Poste and G. L. Nicolson [ed.] Membrane fusion, Cell Surface Reviews, Vol. 5, North Holland Publishing Co., Amsterdam.

Schaeffer, P., B. Gami, and R. D. Hotchkiss. 1976. Fusion of bacterial protoplasts. Proc. Natl. Acad. Sci. USA 73: 2151–2155.

Sell, E. K., and R. S. Krooth. 1973. Tabulation of somatic cell hybrids formed between lines of cultured cells. J. Cell. Physiol. 80: 453–461.

Sipiczki, M., and L. Ferenczy. 1977. Protoplast fusion of *Schizosaccharomyces pombe* auxotrophic mutants of identical mating-type. Mol. Gen. Genet. 151: 77–81.

Solingen, P. van, and J. V. van der Plaat. 1977. Fusion of yeast spheroplasts. J. Bacteriol. 130: 946–947.

Steplewski, Z., and H. Koprowski. 1970. Somatic cell fusion and hybridization, p. 155–191. *In* H. Busch [ed.] Methods in Cancer Research, Vol. 5, Academic Press, New York and London.

Szybalski, W., and V. Bryson. 1952. Genetic studies on microbial cross resistance to toxic agents. I. Cross resistance of *Escherichia coli* to fifteen antibiotics. J. Bacteriol. 64: 489–499.