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SHORT COMMUNICATION

DEMONSTRATION OF LYSOGENY IN STABLE L-FORMS OF *STAPHYLOCOCCUS AUREUS*

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Two stable L-form strains of *Staphylococcus aureus* (EMT-L and 209P-L) were shown to contain different own prophages (α and β, respectively), which were induced by ultraviolet light irradiation.

During studies on intraspecies cell fusion of *Staphylococcus aureus* L-forms (Hirachi, Kurono and Kotani, 1979, 1980; Hirachi et al., 1982), we found that stable L-forms derived from *S. aureus* strains EMT (Tazaki) and 209P spontaneously released phages into the culture fluid that were capable of lysing some of the standard indicator *S. aureus* strains used for phage typing. There are no reports on lysogeny of bacterial L-forms, except those by Taubeneck (1963) on L-forms of *Proteus mirabilis* and by Bertolani, Elberg and Ralston (1975) on L-forms of *Pseudomonas aeruginosa*. Therefore, we investigated the lysogeny of the above two stable L-form strains in further detail with a view to possible induction of prophages by ultraviolet (UV) light irradiation.

The stable L-form strains EMT-L and 209P-L used in this study were obtained in the Department of Microbiology, Yokohama City University School of Medicine, from the respective coccal forms which were serially subcultured there. Each L-form strain was cultured in 6 ml of basal medium—[Brain-

Heart-Infusion broth (Difco Laboratories, Detroit, Mich., USA) supplemented with 4.5% NaCl] for 6 h at 37 C to obtain cell densities of 10^8 to 10^9 cells/ml. Half the culture was centrifuged at 5,000 *g* for 15 min to separate the culture supernatant for tests on spontaneously released phages (spontaneous phage lysate). The other half of the culture was irradiated with UV light to induce prophages. For this, the suspension was transferred to a Petri dish (9 cm diameter) and exposed to a 15-watt germicidal lamp (Toshiba GL 15, Toshiba Ltd., Tokyo), 30 cm above the surface of the medium, for 40 sec with shaking by hand. The irradiated culture was mixed with 3 ml of fresh medium, incubated overnight at 37 C, and then centrifuged at 5,000 *g* for 15 min. The resulting supernatant fluid was used as a test specimen for detection of induced phages (induced phage lysate). No attempt was made to remove or kill intact L-form cells possibly remaining in test specimens, since L-forms should be destroyed during the following phage typing procedures, which were done under conditions of low osmotic pressure.

The lytic activities of the spontaneous and induced phage lysates were tested on 23 standard indicator strains and strain St. 1125 (a gift from Dr. Makoto Ohashi, Department of Microbiology, Tokyo Metropolitan Research Institute of Public Health, Tokyo), by the spotting method (Parker, 1966) on the nutrient agar (Nissui Seiyaku Co., Tokyo) supplemented with 0.04% $CaCl_2$. Table 1 shows that the phage released spontaneously from EMT-L lyzed indicator strain 6, and that released from 209P-L lyzed indicator strains 6, 77, 83A and St. 1125. Table 1 also shows that the induced phage lysates of EMT-L and 209P-L have wider lytic spectra than the respective spontaneous phage lysates. After UV-light irradiation, EMT-L produced phage that lyzed indicator strains 3A, 3C, 53, 54 and 42D in addition to strain 6, while 209P-L strain released phages that were also capable of lysing 52A/79 and 3A in addition to strains 6, 77, 83A and St. 1125. Moreover, assay by the spotting method revealed that the lytic titer of EMT-L against strain 6 and those of 209P-L against strains 6, 77, 83A and St. 1125 were significantly increased by UV light-irradiation (data not shown).

Next we examined whether single or multiple phage species were produced by the test L-form strains after UV light-irradiation and whether the phages induced from the two L-form strains were the same. Phages released by UV-irradiated EMT-L and 209P-L were propagated with indicator strain 6, which was common to both strains, by the soft agar method: briefly, a mixture of 0.2 ml volumes of appropriate dilutions of the induced phage lysate, 0.2 ml of a 6 h culture of strain 6 and 3 ml of melted agar medium consisting of nutrient broth (Nutrient Broth No. 2, Oxoid Ltd., Hants., England, unless otherwise stated) and 0.6% agar were poured onto nutrient agar (Nissui) solidified in Petri dishes. After overnight growth at 37 C, 9 agar blocks each with a well isolated plaque (as different in size and shape as possible) due to propagation of phages were cut out and put into 3 ml of nutrient broth. The broth was inoculated with 0.1 ml of 6 h-culture of indicator strain 6 and incubated at 37 C for 3 h. The culture was centrifuged at 3,000 *g* for 10 min and the supernatant fluid was filtered through a Millipore filter of 0.45 μm pore size (Sartorius-Membrane-Filter G m b h, Göttingen, West Germany). The filtrate was examined for lytic activity on standard indicator strains and strain St. 1125 by the spotting method. With indicator strains that were found to be sensitive by the spotting method, the filtrate was serially diluted 10-fold with nutrient broth and submitted to plaque counting by the soft agar method. Table 2 shows the results of individual titration assays for each plaque. It is indicated that the phage from EMT-L shows single type and that from 209P-L also shows single type. The host ranges of EMT-L phage and 209P-L phage were quite different: the former lyzed strains 6, 54 and 53,

TABLE 1. *Lytic pattern (host range) of phages of stable L-form strains, EMT-L and 209P-L, spontaneously released or induced by ultraviolet light irradiation*

Lytic group	Indicator strain	EMT-L		209P-L	
		Spon.[a]	UV[b]	Spon.	UV
I	29				
	52				
	52A/79				(+)
	80				
II	3A		(+)[c]		(+)
	3C		(+)		
	55				
	71				
III	6	+	+	+	+
	42E				
	47				
	53		+		
	54		+		
	75				
	77			+	+
	83A			+	+
	84				
	85				
Misc.[d]	81				
	94				
	95				
	96				
	42D		(+)		
	St. 1125			+	+

[a] Spontaneously released.
[b] Induced by UV irradiation.
[c] Variable from assay to assay.
[d] Miscellaneous.

whereas the latter lyzed strains 6, 77, 83A and St. 1125, and neither of them lyzed strains 52A/79, 3A, 3C and 42D. These findings strongly suggest that *S. aureus* L-form strains, EMT-L and 209P-L, were lysogenized by their own prophages which were distinct from each other. These prophages were tentatively named α and β, respectively.

The relationship between the prophages lysogenizing EMT and 209P (the parental, coccal forms) and those held by their L-forms were examined as follows. The UV-induced phage lysates of EMT-L and 209P-L were propagated on indicator strain 6 to produce well isolated plaques by the soft agar method as described above. Surviving organisms on each of more than 10 plaques were then inoculated into nutrient broth to isolate substrains immune to and lysogenic for the respective phages.

TABLE 2. *Lytic patterns of phages of S. aureus L-form strains EMT-L and 209P-L induced by UV irradiation and propagated on indicator strain 6*

Phage specimen		Indicator strain					
		6	54	53[a]	77	83A	St. 1125
EMT-L	1	1.3×10^{7}[b] (100)	5.0×10^{6} (38)	2.0×10^{3} (0.015)	—	—	—
	2	1.7×10^{7} (100)	2.5×10^{7} (147)	2.9×10^{3} (0.017)	—	—	—
	3	3.9×10^{6} (100)	2.2×10^{6} (56)	7.2×10^{2} (0.018)	—	—	—
	4	2.0×10^{7} (100)	3.3×10^{7} (165)	1.6×10^{3} (0.008)	—	—	—
	5	3.4×10^{7} (100)	2.2×10^{7} (65)	1.4×10^{3} (0.004)	—	—	—
	6	1.9×10^{6} (100)	3.3×10^{6} (174)	5.7×10^{2} (0.030)	—	—	—
	7	5.9×10^{6} (100)	4.1×10^{6} (69)	2.3×10^{3} (0.039)	—	—	—
	8	1.6×10^{6} (100)	2.0×10^{6} (125)	4.3×10^{2} (0.027)	—	—	—
	9	1.5×10^{6} (100)	1.6×10^{6} (107)	1.3×10^{3} (0.087)	—	—	—
209P-L	1	6.3×10^{8} (100)	—	—	6.0×10^{7} (9.5)	2.5×10^{6} (0.41)	8.4×10^{6} (1.3)
	2	5.7×10^{8} (100)	—	—	2.4×10^{8} (42)	2.5×10^{6} (0.44)	1.5×10^{7} (2.6)
	3	1.8×10^{6} (100)	—	—	1.6×10^{6} (88)	1.9×10^{5} (11)	3.9×10^{4} (2.2)
	4	7.7×10^{8} (100)	—	—	4.2×10^{8} (55)	3.3×10^{6} (0.42)	1.0×10^{7} (1.2)
	5	8.4×10^{8} (100)	—	—	1.6×10^{8} (19)	3.4×10^{6} (0.40)	1.1×10^{7} (1.3)
	6	1.3×10^{7} (100)	—	—	2.1×10^{6} (16)	4.3×10^{5} (3.3)	5.7×10^{4} (0.43)
	7	1.2×10^{8} (100)	—	—	8.0×10^{7} (67)	1.0×10^{7} (8.3)	1.0×10^{6} (0.83)
	8	3.7×10^{6} (100)	—	—	7.1×10^{6} (192)	6.0×10^{5} (16)	3.0×10^{4} (0.81)
	9	3.1×10^{6} (100)	—	—	3.0×10^{6} (98)	3.6×10^{5} (12)	1.4×10^{4} (0.46)

[a] Assayed by the spotting method.

[b] Phage plaque count/ml (relative value, taking the plaque count against strain 6 as 100).

Strains 52A/79, 3A, 3C and 42D were also examined for susceptibility, but none of them were susceptible to test phage specimens.

All substrains lysogenic for the induced phage of EMT-L were susceptible to that of EMT. On the other hand, there were two types of lysogens among those isolated from plaques produced by the induced phage lysate of EMT. One third of them, 6(α) were immune to phage α, the induced phage lysate of EMT-L, while the rest, tentatively named 6(γ), were susceptible to phage α. Assay by the spotting method revealed that lysogens

TABLE 3. *Relation between prophage types of coccal forms and L-forms of S. aureus EMT and 209P*

Substrain[a] derived from indicator 6	Phage induced by UV irradiation of the following coccal and L-forms and isolated by propagation in indicator strain 6				
	EMT		EMT-L	209P	209P-L
	α	γ	α	β	β
6	+	+	+	+	+
6 (α)	−	+	−	+	+
6 (γ)	+	−	+	+	+
6 (β)	+	+	+	−	−

[a] See text for details.
+ : Susceptible to the lytic action of the respective phage.
− : Resistant (immune) to the respective phage.

6(γ) were immune to some (phage γ) of phages induced in the parental EMT propagated on the indicator strain and caused lysis of the 6(α) substrain. These results strongly suggest that the parental strain, EMT, has an additional prophage γ, differing from prophage α harboring in the L-form strain, EMT-L; that is to say, during the successive subculture or the transition from coccal EMT to stable L-forms, EMT lost the prophage γ. Similar analyses indicated that there were no essential differences between prophage types of the coccal forms and L-forms of 209P. These findings are summarized in Table 3.

We believe that this work is the first demonstration that L-form cells of *S. aureus* are lysogenic and contain their own prophages. Studies are in progress on the use of prophages α and β as markers in intraspecies cell fusion of *S. aureus* L-forms.

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