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Abnormal Motility and Fruiting Behavior of *Myxococcus xanthus* Bacteriophage-Resistant Strains Induced by a Clear-Plaque Mutant of Bacteriophage Mx8

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***Myxococcus xanthus* mutants resistant to a clear-plaque derivative of phage Mx8 were isolated. A significant fraction of the mutants, easily recognizable by their colony morphology, were induced by the presence of the phage and may correspond to low-frequency lysogens. They were all defective in cell motility and showed the same nonfruiting phenotype under starvation conditions.**

Several phages active against the bacterium *Myxococcus xanthus* have been isolated and characterized (2, 7, 11). One of them, Mx8, contains double-stranded DNA 56 kilobases in length and acts as a generalized transducing phage. Mx8 produces turbid plaques, and this was considered evidence for a lysogeny-related interaction with the host (7). Recently, Orndorff et al. (9) obtained direct evidence for the integration of Mx8 DNA into the host chromosome, likely through a recombination process involving a circular intermediate of the phage DNA.

We report here the isolation of *M. xanthus* mutants resistant to either wild-type Mx8 or a clear-plaque derivative of Mx8. Some properties of these mutants further support lysogeny by Mx8 and suggest a distinct temperate stage for the clear-plaque variant. In contrast to wild-type Mx8, lysogeny by the clear-plaque variant seems to interfere with cell motility and the multicellular development characteristic of *M. xanthus*.

The standard strain used in this work was DK1050, a developmentally proficient, stable yellow derivative of *M. xanthus* FB (1). Plaque morphology mutants of Mx8 were obtained by *N*-methyl-*N'*-nitro-*N*-nitrosoguandine treatment of an exponentially growing culture of DK1050, in CTT Broth (4), which had been previously infected with Mx8. After cell lysis, phages were plated on a DK1050 cell lawn, and several clear plaques were picked and repeatedly purified. One of the clear-plaque mutants, designated Mx8cp2, was used in this work.

Mutants resistant to Mx8 ($Mx8^r$) or Mx8cp2 ($Mx8cp2^r$) were obtained by plating together, on CTT agar, exponentially growing DK1050 cells and the corresponding phage (see footnotes *a* of Tables 1 and 2). Surviving colonies were picked, and their phage resistance phenotype was retested by a plaque-forming assay. The plaquing efficiencies of all the colonies tested (more than 200) were $<10^{-5}$ relative to the parental strain.

$Mx8^r$ mutants arose at a spontaneous frequency of 1.5×10^{-2} per cell. This high frequency would not be surprising if a lysogenic process were operative, as has been previously proposed (9). If most of the $Mx8^r$ colonies were lysogens and were therefore induced by the phage, their spontaneous frequency in different experiments should not show the large fluctuations that would be expected for preadaptive mutations (6). The result of a Luria and Delbrück (6) fluctuation test for Mx8 resistance (Table 1) clearly established the induced nature of the $Mx8^r$ mutants.

Colonies formed by more than 1,500 $Mx8^r$ mutants in two independent experiments were observed, and none of them were distinguishable from those of the parental strain (DK1050). They showed the characteristic rough surface and irregular edge caused by group and individual cell movements (4) (see Fig. 1). A total of 170 of those $Mx8^r$ colonies were tested for multicellular development on coli-agar plates (3), and all of them fruited normally.

$Mx8cp2^r$ mutants arose at a spontaneous frequency of 3×10^{-6} per cell. Some of them formed colonies like those described for DK1050, but others formed very smooth colonies with well-defined edges; individual cells could be seen in these colonies only very occasionally (see Fig. 1). The proportion of smooth colonies among all the $Mx8cp2^r$ mutants was greatly different in independent experiments. The lowest and highest values found were 16 and 83%, respectively. Smooth colonies showed up on plates later than rough colonies, probably because of a lower growth rate.

When we tried to purify $Mx8cp2^r$ mutants from phages possibly contaminating them on the original selection plates, rough colonies needed only one restreaking. However, phages could be detected in culture supernatants of most of the smooth colonies after many purification steps. Eventually, the colonies seemed to be free of phages, but they were detected again, in some cases, in culture supernatants of descendant single colonies.

The different behavior of rough and smooth $Mx8cp2^r$ mutants with respect to the presence of phages was systematically studied, as follows. A total of 43 smooth and 45 rough spontaneous $Mx8cp2^r$ colonies from five independent experiments were streaked twice for purity, and single colonies from the second streak were cultured in CTT broth. Phages were detected in culture supernatants of 38 of the smooth colonies at a density of $>10^3$ PFU/ml, whereas they were absent in culture supernatants of all rough colonies tested (we could not have detected <100 PFU/ml).

The observations mentioned above could be explained if the smooth $Mx8cp2^r$ mutants were lysogens with a low frequency of spontaneous lytic induction, as described for wild-type Mx8 lysogens (9). The result of a fluctuation test for Mx8cp2 resistance (Table 2) strongly supported the induced nature of the smooth $Mx8cp2^r$ phenotype, as opposed to the preadaptive character of mutations responsible for the rough, fully motile $Mx8cp2^r$ phenotype.

Exponentially growing cells of a smooth $Mx8cp2^r$ mutant were extensively washed and plated at a low density on a lawn of sensitive cells. After 3 days, only 12% of the colonies

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TABLE 1. Fluctuation test for resistance of *M. xanthus* to phage Mx8[a]

Individual cultures		Bulk culture	
Culture no.	No. of Mx8[r] colonies[b]	Culture no.	No. of Mx8[r] colonies[c]
1	446	1	580
2	469	2	576
3	524	3	539
4	494	4	524
5	501	5	588
6	472	6	554
		7	531

[a] An exponentially growing DK1050 culture was diluted in 70 ml of CCT broth. This volume was equally distributed in seven culture flasks and incubated in a shaker at 33°C for 3 days (20 generations). For individual cultures, 0.1 ml of a 100-fold dilution from each of six flasks (ca. 10^6 cells) was mixed with 10^7 Mx8, incubated at room temperature for 1 h, and plated on CTT agar at different dilutions. Seven samples from the last flask (bulk culture) were plated in the same way. Plates were incubated at 33°C for 8 days, and data correspond to the same dilution factor (50-fold) in all cases.
[b] Mean, 484.3; variance, 760.3.
[c] Mean, 556.0; variance, 657.0.

had released phages, detected by clearing of the lawn. Thus, the frequency of spontaneous lytic induction should be very low. As happened with Mx8 lysogens (9), we were unsuccessful in raising that frequency with different doses of UV light.

M. xanthus mutants affected in both cell motility and fruiting have been reported (5). We tested for fruiting a large number of smooth Mx8cp2[r] colonies from seven independent experiments by placing them on coli-agar plates. For comparison, a similar number of rough Mx8cp2[r] colonies from the same experiment were also tested. The results are shown in Table 3. None of the smooth colonies fruited normally, whereas most of the rough colonies showed a fruiting behavior similar to that of the parental strain.

Figure 1 shows normal fruiting by DK1050 and abnormal fruiting by smooth Mx8cp2[r] mutants after developmental induction on PM agar plates (3). Only small differences were observed in the agregation patterns of different smooth

TABLE 2. Fluctuation test for resistance of *M. xanthus* to phage Mx8cp2[a]

Individual cultures				Bulk culture			
Culture no.	No. of Mx8cp2[r] colonies[b]			Sample no.	No. of Mx8cp2[r] colonies[c]		
	Total	Smooth	Rough		Total	Smooth	Rough
1	52	36	16	1	41	21	20
2	29	24	5	2	20	10	10
3	52	25	27	3	21	12	9
4	42	22	20	4	28	20	8
5	41	18	23	5	34	18	16
6	84	20	64	6	26	14	12
7	25	20	5	7	30	20	10
8	35	20	15				
9	27	21	6				

[a] An exponentially growing DK1050 culture was diluted in 100 ml of CTT broth. This volume was equally distributed in 10 culture flasks and incubated in a shaker at 33°C for 3 days (20 generations). For individual cultures, 0.1 ml of a 10-fold dilution from each of nine flasks (ca. 10^7 cells) was mixed with 10^7 Mx8cp2, incubated at room temperature for 1 h, and plated on CTT. Seven samples from the last flask (bulk culture) were plated in the same way. Plate incubation was carried out at 33°C for 8 days.
[b] Means, 43.0 (total); 22.9 (smooth); and 20.1 (rough). Variance, 336.0 (total); 28.9 (smooth); and 335.1 (rough).
[c] Means, 28.6 (total); 16.4 (smooth); and 12.1 (rough). Variance, 53.9 (total); 19.3 (smooth); and 18.8 (rough).

TABLE 3. Fruiting behavior of smooth and rough Mx8cp2[r] mutants

Expt	No. tested	No. of colonies[a]			
		Smooth		Rough	
		Fru[+]	Fru[−]	Fru[+]	Fru[−]
1	48	0	20	28	0
2	78	0	32	41	5
3	22	0	5	15	2
4	75	0	11	57	7
5	61	0	14	39	8
6	78	0	9	57	12
7	185	0	94	86	5

[a] Smooth and rough colonies were placed on coli-agar plates, incubated at 33°C, and observed after six to eight days. Fru[+], Cell aggregation similar to that in control (DK1050) colonies; Fru[−], abnormal or no aggregation. The experiments were different from those in Table 2.

Mx8cp2[r] mutants. These formed loose aggregates of only a few cells which, however, differentiated into highly refractile spores similar to those formed by DK1050. Spores were also relatively frequent outside the loose aggregates formed by the mutants, usually on cell streams. The presence of spores outside fruiting bodies is very rare in DK1050.

When exponentially growing cells of several smooth Mx8cp2[r] mutants were resuspended in PM buffer (3), phage induction, measured as the PFU in the supernatant before and after incubation for 24 h, was not detected. Cell death during that period of time in PM buffer was similar to that in control experiments with the parental strain (DK1050) (data not shown). Thus, the nonfruiting phenotype of these mutants cannot be the result of an artifactual decrease in cell density.

Of 362 rough, fully motile Mx8cp2[r] mutants, 39 could not fruit on coli-agar (Table 3). None of them showed any sign of cell aggregation or spore formation. They were either spontaneous double mutants or mutants affected in a gene involved in both cell-to-cell interaction and resistance to Mx8cp2.

Our results indicate that lysogeny by wild-type Mx8 does not affect motility or fruiting behavior in *M. xanthus*. We propose that the virulent character of Mx8cp2 is caused by a genetic block in the normal lysogenic pathway of Mx8. Among other alternatives, the mutation in Mx8cp2 may affect a gene controlling the lysis-lysogeny decision or the integration of phage DNA into the host chromosome (9). Lysogens for Mx8cp2 would thus arise by an unusual, low-frequency mechanism which would interfere with cell motility and fruiting. If these lysogens are also generated upon infection with wild-type Mx8, they should represent only about 0.01% of the total lysogens and would be difficult to identify.

How the unusual lysogenic process interferes with cell motility and fruiting is an open question. A possible mechanism may be an impairment of the interaction of phage DNA with the main host DNA attachment site which, as happens in phage λ, would bring into play a secondary attachment site (8, 9). The unusual integration event could result in the physical interruption or modification in the expression of a gene involved in both motility and fruiting behavior (5).

Pate et al. (10) reported the appearance of two colony types, spreading and compact, when selecting for phage-resistant mutants of *Cytophaga*, another gliding bacterium. This result is similar to the phenomenon we describe here and may have a similar explanation.

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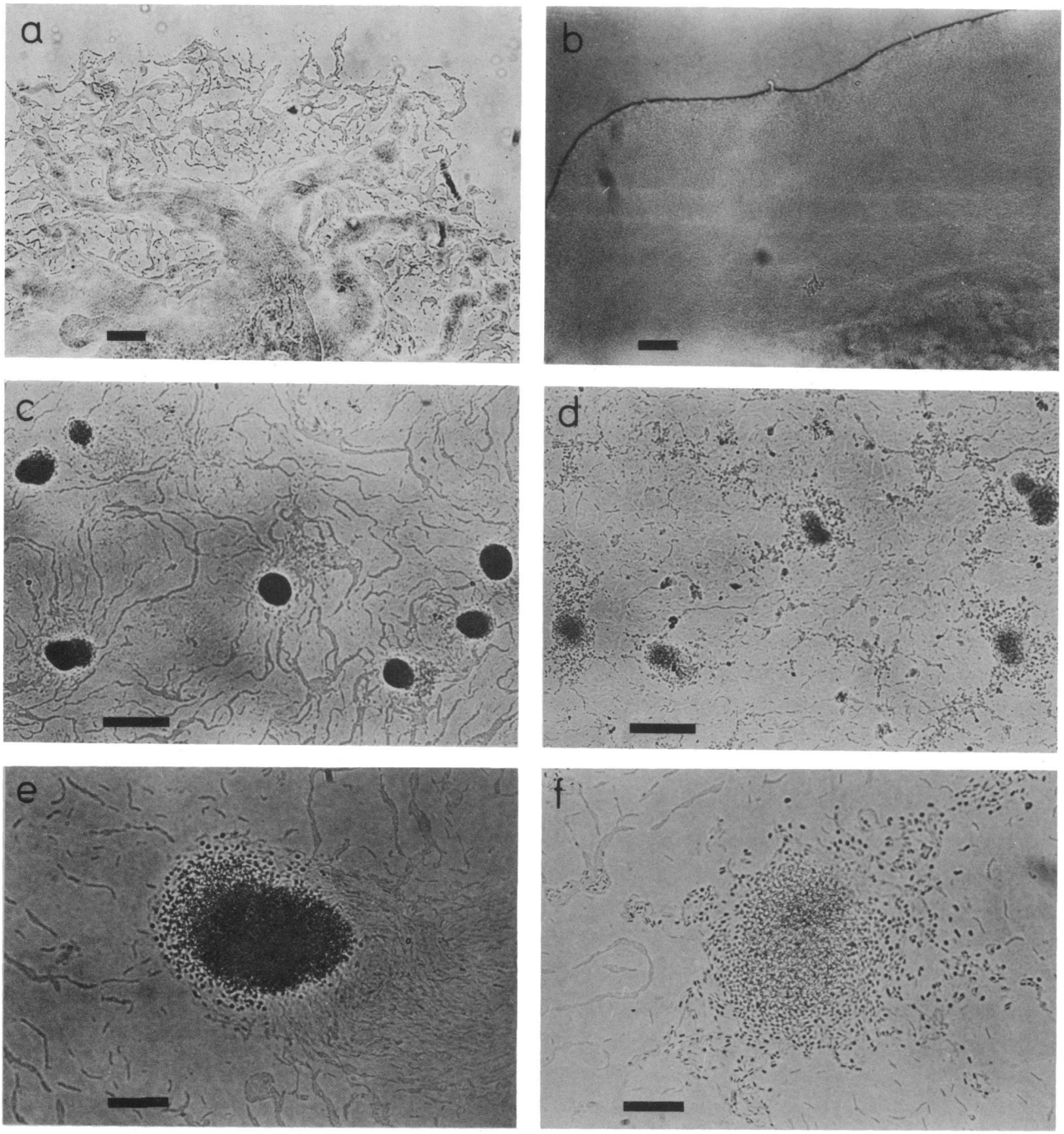

FIG. 1. Abnormal colony morphology and fruiting behavior of smooth Mx8cp2^r mutants. (a) and (b) Edges of colonies of the parental strain and a mutant, respectively, grown on CTT agar for 3 days at 33°C. (c) and (e), Normal fruits formed by DK1050. (d) and (f), Loose aggregates formed by the mutants. The tiny black spheres in (e) and (f) are mature, refringent spores. Fruiting was induced by spotting, on PM agar plates, 20 μl of exponentially growing cells that had been previously washed and resuspended in PM buffer at a density of 10^9 cells per ml. Photographs were taken after 6 days of incubation at 33°C. Bars, 100 μm in (a) through (d) and 20 μm in (e) and (f).

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