CHARACTERIZATION OF HOST-RANGE MUTANTS OF CYANOPHAGE N-1

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Summary. – Fifteen host-range (*h*) mutants of cyanophage N-1 were characterized with reference to their efficiency of plating, time of appearance, morphology and size of plaques on *Nostoc muscorum* and its three phage-resistant (Nm 1/N-1, Nm 2/N-1 and Nm 8/N-1) mutants. While phage N-1 did not adsorb to the three phage-resistant mutants, the *h* mutants differed one from the other in having lower or higher adsorption rate constants on *N. muscorum* or the phage-resistant mutants. The inability of majority of *h* mutants isolated on Nm 1/N-1 to grow in Nm 8/N-1 was shown to be due to a failure of adsorption. The *h* mutants also differed one from the other in their reversion (back mutation) frequencies. The lethal doses (LD_{37}) required to kill 37% of free phage particles after UV-irradiation, heating and ethylenediamine tetraacetate (EDTA) treatment greatly varied. Most of the *h* mutants were found to be considerably more sensitive to UV and thermic inactivation than N-1 while they were resistant to EDTA. The *h* mutants except five of them were unable to multiply at 40°C. The significance of these features is discussed.

Key words: cyanophage N-1; host-range mutants

Introduction

Host-range phenotype along with rapid lysis phenotype has been used in the classical mating and genetic recombination experiments with phage T2. It is easy to recognize the h^+ and *h* alleles in the progeny particles by their distinctive plaques if plated on a mixture of sensitive and T2-resistant *Escherichia coli* B/2 cells. Since *h* mutants have the capacity to lyze both strains of the host they form clear plaques, and since h^+ phages can lyze only sensitive *E. coli* B cells they yield turbid plaques (Hayes, 1970).

A mixed infection of different *h* mutants of T2 does not yield the production of h^+ phenotype, suggesting that *h* mutants do not complement each other. On the other hand, revertants of *h* mutants in superinfection produce *h* phenotype. Genetic analysis of *h* mutants of phage T2 mentioned above showed that the *h* mutation is controlled by a single gene (Hayes, 1970). Such studies on cyanophages are lacking completely. We have earlier reported the isolation of *h* mutants of N-1 occurring spontaneously and after acridine orange (AO) mutagenesis of intracellular N-1 phage (Sarma and Kaur, 1993). As a prerequisite for crossing experiments aiming at the determination of the number of genes regulating this marker, we report here the properties and characterization of *h* mutants of N-1.

Abbreviations: AO = acridine orange; EDTA = ethylenediamine tetraacetate; *h* = host-range; UV = ultraviolet; LPS = lipopolysaccharide

Materials and Methods

Bacterium and phages. The cyanobacterium *Nostoc muscorum* ISU (ATCC 27893) and cyanophage N-1 were used in this study. The host alga and its three phage-resistant mutants were propagated in modified Chu-10 Medium (Safferman and Morris, 1964) with micronutrient solution A6 of Allen and Arnon (1955) where calcium nitrate was replaced by calcium chloride in the same molar concentration. The cultures were maintained in a culture room at 28 ± 1°C and illuminated with day light fluorescent tubes (2 K Lux) at the 14:10 light/dark regime. The preparation, purification and maintenance of lysates of phage N-1 and its *h* mutants were already described (Sarma and Kaur, 1993).

Phage N-1 resistant mutants of *N. muscorum*, i.e. Nm 1/N-1, Nm 2/N-1 and Nm 8/N-1 were used to isolate *h* mutants from the lysates of N-1 (Sarma and Kaur, 1993). Ten such mutants (*h*-1, *h*-3, *h*-5, *h*-7, *h*-9, *h*-11, *h*-13, *h*-24 and *h*-97) screened against Nm 1/N-1, three *h* mutants (*h*-36, *h*-44 and *h*-126) screened against Nm 2/N-1 and two *h* mutants (*h*-49 and *h*-62) screened against Nm 8/N-1 caused lysis of *N. muscorum* and of the respective phage-resistant mutant against which they were screened. All the *h* mutants of N-1 were propagated in their respective phage-resistant mutant of *N. muscorum*. Titers of individual lysates were determined by counting the number of plaques arising out of serial dilutions of the phage strains plated on their respective hosts by the double-agar layer technique (Adams, 1959).

Efficiency of plating. N-1 and the fifteen *h* mutants mentioned above were plated on *N. muscorum* and its three phage-resistant mutants to determine the efficiency of plating, time of appearance, morphology and size of plaques. Since earlier studies (Padhy and Singh, 1978) have indicated that the age of host cells influences the adsorption of N-1, 6-day-old exponentially growing algal cultures were adjusted to an uniform absorbance at 660 nm and were used for phage plating to minimize variations of plaque morphology and size.

Rate of adsorption. Diluted phage lysates of known titer were added to algal cell suspensions (2 x 10^8 cells/ml) at a multiplicity of infection of 0.5 PFU per cell and incubated in standard growth conditions. One-ml-aliquots were withdrawn at intervals of 10 mins during 2 hrs, diluted to 10^{-5} and rapidly centrifuged to pellet the cells. The supernatants were diluted 10-fold and assayed for plaque titer. The latter indicated the number of unadsorbed phage particles. The adsorption rate constant (k) was calculated according to the formula

$$k = 2.3\ Bt\ (P_o/P)$$

where P_o = phage titer at zero time, P = virus not adsorbed at time t in mins, B = concentration of cells per ml.

Reversion of h mutants. Reversion frequency of *h* mutants was detected by plating mixtures of equal volumes of *N. muscorum* and phage-resistant mutants. Revertants to h^+ phenotype were scored by counting the number of turbid plaques. Clear plaques indicated *h* phenotype.

Inactivation experiments. In UV-irradiation experiments (Sarma and Singh, 1995), 0.1-ml-aliquots of phage samples (original titer of 10^8 PFU/ml) were withdrawn after every 30 secs of irradiation, diluted and plated on the respective host. The plates were kept in the dark for 24 hrs and then returned to standard growth conditions. Inactivation experiments at 55°C and in 5 x 10^{-4} mol/l EDTA were performed as described earlier (Sarma and Singh, 1995).

Temperature-sensitive growth of h mutants. The phages under study were allowed to infect *N. muscorum* and its phage-resistant mutants in an incubator illuminated with day light at the 14/10 light/dark regime and maintained at 40 ± 1°C. Phage strains which caused lysis of their hosts were identified directly and those which did not bring about the lysis were then shifted to permissive temperature of 28°C and the lysis was followed.

Results

Efficiency of plating and plaque morphology

Cyanophage N-1 did not grow at all in the three phage-resistant mutants of *N. muscorum*. All the *h* mutants grew in *N. muscorum* with different efficiencies as evidenced by their titers (Table 1). The highest titers of *h* mutants were found with *h*-44 (2 x 10^{12} PFU/ml), *h*-13 (1.7 x 10^{12} PFU/ml), *h*-12 (1.5 x 10^{12} PFU/ml), *h*-24 (1.4 x 10^{12}PFU/ml) and *h*-9 (1.0 x 10^{12} PFU/ml). The lowest titer was noted in case of *h*-126 (AO) (2 x 10^{10} PFU/ml) followed by *h*-5, *h*-7 (6 x 10^{10} PFU/ml) and *h*-1 (7 x 10^{10} PFU/ml). The rest of the *h* mutants multiplied to titers between 1 x 10^{11} and 3.4 x 10^{11} PFU/ml.

All the *h* mutants exhibited multiplication in the phage-resistant mutant cells with an efficiency lowered by 10^5 times. Of the ten *h* mutants isolated from Nm 1/N-1 cells, only *h*-3 multiplied with greater efficiency in Nm 8/N-1 cells (2 x 10^7 PFU/ml). The rest of the *h* mutants isolated from Nm 1/N-1 cells neither developed plaques on Nm 8/N-1 cells nor lyzed them in liquid cultures. Similarly, *h*-11 and *h*-97 (AO mutant) did not grow in Nm 2/N-1 cells. With the exception of *h*-3 and *h*-24 which showed titers of 3.9 x 10^6 and 5.5 x 10^6 PFU/ml, respectively, in Nm 2/N-1 cells, the *h* mutants did not grow to the extent of efficiency observed in Nm 1/N-1 and *N. muscorum* cells.

Mutants *h*-36, *h*-44 and *h*-128 (AO mutant), isolated from Nm 2/N-1 cells, behaved differently. The growth of *h*-44 was found to be highest in Nm 8/N-1 cells (4.56 x 10^7 PFU/ml) followed by Nm 2/N-1 and Nm 1/N-1 cells. In contrast, *h*-36 did not grow in Nm 8/N-1 cells while it exhibited almost similar multiplication in Nm 1/N-1 (3.7 x 10^6 PFU/ml) and Nm 2/N-1 cells (2.5 x 10^6 PFU/ml). Whereas *h*-126 (AO) multiplied similarly well in Nm 1/N-1 and Nm 8/N-1 cells (4.13 x 10^7 and 4.84 x 10^7 PFU/ml, respectively), it grew least in Nm 2/N-1 cells which from it was initially isolated (Table 1). Mutants *h*-49 and *h*-62, isolated in Nm 8/N-1 cells, multiplied with greater efficiency in Nm 1/N-1 and Nm 2/N-1 cells.

The time of appearance of plaques of *h* mutants (isolated from Nm 1/N-1 and Nm 2/N-1 cells) on *N. muscorum* was 3 days while it was 4-5 days on the *N. musorum* mutants with exception of *h*-1 which developed plaques on the three phage-resistant mutant cells after 3 days. The *h* mutants isolated from Nm 8/N-1 cells formed plaques on *N. muscorum* after 3 days but they took 5-6 days to develop plaques on the three phage-resistant mutant cells.

Phage N-1 formed plaques with a slightly yellowish halo on *N. muscorum* (Fig. 1). The morphology of plaques of various *h* mutants on *N. muscorum* was circular (A-II to D-IV) with a turbid halo as in case of *h*-1, *h*-3, *h*-12, *h*-36 and *h*-62 (A-II, A-III, B-IV, D-II) or without a halo as in the

Table 1. Efficiency of plating of *h* mutants of cyanophage N-1 on *N. muscorum* and its phage-resistant mutants

Phage strain	Phage titer			
	N. muscorum (PFU/ml x 10^{-10})	Nm 1/N-1	Nm 2/N-1	Nm 8/N-1
		(PFU/ml x 10^{-5})		
N-1	0.44	0	0	0
h-1	7	3	3	1
h-3	34	100	39	201
h-5	6	1	1	0
h-7	6	1	2	0
h-9	105	43	2	0
h-11	29	3	0	0
h-12	156	50	20	0
h-13	173	42	1	0
h-24	142	9	50	0
h-97 (AO)[1]	10	10	0	0
h-36	14	37	25	0
h-44	202	78	117	456
h-126 (AO)[1]	2	413	24	484
h-49	18	79	63	33
h-62	17	112	108	14

[1]Mutant produced by AO mutagenesis of N-1 while the rest of the *h* mutants appeared spontaneously (Sarma and Kaur, 1993).

Table 2. Adsorption rate constant (k) of different *h* mutants

Phage strain	k x 10^{-8}	
	N. muscorum	Nm 1/N-1
N-1	2.72	0
h-1	1.00	2.70
h-3	1.30	3.00
h-5	0.29	0.38
h-7	0.98	0.93
h-9	1.00	0.87
h-11	0.93	0.87
h-12	0.26	0.47
h-13	0.36	0.54
h-24	1.00	0.80
h-97 (AO)	0.95	0.90
	N. muscorum	Nm 2/N-1
h-36	1.05	0.90
h-44	0.80	0.80
h-126 (AO)	1.00	0.90
	N. muscorum	Nm 8/N-1
h-49	0.29	0.76
h-62	0.30	0.50

For the legend see Table 1.

Table 3. Reversion frequency of *h* mutants of cyanophage N-1 in the phage-resistant mutants of *N. muscorum*

Phage strain	Reversion frequency x 10^{12}		
	Nm 1/N-1	Nm 2/N-1	Nm 8/N-1
h-1	0	0	0
h-3	0	0	0
h-5	8.7	8.6	8.0
h-7	0	0	0
h-9	3.2	1.8	2.4
h-11	2.5	2.0	2.0
h-12	2.4	2.3	2.8
h-13	0	0	0
h-24	1.6	1.6	0
h-97 (AO)	1.0	1.0	0
h-36	0.8	0.6	0.8
h-44	0.7	0.6	1.2
h-126 (AO)	1.8	0.8	0
h-49	13.3	16.6	13.3
h-62	4.4	3.8	0

The maximum standard deviation in the above experiments was ± 5%.

rest. The plaques of *h*-1, *h*-3, *h*-9 (B-II), *h*-12 (B-IV) and *h*-13 (C-I) were slightly turbid while the plaques of the rest of the *h* mutants were clear (A-IV, B-I, B-III, C-II to D-IV).

The plaque morphology of *h* mutants on the three phage-resistant mutants of *N. muscorum* was quite variable (Fig. 2). Mutant *h*-1 formed slightly turbid plaques with a halo on Nm 1/N-1 and Nm 2/N-1 cells while it formed clear plaques on Nm 8/N-1 cells (E-I to E-III). The plaques of *h*-9, *h*-12, *h*-44, *h*-62 and *h*-126 on Nm 1/N-1 cells were turbid (F-II, F-III, G-II, H-IV, J-II). Likewise, the plaques of *h*-44, *h*-62 and *h*-126 on Nm 2/N-1 cells were turbid (G-III, J-I, J-III). The plaques of *h*-1 on Nm 8/N-1 (E-III), *h*-3 on Nm 1/N-1 and Nm 8/N-1 (E-IV, F-I), *h*-36 on Nm 1/N-1 and Nm 2/N-1 (F-IV, G-I), *h*-44 on Nm 8/N-1 cells (G-IV) and *h*-49 on the three phage-resistant cells (H-I to H-III) were clear. Except *h*-49 which formed plaques without a halo on the three phage-resistant cells, the rest of the *h* mutants formed plaques with an yellowish halo.

The plaque diameter of N-1 on *N. muscorum* was 4-7 mm after 6 days of incubation. Amongst the *h* mutants, the smallest plaques were those of *h*-3 and *h*-97 (2.5 mm) while with *h*-5, *h*-11, *h*-24 and *h*-36 the plaque size ranged from 10 to 12 mm. The largest plaques that appeared on *N. muscorum* were those of *h*-62 (15 mm) followed by *h*-49 (13 mm). The plaque size of all the *h* mutants on the three phage-resistant cells ranged from 0.5 to 5.0 mm.

Phage N-1 did not show any adsorption to cells of the three phage-resistant cells. The adsorption rate constants of the different *h* mutant phages differed one from the other (Table 2). Their rate of adsorption to *N. muscorum* was lower than that of N-1. Mutants *h*-1 and *h*-3 showed an increased rate of adsorption to Nm 1/N-1 cells, while the rest of the *h* mutants exhibited almost similar (*h*-7, *h*-9, *h*-11, *h*-24 and *h*-97) or slightly increased rate of adsorption (*h*-5, *h*-12 and *h*-13) to Nm 1/N-1 cells. The adsorption rate constants of

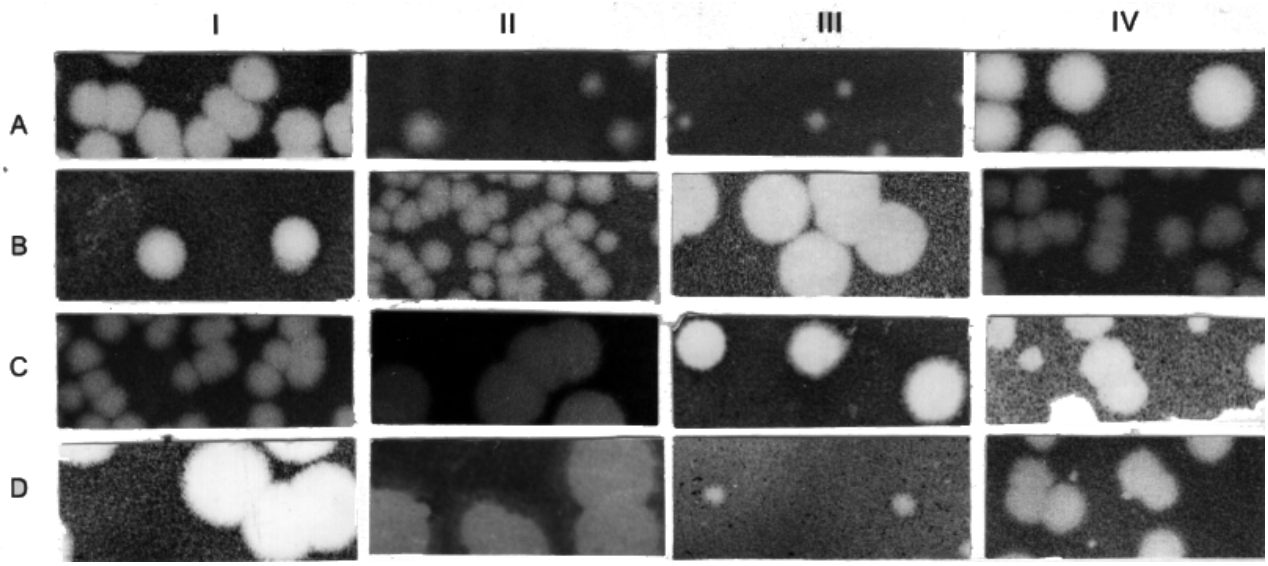

Fig. 1
Plaque morphology of *h* mutants of cyanophage N-1 on *N. muscorum*

Position/*h* mutant:

A-I/N-1	B-I/*h*-7	C-I/*h*-13	D-I/*h*-49
A-II/*h*-1	B-II/*h*-9	C-II/*h*-24	D-II/*h*-62
A-III/*h*-3	B-III/*h*-11	C-III/*h*-36	D-III/*h*-97 (AO)
A-IV/*h*-5	B-IV/*h*-12	C-IV/*h*-44	D-IV/*h*-126 (AO)

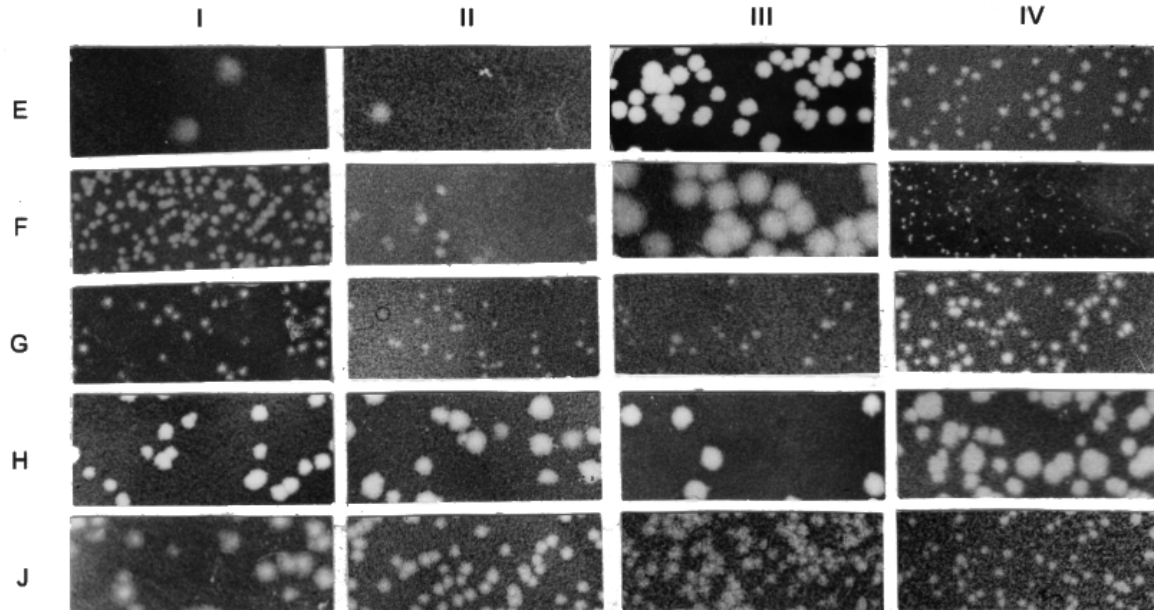

Fig. 2
Plaque morphology of *h* mutants of cyanophage N-1 on the phage-resistant mutants of *N. muscorum*

Position/*h* mutant on cells:

E-I/*h*-1 on Nm 1/N-1	F-I/*h*-3 on Nm 8/N-1	G-I/*h*-36 on Nm 2/N-1	H-I/*h*-49 on Nm 1/N-1	J-I/*h*-62 on Nm 2/N-1
E-II/*h*-1 on Nm 2/N-1	F-II/*h*-9 on Nm 1/N-1	G-II/*h*-44 on Nm 1/N-1	H-II/*h*-49 on Nm 2/N-1	J-II/*h*-126 on Nm 1/N-1
E-III/*h*-1 on Nm 8/N-1	F-III/*h*-12 on Nm 1/N-1	G-III/*h*-44 on Nm 2/N-1	H-III/*h*-49 on Nm 8/N-1	J-III/*h*-126 on Nm 2/N-1
E-IV/*h*-1 on Nm 1/N-1	F-IV/*h*-36 on Nm 1/N-1	G-IV/*h*-44 on Nm 8/N-1	H-IV/*h*-62 on Nm 1/N-1	J-IV/*h*-126 on Nm 8/N-1

majority of *h* mutants isolated from Nm 1/N-1 cells (*h*-3, *h*-7, *h*-9, *h*-11, *h*-12, *h*-13, *h*-24 and *h*-97) were considerably lower (0.02 to 0.08 x 10^{-8}/ml/min) for Nm 8/N-1 cells. Mutant *h*-36 isolated from Nm 2/N-1 cells, did not show any adsorption to Nm 8/N-1 cells (the adsorption rate constant was found to be zero). In these experiments, the *h* mutants were allowed to adsorb to Nm 8/N-1 cells and the unadsorbed particles were assayed on either Nm 1/N-1 (*h*-5, *h*-7, *h*-9, *h*-11, *h*-12, *h*-13, *h*-24 and *h*-97) or Nm 2/N-1 cells (*h*-36).

Revertants of *h* mutants formed turbid plaques on cultures of a mixture of *N. muscorum* and the respective phage N-1-resistant cells while the presence of *h* mutants was indicated by the appearance of clear plaques. The reversion frequency of different *h* mutants is given in Table 3. Mutants *h*-1, *h*-3 and *h*-13 were unique since they did not show any reversion to wild type and did not produce turbid plaques. The rate of reversion appeared to be similar for the three phage-resistant cells. Maximum frequency of revertants appeared in case of *h*-49 followed by *h*-5. The lowest rate of reversion was noted in *h*-36.

Except *h*-5 and *h*-62 most of the *h* mutants were found to be sensitive to UV-light with respect to N-1 particles as reflected by corresponding LD_{37} values. Likewise, their thermic inactivation (at 55°C) was quite rapid with LD_{37} values ranging from 3 to 8.6 mins in contrast to 30 mins for N-1. Another interesting feature of the *h* mutants is their relative resistance to EDTA in comparison to N-1, because the D_{37} values were considerably higher for the *h* mutants than for N-1 (Table 4).

Five of the *h* mutants (*h*-9, *h*-11, *h*-97 AO, *h*-44 and *h*-126) caused lysis of their respective hosts only upon shifting to 28°C suggesting that their multiplication was temperature-sensitive (Table 5).

Table 4. LD_{37} values of inactivation of cyanophage N-1 and its *h* mutants by UV, heating and EDTA

Phage strain	LD_{37} (sec)		
	UV	55°C	EDTA
N-1	30.0	30.0	3.0
h-1	17.2	3.0	39.0
h-3	23.0	5.2	32.0
h-5	42.0	7.8	38.0
h-7	18.4	6.5	29.0
h-9	21.6	5.8	17.8
h-11	14.5	3.2	28.4
h-12	11.2	3.7	24.0
h-13	9.0	6.0	9.7
h-24	26.2	8.6	16.4
h-97 (AO)	18.0	4.8	18.0
h-36	19.5	5.2	14.2
h-44	10.5	3.7	18.0
h-126 (AO)	14.5	3.0	–
h-49	7.5	4.1	10.5
h-62	30.0	3.3	15.3

Table 5. Multiplication of *h* mutants at the restrictive temperature

Host strain	Phage strains exhibiting lysis at 40°C	Phage strains exhibiting lysis after shift from 40°C to 28°C
Nm 1/N-1	*h*-9, *h*-11, *h*-97 *h*-12, *h*-13, *h*-24	*h*-1, *h*-3, *h*-5, *h*-7
Nm 2/N-1	*h*-44, *h*-126	*h*-36
Nm 8/N-1	–	*h*-49, *h*-62

Discussion

The difference between h^+ and *h* phenotypes is an all-or-none phenomenon, i.e. the phage either produces plaques or does not on a particular host cell (Hayes, 1970). All the *h* mutants of N-1 phage described in the present study grew with greater efficiency in *N. muscorum* as compared to that in the respective phage-resistant mutant cells. It is interesting to note that the *h* mutants isolated from Nm 1/N-1 cells did not grow in Nm 8/N-1 cells.

The phage resistance of host cells is mainly explained by an alteration in the receptor sites on the cell wall due to which phage particles no longer get adsorbed and the host cells survive even in the presence of high phage concentration (Hayes, 1970). In our experiments, phage N-1 did not adsorb to the phage-resistant cells signifying that their resistance to N-1 resides in their inability to adsorb it. The cell wall of cyanobacteria has the same fine structure as that of gram-negative bacteria (Goleki, 1979). Peptidiglycan, protein and LPS were shown to be the components of the cell wall of *Anacystis nidulans* (Katz *et al*., 1977). Of these, the LPS component was found to be responsible for the first step of recognition of attachment sites of cyanophage AS-1 (Samimi and Drews, 1978). The altered adsorption rate constants of different *h* mutants found by us may be explained by probable alterations in cell wall components at receptor sites. Cyanophage AS-1-resistant mutant of *A. nidulans* exhibited a slow rate of nutrient uptake and high Cu^{++} uptake leading to a pleiotropic behaviour (Kashyap and Gupta, 1982). This was explained on the basis of alterations in receptor proteins to be involved in the uptake of nutrients (Nikaido, 1979). The three phage-resistant mutants of *N. muscorum* also exhibited a pleiotropic behaviour with reference to the nutrient up-

take and acquired the ability to grow at 40°C, a feature which is lacking in *N. muscorum* (results to be published).

A single cycle of phage growth in a particular bacterial host is known to alter the host-range virtually of all the progeny (Hayes, 1970; Stent, 1963). This is known as the host-controlled modification or phenotypic modification. The factors underlying such a behaviour of a host strain leading to differential phage multiplication are governed by the lysogenization of the host by an unrelated phage. Secondly, the phage adsorption does not pose a problem in the host-controlled modification since it is the phage development cycle that is either triggered or inhibited. Thus, the inability of the *h* mutants (isolated from Nm 1/N-1 cells) to grow in Nm 8/N-1 cells is not due to the host-controlled modification but resides in their inability to adsorb to Nm 8/N-1 cells.

Moreover, the *h* mutants constitute the rare and random plaques that developed on the phage-resistant cells from the 10^{11} PFU per plate.

The *h* mutants also differed one from the other in their reversion frequencies reflecting their independent mutational sites on the N-1 phage genome. Their differences in the efficiency of plating, plaque morphology, rate of adsorption, rate of inactivation by UV-light, heating and EDTA were quite significant. The higher resistance of the *h* mutants to EDTA than that of N-1 could be due to alterations in some proteins of the phage head leading to increased permeability which did not result in rupture of heads upon transfer to distilled water as known in *o*-mutants of T-even phages (Hayes, 1970). Furthermore, the *o*-mutants were shown to be more susceptible than the wild type phage to the lethal action of nitrogen mustard (Brenner and Barnet, 1959). The *h* mutants of N-1 also showed the increased sensitivity to UV light and heating. All the *h* mutants of N-1, whose characterization is reported here, appear to be distinct one from the other and the revertants of some of the *h* mutants might lead in superinfection to the production of h^+ phenotype. Further work is in progress in this direction.

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