PHAGE RESISTANCE IN *Haemophilus influenzae*

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Harm and Rupert in 1963 described a temperate bacteriophage of *Haemophilus influenzae*. They reported that this phage called HP1 produced very turbid plaques on Rd indicator strains. In this laboratory the plaques were initially so turbid and ill-defined that quantitative studies were not possible. Investigations into the nature of this problem indicate that we are here dealing with a highly mutable phage resistance gene which clusters with the cathomycin and streptomycin resistance markers.

EXPERIMENTAL. All strains were *H. influenzae* Rd. Strains 138 lysogenic for phage HP1, and 143, indicator for HP1, were obtained from Dr. Rupert. Clear plaque formers 207 and streptomycin resistant (str^R) 200 were single clones from the latter. Strain 197 is a lysogenic derivative of 200. Strain 62 is insensitive to HP1. Derivatives are a str^R, a cathomycin resistant (cat^R), an erythromycin resistant (ery^R, 50 ug/ml), a viomycin resistant (vio^R) and a $str^R cat^R$ strain.

Media, transformation techniques, preparation of competent cultures and of crude DNA extracts have been described (Stuy, 1960, 1965). Phage HP1 was assayed for by the soft (Levinthal) agar overlay method. Indicator cells were grown

until 4 x 10^9 per ml and 0.05 ml used per plate. Phage was prepared by inducing growing cultures at 10^9 per ml with ultraviolet radiation killing 90-99% of the cells. Purification was accomplished by ultracentrifugation.

Phage resistance is defined as the inability to adsorb phage. Adsorption was measured by mixing purified phage at 2 x 10^8 with washed growing cells at 2 x 10^9 per ml in Difco Brain Heart Infusion containing 3 mM $CaCl_2$. After 7 min at 37 C the suspension was treated with chloroform and plated for free phage. Sensitive cells adsorb over 90%, resistant cells adsorb less than 30% of the added phage.

Phage insensitivity is defined as the inability to produce plaques under standard conditions. Insensitivity of non-lysogenic cultures was invariably found due to phage resistance.

RESULTS. a. Location of the phage resistance gene.

H. influenzae Rd strain 62 and its derivatives (obtained by transformation) do not adsorb phage HP1. They can be successfully transfected with HP1 DNA and are thus classified as phage resistant ($HP1^R$).

HP1 resistance is a genetic marker transferable by transformation. It has been mapped by transformation of strain 200 $str^R cat^S HP1^S$ with DNA($str^S cat^R HP1^R$), of strain 207 $str^S cat^S HP1^S$ with DNA($str^R cat^R HP1^R$), and finally of strain 200 with DNA($cat^R HP1^R$), with DNA($ery^R HP1^R$) and with DNA ($vio^R HP1^R$). Selected antibiotic resistance transformants were examined for phage resistance. It can be seen (Table I) that the $HP1^R$ marker is 55% linked to the cat^R marker, 35% to the str^R marker and not (or very loosely) to the ery^R and vio^R markers.

Table I

Recipient	Donor	Co-transformation to			Total
$s^{R}c^{S}p^{S}$	$s^{S}c^{R}p^{R}$	$c^{R}s^{S}$:19	$c^{R}p^{R}$:15	$s^{S}c^{R}p^{R}$:2	93
$s^{S}c^{S}p^{S}$	$s^{R}c^{R}p^{R}$	$s^{R}c^{R}$:52	$s^{R}p^{R}$:34		94
		$c^{R}s^{R}$:17	$c^{R}p^{R}$:36		63
$an^{S}p^{S}$	$an^{R}p^{R}$	$c^{R}p^{R}$: 8			16
		$e^{R}p^{R}$: 2			37
		$v^{R}p^{R}$: 1			54

Abbreviations: s:str, c:cat, p:phage, an:antibiotic, e:ery, v:vio. The str marker expresses at 50%. First cross was done by Miss Ruth Linn.

Phage sensitivity can also be transferred by transformation. Strain 63 $str^{R}cat^{S}HPl^{R}$ was exposed to DNA ($str^{S}cat^{R}HPl^{S}$). Of 17 cat^{R} transformants, 5 were $str^{R}HPl^{R}$, 9 were $str^{R}HPl^{S}$, 2 were $str^{S}HPl^{R}$, and 1 was $str^{S}HPl^{S}$. The HPl^{S} marker was thus co-transformed with the cat^{R} marker in 10 out of 17 cases.

These results localize the phage resistance marker and show that it is transformed at high efficiency suggesting a small gene mutation.

b. Conversion to phage resistance.

When growing sensitive cells were exposed to a tenfold excess of phage under standard adsorption conditions, the 10-15% surviving bacteria routinely observed consisted of 2/3 resistant non-lysogenic and 1/3 sensitive lysogenic cells. Roughly the same ratio was found when survivors were picked from the centers of plaques. The original indicator

strain 143 showed much greater ratios of resistant surviving bacteria. It is thus clear that the turbidity of the plaques is mainly due to the presence of resistant cells in the indicator cultures.

Every clone of sensitive cells investigated was found to contain a few resistant bacteria. This indicates a high mutation frequency. The expectation, however, that such sensitive cultures would rapidly become resistant upon subculturing was not borne out. Neither was it observed that resistant clones would become sensitive cultures upon continued transfer (Table II). In either case did the fraction

Table II

Generations	Resistant fraction in				
	$HP1^S$ clones		$HP1^R$ clones		
22	3/72	0/71	72/72	-	69/71
33	2/72	0/69	72/72	70/70	71/71
44	1/72	-	-	-	-
55	0/72	-	71/72	-	-
66	0/72	-	-	-	-
77	5/72	2/56	-	59/59	54/56
88	3/72	-	62/67	-	-
99	1/66	-	-	-	-
121	-	3/72	-	69/71	69/72

Cultures, grown from single cells, were continously subcultured by diluting them 2000 times every 24 hr in fresh pre-warmed broth at 37 C. They were then plated in agar and some 70 colonies stabbed for overnight cultures. These were plated in soft agar as indicator cells and spottested with dilute phage suspensions. Most of the resistant clones (columns 2 and 3) were checked for phage adsorption; all were negative. Clone in column 2 was subcultured for another 55 generations at room temperature. It then contained 2/48 resistant cells. Resistance clustered with the cat locus as described above.

of "contaminant" mutants "stabilize" at a few per cent.

Certain experiments indicated that more than genetic conversion was involved. Indeed, it was shown by Mr. W.R. McWilliams that sensitive bacteria grown at 30 C did not at all adsorb phage at either 30 or 37 C. Bacteria grown at 37 C adsorbed 94 and 89% respectively, at these two temperatures. This phenomenon is thus responsible for the inability of sensitive bacteria to produce plaques below 33 C.

DISCUSSION. Young (1967) and Young and Arias (1967) describe a series of phage resistant mutants in Bacillus subtilis. These mutants fall into 3 groups which are genetically clustered on the chromosome between the histidine A_1 and the arginine C loci. They map in a highly mutable region which fact may be of great survival value to the bacteria (Young, pers. comm.). The biochemical mechanism of the resistance has been worked out by Young (1967). The main observation is that all three groups of mutants lack glucose in the cell wall teichoic acid.

Phage HP1 resistance and sensitivity in H. influenzae is analogous to above system in that it shows a very high mutation frequency. The mutation clusters with the cat^R and str^R markers. It is speculated that there are two (or more) pathways in cell wall synthesis. The predominant one at low temperatures does not lead to phage receptor sites. Such sites are synthesized above 33 C. The mutation to resistance blocks the latter pathway. A number of predictions can be made on the basis of this speculation. These are presently being tested.

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