PHAGE-RESISTANT MUTANTS OF P. PESTIS AND THEIR PROPERTIES

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Previously we have reported the biological properties of three phages of P. pestis. According to these results the lytic activity of these phages (EV, 1-17 d'Heralle) was correlated with their absorptive power and the duration of intracellular development. All these phages form a single serological group. The absorption of the main mass of the phage particle takes place in 5-8 min (EV and 1-17) and in 8-10 min (d'Heralle). The discovery of the property of these phages enabled us to use them in experiments to separate the phage-resistant mutants and to determine their characteristics.

The present article describes the determination of the incidence of spontaneous mutation of P. pestis from a phage-sensitive to a phage-resistant strain; we also describe certain properties of the phage-resistant mutants in relation to the properties of the phages.

EXPERIMENTAL METHOD

The possibility of the origin through mutation of phage-resistant strains of P. pestis has been found by means of the "replica" technique [4]. For this purpose colonies of plague bacteria (100 strains) aged 18-24 hours were transferred by a "stamp" to dishes containing agar into which the bacteriophage had been introduced previously. The resistant colonies which appeared in the replica dish were repeatedly explanted with a loop on to an agar plate together with the phage, and were then explanted on to an agar plate without the phage.

The incidence of spontaneous mutation from the phage-sensitive into the phage-resistant form was determined by Newcomb's modification of Luriya and Del'dryuk's method. For this purpose broth cultures of P. postis aged 24 h were introduced onto agar plates already containing the phage. Control explants of the same volume were made from two agar plates without phage. A count of the resistant colony was made after two days; this was done because a count after one day (as recommended by the author) revealed no sign of growth. As in the first method the phage-sensitive mutants which appeared were repeatedly passed through agar plates containing phage, and then the colonies which had grown a second time were emulsified in broth and explanted several times onto agar plates without phage. The incidence of phage-sensitive mutants was calculated from the formula: $a = -(\log 2)(\log P_0)/N$, where a is the mutation incidence per cycle of division of the bacterial cell, P_0 is the number of cultures showing no mutant, and N is the mean number of colonies at the end of the growth in broth before explantation into a dish with the phage.

The properties of the original strains were studied by the usual method for P.pestis [1, 2].

EXPERIMENTAL RESULTS

Three or four colonies of resistant mutant bacteria appeared among the cell population of both the virulent and avirulent strains of P. pestis. The incidence of the phage-sensitive mutants calculated from the formula given was:

$a_{EV} = -(\log 2) \cdot (\log P_0)/N = -(\log 2) \cdot (\log 86)/500 = 6 \cdot 10^{-3}$ in another case the value was $2 \cdot 10^{-3}$

$a_{1-17} = -(\log 2) \cdot (\log P_0)/N = -(\log 2) \cdot (\log 80)/500 = 6 \cdot 10^{-3}$ in another case the value was $2 \cdot 10^{-3}$

$a_{d'heralle} = -(\log 2)(\log P_0)/N = -(\log 2) \cdot (\log [83])/500 = 6 \cdot 10^{-3}$; in another case the value was $2 \cdot 10^{-3}$

From what has been said it can be seen that the number of mutant colonies makes up some percentage or fraction of a percentage of the number of explanted cells, and that the incidence of phage-resistant mutants of P.pestis is insignificant.

TABLE 1. The Sensitivity of Phage-Sensitive Mutants to Bacteriophages

Mutants	Phage			
	EV	1-17	d'Heralle	pseudo-tuberculosis
499/1-17 (1)	—	—	—	+
1309/EV	—	±	±	—
179/EV	—	—	—	—
1107/1-17	—	—	—	—
418/1-17 (1)	—	—	—	+
495/1-17	—	—	—	—
1303/D	—	—	—	+
1378/1-17	±	±	±	±
4 0/EV	—	—	—	—
PB-Bzh/D	—	—	—	—
499/1-17 (2)	—	—	—	±
418/1-17 (2)	—	—	—	±
460/1-17	—	—	—	—

The next step was to study the properties of some of the resistant mutants. They were described according to the accepted scheme [3]. We observed three variants of colonies distinguished by their appearance on agar.

1. Pale yellow with a hillock in the center and a well-shown flat-or scalloped edge – the R-form.

2. Pale yellow colonies having a fine or smooth granularity, center raised, and in most cases an indented or more rarely an even edge – OR-form.

3. Completely smooth colourless colonies with a raised center and even outline – S-form. The growth on broth varied according to the morphology of the colonies on agar. In colonies of the first two types there was an agglutinative growth and a precipitate on the bottom which rose up in clumps when the vessel was shaken. The colonies of the third type as a rule gave a turbidity to the broth. A study of the morphology of the bacteria showed a considerable degree of polymorphism of the Gram-negative bacilli, which ranged from well-marked ovoids to cocco-bacilli. The active mobility of these bacteria observed in the hanging drop was found in four out of the thirteen experimental mutants (418/1-17$_{(1)}$, 460/EV, 499/1-17$_{(1)}$ 1303/D) whereas in the remainder either a Brownian movement or no movement was recorded. The extent to which the mutants were lysed was tested by application of the undiluted phages EV, 1-17, d'Heralle, and pseudotuberculous phage onto the surface culture (see table).

Thus, most mutants are resistant to all the phages and even to the pseudotuberculous phage. Mutant 1309/EV was resistant only to phage EV but was lysed by any two others. The second mutant 1378/1-17 was partially lysed by all phages. The resistance of most mutants to the phages investigated indicates the close antigenic similarity of the phages.

The mutants obtained showed the power to absorb phage particles. It is known that certain resistant variants are able to absorb the phage to whose action they are resistant [3]. In our experiment the mutant 1378/1-17 was found capable of absorbing particles of all phages. When the other variants were explanted by the method of agar layers no sterile spots developed.

Taking into account the finding of many authors that under the action of certain factors and of a phage in particular, the plague bacterium is frequently altered so as to be brought nearer to P. pseudotuberculosis, we have studied the biochemical activity of mutants and their ability to grow in differentially diagnostic media (plain acid agar, peptone-free agar, Otten's medium, Mied's medium number 2, Kolya-bel'kur medium, litmus milk, urea).

Table 2 allows us to distinguish two groups of phage-resistant mutants from their biochemical properties and their ability to grow on differentially diagnostic media. The first includes three mutants 418/1-17$_{(1)}$, 499/1-17$_{(1)}$, and 1303/D. The second group contains all the remainder.

Mutants of the first group which at the time of separation show a pale-yellow surface with hillocks and a well-marked scalloped edge, after 24 hours fermented rhamnose, gave a blue colour with Atten's medium when an infusion of litmus was added, they grew on Maied's No. 2 medium on plain acid agar, they decomposed urea for 1-4 days, reduced nitrate and nitrite, and almost completely reduced methylene blue. On colourless agar the mutants of the first group grew in an 8-fold dilution, whereas the remainder grew in a 2-fold dilution, and variant EP-5Zh/D grew in a 4-fold dilution.

Mutants of both groups decomposed a kolya-bel' medium.

The relation of the mutants of the second group to rhamnose and glycerine deserves attention. Most of them fermented rhamnose but they did so later than mutants of the first group (on the 7-10th day). Three mutants (499 per 1-17$_{(2)}$, 460/1-17, 430/EV) lost the ability to decompose glycerine, whereas the initial cultures fermented this alcohol on the third day. Mutant 1107 did not decompose glycerine until the sixteenth day. From what has been said it follows that in their biochemical activity and ability to grow on differentially diagnostic media the mutants of the first group revealed properties akin to those of P. pseudotuberculosis. A study of the serological properties of the

TABLE 2. Properties of Phage-Resistant Mutants

Strain	Glucose	Maltose	Mannite	Lactose	Sucrose	Glycerine	Rhamnose	Nitrification	de-nitrification	Reduction, blue cell medium Kolya-bel'kur	Kolya-Belkura's medium	Otten's medium	Maied's medium No. 2	Plain acid agar	Peptone-free agar	Urea
490/1—17 (1)	+++	++++	++	—	—	—	+1 c	+	—	irb	++++	Blue stain	Marked growth	Weak growth	8	+3 c
1309 EV	++	++++	++	—	—	+3 c	—	++++	++++	—	++++	Red	—	—	2	—
179/EV	++	++	++	—	—	+3 c	—	—	—	—	++++	Red	—	—	2	—
1107/1—17	++	+++	++	—	—	+16 c	+7 c	++++	++++	—	++++	Red	—	—	2	—
418/1—17 (1)	++	+++	++	—	—	+3 c	+1 c	+	—	irb	++++	Blue	Marked growth	Weak growth	8	+1 c
495/1—17	++	+++	++	—	—	+2 c	+10 c	—	—	irb	++++	Red	—	—	2	—
1309/D	++	+++	++	—	—	+3 c	+1 c	+	—	irb	++++	Blue	Marked growth	Weak growth	8	+4 c
1378/1—17	++	+++	++	—	—	+3 c	—	—	—	—	++++	Red	—	—	2	—
460/EV	+	+	++	—	—	—	+7 c	—	—	—	++++	Red	—	—	2	—
460/1—17	+	++	++	—	—	—	+7 c	—	—	—	++++	Red	—	—	2	—
BP-582b/D	++	+	++	—	—	+3 c	+10 c	—	—	—	++++	Red	—	—	4	—
490/1—17 (2)	++	+	++	—	—	—	—	—	—	—	++++	Red	—	—	2	—
418/1—17 (2)	++	+	++	—	—	+10 c	—	+	+	—	++++	Red	—	—	2	—

Symbols:— and negative reaction; + partial decomposition; ++ weak composition; +++ complete decomposition (intense stain; ++++ well-marked decomposition; a figure marked with the letter "d" indicated the day on which the decomposition occurred; irb incomplete reduction of methylene blue.

mutants obtained was studied by the classical reaction with agglutinating serum; we also investigated the sera obtained which were immune to the mutants; treatment with the original strains and mutants showed that a positive agglutination reaction was given in the same dilutions, both by the original strains and by the mutants.

The final stage in studying the properties of the mutants produced was to test their pathogenicity against rabbits and guinea pigs. Mutants of the first group injected as 0.1 ml of a two-day suspension in physiological saline containing one billion cells caused the rabbits to die within two days. Mutants of the second group were not lethal. Guinea pigs died after receiving an injection of 10,000 million or 1000 million microbial cells of the mutant 1378 per 11 -17, whereas other mutants were not lethal. Repeated injection of 200 Dcl of a virulent strain into the same animals caused death within 4-14 days, showing that the mutants produced had possessed no immunogenic properties.

SUMMARY

An investigation was made into spontaneous mutation from bacteriophage-sensitivity to bacteriophage-resistance in P. pestis strains of different virulents. According to preliminary data, mutants resistant to bacteriophage appeared irrespective of the virulence of the initial strain and of the bacteriophage species used. Variants were isolated from the mutants, and by their chemical properties and capacity to grow on differential-diagnostic media and high pathogenicity for rabbits could be classed as causative agents of pseudotuberculosis.

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