Surface Changes in a Strain of *Rhizobium trifolii* on Mutation to Bacteriophage Resistance

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SUMMARY

A strain of *Rhizobium trifolii* and four phage-resistant derivatives had different mutation frequencies to phage resistance. One mutant adsorbed the phage to which it was resistant; the others showed little or no adsorption. Differences between the strains in the number and nature of ionizable surface groups were revealed by micro-electrophoresis. Two of the resistant mutants were lysed by lysozyme and three by lipase. Lysozyme + EDTA lysed all strains, but at different rates.

INTRODUCTION

Resistance to bacteriophage infection may be acquired by bacteria in three ways. The adsorption of bacteriophages to the bacterial surface may be prevented or penetration of the bacterial wall by bacteriophage nucleic acid may be inhibited. These two kinds of resistance involve changes in the bacterial wall (Weidel, 1958). The third kind of resistance involves the action of a restricting enzyme which degrades bacteriophage nucleic acid after injection (Molholt & Fraser, 1965).

The walls of a few strains of *Rhizobium trifolii* have been partially analysed. Humphrey & Vincent (1962) found mucopeptide components, glucose, rhamnose and a high proportion of amino acids in the wall of one strain. Humphrey & Vincent (1969*a*) have also studied the serological properties of the somatic O antigens from two other strains of *R. trifolii*. Both antigens showed components characteristic of both normal lipopolysaccharide (LPS) structures and also of the capsular K antigens which occur in certain Gram-negative bacteria. Marshall (1966) studied the electrophoretic behaviour of some fast- and slow-growing strains of *Rhizobium*. With one exception, the slow-growing strains had only carboxyl groups, whereas the fast-growing strains had both carboxyl and amino groups in the surface. Humphrey & Vincent (1969*b*) reported that the LPS antigens from two strains of *R. trifolii* showed relative mobilities (from immuno-electrophoresis) similar to those found for whole bacteria. It was suggested that the surface charge characteristics of these two strains were determined by the structures and compositions of the LPS antigens.

Most phage-resistant mutants in *Escherichia coli* acquire resistance by preventing phage adsorption (Weidel, 1958). This paper investigates the surface of a strain of *Rhizobium trifolii* and four rhizobiophage-resistant derivatives.

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METHODS

Phage and bacteria. Phages e and c were originally isolated from soil and form large, clear plaques on susceptible bacteria. The bacterial strains used are all derivatives of the non-capsulated strain W19 isolated from the natural environment. W19/c is a bacterial strain selected for resistance to phage c. W19/e1 and W19/e2 were selected for e resistance, but differ in their pattern of susceptibility to other bacteriophages. W19/ch was obtained by selecting for resistance of W19 to a host-range mutant of phage c able to grow and form plaques on W19/c.

Bacteria were grown in the medium designated GS-YC by Schwinghamer (1960), with the exception that glucose was incorporated at 0.1% to reduce gum formation. For all experiments, bacterial cultures were harvested in late exponential growth phase (bacterial concentration about 2×10^8 /ml).

Phage lysates were prepared by inoculating an exponentially growing bacterial culture with a single phage plaque and usually contained 10^{10} – 10^{11} plaque-forming units (p.f.u.)/ml.

Mutation to phage resistance. A viable count was first performed on a bacterial culture in exponential growth phase. This culture was then infected at high multiplicity by a phage strain in liquid culture for a period of 30 min. The bacteria were then plated and resistant colonies counted. The average proportion of resistant bacteria in five independent cultures was recorded as the mutation frequency.

Adsorption constants. These were measured as described by Adams (1959). After the addition of phage to bacteria, unadsorbed phage were measured at intervals.

Micro-electrophoresis. Electrophoretic mobilities were determined as described by Douglas, Ruddick & Williams (1970). Bacterial suspensions were prepared for electrophoresis by $3 \times$ centrifugation and resuspension in 0.05 M-NaCl at a concentration of about 5×10^9 organisms/ml. For each mobility determination, 1 ml of this suspension was added to 99 ml of the appropriate buffer solution (Douglas *et al.* 1970).

Reagents. *p*-Toluene sulphonyl chloride (PTSC) was recrystallized from petroleum ether and used as a saturated solution in phosphate buffer, pH 7. Washed ($3 \times$) bacteria were treated for 1 h at 4 °C, washed $3 \times$ in Michaelis pH 7 buffer, and finally resuspended in Michaelis buffer. Bacteria which had been subjected to the same procedure in the absence of PTSC were used as controls.

Sodium dodecyl sulphate (SDS) was used as described by Douglas *et al.* (1970).

Enzyme treatments. Lipase (wheat-germ; British Drug Houses (B.D.H.), Godalming, Surrey) was used at 500 p.p.m. in Michaelis pH 7 buffer. Lysozyme (egg-white; B.D.H.) was used at 50 p.p.m. in Michaelis pH 7 buffer. Lysozyme was also used at 50 p.p.m. in 0.05 M-ammonium acetate + 1.0 mM-ethylenediamine-tetra-acetic acid (EDTA). All enzyme treatments were carried out at 25 °C. Exponentially growing bacteria were washed $3 \times$ by centrifugation and finally resuspended at about 5×10^8 /ml in the appropriate enzyme solution. The change in extinction of each suspension was determined at intervals.

RESULTS

Phage-resistant mutants obtained. The proportion of bacteria resistant to phage e in cultures of W19 was approximately 100 times that of bacteria resistant to phage c (Table 1). Also, the frequency of e resistant bacteria in W19/c cultures was approximately the same as in W19 cultures. However, no mutants of W19/e1 or W19/e2 resistant to c were observed. Only one W19/ch resistant mutant colony was isolated.

Table 1. *Frequency of phage resistant bacteria in cultures of each strain*

Strain	Frequency of resistance to phage c	Frequency of resistance to phage e
w19	2.1×10^{-7}	2.2×10^{-5}
w19/c	—	1.4×10^{-5}
w19/e1	$< 10^{-8}$	—
w19/e2	$< 10^{-8}$	—

Table 2. *Adsorption constants of phages c and e to sensitive and resistant bacteria*

Phage	Bacterial strain	Adsorption constant (ml/min)
c	w19	2.2×10^{-9}
	w19/c	3.3×10^{-9}
	w19/ch	4.0×10^{-11}
	w19/e1	3.4×10^{-9}
	w19/e2	4.5×10^{-9}
e	w19	3.3×10^{-10}
	w19/c	4.6×10^{-10}
	w19/ch	$< 10^{-11}$
	w19/e1	$< 10^{-11}$
	w19/e2	$< 10^{-11}$

No lysis was observed when supernatants from cultures of any of the phage-resistant mutants were plated with w19. It is therefore unlikely that any of the phage-resistant mutants are lysogenic for phages c or e.

Adsorption constants. These results are summarized in Table 2. Phage c adsorbed to w19 at a faster rate than phage e. However, although phage c adsorbed to w19/e1 and w19/e2 at essentially the same rate as to w19, phage e was not adsorbed to these two mutant strains. Similar constants were found for the adsorption of phage e to both w19 and w19/c; phage c also adsorbed to w19/c at a similar rate to w19, although resistance was still maintained. No detectable adsorption of phage e to w19/ch was found, and adsorption of phage c was much slower than to w19 or w19/c.

Micro-electrophoresis. All strains lysed to varying degrees at pH values greater than 9, and hence mobilities could not be measured at higher pH values. The mobility versus pH curves showed differences between strains (Fig. 1). Only w19 had fairly strong acidic groups. w19/c and w19/e2 gave similar mobility versus pH responses, as did w19/ch and w19/e1. These latter two strains showed substantial positive mobilities at pH 2.0.

The mobility shifts obtained on treatment with PTSC and SDS are shown in Table 3. w19 showed substantial shifts after treatment with both reagents, indicating that both surface amino groups and surface lipid are present. w19/c and w19/e2 showed only small shifts after PTSC treatment, indicating low frequencies of amino groups in both bacterial surfaces. These strains gave heterogeneous mobility distributions from individual organisms after SDS treatment. This unusual finding suggests non-uniform surface lipid contents within the populations for these two strains. Substantial shifts were obtained for w19/ch and w19/e1 with SDS, but whereas w19/ch showed only a small shift with PTSC, w19/e1 gave a large shift after treatment with this reagent.

Enzyme treatments. The decrease in extinction of bacterial suspensions during incubation with lipase, lysozyme and lysozyme + EDTA are shown in Fig. 2. w19 only showed a significant decrease in extinction when treated with lysozyme + EDTA; lipase and lysozyme

Fig. 1. Electrophoretic mobility versus pH for *Rhizobium trifolii* w19 and four phage-resistant mutants of this strain. —□—□—, w19; —■—■—, w19/c; —○—○—, w19/ch; —●—●—, w19/e1; —△—△—, w19/e2.

Table 3. *Electrophoretic mobilities of bacterial strains before and after chemical treatments*

Bacterial strain	Mobilities (μ /s/volt/cm)		
	Control in Michaelis 7 buffer alone	SDS*	PTSC†
w19	-2.06	-2.94	-2.47
w19/c	-2.21	Heterogeneous	-2.44
w19/e1	-2.73	-3.15	-3.36
w19/e2	-2.04	Heterogeneous	-2.18
w19/ch	-2.47	-3.08	-2.53

* Mobilities were determined in Michaelis pH 7 buffer with 1.0 mM-SDS.

† Mobilities were determined in Michaelis pH 7 buffer after treatment with excess PTSC.

alone had only a small effect. This indicates a normal type of Gram-negative wall structure.

Lipase produced some lysis of w19/ch, w19/e1 and w19/e2, but not w19/c. Hence it is possible that the surface lipid in these first three strains may have a structural role in the walls. This may be due to alterations in the organization of wall polymers such that the underlying mucopeptide layer is disturbed.

Lysozyme attacked w19/ch in conjunction with EDTA or in buffer alone and considerably aggregated w19/e1. The decrease in extinction for this strain was therefore not directly related to lysis. These results suggest the presence of some exposed mucopeptide in the bacterial surfaces. Lysozyme+EDTA lysed all the bacterial strains, but w19/c and w19/e2 were lysed significantly slower than the other strains.

Fig. 2. Decrease in extinction of bacterial cultures during enzyme treatment. Lysozyme produced cellular aggregation for w19/e1. —□—□—, w19; —■—■—, w19/c; —○—○—, w19/ch; —●—●—, w19/e1; —△—△—, w19/e2.

DISCUSSION

The electrophoretic mobility of strain w19 suggests that carboxyl and possibly also phosphate groups are present on the organism surface. Stronger acidic groups are indicated for this strain than for any of the phage-resistant mutants. Lysis of Gram-negative bacteria by lysozyme+EDTA, as found for w19, has been observed previously (Asbell & Eagon, 1966; Gray & Wilkinson, 1965). The mechanism is thought to involve chelation of divalent cations present in the wall and hence disturbance of structures which normally shield the mucopeptide substrate.

The changes bringing about phage resistance in the mutants derived from w19 are likely to arise from alterations in the composition or organization of cell wall polymers. These changes probably affect the outer layers of LPS and lipoprotein which are linked to and overlie the mucopeptide layer. In w19/c, a small change in cell wall composition, which does not drastically alter the overlying layers, is indicated. Hence both phages can still adsorb to this mutant. The change does, however, apparently render the mucopeptide layer less susceptible to the action of lysozyme. Lysozyme may be a component of some bacteriophages and may be involved in phage penetration (Barrington & Kozloff, 1956). Possibly the changed surface of w19/c inhibits penetration of phage c by rendering its lysozyme ineffective.

The differences found for w19/e1 and w19/e2 probably reflect greater alterations in surface composition so that phage e can no longer adsorb to either mutant. Furthermore, lipid in the outer layers appears to have some structural properties, possibly because of altered underlying mucopeptide.

The changes resulting from the w19/ch mutation probably involve considerable abnormalities in wall structure. In consequence, the primary receptor sites for both phage e and phage c are largely destroyed.

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