

ELECTROPHORETIC PROPERTIES OF FAST- AND SLOW-GROWING SPECIES OF *RHIZOBIUM*

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Summary

The electrophoretic mobility of *Rhizobium trifolii* strain TA1 was relatively constant during the early and late phases of growth, but exhibited a temporary sharp increase in the late logarithmic-early stationary phase. The electrophoretic behaviour of slow-growing species of *Rhizobium* was characterized by zero mobility at pH 2.0, and a constant negative mobility between pH 4.0 and 10.7. In contrast, all fast-growing strains, except *R. trifolii* TA1, exhibited slight positive mobility at pH 2.0, a constant negative mobility between pH 4.0 and 9.2, and a marked increase in mobility between pH 9.2 and 10.7. These results suggest that the surfaces of the slow-growing bacteria and strain TA1 contain only acidic (carboxyl) groups, whereas those of the fast-growing strains contain a predominance of acid groups along with some basic (amino) groups.

The electrophoretic behaviour of a number of variants of *R. trifolii* strains SU297 and SU298 apparently is related to antigenic structure, but not to nitrogen-fixing ability. The relationship of surface charge density to the poor expression of lysogeny in one variant (SU298D) is discussed. Variants of *R. trifolii* strain NA34 differing in their infective ability exhibited identical electrophoretic mobilities.

I. INTRODUCTION

Bacteria suspended in electrolyte solutions at pH 7.0 possess a negatively charged surface and, consequently, the cells migrate towards the anode when an electrical field is applied. Variations in polarity and charge density on cell surfaces under different conditions of growth or post-harvest treatment have been studied by the use of microelectrophoretic techniques (Moyer 1936; McQuillen 1950; Plummer and James 1961). In particular, investigations of the electrophoretic mobility of cells at different levels of pH have given an indication of the nature of the surface components of some bacteria (Plummer *et al.* 1962; Gittens and James 1963). The present paper describes an investigation of the electrophoretic properties of various species of *Rhizobium*, including a comparison of some colonial and avirulent (unable to form nodules on clover roots) mutants of strains of *R. trifolii*.

II. MATERIALS AND METHODS

(a) *Bacterial Strains and Cultural Conditions*

The following were employed in a comparison of electrophoretic properties:

R. trifolii strains TA1 and NA34;

R. leguminosarum strains UT11 and TA101;

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R. meliloti strain SU47;

R. lupini strains UT2 (previously termed strain CP1, Marshall 1964) and UT12;

R. japonicum strain QA372;

Rhizobium sp. strain UT53 (isolated from *Acacia mollissima* Willd.).

An assessment of the role of extracellular surface components on the charge properties of the cells was made by comparing mobilities of the colonial variants of *R. trifolii* strains SU297 and SU298 (Vincent 1962). The mobilities of avirulent mutants of *R. trifolii* strain NA34 were compared with virulent isolates of this culture. Previous studies (Marshall, unpublished data) have shown that these avirulent mutants retain the serological characteristics of the parent culture.

Normally, cultures were grown on yeast-mannitol agar at 28°C and harvested in the stationary phase (after 3–4 days for fast-growing and 8–12 days for slow-growing cultures). In studying the mobility of *R. trifolii* TA1 at different phases of growth, the culture was grown in 3 litres of aerated yeast-mannitol broth and aliquots were taken at regular intervals for determinations of mobility. At the same time the absorbance of the culture at 600 $m\mu$ was measured in a Bausch and Lomb Spectronic 20 colorimeter.

(b) Buffer Solutions

Measurements of electrophoretic mobility of *R. trifolii* TA1 at different growth phases were made in a NaH_2PO_4 – Na_2HPO_4 buffer of pH 7.0 and ionic strength (I) = 0.012. The cells grown in yeast-mannitol broth were centrifuged and washed three times in the buffer prior to the measurement of mobilities.

The following buffers (I = 0.015) were used in studies of mobilities at different levels of pH:

pH 2.6–6.0, citric acid– Na_2HPO_4 buffers;

pH 7.0–8.0, NaH_2PO_4 – Na_2HPO_4 buffers;

pH 9.2–10.7, Na_2CO_3 – NaHCO_3 buffers.

Measurements at pH 2.0 were made in 0.01M HCl (I = 0.01). The cells were suspended initially in phosphate buffer (pH 7.0), centrifuged, washed twice in the appropriate buffer, and homogenized in a Ten-Broek homogenizer prior to the measurement of electrophoretic mobility.

(c) Determination of Electrophoretic Mobility

All measurements of electrophoretic mobility were made at 25°C in an assembly similar to that described by Loveday and James (1957). The electrophoresis cell was firmly mounted on the microscope stage and, because of the thickness of the cell, observations were made with a $\times 20$ objective and $\times 15$ oculars using negative phase contrast. A resistance unit based on that recommended by Abramson, Moyer, and Gorin (1942) was used to maintain a constant field strength in the electrophoresis cell and to reverse the polarity of the electrodes when required. Organisms at the lower stationary level in the cell (Abramson, Moyer, and Gorin 1942) were timed over a distance of 260 μ in both directions in order to minimize the effects of drift

and electrode polarization. At least 10 cells of each sample were timed in both directions and the average velocity was used to calculate the electrophoretic mobility as follows:

$$\bar{v} = v/X,$$

where $\bar{v}$ is the electrophoretic mobility ($\mu/\text{sec}/\text{V}/\text{cm}$), v the velocity of the cells (μ/sec), and X the field strength (V/cm). Further,

$$X = i/K_s q,$$

where i is the current (amp), K_s the specific conductivity of the buffer solution, and q the cross-sectional area of the cell (cm^2).

The performance of the apparatus was checked by determining the mobilities of red blood cells. Values of $1.30 \mu/\text{sec}/\text{V}/\text{cm}$ for human erythrocytes in M/15 phosphate buffer (pH 7.4) and $1.04 \mu/\text{sec}/\text{V}/\text{cm}$ for rabbit red blood cells in M/150 phosphate buffer (pH 7.4) containing 4.86% glucose agreed with previously published values (Abramson, Moyer, and Gorin 1942; Hartman, Bateman, and Lauffer 1952).

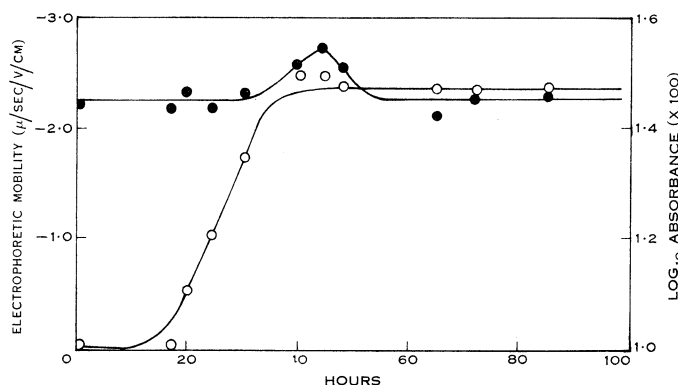


Fig. 1.—Variation of electrophoretic mobility during the growth of *R. trifolii* strain TA1. ○ Absorbance. ● Electrophoretic mobility.

In the calculation of surface charge density, the electrophoretic mobility ($\bar{v}$) was first converted into zeta potential (ζ) by the use of the Helmholtz-Smoluchowski equation:

$$\zeta = (4\pi\eta\bar{v})/D,$$

where η and D are the viscosity and dielectric constant, respectively, in the diffuse double layer. At the low ionic strengths employed in this investigation, the values of η and D were taken as those for water (Plummer and James 1961). The surface charge density (σ) was determined by substituting the values of ζ and electrolyte concentration in the following equation (Abramson, Moyer, and Gorin 1942):

$$\sigma = (NDkT/2000\pi)^{\frac{1}{2}} [\sum c_i (e^{-z_i e \zeta / kT} - 1) + \sum c_j (e^{+z_j e \zeta / kT} - 1)]^{\frac{1}{2}},$$

where c_i and c_j are the concentrations and z_i and z_j the valencies of the cations and anions, respectively, N the Avogadro number, D the dielectric constant, k the Boltzmann constant, T the absolute temperature, and e the electronic charge.

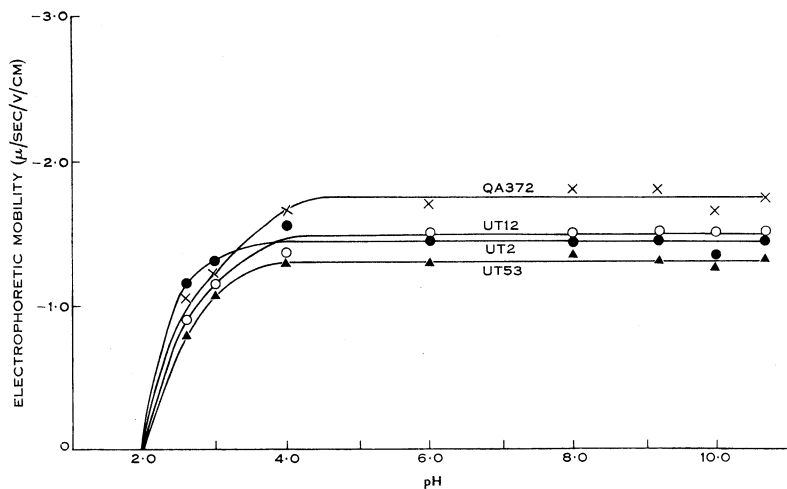


Fig. 2.—pH-mobility curves for the slow-growing strains of *R. japonicum* QA372, *R. lupini* UT2 and UT12, and *Rhizobium* sp. UT53.

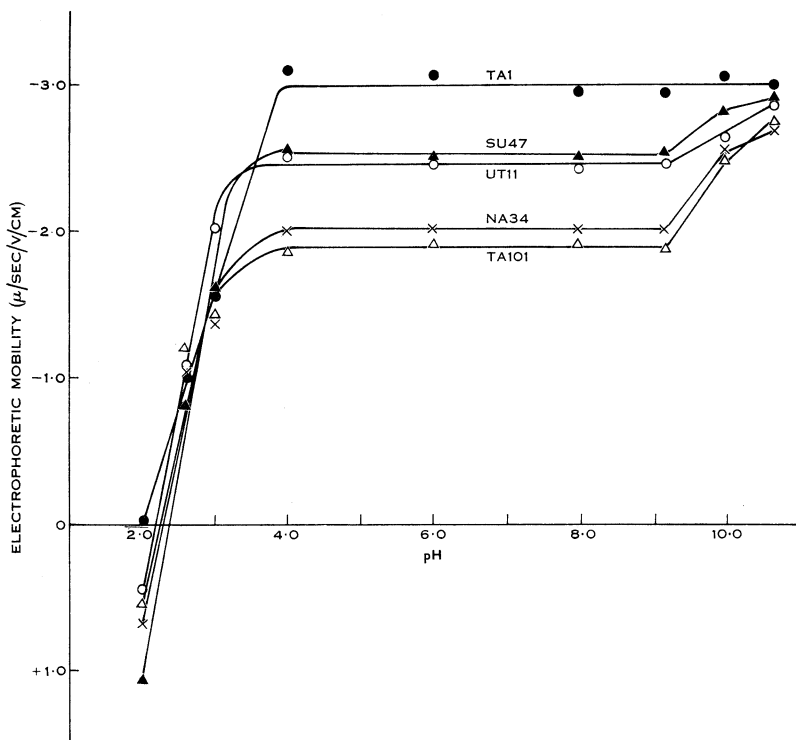


Fig. 3.—pH-mobility curves for the fast-growing strains of *R. trifolii* TA1 and NA34, *R. leguminosarum* UT11 and TA101, and *R. meliloti* SU47.

III. RESULTS

(a) *Variation in Electrophoretic Mobility of R. trifolii TA1 during Growth*

The results presented in Figure 1 show that the mobility is relatively constant during growth except for a sharp increase in the late logarithmic-early stationary phase. Similar results have been observed with *Aerobacter aerogenes* (Plummer and James 1961) and *Streptococcus pyogenes* (Plummer *et al.* 1962). Mobility values generally are stable in the late stationary phase and, consequently, all subsequent measurements were made on cells at this stage of growth.

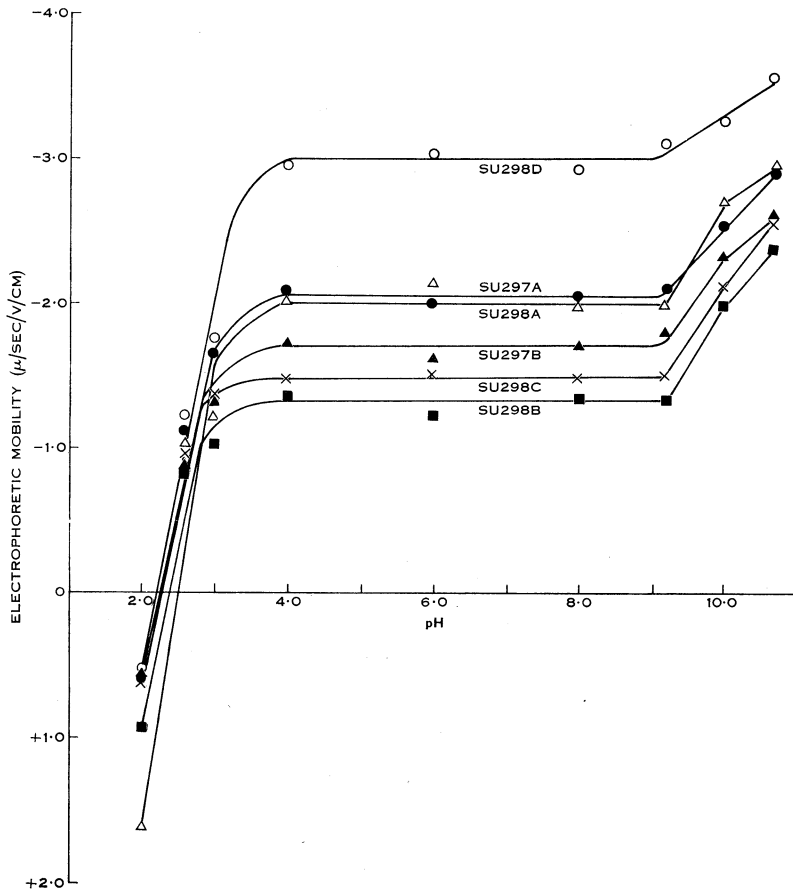


Fig. 4.—pH-mobility curves for the colonial variants of *R. trifolii* strains SU297 and SU298.

(b) *Electrophoretic Mobility of Root Nodule Bacteria at Different Levels of pH*

All the cultures examined exhibited constant electrophoretic mobilities over the pH range from 4.0–9.2 (Figs. 2, 3, and 4). Each culture, however, possessed a characteristic mobility plateau, the value of which ranged from $1.33 \mu/\text{sec}/\text{V}/\text{cm}$ in

Rhizobium sp. strain UT53 to about $3.00 \mu/\text{sec}/\text{V}/\text{cm}$ in *R. trifolii* strains TA1 and SU298D. In general, the fast-growing bacteria had a higher mobility plateau than the slow-growing species.

The root nodule bacteria appear to fall into two major groups according to their electrophoretic behaviour at high and low pH values. The slow-growing strains (*R. lupini*, *R. japonicum*, and *Rhizobium* sp. strain UT53) showed no increase in mobility over the pH range from 9.2 to 10.7 and had zero mobility at pH 2.0. Most fast-growing strains (*R. trifolii*, *R. meliloti*, and *R. leguminosarum*) exhibited a sharp increase in negative charge at very high pH levels, while a slight positive charge was observed at pH 2.0. *R. trifolii* TA1 was exceptional in that its mobility characteristics at high and low pH values resembled those of the slow-growing types.

TABLE 1

COMPARISON OF SURFACE CHARGE DENSITY WITH OTHER REPORTED CHARACTERISTICS OF COLONIAL VARIANTS OF *R. TRIFOLII* STRAINS SU297 AND SU298

Strain	Colony Size*	Nitrogen Fixation (subterranean clover)*	Lysogeny†	Antigenic Constitution‡	Surface Charge Density at pH 7.0 (e.s.u./cm ²)
SU297 A	Large	Effective	—	<i>gt₁p</i>	—1767
SU297 B	Small	Ineffective	—	<i>gt₁</i>	—1667
SU298 C	Large	Effective	+	<i>gt₂p</i>	—1619
SU298 B	Small	Ineffective	+	<i>gt₂</i>	—1559
SU298 A	Large	Effective	+	<i>gp</i>	—1746
SU298 D	Large	Ineffective	—	<i>(t₁)p'</i>	—1892

* Vincent (1962).

† Marshall (1956).

‡ Antigens: *g* = group antigen; *t* = 297 type; (*t₁*) = incomplete 297 type; *t₂* = 298 type; *p* = polysaccharide type; *p'* = "mucoid" type.

In a number of cultures where marked changes in electrophoretic mobility were observed at extreme levels of pH (2.0 and 10.7), the cells were washed in pH 7.0 buffer and the mobilities redetermined. The observed mobility values were similar to those normally obtained at pH 7.0, indicating that the observed changes in surface charge properties at high and low pH levels were reversible and not the result of permanent alterations to the surface components of the cells.

An analysis of variance carried out on the results obtained at pH values of 4.0, 6.0, 8.0, and 9.2 for all cultures showed that the differences in mobility between these four pH values were not significant. Using the pooled results from these pH values, it was found that a difference of $0.16 \mu/\text{sec}/\text{V}/\text{cm}$ between the electrophoretic mobilities of the different cultures was significant at the 5% level.

(c) *Mobility of Colonial Variants of R. trifolii Strains SU 297 and SU298*

The detailed electrophoretic properties of the *R. trifolii* SU297 and SU298 colonial variants are shown in Figure 4, while a comparison is made in Table 1 of the surface charge densities with some other reported characteristics of these cultures.

In comparison with the parental forms, SU297A and SU298C (Vincent 1962), there is a slight but significant reduction in surface charge in the small colony variants SU297B and SU298B that lack a common polysaccharide-type antigen. The absence of the 298 type antigen in SU298A, on the other hand, is associated with a distinct increase in negative charge density at the cell surface. The most significant alteration in mobility, and hence surface charge density, is in SU298D which lacks the group antigen of the parental form and has a modified type of gum production ("mucoid" antigen).

(d) *Mobility of Avirulent Mutants of R. trifolii Strain NA34*

Five out of nine single colony isolates of *R. trifolii* NA34 tested for their ability to nodulate subterranean clover (*Trifolium subterraneum* L. cv. Tallarook) proved to be avirulent. It is apparent from the results in Table 2 that mutation to the avirulent form is not accompanied by any change in the surface charge properties of the cells.

TABLE 2
ELECTROPHORETIC MOBILITY OF VIRULENT AND AVIRULENT ISOLATES OF
R. TRIFOLII NA34
pH 7.0; $I = 0.015$

Isolate	Electrophoretic Mobility (μ /sec/V/cm)					Mean Mobility
Virulent	2.02	1.90	1.98	1.96	—	1.97
Avirulent	1.98	1.94	1.99	1.86	2.04	1.96

IV. DISCUSSION

Alterations in the electrophoretic mobility of bacteria that occur during active growth must be related to some temporary changes in the surface structure of the cells. Plummer and James (1961) have demonstrated that an increase in surface negative charge during active growth of *Aerobacter aerogenes* was related to an increase in capsule size, whilst Plummer *et al.* (1962) and Hill, James, and Maxted (1963) have shown that the increased mobility of *Streptococcus pyogenes* is correlated with the accumulation of hyaluronic acid on the cell surface during active growth. The reason for the increase in surface charge of *R. trifolii* TA1 towards the end of the growth phase has not been investigated, but in view of extensive gum production by this organism the increase in charge may be related to changes in capsule size.

The surface charge characteristics of *R. trifolii* TA1 and of the four slow-growing cultures closely resemble those of *A. aerogenes* (Plummer and James 1961) in that the cells exhibit zero mobility at pH 2.0 and a constant negative surface charge is maintained over the pH range from 4.0 to 10.7. It is likely, therefore, that the surface ionogenic groups of these cultures are exclusively carboxyl in nature. Apparently, most fast-growing species of *Rhizobium* possess a somewhat different ionogenic surface, as evidenced by the slight positive charge on the cells at pH 2.0 and the marked increase in negative charge between pH 9.2 and 10.7. The different

ionogenic surfaces of typical fast- and slow-growing species may be visualized as shown in Figure 5. The slow-growing bacterium is represented as having a simple carboxyl surface, showing the uncharged surface (undissociated carboxyl groups) at pH 2.0 and the identical distribution of ionized carboxyl groups at both pH 7.0 and pH 10.7. The fast-growing bacterium is represented as possessing a predominantly carboxyl surface but with amphoteric properties arising from the presence of a small number of basic (amino) groups characterized by a high isoelectric point (approx. pH 10). At pH 2, the surface will have a small positive charge because the amino groups are positively charged while the carboxyl groups are undissociated. Within the mobility plateau from pH 4.0 to 9.2 the basic groups are still positively charged but the excess of ionized carboxyl groups leads to a net negative charge on the surface. The sharp increase in negative charge beyond pH 9.2 may be explained by the suppression of the ionization of the basic groups.

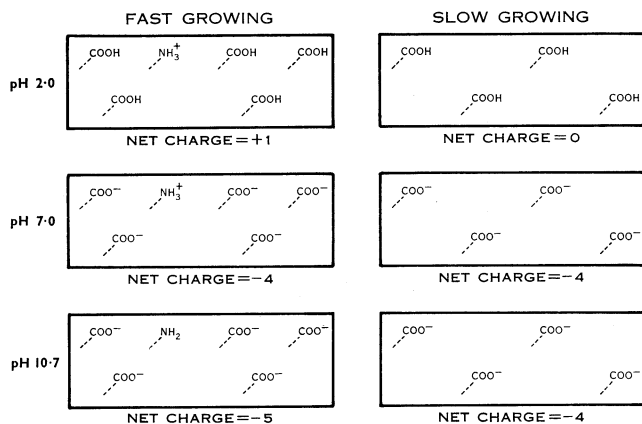


Fig. 5.—Diagrammatic interpretation of the effect of pH on the surface ionogenic groups of fast- and slow-growing species of *Rhizobium* exhibiting comparable electrophoretic mobilities at pH 7.0.

A comparison of the electrophoretic properties of the variants of *R. trifolii* strains SU297 and SU298 provides an opportunity to assess the possible contribution of certain surface antigens to the charge properties exhibited by the cells. The polysaccharide (*p*) antigen may contribute slightly to the overall negative charge of the parent forms (SU297A and SU298C), as mutants lacking this antigen exhibit lower mobilities than the parent cultures. The lack of the 298 type antigen in SU298A is associated with a higher negative charge on the cell surface at pH 7.0. This may result from an unmasking of certain negatively charged groups at the surface or from the loss of positively charged groups associated with the 298 type antigen. For similar reasons, the loss of the group (*g*) antigen may be partly involved in the dramatic increase in surface charge density observed in the case of SU298D. The presence of the "mucoid" (*p'*) antigen also may be associated with this increase in negative charge. Despite the changes in surface charge density, the shape of the pH-mobility curves for these variants was unaltered.

The expression of lysogeny in the SU298 variants may be related to the surface charge properties of these cells. The SU298 types A, B, and C were reported by Marshall (1956) to be lysogenic, the SU297 types A and B being susceptible indicator strains. Marshall reported that SU298D was non-lysogenic, but later Bloomfield (see Vincent 1962) was able to demonstrate very low phage yields from this culture. The lysogenic condition in SU298 is extremely stable and phage production is only induced by a phage-like particle (phage *i*) derived from the indicator strain SU297 (Takahashi and Quadling 1961). In view of this need for a phage-mediated induction of SU298, it is possible that induction of SU298D is reduced to a negligible level because of the failure of phage *i* to adsorb on the more highly charged and probably drastically reorganized surface of this variant.

Tittsler, Lisse, and Ferguson (1932) have suggested a relationship between nitrogen-fixing ability and electrophoretic mobility in strains of *R. meliloti*. The data presented in Table 1 indicates that there is no consistent relationship between surface charge density and the effectiveness of nodulation of subterranean clover by variants of *R. trifolii* strains SU297 and SU298. Likewise, loss of the ability to nodulate subterranean clover in mutants of *R. trifolii* NA34 is not accompanied by an alteration in the electrophoretic mobility of these cells.

V. ACKNOWLEDGMENTS

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