The Role of Motility and Aerotaxis in the Selective Increase of Avirulent Bacteria in Still Broth Cultures of *Pseudomonas solanacearum*

By A. KELMAN AND JUDITH HRUSCHKA

*Department of Plant Pathology, University of Wisconsin, Madison,
Wisconsin, 53706, U.S.A.*

(Received 17 July 1972; revised 21 November 1972)

SUMMARY

A difference in relative motility between virulent and avirulent *Pseudomonas solanacearum* was a possible factor in the shift from virulent to avirulent populations in still broth cultures. Virulent isolates grown on solid media or in tryptone yeast-extract glucose or glycerol broth for 24 to 48 h were mainly non-flagellated or non-motile, whereas avirulent isolates grown under the same conditions were usually flagellated and highly motile. Fimbriae were observed in electron photomicrographs of both types. A rapid preferential increase of avirulent bacteria occurred when mixtures were grown in still, but not in shaken, broth cultures; this relative increase was greatest close to the surface of the medium. Rapid aerotaxis of avirulent bacteria was demonstrated in mixtures of virulent and avirulent bacteria in a semisolid motility agar medium. Positive aerotaxis and high motility apparently favoured rapid increase of avirulent *P. solanacearum* when oxygen became limiting in broth media, in which virulent bacteria were not actively motile.

INTRODUCTION

Rapid loss in virulence in certain culture media characterizes *Pseudomonas solanacearum* E. F. Smith, which causes destructive wilt diseases of many important solanaceous crops (Kelman, 1953). In non-aerated liquid media containing glucose and an organic source of nitrogen, attenuation correlates with the relatively rapid appearance of avirulent or weakly virulent variants (Kelman, 1954, 1956; Husain & Kelman, 1958). On a tetrazolium chloride medium, the irregularly round, fluidal colonies of virulent isolates can be readily distinguished from the small butyrous colonies of avirulent or weakly virulent isolates (B type) that do not form the extracellular polysaccharide characteristically formed by virulent bacteria (Kelman, 1954; Husain & Kelman, 1958). Variants recognizable by colony type appear in cultures derived from single virulent bacteria (Kelman, 1954), but the genetic basis for this change in colony morphology has not been determined.

A wide range of factors has been shown to influence the similar shift from smooth (S) to rough (R) colony types in populations of certain bacteria in non-aerated broth cultures (Braun, 1965). In recent studies with *Pseudomonas solanacearum*, none of the major factors associated with the shift in colony types in other bacteria could be directly related to that observed in non-aerated tryptone yeast-extract glucose (TYG) broth cultures of this pathogen. However, a virulent culture (25-K60) grown on the TZC medium was usually non-motile, whereas the avirulent B types of 25-K60 were highly motile.

Motility presumably aids a bacterium to survive when nutrient and other essential factors such as oxygen become limiting (Carlson, 1962). Quantitative data in support of this concept

have been presented by Old & Duguid (1970, 1971) with motile and non-motile strains of *Salmonella typhimurium* as an adjunct to their detailed studies on selective growth of fimbriate over non-fimbriate bacteria in still culture. Similar observations were made by Smith & Doetsch (1969) in experiments with motile and non-motile strains of *Pseudomonas fluorescens*.

The factors affecting chemotaxis of bacteria have been re-examined recently (Adler, 1966*a*), and the observations of early investigators on aerotaxis confirmed and extended (Sherris, Preston & Shoesmith, 1957; Baracchini & Sherris, 1959). Adler and co-workers (Adler, 1966*a, b*, 1969; Adler & Dahl, 1967; Adler & Templeton, 1967) developed improved quantitative techniques in their careful investigations of motility and chemotaxis of *Escherichia coli*. Certain of these techniques were found to be applicable for motility studies with *Pseudomonas solanacearum*.

This investigation was initiated to determine whether differences in motility between virulent and avirulent *Pseudomonas solanacearum* could be related to the population shifts in non-aerated broth cultures. Preliminary reports on parts of this study have been presented (Kelman & Hruschka, 1970, 1972).

METHODS

General procedures. The isolates of *Pseudomonas solanacearum* mainly used were (i) the virulent fluidal strain 25-K60 (Husain & Kelman, 1958), and (ii) BI-A, an avirulent culture of 25-K60 (Table 1). The avirulent isolate was obtained from a single colony of the butyrous variant type (B) selected from a dilution plating of a culture of 25-K60 on tetrazolium chloride agar medium (TZC) (Kelman, 1954), following growth of a pure clone of the virulent colony type in tryptone yeast-extract-glucose-broth (TYG) in a test tube for 96 h at 32 °C. The TYG broth medium contained (g/l distilled water): tryptone (Difco, Detroit, Michigan, U.S.A.), 10; yeast extract (Difco), 1; glucose, 10. Test isolates were stored as suspensions (approximately 1×10^7 bacteria/ml) in sterile double-distilled water (5 ml) in tightly capped test tubes at approximately 20 °C (Kelman & Person, 1961). Isolates from other hosts and other geographic areas (Table 1) were also examined; avirulent butyrous colony types of these isolates to be used in motility studies were obtained as described above.

Viable counts were made by surface plating on the TZC medium. Cultures were usually incubated at 32 °C for 48 h.

Percentages of avirulent colony types in cultures were determined by either streaking dilute suspensions or making dilutions on TZC plates and examining colony characteristics with oblique lighting (Kelman, 1954; Husain & Kelman, 1958).

In greenhouse tests of relative virulence of cultures, tomato (*Lycopersicon esculentum* Mill.) and tobacco (*Nicotiana tabacum* L.) seedlings were stem-inoculated following procedures outlined by Winstead & Kelman (1952).

Motility studies. Suspensions were prepared from cultures grown on TZC plates for 24 to 48 h or in TYG broth for 24 h at 32 °C and examined in hanging drops in distilled water, TYG broth, or the liquid motility medium (see below) at a 430-fold magnification. Since it was difficult to determine exact percentages of motile bacteria in hanging drops, we used the following motility index scale: 0 = no motile bacteria; 1 = less than 1%; 2 = 1 to 10%; 3 = 11 to 50%; 4 = 51 to 90%; and 5 = 91 to 100%.

Relative motility was also determined in semisolid agar plates following the procedure developed by Adler (1966*a*). The semisolid motility medium (SMM) contained: glucose, 0.1 g; tryptone (Difco), 0.1 g; (ethylenedinitrilo) tetra-acetic acid disodium salt, 0.038 g

(Adler & Templeton, 1967); 10 ml of pH 7.0 phosphate buffer which provided 1.177 g K_2HPO_4 and 0.441 g KH_2PO_4 /l of medium; and 3.5 g agar (Difco)/l distilled water.

To determine whether virulent bacteria that were initially non-motile when grown on TZC agar could become motile, suspensions were prepared in SMM without agar, incubated for 2 to 6 h at 32 °C, and then examined directly in hanging-drop preparations.

Following a procedure similar to that described by Vaituzis & Doetsch (1969), motility track photographs of virulent and avirulent bacteria in water suspensions were taken with a Zeiss photomicroscope with Leica M-3 camera.

Electron microscopy. Electron photomicrographs were made with a JEM Model 7 electron microscope used at an accelerating voltage of 80 kV. Suspensions were washed in double-distilled filter-sterilized water, diluted with sterile 0.5% bovine serum albumin, placed on collodion-coated grids, freeze-dried over $CaCl_2$, and shadowed with carbon-platinum pellets.

Aerotaxis experiments. To determine whether motile bacteria would show aerotaxis, SSM containing approximately 1:1 mixtures of virulent (25-K60):avirulent bacteria (B1-A) (approx. 1×10^8 viable bacteria/ml) was pipetted into sterile glass tubes (5.5 cm long with an inner diam. of 2.5 mm). These tubes were plugged at one end with hard agar and plasticine and open to the air at the other; were incubated upright, one set in air and the second in a container made oxygen-free by placing 3 ml of 2.5 N-NaOH and 0.3 g pyrogallol in a centre well and then flushing with nitrogen. After 6 to 12 h at 32 °C, the agar columns were extruded from the tubes by pushing up the basal plugs. Serial segments approximately 1 mm long were sliced with a razor from the agar column from the top down as the agar was extruded. Each segment was added to a water blank and percentage of avirulent bacteria determined by plating on TZC plates. Resazurin (1.5 µg/ml final concn) was added to a series of control tubes with suspensions in the semisolid agar as an indirect means of monitoring oxygen levels.

RESULTS

Relative motility in hanging-drop preparations. The virulent isolate 25-K60 was non-motile in hanging drops of suspensions prepared in sterile distilled water, TYG broth, or SMM minus agar. In contrast, B1-A and ten other avirulent B colony type cultures (also derived from 25-K60) were actively motile (motility index, 3 to 4) when grown and observed under similar conditions. Similarly, 13 different virulent isolates (203-215) obtained from North Carolina in 1969 also were mainly non-motile (motility index, 0 to 1) when grown on TZC agar plates. In sharp contrast, the variant B colony types selected in platings from TYG broth cultures of each of these isolates were consistently highly motile (motility indices, 3 to 5) (Table 1).

In cultures of several isolates [3 and 98 (banana), 19 (potato-Colombia), 80 (*Solanum phureja*-Colombia), 90 (tobacco-Brazil) and 147 (tobacco-Australia)], B type variants were not detected until after incubation for 5 to 9 days and then only very low numbers were present. The B type colony variants selected at random for testing from these isolates were non-motile. A large number of B type colony variants obtained from various virulent cultures from solanaceous hosts were examined for motility in the course of our studies; non-motile strains were only detected in a few subcultures of these B type cultures. Rapid preferential increase of non-motile B types was not observed in mixtures with 25-K60 in still culture.

In the early phases of the work, all observations on motility were made using suspensions shortly after they were prepared in double-distilled water or TYG broth from 24 to 48-h

Table 1. *Motility of virulent and avirulent cells of different isolates of Pseudomonas solanacearum grown on tetrazolium chloride medium and examined in hanging drops*

Race*	Host	Isolate no.†	Geographic area	Motility‡	
				Virulent	Avirulent
1	Pepper	29 (K132)	Raleigh, North Carolina, U.S.A.	o	+
1	<i>Solanum nigrum</i>	96 (SN196)	Costa Rica	o	+
1	Tobacco	27 (K105)	Quincy, Florida, U.S.A.	o	+
1	Tobacco	77 (B-1)	Japan	o	+
1	Tobacco	90 (ENA571)	Minas Gerais, Brazil	o	o
1	Tobacco	147 (O170)	Queensland, Australia	o	o
1	Tobacco	203-215 (M64-M76)	North Carolina, U.S.A.	o	+
1	Tomato	25 (K60)	Raleigh, North Carolina, U.S.A.	o	+
1	Tomato	26 (K74)	Worth Co., Georgia, U.S.A.	o	+
1	Tomato	30 (K136)	Trinidad	o	+
1	Tomato	84 (ST3)	Climax, Georgia, U.S.A.§	o	+
1	Tomato	88 (ENA557)	Brazil	o	+
2	Banana	3 (S107)	Armuelles, Panama	o	o
2	Banana	98 (B100)	Costa Rica	o	o
2	Plantain	70 (S210)	Ibaque, Colombia	o	+
3	Ginger	151 (092)	Queensland, Australia	o	+
3	Potato	19 (S205)	Tibaitata, Colombia	o	o
3	Potato	134 (K197)	Nairobi, Kenya	o	+
3	Potato	145 (O18A)	Queensland, Australia	o	+
3	Potato	152 (O13A)	Queensland, Australia	o	+
3	Potato	153 (O158)	Queensland, Australia	o	+
3	Potato	198 (LB-6)	Laguna, Philippines	o	+
3	<i>Solanum phureja</i>	80 (S206)	Las Palmas, Colombia	o	o
3	Tomato	143 (002A)	Queensland, Australia	o	+

* Race designation following characteristics defined by Buddenhagen & Kelman (1964).

† Appreciation is expressed to the following who provided designated cultures: W. F. Dudman, 30 (K136); J. S. Cole, 134 (K197); I. Buddenhagen, 96 (SN196), 98 (B100); M. Goto, 77 (B-1); A. C. Hayward, 147 (O170), 153 (O158); R. E. C. Layne, 84 (ST3); C. F. Hobbs, 88 (ENA77), 90 (ENA571); C. E. Main and Alice Hisada, 203 (M64) through 215 (M76); L. Sequeira, 3 (S107), 19 (S205), 70 (S210), 80 (S206); and E. I. Zehr, 198 (LB-6). Cultures 25 (K60), 26 (K74), and 29 (K132) were isolated by the senior author.

‡ Motility was based on microscopic observations of suspensions in hanging drops containing approximately 1×10^6 c/ml made in sterile distilled water from cultures grown on TZC agar at 32 °C for 48 h. o = none or less than 1 % actively motile; + = more than 50 % actively motile.

§ This culture was isolated from an infected plant observed in a field in S.W. Ontario, Canada, planted with seedlings obtained from Climax, Georgia, U.S.A.

cultures grown on TZC agar plates. When hanging drops of virulent suspensions prepared in SMM (minus agar) were examined after 4 h, a high percentage (motility index, 4) became actively motile. This also occurred in TYG broth after 4 h, but bacteria were usually non-motile after 12 to 16 h in TYG broth.

Electron microscopy. Five to 10 out of approximately 500 virulent 25-K60 from 18 to 24 h TZC agar cultures had flagella. In contrast, 80 to 85 % of avirulent organisms had one or more polar flagella (Fig. 1 b).

Motile bacteria were obtained when suspensions of 25-K60 were made in SMM (minus agar) for 3 to 4 h. The flagella present usually appeared to be straight and shorter (Fig. 1 a) than those observed on motile avirulent bacteria. Other investigators have reported that bacteria with straight flagella were non-motile (Iino & Mitani, 1967; Iino, 1969).

The areas immediately adjacent to virulent bacteria appeared to boil for a few seconds after exposure to the electron beam. This was apparently an effect of high temperatures on their slime or extracellular polysaccharide.

Fig. 1. Electron photomicrographs of virulent (a) and avirulent (b) cells of *Pseudomonas solanacearum* showing flagella. Motility track photographs of a suspension of motile avirulent (c) and non-motile virulent (d) *Pseudomonas solanacearum*. Exposure time = 0.5 s.

Polar fimbriae were commonly present on 25-K60 and B1-A. Previously, Fuerst & Hayward (1969) had observed polar fimbriae on biotypes II, III and IV of *Pseudomonas solanacearum*. The cultures (25-K60 and B1-A) examined by electron microscopy in this study belong to biotype I according to Hayward's classification (Hayward, 1964).

Motility track photography. Photographs of suspensions of the avirulent B1-A strain and the virulent 25-K60 were taken shortly after suspensions were prepared from TZC plates. The distinct tracks made by actively motile B1-A are visible as white streaks on the dark background (Fig. 1c). No tracks are evident in the preparations made with virulent organisms (Fig. 1d). Velocity of the avirulent types, calculated from motility track lengths, was approximately 53 $\mu\text{m/s}$ or 34 lengths/s, very similar to values obtained from motility tracks of *Pseudomonas aeruginosa* (Vaituzis & Doetsch, 1969).

Population trends in broth cultures. Variants did not appear in TYG broth cultures of the virulent 25-K60 grown on a shaker, whereas variants appeared and increased rapidly in still cultures (Kelman, 1956). If aerotaxis and/or differences in motility were involved in this rapid increase at limiting oxygen levels, one could expect to find more variants near the surface of the medium in still cultures of mixtures of virulent and avirulent types.

Cultures were sampled at the surface and at different levels below the surface of TYG broth containing 0.075 g Difco agar/l to minimize the effect of movement of medium. The agar also reduced rate of diffusion of oxygen. Suspensions ($1 \times 10^8/\text{ml}$) of 25-K60, B1-A and a mixture of 25-K60 with B1-A in the ratio of 100:1 were prepared in 10 ml portions in test tubes. Resazurin was added in one series of test tubes. As had been observed in a preliminary experiment using broth media, no motile bacteria were present in the surface layer of cultures of 25-K60 alone at 12 h, and less than 1% were motile at 24 h. In marked contrast, B1-A organisms were still actively motile after 48 h (motility index, 4 to 5). In the mixture series, the number of motile bacteria increased rapidly and at 48 h the motility index was 4. The percentage of B type colonies in streaked plates from the mixture was 68%; thus, the ratio of 25-K60:B1-A shifted from 100:1 to approximately 1:2 during this period. This shift was attributable to the preferential increase of the B1-A population rather than the development of other B type variants from 25-K60, since the percentage of avirulent colonies in streaked plates of 25-K60 alone was about 1% after 48 h.

With B1-A alone, a distinct layer or band of growth appeared several mm below the surface of the medium. The position of this band indicated that the high levels of O_2 at the medium/air interface may be inhibitory. This effect has been observed previously with other pseudomonads (Baracchini & Sherris, 1959).

Observations on the patterns of distribution of oxidized resazurin indicated that the population shift had occurred during the period when oxygen concentrations in the medium had decreased to a level limiting growth except close to the surface.

To determine the influence of aeration on motility of 25-K60 and B1-A, TYG broth was dispensed in 25 ml samples into 125 ml flasks. Initial populations were 10^7 bacteria/ml; three flasks of each isolate were placed on a rotary shaker and three were incubated without aeration. Motility was determined at 8, 19, 25 and 96 h. Percentage motility of 25-K60 was low in the shaken (motility index = 2) and non-shaken flasks (motility index, 2) at 8 h and zero at 19 and 25 h. In contrast, 90% or more of the B1-A series (shaken or non-shaken) were actively motile at 8 and 19 h; motility declined only slightly after 25 h. After the initial growth period, no motile organisms were detected in the shake flasks of 25-K60, even after 8 days; furthermore, no mutant B colony types appeared in the streakings made on TZC agar. In marked contrast, motile bacteria were detected after 4 days in the non-shaken flasks and could be directly correlated to the percentage of variant B type colonies on

Table 2. *Relative growth of virulent (25-K60) and avirulent (BI-A) Pseudomonas solanacearum alone and in a mixture in still and shaken cultures**

Culture	Viable bacteria ($\times 10^7$)/ml Growth period (h)			
	24		48	
	Still	Shaken	Still	Shaken
25-K60	20	72	90	1500
BI-A	26	79	180	2000
Mixture of 25-K60:BI-A in ratio of 9:1				
25-K60	19	66	90	1200
BI-A	3.6	7.5	120	280

* Growth medium = TYG broth, 25 ml/250 ml flask, incubated at 30 °C. Initial populations of 25-K60 and BI-A grown separately = 1×10^5 ml; in mixture 25-K60 = approx. 9×10^4 and BI-A = 1×10^4 ml.

Table 3. *Percentage increase in diameter of bacterial migration zones of virulent (25-K60) and avirulent (BI-A) Pseudomonas solanacearum on semisolid motility agar plates with different nitrogen sources*

N source	Concentration (g/l)	Increase in diameter of migration zones (%)*			Avirulent at outer edge of migration zone in mixture plates (%)
		Virulent	Avirulent	Mixture (1:1), virulent:avirulent	
Ammonium chloride	0.50	0	67	133	100
Ammonium nitrate	0.74	0	102	133	100
Asparagine	0.61	0	267	217	100
Casamino acids	1.3	0	233	167	100
Peptone	0.8	0	267	283	100
Tryptone	1.0	0	217	200	100

* Based on average diameter of migration zones in three plates; initial diameters of inoculum drops = 7 ± 1 mm; measurements after 20 h at 32 °C.

streaked plates. This pattern was duplicated when this experiment was repeated with 25-K60 and with five other virulent isolates from North Carolina (203, 205, 209, 212, 214). Lack of aeration *per se* was thus not the primary factor determining motility of virulent 25-K60 in TYG broth. However, lack of aeration was correlated with appearance and rapid increase of the B colony type variants in the non-aerated cultures of 25-K60, as had been noted many times previously.

In a similar experiment, viable counts were made of 25-K60, BI-A and a mixture of 25-K60:BI-A (in ratio of 9:1), grown in TYG broth in still and shake culture. Viable counts from shake cultures of 25-K60 and BI-A after 24 and 48 h were similar (Table 2). However, in the mixture in still cultures the population of BI-A increased from 3.6×10^7 at 24 h to 1.2×10^9 at 48 h, a 33-fold increase. These data provided further evidence that the competitive ability of the motile BI-A was superior to that of K-60 in still culture.

Migration in SMM plates. The relative motility of 25-K60 was compared with that of BI-A in SMM in plates with the following nitrogen sources: tryptone, peptone, Difco Casamino acids, asparagine, ammonium nitrate and ammonium chloride. A single drop containing approximately 1×10^8 bacteria was placed in the centre of each Petri plate. Suspensions used included a 1:1 mixture of BI-A:25-K60, BI-A alone, and 25-60 alone.

After 20 h, the diameter of the migration zone of BI-A had increased from 6 mm

Fig. 2. Relative percentage of avirulent cells of *Pseudomonas solanacearum* in serial segments taken from surface downwards in semisolid motility agar in glass tubes (5.5 cm long with inner diam. of 2.5 mm) 6 h after incubation at 32 °C in air or in nitrogen. Ratio of avirulent:virulent bacteria = 46%:54% in the suspension added to the semisolid agar at the time tubes were filled.

(diameter of inoculum drop) to 22 mm (% increase = 267), with peptone or asparagine as the N source (Table 3). No detectable migration band was formed by 25-K60 during this period. In the plates containing 1:1 mixtures of 25-K60:B1-A, the increase in diameter of migration zones was attributable to the B1-A component; streakings of samples taken from the edges of the migration zones contained only B1-A. Migration of B1-A in plates containing inorganic nitrogen was markedly less rapid than in plates containing organic sources of N.

Avirulent B type bacteria in cultures of various isolates could be detected if distinct migration bands became evident 24 to 48 h after drops of suspensions were placed on the motility medium. Streakings of samples from the edge of the migration zone usually revealed that the B type was present. In those instances in which pure clones of 25-K60 were used (suspensions from single well-isolated colonies from 36 to 48 h TZC plates), migration bands were usually not evident until after 48 to 72 h.

Aerotaxis studies. At zero time in a typical experiment, the ratio of virulent (25-K60) to avirulent (B1-A) was 47:53, and the viable count in the suspension added to the tubes was 9.8×10^8 bacteria/ml. At the end of 6 h, the viable count in the top 1 mm of agar was 15.5×10^8 bacteria/ml indicating that growth and/or migration to the air interface had occurred during the test period.

The percentage of avirulent type in segments taken in descending order from top to

Table 4. Motility and percentage avirulent B type present in surface samples of tryptone yeast extract broth tubes containing glucose or glycerol

Cultures tested	Incubation period (h)	Motility index*		Percentage B type colony present	
		Glucose	Glycerol	Glucose	Glycerol
25-K60	0	0	0	0	0
	26	0	0	0	0.5
B1-A	0	5	5	100	100
	26	4	4	100	100
Initial mixture of 25-K60:B1-A in ratio 25:1	0	1	1	4	4
	26	3	4	44	60

* Based on estimates of percentages of motile bacteria in hanging drops according to following index scale: 0 = none; 1 = less than 1%; 2 = 1 to 10%; 3 = 11 to 50%; 4 = 51 to 90%; 5 = 91 to 100%. Initial populations for 25-K60 and B1-A = 4×10^5 ml. Suspensions prepared from cultures grown on TZC plates for 48 h at 32 °C.

bottom in the tubes in the nitrogen atmosphere showed no distinct migration pattern (Fig. 2). In the tubes in air, however, the percentage of B1-A in the top segment had increased to 70%, whereas in the third segment they had decreased to 32% from the original level of 53%. In the lower sections, the percentage of B1-A in the population was 52 to 55%. These data indicate that positive aerotaxis occurred and that the avirulent type moved more rapidly than the virulent to the zone close to surface. In control tubes containing resazurin, there was little or no colour at the surface of the agar in the nitrogen series, whereas a distinct pink colour extended to 3 to 4 mm in the tubes in the air. After 6 h, a distinct band of massed bacteria appeared just below the meniscus of the SMM in the tubes containing B1-A alone and a clear zone was evident below; 25-K60 formed no such band. Similar visual evidence for positive aerotaxis of the B1-A was obtained when these experiments were repeated with NH_4NO_3 substituted for tryptone in the SMM.

Effect of carbon source on motility. Synthesis of flagella in a motile strain of *Escherichia coli* was inhibited by glucose (Adler & Templeton, 1967); inhibition was related to decrease in levels of cyclic adenosine-3,5-monophosphate (cyclic AMP) (Yakota & Gots, 1970) and was overcome by added cyclic AMP (Dobrogosz & Hamilton, 1971). In our studies, glucose did not inhibit synthesis of flagella by the avirulent form of *Pseudomonas solanacearum*; however, the possibility existed that glucose could affect motility of the virulent form. If glucose repression of one or more enzyme systems (including those involved in flagella synthesis) occurs in 25-K60 and not in B1-A, the generation time of 25-K60 should presumably be shorter than that of B1-A in glucose. Relative growth rates of 25-K60 and B1-A were determined in TYG broth and in the same medium with glycerol substituted for glucose. Cultures were grown in 25 ml of medium/250 ml flask incubated in a water-bath shaker at 32 °C. Initial populations were approximately 10^5 bacteria/ml. Viable counts were made at 2 to 3 h intervals over 13 h. The mean generation times were 57 min for 25-K60 and 56 min for B1-A in tryptone yeast-extract-glucose and 68 and 72 min respectively in the glycerol medium.

The motility of 25-K60 alone and in mixtures with B1-A was examined in tryptone-yeast extract broth with 10 g of either glucose or glycerol/l. No motile 25-K60 was observed in hanging-drop suspensions prepared from samples taken close to the surface of either medium after 26 h (Table 4). In contrast, the B1-A cultures were actively motile, both initially and after 26 h. In the series containing a 25:1 mixture of 25-K60 with B1-A, the

percentage of motile bacteria increased markedly over 26 h. Furthermore, the ratio of 25-K60 to B1-A shifted from 25:1 to 1:37:1 in glucose and 0.67:1 in the glycerol series. These observations confirm the rapid preferential increase of B1-A in mixed cultures with 25-K60 when grown in still culture (Table 2). After 48 to 72 h, the rate of appearance of B type variants from 25-K60 in broth containing glycerol was similar to that observed in cultures with glucose.

Further evidence that a glucose effect was not involved in low motility of 25-K60 was obtained in an experiment to determine relative motility of 25-K60 and B1-A when grown on two different solid media: (i) the peptone (1%, w/v)-Casamino acids (0.1%, w/v) agar, and (ii) tryptone (1%, w/v) yeast-extract (0.3%, w/v) agar containing either glucose or glycerol or without either. No motile 25-K60 was observed in hanging drops of suspensions prepared following growth for 20 h on these various media. In contrast, B1-A from all the media was actively motile. There were no apparent differences in relative motility that could be related to presence or absence of glucose or glycerol. Relative motility of test cultures in TYG broth or migration in SMM containing filter-sterilized glucose were essentially the same as in these media with autoclaved glucose.

Influence of temperature on motility. Temperatures at or above the optimum for growth may induce development of non-motile or non-flagellated forms of many motile bacteria (Kerridge, 1961; Doetsch & Hageage, 1968). Relative motility of *Escherichia coli* was also greater if organisms were grown at 24 to 25 °C rather than at 37 °C (Adler & Templeton, 1967). The optimum for growth of *Pseudomonas solanacearum* is 32 °C (Kelman, 1953), and most of the experiments on growth and motility had been completed at this temperature.

To determine effect of optimal and suboptimal temperatures on motility in *Pseudomonas solanacearum*, suspensions of virulent isolates (25-K60, 204, 208 and 211) and one avirulent isolate (B1-A) were prepared from cultures grown for 48 h on TZC plates at 32 °C. The suspensions (5 ml, containing approximately 1×10^8 bacteria/ml of SMM minus agar) were placed in tubes and incubated for 30 h at 22° and 32 °C. At 4 h, relative motility was higher at 22 °C than at 32 °C for all isolates. After 24 h, motility in suspensions at 32 °C was markedly reduced, but a relatively high percentage at 22 °C were still motile. At 30 h, all the virulent bacteria were non-motile at 32 °C and slightly motile at 22 °C. The motility indices for B1-A were 3 at 22 °C and 2 at 32 °C at 30 h.

Virulence and physiological characteristics of test isolates. In a comparison involving key diagnostic characteristics of *Pseudomonas solanacearum* (Hayward, 1964), selected avirulent butyrous cultures (including B1-A) were essentially indistinguishable from the type species. When injected into tobacco leaves, B1-A and the other B type variants used elicited a typical hypersensitive reaction (Lozano & Sequeira, 1970).

None of the B type isolates from race 1 cultures induced wilting symptoms when stem inoculations were made in tobacco and tomato seedlings, whereas all those classified as virulent on the basis of colony morphology induced the typical disease syndrome. All the B type isolates elicited formation of a localized necrotic lesion in the stem at the point of inoculation at the leaf axil, as has been noted previously (Averre & Kelman, 1964).

DISCUSSION

This investigation provides evidence that rapid selective increase of avirulent versus virulent bacteria in still broth cultures of *Pseudomonas solanacearum* can be attributed to the high motility and capacity for aerotaxis of the avirulent forms. Thus they have a competitive advantage over non-motile virulent forms when oxygen and perhaps other factors

may become limiting. At a time in the growth of a given culture when oxygen was becoming a limiting factor, the virulent types were usually no longer motile.

The difference in motility between virulent isolates and their avirulent variants was not restricted to isolates from the southeastern United States, but also characterized most isolates from solanaceous hosts in other areas of the world (Table 1).

These observations on the competitive advantage provided by motility of avirulent bacteria are similar to results obtained by Smith & Doetsch (1969) with *Pseudomonas fluorescens*. Also, Old & Duguid (1970, 1971) concluded that the selective advantage of motility was a more important factor in the outgrowth of a given clone of *Salmonella typhimurium* than the presence of fimbriae. If both fimbriate and non-fimbriate cultures were motile, the fimbriate bacteria had a distinct selective advantage over bacteria lacking fimbriae. Since fimbriae were observed on both the virulent (25-K60) and avirulent (BI-A) cultures they probably do not play a major role in selective growth of avirulent BI-A in still cultures.

Since the shift from virulent to avirulent types is relatively rapid in broth cultures, it is probable that many early observations by E. F. Smith and others on motility (Kelman, 1953) and electron photomicrographs of flagella (Hodgkiss, 1964) of *Pseudomonas solanacearum* may have been based on cultures containing motile avirulent bacteria.

In contrast to observations with *Escherichia coli* (Adler & Templeton, 1967; Yakota & Gots, 1970), we found no direct evidence that glucose repressed either motility or chemotaxis of avirulent or virulent bacteria.

No references were found in the literature indicating that motility and aerotaxis were involved in the colony type changes (S to R) in other species of motile bacteria (Braun, 1965). A re-evaluation of such studies may be indicated in the light of related research by other investigators (Smith & Doetsch, 1969; Old & Duguid, 1970, 1971) and our own investigations.

This work was supported in part by National Science Foundation Grant GB8288, the Wisconsin Alumni Research Foundation, and the College of Agricultural and Life Sciences, University of Wisconsin, Madison. For helpful suggestions during the investigation we are grateful to C. D. Upper, J. Adler, D. Kerridge and L. Sequeira. We also acknowledge with appreciation assistance of P. H. Williams in preparation of motility track photomicrographs, of G. Gaard and G. de Zoeten for the electron photomicrographs, and of Diane Cuppels for certain laboratory studies.

REFERENCES

- ADLER, J. (1966a). Chemotaxis in bacteria. *Science, New York* **152**, 708-716.
ADLER, J. (1966b). Effect of amino acids and oxygen on chemotaxis in *Escherichia coli*. *Journal of Bacteriology* **92**, 121-129.
ADLER, J. (1969). Chemoreceptors in bacteria. *Science, New York* **166**, 1588-1597.
ADLER, J. & DAHL, M. M. (1967). A method of measuring the motility of bacteria and for comparing random and non-random motility. *Journal of General Microbiology* **46**, 161-173.
ADLER, J. & TEMPLETON, B. (1967). The effect of environmental conditions on the motility of *Escherichia coli*. *Journal of General Microbiology* **46**, 175-183.
AVERRE, C. W., III & KELMAN, A. (1964). Severity of bacterial wilt as influenced by the ratio of virulent to avirulent cells of *Pseudomonas solanacearum* in inoculum. *Phytopathology* **54**, 779-783.
BARACCHINI, O. & SHERRIS, J. C. (1959). The chemotactic effect of oxygen on bacteria. *Journal of Pathology and Bacteriology* **77**, 565-574.
BRAUN, W. (1965). *Bacterial Genetics*, 2nd edn, p. 380. Philadelphia, Pennsylvania: W. B. Saunders.

- BUDDENHAGEN, I. & KELMAN, A. (1964). Biological and physiological aspects of bacterial wilt caused by *Pseudomonas solanacearum*. *Annual Review of Phytopathology* **2**, 202-230.
- CARLSON, F. D. (1962). A theory of the survival value of motility. In *Spermatozoan Motility*. Edited by D. W. Bishop. Washington, D.C.: American Association for the Advancement of Science Publication 72.
- DOBROGOSZ, W. J. & HAMILTON, P. B. (1971). The role of cyclic AMP in chemotaxis in *Escherichia coli*. *Biochemical and Biophysical Research Communications* **42**, 202-207.
- DOETSCH, R. N. & HAGEAGE, G. J. (1968). Motility in procaryotic organisms: problems, points of view and perspective. *Biological Reviews* **43**, 317-362.
- FUERST, J. A. & HAYWARD, A. C. (1969). Surface appendages similar to fimbriae (pili) on *Pseudomonas* species. *Journal of General Microbiology* **58**, 227-237.
- HAYWARD, A. C. (1964). Characteristics of *Pseudomonas solanacearum*. *Journal of Applied Bacteriology* **27**, 265-277.
- HODGKISS, W. (1964). The flagella of *Pseudomonas solanacearum*. *Journal of Applied Bacteriology* **27**, 278-280.
- HUSAIN, A. & KELMAN, A. (1958). Relation of slime production to mechanism of wilting and pathogenicity of *Pseudomonas solanacearum*. *Phytopathology* **48**, 155-165.
- IINO, T. (1969). Genetics and chemistry of bacterial flagella. *Bacteriological Reviews* **33**, 454-475.
- IINO, T. & MITANI, M. (1967). A mutant of *Salmonella* possessing straight flagella. *Journal of General Microbiology* **49**, 81-88.
- KELMAN, A. (1953). The bacterial wilt caused by *Pseudomonas solanacearum*: a literature review and bibliography. *North Carolina Agricultural Experimental Station Bulletin* 99, pp. 194.
- KELMAN, A. (1954). The relationship of pathogenicity of *Pseudomonas solanacearum* to colony appearance in a tetrazolium medium. *Phytopathology* **44**, 693-695.
- KELMAN, A. (1956). Factors influencing viability and variation in cultures of *Pseudomonas solanacearum* (Abstr.). *Phytopathology* **46**, 16-17.
- KELMAN, A. & HRUSCHKA, J. (1970). Relationship between motility and loss in virulence in *Pseudomonas solanacearum* (Abstr.). *Phytopathology* **60**, 1298.
- KELMAN, A. & HRUSCHKA, J. (1972). Differences in motility between virulent and avirulent cells of *Pseudomonas solanacearum*. *Proceedings Third International Conference on Plant Pathogenic Bacteria*, pp. 313-315. Edited by H. P. Mass Geesteranus. Wageningen: Centre for Agricultural Publishing and Documentation.
- KELMAN, A. & PERSON, L. H. (1961). Strains of *Pseudomonas solanacearum* differing in pathogenicity to tobacco and peanut. *Phytopathology* **51**, 158-161.
- KERRIDGE, D. (1961). The effect of environment on the formation of bacterial flagella. *Symposium of the Society for General Microbiology* **11**, 41-68.
- LOZANO, C. & SEQUEIRA, L. (1970). Differentiation of races of *Pseudomonas solanacearum* by a leaf infiltration technique. *Phytopathology* **60**, 833-838.
- OLD, D. C. & DUGUID, J. P. (1970). Selective outgrowth of fimbriate bacteria in static liquid medium. *Journal of Bacteriology* **103**, 447-456.
- OLD, D. C. & DUGUID, J. P. (1971). Selection of fimbriate transductants of *Salmonella typhimurium* dependent on motility. *Journal of Bacteriology* **107**, 655-658.
- SHERRIS, J. C., PRESTON, N. W. & SHOESMITH, J. C. (1957). The influence of oxygen and arginine on the motility of a strain of *Pseudomonas* sp. *Journal of General Microbiology* **16**, 86-96.
- SMITH, J. L. & DOETSCH, R. N. (1969). Studies on negative chemotaxis and the survival value of motility in *Pseudomonas fluorescens*. *Journal of General Microbiology* **55**, 379-391.
- VAITUZIS, Z. & DOETSCH, R. N. (1969). Motility tracks: technique for quantitative study of bacterial movement. *Journal of Applied Microbiology* **17**, 584-588.
- WINSTEAD, N. N. & KELMAN, A. (1952). Inoculation techniques for evaluating resistance to *Pseudomonas solanacearum*. *Phytopathology* **42**, 628-634.
- YAKOTA, T. & GOTS, J. S. (1970). Requirement of cyclic adenosine 3',5' phosphate for flagella formation in *Escherichia coli* and *Salmonella typhimurium*. *Journal of Bacteriology* **103**, 513-516.