Role of Flagellar Motility in the Invasion of Bean Leaves by Pseudomonas phaseolicola

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ABSTRACT

The role of flagellar motility in the invasion of bean leaves by *Pseudomonas phaseolicola* was examined using isogenic pairs of motile and nonmotile mutants. The latter possessed paralysed flagella and did not spread in semisolid medium. They consisted of predominantly nonmotile cells, but contained a few motile individuals presumably because of leakiness of their mutations. Spontaneous motile revertants were obtained and used in comparative inoculation tests. With externally applied inocula, motile strains caused up to 12 times as many lesions than did nonmotile counterparts. Similar results were obtained with wounded and nonwounded leaves. Invasion of water-congested leaves by motile or nonmotile strains was 90 to 400 times greater than of noncongested leaves. Systemic invasion in the plant was not related to motility. When leaves were immersed in motile bacterial suspensions containing 10^6 cells/ml, the initial rates of entry under optimal conditions were 180 to 620 cells/min/cm^2 of leaf tissue. Invasion rates calculated from the gas effusion equation were in good agreement with experimentally measured rates for nonchemotactic organisms, but were somewhat lower for chemotactic *P. phaseolicola*. Also, the long-term distribution of bacteria between inside and outside congested leaf tissue deviated substantially upwards from that predicted for a simple diffusional equilibrium, suggesting that the bacteria were attracted to and concentrated in the leaf tissue.

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Additional key words: paralysed mutants, water congestion, chemotaxis, effusion model.

Flagellar motility in bacteria is believed to confer significant survival advantages by permitting them to explore larger areas of their microenvironments and to respond rapidly to both favorable and unfavorable chemotactic stimuli (6, 22, 25, 33). With plant pathogenic bacteria, motility could significantly contribute to their infection potential by enabling them to reach favorable sites for infection. Invasion of plant tissues by foliar bacterial pathogens generally occurs through natural openings, e.g., stomata, hydathodes, or nectaries (10, 24, 30), when there is water congestion, such as following a rain (8, 14) or heavy dew or under high humidity in the greenhouse (35). These conditions could give motile strains of bacteria considerable advantage over nonmotile strains in the invasion of plants. We know of no bacterial strains in nature that invade plants through stomata that are not motile.

The role of motility in plant pathogenic bacteria was studied using *Pseudomonas phaseolicola,* the cause of the halo blight disease of common bean (*Phaseolus vulgaris* L.). The pathogenic capabilities of mutants selected for immotility were compared with those of isogenic motile revertants in controlled experimental conditions in the greenhouse. In addition, factors which affected the invasion process directly and indirectly, as well as theoretical models for predicting leaf invasion rates, were

TABLE 1. Designation and description of strains of *Pseudomonas phaseolicola*[a] and *Salmonella typhimurium* used to study the role of flagellar motility in the invasion of bean leaves

Strain designation	Relevant phenotype and genotype	Immediate ancestor	Ability to spread in STM[b]	Virulence index[c]
Pseudomonas phaseolicola				
HB36	wild-type motile	...	100	0.12, 0.33, 0.50
HB36Sm500	streptomycin resistant	HB36	95	ND[e]
M1[d]	paralysed *(mot-1)* requires leucine and histidine *(leu⁻, his⁻)*	HB36	10	ND
M3	paralysed *(mot-3)*	HB36	10	0.29
M5	paralysed *(mot-5)*	HB36	13	0.20
M6	paralysed *(mot-6)*	HB36	20	0.30
M7	paralysed *(mot-7)*	HB36	15	0.17
M10	paralysed *(mot-10)*	HB36	18	0.20
M17	paralysed *(mot-17)*	HB36	33	ND
M3R1[f]	motile revertant *(mot-3, rev-1)*	M3	90	0.17
M5R1	motile revertant *(mot-5, rev-1)*	M5	87	0.20
M6R1	motile revertant *(mot-6, rev-1)*	M6	96	0.40
M6R2	motile revertant *(mot-6, rev-2)*	M6	94	ND
M6R3	motile revertant *(mot-6, rev-3)*	M6	ND	ND
M6R4	motile revertant *(mot-6, rev-4)*	M6	ND	ND
M6R5	motile revertant *(mot-6, rev-5)*	M6	ND	ND
M6R6	motile revertant *(mot-6, rev-6)*	M6	ND	ND
M7R1	motile revertant *(mot-7, rev-7)*	M7	10	0.20
M10R1	motile revertant *(mot-7, rev-1)*	M10	98	0.20
M17rev1	motile *(mot⁺)*	M17	97	ND
DA4	motile, non-chemotactic *(che⁻)* multiauxotrophic *(his⁻, try⁻, met⁻)*	SL4012	ND	...
AL39	non-chemotactic *(che⁻)*	SL1634	ND	...

[a]All HB36 mutants were obtained by nitrosoguanidine mutagenesis. Genotype designations are according to Demerec et al. (13).

[b]Spread diameter, measured after incubation of semisolid tryptone medium (STM) plates for 18 h at 20 to 25 C. Data expressed as percent of the diam of spread of the wild-type parent.

[c]Virulence index is the ratio of water-soaked lesions produced to the number of cells infiltrated into the primary leaves. The latter was estimated from the number of viable cells/ml of the suspension used to infiltrate the leaves by multiplying with the intercellular space volume determined as described in the text.

[d]M designates nonmotile mutant.

[e]ND = not determined.

[f]R designates spontaneous revertant.

[g]Strain DA4 was obtained by nitrosoguanidine mutagenesis and strain SL39 by ICR-191 mutagenesis.

examined from a quantitative standpoint.

MATERIALS AND METHODS.—*Culture media.*—Slant and petri dish cultures were prepared on King's medium B (23). Semisolid tryptone media (STM) used to evaluate bacterial motility contained 1% tryptone (Difco) and 0.4% agar (5). Complex liquid media contained 1% proteose peptone No. 3 (Difco). Minimal media contained Hutner's mineral base (9), 0.01% $(NH_4)_2SO_4$, 0.05 M Na-K phosphate buffer, pH 7.0, and appropriate amounts of carbon source. The salt solutions were sterilized by Millipore filtration and sugar solutions were autoclaved separately. All solutions were kept sterile over chloroform. Bacterial turbidities were measured with a Klett-Summerson colorimeter (green filter).

Bacterial strains.—We used *P. phaseolicola* strain HB-36, which causes typical halo blight symptoms on *Phaseolus vulgaris* 'Red Kidney'; i.e., watersoaked lesions in 3-4 days in the leaves and local as well as systemic chlorosis. It carries no known mutations and its nutritional catabolic spectrum is known (32). Two nonchemotactic *Salmonella typhimurium* strains, DA4 and AL39, were kindly provided by R. M. Macnab and D. E. Koshland, Department of Biochemistry, University of California, Berkeley, and by B. A. D. Stocker, Department of Microbiology, Stanford University, School of Medicine, respectively. Other details about these strains are given in Table 1.

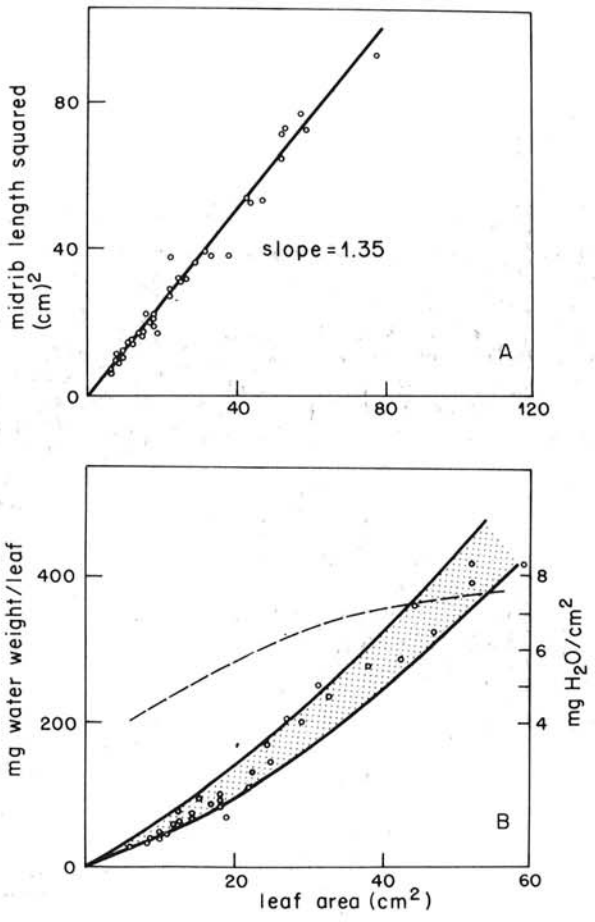

Fig. 1-(A, B). Relationship among leaf area, midrib length, and intercellular space volume in primary leaves of 'Red Kidney' beans.

Induction of mutants.—Cells for mutagenesis were collected from log-phase cultures by centrifugation and washed twice with 0.05 M tris-maleate buffer, pH 5.5. The pellet was finally resuspended in buffer at a concn of approx. 2-4 × 10^8 cells/ml. Mutagenesis was done essentially according to Adelberg et al. (1). To a test tube containing 4.5 ml of cell suspension was added 0.5 ml of 250 μg/μl N-methyl-N′-nitro-N-nitrosoguanidine freshly dissolved in tris-maleate buffer. The cells were incubated at 28 C for 30 min (approximately 35% survival), centrifuged, and washed once with 0.05 M phosphate buffer, pH 6.9, resuspended in 10 ml of complex medium and grown for approximately two generations to allow for phenotypic expression of the induced mutations. The cells were then centrifuged and resuspended in 2 ml of 1% proteose peptone No. 3, and kept in the refrigerator until further use.

Enrichment and selection of nonmotile mutants.—Enrichment for nonmotile mutants was performed according to the method of Armstrong, Adler and Dahl (5). Petri dishes containing STM were inoculated at the center with a small portion of the mutagenized cells. After overnight incubation on the laboratory bench (approximately 20 to 25 C), the cells had multiplied and spread into the medium. Cells from the center of each petri dish were then picked and transferred onto a new medium and the overnight incubation was repeated. After several selection steps, cells from the center of the petri dish were plated for single colony isolations. The motility of each colony was examined with the light microscope and also by stab-inoculation on STM and scoring the diam of spread after overnight incubation. Colonies which showed no motility under the microscope and reduced spreading ability in STM were purified twice on King's medium B and stored on King's B slants in a refrigerator.

Isolation of motile revertants.—Spontaneous motile revertants were obtained by incubating stab-inoculated STM petri dishes for 1 to 2 days by which time the cell population had reached a level of 10^{-8} or greater. Revertants were evident by the appearance of "flares" of bacteria spreading away from the site of growth. Cells taken from the edges of the flares were purified twice, and stored as above.

Electron microscopy.—To determine the presence or absence of flagella, cells from 24-h slant cultures were gently suspended in distilled water, centrifuged at 3,000 *g* for 15 min at room temp, and the pellets resuspended gently in water. Grids were negatively stained with phosphotungstic acid or shadow-casted with platinum.

Preparation of inoculum.—Actively motile cells were obtained from 20- to 24-h King's B slants or from log phase liquid cultures with glucose or arabinose as substrate, along with required amino acids when necessary. Care was exercised during suspension and handling of inocula to minimize damage to flagella. The cell suspensions were diluted with distilled water to give the appropriate cell concentration. To maintain high motility of the cells, 0.1 mM ethylenediaminetetraacetate (EDTA) was added (3).

To prevent the accumulation of revertants, the cultures of nonmotile mutants were routinely streaked and repurified from single colonies.

Plant inoculations.—Inoculations were performed on primary leaves of 11- to 13-day-old Red Kidney bean plants in the greenhouse unless otherwise indicated. Routine testing of pathogenicity was done by rubbing the surface or Carborundum-dusted leaves with a Q-tip dipped in bacterial suspensions (24, 32). To compare the inherent virulence of the various strains under conditions in which motility was not required for entry into the leaf, the inoculum was administered by vacuum infiltration. Interstrain comparisons were made on the basis of a "virulence index"; i.e., the ratio:

$$\frac{\text{number of lesions/cm}^2}{\text{number of cells introduced/cm}^2}$$

The latter quantity was calculated from the known concn of cells in the inoculum suspension and the volume of intercellular space/cm^2 of leaf, determined from Fig. 1-B. This graph was obtained by plotting the difference in fresh wt of a set of 33 primary leaves before and after vacuum infiltration. The intercellular space volume increases with leaf maturity (Fig. 1-B). For young leaves, however, at the stage used for the inoculations the volume was 4-6 μliters/cm^2 leaf.

Lesions were counted 6 to 7 days after inoculation. Whenever necessary, the lesion density was corrected for leaf expansion between the dates of inoculation and lesion counting by an empirical correction factor which was determined from the linear relationship between leaf area and the squared length of the midrib (Fig. 1-A). Thus, by measuring the midrib length at the time of inoculation and again at the time the lesions were counted, the amount of leaf expansion could be determined. In most experiments, the increase in leaf area from time of inoculation until time of data collection was 80-100%.

To determine the infectivity of external inocula, leaves were immersed in or sprayed on both surfaces with bacterial suspensions containing approximately 1 to 6×10^7 cells/ml. The excess inoculum was not rinsed from the leaves in these experiments. The rate of leaf invasion by bacteria through water-congested stomata was studied by immersing leaves, previously infiltrated with water, into bacterial suspensions of approximately 1 to 5×10^4 cells/ml for various periods of time. Unless otherwise indicated, all inocula were prepared and diluted as necessary in 0.1 mM concn of EDTA to preserve good motility (3). The leaves were rinsed thoroughly with running water immediately following the inoculations. This method of inoculation resulted in a uniform distribution of lesions, with good reproducibility. To express the rate-of-entry data in terms of number of bacteria entering per unit time into a cm^2 area rather than as lesions formed per cm^2 per unit of immersion time we multiplied the latter by the inverse of the infection probability which was taken to be equal to the virulence index defined earlier for vacuum-infiltrated cells.

Direct determinations of the rate of entry were also made in similar experiments by grinding leaf disks in a sterile mortar and plating serial dilutions of King's medium B. To counterselect saprophytic contaminants we used a streptomycin-resistant strain of *P. phaseolicola* and added streptomycin to the medium at 500 μg/ml. In experiments involving *Salmonella typhimurium,* counterselection was effected by incubating the plates at 42 C. EDTA was included in the suspending water in these experiments as in the previous ones.

In all cases, the concn of viable cells in the inocula was determined by plating serial dilutions prior to, and/or immediately after, inoculation. To facilitate comparisons between different experiments, strains, or methods of inoculation, lesion densities or colony counts were normalized to an inoculum concn of 10^6 cells/ml.

RESULTS AND DISCUSSION.—*Pathogenic characterization of nonmotile mutants.*—All but one of 17 mutants selected for their inability to spread in STM produced typical water-soaked lesions indistinguishable from those produced by the parent isolate when inoculated by the Carborundum technique. The exception also failed to grow on minimal media and was later shown to contain a double auxotrophic mutation. Mutation to auxotrophy often results in partial or complete loss of pathogenicity (16, 17).

Genotypic characterization.—Flagellar motility in bacteria is under the control of several groups of genetic loci (4, 20). In the Enterobacteriaceae there are one or two genes, called *H* or *hag,* which determine the amino acid sequence of the flagellar protein monomer, flagellin. A group of genes designated *fla* play a role in the production of flagella other than the determination of the primary structure of flagellin. A third group of genes, called *mot,* regulate motility. Fourteen *fla* genes and three *mot* genes have been identified genetically in the Enterobacteriaceae (4, 20). Mutations in the *H* gene result in loss of the flagella (nonflagellated mutants) or alteration of its shape (curly, straight, etc.); *fla*$^-$ mutations also produce a nonflagellated phenotype. Finally, mutants carrying a *mot*$^-$ allele are paralyzed, i.e., nonmotile, though normally flagellated. Electron microscopy revealed that our nonmotile mutants were flagellated, indicating that the mutagenic treatment affected the *mot* gene(s) which control(s) the activation, rather than the formation, of the flagellum.

Although the mutants were stable when grown on solid media or in liquid shake cultures for several months, they reverted when maintained on semisolid agar plates for 2 days or longer. The frequency of reversion, determined approximately from the size of the bacterial population at the time the flares first appeared, was $\leqslant 2 \times 10^{-8}$ to 10^{-9}/cells, indicating that the *mot*$^-$ phenotype was caused by a single mutation, rather than a combination of mutations (31).

Phenotypic characterization.—Nonmotile bacteria multiplied in STM but did not move through the medium except by lateral displacement caused by growth (Table 1). Motile strains spread through the medium and produced chemotactic rings (2, 5). The rate of increase in ring diam was approximately 0.4 mm/h in 0.4% agar. The rate depended both on the agar content and the amount of substrate added to the medium. It decreased exponentially with increasing agar content, a 0.15% increment causing a 50% decrease in the rate of spread. When L-arabinose was used as the carbon source, the rate of ring expansion decreased by approximately 25% when

the concn was increased from 0.1 mM to 1.0 mM.

An interfering class of mutants encountered in our selection process produced filaments with heterogeneous cell lengths. These mutants also produced slowly spreading swarms in STM but exhibited active motility under the microscope. They could not be cultured in liquid minimal medium because of autolysis. The strains grew well, however, in minimal medium containing 0.5% NaCl, or in proteose peptone medium. One isolate produced minicells. The mutants were, therefore, defective in cross-septum formation and wall synthesis. Apparently, because of the elongated shape and the resultant decrease in their ability to spread in STM, they eventually enriched the population during the repeated transfers from the center of the semisolid plates. These mutants were not included in the experiments presented later.

The mutants selected for the study exhibited a leaky phenotype; i.e., they contained a low proportion of motile cells. These cells did not result from genetic reversion since no spreading swarms were produced in STM even when several consecutive subcultures were made daily. Swarms (i.e. revertants) appeared when subculturing was delayed for 1-2 days. The mutants also produced satellite microcolonies surrounding the compact colonies formed at the point of inoculation in STM (29). Their number and size varied somewhat with agar and substrate concn, but generally was characteristic of the mutant strain. Microcolony formation is another indication of mutation leakiness (21, 29). Quadling and Stocker (29) studied this phenomenon in paralysed or nonflagellated bacterial mutants. They suggested that some mutant cells temporarily regained their motility as a result of some "discontinuous intracellular event" which permitted the synthesis (or functioning) of flagella. The frequency of such events was estimated by them as 4×10^{-5} cells/generation. The motile cells migrated through the agar and multiplied, but the progeny were immotile and gave formation to microcolonies.

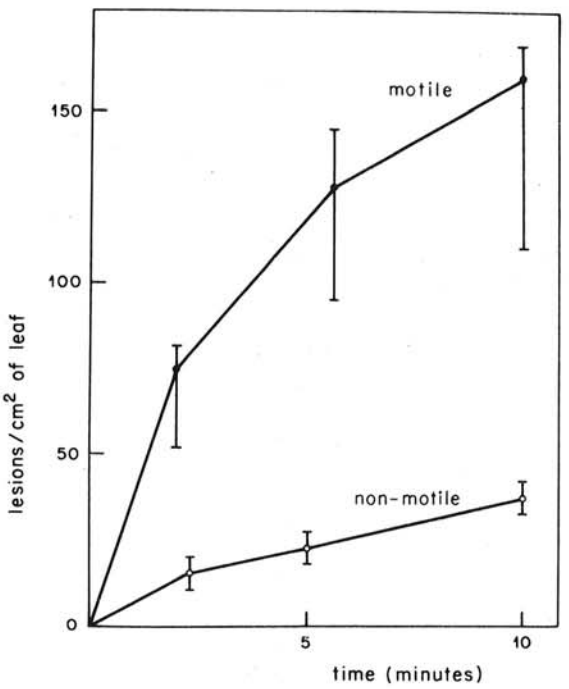

Fig. 2. Increase in lesion numbers/cm^2 in primary bean leaves as a function of immersion time in bacterial suspension. Leaves were preinfiltrated with distilled water containing 0.1 mM EDTA just prior to immersion. Upper curve: M6revl; lower curve: M6. Each point represents the average from six leaves. Vertical lines indicate the actual maximum and minimum values encountered. Values on the ordinate have been corrected upwards to an inoculum concn of 10^6 cells/ml. The actual cell concentration in this experiment was 4×10^5 cells/μl and the highest lesion density was 75/cm^2.

Speed of travel in water.—The speed with which individual cells traveled in water was estimated for the wild-type strain as well as for several of the revertant strains from motility tract photographs. The mean speed (average of the five longest tracks observed) was 25 μm/s. This value is in the same range as those reported for other bacteria (7, 26, 34). Nonmotile mutants produced few or no motility tracks.

Comparative virulence of motile and nonmotile strains.—The "virulence index" (the ratio of water-soaked lesions produced in the primary leaves to the number of viable cells introduced into the leaf) was used as the basis for comparison of virulence among strains. The values obtained with vacuum-infiltrated inocula of wild-type cells were generally between 0.17 and 0.50. Nonmotile mutant and revertant strains also gave values within this range (Table 1). Thus, loss of motility had no effect on the inherent virulence of the strains.

When the inoculum was applied to the leaves externally, however, either by spraying or dipping, differences in the infectivity of *mot*$^+$ and *mot*$^-$ strains were substantial (Fig. 2). Infectivity is defined as the capacity of the pathogen to enter the plant and incite disease. In general *mot*$^+$ strains incited 2 to 12 times more lesions than the respective *mot*$^-$ strains (Tables 2 and 3). In all cases, infectivity of *mot*$^-$ mutants increased upon reversion to *mot*$^+$.

In the experiments presented above, invasion of the leaves was primarily through stomata; care was exercised to avoid mechanical damage to the leaf, since fresh wounds increase leaf susceptibility to bacterial diseases. To determine whether or not the "motility advantage" in infectivity was peculiar to stomatal penetration, leaves were artificially injured by rubbing one-half of each leaf with Carborundum. This caused localized water congestion about the wounds. Leaf surfaces were then sprayed with equally concd suspensions of motile and nonmotile strains. The number of lesions in the wounded half was 9 to 12 times greater than that in the nonwounded half. The motile strains, however, incited several times more lesions per unit area than did nonmotile strains (Table 2).

In addition to inciting localized water-soaked lesions, *P. phaseolicola* invades the plant systemically. We examined whether or not this depended on flagellar motility. Plants inoculated with nonmotile strains became systemically diseased the same as plants inoculated with motile strains. Reisolation from the systemically invaded trifoliate leaves always yielded the original nonmotile strains. Thus, systemic invasion of the plants did not depend upon flagellar motility.

The presence of many undetected mutations in bacteria treated with nitrosoguanidine (31) and the possible occurrence of t-RNA suppressors in revertant strains in general (18, 19) introduce ambiguities in the interpretation of data obtained with mutant and revertant strains, since a simultaneous correction of other genetic lesions unrelated to the phenotypic property being examined by suppressor mutations can occur. To rule out the possibility that the restoration of high infectivity in our revertant strains might be caused by such a mechanism, six independent revertants of one *mot*$^-$ mutant were isolated and compared with the parent M6 as to their infectivity (Table 2). Since external t-RNA suppressor mutations in bacteria occur with a frequency of 18 to 25% (18, 36), it is likely that some of the six revertants did not result from a suppressor mutation. All six revertants showed comparable restoration of infectivity (Table 2). This makes it unlikely that the restoration of high infectivity in our mutants by reversion was caused by a suppressive correction of unsuspected mutations different from the *mot* gene(s).

Differences in the degree of restoration of infectivity by genetic reversion were observed among *mot*$^+$-*mot*$^-$ strain pairs (Table 2). In strains M5, M10, and M17, for example, reversion enhanced infectivity only 2.1- to 2.5-fold, whereas in strains M3, M6, and M7 increases of 4- to 12-fold were recorded. The small differences found in the rate of migration of spontaneous revertants in STM (Table 1) do not account for this large variation which, therefore, must be caused by other properties of the strains. For example, M17 appeared to have a more leaky phenotype than other *mot*$^-$ mutants, and M5 and M10 had a prolonged lag phase on minimal glycerol medium.

Invasion of watercongested leaves by motile bacteria.—Motile bacteria invade leaves readily when there is water congestion; i.e., when a continuous water channel is established between the intercellular spaces and the leaf surface (8, 10, 14). The rate at which invasion takes place is not known.

Assuming that the entry through the stomata is governed by the random motion of the bacteria in the suspending fluid, and that no chemotactic gradients are operative across the stomatal pores, one can calculate the rate of invasion of a unit water-soaked leaf area theoretically from the effusion formula for gases: $R = 1/4N\bar{c}$, where R is the rate of passage of gas molecules through a small orifice, N is the concn of the gas and $\bar{c}$, its average molecular velocity (28). For our purpose, $\bar{c}$ is substituted for by the mean velocity of the bacteria, and N by the number of cells/ml. Mean velocities of several bacteria have been measured either by the motility track method used here, or by more accurate and elaborate techniques (7, 26, 34). They generally vary from approximately 14 μm/s for *Escherichia coli* (7, 34) to 56 μm/s for *P. aeruginosa* (34). As we said previously, *P. phaseolicola* had an average velocity of approximately 25 μm/s. To illustrate the applicability of the effusion formula we shall calculate the rate of invasion for *P. phaseolicola* and *Salmonella typhimurium*. Taking $N = 10^6$ cells/ml and $\bar{c} = 25$ μm/s for the former and 28.8 μm/s for the latter (26) we obtain $R = 33.0 \times 10^{-3}$ cells/min/cm^2 for *P. phaseolicola* and 43.2×10^{-3} cells/min/cm^2 for *S. typhimurium*. These values represent rates of passage of cells through a total orifice cross section of 1.0 cm^2. To be expressed on a unit leaf-area basis they must be multiplied by the proportion of the combined stomatal area per unit leaf area. For many greenhouse-grown plants this proportion varies between narrow limits, i.e., from 0.4 to 1.0% (11). R then becomes 150-375 cells/min/cm^2 leaf and 173-432 cells/min/cm^2 leaf for *P. phaseolicola* and *S. typhimurium*, respectively.

TABLE 2. Restoration of high infectivity of paralysed *Pseudomonas phaseolicola* HB36 mutants by genetic reversion

Isogenic strain pair	Ratio of the no. of lesions (Motile revertant/ paralysed parent)	Method of inoculation[a]
M3R1/M3	7.0[d]	1
	3.5[b]	4
M5R1/M5	2.5[b]	2
	2.5[b]	3
	2.2[b]	4
M6R1/M6	4.8[c]	1
	9.0[d]	2
	7.2[b]	3
M6R2/M6	3.4[b]	2
M6R3/M6	8.4[e]	2
M6R4/M6	7.0[b]	2
M6R5/M6	6.2[d]	2
M6R6/M6	7.0[d]	2
M7R1/M7	5.5[b]	2
M10R1/M10	2.2[c]	1
M17R1/M17	2.1[b]	1

[a]Descriptions of inoculation methods: 1 = leaves were preinfiltrated with water, then immersed for a short period (1 to 5 min) in bacterial suspension, and finally rinsed in running water to remove cells remaining on the leaf surfaces; 2 = leaves in their normal condition were dipped in bacterial suspension and either left on greenhouse bench to dry or placed in 95% relative humidity atmosphere; 3 = as in 2, except that the leaves were wounded by rubbing with Carborundum just prior to being dipped in the bacterial suspension; and 4 = as in 2, except that the inoculum was sprayed onto the leaf surfaces under air pressure.

[b]Data from one experiment.

[c,d,e]Average of data from two, three, and four experiments, respectively.

TABLE 3. Rates of entry of motile and paralysed *Pseudomonas phaseolicola* into water-infiltrated and nonwater-congested bean leaves, as measured by the number of lesions[a] per cm^2 of leaf per min of immersion in inoculum suspension containing 10^6 cells/ml

Strain	Entry rate (lesions per cm^2 of leaf per min of immersion)	
	Infiltrated leaves	Noninfiltrated leaves
HB36	75	...
M3R1	90, 80	0.4
M6R1	34, 11[b]	0.8
M10R1	12[b]	0.09
M17R1	76	...
M3	13, 12	0.1
M6	6.5, 2.5[b]	0.07
M10	5.2[b]	0.04
M17	36	...

[a]The actual numbers have been corrected to a standard viable cell count of 10^6 cells/ml. Inocula ranged from 5×10^4 to 6×10^5 cells/ml.

[b]No EDTA was added to the inoculum.

TABLE 4. Experimental and theoretical entry rates of various bacteria into bean leaves. Data are given for two nonchemotactic strains of *Salmonella typhimurium* and one chemotactic strain of *Pseudomonas phaseolicola*

Strain	Entry rate (cells per cm^2 of leaf per min of immersion)	
	Experimental	Theoretical
Salmonella typhimurium		
DA4 nonchemotactic	300, 375	173-432[c]
AL39 nonchemotactic	391, 490	
Pseudomonas phaseolicola		
indirect method[a]	180, 450[d]	150-375[c]
direct method[b]	430, 620	

[a]Calculated from lesion density data (Table 3) using the virulence index.
[b]Based on direct plating.
[c]Range given is based on the proportion of leaf area that is stomatal area [0.4 to 1.0%, ref. (11)].
[d]Range is based on experimental data.

To test the validity of this model, we measured the rates of entry of two nonchemotactic mutants of *S. typhimurium* into bean leaves following timed immersions in bacterial suspensions as described previously. These strains are unable to respond to chemical gradients of any substance tested, and thus they provided a good tool for testing our model. Experimental entry rates were 300-375 cells/min for one strain and 391-490 cells/min for the other, expressed on a cm^2 area and 10^6 cells/ml inoculum concn (Table 4). These results are essentially within the theoretically predicted range.

Whether or not similar predictions could be applied to the entry of *P. phaseolicola* would depend among other things on the relative contributions of the "random motility vector" and the "chemotactic vector." The speed of the chemotactic response, i.e., the transfer velocity of bacteria in preformed chemotactic gradients is relatively small, compared with the speed of individual bacteria (12). On this basis, one could argue that the random motility of individual bacteria is an overwhelming factor during the initial stomatal invasion, so that theoretical predictions based on the previous model would not be grossly in error. This was examined by comparing the theoretically calculated with the experimentally measured rates of entry, both direct (determined by grinding leaf disks and plating) and indirect (estimated from lesion-density data) (Table 3). The direct method gave rates from 430-620 cells/min whereas the indirect estimates were 180-450 cells/min (under optimal conditions), again expressed on a cm^2 and 10^6 cells/ml inoculum basis. Although there is some indication in these results that the calculations tend to underestimate the rate of entry to some degree, a substantial overlap between theoretical and experimental results is evident (Table 4). Because of the variability in the data and the various uncertainty factors entering into our theoretical calculations we cannot conclude from these experiments that chemotaxis is indeed important during entry. Our examination shows, however, that fair predictions can be made on the basis of the effusion model.

From both theory and experimental evidence, we conclude that when a suspension of *P. phaseolicola* comes into contact with bean leaves and the conditions are optimal for motility, we can expect approximately 0.015% to 0.06% of the bacteria contained in 1 ml of suspension to gain entrance into the water-soaked leaf in 1 min if the surface area available for invasion were 1 cm^2.

The plot of bacterial entry versus time showed a progressive decline as the immersion time was increased (Fig. 2). Thus, the rate figures given previously pertain only to the first few minutes after the inoculum contacted the leaf. One reason for the decline is the gradual equilibration of bacterial concn in the intercellular fluid and the outside suspension. Several bacteria contributing to one lesion at high lesion densities may have contributed to the decline shown in Fig. 2 (highest lesion density was 75 lesions/cm^2), but similar kinetics have been obtained using more dilute inoculum and with lesion densities less than 25 lesions/cm^2. Thus, if entry into the leaf were governed by diffusion (random movements) alone, the final concn of cells inside the water-soaked leaf spaces would eventually reach that on the outside. Equilibration of the concns would occur in about 1 h. Beyond that, the number of bacteria inside the leaf should not increase further. However, when the time of immersion was extended to about 2 h, using inoculum of 2×10^4 cells/ml, the number of lesions which developed per unit area of leaf was 5- to 7-fold higher than that predicted of an equilibrium distribution. Since the generation time of *P. phaseolicola* is longer than 2 h at temp of 20 to 25 C, bacterial multiplication inside the intercellular spaces could not have played a major role. Other reasons that can account for this result include: (i) loss of motility of the bacteria after entry, (ii) physical adhesion and immobilization onto cell walls, (iii) chemotaxis towards the solutes present in the intercellular fluid, (iv) increase in leaf susceptibility caused by prolonged water-soaking, or other reasons. Although additional experiments are needed to determine which of these factors are actually operative, the results show that water-soaked leaf tissue exerts a substantial "concentrating" effect upon motile bacteria present in suspension of the leaf surface.

Importance of leaf water congestion.—The importance of leaf water-soaking in the epidemiology of the wild-fire disease of tabacco (8, 14, 15) and the citrus canker (27) is well established. Water-soaking facilitated both invasion and the spread of the bacteria within the leaf tissues after infection and multiplication had occurred. In the case of

the wild-fire organism, water congestion resulted in a several thousand-fold increased invasion of the leaf tissues (15).

To determine the magnitude of the "predisposing effect" of water congestion in the case of *P. phaseolicola* we compared the rate data presented previously with water-infiltrated leaves with data obtained in a similar way but using normal, noncongested leaves. The lesions developing on such leaves following dipping into bacterial suspensions for varying time periods were irregularly distributed over the leaf blade and tended to concentrate around the margins or around occasional wounds inflicted on the leaves during handling. If the nonrandom lesion distribution is disregarded and the number of lesions per leaf is divided by the total leaf area in cm^2, the lesion density was 0.1-0.8 lesions/cm^2 for motile strains when calculated to an inoculum density of 10^6 cells/ml and the immersion time was equal to 1 min. A comparison between these values and those given previously for water-infiltrated leaves reveals an impressive 80- to 400-fold increase in lesion density caused by water-soaking. Although these comparisons were for motile strains similar results were also obtained with nonmotile ones as well (Table 4). The results show clearly that noncongested leaves are by virtue of their physical structure very resistant to invasion by bacteria.

As previously stated, leaves of crop plants in the field become water-congested after rain or dew or high humidity, but the extent to which this occurs depends on plant species and variety, and the cultural-agronomic practices. Examples of plant species or certain cultivars showing unusual susceptibility to water-soaking with concomitant susceptibility to bacterial diseases of their foliage are well documented (8, 14, 27, 35). A speculative extension of our observations is that plant cultivars which have been bred for resistance to water-soaking should possess a generalized, horizontal type of resistance to leaf-spotting bacterial diseases.

Significance of motility in disease and for P. phaseolicola.—Our findings show that flagellar motility markedly increased the infectivity of *P. phaseolicola* towards bean leaves, either congested or noncongested, wounded or intact. As pointed out earlier, our data probably underestimate the quantitative role of motility in leaf invasion and infectivity in our system, since the mutants we used had a "leaky" phenotype. The question is raised, however, whether natural inoculum from lesions in the field is motile or not. To test this, drops of water were placed on leaves with 7- to 10-day-old lesions and the leaf was punctured with a needle to facilitate exit of the bacteria. Alternatively, leaves were vacuum-infiltrated with water and droplets were placed on the surface of the leaf. Bacterial motility was examined at various intervals. In the first case, the bacteria remained essentially nonmotile even after 1 h. In the infiltrated leaf, however, fully motile populations were seen after 30-60 min. Although differences between lesions from the greenhouse and lesions from the field are conceivable, the observations show that bacteria from lesions are capable of regaining their motility soon after they come into contact with liquid water.

Our results confirm earlier findings that autonomous motility is not absolutely essential for bacterial entry into the leaf (15), which is true regardless of whether these are (macroscopically) water congested or not. There is, however, some disagreement between our conclusions and the suggestion that motile organisms have no advantages over nonmotile ones in entering watersoaked leaves (15).

Since, according to our findings, ingress of the bacteria into water-soaked leaves proceeded very rapidly and approached its maximum in about 1.0 h from the time the initial contact between the leaves and the inoculum was made, leaf invasion in nature can occur very effectively even when water-soaking is only transient. Invasion would also be considerable even when the epiphytic population of the pathogen was as low as 10^3 bacteria/cm^2 and even if proper conditions for motility lasted for only several min.

It has been considered, from an evolutionary viewpoint, that motility would be advantageous to bacterial cells when growth was limited by diffusion because they could "graze and then move on" to other sites (25). However, with low molecular wt organic substrates, such as sugars or amino acids, the diffusion coefficients are great enough so that the cell surface is supplied with nutrients far in excess of the capacity of the uptake systems (25). Nevertheless, diffusion can become limiting when the important substrates are macromolecules, or when bacteria grow as microcolonies (25). These situations occur in the life cycles of many saprophytic bacteria. With most bacterial pathogens of plant foliage, such as *P. phaseolicola,* the life cycles include limited or no saprophytic growth. The main increase in population occurs within the host plant where conditions do not appear to be favorable for motility because of the absence of free water in the intracellular spaces. Accordingly, with *P. phaseolicola* and a number of other bacterial foliar diseases, we have not observed motile bacteria swimming from systemically infected tissues except where water-congested lesions have erupted. Motility, therefore, appears advantageous mainly during ingress and egress of the pathogen from the host plant since, after dissemination by rain, vectors, or other agencies, swimming enables a greater percentage of cells to reach the nutritionally favorable microsites. Because of the diffusion rate for nonmotile cells is minimal, entrance into the leaf would largely depend on chance contact, on other physical forces, such as suction through hydathodes (10), forceful ingress of rain-borne inoculum through stomata (8, 14), and following wounding. It is likely, therefore, that the structure of the leaf favors the survival of motile strains in nature through selection; once inside the leaf, bacteria enjoy special advantages such as ability to attack the leaf and to multiply without competition by saprophytes, protection from ultraviolet irradiation and drought, etc. Furthermore, when considering that many cycles of secondary infection occur during a field epidemic, a motility advantage of the magnitude measured in our experiments would promote a virtually complete replacement of nonmotile strains of *P. phaseolicola* by motile ones.

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