Aspects of Motility and Chemotaxis in Spiroplasmas

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(Received 19 November 1979)

Spiroplasmas, the helical wall-free prokaryotes isolated from plants and insects, are capable of translational motility in viscous media, and the speed of swimming increases with viscosity. In consequence, spiroplasmas show 'viscotactic' behaviour in viscosity gradients. Motility is energy-dependent and is optimal at pH 7. It has also been found that spiroplasmas are capable of chemotaxis, being attracted towards sugars and some amino acids and repelled by hydrophobic amino acids, some organic acids and heavy metals.

INTRODUCTION

Two types of motility have been described for wall-free prokaryotes of the class Mollicutes. A few species (Mycoplasma gallisepticum, M. pneumoniae and M. pulmonis, pathogens of animal respiratory tracts) are able to glide over liquid-covered surfaces at speeds of up to 2 \( \mu \text{m s}^{-1} \) (Bredt, 1974) while the recently discovered helical spiroplasmas from plants and arthropods display what appears to be rotation about the helix axis and flexing of the cell body (Davis & Worley, 1973). Little is known about the mechanisms of mycoplasma motility.

In liquid media of low viscosity the movements of spiroplasmas produce no appreciable translational motility (i.e. considerable 'slippage' takes place) but when the viscosity is increased by incorporating agar or methylcellulose in the medium the organisms rapidly 'screw' their way along (Davis & Worley, 1973; Lee, 1977; Davis, 1978). We have confirmed these observations and have measured the speed at which spiroplasmas swim as a function of viscosity and have shown that spiroplasmas exhibit the phenomenon of viscotaxis recently described for leptospiras (Petrino & Doetsch, 1978). In addition, we have found that spiroplasmas display chemotaxis towards or away from a range of substances. This is the first demonstration of chemotactic behaviour among the Mollicutes.

METHODS

Spiroplasmas. Spiroplasma strain BC3 was obtained from Dr T. B. Clark, who isolated it from diseased honey-bees (Clark, 1977). This strain was used in preference to well-characterized Spiroplasma citri strains to which it is probably closely related (Williamson et al., 1979) because it grows more rapidly and can be harvested and washed by centrifugation without the loss of helical morphology and viability which accompanies such procedures with S. citri (Razin et al., 1973). The spiroplasmas were grown at 32 °C in T medium containing (per litre): PPLO broth (Difco), 21 g; sorbitol, 70 g; glucose, 1 g; fructose 1 g; sucrose, 10 g; phenol red, 20 mg; horse serum (Wellcome no. 3 or Gibco Biocult, mycoplasma-screened), 100 ml. Plates contained the same medium solidified with 1 % (w/v) agar (LAB M, London). Viable counts were made as described by Daniels et al. (1979). To obtain accurate, reproducible counts it was found to be essential to use freshly poured plates dried for 20 to 30 min in a laminar-flow sterile cabinet.

Demonstrations of motility in soft agar. Aluminium cylinders (diam. 2 cm, prepared by sawing 1 cm lengths from Oxoid test tube caps) were placed in the centre of Petri dishes containing base layers of T medium supplemented with 1.5 % agar. Top layers of 10 ml medium containing 0-4 to 0.8 % agar were poured around
the outside of the cylinders. When the agar had set the cylinders were carefully removed and the well in the top layer was filled with molten soft agar maintained at 42 °C to which spiroplasmas (about 10⁷ ml⁻¹) were added shortly before pouring. The plates were incubated at 32 °C and examined daily.

Microscopic observations. A Zeiss Photomicroscope II equipped with a 60 W illuminator and a dark-field optical system was used. Active, exponentially growing, spiroplasma cultures were mixed with stock solutions of polymers dissolved in T medium, drops were placed on slides, covered with coverslips, and the edges of the coverslips were sealed with Vaseline to prevent evaporation from the specimen and consequent rapid streaming of spiroplasmas across the slide.

Two methods were used for determining the speed of swimming of spiroplasmas. (1) Using an eyepiece graticule and a stopwatch the time taken for individual organisms to cover a distance of 10 μm was measured. At least 50 measurements were made to determine the mean swimming speed. (2) Photographs of the swimming spiroplasmas were taken using 10 s exposures of Ilford HP5 or Kodak TriX film (400 ASA). Lengths of ‘motility tracks’ were measured on prints with a map-measuring device, and the speed of swimming was calculated, taking into account the magnification produced by the microscope and photographic systems. Care was taken to focus on organisms swimming freely in the medium, avoiding those in contact with the glass surfaces. Magnifications were checked using a stage micrometer (Graticules Ltd, Tonbridge, Kent).

Demonstration of viscotaxis. A procedure similar to that of Petrino & Doetsch (1978) was used. Sealed tubes made from 10 μl capillaries (Drummond Microcaps or Camlab Disposable Micropipettes) partially filled with the viscous solution being investigated were suspended in spiroplasma cultures. After incubation at 32 °C the capillary tubes were removed, the outside surfaces carefully wiped with tissue moistened with ethanol, the tubes broken and the contents ejected into 0.2 ml T medium. The suspensions were serially diluted and duplicate 10 μl drops were plated so that the total number of viable spiroplasmas which had entered the capillaries could be calculated.

Demonstration of chemotaxis. Exponentially growing spiroplasmas were harvested by centrifugation at 4 °C (10000 g for 10 min), washed by resuspension in cold PSF [10 mM-sodium phosphate buffer, pH 7.0, containing 5% (w/v) sorbitol and 0.1% (w/v) fructose], recentrifuged and finally resuspended in PSF at a concentration of approximately 10⁷ colony-forming units (c.f.u.) ml⁻¹. Migration of spiroplasmas into capillaries containing a range of concentration of substances to be tested dissolved in PSF was investigated using the procedure described for viscotaxis.

Polymers. Methylcellulose (Methocel A4M, premium grade) was a gift from K. & K.-Greef Chemical Group Ltd, Croydon. Polyvinylpyrrolidone (average mol. wt 360000), Ficoll (average mol. wt 400000) and polyethylene glycol (average mol. wt 6000) were obtained from Sigma, Pharmacia and BDH, respectively.

RESULTS

Motility and viscotaxis

In liquid medium spiroplasma strain BC3 showed similar motile behaviour to the corn stunt organism studied by Davis & Worley (1973), in that vigorous rotation and flexing of the cells produced little translational movement. However, when the cells were suspended in the centre of soft agar plates they migrated radially so that after incubation for 4 to 5 d growth, marked by the presence of colonies, was seen in an annular area (Fig. 1). This behaviour was seen with agar concentrations in the top layer between 0.4 and 0.8% (w/v). At higher agar concentrations the spiroplasmas were unable to penetrate the agar appreciably, while lower concentrations were too soft to handle.

When the viscosity of liquid T medium was increased by incorporating methylcellulose (up to 1.5%, w/v), spiroplasmas were seen swimming rapidly through the solution. The speed of swimming increased with increasing viscosity (Fig. 2), a response also observed with leptospiros (Kaiser & Doetsch, 1975) but not with most bacteria which depend on flagellar locomotion (Schneider & Doetsch, 1974). Both methods for measuring speed of swimming gave similar values. The response of spiroplasmas to increasing viscosity was remarkably consistent. Independent experiments to measure swimming speed of cultures at pH 7 supplemented with 0.3% methylcellulose gave mean values of 2.86, 3.14, 3.15 and 3.54 μm s⁻¹. In a single experiment the coefficient of variation was about 10%.

The dependence of speed of swimming in methylcellulose solutions on the pH value of the medium is shown in Fig. 3. The optimum value of 7.0 was used in all subsequent experiments.
Spiroplasma motility and chemotaxis

Fig. 1. Migration of spiroplasmas in soft agar. The spiroplasmas were initially confined to the central area of the 9 cm diameter plate, and migrated through the top layer containing 0.6% (w/v) agar. After incubation for 5 d at 32°C the plate was stained with Dienes' stain to render colonies more easily visible (Daniels et al., 1979).

Fig. 2. Dependence of speed of swimming of spiroplasmas on the concentration of methylcellulose incorporated in T medium. Determinations of speed were made either by timing organisms over a 10 μm path (○) or by measuring 'motility tracks' on photographs exposed for 10 s (●).

Fig. 3. Dependence of speed of swimming of spiroplasmas on pH. A spiroplasma culture was supplemented with methylcellulose (0.3%, w/v) and portions were adjusted to various pH values with phosphate buffers (final concn 50 mM). Speed of swimming was determined by the photographic method.

Lee (1977) also concluded that a pH value of 7 is optimal for motility, from observations of the passage of spiroplasmas through membrane filters.

Energy metabolism was necessary for motility but not for maintenance of helical shape. When spiroplasmas were harvested, washed and suspended in phosphate-buffered sorbitol they appeared as if paralysed, but when fructose (a fermentable substrate) was added, the characteristic movements resumed, in some cases less than 30 s after sugar addition. Addition to cultures of inhibitors of glycolysis [sodium iodoacetate (10⁻⁴ M), sodium arsenate (10⁻¹ M) and sodium fluoride (10⁻² M)] or of membrane-bound ATPase [N,N'-dicyclohexylcarbodiimide (10⁻³ M)] also prevented motility but did not affect helicity. By contrast, potassium cyanide (10⁻² M), which inhibits respiration, and 2,4-dinitrophenol (10⁻³ M), an uncoupler of oxidative phosphorylation, had no discernible effect. In these experiments cultures were examined immediately after adding the cover slips (i.e. within 20 s) to guard against the possibility that dissolved oxygen may have become exhausted thus diverting metabolism to anaerobic pathways and altering the sensitivity of the cells to inhibitors of

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oxidative metabolism. However, \( S. \) \( c. \) \textit{citri} ferments carbohydrates almost exclusively to \(+)-lactic acid (Hawthorne \\& Van Demark, 1977) and possesses no cytochromes (Hollander \textit{et al.}, 1977).

In view of the similarities between spiroplasma and leptospira behaviour in viscous media we anticipated that spiroplasmas would show viscotactic migration in viscosity gradients. Figure 4 shows the accumulation of spiroplasmas in capillaries containing a range of concentrations of methylcellulose. At polymer concentrations greater than 0-3 \% the number of spiroplasmas in capillaries appeared to decrease, which would not be expected from the explanation for viscotaxis advanced below. We suspected that the decreased response was an artefact caused either by an effect of methylcellulose on viability or by incomplete recovery of the viscous capillary contents. The latter explanation proved to be correct: incubating a spiroplasma suspension with 0-6 \% methylcellulose for 2 h at 32 \(^\circ\)C had no effect on the viable count, but if the suspensions were incubated in capillaries the number of c.f.u. recovered decreased at the higher polymer concentrations.

The number of spiroplasmas in the capillaries increased with time to reach a plateau after 30 min. In addition to methylcellulose, viscotactic responses were demonstrated to 0-5 \% solutions of starch, Ficoll, polyethylene glycol and polyvinylpyrrolidone. \textit{Spiroplasma citri} strain SP13 responded similarly to strain BC3.

\textbf{Chemotaxis}

Spiroplasmas suspended in PSF accumulated in capillaries containing PSF supplemented with a number of substances. A range of concentrations of each substance was used and approximate threshold concentrations were determined, i.e. minimum concentrations which induced an accumulation of spiroplasmas significantly higher (for attractants) or lower (for
Table 1. Chemotaxis by spiroplasmas to various substances

Capillaries were filled with solutions of substances to be tested (at various concentrations) dissolved in PSF and adjusted to pH 7.0 (apart from lactic acid). After incubation for 1 h at 32°C in a spiroplasma suspension in PSF, the number of c.f.u. in each capillary was determined. From results similar to those shown in Fig. 5 the substances were classified as attractants or repellents, or neither. Values given are threshold concentrations, below which no response was observed. In most cases the threshold is denoted by the negative logarithm (base 10) of the molar concentration, but in the case of PPLO broth and yeast extract the concentration is given in mg l⁻¹, and in the case of serum as % (v/v). The amino acid mixture contained the amino acids used in Eagle's Basal Medium, supplied as a 100-fold concentrate, and the threshold is given as % (v/v).

<table>
<thead>
<tr>
<th>Attractants</th>
<th>Threshold</th>
<th>Repellents</th>
<th>Threshold</th>
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<tr>
<td>PPLO broth (Difco)</td>
<td>1</td>
<td>CoCl₂</td>
<td>5</td>
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<tr>
<td>Yeast extract (Oxoid)</td>
<td>50</td>
<td>Uracil</td>
<td>5</td>
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<tr>
<td>Foetal calf serum (Seralab)</td>
<td>0.1</td>
<td>Nicotinamide</td>
<td>5</td>
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<tr>
<td>Amino acid mixture (Biocult)</td>
<td>0.01</td>
<td>Lactic acid</td>
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<tr>
<td>D-Fructose</td>
<td>2</td>
<td>L-Histidine</td>
<td>3</td>
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<tr>
<td>D-Glucose</td>
<td>2</td>
<td>L-Isoleucine</td>
<td>5</td>
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<tr>
<td>D-Maltose</td>
<td>2</td>
<td>L-Leucine</td>
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<td>D-Raffinose</td>
<td>2</td>
<td>L-Phenylalanine</td>
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<td>2</td>
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<td>L-Arginine</td>
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<td>L-Cysteine</td>
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<td>L-Glutamate</td>
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<td>Glycine</td>
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<tr>
<td>L-Threonine</td>
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D-Xylose, L-lysine, L-asparagine, cyclic AMP and sodium lactate (pH 7.0) neither attracted nor repelled spiroplasmas.

repellents) than unsupplemented PSF. The substances tested, together with their approximate response thresholds, are listed in Table 1. The thresholds varied by a factor up to 10 between experiments, for reasons which are not understood. However, the number of spiroplasmas accumulating in duplicate capillaries in any experiment did not differ by more than 20%. Saturation of the response was observed at high concentrations of repellents, shown by the curves for tryptophan and valine in Fig. 5, the saturating concentration being typically 100× higher than the threshold. In the case of attractants, saturation was not usually apparent at the highest practicable concentration (about 50 mM for amino acids).

Figure 5 shows typical responses as a function of concentration of substances added to capillaries. The ordinate denotes the ratio of the number of spiroplasmas entering the capillaries containing the substance to the number entering control capillaries containing PSF. Thus a value greater than 1 denotes attraction whereas repulsion produces a value less than 1.

In initial experiments 0.5% methycellulose or 2% polyvinylpyrrolidone was incorporated in the PSF medium used to suspend the cells and to fill the capillaries in the expectation that chemotactic responses might be more easily detected the faster the spiroplasmas were swimming. However, this proved not to be the case: provided that the cell suspension and the capillary contents had the same viscosity the number of spiroplasmas entering capillaries in the presence or absence of attractants did not depend on the polymer concentration (cf. Petrino & Doetsch, 1978) and, moreover, chemotactic responses appeared to be partially inhibited by certain batches of polyvinylpyrrolidone.

In view of the rather high threshold concentration for response to sugars (10⁻² M) we
investigated the possibility that the solutions possessed a viscosity sufficiently high to elicit viscottactic responses, rather than true chemotaxis. Spiroplasma suspensions containing 0.3% methylcellulose were supplemented with some attractants, repellents and chemotactically ineffective substances, namely, methionine, glycine, sucrose, proline, valine, cobaltous chloride and xylose, all at final concentrations of 10 mM, adjusted to pH 7.0. Measurements of swimming speed showed no significant differences from the control unsupplemented suspension.

The number of spiroplasmas in capillaries increased with time and reached a plateau value at 30 min. Incubation was generally continued for 60 min.

Chemotaxis was strongly dependent on the temperature. In an experiment to test this the number of spiroplasmas entering capillaries containing only PSF was 6900 at 20 °C and 6300 at 32 °C, whereas capillaries containing 2.1% PPLO broth in PSF attracted 17000 c.f.u. at 20 °C, 176000 at 32 °C and 171000 at 37 °C. Motility was much less dependent on temperature. There was no significant difference between swimming speed in PSF supplemented with 0.3% methylcellulose measured at 23 °C and at 32 °C, using a warmed microscope slide.

Under our experimental conditions supplementing the PSF buffer with EDTA (up to 10 mM) did not enhance chemotaxis. In E. coli EDTA is required to prevent inhibition of motility by heavy metal ions (Adler & Templeton, 1967), and the lack of effect of the chelating agent on chemotaxis may be related to the insensitivity of spiroplasma motility to Co²⁺ noted above.

In E. coli and Salmonella typhimurium methionine is required for chemotaxis because of the involvement of membrane protein methylation reactions in signal transduction (for review, see Hazelbauer, 1979). Since spiroplasmas are probably auxotrophic for methionine we investigated the effect of a range of methionine concentrations (present in both capillaries and cell suspension) on chemotaxis. It was not possible to demonstrate a dependence on methionine, perhaps because the cells have a relatively large internal pool which is not easily depleted.

**DISCUSSION**

Our experiments have confirmed previous observations on spiroplasma motility and have added a new dimension by demonstrating that spiroplasmas are capable of chemotactic responses to certain chemical stimuli. Motility and chemotaxis are clearly demonstrated by radial migration in soft agar. The spiroplasmas, initially confined to the central area of the plate, moved outwards, presumably responding to gradients of attractants, caused by utilization of nutrients in the medium, and repellents, caused by metabolism (e.g. lactic acid). At the same time the organisms were growing so that the rate of production of acidic metabolites was increasing. Motility diminishes as the pH value is lowered, and this phenomenon probably explains why the spiroplasmas stop swimming and form colonies, rather than continue moving to the edge of the plate. On a smaller scale, motility in agar is illustrated by the rough appearance of normal spiroplasma colonies caused by the establishment of satellite microcolonies by spiroplasmas migrating from the parent colony, compared with the smooth colonies produced by non-motile strains (Townsend et al., 1977a). It should be possible to use the radial migration phenomenon as an aid to isolating non-motile and non-chemotactic mutants.

The behaviour of spiroplasmas in viscous media is very similar to that of leptospiras, in that high viscosity favours translational motility. The response termed viscottaxis by Petrino & Doetsch (1978) (i.e. migration in the direction of a viscosity gradient) follows as a necessary consequence of the dependence of swimming speed on viscosity, because the velocity component of random motion up the gradient will tend to increase in value, whereas that down the gradient will decrease. Thus random motion will be biased to that spiroplasmas accumulate in capillaries containing viscous media.
The natural habitats of spiroplasmas so far described are the interior of phloem sieve tubes, the nectar of flowers and the tissues of arthropods. All these environments are viscous, so it is likely that dispersal of spiroplasmas is facilitated by their ability to swim rapidly in viscous media.

In their general chemotactic properties revealed by our experiments, spiroplasmas are very similar to eubacteria (Adler, 1978). Complex nutrient mixtures, sugars and many amino acids tend to be attractants, whereas hydrophobic amino acids, acidic metabolites and heavy metals are repellents. It is noteworthy that it is not necessary for a substance to be metabolizable for it to serve as a chemoeffector. Of the sugars listed in Table 1 as attractants, only glucose, fructose and maltose can be metabolized by spiroplasmas, whilst sorbose is inhibitory to growth (Morgan & Barnes, 1979). Table 1 also reveals that lactic acid appears to act as a repellent but sodium lactate does not. It is not clear whether repulsion is due to undissociated lactic acid or to \( H^+ \) ions or, at higher concentrations, to inhibition of motility. Further experiments will be required to investigate the extent of homology of the spiroplasma and bacterial chemotactic mechanisms, notably the nature of chemotaxis receptors at the cell surface, and the possible involvement of protein methylation in chemotactic signal processing.

The existence of chemotaxis implies that spiroplasmas are able to bias random motility so as to produce net migration in a preferred direction. The mechanism for viscotaxis discussed above suggests a possible analogous explanation for chemotaxis, namely that the speed of swimming of spiroplasmas is a function of the concentration of effector to which the cells are exposed. Increasing concentrations of attractants would induce faster swimming and repellents would reduce the speed. However, our measurements of swimming speed in the presence of high concentrations of a selection of effectors disprove this hypothesis. In flagellated bacteria, directional bias is accomplished by varying the relative frequency of runs and tumbles, by altering the direction of rotation of the flagella (for review, see Adler, 1978). So far, microscopic observation has furnished insufficient information to enable us to propose a general mechanism but we are investigating the hypothesis that rotation of spiroplasmas about the helix axis may generate ‘runs’, whereas flexing of the cell body may give rise to ‘tumbles’. Thus the frequency of flexing of the cell body would be under the control of the chemotaxis system.

The ability of spiroplasmas to respond to chemical gradients may be an important factor in the natural host cycle. In the phloem of plants there is a concentration gradient of photosynthetic products from roots to shoots (Mason & Maskell, 1928), so that spiroplasmas may tend to migrate to the top of actively photosynthesizing plants. This may explain why spiroplasmas are readily detectable only in young shoots of citrus plants infected by graft inoculation (Laflèche & Bové, 1970). Young shoots are often preferred by feeding insects, and the concentration of spiroplasmas in these tissues may favour acquisition by insects. Finally, in leaf hopper vectors spiroplasmas appear to accumulate to high concentrations in salivary glands (Townsend et al., 1977b), whence they are discharged with saliva into plants. It may be that chemotaxis is responsible for the accumulation of spiroplasmas in the glands.

REFERENCES


