Motility of the diplomonad fish parasite *Spironucleus vortens* through thixotropic solid media

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Investigation of a series of nutrient-supplemented thixotropic gels at successive dilutions that impede the trajectories of a highly vigorous motile flagellated protist, *Spironucleus vortens*, provides insights into both its swimming characteristics and a means for its immobilization. The progress of movement of this organism through the solidified growth medium was monitored by the *in situ* reductive production of a formazan chromophore from a dissolved tetrazolium salt. The physical properties of the gels were measured using an Anton Paar rheometer. The test parameters and measurements included: angular frequency, complex viscosity, complex shear modulus, shear rate and rotational recovery. These rheological characteristics affected the forward velocity of the organism through the gels, during and after multiple resetting, information potentially useful for determination of the dynamic characteristics of flagellar movement and propulsion rates of the organism. Application to separation of single cells, individuals of distinct sizes or the differing species from mixed cultures of motile and non-motile organisms or less actively swimming species was evident. These applications can be used when isolating the parasite from the intestinal contents of its host or from faecal pellets.

INTRODUCTION

The use of surfaces of solid media to separate single organisms prior to growth as colonies dates back to 1729, when cut fruit surfaces to grow fungal colonies were used (Micheli, 1729). Gelatin-containing media (Koch, 1881) liquify at 25 °C, and also behave similarly when culturing proteolytic organisms; therefore, they were not as useful as agar-based compositions (Hesse, 1882). That an aqueous solution of agar (1.5 %) ‘melts’ at 85 °C and sets at 39 °C has been routinely employed for laboratory work ever since. Traditional methods obtain surface colonial growth of microorganisms, whereas separation of individuals from mixed populations that include motile species requires alternative techniques. Surfaces of solid agar media have been used to obtain axenic cultures of slowly motile protists and cyanobacteria (Vaara et al., 1979).

A more adaptable method for viability estimates by the counting of colonies awaited the introduction of a growth medium that incorporates thixotropic biopolymers (Hill, 1998; Mewis & Wagner, 2009). Using that technique, environmental samples suitably diluted were shaken vigorously in flat-sided screw-capped bottles with the thixotropic growth medium that incorporated a tetrizolium salt (TN gel). Dispersed coloured colony-forming units were then counted after incubation of the reset gels. Here we apply this procedure to investigate the swimming behaviour of *Spironucleus vortens*, a powerfully motile diplomonad parasite of ornamental aquarium fish (Williams et al., 2011). We measure the rheological properties of dilutions of the standard TN gel to obtain an indication of the forces necessary to restrict motility or immobilize the organism.

METHODS

Organism. *S. vortens* ATCC 50386 was obtained from Professor J. Kulda, Charles University, Prague. Trophozoites were cultured axenically at 22 °C in 15 ml screw-capped Falcon tubes containing 10 ml TYI-S-33 medium composed of (per litre): 20 g yeast extract (Oxoid) 10 g glucose, 2 g NaCl, 2 g L-cysteine HCl, 0.2 g ascorbic acid (Na salt), 1 g K₂HPO₄, 0.6 g KH₂PO₄ and 23 mg ferric ammonium citrate (Millet et al., 2010, 2011). After autoclaving at 15 p.s.i. (~103.5 kPa) for 20 min, 100 ml heat-inactivated newborn calf serum (Difco) was added, and the pH was adjusted to 6.8 with NaOH.

Abbreviation: TN gel, thixotropic nutrient gel.

Four supplementary movies are available with the online Supplementary Material.
Harvesting. Organisms were harvested by centrifugation at 950 r.p.m. (1000 gav.) for 5 min at room temperature in a bench centrifuge (MSE minor) and washed twice in PBS, pH 7.2 (per litre: 8 g NaCl, 0.2 g KCl, 1.44 g Na2HPO4, 0.24 g KH2PO4). Organisms were resuspended in PBS.

Thixotropic nutrient (TN) gel. TN gel was a proprietary product (ECHA Microbiology). Dilutions of this gel were made as indicated using the S. vortens growth medium in 15 ml stoppered tubes. The TN gel was fluidized by vigorous shaking before dilution (e.g. 0.7+7.0 ml=91 % gel or 3.5+7.0 ml=67 % gel), then shaken again to ensure complete mixing; immediate centrifugation at 1000 gav. for 2 min removed all air bubbles.

Rheological measurements. A combination of rheological tests (Merger, 2006) was employed for samples of TN gel and its two diluted samples. The samples were loaded onto the surface of the stationary test plate of a Physica MCR 101 modular compact rheometer (Anton Paar) and allowed to set at 20 °C for 10 min. This device was then used to determine rotational and oscillatory characteristics of a 1 mm layer of gel using the automated preset operation (Loersch et al., 2008), whereby the rotating (or oscillating) upper plate was actuated.

Rheological terminology. 1. Angular frequency, \( \omega = 2\pi \cdot f \), measured in rad s \(^{-1} \), where \( f \) is the frequency (Hz) of the oscillating circular surface of the rheometer tool.

2. Complex viscosity, \( \eta^* = \tau' / \gamma' \), where \( \tau \) and \( \gamma \) are both time-dependent. \( \tau \) measures the shear stress (force/area), and \( \gamma \) is a measure of the shear rate (velocity/depth of the sample). This is measured by oscillatory tests and is calculated as a result of these changing shear conditions.

3. The complex shear modulus \( G^* \) is \( \tau / \gamma \) where \( \tau \) and \( \gamma \) are both time-dependent. \( G^* \) is a measure of the rigidity of the sample. \( \gamma \) is the shear strain (\( \delta \hat{h} \), where \( \delta \) is the extent of the deflection path, and \( \hat{h} \) is the sample depth). The deflection path is created when the sample ‘lags’ behind the tool during oscillation. The more ‘liquid’ a sample, the further it will lag, \( G^* \), and \( G^* \) are the storage and loss moduli, representing the ‘solid’ (elastic) and ‘liquid’ (viscous) portions of a sample, respectively, and are measured in Pascals. Because oscillation occurs through the degrees of the circle over time, sine curves are created by the preset and the resultant values, the difference being referred to as the phase angle (\( \delta \)). This is the lag time between the preset and resulting measurements, either of shear stress or of shear strain. The lag times vary from 0° (for an ideal solid) to 90° (ideal liquid), and thus 0°<\( \delta <90° \). The loss factor (tan \( \delta \)) is the quotient of the lost (\( G^* \)) and stored (\( G^* \)) energy, and thus: tan \( \delta = G^*/G^* \) and \( G^* = (G^*)^2 + (G^*)^2 \), and hence \( G^* = (\tau / \gamma) \cdot \cos \delta \), and \( G^* = (\tau / \gamma) \cdot \sin \delta \).

4. Viscosity (\( \eta \)) describes the flow resistance of a sample when measured by rotational tests where the shear conditions are constant. It is equal to \( \tau / \gamma \) where \( \tau \) is the shear stress (force/area) and \( \gamma \) is the shear rate. Viscosity is measured in Pascal seconds (Pa.s).

Separation of S. vortens from a bacterial contaminant by u-tube. The differential motility of S. vortens and a motile unidentified bacterial contaminant within the culture was tested using the u-tube method. Thixotropic gel was prepared to 127% the strength of normal gel. This gel was diluted whilst still molten with TYI-S-33 medium to 117%. The gel was then immediately transferred to a sterile glass u-tube (approx. 1 cm in diameter) in a 10 ml volume, allowing a 3 cm headspace on either side of the gel. After the gel had set, 1 ml fresh TYI-S-33 medium was added to one end of the tube, whilst 1 ml of the contaminated S. vortens culture was added at the other end. The tube was then incubated in the dark for 24 h at 24 °C. After incubation, a sample was taken from the end of the tube containing fresh medium and viewed microscopically using 10× and 100× objectives for the presence of S. vortens and/or bacteria.

RESULTS

S. vortens has a prolate cylindrical body of between 8 and 14 μm. The six anterior and two posterior flagella all exceed the body length (Paul et al. 2001). Fig. 1 shows the typical pyriform body shape of the trophozoite. Suspended in its growth medium the organism moves extremely rapidly (>50 μm s\(^{-1} \)) with a strong forward propulsive action, and steered mainly by its posterior flagella, the trophozoite makes frequent sudden changes in direction (>2 Hz). The movement also has a sideways oscillatory component, providing its characteristic ‘wriggling’ movement (see Movies S1–4, available in the online Supplementary Material).

As the concentration of TN gel in the tubes was decreased, S. vortens were able to move more freely and travel further. Movement downwards through the gel was observed for 120 h in the least concentrated gels (74 and 66 %), but in the more concentrated gels (100, 93, 87 and 80 %) no progress was observed (Fig. 2). Comparison of the distribution of the red formazan reduction product in situ within S. vortens in gels of decreasing concentrations after 48 h is shown in Fig. 3. Microscopic examination reveals that the organism swims within the more liquid domains of the thixotropic gel, and that deposition of the formazan is localized within the redox-active organelles, the hydrogenosomes (Millet et al., 2013; Williams et al., 2013a). Movies S3 and S4 provide a real-time comparison of S. vortens motility in culture medium (uninterrupted) versus thixotropic gel, respectively, and Movies S1 and S2 show subcellular formazan localization. In the u-tube method, motile S. vortens cells were recovered from the opposite end of the tube after 24 h of incubation. No bacterial cells were apparent at this point, suggesting that the motility of

![Fig. 1. Scanning electron micrograph of Spironucleus vortens from head lesions of discus showing its eight flagella (six anterior, two posterior). Bar, 5 μm. Adapted from Paul & Matthews (2001).](Image 312x103 to 539x248)
S. vortens is greater than that of the unidentified bacterial contaminant through the thixotropic gel.

Fig. 4 compares the storage and loss moduli of the samples, with shear strain as a function of shear stress as determined by the application of three sessions of ‘frequency sweep’ deformation of TN gel and two dilutions of this gel (i.e. 10+1 and 2+1; gel + growth medium). This choice of gel strengths (i.e. dilutions) was based on the observation that S. vortens did not progress through 100% gel (i.e. in the absence of growth medium), but continued to move vertically through 21 mm in 24 h, and 40 mm over a period of 48 h in the most liquid gel diluted with growth medium (Fig. 3). Thus, the $G'$ line (storage modulus), a property of the ‘solid’ component, and the $G''$ plot representing the ‘liquid’ component show the decreasing strength of the gels on increased dilution. The larger the separation between $G'$ and $G''$ plots, the more stable is the state of the gel. The standard gel that has not been deformed exhibits increased stability at lower frequency levels. The two dilutions of the gel exhibit almost identical spacing between their two portions, suggesting that pre-deformed gel has a constant stability over the observation period. This shows that deformation of the gel by protozoa is unlikely to alter the stability of the medium. Furthermore, to move significantly, S. vortens has to overcome the yield value of the gel (shown below in Fig. 6).

Fig. 5 shows the results of the rotational recovery tests carried out on the gels. The samples were loaded as described previously, but rotation was continuous at preset shear rates. It began with a predetermined low shear rate that creates a constant viscosity in the thixotropic samples. In terms of flow behaviour, viscosity is used to describe the ‘thickness’ of a solution. After a constant viscosity was attained, at 89 s (Fig. 5), the shear rate was increased to a higher value, one that is known to completely deform the gel structure so that the viscosity rapidly falls. The shear rate was programmed so that after 60 s it was returned to its original value. The curve created as the viscosity increases and the sample structure begins to recover represents the recovery characteristics of the gel.

The inclination of the curve at the beginning of the recovery period indicates the speed of initial recovery, and the gradual incline following the curve shows the slow approach to initial viscosity. The higher initial recovery ratio from 2 to 20 s of the 10+1 tube was as expected of the more concentrated gel. However, over a longer period (150 s) the weaker gel has a higher recovery ratio by over 3%. This higher recovery ratio could be because: (a) fewer bonds need to be reformed to attain the original strength of gel, or (b) TN gel is known to have many complex characteristics arising from multiple types of molecular interaction, which could become weakened on dilution.

When the gel breaks, it does so progressively (i.e. it is not brittle), and so does not ‘snap’ (Fig. 6). The kinetics of recovery after deformation show that the weaker gel is slower in its initial reconstitution. The more rapidly S. vortens moves forward, with its characteristic sideways oscillatory motion, the less chance the gel has to recover between the cycles of oscillatory movement of the organism.

Fig. 6 compares yield and flow points of the diluted samples to those of the undiluted TN gel, allowed to set on the rheometer stage immediately following its preparation; also shown is the rheology of the undiluted TN
gel pre-deformed and then tested. The structural differences of the gels are clear, as is the decreasing speed of initial recoveries with higher dilutions (shown in Fig. 5). These plots (Fig. 6) indicate the behaviour of the gels as a function of ‘stress’. The amplitude sweeps show the forces needed to begin breaking the gels, and the forces required to completely deform them. These are referred to as the yield and flow points, respectively.

**DISCUSSION**

In the eukaryotic flagellum, the mechanisms of the sliding microtubule interactions between tubulin and dynein ATPase motors, leading to the 9 + 2 flagellar motion and oscillatory switching, are the subject of a rapidly developing literature (Brokaw, 2009; Mohri et al., 2012). Mechano-signalling between the central pair and radial spoke microtubules regulates flagellar beating, providing responsive dynein control (Oda et al., 2014) and also generates mobility and fluid flow (Mitchell, 2007; Ginger et al., 2008). High-speed microscopic imaging (>800 frames s⁻¹) is necessary to resolve different mechanisms of flagellar movement in the planar and rotational swimming of *Giardia lamblia* (Lenaghan et al., 2011, 2013) and *Tritrichomonas foetus* (Lenaghan et al., 2014).

In a thixotropic gel, motility of *S. vortens* became progressively restricted during the rapid setting process: gels of
increased strengths gave decreasing final progressive swimming capabilities, as indicated by the deposition of the formazan indicator (Bovil et al., 1994). During this experiment no quantification of cell numbers was conducted, and therefore parasite growth within the gel is yet to be established. However, comparison of the continuous progression of the parasite through the gel over the duration of the experiment (120 h) versus the short doubling time of the organism (approximately 2 h; Millet et al., 2011) clearly suggests parasite growth, supported by culture medium components present within the gel. As demonstrated here in the u-tube experiment, thixotropic gels with the correct physical characteristics are capable of separating S. vortens from less actively motile bacterial contaminants within the original culture. Thixotropic attributes of gels are modified by pH, cations as well as other solutes and dispersions, and it is possible that the TN gel properties at the gel/inoculum interface were not identical to those determined for the bulk gels. This could affect initial penetration of the S. vortens into the gels, but not its progress through the gels.

The characteristic ‘wriggling’ motility of S. vortens observed through the thixotropic gel has also been observed in vivo in angelfish intestinal tissue and when co-cultured with mammalian intestinal cells (Caco-2 and HT-29) whereby, rather than attaching to the epithelium, the organism ‘wriggles’ between cells (Williams et al., 2013b). This may provide an important insight into the pathogenicity of this organism, and how it is able to penetrate the epithelial barrier during progression to a systemic infection. The organism is well adapted to its parasitic lifestyle, both in its benign stage in the lumen of the fish gut, when in a stressed host it penetrates the endothelial lining to initiate a systemic infection of the bloodstream, and eventually when it invades tissues and organs (Williams et al., 2011, 2013b). The variations in the physical and chemical consistencies encountered require energetic propulsion for the spread of the infecting organism, and effective motility and adaptability are key attributes for its reproductive fitness and pathogenicity.

A final, important potential application is in high-performance fluorescence microscopy for imaging intracellular and extracellular events, as well as investigating interactions between different cell types in real time. An array of different fluorescent probes and antibodies are available which are directed at specific intracellular molecules or structures and can be used in conjunction with microscopy to elucidate key biological pathways which may be important in pathogenicity. Furthermore, a protocol for expression of GFP fusion proteins in S. vortens has been developed (Dawson et al., 2008), but to date has been underused. For such experiments, the thixotropic nature of the gel is particularly advantageous as it would allow recovery of valuable samples by mechanical disruption and resuspension into culture medium. Use of TN gel in the applications listed also extends to other motile protozoan microparasites of medical and veterinary importance and may help elucidate new chemotherapeutic targets.

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