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ARTICLE

Settlement Behavior of Zoospores of *Ulva linza* During Surface Selection Studied by Digital Holographic Microscopy

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Abstract Settlement of the planktonic dispersal stages of marine organisms is the crucial step for the development of marine biofouling. Four-dimensional holographic tracking reveals the mechanism by which algal spores select surfaces suitable for colonization. Quantitative analysis of the three dimensional swimming trajectories of motile spores of a macroalga (*Ulva linza*) in the vicinity of surfaces functionalized with different chemistries reveals that their search strategy and swimming behavior is correlated to the number of settled spores found in spore settlement bioassays conducted over 45 min. The spore motility and exploration behavior can be classified into different motion patterns, with their relative occurrence changing with the surface chemistry. Based on the detailed motility analysis we derived a model for the surface selection and settlement process of *Ulva* zoospores.

1 Introduction

A critical phase during the life cycle of sessile marine organisms is the planktonic dispersal stage, (larvae in the case of invertebrates such as barnacles and hydroids; motile spores in the case of many seaweeds), during which they settle and attach to a surface. Settlement is a selective process involving reversible contact with the substratum through exploratory behavior in response to a range of surface-associated signals. Having selected a suitable surface the attached larvae/spores then undergo metamorphosis to generate the adult organisms. When the immersed surface is man-made, the resulting colonization is a nuisance phenomenon generally referred to as 'biofouling', which causes major financial and environmental problems in various industries. Ships and leisure vessels, membrane filters, heat exchangers, underwater sensors and aquaculture systems are all subject to biofouling, with the consequence for ships of higher fuel consumption (and hence greenhouse gas and soot emissions) and demand for frequent cleaning [1].

Understanding the complex behavioral mechanisms involved in the recruitment of motile propagules to surfaces is fundamental to control the distribution, abundance and dynamics of organisms on hard substrata in the marine environment, and thus in the design of novel 'antifouling' coatings. The motile, quadriflagellated zoospore of *Ulva linza* with a spore body diameter of 4–5 μm has been extensively studied as a model biofouling organism. In order to complete the life cycle, zoospores must locate a surface and settle on it (i.e., permanently attach to it). Previous investigations by video microscopy (2) revealed that spores show complex swimming behaviors as they approach and make contact with surfaces. The attachment process appears to involve an initial, temporary, and reversible phase of varying duration, and is characterized by a rapid "top-like spinning", although in certain circumstances spores may also settle without spinning. For example, video microscopy has recorded settlement i.e., permanent adhesion, of swimming spores against debris

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and previously adhered spores without any surface exploration or spinning (unpublished observations). Final commitment to irreversible and permanent adhesion involves the discharge of a preformed glycoprotein adhesive from cytoplasmic vesicles, as the cell contracts against the surface. The membrane of the sheaths surrounding the flagella are shed into the water, the axonemes are retracted into the settling spore [2, 3]. The attached spore secretes a cell wall and can then germinate to grow into the macroscopic, easily visible green seaweed.

Settlement of zoospores of *U. linza* is 'selective', i.e., the spores respond to physical and chemical surface cues such as wettability [4–7], topography [8–11], and charge [12, 13], resulting in clear settlement preferences and therefore variable surface colonization by the alga. The question arises as to how spores exert this selectivity—for example; does it involve different behavioral responses in terms of swimming patterns, trajectories and velocities? The challenge to generate such understanding is the three dimensional motion of spores at a velocity of $\approx$25 body lengths per second (150 μm/s) [14]. Holography, as first invented by Gabor in 1948 [15], is capable of imaging such fast, three-dimensional trajectories [16–21]. The physical concept of holography allows the capture of the complete information about objects within a given volume by encoding into an interference pattern. With the advent of lasers and recent developments in digital recording using CCD or CMOS technology, hologram acquisition with video frequency has become accessible (schematic setup in Fig. 1). Such hologram sequences of moving microscopic objects (down to submicrometer size) provide four-dimensional information (three spatial coordinates and the temporal coordinate) [16, 22]. In a previous paper [17] the proof of principle that digital in-line holographic microscopy (DIHM) can be used to capture the motion of zoospores of *U. linza*, was demonstrated. The present paper now extends this approach to analyze, qualitatively and quantitatively, the response of zoospores to three different surface chemistries: acid washed glass microscope cover slips (AWG) and glass cover slips coated with polyethylene glycol (PEG) or tridecafluoroctyl-triethoxysilane (FOTS). Previous studies have shown that spores of *U. linza* distinguish between surfaces coated with FOTS and PEG when presented with a 'choice', resulting is substantially greater settlement densities on the hydrophobic fluorinated surface [6].

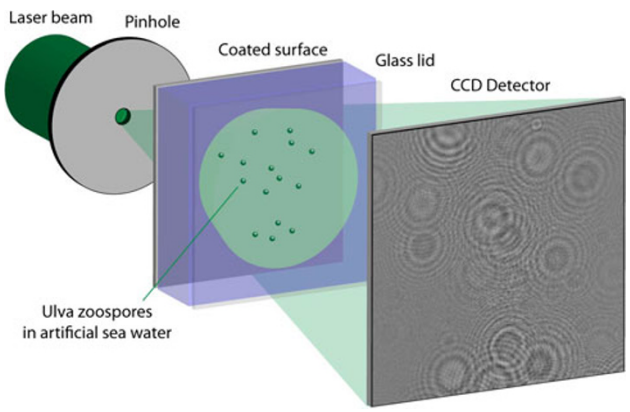

Fig. 1 Schematic setup of the holographic tracking experiment consisting of a pinhole as point source, the sample volume containing the spores and a CCD detector to record the scattering pattern

2 Materials and Methods

2.1 Preparation of Surfaces

The surfaces used to seal the observation chamber and for the motility analysis were either acid washed glass (AWG) cover slips (Carl Roth GmbH) or cover slips coated with FOTS or polyethyleneglycoltriethoxysilane (PEG). FOTS silane was coated on glass cover slips through chemical vapor deposition (CVD) for 2 h at 80 °C [10]. Subsequently samples were rinsed with ethanol (p.A.) and dried with N_2 gas. Polyethylene glycol (PEG) triethoxysilane (PEG2000-urea) was synthesized according to previously published protocols [23]. Prior to coating, glass cover slips were activated by piranha solution (H_2SO_4/H_2O_2 = 3:1). 0.25 mM PEG silane and 2.5 μM triethylamine were dissolved in dried toluene (p.A.). The activated glass cover slips were immersed in this solution and allowed to react for 48 h at 55 °C. Subsequently, the samples were rinsed with ethyl acetate (p.A.) and sonicated in ethyl acetate for 2 min and then rinsed again with ethyl acetate and methanol (p.A.). All surface coatings were characterized by ellipsometry, XPS, and contact angle goniometry. The AWG coverslips were prepared by immersion in 0.1 M HCl for 24 h, before washing extensively in deionised water and blowing dry in a stream of nitrogen. For the standard spore settlement assay, clean-room sealed Nexterion glass slides (Schott) were used as substrate. Slides were reacted with FOTS and PEG as supplied.

2.2 Preparation of zoospore suspension

Fertile plants of *Ulva linza* were collected from the seashore at Llantwit Major, South Wales, UK (51°40′N; 3°48′W) in June 2008, 5 days before full moon. Plants were stored (maximum 48 h) at 4 °C until zoospores were released. Zoospores were released from fertile tips into filtered (0.22 μm) artificial seawater (ASW: Tropic Marin). The spore suspension was filtered into a beaker through 3 layers of nylon mesh (100, 50 and 20 μm) to remove debris. The beaker containing the spore suspension was

plunged into ice, which concentrates the spores (spores swim towards the bottom of the beaker), which were pipetted into another beaker. This procedure was repeated and then the spore suspension was filtered through 2 layers of nylon mesh (20 μm pore size). The spore suspension was kept on a magnetic stirrer and the absorbance at 660 nm measured. The spore suspension was diluted with filtered (0.22 μm) ASW to a final concentration of 1.3×10^4 spores/ml for holography, or 1.0×10^6 spores/ml for standard settlement assays. As only one holographic setup was available, the spores used for the experiments were released at different times. For the FOTS surfaces spores were released 7 h after collection from the seashore, for PEG 21 h and for AWG 30.5 h. One dataset obtained on each of the respective surfaces has been analysed. While the release time after collection did not have a major influence on the observed motion patterns, we noted that the mean velocity of the spores decreased over time.

For the standard assay, 10 ml of freshly released spores were added to individual compartments of sterile Quadriperm dishes each containing a test surface. Three replicates of each test sample were immersed simultaneously. The slides were incubated in darkness for 45 min and then washed gently with ASW to remove unsettled i.e., motile, spores. The three replicates were used to determine the number of settled (attached) spores. Spores were fixed in 2.5 % glutaraldehyde in ASW, washed in deionized water and dried. Spore counts were taken using a Kontron 3000 image analysis system attached to a Zeiss epifluorescence microscope. Spores were visualized by autofluorescence of chlorophyll and counts were recorded for 30 fields of view on each replicate slide as described by Callow et al. [24].

2.3 Digital In-Line Holographic Microscopy

An in-line holographic microscope as developed by the Kreuzer group [16, 22, 25] was used, consisting of a light source, a pinhole, a wet cell and a detector, all arranged on the same optical axis. For illumination a diode pumped solid state laser providing continuous wave (cw) light with a wavelength of 532 nm with a power of 30 mW was used (IMM Messtechnologie, model GLML4C1-30). A commercial (National Apertures) pinhole with a diameter of 500 nm was used to generate the divergent wave front. The detector was an OEM CCD module (Lumenera Lu160M) with a resolution of 1,392 × 1,040 pixels (pixel size 6.45 μm) and a frame rate of 15.4 Hz located 16 mm behind the pinhole. The wet cell had a volume of 50 μl and was made out of Teflon® and sealed with the surfaces of interest. From the recorded holograms, the wavefront was back-propagated through the complete volume via a Kirchhoff-Helmholtz transformation [22]. From such reconstructions, different projections in real space were calculated and spore trajectories were extracted as described previously [14]. For each of the three surfaces, holographic movies at three different time points (immediately and up to 13 min after injections) with a typical duration of 40 s were analyzed. The full data set consisted of 414 spore trajectories containing a total of 39,650 spore positions and correspondingly velocity vectors.

3 Results

Hologram series of zoospores during their exploration of these three surfaces were recorded over a period of 10 min. After reconstruction of three 40 s sequences for each surface, the trajectories were extracted as described previously [14] and used to quantitatively characterize spore motility. All 414 analyzed trajectories are shown in Fig. 2. As we described recently, the divergent nature of the wavefront used for imaging leads to a change of the field of view which increases from the surface into the volume, which is the reason that the volume in which trajectories are acquired appears slightly compressed towards the bottom [14]. The field of view in the surface plane is slightly different as the pinhole-surface distance was slightly

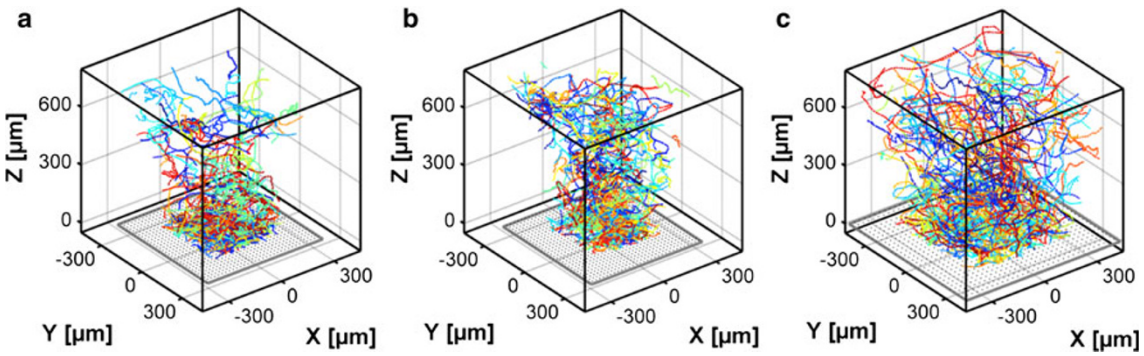

Fig. 2 3D rendered spore trajectories of zoospores in the vicinity of **a** polyethylene glycol coatings (PEG), **b** acid washed glass (AWG) and **c** tridecafluoroctyl-triethoxysilane (FOTS) coated glass surfaces

different in the three data sets. Within the first 2 min from injection, spores were rather homogeneously distributed in the bulk solution and close to the surface. From the analyzed spore positions their velocities can be calculated as the fraction of the distance between two subsequent data points and the time between the two positions. Figure 3a shows the mean velocities at different distances from the surface. The bulk velocity close to the surface was found to be reduced for the fluorinated surface. As only one holograph was available, the experiments on the 3 different surfaces were done at different times after collection of seaweed, each for a total duration of 1 h and multiple replicates were measured. Although freshly released spores were used for each surface, the spores used for the last two assays (glass and PEG) were released up to 24 h later than the spores for the first experiment with FOTS. The overall velocity of the later preparations was decreased by ≈20 %, which may reflect the fact that the seaweed from which the spores were released had been stored for longer. However, this is not a concern for the present study since it is only the change in velocity in relation to different surfaces rather than absolute velocity that is of interest. In all three curves (Fig. 3a) the velocity of the spores was reduced close to the surface; however, this was most strongly seen with the hydrophobic FOTS coating. The calculated deceleration of spores in close proximity (0–30 μm) to the surface with respect to the mean velocity in bulk water is shown as a bar diagram in Fig. 3b. Since in particular on the attractive surfaces, longer surface contacts occasionally occur, the velocity distribution analysis was restricted to freely swimming spores. When exploring a PEG surface, the velocity barely changed (only by 19 %), while at the AWG surface the velocity was reduced by nearly 39 % compared to the bulk velocity. The FOTS surface had the strongest influence on spore velocity and decelerated the spores by nearly 70 %. The increasing deceleration of the spores in the order FOTS > AWG > PEG indicates a stronger initial surface interaction between the spores and the hydrophobic FOTS surface as compared to the PEG coating.

The result of a standard spore settlement assay (after 45 min exposure to a zoospore solution) [24] is shown in Fig. 3c. In line with previous studies, FOTS showed the highest amount of settlement after 45 min and glass (AWG) an intermediate coverage of zoospores, while the PEG coated surface had no settled spores [6, 26]. These results positively correlate with the deceleration of the spores Fig. 3b, i.e., the most 'attractive' surface for settlement showed the greatest spore deceleration.

In addition to the velocity reduction it was observed that spores behaved differently on the three surfaces and responded to the variation in surface chemistry by exhibiting different swimming patterns. Figure 4 shows sample traces for the observed patterns (a) and their schematic

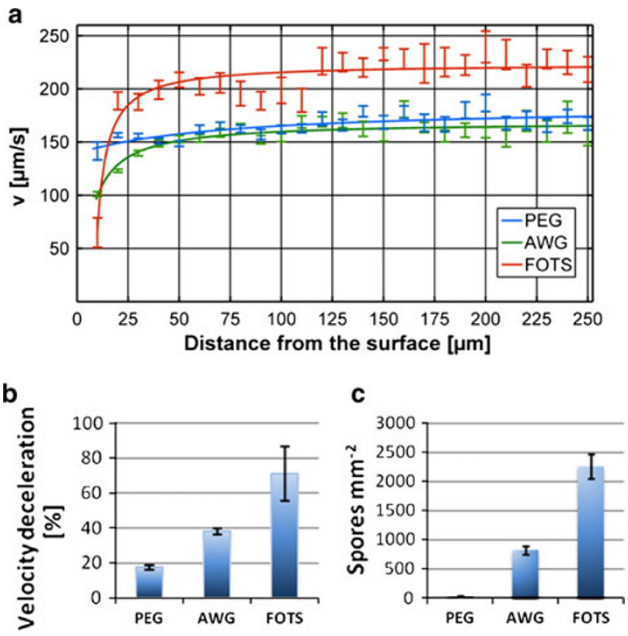

Fig. 3 Interaction of zoospores with polyethylene glycol coating (PEG), acid washed glass (AWG) and tridecafluoroctyl-triethoxysilane (FOTS) coated surfaces. **a** Mean spore velocity during the first 2 min after spore injection at different distances above the surface. **b** Velocity deceleration of exploring spores in close proximity (0–30 μm) of the surface as compared to the speed in the bulk water. **c** Number of spores settled on the surface in a standard assay after 45 min. The glass samples used in this assay were Nexterion glass

representations (b). Some of the traces were previously already correlated to behavioral patterns observed for the brown alga *Hincksia irregularis* by the Amsler group [17, 27]. We use some of the terms to underline the correlation to our data, but also introduced new patterns to account for the behavior observed for spores of *U. linza*. *Pattern 1* is an example of the *orientation* pattern, which occurred in solution with a mean velocity of ≈150 μm/s and involved occasional changes between straight swimming and turning. *Pattern 2* (*'wobbling'*) is characterized by frequent directional changes and a slower velocity of around 50 μm/s. *Pattern 3* shows the *gyration* motion, which is similar to the orientation pattern in solution, but occasionally surface contacts were established. The *gyration* motion can be subdivided into two extreme cases of motion: *hit and run* (*pattern 4*), describes a single surface contact after which the spores immediately left the surface; *hit and stick* (*pattern 5*) describes the situation whereby, as soon as spores contacted the surface, they immediately stopped swimming and stuck to the surface. It is important to note that these 'stuck' spores had stopped moving, but they had not yet committed to permanent settlement as they had neither secreted any permanent adhesive, nor retracted their flagella. Irrespective of whether spores got stuck on the surface as a result of *hit and stick* motion or *gyration*,

soon after the surface contact a *spinning* motion (*pattern 6)* started. This motion involved rapid spinning of the spores around a temporary anchoring point on the surface. This spinning motion could take up to several minutes, but most spores left the surface soon after spinning was initiated and continued exploration. A minority of spores spun for a longer time and finally settled permanently, which involves secretion of adhesive and retraction of the flagella [2, 3].

Figure 4c–e shows the occurrence of the different motion patterns depending on the surface chemistry as they occurred in the volume extending 200 μm away from the surface. For the distributions only the three earliest time points immediately after injection were investigated (≈60 trajectories with a total of ≈5,500 data points). The probability of observing the different motion patterns differed between the surfaces. *Gyration* was detected as the dominant pattern on PEG and on AWG (Fig. 4c, d), which means that spores explored the surfaces and established temporary surface contacts. However, on PEG the probability of observing a *hit and run* event was nearly twice as high (44 %) compared to AWG (23 %), indicating that the PEG surfaces were less attractive to the spores. The situation was different on FOTS and spores exploring the surface showed predominantly the *hit and stick* behavior (Fig. 4e). A *hit and stick* pattern never occurred on PEG and AWG. The high probability of observing a *hit and stick* pattern indicated that the pristine and hydrophobic fluorinated surface attracted spores.

Figure 5 shows the evolution of pattern distribution over time. The earliest time points resemble the same distribution as represented in (Fig. 4c–e. For PEG (a) and Glass (b) no major changes were observed with increasing time. On PEG nearly all spores swam in exploration motions in the categories *gyration* and *hit and run*. No *hit and stick* or *spinning* was observed even at later time points and the high probability of 42 % of observing a *hit and run* event suggests that the surface maintained its repellent nature. For the intermediately attractive AWG surface, the *gyration* pattern dominated (≈70 %) while it was less likely to observe a hit and run motion (≈20 %). The occurrence of *gyration* and *hit and run* remained nearly unchanged over time. However, as settlement continued, increasing numbers of spinning spores could be found (up to 11 % at the last time point). On FOTS an even higher occurrence of spinning spores (up to ≈30 %) was found, which correlates with the nearly tripled number of spores counted in the standard spore assay (Fig. 3c). Noteworthy on the fluorinated chemistry is the change in occurrence of the different motion patterns. The occurrence of the *hit and stick* pattern rapidly decreased and instead *gyration* was observed. Already after 7 min, the probability of finding gyrating spores was similar compared to glass while the occurrence of the *hit and stick* pattern dropped nearly to zero.

The disappearance of the *hit and stick* pattern on FOTS after only 7 min and the very similar occurrence of the different motion patterns on FOTS and AWG suggests that the different spore numbers after a conventional 45 min settlement assay cannot be a consequence only of the initial sticking events. The probabilities of observing *gyration* motions after several minutes were similar on both surfaces and the lack of *hit and stick* implies that the number of spores sticking to the surface ("on-rate") is similar. However it can be seen in Fig. 5 that the percentage of spinning spores at the later time points on FOTS (Fig. 5c) was much higher than on glass (Fig. 5b). Therefore we analyzed the duration of the spinning phase in greater detail. Figure 6 shows the distribution of the durations of

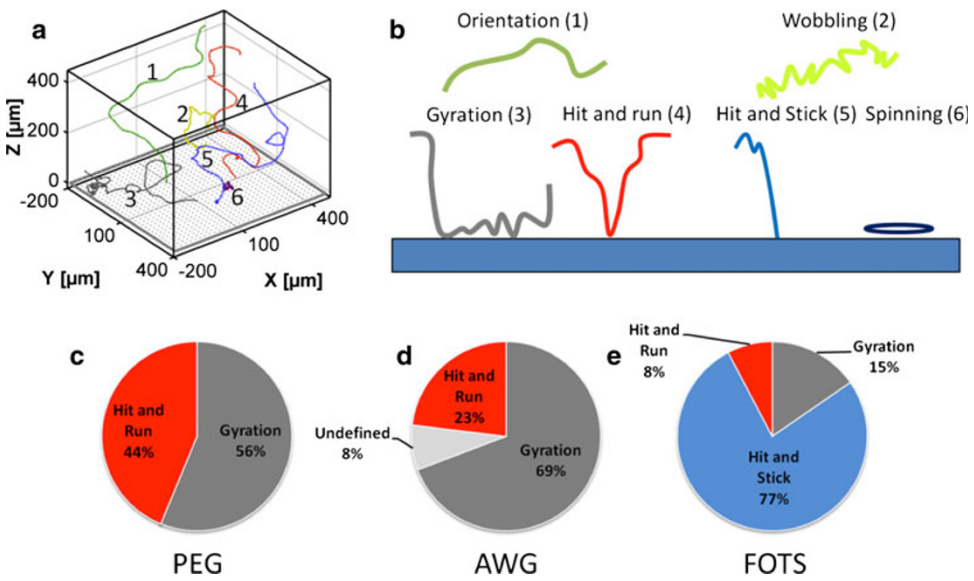

Fig. 4 Analysis of motility patterns: **a** Classification of motion patterns for the movement in solution [*orientation* (*1*) and *wobbling* (*2*)] and in vicinity to the surface [*gyration* (*3*), *hit and run* (*4*), *hit and stick* (*5*) and *spinning* (*6*)]. **b** Schematic sketch of the motion patterns. **c–e** Occurrence of motion patterns in ≈40 s recordings (≈60 traces) immediately after injection on the three different surfaces PEG, AWG, and FOTS, respectively

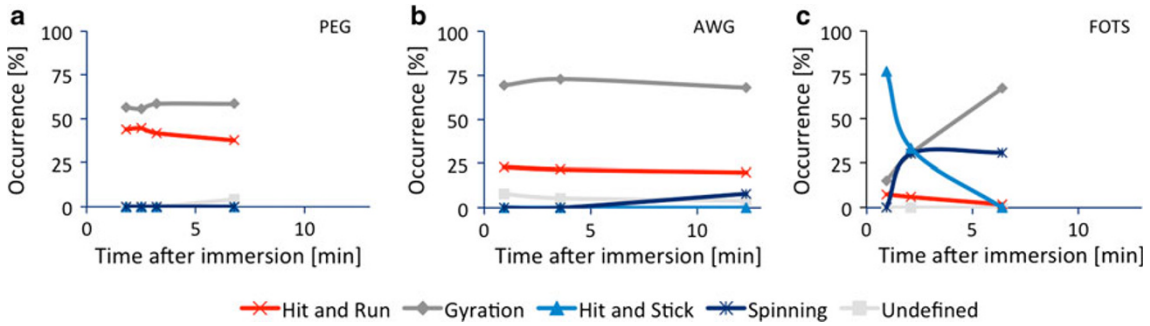

Fig. 5 Occurrence of the different surface exploration motion patterns (in %) **a** on PEG, **b** on AWG, **c** on FOTS for different times after injecting the spore solution

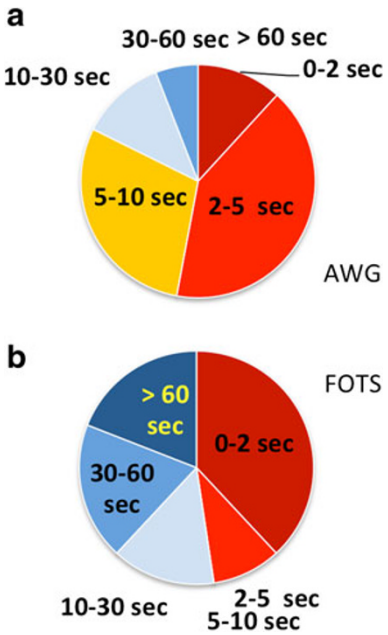

Fig. 6 Distribution of the duration of the *spinning* phase of individual spores on AWG (**a**) and FOTS (**b**). All investigated time points have been used for analysis

the spinning phase and it turns out that *spinning* on AWG mostly lasted seconds while *spinning* on FOTS often took minutes. On AWG, all spores left the surface during the spinning phase. On FOTS, 90 % of the spores left the surface during the spinning phase while 10 % of the spores were spinning for a longer time and only 2 % finally settled permanently. This means that the high percentage of *spinning* spores ($\approx$30 %) on FOTS was caused by the longer *spinning* duration ($\approx$50 % spin longer than 10 s), which implies a smaller probability of the spores leaving the surface (and thus a smaller "off-rate"). The higher chance of proceeding to permanent adhesion on FOTS correlates with the 45 min settlement data. This is an important mechanistic detail as it suggests that spores do not only rely on passive sensing during swimming close to the surface, but additionally use the spinning motion as method to actively probe surface properties.

4 Discussion

The analysis of spore motility by DHIM, coupled with the information gleaned by video microscopy in earlier investigations [2], leads us to the model (Fig. 7), which illustrates the course of events as spores select a suitable surface for settlement. The first step in the selection process involves swimming of the spore towards the surface (a). By swimming close to the surface (b) spores sense the suitability of a surface for settlement and only if positive cues are sensed does a sufficient deceleration occur leading to temporary adhesion via the apical papilla of the spore (c). Soon after this temporary 'sticking' event, spores initiate a spinning motion (d) that varies in length, depending on the surface chemistry. Video evidence suggests that the spinning event may involve the secretion of a small amount of temporary adhesive as an elastic pad [2], although there is no direct biochemical evidence for the existence of this adhesive. Spinning spores may then either initiate permanent adhesion (e), but the majority of spores leave the surface to continue exploration (f). While video microscopy [2] also revealed the importance of the spinning motion, in rare circumstances spores may settle (for example against previously settled spores or debris) without any observable spinning motion. Especially over longer incubation times it is clear from DHIM that the proportion

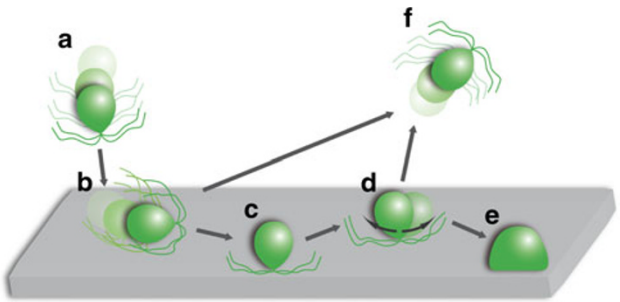

Fig. 7 Schematic representation of the settlement of spores of *U. linza*. **a** approach to the surface, **b** exploration of the surface, **c** initial adhesion, **d** spinning, which may lead to permanent adhesion (**e**) or to spores leaving the surface and continue exploration (**f**)

of spores showing spinning increases, and the duration of their spinning is longer on FOTS than on the less attractive AWG surface. On AWG, the spinning motion is in all cases terminated by the spores leaving the surface again. On FOTS only a very small fraction spun long enough so that permanent commitment to settlement was initiated. It appears as if the spinning motion exerts a force on the temporary surface contact and only if its contact with the surface is strong enough, is permanent settlement initiated. The duration of the spinning phase may thus reflect the strength of the initial temporary bond to the surface. This strategy may be advantageous in nature since it may reduce the likelihood of spores committing to permanent settlement on surfaces to which they adhere weakly, as spores immediately leave such surfaces after initiation of spinning. We propose that spinning is a mechanism used by the spores to probe the ability of the surface to interact with the adhesive and thus used to complement surface selection by exploration behavior.

In summary, insight into the differences in spore settlement density on three different surfaces has been provided by DIHM. Quantitative analysis of DIHM data revealed that the mechanism by which spores select a surface for settlement involves deceleration, followed by a number of different surface probing behaviors, the nature and duration of which vary with the attractiveness of the surface for settlement and permanent adhesion. The spinning phase in particular could serve as a sensing mechanism by which spores probe the surface and predict its ability to bind the permanent adhesive. The exploration behavior of the spores correlates with the final spore coverage after a conventional 45 min settlement assay on the respective surface.

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