

Visualization of flagellar interactions on bacterial carpets

W.R. HESSE & M.J. KIM

Department of Mechanical Engineering and Mechanics, Drexel University, Philadelphia, Pennsylvania, U.S.A.

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Summary

Methods for the in-depth study of the physics of microscale actuation of microfluidics environments by flagellated bacteria ‘teamsters’ have been developed. These methods, which include single and multi-colour fluorescent labelling and electron microscopy allow for the analysis of the effect that individual flagellar filaments have on bacterially driven microstructures, and allow for the investigation of the interaction and coordination of flagellar filaments of neighbouring bacteria on densely packed monolayers of bacteria, ‘bacterial carpets’. We show that the flagella of bacteria that are immobilized on a surface often interact with each other, and that the flagella of these bacteria do not often form multi-flagella bundles that are aligned with the cell body.

Introduction

Flagellated bacteria such as *Salmonella typhimurium*, *Serratia marcescens* and *Escherichia coli* move through their fluidic environment by rotating many flagella filaments (Berg & Anderson, 1973) that are rotated at approximately 100 Hz. The flagella filament is a left-handed helical structure that is approximately 10 µm in length (Namba *et al.*, 1989), 12–25 nm in diameter and made of a single protein, flagellin (Yonekura *et al.*, 2003). The helical diameter and pitch of the ‘normal’ polymorphic form of flagella is about 500 nm and 2.3 µm, respectively (Kamiya *et al.*, 1982). Swarming bacteria, especially *S. marcescens*, have been employed as fluidic mixers (Darnton *et al.*, 2004; Kim & Breuer, 2007a,b, 2008), as actuators in the movement of microscale structures, ‘microbarges’ (Steager *et al.*, 2007; Steager *et al.*, 2008b) and microbeads (Behkam & Sitti, 2007). The swimming behaviour of bacteria, especially *E. coli*, has been extensively studied, with much of this work focused on how flagellar bundles form and come apart in ‘runs’ and ‘tumbles’ as well as

the polymorphic shapes that are produced during bacterial swimming (Turner *et al.*, 2000). Unlike the swimming behaviour, swarming bacteria do not ‘run’ and ‘tumble’, but move constantly forward and the cells collectively form regions of translation and vortical motion, which has been studied with an unlabelled cell technique (Steager *et al.*, 2008a). The swarming behaviour of various bacteria such as *Vibrio parahaemolyticus* (Belas & Colwell, 1982) and *Paenibacillus vortex* (Ingham & Jacob, 2008) have been studied with the scanning electron microscope (SEM), but flagellar interactions of a monolayer of many closely packed bacteria, attached to a common surface has not been shown. Traditionally, the methods of determining the physics behind bacterially actuated microstructures has been with light microscopy, namely phase contrast microscopy (Steager *et al.*, 2008b). This method is suitable for studying how the orientation and distribution of bacteria over a surface controls the surface’s movement, and for the ‘bulk’ movement of the structures, but it does not allow for the study of how the flagella actuate local fluid environment, and how the flagella themselves react under such conditions.

Many methods have been developed for the investigation of bacterial flagella with optical means. Flagella have been visualized with dark-field microscopy (Macnab, 1976), video-enhanced light microscopy (differential interference contrast microscopy) (Block *et al.*, 1991) and fluorescent microscopy (Turner *et al.*, 2000). These methods, however, have not yet been applied to the study of how the flagella of large numbers of coordinated bacteria function.

Fluorescent labelling of flagella has been recently used as a less technically challenging method, as compared to dark-field and differential interference contrast microscopy, to visualize single flagellar filaments on bacteria (Grossart *et al.*, 2000; Turner *et al.*, 2000). This method has also been used to visualize the complex flagella of *Rhizobium lupini* (Scharf, 2002). Fluorescent labelling of flagella has been employed to study flagellar interactions and polymorphic shape change of flagella attached to a single swimming cell (Turner *et al.*, 2000), the torque generated by a cell

(Darnton *et al.*, 2007) and how the flagella align under the influence of an applied flow (Darnton *et al.*, 2004). The method employed by Turner *et al.* (2000) uses fluorescent dyes that have amine-reactive *N*-hydroxysuccinimide (NHS)-esters that form peptide bonds with surface proteins to covalently attach the dye molecules to surface proteins. The method of Grossart *et al.* (2000) was developed to create an easy alternative to staining bacteria with uranyl acetate for imaging flagella with a transmission electron microscope. The concentrated NanoOrange (Invitrogen, Carlsbad, CA, U.S.A.) reagent used in this method binds with the hydrophobic region of proteins. When the dye, which is designed to be non-fluorescent in solution (although in actuality, it does give some background fluorescence since the reagent is used undiluted), binds with protein it becomes fluorescent.

The SEM is able to provide much higher magnifications than fluorescent cell labelling techniques and thus allows for a very high-resolution method of studying the interactions of flagella of neighbouring bacteria on bacterially actuated microstructures. A major obstacle with SEM imaging is that the sample preparation is very critical in order to preserve the bacteria and flagella without artefacts introduced by the sample preparation procedure. The first method of SEM sample preparation of bacteria with the specific intention of imaging the flagella was that of (Matsuguchi *et al.*, 1977), which is largely the basis for SEM sample preparation for imaging bacteria. The disadvantage with this method is that it requires the use of critical point drying (CPD), which is a common, but technically challenging technique for the preparation of biological samples for electron microscopy that protects the morphology and structure of samples as they are dried. Simple air drying damages biological material with the effects of surface tension as the liquid is dried from the specimen. The disadvantage to CPD is that it requires expensive specialized equipment that must be used with proper care. An alternative to this is the use of chemical drying agents such as hexamethyldisilazane (HMDS) as used in our procedure. The use of HMDS does not require any special equipment, other than a chemical hood, and is applied by simply pipetting the liquid over the sample and incubating. The comparison of HMDS to CPD in the preparation of anaerobic biofilms (Araujo *et al.*, 2003) for SEM analysis showed that HMDS was suitable for preserving the delicate structures of the biofilm, but until now, no work has been done to show the preservation of flagella in a process that replaces CPD with HMDS.

In this paper, we present methods for the visualization of bacterial flagellar filaments of *S. typhimurium* and *S. marcescens* for the purpose of studying the interaction of flagella of neighbouring cells on bacterial carpets. We show that fluorescent labelling of cells can be used to study the real-time interaction of flagella, whereas scanning electron microscopy can be used to study the fine details of flagellar interaction at very high magnification. We also show that the flagella of the cells that are part of the carpet do not align with

the major axis of the cell body and form bundles, but rather move and align randomly.

Experimental setup and procedures

Single-colour fluorescent dyeing of cells

Cells of *S. typhimurium* SJW 1103 and *S. marcescens* ATCC 274 (American Type Culture Collection, Manassas, VA, U.S.A.) were labelled with Cy3 amine reactive dye with a slight modification of the method outlined by Turner *et al.* (2000). Cells were grown by adding 100 μ L of a slow thawed glycerol stock of bacteria to 10 mL of Lysogeny broth [1% NaCl, 1% Tryptone, 0.5% yeast extract (w/v)]. The bacteria were incubated at 33°C for 4.5 h with constant shaking. Cells were then harvested by centrifugation for 10–15 min at 2000 \times g to pellet the cells. The pellets were then resuspended by adding 500 μ L of pH 7.0 motility buffer (10 mM M potassium phosphate buffer, 67 mM NaCl, 10^{-4} M ethylenediaminetetraacetic acid) to the centrifuge tube and then gently resuspending the pellet. Once resuspended, 9.5 mL of motility buffer was added and the washing process by centrifugation was repeated twice with pH 7.0 motility buffer and once with pH 7.5 motility buffer. After the final wash, the cells were resuspended to a volume of 200 μ L. Two hundred microliters of pH 7.5 motility buffer was added to one vial of Cy3 dye (GE Healthcare Life Sciences PA23001 Piscataway NJ, USA) and mixed by pipetting the solution a few times. The reconstituted dye and 20 μ L of 1 M NaHCO₃ was added to the suspension of cells and the mixture was mixed by constant shaking in the dark for 90 min at room temperature. The labelled cells were then washed free of unconjugated dye by washing the cells twice with pH 7.0 motility buffer and resuspended to a final volume of 2 mL. For imaging, the cells were diluted 25 times in motility buffer with 0.1 M glucose and 0.002% Tween 20. Cells were imaged by dropping 10 μ L of labelled cells on a 140- μ m-thick #1 slide with or without a cover slide sealed with vacuum grease (Dow Corning high vacuum grease, Midland, MI, U.S.A.) and using a Leica DM-IRB inverted microscope with a 100 \times oil immersion objective and a Leica N2.1 filter cube (Leica, Wetzlar, Germany). A 100 W mercury lamp was used for fluorescent excitation. Images were recorded with the use of a Retiga 4000r CCD camera and QCapturePro software (QImaging, Surrey, BC, Canada).

Cells were also labelled by stained with NanoOrange (Invitrogen) general protein stain with the general method of (Grossart *et al.*, 2000). Briefly, cells were cultured as previously stated and 10 μ L of cells were placed on to a glass slide and 0.5 μ L of NanoOrange undiluted stock solution was added and sealed under a cover slide with vacuum grease. The cells were allowed to stand at room temperature in the dark for 15 min and then imaged. Imaging was done with the same set-up as the cells dyed with Cy3, except a Chroma 41001 filter cube was used in place of the Leica N2.1 filter cube.

Two-colour fluorescent dyeing of cells

Two-colour labelling of cells was done with similar methods to that of the single-colour labelling of cells. First, *S. marcescens* was dyed a single colour with Alexa Fluor 594 (Invitrogen), which is a dye that emits in the red part of the visible spectrum is conjugated on the bacteria as done with the Cy3 in the previous section. Chloramphenicol was then added to stop protein synthesis. This was necessary in order to stop flagellar growth to assure that the flagellar filaments would not be elongated with undyed flagellin protein, which would have created two-colour flagella filaments after the second dyeing process. The bacteria were then labelled again, this time with Alexa Fluor 488 (Invitrogen), which emits in the green part of the visible spectrum. The bacteria were visualized using a Nikon Diaphot 200 inverted microscope with a 60 \times oil immersion objective and a Nikon D-70 digital camera (Nikon, Melville, NY, USA).

Scanning electron microscopy of bacterial carpets

S. marcescens was cultured by inoculating the edge of an agar plate with a saturated culture of bacteria and then incubating at 33 $^{\circ}$ C overnight. Bacterial carpets on microstructures were created by blotting the swarm surface as mentioned in (Steager *et al.*, 2008b). The samples were prepared for imaging by first blotting the microstructures and rinsing with motility buffer (0.01 potassium phosphate buffer pH 7.0, 67 mM NaCl, 10 $^{-4}$ M

EDTA) as done in (Steager *et al.*, 2008b). The monolayer of bacteria was then fixed with 2.5% glutaraldehyde in 0.1 M potassium phosphate buffer pH 7.0 for 2 h. The fixative was replaced every 30 min. The fixing process was followed by rinsing the microstructures in motility buffer three times, each time for 10 min. The rinsed microstructures were then dehydrated with a graded series of acetone (50%, 70%, 90%, 95%, 100% [3 \times]) for 10 min each. The samples were then prepared for drying by treating the microstructures with a series of baths of HMDS. The first bath consisted of two parts acetone/one part HMDS followed by a bath of one part acetone/two parts HMDS and finally two baths of 100% HMDS. The microstructures were incubated in each bath for 15 min. The HMDS treated microstructures were then allowed to air dry and then mounted to aluminium SEM stubs with adhesive tabs and prepared for imaging by sputter coating the sample with Pt/Pd for 10–15 s. The samples were viewed with an FEI XL-30 FE-SEM with an accelerating voltage of 10 kV at various magnifications.

Results and discussion

Fluorescent labelling of cells

Single-coloured cells labelled with Cy3 dye resulted in brightly coloured cell bodies and flagella that were easily discernable (Fig. 1). By contrast, the cells stained with NanoOrange were

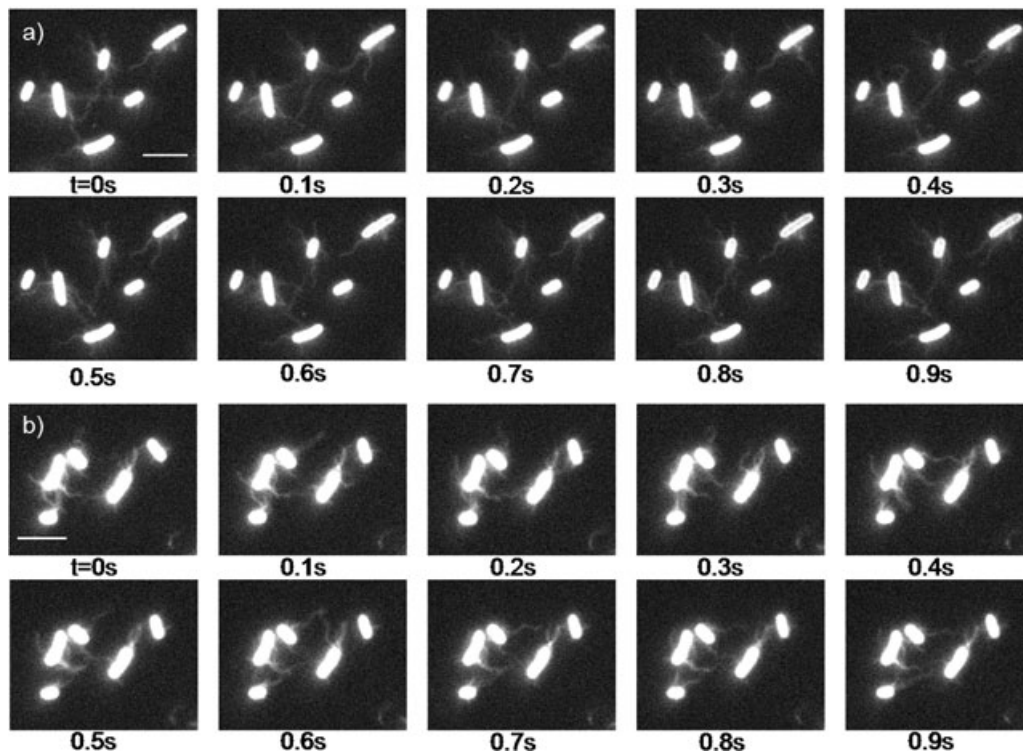


Fig. 1. Sequential images of a bacterial carpet of *S. typhimurium*. The flagella move randomly in the tactically unforced system, and flagella from neighbouring cells can be seen to interact. The scale bar in both (a) and (b) is 5 μ m.

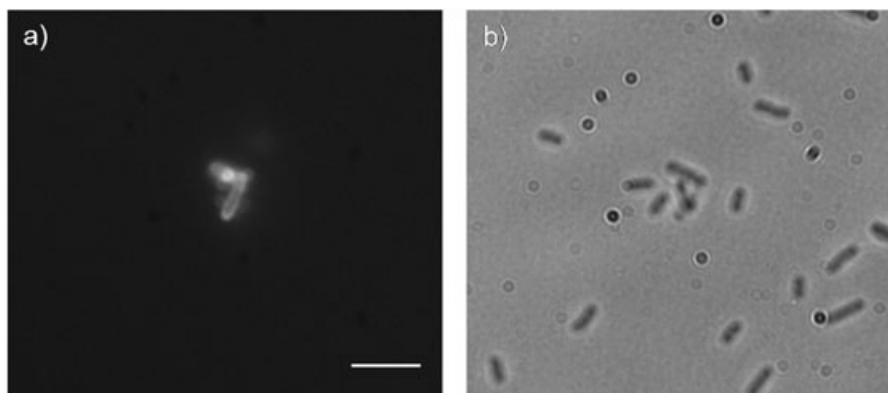


Fig. 2. *S. typhimurium* stained with NanoOrange. (a) fluorescently illuminated cells. (b) same view as (a), but using phase contrast rather than fluorescence. Notice that the bacteria that were fluorescently labelled can be seen in the phase contrast image, but NanoOrange did not label the majority of the cells. The scale bar is 5 μm .

bright, but the flagella were not able to be visualized. This finding was disappointing since the NanoOrange staining process is much quicker than that of the dyes (Cy3 and Alexa Fluor dyes) which rely on the covalent attachment of dye molecules to surface proteins of the bacteria via the formation of peptide bonds between exposed amine groups and the NHS ester of the dye. Also, NanoOrange is mostly non-fluorescent when not attached to protein, thus the removal of unconjugated dye is unnecessary, which also saves time in the process. Extra time for cell staining, as well as an increase in the amount of added NanoOrange reagent was done to improve the results, but these changes did not improve the visibility of flagella, and adding more NanoOrange reagent simply added more background fluorescence. NanoOrange also did not label cells very efficiently (as few as 3% of cells were labelled in some cases), which is evidenced by Fig. 2. When a sample was viewed with phase contrast many more cells could be seen swimming in the field of view than with fluorescence. Many of the cells dyed with either method settled and attached as a monolayer of cells to the glass slide which created an optimal environment for the study of flagellar coordination and interaction on bacterial carpets. It has been reported that there was no discernable correlation between the flow field visualized by fluorescent beads above the bacterial carpet and the orientation of the cells of the carpet (Darnton *et al.*, 2004), which suggested that there are complex flagellar interactions

and coordinations that allow for bacterial carpets to do useful work which can be understood once the flagella are able to be visualized and correlated to the resulting flow field. This theory is strengthened by the evidence found in Table 1. The flagella of closely packed cells in a tactically unforced system move randomly, and do not form ordered bundles that align with the major axis of the cell body, thus the fluid is actuated in a random manner in the vicinity of a particular cell, and has no direct correlation with the orientation of the cell. It was found that 75% of the flagella from cells where the flagella demonstrated directed motion moved in a clockwise fashion. This finding agrees with the model of bacterial movement discussed by (DiLuzio *et al.*, 2005). The cell body rotates clockwise as the flagella rotate counterclockwise (as viewed from the back of the cell) under normal swimming, which makes a cell swim to the right side of a channel when swimming at the bottom of the channel and to the left at the top of the channel. When the cells are immobilized on the glass slide, it is similar to the cell swimming near the floor, thus making the cell try to swim to the right. Since the cell is immobilized and its body cannot rotate, the cell's flagella should make a clockwise motion around the cell body, as seen.

The single-colour fluorescent microscopy method is also suitable for the study of the physics behind the phenomenon of galvanotaxis, the mechanism of which is currently only

Table 1. Comparison of results obtained with the three imaging methods. The error reported for the average number of flagella is the standard deviation. The error for the alignment of flagella and the number of flagellar bundles per cell is the standard deviation of the results obtained from multiple image or movie files.

Method	Flagella per cell	Flagellar bundles per cell	Off axis, alignment (%)
Single-colour fluorescence (<i>S. typhimurium</i>)	2 ± 1	0.3 ± 0.2	88 ± 10
Two-colour fluorescence (<i>S. marcescens</i>)	2 ± 1	0.3 ± 0.1	71 ± 8
SEM (<i>S. marcescens</i>)	7 ± 3	1.8 ± 0.2	79 ± 5

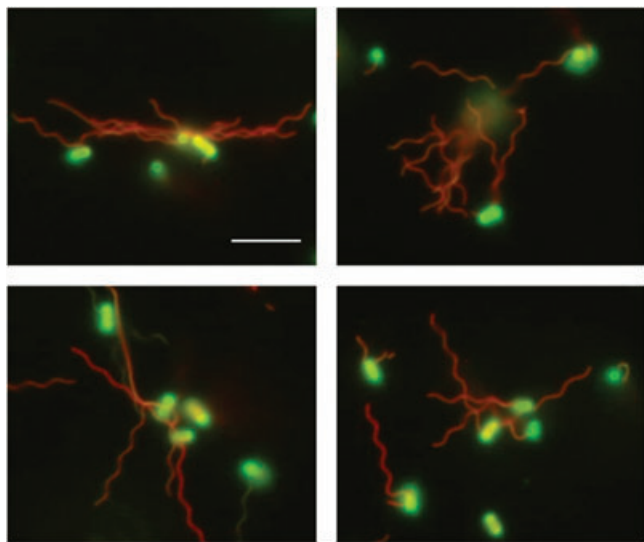


Fig. 3. Two-colour labelling of *S. marcescens* which results in green labelled cell bodies and red labelled flagella. The cells are stuck to the slide to form a bacterial carpet. In each image the flagella of multiple cells are seen to interact. The scale bar represents 5 μm .

hypothesized (Shi *et al.*, 1996). With the single-colour fluorescent labelling method, the true effect of the electric field on the cell body and how the flagella orient can be determined, and the mechanism behind the response can be identified. Since the cells are stuck to the slide, they are not susceptible to the effects of hydrodynamics in the same manner as swimming cells, and electrophoretic effects of the cell body are also eliminated which can be used to determine the effect of an applied electric field on the flagella of bacteria. The alignment of isolated flagella filaments has been reported (Washizu *et al.*, 1992), but the effect of an electric field on flagella that are still attached to cells has not been investigated. Discovery of the mechanism behind galvanotaxis is important due to its possible use as a control methodology for bacterially actuated microstructures.

Two-colour labelling of bacteria resulted in vivid images where cell bodies and flagella can be easily differentiated (Fig. 3). This method was also highly efficient with 85% of the cells displaying the two-colour scheme of green cell bodies and red flagella. The two-colour labelling procedure, which affords all of the benefits of the single-colour labelling procedure, also gives the ability to filter out the cell bodies and leave the flagella visible for analysis of flagellar interaction in very densely packed bacterial carpets, where the interaction might not be clearly visible with single-colour labelling due to the bright cell bodies interfering with the visibility of flagella filaments as they pass over the cell body. Even without any filtering, the flagella and cell bodies are clearly distinct, as seen in Fig. 3. The drawbacks to two-colour labelling versus

single-colour labelling, and thus the reasons why single-colour labelling is still important, is that the process is more time consuming and is twice as expensive as single-colour labelling, since two dyeing procedures are required.

Bacterial carpet preparation for SEM analysis

The flagella of *S. marcescens* were excellently preserved with the use of sample drying with HMDS and air drying (Fig. 4). Table 1 also shows that the preparation of cells for viewing under the electron microscope may be gentler to the cells than the fluorescent drying procedure since the number of flagella per cell was higher for the SEM imaging than the two fluorescent dyeing procedures. A comparison of the results obtained with our procedure to that of (Matsuguchi *et al.*, 1977), which used CPD in the drying of samples, shows that there is very little difference in the morphology of the flagella. An interesting observation of the SEM images of the bacterial carpet on the bottom of the microbaffle was that many adjacent cells had what appear to be bundles of flagella that go between cells and that flagella from adjacent cells intertwine to form separate bundles, which is demonstrated by Fig. 5. The use of the SEM gives much finer detail of the individual flagella filaments than the fluorescent methods, which is due to the filaments not being artificially made to look thicker than they are due to light scattering effects, which explains why more flagellar bundles were identified from the SEM images than from the images of fluorescently dyed cells. Also of interesting note is that raster burn was much less of a problem with the samples prepared with our method rather than with samples that were prepared by simply letting the liquid air dry from the surface.

Conclusion

With the techniques of fluorescent and scanning electron microscopy, the fundamentals regarding the actuation of bacterially driven microstructures can be elucidated. Fluorescent microscopy is useful for the determination of real time flagellar interaction and coordination of bacterial carpets, and how this coordination leads to the movement of microstructures. The fluorescent labelling technique is also useful for mechanistic studies of the various control methodologies, which thus far have largely been studied on the cellular level rather than the flagellar level. Since the ultimate actuation of the fluid is done by the flagella filaments, the knowledge gained from these studies will enhance our understanding of bacterial control behaviours and make these bacterially actuated systems more efficient. The technique of electron microscopy is suitable for studying the fine details of bacterial carpet flagellar interactions, which may be hidden by the light scattering effects of fluorescent microscopy on sub-micrometre structures. The SEM imaging is most important in understanding how the flagella interact in the regions close to the cell body because this region is generally obscured by the brightness of the cell body in fluorescent microscopy.

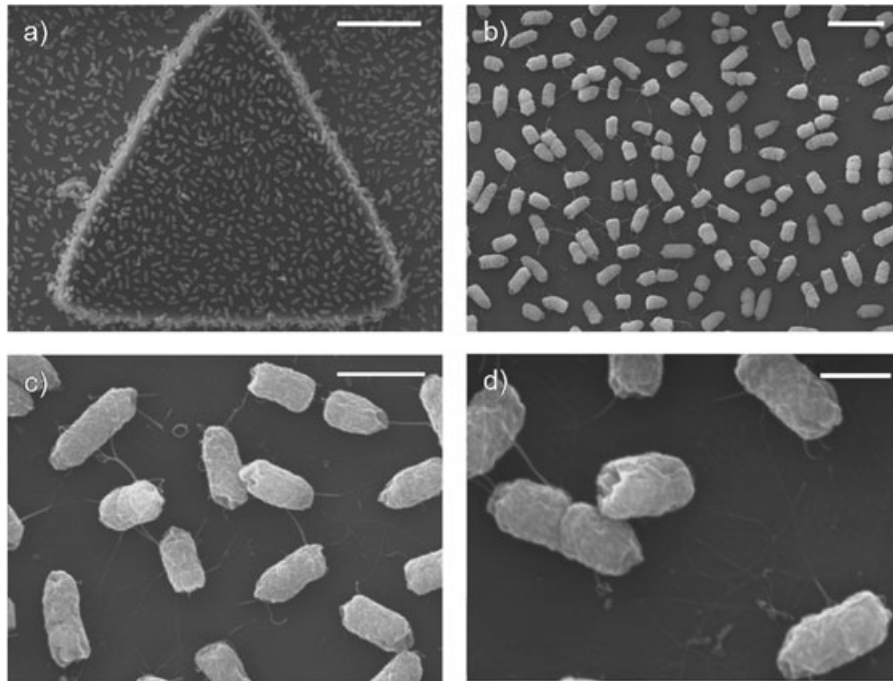


Fig. 4. SEM images of *S. marcescens* attached to an equilateral triangle shaped microbarga. (a)–(d) in order of increasing magnification, 2415 \times , 8000 \times , 25 000 \times , 40 000 \times with scales bars that represent 10 μm , 2 μm , 1 μm , 500 nm, respectively. Bundles of flagella appear to connect cells, and under high magnification [(c) and (d)] bundling of multiple flagella can easily be seen.

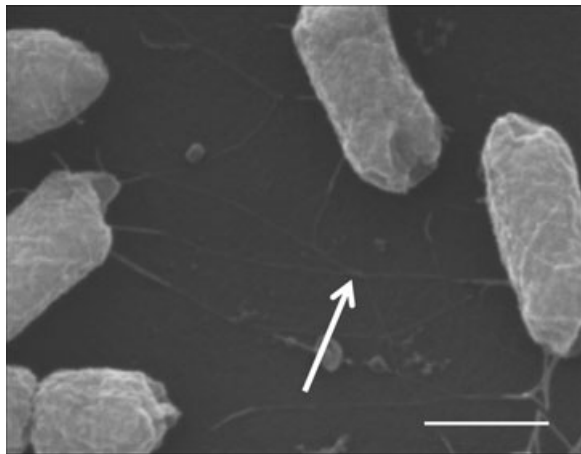


Fig. 5. Enlarged portion of Fig. 4(b), showing an example of the interaction of flagella from adjacent cells. Scale bar is 500 nm.

The use of the SEM also enables multi-scale investigation of bacterial actuation of microstructures through the use of easily and quickly changeable magnifications. The orientation and placement of cells can be directly compared and correlated with the orientation and composition of flagellar bundles in order to determine the interactions (i.e. cellular, flagellar or hydrodynamic) that are important for the ultimate movement of a microstructure in a particular direction with or without rotation.

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