

# A study on twitching motility dynamics in *Ralstonia solanacearum* microcolonies by live imaging

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## Abstract

*Ralstonia solanacearum* is a rod-shaped phytopathogenic bacterium that causes lethal wilt disease in many plants. On solid agar growth medium, in the early hour of the growth of the bacterial colony, the type IV pili-mediated twitching motility, which is important for its virulence and biofilm formation, is prominently observed under the microscope. In this study, we have done a detailed observation of twitching motility in *R. solanacearum* colony. In the beginning, twitching motility in microcolonies was observed as a density-dependent phenomenon that influences the shape and sizes of the microcolonies. No such phenomenon was observed in *Escherichia coli*, where twitching motility is absent. In the early phase of colony growth, twitching motility exhibited by the cells at the peripheral region of the colony was more prominent than the cells towards the centre of the colony. Using a time scale photography and merging those into a video, twitching motility was observed as an intermittent phenomenon that progresses in layers in all directions as finger-like projections at the peripheral region of a bacterial colony. Each layer of bacteria twitches on top of the other and produces a multi-layered film-like appearance. We found that the duration between the emergence of each layer diminishes progressively as the colony becomes older. This study on twitching motility demonstrates distinctly heterogeneity among the cells within a colony regarding their dynamics and the influence of microcolonies on each other regarding colony shape and size.

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## ABSTRACT

*Ralstonia solanacearum* is a rod-shaped phytopathogenic bacterium that causes lethal wilt disease in many plants. On solid agar growth medium, in the early hour of the growth of the bacterial colony, the type IV pili-mediated twitching motility, which is important for its virulence and biofilm formation, is prominently observed under the microscope. In this study, we have done a detailed observation of twitching motility in *R. solanacearum* colony. In the beginning, twitching motility in microcolonies was observed as a density-dependent phenomenon that influences the shape and sizes of the microcolonies. No such phenomenon was observed in *Escherichia coli*, where twitching motility is absent. In the early phase of colony growth, twitching motility exhibited by the cells at the peripheral region of the colony was more prominent than the cells towards the centre of the colony. Using a time scale photography and merging those into a video,

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## KEYWORDS

*Ralstonia solanacearum* , twitching motility, colony morphology, cell density, microcolonies

## ABBREVIATIONS

TFP - Type IV pili, CFU - Colony forming units, BG - Bacto agar-glucose, LB - Luria–Bertani

## 1 | INTRODUCTION

Bacteria have developed a variety of motility mechanisms, which can be categorized into two types: swimming motility in an aqueous medium and twitching motility in a solid medium (Wadhwa *et. al* 2022). Swimming motility is the individual cell movement in an aqueous medium or over a semi-solid surface governed by rotating flagella. Twitching motility is the flagella-independent movement of bacteria in a group over solid surfaces driven by type IV pilus appendages (Liu *et. al* 2001; Corral *et. al* 2020). Lautrop was the first to use the term "twitching motility" in 1961 to refer to the surface movement of *Acinetobacter calcoaceticus* without the use of flagella (Lautrop 1961). The term originates from the discovery that cells moving in this mode of motility appeared as a jerky movement when viewed under the microscope, resembling twitching. Bacterial communities generally follow this mode of motility for rapid colonization on new surfaces under high nutrient availability as well as for the successful formation of biofilm (Ward *et. al* 1997; Ward *et. al* 1999). Several bacteria have been found to exhibit twitching motility among which *Pseudomonas aeruginosa*, *Neisseria gonorrhoeae*, and *Myxococcus xanthus* have been studied extensively (Mattick *et. al* 2002).

*Ralstonia solanacearum* is a Gram-negative,  $\beta$  - proteobacterium, that causes a lethal wilt disease in many host plants (Genin and Boucher, 2002; Phukan *et al.*, 2019; Naik *et al.*, 2023). The bacterium is a soil-borne, systemic phytopathogen but is also known to infect several hosts without causing disease (Phukan *et al.*, 2019; Genin, 2010). It is a species complex armed with a wide array of virulence determinants that allow it to infect over 200 crop species belonging to 53 families (Genin, 2010). It has been observed that twitching motility is one of the important phenomena in this bacterium to colonize inside the host plant as well as disease progression to cause systemic infection (Tans-karsten *et. al* 2001, Corralet. *al* 2020). Out of the several virulence determinants, the TFP besides aiding in twitching motility also mediates diverse processes such as biofilm formation, adhesion, aggregation, horizontal gene transfer and virulence. The TFP are surface-exposed slender appendages that are made of repeating PilA pilin subunits . They are polarly localized in rod-shaped bacteria such as *Pseudomonas aeruginosa* (Talà *et al.* , 2019) and *R. solanacearum* but tend to be peritrichously piliated in cocci-shaped *Neisseria gonorrhoeae* . Albeit executing scores of biological processes, the fundamental mechanism of TFP operations remains unchanged i.e., extension, attachment and retraction. Recent studies on the 'grouped' behaviour of twitching motility on rod-shaped bacteria throw some light on how PilG and PilH aid in bacterial navigation by inducing polarization of the adenosine triphosphatase PilB favouring forward migration and also its reversal upon collision respectively to direct twitching in the response to spatially resolved signals from the TFPs by exercising mechanotaxis .

Although various studies reported about the twitching motility in *R. solanacearum* and the factors associated with it (Kai *et al.*, 2015; Kang *et al.*, 2002; Ray *et al.*, 2015; Singh *et al.*, 2018), its impact on the microcolony shape and size (Bhuyan *et al* 2023) in this bacterium in a density-dependent manner has not been reported yet. More importantly, the dynamics as well as the directionality of twitching motility exhibited by peripheral cells in a colony are yet to be studied adequately. In this manuscript, we have tried to address these questions by observing the twitching motility in the microcolonies through a time-lapse video.

## 2 | MATERIALS AND METHODS

## 2.1 | Chemicals and growth media

Chemicals, bacterial culture medium components, and antibiotics used were procured from Hi-Media, Mumbai, India and SRL, New Mumbai, India. Plastic wares were bought from Tarsons, Kolkata, India; Glasswares were procured from Borosil, Kolkata, India.

## 2.2 | Bacterial strains and growth conditions

*E. coli* DH5 $\alpha$  cells were grown in LB (Hi-Media, Mumbai, India) (contains 1% casein enzymic hydrolysate, 0.5% yeast extract, 1% NaCl) medium at 37 °C. *R. solanacearum* F1C1 was grown in BG medium (Boucher et al., 1985) which contains 1% peptone (Hi-Media, Mumbai, India), 0.1% yeast extract (Hi-Media, Mumbai, India) and 0.1% casamino acids (SRL, New Mumbai, India) supplemented with 5 g/L glucose (Hi-Media, Mumbai, India) at 28 °C. 1.5% agar (Hi-Media, Mumbai, India) was used for preparing solid medium.

## 2.3 | Twitching motility

16  $\pm$  1 h grown *R. solanacearum* F1C1, and 6 h grown *E. coli*DH5 $\alpha$  cells were serially diluted. 5.0  $\mu$ l of this 10<sup>5</sup>CFU ml<sup>-1</sup> concentration was spotted at the centre of their respective nutrient-rich agar plates and incubated at their respective optimal temperatures of growth. The Petri plates containing the *R. solanacearum* F1C1 cells were observed for 10:00-22:20 h of spotting while *E. coli* DH5 $\alpha$  cells were observed within 4:00-8:00 h duration of spotting under Life technologies EVOS FL inverted microscope with 40X magnification using the 4/10 PH condenser annulus.

## 2.4 | Twitching motility rate calculation and preparation of time-lapse video

After 10 h of incubation of *R. solanacearum* strains, the Petri plates containing *R. solanacearum* F1C1 cells were observed for 12 h 20 mins under Life technologies EVOS FL inverted microscope with 40X magnification using the 4/10 PH condenser annulus. During observation, photomicrographs were taken at an interval of every 5 min in JPEG format which was later compiled to create a time-lapse video.

Three random points at the margin of the spotted area were marked and the displacement from these points at each hour was taken and averaged out. The data obtained were plotted on a scatter plot against time to determine the line of best fit that best explains the rate of twitching motility.

## 3 | RESULTS

### 3.1 | Twitching motility influences *R. solanacearum* micro-colony morphology in a density-dependent manner

We studied twitching motility in *R. solanacearum* at different dilutions. Microcolonies of *R. solanacearum* were observed at different dilutions after 16 h. Interestingly we observed that the shape of microcolonies was more like linear shape at higher concentrations ( $\sim 10^5$  CFU ml<sup>-1</sup>) whereas the shape of the microcolonies seems to be circular in shape when the bacterial concentration was low ( $\sim 10^4$  CFU ml<sup>-1</sup>). No such distinct colony morphology variation across different concentrations was observed in the case of *E. coli* where twitching motility is absent (Fig. 1). This suggested that magnitude of twitching motility influences colony morphology in this bacterium. While observing the twitching motility it is obvious that cells at the peripheral region of the spotting area exhibited stronger twitching motility than the cells towards the centre. It might be that bacteria in the peripheral region have dual experiences such as more moisture towards the centre of the spotted area and dryness towards the outward area. It indicated that a more fluidal environment results in lowering the twitching motility.

It is pertinent to note that bacterial cell concentration determines the shape of microcolonies. At higher cell concentrations such as 10<sup>5</sup> CFU ml<sup>-1</sup>, the microcolonies take a slender shape, unlike the lower cell concentrations such as 10<sup>4</sup> CFU ml<sup>-1</sup> where the microcolonies take on a more circular shape (Fig. 1). It seems that at higher cell numbers, individual microcolonies tend to recognize the presence of its adjacent counterparts by quorum sensing and propel to a direction unexplored by other microcolonies attributing it an asymmetric, elongated shape. Besides controlling several other phenotypes across bacterial populations ;

this is a distinct demonstration of quorum sensing affecting the shape of bacterial micro-colonies by piloting asymmetry in the magnitude of twitching motility in all directions. However, the microcolony margins of *E. coli* appear smooth irrespective of their cell concentrations. Therefore, it can be said with conviction that while twitching motility influences microcolony morphology, which in turn is influenced by cell density within a spotted area.

### 3.2 | Dynamics of twitching motility in the peripheral cells

We proceeded to measure the magnitude and directionality of twitching motility in *R. solanacearum*. We observed that these microcolonies grew radially in all directions in successive film-like layers (Fig. 3B). This was evident from the cells exhibiting twitching motility at the edge of the microliter spot. We then studied the dynamics of twitching motility in *R. solanacearum* under 40X magnification by creating a time-lapse video (Supplementary video 1). Twitching motility in *R. solanacearum* F1C1 is generally observed *in vitro* post 12 h of incubation on a nutrient-rich BG media containing 1.5% agar. When bacterial culture is spotted, besides sparse random arrangement of cells within the spotted area, the cells at the border i.e., at the wet-dry interface of the microliter spot area arrange themselves to make a continuous ring-like appearance (Fig. 2A), defining distinctly its perimeter. The cell bodies divide and advance as finger-like projections of individual microcolonies before they ultimately merge into one.

The twitching motility displayed by the bordering cells occurs in a synchronized manner. In the video at the timestamp of 01:03 min and 01:14 min, the twitching motility by the peripheral cells is observed as the emergence of the bacterial cells intermittently from the same region, which gives rise to successive layers. The successive layers of bacterial progression by twitching motility, initiate from the same margin area where the initial layer progression had begun giving the appearance of a multi-layered film (Fig. 2). In a 5.0  $\mu\text{l}$  spot containing 500 cells ( $\sim 10^5$  CFU  $\text{ml}^{-1}$ ) *R. solanacearum*, the formation of the first layer began at around 12:00 h of incubation. Successive formation of layers was initiated at 19:45 h and 21:45 h respectively over the preceding layers from the same point of initiation of the first layer. So, the duration between the emergence of each successive layer of twitching cells is also minimized progressively. Also, each layer of bacterial cells twitches on top of the other (Supplementary video 1).

The pace with which bacteria twitches on solid surfaces is non-uniform making the edges of its colonies uneven. The rate at which twitching motility progresses follows an exponential curve. *R. solanacearum*F1C1 displaces 766  $\mu\text{m}$  in just 22 h (Fig. 2C) (Table 1). However, the displacement is not unidirectional. It occurs in all directions of the bacterial microcolony and at a similar pace (Fig. 3). Twitching motility in *R. solanacearum* F1C1 is not ever continuing. It almost comes to a halt at 7 days post-incubation at room temperature. It has been speculated that at higher cell concentrations when autoinducer levels have reached a threshold, PhcS loses its ability to phosphorylate PhcR, which leads to an upregulated PhcA and a downregulated PehSR. By plotting the displacement of the bacteria against time at every hour we found that the progression of twitching motility in *R. solanacearum* follows an exponential curve. The bacteria were found to migrate from the origin of inoculation by twitching in multi-directions with a displacement of 766  $\mu\text{m}$  within 22 h of incubation (Table 1). Twitching motility was absent in *E. coli*.

## 4 | DISCUSSION

Bacteria have adapted strategies to translocate themselves in solid as well as liquid mediums. In a liquid medium while growth and movement are more of an individual style; but in a colony, the cells translocate as different groups. In this study, we have distinctly demonstrated that while the peripheral cells in a grown-up colony are exhibiting more twitching motility, cells towards the centre of the colony are exhibiting less twitching motility. This heterogeneity and non-uniformity within a colony are prominently observed and presented in this study in the form of twitching motility in *R. solanacearum*. It indicates that within a bacterial colony there exist different niches. The bordering cells joining to make a continuous ring-like structure in the spotted area behave in a synchronized fashion during the twitching motility. The behavior of cells in that region needs further investigation regarding intercellular communication and their synchronization process. There are studies related to different mutation rates within a bacterial colony between peripheral

and central cells because the cells in the former region are considered active in cell division whereas the cells in the latter region are considered non-dividing (Reddy et. al 1997).

Not all bacteria in nature exhibit twitching motility. Twitching motility has been mainly associated with biofilm formation. In *R. solanacearum* colony, the bacteria form films one above the other, which indicates the large number of bacteria that can be accommodated in a given surface area. This is not observed in *Escherichia coli* where twitching motility is absent. A careful observation of microcolonies in different dilutions suggests that microcolonies in a more concentrated culture are more asymmetric than microcolonies in a lower concentrated culture. This happens 16-18 h after spotting the bacterial suspension. This raises an interesting question regarding the sensing of microcolonies to each other and accordingly managing cell division and translocation. A future study on this aspect is likely to reveal interesting aspects of bacterial behavior in the bacterial colony.

Our study on twitching motility dynamics in *R. solanacearum* colony leads to an interesting future perspective to analyze the same in other bacterial colonies such as *Pseudomonas aeruginosa*, *Myxococcus xanthus* and *Neisseria gonorrhoeae*. Exactly the advantage twitching motility provides to the pathogen within a host will be an interesting study in future.

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## CONFLICT OF INTEREST

The authors declare that they do not have any conflict of interest.

## Ethical statement

No human participants and/or animals were used in the study.

**Consent to participate:** Not applicable.

**Consent for publication:** Not applicable.

**Availability of data and material:** Data is available in the article and in the supplementary material.

**Code availability:** Not applicable.

## Author contribution statement

Shuvam Bhuyan, Lukapriya Dutta, Shuhada Begum, Shubhrajyoti Giri and Monika Jain did the experiments and analysed the data; Shuvam Bhuyan and Shuhada Begum did the literature search; Shuvam Bhuyan designed the figures; Manabendra Mandal, and Suvendra Kumar Ray supervised, reviewed, edited.

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**Table 1: Displacement of bacterial cells**

Hours	Displacement ( $\mu\text{m}$ )
<b>10</b>	<b>15.75</b>
<b>12</b>	<b>30.09</b>
<b>14</b>	<b>57.48</b>
<b>16</b>	<b>109.83</b>
<b>18</b>	<b>209.83</b>
<b>20</b>	<b>400.89</b>
<b>22</b>	<b>765.93</b>

## Figure Legends

### Fig. 1. Bacteria of different concentrations at 16hr of incubation

**A.** *R. solanacearum* F1C1 at high ( $\sim 10^5$  CFU ml<sup>-1</sup>) concentrations have slender, finger-like projected microcolonies. **B.** Microcolonies of *R. solanacearum* appear more circular at low ( $\sim 10^4$  CFU ml<sup>-1</sup>) concentrations. **C-D.** Microcolonies of *E. coli* devoid of twitching motility exhibited similar shape at both high and low concentrations.

### Fig. 2. Kinetics of twitching motility

Twitching motility progresses in layers at an exponential rate. **A.** Bacterial cell body position at 10:00 h of incubation. **B.** Displacement of bacterial cells at 22 h post-incubation by forming three overlapping layers. **C.** Rate of twitching motility in *R. solanacearum* F1C1 follows an exponential curve.

**Fig. 3. Twitching motility progresses radially in all directions**

**Figure 1.**

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**Figure 2.**

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**Figure 3.**

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