

Capillary bacterial migration on non-nutritive solid surfaces

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[Received in May 2020; Similarity Check in May 2020; Accepted in August 2020]

Here we describe an additional type of bacterial migration in which bacterial cells migrate vertically across a non-nutritive solid surface carried by capillary forces. Unlike standard motility experiments, these were run on a glass slide inserted into a Falcon tube, partly immersed in a nutrient medium and partly exposed to air. Observations revealed that capillary forces initiated upward cell migration when biofilm was formed at the border between liquid and air. The movement was facilitated by the production of extracellular polymeric substances (EPS). This motility differs from earlier described swarming, twitching, gliding, or surfing, although these types of movements are not excluded. We therefore propose to call it “capillary movement of biofilm”. This phenomenon may be an ecologically important mode of bacterial motility on solid surfaces.

KEY WORDS: *Acinetobacter*; air/liquid interface; *Bacillus*; biofilm; microscopy; *Pseudomonas*; *Staphylococcus*; surface motility

Bacterial motility was first described by Leeuwenhoek (1, 2), and many types of bacterial movement have since been described and characterised in detail. In liquid media, bacteria move constantly by either active use of their flagella (including axial filaments) or passive use of Brownian motion in a fluid (3, 4). Even in a structure attached to a solid surface – known as a biofilm – their cells are not as immobile as one might think.

Several types of bacterial movement across solid surfaces have been characterised in detail. These mostly fall into three categories: *swarming*, *twitching*, and *gliding* (5, 6). *Swarming* is a rapid movement over a surface powered by rotating flagella and is characteristic of bacteria such as *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Salmonella enterica*, and *Proteus mirabilis* (7). *Twitching* is a surface movement of bacterial cells by extension, attachment to a surface, and retraction of their type IV pili. It has been described in bacteria such as *Neisseria gonorrhoeae*, *P. aeruginosa*, *Clostridium perfringens*, or *Acinetobacter baumannii* (6, 8). *Gliding* is movement over a solid surface that does not involve flagella or pili (9). Another type of movement, termed *sliding*, is powered by the pushing force of dividing cells and by additional factors such as biosurfactant and exopolysaccharide production that facilitate the expansion of the bacterial colonies over surfaces (10). Typical “gliders” are *Myxococcus xanthus* or

Flavobacterium johnsoniae, while “sliders” include *Serratia marcescens*, *B. subtilis* or *P. aeruginosa* (10). There is yet another type of collective movement, termed *surfing*, in which bacteria surf on biosurfactants they produce (11, 12). Some bacteria are capable of several types of movement, depending on the surface and environment.

Here, we describe an additional type of bacterial motility, in which bacterial cells migrate from biofilm up across non-nutritive solid surface carried by capillary forces. As soon as we noticed this phenomenon and browsed available literature, we found that this type of motility had not been described before.

The aim of this study was to prove the concept, that is, that bacteria can also move on solid non-nutritive surfaces through capillary action.

MATERIALS AND METHODS

To that end and to further investigate the phenomenon, we ran experiments on glass slides partly immersed in liquid and partly exposed to air (Figure 1), on which we grew an array of bacterial species that differ significantly in physiology, morphology, and motility. One group uses the flagella to move, namely *Bacillus cereus* (4080 LBK), *Bacillus thuringiensis* subsp. *israelensis* (BTI), and *Pseudomonas aeruginosa* (DSM 15680), while the other has no flagella, namely *Acinetobacter junii* (DSM 1532), *A. baumannii* (ATCC 19606), and *Staphylococcus aureus*

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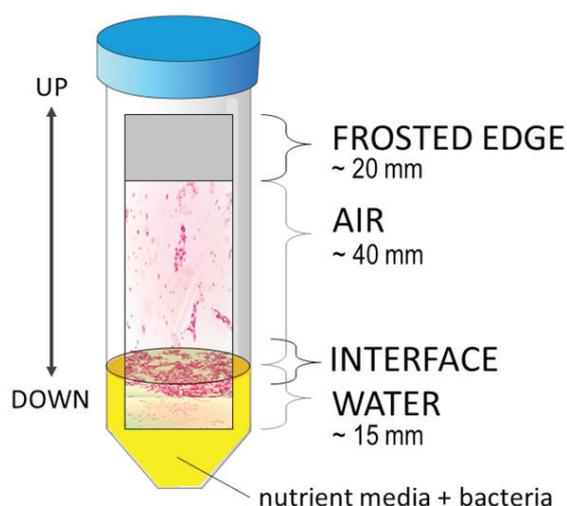


Figure 1 Experimental setup for growing bacterial biofilms on an air/liquid interface

(ATCC 25923). All are capable of aerobic respiration and were subjected to the same experimental setup.

Pure cultures were kept in a Microbank™ system (Pro-Lab Diagnostics, Richmond Hill, Canada) and re-cultivated on Luria-Bertani (LB) agar (tryptone 10 g, yeast extract 5 g, NaCl 5 g, Agar 20 g, deionised water 1000 mL, pH=7±0.2) at 37 °C for 16 h before the experiments started.

Followed suspension of two 10 µL loops of biomass in 10 mL of sterile saline (0.3% NaCl) in 15 mL Falcon tubes. To obtain homogenous suspension, the tubes were vortexed at 45 Hz for 1 min (Kartell Labware, Milano, Italy). Suspension concentrations were ~10⁸ colony forming units per one mL (CFU/mL) of suspension. The CFUs were counted on plates, as follows: 1 mL of suspension was serially diluted in 9 mL of sterile 0.3% NaCl, 0.1 mL dilutions were then inoculated on LB agar, spread with a sterile L-shaped cell spreader, incubated at 37 °C for 24 h, and counted.

Biofilm growth on glass slide

Biofilms were grown on glass microscope slides (75×25 mm, VWR International, Leuven, Belgium) as follows: 1 mL of bacterial suspension (~10⁸ CFU/mL) was

inoculated in 10 mL of LB medium in 50 mL Falcon tubes. Sterilised slides (autoclaved at 121 °C for 20 min) were then vertically inserted in the tubes so that they were partly immersed in the inoculated medium, while the rest was exposed to air (Figure 1). The tubes were lightly capped to let in air and incubated at 37 °C for seven days. Two incubation settings were applied: i) tubes were gently shaken at 50 rpm on an orbital shaker in a vertical position, or ii) tubes were not shaken to exclude the possibility of bacterial cell migration via aerosols.

As soon as we obtained initial results, we ran a new set of experiments described above with *B. cereus* and *A. baumannii* to clarify our findings (Table 1). We selected these two strains as they exhibited the strongest biofilm growth and cell migration in the main experiment.

Additionally, we immersed glass slides in LB medium enriched with 1 mL of carbol-fuchsin solution (Biognost, Croatia) to assess capillary forces by monitoring if and to what extent the nutrient medium would move up the glass slide over the same period of incubation (biofilm growth). To do that, we prepared a different slide for every time point.

Monitoring biofilm growth

After incubation at specified time points, the slides were removed from the tubes and gently washed with sterile saline to remove unattached or loosely attached cells. The bottom of each slide (resting on the microscope stage) was wiped off with a paper cloth soaked in 70 % ethanol, and the top side was prepared for microscopy. The slides were heat-fixed, treated with Alcian blue solution [1 g of Alcian blue (Fluka Analytical, Munich, Germany) dissolved in 10 mL of ethanol and 90 mL of deionised water] for 2 min to visualise biofilm extracellular polymeric substances (EPS), washed with tap water, and then treated with carbol-fuchsin solution for 1 min to visualise bacterial cells, washed with tap water, and viewed with an Olympus CX21 (Tokyo, Japan) light microscope under 1000× magnification. Images were taken with a 5-megapixel mobile phone camera (Samsung Galaxy J1, Seoul, South Korea). Biofilm growth was monitored on the liquid section (that was immersed in

Table 1 Experimental combinations performed in order to explain the migration of bacterial cells up the glass slide

	Biofilm growth (days)	<i>B. cereus</i>		<i>A. baumannii</i>		Carbol-fuchsin
		10 ³ CFU/mL	10 ⁷ CFU/mL	10 ³ CFU/mL	10 ⁷ CFU/mL	
LB medium	1	Exp 1	Exp 2	Exp 17	Exp 18	Exp 33
	3	Exp 3	Exp 4	Exp 19	Exp 20	Exp 34
	7	Exp 5	Exp 6	Exp 21	Exp 22	Exp 35
	10	Exp 7	Exp 8	Exp 23	Exp 24	Exp 36
Sterile saline (0.3 %)	1	Exp 9	Exp 10	Exp 25	Exp 26	Exp 37
	3	Exp 11	Exp 12	Exp 27	Exp 28	Exp 38
	7	Exp 13	Exp 14	Exp 29	Exp 30	Exp 39
	10	Exp 15	Exp 16	Exp 31	Exp 32	Exp 40

the nutrient medium), at the liquid/air “interface” (border), and on the air-exposed (non-immersed) section. Each experiment was done in triplicate. Since the aim of this “proof-of-concept” study was to prove a new type of bacterial motility, the results are described qualitatively, without quantifying biofilm biomass.

Confocal microscopy

It was challenging to obtain good images of *P. aeruginosa* biofilms with classical microscopy due to the vast amounts of EPS produced by the biofilm covering the cells. Instead, we opted for confocal microscopy, which enables viewing and imaging large biomasses, the EPS, and the whole biofilm exhibiting noticeable auto-fluorescence. Confocal images were acquired with a Zeiss LSM880 (Carl Zeiss AG, Oberkochen, Germany) plan apochromatic 20x/0.8 dry objective, and parameters set to avoid pixel intensity saturation and ensure Nyquist sampling in 3440 x 3440 resolution. Bright-field images were acquired with properly calibrated differential interference contrast optics. Autofluorescence of *P. aeruginosa* was excited with 405 nm lasers, and emission was recorded in the range of 410–525 nm.

Swarming and twitching assay

Swarming and twitching surface motility was assessed to check for other types of motility in our bacterial species. We followed the method described by Antunes et al. (8) using Luria-Bertani medium containing 0.5 % agarose. An overnight bacterial culture was suspended in 1mL PBS. With a pipette tip, 10 µL of the bacterial suspension was inoculated to the bottom of the polystyrene Petri dish, tightly wrapped in parafilm to prevent the loss of moisture, and incubated in a humid atmosphere at 37 °C/24 h. Swarming motility was determined at the air-agarose layer while

twitching motility was determined after the removal of the agarose layer and staining the Petri dish with 0.5% crystal violet for 10 min. The longest diameter of the motility was measured. Isolates were grouped into categories based on the average values of motility: < 25 mm poor; 25–50 mm intermediate; > 50 mm highly motile isolates.

RESULTS

Our experiments showed that biofilm formation of all tested bacteria was the strongest at the air/liquid interface and characterised by massive biomass (Figures 2 and 3). All the bacteria also migrated from the biofilm at the air/liquid interface to the clean glass surface. Shaking did not contribute to bacterial presence in the air-exposed zone, as both shaking and non-shaking tests produced practically the same results.

Cells of all the tested bacteria were observed all over the air-exposed section, reaching distances of ~35 mm above the interface in experiments with *B. cereus* (Figure 4A), *A. junii* (Figure 4B), and *A. baumannii* (Figure 5), ~30 mm with *P. aeruginosa* (Figure 6) and *B. thuringiensis* (Figure 7), and ~25 mm with *S. aureus* (Figure 4C).

The air-exposed section of *B. cereus* slides clearly showed that biofilm mostly moved up in a monolayer firmly attached to the surface. When the cells reached a certain position, they sporulated, leaving characteristic microcolonies of endospores engulfed with the EPS matrix (Figures 4A and 8). This can be seen even better with confocal microscopy images of damp slides, which were not heat-fixed. EPS seemed to concentrate around microcolonies that were in the later phase of sporulation and further away from the interface (Figure 7A and B). *B. thuringiensis* produced identical microcolonies as *B. cereus* – endospores “glued” with EPS (Figure 7C).

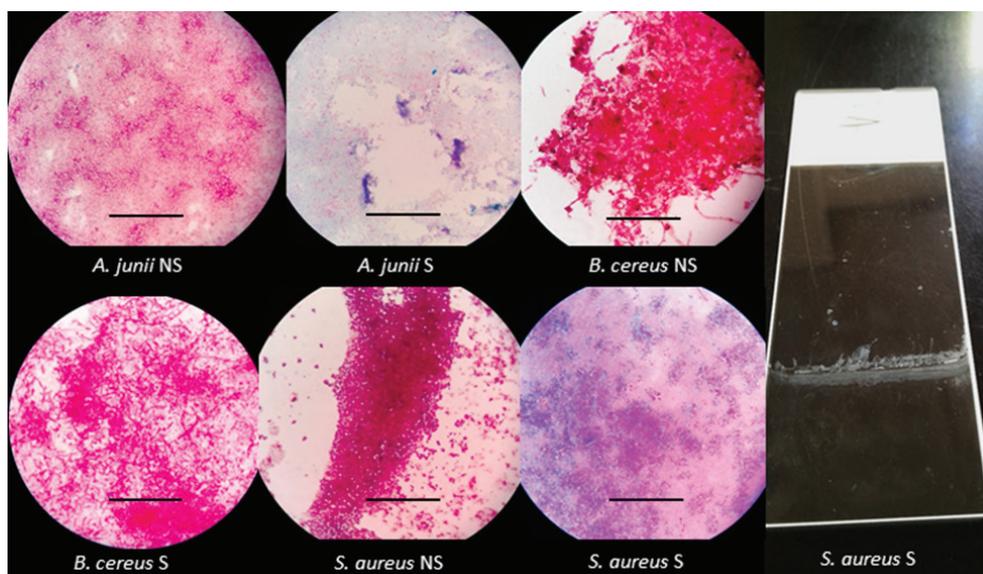


Figure 2 Biofilms grown on glass microscopy slides at the air/liquid interface after 7 days of incubation. Far right: macroscopic view of biofilm formed at air/liquid interface. NS – experiments without shaking; S – experiments with shaking. Scale bar=50 µm

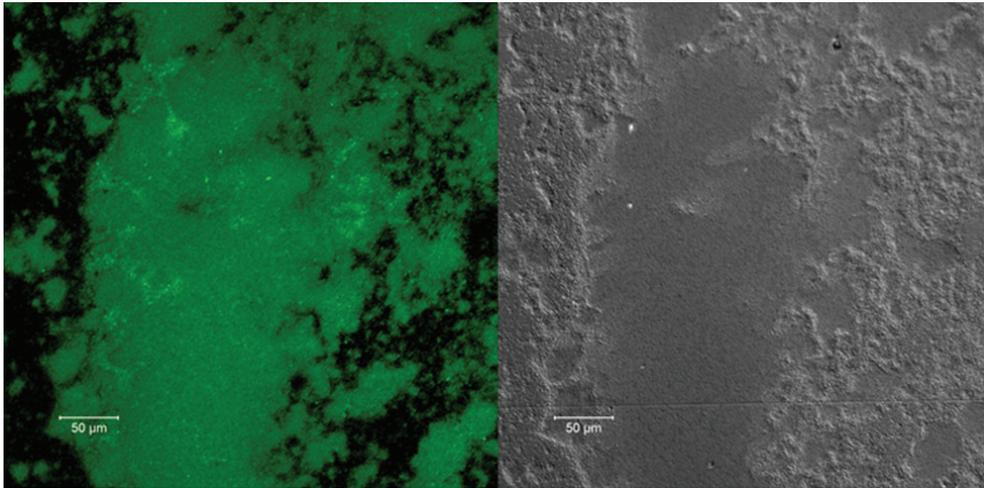


Figure 3 Biofilm of *P. aeruginosa* at the air/liquid interface after 7 days of incubation under confocal microscopy. Left: autofluorescence; Right: bright field. Scale bar=50 µm

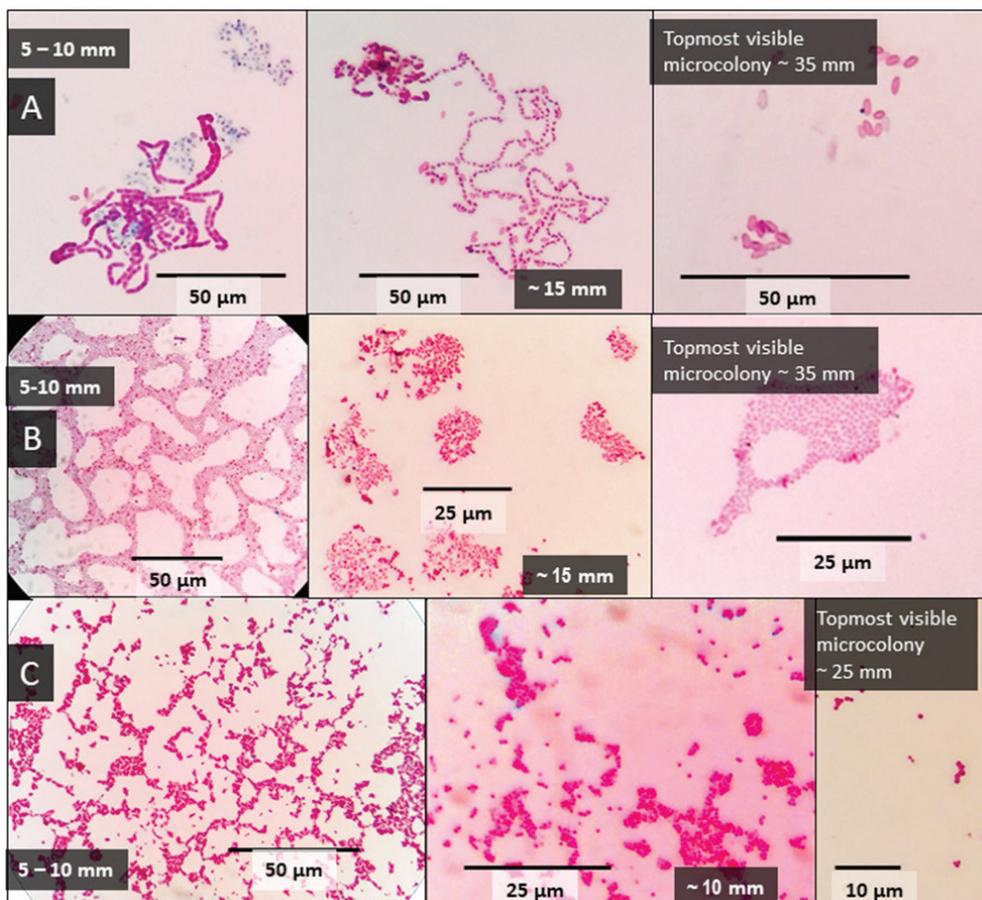


Figure 4 Migration of *B. cereus* (A), *A. junii* (B), and *S. aureus* cells (C) across the air-exposed section of the glass slide imaged after 7 days of incubation. Measures in mm designate the distance crossed up the slide from the air/liquid interface. The “topmost visible microcolony” shows a microcolony or aggregate of cells reaching the farthest away from the interface

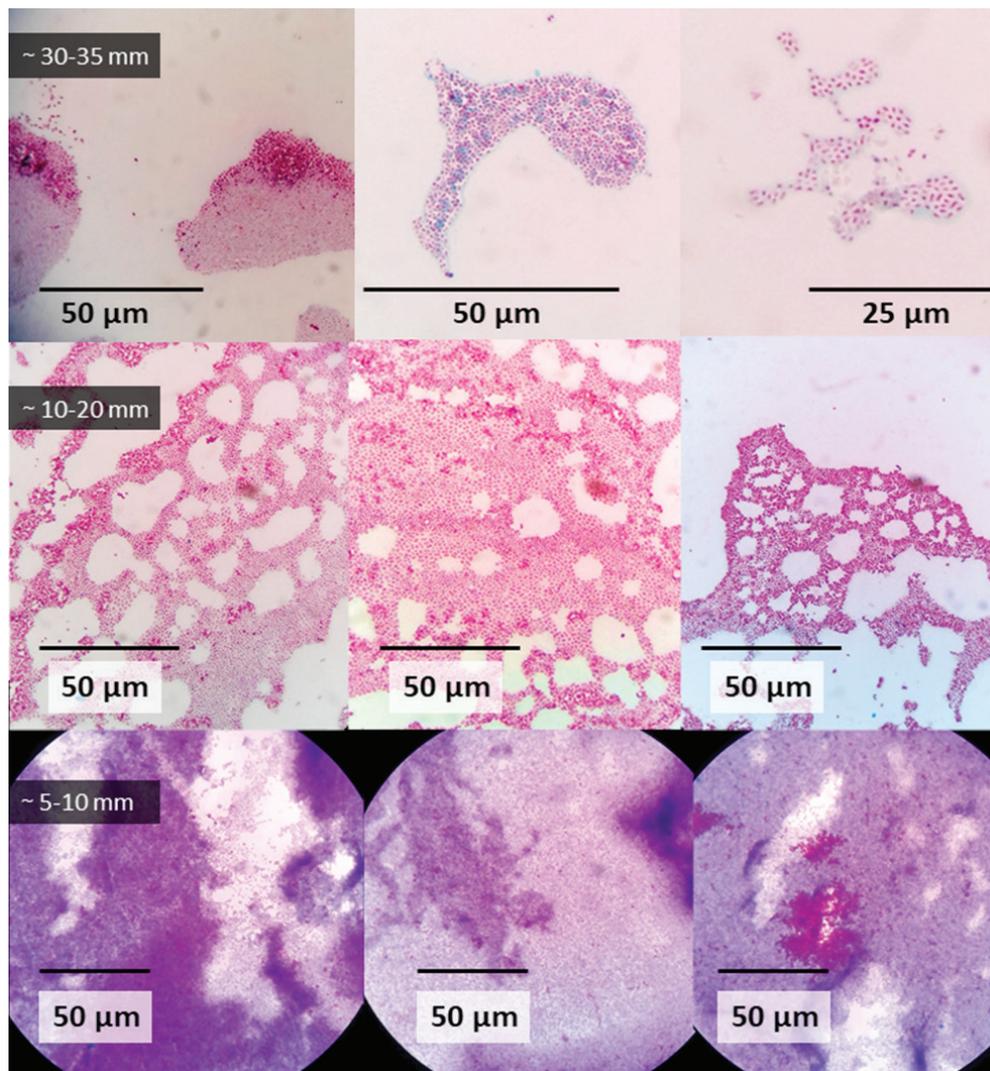


Figure 5 Migration of *A. baumannii* cells across the air zone of glass microscopy slide imaged after 7 days of incubation. Measures in mm designate the distance crossed up the slide from the air/liquid interface. Upper row shows the “topmost visible microcolony”, a microcolony or aggregate of cells reaching the farthest away from the interface. This figure shows several snapshots from the same area (height)

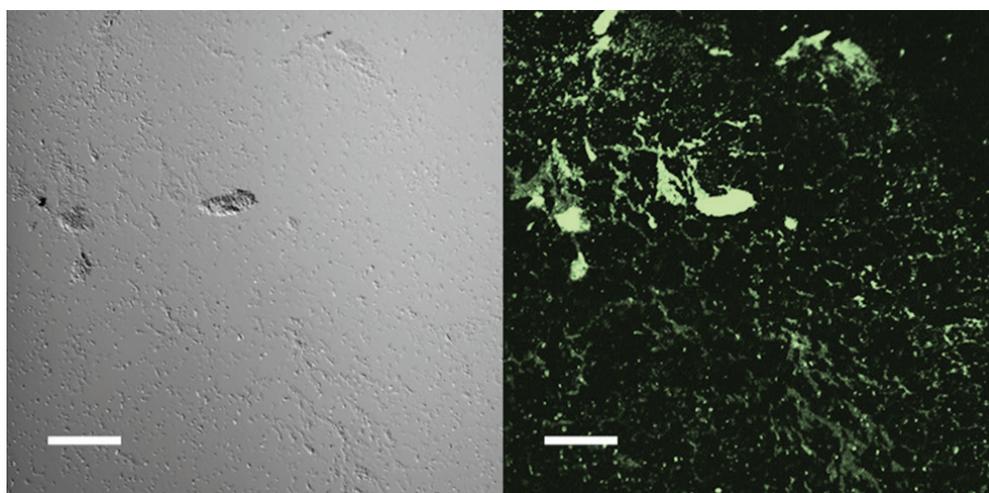


Figure 6 Migration of *P. aeruginosa* cells across the air-exposed section of the glass slide at 20 mm above the air/liquid interface. Image on the right shows superimposed autofluorescence. Scale bar=50 μ m

The upward movement of cells was the clearest in experiments with *A. junii* and *A. baumannii*, whose migration could be seen with the naked eye (Figure 9A and B). EPS, dyed in purple/blue, also visibly moved up the glass slide from the interface (Figure 9C and D). The cells of both bacteria made a specific growth pattern (Figures 4B and 5), very similar to the typical *P. aeruginosa* twitching pattern (13). This growth pattern was visible almost all the way up the glass slide, but the farthest reaching microcolonies (at 35 mm above the interface) were “separated” from it (Figures 4B and 5). However, this growth pattern could also be an artefact of drying and heat-fixing.

P. aeruginosa also showed migration across the glass slide, visible as autofluorescence under the confocal microscope (Figure 6). Microcolonies were visible all the way up to the frosted edge.

S. aureus cells migrated across the air-exposed section (Figure 4C) with microcolonies sometimes surrounded with EPS as a blue halo. Higher up in the air-exposed section, single cells were more common reaching 25 mm above the air/liquid interface (Figure 4C).

Figures 10 and 11 summarise in drawing the results of tests with carbol-fuchsin, designed to reveal bacterial movement with the capillary movement of nutrient medium. The dots, smudges, and spots of coloured nutrient reached as far as 30–35 mm above the air/liquid interface after ten days of incubation. With *A. baumannii* the distance crossed was greater with higher starting bacterial count, and the time needed to cross it was shorter (Figure 10). This relation was not as straightforward with *B. cereus* (Figure 11).

Even when we replaced the nutrient medium with saline, *A. baumannii* reached 10 mm above the interface with the starting concentration of 10^3 CFU/mL and 35 mm with the starting concentration of 10^7 CFU/mL (Figure 10). *B. cereus* reached 5 mm at 10^3 CFU/mL and 10–15 mm at 10^7 CFU/mL (Figure 11).

Routine swarming and twitching assays (Table 2) confirmed swarming of *B. cereus* and *P. aeruginosa* and twitching of *A. junii* and *A. baumannii* in addition to capillary movement.

DISCUSSION

Our results demonstrate that various types of bacteria are capable of moving across an inert, non-nutritive solid surface. However, it is important to point out that the air-exposed section of the glass slide surface was moist from nutrient medium or saline evaporation, and surface moistness is one of the requirements for bacterial movement.

We did not measure the exact amount of condensed water on the air-exposed surface, but it was clearly visible.

At this point, it is intriguing to see what motility mechanism(s) enabled the bacteria to climb vertically up the glass slide. *B. subtilis*, *B. cereus*, and *P. aeruginosa* are model organisms of swarming (14–18). *B. cereus* and *P. aeruginosa* confirmed in our study to move by rotating flagella, which fits the definition of swarming (7). However, our experimental setup differed from the standard swarming assay in that it used a non-nutritive, inert, and smooth surface.

In that respect, the standard “twitching assay” is more similar to our conditions, as it monitors colony spreading over inert surfaces like a Petri dish (19), glass slides (13), or even cellophane (20). However, in all those experiments, inert surfaces were covered by a layer of nutrient-rich agar, and our air-exposed glass slide sections were not, yet the tested strains did twitch across them. This type of movement was the most prominent with *A. baumannii* and *A. junii*, both of which have type IV pili required for twitching motility (8, 21, 22).

S. aureus, in turn, also exhibited movement across the air-exposed glass surface even though it cannot swarm and twitch, having no flagella and type IV pili (4). It is quite likely that it moved by gliding or sliding (4), but we could not verify these mechanisms, as laboratory assays for investigating these types of motilities are based on soft agar plates rather than non-nutritive solid surfaces. We also could not verify whether *B. cereus* and *P. aeruginosa* also moved, at least in part, through these mechanisms for the same reason.

Angelini et al. (11) reported the type of movement most similar to the ones presented in this study. In their experiment, *B. subtilis* biofilm was described as “climbing on the walls of a conical vessel” after pellicle formation, reaching about 8 mm in height following a 20-hour incubation at 30 °C. This “wall-climbing biofilm” shares similarities with our findings, as our bacteria were climbing by forming a monolayer biofilm. Angelini et al. (11) further proposed that their cells were “surfing” on bio-surfactant produced by the pellicle. Even though we did not establish or monitor biosurfactant production in our study, we too can assume that surfing took place, judging by the similarity of the experimental setups between ours and Angelini study, at least in the case of *B. cereus* and *B. thuringiensis*.

The bacterial migration up the glass slide was most likely initiated by capillary forces, since the bacteria migrated even when immersed in saline, in which no multiplication is expected. This migration was probably

Table 2 Swarming and twitching motility determined by standard assays for *B. cereus*, *A. junii*, *A. baumannii* and *P. aeruginosa* and expressed as diameter of the growth area from inoculation spot

	<i>B. cereus</i>	<i>A. junii</i>	<i>A. baumannii</i>	<i>P. aeruginosa</i>
Swarming	82±14 mm	N/A	N/A	16±7 mm
Twitching	N/A	35±12 mm	45±14 mm	51±9 mm

N/A – not applicable

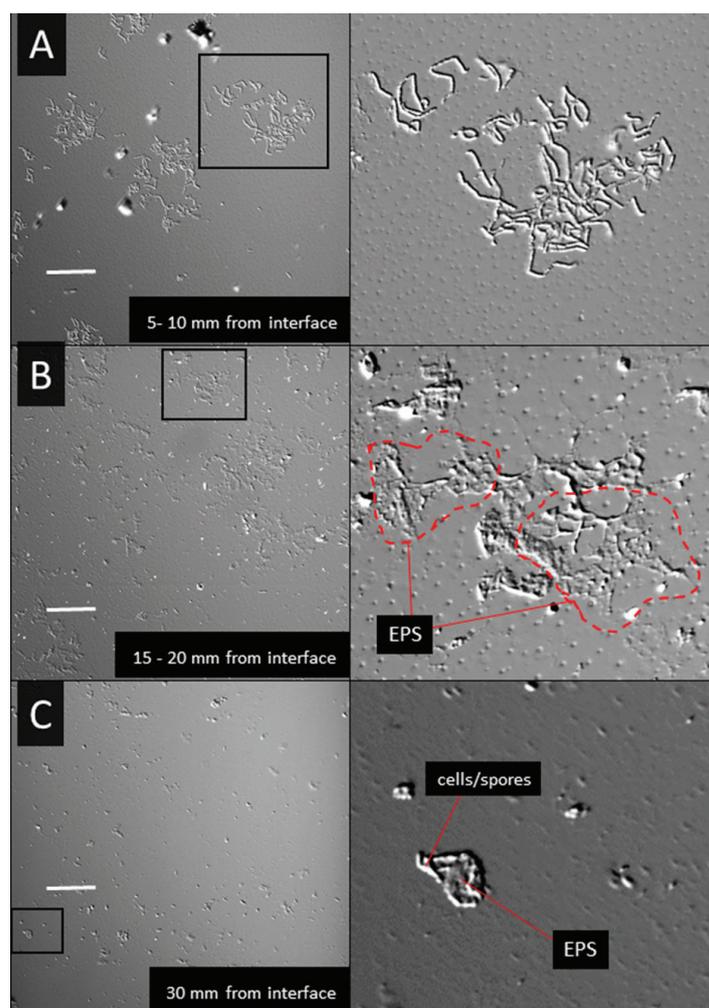


Figure 7 Microcolonies of *B. cereus* (A and B) and *B. thuringiensis* (C) on the air-exposed section of the glass slide after 7 days of incubation. Images on the right show magnified cut-outs. EPS – extracellular polymeric substances. Scale bar=25 μ m

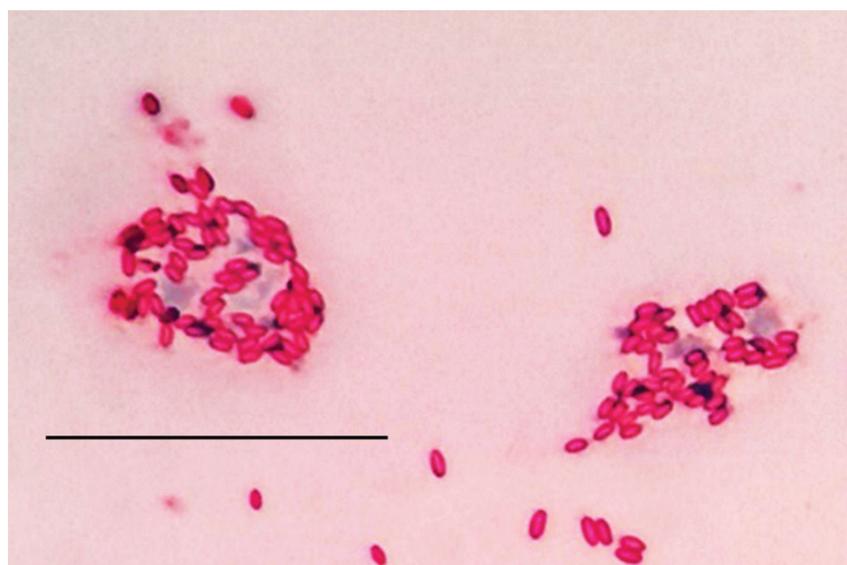


Figure 8 Typical microcolony of *B. cereus* spotted high in the air-exposed section of the glass slide. Scale bar=25 μ m

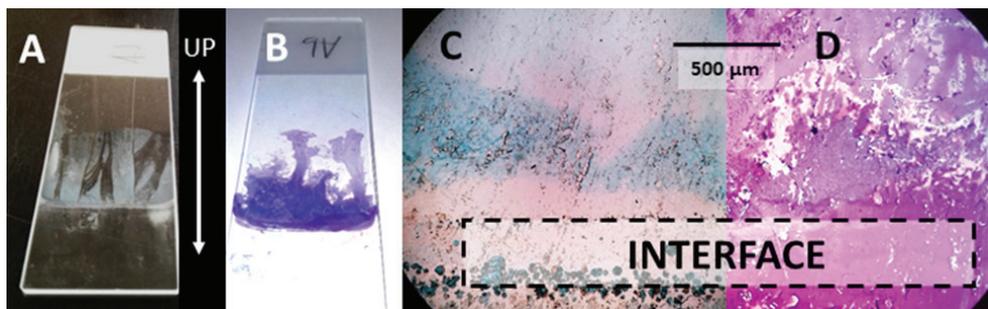


Figure 9 Migration of *A. junii* (A) and *A. baumannii* (B) from the air/liquid interface to the air-exposed section of the glass slide. EPS is dyed in blue/purple to better show spreading up the slide [*A. junii* (C); *A. baumannii* (D)]

facilitated by the production of EPS. Similar observations were reported by Be'er et al. (25) on agar plates and Hennes et al. (26), who argued that bacteria inside a droplet overcame the pinning capillary forces of a water drop on an agar surface, and collectively “surfing” across agar in speeds well above that of mass swarming. They proposed that surfactin lowered surface tension and created inward osmotic flow. In our experiments this may indicate that biofilm formation (and the production of EPS) above the air/liquid interface created a suction force for nutrient media, enabling further cell division, more suction, and step by step upward migration of bacterial cells.

Another question raised here is, do the bacteria just migrate upward from biofilm at the interface, or do they also actively multiply along the way? The presence of microcolonies on the air-exposed section seems to evidence active multiplication (proliferation). This, in turn, suggests that nutrients are transferred from the medium all the way up the glass slide, possibly facilitated by the production of EPS, as argued above. Another explanation could be the “bust and boom” survival strategy, where weak bacterial cells die in unfavourable conditions, and the remaining cells live on their expense (27).

CONCLUSIONS

Even though the tested bacteria differ in morphology, physiology, and dominant types of motility, all of them migrated vertically from biofilm at the air/liquid interface over non-nutritive surface. It is important to note that our experimental conditions differ significantly from standard assays, which enabled us to describe a novel type of motility that seems to be a common property of various bacterial species. We propose to call it “capillary movement of biofilm” to describe this phenomenon. It is further facilitated by biofilm production of EPS.

Further experiments that would involve quantification of cell migration (with imaging software), comparison between motile and non-motile mutants, mixed microbial communities, and determination of physicochemical properties such as capillary forces, transport of nutrients, and the importance of water vapour in the gas phase, could shed more light on this newly described phenomenon.

Acknowledgments

This work has been partly financed by the ERASMUS+ program for partner countries (KA107) and Croatian Science Foundation (project no. IP-2014-09-5656).

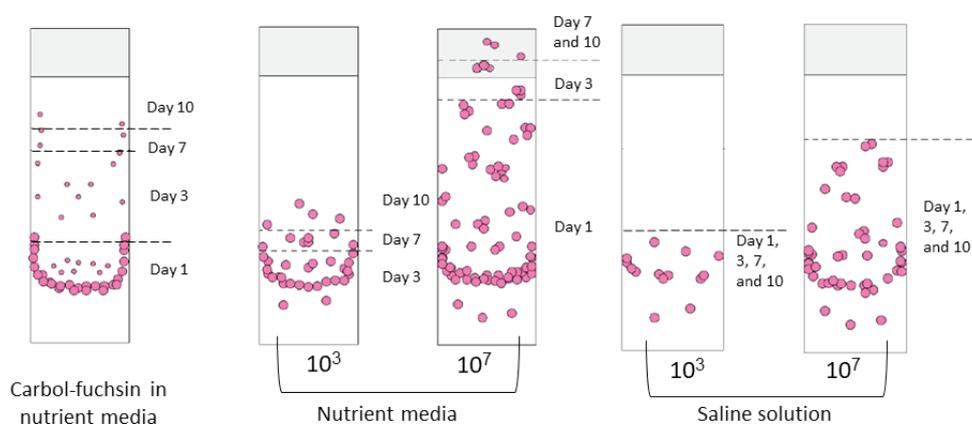


Figure 10 Migration of carbol-fuchsin (left) and *A. baumannii* cells from nutrient media or saline (right) up the glass slide, recreated from experiments listed in Table 1. Starting bacterial concentrations were either 10^3 or 10^7 CFU/mL. The dashed line marks the farthest point reached on the given days of incubation

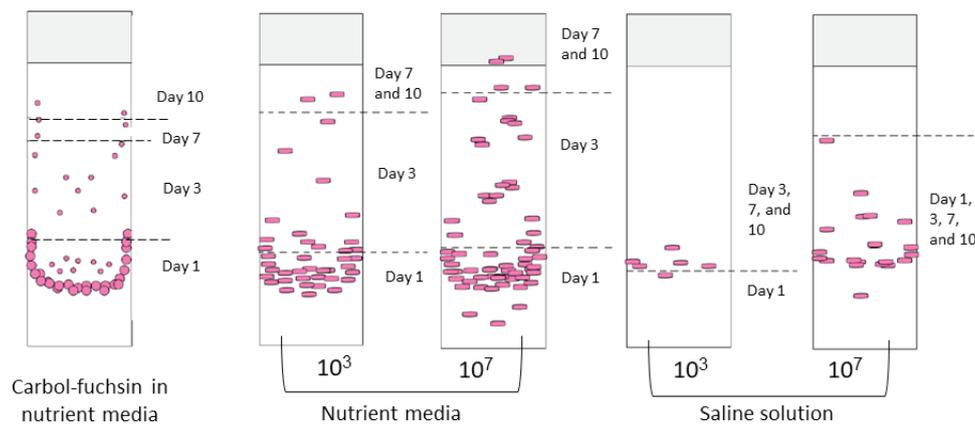


Figure 11 Migration of carbol-fuchsin (left) and *B. cereus* cells from nutrient media or saline (right) up the glass slide, recreated from experiments listed in Table 1. Starting bacterial concentrations were either 10^3 or 10^7 CFU/mL. The dashed line marks the farthest point reached on the given days of incubation

Conflicts of interest

None to declare.

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Pokretljivost bakterija po inertnoj površini posredovana kapilarnim silama

Primijećen je i prikazan dosad neopisan način pokretljivosti bakterija u obliku biofilma. Pokazano je kako se bakterijske stanice iz biofilma formiranoga na razmeđi tekućine i zraka gibaju okomito po staklenom predmetnom stakalcu, inertnoj, nehranjivoj ali djelomično vlažnoj površini. Takva površina, izložena zraku, uvelike se razlikuje od površine hranjivog agara, ili od površine prekrivene hranjivim agarom, kakve se koriste u standardnim testovima za ispitivanje bakterijske pokretljivosti u biofilmu. Sudeći prema opažanjima, stanice se prvotno gibaju posredstvom kapilarnih sila, a kada se formira biofilm, stvaranje izvanstanične polimerne tvari (EPS) potiče daljnju migraciju bakterija. Takva pokretljivost opažena je kod svih testiranih a fiziološki bitno različitih bakterijskih vrsta. Kako se eksperimentalni postav značajno razlikuje od standardnih testova praćenja bakterijske pokretljivosti, kao što su rojenje (*swarming*), trzanje (*twitching*) ili klizanje po površini (*gliding*, *sliding* ili *surfing*), predložen je generički naziv “kapilarna pokretljivost biofilma”. Ovdje opisani fenomen mogao bi biti značajan ekološki čimbenik pokretljivosti bakterija u okolišu.

KLJUČNE RIJEČI: *Acinetobacter*; *Bacillus*; biofilm; razmeđa tekućine i zraka; mikroskopija; površinska pokretljivost; *Pseudomonas*; *Staphylococcus*